UNIVERSITY OF THESSALY
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PhD Thesis

GENOMIC AND TRANSCRIPTOMIC ANALYSIS OF THE OLIVE FLY REPRODUCTIVE SYSTEM, AIMING AT NOVEL CONTROL METHODS

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ΓΟΝΙΔΙΩΜΑΤΙΚΗ ΚΑΙ ΜΕΤΑΓΡΑΦΟΜΙΚΗ ΑΝΑΛΥΣΗ ΤΟΥ ΑΝΑΠΑΡΑΓΩΓΙΚΟΥ ΣΥΣΤΗΜΑΤΟΣ ΤΟΥ ΔΑΚΟΥ ΤΗΣ ΕΛΙΑΣ, ΜΕ ΣΤΟΧΟ ΚΑΙΝΟΤΟΜΟΥΣ ΜΕΘΟΔΟΥΣ ΕΛΕΓΧΟΥ ΤΟΥ ΕΝΤΟΜΟΥ

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Στην ανηψούλα μου………

Abstract

The olive fruit fly, *Bactrocera oleae*, is the most important pest of cultivated olives causing significant production losses and olive fruit impoverishment. After mating, the female insect, deposits its eggs in the olive fruit where the developing larvae feed and grow. In most insects, mating is a prerequisite for reproduction and, thus, critical to the maintenance of their population and the continuation of their species.

Currently, olive fly control mostly relies on insecticide spraying. However, the use of insecticides has led to resistance development and environmental damages, rendering the design of new alternative methods of control a necessity. Targeting the reproductive success of the olive fly is a promising method for pest control as manipulation of the reproductive system could affect the destructive activity of the fly. At present, genomic and transcriptomic data of the fly's reproductive system is practically non-existent.

Based on the above, a comprehensive analysis of the reproductive system was performed focusing on the identification of genes related to post-mating response. Specifically, RNAseq was performed for: 1. testes and accessory glands with ejaculatory bulb from virgin (7^{th} day old insects) and mated male insects and 2. lower reproductive tract from virgin (7^{th} day old insects) and mated female insects. Comparison of the transcriptomes between virgin and mated insects resulted in the identification of genes that are differentially expressed after mating. In testes 107 genes were up-regulated and 345 genes were down-regulated, while in male accessory glands with ejaculatory bulb 1,608 genes were up-regulated and 383 genes were down-regulated in mated insects. In females 1,705 genes were up-regulated and 120 genes were down-regulated in mated insects.

The top 100 most highly differentially expressed genes from each comparison were further annotated to the newly sequenced olive fly genome and functionally annotated through the Gene Ontology database. Annotations showed an alteration in metabolic, catalytic and cellular processes in the mated tissues. The identified genes encoded proteins implicated in immune response, mucins, antigen 5 proteins, proteases inhibitors and proteins with putative secretory activity.

Several genes were further selected for validation through qRT-PCR. For testes nine reproductive loci were considered. Results showed significant overexpression in mated flies of genes *c58283, c37552, hemolectin, mucin* and *cation transporter,* while significant downregulation was detected for *scribbler*. qRT-PCR did not confirm the expression profile of *c15699* and *c52071* genes obtained from RNAseq and *c42528* showed very low expression. For male accessory glands six reproductive loci were analyzed and confirmed their overexpressin in mated flies through qRT-PCR: *timeless*, *c52416*, *c57257*, *c52655*, *yellow-g* and *c53574.* Furthermore, we determined the expression profile of the selected genes from the first day of the insect eclosion to DAY 7. If a gene codes for a protein in the seminal fluid that is important for mating, it should be expressed earlier so that the protein will be present at the time of mating.

For the female lower reproductive tract six loci were analyzed. The results of the qRT-PCR confirmed the upregulation for *lingerer, bestrophin-2, ornithine decarboxylase genes* but did not confirm the RNAseq results for *troponin C* and *glutathione S-transferase epsilon class* genes. *Yolk protein-2* had low expression. The expression profile of the selected genes was determined in 7-day old virgin females and at five time points (0, 3, 6, 9, 12, 24 h) after mating. Expression profiles of the 6 genes were different. For example, *ornithine decarboxylase antizyme* showed an increasing expression with the highest expression 24 hours after mating, while *bestrophin-2* and *lingerer* expression peaked after 12 hours and fell afterwards.

Functional analysis through RNAi silencing was performed through RNAi injections or RNAi feeding. For *yellow-g* (from the male accessory glands) and *troponin C* (from the lower female reproductive tract) transfer of the dsRNA to insects was achieved through injections. Transient silencing through feeding was used for silencing of the *sex peptide receptor* (*spr*) based on its known involvement in reproduction in other insects.

For RNAi silencing through injections, results indicated high percentage of silencing in the insect, reaching 81% for *yellow-g* and 70% for *troponin-C.* Furthermore, mating experiments showed that transient silencing of the two genes had an impact on reproduction, since the oviposition rate of injected females was significantly reduced. For RNAi silencing through feeding, there was 90% and 40% downregulation of the *spr* gene on the female reproductive tract and head, respectively, resulting in significantly lower oviposition rate and reduced longevity compared to controlled flies.

This thesis constitutes the first comprehensive analysis of the reproductive system of *B. oleae,* identifying genes that could be target for the development of new intervention methods. Moreover, it demonstrated the first successful application of RNAi-feeding in *B. oleae,* giving new prospects to the use of this molecular tool.

Περίληψη

Ο δάκος της ελιάς, *Bactrocera oleae*, είναι ο σημαντικότερος εχθρός των ελαιοκαλλιεργειών προκαλώντας την ποσοτική και ποιοτική υποβάθμιση της παραγωγής. Τα συζευγμένα θηλυκά έντομα, ωαποθέτουν στους καρπούς, εντός των οποίων εκκολάπτονται οι προνύμφες και αναπτύσσονται τρεφόμενες από το εσωτερικό του. Η σύζευξη επομένως, στα περισσότερα έντομα, είναι απαραίτητη προϋπόθεση για την αναπαραγωγή και κατ' επέκταση για τη διατήρηση του πληθυσμού τους.

Σήμερα, η καταπολέμησή του εντόμου γίνεται κυρίως με τη χρήση εντομοκτόνων. Η αλόγιστη χρήση όμως των εντομοκτόνων έχει οδηγήσει στην ανάπτυξη φαινομένων ανθεκτικότητας των εντόμων αλλά και σε περιβαλλοντικές συνέπειες καθιστώντας απαραίτητη την ανάγκη για την εύρεση αποτελεσματικότερων και φιλικότερων προς το περιβάλλον μεθόδων ελέγχου. Το αναπαραγωγικό σύστημα του εντόμου θα μπορούσε να είναι ένας πολλά υποσχόμενος στόχος για την ανάπτυξη τέτοιων μεθόδων. Εμποδίζοντας είτε τη διαδικασία της σύζευξης είτε μειώνοντας την αναπαραγωγική ικανότητα των εντόμων, αναπόφευκτα θα υπάρξει και ελάττωση του πληθυσμού. Ωστόσο, μέχρι σήμερα, ελάχιστες είναι οι πληροφορίες για το αναπαραγωγικό σύστημα του δάκου της ελιάς σε γονιδιωματικό και μεταγραφικό επίπεδο.

Στα πλαίσια της παρούσας διατριβής, πραγματοποιήθηκε ανάλυση του αναπαραγωγικού συστήματος με έμφαση στην ταυτοποίηση γονιδίων που εμπλέκονται στη μετα-συζευκτική δραστηριότητα του εντόμου. Συγκεκριμένα, πραγματοποιήθηκε μεταγραφομική ανάλυση των ακόλουθων ιστών: 1. όρχεις και βοηθητικοί αδένες μαζί με εκσπερματική βαλβίδα από παρθένα (7 ημερών) και συζευγμένα αρσενικά έντομα αντίστοιχα και 2. αναπαραγωγικό σύστημα των θηλυκών (εκτός από ωοθήκες) από παρθένα και συζευγμένα θηλυκά έντομα αντίστοιχα. Η σύγκριση του μεταφραφώματος των ιστών μεταξύ παρθένων και συζευγμένων εντόμων ανέδειξε γονίδια που παρουσίασαν διαφορική έκφραση μετά τη σύζευξη. Για ότι αφορά τον ιστό των όρχεων εντοπίστηκαν 107 γονίδια να υπερ-εκφράζονται και 345 να υπο-εκφράζονται στα συζευγμένα έντομα. Αντίστοιχα, στους ιστούς των βοηθητικών αδένων με εκσπερματική βαλβίδα εντοπίστηκαν 1,608 γονίδια να υπερεκφράζονται και 383 να υποεκφράζονται, ενώ στο θηλυκό αναπαραγωγικό σύστημα 1,705 γονίδια να υπερεκφράζονται και 120 γονίδια να υποεκφράζονται στα συζευγμένα έντομα.

Τα 100 πρώτα υπερεκφραζόμενα γονίδια από κάθε σύγκριση επισημειώθηκαν στο πρόσφατα αλληλουχημένο γονιδίωμα του δάκου της ελιάς και πραγματοποιήθηκε κατηγοριοποίηση τους με βάση τους όρους γονιδιακής οντολογίας (Go annotation). Από τη διαδικασία αυτή εντοπίστηκε μια αύξηση των βιολογικών διαδικασιών που συμμετέχουν σε μεταβολικά, κυτταρικά και καταλυτικά μονοπάτια. Η πλειοψηφία των γονιδίων κωδικοποιεί πρωτεΐνες που συμμετέχουν στην ανοσοαπόκριση, αναστολείς πρωτεασών, εκκριτικές πρωτεΐνες και μουκίνες.

Στη συνέχεια επαληθεύτηκαν τα αποτελέσματα της μεταγραφομικής ανάλυσης μέσω πραγματοποίησης ποσοτικής qRT-PCR σε ομάδα γονιδίων από κάθε ιστό. Για τον ιστό των όρχεων αναλύθηκαν 9

γονίδια από τα οποία επαληθεύτηκε η υπερέκφραση των *c58283, c37552, hemolectin, mucin* και *cation transporter* και η υποέκφραση του *scribbler*. Δεν επαληθεύτηκε η έκφραση για τα *c15699, c52071* ενώ το *c42528* έδωσε πολύ χαμηλή έκφραση. Για τον ιστό των βοηθητικών αδένων με εκσπερματική βαλβίδα επαληθεύτηκε η υπερέκφραση και των 6 γονιδίων που επιλέχθηκαν (*timeless*, *c52416*, *c57257*, *c52655*, *yellow-g* και *c53574).* Επιπλέον καθορίστηκε το προφίλ έκφρασης των επιλεγμένων γονιδίων από την πρώτη μέρα έκδυσης των ενήλικων εντόμων μέχρι και την έβδομη μέρα ζωής τους (σεξουαλικώς ώριμα έντομα). Αν ένα γονίδιο κωδικοποιεί μια πρωτεΐνη που συμμετέχει στην αναπαραγωγή θα πρέπει να εκφράζεται κατά τη σεξουαλική ωρίμανση του εντόμου ώστε αυτή να είναι διαθέσιμη κατά τη διάρκεια της σύζευξης. Σε συμφωνία με την παραπάνω υπόθεση, τα περισσότερα γονίδια παρουσίασαν μέγιστη έκφραση πριν από την ημέρα που πραγματοποιήθηκε η συλλογή ιστών για τη μεταγραφομική ανάλυση.

Για το θηλυκό αναπαραγωγικό σύστημα αναλυθήκαν 6 γονιδιακοί τόποι. Τα αποτελέσματα της qRT-PCR επιβεβαίωσαν την υπερέκφραση των *lingerer, bestrophin-2, ornithine decarboxylase genes* ενώ δεν επιβεβαίωσαν την υπερέκφραση των γονιδίων *troponin C* και *glutathione S-transferase epsilon class.* Η *yolk protein-2* είχε ελάχιστη έκφραση. Για τα επιλεγμένα αυτά γονίδια καθορίστηκαν επιπλέον τα επίπεδα έκφρασής τους μέσω ποσοτικής qRT-PCR σε αναπαραγωγικούς ιστούς παρθένων θηλυκών εντόμων ηλικίας 7 ημερών και σε διάφορες χρονικές στιγμές μετά τη σύζευξη (0, 3, 6, 9, 12, 24 h). Τα προφίλ έκφρασής τους ωστόσο παρουσίασαν διαφοροποιήσεις. Για παράδειγμα το γονίδιο *ornithine decarboxylase antizyme* έδειξε αυξημένη έκφραση 24 ώρες μετά τη σύζευξη ενώ τα γονίδια *bestrophin-2* και *lingerer* 12 ώρες μετά.

Επιπλέον πραγματοποιήθηκε λειτουργική ανάλυση με παροδική σίγηση των γονιδίων είτε μέσω της έγχυσης δίκλωνων μορίων RNA στην αιμολέμφο είτε μέσω της τροφής. Παράλληλα καταγράφηκε η φαινοτυπική επίδραση της σίγησης στην αναπαραγωγική δραστηριότητα των εντόμων (καταγραφή σύζευξης και ωαπόθεσης). Για τα γονίδια *yellow-g* (από τους αρσενικούς βοηθητικούς αδένες) και *troponin C* (από το κατώτερο θηλυκό αναπαραγωγικό σύστημα) η έγχυση του dsRNA στα έντομα έγινε μέσω μικροενέσεων. Η παροδική σίγηση μέσω τροφής χρησιμοποιήθηκε για την αποσιώπηση του υποδοχέα του συζευκτικού πεπτιδίου (spr) λόγω της γνωστής συμμετοχής του στην αναπαραγωγή άλλων εντόμων.

Για τα γονίδια *yellow-g* και *troponin-C* καταγράφηκε 81% και 70% σίγηση αντίστοιχα. Επίσης παρατηρήθηκε μείωση του ρυθμού ωαπόθεσης των εντόμων υποδηλώνοντας την πιθανή συμμετοχή των συγκεκριμένων γονιδίων στην αναπαραγωγή. Το ποσοστό σίγησης του υποδοχέα του συζευκτικού πεπτιδίου (spr) μέσω τροφής καθορίστηκε στο 90% στο αναπαραγωγικό σύστημα και 40% στο κεφάλι του εντόμου. Επίσης παρατηρήθηκε μείωση της ωαπόθεσης των εντόμων σε σύγκριση με τα έντομα ελέγχου.

Συνολικά, μέσω της παρούσας διδακτορικής διατριβής πραγματοποιήθηκε η πρώτη ανάλυση του αναπαραγωγικού συστήματος του δάκου της ελιάς, ταυτοποιώντας γονίδια που θα μπορούσαν να αποτελέσουν στόχους για την ανάπτυξη καινοτόμων μεθόδων καταπολέμησης του εντόμου. Παράλληλα, καταγράφηκε και η πρώτη επιτυχημένη εφαρμογή της παροδικής αποσιώπησης γονιδίων μέσω της τροφής σε ενήλικα έντομα *B. oleae*, δίνοντας μια καινούρια προοπτική για τη χρήση της μεθόδου ως μοριακό εργαλείο.

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Όσο μοναχικός κι αν φαίνεται ο δρόμος για την ολοκλήρωση μιας διδακτορικής διατριβής, δε μπορώ να πω παρά ένα μεγάλο ευχαριστώ….

Σε όλα τα άτομα που πέρασαν από το εργαστήριο και συμβιώσαμε έστω και για μικρό χρονικό διάστημα μαζί. Μαζί τους έμαθα να εκτιμώ και να σέβομαι τη λέξη «συνεργασία». Ιδιαίτερο ευχαριστώ στην Μαρία Αδαμοπούλου, την Στέλλα Γαλατίδου και την Άννα Αγγελοπούλου για την τέλεια συνεργασία που είχαμε στις πιο δύσκολες χρονικά περιόδους που πέρασα στο διδακτορικό. Η πρακτική αλλά και ψυχολογική τους βοήθεια ήταν πολύτιμη.

Στις «συντρόφισσες» μου στο εργαστήριο, την δρ Σαγρή Έφη (η οποία είναι τώρα μια υπέροχη μανούλα) και την υποψήφια διδάκτορα Κοσκινιώτη Γιώτα (την «επαναστάτρια» του εργαστηρίου) για την αλληλοστήριξη και τις ατελείωτες ώρες γέλιου και συζητήσεων, εντός και εκτός εργαστηρίου. Χωρίς εσάς το «εργαστήριο» για μένα θα ήταν απλά ένας χώρος εργασίας, εσείς το κάνατε κάτι πολύ περισσότερο και ελπίζω η επαφή μας να συνεχιστεί, ανεξαρτήτου διαφορετικής διαδρομής στη ζωή.

Στην δρ Τσουμάνη Κωνσταντίνα, η οποία είχα την τύχη να είναι από τα πρώτα άτομα που γνώρισα στα πλάισια του ερευνητικού χώρου και μπορώ να πω ότι έβαλε κι αυτή τα πρώτα «λιθαράκια» για να αγαπήσω την έρευνα. Χαίρομαι να τη βλέπω να εξελίσσεται και να προοδεύει στον ακαδημαικό τομέα που τόσο αγαπά και υπηρετά με ζήλο και επαγγελματισμό. Η θετικότητά της και η ικανότητά της να αντιμετωπίζει οποιοδήποτε εμπόδιο ως ένα μέσο για να εξελιχθεί, αποτελεί πηγή έμπνευσης. Φυσικά, δε θα μπορούσα να μην αναφερθώ στα υπέροχα μας ταξίδια που παρόλο που γίνονταν στα πλαίσια εργαστηριακών υποχρεώσεων, κατέληγαν πάντα να είναι μια «ζηλευτή» ιστορία για τους εργαστηριακούς και μη φίλους μας. Ευχαριστώ για την ένεση «θετικής ενέργειας» στην τελική ευθεία.

Φυσικά, δε θα μπορούσα να παραδώσω αυτή τη διατριβή χωρίς να ευχαριστήσω τα άτομα που με στήριξαν και πίστεψαν σε μένα, κάποιες φορές, περισσότερο από όσο πίστευα εγώ στον εαυτό μου. Τους γονείς μου, Γιαννάκη και Δέσποινα, για όλη την αγάπη

και τις αξίες που μου έδωσαν. Ο αλληλοσεβασμός τους και η δύναμή τους στην αντιμετώπιση όλων των δυσκολιών της ζωής ως «ένα σώμα» είναι υποδείγμα ζωής και αγάπης για μένα. Φυσικά, τους «μεταλλάδες» της οικογένειας, την αδελφή μου Ραφαέλα και τον σύντροφό της Σπύρο, για την στήριξη και την αγάπη τους. Νιώθω τυχερή που τους αποκαλώ όλους «οικογένεια» και ακόμη πιο τυχερή που σύντομα θα αποκτήσουμε ακόμη ένα μέλος, την ανηψούλα μου. Μπορεί να μην έχει γεννηθεί ακόμη, αλλά ήδη η χαρά είναι ζωγραφισμένη στα πρόσωπα όλων μας. Το λιγότερο που θα μπορούσα να κάνω είναι να της αφιερώσω αυτή τη διατριβή. Ελπίζω να σας κάνω συνέχεια όλους περήφανους.

Για το τέλος, κράτησα δυο άτομα για τα οποία τα λόγια είναι λίγα για να εκφράσω την χαρά που μπορώ να τους αποκαλώ κι αυτούς «οικογένεια». Τον Γιώργο, τον «τζεπέτο» της καρδιάς μου, ο οποίος αποτέλεσε ένα από τους παράγοντες που αυτή η διατριβή έφτασε στο τέλος της και κυρίως που η συγγραφέας της διατριβής αυτής είναι χαρούμενη και ευτυχισμένη. Το ευχαριστώ είναι λίγο για όλα αυτά που μου πρόσφερε, ελπίζω μόνο, η ζωή να μου δώσει την ευκαιρία να του τα επιστρέψω όλα και με το παραπάνω.

Κλείνω με το άτομο που πραγματικά με «γαλούχησε» στον τομέα της έρευνας, την δρ Κακάνη Εύη. Ένα αξιοθαύμαστο άτομο, το οποίο ήταν πάντα πρόθυμο να με βοηθήσει και να με καθοδηγήσει από τα πρώτα μου βήματα ως προ- πτυχιακή φοιτήτρια. Η οξυδέρκεια της και η ερευνητική της ικανότητα είναι προσόντα που θαύμασα από την πρώτη στιγμή και η επαγγελματική της πορεία με κάνει να νιώθω μόνο περηφάνια. Πιο πολύ, όμως, χαίρομαι που η σχέση μας ξεπέρασε τα εργαστηριακά δρώμενα. Μαζί καταφέραμε να μας ενώνουν πολύ περισσότερα πράγματα στη ζωή, καθιστώντας την σήμερα, μια από τους πιο κοντινούς μου ανθρώπους. Περιμένω σύντομα να ανταμώσουμε και πάλι.

Είναι όμορφο να κλείνει αυτός ο κύκλος…. Ένας κύκλος γεμάτο ωραίες αναμνήσεις όπου ακόμη και οι πιο δύσκολες στιγμές να φαίνονται μικρές, μπροστά στο τέλος. Όμως, κάθε τέλος είναι και μια καινούρια αρχή γι΄αυτό και θα κλείσω με μια ευχή: ότι και να έρθει στη ζωή να είναι αληθινό, ουσιαστικό, έντονο, γεμάτο όμορφα συναισθήματα και να είμαστε τυχεροί να έχουμε αληθινούς ανθρώπους δίπλα μας να τα μοιραζόμαστε.

Γρηγορίου Μαρία-Ελένη

Λάρισα, 2018

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List of Tables, Charts and Figures

1.INTRODUCTION

1.Introduction

1.1 The olive tree

The olive tree, scientifically known as *"Oleae europea",* is an evergreen tree and belongs to the family *Oleaceae*. The indigenous olive tree (wild olive tree) first appeared in the eastern Mediterranean and its cultivation started more than 7000 years ago in the Mediterranean basin. Nowadays, olive trees can be found all over the world (Fares et al., 2011).

Over the centuries, the olive tree has a central role in many aspects of people's everyday life and habits. The fruit, the oil and the branches of olives trees have been used in trade, nourished generation after generation helping people grow healthily, providing longevity and protection from several illnesses (Trichopoulou and Dilis, 2007).

Today, the olive tree remains one of the most important crops, especially in the Mediterranean countries. More than eight million hectares of olive trees are cultivated worldwide among which the Mediterranean basin presents around 98% of them (Peralbo-Molina and de Castro, 2013) (Chart 1.1). Led by Spain producing 5,276,899 metric tons of olive oil annually, the world's top olive producers form a ring around the Mediterranean Sea. Italy is the second largest olive producing country with 3,220,674 metric tons annually followed by Greece with 2,232,412 metric tons of olive production annually. Other countries that cultivate olive trees ranked according to their production of olive oil are Tunisia, Turkey, Syria, Morocco, Portugal, Algeria, Argentina, Jordan, Lebanon, Libya, Israel and the United States ((Food and Agriculture Organization (FAO)).

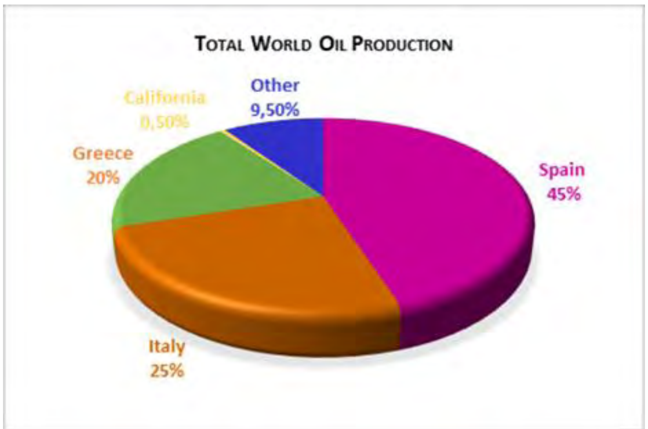


Chart 1.1: Percentage production of the total world olive oil production. First country is Spain 45%, followed by Italy 25%, Greece 20%, California 0,05% and Other countries 9,5%. Because of its olive production California is thought to be the Mediterranean of the United States.

Based on the International Olive oil council (IOC), the world olive oil production for the year 2016/2017 is assessed at 2,539,000 tonnes, down 20% compared with the previous crop year. Fortunately, this is not the case for current years' production. According to the IOC Statistics, world olive oil production in 2017/2018 is expected to increase by 14% to around 2,894,000 tones (IOC newsletter November 2017).

1.1.1 The olive tree and the Greeks

To Greeks, the olive tree is a blessed, valuable gift of nature, connected with their history and culture. In Greek mythology, the olive tree was the sacred tree of the city of Athens. During the dispute of the Gods as to who will be the patron of Athens, people had to choose between the gifts from two Olympic Gods: the olive tree from Athena, the Goddess of wisdom and the horse from Poseidon, the God of the sea. Ancient Greeks considered the olive tree more valuable than the horse, chose Athena and renamed their city to honor the goddess (Figure 1.1).

Figure 1.1: Bronze relief presenting Poseidon and Athena with their gifts to Ancient Greeks. This museum piece is exhibited at the National museum of Renaissance in Paris, France.

This gift to the people of Athens may be a myth but even in modern times, the olive tree has a relation to Greek culture and habits. To honor the olive and its symbolism,

in 2017, the 15th of November was called "The World Olive Day in Crete" recognized by the International Olive Council.

Olive trees fruit and extract, olives and olive oil respectively, are cornerstones of the Mediterranean diet with numerous proven health benefits (Faig et al., 2011; Servili et al., 2004). Nearly half of greek olive oil production is exported to other countries, while the rest

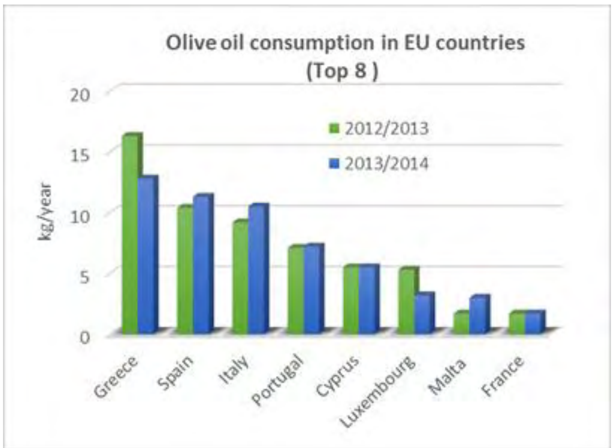


Chart 1.2: Per capita Olive oil consumption in EU countries (kg). Greece remains the first country of olive oil consumption for the years 2012/2013 and 2013/2014.

is domestically used, mainly for cooking purposes. Indeed, Greeks consume more olive oil per capita than anyone else in the world. Chart 1.2 represents the top eight countries of the olive oil consumption in Europe (EU) for the years 2012/2013, 2013/ 2014.

In respect to olive cultivation and according to the Hellenic Statistical Authority 11.111,3 hectares of the 32.825,2 hectares of the round up cultivated land in 2015 of Greece was devoted to olive growing. Peloponnese produces 65% of the Greek olive production, followed by Crete and the Aegean and Ionian Islands. Greece is the world's top producer of black olives and has more varieties of olives than any other country. Moreover, Greece is famous not only for the olives varieties but for the quality of the olive oil, too. About 80% of olive oil is extra virgin.

1.1.2 Olive tree pests and diseases

As with any other part of the environment, olive trees have pests and diseases. The creatures that we call pests and the organisms that cause disease only become "pest and diseases" when their activities start to damage crops and affect yields (Stoll 1986).

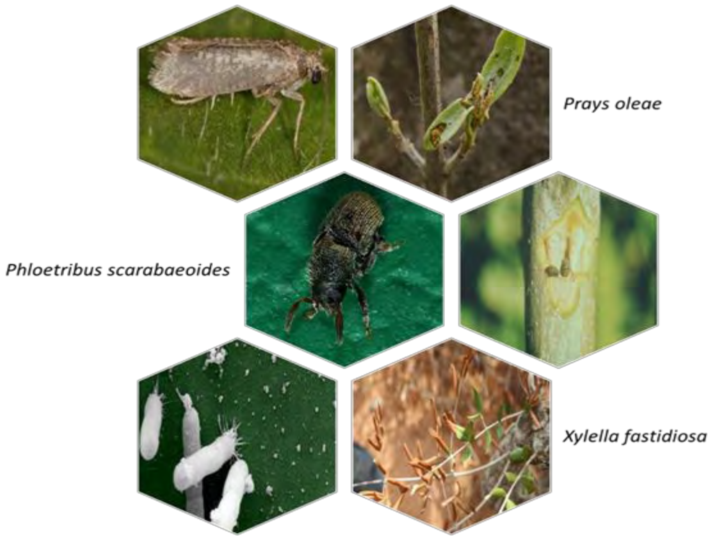


Figure 1.2: Pests and diseases of olive trees. On the left side are pictures of the adults *Prays oleae*, *Phloetribus scarabaecoides* and the bacterium *Xylella fastidiosa*. On the right side are pictures of infested olive trees.

A well-known olive tree pest is *Prays oleae*. *Prays oleae* is a moth with three generations per year. From each generation, larvae develop in a separate body of the olive tree. The females lay the eggs on the flower buds and the first generation larvae feed on the buds and flowers. The second generation larvae live and bore into the kernel of olive fruit and the third generation larvae make mines in the olive leaves (Figure 1.2). *Prays oleae* can reduce the olive production by 49 to 63% (Ramos et al., 1998; Patanita et al., 2004; Ait Mansour et al., 2017).

Another olive pest is *Phloetribus scarabaeoides,* a beetle that has 2-4 generations per year. *Phloetribus scarabaeoides* hosts are *Oleae europea* and other *Oleaceae*, like *Fraxinus*, *Ligustrum*, *Syringa,* and *Phyllirea*. Adult females bore through the bark of the tree and excavate a transverse tunnel on either side of the entry point (Figure 1.2). Inside the branch, each female can lay up to 60 eggs. As larvae hatch, they bore up or down from the entrance tunnel underneath the bark. This feeding causes partial to complete girdling of the branch; thereby structurally weakening it as well as damaging vasculature. Larvae pupate inside the feeding galleries (Ruiz et al., 1993).

A newly introduced in Europe pathogen that causes the Olive Quick Decline Syndrome (OQDS) disease is *Xylella fastidiosa*. Olive Quick Decline Syndrome causes withering and desiccation (extreme dryness) of terminal shoots, which then expands to the rest of the canopy, causing the tree to collapse and die (Figure 1.2). In Europe, *Xylella fastidiosa* was first spotted in Puglia, Italy, in 2013, while in America *Xylella* is endemic and has a broad range of vectors and host plants (Baldi et al., 2017).

However, the most important insect pest of the olive tree is the olive fruit fly, *B. oleae*.

1.2 Olive fruit fly

1.2.1 The origins of fruit fly

The olive fruit fly, *B. oleae*, belongs to the *Tephritidae* family and the genus Bactrocera (detailed scientific classification is shown in Table 1.1).

The family *Tephritidae* constitutes a group of agricultural pests of worldwide

Scientific classification	
Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Tephritidae
Genus	Bactrocera
Subgenus	Daculus
Species	Bactrocera oleae

Table 1.1: Scientific classification of *B. oleae*, the major insect pest of olive trees.

importance that attack a wide range of fruits and vegetables (White and Elson-Harris, 1992). The genus Bactrocera comprises 651 described species. It is the most economically significant fruit fly genus with at least 50 species considered to be important pests including *B. oleae* (Vargas et al., 2015). The majority of these flies are highly polyphagous like *B. dorsalis* (Hendel), *B. tryoni* (Froggatt) and *B. zonata* (Saunders). In contrast, the olive fly is strictly monophagous, closely associated with the olive tree. Therefore, distribution of the fly is linked to the olive tree cultivation.

The olive fly is mainly spread in the Mediterranean basin. However, there are reports of the fly from various parts of the world, including South and Central Africa, Near and the Middle East, California and Central America (Augustinos et al., 2002; Rice et al., 2003; Nardi et al., 2005).

Analyses of natural olive fly populations support its subdivision into three groups: Pakistan, Africa and Mediterranean plus America (Nardi et al., 2005). Based on genetic data, Pakistani *B. oleae* may constitute a separate evolutionary entity. With regard to Africa, it has been suggested that sub-Saharan Africa is the local center of diversification (Nardi et al., 2010). Within the Mediterranean population, three geographically distinct genetic groups have been described: eastern (Cypriot/Israeli), central (Greek/Italian) and western (Iberian) groups by Augustinos et al., (2005) and Zygourides et al., (2009), albeit with very high overall gene flow. Similarly, Nardi et al., (2010) described the occurrence of at least two groups, with a third weakly supported subdivision, with medium levels of gene flow (Figure 1.3).

1.2.2 Biological cycle and morphology

B. oleae is a holometabolous insect, undergoing complete metamorphosis. The life cycle of the insect includes four stages: egg, larva, pupa, and adult (Figure 1.4).

At the first stage, the egg is small (around 0.7 to 1.2 mm long), white and elongated (Figure 1.4a).

At the second stage, the larva has a conical-cylindrical and narrow front (Figure 1.4b). The larva undergoes three

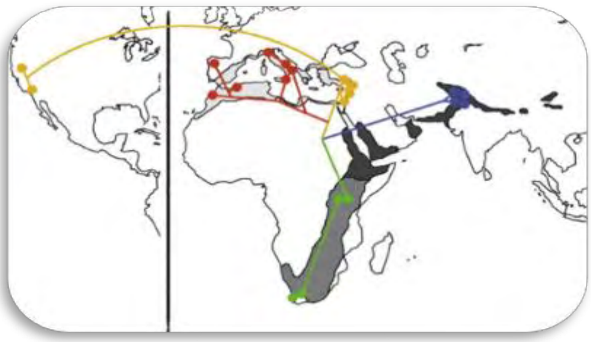


Figure 1.3: Co-distribution of olive and olive fly. The distribution of non-invasive olive lineages is shaded. Light grey: Oleae europea subsp. europea; Mid grey: *O. europea* subsp. cuspidata, tropical African group; Dark grey: *O. europea* subsp. cuspidata, North-Eastern African/Asian group (based on Besnard et al., 2007a). Circles represent olive fly genomes sampled, color coded according to their genetic group (see text). Blue: Pakistani group; Green: African Group; Red: Central/Western Mediterranean group; Orange: Eastern Mediterranean group (Nardi et al.,2010)

developmental stages (first, second and third instar stage). The different shapes of the frontal stigmas allow determination of the larvae of the second and third stages. The mature larva (third stage) is 7-8mm long, white-yellowish in color, elongated and subconical. In all stages, larva lacks a subhypostomal skeleton, is wingless and its form and habits are suited for growth and development rather than reproduction.

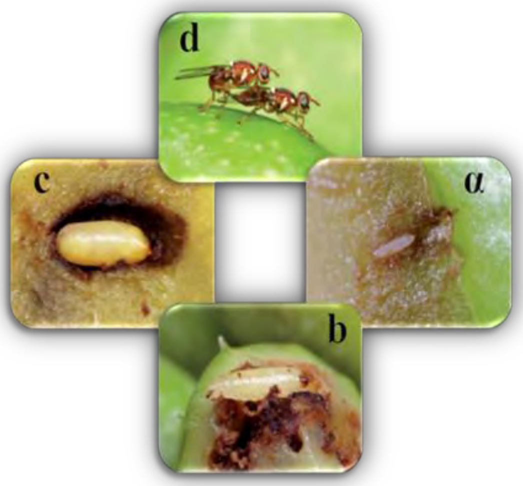


Figure 1.4: The life cycle of *B. oleae* consists of four stages: a) egg, b) larva, c) pupa, d) adult insect (male and female)

The pupal stage takes place inside the puparium, an elliptical shell formed by the last exuvial transformation of the larva. Depending on the age of the pupa the color of the puparium varies from creamy-white to yellow-brown (Figure 1.4c).

At the fourth stage, the adult insect is about 5mm long. It has wings that are transparent, with a brown spot at the tip of each wing. The thorax and abdomen of the adult fly are mostly dark-brown to black, with yellow-brown markings and short, silvery hairs (Weems and Nation, 1999). The head is blonde-yellow with two black spots under the small pair of antennas. (Figure 1.4d and 1.5). The visible sex diversity is based on the serrated ovipositor that is used by the females to pierce the skin of fruits during oviposition (Figure 1.5) (Tzanakakis, 2005).

B. oleae is a multivoltine species based on different environmental conditions, such as climatic factors and host availability (Yokoyama et al., 2012). In Greece, there have been reports of 3-4 generations per year (Kapatos and Fletcher, 1984; Tzanakakis, 1989). In other areas with abundant host plants, like California, the olive fruit fly may produce up to six generations per year (Rice, 2000).

In the Mediterranean basin, the first generation of the insect appears at the end of May (end of spring). At this point, female insects oviposit on the "leftover" olives from the previous year or wait until the new olive

Figure 1.5: The female (left) and the male (right) *B. oleae* insects. The visible ovipositor of the female insect is used to lay eggs on the olive fruit.

fruit is ideal for the development of the larva (pit hardening).

The second generation of the insect appears in the summer (June-July). Given suitable seasonal temperatures (25ºC), the olive fly can complete its biological cycle within 30 days (Tzanakakis, 1989). After oviposition, the first instar larvae enclose in 3-7 days. The larval stage lasts 10 to 15 days while the pupal stage lasts 8-10 days. In summer till mid-fall, eggs complete their development until the adult stage in the olive fruit; while later in fall, larvae leave the fruit and pupate in the soil (Tsitsipis and Kontos 1983).

Factors that are critical for olive infestation are the ideal temperature (20-25ºC), high humidity (60-80%) (Tsitsipis 1980; Fletcher and Kapatos 1983) and the type of olive cultivar. Different cultivars show different susceptibilities to the insect, with some cultivars having systematically low infestation levels, while others, within the same agro-ecosystem, are usually more heavily affected (Latinovic et al., 2013). Several physical and chemical factors, such as the size and the color of the fruit (Burrack et al., 2008; Genc, 2016), and the fatty acid composition (Gonçalves et al., 2012), respectively, interact for this olive fly/olive tree relation.

1.2.3 Impact on Crop

The olive fruit flies reduce the olive production in several ways. The female olive flies lay eggs into the olive fruits leaving an oviposition "sting" on the fruit surface. This may easily become a point of entry of secondary bacteria and fungi, leading to the appearance and development of other olives diseases (Latinovic et al., 2013; Malheiro et al., 2015).

As the larvae consume the olive pulp, they cause reduction of oil yield because of the increase of olive oil acidity and peroxide value (Pereira et al., 2004). Olive oil quality decreases as levels of acid increase (Gomez et al., 2008). Moreover, when the immature fruit is stung it may be aborted prior to harvest (Tzanakakis, 2006).

From all of the above, the olive production can be decreased by 80% in areas of the world where the olive fruit fly is established and not controlled. In Greece, the damage is estimated to 40-50% of the olive crop every year (Mazomenos et al., 2002; Haniotakis, 2005).

The damage of the olive fly therefore has a serious economic impact. Olives affected by the fly lose their market value for table consumption and oil production as the economic thresholds in table olives are extremely low (less than 1%) (Rice, 2000). The economic thresholds is the density of a pest at which a control treatment will provide an economic return. The economic impact of the damage can be as high as 800 million dollars per year worldwide (Montiel Bueno and Jones 2002).

1.3 Management of the olive fly

As olive fly is intimately linked to olive tree cultivation, countries producing olive oil developed methods to minimize the impact of the insect’s infestation. A broad-based approach that integrates practices for the control of pests is called Integrated Pest Management or IPM.

IPM considers all available pest control techniques and other measures that discourage the development of pest populations while minimizing the risks to human health and the environment (Food and Agriculture Organization (FAO)). IPM requires competences in three areas: prevention, monitoring, and intervention.

Prevention regards the selection of the appropriate variety and location for the crop (depending on the climate, soil, and topography of the field). However, regarding olive trees, prevention is limited as they are evergreen trees with no limitation in the locations. For this reason, more attention is given to monitoring and intervention.

Monitoring refers to the observation of the field in order to locate, identify and rank the severity of pest infestation. The goal is to determine when and what action should be taken to maximize crop production and quality and minimize the loss of the production due to pests and diseases. This way of monitoring decreases the possibility of using the wrong pesticide, or a pesticide that is not really needed (Pontikakos et al., 2012).

Tools like pheromone traps, diagnostics, and forecasting systems can assist with such monitoring in a timely and accurate way. Traps used for monitoring olive fly populations are the yellow sticky traps or McPhail-type trap glass or plastic (Haniotakis, 2005). Yellow sticky traps are baited with spirochetal sex-pheromone lures (attractive to male flies) or ammonium carbonate, ammonium bicarbonate, or the diammonium phosphate (attractive to both sexes). McPhail-type trap glass or plastic are baited with torula yeast lures. Torula yeast lures attract more female than male olive flies (McPhail, 1939).

Intervention aims at the reduction of the economic damage of a pest to acceptable levels by decreasing pest population and involves biological, chemical and genetic control methods.

1.3.1 Biological control

Biological control of the olive fly is based on natural enemies of the insect such as ectoparasitoids. A well-known highly polyphagous ectoparasitoid is *Eupelmus urozonus DALM* which attacks late instar larvae and pupae (Bigler et al., 1986; Kapatos and Fletcher, 1986*).* A number of pteromalids have also been associated with olive fruit fly like *Cyrtoptyx latipes* which attacks the larval stage of the olive fruit fly (Silvestri, 1914).

However, biological control is efficient when crops are grown in controlled environments like greenhouses and plastic tunnels or in the open field conditions at very low pest intensities. For this reason, other interventions are often required such as chemical control.

1.3.2 Chemical control

Chemical control involves the use of insecticides. Insecticides used for olive fly control are organophosphate (OP) like dimethoate and fenthion (Skouras et al., 2007), pyrethroids like lamba-cyhalothrin, alphacypermethrin (Margaritopoulos et al., 2008) and the naturalyte spinosad (Thomas et al., 2005).

Traditionally, control of the olive fly is based on the cover or bait sprays with chemical insecticides. The main difference between cover and bait spray is their application; cover sprays should cover all the trees while bait sprays should be applied at a rate of 1 to 3 fluid ounces per tree in a coarse spray or stream to a small portion of foliage. With bait spray there is no need to cover the whole tree, because the adult flies are attracted, feed on it, and die. In this way, the reproductive activity is interrupted minimizing the infestation of the trees (Tzanakakis, 1989).

The extensive use of insecticides, however, is posing a serious threat to the environment. Chemical insecticides are not species-specific (Aktar et al., 2009) having a direct impact on a human with potentially serious health effects for the high-risk group in each country (WHO, 1990). The world-wide deaths and chronic diseases due to pesticide poisoning number about 1 million per year (Environews Forum, 1999). Moreover, they impose a serious negative impact on the environment leading to the destruction of biodiversity. Many birds, aquatic organisms, and animals are under the threat of harmful pesticides for their survival (Denholm and Rowland 1992).

More importantly, though, the intensive application of insecticides over many years has led to the development and selection of insecticide resistance. Insecticide resistance can occur if a small proportion of the insect population is able to survive treatment with insecticide. These rare individuals can reproduce and pass on their resistance to the offspring. If an insecticide with the same mode of action is repeatedly used, then an even greater proportion will survive (Vontas et al., 2011) leading to decreased effectiveness of the control method. Insecticide resistance of the olive fly has been detected to organophosphates (Skouras et al., 2007) and pyrethroids (Margaritopoulos et al., 2008).

The mechanism of resistance to organophosphates (OPs) in *B. oleae* has been extensively studied and has been attributed to target site mutations in the acetylcholin-esterase gene (AChE). Two of these are point mutations that reside in the catalytic gorge of the enzyme (Vontas et al., 2002) while the third one is a small deletion located in the carboxyl-terminal of the enzyme (Kakani et al., 2008; Kakani et al. 2010). The mechanism for resistance in pyrethroids implicates enhanced MFO activities in association with α-cypermethrin resistance (Margaritopoulos et al., 2008). For spinosad resistance, whole transcriptome analysis of spinosad susceptible and resistant olive flies indicated that several immune system loci, as well as elevated energy requirements of the resistant flies, are implicated in the detoxification process (Sagri et al., 2014).

1.3.3 Genetic control

In 1964, the WHO defined genetic control as “the use of any condition or treatment that can reduce the reproductive potential of noxious forms (of the insect) by altering or replacing the hereditary material” (World Health organization, 1964). This definition includes two types of genetic control methods: 1. ones aiming at “population suppression”, reducing the numerical size of the pest population and 2. the control methods that aim at “population replacement” that is to change the pest population to a less harmful form.

The first genetic control technique that has been used widely as a pest control method is the Sterile Insect Technique or SIT.

The principles of SIT have been the base for the development of different other methods such as Release of Insects carrying a Dominant-Lethal" (RIDL) and Incompatible Insect Technique (IIT).

1.3.3.1 Sterile Insect Technique (SIT)

Sterile Insect Technique or SIT is an alternative, environmentally friendly and species-specific method of pest control that aims at the suppression of insect population. SIT is based on the mass rearing of a target-species, the sterilization of these mass-reared insects and the release of sterilized, preferably male-only insects in specific areas (Figure 1.6). The sterilization of the male insects is based on irradiation. Competition for mating between wild and sterile males results in a decrease in the number of fertile matings and a decline in the overall population size (Knipling et al., 1955). In theory, if continued releases are performed over several consecutive generations, the population will progressively be reduced and, eventually, a total eradication could occur. The sterile insect technique was first applied on an area-wide basis to eradicate the New World screwworm *Cochliomyia hominivorax* in the USA, Mexico, and Central America, after World War II (Knipling, 1955). Since then, it has been successfully implemented against several different insect pests including *Tephritidae*: *B. curcubitae* (Iwahasi 1977), *B. tryoni* (Fisher 1994) and *C. capitata* (Hendricks et al., 1983).

Figure 1.6: Diagram of the Sterile Insect Technique (SIT) method (New Scientist ©)

For *B. oleae,* there were two SIT unsuccessful attempts. The first one was in the early '70s where 150,000 insects (both sexes) were sterilized by gamma-irradiation and released in specific areas. However, at the end of the season olives were as highly infested as in the two nearby control plantations. The second attempt was in the late 70's in a small Greek island but with the same results (Economopoulos 1972; Economopoulos et al. 1977; Economopoulos and Zervas., 1982). After these unsuccessful efforts, the SIT program for olive fly was abandoned.

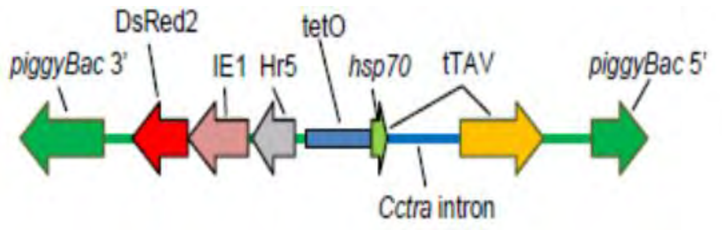


Figure 1.7: Diagrammatic representation of the OX3097 transposon. OX3097 comprises a fluorescent marker (hr5-IE1-DsRed2), and the female specific tTAV expression system (tetO-Dmhsp70 minimal promoter-Cctra: tTAV Sex-specific alternative splicing of the Cctra intron leads to production of tTAV and the initiation of a lethal tTAV positive-feedback loop in females only Thomas et al., 2012

There were specific problems that led to the failure of the projects. First, the non-standardized procedure for the mass rearing of the insects (Economopoulos and Zervas., 1982; Estes et al., 2011). *B. oleae* could be reared on an artificial diet under laboratory conditions, however, its large-scale mass rearing, needed for SIT, was difficult to be accomplished. The problem was based on the larval stage as *B. oleae* larvae are very sensitive to dietary changes such as pH and preservatives (Tzanakakis, 1989; Cohen, 2003; Lance and McInnis, 2005). Second, the different mating times of the released and wild population. Specifically, the laboratory-reared flies mated several hours before scotophase whereas wild flies mated the last two hours of the photophase. Third, even though radiation was quite effective, it caused somatic damage to the insect reducing the competitive hen of male flies to mate with wild females (Economopoulos and Zervas, 1982). Finally, the release of both sexes in the field led to the opposite results as laboratory-reared females also infested olive trees. Indeed, in the closely related species *C. capitata* (Medfly), a release of male-only insects gave a three- to five-fold improvement in the performance of released radiation-sterilized males (Rendon et al., 2004).

Nonetheless, over the years, there have been improvements in olive fly mass-rearing (Ras et al., 2017). Together with the progress in the molecular biology of the insect, there has been a renewed interest of SIT application in the olive fly (Estes et al., 2011). Moreover, the development of new genetic tools gave the opportunity to update the classic method of SIT in a modern and more efficient way enhancing it at three levels: genetic sexing, sterilization and monitoring.

As it was mentioned above, two strategies that are based on the SIT are the "Release of Insects carrying a Dominant-Lethal" (RIDL) and the Incompatible insect technique (IIT).

1.3.3.2 Release of Insects carrying a Dominant-Lethal (RIDL)

RIDL is a strategy related to SIT but with a dominant lethal transgene inserted into the insect genome, thus replacing the need for radiation exposure. In this method, "sterilization" of the released insects is induced not by irradiation but by homozygosity for a dominant lethal gene. One version of RIDL system involves the mass release of insects carrying a female-specific lethal transgene (fsRIDL). Successful application of this method was carried out in *Ceratitis capitata* (Fu et al., 2007) and *Aedes aegypti* (Carvalho et al., 2015).

The construct used for RIDL system in the Medfly was based on the sex-specific alternative splicing of the *transformer* (*tra*) gene. Specifically, the two sex-determination genes of insects are *tra* and the *doublesex* (*dsx*). The *tra* gene is regulated by alternative splicing in females producing a functional TRA protein while in the male is interrupted by additional exons that contain early stop codons. Based on this feature, they isolated the female-specific sequence that induces the alternative splicing into a tTAV coding region which was previously shown that induces dominant lethality in the insects (Gong et al., 2005) and developed two constructs, LA3077, and LA3097. These constructs carry also, a fluorescent marker (DsRed2) to allow detection of transgenic individuals and a self-limiting genetic trait that is repressed by tetracycline (tetO). In the absence of tetracycline (for example in nature), the tetracycline transactivator (tTAV) accumulates and results in female lethality at pupal stage.

In 2012, using the same construct as in Medfly, the first fsRIDL olive fly strain was developed (Ant et al., 2012). The insect strain, OX3097D-Bol, (Figure 1.7) resulted in female death at larval and early pupal stages in the absence of tetracycline. The potential release of OX3097D-Bol males can give the opportunity to mate with wild females and produce progeny that would die at the larval/pupal stages. On one hand, this technique is species-specific. On the other hand, it also seems environmentally safe since the engineered strain OX3097D-Bol does not have any impact on non-target organisms that either predate or parasitize olive flies (Marubbi et al., 2017). Until today, this technique has not been tested in the field for *B. oleae*.

While transgenic approaches have renewed interest in SIT, the use of genetically-modified (GM) insects will require addressing public concerns about the possible impacts in nature. Such concerns led to the development of alternative non-GM methods.

1.3.3.3 Incompatible Insect Technique (IIT)

A control method that suppresses the pest population and does not use transgenic approaches is the Incompatible Insect Technique (IIT) (Boller and Bush 1976; Bourtzis and Robinson 2006). This technique relies on bacterial endosymbionts named *Wolbachia*. *Wolbachia* is an obligatory, intracellular, maternally transmitted α-proteobacterium of the *Rickettsiaceae* family, infecting many arthropod and nematode species. *Wolbachia* symbionts act as reproductive parasite inducing cytoplasmic incompatibility (CI), male killing, feminization or parthenogenesis of the host (Stouthamer et al. 1990; Hoffmann and Turelli 1997; Rigaud 1997; Hurst et al. 1999). Cytoplasmic incompatibility is a type of conditional sterility. Specifically, sperm from Wolbachia-infected males is incompatible with eggs from females that do not harbor the same *Wolbachia* type resulting to embryonic lethality (Wenner and O'Neill et al. 1997; Werren 1997; Charlat et al. 2002; Bourtzis et al. 2003).

IIT method can be used as suppression method since *Wolbachia* is not paternally transmitted. When there is only male insect release, the infection type present in the released strain does not become established in the field. As the size of the field population decreases due to incompatible matings, the proportion of males of the released strain increases. Similar to conventional SIT, the increasing ratio of incompatible matings over time can lead to population suppression.

IIT method can be used as a replacement technique, too. Specifically, in mosquitoes, releasing males and females carrying *Wolbachia* will both mate with wild-type mosquitoes leading to all infected offspring. It has been shown that vector-borne diseases like Dengue, Zika, and malaria that are transmitted to human through mosquito cannot develop in *Wolbachia*-infected adult mosquitoes and so these viral diseases cannot be transmitted to humans (Callaway 2016).

This strategy was first introduced by Boller and colleagues who performed a study of the incompatible races of European cherry fruit fly *R. cerasi* (Boller and Bush 1974; Boller et al. 1976), followed by a small field trial by Russ and Faber (1978). Nowadays, it is widely used to reduce *Aedes aegypti* mosquito populations and the viruses they transmit such as Zika, dengue and chikungunya (World Mosquito Program). In *B. oleae*, lines were transinfected with the Wolbachia strain wCer2 in 2011 (Apostolaki et al., 2011) but no field trials were performed.

With the emergence of the gene disruption technology two new techniques have been discovered. The CRISPR-gene drive system and the RNA interference (RNAi) are rapidly expanding in many facets of biological research. Moreover, agriculture researchers are developing ways to include these technologies in the integrated pest management (IPM) (Baum et al., 2007; Huvenne et al., 2010; Noh et al., 2012).

1.4 Gene drive systems

Gene drive systems have the power to push the desired trait through an entire population of animals. In 2003, Austin Burt noticed some selfish genetic elements that are naturally present in organisms and enhance their own transmission relative to the rest genes, in a non-Mendelian way (Burt, 2003). This phenomenon is called homing and he proposed that these elements, called homing endonucleases genes (HEGs), could be used to spread into a population trait that can manipulate an organism’s fitness and reproduction. He believed that the release of just a few individuals within a population could lead to complete invasion of the gene drive cassette within 15–20 generations.

Eight years later, the first successful engineering of a HEG-based gene drive in *An. gambiae* was reported (Windbichler et al., 2011). The major disadvantage at this stage was that the locations in the genome where the gene drive could move were pre-established in transgenic lines by random transposon-mediated integration, so specific genes could not be targeted for modification (Labbe et al., 2010; Isaacs et al., 2012).

In 2002, an alternative type of nucleases was identified, the zinc-finger nucleases (ZFNs) (Beerli et al., 2002). ZFNs seemed to be modular and more-straightforward than the HEGs. Zinc-finger domains could recognize the shapes of nucleotide triplets in the major groove of a DNA double-helix and could be engineered to recognize an 18 nucleotide sequence such that a whole array of protein effectors could be recruited to a very specific site in the genome (Liu et al., 1997; Berli et al., 2002; Miller et al., 2007). However, the cost of a ZFN and the low success rate was still prohibitive to the wider use of the technology.

In 2010, transcription activator-like effector nucleases (TALENs) were identified. These nucleases were affordable and could be easily engineered. Moreover, they were modular and they could be encoded on a plasmid by cloning in relatively more efficient way in comparison to ZFNs (Christian et al., 2010). The recognition of each nucleotide on a DNA target was encoded in the 12th and 13th amino acid of each 34 amino-acid repeat; a peptide stretch of 18 or 19 repeats could be engineered to recognize any nucleotide sequence (Boch et al., 2009, Moscou et al., 2009). Gene-editing in *Ae. aegypti* and *An. stephensi* using ZFNs and TALENs were reported in 2013 (Aryan et al., 2013; Smidler et al., 2013).

However, the breakthrough of the gene-drive technology came in 2012 when CRISPR-based gene drive was identified (Zetsche et al., 2015). The word CRISPR means "Clustered Regularly Interspersed Palindromic Repeats" and refers to the widespread loci in bacterial and archaeal genomes that store sequences of parasitic nucleic acid to which they were previously exposed (Deveau et al., 2010). These are co-located on the bacterial genome with Cas (CRISPR associated) genes which are expressed and used to target and destroy incoming parasites with homology to small RNAs derived from the CRISPR loci. Cas9 was borrowed from the bacteria *Streptococcus pyogenes* (Jinek et al., 2012) and can target any region of a genome by

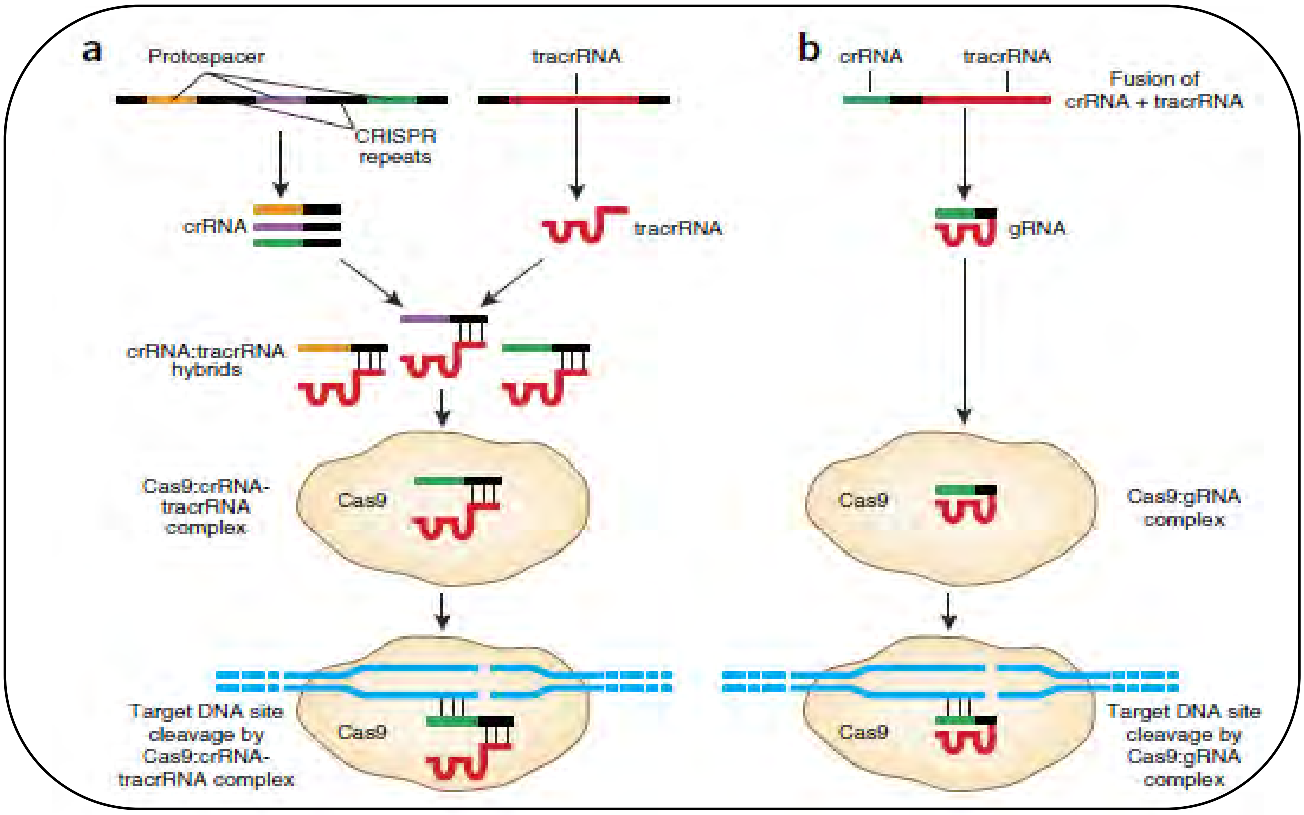


Figure 1.8 Naturally occurring and engineered CRISPR-Cas systems. (a) Naturally occurring CRISPR systems incorporate foreign DNA sequences into CRISPR arrays, which then produce crRNAs bearing "protospacer" regions that are complementary to the foreign DNA site. crRNAs hybridize to tracrRNAs (also encoded by the CRISPR system) and this pair of RNAs can associate with the Cas9 nuclease. crRNA-tracrRNA: Cas9 complexes recognize and cleave foreign DNAs bearing the protospacer sequences. (b) The most widely used engineered CRISPR-Cas system utilizes a fusion between a crRNA and part of the tracrRNA sequence. This single gRNA complexes with Cas9 to mediate cleavage of target DNA sites that are complementary to the 5' 20 nucleotides of the gRNA and that lie next to a PAM sequence (Sanders and Jound, 2014).

encoding a homologous ~20 nucleotides on a modified single guide RNA (sgRNA). This sgRNA will localize Cas9 to the target site for double-stranded cleavage of the DNA (Jinek et al., 2012).

CRISPR-based gene drive spreads a specific DNA cassette into the target species and it contains three elements: a gene encoding the bacterial Cas-9 protein, a gene coding a guide RNA that targets a particular site in the genome and flanking sequences which allow the cassette to insert at a given target site. The gene drive cassette can transport a payload gene into the target genome or it can be integrated to a specific position within the genome to knock out a gene. If the targeted position is a gene essential for reproduction, it can disrupt its physiology and behavior leading theoretically to the extinction of the species (Burt, 2003) (Figure 1.8).

Shortly after the CRISPR-based technology was released, there have been several reports of successfully engineered insects, from *D. melanogaster* with a remarkable 96% homing efficiency (Gantz and Bier 2015) to various mosquitoes (Gantz et al., 2015; Hammond et al., 2016). In *A. stephensi*, for example, the ability of a homing modification drive system to spread a large payload containing an antimalarial single-chain antibody was demonstrated (Gantz et al., 2015). In another study in *A. gambiae*, researchers created a suppression drive targeting female fertility genes (Hammond et al., 2016). This drive was successfully transmitted to offspring, although its population suppression capability was limited because heterozygous disruption of the target genes greatly reduced female fertility. In 2017, there was the first report of successfully engineered *C. capitata* using the CRISP-Cas9 technology (Meccariello et al., 2017).

As it comes to the targeted DNA sequence, there is no limitation. This gives the technology a broad range of applications, for example, holding invasive species at bay, ensuring plants remain sensitive to hermicides and as a control method for pest species (Gantz et al., 2015). Compared to other pest management techniques, it is cheaper, more precise and less controversial as the use of pesticides. These characteristics make gene drive-mediated pest control attractive for agribusiness because it allows direct manipulation of pest species (Courtier-Orgogonzo et al., 2017).

However, The National Academies of Sciences, Engineering and Medicine released a report outlining the hazards to be considered when thinking about gene drives (NASEM, 2016). Based on the report, not all pest species seem to be suitable for control using gene drives. In order for gene drives to work, pests need to reproduce sexually and have short generation times. The effectiveness of gene drives deployed for pest control will also depend on the breeding structure of the target pest as well as on its geographic distribution and degree of gene flow (NASEM, 2016).

While there is no permission to release genetically modified organisms with a gene drive in the field, the US National Academy of Sciences, Engineering and Medicine recently approved research on gene drive and called for carefully controlled field trials (NASEM, 2016). Moreover, the Gates Foundation and the Indian Tata Group invested more than US$140 million in gene drive research for controlling disease vectors and improving crop productivity. Recently, the US Defense Advanced Research Projects Agency (DARPA) announced US$65 million in funding to scientists studying gene-drive technologies (Callaway, 2017). Besides research programs, companies such as Bayer, Dupont, and Monsanto have signed license agreements with biotech companies to use the CRISPR/Cas-9 technology (Begley et al., 2016).

European commission proposes changes on the Directive 2001/18/EC for the GMOs release to alow the freedom of restrict or prohibit use of Authorized GMOs. Their opinion regards that unlike transgenesis, mutagenesis (including CRISP-Cas9 technology) does not entail the insertion of foreign DNA into a living organism but alterates the genome of a living species. However, the Advocate General Bobek recently released his opinion that organisms obtained by mutagenesis can be a GMO as the alteration of the genome is not occur naturally. (Press release No 04/18). The case is still pending on the Court of Justice of the European Union.

1.5 RNAi

RNA interference (RNAi) is a term used to describe a number of gene silencing phenomena characterized by the specific binding of short RNAs (20-30 nucleotides in length) to target sequences (Fire et al., 1998). The first report of an RNAi-like gene silencing was in the late 1980s when researchers tried to overexpress the *chalcone synthase* in the violet petunia flowers for a deeper violet color. *Chalcone synthase* is a component of the pathway responsible for violet coloration in petunia flowers. However, they unexpectedly obtained white flowers (Napoli et al., 1990) with 50 times lower expression of *chalcone synthase* in contrast with the wild-type flowers. Their explanation was that this effect was caused by the exogenous transgene suppressing the endogenous gene giving this process the name "co-suppression".

In animals, the first report about RNAi was obtained by Guo and Kemphues in a series of experiments conducted with RNA antisense on the nematode *C. elegans* (Guo and Kemphues., 1995). They attempted to knock down gene expression by introducing antisense RNA for the *partition 1* (*PAR-1*) gene. As a control, they used RNA sense of *par-1* gene. Surprisingly, they noticed that sense RNA was also impaired with *par-1* and induced silencing. However, only in 1998, Andrew Fire and Craig Mello explained the phenomenon by studying interference towards the *C. elegans unc-22* gene. They carefully purified the RNA antisense, RNA sense, and double-stranded RNA (ds-RNA) for the *unc-22* gene and compared their ability to interfere with the endogenous gene expression. Their results showed that gene interference single-stranded RNAs (either sense or antisense) were between 10 and 100 times less effective than dsRNA. They named this silencing phenomenon RNA interference (RNAi) (Fire et al., 1998). The two researchers were awarded the 2006 Nobel Prize in Physiology or Medicine for their discovery.

The mechanism of RNAi in insects was extensively studied in *D. melanogaster*. The double-stranded RNA (dsRNA) is cleaved into fragments of ∼21 nucleotides (the small interfering RNAs, or siRNAs) by the enzyme Dicer. The siRNAs are unwinding and loaded on to RNA-induced silencing complex (RISC) via an RLC (RISC-loading complex), which contains Dicer-2 (Dcr-2) and a partner protein R2D2 (Tomari and Zamore, 2005).

RNAi in insects allowed the analysis of gene function in non-model insects (Mito et al., 2011). Prior to the advent of RNAi technologies, it was difficult to perform any analysis of gene function outside of the few genetic model insects such as *D. melanogaster* and *T. castaneum* (Hughes and Kaufman, 2000). Furthermore, genome data has become more readily available revealing a large array of genes with unknown functions, leading to the problem of how to unveil the functions of these new genes (Belles, 2010). With RNAi, the expression of a given gene can be disrupted, and the phenotypic effects shed light on its function; thus, a phenotype is automatically linked to a precise DNA sequence (Belles., 2010).

As it comes to RNAi delivery in insects there are two types. First, the endogenous RNAi, which it is generated within cells. Second, the exogenous RNAi, where short RNAs of exogenous origin bind specifically to endogenous target RNA sequences leading to cleavage of the targeted endogenous RNA. The delivery of the exogenous RNAi can be achieved either by injections or by feeding.

In RNAi studies of gene function in insects the trigger is typically introduced by injection for example in larvae and adults (Rajagopal et al., 2002; Arakane et al., 2005; Tsao et al., 2009) and in embryos (Liu et al., 2008; Lemke and Schmidt-Ott, 2009; Pan et al., 2009). Injections, however, are not always easy to administer and may induce mortality in the test insects (Bucher et al., 2002). There has, therefore, been interest in achieving RNAi

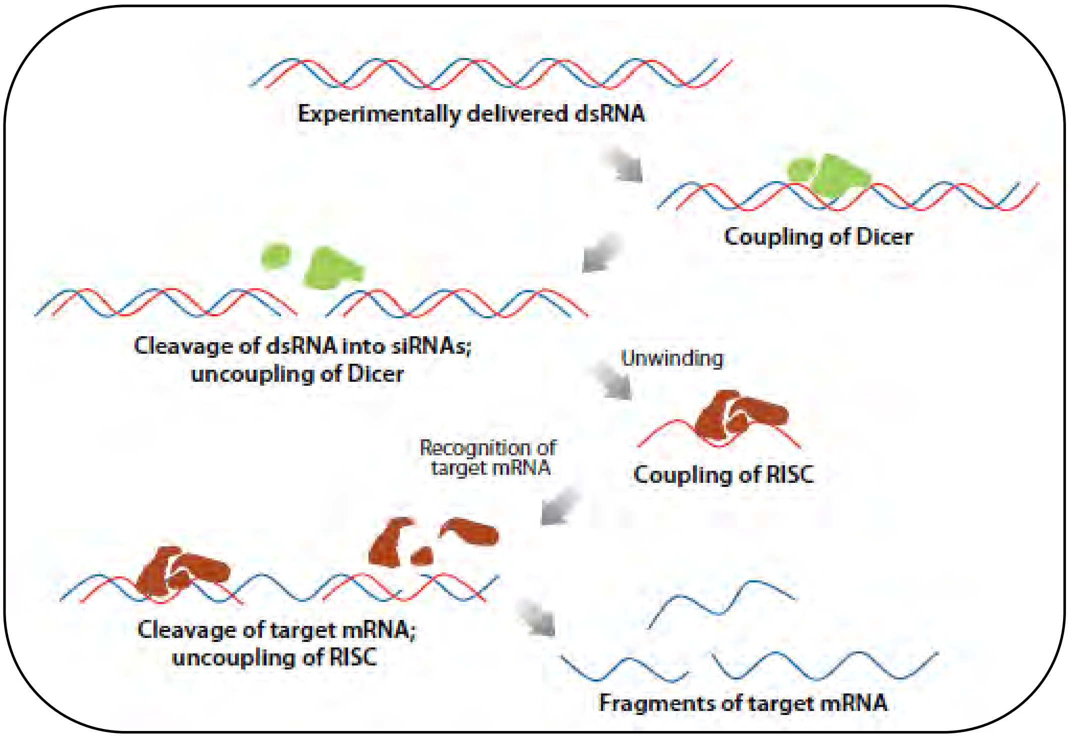


Figure 1.9: Basic mechanisms of RNA interference (RNAi). The double-stranded RNA (dsRNA) is cleaved into fragments of ~21 nucleotides (the small interference or siRNAs) by the enzyme Dicer. The siRNAs unwind, and the antisense strand couples to the RNA-induced silencing complex (RISC) and conveys it to the target mRNA. Then RISC couples to the target mRNA, blocking and degrading it (Belles., 2010).

by oral delivery of the RNAi trigger, since this is a less invasive technique. Successful oral RNAi was first demonstrated by Turner et al. (2006), who fed larvae of the light brown apple moth, *E. postvittana*, dsRNA for a larval gut *carboxylesterase* gene and found a substantial reduction in *carboxylesterase* transcript levels after two days.

The RNAi approach to control insect pests had been considered for many years, but the application of this technology was just realized after it was shown that ingestion of dsRNA would trigger RNAi. Recent studies demonstrated the feasibility of using RNAi-based strategies to reduce insect pests. In 2007, an RNAi-plant mediated pest control was developed for transgenic plants producing dsRNAs against specific insect genes, with the consequent effect on the target species (Baum et al., 2007; Mao et al., 2007). The main requirements to generate successful RNAi insect-resistant transgenic plants are: 1. identification of a specific gene with an essential function in the insect to be knocked down or knocked out; and 2. dsRNA delivery by oral ingestion that must be uptaken by the insect cells, and spread systemically. Moreover, a 90% sterilized male mosquito population was produced through RNAi feeding using sex-sorting genes and genes involved in male reproduction (Whyard et al., 2015) while sperm less *Bactrocera dorsalis* males were developed by feeding dsRNA to target genes important for the germ cell differentiation or genes related to azoospermia (Waqar et al., 2017).

In conclusion, almost all intervention methods, from typical traps to genetic control methods have the same goal: to interfere with the reproductive capacity of the insect and suppress their population. The main biological system that is responsible for reproduction is the reproductive system (Gilmore 1989).

1.6 Reproductive system

B. oleae species is bisexual and biparental, meaning that one egg from a female and one sperm from a male fuse to produce a diploid zygote.

1.6.1 Mating system and behavior

The term “mating system” is used to describe how mating and fertilization are achieved. The most fundamental categorization of the mating system uses the number of mates that each sex has within a defined time period. Female olive flies are oligogamous and mate 1-3 times during their lifetime (Tzanakakis et al., 1968; Cavalloro and Delrio, 1970; Zouros and Krimbas, 1970). Male olive fruit flies are polygamous and can mate daily if receptive females are available (Zervas, 1982).

Courtship displays are similar among Tephritid species. Including olive fly, both male and female display seven behaviors during courtship besides walking, staying still and preening: enation, supination, twirl, swaying, sidestepping, approach and touching. The male also performs wing buzzing, alternating legs, and mounting, and the female may extrude her ovipositor during courtship. Females do not display obvious receptivity behaviors.

However, there is apparently at least one behavioral element unique to each Tephritid species (Headricks and Goeden., 1994), Benelli et al., (2012) divided the courting sequence of the olive fly insect into three main phases: (1) Initial phase: ends with the male's arrestment (visual and olfactory cues play an important role); (2) Close-range phase: includes male wing vibrations, and (3) Final contact phase: copulation attempts (tactile cues probably dominate). Olive flies share the common courting behaviors such as wing buzzing, swaying, supination, enation, etc., with other Tephritid, but they have also unique behaviors. For example, the alternating legs behavior performed just before, during, and/or after buzzing its wings appears to be characteristic. Another characteristic of *B. oleae* is that both male and female secrete sexual pheromones (Canale et al., 2013).

The interplay between males and females is also due to bimolecular interactions. As behavior is a very flexible phenotype, the various aspects of –omics techniques, including genomic, transcriptomic, and proteomic, have been selected to identify genes or gene networks involved in the control of mating. Mating interactions represent the major arena within which many aspects of sexually antagonistic gene action may play out (Chapman et al., 1995). However, the first step is the acknowledgment of the physiology of the reproductive system of the insect of interest (both sexes).

1.6.2 Male reproductive system

In general, the male reproductive system contains a pair of testes and accessory glands which connect into a common large chamber. From this chamber starts a long ejaculatory duct which ends in the erecting and pumping organ (Hanna 1938).

1.6.2.1 Testes:

In insects, testes are the organs where sperm is produced. Each testis is subdivided into hundreds of follicles, the sperm productive cells. At the end of each follicle, there is a group of germ cells called spermatogonia that are divided by mitosis and increased in size to form spermatocytes.

Each spermatocyte undergoes meiosis: this yields four haploid spermatids which develop into mature spermatozoa as they progress further along through the follicle. A thin long duct leads the mature sperm away from each testis. Ducts are joined near the midline of the body. There, they form a single ejaculatory duct that leads out of the body through the male's copulatory organ, the aedeagus.

Sperm is of vital importance for fertilization of the egg. The presence of the sperm in the female reproductive system causes several post-mating effects on female flies. This function is called "sperm effect". The sperm effect is mediated by the binding of male accessory gland peptides (more details below) to the sperm cell flagella; sperm tails carry seminal proteins into the female sperm storage organs, where the gradual release of peptides from the sperm tails maintains the post-mating response.

Among insects, the testis transcriptome has been studied in detail in *D. melanogaster* (Andrews et al., 2000; Parisi et al., 2003; Mikhaylova et al., 2008), *B. mori* (Arunkumar et al., 2009), *A. gambiae* (Baker et al., 2011) and *C. capitata* (Scolari et al., 2012).

More recently, the first dataset on testis transcriptome for *B. oleae* was published (Sagri et al., 2014). Specifically, the *growth arrest-specific protein* 8 (*gas8*), *sex-determining protein* (*fem-1*) and *lost boy* (*lobo*) genes have been identified that are implicated in spermatogenesis and sperm motility.

1.6.2.2 Male Accessory Glands (MAGs):

1.6.2.2.1 Morphology

Male accessory glands are the secretory organs of the male reproductive system.

Specifically, *B. oleae* has two types of glands: the ectoderm- and the mesoderm-derived. The ectodermic glands have a spongy appearance and they are further distributed to one dorsal and two ventral pairs. They measure about 50 mm in diameter and 0.3–1.5 mm in length. The glands of the dorsal pair are long (1–1.5 mm) and generally branched at different levels along their length. On the ventral side, a pair of glands, 0.3–0.6 mm in length, usually branched, can be sometimes asymmetrically substituted on one side by a single 50 mm long lobation. The third pair of ectodermic glands is constituted of single or distally branched units, of variable length but generally longer with respect to the glands of the previous pair. The mesodermal glands are two sac-like structures 600–800 mm long and 80 mm in diameter. They enter the common chamber of the ejaculatory duct between the dorsal and the ventral pairs of ectodermic glands (Figure 1.10) (Marchini et al., 2006).

Male accessory glands produce and secrete an organic fluid that is transferred to females along with sperm, during mating. Male accessory gland secretions together with sperm are called seminal fluid or semen.

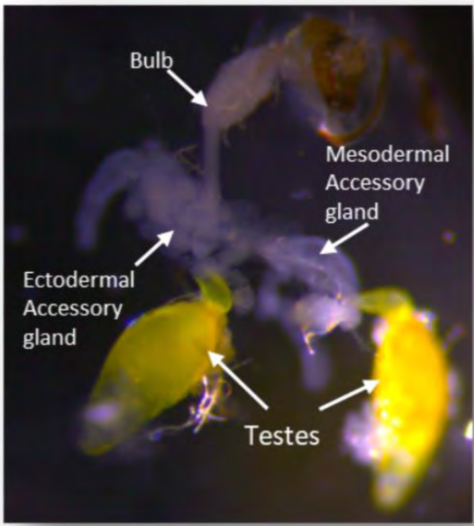


Figure 1.10: Male reproductive system of *B. oleae*.

1.6.2.2.2 Secretions of male accessory glands

In general, the secretions of male accessory glands represent numerous protein classes (Poiani et al., 2006) and they are multifunction, affecting both male and female insects.

In respect to the male insect, they have two major functions: 1. production of a liquid medium that sustains and nourishes mature sperm while they are in the male’s genital system and 2. production of proteins that encase sperm and protect them as they are delivered to the female’s body.

Through mating, they are transferred to females where they induce different behavioral and physiological effects on the female insect. Effects that have been identified in several insect species are: repression of sexual receptivity to further mating (Craig et al., 1967; Radhakrishnan and Taylor, 2007; Shutt et al., 2010; Abraham et al., 2011), egg-laying stimulation, increased feeding and sleeping activity, induction of immune responses and decreased longevity (Cavalloro and Delrio 1970; Delrio and Cavalloro 1979; Chen 1984; Jang 1995; Miyatake et al., 1999).

Due to their multifunction role, the secretions of the accessory glands are a matter of great interest and discussion. In recent years, with the aid of new technologies in sequencing and proteomic approaches, different proteins have been identified and analyzed. Generally, functional categories that are present in high levels in male accessory gland proteins are proteases, peptidases, serpins and protease inhibitors. Although the functional classes are conserved across species, the male accessory gland-expression of individual genes rarely is. Genes expressed in the accessory glands exhibit rapid evolutionary change and gene expansion (Begun et al., 2006) showing their critical role in encoding products that underlie striking, fitness-related phenotypes.

A various number of seminal fluid proteins have been identified in different *Drosophilidae*: 146 proteins for *D. melanogaster*, 125 proteins for *D. simulans* and 115 proteins for *D.* yakuba (Findlay et al., 2008; Findlay et al., 2009). The molecular and physiological functions of these substances have been most extensively investigated in *D. melanogaster* (McGraw et al., 2004; Ravi and Wolfner, 2007).

The *D. melanogaster's* male accessory glands are composed of proteins, carbohydrates, lipids and peptides with putative hormonal function (Gillot et al., 2003). From this category, a 36-amino-acid peptide, sex peptide or Acp70 has been well studied. This peptide is showed to be responsible for the inhibition of remating and increased egg production (Liu and Kubli, 2003; Chapman et al., 2003), decreased longevity, alteration of locomotion and feeding behaviors and stimulation of the immune system (Isaac et al., 2010).

Sex peptide is bound to the tails of sperm and transferred to females. After mating, it is detectable in the female's hemolymph (Pilpel et al., 2008) and reproductive tract. There, the active region of sperm-bound sex peptide is gradually cleaved from sperm, presumably freeing it to induce its long-term effects on the post-mating response (Peng et al., 2005). Specifically, sex peptide is detected by sensory neurons where it binds to a specific G-protein coupled receptor, called sex peptide receptor, leading to the alteration of female physiology and behavior (Yapici et al., 2008).

The female *D. melanogaster* flies that did not receive sex peptide during mating failed to release sperm efficiently (Avila et al., 2010). However, the removal of four other accessory gland proteins CG9997, CG1652, CG1656, G17575 that encode a serine protease, two C-type lectins and a cysteine-rich secretory protein respectively seemed to be required for the localization of the sex peptide to sperm showing the interaction between the molecules of the secretions (Ram et al., 2009). Another well studied accessory gland protein from *D. melanogaster* is Acp26Aa or ovulin. Acp26Aa is a regulator of ovulation and oviposition and it is transferred to females as a prohormone where it is processed by a seminal attacin-like protease (Chapman et al., 2008).

In the major malaria vector *A. gambiae,* 46 male accessory gland proteins have been identified. Twenty-five of them were orthologues of *D. melanogaster* accessory gland proteins with very low homology, ranging from 19-29% confirming the rapid evolution of reproductive genes (Dottorini et al., 2007). In spermless males, it has been shown that sperm is not required to induce oviposition or refractoriness to further mating (Thailayil et al., 2011). Interestingly, the switch for the post-mating effects on the female *A. gambiae* seems to be a steroid hormone named 20-hydroxyecdysone. The hormone 20- hydroxyecdysone is sexually transferred from the males and interacts with a female protein regulating oogenesis (Pondeville et al., 2008; Baldini et al., 2013) and loss of the female's susceptibility to further mating (Gabrieli et al.,2014; Mitchell et al 2015). Specifically, 20-hydroxyecdysone activates the transcription of vitellogenin (Vg) in the female fat body, an important ingredient of the growing oocytes.

In the major vector of dengue fever, *A. aegypti*, 63 putative proteins have been identified (Sirot et al., 2009). Most of the proteins identified fall into similar biochemical protein classes as male-derived reproductive proteins in other insects (Sirot et al., 2009). A partially purified protein, named matrone (Craig 1967) had a variety of effects on female reproductive and feeding behavior (Lee and Klowden 1999). Matrone seems to be the switch for a variety of effects on female reproductive and feeding behavior (Lee and Klowden 1999; Gillot, 2003.) Matrone is a 7.6 kDa peptide that apparently reduces female host-seeking behavior (Lee and Klowden, 1999).

Respectively, in *Tephritid* fruit flies, male accessory gland fluids (AGFs) have been shown to play a role in inhibiting remating, such as in *C. capitata, B. cucurbitae* and *B. tryoni* (Jang et al., 1999; Kuba and Itô 1993; Miyatake et al., 1999; Radhakrishnan and Taylor, 2007). A substance of sex peptide is strongly suggested to be responsible for suppression of female receptivity in *C. capitata* and *B. oleae* (Kuba and Itô 1993). However, isolation and electrophoresis of low molecular proteins from these insects did not detect any peptide with a mass compatible with that of the sex peptide from Drosophila (about 3 kDa) (Marchini et al., 2016).

The male accessory glands of *C. capitata* were previously studied by Hanna (1938) and Cavalloro and Delrio (1979). The secretions of these glands, when injected into virgin females, appear to influence their behavior and to increase the number of eggs laid as compared to typically mated females (Jang, 1995).

In *C. capitata,* two studies have identified genes expressed in the male accessory glands (Davies et al., 2006; Scolari et al., 2012). The identified transcripts were not homologs of genes encoding known accessory gland proteins in *D. melanogaster*, but they encoded proteins that fall into known functional categories (Davies et al., 2006).

More recently, through the whole genome sequencing of *C. capitata*, 459 genes were annotated and grouped into 17 functional classes based on Drosophila seminal fluids for both sexes (Papanikolaou et al., 2016). These accessory gland proteins consisted mainly of putative proteolysis regulators (proteases and protease inhibitors), lipid modifiers (lipases), sperm-binding candidates (Cysteine-RIch Secretory Proteins, CRISPs), antioxidants, carbohydrate-binding proteins (lectins), and many other small peptides and prohormones.

Another role of accessory gland proteins of *C. capitata* is to stimulate the recognition of the host fruit. During an experiment, unmated, laboratory-reared, virgin females chose the odor of male-produced pheromone over host fruit odor in a dual-choice flight tunnel bioassay. Mated females, on the other hand, chose the host fruit odor over the male-produced pheromone and deposited significantly greater amounts of fertile eggs if given the opportunity. However, virgin females injected with accessory gland fluid (AGF) from sexually mature males "switched" their response from choosing the pheromone odor to choosing host fruit odor in the flight tunnel bioassay and exhibited egg-laying behavior typical of mated females (Jang et al., 1995; Jang et al., 2002).

Despite the crucial role of male accessory gland components in regulating aspects of reproduction, elucidation of the seminal proteome of *Bactrocera* species has only been established in the last few years. Wei and his collaborators (2015) published the first proteome analysis of male accessory gland secretion in oriental fruit flies, *B. dorsalis*. Moreover, the detection of immune-related genes in male accessory gland was detected and studied (Lung et al., 2001; Belardinelli et al., 2005; Wong et al. 2008). In 2017, through comparative transcriptome analysis of three *B. dorsalis* (Diptera: Tephritidae) organs, functional genes in the male accessory glands and ejaculatory duct were identified (Tian et al. 2017).

1.6.3 Female reproductive system

The female reproductive system consists of a pair of ovaries. Each ovary empties the mature oocytes to the lateral oviduct which unite to form the uterus. The opening of the uterus is called gonopore and opens into the genital chamber. Two types of ectodermal glands open into the genital chamber. The first is the spermathecal and the second is the accessory glands of the female system (Figure 1.11).

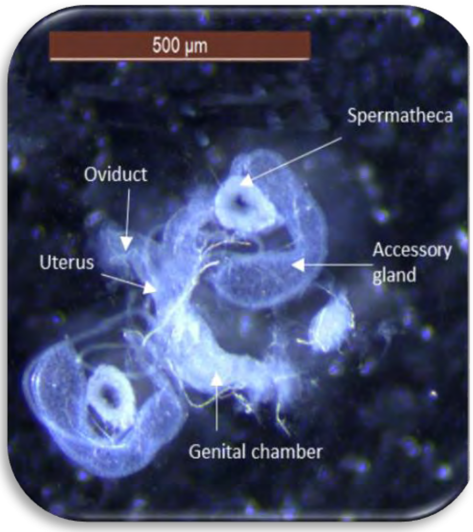


Figure 1.11: Female reproductive system of *B. oleae*.

1.6.3.1 Ovaries

The ovaries are the organs that produce egg cells. Each ovary is composed of a cluster of egg or ovarian tubes, the ovarioles. Each ovariole consists of a terminal filament, a germarium, a vitellarium and a pedicel. Germarium is the counterpart of spermatogonia in the male reproductive system. Through mitosis, they give rise to oocytes. Oocytes grow by deposition of yolk, in a process known as vitellogenesis, as they go through the vitellarium to the pedicel. The youngest oocytes occur near the germarium and the most mature near the pedicel. The six-stage ovarian development of *B. oleae* was well reported by Fletcher et al (1978) and includes previtellogenesis (1-2 stages), vitellogenesis (3-4 stages), gravid (5 stage) and parous (6 stage).

1.6.3.2 Spermathecae

Spermathecae are tubes or sacs in which sperm can be stored between the time of mating and the time of egg fertilization. The number of spermathecae varies among insects. *Anopheles* mosquitoes have a single spermatheca while Drosophila, Aedes, and Culex have three spermathecae (Clements et al., 1992). Female *B. oleae* has two spermathecae (Figure 1.11). In general, each spermatheca is composed of the duct, reservoir, muscular pump and spermathecal gland.

After copulation, sperm migrates from the genital chamber of the female reproductive tract, through the spermathecal duct, and into the reservoir (Tombes and Roppel., 1972; Bailey and Nuhardiyati., 2005; Oppelt and Heinze, 2007). When an egg is released, the sperm retraces this route and fertilize it (Lefevre and Jonsson., 1962; Tombes and Roppel., 1972; reviewed by Chapman., 2013).

Spermatheca plays the role of the “bodyguard” for the sperm. Inside it, the sperm is protected from mechanical damage, contact with the female hemolymph, and putatively from free radicals such as reactive oxygen species (Collins et al., 2004; Al-Lawati et al., 2009; King et al., 2011).

The sperm constantly moves inside the spermatheca, swirling within the spermathecal lumen, which organizes and stores them for release at the appropriate time during fertilization (Jones, 1973; Werner et al., 2007; Dallai et al., 2014). Sperm cells are nourished and preferentially selected, beginning when the sperm cells migrate to the spermathecal reservoir and continuing until fertilization occurs (Ward, 2000; Franck et al., 2002; Bloch et al., 2003). Thus, the storage of sperm creates an opportunity for post-copulatory sperm competition and female cryptic choice of specific sperm cells for fertilization, altering the genetic background of offspring (Klowden and Chambers, 2004).

Spermathecae have been extensively analyzed in *Drosophilidae* family. The male accessory gland proteins of *D. melanogaster* that are transferred to female after mating have a short lifespan, they are degraded 7 hours after mating (Wolfner et al., 2011). Thereafter, spermatheca appears to sustain the sperm with its own glandular secretions (Schnakenberg et al., 2012). The genes expressed in the spermatheca of mated *D. simulans* illustrate the intricate structure and function of the spermatheca. Eleven genes have been shown to encode serine proteases, protein carriers, or antimicrobial and energy metabolism-related proteins. These genes are associated with processes that establish an environment suitable for the allocation and nutrition of sperm within the spermatheca (Prokupec al., 2008).

In *A. gambiae,* surgical removal of the spermatheca from mated females resulted in inhibition of oviposition while implantation of a mated spermatheca into virgin females did not stimulate egg laying or loss of sexual receptivity (Klowden et al., 2001; Klowden et al., 2006).

1.6.3.3 Secretions of female accessory glands

The female accessory glands produce a secretory material that serves a number of functions: acting as a lubricant for egg passage, as protective oothecal coverings, or as glues to attach eggs to various substrates (Soltani et al., 1987). In addition, they also produce the gelatinous sheaths around the eggs and nutrition (Adiyodi and Adiyodi, 1988; Romoser and Stoffolano, 1988). The female accessory glands of the sandfly *P. papatasi* secrete a protein with lipase and antibacterial activities (Rosetto et al., 2003). Female accessory glands of the house fly *M. domestica* produce secretions that induce contraction of the oviducts during oviposition (Wagner et al.,1993) and contribute to the fertilization process, modifying the micropyle cap of the eggs and taking part in the acrosomal reaction (Degrugillier, 1985).

In *Ceratitis* genus, immunity genes have been identified to be present in the female accessory glands. Specifically, the antimicrobial ceratotoxin peptide family (seven genes) was found to protect the reproductive tract from the bacterial infection during mating. These peptides have also been found on the surface of oviposited eggs where they may create a microbiologically controlled environment that favors early larval development (Marchini et al., 1991; Marchini et al., 1997; Marchini et al., 2002). Unlike most insect antibacterial peptides, ceratotoxins are not induced by bacterial infection, but they are expressed in the female reproductive accessory glands of adult insects (Marchini et al., 1995; Rosetto et al., 1996) in response to juvenile hormone stimulation (Manetti et al., 1997). Expression of immunity genes was also found in the transcriptomic analysis of the female accessory glands and spermathecae of virgin *B. oleae*, too. (Sagri et al., 2014).

1.6.3.4 Post mating response in female fly

Genome-wide research into the post-mating response in females has been performed in model species such as *D. melanogaster* (Lawniczak et al., 2004; Mack et al., 2006), the honeybee queen *A. mellifera* (Kocher et al., 2008; Kocher et al., 2009; Manfredini et al., 2015), *C. capitata* (Gomulski et al., 2012), *A. gambiae* (Rogers et al., 2009) and *A. aegypti* (Alfonso- Parra et al., 2016) which have revealed that the post-mating response differs among species as well as the time following mating.

To obtain a full coverage of the transcriptome changes during mating for *D. melanogaster,* McGraw et al., (2004) compared virgin females to females mated with 1. Wild-type males, 2. Mutant males who lack sperm and 3. Mutant males who lack accessory gland proteins (McGraw et al., 2004). About 13% of the genome (1500 genes) changed expression in response to mating. Only 160 loci were influenced by male accessory gland proteins, 500 genes by sperm and the rest (~1000 genes) by other aspects like courtship and copulation. This possibly suggests that female counter responses were detected rather than male manipulation effects. For this reason, McGraw et al examined the time course of these changes over 24 hours. They noticed that as the time increases, the number of genes showing changes decrease.

In 2006, Mack et al., focused on gene and proteomic changes in the lower female reproductive tract of *D. melanogaster*, where gene expression associated with sperm storage and sperm competition might be expected to be concentrated. For this reason, they compared virgin females to females after specific time points after mating: immediately after mating and 3, 6 and 24 hours after mating. At around 6 hours after mating, there was a pronounced peak (539 genes) in the number of differentially expressed RNAs after mating. After that time point, most of the seminal fluid proteins had a low or undetectable level of expression (Peng et al., 2005; Ravi et al., 2005). These results suggest that there is a two-stage response to mating, from early male-induced changes to longer-term sperm storage and female reproduction effects.

In *D. melanogaster*, post-copulatory effects in gene expression are generally of small-scale (<2fold) (Lawniczak et al., 2004; Mack et al., 2006; McGraw et al., 2008) except one functional class that shows a consistently strong response to mating. This functional class is the immune genes, in particular, antimicrobial peptides that are highly induced (McGraw et al., 2008; McGraw et al., 2004; Domanitskaya et al., 2007; Peng et al., 2005). Unlike Drosophila, *A. gambiae* females undergo prominent transcriptional changes after mating (Rogers et al., 2008). The number of genes differentially expressed in mated females increased in time between 2 hours, 6 hours and 24 hours after mating. The majority of the genes were found to be expressed exclusively in a single tissue, mostly on the lower reproductive system (reproductive tract and spermatheca) of the female insect with no expression on the ovaries (Rogers et al., 2008). Surprisingly, some of the mating response genes were expressed primarily or exclusively in the gut. The gut is an important endocrine tissue, releasing peptides similar to the gut-brain hormones of vertebrates that are crucial modulators of reproductive physiology (Zitnan et al., 1993).

Moreover, a comparison between mated and virgin female *A. gambiae* insects at 3, 12, and 24 hours after mating identified 708 genes induced and 412 genes repressed by mating across the different time points. Most of these genes are involved in metabolic processes (Gabrielli et al., 2014).

Recently, the post-mating response of *A. aegypti* was investigated (Parra et al., 2016). Investigators found that 280 genes in the female reproductive tract are affected by mating. The nature of the predicted products of many of these genes suggested roles in priming the reproductive tract for egg development, protecting the female against bacterial infections or processing the blood meal.

Transcriptional changes in several genes that may be involved in female sexual maturity and mating were also identified in *C. capitata based* on microarray data (Gomulski et al., 2012). However, the cDNA library of *C. capitata* was limited to the head of the female.

In *B. dorsalis* differentially expressed genes between mated and mature virgin females were identified by Zheng et al., (2016). Out of 83 transcripts that displayed significant transcriptional changes between virgin mature and mated females 24 hours post-mating, 65 (78 %) were more abundant in mated females, while only 18 (22 %) were more abundant in virgin mature females. During mating, statistically significant differences among enriched biological process GO terms included those involved in the response to a stimulus, as well as immune system, developmental, cellular, biological regulation, and metabolic processes. Of these transcripts, those that are related to the response to stimulus and immune system process were the largest group (Zheng et al., 2016).

1.7 Scope

The most important insect pest of the olive tree is the olive fruit fly*, B. oleae.* Currently control methods of the insect rely on chemical insecticides. However, the development of insecticide resistance and the negative impact of chemical control in nature are calling for the development of novel tools.

Alternative methods of pest control may be based on the manipulation of the reproductive system of an insect, since this system is responsible for its reproductive success. The identification and functional characterization of the molecular pathways regulating the female post-mating biology could help the identification of target-genes. To accomplish this, a good knowledge of the olive fly's reproductive system is required. Until today, this information is limited.

To fill this gap of knowledge the goal of this thesis was to perform genomic and transcriptomic analysis of the male and female reproductive system of the olive fly in virgin and mated flies, focusing on the identification of genes implicated in the reproductive activity of the insect. We further analyzed selected genes and performed functional analysis through RNAi silencing. To distinguish whether transient silencing of the selected genes had an impact on reproduction we performed mating experiments and recorded their oviposition rate and longevity.

Apart from advancing the knowledge on insect reproduction, we also opted for generating new tools that could ultimately be used to interfere with the fly's reproductive capacity and thus help at its population control.

2.MATERIALS-METHODS

2. Materials and methods

2.1 Fly culture and stock

The laboratory strain of the olive fly is part of the original stock from the Department of Biology, "Democritus" Nuclear Research Centre, Athens, Greece, and has been reared in our laboratory for over 20 years. The flies are reared at 25°C with a 12h light/12h dark photoperiod and humidity 65% as described by Tzanakakis 1989.

2.1.1 Adult rearing

The adult flies are reared in laboratory cages with diameter 30*30*30cm^3 with wax cones inside for oviposition. Three sides of the cages are covered with netting to allow air flow. The top parts have one hole (diameter of 8 cm) to put the wax cones (8 cm diameter × 30 cm height) in the cages. Each cone is covered with a plexi glass lid, on which a piece of sponge is placed glued to maintain humidity. Inside the cage there is a petri-plate for the adult food which contains sugar, hydrolyzed yeast, and egg yolk in a ratio of 8:2:0.6 (Economopoulos and Tzanakakis, 1967; Tsitsipis and Kontos, 1983) and a water supplier, a small plastic bottle of water with a sponge on the edge (Tzanakakis 1989).

2.1.2 Egg collection

Adult insects are sexually mature five days after their emergence. However, high rate of oviposition is observed after the first week. To collect the eggs, the cones are rinsed interiorly with distilled water (dH_2O) and collected in a petri dish at the bottom part of the oviposition cones.

The collected eggs are placed on a white filter paper soaked in 0.3% propionic acid (Manoukas and Mazomenos 1977) for 48-72 hours in a high humidity rate (90%). Propionic acid is important for the preservation of the eggs.

2.1.3 Larval rearing

After the egg hatching, larvae are transferred to small plastic trays with food until their pupation (Tzanakakis., 1989). The larval diet is based on a diet described by (Tsitsipis, 1975). For 1 kg of larval diet the following ingredients are used: tap water (550 mL), extra virgin olive oil (20 mL), Tween® 80 emulsifier (7.5 mL), potassium sorbate (0.5 g), Nipagin® (2 g), sugar (20 g), brewer's yeast (75 g), soy hydrolysate (30 g), hydrochloric acid 2N (30 mL) and cellulose powder (275 g). The critical issue in this stage is the overcrowding of the larvae in the food trays. No more than 20 larvae should be placed in each tray as overcrowding can cause competition phenomenon. This stage lasts 8-10 days.

2.1.4 Pupal collection

Pupae are collected from the small plastic trays in a Petri plate and placed in an adult holding cages for adult emergence (Tzanakakis 1989). The newly emerge flies flag the new generation of the laboratory strain. Each generation lasts one month.

2.2 Nucleic acid isolation

2.2.1 DNA isolation

The DNA isolation is accomplished with The Wizard® Genomic DNA Purification Kit according to the manufacture's guideline.

The procedure is based on a three-step process (Chomczynski et al., 1992). The first step is the lyses of the cells and the nuclei from the Nuclei Lysis Solution. The second step is the remove of the cellular proteins by salt precipitation, which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by 2-propanol precipitation.

DNA purified with this system is suitable for a variety of applications, including amplification, digestion with restriction endonucleases and membrane hybridizations (e.g., Southern and dot/slot blots).

Method:

1. Homogenize the tissue using a pestle in 600µl of Nuclei Lysis Solution, and vortex 1–3 seconds to wet the tissue.
2. Incubate at 65°C for 15 minutes.
3. Add 3µl of RNase Solution to the cell lysate, and mix the sample by inverting the tube 2–5 times. Incubate the mixture at 37°C for 15 minutes. Allow the sample to cool to room temperature for 5 minutes before proceeding.
4. Add 200µl of Protein Precipitation Solution, and vortex vigorously at high speed for 20 seconds.
5. Centrifuge for 3 minutes at 13,000–16,000 × g. The precipitated proteins will form a tight pellet.
6. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5ml micro centrifuge tube containing 600µl of room temperature 2-propanol.

** Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein***

7. Gently mix the solution by inversion until thread-like strands of DNA form a visible mass.
8. Centrifuge at 13,000–16,000 × g for 1 minute at room temperature.
9. Carefully decant the supernatant. Add 600µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA. Centrifuge at 13,000–16,000 × g for 1 minute at room temperature.
10. Carefully aspirate the ethanol using a pipette. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into the pipette.
11. Invert the tube onto clean absorbent paper and air-dry the pellet for 15 minutes.
12. Add 100µl of DNA Rehydration Solution and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.
13. Store the DNA at 2–8°C.

Materials:

• 1.5ml micro centrifuge tubes • micro centrifuge tube pestle or mortar and pestle • RNase solution • water bath, 65°C • water bath, 37°C • 2-propanol, room temperature • 70% ethanol, room temperature • Nuclei Lysis Solution • Protein Precipitation Solution • DNA Rehydration Solution

2.2.2 RNA isolation

For the isolation of RNA, a quick and convenient method is used, based on the TRI Reagent® material (Sigma- Aldrich) following the instructions of the manufacturer with minor modifications.

This procedure is an improvement of the single-step method reported by Chomczynski and Sacchi (1987) for total RNA isolation. TRI Reagent® performs well with large or small amounts of tissue.

The reagent is a mixture of guanidine thiocyanate and phenol in a monophasic solution that can dissolve DNA, RNA, and protein from a homogenized sample of tissue. After adding chloroform or 1-bromo 3-chloropropane (BCP) and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases. One ml of TRI Reagent® is sufficient to isolate RNA, DNA, and protein from 50–100 mg of tissue. The resulting RNA is intact with little or no contaminating DNA and protein.

Method:

1. Homogenize the tissue samples in TRI Reagent® (1 ml per 50–100 mg of tissue).
2. Allow samples to stand for 5 minutes at room temperature for complete dissociation of nucleoprotein complexes. Centrifuge the resulting mixture at 12,000x g for 15 minutes at 4 °C and transfer aqueous phase.
3. Add 0.1 ml of 1-bromo-3-chloropropane (BCP) or 0.2 ml of chloroform per ml of TRI Reagent® used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 2–15 minutes at room temperature.
4. Centrifuge the resulting mixture at 12,000x g for 15 minutes at 4°C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).
5. Transfer the aqueous phase to a fresh tube and add 0.5 ml of 2-propanol per ml of TRI Reagent used in Sample Preparation (step 1) and mix. Allow the sample to stand for 5–10 minutes at room temperature.
6. Centrifuge at 12,000x g for 10 minutes at 4 °C. The RNA precipitate will form a pellet on the side and bottom of the tube.

Store the interphase and organic phase at 4 °C for subsequent isolation of the DNA and proteins

7. Remove the supernatant and wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent® used in Sample Preparation, step 1. Vortex the sample and then centrifuge at 7,500x g for 5 minutes at 4 °C.
8. Briefly dry the RNA pellet for 5–10 minutes by air drying or under a vacuum.
9. Add an appropriate volume of ddH_20 solution to the RNA pellet.

To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60 °C for 10–15 minutes

Materials:

• TRI Reagent® •1-bromo-3-chloropropane (BCP) • chloroform •1.5ml micro centrifuge tubes • micro centrifuge tube pestle • water bath, 65°C • water bath, 37°C • 2-propanol, room temperature • 70% ethanol, room temperature • chloroform or 1-Bromo-3-chloropropane • 2-Propanol • 75% Ethanol • 1 mM sodium phosphate, pH 8.2 • 0.5% SDS solution • ddH_2O

2.2.3 Plasmid isolation

The plasmid isolation is performed through alkaline lysis in combination with the detergent SDS (Birnhoim and Doly, 1979).

Exposure of bacteria suspension to the strongly anionic detergent at high pH opens the cell wall, denatures chromosomal DNA and proteins, and releases plasmid DNA into the supernatant. Although the alkaline solution completely disrupts base pairing, the strands of close circular plasmid DNA are unable to separate from each other because they are topologically intertwined. During lysis, bacteria proteins, broken cell wells and denatured chromosomal DNA become enmeshed in large complexes that are efficiently precipitated from solution when sodium ions are replaces by potassium ions (Ish-Horowicz and Burke, 1981). The native plasmid DNA can be recovered from the supernatant after the remove of the denatured material by centrifugation.

Method:

1. Inoculate 2ml of LB Broth containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37^{o}C with vigorous shaking.
2. Pour 1.5 ml of the culture into a micro centrifuge tubes. Centrifuge at 12,000x g for 30 seconds at 4 ^{o}C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 100μl of ice-cold GET (Solution I) by vigorous vortexing. Tore the tube in room temperature for 5 minutes.
5. Add 200μl of freshly prepared Alkali (Solution II) to the bacteria suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. Store the tube in ice for 7-8 minutes.
6. Add 150μl of ice-cold CH_3COOK (Solution III). Close the tube and invert it several times so that the lysis from the previous step will stop. Store the tube in ice for 3-5 minutes.
7. Centrifuge the bacteria lysate at 12,000x g for 5 minutes at 4^{o}C in a microfuge. Transfer the supernatant to a fresh tube.
8. Precipitate nucleic acids from the supernatant by adding 1 volume of 2-propanol 100%. Mix the solution by vortexing and then allow the mixture to stand for 15 minutes in room temperature.
9. Collect the precipitated nucleic acids by centrifugation at 12,000x g for 5 minutes at 4^{o}C in a microfuge.
10. Remove the supernatant by gentle aspiration.
11. Add 0.5 volume of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at 12,000x g for 2 minutes at 4^{o}C in a microfuge.
12. Again remove all the supernatant by gentle aspiration as the pellet sometimes does not adhere tightly to the tube.
13. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has

evaporated and no fluid is visible in the tube.

14. Dissolve the nucleic acids in 50µl of TE (pH 8.0) containing 20µg/ml DNAse-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds. Store the DNA solution at -20°C.

Materials:

• GET (Solution I): 1.5ml glucose 50mM, 25mM TrisCl (pH 8.0), 10mM EDTA (pH 8.0) • Alkali (solution II): 0,2N NaOH, 1% SDS • CH3COOK (solution III): 5M potassium acetate, glacial acetic acid• water bath, 65°C • micro centrifuge tubes • 2-propanol, room temperature • 70% ethanol, room temperature • RNase A (pancreatic RNase) • LB Broth •0.5% SDS solution • TE buffer (Tis HCl pH 8.0, EDTA pH 8.0)

2.3. Phenol: chloroform extraction

A widely used method to isolate RNA, DNA or proteins is the phenol-chloroform extraction. It is a method that separates mixtures of molecules based on the differential solubilities of the individual molecules in two different immiscible liquids (Stenesh et al., 1979).

The extraction of nucleic acids involves adding an equal volume of phenol-chloroform to an aqueous solution of lysed cells or homogenized tissue, mixing the two phases, and allowing the phases to separate by centrifugation. Chloroform mixed with phenol is more efficient at denaturing proteins than either reagent is alone. The phenol-chloroform combination reduces the partitioning of poly(A)+ mRNA into the organic phase and reduces the formation of insoluble RNA protein complexes at the interphase.

Method:

1. Add one volume of phenol: chloroform (1:1) to your sample, and vortex or shake by hand thoroughly for approximately 20 seconds.
2. Centrifuge at room temperature for 5 minutes at 16,000x g. Carefully remove the upper aqueous phase to a fresh tube.
3. Add one volume of chloroform, vortex and centrifuge at room temperature for 5 minutes at 16,000× g.
4. Repeat the step 3.
5. Transfer the aqueous phase in a fresh tube and perform ethanol precipitation.

2.4 Ethanol precipitation

Ethanol precipitation is a widely used technique to purify or concentrate nucleic acids. This is accomplished by adding salt and ethanol to a solution containing DNA or RNA (Sambrook et al., 1989). In the presence of salt (in particular, monovalent cations such as sodium ions (Na^+)), ethanol efficiently precipitates nucleic acids. The purified precipitate can be collected by centrifugation, and then suspended in a volume of choice.

Method:

1. Add 1X volume of 2-propanol and 3M of CH_3COONa in the DNA sample and mix well.
2. Incubate at room temperature for 15 minutes.
3. Centrifuge for 20 minutes at 14,000x g.

4. Decant supernatant carefully without disturbing the pellet.
5. Wash by adding 0,5X volume of 70% ethanol and vortex 3 times.
6. Centrifuge for 5 minutes at 14,000x g.
7. Remove the residual ethanol and air dry the pellet. Re-suspend in appropriate volume of ddH_2O or TE buffer.

Materials:
• 2- propanol • CH_3COONa (pH 5.2, 3.0 M) • Ethanol 70% • ddH_2O or TE buffer

2.5 Cloning into Plasmid Vector

Cloning of double-stranded DNA molecules into plasmid vector is one of the most commonly employed techniques in molecular biology. The procedure is used for sequencing, building libraries of DNA molecules, expressing coding and non-coding RNA, and many other applications.

The procedure involves ligating dsDNA into a plasmid. If both the insert and linearized plasmid have no overhanging bases at their termini, then then it is called "blunt-end". If the insert and the plasmid have complementary ends its called "cohesive".

There are three critical steps for the successful cloning. First, the preparation of the selected vector. Second, the designing of the insert. The blunt-ended insert needs to be phosphorylated. When the ends of the insert are not blunt, a polishing or filling reaction is required. Third, the ligation conditions. In blunt-end ligations, the association of 5' phosphate groups and 3' hydroxyl groups are more transient than in cohesive-end ligations. Because they lack the hydrogen bond stabilization of cohesive ends, blunt-end ligations are more sensitive to reaction conditions, especially to the concentrations of the reaction components. T4 ligase quality and concentration are also important. Blunt-end ligations typically take place in the presence of higher concentrations of ligase than cohesive-end ligations.

2.5.1 Preparation of the cloning vector

Cloning vectors have three common properties: 1. A selectable marker, which is almost always antibiotic resistant; 2. An origin of DNA replication to allow vector propagation and 3. A MCS or polylinker that contains a number of restriction sites to clone foreign DNA.

The first step of the preparation of the cloning vector includes the digestion of the vector by the restriction enzymes. Restriction enzymes are proteins that cut DNA at specific recognition sites. There are two types of restriction enzymes based on the way they cut the target DNA: the blunt-end cutters which cut both strand of the target DNA at the same spot creating blunt ends and the sticky end cutters that cut both strand of the target DNA at different spots creating 3'- or 5'- overhangs of 1 to 4 nucleotides (sticky-ends).

The second step is the dephosphorylation of the ends using alkaline phosphatase. The third step is the purification of the digested vector by agarose electrophoresis to remove residual nicked and supercoiled vector DNA and the small piece of DNA that was cut out by the digestions. This usually reduces strongly the background of non-recombinants due to the very efficient transformation of undigested vector.

2.5.1.1 Digestion of the cloning vector

Method:

1. Mix the following ingredients in an eppendorf tube: the appropriate amount of DNA plasmid, 1x restriction enzyme buffer, ddH_2O until the final volume of the reaction. Last step is to add the appropriate units of enzyme.

*** One unit is defined as the amount of enzyme required to digest 1 µg of λ DNA in 1 hour at 25°C in a total reaction volume of 50 µl.***

2. Mix gently by tapping the tube or pipetting the solution up and down and incubate the reaction at the suitable temperature for the activity of the enzyme.

*** Different enzyme have different temperatures and incubation times. Information is given by the manufacturer. ***

3. To terminate the reaction, add 0.5µl EDTA that deactivates the enzyme.
4. For complete deactivation of the enzyme, incubate the reaction to high temperatures based on the manufactures of the enzyme guidelines.
5. To separate the digested vector from the small DNA fragment that was removed by the digestion, the entire sample is purified using preparative agarose gel electrophoresis (see 2.9).

Materials:

• 10x restriction enzyme buffer • DNA plasmid • water bath, 65°C • water bath, 37°C • 2-propanol, room temperature • 70% ethanol, room temperature • Restriction enzyme • EDTA (0.5M)

2.5.1.2 Dephosphorylation of the digested cloning vector

If the vector is cut with a single restriction enzyme, chances are much higher that the vector relegates back on itself rather than on an added DNA fragment. This results in a high fraction of "empty clones", or background. This is characteristic for the blunt end ligations.

For this reason, a step of dephosphorylation is achieved with the use of alkaline phosphatase. These enzymes dephosphorylate the DNA termini of the digested vectors so they cannot be ligated by DNA ligase.

Method:

1. Mix gently the digested plasmids with 1X CIAP buffer, 1-unit alkaline phosphatase and add ddH_2O to final volume of the reaction.
2. Incubate the mixture for 20 minutes at 37°C.
3. Add 1 unit of alkaline phosphatase and incubate the mixture for another 20 minutes.
4. Extract the dephosphorylated plasmid through phenol: chloroform (see 2.3) and precipitate it with ethanol precipitation (see 2.4).
5. Dissolve the nucleic acids in the appropriate amount of ddH_2O for concentration of 50 ng/µl.

Materials:

• CIAP buffer • water bath, 65°C • water bath, 37°C • 2-propanol, room temperature • 70% ethanol, room temperature •phenol •chloroform

2.5.1.3 Addition of deoxythymidine (T) residues to the vector

PCR products are usually amplified using a Taq DNA polymerase which adds a single deoxyadenosine to the 3' end of the product. If you add deoxythymidine (T) residues to the linearized vectors, the insert will ligate into the vector efficiently as they have complementary ends.

Method:

1. To a linear plasmid vector (20ul) add: 2mM dTTPs, 1x Taq Buffer, 1.5mM $MgCl_2$, 2 units Taq polymerase and ddH_2O until the final volume of the reaction (50μl).
2. Incubate the mixture at 72^{o}C for 2.5hours.
3. Extract the dephosphorylated plasmid through phenol: chloroform (see 2.3) and precipitate it with ethanol precipitation (see 2.4).
4. Dissolve the nucleic acids in the appropriate amount of ddH_2O for concentration of 50 ng/μl.

Materials:

• linear plasmid vector • dTTPs • 10x Taq Buffer •$MgCl_2$ • Taq polymerase • water bath, 37°C • 2-propanol, room temperature • 70% ethanol, room temperature •phenol •chloroform

2.5.2 Ligation

On this step the insert DNA (gene or fragment of interest) is connected to the compatibly digested plasmid vector. This is performed by the T4 DNA ligase enzyme. The DNA ligase catalyzes the formation of covalent phosphodiester linkages which permanently joins the nucleotides together. After ligation, the insert DNA is physically attached to the vector and the complete plasmid can be transformed into bacterial cells for propagation.

Before setting up the ligation reaction itself, it is important to determine the amount of insert and plasmid vector to use for the ligation reaction. The volume of vector DNA and insert DNA used in the ligation vary depending on the size of each and their concentration. For most standard cloning (where the insert is smaller than the vector) a 3 insert: 1 vector ratio is used.

The formula to calculate the appropriate amount of the ingredients is:

$$\text{ngDNA}= \frac{\text{ng plasmid x bp DNA x ratio}}{\text{bp plasmid}}$$

Method:

1. Add in an eppendorf type tube the following ingredients: 50ng of the plasmid vector, 1x ligase buffer, 1 unit of T4 ligase and ddH_2O until the final volume (20μl).

Add 5units of enzyme and 2μl of 50% PEG if the reaction is for blunt-end ligation

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. Incubate at 16°C overnight and 1 hour at 22°C.
4. To inactivate the reaction, incubate for 10 minutes at 65°C.
5. Save the recombinant plasmin in -20^{o}C.

Materials:

• plasmid vector • 10x T4 Ligase Buffer •$MgCl_2$ • T4 Ligase enzyme

2.5.3 Preparation and transformation of competent E. coli

The recombinant vector gets into *E. coli* cells by a process called transformation. The *E. coli* cells have to be made competent to take up the circular vector. The method for the preparation of competent cells depends on the transformation method used.

2.5.3.1 Electro-competent cells

Electro-competent cells are used for when the transformation is achieved through the exposure to electrical charge. The change of the electrical charge destabilizes the membranes of E. coli and forms transient membrane pores through which DNA molecules can pass (Neumann et al., 1982).

This method is called electroporation and it is the easiest, fastest, most efficient and most reproducible method for transformation of bacteria cells with DNA. Various parameters can be optimized like strength of the electrical field, the length of the electrical pulse and the concentration of DNA to achieve a transformation efficiency of 1010 transformant/μg of DNA.

2.5.3.1.1 Preparation of electro-competent cells

Method:

1. Inoculate 1 colony from a fresh plate of the strain to be made electro competent into 10 ml of LB-Broth in a 125 ml flask and incubate for 16-18 hours at 37^{o}C and 250x rpm.

For the LB Broth Medium stir to suspend 20g powder in 1L water. Autoclave for 15 minutes at 121C to sterilize. Allow to cool before making additions, such as antibiotics

2. Have ready 2, 1 L flasks containing 250 ml each of LB-Broth pre-warmed to 37°C. Add two drops of the overnight culture to each of the flasks.
3. Shake at 37°C and 250x rpm until the cultures reach an OD600 of 0.5-0.7. Be sure to turn on centrifuge and cool rotor to 4°C well in advance of harvesting cells. Be sure to place 1 L of 10% glycerol on ice well in advance of harvesting cells.
4. Place cultures on ice for 15 minutes. From this point on the cultures must be kept ice cold. Pour each 250 ml culture into chilled 500 ml (or 1000 ml) centrifuge bottles.
5. Centrifuge at 5000x rpm for 10 min. Pour off the supernatant and aspirate any residual broth.
6. Add 250 ml of glycerol to each of the centrifuge bottles and completely suspend the cells by pipetting up and down.
7. Centrifuge at 5000x rpm for 10 min. Pour off the supernatant, it is not necessary to aspirate. Completely suspend the cells in 250 ml glycerol and re-centrifuge.
8. Pour off the supernatant and suspend the cells in the residual glycerol by pipetting up and down.
9. At this point you can electroporate or freeze the cells away. To freeze, add 100 microliters of the culture to micro centrifuge tubes on ice. Once you have used all of the culture, transfer the tubes to dry ice for 10 minutes. Once the cultures are frozen, transfer them to a -80°C freezer.

2.5.3.1.2 Electroporation

The electroporation method was first used for the introduction of DNA in eukaryotic cells (Neuman et al., 1982).

Method:

1. Thaw amount of the competent *E. coli* cells on ice for 10 minutes.
2. Turn on electroporator and set to 13.8 kv for 5-6m second.
3. Place recovery SOC in 37°C water bath.
4. Pre-warm LB-antibiotic plates at 37°C.
5. Place appropriate number of micro centrifuge tubes and 1 mm-electroporation cuvettes on ice.
6. Add 1 μl of a 10 pg/μl DNA solution to the cells in the micro centrifuge tube. Incubate for 1 minute.
7. Transfer the DNA-cell mixture to the cold cuvette, wipe water from exterior of cuvette and place in the electroporation module and press pulse.
8. Immediately add 1000 μl of 37°C SOC, mix by pipetting up and down once and transfer to a 15 ml-falcon tube.
9. Rotate in the 37°C incubator for 1 h.
10. Make appropriate dilutions and overlay petri plates (90mm) that contain LB agar with the appropriate antibiotics that the recombinant plasmid has resistance (e.g. ampicillin). Incubate the plates overnight at 37°C.

*** If you will perform blue-white selection add 30μl X-gal and 3 μl IPTG before the overlay of the cells. ***

Materials:

• S.O.C medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose • LB Broth (10 g/L Tryptone, 5 g/L Yeast Extract 5 g/L NaCl) • 10% glycerol • DNA solution • antibiotic

2.5.3.2 Chemically competent cells

The entrance of nucleic acids to the bacteria cells is also accomplished by chemical method. The chemical method includes the wash of the *E. coli* cell in cocktails of simple salt solutions to achieve a state of competence during which DNA molecules may be admitted to the cell. Most of the chemical methods currently used for bacteria transformation are based on the observations of Mendel and Higa (1970), who showed that bacteria treated with ice-cold solutions of $CaCl_2$, and then briefly heated to 37^{o}C or 42^{o}C could be transfected with bacteriophage λ DNA.

The method described is a transformation protocol described by Cohen et al., 1972 and yields competent cells that generate 10^6 to 10^7 transformed colonies/μg of supercoiled plasmid DNA.

2.5.3.2.1 Preparation of chemical competent cells

Method:

Inoculate 3 ml LB medium with the appropriate *E. coli* strain and incubate the culture overnight at 37°C at 210x rpm.

*** For the RNAi feeding assay we used the *E. coli* HT115 (DH3) strain. ***

2. Add the overnight culture to 500 ml LB-Broth medium and incubate the culture at until the absorbance at 600 nm was approx. 0.5 (between 0.4 and 0.6).
3. Chill the culture for at least 10 min on ice.
4. Centrifuge the cell suspension for 10 min at 4,500 rpm at 4°C.
5. Gently resuspend the pellet in 0.5x volume of the started material in $CaCl_2$ 50Mm.
6. Incubate the cell suspension on ice for 30 min.
7. Centrifuge for 10 min at 4000 rpm at 4°C.
8. Gently resuspend the pellet in 0.1x volume of the started material in $CaCl_2$ and add 1.4 ml glycerol 10%.
9. Incubate the cell suspension on wet ice for at least 10 min.
10. Aliquot the cell suspension at 600 μl per tube glycerol 10% and store the tubes at -80°C.

2.5.3.2.2. Chemical transformation

Method:

1. Thaw amount of the competent *E. coli* cells on ice.
2. Add 1 μl of a 10 pg/μl DNA solution (in DI water) to the cells in the micro centrifuge tube. Incubate for 30 minutes.
3. Transfer the mixture in a water bath at 42 °C for 2 minutes.
4. Transfer again on ice and add 1ml of SOC medium.
5. Rotate in the 37°C incubator for 1 h.
6. Centrifuge at 4000x rpm for 2 minutes.
7. Resuspend the pellet in 200μl SOC.
8. Make appropriate dilutions and overlay petri plates (90mm) that contain LB agar with the appropriate antibiotics that the recombinant plasmid has resistance (e.g. ampicillin). Incubate the plates overnight at 37°C.

Materials:

• S.O.C medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose • LB Broth (10 g/L Tryptone, 5 g/L Yeast Extract 5 g/L NaCl) • 10% glycerol • DNA solution • antibiotic • $CaCl_2$ 50Mm

2.5.4 Screening and identification of recombinant clone

Based on the cloning plasmid vector you choose the screening and identification of the recombinant cells differs.

2.5.4.1 Blue-white screening

Blue-white screening is a widely used technique to examine successful cloning. In this method the insert is cloned into a vector containing a *lacZα* sequence encoding the α-peptide, a functional subunit of the β-galactosidase enzyme in the multiple cloning site.

The competent cells used for the transformation of this plasmids should have the lac*ZΔM15* mutation. An empty vector will produce blue colonies since the activity of the β-galactosidase remains intact. The colorless X-gal (lactose analog) provided in the screening plates is hydrolyzed by β-galactosidase to form a blue pigment (5,5'-dibromo-4,4'-dichloro-indigo). If the vector contains the DNA insert disrupting the *lacZα* sequence, then the α-peptide will not be expressed and X-gal will not be hydrolyzed and the colonies that have the DNA insert will be white.

2.5.4.2 Colony PCR

Another way of screening the colonies for the presence of the DNA insert is by using PCR. The primers may be insert-specific, vector-specific, or both to detect the insert. Colony screening by PCR is suitable for inserts shorter than 3 kb. Amount of the individual colony can be directly subjected to the PCR mix and perform PCR (see 2.6). The remaining portion of the colony may be used to inoculate a culture plate or liquid LB media with appropriate antibiotic for downstream applications.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Standard PCR reaction

A standard Polymerase Chain Reaction (PCR) is an *in vitro* method that allows a single, short region of a DNA molecule (single gene perhaps) to be copied multiple times (Saiki et al. 1985).

A typical PCR reaction requires the DNA template, a pair of DNA oligonucleotide (oligo) primers, free nucleotides (dNTPs), enzyme Taq DNA polymerase and the reaction Buffer.

The template DNA could be a fragment of specific sequence or genomic DNA and cDNA. Based on the DNA segment to be amplified, two unique single stranded DNA oligonucleotide (oligo) primers are required. The primers anneal to the regions upstream (5’) and downstream (3’) of the sequence. For the efficiency of the primer is essential, that the 3’ end of the primer corresponds completely to the template DNA strand so elongation can proceed. Usually a guanine or cytosine is used at the 3’ end, and the 5’ end of the primer usually has stretches of several nucleotides. Also, both of the 3’ ends of the hybridized primers must point toward one another.

Another important aspect is the size of the primer. Short primers are mainly used for amplifying a small, simple fragment of DNA. On the other hand, a long primer is used to amplify a eukaryotic genomic DNA sample. The size of the primer should be between 18-24 bases. Moreover, the sequence of the primer should have 40-60% G/C and the melting temperature (Tm) at 50-60°C. The structure of the primer should be relatively simple and contain no internal secondary structure to avoid internal folding.

The basic enzyme for the reaction is a DNA polymerase isolated from the thermophilic bacterium, *T. aquaticus (Taq)*. *Taq* polymerase can withstand many heating and cooling cycles, which would denature DNA polymerases from other species.

The free nucleotides (dNTPs) are the building blocks added one at a time to the new DNA strand by the DNA polymerase.

The reaction buffer contains $MgCl_2$ which provides an optimal and stable chemical environment for the DNA polymerase to work adequately. Divalent cations such as Mg^{2+} and Mn^{2+} stabilize the buffer solution. The buffers usually come to a concentration of 10X based on the provided guidelines.

Method:

1. Place all the reagents on ice.
2. In a PCR tube (200μl) add: the DNA template, Buffer (1x), dNTPs (10Mm each), (0,4- 0,6 μM ο καθένας), the enzyme Taq DNA polymerase (1 unit) and ddH_2O until the final volume of the reaction (25ul).

3. The PCR tube is placed in a PCR machine.

A basic PCR program has six steps:

1. Initial Denaturation for 2 minutes at 94°C: This initiation step heats the double stranded DNA template strand to the point where the strands start denaturing and the hydrogen bonds are broken between the nucleotide base pairs.
2. Denature 30 seconds at 94°C: Continued denaturation of double stranded DNA.
3. Anneal primers for 30 seconds at annealing temperature (Ta): The Ta is calculated as the melting temperature (Tm) minus 5 (Ta=Tm-5).
4. Extend DNA for 1 minute at 74°C: The Taq polymerase has an optimal temperature around 70-75°C so this step enables the DNA polymerase to synthesize and elongate the new target DNA strand accurately and rapidly.
5. Repeat steps 2-5 for 25-30 times.
6. Final extension for 5 minutes at 74°C: A final extension to fill-in any protruding ends of the newly synthesized strands.

Materials:

• DNA template (20-40ng) • Primers (10pmol/μl each) • dNTPs (10mM each) • Enzyme Taq DNA polymerase (5units/μl) • Reaction Buffer (10x) • ddH_2O

2.6.2 Real time PCR

Real-time PCR (also known as quantitative or qRT-PCR) allows accurate quantification of starting amounts of DNA, cDNA, and RNA targets. During each cycle of the qRT-PCR a fluorescence is measured giving you the opportunity to measure the amount of your PCR product as it is proportional to the amount of the dye. PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA or fluorescently labeled sequence-specific probes.

The most common fluorescent dye is called SYBR Green. This dye binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding. Detection takes place in the extension step of real-time PCR. Signal intensity increases with increasing cycle number due to the accumulation of PCR product.

Method:

1. In transparent low profile strips add the desired amount of DNA (1 μl), the specific pair of primers for the amplified region and the master-mix mixture as it is provided by the manufacturer. The master mix contains buffer, dNTPs, the DNA polymerase, and the SYBR Green dye. Add ddH_2O until the final volume of the reaction.
2. Place the strips in the PCR real-time machine.

2.6.3 Reverse transcription

The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). The enzyme used for the reaction is MMLV RT with reduced RNase H activity and increased thermal stability. The enzyme is purified to near homogeneity from E. coli containing the modified pol gene of Moloney Murine Leukemia Virus (Kotewicz et al., 1985).

Method:

1. In a nuclease-free microcentrifuge tube add the following components for a 20-µl reaction volume: Oligo(dT)12-18 (500 µg/mL) or 1 µl 50–250 ng random primers or 2 pmole gene-specific primer (GSP), 1 ng to 5 µg total RNA or x µl, 1–500 ng of mRNA, 1 µl dNTP Mix (10 mM each), 1 µl ddH_2O to 12 µl.
2. Heat the mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation.
3. Add 5X First-Strand Buffer 4 µl, 0.1 M DTT 2 µl, RNaseOUT™ (40 units/µl)
4. Mix the contents of the tube gently.
5. Incubate at 42°C for 2 min.
6. Add 1 µl (200 units) of SuperScript™ II RT and mix by pipetting gently up and down.
7. Incubate tube at 25°C for 10 min.
8. Incubate at 42°C for 50 min.
9. Inactivate the reaction by heating at 70°C for 15 min.

Materials:

• Oligo(dT) (500 µg/mL) or 50–250 ng random primers or 2 pmole gene-specific primer (GSP) • RNA template (1 ng to 5 µg total RNA or x µl, 1–500 ng of mRNA) • Primers (10pmol/µl each) • dNTPs (10mM each) • SuperScript™ II Reverse transcriptase (5units/µl) • 5x Reaction Buffer (250 mM Tris-HCl, pH 8.3 at room temperature; 375 mM KCl; 15 mM $MgCl_2$) • ddH_2O

2.7 In vitro transcription

In vitro transcription is a procedure that allows the template-directed synthesis of RNA molecules of any sequence from short oligonucleotides to those of several kb in µg to mg quantities. It is based on the engineering of a template that includes a bacteriophage promoter sequence (e.g. T7 promoter) upstream of the sequence of interest followed by transcription using the corresponding RNA polymerase. The protocol followed in this thesis was based on the MEGAscript® Kit from Invitrogen.

In vitro transcription is used for the synthesis of large amounts of unlabeled or low specific activity RNA for a variety of uses including in vitro translation, antisense/ microinjection studies, and isolation of RNA binding proteins.

Method:

1. Thaw the frozen reagents in ice. Only the 10X Reaction Buffer should be at room temperature as it contains spermidine that can coprecipitate the template DNA.
2. For 20µl final volume of reaction add in the specific order the: PCR-product template, 2 µl ATP solution, 2 µl CTP solution, 2 µL GTP solution, 2 µL UTP solution, ddH_2O, 2 µL 10X Reaction Buffer (1 µl) and 2 µL Enzyme Mix.

*** Add the 10X Reaction Buffer after the water and the ribonucleotides are already in the tube. ***

3. Mix thoroughly by gently flicking the tube or pipette the mixture up and down gently.
4. Centrifuge the tube briefly to collect the reaction mixture at the bottom of the tube.
5. Incubate at 37°C for 2–4 hours.
6. To stop the reaction, add 115 µl Nuclease-free Water and 15 µl Ammonium Acetate Stop Solution and mix thoroughly.
7. Extract with an equal volume of phenol/chloroform and then with an equal

volume of chloroform. Recover aqueous phase and transfer to new tube.

8. Precipitate the RNA by adding 1 volume of 2-propanol and mixing well.
9. Chill the mixture for at least 15 min at –20°C. centrifuge at 4°C for 15 min at maximum speed to pellet the RNA.
10. Carefully remove the supernatant solution and resuspend the RNA in a solution appropriate for your application. Store frozen at –20°C or –70°C.

Materials:

• Enzyme Mix • DNA template that has the correct RNA polymerase promoter site upstream of the sequence to be transcribed • 10X Reaction Buffer • dNTPs (10mM each) • Enzyme Taq DNA polymerase (5units/μl) • Ammonium Acetate Stop Solution (5 M ammonium acetate, 100mM EDTA) • ddH_2O • 2-propanol• Phenol • Chloroform

2.8 DNase treatment

The presence of contaminating genomic DNA (gDNA) in RNA preparations is a frequent cause of false positives in RT-PCR-based assays aimed at gene expression analysis. two alternative methods for RNA treatment with DNase are described. The choice of the most suitable method is largely dependent on the availability of starting RNA.

The conventional DNase treatment requires a step of phenol/chloroform. However, this treatment causes 50% RNA loss so it is suitable only when an RNA solution is not pure and large RNA amounts are available. For this reason, the DNase digestion followed by enzyme heat inactivation is more suitable, especially when an RNA starting quantity is very low. This method may be very useful when an RNA has been extracted from small biopsies or cytologic specimens. Here, we use the TURBO DNA-free™ Kit Ambion-Invitrogen for the DNAse treatment.

Method:

1. Add 0.1 volume 10x TURBO DNase Buffer and 1 µl TURBO DNase to the RNA, and mix gently.
2. Incubate at 37°C for 20–30 min.
3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume) and mix well.

***Always resuspend the DNase Inactivation Reagent by flicking or vortexing the tube before dispensing it. ***

4. Incubate for 5 minutes at room temperature while mixing occasionally.
5. Centrifuge at 10,000× g for 1.5minutes and transfer the RNA to a fresh tube.

Materials:

• 10x TURBO DNase Buffer • DNase Inactivation Reagent • RNA• TURBO DNase (2 Units/µl)

2.9 Gel electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. (Kryndushkin et al., 2003). Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward positive pole. The migration flow is determined by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired "band" from a stained gel viewed with a UV transilluminator.

To view the gel with UV a fluorescent dye, named Ethidium bromide, is currently used. Ethidium bromide intercalates between nucleic acids bases and provides easily detection of nucleic acid fragments in gels (Sharp et al. 1973). The gel subsequently is being illuminated with an ultraviolet lamp usually by placing it on a light box.

2.9.1 Preparation of the gel

1. Prepare a solution of agarose in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA sample.
2. If using a glass bottle, loose the cap. Heat the mixture in a microwave oven until the agarose dissolves.
3. When the melted gel has cooled at 55°C, add ethidium bromide to a final concentration of 0.5 μg/ml. Mix the gel solution thoroughly by gentle swirling.
4. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.
5. Pour the warm agarose solution into the mold.
6. Allow the gel to polymerize completely (20-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.

2.9.2 Run the gel

1. Place the gel into the electrophoresis device and enough electrophoresis buffers to cover the gel.
2. Mix the sample by loading dye with a ration 1:5 or 1:10. Slowly load the sample mixture into the slots of the submerged gel. Add also a size standard into slot.
3. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead).

If the leads have been attached correctly, bubbles should be generated at the anode and cathode, and within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel

4. Run the gel until the bromophenol blue and xylene cyanol FF have migrated for distance through the often to the last third of the gel.
5. When the DNA samples or dyes have migrated for a sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank. Otherwise, stain the gel by immersing it in electrophoresis buffer or H_2O containing ethidium bromide (0.5 μg/ml) for 20-45 minutes at room temperature.

Materials:

• TBE (10x stock solution in 1 liter of H2O: 48.4 g Tris base, 5g of boric acid, 40 ml of 0.5 M EDTA (pH 8.0) • 6x Gel-loading Buffer (0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF 40% (w/v) sucrose in H_2O) • Ethidium Bromide (1 g of ethidium bromide to 100 ml of H2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the 1% (10 mg/ml) solution to a dark bottle and store at room temperature).

2.9.3 Gel extraction

The GF-1 Gel DNA Recovery (extraction) Kit is a system designed for rapid purification of DNA bands ranging from 100bp to 10kb from all grades of agarose gel in TAE (Tris-acetate/ EDTA) or TBE (Tris-borate/ EDTA). The method includes the solubilization of the agarose gel in the Buffer GB and the transfer of the DNA solution on a filter membrane for efficient recovery of highly pure material.

Method:

1. Determine the net weight of gel slice and add 1 volume of Buffer GB to 1 volume of gel (A gel slice of mass 0.1g will have a volume of 100μl).
2. Centrifuge the tube briefly to make sure the gel slice stays at the bottom of the tube.
3. Incubate at 50°C until gel has melted completely. Mix occasionally to ensure complete solubilization.
4. Transfer the sample into a column assembled in a clean collection tube.
5. Centrifuge at 10,000x g for 1 min. Discard flow through.
6. Repeat for any remaining sample from step 4.
7. Add 650μl Wash Buffer into the column.
8. Centrifuge at 10,000x g for 1 min. Discard flow through.

Ensure that ethanol has been added into the Wash Buffer before

9. Column drying Centrifuge the column at 10,000 x g for 1 min to remove residual ethanol. This step has to be carried out to remove all traces of ethanol as residual ethanol can affect the quality of DNA and may subsequently inhibit enzymatic reactions.
10. Place the column into a clean microcentrifuge tube. Add 30 - 50μl Elution Buffer, TE buffer or sterile water directly onto column membrane and stand for 2 min. For DNA fragments larger than 8kb, use preheated elution buffer at 65°C - 70°C for better elution efficiency.
11. Centrifuge at 10,000x g for 1 min to elute DNA. Store DNA at 4°C or -20°C.

For higher yield, elute DNA in 50μl and for higher concentration, elute DNA in smaller volume. Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution

12. Store DNA at -20°C.

2.10 Microinjection technique

The use of microinjection as a biological procedure began in the early twentieth century. By the 1990s, its use had escalated significantly and it is now considered a common laboratory technique introducing a small amount of a substance into a small target (Lacal et al., 1999). The insect microinjection has three steps. First, the preparation of the injector which includes wash of all the components with ethanol and preparation of the needle. Second, filling the needle with the substance you want to introduce to third injecting the insects

Method:

Preparation of the injector

1. Clean injection area with 70% ethanol.
2. Plug power cord into mains.
3. Plug power source into top of Nanoject II Module.
4. Plug injector into top of Nanoject II Module.
5. Unscrew black collet on injector and carefully tap out 2 black O-rings and 1 white spacer.
6. Wash all components with ethanol.
7. Fully extend plunger by holding EMPTY and pressing FILL once to increase speed.
8. Place small black O-ring on plunger and carefully push to Base.
9. Place white spacer on plunger with indentation towards tip.
10. Choose glass needle and break off tip using forceps under microscope.
11. Backfill glass needle with mineral oil using a syringe with yellow needle, VERY slowly to avoid bubbles until a drop comes out of the tip.
12. Continue backfilling as needle is removed to avoid air pocket.
13. Insert glass needle through BLACK collet.
14. Place LARGE BLACK O-ring on end of glass needle such that the domed side faces tip.
15. Insert injector plunger into back of needle and pull all the way down to base Tighten collet onto injector.

Filling the needle with the injected materials

16. Cut a square of parafilm and place under microscope.
17. Pipette 3-4 μl drop of liquid material onto parafilm.
18. Clean oil carefully from tip of needle.
19. Position point of needle in the liquid drop (hold injector vertically).
20. Suck up dsRNA by holding FILL (press EMPTY once to increase speed). Stop before complete to avoid air bubbles.

Injecting the insects

21. Fill petri dishes with water and freeze them in -80°C.
22. Place one cup (10-15 insects) for 1-2 minutes in a box full of ice to anaesthetize insects.
23. Place whatman filter paper on a frozen petri dish.
24. Place insects on the petri dish.
25. Inject insect in one of two sites (Between the thorax and first abdominal segment or Unpigmented area below the mesonotum) by piercing cuticle and pressing foot pedal.

2.11 Feeding assay

The protocol used for the RNAi feeding was based on the protocol described by Li et al. (2011).

Method:

Preparation of the plasmid

1. Amplify part of the coding region from your target gene by RT-PCR using specific primers. Most of the RNAi experiments use 300-520bp fragment.
2. Clone the PCR products into MCS of L4440 plasmid (see 2.5). The L4440 plasmid has

two T7 promoters in inverted orientation flanking the multiple cloning site.

3. Prepare HT115 (DE3) competent cells lacking RNase III using standard $CaCl_2$ methodology and transform them with recombinant plasmid L4440 (see 2.5.3.2).

Bacterial culture

4. Culture 2ml of the transformed bacteria at 37ºC with shaking at 210x rpm overnight
5. Transfer the culture in 100ml YT buffer supplemented with 75 mg/ml ampicillin plus 12.5 mg/ml tetracycline and culture at 37ºC, 250x rpm until OD_{600} = 0.5 (3.5-4 hours)
6. Add IPTG to 0.4mM (final concentration) for 4hours (IPTG induces the synthesis of T7 polymerase)

Bacterial feeding experiments

1. Centrifuge for 10 minutes at 5000x g and dilute the pellet in 200μl H_2O.
2. Mix the dilution with 2gr of artificial diet and feed the insects.

RNAi experiments should be performed in two different groups. Group 1 will be fed for the gene of interest and group 2 will be fed for GFP (a sequence with no homology on insects' genomes). Use the control group to determine your silencing percentage and phenotype

2.12 Next-generation sequencing

Next-generation sequencing (NGS) involves all the described number of different modern sequencing technologies. Here, are analyzed two of them: Illumina and Ion Proton sequencing.

2.12.1 Illumina library preparation and sequencing

The RNAseq for the testes was performed by the Illumina Hi-Seq 2000 using the Illumina TruSeq RNA Sequencing protocol at the Genome Quebec in Canada.

To perform Illumina sequencing the first step is to prepare the cDNA libraries (Kozarewa et al., 2009). An isolation of total RNA (>200ng) is performed for the analyzed samples using the TRI Reagent® material (see 2.2.2). Then, an RNA extraction is followed by an additional DNase treatment using the TURBO DNA-free Kit (see 2.8). Polyadenylated RNA (polyA-RNA) is isolated through Dynabeads Oligo(dT) kit (Ambion, Life Technologies Corporation) and randomly fragment by chemical hydrolysis at 94°C for 5 minutes. Then a removal of phosphatase groups from the fragments' ends is required with antarctic phosphatase followed by treatment with T4 polynucleotide kinase to add a Pi at the 5' end of each fragment.

The resulting RNA fragments are hybridized and ligated to the P1 and P2 adaptor sequences and the RNA produced is reverse transcribed to cDNA which then is amplified in a 15-cycle PCR. At this step, the use of different barcoded 3' PCR primers from the selection included in the SOLiD barcoding kit allows the preparation of cDNA libraries for multiplex sequencing. PCR is carried out to amplify each read, creating a spot with many copies of the same read.

From the cDNA produced, the preferable fragments are selected (in this thesis 200-300bp) with two rounds of magnetic bead purification (Agencourt AMPure XP Reagent, Beckman Coulter).

The quality and size of the purified cDNA library is assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc.) and with quantitative PCR using the Library Quant Kit ABI Solid (KAPA Biosystems). A multiplex library mix (500pM) is used to prepare a full-slide for analysis on the SOliD 4 Sequencing System (Applied Biosystems. The library sequencing is performed on the SOLiD 4 Sequencing System using the SOLiD Total RNA-Seq Kit, Life Technologies Corporation.

The logic of the sequencing is simple. The prepared slice is flooded with nucleotides and DNA polymerase. Each nucleotide is labeled with different a fluorescence dye and a terminator so that one nucleotide will be added at each cycle. An image is taken of the slide. In each read location, there will be a fluorescent signal indicating the base that has been added. Before each cycle the terminators are removed allowing the next base to be added, and the fluorescent signal is removed, preventing the signal from contaminating the next image. Computers are then used to detect the base at each site in each image and these are used to construct a sequence. All of the sequence reads will be the same length, as the read length depends on the number of cycles carried out.

2.12.2 Ion proton library preparation and sequencing

The RNAseq for the male (male accessory gland with ejaculatory bulb) and female reproductive tissues (lower reproductive tract) was sequenced on the Ion Proton™ system for Next-Generation Sequencing at the Fleming Institute (Greece) using the Ion Torrent™ Ion Chef™ automated.

The method for the library preparation for Ion proton is not too different from the above. An isolation of total RNA is performed for the analyzed samples using the TRI Reagent® material (see 2.2.2). Then, an RNA extraction is followed by an additional DNase treatment using the TURBO DNA-free Kit (see 2.8). Polyadenylated RNA (polyA-RNA) is isolated through Dynabeads Oligo(dT) kit (Ambion, Life Technologies Corporation) and the fragmentation is performed (chemical or mechanical) using a sonicator (Covaris S2). As in other kinds of next-generation sequencing, the input DNA or RNA is fragmented, this time ~200bp. Adaptors are added and one molecule is placed onto a bead. The molecules are amplified on the bead by emulsion PCR (One Touch 2 emulsion) and each bead is placed into a single well of a slide. The quality and size of the purified cDNA library is assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc.) and Nanodrop. The library sequencing is performed on the Ion Torrent PROTON Sequencing System.

The logic of the sequencing in Ion proton has specific differences compared to Illumina. The Ion proton does not make use of optical signals (Rothberg et al., 2011). It is based on the fact that each addition of dNTP by DNA polymerase releases an H^+ ion. The prepares slides is flooded with a single species of dNTP along with buffers and polymerase. The pH is detected in each of the wells, as each H^+ ion released will decrease the pH. The addition of one or more bases to the sequence is mirrorize on the pH changes allowing to determine if that base, and how many thereof, is added to the sequence read. Before each PCR cycle the dNTPs are washed away. All of the sequence reads from Ion proton are different lengths, because different numbers of bases are added with each cycle.

2. 13 Bioinformatics

2.13.1 BLAST

The comparisons of the sequences were performed using the BLAST (The Basic Local Alignment Search) program (Altschul et al., 1990). BLAST finds a suite of programs provided by NCBI for aligning query sequences against those present in a selected target database.

The program finds region of local similarity between sequences through comparison of nucleotide to sequence databases of nucleotides (BLASTn), or protein sequences to databases of proteins (BLASTp) and calculates the statistical significance of matches. Moreover, it gives you the opportunity to use BLASTx for identifying potential protein products encoded by a nucleotide query, tBLASTn, for identifying database sequences encoding protein similar to query or tBLASTx for identifying nucleotide sequences similar to query based on the coding potential.

Moreover, the program Primer-Blast gives you the opportunity to design pair of primers for your amplifications through PCR or qRT-PCR (see 2.6.1).

2.13.2 BLAST2GO

BLAST2GO is a bioinformatic platform for high-quality functional annotation and analysis of genomic datasets It is user friendly and gives you the opportunity to annotate thousands of sequences, in multiple projects and interrogate the biological meaning with different graphical and statistical functions.

2.13.3. **GraphPad Prism**

GraphPad Prism is a scientific 2D software for graphing and statistics. Data of this thesis were analyzed using the Student's t-test in this program. Results were expressed as mean ± standard error and a p value of < 0.05 was considered statistically significant.

2.13.4 E-RNAi3

E-RNAi is a tool for the design and evaluation of RNAi reagents for a variety of species. It can be used to design and evaluate long dsRNAs (including esiRNAs) as well as siRNAs. (http://www.dkfz.de/signaling/e-rnai3/idseq.php). Typically, RNAi experiments are done with dsRNA 400bp and larger, 200bp is the minimum size for dsRNA recommended. (Horn et al., 2010).

2.14 Peptidomic analysis

2.14.1 Sample Preparation for LC/MS

The reproductive system of virgin and mated flies was dissected and directly transferred into 30-40 μl ice-cold 90% methanol/ 9% water/ 1% trifluoroacetic acid (TFA) (v/v/v) in an eppendorf microtube. Each sample was consisted of tissues from 40 insects performed in two biological replicates. The isolation of the peptides was performed using the ZipTip pipette according to the manufacture's guideline. The eluted supernatant was transferred to a new tube, centrifuged and dried in a Speed-Vac and stored at -20^{o}C.

2.14.2 Mass Spectrometry analysis

Re-suspended peptides in 0.1% TFA were vortexed and sonicated, each for 5 minutes followed by 10-minute centrifugation at 13,000x rpm. After centrifugation the supernatant was transferred to a new tube and injected for reverse phase chromatography separation. The peptide samples were spotted on the MALDI plate with the use of a robot-based target spotting (Proteineer fc II; Bruker Dalton- ics), samples were then analyzed with a matrix-assisted laser deionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometer (Ultraflex- treme; Bruker Daltonics). Tandem mass spectrometry (MS/MS) spectra containing precursor masses corresponding to possible cross-linked peptides were analyzed using a combination of Biotools and Flex-Analysis software (Bruker Daltonics). For peptide identification, MS/MS spectra were analyzed with the use of MASCOT software 2.4.1. and followed by manual validation which includes a thorough manual analysis of each spectrum, accurate identification of series of b- and y- ions and ammonium ions confirming sequences.

2.15 Fertility assays

On the 7th day after eclosion males were allowed to mate with virgin females. Mating that lasts >1 hour is considered successful (Zervas et al., 1982). When the insects separate from each other, males are discarded and females are allowed to lay eggs for twelve days in separate cages. Oviposition rate is calculated based on the daily egg count for each female fly. Experiments are repeated three times. Experiments were repeated three times.

2.16 Adult survival experiment

Insects fed with ds-SPR and ds-GFP are placed in different cages with the change to fresh food at the interval of 24h. Mortality is monitored every 24 hours and dead insects are counted and removed from the cages.

3. RESULTS

3.Results

3.1 Transcriptome sequencing analysis of the reproductive system

In order to identify genes that may play a role in mating, a comprehensive analysis of the transcriptome of male and female reproductive tissues from sexually mature virgin and mated olive flies was performed. The tissues selected for the analysis were: 1. the male accessory glands and testes that produce the seminal fluid transferred to females during mating and 2. the lower female reproductive tract, comprising from the spermathecae, where sperm is stored until the time of fertilization, the uterus and the female accessory glands that produce a multi-function material for protection and nutrition of the zygote after fertilization.

In order to obtain tissues from sexually mature virgin flies, dissections were performed at the 7th day after eclosion. The sexual maturation of the insects was determined by their ability to mate and give offspring. The laboratory strain of the olive flies used for the experiments was sexually mature and could mate successfully giving offspring on the selected day.

The dissection of the tissues from mated olive flies was performed 12 hours after mating. Specifically, on the 7th day after eclosion we mixed virgin male and female flies to mate. To consider a mating successful, it should last >1hour (Zervas et al., 1982). When the insects separated from each other, we kept them in different cages for 12 hours before we perform dissections. In *D. melanogaster* the highest post-mating gene expression occurs after 6 hours (Mack et al., 2006) and in *C. capitata* there is a general increase in the transcriptional activity only after three repeated matings (Scolari et al., 2012). As there is no such evidence for the reproductive tissues of *B. oleae*, two pieces of information guided our decision. Firstly, male olive flies can remate at least 24 hours after previous mating (Tsiropoulos 1970) and, secondly, oviposition of the mated females also starts 24 hours after mating. Given that, we considered that the most appropriate time to collect the tissues would be 12 hours after mating, since we hypothesized that at that point there should be high transcription rate of reproductive genes.

The collected tissues per sample included:

1. Female lower reproductive tract tissues from sexually mature virgin females (two biological samples).
2. Female lower reproductive tract tissues from mated females at 12h post mating (two biological samples).
3. Male reproductive tissues from male accessory glands with ejaculatory bulb (two biological samples).
4. Male reproductive tissues from mated males 12h post mating (two biological samples) and testes (one biological sample).
5. Gut tissues from virgin males to be used as a control. Gut is a tissue from the digestive system of the insect (one biological sample) that should not be affected by mating.

The analyzed tissues are shown in Table 3.1. Total RNA of ~1μg (50 insects) from each sample was isolated followed by subsequent DNA removal. The integrity of RNA was assessed by 1% agarose gel

System	Tissues	Virgin	Mated
Male reproductive	testes	-	M_TESTES
	accessory gland with ejaculatory bulb	V_MALE_1	M_MALE_1
		V_MALE_2	M_MALE_2
Female reproductive	lower reproductive tract	V_FEMALE_1	M_FEMALE_1
		V_FEMALE_2	M_FEMALE_2
Gustatory	gut	GUT-1 (male)	-

Table 3.1: Samples used for the RNAseq of *B. oleae*. Numbers (1 and 2) indicate the different biological replicates of the tissues.

electrophoresis and the purity of all RNA samples was evaluated at Fleming Institute (Greece) with the use of Agilent 2100 Bioanalyzer and NanoDrop™ 2000 (S6.1). Electrophoresis of the samples is presented in Figure 3.1.

mRNA transcripts from the samples were used to construct cDNA libraries for sequencing analysis. The cDNA libraries of accessory glands and ejaculatory bulb from virgin and mated male flies, the lower reproductive tract from virgin and mated female flies and the gut tissue from virgin male flies were sequenced on the Ion Proton™ system for Next-Generation

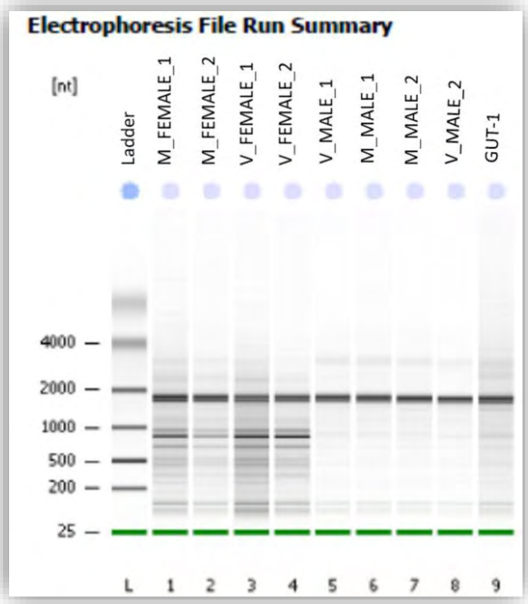


Figure 3.1: Electrophoresis Run Summary of the samples for RNAseq.

Sequencing at the Fleming Institute (Greece) using the Ion Torrent™ Ion Chef™ automated platform. The cDNA library obtained from the testes of mated male insect (M_TESTES) was sequenced by Illumina Hi-Seq 2000 using the Illumina TruSeq RNA Sequencing protocol at the Genome Quebec in Canada.

RNAseq read mapping was performed in collaboration with Dr. Martin Rezsco. A single representative *de novo* transcriptome assembly was generated from a concatenation of the libraries obtained with the Illumina platform using the Trinity pipeline (Haas et al. 2013). After assembly, transcript and unigene level expression values were calculated using RSEM (Li et al 2011) for the libraries sequenced with the Ion Proton. The average length of read was 669,78 bp and the sequenced 203,690,146 bp gave 255,077 genes. From the libraries V_MALE and M_MALE we obtained 11,452 transcripts and

Summary of the total *B. oleae* transcriptome	
Total base pairs	203,690,146
Total trinity "genes"	255,077
Average read length	669,78
Percent GC	37,47
median contig length	340

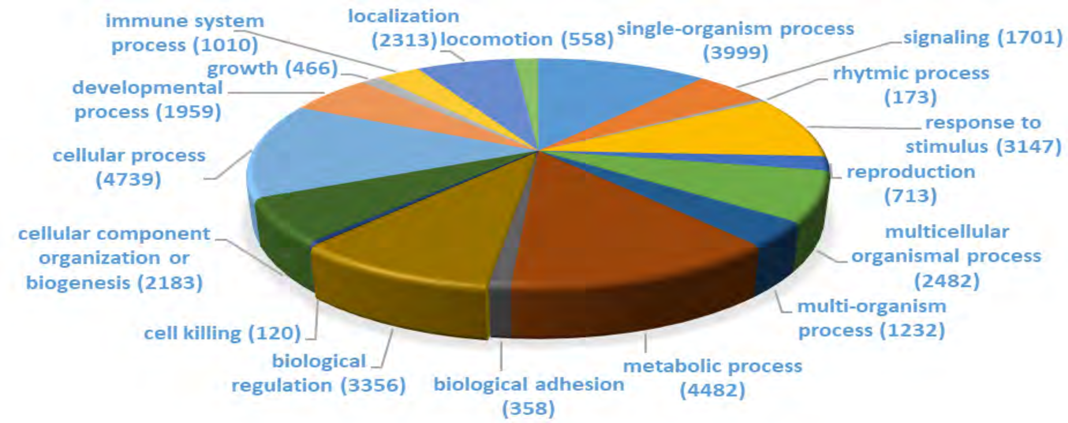
Table 3.2: Assembly statistics of the Illumina and Ion proton sequencing.

from the libraries V_FEMALE and M_FEMALE we obtained 10,478 transcripts. The assembly statistics of the Illumina and Ion proton transcriptome are presented in Table 3.2.

Moreover, we performed functional annotation of the assembled transcripts for the Ion Proton^{TM} transcriptomes based on the gene ontology (GO) categorization and using BLAST2GO. The GO analysis performed for the category of biological process level II is shown in Figure 3.2 and represents all the transcriptome sequences obtained for the male and female reproductive system.

Specifically, the transcriptome from both sexes is distributed to the same GO terms indicating a similar biological profile of the genes expressed in both reproductive systems. Within the GO Biological Process, the metabolic, cellular and single-organism processes were the most representative terms. Interestingly, only 713 out of 11,452 transcripts from the male tissues and 693 out of 10,478 transcripts from the female tissues have the GO term of reproduction.

A. Male accessory glands/ ejaculatory bulb



B. Lower female reproductive

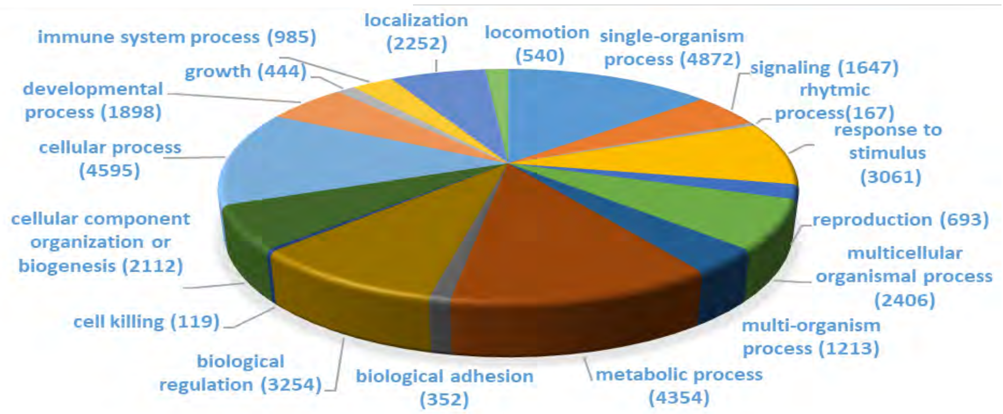


Figure 3.2: Distribution of the olive fly transcriptome sequences in Gene Ontology Biological Process Categories Level II. Unknown sequences were excluded from the analysis. GO categories with less hits in less than three genes are not indicated.

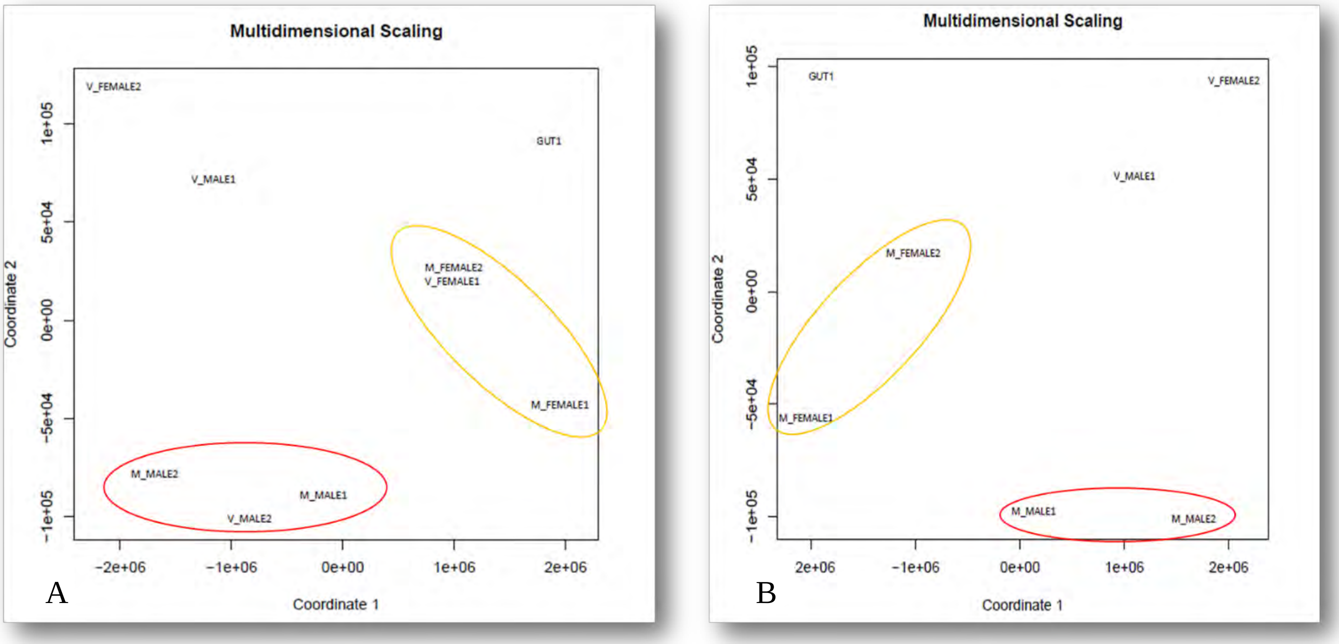


Figure 3.3: Multidimensional plots for all the samples sequenced by Ion Proton. Colored circles indicate groups of biological replicates. Yellow for mated female flies and red for male flies. A. The samples V_FEMALE_1 and V_MALE_2 are grouped with the mated flies and not with their biological replicates V_FEMALE_2 and V_MALE_1, respectively. B. Multidimensional plot analysis after omission of the V_FEMALE_1 and V_MALE_2 samples. The different groups are better distinguished.

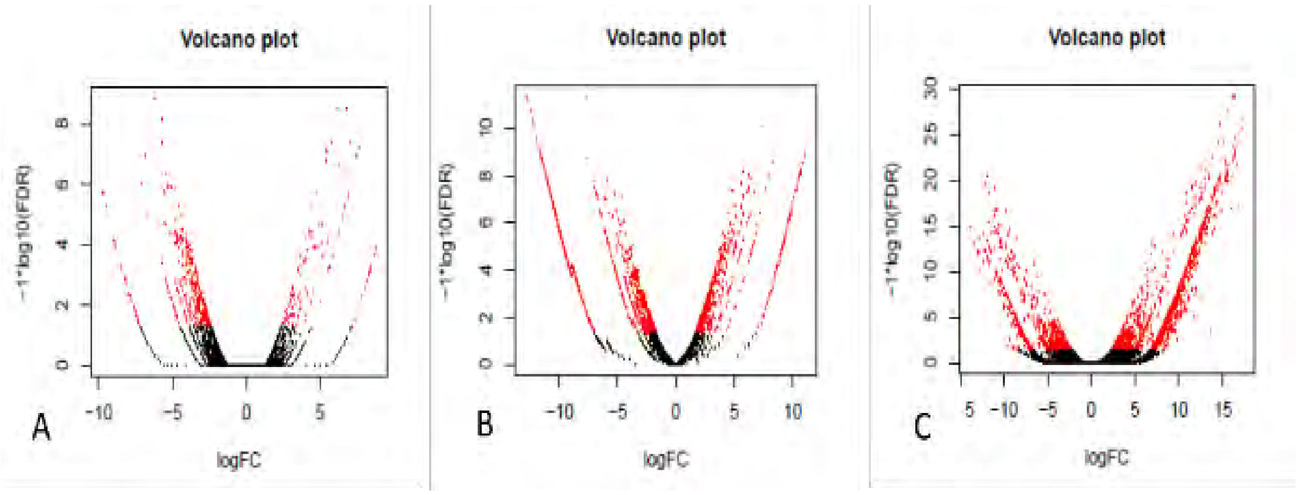


Figure 3.4: Volcano plots represent the differentially expressed genes between virgin and mated flies in the three dissected tissues: a. testes, b. male accessory glands with ejaculatory bulb, c. lower female reproductive tract. The Y axis represents significance and the X axis represents logarithmic fold change. The red dots represent differentially expressed genes (p value < 0.05).

3.1.1 Differentially expressed genes

Prior to the identification of the differentially expressed genes, we performed multidimensional-scaling plots to ensure the similarities between the biological replicates (Figure 3.3A). The plot showed that the samples of V_MALE_2 and V_FEMALE_1 were closer to the samples of mated flies than to their replicates. For this reason, and even though we lost statistical power, we decided to omit these two samples from the subsequent analysis. The new dimensional plot showed better results as only the remaining biological replicates were grouped together (Figure 3.3B).

To identify the differentially expressed genes, transcripts obtained from the analysis above were ranked based on their differential expression between virgin and mated insects. The M_TESTES transcriptome was compared to a previously annotated transcriptomic analysis of testes from three-day old virgin male olive flies (Sagri et al., 2016).

Differential expression between samples was assessed using the edgeR algorithm (Robinson et al. 2010) with a stringed cutoff (p value < 0.05). Distribution of transcripts can be seen in the Volcano plots with p value < 0.05 (Figure 3.4). The red dots indicate differentially expressed genes.

Further examination of the fold change differences revealed that 1,608 genes were up-regulated in male accessory glands with ejaculatory bulb, while 383 genes were down-regulated. In testes 107 genes were up-regulated and 345 genes were down-regulated. In females 1,705 genes were up-regulated in female mated insects while 120 genes were down-regulated (Figure 3.5). The entire lists of all significantly (p<0.05) up-regulated and down-regulated genes in the reproductive tissues are listed for male tissues in S6.2 and S6.3 and for the female tissues in S6.4.

In addition, we performed functional annotation of the top 100 most highly differentially expressed transcripts in the three different tissues based on the Gene Ontology (GO) categorization level II using BLAST2GO. The GO categorization is presented in Figure 3.6 and involves biological process (BP) and molecular function (MF).

The tissue of testes gave the fewest terms hits of the GO annotation. This may be due to the fact that testes are dedicated to sperm production, a significant function that allows very little variety for other roles (i.e., biological processes or molecular functions) for the genes expressed in this tissue.

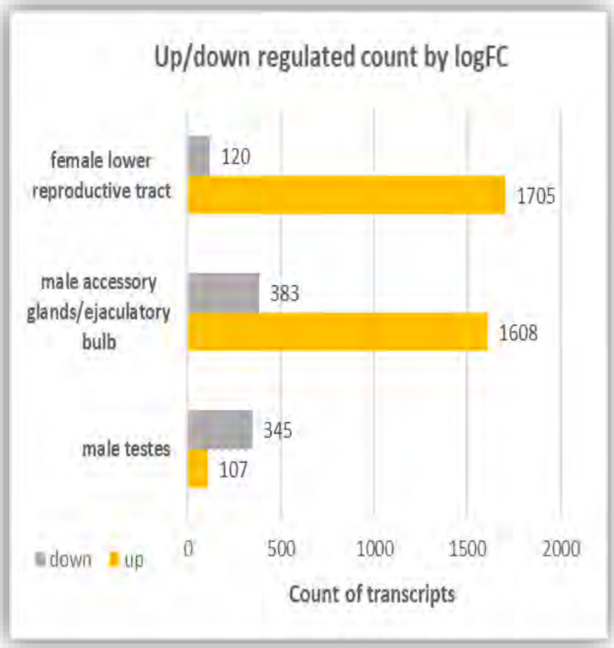


Figure 3.5: Total genes up- and down- regulated in the reproductive tissues of mated *B. oleae* insects

For the top 100 differentially expressed transcripts the three most abundant GO terms were the same for male accessory glands with ejaculatory bulb and female tissues. Cellular and metabolic processes along with the biological regulation were the most abundant GO terms. These findings can be related to the fact that the dissected tissues were from mated insects. For males, this is a time point when these tissues are transcriptionally active in order to replenish the seminal fluid. For females, it is the beginning of post mating responses, including oviposition. This can be also concluded from the different GO terms. Specifically, in male accessory glands with ejaculatory bulb the GO terms cell proliferation and biological adhesion may reflect the reproduction of cell tissues. While in female tissues the GO terms growth and rhythmic process may indicate the biosynthetic production and the preparation of the female insect to oviposit.

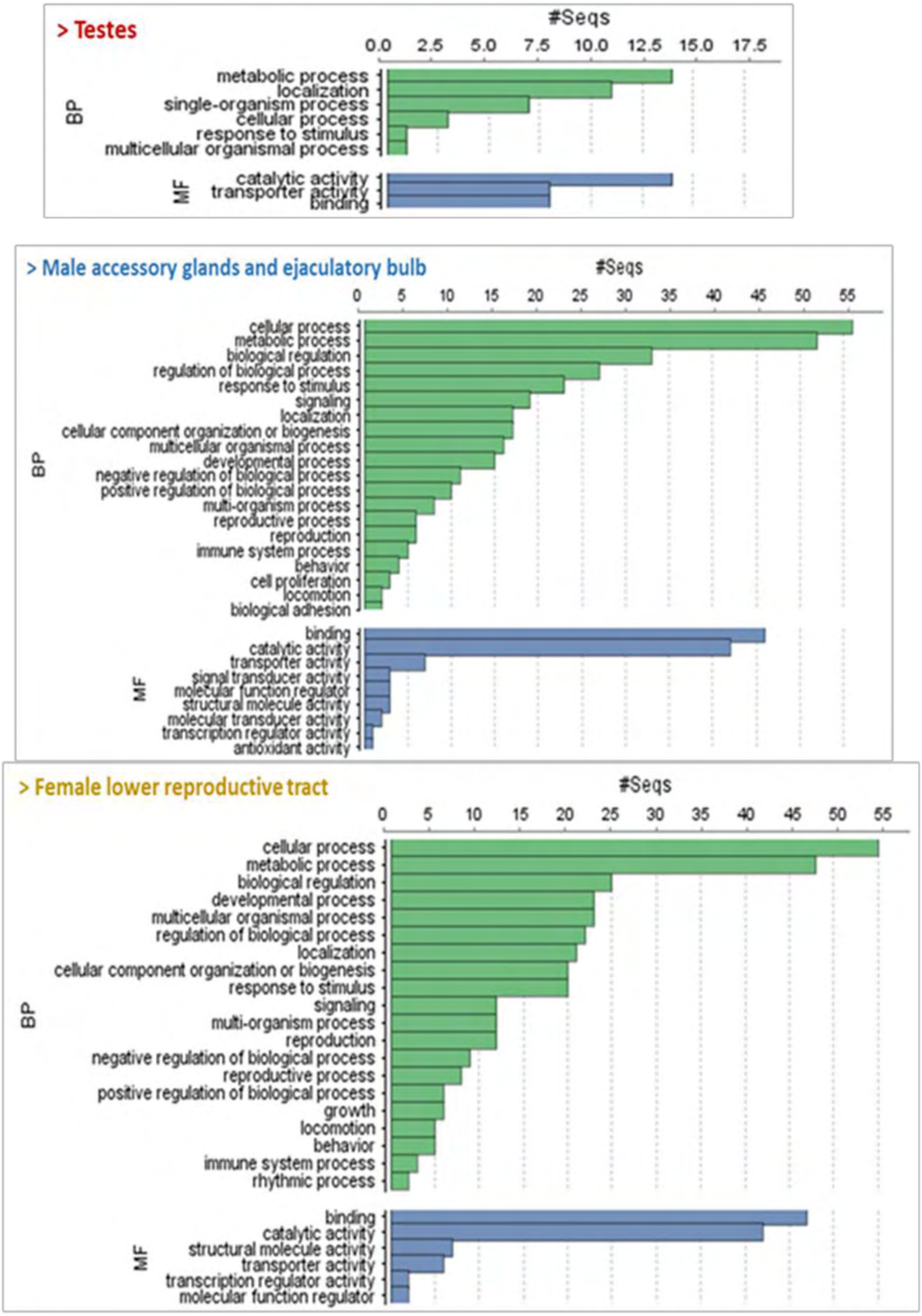


Figure 3.6: Functional annotation of the top 100 differentially expressed genes in *B. oleae* reproductive tissues showing top 20 hits of different category. BP, biological process; MF, molecular function.

Furthermore, in the Molecular Function annotation, all three tissues share the same GO terms except 3 that only the male accessory glands with ejaculatory bulb have. The antioxidant activity in male accessory glands and ejaculatory bulb may indicate that reproductive process causes oxidative stress to the male fly, a negative impact that has been documented previously in *A. gambiae* (DeJong et al., 2007) and *D. melanogaster* (Williams et al., 2005). Moreover, the signal and molecular transducer activity terms show that genes in male tissues may encode molecules in order to trigger the post-mating response to the female fly.

3.2 Genomic analysis of the reproductive genes

The top 100 differentially expressed genes were also annotated in the recently sequenced genome of the olive fly (https://i5k.nal.usda.gov/Bactrocera_oleae). We queried (BLASTn, e-value <10^{-10}) the genome scaffolds using the gene transcripts. The predicted amino acid sequences of the identified gene models were considered for annotation, if they gave significant reciprocal BLASTp (e-value <10^{-10}) hits in the NCBI database to known sequences. The gene names for the annotated sequences were given based on the nomenclature of the *D. melanogaster* homologues. The annotated genes are present in the supplementary file S6.5 for the tissue of testes, S6.6 for the male accessory glands with ejaculatory bulb and S6.7 for the female lower reproductive tract.

Interestingly, only 40 genes gave hit to the GO categories Level II for Biological process and Molecular function from the testes transcriptome. This may indicate that the function of several genes in testes is still unknown.

Many of the proteins fall into functional categories known to be present in reproductive systems of other insects like proteases, proteases inhibitors, mediators of immune response, and proteins involved in lipid metabolism. For example, in male accessory glands three genes *attacin–A*, *ryanodine receptor* and *heixuedian* are implicated in the immune response while in female tissues *seprin42Da* and *CG9676-like* genes, that encode a protease and a protease inhibitor, have been identified. In testes, genes that encode proteins implicated in metabolism processes were identified like *CG34189-like,* a *trypsin Inhibitor-like enzyme*, *black* gene, a pyridoxal phosphate-dependent decarboxylase and *CG8303-like* which belongs to the fatty Acyl-CoA reductase family.

Moreover, we queried (tBLASTn, e-value <10^{-10}) the genome scaffolds using the amino acid sequences of the 139 characterized *D. melanogaster* SFPs (Findlay et al., 2008). Only 43 of the Drosophila genes gave significant hits to the olive fly genome. The predicted amino acid sequences of the identified olive fly models were considered for annotation if they gave significant reciprocal BLASTp (e-value <10^{-10}) hits in the NCBI database. All the homologous genes were grouped into 17 functional classes based on the categories defined for *D. melanogaster* (Findlay et al., 2009) and *C. capitata* (Papanikolaou et al., 2016) seminal fluid proteins (Table 3.3). None of the annotated genes belong to the top 100 significantly expressed genes annotated above. This may indicate the rapid evolution of the

	Bo	Scaffold	Functional Class
1	alphaTub84B	NW_013581225.1.2	sperm protein
2	betaTub85D	NW_013581217.1.128	sperm protein
3	Ccp84Ad	NW_013583085.1.13	chitin binding
4	Cdlc2	NW_013581488.1.1	sperm protein
5	CG10407-like	NW_013581506.1.10	unknown function
6	CG10433-like	NW_013581250.1.1	defense/immunity
7	CG10730-like	NW_013581262.1.37	unknown function
8	CG11598-PB-like	NW_013581987.1.8	lipid metabolism
9	CG11864-like	NW_013581224.1.5	protease
10	CG13340-like	NW_013581355.1.7	protease
11	CG15031-like	NW_013581935.1.1	unknown function
12	CG15116-like	NW_013581511.1.8	defense/immunity
13	CG15117-like	NW_013582880.1.1	carbohydrate metabolism
14	CG17843-like	NW_013581762.1.2	oxidative stress response
15	CG17919-like	NW_013581353.1.17	signal transduction
16	CG18135-like	NW_013581267.1.22	unknown function
17	CG18284-like	NW_013581453.1.14	lipid metabolism
18	CG18628-like	NW_013581453.1.14	unknown function
19	CG2852-like	NW_013581924.1.1	protein modification
20	CG3153-like	NW_013581262.1.25	unknown function
21	CG31704-like	NW_013582946.1.1	protease inhibitor
22	CG31758-like	NW_013599804.1.1	protease inhibitor
23	CG4847-like	NW_013581323.1.30	protease
24	CG5162-like	NW_013586638.1.2	lipid metabolism
25	CG6426-like	NW_013581234.1.7	defense/immunity
26	CG6461-like	NW_013581236.1.27	protease
27	CG8102-like	NW_013582722.1.1	sperm protein
28	CG9168-like	NW_013581262.1.39	unknown function
29	CG9975-like	NW_013582087.1.1	unknown function
30	Cpr51A	NW_013581314.1.29	chitin binding
31	Cpr67Fb	NW_013584326.1.1	chitin binding
32	Egm	NW_013581265.1.12	oxidative stress response
33	Est-6	NW_013581551.1.20	lipid metabolism
34	Hexo2	NW_013581256.1.14	carbohydrate metabolism
35	mfas-PB	NW_013581231.1.21	signal transduction
36	NUCB1	NW_013581534.1.9	calcium binding
37	Peb	NW_013581247.1.21	post-mating behavior
38	Peritrophin-A	NW_013581236.1.13	chitin binding
39	Phm	NW_013583124.1.1	protein modification
40	regucalcin	NW_013581216.1.74	defense/immunity
41	Spn1	NW_013581585.1.8	protease inhibitor
42	trx	NW_013581231.1.1	DNA interactions
43	Or82a	NW_013581351.1.3	odorant binding

Table 3.3: Annotated genes of *B. oleae* based on the homology of known seminal fluid proteins in *D. melanogaster* (Mueller et al., 2004).

reproductive proteins compared to other protein classes and the divergence between different organisms (Begun et al., 2006).

The annotated genes obtained from this procedure encode proteins that belong to the conserved functional classes such as proteases and proteases inhibitors, lipases, sperm-binding proteins and antioxidants (Mueller et al., 2004). Four sperm proteins were annotated including the testes-specific protein betaTub85D. Interestingly, an odorant binding receptor, or82a, seemed to be annotated, indicating the involvement of the olfactory system in reproduction.

3.3 Expression analysis of selected genes

3.3.1 Validation of the RNAseq and expression profile of selected loci

In order to validate the differential gene expression between virgin and mated insects that was determined by RNAseq analysis, qRT-PCR was performed for selected genes. The genes were selected based on their differential expression data from RNAseq.

3.3.1.1 Testes

Testes			
Gene	**LogFC**	**Primers**	**product size**
c15699	8,750	5'-CGAGAATATAAACGAACCTG-3' 5'-ATCACTTCAACTCTCTCTGTC-3'	150
c58283	8,639	5'-AGTGAGTGATCCTGTACTGTC-3' 5'-TCGGTATACTCTACCTATCCAC-3'	98
mucin	8,167	5'-CCAACCGACACAACGAAAGG-3' 5'-TGGCAAAGCCGCCAAAATAC-3'	125
hemolectin	7,629	5'-CCAAATGCACAATTCACCAC-3; 5'-GCATCGTTCAGCACATATCC-3'	140
c37552	7,480	5'-AGCGAAATAGTCCAGTTAGGTG-3' 5'-CCACACCAAACGATTACGGC-3'	82
cation transporter	6,597	5'-ACTAAGTTTGGGTGTAACCG-3' 5'-GTGATACTTTCCGTAGTTTG-3'	120
c42518	5,468	5'-GGCACCACATAAACTCTAAC-3' 5'-TGCACTCCGCTAATTGCC-3'	100
scribbler isoform J	-3,327	5'-GGTTTACTCCTTGCGTTGCC-3' 5'-CGGACCTCAAAACGATGCAC-3'	83
c52071	-4,764	5'-GCGCTTCATCATCCACAGAC-3' 5'-CGCTGTTAATACGCCACGC-3'	135

Table 3.4: List of the analyzed for the validation of the differential expressed genes from the RNAseq result. The name used is based on their homologue in *D. melanogaster*. Genes that have no hits are presented with their transcript name. Positive value of logFC represents the overexpression of the genes in mated flies while negative value of logFC represents the overexpression of the genes in virgin flies. The primers used for the qRT-PCR experiments and their product size are presented.

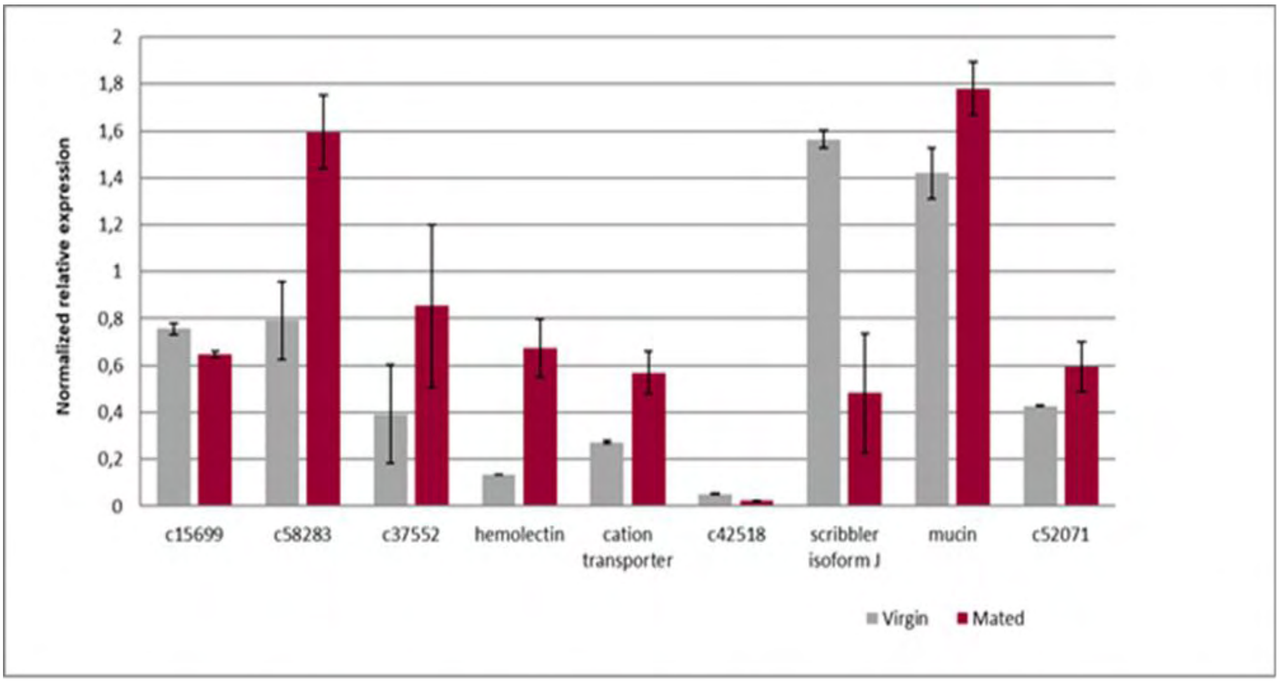


Figure 3.7: Validation of the expression difference between mated and virgin insects. Mean values ± standard error of data from three biological replicates is shown.

For the validation of the RNAseq we isolated testes from 3 day-old virgin males (virgin) and from males 12 hours after one mating (mated). Each sample used for qRT-PCR validation consisted of pooled tissues from ten insects and three biological replicates. Total RNA was extracted and qRT-PCR was performed for each sample. The appropriate housekeeping gene that was used for the normalization of the results was *rpl19* (S6.8) for male tissues, as it was demonstrated in Sagri et al., 2017. Differential gene expression between virgin and mated insects was determined by comparing the expression of the gene of interest in virgin insect on DAY 7 with its expression in the mated insect. For testes, primers were designed for the analysis of nine genes (Table 3.4).

In Table 3.4, positive logFC values correspond to overexpression in mated insects, whereas negative values correspond to down regulation. qRT-PCR results were consistent with those of RNAseq in 7 out of 9 genes in mated insects. The genes *c58283, c37552, hemolectin, mucin* and *cation transporter* showed overexpression in mated insects while the *scribbler* showed down-regulation (Figure 3.7). Instead, qRT-PCR results for *c15699* and *52071* were not consistent with RNAseq results, whereas *c42528* showed very low expression.

3.3.1.2 Male accessory glands with ejaculatory bulb

We isolated male accessory glands including the ejaculatory bulb from 7-day old virgin males and from mated insects twelve hours after one mating. Each sample used for qRT-PCR validation consisted of pooled tissues of ten insects and three biological replicates. Total RNA was extracted and qRT-PCR was performed for each sample. Primers were designed for the analysis of six genes (Table 3.5). On Table 3.5 negative logFC values present overexpression in mated flies while positive logFC values represent overexpression in virgin flies. Validation of the RNAseq results through qRT-PCR confirmed the overexpression of all 6 genes: *timeless*, *c52416*, *CG2254-like*, *brunelleschi*, *yellow-g* and *c53574* (Figure 3.8). *rpl19* (S6.8) was used as a housekeeping gene for the normalization

Male accessory glands and ejaculatory bulb			
Gene	LogFC	Primers	product size
brunelleschi	-9,529	5'-AAGCGAGGTAACACTACGGC-3' 5'-GATTACCGTTTGTGGCAGCG-3'	75
CG2254-like	-12,304	5'-TATGCACATATGTATGCACATGAAA-3' 5'-ATGTTGCGCGCGTCTTTAG-3'	73
timeless	-11,708	5'-TGGCGGCGGACGTATAATAG-3' 5'-AAGTGCTCCCGTAGTTGGTG-3'	87
c52416	-9,696	5'-CGCTGTCACCACTGACTATGGC-3' 5'-TCCTCTGTCACCAGCTCAGAAAC-3'	111
c53574	-11,667	5'-GCATTTGCTGGCGCTTATCA-3' 5'-GCACAAACGGAAAGATGGCA-3'	112
yellow-g	-10,490	5'-TTGCGTGTTGGACAGGGTGC-3' 5'-AATTCGTGCCACCATCGGCG-3'	97

Table 3.5: List of the genes analyzed from the tissue of male accessory glands with the ejaculatory bulb. The name used is based on their homologue in *D. melanogaster*. Genes that have no hits are presented with their transcript name. Positive logFC value presents overexpression of the gene in mated flies while negative logFC value presents overexpression of the gene in virgin flies. The primers used for the qRT-PCR experiments and their product size are also shown.

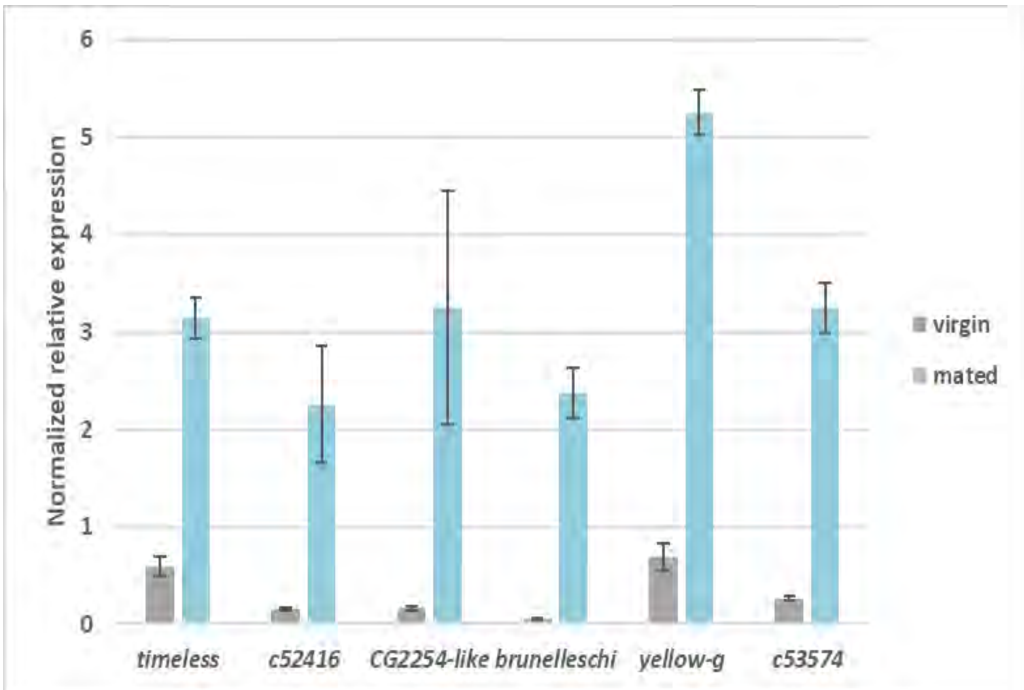


Figure 3.8: Validation of the expression difference in the male accessory glands with ejaculatory bulb between virgin and mated insects. Mean values ± standard error of triplicate data from three biological replicates are shown.

of the results.

Furthermore, we determined the expression profile of the selected genes in the reproductive tissue from the first day of the insect eclosion (DAY 0) to the 7th day (DAY 7). If a gene codes for a protein in the seminal fluid that is important for mating, it should be expressed earlier so that the protein will be present at the time of mating.

In Figure 3.9 the expression profiles of the selected genes are presented. Interestingly, the highest expression of most of the genes was detected in different days before the selected day for dissection. The genes *timeless, c52416* and *c53574* show high expression on the DAY 0 while the rest days the expression is stable. The genes *brunelleschi* and *yellow-g* showed their highest expression in virgin males on DAY 5 and the gene *CG2254-like* on DAY 6.

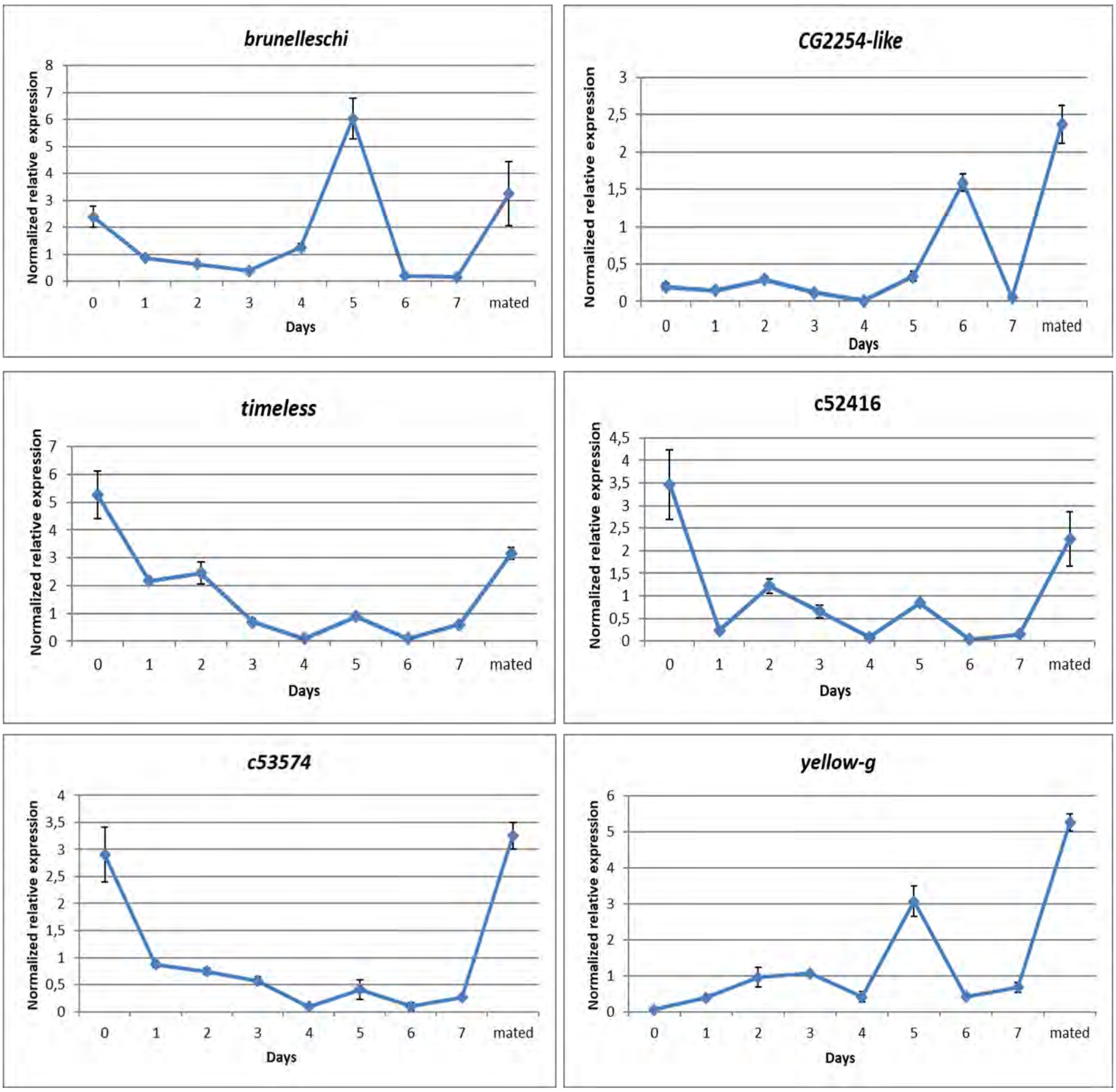


Figure 3.9: Expression profiles of the selected genes from the first day of the eclosion (DAY 0) until DAY 7. Mean values ± standard error of triplicate data from three biological replicates are shown

3.3.1.3 Female lower reproductive tract

For the validation of the RNAseq results of the lower female reproductive tract, we isolated tissues from 7-day old virgin females and from mated females, twelve hours after one mating. Primers were designed for the analysis of 6 genes in the female lower reproductive tract and qRT-PCR was performed for each sample (Table 3.6). Each sample used for qRT-PCR validation consisted of pooled tissues from ten insects and three biological replicates (Figure 3.10). The housekeeping gene used for the normalization of the results was GAPDH (S6.8) for the female tissues (Sagri et al., 2017). The results of the qRT-PCR showed that *troponin C* had high expression (18 fold) in virgin flies in contrast to the RNAseq results. The *glutathione S-transferase epsilon class* gene showed very low expression. The other four genes, *lingerer*, *yolk prote in-2, bestrophin-2* and *ornithine decarboxylase antizyme* were confirmed as they showed overexpression in qRT-PCR.Moreover, the expression profile of the selected genes was determined in 7-day old virgin females and at five time points (0, 3, 6, 9, 12, 24 h) after mating (Figure 3.11). Expression profiles of the 6 genes differ. The gene *troponin C* showed limited expression after mating. The *ornithine decarboxylase antizyme* showed an increasing expression with the highest at 24 hours after mating, while *bestrophin-2* (10-fold) and *lingerer* (10-fold) showed highest expression at 12 hours. The *yolk protein-2* showed 2-fold overexpression 9 hours after mating and *glutathione S-transferase* showed highest expression immediately after mating (0 Hours). This may indicate the different roles each gene could demonstrate in the female reproductive system.

The next step was to investigate the functional role of selected genes to identify if they play a role in the mating procedure or the post-mating response of the female. To this end, we performed silencing through RNA interference (RNAi) and we recorded: 1. their behavior during mating and 2. the female post mating responses that may include oviposition rate, sex ratio of the progeny, longevity, and total number of eggs laid.

Lower female reproductive tract			
Gene	LogFC	Primers	Product size
troponin C	-14,991	5'-AAAACCAAGCCCATCCACC-3' 5'-GCGATTTGTTCGGGAGTCAG-3'	98
yolk protein-2	-14,608	5'-CGCGTATAGCCTAAAACCCAC-3' 5'-TGCAGGGTGATATCCTCCAC-3'	80
lingerer	-11,727	5'-CGCGTATAACTCGAGCGACTCC-3' 5'-GCGGCAGCTAATCGTCAATGC-3'	124
glutathione S-transferase epsilon class	8,820	5'-ATGGCTTACCTGTCTAAATG-3' 5'-GTTTATTCCTCACTTTCACC-3'	114
bestrophin 2	-11,717	5'-AGGACATCCGACAACAACGGC-3' 5'-ATATTTGTGGTGACGGGCGCAG-3'	105
ornithine decarboxylase antizyme	-14,298	5'-ACGTTGCAATGCCTGACAAG-3' 5'-AACAACTGCGTCGACATCCA-3'	101

Table 3.6: List of the genes analyzed from the lower female reproductive tract. The name used is based on their homologue in *D. melanogaster.* Positive logFC value represents overexpression of the genes in mated flies while negative logFC value represents overexpression of the genes in virgin flies. The primers used for the qPCR experiments and their product size are also shown.

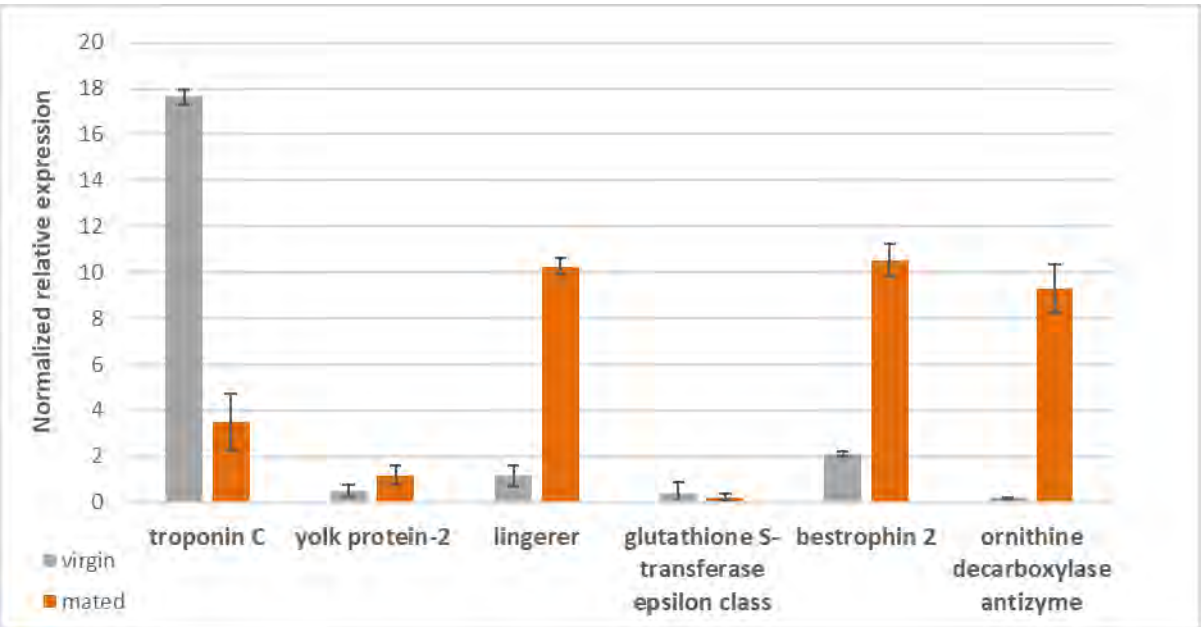


Figure 3.10: Validation of the expression difference between virgin and mated insects. Mean values ± standard error of triplicate data from three biological replicates are shown.

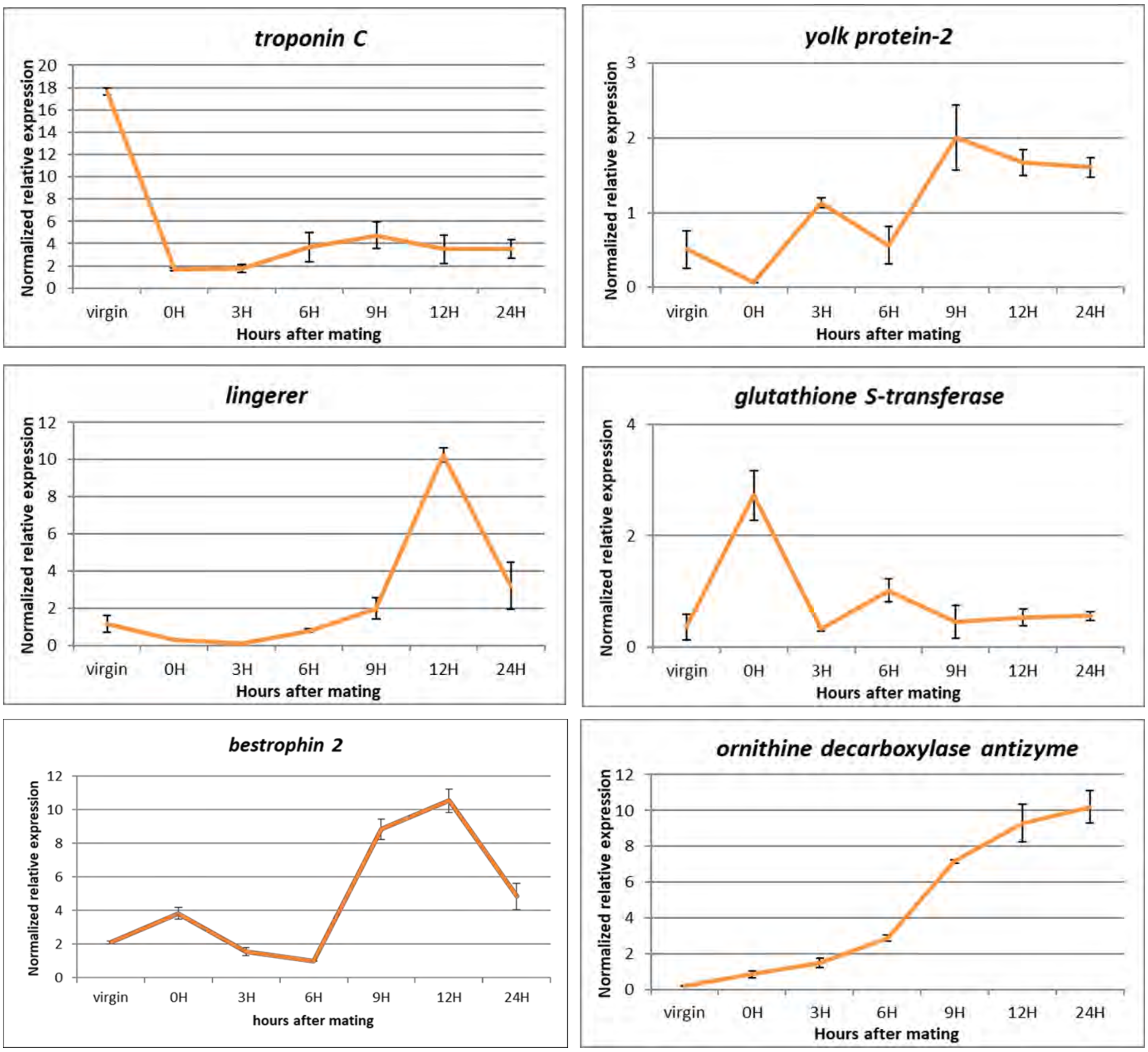


Figure 3.11: Expression profile of the selected genes from the virgin flies and several time-points after mating (0,3,6 9, 12, 24hours). The error bars show the standard error of the mean between the three biological samples.

3.4 Gene silencing through RNAi

RNAi technology was used to induce gene silencing. This was done with two different ways: through injections to the insects of dsRNA and through feeding the insects with dsRNA producing bacteria.

3.4.1 Gene silencing through injections

For the RNAi silencing we chose two genes based on the logFC value from the RNAseq, the *yellow-g* for the tissue of male accessory glands with ejaculatory bulb and the *troponin-C* for the female lower reproductive tract. Even though *troponin-C* was not confirmed by the qRT-PCR, the high expression of the gene made it a good candidate for the silencing experiments. The first step for the RNAi was to produce the dsRNA through *in vitro* transcription using T7 polymerase. Primers were designed using the E-RNAi web application (Horn et al., 2010) that contained the recognition site for T7 RNA polymerase.

3.4.1.1 Silencing of *yellow-g* gene

For the yellow dsRNA synthesis, we isolated a 498 bp clone of *yellow-g* cDNA through PCR amplification. The primers with the respective recognition site for T7 RNA polymerase (small letters) were: ds_yellow_F 5′-taatacgactcactataggg-CATTTACGTCCAATCCGGTC and ds_yellow_R 5′-taatacgactcactataggg-TCGCCGGCTATACGTAGA (Figure 3.12). A green fluorescent protein (GFP) gene clone was used as template to synthesize the respective dsRNA used as a non-target control. The control gene was a GFP fragment that is not present in the *B. oleae* genome. The sequence was amplified using GFP-forward 5′-taatacgactcactataggg-CCGCCAGTGTGCTGGAA-3′ and GFP-reverse 5′-taatacgactcactataggg-GATATCTGCAGAATTCGCC-3′ through PCR reaction and isolated for *in vitro* transcription.

ATGCAACTGCAGCTGCACACCAACTTACAACAACAAATCCCGCTAATACTCTTTGCTGCCACACTTGCCACCCTACTCCG
GCCCACACCGGCTAGCGCCGCCTACTCCGCTGATTTGCACGATGACCCTGCTGACGTAGCCGACACCGCTGCTGCTGAC
TATGACAGCTACGGCCACCACAGCAACAAGTATGTGAGTAAGTGCGATAAAAATCCATTGGCTGCACTCACCTTCCAGC
ds_yellow_F →
TGAGCGGCAACAACCTGCAGTGGCCTTGCGAGTCGACCAAGAGCATTTACGTCCAATCCGGTCGGTATGTGCCGCGCA
ATGTGATCGTGACGCGCGCCCAACTGCGACGTGATGACGCCTTTGTGGCACTACCGCGCTATAAACAGGGTGTGCCATT
TACATTGGGTCGCGTGCAATTGAAGCGGGGTCAGTGTGTGGCCAAGATTGCGCCATATCCGTGCTGGGCCATACAGGA
GGAGGGCAATTGTCAGGCGTTGCAGTCCGTCGTCGACATCGCTGTGGATCCCAATGGTCTCCTCTGGGCTTTGGACGTG
GGACTTGTGAACACATTGGAACAACCGATACGCCGTTGTGGACCAAAAATTGTAGCAATCAACACGGCTGACAACAAA
GTAGTAAAGATCATCGACCTGAGTGATCTCGTAACGGCTGAGTCCCGCTTGCAGTTCATAGTGGTGGACTACTCCAAGG
← ds_yellow_R
ACAACAAGCCGTTCGTATATGTTGCTGACGCCGGAGCACGCAGCATACTCGTCTACGATATAGCCGGCGACAAATCGTA
TCGCATCGTTTTACCTAAAGCCACTGCACCTACTACCACCGATGTGCTTTATATGGCGCTCACCGCCACACCGGATGGCA
Bo_yellow_F →
CATCGACACTGTACTTCACGTATCTTAGCTCACCGCGTCTGTACTCCATACGTGGACAGTATTTGCGTGTTGGACAGGGT
← Bo_yellow_R
GCCGGTTCGATTGTCGATGTGGGCGCCAAGCCTTATGGCAAGCAAATGGTGCTGCTCGGCGCCGATGGTGGCACGAAT
TTGTTCTTCCGTTACAAGGGTGAAAACGACATATATATGTGGAACTCGGAGACTTGCTTCAAATCAGCCAATTTAAAAGA
TGTACAACATGGCGGTGATTGTCGGCTGTCGACACAGGTGCTGCCCGGCCATAGGCGCTTCATGTGGGCTTTGGAGAG
CAATTTCCATGATTTCATATCGGAACGAACGGGTTGCAATGGTGCATCGATTGTTCTGCATCCCGTAGTGCGCGAATGTA
ATGACTAAGGGTCGCAGTGAGATGGTAGTTTTAAGTGAATTGTTTGAAGTTTTTAAAAAATGTGTTTTTTTGTTTTTGAGT
TTACAAATTATGTTCGTCT

Figure 3.12: Partial sequence of the potential yellow-g gene of *B. oleae.* Green sequences and arrows: the primers used for qRT-PCR, Yellow sequences and arrows: the primers used for the ds-RNA.

The amplified products were visualized and retrieved after agarose gel electrophoresis. *In vitro* transcription reactions were performed using the Ambion MEGAscript Kit. The resulting dsRNA was isolated and diluted to a final concentration of 10 μg/μl. The dsRNA quality was assessed by agarose gel electrophoresis (Figure 3.13).

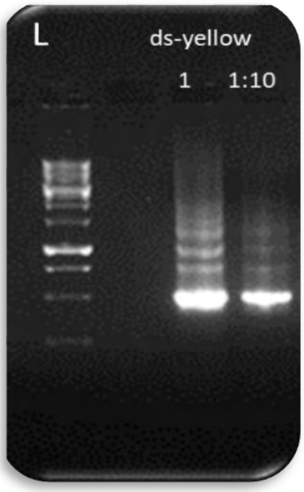


Figure 3.13: Electrophoresis of the yellow dsRNA

After the preparation of the dsRNA and according to the expression profile of the *yellow-g* gene, we injected male insects based on the following principles:

- After the eclosion of the flies, we separated males from females to different cages.
- On DAY 0 we injected male flies with ds-*yellow-g*.
- In order to detect the effect of silencing, samples were collected on the day of the highest expression (DAY 5) and on the day of the mating experiments (DAY 7).
- The experiments were repeated for the control GFP ds-RNA.

Each biological sample consisted of pooled tissues from ten insects and three biological replicates were performed. Yellow knockdown efficiency was assessed by RT-PCR using the Bo_yellow_F and Bo_yellow_R specific primers (Figure 3.12). Specifically, we dissected male accessory glands with ejaculatory bulb from ten insects of two biological replicates. Maximum reduction occurred on DAY 7 at 81% while on DAY 5 the expression of the gene was reduced at 46% compared to ds-GFP control flies (Figure 3.14).

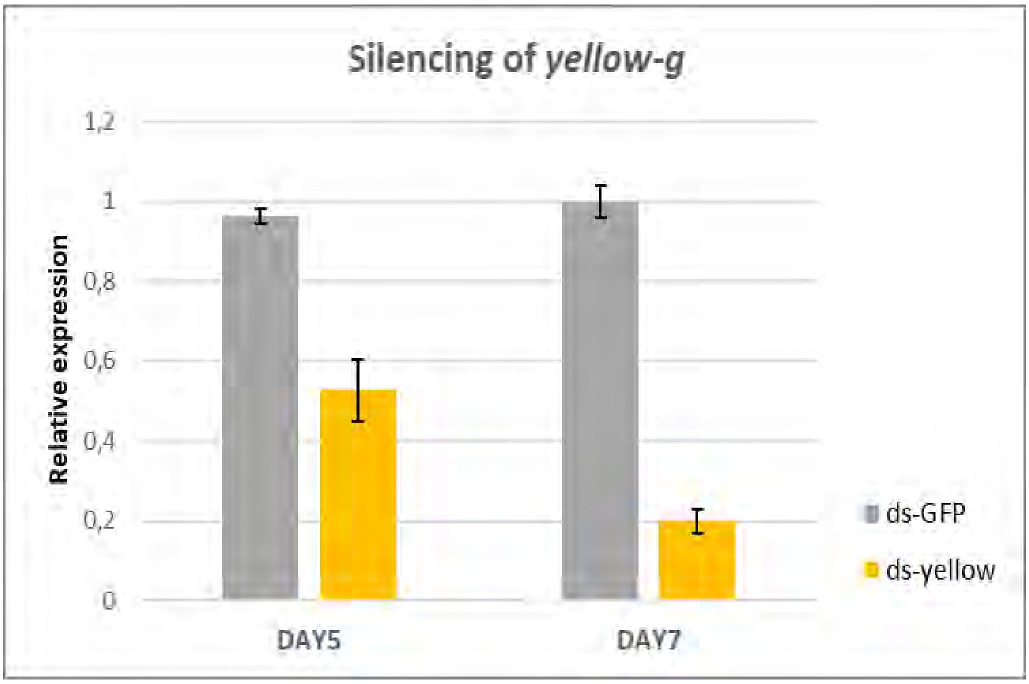


Figure 3.14: The yellow bars show the silencing of the *yellow-g* gene. The grey bars show the expression of the gene in the control group. Mean values ± standard error of triplicate data from three biological replicates is shown.

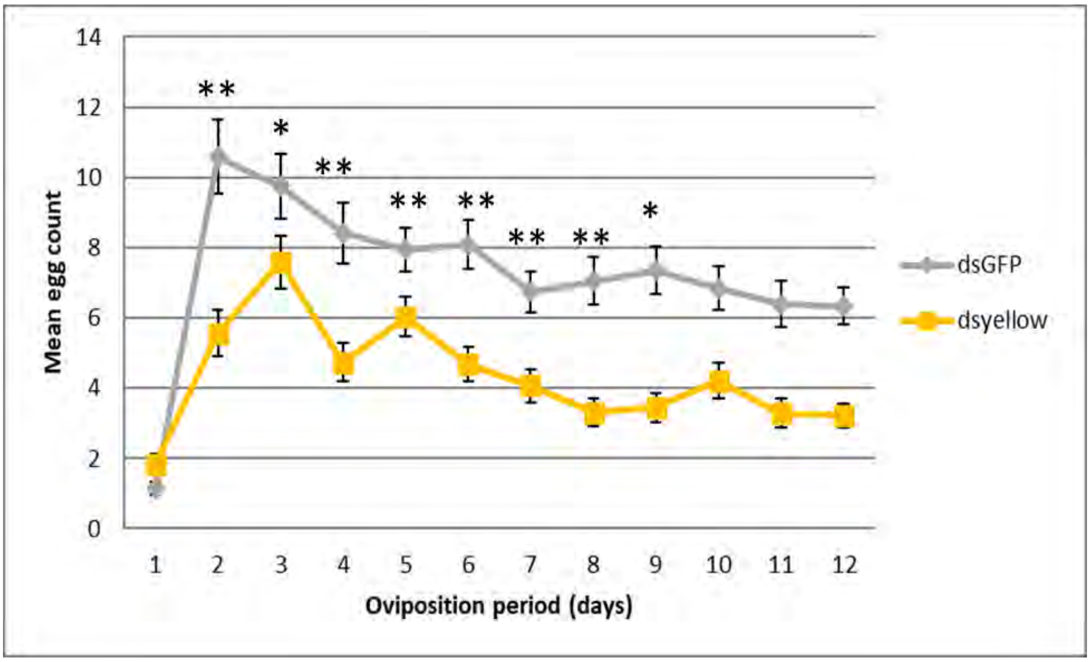


Figure 3.15: The mean daily egg count for the females mated with males injected with ds yellow (yellow line) and males injected with ds-GFP (grey line). The * indicates statistical significant difference for p value < 0.05.

The silencing percentages show the successful silencing of the gene of interest. However, to distinguish whether the transient silencing of *yellow-g* had an impact on the reproduction we performed mating experiments with virgin female flies.

Specifically, two groups of 30 male insects were injected with ds-yellow and ds-GFP, respectively, were mixed with virgin female flies of the same age and were allowed to mate. Successful mating should last >1hour (Zervas et al., 1982). Each one of the mated female flies was placed in an isolated cage where we recorded the oviposition rate of the insect. The mean daily egg count is presented in Figure 3.15.

The egg laying rate for the group of females which mated with ds-*yellow-g* injected males is characterized by a peak egg laying on day 3 followed by a regressive phase, with the lowest counts recorded on day 12. In addition, the number of eggs laid from the same group was significantly lower for the second day until day 9 compared to control females. The lower oviposition rate of the females may indicate that *yellow-g* gene may encode a protein that is part of the seminal fluid and triggers the mated female to start oviposition.

Moreover, we recorded the sex ratio of the offspring in an attempt to distinguish if the transient silencing of the gene may influence the sex differentiation cascade of the progenies. However, no significant difference was detected (Figure 3.16). The offspring of the male insects injected with ds-*yellow* had similar sex ratio compared to the control group.

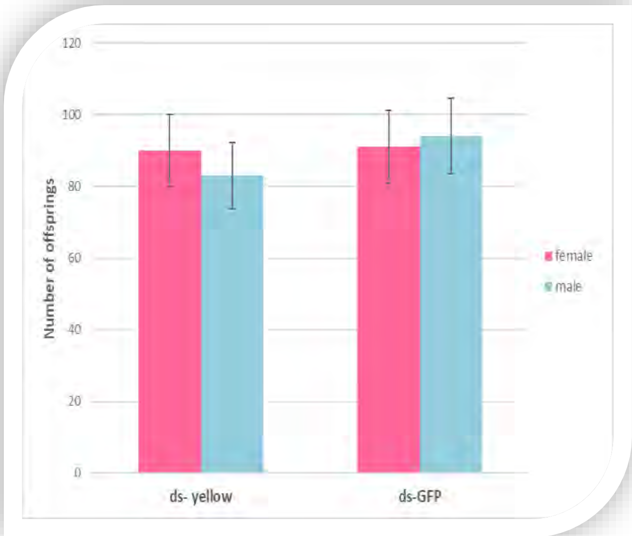


Figure 3.16: Sex ratio of the offspring. The pink bar represents the number of female insects and the grey bar represents the number of male insects. There is no significant difference for p value < 0.05.

3.4.1.2 Silencing of *troponin C* gene

For the *troponin C* dsRNA synthesis, we isolated a 454bp clone of *troponin C* cDNA through PCR amplification. The primers used for the ds-RNA amplification were: T7_troponinC_F 5′-taatacgactcactataggg-GATGAGGATCTGACTCCCGA and T7_troponinC_R 5′-taatacgactcactataggg-ATTCGCCAGTCATCATTTCC (Figure 3.17). The non-target control was the same as above. Following the *in vitro* transcription, we isolated the ds-RNA and diluted it to a final concentration of 10μg/μl.

To define the most appropriate day to perform the injections of the female flies we determined the expression profile of the gene of interest from the first day after eclosion (DAY 0) until DAY 7. Each biological sample consisted of pooled tissues from ten insects and three biological replicates were performed The dissected tissues were the lower female reproductive tract. The qRT-PCR was performed using the pair of primers used

AGTATCGCCCGCTGAAGAGCCCAATTGTCCGTCTGTGAGGCAAAGTAAAAGTCAGTTTTGAA
Bo_troponinC_F
AAATCAAATCAAAAACCAAGCCCATCCACCGGACATCACCGCAAACACACAACCAAGCCAAT
Bo_troponinC_R
ATGAGTGATACATTTATTGATGAGGATCTGACTCCCGAACAAATCGCCGTGTTGCAAAAGGC
T7_troponinC_F
CTTCAACAGTTTCGATCACCAAAAATGCGGCAGCATCTCAACTGAAATGGTCGCTGATATTTT
GCGCCTCATGGGTCAGCCCTTTGACAAGAAGATCTTGGAGGAGCTCATCGATGAAGTTGATG
AAGACAAGTCTGGTCGCTTGGAATTCGAGGAATTCGTTCAACTTGCCGCCAAATTCATCGTTG
AGGAGGATGACGAAGCCATGCAAAAGGAATTGCGTGAAGCCTTCCGTCTGTACGACAAACA
AGGCAATGGCTACATTCCTACCTCTTGCTTGCGTGAAATCTTGCGTGAATTGGACGATCAGCT
GACCAATGAAGAGTTGGACATCATGATTGAGGAAATCGATTCTGATGGTTCCGGCACCGTCG
T7_troponinC_R
ATTTCGATGAATTTATGGAAATGATGACTGGCGAATAAATATTTATTTTAATACCTAAATACTA
GAAAGTTTTTCATCTTGTATATATACACCTAAATACAATTGTAGCTAAATACCTAATACACTCA
CACACAAATACACATGTAAATACCAGAACATCGAATGGCGGTATATTAACACCGCAATGGCA
ACCTTGCCAGAAAACAGATATGTACTTACGTATATAAACACATACACCTTATTTATTGTATAAT
GTAACAGAGCTTTGGCAAATAAATTTGTTTTATCTTCTTTTTGTAAAAA

Figure 3.17: Partial sequence of the potential *troponin C* gene of *B. oleae.* Blue sequences and arrows: the primers used for qRT-PCR, Red sequences and arrows: the primers used for the ds-RNA. The green sequence is common for the primers Bo_troponinC_R and T7_troponinC_F.

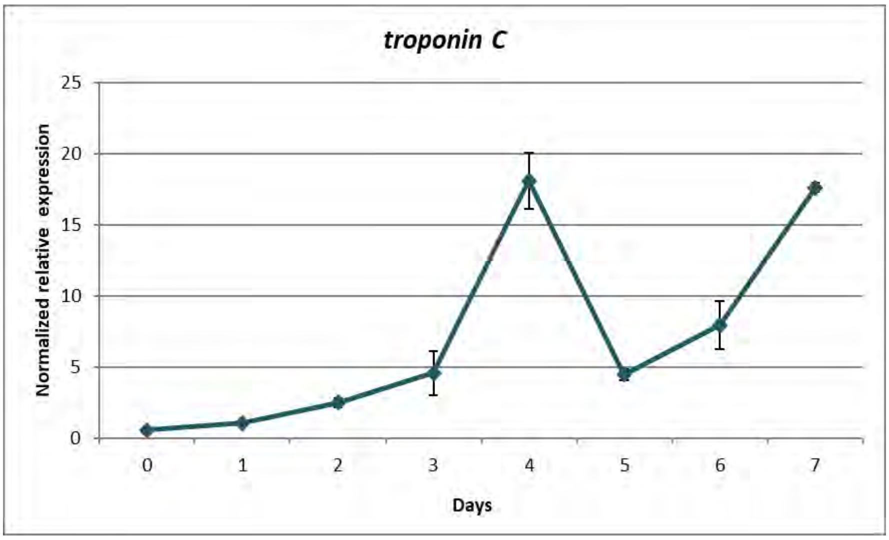


Figure 3.18: Expression profile of the *troponin C* gene from the first day of eclosion (DAY O) until the DAY 7. The error bars show the standard error of the mean between the three biological samples.

for the expression profile from mated female insects Bo_troponinC_F and Bo_troponinC_R.

Based on the expression profile, the highest day of expression for *troponin C* gene was the DAY 4 (18-fold) (Figure 3.18). As the expression of the gene starts from the first day of the insect, we injected the female flies on DAY 0.

For the injection experiment we set the following principles:

- After the eclosion of the flies, we separated males from females to different cages.
- On DAY 0 we injected female flies with troponin C ds-RNA.
- In order to detect the effect of silencing, samples were collected on DAY 4 (highest expression) and DAY 7 (mating experiments).

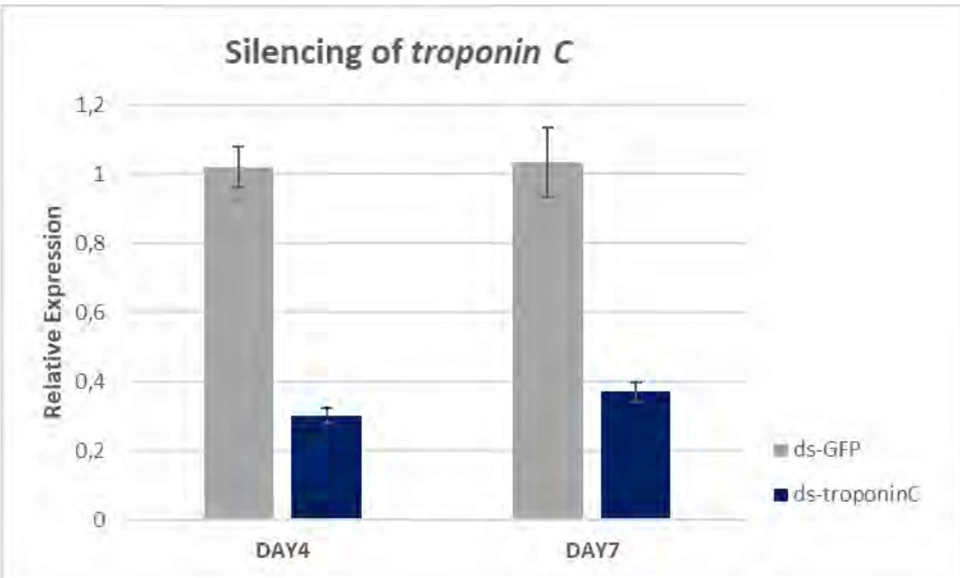


Figure 3.19: The blue bars demonstrate the silencing of the *troponin C* gene. The grey bars represent the expression of the gene in the control group. Mean values ± standard error of triplicate data from three biological replicates is shown.

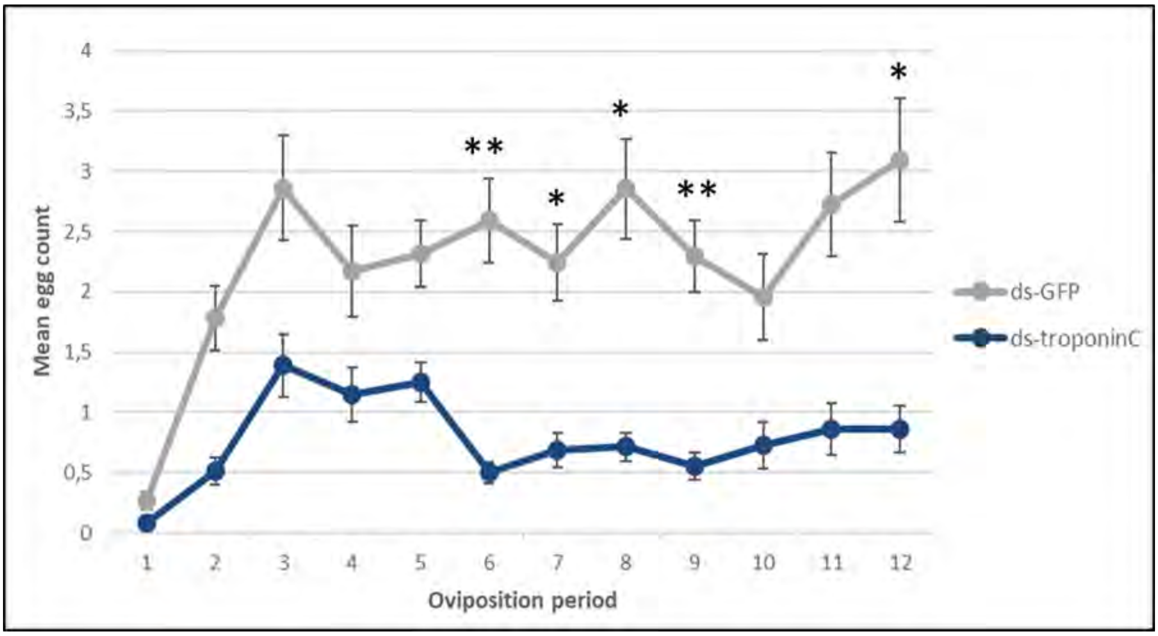


Figure 3.20: The blue line shows the oviposition rate of the females I injected with ds- troponin C and the grey line shows the oviposition of the control group.

- On DAY 7 injected females were mixed with male insects of the same age and were allowed to mate.

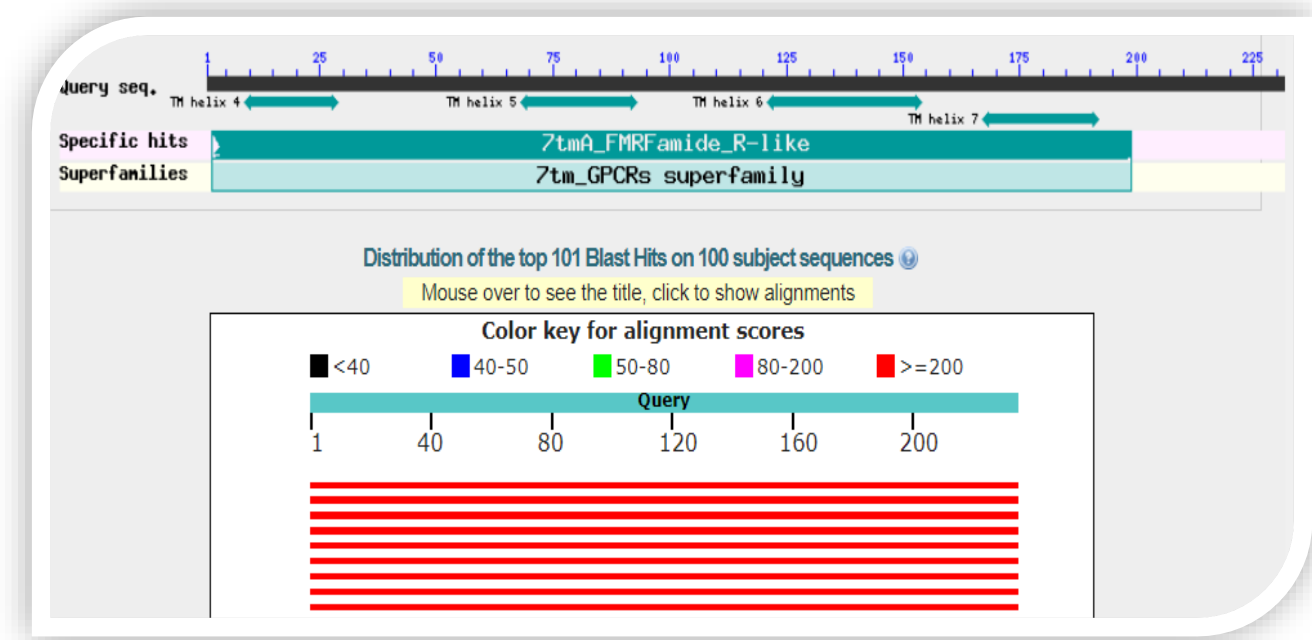
The efficiency of the gene silencing by RNAi was evaluated by qRT-PCR. Maximum reduction was assessed on DAY 4 (70%) and it was maintained till at least DAY 7 (64%) (Figure 3.19). To determine if this degree of silencing is sufficient to affect the post mating response, we recorded the oviposition rate of 36 females injected with ds-yellow compared to 36 females injected with ds-GFP (control group).

Based on the results of the oviposition rate, the females injected with ds-troponin C laid statistically significant fewer eggs compared to the control group from DAY 6 until DAY 11 (Figure 3.20). Specifically, the total oviposition of the females injected with ds-*troponin C* was 279 eggs in contrast to the control flies where the total number of eggs was 795. This may indicate that troponin C may contribute to the post mating response of female insects by muscle constructions that could help egg laying.

3.4.2 RNAi silencing through feeding

To perform RNAi silencing through feeding in *B. oleae* we selected a gene target, the *sex peptide receptor* (*spr*), that has a well demonstrated role in the reproduction of other insects such as *D. melanogaster* (Avila et al., 2015) and *B. dorsalis* (Zheng et al., 2015). The *sex peptide receptor* and the *sex peptide* are the regulators of the post-mating behavioral switch in *D. melanogaster* (Yapici et al., 2008).

3.4.2.1 Cloning of partial CDS of the potential B. oleae sex peptide receptor (spr) gene



	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	sex peptide receptor [Ceratitis capitata]	397	397	100%	4e-135	85%	XP_020712820.1
☐	sex peptide receptor [Drosophila melanogaster]	367	367	100%	9e-127	73%	ADO40410.1
☐	sex peptide receptor [Drosophila hydei]	367	367	100%	2e-126	74%	XP_023175892.1

Figure 3.21: BLAST results of the *B. oleae sex peptide receptor* sequence. The sequence shows 85% similarity to the sex peptide receptor of *C. capitata*, and 73% of *D. melanogaster.*

In order to identify the *spr* sequence of *B. oleae*, the genomic scaffolds of the olive fly were queried with the protein sequence of the *D. melanogaster* SPR and gave hits in specific scaffolds. The final sequence was obtained after the confirmation through reciprocal BLASTp (e value $<10^{-10}$) hits in NCBI nr database to sequences belonging to known *spr* genes (Figure 3.21).

Based on the obtained sequence, primers were designed (Bo_SPR_F1/R1) to amplify a partial sequence of the target gene (Figure 3.22) through RT-PCR reaction using as template cDNA from female insects. The 666 bp PCR product was electrophoresed in an agarose gel (1%) and the product (Figure 3.23) was isolated and cloned into the MCS of the plasmid vector L4440. The L4440 plasmid has two T7 promoters, in inverted orientation, flanking the multiple cloning site. The same procedure was followed for the construction of the control plasmid for the experiments, using a GFP fragment that is not present in *B. oleae*. The constructs L4440-SPR and L4440-GFP containing the *spr* and *GFP* inserts, respectively, were verified by sequencing

Based on the sequencing results, the 666bp PCR product of the potential *spr* of *B. oleae* showed 75% identity with Dm_SPR. Using the UniProt database, it was shown that transmembrane helices TM 4-5-6-7 and the Bo_SPR sequence codes for the extracellular parts.

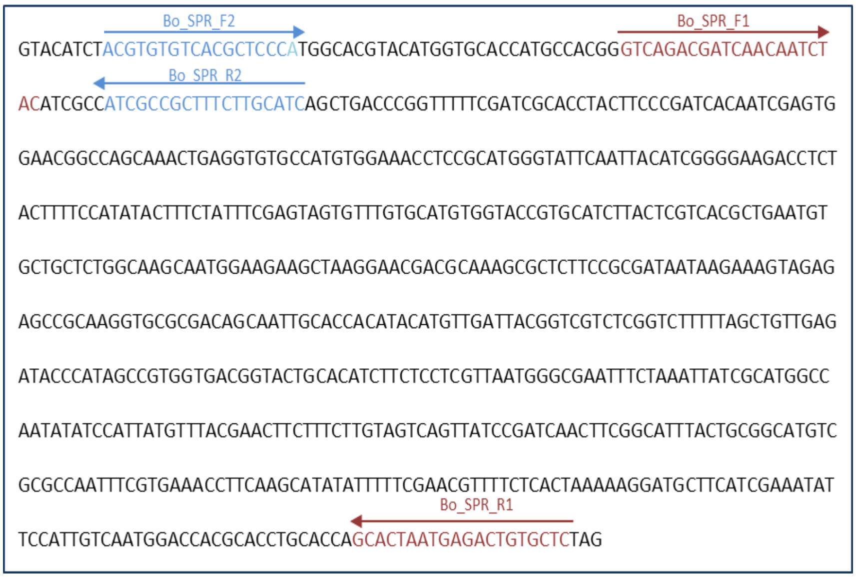


Figure 3.22: Partial sequence of the potential *B. oleae spr* gene. Blue sequences and arrows: the primers used for qRT-PCR, Red sequences and arrows: the primers used for amplification of the *spr* sequence.

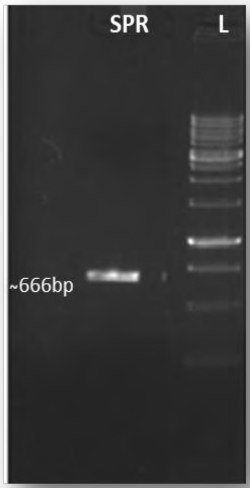


Figure 3.23: PCR amplification of sex peptide receptor (SPR) of *Bactrocera oleae*

Subsequently, we transformed HT115(DE3) competent cells with the feeding constructs. HT115(DE3) cells have IPTG inducible expression of the phage T7 polymerase and lack RNAse III, therefore dsRNA is protected from degradation. However, as it was the first time that this procedure was applied in B. oleae, we had to verify that the flies did, in fact, consume the given bacterial strain.

3.4.2.2 Ingestion of dsRNA-expressing bacteria induced RNAi

In order to verify that the bacteria are consumed by the adult olive flies, HT115(DE3) bacteria were colored by Coomassie Brilliant Blue Dye and fed to the insects. After 24 hours, the abdomen of the insect was turned blue and midgut dissections showed the blue color clearly, demonstrating that bacteria introduced in adult food are ingested by the olive fly (Figure 3.24).

3.4.2.3 Expression profile of the Bo_SPR

Given that RNAi feeding should start before the expression of the *spr* gene, it was important to determine the expression profile of the *spr* gene. We analyzed the expression profile of the Bo_SPR in the female insects and lower reproductive tract including spermathecae, uterus and accessory glands. We chose these tissues based on the experiments performed by Yapici et al., 2008

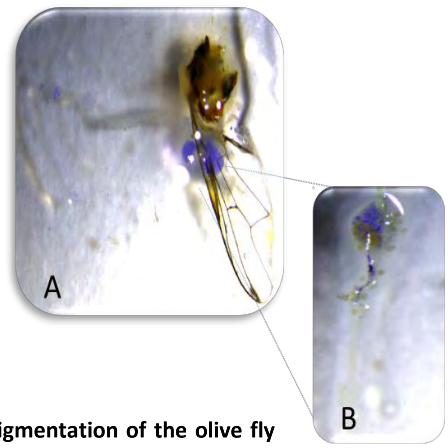


Figure 3.24: Pigmentation of the olive fly midgut. The abdomen of the fly turned blue (A), confirming the ingestion of bacteria. The dissected midgut is shown in (B).

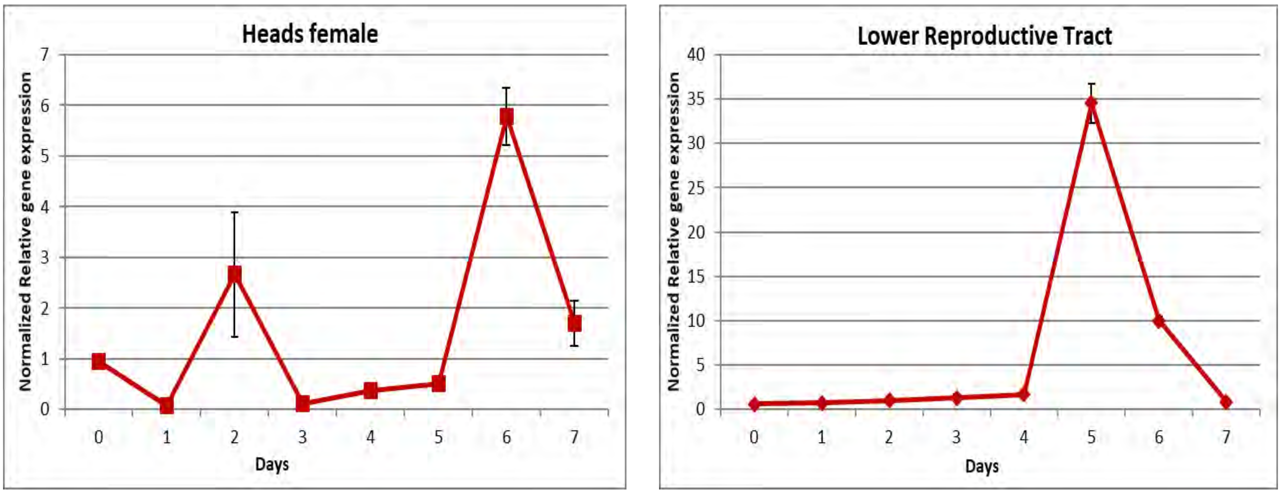


Figure 3.25: Expression profile of the *spr* gene in the head (A) and the reproductive tract of female *B. oleae (B)*

in *D. melanogaster* that revealed high levels of SPR expression in the female reproductive organs, in particular in the spermathecae, and the lower oviduct and in the female heads while SPR could not be detected in the male re productive organs.

Each biological sample consisted of pooled tissues from ten insects and three biological replicates were performed. Tissues from virgin females were isolated from their first day as adult insects (DAY 0) until the day the mating experiments occurred (DAY 7). DAY 7 was chosen as preliminary experiments showed that our laboratory strain is sexually mature one week after the emergence as adult. Total RNA was extracted and qRT-PCR was performed using the primers Bo_SPR_F2/R2. The housekeeping genes used for normalization were *rpl19* (S6.8) for the female heads and *GAPDH* (S6.8) for the female reproductive system based on the publication of Sagri et al., 2017.

The expression profile of the *spr* gene over a 7-day period in the reproductive system and in the female heads is shown in Figure 3.25. The two samples showed different expression profiles of the gene. In female heads, there is a 2.5x expression spike of the *spr* gene on DAY 2, while the highest expression (~6x) is on DAY 6. In the female

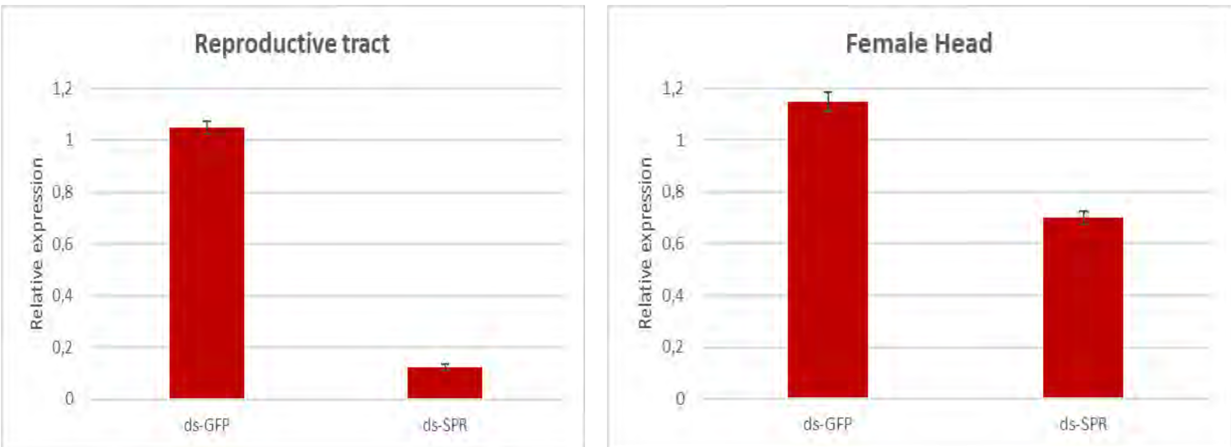


Figure 3.26: Left panel presents the silencing of the *spr* gene in the reproductive tract and right panel presents the *spr* silencing in the female heads. Samples were collected at DAY 5 of RNAi feeding. All experiments were performed in duplicate.

reproductive tract *spr* expression reaches a much higher level (~35x) on DAY 5. The expression profile of *spr* in the female reproductive system follows the pattern of the sexual maturation of the insect.

3.4.2.4 RNAi silencing of the Bo_SPR

Since our interest was on the post-mating response of the female flies, it was important that females remained virgin until the day of the mating experiment. For this reason, upon emergence females were separated from males and were kept to individual cages until the mating experiments. In order to achieve the maximum *spr* silencing effect, the ds-RNA containing food was changed daily (Li et al., 2015).

To guarantee suppression of any *spr* expression that could occur either before or after mating (and could consequently elicit the post-mating behavior), ds-RNA containing food was provided throughout the life of the insect (both before and after mating). Mating experiments were performed on DAY 7. Female flies fed with ds-RNA were allowed to mate once with virgin males and after the end of copulation each female was kept in a separate cage where oviposition could be followed. Control experiments using ds_GFP were performed in parallel.

To validate the percentage of *spr* silencing, samples were collected on DAY 5 (day of the highest *spr* expression in the female reproductive tract). Specifically, reproductive tract and head were dissected from ten insects, RNA was extracted, cDNA was synthesized and qRT-PCR was performed with the Bo_SPR_F2/R2 primers in two biological replicates. Maximum reduction occurred in the female reproductive tract at 90%, while on the female head *spr* expression was reduced at 40% compared to ds-GFP control flies (Figure 3.26). This indicates the successful inhibition of the *spr* gene through the method of RNAi feeding.

3.4.2.5 Phenotype of the RNAi silencing

The SPR protein is involved in the post-

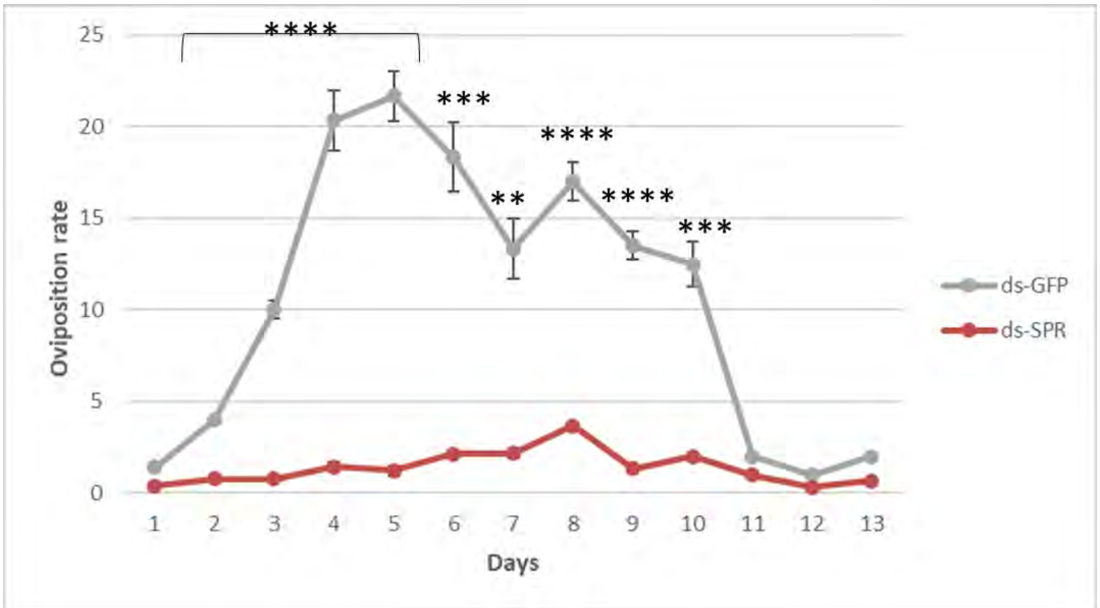


Figure 3.27: Oviposition rate of ds-GFP and ds-SPR fed flies daily. The * indicates statistical significance for p<0.05, ** for p<0.01, * for p<0.001, **** for p<0.0001.**

mating response of *D. melanogaster* flies, including increase in oviposition and decrease of female longevity (Avila et al., 2010; Wigby et al., 2005). To investigate if the silencing of the *spr* gene has a similar impact in olive fly, we tested the oviposition rate and the survival rate between the ds-SPR group and the ds-GFP group (control). Ten insects from each group were tested.

The oviposition rate of the insects fed with ds-SPR was lower than that of the insects fed with ds-GFP. Figure 3.27 shows that ds-SPR females oviposited significantly fewer eggs compared to control females from DAY2 until DAY11. This shows that sex peptide receptor plays a significant role in the oviposition rate of the insect females. Increased egg production is one of the post-mating responses that sex peptide induces to the females (Liu et al., 2003; Chapman et al., 2003). This may indicate that sex peptide (SP) has a similar role in reproduction for olive fly. changes (Figure 3.27).

Moreover, RNA silencing of the *spr* gene seemed to increase longevity of female insects (Figure 3.28). More than 50% of the females of ds-SPR were alive at the end of the experiment, while all ds-GFP females died by DAY 20. This observation comes to agreement with experiments in *D. melanogaster* (Chapman et al., 2003). They indicated that females continuously exposed to SP-deficient males had significantly higher lifetime span.

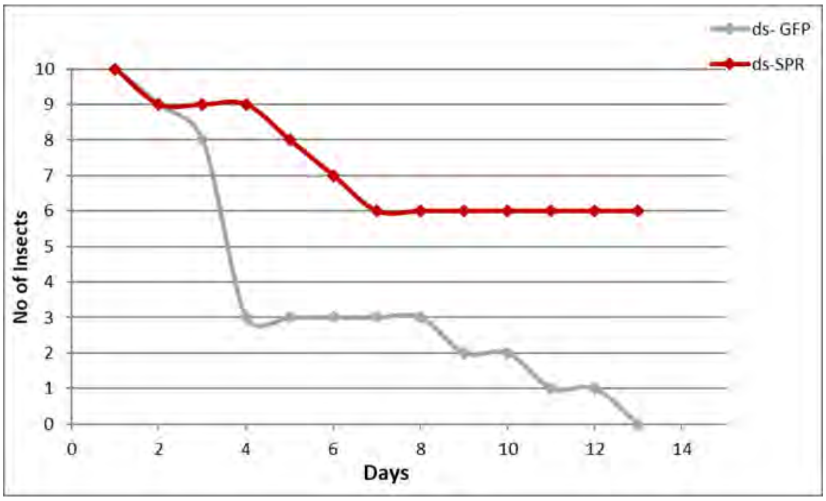


Figure 3.28: Survival of ds-SPR (red line) and ds-GFP (grey line) fed female insects. Female insects fed with ds-SPR lived longer than control insects.

3.5 Validation of olfactory differential expression in reproductive system

Odor recognition is a coordinated process requiring the combined specificities contributed by odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) as well as odorant receptors (ORs). Insect odorant-binding proteins (OBPs) are soluble proteins surrounding the extracellular lymph of olfactory neurons (Pelosi et al., 1995). OBPs are capable of binding and solubilizing small hydrophobic molecules from the environment and therefore transport them to the underlying ORs, which are expressed on peripheral olfactory receptor neurons. Insect ORs are either ionotropic receptors (IRs) or seven-transmembrane proteins (ORs) with an inverse topology compared to GPCRs, that form heterodimers of a ligand-binding OR and a ubiquitous highly conserved co-receptor named Orco (Vosshall et al., 2011). While OR expression in olfactory tissues is well-established, the distribution of ORs beyond the olfactory system has also been documented in different mammalian species (Vanderhaeghen et al., 1993; Vanderhaeghen et al., 1997), suggesting that ORs may play an important role in the ectopic expression of nonchemosensory tissues.

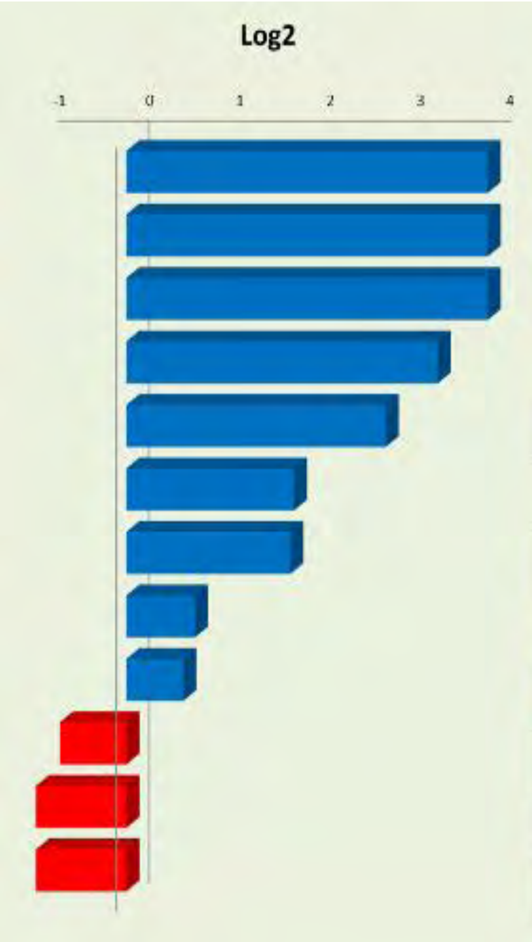
This comes to an agreement with several studies reporting the expression of such genes in the male accessory glands and testes of multiple species. OR expression has been documented in human and mouse germ cells (Spehr et al.,2003; Spehr et al., 2004; Spehr et al., 2006; Fukuda et al., 2004; Veitinger et al., 2011) and recently in mosquitoes (Pitts et al., 2014). Similarly, other non-olfactory functions have been reported for OBP-like proteins including the B proteins of Tenebrio molitor accessory glands (Paesen et al., 1995), the male-specific serum proteins of Ceratitis capitata (Thymianou et al., 1998), and the heme-binding protein of Rhodnius prolixus (Paiva-Silva et al., 2002). These studies demonstrate that OBPs are not restricted to olfaction and are likely to be involved in wide-ranging physiological functions having general carrier capabilities with broad specificity for lipophilic compounds (Foret et al., 2006).

With that in mind, we opted to explore the expression of various olfactory-related genes in the reproductive systems of *B. oleae*. In order to get a deeper insight into the involvement of olfactory genes in olive fly reproduction, the relative expression of five olfactory genes was further analyzed in female FAGs/spermathecae, male testes and male accessory glands (MAGs), before and twelve hours after mating. The primers used for the qRT-PCR are presented in Table 3.7. The classification of the genes under investigation is presented in Figure 3.29.

Primers	Sequences	Tm	Product size
obp8a	5'-AAGGCGAATACGGAAGTGC-3' 5'-CTGACCCACCTGACTGTTTAGC-3'	55	113
obp83a	5'-ACAGAGGAGGCAATTAAG-3' 5'-ATCACCGTTATCATCCAC-3'	55	114
obp19a	5'-AAGGAGGATTATCGCAAC-3' 5'-AATTAGAAGGGCATAAGACG-3'	55	89
os-d	5'-CCTGGACGAGGTTTTGAGC-3' 5'-TTGATATAGCGTCGGGGAGTATC-3'	55	121
or10	5'-AGCTCTTCAATTTCTTGTTGCTGT-3' 5'-CATCGCTTGAGCCATTCTTCG-3'	55	100
rpl19	5'-AACAAACGTGTACTGATGG-3' 5'-CACGTACTTTATGTCGTCTG-3'	55	138
1433z	5'-GGTCTAGCACTAAACTTTTC-3' 5'-TGAGTCTTTGTATGAGTCC-3'	55	103

Table 3.7: The primers used for the qRT-PCR of the olfactory genes.

Based on the qRT-PCR results, the *obp83a*, *obp8a* and *obp19a* genes are over-expressed in MALE tissue (Figure 3.30). The *obp83a* and *obp8a* are over-expressed before mating in testes while *obp83a* and *obp19a* are over-expressed after mating in FAGs/spermathecae (Figure 3.30). All three genes are characterized by a GOBP (general odorant binding protein) domain that is also found in their orthologues in Drosophila melanogaster. This structural domain is found in pheromone binding proteins, which exist in extracellular fluid surrounding odorant receptors (Vogt et al., 1991). The presence of these OBPs in the reproductive tissues implicates their interaction with other substrates except the olfactory system as transporters in the post-mating events in the male reproductive system. In fact, *D. melanogaster'*s *.obp8a* shows the highest levels of expression in male accessory glands (Arya et al 2010; Zhou et al., 2009) and has been associated with non-olfactory functions such as RNA transcription (Kodik et al., 1995). *os-d* is over-expressed in MALE tissue (Figure 3.29) while qRT-PCR showed similar expression patterns in mature FAGs/spermathecae, MAGs and testes, but no



Gene name	GO analysis (Biological Process)	GO analysis (Molecular Function)	InterPro (IP)
obp19a	Olfactory behavior	Odorant binding	GOBP domain
jump6	Chemosensory jump behavior	Odorant binding	POU-specific domain
os-d	Sensory perception of chemical stimulus	Odorant binding	Ejaculatory bulb-specific protein 3
obp56g	Sensory perception of chemical stimulus	Odorant binding	GOBP domain
or10	Sensory perception of smell	Odorant binding	-
obp83g	Sensory perception of chemical stimulus	Odorant binding	GOBP domain
obp99a	Sensory perception of smell	Odorant binding	GOBP domain
obp8a	sensory perception of chemical stimulus	Odorant binding	GOBP domain
obp83a	Sensory perception of chemical stimulus	Pheromone binding	GOBP domain
obp83ef	sensory perception of chemical stimulus	Odorant biding	GOBP domain
glr1k	Ion transport	Kainate selective receptor activity	NMDA receptor; inotropic glutamate receptor
glr2k	Ion transport	Kainate selective receptor activity	NMDA receptor; inotropic glutamate receptor

Figure 3.29: Functional annotation of differentially expressed olfactory genes in olive fly reproductive tissues. At the left part of the figure, the expression levels of the differentially expressed olfactory genes (Log2, fold change) are shown, as resulted from the RNA-seq analysis. The up-regulated genes in males are depicted in blue bars and the up-regulated genes in females in red bars. At the right part of the figure, the Gene Ontology (GO) classification of the same genes for the ontologies: Biological Process (BP), Molecular Function (MF) and Interpro (IP) protein domains is listed. Gene names are based on the nomenclature of the Drosophila melanogaster homologues.

expression in MAGs before mating (Figure 3.30). Os-D is a chemosensory protein (CSP) that encodes the antennal protein 10 in *D. melanogaster*. CSPs are secreted in the sensillum lymph of insect chemosensory sensilla and some OS-D like proteins bind short to medium chain length fatty acid derivatives with low specificity (Nagnag-Le et al., 2000; Jacquin-Joly et al., 2001). Their specific function remains uncertain (Wanner et al., 2004) suggesting a more general physiological function relating to the transport/solubility of hydrophobic ligands in various tissues. *or10* showed expression in male tissues (Figure 3.29) while qRT-PCR detected same transcriptional profiles in all three tissues before and after mating (Figure 3.30). or10 encodes an olfactory receptor protein and has a G-protein coupled receptor activity. The expression of ORs in testes has been reported for a number of species Fukuda et al, 2004; Walensky et al., 1998). ORs' function in mammalian sperm is thought to regulate motility in response to exogenous signals derived from the existence of sperm-egg chemotaxis in invertebrates. The small peptides, speract and resact, are secreted by sea urchin eggs and attract spermatozoa in a species-specific manner by stimulating sperm motility and respiration (Suzuki et al., 1984; Parmentier et al, 1992). The presence of a similar chemoreceptor may be essential in female spermatheca in order to establish a concentration gradient of a putative chemo-attractant. Since female accessory glands and spermatheca were dissected together, we are not able at this point to establish which exact tissue is the source of the observed expression of *or10*.

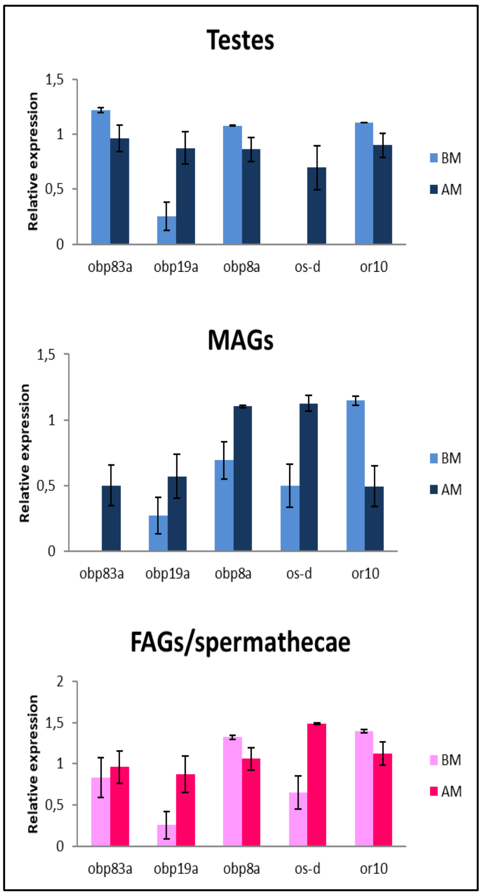


Figure 3.30: Relative expression profiles of differentially expressed olfactory genes in the olive fly reproduction system. Expression profiles of five olfactory genes [odorant binding proteins *obp83a*, *obp19a*, *obp8a*, chemosensory protein, *osd*, and odorant receptor 10, *or10*] as determined by qRT-PCR in three different tissues: Testes (a), MAGs (b) and FAGs/spermatheca (c) before (BM) and after (AM) mating. Standard error of the mean of five biological replicates is depicted in bars. No significant difference (for $P < 0.05$) was detected. *Rpl19* and *14-3-3z* genes were used as a reference in MAGs and testes while actin3 and a-tubulin in FAGs/spermathecae.

3.6 Peptidomics

Using transcriptomics technology, we adopted a holistic view of the genes expressed in the reproductive system of *B. oleae* in virgin and mated insects. A combination of this generated transcriptomic data with different –omic technologies will help the clarification of this complex system.

Such systems biology approaches have offered insights in several physiological processes in insects. A characteristic example is the combination of genetics and peptidomics to characterize the role of amidating enzyme in peptide processing in Drosophila (Pauls et al., 2014). Peptidomics targets the comprehensive qualitative and quantitative analysis of all peptides that are derived endogenously in a biological sample (Schulte et al., 2005).

The first insect peptidomics analysis was published in 2002 where the first neuropeptides were identified from an extract of the central nervous system of Drosophila larvae (Baggerman et al., 2002). In 2009, the first peptidome from adult insect brain was published (Yew et al., 2009). The peptide signaling through G-protein-coupled receptors is widely used in insects.

As it comes to reproduction, an example of the modulation of behavior by peptides, is the regulation of female reproductive behavior in D. melanogaster by sex peptide (SP). As mentioned previously, SP is a small peptide present in the male seminal fluid. Upon mating, SP is transferred to the female, where it triggers dramatic changes in reproduction (Carvalho et al., 2006; Baggerman et al., 2002). Within females, SP activates a specific G protein-coupled receptor (SPR) (Yapici et al., 2008) in a small set of internal sensory neurons of the female reproductive tract (Häsemeyer et al., 2009; Yang et al., 2009).

In an attempt to identify peptides that are involved in olive fly mating we performed peptidomic analysis of the reproductive system of *B. oleae*. Specifically, we dissected the testes and male accessory glands with ejaculatory bulb from virgin and mated insects and the lower female reproductive tract from virgin and mated female insects. Each sample consisted of tissues from 25 insects and they were duplicate biological.

In table 3.8 the results from the peptidomic analysis of virgin and mated female flies are presented. From the peptidome of the lower female reproductive tract, we have identified 49 peptides from the virgin insects and 23 peptides from the mated female insects. The proteins MCM5_DROME (DNA replication licensing factor Mcm5), SPNE_DROAN (Probable ATP-dependent RNA helicase Spindle-E), THR_DROVI (Protein three rows) and OR35A_DROME (Odorant receptor 35a) were identified in both virgin and mated flies.

The MCM5_DROME is the putative replicative helicase essential for 'once per cell cycle' DNA replication initiation and elongation in eukaryotic cells (Su et al., 1997). The SPNE_DROAN plays a central role during oogenesis by repressing transposable elements and preventing their mobilization, which is essential for the germline integrity (Specchia et al., 2017). The THR_DROVI is a maternally derived protein (Jaeger et al., 2004) that is involved in the formation and maintenance of epithelial structures for the zygotes. The presence of the Odorant receptor 35a is not surprising as the odorant receptor repertoire encodes a large collection of odor stimuli that vary widely in identity, intensity, and duration.

The peptidomics analysis of the other tissues is in progress. Future work will be focused on the analysis of the peptidomic data that will be obtained from the different tissues focusing on the identification of molecules that are involved in the reproductive system.

Peptides identified in the transcriptomic analysis of female insects				
	Virgin		Mated	
N	Protein code	Peptide	Protein code	Peptide
1	OR35A_DROME	QAGLKILQ	OR35A_DROME	QAGLKILQ
2	THR_DROVI	NAAKLIQV	THR_DROVI	NAAKLIQV
3	SPNE_DROAN	KAAVKII	SPNE_DROAN	KAAVKII
4	NAA25_DROME	QAVALQL	BRM_DROME	VGARQRITAA
5	FBRL_DROER	KLAAAVLGGV	Y7065_DROME	SASAGGGGGVVGA
6	RS3A_DROSE	SEGAVIDRPEGYEPPVQE	TRX_DROVI	QLGLAQIAR
7	RS3A_DROSE	DIDPERSF	TRX_DROME	ACALVSPGGSSQGG
8	C28A5_DROME	QLLVINP	TRX_DROME	PQPTATP
9	C28A5_DROME	KPTPIMS	MCM5_DROME	VVGVRAP
10	ACT3_DROME	ISKEEYDESGPGIVHRK	HEAT1_DROME	KPTAQQLI
11	MCM5_DROME	VVGVRAP	DAAF1_DROPE	EAASGDVDSIVK
12	MCM5_DROME	ITAPRPEH	MCTS1_DROME	DLILPKK
13	SIF1_DROME	VVVGGLGVAKP	FACR2_DROME	KVVPVV
14	Y3800_DROME	GGGGGPGGVGGGGGGGGGGMRGNDGGGMRR	DSCL_DROME	LLQVKVP
15	AIMP2_DROME	NLAKVPVNPALPK	DSCL_DROME	LTHTLIVQVP
16	BCD_DROME	ASACRVLVK	TITIN_DROME	KVREKVVKT
17	ROP_DROME	RNIVPILL	TITIN_DROME	IDNVGGIH
18	PUR4_DROME	KPAPKDLEQ	WHITE_DROME	QVLAVVP
19	DOT1L_DROME	KQLKNLPE	KLP68_DROPS	MPNVRNI
20	DOT1L_DROME	IIEVPPP	MMS22_DROME	KLVARP
21	DOT1L_DROME	LRGPRL	ATPA_DROME	KGIRPAINV
22	ANKHM_DROME	KVQVPVNAI	BX42_DROME	RRLVARGAP
23	WNT4_DROME	KLGIIVPGGQGLP	EIF3C_DROAN	TIELVLQY
24	GAWKY_DROME	QPTSQQQQP		
25	CP9F2_DROME	LKGLNKILKV		
26	WDR48_DROGR	IRGGAAIK		
27	RTBS_DROME	LKVNPQK		
28	MON2_DROME	KVARAKPQ		
29	MON2_DROME	QVLFPLLDNVRALSS		
30	CLH_DROME	AALAPKAIL		
31	SUCA_DROME	KLVGGISPKKGGTQHLGL		
32	CAZ_DROME	GGGGGGGRYDRGGGGGGGGGGGNVQPR		
33	PDFR_DROME	VRAAIVLLPL		
34	SUV39_DROME	KDVPKP		
35	RBGPR_DROME	PLQAAAKLIQKVGR		
36	MSH6_DROME	VRTVLGGILKEPVP		
37	C5210_DROME	AKELPNK		
38	2AAA_DROME	KLLLPTVLL		
39	BOP1_DROPE	KVVPVV		
40	MED15_DROME	PTQRVPL		
41	MED15_DROME	APVPGGPGTA		
42	MED15_DROME	TLQSPVANHTL		
43	AP3D_DROME	GRLIAEQLLDVAIRVPV		
44	GBB2_DROME	NKVQIIPL		
45	RL7A_DROME	QLFEKRPK		
46	PCAT1_DROME	VQPVLLK		
47	GBB2_DROME	NKVQIIPL		
48	RL7A_DROME	QLFEKRPK		
49	PCAT1_DROME	VQPVLLK		

Table 3.8: List of the peptides identified in the virgin and mated female insects.

4. DISCUSSION

4. Discussion

The olive fruit fly, *B. oleae*, is the major arthropod pest of commercial olive production, causing extensive damage to olive crops worldwide. As it was analyzed in the Introduction, olive fly control mostly relies on insecticide spraying. However, extensive insecticide resistance in olive fly populations is jeopardizing chemical control efforts and calls for the development of novel tools.

Targeting the reproductive success of the olive fly is a promising method for pest control as a possible manipulation of the reproductive system could affect the destructive activity of the fly.

For *B. oleae*, several transcriptome datasets have analyzed genes involved in detoxification (Pavlidi et al., 2013), insecticide resistance (Sagri et al., 2014), digestion or food recognition (Pavlidi et al., 2017). In 2014, the first transcriptomic analysis of the reproductive system of the olive fly was presented (Sagri et al., 2014). The analyzed tissues were limited in the testes for the male reproductive system and in the female accessory glands and spermathecae for the female reproductive system. The insects used in that analysis were immature virgin flies and the focus was on the identification of sex differentiation genes (Sagri et al., 2014). However, there was no information on the molecular factors and mechanisms that shape the reproductive success on the olive fly.

In the present thesis, our aim was to shed light on the processes that trigger female post-mating responses. Apart from the fundamental knowledge that this would offer, such understanding could be potentially transformed into novel tools for the control of *B. oleae* field populations.

4.1 Transcriptomic analysis of reproductive tissues

We initially performed a transcriptomic analysis of the reproductive tissues focusing on the identification of genes that may play a role in the post-mating response of the insect. Sequencing was performed in the reproductive tissues of the olive fly from virgin and mated insects. Specifically, testes and male accessory glands with ejaculatory bulb of male insects and the lower reproductive tract (uterus, accessory glands and spermathecae) of female insects constituted our samples. Since we were specifically interested in the male's potential to prime the female reproductive system, we examined changes that occurred in the female reproductive tract (ovaries excluded) as this is the primary site of interaction between the male ejaculate and the female. Ovaries were chosen for the developmental analysis as it is described for *D. melanogaster* (Graveley et al., 2010), *A. aegypti* (Akbari et al., 2013) and *B. dorsalis* (Geib et al., 2014).

The transcriptomic analysis yielded 11,452 male and 10,478 female transcripts. More transcripts were obtained from the tissues of mated insects (~5000 transcripts from each library) compared to virgin. This was not surprising as mated insects were regulated for the post-mating response including changes in insect behavior, physiology and gene expression.

Functional annotation was performed for all the transcripts from male and female tissues based on gene ontology (GO) categorization for biological process level II. The transcriptome profile obtained from this analysis showed a homogenization in the GO term hits between the two sexes. One explanation is that the samples were collected from already sexually mature insects. Genes that are sex-biased and responsible for the morphological difference or the maturation of the tissues are not represented in this transcriptomic analysis as they may be expressed in other developmental or adult maturation stages e.g. egg and larvae.

The most representative GO terms in both sexes were the metabolic, cellular and single-organism processes. Interestingly, only 713 out of 11,452 transcripts from the male tissues and 693 out of 10,478 transcripts from the female tissues have the GO term of reproduction indicating the limited information that is available for the genes expressed in the reproductive tissues of the insects resulting in reduced GO hits of the term "reproduction".

The presence of the "metabolic process" term reflects the high amount of energy that reproduction demands. In insects, when energy reserves have to be mobilized, hormonal activation of catabolic enzymes causes the breakdown of lipid and glycogen stores (Lorenz et al., 2009).

4.2 Transcriptional Profiles of Mating-Responsive Genes

The transcriptomes obtained by the virgin insects were compared to the transcriptome collected from the mated insects in order to identify genes that change their transcript abundance in response to mating and encode crucial proteins for the male and female reproductive success. Multidimensional-scaling plots ensured the similarity of the biological samples to one another, as biological replicates clustered together. However, two replicates of the virgin tissues, one from each sex, were grouped with the tissues obtained by the mated insects. This could be attributed to a variety of technical and biological factors for example differences in the amount of RNA, library preparation, operators, and procedures for sample extraction, preservation, or storage (Peixoto et al., 2015). Proper normalization, the transformation of values that allows comparisons between samples, has been shown to critically impact the analysis of high-throughput data (Dillies et al., 2013). However, the use of RNA-seq to study gene expression can be also influenced by a variety of biological factors such as time of day, differences in responsiveness between individuals and cell-type heterogeneity. Given this, we decided to omit these two samples from the following analysis even at the expense of statistical power.

4.2.1 Testes

Transcriptomic analysis of the *B. oleae* testes from three-day old virgin flies has been analyzed previously (Sagri et al., 2016). The transcriptomic data from that analysis were used for the comparison of the obtained data from mated insects.

Comparison of the transcriptomes revealed that 107 genes were up-regulated and 345 genes were down-regulated in the testes of mated males. This number of regulated genes is smaller compared to the other reproductive tissues. As it was demonstrated in *D. melanogaster,* the spermatozoa are generally metabolically quiescent and transcriptionally silent in the adult insects (Olivieri et al., 1965) and therefore the derived transcriptional information is limited (Wasbrough et al., 2010).

From the functional annotation based on Gene Ontology (GO) categorization level II the tissue of testes gave the smallest terms hits demonstrating homogeneity of the genes with regard to their biological process and molecular function. The GO terms with the most abundant hits were similar to those obtained for the *B. dorsalis* testes (Wei et al., 2015). Comparing the results from both insects, in *B. dorsalis* the most abundant GO term was “cellular process” while in *B. oleae* “metabolic process”. This difference obviously reflects the difference in the analyzed samples. In *B. dorsalis* the authors followed spermatogenesis in adult male flies at different ages, whereas in *B. oleae* we focused on differential expression of genes before and after mating of the flies.

Regarding the “molecular function” classification, the main groups involved “binding” and “catalytic activity”. These groups were also identified as the most abundant in *B. dorsalis* (Wei et al., 2015) and *C. capitata* (Scolari et al., 2012) showing a conservation of the functions that are altered during mating in the insects.

Moreover, we annotated the genes on the recently sequenced genome of the olive fly. Also, 13 genes were annotated as unknown function genes. There was no homology with known sequences from other insects. Studies in numerous other insects have shown that the genes expressed in the reproductive tissues are among the most rapidly evolving genes, especially in males (Panhuis et al., 2006; Clark et al., 2006; Haerty et al., 2007; Scolari et al., 2012). These genes could be novel or fast-evolving sequences and may have a potential role in sexual selection and speciation and, therefore, represent ideal subjects for future evolutionary genetic studies for this species.

Two genes, *antigen 5-related 2* and *mucin*, are involved in immune response, both in testis and in sperm development. Antigen 5-related 2 belongs to the large CAP family. Several members of this family in Drosophila are preferentially expressed in males and some within primary spermatocytes (Haynes et al., 1997). Antigen 5-related 2 has also been detected in the accessory glands of *C. capitata* (Davies et al., 2006). It has been proposed that the proteins of this family may act either mediating interactions between germ-line and somatic cells within the male or between the sperm and egg (Kovalick et al., 2005).

Mucin belongs to a family of large glycosylated macromolecules capable of forming enormous networks that act as selective barriers (Syed et al., 2008). Mucins have been shown to participate, together with other proteins and lipids, in the formation of mating plugs, often produced within the female reproductive tract during or shortly after mating (Avila et al., 2011). Mating plugs induce the post-mating response in several insects by preventing remating and helping sperm storage (Lung et al., 2001; Rogers et al., 2009). The olive fly does not produce a mating plug but mucins may have a sperm protection function, or may have a role in the differentiation and renewal of the epithelium and modulation of cell adhesion, immune response, and cell signaling (Wesseling et al., 1995; Chaturvedi e al., 2008).

Two cytochrome P450 genes (*Cyp6a16* and *Cyp313a4*) were upregulated in the testes of mated insects. Insect cytochrome P450s comprise a diverse class of enzymes involved in detoxification and biosynthesis of ecdysteroids and juvenile hormones (JHs) (Feyereisen et al., 1999; Wilson et al., 2003). JHs and ecdysteroids control insect development during larval and pupal stages and have gonadotropic function in the adult stages (Hardie, 1995). Yu and Terriere suggested that insect P450s were involved in reproduction via control of hormone titers (Hodgson, 1985). Thus, one possible role for these P450 is the regulation of the ecdysteroids in testes.

4.2.2 Male accessory glands with ejaculatory bulb

In the order of Diptera that *B. oleae* belongs, male accessory glands secretions are transferred to the female during mating along with the sperm produced in the testes, affecting the female fly in two main behavioral and physiological characteristics: repression of sexual receptivity to further mating and egg laying stimulation (Delrio and Cavalloro 1979; Chen 1984; Jang 1995; Miyatake et al., 1999). Even though the morphology and ultrastructure of the male accessory glands with ejaculatory bulb of *B. oleae* has been analyzed by Marchini et al (2006), up to date there is no molecular information.

In the present thesis, we identified 11,452 new transcripts that are expressed in the male accessory glands of the olive fly. From the 11,452 genes, 1,608 genes were up-regulated while 383 genes were down-regulated in mated insects.

Moreover, we annotated the genes on the recently sequenced genome of the olive fly and analyzed their distribution to a functional annotation using GO analysis level II. All the genes showed high similarity with homology sequences in *D. melanogaster*.

The GO analysis of the top 100 genes in response to biological processes and molecular function showed enrichment of the terms of “metabolic processes” and “biological regulation”. As sexually mature males are actively involved in pheromone emission and female courting, it is of no surprise that they show significant enrichment of these GO terms. These processes indicate the high energy investment required in mating, as it was also observed in *C. capitata* (Gomulski et al., 2012).

An increased expression of immunity related genes, *attacin-A* and *catalase* was detected in these tissues. *Attacin-A* encodes an antimicrobial peptide (AMP) that has been involved in insect immunity (Yi et al., 2014). Moreover, *catalase* encodes for a detoxification enzyme that detoxifies the insects from reactive oxygen species. In *A. gambiae,* a systemic reduction in catalase activity by dsRNA-mediated knockdown resulted in significant reduction of oviposition, indicating that catalase plays a central role in protecting oocytes and early embryos from reactive oxygen species (ROS) damage (Magalhaes et al., 2008). The presence of the gene in the male tissues indicates a similar role for the gene in *B. oleae.*

Interestingly, three genes that may be implicated in the foraging behavior have been identified. Foraging behavior involves memory/learning, visual and olfactory functions (Drew et al., 2000). The genes of gustatory receptors 32a and 21a and *scribbler* gene were upregulated in the reproductive tissues. This is in agreement with several studies reporting the expression of such genes in male accessory glands and testes of multiple species (Allen et al., 2008; Chapman et al., 2008; Zhou et al., 2009; Edwards et al., 2009). *Scribbler* was also identified in testes. However, while in male accessory glands with ejaculatory bulb it was upregulated, in testes it was downregulated. *Scribbler* encodes two transcripts widely expressed in the sensory nervous system and it was found to play a role in the food search behavior (Suster et al., 2004). Further examination of its role in the olive fly is necessary.

Finally, *ryanodine receptor* was upregulated in the male tissues. Ryanodine receptor belongs to a distinct class of ligand-gated calcium channels controlling the release of calcium from intracellular stores. They are located on the sarcoplasmic reticulum of muscle and the endoplasmic reticulum of neurons and many other cell types. Ejaculatory bulb is a muscle tissue whose contraction helps the transfer of the seminal fluid to the female insect (Guiraudie et al., 2007). The presence of a ryanodine receptor indicates that contraction maybe depended on ligand-gated calcium channels.

4.3.2.1 Expression profile of selected genes in males

Our transcriptional analysis showed that the male response to mating in *B. oleae* translates into substantial transcriptional changes. A more detailed follow-up expression profile analysis from insects during sexual maturation and mated was performed for 6 mating-responsive genes (*brunelleschi*, *CG2254-like*, *timeless, c52416, c53574* and *yellow-g*). Our working hypothesis was that a gene that encodes a protein in the seminal fluid that is important for mating, should be expressed earlier so that the protein will be present at the time of mating. In fact, most of the aforementioned genes showed highest expression before mating, thus confirming our hypothesis.

Brunelleschi and *yellow-g* showed highest expression on DAY-5 after adult emergence, while *CG2254-like* showed highest expression on DAY-6. *Brunelleschi* belongs to the TRAPII complex which is involved in vesicle trafficking in the secretory pathway (Robinett et al., 2009). As the male accessory glands are the secretory tissues for the reproductive system, *brunelleschi* is involved in the maturation of the accessory gland tissue to produce the secretory proteins of the seminal fluid.

Yellow-g belongs to the MRJP/YELLOW family that includes the major royal jelly proteins and the yellow proteins. The *yellow* gene family has been associated with behavior (Dow 1976; Wilson et al., 1976; Burnet and Wilson 1980; Drapeau et al., 2003; Drapeau et al., 2006; Prud'homme et al., 2006), pigmentation (Han et al. 2002; Wittkopp, True, et al., 2002; Wittkopp, Vaccaro, et al., 2002; Prud'homme et al., 2006), and sex-specific reproductive maturation (Drapeau et al., 2006) in *D. melanogaster* and *A. mellifera*.

CG2254-like encodes for a dehydrogenase that is localized in the lipid droplets, organelles that store lipids and have a significant role in metabolism and membrane synthesis (Thul et al., 2013). The ejaculatory bulb is a muscle tissue and its contractions help to transfer the seminal fluid to the female flies during mating. During mating, the tissue has high energy demands and the presence of lipid droplets give them an alternative source of energy. Moreover, these lipid organelles could serve as a source of substrate for steroid hormone synthesis such as ecdysteroid hormone that play a significant role in reproduction.

Timeless showed an increase in its expression after mating, indicating possibly a role in the rhythmic cycle. *Timeless* along with *per* (period) regulate the circadian cycle of insects. Knockout of *timeless* in male *D. melanogaster* showed a change in mating time (Beaver, 2003). In *S. litorralis* it has been demonstrated that the sperm release rhythm is controlled by an intrinsic circadian mechanism located in the reproductive system (Gvakharia et al., 2013).

4.2.3 Female lower reproductive tract

The female reproductive tract is constituted by the spermathecae (where sperm is stored), the uterus (where the seminal fluid is transferred) and the accessory glands (the secretory tissue of the reproductive system). Although, the reproductive tract of the female insect contains secretory tissue, to date female reproductive genes have been comprehensively studied in very few taxa compared to the male reproductive tissues (Lawniczak et al., 2004; Mack et al., 2006; Rogers et al., 2008).

A transcriptomic analysis of female accessory glands and spermathecae in virgin olive flies has been analyzed previously (Sagri et al., 2014). However, the approach was focused on the identification of genes differentially expressed between the sexes. Here, we obtained 10,478 new transcripts from which 1,705 genes were up-regulated while 120 genes were down-regulated in mated flies. This is similar with the results in *D. melanogaster* after analysis of transcriptome the lower reproductive system in several time points after mating (Mack et al. 2006). Other works in *B. dorsalis* and *C. capitata*, however, showed significantly lower transcriptional changes after mating. Only 65 and 32 transcripts were altered in abundance in *B. dorsalis* and *C. capitata*, respectively (McGraw et al., 2004; Zheng et al., 2016).

Two possible explanations may account for this difference. The first one regards the analyzed tissues. In *B. oleae* and *D. melanogaster* the lower reproductive tract was analyzed, whereas in *B. dorsalis* and *C. capitata* the whole body was analyzed. The second reason regards the time when tissues were collected. In *B. oleae* tissues were collected 12 hours after one mating, in *D. melanogaster* tissues were collected at several time points (0, 3, 6 and 24 hour) after mating, while in *B. dorsalis* and *C. capitata* the analyzed time point was 24 hours after mating. This shows the variability of the post-mating transcriptional changes in different insect species and time points.

As it was extensively analyzed in many Drosophila species, the female reproductive genes encode proteases, proteases inhibitors and genes related to immune response and energy metabolism (Mack et al. 2006;

Lawniczak et al., 2004; McGraw et al., 2008; Prokupek et al., 2008). Unsurprisingly, these genes were also observed in the GO annotation of the upregulated genes in the lower female reproductive tract of *B. oleae*.

A protease, *serpin42Da* and a protease inhibitor *CG9676-like* were identified. In *D. melanogaster* proteases and protease inhibitors have been shown to be required for activation of ovulation-inducing seminal fluid proteins (McGraw et al., 2004).

Four genes encoding ATPase and NADH dehydrogenases that play a role in energy metabolism were found upregulated in mated females. Energy metabolism genes were highly expressed in the spermatheca of the ant queen, suggesting high energy costs of spermatheca function (Gotoh et al., 2017). This indicates that *B. oleae* spermatheca has high energy demands.

A gene related to immune response had an overexpression in the mated female tissues. The *defensin* gene, encodes an antibacterial peptide (AMP). Genes encoding AMPs have been observed in the male accessory gland with ejaculatory bulb, too, indicating that the AMPs play a significant role in the immune response of the olive fly. Interestingly, in *C. capitata*, the *defensin* gene was upregulated in the abdomen of mature virgin females compared to mature mated insects (Gomulski et al., 2012). Moreover, Gomulski et al (2012) showed that the medfly does not appear to activate any immune gene expression after mating displaying a greater similarity to its more distant evolutionary relatives, *A. gambiae* and *A. mellifera* (Kocher et al., 2008; Rogers et al., 2008). This is not the case for the olive fly. Even though *B. oleae* and *C. capitata* are closely related they show different post-mating immunity response. Olive fly shows more similarity with Drosophila species. In mated *D. melanogaster* females, the immune response is activated by the sperm and seminal fluid components. It has been suggested that this immune response is part of a sexually antagonistic arms race in which the male produces increasingly potent signal molecules that modify the behavior and physiology of the female away from reproductive receptivity towards fecundity (Chapman et al., 1995).

4.3.3.1 Expression profile of selected genes in females

The transcriptional analysis of the post-mating response in female olive flies showed extensive transcriptional changes. A follow-up expression profile analysis during time-points after mating was performed for 6 mating-responsive genes (*troponin C, yolk-protein 2, lingerer, glutathione S-transferase epsilon class, bestrophin 2, ornithine decarboxylase antizyme*). The analysis showed that most genes presented an increasing expression after mating, while two genes, *troponin C* and *glutathione S-transferase epsilon class*, showed overexpression in virgin female flies.

Troponin C pays a significant role in muscle contractions. In *Pieris rapae*, the small cabbage white butterfly, it was identified as a component of the bursa copulatrix female reproductive tissue that is responsible for the digestion of the nutrient-rich spermatophore produced by the male accessory glands (Meslin et al., 2015). The identification of this protein indicates its involvement as a muscle protein in the contraction of the female reproductive system of the olive fly, probably aiding the digestion of the seminal fluid proteins that are transferred to the female during mating. The overexpression in virgin flies may indicate that the protein should be present in the reproductive tract of the female insect to digest the seminal fluid when the mating occurs.

Glutathione S-transferase epsilon class is a predicted intracellular or membrane-bound protein (Bloch et al., 2011). Predicted intracellular proteins have been reported in the reproductive system of *D. melanogaster* (Walker et al., 2006), *A. mellifera* (Baer et al., 2009) and *A. aegypti* (Jones et al., 1965). For *A. mellifera* and *A. aegypti* it has been suggested that these proteins may be secreted through non-standard secretion routes such as apocrine and holocrine secretion (Dapples et al., 1974; Ramalingam et al., 1983). Macro apocrine secretion has been reported in the *B. oleae* male reproductive system (Marchini et al., 2006).

Yolk protein-2 showed overexpression 9 hours after mating indicating its supporting role in embryonic development. The homologue of this gene in *D. melanogaster* is expressed almost exclusively in females and it was associated with a female-sterile mutation (Williams et al., 2005; Goldman et al., 2007). The *yolk protein-2* gene encodes for a precursor of the major egg storage protein, the vitelline. There are three main factors that regulate vitellogenesis in *D. melanogaster*: a brain factor, an ovarian factor that stimulates fat bodies vitelline synthesis and a thoracic factor that is involved in the uptake of the vitelline by the ovaries (Handler et al., 1977; Postlethwait et al., 1979). Vitellogenins have also been implicated with the transport of various molecules including sugars, lipids and hormones in insects (Sappington and Raidhel, 1998).

The *lingerer* gene showed upregulation 12 hours after mating. Mutations of *lingerer* in male *D. melanogaster* result in abnormal matings and the "stuck" phenotype where males could not be separated from the females after the end of mating. It has been also identified as a maternal gene expressed in *D. melanogaster* early embryos (Kuniyoshi et al., 2002).

A similar expression profile has been demonstrated for the *bestrophin 2* gene. In *D. melanogaster* it encodes for an oligomeric transmembrane protein that is thought to act as chloride channel (Tavsanli et al., 2001). It helps in the transportation of small molecules that are transferred in the female flies as part of the seminal fluid during mating.

An upregulation of an Ornithine decarboxylase antizyme (ODC-AZ) was observed in mated females. ODC-AZ binds and destabilizes the ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis (Cayre et al., 1996). Correlative changes between hormone levels and polyamine metabolism were described in several insects. For example, 20-hydroxyecdysone increases ODC activity in silk moth pupal tissues (Wyatt et al., 1973) and juvenile hormone stimulates ODC activity during vitellogenesis in *D. melanogaster* (Birnhaum and Gilbert, 1990). Ornithine decarboxylase antizyme is an inhibitor of ODC. Inhibition of ODC activity causes impaired vitellogenesis in *A. aegypti* (Kogan et al., 2000) and oviposition delay in *A. domesticus* (Wyatt et al., 1973). The observed upregulation of their inhibitor indicates that ODC-AZ is probably involved in the control of ODCs levels in mated female olive flies.

4.3 Functional analysis of mating regulated genes through RNAi

4.3.1 RNAi silencing through injection

Two genes, *yellow-g* and *troponin-C,* that were identified as important for mating through transcriptomic analysis of the reproductive system and were selected for further validation of their role in the mating response, using RNA interference through injection. The results indicated high percentage of silencing in the insect, reaching 81% for *yellow-g* and 70% for *troponin-C.*

The successful response of the fly to the RNAi process should not be taken for granted, as RNAi response differs between different insects and different genes. In Drosophila, RNAi-mediated gene knockdown through microinjection was only localized to the site of dsRNA delivery and effects were temporally limited (Daniel et al., 2008). On the contrary, injection of dsRNA into adult abdomen of *B. dorsalis* successfully inhibited the expression of *doublesex* gene in ovaries (Chen et al., 2008). Our RNAi experiments demonstrated a more generalized inhibition in the olive fly, more like that observed in *B. dorsalis* than that in *D. melanogaster*.

Additionally, the phenotypic impact of the silencing of these genes was remarkable as they reduced the fertility of the insects showing that they play a significant role in the post-mating response.

The *yellow-g* gene as it was reported earlier, belongs to the MRJP/YELLOW family that includes the major royal jelly proteins and the yellow proteins. In *D. melanogaster* it was demonstrated that *yellow-g* is needed for proper egg formation, possibly for the production of a structurally sound vitelline membrane, or to catalyze the crosslinking of eggshell layers for the rigidity of the egg (Claycomb et al., 2004). An orthologue of Drosophila *yellow-g* gene in *A. gambiae* was targeted through CRISPR-Cas9 technology and resulted in female-sterility phenotype (Hammond et al., 2016). In our study we present the first report of the upregulation of the *yellow-g* gene in mated male insects. To distinguish whether the transient silencing of *yellow-g* had an impact on the reproduction we performed mating experiments with virgin female flies. The results showed that the mated females had a significant lower reproductive rate compared to the control flies. This report shows that *yellow-g* could be a good candidate for effective *B. oleae* pest control target.

Troponin-C is the major component of the tropomyosin–troponin complex (Tm–Tn) on the actin of the striated muscles of insects. *Troponin-C* gene along with other classical muscle-related genes (Troponin T, myosin, tropomyosin and myofilin) is highly expressed in the female lower reproductive tract and the bursa copulatrix of Lepidoptera. The bursa copulatrix provides unique and specific digestive functionality in the female reproductive tract of Lepidoptera as it digests the spermatophore that is produced by the male accessory glands (Karlson et al.,1996; Meslin et al., 2015). The evolutionary distance between the olive fly and Lepidoptera allows us to speculate that digestion of the seminal fluid might be completed by the lower reproductive system in the olive fly as well. The presence of *troponin C* in the female reproductive tract shows that it is used for the contraction of the female reproductive system helping the movement of the sperm to the spermathecae.

The transient silencing of the *troponin-C* in the female insects caused lower oviposition rate compared to control. This finding validates the previous speculation on the important role of the gene in the reproductive system of the olive fly.

4.3.2 Gene silencing through dsRNA feeding

The gene selected for RNAi silencing through feeding was the *sex peptide receptor* or *spr* gene. Sex peptide receptor is a G-protein-coupled receptor (GPCR) required in the nervous system for the post-mating behavioral switch triggered by the sex peptide in *D. melanogaster* (Yapici et al., 2008). This behavioral switch includes an increase in feeding, a change in food choice and sleep, stimulation of the immune system (Peng et al., 2005; Carvalho et al., 2006; Domanitskaya et al., 2007; Ribeiro et al., 2010; Isaac et al., 2010; Haussmann et al., 2015) increase in oviposition and decrease of female longevity (Avila et al., 2010; Wigby et al., 2005).

Sex peptide or SP is a 36-amino-acid peptide produced in the male accessory gland in all Drosophilidae (Chen et al., 1988; Chapman et al., 2003; Liu et al., 2003). Even though orthologue SP genes are difficult to identify outside the Drosophilidae mainly because of their small size, this is not the case for the sex peptide receptor.

Putative sex peptide receptor orthologues have been identified in most insect genomes including the mosquitoes *A. aegypti* and *A. gambiae*, the moth *B. mori*, the beetle *T. castaneum* (Yapici et al., 2008) and the fruit fly *B. dorsalis* (Zheng et al., 2012). In the transcriptomic analysis demonstrated in this thesis there was no identification of the *sex peptide* gene.

Gene silencing through RNAi feeding is mediated through feeding with specific *E. coli* bacterial strain engineered to produce specifically designed dsRNA. RNAi feeding has been successfully reported in *C. elegans* (Timmons et al., 2000), *E. histolytica* (Solis et al., 2009) and *S. exigua* (Tian et al., 2009). However, in *D. melanogaster*, feeding yeast cells engineered to express double-stranded RNA to the flies failed to work (Gura et al., 2000). These facts made people believe that in Diptera, feeding dsRNA cannot induce RNAi. However, in 2011 RNAi feeding was achieved in *B. dorsalis* indicating that feeding dsRNA-expressing bacteria can achieve RNAi silencing in different species. Here, we report the first dsRNA-feeding assay for *B. oleae* adults.

Feeding adult olive fly females dsRNA targeting the sex peptide receptor induced 90% and 40% downregulation of the gene on the female reproductive tract and head, respectively. In *B. dorsalis* feeding ds-RNA of the sex peptide receptor induced only 52% downregulation (Zheng et al., 2015). The difference in the silencing results may be due to the different examined tissues. Zheng and co-workers (2015) used the whole insect body to determine silencing efficiency, instead we determined silencing effect at the target tissue (female head and reproductive tract). Our findings indicate that silencing efficiency maybe different at different tissues of the same insect.

Feeding insects with dsRNA of the sex peptide receptor produced female flies with greatly reduced fertility. The number of eggs laid by female flies decreased significantly after *spr* gene silencing. This result is consistent with research in *D. melanogaster* where females lacking the SPR failed to respond to SP and continued to show virgin behaviors laying very few eggs after mating (Yapici et al., 2008). RNAi of the sex peptide receptor of the *H. armigera* (Haspr) induced suppression of sex pheromone production and reduced the egg-laying response of mated females compared with virgin females (Hanin et al., 2012). These results suggested that SPR has an important function in postmating and general reproductive behavior of insects.

RNAi by continuous feeding dsRNA can significantly impact target gene expression and its functions in the olive fly.

4.4 Concluding remarks

4.4.1 The reproductive system of *B. oleae*

This thesis was focused on some facets of the reproductive processes in the olive fly. The underlying premise is that elucidation of such processes would not only shed light on fundamental questions of the fly's reproductive biology but also provide new tools for its control.

Until today, molecular and functional information on the proteins secreted in the olive fly reproductive apparatus was limited. Within the transcriptome dataset generated in the present thesis, a subset of transcripts was identified that, on the basis of their tissue-specificity, may encode olive fly reproductive proteins. Comparison of mated and virgin fly transcriptomes identified a change in transcriptional activity of 452 loci in testes, 1,991 loci in male accessory glands with ejaculatory bulb and 1,825 loci in female lower reproductive system. This transcriptional activity of mated olive flies is characterized by rapid cell proliferation and secretory activity, as supported by the categorization of the transcripts in functional classes related to biological regulation, metabolic and cellular processes.

In general, through comparative structural modeling it was shown that the major functional classes of the reproductive proteins are conserved even between mammalian and Drosophila despite the differences in reproductive strategies (Mueller et al., 2004). As it comes to olive fly, indeed the general transcriptomic profile of the analyzed tissues was similar to other diptera reproductive systems such as *C. capitata* (Gomulski et al., 2012; Scolari et al., 2012). However, a more detailed analysis of the transcripts showed that there is diversity in the mating response among species. Specifically, comparing to *C. capitata,* an insect that belongs to the same family with olive fly, there were two distinct differences. Firstly, there was a profound alteration in transcripts in one time mated *B. oleae* insects while a similar alteration was detected in three times mated *C. capitata* insects. Secondly, in *B. oleae* there was a modification of the immunity response of the reproductive tissues while in *C. capitata* there was not (Gomulski et al., 2012). Similarly, comparison of *D. simulans* male accessory gland proteins with their orthologues in its close relative *D. melanogaster* demonstrated rapid divergence of many of these reproductive genes (Swanson et al., 2002). The divergence of the reproductive genes is based on the important role that they play in ensuring the successful mating and fertilization (Braswell et al. 2006; Tian et al., 2017).

The extensive transcriptome resources we gained through this research will improve the on-going annotation of the olive fly genome. The obtained data will help genomic data from other Tephritid species of agricultural importance, opening new ways for comparative genomics and barcoding for species identification (Schultz et al., 1998; Pearson et al., 1997; Nielsen et al., 1997).

The transcriptional profiles of a group of genes identified in the reproductive tissues of *B. oleae* were analyzed further and showed mating-induced changes most probably related to replenishment of their protein products after mating for the males and inducing several post-mating processes for the females. However, the very complex transcriptional profile of several of these genes necessitates further characterization. A key focus for future studies is a better understanding of the molecules and the processes that are derived in the reproduction of olive fly.

Systems biology approaches can contribute to the clarification of the seminal fluid components and their regulations. Combining data from different –omic technologies, such as transcriptomic, proteomic, peptidomic and metabolomic analyses, will help to establish a more comprehensive picture. The integrative nature of systems biology approaches provides more power in prediction of key targets for functional testing. The advances in the –omic technologies can bridge the gap between reproductive phenotypes and their molecular mechanisms (Findlay et al., 2010). In *Heliconius* butterfly 52 different accessory gland proteins have been identified by a combination of EST and proteome analyses, including the identification of chymotrypsin, proteinase inhibitor and hormone binding proteins (Walters et al., 2010). Similar work is in progress in our laboratory, in an effort to combine our present transcriptomic data with proteomic or peptidomic analyses of the reproductive tissues.

4.4.2. Transient silencing of selected reproductive genes

An interesting outcome of the research is the significant phenotypes that were observed through the RNAi experiments. Transient silencing was performed on three genes (*yellow-g*, *troponin C* and *spr*) and the results showed a statistically significant modification on the behavior of the female insects. Our observations were focused on oviposition rate and longevity of female flies. Transient silencing of the aforementioned genes resulted in lower oviposition rate and longevity compared to the control flies.

Reproductive genes encode several proteins that have roles in modulating many female behavioral and physiological processes across a wide range of insect species including transcriptional and reproductive tract structural changes, upregulation of antimicrobial peptide genes, altered receptivity to remating, sperm storage, mating plug formation, postmating feeding and female activity levels (Avila et al., 2011). These post-mating behavioral changes are the result of the alteration of a cascade of genes. The phenotypic observations that were recorded in this thesis, show that the studied genes may have a key role in the alteration of oviposition or longevity of the olive fly.

However, to better demonstrate and verify their role, future direction of the project could be the generation of knock out lines for the genes of interest. Since RNAi technology knocks down but not out the targeted transcript, it produces phenotypes which not necessarily mirror the complete loss-of-function of the targeted gene. In *A. gambiae*, the antiparasitic role of TEP1 was discovered and characterized using RNA interference assays (Blandin et al., 2004). To confirm the pivotal role of TEP1, they produced knock out lines using TALENS technology where they observed that all mutations in a homozygous state resulted in a similar phenotype (Smidler et al., 2013). On this account, complete knock out of the genes under study, for instance by CRISPR technology, will demonstrate whether these genes are responsible for the resulting phenotype, or they are involved in a cascade of genes associated with the phenotype.

Such knowledge of the key components in the reproduction and their behavior during the mating response will give the power to identify beneficial targets for pest management techniques. The destructive success of the insect is based on its high reproductive rate. Interfering with the outcome of mating would have a strong impact on the size of natural fly populations.

4.4.3. Implementation in control methods

Sterile insect technique (SIT) is an established method of insect population control for several insect species including fruit flies, tsetse fly, screwworm, moths and mosquitoes. In olive fly, there were two unsuccessful SIT efforts with specific key problems (Economopoulos and Zervas, 1982). One of them was that sterilization was mainly caused through radiation, a procedure that causes somatic damages to the insects reducing the competitive ability of male flies to mate with wild females (Proverbs et al., 1969). Alternative methods for sterilization of the insects can enhance the efficiency of the SIT programs.

RNAi technology could prove a powerful molecular tool to induce sterilization for SIT purposes. RNAi technology could be more effective and species-specific due to its target specificity that would preserve male fitness (Waqar et al., 2017). Moreover, it could be applied to both mass-reared and recently captured field insects, avoiding the need to use highly inbred mass-reared colonies with known competitiveness problems (Lance et al., 2005). A promising result for this application was published recently for *B. dorsalis*, where spermless males were developed by interfering with germ cell differentiation and azoospermia related genes (*boul*, *zpg*, *dsx*^{M}, *fzo* and *gas8*). Knock down of target genes significantly affected the reproductive ability of males and reduced egg-hatching while different combinations of the selected genes resulted in 85.4% male sterility (Waqar Ali et al., 2017). In this thesis, RNA inhibition through dsRNA feeding was successfully implemented to the olive fly reducing the expression of the *spr* gene showing that this method could be used in *B. oleae*, too. Implementation of bacteria producing dsRNA for specific reproductive genes in the artificial diet of the insects could be an alternative sterilization method. However, in order for this approach to be commercially used, many barriers have to be conquered. For example, the selected gene should confer nearly 100% sterilization, the species-specificity of the selected gene should be confirmed and off-target effects should be tested (Wei et al., 2015).

Another application of the RNAi technology that is under rapid development recently is the dsRNA insecticides. In 2017, EPA (United states Environmental Protection Agency) approved the first plant-incorporated protectant (PIP) of its kind based on RNA interference technology. Specifically, EPA approved four SmartStax Pro seed products with the RNAi-based PIP production. PIPs are pesticides that plants are able to produce themselves, thanks to modifications to plants genes that can incorporate DvSnf7 dsRNA. This dsRNA is specific to the western corn rootworm *Snf7* gene. When a DvSnf7 dsRNA-containing plant is consumed by a corn rootworm, the dsRNA will initiate RNAi within the worm, leading to the suppression of this critical gene and, eventually, rootworm's demise (EPA 2017).

The delivery of dsRNA to pests through transgenic plants is not possible for the control of the olive fly, as olive tree is a centenarian tree. However, if the insecticidal dsRNA could be delivered as a spray or powder, this approach could be conceivable. The foliar application of Colorado potato beetle actin dsRNA was highly effective against the beetle, since potato plants were protected for at least 28 days under greenhouse conditions (Miguel et al., 2015). Nonetheless, several important issues should be considered for this new type of pest control. First dsRNA is a fragile biological molecule, so a formulation should be developed to pack the molecule until it is delivered to its target gene. Second, issues such as persistence in the environment and impact at non-target organisms need to be researched thoroughly.

The high specificity of dsRNA (having the potential to be species specific) makes RNAi an extremely promising pest management technology. In this research, we proved that this technology could be used for the control of olive fly as we successfully reduced the expression of specific genes.

Gene-drive technologies can also be recruited for pest management control, using as targets reproductive genes identified in this research. One possible gene drive approach is to use selfish genes like CRISPR/Cas9 nucleases that will spread a single characteristic in the genome through non-Mendelian inheritance. If the introduced driving gene disrupts a fertility gene, it could suppress the population or even collapse it (Burt et al., 2003). Such a synthetic gene drive was generated for *A. gambiae* mosquitoes using the gene editing technology CRISPR/Cas9 to target genes involved in egg production to reduce the number of offspring (Hammond et al., 2016). CRISPC/Cas9 nuclease copied the gene drive onto both chromosomes during sperm and egg formation and in just four generations the new genes spread through the mosquito population. However, as the gene drive continued, mutations gradually arose and blocked the engineered genes restoring females' fertility. However, as it was analyzed in the Introduction, gene drive systems have still many obstacles to overcome before they could be used in the field.

Alternatively, reproductive genes identified in this project could be the base for the design of chemosterilants. These compounds would mimic the function of these proteins resulting to inhibition of their role in the cell. For example, it was shown that when a compound that encodes an agonist of the steroid hormone 20-hydroxyecdysone was applied to *A. gambiae* females, it disrupted multiple biological processes such as lifespan, insemination and egg production, that are key to the ability of mosquitoes to transmit malaria (Childs et al., 2016).

In conclusion, the wealth of data generated here including the categorization of a large number of reproductive genes, the elucidation of the role of several of them and the application of techniques like dsRNA feeding will provide targets for genetic sterility and novel means for chemical sterilization in mass rearing facilities and will pave the way of developing novel tools for the control of the insect.

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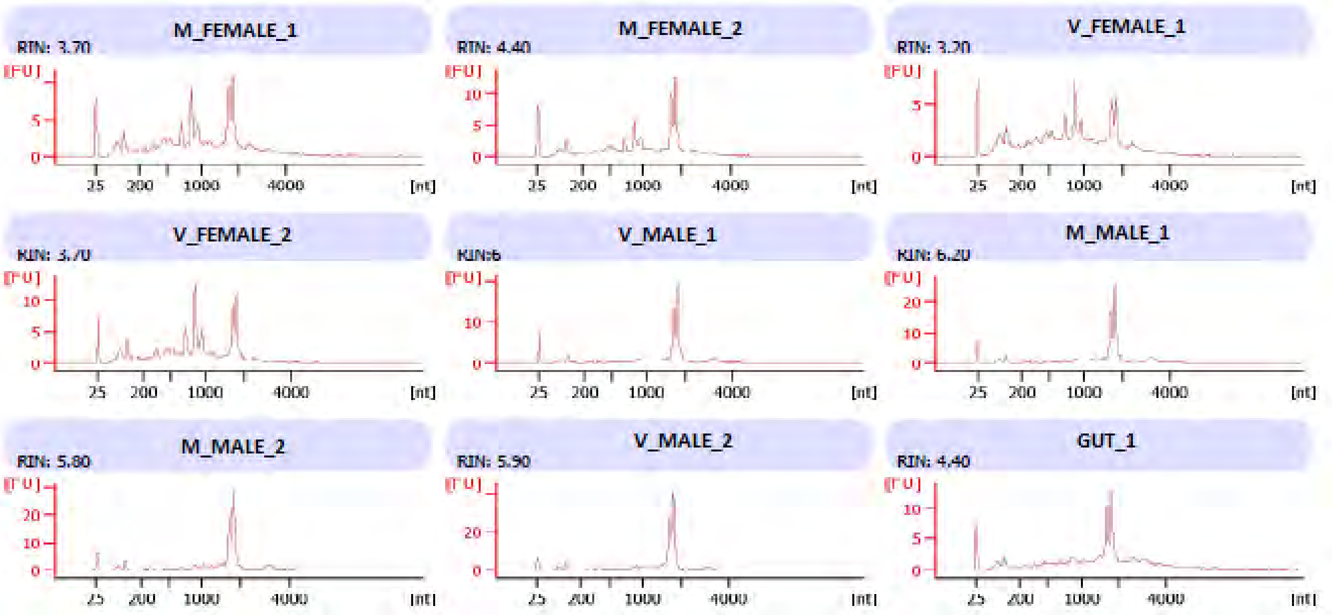
6.SUPPLEMENTARY

6.Supplementary

6.1 Summary of the results from the Nanodrop and Bioanalyser for the sequenced samples from Ion Torrent system



Sample Name	Sample Comment	Status	Result Label	Result Color
M_FEMALE_1		✔	RIN: 3.70	
M_FEMALE_2		✔	RIN: 4.40	
V_FEMALE_1		✔	RIN: 3.20	
V_FEMALE_2		✔	RIN: 3.70	
V_MALE_1		✔	RIN:6	
M_MALE_1		✔	RIN: 6.20	
M_MALE_2		✔	RIN: 5.80	
V_MALE_2		✔	RIN: 5.90	
GUT_1		✔	RIN: 4.40	

Chip Lot # **Reagent Kit Lot #**

Chip Comments :

Electrophoresis Assay Details

General Analysis Settings

Number of Available Sample and Ladder Wells (Max.) : 13
Minimum Visible Range [s] : 17
Maximum Visible Range [s] : 70
Start Analysis Time Range [s] : 19
End Analysis Time Range [s] : 69
Ladder Concentration [ng/µl] : 150
Lower Marker Concentration [ng/µl] : 0
Upper Marker Concentration [ng/µl] : 0
Used Lower Marker for Quantitation
Standard Curve Fit is Logarithmic
Show Data Aligned to Lower Marker

Integrator Settings

Integration Start Time [s] : 19
Integration End Time [s] : 69
Slope Threshold : 0.6
Height Threshold [FU] : 0.5
Area Threshold : 0.2
Width Threshold [s] : 0.5
Baseline Plateau [s] : 6

Filter Settings

Filter Width [s] : 0.5
Polynomial Order : 4

Ladder

Ladder Peak	Size
1	25
2	200
3	500
4	1000
5	2000
6	4000

6.2 The results from the RNAseq analysis of the testes tissue (Table 6.1)

N	gene_id	logFC	V_TESTES	M_Testes	PValue
1	c15699_g1	8,750	0	54	2,97E-07
2	c58283_g1	8,639	0	50	5,43E-07
3	c11986_g1	8,422	0	43	1,72E-06
4	c38051_g1	8,354	0	41	2,45E-06
5	c52158_g4	8,318	0	40	2,94E-06
6	c97454_g1	8,167	0	36	6,32E-06
7	c57629_g2	8,041	0	33	1,17E-05
8	c128061_g1	7,856	0	29	2,82E-05
9	c52274_g1	7,699	0	26	5,79E-05
10	c123143_g1	7,699	0	26	5,79E-05
11	c44387_g1	7,629	1	225	1,15E-11
12	c37552_g1	7,480	1	203	3,00E-11
13	c56753_g2	7,323	0	20	0,00029
14	c14215_g1	7,323	0	20	0,00029
15	c13478_g1	7,172	0	18	0,00053
16	c38273_g1	7,057	2	286	6,97E-12
17	c32508_g1	6,903	1	136	1,17E-09
18	c47470_g1	6,849	1	131	1,64E-09
19	c24782_g1	6,776	6	679	1,89E-13
20	c50402_g1	6,698	1	118	4,15E-09
21	c36907_g1	6,597	2	208	1,32E-10
22	c45607_g1	6,544	1	106	1,06E-08
23	c84195_g1	6,544	1	106	1,06E-08
24	c44747_g1	6,323	3	253	8,63E-11
25	c51337_g1	6,308	1	90	4,37E-08
26	c97069_g1	6,191	1	83	7,86E-08
27	c39724_g1	6,104	35	2445	2,16E-13
28	c35561_g1	5,839	1	65	5,79E-07
29	c25108_g1	5,745	21	1146	5,13E-12
30	c122821_g1	5,650	1	57	1,63E-06
31	c34116_g1	5,603	16	793	2,24E-11
32	c123047_g1	5,599	1	55	2,14E-06
33	c34524_g1	5,584	2	103	6,99E-08
34	c42518_g1	5,468	2	95	1,39E-07
35	c122834_g1	5,395	21	899	5,12E-11
36	c15819_g1	5,348	79	3261	1,64E-11
37	c55481_g1	5,202	2	79	5,82E-07
38	c72383_g1	5,109	3	109	1,60E-07
39	c58415_g1	5,094	5	177	1,98E-08
40	c34152_g1	5,070	5	174	2,30E-08
41	c15924_g1	4,989	1	36	4,69E-05
42	c48988_g3	4,926	7	219	1,63E-08
43	c54167_g1	4,811	4	117	2,66E-07
44	c39853_g1	4,709	66	1750	1,18E-09
45	c33022_g1	4,682	5	133	2,37E-07
46	c52085_g2	4,661	5	131	2,69E-07
47	c48380_g1	4,589	6	149	2,13E-07
48	c49725_g1	4,572	2	51	1,71E-05
49	c123043_g1	4,477	416	9376	3,08E-09
50	c48988_g1	4,468	11	249	7,14E-08
51	c55436_g1	4,392	18	385	3,97E-08
52	c96078_g1	4,360	2	44	4,92E-05
53	c53162_g1	4,327	2	43	5,78E-05
54	c13906_g1	4,258	30	583	4,19E-08
55	c97206_g1	4,247	31	598	4,29E-08
56	c84109_g1	4,201	3	58	2,20E-05
57	c49026_g1	4,140	13	234	3,46E-07
58	c22765_g1	4,110	2	37	0,0002
59	c33506_g1	4,091	4	71	1,35E-05
60	c45977_g1	4,086	11	191	7,01E-07
61	c42936_g2	3,983	253	4049	7,56E-08
62	c123354_g1	3,946	2	33	0,0003
63	c36293_g1	3,945	14	220	9,32E-07
64	c48988_g2	3,911	15	230	9,93E-07
65	c112992_g1	3,740	22	299	1,44E-06
66	c55736_g1	3,724	4	55	8,71E-05
67	c109541_g1	3,698	81	1065	5,64E-07
68	c50185_g1	3,694	5	67	5,01E-05
69	c31533_g1	3,694	16	211	3,07E-06
70	c21199_g1	3,606	8	100	2,30E-05
71	c43247_g1	3,559	5	61	9,81E-05
72	c32887_g1	3,528	4	48	0,0002
73	c46266_g1	3,502	51	586	2,34E-06
74	c110791_g1	3,497	4	47	0,0003
75	c49500_g1	3,482	9	103	3,33E-05
76	c39986_g1	3,468	129	1446	1,94E-06
77	c48959_g1	3,369	4	43	0,0005
78	c55551_g1	3,318	10	102	6,17E-05
79	c44647_g1	3,240	7	68	0,0002
80	c111644_g1	3,217	17	161	3,86E-05
81	c44955_g1	3,189	31	287	2,12E-05
82	c27147_g1	3,171	27	247	2,65E-05
83	c49730_g1	3,164	87	790	1,32E-05
84	c54159_g1	3,153	9	82	0,0002
85	c41732_g1	3,144	174	1557	1,14E-05
86	c48496_g1	3,130	14	125	8,41E-05
87	c43362_g1	3,127	6	54	0,0005
88	c56485_g2	3,100	6	53	0,0005
89	c55628_g1	3,095	283	2448	1,39E-05
90	c26491_g1	3,062	67	567	2,38E-05
91	c42352_g1	3,043	12	101	0,0002
92	c59379_g1	3,043	12	101	0,0002
93	c52144_g1	3,024	35	289	4,60E-05
94	c57220_g4	2,965	28	222	7,64E-05
95	c51861_g2	2,956	33	260	6,98E-05
96	c41816_g1	2,924	15	116	0,0002
97	c84264_g1	2,885	11	83	0,0004
98	c34087_g1	2,852	17	125	0,0003
99	c13969_g1	2,791	70	491	9,98E-05
100	c39639_g1	2,757	36	247	0,0002

101	c26571_g1	2,734	25	169	0,0003
102	c29083_g1	2,688	360	2348	0,0001
103	c52914_g1	2,646	40	254	0,0003
104	c58240_g1	2,589	38	232	0,0004
105	c122942_g1	2,575	227	1369	0,0002
106	c19100_g1	2,474	160	900	0,0004
107	c122679_g1	2,415	221	1193	0,0005
108	c50538_g1	-2,387	2787	539	0,0005
109	c55344_g2	-2,417	935	177	0,0005
110	c57765_g3	-2,436	2696	504	0,0004
111	c47407_g1	-2,445	2083	387	0,0004
112	c56265_g1	-2,457	1753	323	0,0004
113	c43732_g1	-2,457	641	118	0,0005
114	c51276_g1	-2,479	954	173	0,0004
115	c46996_g1	-2,501	549	98	0,0004
116	c55344_g1	-2,520	1520	268	0,0003
117	c56371_g1	-2,526	251	44	0,0005
118	c44092_g1	-2,553	627	108	0,0003
119	c57759_g4	-2,554	1504	259	0,0002
120	c55206_g1	-2,559	519	89	0,0003
121	c57355_g1	-2,577	779	132	0,0002
122	c31286_g1	-2,593	603	101	0,0002
123	c56249_g1	-2,594	1075	180	0,0002
124	c56992_g5	-2,615	152	25	0,0005
125	c55353_g1	-2,623	981	161	0,0002
126	c57733_g1	-2,624	2900	476	0,0002
127	c47427_g1	-2,634	154	25	0,0005
128	c57763_g1	-2,637	388	63	0,0003
129	c57312_g1	-2,639	253	41	0,0003
130	c53079_g2	-2,644	186	30	0,0004
131	c58068_g1	-2,650	2191	353	0,0001
132	c110096_g1	-2,654	150	24	0,0005
133	c49218_g1	-2,655	231	37	0,0003
134	c45643_g1	-2,656	468	75	0,0002
135	c55193_g1	-2,661	207	33	0,0003
136	c43987_g1	-2,667	145	23	0,0005
137	c53267_g1	-2,670	246	39	0,0003
138	c52375_g1	-2,673	1702	270	0,0001
139	c48857_g2	-2,673	650	103	0,0002
140	c56986_g1	-2,686	236	37	0,0003
141	c53096_g1	-2,696	276	43	0,0002
142	c84182_g1	-2,699	129	20	0,0005
143	c57475_g1	-2,699	1156	180	0,0001
144	c49934_g1	-2,716	228	35	0,0002
145	c52618_g1	-2,721	294	45	0,0002
146	c56982_g1	-2,730	617	94	0,0001
147	c58058_g1	-2,730	1463	223	0,0001
148	c53383_g1	-2,733	395	60	0,0002
149	c30480_g1	-2,740	146	22	0,0003
150	c26571_g2	-3,940	546,4209	-125,508	0,0002
151	c50698_g3	-2,746	299	45	0,0002
152	c57476_g1	-2,758	101	15	0,0005
153	c57488_g2	-2,764	880	131	9,55E-05
154	c41118_g1	-2,764	276	41	0,0002
155	c46296_g1	-2,770	149	22	0,0003
156	c55370_g2	-2,771	574	85	0,0001
157	c58044_g1	-2,778	8328	1228	6,96E-05
158	c54470_g1	-2,786	2339	343	7,27E-05
159	c55938_g1	-2,792	822	120	8,37E-05
160	c97629_g1	-2,794	227	33	0,0002
161	c52929_g2	-2,797	516	75	0,0001
162	c52011_g1	-2,809	167	24	0,0002
163	c40049_g1	-2,831	268	38	0,0001
164	c47671_g1	-2,840	291	41	0,0001
165	c57095_g3	-2,842	1000	141	6,20E-05
166	c33415_g1	-2,844	164	23	0,0002
167	c49275_g1	-2,859	180	25	0,0002
168	c46960_g1	-2,860	948	132	5,69E-05
169	c49571_g1	-2,872	869	120	5,48E-05
170	c40254_g1	-2,884	1022	140	4,97E-05
171	c29046_g1	-2,885	1198	164	4,80E-05
172	c57465_g1	-2,887	688	94	5,38E-05
173	c52535_g1	-2,891	2442	333	4,16E-05
174	c85185_g1	-2,900	3779	512	3,80E-05
175	c50803_g1	-2,904	2331	315	3,88E-05
176	c55911_g2	-2,904	97	13	0,0003
177	c23092_g1	-2,943	434	57	5,39E-05
178	c74646_g1	-2,963	101	13	0,0002
179	c46348_g1	-2,978	507	65	4,19E-05
180	c57289_g3	-2,996	127	16	0,0001
181	c40895_g1	-2,996	127	16	0,0001
182	c54701_g1	-2,997	482	61	3,91E-05
183	c51200_g1	-2,997	277	35	5,71E-05
184	c48389_g2	-3,012	6602	828	2,00E-05
185	c56712_g2	-3,018	305	38	4,80E-05
186	c50003_g1	-3,018	113	14	0,0002
187	c41212_g1	-3,019	73	9	0,0004
188	c27294_g1	-3,040	1440	177	2,03E-05
189	c57161_g1	-3,041	839	103	2,26E-05
190	c53386_g2	-3,074	367	44	3,20E-05
191	c62696_g1	-3,077	101	12	0,0001
192	c55202_g1	-3,081	327	39	3,36E-05
193	c51547_g1	-3,086	496	59	2,46E-05
194	c57780_g1	-3,106	69	8	0,0003
195	c55013_g1	-3,117	241	28	3,83E-05
196	c56822_g1	-3,118	61	7	0,0004
197	c42551_g1	-3,129	3640	421	1,10E-05
198	c57275_g2	-3,141	245	28	3,37E-05
199	c48497_g1	-3,155	617	70	1,53E-05
200	c10274_g1	-3,155	133	15	7,01E-05

201	c52211_g3	-3,157	583	66	1,57E-05
202	c54336_g3	-3,160	107	12	9,91E-05
203	c54965_g1	-3,164	249	28	2,97E-05
204	c64784_g1	-3,187	55	6	0,0004
205	c52365_g1	-3,187	136	15	5,94E-05
206	c122852_g1	-3,187	226	25	2,97E-05
207	c34646_g1	-3,195	182	20	3,75E-05
208	c39315_g1	-3,200	219	24	2,90E-05
209	c10933_g1	-3,203	92	10	0,0001
210	c39172_g1	-3,216	1232	134	8,00E-06
211	c57699_g1	-3,221	84	9	0,0001
212	c97168_g1	-3,229	279	30	1,90E-05
213	c41861_g1	-3,233	94	10	9,67E-05
214	c40163_g1	-3,238	57	6	0,0003
215	c52020_g1	-3,243	104	11	7,65E-05
216	c53271_g1	-3,257	9694	1026	4,90E-06
217	c45672_g1	-3,257	105	11	7,13E-05
218	c28791_g1	-3,263	58	6	0,0003
219	c54949_g3	-3,264	134	14	4,46E-05
220	c54630_g1	-3,289	146	15	3,49E-05
221	c37731_g1	-3,292	921	95	5,71E-06
222	c57149_g1	-3,297	516	53	8,14E-06
223	c42373_g1	-3,321	90	9	7,76E-05
224	c52287_g1	-3,323	100	10	6,17E-05
225	c47613_g1	-3,324	387	39	8,90E-06
226	c52991_g1	-3,327	1806	182	4,00E-06
227	c56631_g1	-3,345	1136	113	3,99E-06
228	c44673_g1	-3,349	263	26	1,14E-05
229	c48961_g1	-3,349	122	12	3,77E-05
230	c44690_g1	-3,351	102	10	5,34E-05
231	c40912_g1	-3,361	123	12	3,54E-05
232	c52088_g1	-3,369	42	4	0,0005
233	c51738_g2	-3,380	2594	252	2,81E-06
234	c57460_g1	-3,385	889	86	3,47E-06
235	c45148_g1	-3,389	84	8	7,81E-05
236	c58211_g1	-3,396	230	22	1,12E-05
237	c19115_g1	-3,396	74	7	0,0001
238	c50248_g1	-3,405	64	6	0,0001
239	c49755_g1	-3,407	1448	138	2,65E-06
240	c47531_g1	-3,412	138	13	2,33E-05
241	c46178_g1	-3,417	54	5	0,0002
242	c40189_g1	-3,421	160	15	1,73E-05
243	c53900_g1	-3,434	76	7	8,62E-05
244	c44081_g1	-3,436	44	4	0,0004
245	c57523_g2	-3,440	205	19	1,08E-05
246	c57335_g3	-3,443	227	21	9,15E-06
247	c57768_g1	-3,448	2407	223	1,92E-06
248	c33133_g1	-3,468	756	69	2,63E-06
249	c56821_g1	-3,469	1938	177	1,77E-06
250	c56302_g1	-3,493	235	21	6,94E-06

251	c52329_g1	-3,561	83	7	4,53E-05
252	c48371_g1	-3,564	165	14	8,97E-06
253	c48474_g1	-3,573	119	10	1,68E-05
254	c37881_g1	-3,578	320	27	3,05E-06
255	c27193_g1	-3,578	84	7	4,15E-05
256	c46734_g1	-3,587	37	3	0,0005
257	c48397_g1	-3,591	49	4	0,0002
258	c44557_g1	-3,594	1159	97	9,80E-07
259	c57187_g5	-3,603	758	63	1,26E-06
260	c40503_g1	-3,608	146	12	9,48E-06
261	c57459_g1	-3,609	122	10	1,38E-05
262	c57429_g1	-3,626	160	13	7,35E-06
263	c48199_g1	-3,643	594	48	1,20E-06
264	c48656_g1	-3,644	162	13	6,66E-06
265	c55380_g1	-3,648	51	4	0,0001
266	c57565_g3	-3,648	51	4	0,0001
267	c36326_g1	-3,654	188	15	4,83E-06
268	c46862_g1	-3,654	188	15	4,83E-06
269	c42390_g1	-3,661	939	75	8,06E-07
270	c42834_g1	-3,661	189	15	4,63E-06
271	c51291_g2	-3,699	40	3	0,0003
272	c45839_g1	-3,699	40	3	0,0003
273	c49521_g1	-3,708	79	6	3,24E-05
274	c54522_g2	-3,721	197	15	3,31E-06
275	c34264_g1	-3,732	645	49	6,94E-07
276	c56554_g1	-3,734	396	30	1,10E-06
277	c33419_g1	-3,735	41	3	0,0002
278	c55084_g2	-3,749	2526	190	3,29E-07
279	c57026_g1	-3,759	122	9	7,85E-06
280	c44596_g1	-3,775	1057	78	4,02E-07
281	c54063_g1	-3,783	56	4	7,67E-05
282	c63028_g1	-3,787	288	21	1,27E-06
283	c55915_g2	-3,792	207	15	2,21E-06
284	c26153_g1	-3,803	43	3	0,0002
285	c43509_g2	-3,822	393	28	7,12E-07
286	c53338_g1	-3,833	58	4	5,98E-05
287	c42248_g2	-3,837	2953	209	1,91E-07
288	c57759_g1	-3,862	246	17	1,19E-06
289	c56175_g1	-3,866	117	8	5,88E-06
290	c38916_g1	-3,869	45	3	0,0001
291	c56951_g2	-3,871	638	44	3,30E-07
292	c57056_g1	-3,875	1075	74	2,26E-07
293	c53374_g2	-3,907	150	10	2,70E-06
294	c54496_g1	-3,911	418	28	4,21E-07
295	c51614_g1	-3,931	47	3	9,86E-05
296	c53737_g1	-3,964	264	17	6,35E-07
297	c44883_g1	-3,979	33	2	0,0003
298	c22879_g1	-3,979	33	2	0,0003
299	c46130_g1	-3,997	65	4	2,61E-05
300	c84227_g1	-4,003	192	12	1,04E-06

301	c55216_g1	-4,014	449	28	2,23E-07
302	c29685_g1	-4,019	66	4	2,33E-05
303	c57856_g2	-4,020	50	3	6,41E-05
304	c40300_g1	-4,022	34	2	0,0003
305	c43713_g1	-4,022	34	2	0,0003
306	c56612_g1	-4,022	34	2	0,0003
307	c52848_g1	-4,033	196	12	8,73E-07
308	c51509_g2	-4,041	67	4	2,08E-05
309	c52465_g2	-4,043	116	7	3,20E-06
310	c55977_g3	-4,053	166	10	1,18E-06
311	c32079_g1	-4,054	560	34	1,42E-07
312	c28809_g1	-4,056	117	7	2,99E-06
313	c34934_g1	-4,063	35	2	0,0002
314	c58025_g1	-4,076	152	9	1,35E-06
315	c44543_g1	-4,083	203	12	6,51E-07
316	c51015_g1	-4,088	86	5	7,66E-06
317	c54053_g2	-4,092	238	14	4,45E-07
318	c51721_g1	-4,104	36	2	0,0002
319	c51754_g1	-4,104	53	3	4,23E-05
320	c56210_g2	-4,104	53	3	4,23E-05
321	c45019_g1	-4,104	53	3	4,23E-05
322	c56234_g1	-4,107	513	30	1,18E-07
323	c57830_g1	-4,124	226	13	4,32E-07
324	c57952_g1	-4,128	1125	65	5,19E-08
325	c28857_g1	-4,143	264	15	2,79E-07
326	c51224_g1	-4,143	526	30	9,46E-08
327	c52025_g1	-4,153	90	5	4,93E-06
328	c56528_g1	-4,155	407	23	1,27E-07
329	c52729_g1	-4,182	38	2	0,0001
330	c53747_g1	-4,183	56	3	2,84E-05
331	c56928_g1	-4,183	56	3	2,84E-05
332	c71597_g1	-4,185	92	5	4,15E-06
333	c46777_g1	-4,209	57	3	2,50E-05
334	c52966_g1	-4,274	117	6	1,41E-06
335	c53631_g1	-4,279	175	9	4,18E-07
336	c20346_g1	-4,283	60	3	1,71E-05
337	c42212_g1	-4,296	177	9	3,80E-07
338	c54800_g1	-4,308	159	8	4,88E-07
339	c53638_g1	-4,314	81	4	4,90E-06
340	c42132_g1	-4,316	278	14	1,14E-07
341	c51579_g1	-4,328	578	29	3,19E-08
342	c109590_g1	-4,359	43	2	5,78E-05
343	c44734_g1	-4,370	309	15	7,17E-08
344	c56412_g1	-4,375	474	23	3,27E-08
345	c56056_g6	-4,378	2406	117	7,58E-09
346	c33376_g1	-4,398	65	3	9,39E-06
347	c36801_g1	-4,406	212	10	1,51E-07
348	c44337_g1	-4,425	45	2	4,20E-05
349	c57307_g1	-4,429	407	19	3,24E-08
350	c54748_g1	-4,431	237	11	1,02E-07
351	c57630_g3	-4,463	68	3	6,66E-06
352	c39905_g1	-4,484	69	3	5,95E-06
353	c55854_g1	-4,530	231	10	7,18E-08
354	c58085_g1	-4,544	1617	70	3,63E-09
355	c54804_g1	-4,553	26	1	0,0004
356	c57630_g5	-4,553	26	1	0,0004
357	c25218_g1	-4,558	189	8	1,13E-07
358	c52207_g1	-4,563	143	6	2,69E-07
359	c55977_g2	-4,576	50	2	1,98E-05
360	c47541_g1	-4,589	98	4	9,87E-07
361	c79516_g1	-4,633	52	2	1,48E-05
362	c55430_g1	-4,633	52	2	1,48E-05
363	c57146_g2	-4,636	175	7	1,06E-07
364	c54930_g1	-4,642	151	6	1,70E-07
365	c23816_g1	-4,654	202	8	6,35E-08
366	c22300_g1	-4,660	28	1	0,0002
367	c53649_g1	-4,660	28	1	0,0002
368	c39523_g1	-4,660	53	2	1,29E-05
369	c49786_g1	-4,710	29	1	0,0002
370	c51664_g1	-4,710	29	1	0,0002
371	c57146_g1	-4,723	212	8	4,17E-08
372	c46693_g1	-4,735	319	12	1,26E-08
373	c51676_g1	-4,748	136	5	1,73E-07
374	c56696_g1	-4,748	136	5	1,73E-07
375	c43721_g2	-4,759	30	1	0,0002
376	c41006_g1	-4,759	30	1	0,0002
377	c52071_g1	-4,764	2473	92	6,98E-10
378	c57080_g3	-4,790	58	2	6,56E-06
379	c46897_g1	-4,806	31	1	0,0001
380	c36988_g1	-4,806	31	1	0,0001
381	c55953_g1	-4,829	172	6	5,58E-08
382	c52350_g1	-4,896	33	1	8,44E-05
383	c56933_g1	-4,905	122	4	1,63E-07
384	c50418_g1	-4,939	34	1	6,91E-05
385	c16849_g1	-5,060	37	1	3,89E-05
386	c56745_g4	-5,064	632	19	5,85E-10
387	c41322_g1	-5,099	38	1	3,23E-05
388	c36354_g1	-5,099	38	1	3,23E-05
389	c44172_g1	-5,099	580	17	5,84E-10
390	c49931_g1	-5,103	140	4	5,06E-08
391	c53943_g1	-5,208	41	1	1,89E-05
392	c51110_g1	-5,310	44	1	1,14E-05
393	c27665_g1	-5,342	45	1	9,66E-06
394	c22339_g1	-5,342	45	1	9,66E-06
395	c57729_g1	-5,374	374	9	4,94E-10
396	c36680_g1	-5,404	47	1	7,02E-06
397	c52158_g5	-5,404	47	1	7,02E-06
398	c58077_g1	-5,414	511	12	1,69E-10
399	c54336_g2	-5,462	136	3	2,09E-08
400	c53791_g1	-5,464	49	1	5,15E-06

401	c57138_g1	-5,483	138	3	1,84E-08
402	c51609_g1	-5,543	236	5	1,38E-09
403	c122664_g1	-5,614	248	5	8,82E-10
404	c97540_g1	-5,732	59	1	1,24E-06
405	c35555_g1	-5,751	1039271	19529	6,46E-13
406	c49566_g1	-5,752	965	18	7,32E-12
407	c96830_g1	-6,182	568535	7923	3,55E-14
408	c53744_g1	-6,603	108	1	7,70E-09
409	c55702_g3	-6,837	240	2	3,23E-11
410	c56529_g2	-7,095	152	1	3,80E-10
411	c56969_g1	-7,188	18	0	0,0005
412	c50940_g1	-7,188	18	0	0,0005
413	c39646_g1	-7,266	19	0	0,0004
414	c54754_g1	-7,266	19	0	0,0004
415	c37426_g1	-7,266	19	0	0,0004
416	c50234_g1	-7,266	19	0	0,0004
417	c49497_g1	-7,266	19	0	0,0004
418	c127908_g1	-7,339	20	0	0,0003
419	c57032_g1	-7,409	21	0	0,0002
420	c45809_g1	-7,409	21	0	0,0002
421	c87715_g1	-7,409	21	0	0,0002
422	c25757_g1	-7,476	22	0	0,0002
423	c36221_g1	-7,476	22	0	0,0002
424	c56239_g1	-7,476	22	0	0,0002
425	c43357_g1	-7,476	22	0	0,0002
426	c23167_g1	-7,540	23	0	0,0001
427	c30197_g1	-7,540	23	0	0,0001
428	c54896_g1	-7,601	24	0	9,64E-05
429	c58091_g1	-7,770	27	0	4,53E-05
430	c14610_g1	-7,770	27	0	4,53E-05
431	c61895_g1	-7,822	28	0	3,57E-05
432	c129997_g1	-7,822	28	0	3,57E-05
433	c11533_g1	-7,822	28	0	3,57E-05
434	c44875_g1	-7,873	29	0	2,82E-05
435	c47892_g7	-7,873	29	0	2,82E-05
436	c26015_g1	-7,921	30	0	2,25E-05
437	c46922_g1	-7,921	30	0	2,25E-05
438	c15769_g1	-7,921	30	0	2,25E-05
439	c48096_g1	-8,143	35	0	7,72E-06
440	c47807_g1	-8,183	36	0	6,32E-06
441	c32888_g1	-8,223	37	0	5,19E-06
442	c55216_g2	-8,261	38	0	4,28E-06
443	c43606_g1	-8,405	42	0	2,05E-06
444	c53237_g1	-8,472	44	0	1,45E-06
445	c37751_g1	-8,793	55	0	2,57E-07
446	c39173_g1	-8,870	58	0	1,68E-07
447	c56973_g2	-8,918	60	0	1,28E-07
448	c61964_g1	-8,966	62	0	9,84E-08
449	c111150_g1	-9,502	90	0	3,94E-09
450	c53286_g1	-9,683	102	0	1,34E-09
451	c52187_g1	-9,724	105	0	1,04E-09

6.3 The results from the RNAseq analysis of the male accessory gland with ejaculatory bulb tissue (Table 6.2)

N	gene_id	logFC	M_MALE_1	M_MALE_2	V_MALE_1	PValue
1	c31616_g1	-13,051	916,98	1499	0	1,03E-16
2	c52655_g1	-12,304	111	1144	0	1,58E-14
3	c51710_g1	-12,110	1264,95	329,99	0	5,89E-14
4	c47341_g2	-11,860	1021,93	301	0	3,11E-13
5	c47596_g1	-11,831	964,99	316	0	3,79E-13
6	c52892_g1	-11,801	1389	54	0	4,63E-13
7	c57023_g2	-11,708	664	418,42	0	8,58E-13
8	c23397_g1	-11,707	625	439,98	0	8,58E-13
9	c47442_g1	-11,700	687,96	399,98	0	8,94E-13
10	c53574_g1	-11,667	623	419	0	1,11E-12
11	c40374_g1	-11,645	668,89	381	0	1,30E-12
12	c47533_g1	-11,578	775	284,98	0	2,03E-12
13	c55275_g2	-11,254	587,16	245,93	0	1,68E-11
14	c41928_g1	-11,230	453	314	0	1,94E-11
15	c45555_g1	-11,191	25	545,7	0	2,43E-11
16	c57217_g1	-11,185	578	223,99	0	2,62E-11
17	c84745_g1	-11,180	523,75	253	0	2,72E-11
18	c57024_g2	-11,153	687,93	148	0	3,24E-11
19	c53204_g1	-11,101	432,95	276,3	0	4,53E-11
20	c53812_g1	-11,098	578	191	0	4,63E-11
21	c57131_g1	-11,024	567	171	0	7,44E-11
22	c39257_g2	-11,006	334	299,98	0	8,27E-11
23	c10660_g1	-10,976	477	207	0	1,00E-10
24	c47311_g1	-10,965	366,88	266,85	0	1,07E-10
25	c72530_g1	-10,960	321	291,9	0	1,12E-10
26	c53055_g1	-10,948	478	197	0	1,22E-10
27	c55859_g1	-10,922	365,31	254	0	1,43E-10
28	c39648_g1	-10,914	346,68	261,99	0	1,50E-10
29	c57875_g1	-10,907	338	264,94	0	1,57E-10
30	c53746_g1	-10,849	377	223,97	0	2,27E-10
31	c58086_g1	-10,834	551,96	118	0	2,50E-10
32	c54574_g1	-10,788	245,57	282	0	3,34E-10
33	c43349_g1	-10,772	434	168	0	3,69E-10
34	c55965_g3	-10,762	379	197	0	3,97E-10
35	c54053_g5	-10,746	418,95	168,98	0	4,39E-10
36	c52465_g1	-10,683	358	187,03	0	6,46E-10
37	c55810_g1	-10,667	334,99	195,87	0	7,18E-10
38	c33324_g1	-10,650	322	198,87	0	7,99E-10
39	c54844_g2	-10,645	354	178,89	0	8,21E-10
40	c26099_g1	-10,644	387,99	159	0	8,43E-10
41	c58149_g1	-10,630	274,98	221	0	9,14E-10
42	c51872_g1	-10,620	372	161,93	0	9,66E-10
43	c30784_g2	-10,593	242,96	229,74	0	1,14E-09
44	c10274_g1	-10,574	281	203	0	1,31E-09
45	c56997_g1	-10,563	405	128	0	1,39E-09
46	c32538_g1	-10,544	263	206	0	1,55E-09
47	c54799_g1	-10,532	245,81	212,95	0	1,69E-09
48	c57176_g2	-10,527	314	171,83	0	1,74E-09
49	c43839_g1	-10,525	236	216,93	0	1,74E-09
50	c52007_g1	-10,520	400	119,78	0	1,85E-09
51	c57583_g1	-10,516	252,94	204,54	0	1,85E-09
52	c54018_g2	-10,500	293,99	176,73	0	2,08E-09
53	c54728_g1	-10,490	201	229	0	2,14E-09
54	c49066_g1	-10,481	293	173	0	2,34E-09
55	c45487_g1	-10,480	245	200,93	0	2,34E-09
56	c47712_g1	-10,475	270	184,99	0	2,41E-09
57	c52931_g1	-10,454	353,98	130,93	0	2,80E-09
58	c49237_g1	-10,379	342	121	0	4,33E-09
59	c34297_g1	-10,372	329	126,93	0	4,61E-09
60	c57747_g1	-10,353	314,62	131	0	5,07E-09
61	c56234_g1	-10,352	378	93,97	0	5,24E-09
62	c53020_g1	-10,341	196	198	0	5,41E-09
63	c56726_g1	-10,275	183,99	190,97	0,35	8,33E-09
64	c57660_g1	-10,269	368	82	0	8,62E-09
65	c50703_g1	-10,253	301	118	0	9,55E-09
66	c50574_g1	-10,229	286	122	0	1,10E-08
67	c54908_g1	-10,222	321	100	0	1,13E-08
68	c55183_g1	-10,211	302	109	0	1,22E-08
69	c55990_g1	-10,197	246	138,72	0	1,35E-08
70	c56768_g6	-10,193	259,99	130	0	1,35E-08
71	c46700_g1	-10,187	183	174,27	0	1,40E-08
72	c34700_g1	-10,171	313,87	94,1	0	1,56E-08
73	c56128_g2	-10,162	185	167,7	0	1,62E-08
74	c54701_g1	-10,148	383	48,98	0	1,81E-08
75	c47569_g1	-10,135	206,94	150	0	1,94E-08
76	c55175_g1	-10,133	237	131,62	0	1,94E-08
77	c52369_g1	-10,114	202,21	149	0	2,17E-08
78	c48551_g1	-10,098	231	129	0	2,43E-08
79	c71420_g1	-10,082	242,97	119	0	7,02E-09
80	c53306_g2	-10,079	215,33	134,95	0	7,02E-09
81	c51656_g1	-10,069	30	242	0	7,02E-09
82	c37891_g1	-10,067	206	138,03	0	7,58E-09
83	c55308_g1	-10,065	231	123	0	7,58E-09
84	c54308_g1	-10,054	294	84,03	0	8,20E-09
85	c46027_g1	-10,047	238,69	114,56	0	8,52E-09
86	c53094_g7	-10,046	186,15	146	0	8,52E-09
87	c56461_g1	-10,013	258	98	0	1,04E-08
88	c47034_g1	-10,009	203,93	128,99	0	1,08E-08
89	c56555_g3	-10,008	174,57	145,68	0	1,08E-08
90	c56397_g3	-10,007	195	134	0	1,08E-08
91	c57800_g1	-10,002	201,98	128,97	0	1,13E-08
92	c10628_g1	-9,996	190	134,67	0	1,17E-08
93	c36907_g1	-9,994	18	235,99	0	1,08E-08
94	c49121_g2	-9,994	203	126,84	0	1,17E-08
95	c55037_g1	-9,987	268,93	87	0	1,22E-08
96	c43052_g1	-9,972	195	128	0	1,33E-08
97	c51676_g1	-9,972	178	138	0	1,33E-08
98	c46198_g1	-9,971	222	111,74	0	1,33E-08
99	c23655_g1	-9,968	169,97	142	0	1,38E-08
100	c55935_g1	-9,959	205	119,71	0	1,44E-08
101	c47358_g2	-9,947	275,98	76	0	1,56E-08
102	c57740_g4	-9,936	207	115	0	1,70E-08
103	c84178_g1	-9,911	77,98	187	0	1,85E-08
104	c24730_g1	-9,895	265,32	74	0	2,20E-08
105	c49709_g1	-9,893	172,98	128,37	0	2,20E-08
106	c54174_g1	-9,889	298,99	52,99	0	2,30E-08
107	c47367_g1	-9,888	81,83	181	0	2,20E-08
108	c36529_g1	-9,884	196,36	112,98	0	2,30E-08
109	c109723_g1	-9,883	233	91	0	2,30E-08
110	c39363_g1	-9,879	176	124	0	2,40E-08
111	c44810_g1	-9,871	201	108	0	2,50E-08
112	c48513_g1	-9,868	48	198	0	2,40E-08
113	c53513_g1	-9,864	245	81	0	2,62E-08
114	c53176_g1	-9,845	134,86	142,99	0	2,86E-08
115	c57054_g1	-9,842	207	100	0	2,99E-08
116	c42361_g1	-9,838	260	68	0	2,99E-08
117	c57559_g1	-9,822	168	119,79	0	3,27E-08
118	c42395_g1	-9,819	211,04	93,99	0	3,42E-08
119	c56756_g1	-9,818	216	91	0	3,42E-08
120	c36028_g1	-9,817	116	150	0	3,27E-08

121	c43148_g1	-9,812	156,99	125	0	3,42E-08
122	c47769_g1	-9,812	265	61	0	3,58E-08
123	c56109_g1	-9,807	212,65	91	0	3,58E-08
124	c57289_g3	-9,800	181	108,94	0	3,74E-08
125	c55614_g1	-9,798	128	140	0	3,74E-08
126	c23039_g1	-9,794	158,95	121	0	3,92E-08
127	c58429_g1	-9,785	150	124,96	0	4,10E-08
128	c55049_g1	-9,784	159,99	118,98	0	4,10E-08
129	c54905_g1	-9,781	220	82,9	0	4,30E-08
130	c56979_g1	-9,770	197	94,76	0	4,50E-08
131	c46230_g1	-9,766	186	101	0	4,71E-08
132	c57287_g3	-9,756	223,94	77	0	4,94E-08
133	c57544_g1	-9,751	156,99	116	0	4,94E-08
134	c54779_g3	-9,751	211	84	0	5,18E-08
135	c50340_g1	-9,746	191	94,94	0	5,18E-08
136	c54327_g1	-9,745	174	104,99	0	5,18E-08
137	c49506_g1	-9,743	204,28	87	0	5,43E-08
138	c55988_g1	-9,737	198,83	89,03	0	5,43E-08
139	c43974_g1	-9,735	158	112,98	0	5,43E-08
140	c122920_g1	-9,733	102	145,82	0	5,43E-08
141	c53769_g1	-9,722	187	94	0	5,97E-08
142	c49458_g1	-9,715	219	74	0	6,26E-08
143	c56560_g1	-9,710	174	100	0	6,57E-08
144	c46120_g1	-9,709	157	110	0	6,57E-08
145	c51692_g1	-9,709	199	85	0	6,57E-08
146	c56087_g1	-9,703	132	123,98	0	6,57E-08
147	c56786_g4	-9,700	256	50	0	6,89E-08
148	c36776_g1	-9,699	195	86	0	6,89E-08
149	c52416_g1	-9,696	191	88,24	0	6,89E-08
150	c49617_g1	-9,691	210	76	0	7,23E-08
151	c53862_g1	-9,688	135	120	0	7,23E-08
152	c47841_g1	-9,679	137,94	116,97	0	7,60E-08
153	c49752_g1	-9,674	247,76	51	0	7,98E-08
154	c72383_g1	-9,669	6	194	0	7,23E-08
155	c54746_g5	-9,668	271,97	36	0	8,38E-08
156	c38329_g1	-9,662	178	91,36	0	8,38E-08
157	c36507_g1	-9,659	162	100,11	0	8,81E-08
158	c53032_g1	-9,653	132	117	0	8,81E-08
159	c47521_g1	-9,647	60	158,95	0	8,81E-08
160	c58154_g3	-9,640	215	66,43	0	9,73E-08
161	c53847_g1	-9,626	227	56,99	0	1,08E-07
162	c54326_g1	-9,598	150	99	0	1,25E-07
163	c57202_g1	-9,595	210	62,51	0	1,25E-07
164	c54309_g1	-9,592	122	114,98	0	1,25E-07
165	c55372_g1	-9,591	125	113	0	1,25E-07
166	c56198_g1	-9,590	214	59,94	0	1,32E-07
167	c57339_g1	-9,583	203,99	65	0	1,39E-07
168	c55488_g1	-9,581	206,99	63	0	1,39E-07
169	c29799_g1	-9,581	143	101	0	1,39E-07
170	c37516_g1	-9,573	132,98	105,96	0	1,39E-07
171	c54688_g2	-9,568	141,96	99,98	0	1,46E-07
172	c50533_g1	-9,555	161	87	0	1,63E-07
173	c56117_g3	-9,547	161	86	0	1,63E-07
174	c27746_g1	-9,546	144	95,99	0	1,63E-07
175	c71442_g1	-9,542	165	82,97	0	1,71E-07
176	c32929_g1	-9,542	227	46	0	1,71E-07
177	c57257_g1	-9,529	174	76,08	0	1,81E-07
178	c52857_g1	-9,526	179,94	72,18	0	1,91E-07
179	c71604_g1	-9,525	205	56,58	0	1,91E-07
180	c55825_g1	-9,523	171,01	77,18	0	1,91E-07
181	c25284_g1	-9,519	140	95,41	0	1,91E-07
182	c57571_g4	-9,517	168	77,95	0	2,01E-07
183	c51418_g1	-9,511	192	63	0	2,12E-07
184	c49047_g1	-9,510	184,98	66,88	0	2,12E-07
185	c24597_g1	-9,509	111	110,99	0	2,01E-07
186	c57828_g1	-9,508	178	71	0	2,12E-07
187	c55077_g1	-9,507	156	84	0	2,12E-07
188	c49465_g1	-9,500	141	92	0	2,12E-07
189	c10586_g1	-9,492	74	131	0	2,24E-07
190	c52312_g1	-9,488	199	56	0	2,37E-07
191	c57338_g3	-9,487	162	78,32	0	2,37E-07
192	c54951_g2	-9,482	176	69	0	2,50E-07
193	c54954_g1	-9,478	207	50	0	2,50E-07
194	c56282_g1	-9,475	150,93	83	0	2,50E-07
195	c52101_g1	-9,470	182	64	0	2,65E-07
196	c34968_g1	-9,468	148	84	0	2,65E-07
197	c56584_g5	-9,451	133	91	0	2,80E-07
198	c55513_g3	-9,445	124,61	95	0	2,96E-07
199	c54036_g1	-9,444	150	80	0	2,96E-07
200	c32455_g1	-9,441	161	72,99	0	3,13E-07
201	c46987_g1	-9,439	144	82,59	0	3,13E-07
202	c45548_g1	-9,435	127,98	92	0	3,13E-07
203	c39884_g1	-9,419	48	138	0	3,31E-07
204	c54070_g1	-9,418	93	111,45	0	3,50E-07
205	c51292_g1	-9,410	72,9	122	0	3,50E-07
206	c57127_g1	-9,408	153	73,98	0	3,71E-07
207	c49531_g1	-9,405	144	79	0	3,71E-07
208	c39796_g1	-9,404	188,99	52	0	3,93E-07
209	c44934_g1	-9,398	121	92	0	3,93E-07
210	c52661_g1	-9,398	160,71	68	0	3,93E-07
211	c53337_g1	-9,396	134	84	0	3,93E-07
212	c55043_g1	-9,394	151,87	73	0	3,93E-07
213	c54597_g1	-9,390	111,19	97	0	4,16E-07
214	c45909_g1	-9,387	174	59	0	4,16E-07
215	c10568_g1	-9,386	142	78	0	4,16E-07
216	c52914_g1	-9,384	8	157,99	0	3,71E-07
217	c58030_g1	-9,378	169	61	0	4,41E-07
218	c56800_g1	-9,372	76	116	0	4,41E-07
219	c34576_g1	-9,372	86	110	0	4,41E-07
220	c50975_g1	-9,366	130	83	0	4,67E-07
221	c53043_g1	-9,352	154	67	0	5,26E-07
222	c55223_g1	-9,350	141,65	74	0	5,26E-07
223	c51209_g1	-9,348	115	90	0	5,26E-07
224	c58938_g1	-9,345	116,01	89	0	5,26E-07
225	c48323_g1	-9,344	170,97	56	0	5,26E-07
226	c45033_g1	-9,339	180	50	0	5,58E-07
227	c32761_g1	-9,337	113	89,98	0	5,58E-07
228	c41198_g1	-9,335	131	79,18	0	5,58E-07
229	c55117_g1	-9,333	144	71	0	5,58E-07
230	c53527_g1	-9,333	148,52	68	0	5,58E-07
231	c50085_g1	-9,332	102	95,82	0	5,58E-07
232	c53840_g1	-9,328	123	83	0	5,92E-07
233	c44418_g1	-9,324	134	75,96	0	5,92E-07
234	c57220_g4	-9,323	17	146	0	5,58E-07
235	c51512_g1	-9,321	140	72	0	5,92E-07
236	c51138_g1	-9,317	171	53	0	6,29E-07
237	c48978_g1	-9,309	153	63	0,45	6,68E-07
238	c43752_g1	-9,306	129	77	0	6,68E-07
239	c53370_g1	-9,304	172	51	0	6,68E-07
240	c50866_g1	-9,304	91,98	99,43	0	6,68E-07

241	c48591_g1	-9,296	159	58	0	7,10E-07
242	c46050_g1	-9,296	89	100	0	6,68E-07
243	c54493_g2	-9,294	112	86	0	7,10E-07
244	c54940_g1	-9,287	124	78	0	7,10E-07
245	c27232_g1	-9,285	127	76,44	0	7,55E-07
246	c47829_g2	-9,283	100	92	0	7,55E-07
247	c55348_g1	-9,281	158	57,03	0	7,55E-07
248	c46703_g1	-9,276	132	72	0,19	8,03E-07
249	c55949_g1	-9,275	66,55	111	0	7,55E-07
250	c57701_g1	-9,272	133	71	0	8,03E-07
251	c44049_g1	-9,264	140	65,97	0	8,55E-07
252	c57848_g1	-9,262	148	61	0	8,55E-07
253	c57745_g1	-9,261	161	53,25	0	8,55E-07
254	c40654_g1	-9,260	106	85,81	0	8,55E-07
255	c51136_g1	-9,256	117	79	0	8,55E-07
256	c42808_g1	-9,256	92	94	0	8,55E-07
257	c49462_g1	-9,251	136	67	0	9,10E-07
258	c54688_g1	-9,248	79	101	0	9,10E-07
259	c71410_g1	-9,248	59	113	0	8,55E-07
260	c58230_g1	-9,241	161	50,61	0	9,69E-07
261	c42127_g1	-9,237	157	53	0	9,69E-07
262	c57784_g1	-9,236	170	45	0	9,69E-07
263	c41973_g1	-9,233	143	61	0	9,69E-07
264	c52946_g1	-9,233	92,98	91	0	9,69E-07
265	c13906_g1	-9,227	4	144	0	9,10E-07
266	c54049_g1	-9,227	122	72,97	0	1,03E-06
267	c56909_g3	-9,223	113	78	0	1,03E-06
268	c56302_g1	-9,221	126	70	0	1,03E-06
269	c26755_g1	-9,221	116,21	76	0	1,03E-06
270	c47182_g1	-9,216	77	98,7	0	1,03E-06
271	c50571_g1	-9,207	96,99	85,86	0	1,10E-06
272	c57411_g1	-9,206	175	39	0	1,17E-06
273	c55732_g1	-9,205	114,94	75,28	0	1,17E-06
274	c48948_g1	-9,203	108	78,98	0	1,17E-06
275	c55472_g1	-9,201	90,97	89	0	1,17E-06
276	c47914_g1	-9,200	134,05	63	0	1,17E-06
277	c38251_g1	-9,197	117	73	0	1,17E-06
278	c56986_g2	-9,196	144,99	56	0	1,25E-06
279	c53439_g1	-9,194	138	59,55	0	1,25E-06
280	c25322_g1	-9,187	122	69	0	1,25E-06
281	c54951_g1	-9,187	102	81	0	1,25E-06
282	c109602_g1	-9,185	95	85	0	1,25E-06
283	c47291_g1	-9,183	127,78	65	0	1,33E-06
284	c56676_g1	-9,181	136	60	0	1,33E-06
285	c34861_g1	-9,181	131	63	0	1,33E-06
286	c55357_g1	-9,175	105	77,99	0	1,33E-06
287	c53808_g2	-9,174	167,97	40	0	1,42E-06
288	c52669_g1	-9,170	91	86	0	1,33E-06
289	c122989_g1	-9,169	134	60	0	1,42E-06
290	c55220_g3	-9,169	182	31	0	1,42E-06
291	c44767_g1	-9,166	102	79	0	1,42E-06
292	c48858_g1	-9,165	134,98	59	0	1,42E-06
293	c57233_g1	-9,162	166	40,17	0	1,52E-06
294	c57683_g1	-9,153	143	53	0	1,52E-06
295	c56138_g2	-9,147	141,97	52,99	0	1,62E-06
296	c54430_g1	-9,145	92	82,95	0	1,62E-06
297	c36286_g1	-9,137	108,98	72	0	1,62E-06
298	c39441_g1	-9,135	102	75,88	0	1,74E-06
299	c48399_g1	-9,127	114	67,65	0	1,74E-06
300	c51847_g1	-9,127	99	77	0	1,74E-06

301	c53384_g1	-9,122	80	88	0	1,74E-06
302	c50336_g1	-9,121	55	103	0	1,74E-06
303	c54144_g1	-9,121	55	103	0	1,74E-06
304	c52447_g2	-9,118	5	133	0	1,62E-06
305	c32480_g1	-9,118	101	75	0	1,86E-06
306	c49755_g1	-9,115	184,99	24	0	1,99E-06
307	c54339_g3	-9,114	159,99	39	0	1,99E-06
308	c55069_g1	-9,105	104	71,99	0	1,99E-06
309	c55735_g1	-9,105	94	78	0	1,99E-06
310	c47662_g1	-9,103	150	44	0	2,12E-06
311	c33943_g1	-9,100	122,96	60	0	2,12E-06
312	c44704_g1	-9,093	121,99	60	0	2,12E-06
313	c51421_g1	-9,086	154	40	0	2,28E-06
314	c49443_g1	-9,085	124	58	0	2,28E-06
315	c52988_g1	-9,080	196	14	0	2,44E-06
316	c55362_g1	-9,071	107,41	67	0	2,44E-06
317	c57651_g2	-9,070	130	53	0	2,44E-06
318	c57826_g1	-9,066	141,15	46,24	0	2,44E-06
319	c47425_g1	-9,062	142	45	0	2,61E-06
320	c47867_g1	-9,056	118	59	0	2,61E-06
321	c55125_g1	-9,055	130,85	50,99	0	2,61E-06
322	c54324_g2	-9,052	76,26	84	0	2,61E-06
323	c57070_g1	-9,049	112	62	0	2,80E-06
324	c57734_g1	-9,049	102,31	67,99	0	2,80E-06
325	c43968_g1	-9,046	90	75	0	2,80E-06
326	c45007_g1	-9,045	42	104	0	2,61E-06
327	c35166_g1	-9,041	81	80	0	2,80E-06
328	c51973_g1	-9,037	168	27,02	0	3,00E-06
329	c48797_g1	-9,032	116	58	0	3,00E-06
330	c49237_g2	-9,032	149	38	0	3,00E-06
331	c56548_g1	-9,026	102	66	0	3,00E-06
332	c84119_g1	-9,016	84	76	0	3,22E-06
333	c57095_g1	-9,015	140	41,99	0	3,22E-06
334	c36475_g1	-9,013	133	46	0	3,46E-06
335	c49523_g1	-9,012	123	51,93	0	3,46E-06
336	c53918_g1	-9,010	131	47	0	3,46E-06
337	c47196_g1	-9,003	129,99	47	0	3,46E-06
338	c51021_g1	-9,000	123	50,95	0	3,46E-06
339	c20684_g1	-8,993	117	54	0	3,72E-06
340	c53617_g1	-8,991	119,63	51,99	0	3,72E-06
341	c55129_g1	-8,990	77	78	0	3,72E-06
342	c43940_g1	-8,990	100	64	0	3,72E-06
343	c10519_g1	-8,986	111	57	0	3,72E-06
344	c25201_g1	-8,984	118,99	52	0	4,00E-06
345	c53541_g1	-8,983	136,96	41	0	4,00E-06
346	c57315_g1	-8,979	59	88	0	3,72E-06
347	c53532_g1	-8,979	58,99	88	0	3,72E-06
348	c42772_g1	-8,978	133	43	0	4,00E-06
349	c57796_g2	-8,973	96	65	0	4,00E-06
350	c40914_g1	-8,971	127	46	0	4,30E-06
351	c42417_g1	-8,970	111,99	55	0	4,30E-06
352	c54008_g1	-8,968	148	33	0	4,30E-06
353	c47378_g1	-8,965	113	54	0	4,30E-06
354	c46715_g1	-8,959	75,9	76	0	4,30E-06
355	c53163_g1	-8,958	66	82	0	4,30E-06
356	c56913_g6	-8,957	74	77	0	4,30E-06
357	c109657_g1	-8,956	115	52	0	4,63E-06
358	c26619_g1	-8,955	128	44	0	4,63E-06
359	c55907_g1	-8,940	126	43,93	0	4,98E-06
360	c52088_g1	-8,939	93	63,94	0	4,98E-06

361	c56114_g2	-8,939	139	36	0	4,98E-06
362	c50508_g1	-8,937	91	64,59	0	4,98E-06
363	c42861_g1	-8,935	76	74	0	4,98E-06
364	c97179_g1	-8,934	107	55	0	4,98E-06
365	c56931_g1	-8,933	120	47	0	4,98E-06
366	c53026_g1	-8,932	46	92	0	4,63E-06
367	c49037_g1	-8,932	45,98	92	0	4,63E-06
368	c28991_g1	-8,930	123	44,97	0	5,37E-06
369	c55293_g1	-8,930	95	62	0	4,98E-06
370	c52836_g2	-8,928	75	74	0	4,98E-06
371	c36438_g1	-8,924	81	70	0	5,37E-06
372	c48395_g1	-8,923	116,98	48	0	5,37E-06
373	c72899_g1	-8,919	133	38	0	5,37E-06
374	c55809_g5	-8,912	109	52	0	5,78E-06
375	c47590_g1	-8,911	121,64	44	0	5,78E-06
376	c57122_g1	-8,911	94	61	0	5,78E-06
377	c50547_g1	-8,910	107	53	0	5,78E-06
378	c46900_g1	-8,895	23	103	0	5,78E-06
379	c52633_g2	-8,887	103,99	53	0	6,73E-06
380	c55142_g5	-8,885	60,85	79	0	6,24E-06
381	c54468_g2	-8,883	156	21	0	6,73E-06
382	c52127_g1	-8,879	98	56	0	6,73E-06
383	c36293_g1	-8,874	35	93,96	0	6,24E-06
384	c57032_g2	-8,873	106,99	50	0	7,27E-06
385	c10553_g1	-8,872	61	78	0	6,73E-06
386	c57501_g1	-8,870	87	61,77	0,45	7,27E-06
387	c55764_g1	-8,869	100	53,91	0	7,27E-06
388	c57386_g1	-8,869	99,99	53,82	0	7,27E-06
389	c52330_g1	-8,864	160	17	0	7,85E-06
390	c57484_g1	-8,861	116,95	43	0	7,27E-06
391	c57010_g1	-8,857	122,99	39	0	7,85E-06
392	c34677_g1	-8,856	72	70	0	7,27E-06
393	c49638_g1	-8,852	101	52	0	7,85E-06
394	c57126_g1	-8,851	95,89	55	0	7,85E-06
395	c56727_g1	-8,850	149,98	22	0	7,85E-06
396	c49394_g1	-8,841	57	78	0	7,85E-06
397	c54606_g1	-8,840	52	80,69	0	7,85E-06
398	c49389_g1	-8,834	43	86	0	7,85E-06
399	c12401_g1	-8,833	38	89	0	7,85E-06
400	c36775_g1	-8,832	105	48	0	8,48E-06
401	c55023_g1	-8,819	122,97	36	0	9,18E-06
402	c34513_g1	-8,818	118	38,81	0	9,18E-06
403	c24774_g1	-8,815	128,96	32	0	9,93E-06
404	c55121_g1	-8,809	125	34	0	9,93E-06
405	c44747_g1	-8,808	12	103	0	8,48E-06
406	c29575_g1	-8,798	104	46	0	1,08E-05
407	c58198_g3	-8,792	82	58,77	0	1,08E-05
408	c52057_g1	-8,792	94,75	51	0	1,08E-05
409	c25307_g1	-8,788	87,99	55	0	1,08E-05
410	c52778_g1	-8,786	96	49,93	0	1,08E-05
411	c51163_g1	-8,786	96	49,99	0	1,08E-05
412	c50698_g2	-8,786	126,97	31	0	1,17E-05
413	c57547_g1	-8,778	95	50	0	1,17E-05
414	c57306_g8	-8,777	151,99	15	0	1,17E-05
415	c26497_g1	-8,772	91	52	0	1,17E-05
416	c55702_g2	-8,768	48	77,64	0	1,17E-05
417	c56310_g1	-8,767	122,98	32	0	1,26E-05
418	c45312_g1	-8,747	83	54,59	0	1,37E-05
419	c55072_g1	-8,746	109	38,63	0	1,37E-05
420	c36246_g1	-8,744	55	72	0	1,26E-05
421	c57885_g2	-8,744	112	37	0	1,37E-05
422	c33259_g1	-8,741	115	35	0	1,37E-05
423	c71328_g1	-8,740	79	57	0	1,37E-05
424	c56692_g1	-8,740	104,99	41	0	1,37E-05
425	c55837_g1	-8,738	95	47	0	1,37E-05
426	c57362_g2	-8,733	114,09	35	0	1,49E-05
427	c56063_g4	-8,732	78	57	0	1,49E-05
428	c84143_g1	-8,731	103,99	40,96	0	1,49E-05
429	c49492_g1	-8,731	86	52	0	1,49E-05
430	c57034_g1	-8,727	141	18	0	1,49E-05
431	c54643_g1	-8,727	79	56,01	0	1,49E-05
432	c56291_g1	-8,726	118	32	0	1,49E-05
433	c42178_g1	-8,722	67	63	0	1,49E-05
434	c56371_g1	-8,720	101	41,99	0	1,62E-05
435	c54813_g2	-8,719	82,98	53	0	1,49E-05
436	c39708_g1	-8,716	81	54	0	1,62E-05
437	c35604_g1	-8,715	76	57	0	1,62E-05
438	c56498_g2	-8,709	59	67	0	1,62E-05
439	c52780_g2	-8,708	85	51	0	1,62E-05
440	c40857_g1	-8,706	100,99	41	0	1,62E-05
441	c57531_g2	-8,703	86	49,97	0	1,62E-05
442	c39856_g1	-8,703	99	42	0	1,76E-05
443	c45720_g1	-8,702	94	45	0	1,76E-05
444	c55962_g1	-8,700	71	59	0	1,62E-05
445	c10085_g1	-8,698	48	73,08	0	1,62E-05
446	c27264_g1	-8,696	64,5	62,99	0	1,76E-05
447	c45387_g1	-8,694	28	85	0	1,62E-05
448	c33638_g1	-8,694	111	34	0	1,76E-05
449	c57969_g2	-8,690	104	38,46	0	1,76E-05
450	c54032_g1	-8,689	60	65	0	1,76E-05
451	c49141_g2	-8,683	69	58,88	0	1,76E-05
452	c56659_g1	-8,682	64	62,28	0	1,76E-05
453	c54013_g4	-8,680	85	48,92	0	1,91E-05
454	c45595_g1	-8,680	84,87	49	0	1,91E-05
455	c55407_g3	-8,675	112	32	0	1,91E-05
456	c54485_g1	-8,671	123	25	0	2,08E-05
457	c46714_g1	-8,665	80	51	0	2,08E-05
458	c47404_g1	-8,664	114	30,48	0	2,08E-05
459	c56291_g2	-8,664	127	22	0	2,08E-05
460	c109573_g1	-8,663	96	41,28	0	2,08E-05
461	c42201_g1	-8,660	99	38,89	0	2,08E-05
462	c31444_g1	-8,657	58	64	0	2,08E-05
463	c57615_g2	-8,656	92	43	0	2,08E-05
464	c50815_g1	-8,654	43	73	0	2,08E-05
465	c55150_g1	-8,645	68	57	0	2,27E-05
466	c39254_g1	-8,639	95	39,96	0	2,27E-05
467	c47446_g1	-8,633	130	18	0	2,47E-05
468	c34065_g1	-8,631	73	53	0	2,47E-05
469	c52804_g1	-8,629	114,92	26,98	0	2,47E-05
470	c54525_g1	-8,627	105	33	0	2,47E-05
471	c40720_g1	-8,627	79	49	0	2,47E-05
472	c57589_g2	-8,624	69	55	0	2,47E-05
473	c54247_g1	-8,617	99	36	0	2,70E-05
474	c53551_g1	-8,616	73	52	0	2,47E-05
475	c84264_g1	-8,616	0	97	0	2,08E-05
476	c57879_g4	-8,613	84	45	0	2,70E-05
477	c55294_g1	-8,610	82	45,78	0	2,70E-05
478	c56077_g1	-8,604	96	37	0	2,70E-05
479	c57939_g1	-8,600	68	54,35	0	2,70E-05
480	c50604_g1	-8,600	89	41	0	2,95E-05

481	c53292_g1	-8,594	69	52,57	0	2,95E-05
482	c55509_g3	-8,594	56	61,41	0	2,95E-05
483	c57935_g3	-8,589	83	44	0	2,95E-05
484	c56342_g1	-8,588	91	39	0	2,95E-05
485	c57938_g6	-8,585	81	45	0	2,95E-05
486	c55796_g1	-8,584	76	48	0	2,95E-05
487	c55035_g1	-8,574	62	55,99	0	3,22E-05
488	c56510_g1	-8,573	69,99	51	0	3,22E-05
489	c42321_g1	-8,571	73	49	0	3,22E-05
490	c56623_g1	-8,557	2	92	0	2,95E-05
491	c48854_g1	-8,553	63	54	0	3,52E-05
492	c55941_g1	-8,551	100	31	0	3,85E-05
493	c46894_g1	-8,547	85	40	0	3,85E-05
494	c49117_g1	-8,544	87,9	38	0	3,85E-05
495	c56051_g2	-8,542	112	23	0	3,85E-05
496	c53508_g1	-8,542	57	57	0	3,52E-05
497	c49092_g1	-8,541	85,98	39	0	3,85E-05
498	c25207_g1	-8,538	76,5	45	0	3,85E-05
499	c39355_g1	-8,534	53,39	59	0	3,85E-05
500	c50357_g1	-8,533	82	41	0	3,85E-05
501	c55498_g2	-8,533	47,97	62	0	3,85E-05
502	c42360_g1	-8,531	85	39	0	3,85E-05
503	c54176_g1	-8,522	76,5	44	0	4,22E-05
504	c30784_g1	-8,521	84	39	0	4,22E-05
505	c56449_g1	-8,521	84	38,58	0	4,22E-05
506	c46442_g1	-8,520	50	60	0	4,22E-05
507	c56722_g3	-8,518	103	27	0	4,22E-05
508	c58278_g1	-8,512	75	44	0	4,22E-05
509	c56896_g1	-8,511	112,22	21	0	4,63E-05
510	c23843_g1	-8,507	47	61	0	4,22E-05
511	c53257_g2	-8,506	96,91	30	0	4,63E-05
512	c48488_g1	-8,505	63	51	0	4,63E-05
513	c41752_g1	-8,504	79	41	0	4,63E-05
514	c57572_g2	-8,504	58	54	0,08	4,63E-05
515	c55514_g1	-8,504	108	23	0	4,63E-05
516	c26811_g1	-8,495	112	20	0	5,08E-05
517	c53396_g1	-8,495	120	15	0	5,08E-05
518	c40771_g2	-8,490	55	55	0	4,63E-05
519	c55130_g2	-8,488	79	40	0	5,08E-05
520	c40141_g1	-8,488	108	22	0	5,08E-05
521	c10550_g1	-8,487	95	30	0	5,08E-05
522	c23548_g1	-8,482	71,99	44,03	0	5,08E-05
523	c53671_g2	-8,481	88	34	0	5,08E-05
524	c42782_g1	-8,480	46,36	59,95	0	5,08E-05
525	c55958_g5	-8,472	79	39	0	5,57E-05
526	c52188_g1	-8,472	79	39	0	5,57E-05
527	c56370_g1	-8,470	45	60	0	5,08E-05
528	c49951_g1	-8,465	59,18	51	0	5,57E-05
529	c34100_g1	-8,463	91	31	0	5,57E-05
530	c26857_g1	-8,461	94	29	0	5,57E-05
531	c46269_g1	-8,458	97	27	0	6,12E-05
532	c56598_g1	-8,456	108	20	0	6,12E-05
533	c15611_g1	-8,451	85	34	0	6,12E-05
534	c50850_g1	-8,450	63,67	47,25	0	6,12E-05
535	c41767_g1	-8,449	88	32	0	6,12E-05
536	c51241_g1	-8,448	104	22	0	6,12E-05
537	c51662_g1	-8,447	83	35	0	6,12E-05
538	c50095_g1	-8,446	61,83	47,98	0	6,12E-05
539	c57645_g1	-8,446	70	43	0	6,12E-05
540	c44325_g1	-8,445	78	38	0	6,12E-05
541	c51899_g1	-8,443	81	36	0	6,12E-05
542	c46981_g1	-8,441	120,83	11	0	6,74E-05
543	c36220_g1	-8,440	47	57	0	6,12E-05
544	c57414_g1	-8,439	71	42	0	6,12E-05
545	c53938_g1	-8,437	82	35	0	6,74E-05
546	c52220_g1	-8,436	61	48	0	6,12E-05
547	c57094_g2	-8,435	77	38	0	6,74E-05
548	c56831_g2	-8,435	77	38	0	6,74E-05
549	c57759_g4	-8,434	101	23	0	6,74E-05
550	c57185_g6	-8,430	83	34	0	6,74E-05
551	c57488_g9	-8,426	44	58	0	6,12E-05
552	c55033_g1	-8,423	54,99	51	0	6,74E-05
553	c57758_g1	-8,422	78,96	36	0	6,74E-05
554	c51814_g1	-8,419	98	24	0	7,42E-05
555	c47730_g1	-8,418	76,56	37	0	6,74E-05
556	c56207_g1	-8,417	56	49,88	0	6,74E-05
557	c57045_g1	-8,414	67	42,7	0	6,74E-05
558	c9502_g1	-8,412	78	36	0	7,42E-05
559	c44082_g1	-8,409	97	24	0	7,42E-05
560	c52656_g2	-8,403	422	1553,83	1	3,35E-15
561	c38257_g1	-8,399	72	39	0	7,42E-05
562	c55715_g1	-8,399	80	34,09	0	7,42E-05
563	c49432_g1	-8,397	59	46,9	0	7,42E-05
564	c58241_g1	-8,397	74,82	37	0	7,42E-05
565	c50445_g1	-8,388	63	44	0	8,17E-05
566	c110061_g1	-8,388	63	44	0	8,17E-05
567	c50994_g1	-8,388	63	44	0	8,17E-05
568	c42413_g1	-8,388	79	34	0	8,17E-05
569	c48581_g1	-8,387	50	52	0	7,42E-05
570	c55680_g3	-8,386	58	47	0	8,17E-05
571	c26764_g1	-8,384	77	35	0	8,17E-05
572	c51056_g1	-8,380	43	56	0	8,17E-05
573	c13970_g1	-8,380	59	45,99	0	8,17E-05
574	c42618_g1	-8,376	57	47	0	8,17E-05
575	c52509_g1	-8,371	62,95	43	0	9,02E-05
576	c56042_g1	-8,364	80	32	0	9,02E-05
577	c45690_g1	-8,362	59	44,58	0	9,02E-05
578	c53569_g1	-8,362	74,99	35	0	9,02E-05
579	c42623_g1	-8,360	46	53	0	9,02E-05
580	c52058_g2	-8,358	57	46	0	9,02E-05
581	c57459_g1	-8,353	87	26,65	0	9,95E-05
582	c52378_g1	-8,349	69	38	0	9,95E-05
583	c53291_g1	-8,342	78	32	0	9,95E-05
584	c56991_g1	-8,338	83,99	28	0	9,95E-05
585	c43295_g1	-8,333	34	59	0	9,95E-05
586	c45656_g1	-8,329	48	50	0	9,95E-05
587	c25138_g1	-8,329	48	50	0	9,95E-05
588	c51253_g1	-8,327	75	33	0	1,10E-04
589	c27180_g1	-8,325	102	16	0	1,10E-04
590	c19595_g1	-8,325	86	26	0	1,10E-04
591	c54015_g1	-8,324	78	31	0	1,10E-04
592	c54038_g1	-8,322	97	19	0	1,10E-04
593	c37542_g1	-8,322	49	49	0	1,10E-04
594	c57561_g1	-8,320	68	37	0	1,10E-04
595	c30098_g1	-8,319	12	72	0	9,95E-05
596	c54042_g1	-8,318	63	40,03	0	1,10E-04
597	c52004_g1	-8,313	61	40,85	0	1,10E-04
598	c56942_g1	-8,311	72	34	0	1,22E-04
599	c56341_g1	-8,306	70	35	0	1,22E-04
600	c44849_g1	-8,304	57	43	0	1,22E-04

601	c33624_g1	-8,301	52	46	0	1,22E-04
602	c57737_g1	-8,297	74	32	0	1,22E-04
603	c52651_g2	-8,297	73,99	32	0	1,22E-04
604	c26639_g1	-8,296	34	57	0	1,10E-04
605	c53109_g1	-8,295	69	35	0	1,22E-04
606	c53970_g1	-8,289	86	24	0	1,35E-04
607	c42588_g1	-8,287	38	54	0	1,22E-04
608	c16657_g1	-8,281	71	32,94	0	1,35E-04
609	c123090_g1	-8,280	97,66	16	0	1,35E-04
610	c42248_g1	-8,279	66	36	0	1,35E-04
611	c52215_g2	-8,275	96	17	0	1,49E-04
612	c40773_g1	-8,274	55,98	42	0	1,35E-04
613	c21944_g1	-8,269	113	6	0	1,49E-04
614	c53555_g1	-8,267	73	31,28	0	1,49E-04
615	c26480_g1	-8,267	65	36	0	1,49E-04
616	c58121_g1	-8,265	76	29	0	1,49E-04
617	c55655_g6	-8,261	82	25	0	1,49E-04
618	c15369_g1	-8,261	82	25	0	1,49E-04
619	c56605_g1	-8,259	58	40	0	1,49E-04
620	c46275_g1	-8,259	42	50	0	1,35E-04
621	c52773_g1	-8,258	69	33	0	1,49E-04
622	c42815_g1	-8,253	32	56	0	1,35E-04
623	c48363_g1	-8,246	76	28	0	1,66E-04
624	c32215_g1	-8,245	52	43	0	1,49E-04
625	c52367_g1	-8,244	79	26	0	1,66E-04
626	c34663_g1	-8,239	34	54	0	1,49E-04
627	c54027_g1	-8,235	74,97	28	0	1,66E-04
628	c57638_g1	-8,231	62	36	0	1,66E-04
629	c47163_g1	-8,225	78,98	25	0	1,84E-04
630	c55465_g1	-8,224	90	18	0	1,84E-04
631	c46214_g1	-8,220	69	31	0	1,84E-04
632	c50344_g1	-8,217	37	51	0	1,66E-04
633	c55632_g1	-8,214	85,95	20	0	1,84E-04
634	c48431_g1	-8,213	70	30	0	1,84E-04
635	c48899_g1	-8,212	35	52	0	1,66E-04
636	c54235_g1	-8,207	60	36	0	1,84E-04
637	c41888_g1	-8,204	81,96	22	0	2,05E-04
638	c46095_g1	-8,204	55	38,96	0	1,84E-04
639	c39797_g1	-8,203	47	44	0	1,84E-04
640	c53249_g1	-8,201	77	24,98	0	2,05E-04
641	c40382_g1	-8,200	69	30	0	2,05E-04
642	c52555_g2	-8,199	107	6	0	2,05E-04
643	c46885_g1	-8,199	80	23	0	2,05E-04
644	c51359_g1	-8,197	37	50	0	1,84E-04
645	c46449_g1	-8,192	81	22	0	2,05E-04
646	c13996_g1	-8,189	57	37	0	2,05E-04
647	c56569_g1	-8,188	87	18	0	2,05E-04
648	c37720_g1	-8,185	63,33	33	0	2,05E-04
649	c31603_g1	-8,185	63	33	0	2,05E-04
650	c29763_g1	-8,183	66	31	0	2,05E-04
651	c37461_g1	-8,179	53	38,55	0	2,05E-04
652	c51800_g1	-8,179	53	39	0	2,05E-04
653	c57378_g1	-8,175	59	35,13	0	2,28E-04
654	c56930_g1	-8,174	78	23	0	2,28E-04
655	c52839_g1	-8,172	53,98	38	0	2,28E-04
656	c52878_g1	-8,169	76	24	0	2,28E-04
657	c47836_g1	-8,167	98	10	0	2,28E-04
658	c49256_g1	-8,165	63	32	0	2,28E-04
659	c53726_g1	-8,159	91	14	0	2,54E-04
660	c122935_g1	-8,156	67	29,26	0	2,28E-04

661	c27926_g1	-8,155	78	22	0	2,54E-04
662	c36366_g1	-8,154	70	27	0	2,54E-04
663	c45396_g1	-8,151	54	37	0	2,28E-04
664	c42729_g1	-8,150	27	54	0	2,28E-04
665	c23016_g1	-8,148	68	28	0	2,54E-04
666	c50715_g2	-8,146	52	38	0	2,54E-04
667	c47389_g1	-8,143	47	41	0	2,54E-04
668	c11914_g1	-8,141	69	26,78	0	2,54E-04
669	c57553_g1	-8,141	50	39	0	2,54E-04
670	c41557_g1	-8,139	53	37	0	2,54E-04
671	c54661_g2	-8,137	74,9	23	0	2,54E-04
672	c34286_g1	-8,132	43	43	0	2,54E-04
673	c52306_g2	-8,131	54	36	0	2,54E-04
674	c55797_g1	-8,128	68	27	0	2,83E-04
675	c54812_g1	-8,128	49	39	0	2,54E-04
676	c53545_g3	-8,125	63	30	0	2,83E-04
677	c40254_g1	-8,123	55	35	0	2,83E-04
678	c47246_g1	-8,123	66	27,99	0	2,83E-04
679	c23080_g1	-8,123	66	27,97	0	2,83E-04
680	c52030_g1	-8,123	65,99	28	0	2,83E-04
681	c55731_g1	-8,121	58	32,9	0	2,83E-04
682	c57837_g1	-8,121	88	14	0	2,83E-04
683	c52122_g1	-8,119	61	31	0	2,83E-04
684	c39196_g1	-8,117	44,86	41,01	0	2,83E-04
685	c54239_g1	-8,116	56	34,29	0	2,83E-04
686	c55330_g2	-8,112	51	36,99	0	2,83E-04
687	c27140_g1	-8,112	50,87	37	0	2,83E-04
688	c42248_g2	-8,112	92	11	0	3,17E-04
689	c51964_g1	-8,110	54	35	0	2,83E-04
690	c50878_g1	-8,104	63	29	0	3,17E-04
691	c53784_g3	-8,099	49,99	36,94	0	3,17E-04
692	c56512_g1	-8,096	64	28	0	3,17E-04
693	c57345_g1	-8,090	72,97	22	0	3,17E-04
694	c50483_g1	-8,090	43	41	0	3,17E-04
695	c56760_g1	-8,089	65	27	0	3,17E-04
696	c54120_g1	-8,087	56,98	32	0	3,17E-04
697	c57039_g2	-8,085	30	48,96	0	3,17E-04
698	c44282_g1	-8,081	66	26	0	3,54E-04
699	c48720_g1	-8,081	77	19	0	3,54E-04
700	c49223_g1	-8,080	47	38	0	3,17E-04
701	c50539_g1	-8,068	65	26	0	3,54E-04
702	c53003_g1	-8,068	65	26	0	3,54E-04
703	c26695_g1	-8,065	79	17	0	3,54E-04
704	c56232_g1	-8,063	40,91	41	0	3,54E-04
705	c50163_g1	-8,062	52	34,12	0	3,54E-04
706	c18743_g1	-8,059	47	37	0	3,54E-04
707	c10879_g1	-8,055	42	40	0	3,54E-04
708	c26365_g1	-8,054	53	33	0	3,54E-04
709	c52602_g1	-8,051	37	43	0	3,54E-04
710	c42923_g1	-8,047	43	39	0	3,54E-04
711	c58083_g1	-8,046	75,99	18	0	3,97E-04
712	c24147_g1	-8,042	71	21	0	3,97E-04
713	c53153_g1	-8,036	102	1	0	4,45E-04
714	c49965_g4	-8,035	61	27	0	3,97E-04
715	c24274_g1	-8,035	61	27	0	3,97E-04
716	c43416_g1	-8,031	45	37	0	3,97E-04
717	c57042_g1	-8,029	81	14	0	4,45E-04
718	c55927_g3	-8,025	54	30,99	0	4,45E-04
719	c45061_g1	-8,025	43	38	0	3,97E-04
720	c24411_g1	-8,023	57	28,92	0	4,45E-04

721	c56382_g1	-8,017	77	16	0	4,45E-04
722	c56772_g1	-8,015	47	35	0	4,45E-04
723	c56399_g1	-8,011	64	24	0	4,45E-04
724	c50056_g1	-8,007	48	34	0	4,45E-04
725	c52406_g1	-8,004	28,98	46	0	4,45E-04
726	c45516_g1	-7,999	60	26	0	4,99E-04
727	c57624_g6	-7,998	38	39,97	0	4,45E-04
728	c23672_g1	-7,989	64	23	0	4,99E-04
729	c49256_g2	-7,984	59	26	0	4,99E-04
730	c48885_g1	-7,975	74	15,99	0	5,61E-04
731	c57800_g2	-7,975	74	16	0	5,61E-04
732	c57759_g2	-7,971	44	35	0	4,99E-04
733	c56216_g3	-7,971	80	12	0	5,61E-04
734	c50599_g1	-7,969	72	17	0	5,61E-04
735	c58213_g1	-7,968	86	8	0	5,61E-04
736	c57296_g1	-7,966	53	29	0	5,61E-04
737	c56287_g2	-7,965	78	13	0	5,61E-04
738	c51786_g1	-7,964	67	19,99	0	5,61E-04
739	c40687_g1	-7,964	67	20	0	5,61E-04
740	c46491_g1	-7,964	56	27	0	5,61E-04
741	c49956_g1	-7,956	32	42	0	5,61E-04
742	c53600_g1	-7,952	63	22	0	6,32E-04
743	c43981_g3	-7,951	52	29	0	5,61E-04
744	c42555_g1	-7,948	69	18	0	6,32E-04
745	c46325_g1	-7,947	58	25	0	6,32E-04
746	c56238_g1	-7,945	49,86	30	0	6,32E-04
747	c39309_g1	-7,944	63,99	21	0	6,32E-04
748	c57533_g1	-7,943	77,9	12	0	6,32E-04
749	c52997_g1	-7,942	42	35	0	5,61E-04
750	c54747_g1	-7,942	67	19	0	6,32E-04
751	c50948_g1	-7,935	65	20	0	6,32E-04
752	c54581_g1	-7,933	68	18	0	6,32E-04
753	c41233_g1	-7,925	69	17	0	7,12E-04
754	c56142_g2	-7,924	58	24	0	6,32E-04
755	c49916_g1	-7,921	25	45	0	6,32E-04
756	c45675_g1	-7,919	28	43	0	6,32E-04
757	c43849_g1	-7,915	34	39	0	6,32E-04
758	c43115_g1	-7,913	51	28	0	7,12E-04
759	c84559_g1	-7,911	1	60	0	5,61E-04
760	c54975_g2	-7,911	53,99	26,03	0,43	7,12E-04
761	c58069_g1	-7,904	52	27	0	7,12E-04
762	c31515_g1	-7,904	66	18	0	7,12E-04
763	c57406_g1	-7,903	41	34	0	7,12E-04
764	c57216_g1	-7,901	58	23	0	7,12E-04
765	c52765_g1	-7,900	33	39	0	7,12E-04
766	c40022_g1	-7,889	23	45	0	7,12E-04
767	c56686_g3	-7,889	65	18	0	8,03E-04
768	c44266_g1	-7,887	40	34	0	7,12E-04
769	c53878_g2	-7,885	57	23	0	8,03E-04
770	c43838_g1	-7,880	38	35	0	8,03E-04
771	c43290_g1	-7,880	52	26	0	8,03E-04
772	c55416_g1	-7,870	56	23	0	8,03E-04
773	c53879_g1	-7,865	65	17	0	9,08E-04
774	c55060_g1	-7,856	38	34	0	8,03E-04
775	c52935_g1	-7,853	86	3	0	9,08E-04
776	c53608_g1	-7,850	33	36,72	0	8,03E-04
777	c52608_g1	-7,849	78	8	0	9,08E-04
778	c56826_g1	-7,849	64	16,84	0	9,08E-04
779	c49577_g2	-7,849	49,98	26	0	9,08E-04
780	c54510_g3	-7,847	39	33	0	9,08E-04
781	c45160_g1	-7,843	31	38	0	9,08E-04
782	c54496_g1	-7,842	62	18	0	9,08E-04
783	c51736_g1	-7,842	48	26,99	0	9,08E-04
784	c42232_g1	-7,837	57	21	0	1,03E-03
785	c57623_g6	-7,837	57	21	0	1,03E-03
786	c55132_g1	-7,835	46	28	0	9,08E-04
787	c53280_g1	-7,835	46	28	0	9,08E-04
788	c47582_g1	-7,834	32	37	0	9,08E-04
789	c55872_g1	-7,833	49	26	0	1,03E-03
790	c40824_g1	-7,829	41	31	0	9,08E-04
791	c47525_g1	-7,826	47	27	0	1,03E-03
792	c56609_g1	-7,826	47	27	0	1,03E-03
793	c59379_g1	-7,825	2	56	0	8,03E-04
794	c46706_g1	-7,824	19	45	0	9,08E-04
795	c55486_g1	-7,824	19	45	0	9,08E-04
796	c54323_g2	-7,824	50	25	0	1,03E-03
797	c45247_g2	-7,822	39	32,11	0	1,03E-03
798	c52573_g1	-7,818	45	28	0	1,03E-03
799	c28092_g1	-7,818	31	37	0	1,03E-03
800	c57284_g2	-7,817	47,99	26	0	1,03E-03
801	c43872_g1	-7,814	54	22	0	1,03E-03
802	c45092_g1	-7,813	40	31	0	1,03E-03
803	c47595_g1	-7,812	88	0	0	1,17E-03
804	c48097_g1	-7,809	46	27	0	1,03E-03
805	c54179_g1	-7,809	32	36	0	1,03E-03
806	c50715_g3	-7,807	66	14	0	1,17E-03
807	c53533_g1	-7,807	66	14,49	0	1,17E-03
808	c44053_g1	-7,800	47,17	26	0	1,17E-03
809	c56375_g1	-7,797	22	42	0	2,61E-04
810	c48299_g1	-7,796	56	20	0	1,17E-03
811	c110033_g1	-7,791	48	25	0	2,98E-04
812	c47193_g1	-7,791	48	25	0	2,98E-04
813	c53765_g8	-7,785	43	28	0	2,98E-04
814	c58179_g1	-7,779	38	30,92	0	2,98E-04
815	c53794_g1	-7,779	55	20	0	2,98E-04
816	c46238_g1	-7,776	61	15,99	0	3,41E-04
817	c55009_g1	-7,775	47	25	0	2,98E-04
818	c47666_g1	-7,773	16	45	0	2,61E-04
819	c56977_g3	-7,769	42,21	28	0	3,41E-04
820	c42518_g1	-7,769	42	28	0	3,41E-04
821	c54167_g2	-7,767	28	37	0	2,98E-04
822	c53843_g1	-7,766	48	24	0	3,41E-04
823	c51967_g1	-7,765	30,99	35	0	2,98E-04
824	c43025_g1	-7,758	46	25	0	3,41E-04
825	c53676_g1	-7,758	46	25	0	3,41E-04
826	c56148_g1	-7,758	29	36	0	2,98E-04
827	c39949_g1	-7,757	66	12	0	3,41E-04
828	c28798_g1	-7,755	52	21	0	3,41E-04
829	c31520_g1	-7,755	51,98	21	0	3,41E-04
830	c56023_g1	-7,750	44	26	0	3,41E-04
831	c19561_g1	-7,749	64	13	0	3,90E-04
832	c56392_g1	-7,744	56	18	0	3,90E-04
833	c39298_g1	-7,743	59	16	0	3,90E-04
834	c33331_g1	-7,737	54	19	0	3,90E-04
835	c57764_g2	-7,735	57	16,73	0	3,90E-04
836	c57653_g1	-7,732	46	24	0	3,90E-04
837	c44092_g1	-7,731	66	11	0	3,90E-04
838	c52434_g1	-7,728	35	31	0	3,90E-04
839	c50586_g1	-7,722	47	23	0	3,90E-04
840	c48887_g1	-7,721	30	34	0	3,90E-04

841	c58082_g1	-7,721	50	21	0	3,90E-04
842	c56194_g1	-7,719	52,53	19	0	3,90E-04
843	c56848_g3	-7,718	36	30	0	3,90E-04
844	c48943_g1	-7,718	36	30	0	3,90E-04
845	c44533_g1	-7,718	56	17	0	3,90E-04
846	c96869_g1	-7,717	39	28	0	3,90E-04
847	c58054_g10	-7,710	34	31	0	3,90E-04
848	c52660_g1	-7,709	57	16	0	4,48E-04
849	c50657_g1	-7,707	20	40	0	3,90E-04
850	c39617_g1	-7,707	40	27	0	3,90E-04
851	c39362_g1	-7,703	69	8	0	4,48E-04
852	c55286_g1	-7,703	68,92	8	0	4,48E-04
853	c48515_g2	-7,701	35	29,99	0	3,90E-04
854	c50941_g1	-7,694	50	19,84	0	4,48E-04
855	c56747_g1	-7,694	30	33	0	4,48E-04
856	c57218_g2	-7,687	45,06	22,9	0	4,48E-04
857	c52831_g1	-7,684	31	32	0	4,48E-04
858	c33392_g1	-7,679	43	24	0	4,48E-04
859	c49610_g1	-7,670	41	25	0	5,15E-04
860	c55759_g1	-7,669	21	38	0	4,48E-04
861	c53595_g1	-7,669	66,72	8	0	5,15E-04
862	c54516_g1	-7,665	52,74	17	0	5,15E-04
863	c56558_g1	-7,662	39	26	0	5,15E-04
864	c110791_g1	-7,661	65	9	0	5,15E-04
865	c38225_g1	-7,659	45	22	0	5,15E-04
866	c44573_g1	-7,659	45	22	0	5,15E-04
867	c56807_g1	-7,649	0	51	0	3,90E-04
868	c55743_g1	-7,646	54,92	15	0	5,93E-04
869	c34353_g1	-7,644	12	43	0	4,48E-04
870	c34152_g1	-7,644	12	43	0	4,48E-04
871	c55195_g1	-7,641	44	22	0	5,93E-04
872	c53723_g2	-7,636	33	29	0	5,15E-04
873	c57270_g1	-7,634	39	25	0	5,93E-04
874	c38009_g2	-7,634	39	25	0	5,93E-04
875	c49158_g1	-7,626	33,79	28	0	5,93E-04
876	c57887_g1	-7,626	60	11	0	5,93E-04
877	c50911_g1	-7,625	37	26	0	5,93E-04
878	c46163_g1	-7,623	43	22	0	5,93E-04
879	c52996_g1	-7,623	43	22	0	5,93E-04
880	c54036_g2	-7,623	43	22	0	5,93E-04
881	c53054_g1	-7,618	29	31	0	5,93E-04
882	c45279_g1	-7,615	38	25	0	5,93E-04
883	c18631_g1	-7,613	44	21	0	5,93E-04
884	c56186_g1	-7,613	44	21	0	5,93E-04
885	c51015_g1	-7,612	47	19	0	6,84E-04
886	c122528_g1	-7,612	46,93	19	0	6,84E-04
887	c43163_g1	-7,608	56	12,93	0	6,84E-04
888	c50988_g1	-7,608	30	30	0	5,93E-04
889	c48444_g1	-7,607	59	11	0	6,84E-04
890	c33914_g1	-7,605	39	24	0	6,84E-04
891	c32837_g1	-7,604	42	22	0	6,84E-04
892	c40432_g1	-7,601	22	35	0	5,93E-04
893	c50412_g1	-7,593	17,2	37,99	0	5,93E-04
894	c8866_g1	-7,592	49	17	0	6,84E-04
895	c57215_g1	-7,589	58	11	0	6,84E-04
896	c52618_g1	-7,586	38,31	24	0	6,84E-04
897	c35611_g1	-7,580	24	33	0	6,84E-04
898	c51600_g1	-7,575	42	21	0	7,91E-04
899	c52818_g5	-7,572	19	36	0	6,84E-04
900	c54971_g8	-7,570	25	32	0	6,84E-04
901	c45761_g1	-7,568	31	28	0	6,84E-04
902	c55896_g1	-7,567	34	26	0	6,84E-04
903	c56155_g1	-7,566	37	24,44	0	7,91E-04
904	c47543_g1	-7,563	49	16	0	7,91E-04
905	c43300_g1	-7,560	58	10	0	7,91E-04
906	c46227_g1	-7,555	6	43,63	0	5,93E-04
907	c25629_g2	-7,554	44	19	0	7,91E-04
908	c55446_g1	-7,553	47	17,03	0	7,91E-04
909	c39368_g1	-7,553	50	15	0	7,91E-04
910	c46456_g1	-7,552	53	13	0	7,91E-04
911	c26901_g1	-7,552	18	36	0	6,84E-04
912	c26006_g1	-7,551	56	11	0	9,17E-04
913	c31091_g1	-7,548	30	28	0	7,91E-04
914	c46859_g1	-7,544	45	18	0	7,91E-04
915	c24254_g1	-7,531	55	11	0	9,17E-04
916	c58114_g1	-7,531	58	9	0	9,17E-04
917	c57491_g1	-7,523	50	13,99	0	9,17E-04
918	c53931_g1	-7,523	50	14	0	9,17E-04
919	c56592_g1	-7,516	35,97	23	0	9,17E-04
920	c55930_g1	-7,515	39	21	0	9,17E-04
921	c44867_g1	-7,514	45	17	0	9,17E-04
922	c49331_g1	-7,512	51	13	0	1,06E-03
923	c49374_g1	-7,512	51	13,39	0	1,06E-03
924	c25218_g2	-7,512	54	11	0	1,06E-03
925	c54724_g1	-7,503	45,96	16	0	1,06E-03
926	c58154_g4	-7,502	52	12	0	1,06E-03
927	c48884_g1	-7,497	26	29	0	9,17E-04
928	c54264_g1	-7,495	38	21	0	1,06E-03
929	c39222_g1	-7,495	38	21	0	1,06E-03
930	c56337_g1	-7,486	30	25,93	0	1,06E-03
931	c56200_g1	-7,486	30	25,99	0	1,06E-03
932	c57956_g1	-7,484	39	20	0	1,06E-03
933	c98263_g1	-7,483	48	14	0	1,06E-03
934	c109750_g1	-7,482	51	12,35	0	1,06E-03
935	c52230_g1	-7,481	54	10	0	1,06E-03
936	c56661_g2	-7,480	60	6	0	1,24E-03
937	c57632_g1	-7,474	37	21	0	1,06E-03
938	c53453_g1	-7,474	40	19	0	1,06E-03
939	c56213_g1	-7,471	58	7	0	1,24E-03
940	c47758_g1	-7,466	20	31,85	0	1,06E-03
941	c56209_g1	-7,465	29	26	0	1,06E-03
942	c55002_g1	-7,463	41	18	0	1,24E-03
943	c57895_g1	-7,453	39	19	0	1,24E-03
944	c34922_g1	-7,453	38,95	19	0	1,24E-03
945	c52605_g1	-7,452	42	17,01	0	1,24E-03
946	c47514_g1	-7,452	42	17	0	1,24E-03
947	c57505_g2	-7,450	60	5	0	1,24E-03
948	c49501_g1	-7,445	16	34	0	1,06E-03
949	c52168_g1	-7,443	28	26	0	1,24E-03
950	c51666_g19	-7,439	64	2	0	1,44E-03
951	c50711_g1	-7,433	23	28,92	0	1,24E-03
952	c54515_g1	-7,431	38	19	0	1,24E-03
953	c57011_g1	-7,431	44	15	0	1,44E-03
954	c58225_g1	-7,430	47	13	0	1,44E-03
955	c46117_g1	-7,425	0	43,98	0	9,17E-04
956	c51711_g1	-7,420	45	14	0	1,44E-03
957	c52212_g1	-7,419	51	10,13	0	1,44E-03
958	c34530_g1	-7,419	57	6	0	1,44E-03
959	c55990_g3	-7,411	22	29,23	0	1,24E-03
960	c48798_g1	-7,410	30,99	23	0	1,44E-03

961	c53421_g1	-7,410	34	20,82	0	1,44E-03
962	c34582_g1	-7,409	52	9	0	1,44E-03
963	c57204_g1	-7,400	14	33,99	0	1,24E-03
964	c44262_g1	-7,400	26	26	0	1,44E-03
965	c40209_g1	-7,399	29	24	0	1,44E-03
966	c49112_g1	-7,399	32	22	0	1,44E-03
967	c10610_g1	-7,399	35	20	0	1,44E-03
968	c37475_g2	-7,388	30	23	0	1,44E-03
969	c55840_g1	-7,387	44,99	13	0	1,69E-03
970	c84063_g1	-7,377	25	26	0	1,44E-03
971	c57159_g1	-7,377	31	21,5	0	1,69E-03
972	c57851_g1	-7,377	40	16	0	1,69E-03
973	c55605_g1	-7,365	32	21	0	1,69E-03
974	c15646_g1	-7,365	44	13	0	1,69E-03
975	c58520_g1	-7,365	56	5	0	1,69E-03
976	c32808_g1	-7,354	48	10	0	1,98E-03
977	c56024_g1	-7,354	42	14	0	1,69E-03
978	c57473_g1	-7,354	36	17,99	0	1,69E-03
979	c50211_g1	-7,354	33	20	0	1,69E-03
980	c56300_g1	-7,354	21	28	0	1,69E-03
981	c56742_g1	-7,343	48,96	9	0	1,98E-03
982	c41200_g1	-7,343	40	15	0	1,98E-03
983	c44608_g1	-7,343	34	19	0	1,69E-03
984	c46039_g1	-7,343	31	21	0	1,69E-03
985	c50148_g1	-7,343	28	23	0	1,69E-03
986	c46404_g1	-7,342	25	25	0	1,69E-03
987	c57087_g1	-7,332	47,1	10	0	1,98E-03
988	c50276_g1	-7,331	38	16	0	1,98E-03
989	c52207_g1	-7,331	29	22	0	1,98E-03
990	c56913_g4	-7,321	45	11	0	1,98E-03
991	c54212_g1	-7,308	34	18	0	1,98E-03
992	c54846_g3	-7,308	31	20	0	1,98E-03
993	c38895_g1	-7,296	32	19	0	2,32E-03
994	c57322_g2	-7,295	23	25	0	1,98E-03
995	c50967_g1	-7,294	20	27	0	1,98E-03
996	c54346_g1	-7,286	48	8	0	2,32E-03
997	c51703_g1	-7,286	48	8,01	0	2,32E-03
998	c49929_g1	-7,285	38,72	14	0	2,32E-03
999	c57398_g1	-7,284	30	20,07	0	2,32E-03
1000	c50923_g1	-7,283	27	21,99	0	2,32E-03
1001	c15585_g1	-7,282	18	28	0	1,98E-03
1002	c53623_g2	-7,274	43	11	0	2,32E-03
1003	c57672_g2	-7,273	34	17	0	2,32E-03
1004	c19096_g1	-7,271	28	21	0	2,32E-03
1005	c36300_g1	-7,271	28	20,99	0	2,32E-03
1006	c51978_g1	-7,263	47	8	0	2,74E-03
1007	c50645_g1	-7,258	22,92	24	0	2,32E-03
1008	c44491_g1	-7,258	20	26	0	2,32E-03
1009	c52756_g1	-7,257	17	28	0	2,32E-03
1010	c49571_g2	-7,250	39	13	0	2,74E-03
1011	c24327_g2	-7,248	30	19	0	2,74E-03
1012	c109557_g1	-7,246	24	23	0	2,32E-03
1013	c56226_g1	-7,246	24	23	0	2,32E-03
1014	c54445_g1	-7,245	21	24,85	0	2,32E-03
1015	c33096_g1	-7,245	21	25	0	2,32E-03
1016	c54218_g2	-7,244	15	29	0	2,32E-03
1017	c53521_g1	-7,237	37	14	0	2,74E-03
1018	c53713_g1	-7,235	28	20	0	2,74E-03
1019	c50942_g1	-7,233	22	24	0	2,74E-03
1020	c56199_g2	-7,232	16	28	0	2,32E-03

1021	c54268_g1	-7,227	44	9	0	3,23E-03
1022	c57185_g4	-7,211	33	16	0	3,23E-03
1023	c56031_g1	-7,210	30,05	18	0	2,74E-03
1024	c30327_g1	-7,210	30	18	0	2,74E-03
1025	c46695_g1	-7,210	26,62	19,96	0	2,74E-03
1026	c43466_g1	-7,201	40	11	0	3,23E-03
1027	c48592_g1	-7,201	0	38	0	1,98E-03
1028	c45991_g2	-7,196	25	21	0	3,23E-03
1029	c45857_g1	-7,196	25	21,13	0	3,23E-03
1030	c57858_g2	-7,188	38,04	12	0	3,23E-03
1031	c57878_g2	-7,188	38	12	0	3,23E-03
1032	c43981_g1	-7,187	35	14	0	3,23E-03
1033	c57285_g1	-7,187	35	14	0	3,23E-03
1034	c57100_g2	-7,186	502,72	484,77	0,99	1,13E-11
1035	c57948_g1	-7,185	29	17,9	0	3,23E-03
1036	c50726_g1	-7,184	26	20	0	3,23E-03
1037	c53018_g1	-7,184	26	20	0	3,23E-03
1038	c50739_g1	-7,178	11	30	0	2,74E-03
1039	c58206_g1	-7,177	42	9	0	3,83E-03
1040	c58174_g2	-7,177	42	9	0	3,83E-03
1041	c38314_g1	-7,175	36	13	0	3,23E-03
1042	c56540_g1	-7,174	33	15	0	3,23E-03
1043	c35853_g1	-7,171	27	19	0	3,23E-03
1044	c50126_g1	-7,170	24	21	0	3,23E-03
1045	c56845_g1	-7,163	37	12	0	3,83E-03
1046	c57306_g5	-7,161	34	14	0	3,83E-03
1047	c48175_g1	-7,160	31	16	0	3,83E-03
1048	c55133_g1	-7,156	22	22	0	3,23E-03
1049	c58018_g3	-7,155	19	24,2	0	3,23E-03
1050	c56479_g1	-7,155	19	24	0	3,23E-03
1051	c46469_g1	-7,146	767,97	310,85	1	1,47E-11
1052	c44180_g1	-7,132	27	18	0	3,83E-03
1053	c43895_g1	-7,131	24	20	0	3,83E-03
1054	c49057_g1	-7,124	37	11	0	4,54E-03
1055	c57271_g1	-7,124	37	11	0	4,54E-03
1056	c40801_g1	-7,121	31	15	0	3,83E-03
1057	c52586_g1	-7,119	28	17	0	3,83E-03
1058	c54893_g1	-7,118	25	19	0	3,83E-03
1059	c39599_g2	-7,116	22	20,98	0	3,83E-03
1060	c56181_g1	-7,112	38	10	0	4,54E-03
1061	c15308_g1	-7,111	13	26,99	0	3,83E-03
1062	c47150_g1	-7,108	32	14	0	4,54E-03
1063	c48009_g2	-7,108	31,81	14	0	4,54E-03
1064	c46160_g1	-7,104	48	3	0	4,54E-03
1065	c47626_g1	-7,104	48	3	0	4,54E-03
1066	c14157_g1	-7,097	36	11	0	4,54E-03
1067	c50027_g1	-7,094	30	15	0	4,54E-03
1068	c32783_g1	-7,085	15	24,9	0	3,83E-03
1069	c39644_g1	-7,083	34	12	0	4,54E-03
1070	c50399_g1	-7,076	44	5	0	5,41E-03
1071	c55270_g1	-7,075	22	20	0	4,54E-03
1072	c53404_g3	-7,075	22	19,99	0	4,54E-03
1073	c45750_g1	-7,067	10	28	0	3,83E-03
1074	c57321_g1	-7,065	48	2	0	5,41E-03
1075	c48904_g1	-7,061	42	6	0	5,41E-03
1076	c54365_g1	-7,057	36	10	0	5,41E-03
1077	c56233_g1	-7,049	42,76	5	0,49	5,41E-03
1078	c56883_g2	-7,044	37	9,15	0	5,41E-03
1079	c52792_g1	-7,044	17,78	22	0	4,54E-03
1080	c48425_g1	-7,040	50	0	0	6,45E-03

1081	c38003_g1	-7,040	31	13	0	5,41E-03
1082	c58057_g4	-7,038	28	14,96	0	5,41E-03
1083	c51169_g4	-7,038	28	15	0	5,41E-03
1084	c50185_g1	-7,030	0	33,93	0	3,83E-03
1085	c54664_g1	-7,027	32	12	0	5,41E-03
1086	c55919_g7	-7,025	29	14	0	5,41E-03
1087	c53614_g1	-7,025	28,98	14	0	5,41E-03
1088	c50214_g1	-7,022	26	16	0	5,41E-03
1089	c55170_g4	-7,020	23	18,2	0	5,41E-03
1090	c53939_g1	-7,004	21	19	0	5,41E-03
1091	c53092_g3	-7,001	34	10	0	6,45E-03
1092	c43078_g1	-6,993	41	4,95	0	6,45E-03
1093	c49643_g1	-6,987	35	9	0	6,45E-03
1094	c51249_g1	-6,985	32	11	0	6,45E-03
1095	c49517_g1	-6,985	16	22	0	6,45E-03
1096	c57735_g1	-6,985	16	22,49	0	6,45E-03
1097	c57008_g1	-6,982	29	13	0	6,45E-03
1098	c53092_g1	-6,982	29	13	0	6,45E-03
1099	c109529_g1	-6,979	26	15	0	6,45E-03
1100	c45370_g1	-6,976	7	27,88	0	5,41E-03
1101	c55490_g1	-6,968	14	23	0	6,45E-03
1102	c55911_g4	-6,963	24	16	0	6,45E-03
1103	c57500_g1	-6,958	5,37	29	0	5,41E-03
1104	c53999_g1	-6,957	18	20	0	6,45E-03
1105	c124540_g1	-6,956	47	0	0	7,73E-03
1106	c31075_g1	-6,952	28	13	0	7,73E-03
1107	c54105_g2	-6,949	24,98	15	0	7,73E-03
1108	c57426_g1	-6,947	9	26	0	6,45E-03
1109	c49215_g1	-6,939	16	21	0	6,45E-03
1110	c54769_g1	-6,938	29	12	0	7,73E-03
1111	c57214_g1	-6,938	28,87	12	0	7,73E-03
1112	c43033_g1	-6,936	13	22,99	0	6,45E-03
1113	c30979_g1	-6,936	12,92	23	0	6,45E-03
1114	c54152_g1	-6,935	26	14	0	7,73E-03
1115	c29535_g1	-6,934	39	5	0	9,28E-03
1116	c31394_g2	-6,932	23	16	0	7,73E-03
1117	c57331_g1	-6,921	27	13	0	7,73E-03
1118	c36298_g1	-6,918	24	15	0	7,73E-03
1119	c57078_g1	-6,918	23,97	14,96	0	7,73E-03
1120	c57240_g1	-6,914	20,95	17	0	7,73E-03
1121	c51532_g1	-6,914	20,89	17	0	7,73E-03
1122	c56474_g1	-6,911	18	19	0	7,73E-03
1123	c57727_g1	-6,911	31	10	0	9,28E-03
1124	c53115_g1	-6,907	28	12,01	0	9,28E-03
1125	c53172_g1	-6,904	25	14	0	9,28E-03
1126	c41580_g1	-6,900	22	16	0	7,73E-03
1127	c44937_g1	-6,900	22	16	0	7,73E-03
1128	c45174_g1	-6,893	16	20	0	7,73E-03
1129	c45302_g1	-6,886	23	15	0	9,28E-03
1130	c55982_g1	-6,883	33	8	0	9,28E-03
1131	c56089_g1	-6,876	27	12	0	9,28E-03
1132	c39345_g1	-6,874	13,99	21	0	7,73E-03
1133	c44954_g1	-6,872	24	13,98	0	9,28E-03
1134	c52373_g1	-6,869	34	7	0	1,12E-02
1135	c49409_g2	-6,864	18	18	0	9,28E-03
1136	c41121_g1	-6,862	5	27	0	7,73E-03
1137	c51475_g1	-6,862	28	11	0	9,28E-03
1138	c53052_g4	-6,858	25	13	0	9,28E-03
1139	c43089_g1	-6,856	35	6	0	1,12E-02
1140	c53721_g1	-6,854	22,24	15	0	9,28E-03
1141	c30118_g1	-6,852	32	8	0	1,12E-02
1142	c57046_g7	-6,851	9	24	0	9,28E-03
1143	c57771_g1	-6,849	19	17	0	9,28E-03
1144	c35816_g1	-6,845	16	19	0	9,28E-03
1145	c56750_g1	-6,843	26	12	0	1,12E-02
1146	c46899_g1	-6,843	26	12	0	1,12E-02
1147	c49449_g1	-6,837	33	7	0	1,12E-02
1148	c50364_g1	-6,836	10	23	0	9,28E-03
1149	c52817_g1	-6,835	20	16	0	1,12E-02
1150	c55589_g1	-6,835	20	16	0	1,12E-02
1151	c54262_g1	-6,833	30	9	0	1,12E-02
1152	c42008_g2	-6,832	40	2	0	1,12E-02
1153	c46478_g1	-6,830	17	18	0	9,28E-03
1154	c55746_g1	-6,825	24	13	0	1,12E-02
1155	c109999_g1	-6,820	21	15	0	1,12E-02
1156	c57718_g1	-6,816	8	24	0	9,28E-03
1157	c34557_g1	-6,815	18	17	0	1,12E-02
1158	c51753_g1	-6,810	25	12	0	1,12E-02
1159	c45735_g1	-6,806	12	21	0	1,12E-02
1160	c53730_g1	-6,806	12	21	0	1,12E-02
1161	c50121_g1	-6,805	22	14	0	1,12E-02
1162	c56434_g1	-6,801	19	16	0	1,12E-02
1163	c47490_g1	-6,800	29	9	0	1,35E-02
1164	c56140_g1	-6,796	6	25	0	9,28E-03
1165	c58172_g1	-6,795	26	10,85	0	1,35E-02
1166	c52389_g1	-6,791	23	13	0	1,12E-02
1167	c57110_g1	-6,771	31	7	0	1,35E-02
1168	c55582_g2	-6,771	21	14	0	1,35E-02
1169	c51699_g1	-6,770	11	21	0	0,011
1170	c57237_g4	-6,761	25	11	0	0,014
1171	c57750_g1	-6,761	25	11	0	0,014
1172	c109742_g1	-6,757	31,99	6	0	0,014
1173	c55186_g1	-6,756	554,35	255,96	1	0,000
1174	c48979_g1	-6,756	22	13	0	0,014
1175	c55170_g11	-6,756	22	12,85	0	0,014
1176	c52276_g1	-6,755	12	20	0	0,011
1177	c54011_g1	-6,749	9	22	0	0,011
1178	c36281_g1	-6,745	16	17	0	0,014
1179	c14192_g1	-6,742	33,13	5	0	0,016
1180	c50732_g1	-6,739	13	19	0	0,014
1181	c49246_g1	-6,737	30	7	0	0,016
1182	c53357_g1	-6,730	17	16	0	0,014
1183	c58074_g1	-6,726	24	10,98	0	0,016
1184	c29104_g1	-6,722	30,99	6	0	0,016
1185	c21277_g1	-6,719	37,95	1	0	0,016
1186	c54115_g1	-6,716	28	8	0	0,016
1187	c56675_g1	-6,716	27,98	8	0	0,016
1188	c52467_g1	-6,711	25	10	0	0,016
1189	c57961_g1	-6,705	22,16	12	0	0,016
1190	c58134_g1	-6,705	22	12	0	0,016
1191	c39688_g2	-6,705	22	12	0	0,016
1192	c49414_g2	-6,701	29	6,99	0	0,016
1193	c71523_g1	-6,701	29	7	0	0,016
1194	c42176_g1	-6,698	36	2	0	0,020
1195	c50490_g1	-6,695	26	9	0	0,016
1196	c122431_g1	-6,692	16	16	0	0,016
1197	c54831_g1	-6,689	23	11	0	0,016
1198	c56715_g1	-6,689	23	11	0	0,016
1199	c96846_g1	-6,678	34	3	0	0,020
1200	c30933_g1	-6,674	24	10	0	0,016

1201	c56785_g1	-6,674	24	10	0	0,016
1202	c53050_g1	-6,668	21	12	0	0,016
1203	c45667_g1	-6,665	28	7,13	0	0,020
1204	c27311_g1	-6,659	1	26	0	0,014
1205	c57908_g1	-6,659	25	9	0	0,020
1206	c40499_g1	-6,659	25	9	0	0,020
1207	c55056_g1	-6,657	8	21	0	0,016
1208	c55765_g1	-6,657	8	20,99	0	0,016
1209	c33840_g1	-6,654	15	16	0	0,016
1210	c48127_g1	-6,652	22	10,95	0	0,020
1211	c56372_g1	-6,652	22	11	0	0,020
1212	c58160_g1	-6,650	29	6	0	0,020
1213	c36439_g1	-6,649	5	23	0	0,014
1214	c55542_g1	-6,647	12	18	0	0,016
1215	c57445_g1	-6,643	26,43	8	0	0,020
1216	c123047_g1	-6,641	33	3	0	0,020
1217	c24374_g1	-6,638	16	15	0	0,020
1218	c49375_g1	-6,638	16	15	0	0,020
1219	c53439_g2	-6,635	30	5	0	0,020
1220	c42834_g2	-6,633	6	22	0	0,016
1221	c56783_g1	-6,631	13	17	0	0,016
1222	c44620_g1	-6,628	445	270	1	0,000
1223	c23833_g1	-6,628	27	7	0	0,020
1224	c51967_g2	-6,624	10	19	0	0,016
1225	c27671_g1	-6,621	24	9	0	0,020
1226	c109598_g1	-6,615	14	16	0	0,020
1227	c45259_g1	-6,614	21	11	0	0,020
1228	c49084_g1	-6,606	18	13	0	0,020
1229	c55628_g2	-6,605	339	322,93	1	0,000
1230	c51814_g2	-6,599	8	19,97	0	0,020
1231	c54953_g1	-6,599	15	15	0	0,020
1232	c55149_g2	-6,599	15	15	0	0,020
1233	c11450_g1	-6,557	7	20	0	0,020
1234	c96899_g1	-6,540	1	24	0	0,016
1235	c57944_g1	-6,522	463	221,95	1	0,000
1236	c56469_g1	-6,463	417	229,01	1	0,000
1237	c23602_g1	-6,427	459	193	1	0,000
1238	c57311_g1	-6,133	351	171,42	1	0,000
1239	c53192_g1	-6,056	361,87	144,83	1	0,000
1240	c55389_g3	-6,008	262,98	190,99	1	0,000
1241	c43382_g1	-5,988	3323	43	5,88	0,000
1242	c122426_g1	-5,954	519	345,92	2	0,000
1243	c15658_g1	-5,917	239	183,99	1	0,000
1244	c55101_g2	-5,887	336,94	120	1	0,000
1245	c45950_g1	-5,854	270	152	1	0,000
1246	c55094_g2	-5,847	266	153	1	0,000
1247	c53824_g1	-5,835	275	145	0,84	0,000
1248	c57671_g1	-5,831	224	174	1	0,000
1249	c49173_g1	-5,747	217,01	161,04	1	0,000
1250	c49608_g1	-5,589	268	100,9	1	0,000
1251	c48875_g2	-5,587	230	123,46	1	0,000
1252	c56350_g1	-5,553	218	124	1	0,000
1253	c48317_g1	-5,538	435,97	232	2	0,000
1254	c47895_g1	-5,528	935,97	410	4	0,000
1255	c49323_g1	-5,501	533,83	163	2	0,000
1256	c38325_g1	-5,439	215,98	106	1	0,000
1257	c52176_g1	-5,361	184,99	111,99	1	0,000
1258	c54151_g2	-5,317	485	337,86	3	0,000
1259	c56989_g1	-5,259	226,97	72,13	1,3	0,000
1260	c57012_g2	-5,223	236,89	61	1	0,000

1261	c52387_g1	-5,210	121	127,88	1	0,000
1262	c57993_g1	-5,182	206,93	72,93	1	0,000
1263	c49256_g3	-5,085	110	118	1	0,000
1264	c56473_g1	-5,085	203,99	62	1	0,000
1265	c30202_g1	-5,052	487	232,99	3	0,000
1266	c52880_g2	-5,028	187	65	1	0,000
1267	c71326_g1	-5,013	256,93	187,96	2	0,000
1268	c51521_g1	-5,001	111	107,3	1	0,000
1269	c52261_g1	-5,000	151	83	1	0,000
1270	c43343_g2	-4,989	249	187	2	0,000
1271	c57410_g2	-4,982	99	112	1	0,000
1272	c50627_g1	-4,977	707,98	399,97	5	0,000
1273	c54222_g1	-4,956	144	82	1	0,000
1274	c56935_g1	-4,929	184	55	1	0,000
1275	c53372_g1	-4,889	187,96	48	1	0,000
1276	c56129_g1	-4,864	169,75	56	1	0,000
1277	c35155_g1	-4,804	273	133	2	0,000
1278	c52069_g1	-4,789	145	63	0,74	0,000
1279	c51859_g2	-4,785	146	62	1	0,000
1280	c45382_g1	-4,767	376	205	3	0,000
1281	c53478_g1	-4,735	134	64	1	0,000
1282	c58237_g1	-4,709	346,98	205	3	0,000
1283	c51542_g1	-4,675	1302	425	9	0,000
1284	c48420_g1	-4,639	160	39	1	0,000
1285	c52737_g1	-4,636	85	84	1	0,000
1286	c10566_g1	-4,586	162	156,99	2	0,000
1287	c53816_g3	-4,584	125	54,79	0,66	0,000
1288	c56891_g1	-4,582	379	151,58	3	0,000
1289	c53620_g1	-4,550	143	41	1	0,000
1290	c57338_g4	-4,511	88	71	1	0,000
1291	c43425_g1	-4,505	327	162,98	3	0,000
1292	c49669_g1	-4,489	130,56	158,54	2,5	0,000
1293	c57017_g1	-4,476	93	65	1	0,000
1294	c51424_g1	-4,441	98	59,11	1,02	0,000
1295	c54437_g1	-4,435	118	158	2	0,000
1296	c57114_g1	-4,417	645	285	6	0,000
1297	c51265_g2	-4,406	94,93	58	1	0,000
1298	c54902_g1	-4,369	100	52	1	0,000
1299	c41895_g1	-4,332	161	117	2	0,000
1300	c54886_g1	-4,326	523	213	5	0,000
1301	c8189_g1	-4,318	260,16	158,88	3	0,000
1302	c50650_g1	-4,315	253	60	2	0,000
1303	c38514_g1	-4,287	62	69	1	0,000
1304	c55956_g1	-4,263	210,98	176,12	3	0,000
1305	c55795_g3	-4,261	85	53	0,99	0,000
1306	c57565_g3	-4,229	210,97	169	3	0,000
1307	c46562_g1	-4,214	275,93	128	3	0,000
1308	c47887_g1	-4,162	85	46	1	0,001
1309	c50395_g1	-4,131	80	46,96	0,72	0,001
1310	c47898_g1	-4,122	290	191	4,22	0,000
1311	c10120_g1	-4,104	69	51,99	1	0,001
1312	c57583_g2	-3,995	695	170	7	0,000
1313	c51752_g1	-3,983	302	150,84	4	0,000
1314	c56222_g1	-3,942	269	161	4	0,000
1315	c55022_g3	-3,940	96	25	1	0,001
1316	c50481_g1	-3,934	30535,96	6634,93	311	0,000
1317	c55391_g1	-3,934	388	168,34	5	0,000
1318	c56319_g1	-3,889	391	230,09	6	0,000
1319	c47668_g1	-3,889	178,04	127	3	0,000
1320	c42523_g1	-3,874	2056,99	756,95	26	0,000

1321	c44589_g1	-3,869	1031	449	14	0,000
1322	c48878_g2	-3,858	58	44,22	0,63	0,002
1323	c84052_g1	-3,822	535	269,99	8	0,000
1324	c57516_g2	-3,818	1712	1309,86	32	0,000
1325	c44183_g2	-3,816	211	96	3	0,000
1326	c25398_g1	-3,815	59	40,8	1	0,001
1327	c57918_g1	-3,803	50	46	1	0,001
1328	c23731_g1	-3,778	267	127,97	4	0,000
1329	c57728_g2	-3,778	202	165,74	4	0,000
1330	c46499_g1	-3,763	152	123	3	0,000
1331	c10794_g1	-3,750	9	68,98	1	0,001
1332	c10169_g1	-3,692	122,99	130	3	0,000
1333	c57972_g2	-3,685	80	21	1	0,002
1334	c50100_g1	-3,680	100	76	2	0,000
1335	c50506_g1	-3,679	128	59	2	0,000
1336	c58285_g1	-3,679	257,96	180	4,98	0,000
1337	c11557_g1	-3,640	211,99	70	3	0,000
1338	c57208_g1	-3,622	106	67	2	0,000
1339	c58162_g2	-3,602	140,88	43,98	2	0,000
1340	c56996_g1	-3,599	55,99	32	1	0,003
1341	c50665_g2	-3,596	150	38	2	0,000
1342	c50153_g1	-3,579	98	68	2	0,000
1343	c47466_g1	-3,556	45	37	1	0,003
1344	c49648_g2	-3,536	414,07	294,98	9	0,000
1345	c54237_g1	-3,514	75	16	1	0,004
1346	c49895_g1	-3,503	424	103	6	0,000
1347	c51159_g1	-3,498	154	86	3	0,000
1348	c55076_g1	-3,489	56	26,99	1	0,004
1349	c52950_g2	-3,486	126	43	2	0,001
1350	c53447_g1	-3,483	268	132	5	0,000
1351	c45110_g1	-3,461	76	13	1	0,004
1352	c97073_g1	-3,451	168	72	3	0,000
1353	c50986_g2	-3,449	139	32	2	0,001
1354	c32839_g1	-3,430	1021	16	11	0,000
1355	c45955_g1	-3,427	118	42,96	2	0,001
1356	c37503_g1	-3,421	424,98	302	10	0,000
1357	c46416_g1	-3,403	402,98	361,84	11	0,000
1358	c55397_g1	-3,401	131	33	1,69	0,001
1359	c43077_g1	-3,395	285,77	212,38	7	0,000
1360	c32709_g1	-3,373	211	91	4	0,000
1361	c48509_g1	-3,363	1103,82	359,99	19	0,000
1362	c52157_g1	-3,360	161,64	118,07	4	0,000
1363	c46396_g1	-3,347	478,36	140,44	8	0,000
1364	c49547_g1	-3,340	84	57	2	0,001
1365	c58098_g1	-3,327	43,91	28	1	0,008
1366	c25900_g1	-3,318	61,96	16	1	0,008
1367	c56130_g1	-3,314	675,95	219	12	0,000
1368	c51933_g1	-3,309	309	125,96	6	0,000
1369	c58203_g1	-3,290	154	62	3	0,000
1370	c52265_g1	-3,277	125	78	3	0,000
1371	c51719_g1	-3,273	107	38	2	0,001
1372	c57589_g1	-3,251	387	166,17	8	0,000
1373	c54266_g1	-3,230	158	101,99	4	0,000
1374	c57699_g1	-3,224	228,73	59	4	0,000
1375	c50415_g1	-3,201	328	140	7	0,000
1376	c41380_g1	-3,200	181,99	131	5	0,000
1377	c26573_g1	-3,195	71,93	54	2	0,002
1378	c45821_g1	-3,176	66	8	1	0,014
1379	c10667_g1	-3,165	110,98	74,8	3	0,000
1380	c49848_g2	-3,165	106	78,49	3	0,000

1381	c34184_g1	-3,163	313	139,87	7	0,000
1382	c55628_g1	-3,152	301	2574,95	61	0,000
1383	c46841_g1	-3,130	589	330,48	15	0,000
1384	c52305_g1	-3,109	117	66,18	3	0,001
1385	c42941_g2	-3,100	497,99	368,75	15	0,000
1386	c56779_g2	-3,100	586	405	17	0,000
1387	c45746_g1	-3,097	87	83	3	0,001
1388	c58233_g1	-3,086	581	228,03	13	0,000
1389	c57083_g1	-3,084	56,96	56	2	0,003
1390	c57605_g1	-3,080	97	31	2	0,003
1391	c56722_g2	-3,058	319	284,41	10,7	0,000
1392	c44046_g1	-3,052	649	260	15	0,000
1393	c57480_g1	-3,049	869,01	174	16	0,000
1394	c52188_g2	-3,019	140	85,6	4	0,001
1395	c52993_g1	-3,010	124,8	52	2,94	0,001
1396	c48575_g2	-3,000	224,99	157	7	0,000
1397	c54611_g1	-2,996	221,98	116,99	6	0,000
1398	c55692_g1	-2,995	492	244,88	13	0,000
1399	c58347_g1	-2,985	251	97,99	6	0,000
1400	c49611_g1	-2,972	107,99	59	3	0,001
1401	c57241_g1	-2,970	520,98	178,98	12	0,000
1402	c45419_g1	-2,962	270	122,89	7	0,000
1403	c35068_g1	-2,941	139,97	77	4	0,001
1404	c49418_g2	-2,929	575,99	288,92	16	0,000
1405	c48817_g1	-2,927	182	129	6	0,000
1406	c55912_g1	-2,914	129,73	80	4	0,001
1407	c58058_g1	-2,907	757,99	251,43	18	0,000
1408	c49278_g1	-2,902	300	208,62	10	0,000
1409	c57149_g1	-2,887	657	300	17,8	0,000
1410	c54978_g1	-2,886	149,79	64,89	4	0,001
1411	c54679_g1	-2,862	379,99	411,1	17	0,000
1412	c26676_g1	-2,856	440,99	188,99	12	0,000
1413	c47346_g1	-2,847	113	157	6	0,000
1414	c84087_g1	-2,840	757,31	366	22	0,000
1415	c30304_g1	-2,838	202	66	5	0,001
1416	c45428_g1	-2,833	340,96	94,09	8	0,000
1417	c42822_g1	-2,831	305,99	186,95	10	0,000
1418	c58017_g1	-2,828	285,84	233,87	11	0,000
1419	c54457_g1	-2,823	706	241,99	18,2	0,000
1420	c54671_g9	-2,821	1297,73	476	34	0,000
1421	c54698_g1	-2,799	243,99	143,74	8	0,000
1422	c57460_g2	-2,793	340,96	157,45	9,98	0,000
1423	c30138_g1	-2,792	349,9	538,99	21	0,000
1424	c57382_g1	-2,791	74	65,46	3	0,003
1425	c47100_g1	-2,774	28480,97	3280	564	0,000
1426	c36605_g1	-2,769	198	95	6	0,001
1427	c54240_g1	-2,768	281	151	8,98	0,000
1428	c54395_g1	-2,755	203	90,01	6	0,001
1429	c55536_g1	-2,754	124	67	4	0,002
1430	c47897_g1	-2,724	100,85	77,99	4	0,002
1431	c84251_g1	-2,720	87	86	4	0,002
1432	c55170_g12	-2,710	165	38	4	0,003
1433	c36021_g1	-2,705	252,85	154	9	0,000
1434	c57361_g1	-2,695	900	273	24	0,000
1435	c44548_g1	-2,688	229	65	6	0,001
1436	c56292_g1	-2,687	99	42	3	0,004
1437	c54538_g1	-2,676	642,69	572,98	29	0,000
1438	c35673_g1	-2,664	41	76	3	0,004
1439	c34305_g1	-2,663	53,97	68	3	0,005
1440	c44191_g1	-2,642	166	31	4	0,003

1441	c57858_g3	-2,637	287	87,74	8	0,001
1442	c57829_g1	-2,615	39	42	2	0,018
1443	c55277_g1	-2,605	268	125	9	0,001
1444	c51968_g1	-2,604	54	32	2	0,020
1445	c57575_g2	-2,578	677,57	339	24	0,000
1446	c51177_g1	-2,574	109,01	28,23	3	0,007
1447	c52177_g1	-2,569	115,98	177	8	0,001
1448	c54398_g1	-2,564	251	127	8,99	0,001
1449	c50088_g1	-2,556	77,72	46	3	0,007
1450	c57014_g2	-2,549	280,47	196,82	12	0,001
1451	c46072_g1	-2,543	641	105	16	0,000
1452	c57285_g3	-2,519	87,01	38	3	0,009
1453	c29083_g1	-2,496	1372	244	36	0,000
1454	c42741_g1	-2,486	142,34	148,31	8	0,001
1455	c53426_g6	-2,463	114	47	4	0,007
1456	c50648_g1	-2,461	100	84	5	0,004
1457	c46405_g1	-2,460	89	62	4	0,007
1458	c57624_g4	-2,454	159,99	104,22	7	0,002
1459	c55667_g1	-2,453	381	114,97	12	0,001
1460	c55565_g1	-2,453	357	157	13	0,001
1461	c52349_g1	-2,452	134	90,91	6	0,003
1462	c53117_g1	-2,446	231	88,98	8	0,002
1463	c84044_g1	-2,443	644	348,98	26	0,000
1464	c53225_g3	-2,437	68	73	4	0,008
1465	c41358_g1	-2,427	985	608,95	43	0,000
1466	c71344_g1	-2,425	109	75	5	0,005
1467	c52108_g1	-2,424	167	40	5	0,005
1468	c50692_g1	-2,420	147	243,99	12	0,001
1469	c54041_g1	-2,408	123	65	5	0,005
1470	c56235_g1	-2,403	163	68	6	0,003
1471	c54316_g1	-2,398	641	408,93	28,9	0,000
1472	c57856_g1	-2,398	660,97	262,95	24	0,001
1473	c10659_g1	-2,396	607	213	21	0,001
1474	c44257_g1	-2,394	578,89	389	27	0,001
1475	c53066_g1	-2,392	242	208,99	13	0,001
1476	c46085_g1	-2,377	808,11	353,73	31	0,001
1477	c56571_g1	-2,375	101	48	4	0,007
1478	c43981_g2	-2,353	333	94	11	0,002
1479	c50640_g1	-2,347	179	131	9	0,002
1480	c47222_g2	-2,345	190	98	7,98	0,003
1481	c43198_g1	-2,345	349	239	17	0,001
1482	c52161_g1	-2,342	198,58	66	7	0,004
1483	c54488_g1	-2,342	534,97	182,41	19	0,001
1484	c42617_g1	-2,338	71,97	63	4	0,008
1485	c96840_g1	-2,327	393	284,99	20	0,001
1486	c55000_g2	-2,326	717,97	733,75	45	0,001
1487	c34714_g1	-2,324	644,98	444,04	32	0,001
1488	c53583_g2	-2,316	142,98	95,58	7,02	0,004
1489	c55864_g2	-2,313	88	25	3	0,018
1490	c57050_g2	-2,308	549,52	262,6	23	0,001
1491	c54423_g1	-2,305	99	44	4	0,009
1492	c42021_g1	-2,287	149	138,94	9	0,003
1493	c54578_g1	-2,279	210,99	150,98	11	0,002
1494	c41087_g1	-2,278	53	45	3	0,020
1495	c48374_g1	-2,276	451,97	232,78	20	0,001
1496	c56893_g2	-2,266	83	51,29	4	0,010
1497	c49206_g1	-2,247	314	108,99	12	0,002
1498	c57573_g2	-2,246	151	57,83	6	0,006
1499	c37106_g1	-2,242	306	137	13	0,002
1500	c50637_g1	-2,242	112	81	6,2	0,006

1501	c55965_g5	-2,237	109	33	4	0,012
1502	c50854_g1	-2,233	641,55	586,91	40	0,001
1503	c25365_g1	-2,212	930,96	688,87	51,9	0,001
1504	c55516_g1	-2,199	314	333,99	22	0,001
1505	c71560_g1	-2,196	396	97,97	14	0,002
1506	c55008_g1	-2,186	383,99	125,96	15	0,003
1507	c45565_g1	-2,172	112	74	6	0,008
1508	c53305_g1	-2,167	459,94	215	21	0,002
1509	c32546_g1	-2,166	249	108	10,5	0,004
1510	c52075_g1	-2,163	458	192	20	0,002
1511	c49935_g1	-2,162	194,89	139	10,9	0,004
1512	c43718_g1	-2,158	562	244	25	0,002
1513	c39500_g1	-2,150	155	115	9	0,005
1514	c56982_g1	-2,137	141	76	7	0,007
1515	c71416_g1	-2,134	900	731	56	0,001
1516	c38825_g1	-2,129	68	51	4	0,017
1517	c56248_g1	-2,124	149	46,73	6	0,011
1518	c122580_g1	-2,122	301,86	181,2	16	0,003
1519	c34387_g1	-2,119	300,95	202,96	16,9	0,003
1520	c56786_g5	-2,114	2250,87	1016,27	105	0,001
1521	c35450_g1	-2,113	265,99	332	22	0,002
1522	c53141_g1	-2,113	220	138	12	0,004
1523	c50790_g1	-2,086	94,99	98	7	0,008
1524	c54722_g1	-2,085	89,99	78,99	6	0,012
1525	c109627_g1	-2,084	255	133,85	13,2	0,004
1526	c34110_g1	-2,084	526,41	451,84	35	0,002
1527	c57088_g1	-2,080	348	186,99	18	0,003
1528	c52445_g1	-2,079	81	62	5	0,018
1529	c23862_g1	-2,078	2017	1583,76	128	0,001
1530	c41421_g1	-2,077	79	63	5	0,018
1531	c53403_g1	-2,068	171	94	9,35	0,007
1532	c54682_g5	-2,062	162	77	8	0,009
1533	c55862_g1	-2,060	173,19	156,03	12	0,005
1534	c37914_g1	-2,054	196	34	7	0,010
1535	c55689_g1	-2,037	3587,84	90	103	0,002
1536	c53801_g1	-2,036	943,59	458,43	48,3	0,002
1537	c56445_g1	-2,029	155	55,87	7	0,011
1538	c49720_g1	-2,024	196	157	13	0,006
1539	c56873_g1	-2,017	176	126	11	0,006
1540	c46581_g1	-2,014	1892,88	1210,18	112	0,002
1541	c56045_g1	-2,013	860,93	386,88	43	0,002
1542	c56324_g1	-2,006	141	41	5,97	0,016
1543	c57314_g1	-1,982	940,84	262	40	0,003
1544	c57525_g2	-1,981	320	137	16	0,006
1545	c51784_g1	-1,968	1280,81	852,98	79,5	0,002
1546	c51362_g1	-1,956	437	143	20	0,005
1547	c50386_g1	-1,942	302,98	216,98	20	0,005
1548	c56392_g2	-1,939	105	77	7	0,015
1549	c23688_g1	-1,935	230	199	17	0,006
1550	c52695_g1	-1,934	162	82,09	9	0,013
1551	c57811_g1	-1,933	183	148	13	0,008
1552	c52987_g1	-1,926	133,93	77,98	7,86	0,017
1553	c57822_g1	-1,912	366	229,79	23,1	0,006
1554	c51589_g2	-1,906	604	205	28,9	0,005
1555	c49765_g1	-1,894	1634,99	797	92	0,003
1556	c57968_g1	-1,886	465,96	276,58	29	0,005
1557	c57346_g1	-1,876	114,95	65	7	0,020
1558	c25467_g2	-1,872	56	119	8	0,015
1559	c55540_g3	-1,862	526	455	41	0,005
1560	c52782_g1	-1,847	1165,99	919	87	0,004

1561	c51358_g4	-1,844	703,99	86,77	27	0,006
1562	c33443_g1	-1,839	339	77	15	0,010
1563	c57306_g4	-1,837	102,95	123,99	10	0,015
1564	c49452_g1	-1,834	101	143,18	11	0,013
1565	c52884_g1	-1,833	369,59	130,67	19	0,008
1566	c57566_g4	-1,822	1107,57	584,56	68	0,005
1567	c37474_g1	-1,810	536,59	333	36	0,007
1568	c42274_g1	-1,808	104,7	83	8	0,020
1569	c32403_g1	-1,795	223,81	154	16	0,012
1570	c25331_g1	-1,787	276	122,03	16	0,013
1571	c57178_g1	-1,780	343,92	203,97	23	0,009
1572	c51585_g1	-1,772	702	273	39	0,007
1573	c51917_g1	-1,771	188	65	9,71	0,020
1574	c46274_g1	-1,769	661,99	364	43	0,007
1575	c50915_g1	-1,766	119,94	191,87	15	0,013
1576	c53542_g1	-1,756	1114	715,47	79	0,006
1577	c47762_g1	-1,754	491	334,99	36	0,009
1578	c57100_g1	-1,753	351	158	21	0,011
1579	c49411_g1	-1,753	823,76	210,87	40	0,008
1580	c51782_g1	-1,740	2135,97	2314,71	209	0,006
1581	c56356_g1	-1,734	234	84,8	13	0,018
1582	c55400_g2	-1,714	386	195,32	25	0,012
1583	c15618_g1	-1,701	121	129,99	12	0,020
1584	c47909_g1	-1,692	97	142,99	12	0,020
1585	c56584_g3	-1,690	871,98	629,99	69	0,009
1586	c51849_g1	-1,687	613,57	595,87	58	0,009
1587	c57352_g1	-1,671	2070,88	815,94	124	0,008
1588	c51856_g1	-1,667	301	166	21	0,016
1589	c35185_g1	-1,648	5528	1698	306	0,008
1590	c53268_g1	-1,641	906,92	1566,84	132	0,010
1591	c50396_g3	-1,627	511	240	34	0,014
1592	c33044_g1	-1,620	592,58	314,51	42	0,014
1593	c96827_g1	-1,615	720	488,94	58	0,013
1594	c47020_g1	-1,612	971,04	481,88	66,7	0,012
1595	c55727_g4	-1,600	16983,61	6380,6	1048	0,010
1596	c56753_g1	-1,586	591	254,77	39	0,016
1597	c51017_g1	-1,576	477,35	180	30	0,019
1598	c56741_g1	-1,550	1359,73	1180,92	132	0,014
1599	c50761_g3	-1,548	954	298	57	0,016
1600	c46488_g2	-1,529	1027,93	566,84	79	0,017
1601	c54618_g1	-1,518	1304,98	1067,99	125	0,016
1602	c30806_g1	-1,514	549,91	394,98	49	0,020
1603	c44570_g1	-1,511	446,99	770,97	71	0,018
1604	c54301_g1	-1,502	1140,85	638,89	90	0,018
1605	c71380_g1	-1,498	768	663	77	0,019
1606	c50040_g1	-1,482	3208,98	60	134	0,018
1607	c57332_g3	-1,458	1577,85	780,55	121	0,021
1608	c55492_g4	1,291	257	222	178	0,020
1609	c47130_g1	1,292	235,87	211	167	0,020
1610	c41582_g1	1,320	359	107,99	155	0,017
1611	c54696_g1	1,325	208	68	93	0,019
1612	c39759_g1	1,327	430	222	232	0,015
1613	c49578_g1	1,328	599	331,16	334	0,015
1614	c58111_g1	1,332	230	93,32	112	0,017
1615	c53382_g1	1,339	187	56	82	0,018
1616	c52205_g2	1,350	123	46	59	0,021
1617	c57276_g5	1,354	134	91,99	85	0,018
1618	c56939_g1	1,364	292,97	113	143	0,014
1619	c16562_g1	1,372	117	88	79	0,017
1620	c51316_g1	1,384	276,81	76,9	122	0,013
1621	c57145_g1	1,386	94,99	66	62	0,017
1622	c54210_g1	1,387	159	21	59	0,017
1623	c56272_g1	1,390	111	54	61	0,017
1624	c56069_g1	1,390	263	148,55	155	0,012
1625	c48009_g1	1,392	278	168,71	170	0,012
1626	c39584_g1	1,395	142,33	144,99	116,8	0,013
1627	c56499_g1	1,395	89	39	47	0,020
1628	c55095_g1	1,402	359	198,99	211	0,011
1629	c49653_g1	1,403	96,97	80,87	71	0,016
1630	c23145_g1	1,409	391,97	145	194	0,010
1631	c57125_g1	1,411	319,98	145,93	173	0,011
1632	c33554_g2	1,411	151	78,98	87	0,013
1633	c26950_g1	1,423	88,02	73,09	65	0,015
1634	c19925_g1	1,426	254	276	223	0,009
1635	c57841_g1	1,427	105	30,7	49	0,016
1636	c55820_g1	1,427	217	210,44	176,7	0,010
1637	c42893_g1	1,432	177	108,94	112	0,011
1638	c54633_g1	1,444	56	35	36	0,019
1639	c53977_g1	1,450	1193,76	371	568,7	0,007
1640	c53015_g1	1,452	4921,81	3514,67	3420	0,007
1641	c52776_g1	1,463	257	58,98	113	0,009
1642	c49046_g1	1,465	65,34	36	40	0,016
1643	c54809_g2	1,476	92,8	56	60	0,012
1644	c48536_g1	1,482	61	30	36	0,017
1645	c29913_g1	1,483	153	77	91	0,009
1646	c55686_g3	1,485	162	101	107	0,008
1647	c50253_g1	1,489	278,79	147	170	0,007
1648	c36055_g1	1,490	99	55	62	0,011
1649	c45414_g1	1,495	90	106	86,99	0,009
1650	c40964_g1	1,498	123,99	54	70	0,010
1651	c42539_g2	1,500	213,97	101	125	0,007
1652	c50420_g1	1,507	183	91	110	0,007
1653	c51319_g1	1,511	589,59	379,23	402,6	0,005
1654	c54547_g1	1,512	601	229,46	323	0,005
1655	c49420_g1	1,513	154	81,99	96	0,008
1656	c33815_g1	1,513	204	147,81	149	0,007
1657	c46174_g1	1,516	166	60	88	0,008
1658	c43853_g1	1,516	72,99	45	49	0,012
1659	c53718_g1	1,520	67	30	39	0,014
1660	c37731_g1	1,520	61	54	50,02	0,011
1661	c47652_g1	1,522	37	36	32	0,018
1662	c50864_g1	1,531	318	58	138	0,006
1663	c53019_g1	1,539	133	79	88,98	0,007
1664	c34247_g1	1,543	331	262,98	260	0,005
1665	c55022_g1	1,546	111	14	45,99	0,009
1666	c53426_g5	1,553	142,96	82	95	0,006
1667	c51094_g1	1,563	299,99	166	196,8	0,005
1668	c51228_g2	1,563	132	54	75,99	0,007
1669	c10746_g1	1,581	146	103	110	0,005
1670	c109618_g1	1,592	131	157,97	138	0,005
1671	c48866_g1	1,600	365,73	166,5	224,9	0,004
1672	c48531_g1	1,602	189,94	253	216	0,004
1673	c23172_g1	1,607	76	20	39	0,008
1674	c45563_g1	1,611	155	92	109	0,004
1675	c57282_g4	1,611	83	52	60	0,006
1676	c71572_g1	1,616	146,96	47	80	0,005
1677	c9297_g1	1,616	246	83	136	0,004
1678	c57960_g1	1,626	1634,75	671,01	978,9	0,003
1679	c53395_g1	1,632	108	30	56,99	0,005
1680	c57723_g1	1,633	2112586,69	574876,11	1E+06	0,002
1681	c50684_g1	1,636	139,85	51	81	0,004

1682	c54765_g5	1,639	46	29	34	0,009
1683	c44904_g1	1,643	87	54	64	0,005
1684	c18689_g1	1,646	160	53	90	0,004
1685	c58033_g1	1,647	89	22	46	0,006
1686	c56922_g1	1,647	134	94,97	106	0,004
1687	c41707_g1	1,654	169	134	143	0,003
1688	c47718_g1	1,662	1385,96	760,93	971	0,002
1689	c44902_g1	1,670	617	459	509,7	0,002
1690	c32781_g1	1,676	225	192	202	0,002
1691	c52727_g1	1,677	166	156	158	0,003
1692	c36796_g1	1,680	65	55,01	58	0,004
1693	c42796_g1	1,681	66,98	29	43	0,006
1694	c52797_g1	1,681	195	74	118	0,003
1695	c53976_g1	1,685	50	71,98	63	0,005
1696	c54424_g4	1,689	95,64	63	75	0,003
1697	c49504_g1	1,694	184,96	67	111	0,003
1698	c51931_g1	1,696	98	27	54	0,004
1699	c10737_g1	1,698	48,99	27,97	36	0,007
1700	c56585_g1	1,711	899,31	460,27	630,3	0,002
1701	c49378_g1	1,713	273	111,98	174	0,002
1702	c58152_g1	1,719	168	63	104	0,002
1703	c46374_g1	1,721	56	34	43	0,005
1704	c32558_g1	1,722	66,89	59	63	0,004
1705	c57173_g1	1,737	59	5	27	0,006
1706	c39444_g1	1,742	80,97	133,66	118,4	0,002
1707	c54013_g1	1,748	107,01	41,98	69	0,003
1708	c50501_g1	1,756	129	95,58	113	0,002
1709	c23940_g1	1,758	52	55	56	0,003
1710	c45882_g1	1,759	624,85	517,85	585	0,001
1711	c52162_g1	1,767	147,47	60,97	98	0,002
1712	c33415_g1	1,785	159	55	100	0,002
1713	c46084_g2	1,787	150,99	127	145	0,001
1714	c49025_g1	1,789	79	24	48	0,003
1715	c22919_g1	1,794	72	48	61	0,002
1716	c49559_g1	1,807	108,99	53	79,89	0,002
1717	c23689_g1	1,807	16	36	30	0,006
1718	c54844_g1	1,810	171,42	125	154	0,001
1719	c41890_g1	1,816	119	61	90	0,001
1720	c37476_g1	1,821	120	82	105	0,001
1721	c51148_g1	1,823	252	113	180	0,001
1722	c52863_g1	1,827	39,96	18	29	0,005
1723	c52291_g1	1,831	168,88	75	121	0,001
1724	c56823_g2	1,834	111	53,95	83	0,001
1725	c55213_g1	1,848	67	55	66	0,002
1726	c58151_g4	1,854	29	1	14	0,009
1727	c47506_g1	1,858	111	52	83	0,001
1728	c29222_g1	1,859	243,99	97	170	0,001
1729	c58072_g6	1,866	159,73	220,92	224	0,001
1730	c57184_g1	1,867	455,08	244,61	364	0,001
1731	c54228_g1	1,869	63	100	97	0,001
1732	c57966_g1	1,869	408,87	165,69	288,7	0,001
1733	c55006_g2	1,871	746,97	316,99	538	0,001
1734	c57392_g1	1,878	108	45	78	0,001
1735	c54576_g1	1,880	105,99	66	91,93	0,001
1736	c55789_g1	1,896	267	27	134	0,001
1737	c84246_g1	1,907	362,99	174	283	0,000
1738	c47095_g1	1,911	47	41	50	0,002
1739	c56595_g2	1,916	261,99	194	255,9	0,000
1740	c53476_g1	1,927	246	119,98	196	0,000
1741	c12732_g1	1,928	256	254,95	301	0,000

1742	c53468_g1	1,931	106	37,95	75	0,001
1743	c50877_g1	1,933	50	106	100	0,001
1744	c57655_g3	1,937	72	90,89	99	0,001
1745	c47210_g1	1,945	119	29	75	0,001
1746	c48555_g1	1,947	10	12,47	13	0,019
1747	c57957_g1	1,963	62	28,92	50	0,001
1748	c55208_g1	1,965	86,94	20	55	0,001
1749	c56636_g1	1,970	180	87,62	148	0,000
1750	c50534_g1	1,984	63	26	49	0,001
1751	c58119_g1	1,996	253,55	94,97	190	0,000
1752	c51999_g1	2,000	89	41	73	0,001
1753	c39331_g2	2,001	61,99	55	71	0,001
1754	c58057_g7	2,002	84,39	40	70	0,001
1755	c46473_g1	2,003	128	68	111,7	0,000
1756	c57572_g4	2,004	112	45	87	0,000
1757	c48549_g1	2,004	101	87,89	115	0,000
1758	c55784_g1	2,006	97	36,96	74	0,001
1759	c50327_g3	2,011	284	62	180	0,000
1760	c52010_g1	2,012	27	3	16	0,005
1761	c31842_g1	2,012	136	48	101	0,000
1762	c57664_g1	2,022	28	27	34	0,002
1763	c56307_g2	2,026	412,52	166,13	323,9	0,000
1764	c47933_g1	2,032	123,07	62,98	107,6	0,000
1765	c47923_g1	2,036	158	67	128	0,000
1766	c55911_g2	2,039	199	118,99	189	0,000
1767	c56456_g1	2,049	68	29,08	56	0,001
1768	c53392_g1	2,052	49	37	53	0,001
1769	c23671_g1	2,052	182	112	177	0,000
1770	c58103_g2	2,054	102,99	92,98	124	0,000
1771	c50407_g1	2,067	99	14,55	61	0,000
1772	c52247_g1	2,080	89	18	59	0,000
1773	c34056_g1	2,086	358	53	218	0,000
1774	c57592_g3	2,103	89	83	113	0,000
1775	c54149_g1	2,114	85	41	77	0,000
1776	c51628_g1	2,126	12	26	27	0,002
1777	c53085_g1	2,128	113	47	97	0,000
1778	c123192_g1	2,139	21	33	38	0,001
1779	c39927_g1	2,151	32	18	32	0,001
1780	c51920_g1	2,166	41	24,99	43	0,001
1781	c37676_g1	2,169	5	31	28	0,002
1782	c54212_g3	2,176	65	4	39	0,000
1783	c52859_g1	2,180	94	17	65	0,000
1784	c55244_g1	2,195	352,31	219,98	380,7	0,000
1785	c10803_g1	2,202	24	44	51	0,000
1786	c50000_g1	2,215	123,99	55	116	0,000
1787	c54371_g1	2,215	81	111	143	0,000
1788	c52205_g1	2,219	94	47	92,89	0,000
1789	c41099_g1	2,220	48	29	52	0,000
1790	c58202_g2	2,221	14	19	24	0,002
1791	c42080_g1	2,226	87	53	94,96	0,000
1792	c50448_g1	2,244	64,05	37	69	0,000
1793	c55257_g1	2,275	34	11	30	0,000
1794	c55271_g1	2,279	48	21	47	0,000
1795	c55993_g1	2,280	229	84,75	208	0,000
1796	c54102_g1	2,285	81	61	103	0,000
1797	c50070_g1	2,293	242,78	212,78	339,9	0,000
1798	c52632_g1	2,301	35	12	31,93	0,000
1799	c49958_g1	2,311	123	87	154	0,000
1800	c43032_g1	2,318	64,99	41	77	0,000
1801	c47839_g2	2,339	74,98	42	85,3	0,000

1802	c45491_g1	2,341	63	41,99	78	0,000
1803	c35129_g1	2,346	40	13	37	0,000
1804	c54886_g2	2,346	68	50	89	0,000
1805	c45168_g1	2,355	13	0	9	0,005
1806	c49417_g1	2,359	158,86	49	143	0,000
1807	c10186_g1	2,362	0	18	16	0,004
1808	c48671_g1	2,371	13	7	15	0,002
1809	c56647_g1	2,372	45,99	19	47	0,000
1810	c57329_g2	2,373	44	44	70	0,000
1811	c43034_g1	2,375	108	105	170	0,000
1812	c34548_g1	2,378	376	182	408	0,000
1813	c28634_g1	2,378	25	6	22	0,001
1814	c39173_g1	2,390	107	18,68	85	0,000
1815	c56866_g1	2,392	91	57	113	0,000
1816	c54926_g1	2,393	93,67	63,99	122	0,000
1817	c56428_g1	2,394	66,09	25	65,9	0,000
1818	c50832_g1	2,399	103	62	126	0,000
1819	c49777_g1	2,404	32	23	43	0,000
1820	c46983_g1	2,433	49	18	50	0,000
1821	c34377_g1	2,439	72	32	79	0,000
1822	c57781_g1	2,459	88,99	30	89	0,000
1823	c57181_g1	2,459	97	70	136	0,000
1824	c52241_g1	2,473	84	39	96	0,000
1825	c45053_g1	2,473	48	29	62	0,000
1826	c58054_g5	2,481	87,84	36	96	0,000
1827	c15726_g1	2,485	142	75	173	0,000
1828	c38563_g1	2,498	31	51	75	0,000
1829	c57742_g1	2,501	96	42	109	0,000
1830	c47409_g1	2,501	56,28	0	39	0,000
1831	c54341_g1	2,505	52,99	32,65	70,67	0,000
1832	c47791_g1	2,512	97	20	87	0,000
1833	c12925_g1	2,527	52	40	79	0,000
1834	c58307_g1	2,529	16	5	16,88	0,001
1835	c15822_g1	2,535	45,99	47	83	0,000
1836	c43737_g1	2,556	76,99	31	88	0,000
1837	c43850_g1	2,559	56,71	28	71	0,000
1838	c48870_g1	2,568	6	1	6	0,018
1839	c49792_g1	2,568	33	10	35	0,000
1840	c48768_g1	2,588	15	8	20	0,000
1841	c55562_g1	2,595	70	42	98	0,000
1842	c51998_g1	2,608	56	30	75	0,000
1843	c51422_g1	2,610	39	16	47	0,000
1844	c57236_g1	2,617	176	37	169	0,000
1845	c39318_g1	2,625	53	38	83	0,000
1846	c47300_g1	2,632	37	14	44	0,000
1847	c57655_g1	2,643	78,01	41	106	0,000
1848	c23742_g1	2,644	191	87	243	0,000
1849	c23863_g1	2,657	1,68	26,55	32	0,000
1850	c41090_g1	2,670	67,93	28	85	0,000
1851	c57453_g1	2,672	156,91	47,99	175	0,000
1852	c51282_g3	2,675	156	36	160	0,000
1853	c16054_g1	2,678	5	9	14	0,002
1854	c47642_g1	2,689	28	16	41	0,000
1855	c71388_g1	2,702	52	39	88	0,000
1856	c35133_g1	2,703	42	24	62	0,000
1857	c56455_g1	2,711	46	31	74	0,000
1858	c50492_g1	2,713	6	7	13	0,001
1859	c57566_g1	2,722	167	17	150	0,000
1860	c55256_g1	2,725	137	31,07	145	0,000
1861	c44892_g1	2,731	77	82	164	0,000

1862	c52193_g1	2,757	202	53	227	0,000
1863	c23571_g1	2,764	95	52	142,9	0,000
1864	c54276_g1	2,766	99	51	145	0,000
1865	c44096_g1	2,777	26	28	57	0,000
1866	c26184_g1	2,779	2	8	11	0,003
1867	c47674_g1	2,791	21,71	30,99	58	0,000
1868	c97754_g1	2,799	71	0	59,99	0,000
1869	c51691_g1	2,818	81	25	101	0,000
1870	c56941_g4	2,826	14	30	51	0,000
1871	c47667_g1	2,826	57	20	75	0,000
1872	c51193_g1	2,830	44,27	16	59	0,000
1873	c51563_g1	2,830	1,72	6,3	9,31	0,007
1874	c37765_g1	2,833	64	16	76	0,000
1875	c50715_g4	2,834	24	20	47	0,000
1876	c51118_g1	2,844	42	13	54	0,000
1877	c51686_g1	2,846	46	18	64	0,000
1878	c109632_g1	2,847	41	22	65	0,000
1879	c55812_g1	2,850	23,97	3	26	0,000
1880	c54765_g3	2,886	59	23	84	0,000
1881	c51406_g1	2,897	51,98	19,29	73	0,000
1882	c48994_g1	2,912	21	15,1	40	0,000
1883	c58163_g1	2,914	6,57	3,11	10,84	0,001
1884	c55099_g1	2,922	24,99	9	36	0,000
1885	c57912_g1	2,922	34	8	43	0,000
1886	c56860_g1	2,943	61	10	71	0,000
1887	c53165_g1	2,959	51	49	119	0,000
1888	c26072_g1	2,965	16,99	5	24	0,000
1889	c47541_g1	2,980	44	24	77	0,000
1890	c33027_g1	2,984	4	11	19	0,000
1891	c41871_g1	2,987	56	46,96	123	0,000
1892	c56204_g1	2,991	88	18	110	0,000
1893	c56643_g1	2,999	110	38	161	0,000
1894	c43561_g1	3,017	56,86	45,95	125	0,000
1895	c37440_g1	3,018	59,98	25	96	0,000
1896	c53973_g1	3,022	23	10	38	0,000
1897	c56974_g1	3,023	39	8	51	0,000
1898	c96874_g1	3,031	28	7	39	0,000
1899	c48522_g1	3,032	66	62	160	0,000
1900	c52846_g1	3,043	34	15	57	0,000
1901	c56072_g1	3,048	21	13	41	0,000
1902	c53327_g1	3,081	86	35	142	0,000
1903	c57306_g1	3,083	37,02	23	74	0,000
1904	c53469_g3	3,112	30	14,12	54	0,000
1905	c50620_g1	3,172	26	19	60	0,000
1906	c42211_g1	3,198	3,02	1,5	7	0,005
1907	c50607_g1	3,223	23	46	106	0,000
1908	c54682_g2	3,249	31,58	7	50	0,000
1909	c58109_g1	3,263	47	3	61	0,000
1910	c53315_g1	3,266	53	25	106	0,000
1911	c46003_g1	3,287	52,99	18	95	0,000
1912	c41073_g1	3,287	34	3	46,95	0,000
1913	c56439_g1	3,329	47	16	87	0,000
1914	c26871_g1	3,331	31	26,97	88	0,000
1915	c52568_g1	3,334	48	19	94	0,000
1916	c46910_g1	3,388	29,99	10	58	0,000
1917	c43825_g1	3,412	26	17	67	0,000
1918	c23807_g1	3,418	42,52	30	115,5	0,000
1919	c57649_g1	3,423	45	36	130	0,000
1920	c48936_g2	3,449	7	8	25	0,000

1921	c33687_g1	3,450	38	30	111	0,000
1922	c57755_g2	3,481	79,99	56	223,9	0,000
1923	c55761_g1	3,512	13	12	43	0,000
1924	c57466_g1	3,526	2	3	8,69	0,001
1925	c54339_g4	3,534	18	11	49	0,000
1926	c49810_g1	3,567	13	15	51	0,000
1927	c42884_g1	3,609	0	4	8	0,002
1928	c52721_g1	3,657	2	6	16	0,000
1929	c54830_g1	3,682	4	15	40	0,000
1930	c57988_g6	3,790	13	3	31	0,000
1931	c42832_g2	3,844	14	3	34	0,000
1932	c57197_g1	3,868	15,99	9	53	0,000
1933	c54424_g5	3,869	15	14	64	0,000
1934	c22881_g1	3,870	0	2	5	0,007
1935	c45384_g1	3,870	0	2	5,18	0,007
1936	c56289_g1	3,890	3	10	31	0,000
1937	c30879_g1	3,895	28	1	56	0,000
1938	c47907_g1	3,921	34	26,8	138	0,000
1939	c114314_g1	3,921	42	4	90	0,000
1940	c42285_g1	3,931	4	10	34	0,000
1941	c44214_g1	3,941	1	13	36	0,000
1942	c53664_g1	3,942	2	0	5,46	0,007
1943	c71324_g1	3,974	8	12	48,98	0,000
1944	c51557_g1	3,988	12	2	31	0,000
1945	c46527_g1	3,995	16	23	98	0,000
1946	c57908_g2	4,015	29	8	81,98	0,000
1947	c53733_g1	4,070	2	8	27	0,000
1948	c52934_g1	4,086	155	66	521	0,000
1949	c39874_g1	4,101	53	17	163,9	0,000
1950	c56415_g1	4,130	3,72	9	35,85	0,000
1951	c40063_g1	4,145	27	23	133	0,000
1952	c58037_g1	4,174	15,16	6	54	0,000
1953	c52696_g1	4,208	20	15	96	0,000
1954	c54919_g1	4,225	16	16	90,95	0,000
1955	c49167_g1	4,292	3	10	41	0,000
1956	c14276_g1	4,343	8	1	26	0,000
1957	c51761_g1	4,380	0	7	24	0,000
1958	c57185_g8	4,421	1	0	3,56	0,006
1959	c51941_g1	4,427	12	4	48,83	0,000
1960	c56286_g1	4,543	0	2	8	0,000
1961	c14760_g1	4,615	2	0	8	0,000
1962	c40774_g2	4,625	0,97	7	32	0,000
1963	c116579_g1	4,645	4	0	15	0,000
1964	c50147_g1	4,753	19	1	72	0,000
1965	c57098_g1	4,758	0	11	50	0,000
1966	c20831_g1	4,771	5	6	46	0,000
1967	c56730_g2	5,100	11	16	145	0,000
1968	c57577_g7	5,178	1	15	98	0,000
1969	c47944_g2	5,241	0	17	111	0,000
1970	c48876_g1	5,242	3	4	41	0,000
1971	c87135_g1	5,354	3	0	18,99	0,000
1972	c103700_g1	5,369	1074	102	5861	0,000
1973	c39138_g1	5,442	4	10	97	0,000
1974	c23373_g1	5,561	3	1	29	0,000
1975	c53753_g1	5,652	9	1	70,95	0,000
1976	c55948_g2	6,016	0	0	3,14	0,014
1977	c110324_g1	6,129	11	0	106	0,000
1978	c52530_g1	6,278	0	1,37	15,22	0,000
1979	c44994_g1	6,676	0	5	84	0,000
1980	c55058_g1	6,744	0	0	4,93	0,001
1981	c49874_g1	6,811	0	4	74	0,000
1982	c57242_g4	6,911	3,29	0	55,82	0,000
1983	c43804_g1	7,005	0	0	6	0,000
1984	c53455_g1	7,013	0	0,82	25,36	0,000
1985	c52925_g1	7,417	0	0,29	7,87	0,000
1986	c56919_g1	7,828	0	1	44,09	0,000
1987	c20195_g1	8,582	0	0	17,87	0,000
1988	c53122_g2	9,455	0	0	33	0,000
1989	c56947_g1	9,658	0	0	38	0,000
1990	c56744_g1	9,934	0	0	46	0,000
1991	c57839_g1	10,054	0	0	50	0,000

6.4 The results from the RNAseq analysis of the lower female reproductive tract tissue (Table 6.3)

N	gene_id	M_FEMALE_1	M_FEMALE_2	V_FEMALE_2	logFC	PValue
1	c52845_g1	11402,59	5505,82	0	-15,445	1,41E-14
2	c55628_g1	9669,81	3713,62	0	-15,097	1,48E-13
3	c42759_g2	7576,96	4660,95	0	-14,991	3,01E-13
4	c51837_g1	5851,97	5356,98	0	-14,886	6,09E-13
5	c53966_g8	6324,88	4085,97	0	-14,761	1,05E-12
6	c44387_g1	6282,94	4012	0	-14,744	1,18E-12
7	c30526_g1	2803,99	6153,99	0	-14,608	2,90E-12
8	c53346_g1	5313	4002	0	-14,608	2,90E-12
9	c47567_g2	4495,89	3677,85	0	-14,424	9,85E-12
10	c71357_g1	3570,25	3997,9	0	-14,331	1,84E-11
11	c53746_g2	3519,99	3896,97	0	-14,301	2,24E-11
12	c53092_g2	3133,93	4213,95	0	-14,298	2,28E-11
13	c54618_g1	3496,35	3775,4	0	-14,271	2,72E-11
14	c47917_g2	5081,99	2067,97	0	-14,195	3,31E-11
15	c53040_g1	3013,94	3809	0	-14,188	3,48E-11
16	c54829_g2	4335,89	2623,59	0	-14,176	3,77E-11
17	c57143_g2	4068,84	2555,78	0	-14,107	5,92E-11
18	c55918_g1	3073,67	3214,22	0	-14,060	8,10E-11
19	c52436_g1	3570,88	2568,93	0	-14,005	1,16E-10
20	c21198_g1	4097,79	1878,96	0	-13,942	1,74E-10
21	c53599_g1	3683,59	2225,84	0	-13,940	1,76E-10
22	c51323_g1	2910,6	2637,84	0	-13,871	2,76E-10
23	c27262_g1	2850,69	2688,94	0	-13,871	2,76E-10
24	c10647_g1	2669,33	2838,31	0	-13,869	2,80E-10
25	c56768_g3	3549,6	2074,92	0	-13,867	2,85E-10
26	c45452_g1	2770,8	2442,9	0	-13,780	3,67E-10
27	c29117_g1	3270,08	1915,98	0	-13,750	4,48E-10
28	c44594_g1	2842,83	2199,95	0	-13,725	5,25E-10
29	c58085_g1	2890,92	2053,98	0	-13,692	6,54E-10
30	c52934_g1	3670,99	1342,83	0	-13,678	7,10E-10
31	c57634_g1	3203,93	1685,84	0	-13,660	8,00E-10
32	c58253_g1	2750,98	1864,95	0	-13,590	1,26E-09
33	c51907_g4	1948,17	2482,17	0	-13,565	1,47E-09
34	c32492_g1	2156,9	2273,48	0	-13,555	1,57E-09
35	c52931_g1	2911,86	1529,98	0	-13,521	1,96E-09
36	c23076_g1	2498,42	1741,96	0	-13,469	1,99E-09
37	c46559_g1	2045,08	2014,65	0	-13,425	2,64E-09
38	c42417_g1	2344,91	1745,24	0	-13,420	2,73E-09
39	c44954_g1	2379	1541	0	-13,352	4,22E-09
40	c44953_g1	1890,95	1925,99	0	-13,338	4,60E-09
41	c43875_g1	2650,71	1264,91	0	-13,334	4,69E-09
42	c49340_g1	2569,65	1268,99	0	-13,307	5,58E-09
43	c54856_g2	2322,74	1460,95	0	-13,299	5,89E-09
44	c51944_g2	1978,05	1749,99	0	-13,296	5,99E-09
45	c50798_g2	2372,02	1405,99	0	-13,294	6,10E-09
46	c50481_g1	2358,85	1288	0	-13,239	8,61E-09
47	c10600_g2	1367,97	2136,94	0	-13,238	8,69E-09
48	c96905_g1	1662,85	1838,54	0	-13,218	9,80E-09
49	c50073_g1	1638,08	1807,95	0	-13,195	1,14E-08
50	c54629_g1	1974,67	1488	0	-13,181	1,24E-08
51	c55864_g1	2066,9	1355	0	-13,156	1,05E-08
52	c57594_g1	1904,63	1490,99	0	-13,155	1,06E-08
53	c54659_g1	1873,08	1503,62	0	-13,148	1,10E-08
54	c58082_g2	1984,96	1395,27	0	-13,142	1,15E-08
55	c48434_g1	1781,41	1535,57	0	-13,126	1,27E-08
56	c84110_g1	1445	1801,88	0	-13,116	1,36E-08
57	c53327_g2	1912,71	1283,96	0	-13,059	1,94E-08
58	c56913_g5	1884,63	1284,75	0	-13,048	2,09E-08
59	c49653_g2	1901,63	1266,39	0	-13,046	2,11E-08
60	c56580_g1	1996,56	1130,99	0	-13,019	2,49E-08
61	c46189_g1	1556,43	1483,95	0	-13,006	2,72E-08
62	c49765_g1	1983	1073,99	0	-12,984	3,12E-08
63	c55509_g7	1508,25	1456,78	0	-12,971	3,37E-08
64	c58054_g1	1967,96	1032,9	0	-12,955	3,72E-08
65	c56851_g2	1392,46	1478,65	0	-12,929	4,35E-08
66	c46581_g1	1328,07	1524,84	0	-12,925	4,50E-08
67	c53123_g1	1648,28	1196,99	0	-12,895	5,39E-08
68	c55319_g1	1605,99	1220,97	0	-12,889	5,58E-08
69	c46085_g1	1965,82	908	0	-12,887	5,65E-08
70	c57012_g1	1420,85	1362,98	0	-12,879	5,91E-08
71	c57266_g3	1711,25	1104,95	0	-12,874	6,12E-08
72	c27268_g1	1507,24	1261,73	0	-12,864	4,68E-08
73	c49662_g2	1705,66	1067,5	0	-12,851	5,08E-08
74	c52754_g1	1522,43	1155	0	-12,810	6,61E-08
75	c42687_g1	1049,96	1536,9	0	-12,796	7,11E-08
76	c42956_g1	1367,61	1258,99	0	-12,793	7,29E-08
77	c27318_g1	994,92	1536,98	0	-12,768	8,45E-08
78	c43468_g1	1529,37	1031,56	0	-12,740	1,01E-07
79	c50627_g1	1661,93	878,91	0	-12,716	1,17E-07
80	c48875_g2	1282,96	1192	0	-12,708	1,24E-07
81	c47214_g1	1560,99	947,17	0	-12,704	1,27E-07
82	c12311_g1	1065,74	1355,44	0	-12,693	1,35E-07
83	c54043_g1	1660,81	804,43	0	-12,667	1,58E-07
84	c55085_g1	1584	867,94	0	-12,666	1,58E-07
85	c53746_g1	1235,96	1156,24	0	-12,659	1,67E-07
86	c23095_g1	1053,67	1290,95	0	-12,645	1,81E-07
87	c46280_g1	1553,55	861	0	-12,645	1,81E-07
88	c51868_g1	1318,09	1033	0	-12,625	2,04E-07
89	c47782_g1	1552,64	820,07	0	-12,617	2,16E-07
90	c54301_g1	1337,75	970,94	0	-12,594	1,77E-07
91	c42523_g1	1211,84	1079	0	-12,594	1,77E-07
92	c50891_g1	1504,84	820,94	0	-12,590	1,82E-07
93	c57564_g1	1212,13	1033,88	0	-12,563	2,12E-07
94	c57765_g3	882,94	1301,99	0	-12,553	2,25E-07
95	c52177_g1	1417,14	841,81	0	-12,552	2,28E-07
96	c50839_g1	1066,86	1118,94	0	-12,536	2,52E-07
97	c52840_g1	997,28	1153,99	0	-12,518	2,79E-07
98	c36177_g1	719	1302,93	0	-12,452	4,16E-07
99	c54520_g1	1312,82	782,95	0	-12,444	4,35E-07
100	c47718_g1	1001,52	1046,2	0	-12,441	4,42E-07
101	c57577_g2	1192,93	849,99	0	-12,417	5,15E-07
102	c57050_g2	1017,93	961,3	0	-12,386	6,21E-07
103	c31514_g1	1038	942	0	-12,385	6,21E-07
104	c55621_g1	1042,95	929	0	-12,378	6,51E-07
105	c57575_g2	962,84	980,94	0	-12,365	7,04E-07
106	c47020_g1	951,66	966,97	0	-12,346	5,60E-07
107	c54013_g3	1072,25	848,99	0	-12,334	5,97E-07
108	c47106_g2	1126,03	782,06	0	-12,317	6,59E-07
109	c57856_g1	1042,55	837	0	-12,303	7,15E-07
110	c52600_g1	895,14	934	0	-12,278	8,31E-07
111	c43569_g1	1163,99	676,99	0	-12,256	9,52E-07
112	c56278_g2	1018,96	801,41	0	-12,255	9,52E-07
113	c8808_g1	916,51	886,96	0	-12,254	9,68E-07
114	c53801_g1	969,77	832,99	0	-12,247	1,00E-06
115	c50743_g1	935,48	860,84	0	-12,245	1,02E-06
116	c56753_g1	856,44	927	0	-12,243	1,02E-06
117	c46274_g1	1310,34	531	0	-12,238	1,06E-06
118	c8815_g1	887,08	888	0	-12,233	1,09E-06
119	c54746_g6	1043,42	752,64	0	-12,232	1,09E-06
120	c56779_g2	1026	760,95	0	-12,226	1,13E-06

121	c40827_g1	1092,98	687,88	0	-12,212	1,23E-06
122	c51710_g1	934,6	821,93	0	-12,211	1,23E-06
123	c53296_g1	1030,67	735,98	0	-12,208	1,28E-06
124	c47323_g1	1088,14	681,69	0	-12,203	1,30E-06
125	c33421_g1	1182,94	585,99	0	-12,190	1,40E-06
126	c96851_g1	977,83	727	0	-12,158	1,70E-06
127	c55329_g1	504	1134,37	0	-12,158	1,67E-06
128	c50429_g1	896,06	796,94	0	-12,158	1,70E-06
129	c53244_g1	962,39	735,71	0	-12,154	1,73E-06
130	c43092_g1	989,97	711	0	-12,153	1,76E-06
131	c56594_g1	1010,09	692,99	0	-12,152	1,76E-06
132	c26938_g1	982,18	707,55	0	-12,144	1,83E-06
133	c43056_g1	888,69	785	0	-12,141	1,86E-06
134	c58017_g2	943,45	733,85	0	-12,137	1,93E-06
135	c39949_g1	1081,36	603,99	0	-12,126	2,04E-06
136	c51284_g1	838,79	806,24	0	-12,121	2,11E-06
137	c56063_g3	869,82	763	0	-12,105	1,63E-06
138	c53812_g1	920,99	717,28	0	-12,103	1,63E-06
139	c51907_g1	890,37	743	0	-12,102	1,67E-06
140	c48898_g1	332,9	1220,76	0	-12,102	1,63E-06
141	c48550_g2	757	851,98	0	-12,097	1,70E-06
142	c41090_g1	1056,87	591,99	0	-12,095	1,73E-06
143	c46953_g1	996,58	641,97	0	-12,094	1,73E-06
144	c44589_g1	660,99	929,96	0	-12,093	1,73E-06
145	c51247_g1	764,6	833	0	-12,086	1,83E-06
146	c57693_g1	780	804,02	0	-12,070	2,01E-06
147	c46698_g1	807,98	758	0	-12,048	2,26E-06
148	c34532_g1	931,53	648,86	0	-12,046	2,30E-06
149	c52861_g2	852,29	711,9	0	-12,040	2,39E-06
150	c56741_g1	881	678	0	-12,031	2,49E-06
151	c47790_g1	891,95	662	0	-12,024	2,59E-06
152	c37475_g1	1046,97	524	0	-12,019	2,69E-06
153	c53388_g1	798	725,91	0	-12,007	2,86E-06
154	c55275_g2	914,98	624,19	0	-12,006	2,91E-06
155	c56555_g3	876,75	654,55	0	-12,004	2,91E-06
156	c57792_g1	879,95	652,21	0	-12,004	2,91E-06
157	c57914_g1	1063,71	486,82	0	-11,997	3,03E-06
158	c25210_g1	1026,98	512	0	-11,990	3,15E-06
159	c56205_g3	811,96	695	0	-11,988	3,22E-06
160	c10618_g1	921,07	600,88	0	-11,987	3,22E-06
161	c55863_g1	751,99	743,96	0	-11,985	3,28E-06
162	c56656_g2	860,99	645	0	-11,980	3,35E-06
163	c53758_g3	933,6	577	0	-11,974	3,49E-06
164	c51418_g1	774	701,99	0	-11,961	3,78E-06
165	c45998_g1	854,96	630,72	0	-11,960	3,78E-06
166	c58077_g1	931,99	563,94	0	-11,959	3,78E-06
167	c53741_g1	785	690	0	-11,958	3,78E-06
168	c55326_g1	873,78	607,94	0	-11,952	3,94E-06
169	c53734_g1	924,87	556,7	0	-11,945	4,10E-06
170	c26940_g1	890,94	586	0	-11,945	4,10E-06
171	c38209_g1	849,97	612	0	-11,935	4,37E-06
172	c52656_g2	994,92	487	0	-11,934	4,37E-06
173	c42841_g1	754,38	685	0	-11,925	4,64E-06
174	c45726_g1	887,96	562,97	0	-11,917	4,84E-06
175	c55000_g2	706,32	713,98	0	-11,911	4,94E-06
176	c16060_g1	926,37	521,95	0	-11,908	5,05E-06
177	c43839_g1	908,44	533,88	0	-11,904	5,16E-06
178	c58086_g1	530,98	855,46	0	-11,901	5,26E-06
179	c58369_g1	911,8	523	0	-11,896	5,49E-06
180	c56114_g2	313	1030	0	-11,887	3,92E-06

181	c56105_g1	901,19	523,99	0	-11,886	4,00E-06
182	c11918_g1	921,25	501,82	0	-11,881	4,18E-06
183	c56435_g1	814	591	0	-11,878	4,27E-06
184	c57563_g2	760,84	631,99	0	-11,873	4,36E-06
185	c55406_g1	772,94	620,96	0	-11,872	4,36E-06
186	c47822_g1	802	595	0	-11,871	4,36E-06
187	c55697_g1	979,72	440	0	-11,868	4,45E-06
188	c53700_g1	727,89	652,98	0	-11,864	4,55E-06
189	c50073_g2	766,99	617,99	0	-11,863	4,65E-06
190	c50396_g3	936,72	470,98	0	-11,862	4,65E-06
191	c52661_g1	959,99	443	0	-11,852	4,86E-06
192	c52717_g2	831,99	551,84	0	-11,851	4,96E-06
193	c53977_g1	785,94	589	0	-11,848	4,96E-06
194	c36219_g1	812,95	565	0	-11,848	5,07E-06
195	c45281_g1	819,97	557,99	0	-11,846	5,07E-06
196	c23641_g1	876,42	508	0	-11,844	5,07E-06
197	c49783_g2	707,99	650	0	-11,842	5,19E-06
198	c50748_g1	811,96	558,72	0	-11,840	5,30E-06
199	c84144_g1	567	769	0	-11,839	5,30E-06
200	c40681_g2	691,97	655	0	-11,831	5,54E-06
201	c31463_g1	804,92	556,95	0	-11,830	5,54E-06
202	c49086_g2	841,89	523,7	0	-11,829	5,54E-06
203	c49625_g2	739,15	610,65	0	-11,827	5,66E-06
204	c38927_g1	812	547	0	-11,826	5,66E-06
205	c54608_g1	833,99	525,7	0	-11,823	5,79E-06
206	c57314_g1	909,6	456,94	0	-11,819	5,92E-06
207	c54078_g1	720,21	618,95	0	-11,818	5,92E-06
208	c44374_g1	701,17	634,58	0	-11,817	5,92E-06
209	c43515_g1	774,43	570,92	0	-11,816	6,05E-06
210	c55265_g1	784,96	557	0	-11,810	6,19E-06
211	c55179_g1	842,78	504,02	0	-11,807	6,33E-06
212	c54316_g1	840	504,95	0	-11,805	6,47E-06
213	c55607_g1	751	577	0	-11,800	6,62E-06
214	c46567_g1	745,21	573,98	0	-11,790	6,92E-06
215	c55648_g1	586	708,59	0	-11,788	7,08E-06
216	c57480_g1	805	519,26	0	-11,786	7,08E-06
217	c51784_g1	702,91	602	0	-11,780	7,40E-06
218	c58060_g1	658,81	634,39	0	-11,773	7,57E-06
219	c49066_g1	955,69	377,99	0	-11,772	7,75E-06
220	c57875_g1	587,99	690,17	0	-11,767	7,93E-06
221	c24207_g1	668,65	619,99	0	-11,767	7,93E-06
222	c71416_g1	581,88	690,96	0	-11,762	8,11E-06
223	c45991_g5	695,94	590,61	0	-11,760	8,30E-06
224	c25344_g1	887,98	423,97	0	-11,757	8,30E-06
225	c55186_g1	732	554,81	0	-11,754	8,49E-06
226	c51856_g1	432,75	810,99	0	-11,753	8,49E-06
227	c53461_g1	736,96	546,99	0	-11,749	8,69E-06
228	c50756_g1	639,99	628	0	-11,746	8,89E-06
229	c56319_g1	555,74	699,93	0	-11,746	8,89E-06
230	c54168_g1	616	647,92	0	-11,746	8,89E-06
231	c54648_g1	524,99	722,98	0	-11,742	9,10E-06
232	c49519_g2	810,98	473,75	0	-11,738	9,31E-06
233	c58165_g1	788,29	490,99	0	-11,734	9,53E-06
234	c52129_g1	567,99	674	0	-11,727	9,98E-06
235	c57877_g1	736,99	521,92	0	-11,718	1,05E-05
236	c56786_g4	696,99	556	0	-11,718	1,05E-05
237	c39536_g1	718,93	537,46	0	-11,718	1,05E-05
238	c56891_g1	693,82	558	0	-11,717	1,05E-05
239	c42721_g1	739,29	511,11	0	-11,707	1,12E-05
240	c54044_g1	561	661,99	0	-11,704	1,12E-05

241	c57563_g1	697,88	541,94	0	-11,701	1,15E-05
242	c51362_g1	625,91	601	0	-11,698	1,18E-05
243	c55522_g1	835,76	413,99	0	-11,689	1,23E-05
244	c47397_g1	608	608,98	0	-11,688	1,23E-05
245	c50433_g2	803,96	438,95	0	-11,686	1,23E-05
246	c26676_g1	654,9	567	0	-11,686	1,26E-05
247	c57241_g1	752,24	483,26	0	-11,685	1,26E-05
248	c57125_g1	679	544	0	-11,683	1,26E-05
249	c49411_g1	716	509,98	0	-11,680	1,29E-05
250	c57386_g11	817,93	420,99	0	-11,679	1,29E-05
251	c37540_g1	772	458,98	0	-11,677	1,33E-05
252	c50662_g1	674,68	530,84	0	-11,662	9,87E-06
253	c84123_g1	671	532	0	-11,659	1,01E-05
254	c50703_g1	771,73	445,14	0	-11,658	1,01E-05
255	c54032_g1	846,99	378	0	-11,655	1,04E-05
256	c41105_g1	863,06	352,99	0	-11,640	1,12E-05
257	c53755_g9	641,86	535,96	0	-11,631	1,17E-05
258	c42476_g1	746,98	445	0	-11,630	1,17E-05
259	c53066_g1	655	518	0	-11,622	1,23E-05
260	c56350_g1	381,98	745,57	0	-11,613	1,29E-05
261	c57088_g1	655,43	508,98	0	-11,610	1,33E-05
262	c47442_g1	673,99	482,26	0	-11,596	1,43E-05
263	c53173_g1	477,98	649,61	0	-11,595	1,43E-05
264	c53264_g2	542,92	589,73	0	-11,589	1,47E-05
265	c53386_g2	539,96	587,88	0	-11,583	1,54E-05
266	c34297_g1	356	740	0	-11,575	1,58E-05
267	c55984_g1	683,77	457,95	0	-11,575	1,63E-05
268	c122422_g1	658,47	476,54	0	-11,570	1,67E-05
269	c17482_g1	515,99	591,95	0	-11,560	1,76E-05
270	c56348_g10	546,23	565,99	0	-11,560	1,76E-05
271	c49173_g1	697,35	431,92	0	-11,554	1,80E-05
272	c52369_g1	663,98	455	0	-11,547	1,90E-05
273	c51794_g1	705,94	417	0	-11,544	1,90E-05
274	c46866_g1	725	400	0	-11,543	1,90E-05
275	c56123_g1	579,16	525	0	-11,542	1,95E-05
276	c47321_g2	679,98	435,97	0	-11,539	1,95E-05
277	c55303_g1	407,96	662,99	0	-11,529	2,05E-05
278	c52947_g1	595,18	501,97	0	-11,529	2,05E-05
279	c56599_g1	570,94	520,39	0	-11,525	2,11E-05
280	c34247_g1	746,33	369	0	-11,525	2,11E-05
281	c40256_g1	652	448,65	0	-11,523	2,16E-05
282	c46951_g2	439,36	630,93	0	-11,522	2,16E-05
283	c46152_g1	528,97	548,93	0	-11,515	2,22E-05
284	c57488_g12	709,59	393	0	-11,515	2,22E-05
285	c50551_g1	558,99	514,85	0	-11,503	2,40E-05
286	c52627_g1	649,95	434,79	0	-11,501	2,40E-05
287	c55008_g1	501,25	562,99	0	-11,501	2,40E-05
288	c40909_g1	421,92	630	0	-11,499	2,40E-05
289	c49237_g1	552,27	515,99	0	-11,496	2,47E-05
290	c46283_g1	669,97	412,88	0	-11,494	2,54E-05
291	c57907_g1	461,65	585,98	0	-11,485	2,60E-05
292	c57131_g1	529	526,99	0	-11,483	2,68E-05
293	c51319_g1	541,83	512,35	0	-11,478	2,75E-05
294	c58181_g2	605,48	457,38	0	-11,476	2,75E-05
295	c49441_g1	700,89	372,24	0	-11,473	2,82E-05
296	c37945_g1	661,99	401,99	0	-11,467	2,90E-05
297	c26783_g1	513,99	528	0	-11,466	2,90E-05
298	c50926_g1	388,99	634	0	-11,463	2,98E-05
299	c27309_g1	562,38	483,87	0	-11,462	2,98E-05
300	c54998_g2	534	505,82	0	-11,458	3,06E-05

301	c55753_g1	691,7	369,68	0	-11,458	3,06E-05
302	c57592_g2	524	513	0	-11,456	3,06E-05
303	c8482_g1	624,69	423,77	0	-11,453	3,15E-05
304	c57012_g2	560,19	476	0	-11,447	3,23E-05
305	c49935_g1	616,56	424,95	0	-11,444	3,32E-05
306	c47589_g1	688,06	363,41	0	-11,443	2,28E-05
307	c55705_g1	526	498	0	-11,436	2,34E-05
308	c55342_g1	584,35	444,94	0	-11,431	2,41E-05
309	c8759_g1	683,92	358,91	0	-11,431	2,41E-05
310	c55710_g1	487,8	525,86	0	-11,429	2,48E-05
311	c57925_g1	599,54	423,34	0	-11,419	2,62E-05
312	c56109_g1	707,93	329,91	0	-11,418	2,62E-05
313	c50784_g1	573,99	444,88	0	-11,418	2,62E-05
314	c34064_g1	594,96	424,7	0	-11,415	2,62E-05
315	c37474_g1	540,99	469,99	0	-11,413	2,69E-05
316	c32781_g1	647,48	376,97	0	-11,410	2,69E-05
317	c25331_g1	390,68	593,98	0	-11,405	2,77E-05
318	c39556_g1	517	484	0	-11,403	2,85E-05
319	c49699_g1	537,89	464,99	0	-11,401	2,85E-05
320	c27562_g2	458	528,98	0	-11,394	2,93E-05
321	c44842_g1	538	457,97	0	-11,390	3,02E-05
322	c54175_g1	612,99	392	0	-11,388	3,10E-05
323	c57041_g1	412,11	564	0	-11,387	3,10E-05
324	c55306_g1	485,54	497,51	0	-11,383	3,19E-05
325	c57655_g1	516,96	468,81	0	-11,379	3,19E-05
326	c24824_g1	499,97	482,83	0	-11,378	3,19E-05
327	c54880_g1	633,52	362,99	0	-11,371	3,38E-05
328	c57861_g1	487	488,99	0	-11,370	3,38E-05
329	c96827_g1	492	483	0	-11,367	3,38E-05
330	c58149_g1	519,97	455,73	0	-11,363	3,48E-05
331	c51106_g1	522	453,99	0	-11,362	3,48E-05
332	c56898_g1	499,13	472	0	-11,359	3,58E-05
333	c35296_g1	335,83	612	0	-11,359	3,58E-05
334	c51285_g1	374	573,8	0	-11,351	3,69E-05
335	c55683_g1	554,01	418,94	0	-11,350	3,80E-05
336	c53305_g1	492,96	470	0	-11,348	3,80E-05
337	c30806_g1	477,12	479	0	-11,340	4,03E-05
338	c57588_g1	495,73	459,5	0	-11,336	4,03E-05
339	c55188_g1	546	416,91	0	-11,336	4,03E-05
340	c53740_g1	576,52	390,46	0	-11,335	4,03E-05
341	c26900_g1	451	497,94	0	-11,335	4,15E-05
342	c49817_g1	706,66	275,88	0	-11,332	4,15E-05
343	c55226_g1	528,88	428,82	0	-11,332	4,15E-05
344	c56902_g3	319,97	606,96	0	-11,329	4,15E-05
345	c56045_g1	490,96	456,98	0	-11,324	4,40E-05
346	c57966_g1	563,99	392	0	-11,320	4,40E-05
347	c46488_g2	540	412	0	-11,319	4,40E-05
348	c39708_g1	633,68	326	0	-11,311	4,67E-05
349	c47734_g2	580,99	371	0	-11,310	4,67E-05
350	c46428_g1	481,97	456	0	-11,309	4,67E-05
351	c30202_g1	541	399,97	0	-11,301	4,95E-05
352	c55662_g1	467,96	462	0	-11,299	4,95E-05
353	c57651_g2	566,99	373,98	0	-11,295	5,10E-05
354	c56400_g1	477,8	448,99	0	-11,292	5,10E-05
355	c51717_g1	557	380,99	0	-11,292	5,10E-05
356	c42567_g1	534,9	397,95	0	-11,289	5,26E-05
357	c56595_g2	548	385,9	0	-11,287	5,26E-05
358	c48604_g2	379	531	0	-11,287	5,26E-05
359	c122672_g1	527,99	400,81	0	-11,284	5,42E-05
360	c54861_g1	614,36	325,43	0	-11,280	5,42E-05

361	c10705_g1	632	306,86	0	-11,276	5,58E-05
362	c53851_g2	528,91	392,96	0	-11,272	5,75E-05
363	c52935_g1	486	428	0	-11,268	5,75E-05
364	c55026_g1	356,99	537	0	-11,265	5,93E-05
365	c56893_g1	527,86	388,98	0	-11,263	5,93E-05
366	c55462_g1	570,14	351,43	0	-11,260	6,12E-05
367	c54836_g2	652,99	275,75	0	-11,254	6,30E-05
368	c57217_g1	447,94	452	0	-11,253	6,30E-05
369	c54488_g2	584	335	0	-11,253	6,30E-05
370	c48819_g1	547	366	0	-11,252	6,30E-05
371	c52120_g1	522,46	385,97	0	-11,249	6,50E-05
372	c58342_g1	491,12	410,94	0	-11,246	6,50E-05
373	c51973_g1	538	370	0	-11,245	6,50E-05
374	c46160_g1	433,97	459	0	-11,245	6,70E-05
375	c56002_g1	441,83	452,09	0	-11,244	6,70E-05
376	c55680_g1	534	368,99	0	-11,238	6,91E-05
377	c53091_g1	468,97	423,89	0	-11,236	6,91E-05
378	c55917_g1	312,86	556,87	0	-11,234	6,91E-05
379	c10659_g1	546,95	354,95	0	-11,233	7,13E-05
380	c71595_g1	477,94	413,88	0	-11,232	7,13E-05
381	c54971_g3	585,95	321	0	-11,232	7,13E-05
382	c56575_g1	471,79	416	0	-11,227	7,35E-05
383	c46423_g1	516,03	377,92	0	-11,226	7,35E-05
384	c56036_g1	522,25	371,62	0	-11,225	4,99E-05
385	c46636_g1	460,63	423,91	0	-11,224	4,99E-05
386	c71437_g1	465	413,96	0	-11,213	5,31E-05
387	c57968_g1	459,51	416,99	0	-11,210	5,31E-05
388	c57555_g1	316,24	536,99	0	-11,203	5,48E-05
389	c46396_g1	321,73	529,98	0	-11,200	5,66E-05
390	c25201_g1	625,5	268	0	-11,199	5,66E-05
391	c55859_g1	518	360	0	-11,198	5,84E-05
392	c55357_g1	454	412,99	0	-11,194	5,84E-05
393	c57713_g1	434,47	428,93	0	-11,192	6,03E-05
394	c48374_g1	369,13	483,9	0	-11,190	6,03E-05
395	c57759_g1	445	415	0	-11,184	6,23E-05
396	c44934_g1	88	721	0	-11,182	6,03E-05
397	c42889_g1	473,4	389,17	0	-11,180	6,23E-05
398	c46536_g1	521,71	346	0	-11,179	6,43E-05
399	c45802_g1	386,82	461,99	0	-11,178	6,43E-05
400	c37520_g1	430	424	0	-11,176	6,43E-05
401	c57648_g1	577	295,93	0	-11,174	6,43E-05
402	c55540_g3	438	411,95	0	-11,167	6,86E-05
403	c50533_g1	573,96	294,99	0	-11,167	6,86E-05
404	c55965_g3	474,99	380	0	-11,167	6,86E-05
405	c56485_g2	492	364,99	0	-11,166	6,86E-05
406	c35181_g1	459,22	393	0	-11,165	6,86E-05
407	c49648_g2	421,21	421,99	0	-11,159	7,09E-05
408	c53718_g2	457	390,5	0	-11,157	7,09E-05
409	c51611_g2	501	349,98	0	-11,153	7,32E-05
410	c54910_g1	319,16	502,99	0	-11,146	7,56E-05
411	c57211_g1	527,32	324	0	-11,146	7,56E-05
412	c51653_g1	370,01	458	0	-11,144	7,56E-05
413	c48353_g1	428,24	407	0	-11,142	7,81E-05
414	c55792_g1	322	497,98	0	-11,142	7,81E-05
415	c44620_g1	388	438,47	0	-11,136	8,08E-05
416	c48009_g1	544,95	301,1	0	-11,132	8,08E-05
417	c58058_g1	478,99	356,92	0	-11,131	8,35E-05
418	c58017_g1	413,04	413	0	-11,129	8,35E-05
419	c51017_g1	394	428	0	-11,127	8,35E-05
420	c56751_g5	492,55	342,21	0	-11,125	8,35E-05
421	c14616_g1	477	355	0	-11,124	8,63E-05
422	c53952_g1	461	361,65	0	-11,111	9,22E-05
423	c57065_g2	286,22	511,97	0	-11,110	9,22E-05
424	c54090_g2	432,34	385,22	0	-11,107	9,22E-05
425	c55095_g1	354,97	450,94	0	-11,107	9,22E-05
426	c24239_g1	527,97	301	0	-11,104	9,53E-05
427	c57583_g1	310,63	484	0	-11,097	9,85E-05
428	c52100_g1	378,11	421,99	0	-11,089	1,02E-04
429	c34110_g1	515,99	301	0	-11,085	1,05E-04
430	c55691_g1	452,95	352,97	0	-11,080	1,05E-04
431	c50386_g1	412	388	0	-11,080	1,05E-04
432	c58170_g1	458,49	346,84	0	-11,077	1,09E-04
433	c52816_g1	512,54	299	0	-11,076	1,09E-04
434	c52905_g1	479,32	328	0	-11,075	1,09E-04
435	c50190_g1	457,99	345,99	0	-11,075	1,09E-04
436	c53947_g1	301,93	480	0	-11,075	1,09E-04
437	c46469_g1	450,98	350	0	-11,071	1,13E-04
438	c51782_g1	505,99	302	0	-11,070	1,13E-04
439	c52891_g1	446,73	351,65	0	-11,068	1,13E-04
440	c49895_g1	435	358,95	0	-11,062	1,17E-04
441	c54340_g1	557	253	0	-11,060	1,17E-04
442	c57237_g4	586,79	226	0	-11,057	1,21E-04
443	c51693_g1	487,99	308,93	0	-11,053	1,25E-04
444	c57329_g1	403,99	379	0	-11,049	1,25E-04
445	c44309_g1	405,48	377,83	0	-11,048	1,25E-04
446	c15579_g1	354,98	421	0	-11,048	1,25E-04
447	c53693_g1	458	331	0	-11,046	1,29E-04
448	c52391_g1	344	429,25	0	-11,045	1,29E-04
449	c49404_g1	402,85	377,95	0	-11,045	1,29E-04
450	c44902_g1	409,62	370,46	0	-11,041	1,29E-04
451	c57366_g1	496,95	295	0	-11,041	1,29E-04
452	c48540_g1	467,99	318,94	0	-11,039	1,34E-04
453	c54170_g1	320	445,88	0	-11,038	1,34E-04
454	c58064_g4	465,2	319,89	0	-11,036	1,34E-04
455	c57508_g1	389,99	383,98	0	-11,035	1,34E-04
456	c45020_g1	455,98	326,97	0	-11,034	1,34E-04
457	c54685_g1	424,7	352,96	0	-11,033	1,39E-04
458	c55320_g1	390,76	381,32	0	-11,030	1,39E-04
459	c55929_g1	490,99	292,96	0	-11,026	1,43E-04
460	c47502_g1	430	341	0	-11,017	1,48E-04
461	c51926_g1	500,92	279	0	-11,015	1,48E-04
462	c55175_g1	482	294	0	-11,013	1,54E-04
463	c55853_g1	422	345	0	-11,011	1,54E-04
464	c54696_g1	336	415,99	0	-11,005	1,07E-04
465	c54018_g2	391	367,66	0	-11,004	1,07E-04
466	c55959_g1	443	320	0	-10,997	1,11E-04
467	c57988_g9	343	405,61	0	-10,997	1,11E-04
468	c57776_g4	467	299	0	-10,996	1,11E-04
469	c50372_g1	399,09	357	0	-10,995	1,11E-04
470	c42532_g1	327,99	418	0	-10,995	1,11E-04
471	c48551_g1	271,99	465,91	0	-10,995	1,11E-04
472	c56900_g1	328,65	416,2	0	-10,993	1,11E-04
473	c56988_g1	289,99	447,99	0	-10,990	1,15E-04
474	c49064_g1	379	371	0	-10,989	1,15E-04
475	c57100_g1	355,53	390	0	-10,987	1,15E-04
476	c57764_g2	459,96	297	0	-10,980	1,19E-04
477	c47034_g1	403	345	0	-10,978	1,23E-04
478	c50831_g1	0,82	690,99	0	-10,978	1,11E-04
479	c57783_g1	379,72	361,98	0	-10,972	1,23E-04
480	c45303_g1	461,37	292	0	-10,971	1,28E-04
481	c53055_g1	299	428,99	0	-10,966	1,28E-04

482	c39573_g1	469,99	280	0	-10,962	1,33E-04
483	c54576_g1	277,99	443,56	0	-10,960	1,33E-04
484	c39584_g1	500,9	247,96	0	-10,951	1,37E-04
485	c51997_g1	435,81	303	0	-10,949	1,43E-04
486	c56792_g1	380,8	350	0	-10,948	1,43E-04
487	c56024_g2	342,53	381,51	0	-10,947	1,43E-04
488	c57178_g1	394,92	336,32	0	-10,944	1,43E-04
489	c57944_g1	405,88	325	0	-10,941	1,48E-04
490	c71475_g1	407,27	322,86	0	-10,938	1,48E-04
491	c57311_g1	494	248,3	0	-10,938	1,48E-04
492	c47027_g1	389,75	335,97	0	-10,935	1,53E-04
493	c45371_g1	389,44	335	0	-10,931	1,53E-04
494	c29222_g1	172	518,27	0	-10,923	1,59E-04
495	c44190_g1	372,95	342,9	0	-10,918	1,65E-04
496	c56503_g1	448,18	278	0	-10,918	1,65E-04
497	c49748_g1	371,63	343	0	-10,917	1,65E-04
498	c52471_g1	371,9	342,97	0	-10,917	1,65E-04
499	c55692_g1	424	296,98	0	-10,914	1,71E-04
500	c54225_g1	254,98	441,99	0	-10,913	1,71E-04
501	c54671_g9	468,69	255,94	0	-10,909	1,71E-04
502	c35070_g1	360	349	0	-10,907	1,71E-04
503	c55293_g5	384,18	324	0	-10,898	1,85E-04
504	c55825_g1	389	317,98	0	-10,894	1,85E-04
505	c53684_g1	384,39	321,94	0	-10,893	1,85E-04
506	c52162_g1	327,46	370	0	-10,891	1,92E-04
507	c44570_g1	510,96	211	0	-10,890	1,92E-04
508	c41097_g1	422,14	286,78	0	-10,888	1,92E-04
509	c57608_g1	422,87	285,64	0	-10,888	1,92E-04
510	c54268_g1	342	355,46	0	-10,886	1,92E-04
511	c57227_g1	372,08	329	0	-10,886	1,92E-04
512	c84044_g1	416	287	0	-10,877	2,07E-04
513	c57140_g1	504,34	210	0	-10,874	2,07E-04
514	c55565_g1	313,75	373,45	0	-10,873	2,07E-04
515	c56336_g2	400,05	295,99	0	-10,866	2,15E-04
516	c57728_g2	271,75	404,8	0	-10,863	2,15E-04
517	c53447_g1	440,99	258,95	0	-10,862	2,23E-04
518	c58237_g1	361,92	324,97	0	-10,858	2,23E-04
519	c48980_g1	419,27	274,96	0	-10,855	2,23E-04
520	c50238_g1	378,96	309	0	-10,854	2,32E-04
521	c41895_g1	351,99	331,67	0	-10,854	2,32E-04
522	c57540_g1	343,35	338	0	-10,850	2,32E-04
523	c56270_g1	375	309,92	0	-10,849	2,32E-04
524	c51849_g1	396,96	291	0	-10,849	2,32E-04
525	c51916_g1	475,99	220	0	-10,842	2,41E-04
526	c14157_g2	326	346	0	-10,835	2,50E-04
527	c41081_g1	359	317,44	0	-10,833	2,50E-04
528	c55667_g1	341,84	330,51	0	-10,832	2,60E-04
529	c54679_g1	372,98	304,31	0	-10,831	2,60E-04
530	c42211_g1	495	195	0	-10,822	2,70E-04
531	c54578_g1	308,97	352,95	0	-10,817	2,70E-04
532	c55509_g4	481,32	203,98	0	-10,815	2,81E-04
533	c56041_g1	396	276,97	0	-10,815	2,81E-04
534	c34573_g1	325,95	336	0	-10,812	2,81E-04
535	c71560_g1	365,89	301	0	-10,810	2,81E-04
536	c41096_g1	374	293,81	0	-10,810	2,81E-04
537	c33815_g1	361,89	303	0	-10,807	2,92E-04
538	c25365_g1	413,06	259	0	-10,807	2,92E-04
539	c53824_g1	353	309,52	0	-10,805	2,92E-04
540	c71380_g1	402,99	266	0	-10,803	2,92E-04
541	c44257_g1	401,8	266	0	-10,801	3,04E-04
542	c55492_g4	412	253	0	-10,790	3,16E-04
543	c54547_g1	414	251	0	-10,790	3,16E-04
544	c57715_g4	429,93	237,21	0	-10,789	3,16E-04
545	c56474_g1	437,89	230	0	-10,789	3,16E-04
546	c44265_g1	333	320	0	-10,788	2,10E-04
547	c45882_g1	404,26	258,41	0	-10,786	2,10E-04
548	c55001_g1	377	281	0	-10,785	2,10E-04
549	c54844_g2	335,98	316	0	-10,785	2,18E-04
550	c55059_g1	355	298,94	0	-10,783	2,18E-04
551	c42893_g1	348,99	304	0	-10,783	2,18E-04
552	c44418_g1	199	432,99	0	-10,782	2,18E-04
553	c47490_g1	480,99	190	0	-10,782	2,18E-04
554	c52055_g1	214,55	419,22	0	-10,782	2,18E-04
555	c31518_g1	361,82	292	0	-10,781	2,18E-04
556	c71454_g1	379,99	275,95	0	-10,780	2,18E-04
557	c58204_g1	463,3	202	0	-10,774	2,27E-04
558	c48495_g1	361	289	0	-10,772	2,27E-04
559	c47345_g1	399	256	0	-10,771	2,27E-04
560	c55820_g1	251,09	381,71	0	-10,767	2,36E-04
561	c50340_g1	374,99	274,71	0	-10,767	2,36E-04
562	c52485_g2	379,53	269,81	0	-10,765	2,36E-04
563	c43349_g1	329	312,98	0	-10,763	2,36E-04
564	c47895_g1	357,93	288	0	-10,763	2,36E-04
565	c40062_g1	319,99	320,08	0	-10,761	2,36E-04
566	c56130_g1	352	291,06	0	-10,758	2,46E-04
567	c41582_g1	276,95	355	0	-10,756	2,46E-04
568	c43718_g1	422	228	0	-10,751	2,56E-04
569	c48317_g1	452,31	199,96	0	-10,746	2,56E-04
570	c33443_g1	315,99	316,95	0	-10,745	2,56E-04
571	c24109_g1	285,99	340	0	-10,739	2,67E-04
572	c44798_g1	241,85	376,97	0	-10,736	2,67E-04
573	c54266_g1	262	358,81	0	-10,734	2,78E-04
574	c54698_g1	370,99	264,94	0	-10,734	2,78E-04
575	c52923_g1	365,9	268,99	0	-10,733	2,78E-04
576	c50788_g1	297,78	326,86	0	-10,732	2,78E-04
577	c57286_g1	278,97	342,56	0	-10,731	2,78E-04
578	c57640_g4	367,11	266	0	-10,728	2,90E-04
579	c56199_g3	326	301,25	0	-10,727	2,90E-04
580	c49739_g1	388	247	0	-10,726	2,90E-04
581	c54871_g1	395,59	240	0	-10,726	2,90E-04
582	c56889_g1	304,28	319,02	0	-10,725	2,90E-04
583	c54728_g1	367	263,98	0	-10,723	2,90E-04
584	c47358_g2	287,83	332	0	-10,723	2,90E-04
585	c84246_g1	340,48	287	0	-10,722	2,90E-04
586	c51579_g1	417	218	0	-10,716	3,02E-04
587	c56909_g3	266	347,62	0	-10,716	3,02E-04
588	c55516_g1	235	373,97	0	-10,714	3,02E-04
589	c56454_g1	369,15	256	0	-10,707	3,15E-04
590	c55624_g1	383,98	243	0	-10,707	3,15E-04
591	c49571_g2	386,62	239	0	-10,704	3,15E-04
592	c46416_g1	336	282,12	0	-10,701	3,28E-04
593	c54194_g1	331	286,02	0	-10,701	3,28E-04
594	c34700_g1	336	278,99	0	-10,694	3,42E-04
595	c45456_g1	394,99	228	0	-10,693	3,42E-04
596	c57477_g3	273	333,29	0	-10,693	3,42E-04
597	c71326_g1	230,98	368,99	0	-10,693	3,42E-04
598	c38436_g1	1	564,92	0	-10,687	3,15E-04
599	c47533_g1	254	347	0	-10,687	3,42E-04
600	c53545_g1	304,87	303	0	-10,687	3,42E-04

601	c26904_g1	302	305	0	-10,685	3,57E-04
602	c48525_g1	345,99	267,42	0	-10,685	3,57E-04
603	c39814_g1	349,35	260,95	0	-10,676	3,72E-04
604	c55072_g1	157	425,76	0	-10,675	3,57E-04
605	c46153_g1	360,72	249,99	0	-10,675	3,72E-04
606	c15869_g1	362	249	0	-10,674	3,72E-04
607	c47762_g1	370	242	0	-10,674	3,72E-04
608	c43425_g1	309,74	292,77	0	-10,672	3,72E-04
609	c40374_g1	321	282	0	-10,668	3,89E-04
610	c49752_g1	347	259	0	-10,667	3,89E-04
611	c50327_g3	349,97	255,91	0	-10,666	3,89E-04
612	c56729_g1	329,02	274,35	0	-10,665	3,89E-04
613	c48034_g1	267,71	325,87	0	-10,664	3,89E-04
614	c31661_g1	347,98	256	0	-10,661	3,89E-04
615	c50643_g1	229	358	0	-10,660	3,89E-04
616	c54408_g2	143	429	0	-10,652	4,05E-04
617	c58033_g1	359,99	241,32	0	-10,649	4,23E-04
618	c33324_g1	290	301,35	0	-10,648	4,23E-04
619	c55276_g1	404,97	199,99	0	-10,643	4,23E-04
620	c46942_g1	422,76	181,71	0	-10,637	4,42E-04
621	c44028_g1	118	443	0	-10,632	4,42E-04
622	c56585_g1	314,81	273,18	0	-10,631	4,61E-04
623	c51587_g1	376,96	219	0	-10,630	4,61E-04
624	c32004_g1	268,97	312	0	-10,630	4,61E-04
625	c57363_g1	358,99	233,79	0	-10,628	4,61E-04
626	c55935_g1	326	261	0	-10,625	4,81E-04
627	c40796_g1	338,8	249	0	-10,622	4,81E-04
628	c52060_g1	313,87	270	0	-10,621	4,81E-04
629	c57289_g3	353,67	233	0	-10,614	5,03E-04
630	c57984_g2	325	254	0	-10,604	5,25E-04
631	c51652_g1	224,99	339,67	0	-10,603	5,25E-04
632	c55394_g2	279,99	292	0	-10,601	5,25E-04
633	c49669_g1	406,87	180,32	0	-10,594	5,49E-04
634	c55906_g1	268,99	296,98	0	-10,589	5,49E-04
635	c53574_g1	389	193	0	-10,588	5,73E-04
636	c47443_g1	195	360	0	-10,587	5,49E-04
637	c52988_g1	400,85	182	0	-10,586	5,73E-04
638	c47750_g1	309,99	260	0	-10,585	5,73E-04
639	c57784_g1	306,92	261,94	0	-10,583	5,73E-04
640	c71422_g1	0	525	0	-10,579	5,25E-04
641	c36576_g1	375	201	0	-10,577	5,99E-04
642	c54053_g5	317	248,59	0	-10,571	6,26E-04
643	c51676_g1	298	265	0	-10,570	6,26E-04
644	c50866_g2	308	255,88	0	-10,569	6,26E-04
645	c50070_g1	203	346	0	-10,568	6,26E-04
646	c52223_g1	416	162	0	-10,566	4,10E-04
647	c55559_g1	268,5	288,33	0	-10,562	4,10E-04
648	c57509_g1	233	317,67	0	-10,562	4,10E-04
649	c53192_g1	331	232,98	0	-10,560	4,29E-04
650	c57144_g1	287,72	269,41	0	-10,557	4,29E-04
651	c47367_g1	238	312,48	0	-10,557	4,29E-04
652	c58082_g3	319,77	240	0	-10,553	4,29E-04
653	c57539_g1	317,85	240,82	0	-10,551	4,48E-04
654	c42527_g1	282	270	0	-10,546	4,48E-04
655	c46282_g1	245,62	301	0	-10,545	4,48E-04
656	c58084_g2	308,86	243,95	0	-10,538	4,69E-04
657	c48978_g1	179	353,86	0	-10,532	4,69E-04
658	c55822_g1	375	184	0	-10,529	4,91E-04
659	c56756_g1	337	215,15	0	-10,524	5,15E-04
660	c56763_g2	227	308,8	0	-10,522	5,15E-04
661	c39572_g1	371	184	0	-10,519	5,15E-04
662	c57262_g1	337,73	212	0	-10,518	5,15E-04
663	c56719_g1	259,92	279	0	-10,517	5,15E-04
664	c44151_g1	279	262	0	-10,515	5,15E-04
665	c53538_g1	315	230	0	-10,513	5,39E-04
666	c57187_g5	311	233	0	-10,511	5,39E-04
667	c58662_g1	275	264	0	-10,511	5,39E-04
668	c45428_g1	319,45	224,99	0	-10,508	5,39E-04
669	c56728_g1	249	284,96	0	-10,507	5,39E-04
670	c26742_g1	343,2	202,7	0	-10,504	5,64E-04
671	c29913_g1	228	301,99	0	-10,504	5,39E-04
672	c55400_g2	256,95	277	0	-10,504	5,64E-04
673	c29658_g1	235	295	0	-10,501	5,64E-04
674	c51933_g1	388	161,99	0	-10,498	5,64E-04
675	c47222_g2	201,72	321,76	0	-10,497	5,64E-04
676	c54000_g1	269,11	263	0	-10,493	5,91E-04
677	c49378_g1	309	227,88	0	-10,492	5,91E-04
678	c84189_g1	217	306,82	0	-10,491	5,91E-04
679	c50882_g1	263	267	0	-10,490	5,91E-04
680	c55183_g1	222,76	300,99	0	-10,489	5,91E-04
681	c59210_g1	325	213	0	-10,488	5,91E-04
682	c55405_g1	254,19	273,98	0	-10,488	5,91E-04
683	c35218_g1	208	311,87	0	-10,483	6,20E-04
684	c54451_g6	229,97	292,95	0	-10,483	6,20E-04
685	c26619_g1	272,02	255,97	0	-10,481	6,20E-04
686	c55812_g2	358,8	179	0	-10,475	6,50E-04
687	c52727_g1	254	269	0	-10,473	6,50E-04
688	c54372_g1	300	228,99	0	-10,472	6,50E-04
689	c49094_g1	230,92	287,99	0	-10,471	6,50E-04
690	c52261_g1	262,81	260	0	-10,469	6,50E-04
691	c53705_g1	294,31	231,72	0	-10,466	6,81E-04
692	c50646_g1	287,63	236,98	0	-10,465	6,81E-04
693	c52111_g1	283,75	239,97	0	-10,464	6,81E-04
694	c49565_g1	332	197,99	0	-10,462	6,81E-04
695	c57023_g2	340,43	191,49	0	-10,462	6,81E-04
696	c52773_g1	100	395,97	0	-10,456	6,81E-04
697	c56392_g1	224,85	287	0	-10,452	7,14E-04
698	c55322_g1	223	288	0	-10,450	7,14E-04
699	c55000_g1	296	223	0	-10,444	7,49E-04
700	c57631_g2	265,96	247,93	0	-10,441	7,49E-04
701	c12847_g1	339,91	184	0	-10,441	7,49E-04
702	c50574_g1	312	208	0	-10,440	7,49E-04
703	c52009_g1	284	231,73	0	-10,440	7,49E-04
704	c53327_g1	321	200	0	-10,440	7,49E-04
705	c43797_g1	284,91	230,93	0	-10,439	7,49E-04
706	c36358_g1	259,67	252	0	-10,438	7,49E-04
707	c9297_g1	213	291	0	-10,433	7,86E-04
708	c53605_g1	224,13	280	0	-10,428	7,86E-04
709	c56584_g5	191	307	0	-10,424	7,86E-04
710	c42941_g2	263,88	244	0	-10,424	8,24E-04
711	c49409_g3	403,99	121,99	0	-10,420	8,24E-04
712	c54297_g1	222,4	278,01	0	-10,417	8,24E-04
713	c47608_g1	310,82	199,98	0	-10,413	8,65E-04
714	c45727_g1	260,95	243	0	-10,413	8,65E-04
715	c53334_g1	333,9	180	0	-10,413	8,65E-04
716	c55081_g1	212,16	285,09	0	-10,412	8,65E-04
717	c52946_g1	135,71	350,33	0	-10,410	8,65E-04
718	c57460_g2	247	253	0	-10,406	8,65E-04
719	c42741_g1	313,95	194,9	0	-10,406	8,65E-04
720	c34184_g1	231	266,46	0	-10,404	9,08E-04

721	c57152_g1	358	155	0	-10,399	9,08E-04
722	c51680_g1	272,89	228	0	-10,398	9,08E-04
723	c57939_g1	327	178,68	0	-10,391	9,53E-04
724	c55168_g5	292,56	207	0	-10,387	9,53E-04
725	c34387_g1	271,96	225	0	-10,386	9,53E-04
726	c31547_g1	326	178	0	-10,385	9,53E-04
727	c39794_g1	313,77	187,92	0	-10,384	1,00E-03
728	c56944_g1	291,96	206	0	-10,381	1,00E-03
729	c38990_g2	178	304	0	-10,380	1,00E-03
730	c52465_g1	209	277	0	-10,379	1,00E-03
731	c52188_g2	318,66	181,96	0	-10,379	1,00E-03
732	c49299_g1	262,96	229,97	0	-10,378	1,00E-03
733	c57727_g1	295	202,24	0	-10,376	1,00E-03
734	c42943_g1	281,46	213,98	0	-10,376	1,00E-03
735	c54774_g2	209	275,97	0	-10,376	1,00E-03
736	c46603_g1	349,24	154,99	0	-10,375	1,00E-03
737	c19475_g1	230,39	256,82	0	-10,373	1,05E-03
738	c52058_g3	320	179	0	-10,372	1,05E-03
739	c49323_g1	178	301	0	-10,370	1,05E-03
740	c46209_g1	234	252	0	-10,368	1,05E-03
741	c55101_g2	301	193	0	-10,364	1,05E-03
742	c55389_g3	300,97	193	0	-10,364	1,05E-03
743	c52312_g1	282	209	0	-10,363	1,05E-03
744	c40654_g1	257,97	229	0	-10,361	1,10E-03
745	c58057_g8	363	138	0	-10,359	1,10E-03
746	c56076_g1	303,96	187,98	0	-10,357	1,10E-03
747	c55685_g1	331,97	162,98	0	-10,354	1,10E-03
748	c52176_g1	267,86	217,99	0	-10,353	1,10E-03
749	c49628_g2	263,22	222	0	-10,352	1,16E-03
750	c46473_g1	208,69	268	0	-10,350	1,16E-03
751	c57745_g1	194,99	280,04	0	-10,350	1,16E-03
752	c39759_g1	291,99	196	0	-10,349	1,16E-03
753	c40487_g1	148	319,54	0	-10,348	1,16E-03
754	c122703_g1	251	230,88	0	-10,348	1,16E-03
755	c56842_g1	152,65	314,99	0	-10,346	1,16E-03
756	c55162_g1	263,99	219	0	-10,345	1,16E-03
757	c58344_g1	333,68	157,95	0	-10,343	1,16E-03
758	c51285_g2	341,81	150,98	0	-10,343	1,16E-03
759	c52929_g1	183,8	287	0	-10,342	1,16E-03
760	c55687_g1	255	222,94	0	-10,333	7,88E-04
761	c36028_g1	225,48	248	0	-10,330	7,88E-04
762	c55027_g1	169,07	295,81	0	-10,329	7,88E-04
763	c30138_g1	312	170	0	-10,321	8,29E-04
764	c52291_g1	156	303,99	0	-10,319	8,29E-04
765	c45909_g1	234,95	235	0	-10,316	8,72E-04
766	c55540_g1	261	209,78	0	-10,307	9,19E-04
767	c51752_g1	240,86	227	0	-10,307	9,19E-04
768	c49176_g1	241,17	227	0	-10,307	9,19E-04
769	c54695_g1	190,87	269,52	0	-10,306	9,19E-04
770	c32489_g1	287	186	0	-10,302	9,19E-04
771	c51285_g3	238,93	227	0	-10,301	9,19E-04
772	c18689_g1	183	275	0	-10,300	9,19E-04
773	c39363_g1	265	204	0	-10,299	9,19E-04
774	c46987_g1	245,44	221,34	0	-10,298	9,19E-04
775	c48323_g1	289	183	0	-10,298	9,19E-04
776	c58072_g3	289	183	0	-10,298	9,19E-04
777	c109723_g1	210,97	250	0	-10,297	9,19E-04
778	c43148_g1	273	196,33	0	-10,295	9,68E-04
779	c52764_g1	302,99	169,78	0	-10,295	9,68E-04
780	c44191_g1	254,9	211	0	-10,294	9,68E-04
781	c53403_g1	278	191	0	-10,293	9,68E-04
782	c45365_g1	277,88	190,96	0	-10,293	9,68E-04
783	c44312_g1	212,66	247	0	-10,293	9,68E-04
784	c49409_g2	345,99	132	0	-10,292	9,68E-04
785	c109567_g1	229	231,89	0	-10,289	9,68E-04
786	c56094_g1	247,19	214,96	0	-10,284	1,02E-03
787	c37338_g1	247	215	0	-10,284	1,02E-03
788	c51389_g1	276,97	189	0	-10,283	1,02E-03
789	c56235_g1	271	194	0	-10,283	1,02E-03
790	c49462_g1	241,96	219	0	-10,283	1,02E-03
791	c54683_g1	271	192,92	0	-10,279	1,02E-03
792	c57701_g1	121	321,93	0	-10,278	1,02E-03
793	c42347_g1	290,98	175	0	-10,277	1,02E-03
794	c54395_g1	214,65	240	0	-10,275	1,02E-03
795	c57826_g1	252	207,93	0	-10,275	1,07E-03
796	c38825_g1	173,99	273,96	0	-10,270	1,07E-03
797	c56684_g2	240	216,99	0	-10,270	1,07E-03
798	c51148_g1	236	220	0	-10,269	1,07E-03
799	c39922_g1	200	251	0	-10,268	1,07E-03
800	c50991_g1	244	212,99	0	-10,268	1,07E-03
801	c15074_g1	285,92	176	0	-10,266	1,07E-03
802	c57034_g1	266	192,99	0	-10,265	1,07E-03
803	c28798_g1	220,9	231	0	-10,262	1,13E-03
804	c46169_g1	347,95	120,95	0	-10,261	1,13E-03
805	c47667_g1	339	128	0	-10,258	1,13E-03
806	c57501_g1	282	177	0	-10,257	1,13E-03
807	c49671_g1	309,71	152	0	-10,254	1,13E-03
808	c51859_g2	226	223	0	-10,249	1,19E-03
809	c51490_g1	252	200	0	-10,247	1,19E-03
810	c58057_g2	215,99	231	0	-10,247	1,19E-03
811	c27264_g1	233,58	214,98	0	-10,246	1,19E-03
812	c41739_g1	293	164	0	-10,245	1,19E-03
813	c55488_g1	265,4	187	0	-10,241	1,19E-03
814	c47130_g1	245,76	203	0	-10,240	1,26E-03
815	c57259_g2	212,23	231,78	0	-10,239	1,26E-03
816	c15618_g1	185,38	254,89	0	-10,238	1,26E-03
817	c56356_g1	306	149	0	-10,232	1,26E-03
818	c52305_g1	255	193,27	0	-10,232	1,26E-03
819	c23697_g1	254,55	193,03	0	-10,232	1,26E-03
820	c56979_g1	304	150	0	-10,230	1,26E-03
821	c56948_g1	150,99	280,93	0	-10,226	1,33E-03
822	c53776_g1	262	184,98	0	-10,225	1,33E-03
823	c56746_g1	235,85	207	0	-10,224	1,33E-03
824	c24327_g2	52	364,92	0	-10,221	1,26E-03
825	c48422_g1	222	217,64	0	-10,220	1,33E-03
826	c47341_g2	275,96	170	0	-10,215	1,40E-03
827	c55121_g1	95	325,98	0	-10,214	1,33E-03
828	c46703_g1	169,99	260,61	0	-10,213	1,40E-03
829	c48854_g1	265	179	0	-10,213	1,40E-03
830	c12732_g1	187	246	0	-10,212	1,40E-03
831	c53842_g1	252,81	189,42	0	-10,212	1,40E-03
832	c45505_g1	254,15	188	0	-10,211	1,40E-03
833	c48531_g1	182,82	249	0	-10,211	1,40E-03
834	c45624_g1	258	184	0	-10,210	1,40E-03
835	c43077_g1	267	176	0	-10,209	1,40E-03
836	c41928_g1	254,76	186,29	0	-10,207	1,40E-03
837	c55037_g1	247,03	192	0	-10,204	1,48E-03
838	c57640_g2	191,91	239	0	-10,203	1,48E-03
839	c52987_g1	269,87	170,99	0	-10,200	1,48E-03
840	c57577_g8	269,28	171	0	-10,197	1,48E-03

841	c42653_g1	201,67	227,99	0	-10,194	1,48E-03
842	c54844_g1	234,61	198,91	0	-10,192	1,56E-03
843	c47898_g1	279,94	159,98	0	-10,192	1,56E-03
844	c36726_g1	263	173,98	0	-10,189	1,56E-03
845	c50637_g1	213	216,99	0	-10,189	1,56E-03
846	c23145_g1	221	210	0	-10,188	1,56E-03
847	c55117_g1	174,99	248,63	0	-10,186	1,56E-03
848	c54799_g1	219,07	210,9	0	-10,186	1,56E-03
849	c39257_g2	262,22	173	0	-10,183	1,56E-03
850	c45950_g1	205	220,99	0	-10,178	1,65E-03
851	c40625_g1	234,66	195	0	-10,178	1,65E-03
852	c50608_g1	163,17	256,97	0	-10,177	1,65E-03
853	c55993_g1	253	179	0	-10,176	1,65E-03
854	c58154_g3	272,79	160,99	0	-10,173	1,65E-03
855	c51979_g1	233	195,44	0	-10,172	1,65E-03
856	c47346_g1	240,98	188	0	-10,171	1,65E-03
857	c42822_g1	222,98	203	0	-10,169	1,74E-03
858	c122430_g1	246,58	181,94	0	-10,168	1,74E-03
859	c50832_g1	154	261,98	0	-10,167	1,65E-03
860	c45382_g1	256,91	172,54	0	-10,167	1,74E-03
861	c53532_g2	170	247,96	0	-10,167	1,74E-03
862	c54908_g1	195,93	225	0	-10,165	1,74E-03
863	c57811_g1	206	215	0	-10,159	1,74E-03
864	c45782_g1	256	171	0	-10,156	1,84E-03
865	c50872_g1	164	249	0	-10,151	1,84E-03
866	c54688_g2	168,46	245	0	-10,149	1,84E-03
867	c55042_g1	211,79	206,95	0	-10,149	1,84E-03
868	c43854_g1	192	223,74	0	-10,148	1,84E-03
869	c55554_g2	229	190,71	0	-10,144	1,94E-03
870	c55743_g1	260	163,99	0	-10,143	1,94E-03
871	c57176_g2	251,98	170,04	0	-10,140	1,94E-03
872	c58152_g1	181	231,08	0	-10,139	1,94E-03
873	c53172_g1	335,93	97	0	-10,138	1,94E-03
874	c49418_g2	276,99	147	0	-10,135	1,94E-03
875	c56571_g1	142	263	0	-10,133	1,94E-03
876	c54036_g1	210,35	204	0	-10,132	2,05E-03
877	c54746_g5	232	185	0	-10,131	2,05E-03
878	c56997_g1	186	224	0	-10,129	2,05E-03
879	c56879_g1	225	190	0	-10,128	2,05E-03
880	c55658_g1	58	333,9	0	-10,127	1,94E-03
881	c52416_g1	209	202,99	0	-10,125	2,05E-03
882	c57599_g1	261,85	157	0	-10,124	2,05E-03
883	c36507_g1	170,98	235	0	-10,121	2,05E-03
884	c122420_g1	283	137	0	-10,117	2,17E-03
885	c56205_g4	299,99	122	0	-10,115	2,17E-03
886	c56939_g1	201,98	206	0	-10,113	2,17E-03
887	c32709_g1	203	205	0	-10,113	2,17E-03
888	c15554_g1	217,82	192	0	-10,112	2,17E-03
889	c56733_g1	182	223	0	-10,112	2,17E-03
890	c57187_g3	286,24	133	0	-10,111	2,17E-03
891	c53558_g1	234,99	176,9	0	-10,111	2,17E-03
892	c55076_g1	222	188	0	-10,110	2,17E-03
893	c56722_g2	241,38	169,81	0	-10,104	1,46E-03
894	c55677_g1	177	225	0	-10,103	1,46E-03
895	c57187_g1	244	167	0	-10,103	1,46E-03
896	c51292_g1	260,99	152	0	-10,101	1,46E-03
897	c47850_g1	246,99	164	0	-10,101	1,46E-03
898	c39218_g1	227,85	180	0	-10,100	1,46E-03
899	c71442_g1	185,92	214,99	0	-10,095	1,46E-03
900	c45175_g1	173	226	0	-10,094	1,46E-03

901	c55686_g3	195	206,99	0	-10,094	1,55E-03
902	c54308_g1	197	205,49	0	-10,093	1,55E-03
903	c54371_g1	140,54	250,6	0	-10,084	1,55E-03
904	c46084_g2	244,93	161	0	-10,083	1,55E-03
905	c52371_g1	213,32	188,37	0	-10,080	1,64E-03
906	c39152_g1	220,9	181	0	-10,080	1,64E-03
907	c15611_g1	162,63	231	0	-10,080	1,64E-03
908	c58241_g1	217	184	0	-10,078	1,64E-03
909	c57531_g1	158,53	233,95	0	-10,078	1,64E-03
910	c27180_g1	206,57	192	0	-10,076	1,64E-03
911	c57100_g2	283,99	124	0	-10,070	1,64E-03
912	c39960_g1	188,92	205,86	0	-10,070	1,64E-03
913	c44284_g1	220	178,19	0	-10,065	1,74E-03
914	c54828_g1	96	284,92	0	-10,065	1,64E-03
915	c50787_g1	188	205	0	-10,063	1,74E-03
916	c51794_g2	249	152	0	-10,061	1,74E-03
917	c44548_g1	241,96	158,03	0	-10,061	1,74E-03
918	c23397_g1	177,59	212	0	-10,056	1,74E-03
919	c55613_g6	172,72	215,51	0	-10,055	1,74E-03
920	c41234_g1	218,96	174,99	0	-10,050	1,84E-03
921	c56676_g1	221	173,37	0	-10,049	1,84E-03
922	c27163_g1	266,98	133	0	-10,048	1,84E-03
923	c55810_g1	156,76	228	0	-10,048	1,84E-03
924	c51164_g1	188,25	201	0	-10,047	1,84E-03
925	c56560_g1	197	193	0	-10,046	1,84E-03
926	c41890_g1	174	212	0	-10,042	1,84E-03
927	c36021_g1	197	191,53	0	-10,042	1,95E-03
928	c56008_g1	202	187	0	-10,039	1,95E-03
929	c53236_g1	201,73	186,73	0	-10,039	1,95E-03
930	c55613_g3	224,85	167	0	-10,039	1,95E-03
931	c54242_g1	255	141	0	-10,038	1,95E-03
932	c55614_g1	217	173	0	-10,035	1,95E-03
933	c40857_g1	167	216	0	-10,034	1,95E-03
934	c24730_g1	165,98	216	0	-10,031	1,95E-03
935	c53163_g1	153	227	0	-10,030	1,95E-03
936	c34548_g1	197,8	187,97	0	-10,029	1,95E-03
937	c41146_g1	146	232	0	-10,026	2,07E-03
938	c49709_g1	221,36	167	0	-10,025	2,07E-03
939	c52327_g1	201	184,47	0	-10,024	2,07E-03
940	c56526_g1	278	117	0	-10,022	2,07E-03
941	c57024_g2	237,99	151	0	-10,019	2,07E-03
942	c58072_g6	235	152,74	0	-10,017	2,07E-03
943	c54300_g2	165,56	211,99	0	-10,015	2,07E-03
944	c57740_g4	213	170,26	0	-10,009	2,20E-03
945	c43034_g1	191,2	188,9	0	-10,009	2,20E-03
946	c32002_g1	190,65	189	0	-10,009	2,20E-03
947	c24475_g1	218	165	0	-10,006	2,20E-03
948	c31400_g1	167,88	208	0	-10,005	2,20E-03
949	c57338_g4	205,97	175	0	-10,005	2,20E-03
950	c49231_g1	170	206	0	-10,004	2,20E-03
951	c45608_g1	211	169	0	-9,998	2,34E-03
952	c53816_g3	177,98	197	0	-9,996	2,34E-03
953	c50901_g1	224	157,05	0	-9,995	2,34E-03
954	c54736_g1	216,77	162,03	0	-9,990	2,34E-03
955	c53769_g1	195	180,84	0	-9,990	2,34E-03
956	c57969_g2	189,88	184,88	0	-9,989	2,34E-03
957	c54905_g1	179	192	0	-9,979	2,48E-03
958	c44672_g1	188,28	184	0	-9,978	2,48E-03
959	c54801_g1	218	158	0	-9,977	2,48E-03
960	c54953_g1	89	269	0	-9,975	2,48E-03

961	c54222_g1	147,99	217,95	0	-9,975	2,48E-03
962	c50560_g1	207	166,99	0	-9,975	2,48E-03
963	c52157_g1	222,96	152,78	0	-9,975	2,48E-03
964	c54808_g2	178	190,97	0	-9,971	2,64E-03
965	c56238_g1	212,99	159,63	0	-9,968	2,64E-03
966	c40865_g1	177	191	0	-9,967	2,64E-03
967	c56069_g1	211	160,99	0	-9,965	2,64E-03
968	c53355_g1	189	180	0	-9,965	2,64E-03
969	c56982_g1	203,92	167	0	-9,964	2,64E-03
970	c29766_g1	227	146	0	-9,960	2,80E-03
971	c55632_g1	201	167,96	0	-9,958	2,80E-03
972	c49740_g1	179	186,57	0	-9,958	2,80E-03
973	c26797_g1	232	141	0	-9,957	2,80E-03
974	c50640_g1	191	173,9	0	-9,947	2,80E-03
975	c52108_g1	228	142	0	-9,946	2,80E-03
976	c32403_g1	226,93	142	0	-9,943	2,98E-03
977	c15658_g1	219,68	148	0	-9,943	2,98E-03
978	c45373_g1	256,69	115,95	0	-9,942	2,98E-03
979	c43369_g1	188	174,99	0	-9,940	2,98E-03
980	c45565_g1	162,01	197	0	-9,938	2,98E-03
981	c47134_g1	199,79	164	0	-9,937	2,98E-03
982	c47237_g1	201,08	163	0	-9,936	2,98E-03
983	c50775_g1	245,99	123,99	0	-9,936	2,98E-03
984	c57014_g2	238,54	130	0	-9,936	2,98E-03
985	c71411_g1	206	158	0	-9,934	2,98E-03
986	c51178_g1	164	193,98	0	-9,932	2,98E-03
987	c53368_g2	179	181	0	-9,932	2,98E-03
988	c54954_g1	208	154,96	0	-9,928	3,17E-03
989	c53038_g1	172	185,98	0	-9,928	3,17E-03
990	c56234_g1	204	158,06	0	-9,926	3,17E-03
991	c57238_g1	202	159	0	-9,923	3,17E-03
992	c36278_g1	236	129	0	-9,920	3,17E-03
993	c56636_g1	185	172,87	0	-9,920	3,17E-03
994	c57935_g3	170	185	0	-9,916	3,37E-03
995	c54902_g1	120	228	0	-9,915	3,17E-03
996	c57822_g1	216,05	144,78	0	-9,915	3,37E-03
997	c34913_g1	194,99	162	0	-9,910	3,37E-03
998	c97179_g1	198	158,97	0	-9,908	3,37E-03
999	c109627_g1	205,96	152	0	-9,908	3,37E-03
1000	c55512_g3	100	242,95	0	-9,905	3,37E-03
1001	c56768_g6	211	147	0	-9,905	3,37E-03
1002	c57411_g1	192	162,92	0	-9,903	3,37E-03
1003	c36479_g1	184,92	168,98	0	-9,903	3,37E-03
1004	c49256_g3	195	160	0	-9,901	3,59E-03
1005	c43198_g1	227	132	0	-9,900	3,59E-03
1006	c39193_g1	191	163	0	-9,899	3,59E-03
1007	c46072_g1	180	172	0	-9,897	3,59E-03
1008	c51136_g1	144	202,98	0	-9,896	3,59E-03
1009	c53620_g1	183,07	168	0	-9,891	3,59E-03
1010	c39648_g1	265,76	96	0	-9,889	3,59E-03
1011	c46841_g1	170,99	177,94	0	-9,889	3,59E-03
1012	c52075_g1	170	177,98	0	-9,885	3,82E-03
1013	c35155_g1	207,65	145	0	-9,884	3,82E-03
1014	c42270_g1	216	138	0	-9,884	3,82E-03
1015	c56000_g1	187,07	161,98	0	-9,879	3,82E-03
1016	c56584_g4	212,25	140	0	-9,878	3,82E-03
1017	c57758_g1	136	205	0	-9,874	3,82E-03
1018	c10749_g1	232	122	0	-9,874	3,82E-03
1019	c54014_g2	202,98	146,62	0	-9,874	3,82E-03
1020	c71420_g1	248	108	0	-9,874	3,82E-03
1021	c57589_g1	196,59	152	0	-9,873	3,82E-03
1022	c54157_g1	148	193,97	0	-9,872	4,07E-03
1023	c71344_g1	239,97	113,99	0	-9,870	4,07E-03
1024	c48549_g1	156	186	0	-9,867	4,07E-03
1025	c31286_g1	182	162,12	0	-9,860	2,54E-03
1026	c56307_g2	212,34	135,71	0	-9,860	2,54E-03
1027	c54309_g1	206	141	0	-9,859	2,54E-03
1028	c49902_g1	207	139,96	0	-9,858	2,54E-03
1029	c55151_g1	231	119	0	-9,857	2,71E-03
1030	c39500_g1	186,87	157	0	-9,857	2,71E-03
1031	c56071_g1	217	131	0	-9,857	2,71E-03
1032	c52695_g1	181	161,99	0	-9,856	2,71E-03
1033	c49932_g2	130,3	205,96	0	-9,856	2,54E-03
1034	c54951_g1	212	134	0	-9,851	2,71E-03
1035	c58347_g1	231,99	116	0	-9,847	2,71E-03
1036	c50861_g1	218,61	127	0	-9,846	2,71E-03
1037	c56129_g1	170	169	0	-9,845	2,71E-03
1038	c25307_g1	137	197	0	-9,842	2,71E-03
1039	c49121_g2	125	206,92	0	-9,840	2,71E-03
1040	c53384_g1	148,2	187	0	-9,840	2,89E-03
1041	c53426_g5	209	134	0	-9,839	2,89E-03
1042	c55223_g1	159,08	176,9	0	-9,838	2,89E-03
1043	c55496_g1	172,99	163,96	0	-9,834	2,89E-03
1044	c53306_g2	204,9	136	0	-9,832	2,89E-03
1045	c54643_g1	205	135,99	0	-9,832	2,89E-03
1046	c56063_g4	213	129	0	-9,832	2,89E-03
1047	c30098_g1	132	198	0	-9,827	2,89E-03
1048	c50591_g1	177	159	0	-9,826	3,09E-03
1049	c46475_g1	177,6	157,57	0	-9,826	3,09E-03
1050	c22439_g1	149	183	0	-9,826	3,09E-03
1051	c49278_g1	203,97	135	0	-9,823	3,09E-03
1052	c58082_g4	182,83	153	0	-9,823	3,09E-03
1053	c57699_g1	234,67	108	0	-9,823	3,09E-03
1054	c51421_g1	118,98	208	0	-9,821	3,09E-03
1055	c57573_g2	142	188	0	-9,821	3,09E-03
1056	c32766_g1	141,64	188	0	-9,821	3,09E-03
1057	c51476_g1	128	199,93	0	-9,820	3,09E-03
1058	c31551_g2	174	159,99	0	-9,819	3,09E-03
1059	c49420_g1	153	178	0	-9,818	3,09E-03
1060	c47492_g1	167,99	165	0	-9,818	3,09E-03
1061	c57128_g3	162,87	169,03	0	-9,817	3,09E-03
1062	c49608_g1	170,99	161,57	0	-9,816	3,09E-03
1063	c55678_g1	172	161	0	-9,816	3,09E-03
1064	c42178_g1	150	180	0	-9,816	3,09E-03
1065	c42395_g1	162	168,84	0	-9,813	3,09E-03
1066	c54276_g1	91	230	0	-9,811	3,09E-03
1067	c52666_g1	195	140	0	-9,811	3,30E-03
1068	c48575_g2	181	152	0	-9,810	3,30E-03
1069	c52349_g1	123	202	0	-9,809	3,09E-03
1070	c84298_g1	221	117	0	-9,808	3,30E-03
1071	c57615_g2	207	128,94	0	-9,808	3,30E-03
1072	c24295_g1	201	134,39	0	-9,807	3,30E-03
1073	c45563_g1	172	159	0	-9,806	3,30E-03
1074	c55735_g1	172,51	158	0	-9,806	3,30E-03
1075	c55077_g1	187,99	145	0	-9,806	3,30E-03
1076	c49160_g1	166	164	0	-9,805	3,30E-03
1077	c40045_g1	161	168	0	-9,804	3,30E-03
1078	c57922_g1	139,99	185,74	0	-9,803	3,30E-03
1079	c57725_g1	165,99	163,28	0	-9,801	3,30E-03
1080	c54324_g2	159	168,85	0	-9,801	3,30E-03

1081	c57544_g1	176	154	0	-9,799	3,30E-03
1082	c36360_g1	198,88	132,99	0	-9,794	3,53E-03
1083	c40071_g1	223,39	112,15	0	-9,793	3,53E-03
1084	c48948_g1	198	133	0	-9,790	3,53E-03
1085	c53635_g1	213	119,72	0	-9,790	3,53E-03
1086	c42438_g1	174,99	152	0	-9,786	3,53E-03
1087	c34502_g1	198	132	0	-9,785	3,53E-03
1088	c24986_g1	173,96	152,24	0	-9,781	3,53E-03
1089	c55911_g2	152	171	0	-9,781	3,53E-03
1090	c57114_g1	211,98	119	0	-9,781	3,53E-03
1091	c56922_g1	136	184	0	-9,777	3,78E-03
1092	c55345_g1	146	175,32	0	-9,776	3,78E-03
1093	c8189_g1	184	142	0	-9,775	3,78E-03
1094	c57149_g1	162	161	0	-9,775	3,78E-03
1095	c36529_g1	195	132	0	-9,773	3,78E-03
1096	c57276_g5	128,89	188,96	0	-9,772	3,78E-03
1097	c34861_g1	115	201	0	-9,772	3,78E-03
1098	c29347_g1	235	97	0	-9,771	3,78E-03
1099	c56324_g1	200	127	0	-9,769	3,78E-03
1100	c33415_g1	148,87	171	0	-9,769	3,78E-03
1101	c15576_g1	137,52	180	0	-9,766	3,78E-03
1102	c39441_g1	103,33	210,34	0	-9,765	3,78E-03
1103	c54682_g5	104,98	208	0	-9,764	3,78E-03
1104	c55907_g1	153,04	166	0	-9,762	4,04E-03
1105	c37516_g1	153,92	165	0	-9,761	4,04E-03
1106	c46405_g1	131,98	183,99	0	-9,761	4,04E-03
1107	c50002_g1	185	138	0	-9,760	4,04E-03
1108	c40821_g1	147,89	170	0	-9,760	4,04E-03
1109	c47692_g1	91	219	0	-9,758	3,78E-03
1110	c57054_g1	155	163	0	-9,755	4,04E-03
1111	c55049_g1	150	167	0	-9,754	4,04E-03
1112	c55213_g1	189	133	0	-9,752	4,04E-03
1113	c51484_g1	166,81	152	0	-9,752	4,04E-03
1114	c58230_g1	197	126	0	-9,752	4,04E-03
1115	c55256_g1	168	151	0	-9,752	4,04E-03
1116	c47601_g1	104	205,35	0	-9,745	4,04E-03
1117	c52776_g1	197,11	124	0	-9,742	4,32E-03
1118	c57828_g1	160,73	154,97	0	-9,742	4,32E-03
1119	c34968_g1	184	135	0	-9,741	4,32E-03
1120	c43974_g1	176,98	140,95	0	-9,741	4,32E-03
1121	c54951_g2	142	171	0	-9,740	4,32E-03
1122	c56473_g1	128,27	183	0	-9,739	4,32E-03
1123	c43307_g1	113,98	195	0	-9,738	4,32E-03
1124	c53340_g1	167,04	149	0	-9,738	4,32E-03
1125	c52826_g1	164	151	0	-9,735	4,32E-03
1126	c96838_g1	180,76	135,56	0	-9,733	4,32E-03
1127	c122472_g1	152	161	0	-9,733	4,32E-03
1128	c32453_g1	167,91	147	0	-9,732	4,32E-03
1129	c52261_g4	236	87,99	0	-9,731	4,32E-03
1130	c57306_g2	168	145,99	0	-9,727	4,63E-03
1131	c55170_g12	183,52	132	0	-9,726	4,63E-03
1132	c47712_g1	170	144	0	-9,726	4,63E-03
1133	c57655_g3	120	186,54	0	-9,725	4,63E-03
1134	c53617_g1	137	172	0	-9,723	4,63E-03
1135	c57571_g4	160	150,99	0	-9,718	4,63E-03
1136	c45092_g1	227,4	93	0	-9,717	4,63E-03
1137	c56198_g1	74	224,99	0	-9,716	4,63E-03
1138	c53555_g1	120	184,98	0	-9,715	4,63E-03
1139	c55150_g1	168	143	0	-9,712	4,96E-03
1140	c55990_g3	27	265	0	-9,712	4,32E-03
1141	c57276_g1	168,79	142	0	-9,712	4,96E-03
1142	c44810_g1	176	134,9	0	-9,707	4,96E-03
1143	c36265_g1	207,93	106,52	0	-9,705	4,96E-03
1144	c26471_g1	164	144,99	0	-9,705	4,96E-03
1145	c52193_g1	122	181	0	-9,703	4,96E-03
1146	c53020_g1	197	116	0	-9,703	4,96E-03
1147	c46566_g1	211,86	102,66	0	-9,703	4,96E-03
1148	c57638_g1	115,55	186	0	-9,702	4,96E-03
1149	c46174_g1	155	152	0	-9,701	4,96E-03
1150	c55764_g1	148,7	156,94	0	-9,700	4,96E-03
1151	c54176_g1	164,32	143,99	0	-9,700	4,96E-03
1152	c58030_g1	129	174	0	-9,698	4,96E-03
1153	c53377_g1	167	141	0	-9,698	5,31E-03
1154	c39309_g1	200,14	111,78	0	-9,696	5,31E-03
1155	c53094_g7	106	193	0	-9,694	5,31E-03
1156	c50542_g1	161,27	145	0	-9,692	5,31E-03
1157	c52101_g1	139,74	162,99	0	-9,691	5,31E-03
1158	c47196_g1	128	173	0	-9,689	5,31E-03
1159	c53375_g1	181,99	126	0	-9,688	5,31E-03
1160	c54254_g1	220	93	0	-9,687	5,31E-03
1161	c51736_g1	125	175	0	-9,686	5,31E-03
1162	c56722_g3	170,99	135	0	-9,685	5,31E-03
1163	c51193_g1	111,95	185,92	0	-9,685	5,31E-03
1164	c38269_g1	187,09	121	0	-9,684	5,31E-03
1165	c50904_g1	195	114	0	-9,684	5,31E-03
1166	c52313_g1	145	157	0	-9,682	5,31E-03
1167	c56254_g1	191	117	0	-9,681	5,69E-03
1168	c30304_g1	200	109	0	-9,680	5,69E-03
1169	c30953_g1	179,06	127	0	-9,680	5,69E-03
1170	c53527_g1	144	157	0	-9,678	5,69E-03
1171	c55809_g5	176	129	0	-9,676	5,69E-03
1172	c53808_g2	155	147	0	-9,676	5,69E-03
1173	c50138_g1	185	121,01	0	-9,675	5,69E-03
1174	c58181_g1	147	153	0	-9,671	5,69E-03
1175	c52403_g1	197	109	0	-9,667	5,69E-03
1176	c52467_g1	198,06	107,85	0	-9,666	5,69E-03
1177	c55912_g1	156	144,06	0	-9,665	5,69E-03
1178	c56692_g1	195	110	0	-9,663	6,11E-03
1179	c57208_g1	173	129	0	-9,663	6,11E-03
1180	c47378_g1	180,54	122	0	-9,663	6,11E-03
1181	c36438_g1	106,96	186	0	-9,662	5,69E-03
1182	c57778_g4	156,89	141,93	0	-9,659	6,11E-03
1183	c30784_g2	158	141	0	-9,658	6,11E-03
1184	c40016_g1	183	119	0	-9,656	6,11E-03
1185	c49206_g1	138,69	155,95	0	-9,650	6,11E-03
1186	c39135_g1	142,88	152	0	-9,648	6,11E-03
1187	c50501_g1	140	154	0	-9,645	6,55E-03
1188	c84745_g1	104	185	0	-9,644	6,11E-03
1189	c47662_g1	150,97	144	0	-9,642	6,55E-03
1190	c23731_g1	144	149,97	0	-9,642	6,55E-03
1191	c57257_g1	137,94	155	0	-9,641	6,55E-03
1192	c52737_g1	147,97	146	0	-9,639	6,55E-03
1193	c54488_g1	174	122	0	-9,631	6,55E-03
1194	c54611_g1	122,07	167,05	0	-9,631	6,55E-03
1195	c45117_g1	151,73	141	0	-9,631	6,55E-03
1196	c52880_g2	145	147	0	-9,630	6,55E-03
1197	c52241_g1	87,84	196	0	-9,629	6,55E-03
1198	c109657_g1	135,98	154	0	-9,626	7,03E-03
1199	c42425_g1	123	164,99	0	-9,625	7,03E-03
1200	c42588_g1	110	175	0	-9,618	7,03E-03

1201	c27304_g1	208	89,99	0	-9,617	7,03E-03
1202	c54978_g1	155,01	135	0	-9,613	7,03E-03
1203	c48768_g1	28,97	244	0	-9,612	6,55E-03
1204	c54765_g3	112	172	0	-9,611	7,03E-03
1205	c55659_g1	172,99	119	0	-9,610	7,55E-03
1206	c42232_g1	185	108	0	-9,607	7,55E-03
1207	c15822_g1	143	144	0	-9,605	4,63E-03
1208	c57559_g1	159	130	0	-9,604	4,63E-03
1209	c44831_g2	69,92	207	0	-9,604	4,30E-03
1210	c46562_g1	174,83	116	0	-9,604	4,63E-03
1211	c54339_g3	119	164	0	-9,601	4,63E-03
1212	c51358_g4	212	83	0	-9,598	4,63E-03
1213	c53117_g1	153,99	133	0	-9,597	4,63E-03
1214	c50650_g1	177	113	0	-9,597	4,63E-03
1215	c54247_g1	136,7	147	0	-9,593	4,63E-03
1216	c24805_g1	167	121	0	-9,593	4,99E-03
1217	c43052_g1	145	140	0	-9,593	4,99E-03
1218	c57993_g1	167,98	120,08	0	-9,593	4,99E-03
1219	c53439_g1	146,99	137,87	0	-9,592	4,99E-03
1220	c32858_g1	155	131	0	-9,591	4,99E-03
1221	c49559_g1	149	136	0	-9,590	4,99E-03
1222	c33261_g1	159,53	126,47	0	-9,587	4,99E-03
1223	c55023_g1	86	190	0	-9,587	4,99E-03
1224	c13933_g1	143,99	138,62	0	-9,583	4,99E-03
1225	c54013_g1	100,96	176	0	-9,582	4,99E-03
1226	c43343_g2	161	124,19	0	-9,581	4,99E-03
1227	c51845_g2	168,99	117	0	-9,581	4,99E-03
1228	c55990_g1	191,97	96,96	0	-9,580	4,99E-03
1229	c52069_g1	140	142	0	-9,580	4,99E-03
1230	c52030_g1	87,92	187,21	0	-9,580	4,99E-03
1231	c34702_g1	136	144	0	-9,572	5,37E-03
1232	c56510_g1	161	122,23	0	-9,570	5,37E-03
1233	c33638_g1	109	167	0	-9,570	5,37E-03
1234	c53862_g1	192	95	0	-9,569	5,37E-03
1235	c51316_g1	175,99	108	0	-9,565	5,37E-03
1236	c53479_g1	170,98	111,6	0	-9,563	5,37E-03
1237	c46120_g1	169,25	112,99	0	-9,559	5,37E-03
1238	c45690_g1	129,15	145,88	0	-9,550	5,79E-03
1239	c55094_g2	137,25	139	0	-9,549	5,79E-03
1240	c55939_g3	123	151	0	-9,549	5,79E-03
1241	c55038_g1	164,89	114,49	0	-9,545	5,79E-03
1242	c52815_g1	159,28	118,97	0	-9,544	5,79E-03
1243	c50029_g1	197	86	0	-9,544	5,79E-03
1244	c46894_g1	96	173	0	-9,541	5,79E-03
1245	c53476_g1	172	107	0	-9,540	5,79E-03
1246	c26573_g1	136	137,7	0	-9,539	5,79E-03
1247	c34714_g1	159	118	0	-9,538	5,79E-03
1248	c19430_g1	138	136	0	-9,537	6,24E-03
1249	c42021_g1	137,13	136	0	-9,532	6,24E-03
1250	c38351_g1	151,83	123	0	-9,532	6,24E-03
1251	c53337_g1	168	108,99	0	-9,532	6,24E-03
1252	c50986_g2	124	147	0	-9,531	6,24E-03
1253	c55474_g1	162,99	113	0	-9,530	6,24E-03
1254	c57420_g1	135	137	0	-9,528	6,24E-03
1255	c56727_g1	123	147	0	-9,526	6,24E-03
1256	c122580_g1	168,92	107,21	0	-9,525	6,24E-03
1257	c57042_g1	42	216,97	0	-9,525	5,79E-03
1258	c10879_g1	207,01	73	0	-9,519	6,24E-03
1259	c56282_g1	125	143,65	0	-9,519	6,24E-03
1260	c48536_g1	177,99	98	0	-9,518	6,74E-03
1261	c50915_g1	222	59	0	-9,513	6,74E-03
1262	c56989_g1	146,99	122,97	0	-9,508	6,74E-03
1263	c53726_g1	133,97	134	0	-9,506	6,74E-03
1264	c56772_g1	74,7	185	0	-9,505	6,74E-03
1265	c55348_g1	121	144,83	0	-9,505	6,74E-03
1266	c26695_g1	101	162	0	-9,503	6,74E-03
1267	c53478_g1	128	138	0	-9,499	7,27E-03
1268	c33554_g2	136,93	130	0	-9,498	7,27E-03
1269	c57244_g1	166,59	103,96	0	-9,498	7,27E-03
1270	c42490_g1	102	159,98	0	-9,496	7,27E-03
1271	c49452_g1	74,92	182,97	0	-9,494	6,74E-03
1272	c49458_g1	164,98	105	0	-9,494	7,27E-03
1273	c54341_g1	164,85	105	0	-9,494	7,27E-03
1274	c44767_g1	158,04	111	0	-9,493	7,27E-03
1275	c40268_g1	144	123	0	-9,493	7,27E-03
1276	c55715_g1	160,48	108,89	0	-9,492	7,27E-03
1277	c52105_g1	130,99	134	0	-9,491	7,27E-03
1278	c55514_g1	88	171	0	-9,490	7,27E-03
1279	c57045_g1	114	148,03	0	-9,487	7,27E-03
1280	c50007_g1	159	108,99	0	-9,487	7,27E-03
1281	c49617_g1	167,88	101,01	0	-9,486	7,27E-03
1282	c45487_g1	147	119,18	0	-9,484	7,27E-03
1283	c51359_g1	152	114	0	-9,481	7,85E-03
1284	c53019_g1	159,94	106,66	0	-9,480	7,85E-03
1285	c57236_g1	103	155,62	0	-9,478	7,27E-03
1286	c30880_g1	118	143	0	-9,478	7,85E-03
1287	c52057_g1	147,74	117	0	-9,478	7,85E-03
1288	c47485_g2	178,76	89,89	0	-9,477	7,85E-03
1289	c57592_g3	159,98	106	0	-9,474	7,85E-03
1290	c56458_g1	125,99	135	0	-9,472	7,85E-03
1291	c55628_g2	171,38	96	0	-9,472	7,85E-03
1292	c56341_g1	104	154	0	-9,471	7,85E-03
1293	c53204_g1	164,89	101	0	-9,470	7,85E-03
1294	c24345_g1	129	132,04	0	-9,469	7,85E-03
1295	c39162_g1	125	135	0	-9,466	7,85E-03
1296	c54326_g1	141,38	120,99	0	-9,466	7,85E-03
1297	c50903_g1	136	123,98	0	-9,458	8,49E-03
1298	c10085_g1	129,99	129	0	-9,457	8,49E-03
1299	c42539_g2	110,92	145	0	-9,454	8,49E-03
1300	c57796_g2	108	147	0	-9,450	8,49E-03
1301	c51282_g3	130,92	127	0	-9,450	8,49E-03
1302	c46442_g1	115,99	140	0	-9,450	8,49E-03
1303	c34124_g1	143	115,99	0	-9,446	8,49E-03
1304	c54050_g1	8	233	0	-9,446	7,27E-03
1305	c56461_g1	137	120,66	0	-9,445	8,49E-03
1306	c48586_g1	115	140	0	-9,445	8,49E-03
1307	c56931_g1	118,01	137	0	-9,442	8,49E-03
1308	c31076_g1	97	155	0	-9,441	8,49E-03
1309	c44533_g1	97	154,97	0	-9,441	8,49E-03
1310	c57181_g1	152,95	106	0	-9,438	9,18E-03
1311	c51159_g1	164	96	0	-9,435	9,18E-03
1312	c31842_g1	120,93	132,97	0	-9,434	9,18E-03
1313	c57800_g1	156,65	101	0	-9,429	9,18E-03
1314	c51458_g1	164,99	94	0	-9,428	9,18E-03
1315	c52428_g1	113,83	138,32	0	-9,427	9,18E-03
1316	c47521_g1	160	98	0	-9,426	9,18E-03
1317	c54872_g2	109,84	141,11	0	-9,424	9,18E-03
1318	c55066_g1	163	94,52	0	-9,424	9,18E-03
1319	c58111_g1	171,99	86,99	0	-9,423	9,18E-03
1320	c57565_g3	149,96	106,3	0	-9,422	9,18E-03

1321	c7777_g1	151,19	105,03	0	-9,422	9,18E-03
1322	c23688_g1	151	104,95	0	-9,422	9,18E-03
1323	c46019_g1	144	111	0	-9,421	9,18E-03
1324	c55766_g1	40	201	0	-9,420	9,18E-03
1325	c53942_g1	140	114	0	-9,418	9,93E-03
1326	c54701_g1	83	163	0	-9,416	9,18E-03
1327	c54616_g1	106	142	0	-9,409	9,93E-03
1328	c57094_g2	115	134	0	-9,408	9,93E-03
1329	c55989_g2	161	94	0	-9,407	9,93E-03
1330	c57233_g1	144,4	108	0	-9,403	9,93E-03
1331	c57039_g2	182,2	75	0	-9,402	9,93E-03
1332	c10746_g1	138	112,92	0	-9,401	9,93E-03
1333	c56106_g3	145,86	105,99	0	-9,401	9,93E-03
1334	c45294_g1	125	124	0	-9,400	9,93E-03
1335	c57887_g1	163	91	0	-9,399	1,07E-02
1336	c54437_g1	148,97	102,99	0	-9,399	1,07E-02
1337	c34719_g1	106	139,99	0	-9,397	1,07E-02
1338	c57624_g4	137,98	112	0	-9,395	1,07E-02
1339	c45412_g1	139,6	110	0	-9,394	1,07E-02
1340	c52378_g1	113,48	133	0	-9,391	1,07E-02
1341	c23207_g1	190	66	0	-9,390	1,07E-02
1342	c56445_g1	160	92	0	-9,390	1,07E-02
1343	c44560_g1	147	103	0	-9,388	1,07E-02
1344	c33984_g1	158	93	0	-9,385	1,07E-02
1345	c57083_g1	113	132	0	-9,385	1,07E-02
1346	c56031_g1	151,07	99	0	-9,384	1,07E-02
1347	c54493_g2	144	105	0	-9,384	1,07E-02
1348	c57525_g2	85	155,99	0	-9,383	1,07E-02
1349	c52884_g2	124	122,26	0	-9,382	1,07E-02
1350	c49777_g1	147	102	0	-9,382	1,07E-02
1351	c52993_g1	102,02	141,48	0	-9,381	1,07E-02
1352	c55968_g1	149,96	99	0	-9,379	1,16E-02
1353	c46969_g1	95	146	0	-9,375	1,16E-02
1354	c54870_g1	111	131	0	-9,368	1,16E-02
1355	c22919_g1	119	124	0	-9,367	1,16E-02
1356	c39254_g1	156,63	90,51	0	-9,367	1,16E-02
1357	c54049_g1	98	142	0	-9,366	1,16E-02
1358	c42321_g1	114,9	127	0	-9,364	1,16E-02
1359	c46027_g1	158	89	0	-9,360	1,16E-02
1360	c43968_g1	98	140,98	0	-9,359	1,16E-02
1361	c25322_g1	114	126,98	0	-9,359	1,16E-02
1362	c57338_g3	160,52	86,11	0	-9,357	1,26E-02
1363	c27232_g1	116	125	0	-9,357	1,26E-02
1364	c55160_g1	161,97	84,99	0	-9,356	1,26E-02
1365	c50571_g1	141,99	102	0	-9,354	1,26E-02
1366	c53541_g1	105	134	0	-9,353	1,26E-02
1367	c42623_g1	63	170	0	-9,351	1,16E-02
1368	c47541_g1	161,96	84	0	-9,350	1,26E-02
1369	c57313_g1	140	103	0	-9,349	1,26E-02
1370	c48858_g1	125	115,99	0	-9,349	1,26E-02
1371	c57466_g2	104,94	132,85	0	-9,347	1,26E-02
1372	c57530_g1	142,84	99,89	0	-9,347	1,26E-02
1373	c56322_g1	141,32	101	0	-9,342	1,26E-02
1374	c84178_g1	138,9	101,96	0	-9,337	1,26E-02
1375	c57841_g1	105,68	129,95	0	-9,333	1,37E-02
1376	c19925_g1	154	88	0	-9,331	8,21E-03
1377	c58103_g2	164,11	79	0	-9,329	8,21E-03
1378	c55043_g1	149	92	0	-9,329	8,21E-03
1379	c39594_g1	134	105	0	-9,329	8,21E-03
1380	c98332_g1	119	118	0	-9,329	8,21E-03
1381	c35450_g1	143,99	95,97	0	-9,327	8,21E-03
1382	c56080_g1	107,99	126,6	0	-9,325	8,21E-03
1383	c34576_g1	106	128	0	-9,320	8,21E-03
1384	c55673_g1	98,91	134	0	-9,320	8,21E-03
1385	c54525_g1	85	146	0	-9,319	8,21E-03
1386	c40934_g1	2	218	0	-9,319	6,95E-03
1387	c36605_g1	139,99	98	0	-9,317	8,21E-03
1388	c27926_g1	72,95	156	0	-9,316	8,21E-03
1389	c53292_g1	82,47	147,91	0	-9,315	8,21E-03
1390	c55699_g1	143	95	0	-9,314	8,93E-03
1391	c51209_g1	106,93	126	0	-9,313	8,93E-03
1392	c55000_g3	123	111	0	-9,305	8,93E-03
1393	c52130_g1	155	82,99	0	-9,304	8,93E-03
1394	c54926_g1	95	135	0	-9,303	8,93E-03
1395	c26857_g1	148,99	88	0	-9,302	8,93E-03
1396	c52884_g1	126,97	107	0	-9,302	8,93E-03
1397	c56386_g1	137	97,99	0	-9,300	8,93E-03
1398	c56382_g1	94	134,73	0	-9,298	8,93E-03
1399	c42456_g1	147	89	0	-9,298	8,93E-03
1400	c55586_g1	172	67	0	-9,296	8,93E-03
1401	c96939_g1	0	215,56	0	-9,294	7,55E-03
1402	c57957_g1	61	163,16	0	-9,293	8,93E-03
1403	c51118_g1	108	122	0	-9,292	8,93E-03
1404	c47829_g2	155,62	80	0	-9,290	9,73E-03
1405	c53806_g1	125,97	105,82	0	-9,289	9,73E-03
1406	c57561_g1	66,01	158	0	-9,289	8,93E-03
1407	c38329_g1	113	117	0	-9,288	9,73E-03
1408	c56272_g1	83	143	0	-9,287	9,73E-03
1409	c55343_g1	136	97,11	0	-9,287	9,73E-03
1410	c10553_g1	86	139,99	0	-9,285	9,73E-03
1411	c53176_g1	100	127	0	-9,279	9,73E-03
1412	c57126_g1	146	87	0	-9,278	9,73E-03
1413	c48817_g1	124	105,98	0	-9,278	9,73E-03
1414	c57392_g1	118	110,96	0	-9,276	9,73E-03
1415	c40234_g1	111,98	116	0	-9,275	9,73E-03
1416	c56299_g1	0	212,95	0	-9,274	8,21E-03
1417	c96846_g1	115	113	0	-9,272	9,73E-03
1418	c53354_g1	132	97,96	0	-9,271	9,73E-03
1419	c31243_g1	125	104	0	-9,270	9,73E-03
1420	c55069_g1	148	84,23	0	-9,270	9,73E-03
1421	c56499_g1	144	87	0	-9,267	1,06E-02
1422	c26950_g1	131	98	0	-9,265	1,06E-02
1423	c33943_g1	124	104	0	-9,264	1,06E-02
1424	c56392_g2	110	116	0	-9,263	1,06E-02
1425	c46899_g1	148	83	0	-9,263	1,06E-02
1426	c50344_g1	80	141,99	0	-9,263	1,06E-02
1427	c46214_g1	89	133,82	0	-9,262	1,06E-02
1428	c44722_g1	128	98,88	0	-9,254	1,06E-02
1429	c52265_g1	145	84	0	-9,252	1,06E-02
1430	c37891_g1	138,3	89,88	0	-9,252	1,06E-02
1431	c48410_g1	125,61	100	0	-9,249	1,06E-02
1432	c55142_g5	104	119	0	-9,248	1,06E-02
1433	c54824_g1	108,52	114	0	-9,244	1,16E-02
1434	c54431_g1	88	132	0	-9,242	1,16E-02
1435	c23742_g1	111	111,98	0	-9,242	1,16E-02
1436	c54430_g1	120,91	102,98	0	-9,240	1,16E-02
1437	c47317_g1	137	89	0	-9,239	1,16E-02
1438	c10568_g1	117,99	104,99	0	-9,235	1,16E-02
1439	c56117_g3	119,46	104	0	-9,234	1,16E-02
1440	c43737_g1	127	97	0	-9,234	1,16E-02

1441	c53395_g1	136	89	0	-9,233	1,16E-02
1442	c47506_g1	106	114,58	0	-9,232	1,16E-02
1443	c53671_g1	91	128	0	-9,232	1,16E-02
1444	c52161_g1	98,98	120,98	0	-9,232	1,16E-02
1445	c53513_g1	104	116	0	-9,227	1,16E-02
1446	c41696_g1	142	83	0	-9,227	1,16E-02
1447	c55075_g2	165,99	62	0	-9,226	1,16E-02
1448	c57339_g1	121	101,33	0	-9,226	1,16E-02
1449	c54886_g1	152	74	0	-9,225	1,16E-02
1450	c47291_g1	146	79	0	-9,224	1,16E-02
1451	c16562_g1	144	80	0	-9,218	1,26E-02
1452	c41380_g1	154	71	0	-9,216	1,26E-02
1453	c49606_g1	142	81,14	0	-9,213	1,26E-02
1454	c56204_g1	153	71	0	-9,210	1,26E-02
1455	c48799_g1	71	142	0	-9,209	1,26E-02
1456	c51228_g2	115,93	102	0	-9,202	1,26E-02
1457	c50153_g1	85,9	128,12	0	-9,202	1,26E-02
1458	c84052_g1	109	108	0	-9,201	1,26E-02
1459	c39173_g1	117	101	0	-9,201	1,26E-02
1460	c49504_g1	140	81	0	-9,201	1,26E-02
1461	c56866_g1	112	105	0	-9,199	1,38E-02
1462	c71604_g1	74	137,96	0	-9,199	1,26E-02
1463	c45720_g1	90,99	123	0	-9,197	1,38E-02
1464	c56292_g1	84,57	128	0	-9,195	1,38E-02
1465	c41198_g1	110	105,85	0	-9,193	1,38E-02
1466	c54424_g4	112	104	0	-9,192	1,38E-02
1467	c57781_g1	129,01	89	0	-9,190	1,38E-02
1468	c41973_g1	101	113	0	-9,188	1,38E-02
1469	c56087_g1	141	77,92	0	-9,186	1,38E-02
1470	c53085_g1	104	110	0	-9,185	1,38E-02
1471	c56397_g3	120,99	94,83	0	-9,183	1,38E-02
1472	c54485_g1	101	112	0	-9,180	1,38E-02
1473	c56686_g3	73	136	0	-9,178	1,38E-02
1474	c56800_g1	118,65	95,66	0	-9,178	1,38E-02
1475	c34140_g1	116	97,63	0	-9,173	1,50E-02
1476	c41767_g1	63,12	143,88	0	-9,173	1,38E-02
1477	c53583_g2	133,75	82,19	0	-9,171	1,50E-02
1478	c55132_g1	104	108	0	-9,170	1,50E-02
1479	c71328_g1	89	121	0	-9,170	1,50E-02
1480	c54641_g2	37	166	0	-9,169	1,38E-02
1481	c55655_g4	136	80	0	-9,169	1,50E-02
1482	c38325_g1	122	92	0	-9,168	1,50E-02
1483	c57075_g3	106,98	105	0	-9,167	1,50E-02
1484	c48910_g1	115	98	0	-9,167	1,50E-02
1485	c56712_g2	69,5	137	0	-9,166	1,50E-02
1486	c52174_g1	94	116	0	-9,165	1,50E-02
1487	c55778_g1	133	81,97	0	-9,164	1,50E-02
1488	c42772_g1	111	100,96	0	-9,164	1,50E-02
1489	c56865_g1	72,8	134	0	-9,164	1,50E-02
1490	c53508_g1	145	71	0	-9,160	1,50E-02
1491	c55987_g1	114,67	97	0	-9,160	1,50E-02
1492	c54398_g1	206,81	17	0	-9,159	1,38E-02
1493	c57531_g2	131	83	0	-9,159	1,50E-02
1494	c55407_g3	93	116,49	0	-9,159	1,50E-02
1495	c57649_g1	132	82	0	-9,158	1,50E-02
1496	c45595_g1	88,3	120	0	-9,156	1,50E-02
1497	c55890_g1	147	68	0	-9,151	1,50E-02
1498	c52307_g1	65	139	0	-9,149	1,50E-02
1499	c50085_g1	111	99	0	-9,149	1,64E-02
1500	c56028_g1	97,03	110,98	0	-9,148	1,64E-02
1501	c32558_g1	106,02	103	0	-9,146	1,64E-02

1502	c46715_g1	121,83	89	0	-9,146	1,64E-02
1503	c54013_g4	108	101	0	-9,144	1,64E-02
1504	c50448_g1	124,99	85,99	0	-9,143	1,64E-02
1505	c56643_g1	127,97	83	0	-9,140	1,64E-02
1506	c49448_g1	67	136	0	-9,140	1,64E-02
1507	c47095_g1	99	108	0	-9,138	1,64E-02
1508	c55224_g1	99	108	0	-9,138	1,64E-02
1509	c36776_g1	115	93,99	0	-9,138	1,64E-02
1510	c36339_g1	138	74	0	-9,138	1,64E-02
1511	c52463_g1	155	59	0	-9,136	1,64E-02
1512	c10628_g1	125	85	0	-9,135	1,64E-02
1513	c49153_g1	103	104,24	0	-9,134	1,64E-02
1514	c46230_g1	127,94	82	0	-9,132	1,64E-02
1515	c26497_g1	106,87	99,74	0	-9,131	1,64E-02
1516	c49417_g1	103	103	0	-9,127	1,64E-02
1517	c54862_g1	135,17	75	0	-9,126	1,79E-02
1518	c51814_g1	99	105,81	0	-9,123	1,79E-02
1519	c55513_g3	131	77,82	0	-9,122	1,79E-02
1520	c47867_g1	70	130,99	0	-9,122	1,64E-02
1521	c54218_g2	63	136,87	0	-9,121	1,64E-02
1522	c54210_g1	110	96	0	-9,120	1,79E-02
1523	c23655_g1	117,78	89	0	-9,120	1,79E-02
1524	c51115_g1	126	82	0	-9,119	1,79E-02
1525	c34100_g1	81	121	0	-9,119	1,79E-02
1526	c51138_g1	112	94	0	-9,118	1,79E-02
1527	c56213_g1	51,12	147	0	-9,118	1,64E-02
1528	c55362_g1	97	106,89	0	-9,118	1,79E-02
1529	c47969_g1	105	100	0	-9,118	1,79E-02
1530	c52143_g1	106	99	0	-9,117	1,79E-02
1531	c53657_g2	91	112	0	-9,116	1,79E-02
1532	c57145_g1	99,96	103,74	0	-9,115	1,79E-02
1533	c53545_g3	93	110	0	-9,114	1,79E-02
1534	c45033_g1	125	82	0	-9,113	1,79E-02
1535	c47182_g1	110	94,99	0	-9,113	1,79E-02
1536	c52862_g1	132,99	75	0	-9,113	1,79E-02
1537	c57386_g1	122	83,99	0	-9,108	1,79E-02
1538	c45387_g1	153,87	56	0	-9,107	1,79E-02
1539	c40964_g1	123,97	82	0	-9,106	1,79E-02
1540	c53490_g1	105	98	0	-9,102	1,79E-02
1541	c44053_g1	66	131,1	0	-9,096	1,79E-02
1542	c41007_g1	25	166	0	-9,091	1,79E-02
1543	c44892_g1	141,87	57	0	-9,034	1,38E-02
1544	c56120_g1	118,98	77	0	-9,034	1,38E-02
1545	c48399_g1	112	83	0	-9,033	1,38E-02
1546	c49720_g1	138,98	59,35	0	-9,029	1,38E-02
1547	c48555_g1	86,11	105	0	-9,028	1,38E-02
1548	c45362_g1	102	91	0	-9,027	1,38E-02
1549	c52969_g1	87,24	104,05	0	-9,027	1,38E-02
1550	c45708_g1	88,86	101,99	0	-9,025	1,38E-02
1551	c37106_g1	127,96	68	0	-9,024	1,38E-02
1552	c34305_g1	104,99	88	0	-9,024	1,38E-02
1553	c55277_g1	137	60	0	-9,023	1,38E-02
1554	c32215_g1	67	120	0	-9,016	1,52E-02
1555	c54228_g2	98	92,97	0	-9,015	1,52E-02
1556	c56371_g1	101	90	0	-9,012	1,52E-02
1557	c44904_g1	95	95	0	-9,011	1,52E-02
1558	c55035_g1	73	114	0	-9,009	1,52E-02
1559	c29083_g1	113	79	0	-9,008	1,52E-02
1560	c47897_g1	98	92	0	-9,007	1,52E-02

1561	c54070_g1	91,43	97,99	0	-9,007	1,52E-02
1562	c50878_g1	61	123,87	0	-9,006	1,52E-02
1563	c32455_g1	102	88	0	-9,003	1,52E-02
1564	c55220_g3	56	128	0	-9,003	1,52E-02
1565	c32480_g1	96	92,95	0	-9,001	1,52E-02
1566	c23833_g1	34,98	146	0	-9,001	1,52E-02
1567	c37504_g1	62	121,98	0	-8,997	1,52E-02
1568	c52367_g1	124	68	0	-8,996	1,52E-02
1569	c52004_g1	85,99	101	0	-8,996	1,52E-02
1570	c42127_g1	94	94	0	-8,995	1,68E-02
1571	c57306_g4	102,82	86	0	-8,994	1,68E-02
1572	c51733_g1	128,9	63	0	-8,991	1,68E-02
1573	c48513_g1	145	48,75	0	-8,990	1,68E-02
1574	c47404_g1	130	62	0	-8,990	1,68E-02
1575	c52831_g1	85,78	100	0	-8,987	1,68E-02
1576	c23039_g1	71	113	0	-8,987	1,68E-02
1577	c57624_g1	110,95	78	0	-8,985	1,68E-02
1578	c56291_g2	67	116	0	-8,983	1,68E-02
1579	c46524_g1	122	68	0	-8,982	1,68E-02
1580	c40022_g1	122	68	0	-8,982	1,68E-02
1581	c57734_g1	69	113,98	0	-8,981	1,68E-02
1582	c57653_g1	78,56	104,92	0	-8,978	1,68E-02
1583	c39927_g1	81	103	0	-8,976	1,68E-02
1584	c51189_g1	88,99	96	0	-8,976	1,68E-02
1585	c46050_g1	98	87,99	0	-8,974	1,68E-02
1586	c16657_g1	82,92	100,95	0	-8,974	1,68E-02
1587	c44092_g1	83	101	0	-8,974	1,68E-02
1588	c57382_g1	113,51	73,84	0	-8,974	1,68E-02
1589	c49610_g1	70	112	0	-8,971	1,68E-02
1590	c10169_g1	123,62	65	0	-8,971	1,68E-02
1591	c26673_g1	101,87	83,99	0	-8,970	1,68E-02
1592	c47847_g1	88	95,97	0	-8,969	1,68E-02
1593	c43940_g1	118,91	69	0	-8,968	1,68E-02
1594	c48671_g1	50	129	0	-8,968	1,68E-02
1595	c44737_g1	41,99	135	0	-8,960	1,68E-02
1596	c31616_g1	9035,99	13684,92	1	-7,926	1,98E-14
1597	c26772_g1	6914,99	1642,99	1	-6,425	4,34E-10
1598	c36796_g1	3474,71	1692	1	-5,728	3,08E-08
1599	c11124_g1	3156,57	1856	1	-5,694	3,82E-08
1600	c50761_g3	2598,92	1294	1	-5,321	3,79E-07
1601	c46036_g2	4781,66	2312,96	1,99	-5,188	2,15E-08
1602	c56113_g1	1833,91	1588,57	1	-5,165	9,71E-07
1603	c96847_g1	1074,72	2225,98	1	-5,158	1,01E-06
1604	c57332_g3	2053,93	1376,09	1	-5,154	1,04E-06
1605	c54405_g1	1924,79	1458,48	1	-5,140	9,34E-07
1606	c26670_g1	5045,9	4425,96	3	-5,053	9,09E-09
1607	c53364_g1	2342,99	3346,69	2	-4,928	1,09E-07
1608	c49675_g1	1807,74	1129,79	1	-4,927	3,31E-06
1609	c49801_g1	1527,54	1335,34	1	-4,908	3,67E-06
1610	c52007_g1	3773,78	1580,99	2	-4,775	2,80E-07
1611	c47303_g1	2882,78	2057,73	2	-4,686	4,80E-07
1612	c41162_g1	1368,74	1052,7	1	-4,659	1,27E-05
1613	c10660_g1	3892,79	1052,25	2	-4,641	6,28E-07
1614	c14192_g1	19853,08	6123,27	11	-4,582	3,55E-09
1615	c56877_g1	1680	642	1	-4,563	2,19E-05
1616	c51321_g1	925,72	1282,98	1	-4,559	2,25E-05
1617	c56973_g1	11023,9	10445,72	9,8	-4,502	7,30E-09
1618	c31126_g1	4813,79	5273,94	5	-4,420	7,30E-08
1619	c34906_g1	5929,9	4171,57	5	-4,397	8,39E-08
1620	c33259_g1	1148,99	856,72	1	-4,386	5,81E-05
1621	c109563_g1	2012,9	1891,95	2	-4,362	2,89E-06
1622	c57566_g4	1129,99	710,97	1	-4,253	1,00E-04
1623	c19814_g1	3957,96	3178,81	4	-4,225	4,85E-07
1624	c122464_g1	12809,7	12723,95	16	-4,077	4,74E-08
1625	c57020_g1	1094,96	539	1	-4,068	2,63E-04
1626	c55495_g3	4369,33	6148,87	7	-4,008	3,61E-07
1627	c47094_g1	9009,42	5977,11	10,04	-3,963	2,15E-07
1628	c53904_g1	895,81	545	1	-3,898	5,23E-04
1629	c71341_g1	1198,71	2897,77	3	-3,896	8,29E-06
1630	c37675_g1	4258,89	10137,98	11	-3,835	3,88E-07
1631	c57171_g1	7135,76	3836,95	8	-3,825	7,85E-07
1632	c53094_g4	1788,44	957,96	2	-3,824	5,33E-05
1633	c47318_g1	3759,1	4196,37	6	-3,815	1,66E-06
1634	c57475_g1	3413,96	3196,36	5	-3,801	2,87E-06
1635	c40774_g1	4776,14	5451,66	8	-3,764	1,13E-06
1636	c54734_g1	14331,96	12683,97	21	-3,759	2,43E-07
1637	c39277_g1	2158,9	2889,78	4	-3,754	7,22E-06
1638	c50896_g1	901,07	3823,24	4	-3,708	9,34E-06
1639	c52782_g1	728,99	456,95	1	-3,619	1,74E-03
1640	c50545_g1	6455,4	3974,99	9	-3,589	2,47E-06
1641	c96868_g1	4583,01	3290,85	7	-3,554	5,14E-06
1642	c43517_g1	5536,89	4314,88	9	-3,519	3,72E-06
1643	c46691_g2	2815,02	1346,83	4	-3,419	4,75E-05
1644	c56779_g4	461	532,99	1	-3,397	4,07E-03
1645	c42820_g1	1579,98	1332	3	-3,349	1,54E-04
1646	c47729_g4	7901,51	4884,88	14	-3,245	8,58E-06
1647	c53966_g2	1954,93	1628,59	3,97	-3,233	1,20E-04
1648	c46386_g1	522,83	376,99	1	-3,228	7,12E-03
1649	c15133_g1	2815,65	2486,83	6	-3,217	4,82E-05
1650	c57540_g2	1049,28	679,31	2	-3,166	1,24E-03
1651	c10634_g1	7291,94	8674,95	19	-3,161	9,53E-06
1652	c29272_g1	2151,71	1280,02	4	-3,152	1,84E-04
1653	c47773_g1	1405,98	1087,64	3	-3,121	4,57E-04
1654	c44943_g1	471,81	359,98	1	-3,118	9,86E-03
1655	c56762_g4	1403,49	1065,45	3,16	-3,104	4,92E-04
1656	c51296_g1	29748,38	25431,65	68	-3,093	5,87E-06
1657	c71319_g1	267,74	522	1	-3,092	1,07E-02
1658	c58095_g1	943,97	688,59	2	-3,091	1,57E-03
1659	c41358_g1	2380,03	1639	4,94	-3,066	1,65E-04
1660	c46881_g1	1358,69	970,82	3	-3,018	7,64E-04
1661	c50253_g1	401,92	362,99	1	-3,006	1,33E-02
1662	c37438_g1	9020,17	3879,23	17	-2,960	3,34E-05
1663	c44410_g1	5217,42	3585,95	12	-2,935	6,08E-05
1664	c51845_g4	1075,98	1091,76	3	-2,933	1,05E-03
1665	c57516_g2	4210,47	3696,89	11	-2,919	7,64E-05
1666	c52641_g1	3306,5	1739,82	7	-2,895	1,90E-04
1667	c109566_g1	12752,83	13865,97	38	-2,894	2,48E-05
1668	c10543_g1	7601,91	8893,97	25	-2,812	5,29E-05
1669	c41220_g1	927	1044,95	2,94	-2,803	1,77E-03
1670	c51099_g2	38384,64	23007,88	94	-2,760	3,58E-05
1671	c43584_g1	759,16	505,99	2	-2,718	6,73E-03
1672	c53380_g2	8404,67	9659,73	30	-2,679	9,41E-05
1673	c56418_g1	850,99	947,87	3	-2,669	3,32E-03
1674	c38993_g1	2310,3	3587,91	10	-2,665	3,19E-04
1675	c46700_g1	1805,71	585,55	4	-2,603	2,24E-03
1676	c43581_g2	9683,88	3797,88	23	-2,583	1,93E-04
1677	c52990_g1	2261,08	1215,71	6,08	-2,581	1,10E-03
1678	c42642_g1	915,93	771,45	3	-2,561	4,92E-03
1679	c27278_g1	4193,97	5668,96	18	-2,551	2,78E-04
1680	c35005_g2	962,27	636,77	3	-2,471	6,65E-03

1681	c55006_g2	1032,78	555,99	3	-2,451	7,25E-03
1682	c36468_g5	2853,51	3310,87	12	-2,450	7,26E-04
1683	c39630_g1	1934,49	2609,92	9	-2,433	1,17E-03
1684	c24424_g1	11526,36	8052,96	38	-2,426	3,09E-04
1685	c109579_g1	942,05	484,97	3	-2,294	1,30E-02
1686	c52011_g1	667,95	709	3	-2,281	1,36E-02
1687	c71688_g1	710,68	646,99	3	-2,253	1,54E-02
1688	c54058_g1	1177,49	1054,96	5	-2,232	6,69E-03
1689	c54274_g1	2306,63	1281,82	8	-2,214	3,66E-03
1690	c23686_g1	5940,84	7839,82	32	-2,203	1,04E-03
1691	c45178_g1	4008,24	2858,95	16	-2,164	2,08E-03
1692	c57583_g2	869,34	794,51	4	-2,132	1,42E-02
1693	c47898_g2	3691,69	3983,95	19	-2,099	2,40E-03
1694	c56770_g1	1541,84	1249,99	7	-2,065	8,05E-03
1695	c43562_g1	6305,48	7645,99	36	-2,046	2,08E-03
1696	c25467_g1	203397,2	59273,2	691,99	-1,943	2,10E-03
1697	c47992_g1	2057	2596,26	13	-1,933	6,83E-03
1698	c56133_g1	13009,7	9215,55	61	-1,927	2,97E-03
1699	c54123_g3	9926,98	14040	70	-1,875	3,62E-03
1700	c50790_g2	7707,93	7867,91	46	-1,842	4,81E-03
1701	c42746_g1	5048,76	8312,8	41,71	-1,777	6,61E-03
1702	c53698_g2	6782,99	4102,92	33,7	-1,732	8,65E-03
1703	c58238_g3	29046,98	32778,92	214	-1,618	9,74E-03
1704	c43857_g2	7777,98	13538,97	79	-1,543	1,51E-02
1705	c54742_g1	4291,33	2497,97	187	1,410	9,87E-03
1706	c55046_g1	867,97	501	42	1,566	7,83E-03
1707	c51812_g1	728,94	411	35	1,568	9,08E-03
1708	c55881_g1	1271,1	1321,89	82	1,578	5,01E-03
1709	c57979_g1	381	354,99	24	1,628	1,00E-02
1710	c52989_g1	1849,78	1419,81	110	1,684	2,43E-03
1711	c56584_g3	850	645,25	53	1,760	2,36E-03
1712	c57747_g1	201	154,99	13	1,801	1,30E-02
1713	c37995_g1	791,49	479,76	46	1,801	2,11E-03
1714	c24300_g1	499,89	436,98	35	1,827	2,45E-03
1715	c54779_g3	195	867	44	1,902	1,25E-03
1716	c56952_g1	1819,82	1095,96	118	1,963	4,06E-04
1717	c42736_g1	476,94	374,17	35	1,972	1,11E-03
1718	c58134_g1	1111,69	560,98	69	2,000	4,45E-04
1719	c23656_g1	128,98	127,85	11	2,016	8,24E-03
1720	c57605_g1	162	360	25,94	2,195	5,35E-04
1721	c54682_g3	189	140	16	2,216	1,59E-03
1722	c51910_g1	2661,98	2363,76	251	2,246	3,82E-05
1723	c53268_g1	500,91	449,84	48	2,260	1,17E-04
1724	c54463_g2	582,11	426,54	54	2,356	5,12E-05
1725	c42361_g1	329,9	367	38	2,360	9,19E-05
1726	c54468_g2	86	141	13	2,411	1,35E-03
1727	c42782_g1	194,99	191	22	2,430	2,18E-04
1728	c57484_g1	128	216	20	2,431	3,14E-04
1729	c39298_g1	612,98	326	54	2,476	2,17E-05
1730	c49219_g1	108,95	79	11	2,482	1,57E-03
1731	c51652_g2	498	308	47	2,488	2,52E-05
1732	c39199_g1	1311,59	563,66	111	2,528	6,05E-06
1733	c31940_g1	435,9	409,99	52	2,542	1,37E-05
1734	c57284_g1	283,67	280,73	35	2,550	3,01E-05
1735	c49653_g1	222	198,99	26	2,551	6,63E-05
1736	c56315_g3	100	111,26	14	2,642	3,55E-04
1737	c84190_g1	356	242	40	2,681	8,66E-06
1738	c38882_g1	198	180,49	27	2,760	1,58E-05
1739	c27267_g1	276,69	332,83	45	2,792	2,91E-06
1740	c51872_g1	576,53	668,97	97	2,871	3,73E-07
1741	c55776_g1	1	80	7	2,941	3,82E-05
1742	c19787_g1	658,91	339,68	80	2,955	2,29E-07
1743	c50415_g1	235,95	350	49	2,962	5,90E-07
1744	c71572_g1	176	251	36	2,975	1,28E-06
1745	c53266_g1	709,09	496,14	101	3,005	1,05E-07
1746	c52251_g1	144	168,25	27	3,023	2,81E-06
1747	c55656_g1	73	84	14	3,067	5,41E-05
1748	c46247_g1	56	76	12	3,086	1,26E-04
1749	c53438_g1	971	1014,88	180,71	3,104	2,33E-08
1750	c53257_g1	70	69	13	3,143	5,83E-05
1751	c35299_g1	386,3	413	75	3,146	4,69E-08
1752	c53083_g1	1000,8	809,96	175	3,203	9,20E-09
1753	c53144_g1	155	118	27	3,238	5,72E-07
1754	c54457_g1	467	705	120	3,253	8,36E-09
1755	c54646_g1	840,19	556,54	139	3,256	6,72E-09
1756	c50577_g1	2531,84	2251,97	483	3,261	2,89E-09
1757	c53629_g1	258	144,99	42	3,330	4,02E-08
1758	c51080_g1	126	70	21	3,368	1,04E-06
1759	c51692_g1	188	221	46	3,401	1,65E-08
1760	c14005_g2	4035	2302,52	711	3,437	4,12E-10
1761	c45564_g1	346,72	295,99	74	3,452	2,69E-09
1762	c84079_g1	539,71	305,93	98,33	3,483	1,10E-09
1763	c54144_g1	132,72	33	19	3,494	8,71E-07
1764	c54397_g1	181	224	49	3,504	5,28E-09
1765	c53532_g3	265,75	226,83	62	3,579	1,27E-09
1766	c52653_g1	131	150	36	3,590	8,63E-09
1767	c50506_g1	312,98	173,21	64	3,668	4,92E-10
1768	c33810_g2	33	26	8	3,684	1,40E-04
1769	c49686_g1	27,17	24,12	6,91	3,695	2,72E-04
1770	c31520_g1	71,57	79,98	21	3,700	1,20E-07
1771	c51512_g1	157,91	125	45	3,921	1,67E-10
1772	c52696_g1	53	41	15	3,923	5,77E-07
1773	c54042_g1	119,99	110,98	37	3,923	4,48E-10
1774	c57790_g3	194	184,46	62	3,957	2,79E-11
1775	c55244_g1	540,95	558,99	180,73	3,957	3,08E-12
1776	c51276_g18	357	369	130,99	4,090	1,09E-12
1777	c36300_g1	35	29	12	4,150	1,44E-06
1778	c39444_g1	65,44	15,39	15,87	4,292	3,84E-08
1779	c10762_g2	306,79	200,89	109	4,364	7,22E-14
1780	c48522_g1	111	145	57	4,381	4,92E-13
1781	c43382_g2	15	2	4	4,489	1,70E-03
1782	c52058_g2	118	73	45	4,499	6,00E-13
1783	c49047_g1	202	171	92	4,551	1,34E-14
1784	c56291_g1	164,83	132,63	81	4,694	4,71E-15
1785	c46847_g1	76,02	139,68	66	4,821	2,60E-15
1786	c56959_g1	5,42	0	2	5,169	1,31E-02
1787	c56893_g2	115	77	79	5,300	5,19E-18
1788	c32378_g1	13	98,38	52	5,395	3,45E-17
1789	c84143_g1	85	63	66	5,410	3,52E-18
1790	c47324_g1	1,99	1,8	1,84	5,445	1,31E-02
1791	c26502_g2	17	23	19	5,468	5,68E-12
1792	c46661_g1	3,49	0,9	2,69	6,038	2,91E-04
1793	c18631_g1	30	30	45	6,141	6,82E-20
1794	c55650_g1	12	16,94	23	6,203	5,49E-16
1795	c51276_g6	6	24	25,99	6,303	5,42E-17
1796	c52890_g1	2	1	2,68	6,409	2,91E-04
1797	c57109_g1	34	20	66	6,866	1,26E-24
1798	c54365_g1	8	5	19	7,085	1,67E-16
1799	c52517_g1	2	1	5	7,145	1,14E-05
1800	c39339_g1	3,98	0	9,4	7,631	1,66E-08

1801	c50124_g1	17	9	75	8,094	1,94E-30
1802	c46260_g1	6	9	46	8,140	2,23E-28
1803	c49026_g1	5	9	53	8,438	5,60E-30
1804	c55419_g1	1	10	53	8,757	5,80E-30
1805	c56514_g1	14	32	226	8,820	3,92E-37
1806	c58545_g1	0	0	1,99	9,004	6,17E-03
1807	c58307_g1	9	7	91	9,049	3,02E-34
1808	c134138_g1	0	0	2,84	9,588	5,97E-04
1809	c14276_g1	5	0	43	9,593	1,63E-27
1810	c38226_g1	2	2	43	9,868	1,71E-27
1811	c55028_g1	0	0	3,99	10,003	1,45E-04
1812	c31740_g1	0	0	4	10,003	1,45E-04
1813	c46433_g1	2	4	72	10,059	3,51E-33
1814	c37120_g1	1	18	231	10,095	6,17E-42
1815	c84017_g1	2	0	34	10,423	1,77E-24
1816	c50147_g1	14	1	221	10,454	6,92E-44
1817	c103700_g1	392	110	8815	10,757	8,54E-52
1818	c10794_g1	4	5	200	10,981	5,64E-43
1819	c58232_g1	0	0	9,97	11,324	7,17E-09
1820	c114314_g1	12	1	389	11,467	5,71E-49
1821	c77614_g1	0	0	19	12,250	2,53E-15
1822	c129600_g1	0	0	38	13,250	6,61E-26
1823	c53455_g1	0	0	45,13	13,493	5,38E-28
1824	c47749_g1	0	1	173	13,545	1,24E-41

6.5 The annotated genes from the transcriptomic analysis of testes (Table 6.4)

		B. oleae transcriptome Tissue: testes			
N	transcript_id	Annotation name	gene_id	logFC	PValue
1	c15699_g1	*dikar*	NW_013581252.1.3	8,750	3,0E-07
2	c582833_g1		not predicted	8,639	5,4E-07
3	c11986_g1	*Octopamine receptor in mushroom bodies*	NW_013581214.1.89	8,422	1,7E-06
4	c38051_g1	*mucin-2*	NW_013581220.1.46	8,354	2,5E-06
5	c52158_g4		not predicted	8,318	2,9E-06
6	c97454_g1		not predicted	8,167	6,3E-06
7	c57629_g2		not predicted	8,041	1,2E-05
8	c128061_g1		not predicted	7,856	2,8E-05
9	c52274_g1		not predicted	7,699	5,8E-05
10	c123143_g1	*CG10911-like*	NW_013581220.1.59	7,699	5,8E-05
11	c44387_g1	*CG34189-like*	NW_013583061.1.3	7,629	1,1E-11
12	c37552_g1		not predicted	7,480	3,0E-11
13	c56753_g2	*Heat shock protein 23*	NW_013581228.1.43	7,323	2,9E-04
14	c14215_g1		not predicted	7,323	2,9E-04
15	c13478_g1	*antigen 5-related 2*	NW_013581440.1.14	7,172	5,3E-04
16	c38273_g1	*CG14958-like*	NW_013581493.1.7	7,057	7,0E-12
17	c32508_g1		not predicted	6,903	1,2E-09
18	c47470_g1		not predicted	6,849	1,6E-09
19	c24782_g1	*CG34426-like*	NW_013581493.1.9	6,776	1,9E-13
20	c50402_g1		not predicted	6,698	4,1E-09
21	c36907_g1	*CG16727-like*	NW_013581259.1.32	6,597	1,3E-10
22	c45607_g1	*CG9259-like*	NW_013581453.1.8	6,544	1,1E-08
23	c84195_g1	*CG2157-like*	NW_013581212.1.127	6,544	1,1E-08
24	c44747_g1	*CG6337-like*	NW_013582004.1.1	6,323	8,6E-11
25	c51337_g1		not predicted	6,308	4,4E-08
26	c97069_g1	*uncharacterized protein F12A10.7-like*	NW_013581215.1.52	6,191	7,9E-08
27	c39724_g1	*uncharacterized protein LOC106615417*	6178_t	6,104	2,2E-13
28	c35561_g1	*location of vulva defective 1*	NW_013581251.1.58	5,839	5,8E-07
29	c25108_g1	*uncharacterized protein LOC106615433*	6172_t	5,745	5,1E-12
30	c122821_g1	*vacuolar H[+] ATPase 100kD subunit 2*	NW_013583611.1.1	5,650	1,6E-06
31	c34116_g1		not predicted	5,603	2,2E-11
32	c123047_g1	*CG31789-like*	NW_013581220.1.134	5,599	2,1E-06
33	c34524_g1	*uncharacterized protein NW_013591147.1.1*	NW_013591147.1.1	5,584	7,0E-08
34	c42518_g1		not predicted	5,468	1,4E-07
35	c122834_g1	*uncharacterized protein LOC106615425*	6177_t	5,395	5,1E-11
36	c15819_g1	*uncharacterized protein LOC106615424*	NW_013581248.1.9	5,348	1,6E-11
37	c55481_g1		not predicted	5,202	5,8E-07
38	c72383_g1	*CG8560-like*	NW_013583355.1.2	5,109	1,6E-07
39	c58415_g1	*CG15043-like*	NW_013581513.1.17	5,094	2,0E-08
40	c34152_g1	*Cyp6a16*	NW_013583451.1.1	5,070	2,3E-08
41	c15924_g1	*CG31233-like*	NW_013582303.1.1	4,989	4,7E-05
42	c48988_g3	*uncharacterized protein LOC106615425*	NW_013581248.1.11	4,926	1,6E-08
43	c54167_g1	*CG15406-like*	NW_013583385.1.2	4,811	2,7E-07
44	c39853_g1		not predicted	4,709	1,2E-09
45	c33022_g1	*uncharacterized protein LOC106615431*	NW_013581248.1.10	4,682	2,4E-07
46	c52085_g2		not predicted	4,661	2,7E-07
47	c48380_g1		not predicted	4,589	2,1E-07
48	c49725_g1	*CG4363-like*	NW_013581215.1.132	4,572	1,7E-05
49	c123043_g1	*uncharacterized protein LOC106627291*	NW_013581220.1.47	4,477	3,1E-09
50	c48988_g1	*uncharacterized protein NW_013581248.1.14*	NW_013581248.1.14	4,468	7,1E-08

51	c55436_g1	*CG31106-like*	NW_013581235.1.12	4,392	4,0E-08
52	c96078_g1		not predicted	4,360	4,9E-05
53	c53162_g1		not predicted	4,327	5,8E-05
54	c13906_g1	*CG33282-like*	NW_013581214.1.54	4,258	4,2E-08
55	c97206_g1	*CG33998-like*	NW_013581220.1.132	4,247	4,3E-08
56	c84109_g1	*CG10650-like*	NW_013581251.1.59	4,201	2,2E-05
57	c49026_g1	*beta-site APP-cleaving enzyme*	NW_013581223.1.72	4,140	3,5E-07
58	c33506_g1	*acanthoscurrin-1-like*	NW_013581221.1.16	4,110	1,6E-04
59	c45977_g1	*CG42235-like*	NW_013581576.1.7	4,091	1,3E-05
60	c42936_g2	*CG10031-like*	NW_013581471.1.3	4,086	7,0E-07
61	c123354_g1	*CG10911-like*	NW_013581220.1.59	3,983	7,6E-08
62	c36293_g1	*Cyp6a16*	NW_013583451.1.1	3,946	3,4E-04
63	c48988_g2	*uncharacterized protein LOC106615425*	NW_013581248.1.11	3,945	9,3E-07
64	c112992_g1	*CG3168-like*	NW_013583488.1.2	3,911	9,9E-07
65	c55736_g1	*CG30375-like*	19027_t	3,740	1,4E-06
66	c109541_g1	*CG15096-like*	NW_013581220.1.91	3,724	8,7E-05
67	c50185_g1	*SLC22A*	NW_013581465.1.4	3,698	5,6E-07
68	c31533_g1	*CG4363-like*	NW_013581215.1.131	3,694	5,0E-05
69	c21199_g1		not predicted	3,694	3,1E-06
70	c43247_g1		not predicted	3,606	2,3E-05
71	c32887_g1	*nucleolar protein 3-like*	NW_013581215.1.64	3,559	9,8E-05
72	c46266_g1	*black*	NW_013581211.1.19	3,528	2,2E-04
73	c110791_g1	*Cyp313a4*	NW_013581299.1.20	3,502	2,3E-06
74	c49500_g1	*tetraspanin 47F*	NW_013582559.1.4	3,497	2,6E-04
75	c39986_g1	*CG5246-like*	NW_013581294.1.10	3,482	3,3E-05
76	c48959_g1		not predicted	3,468	1,9E-06
77	c55551_g1		not predicted	3,369	4,6E-04
78	c44647_g1	*immune induced molecule 33*	NW_013581471.1.2	3,318	6,2E-05
79	c111644_g1	*CG13308-like*	NW_013581493.1.12	3,240	1,9E-04
80	c44955_g1	*CG5399-like*	NW_013581371.1.4	3,217	3,9E-05
81	c27147_g1	*Ecdysone-dependent gene 91*	NW_013581239.1.59	3,189	2,1E-05
82	c49730_g1	*urate oxidase*	NW_013581246.1.40	3,171	2,6E-05
83	c54159_g1	*CG33514-like*	NW_013581217.1.22	3,164	1,3E-05
84	c41732_g1	*serine-aspartate repeat-containing protein I-like*	NW_013581209.1.24	3,153	1,6E-04
85	c48496_g1	*CG9380-like*	NW_013583883.1.2	3,144	1,1E-05
86	c43362_g1		not predicted	3,130	8,4E-05
87	c56485_g2	*CG8303-like*	NW_013581233.1.1	3,127	4,7E-04
88	c55628_g1	*Neural Lazarillo*	NW_013581373.1.1	3,100	5,3E-04
89	c26491_g1	*uncharacterized protein LOC106624419*	NW_013582993.1.1	3,095	1,4E-05
90	c42352_g1	*CG8323-like*	NW_013581250.1.4	3,062	2,4E-05
91	c59379_g1	*alpha esterase-4*	NW_013581369.1.6	3,043	1,7E-04
92	c52144_g1		not predicted	3,043	1,7E-04
93	c57220_g4	*Ugt36Ba*	NW_013581212.1.175	3,024	4,6E-05
94	c51861_g2	*CG8834-like*	NW_013581215.1.3	2,965	7,6E-05
95	c41816_g1	*uncharacterized protein LOC106623971*	NW_013582629.1.1	2,956	7,0E-05
96	c84264_g1	*Sodium-dependent multivitamin transporter*	NW_013581377.1.15	2,924	2,1E-04
97	c34087_g1	*CG5096-like*	NW_013581248.1.32	2,885	4,1E-04
98	c13969_g1		not predicted	2,852	2,5E-04
99	c39639_g1		not predicted	2,791	1,0E-04
100	c26571_g1		not predicted	2,757	1,8E-04

6.6 The annotated list of genes from the transcriptomic analysis of male accessory glands with ejaculatory bulb (Table 6.5)

		B. oleae transcriptome Tissue: male accessory glands, ejaculatory bulb			
N	transcript_id	*Annotation name*	gene_id	logFC	PValue
1	c31616_g1	*attacin-A*	NW_013581217.1.68	-13,051	1,03E-16
2	c52655_g1	*CG2254-like*	NW_013581268.1.20	-12,304	1,58E-14
3	c51710_g1	*CG31729-like*	NW_013581210.1.33	-12,110	5,89E-14
4	c47341_g2	*CG31798-like*	NW_013581459.1.12	-11,860	3,11E-13
5	c47596_g1	*CG10096-like*	NW_013581245.1.66	-11,831	3,79E-13
6	c52892_g1	*CG10435-like*	NW_013581672.1.3	-11,801	4,63E-13
7	c57023_g2	*timeless*	NW_013581222.1.24	-11,708	8,58E-13
8	c23397_g1	*CG4666-like*	NW_013581218.1.116	-11,707	8,58E-13
9	c47442_g1	*sorting nexin 3*	NW_013581987.1.5	-11,700	8,94E-13
10	c53574_g1	*CG7840-like*	NW_013588004.1.1	-11,667	1,11E-12
11	c40374_g1	*catalase*	NW_013581552.1.5	-11,645	1,30E-12
12	c47533_g1	*Nuclear protein localization 4*	NW_013585174.1.1	-11,578	2,03E-12
13	c55275_g2	*CG43693-like*	NW_013581324.1.28	-11,254	1,68E-11
14	c41928_g1	*mus81*	NW_013581254.1.18	-11,230	1,94E-11
15	c45555_g1	*CG3690-like*	NW_013584662.1.1	-11,191	2,43E-11
16	c57217_g1	*twenty four*	NW_013581628.1.11	-11,185	2,62E-11
17	c84745_g1	*Nuclear polyadenosine RNA-binding 2-RA*	NW_013581231.1.68	-11,180	2,72E-11
18	c57024_g2	*Na+/H+ hydrogen exchanger 2*	NW_013581246.1.67	-11,153	3,24E-11
19	c53204_g1	*CG3394-like*	NW_013581407.1.1	-11,101	4,53E-11
20	c53812_g1		not predicted	-11,098	4,63E-11
21	c57131_g1	*CG34408-like*	NW_013582394.1.4	-11,024	7,44E-11
22	c39257_g2	*CG3420-like*	NW_013581226.1.64	-11,006	8,27E-11
23	c10660_g1	*Tetraspanin 42Ee*	NW_013581220.1.29	-10,976	1,00E-10
24	c47311_g1	*Gustatory receptor 32a*	NW_013581242.1.18	-10,965	1,07E-10
25	c72530_g1		not predicted	-10,960	1,12E-10
26	c53055_g1	*Isoleucyl-tRNA synthetase*	NW_013581248.1.21	-10,948	1,22E-10
27	c55859_g1	*galaktokinase*	NW_013583755.1.2	-10,922	1,43E-10
28	c39648_g1	*CG7322-like*	NW_013581236.1.21	-10,914	1,50E-10
29	c57875_g1		not predicted	-10,907	1,57E-10
30	c53746_g1	*Imaginal disc growth factor 3-RB*	NW_013581222.1.28	-10,849	2,27E-10
31	c58086_g1	*domino*	13374_t	-10,834	2,50E-10
32	c54574_g1	*juvenile hormone epoxide hydrolase 2*	NW_013581209.1.139	-10,788	3,34E-10
33	c43349_g1	*c43349_g1*	18614_t	-10,772	3,69E-10
34	c55965_g3	*Dopamine/Ecdysteroid receptor-RA*	NW_013581302.1.11	-10,762	3,97E-10
35	c54053_g5	*Signal-transducer and activator of transcription protein at 92E*	NW_013581294.1.27	-10,746	4,39E-10
36	c52465_g1	*Guanine nucleotide exchange factor in mesoderm*	NW_013581692.1.2	-10,683	6,46E-10
37	c55810_g1	*CG3376-like*	NW_013581229.1.55	-10,667	7,18E-10
38	c33324_g1	*Small ribonucleoprotein particle protein SmB*	NW_013581247.1.17	-10,650	7,99E-10
39	c54844_g2	*Glucose transporter 1*	NW_013581411.1.14	-10,645	8,21E-10
40	c26099_g1	*CG30008-LIKE*	NW_013581852.1.2	-10,644	8,43E-10
41	c58149_g1	*expanded*	NW_013581242.1.89	-10,630	9,14E-10
42	c51872_g1	*CG43143-like*	NW_013582061.1.2	-10,620	9,66E-10
43	c30784_g2	*Methylthioadenosine phosphorylase*	NW_013581238.1.9	-10,593	1,14E-09
44	c10274_g1	*echinoid*	NW_013581222.1.2	-10,574	1,31E-09
45	c56997_g1	*Gustatory receptor 21a*	NW_013584380.1.1	-10,563	1,39E-09
46	c32538_g1	*CG12321-like*	NW_013581448.1.7	-10,544	1,55E-09
47	c54799_g1	*Cysteine string protein*	NW_013581269.1.4	-10,532	1,69E-09
48	c57176_g2	*wide awake*	NW_013581262.1.13	-10,527	1,74E-09
49	c43839_g1	*Gamma-interferon-inducible reductase 1*	NW_013581310.1.15	-10,525	1,74E-09
50	c52007_g1	*Pyruvate carboxylase*	NW_013581314.1.23	-10,520	1,85E-09

51	c57583_g1	*Abl tyrosine kinase*	NW_013581213.1.160	-10,516	1,85E-09
52	c54018_g2	*CG17746-like*	NW_013582369.1.3	-10,500	2,08E-09
53	c54728_g1	*yellow-g*	NW_013581221.1.69	-10,490	2,14E-09
54	c49066_g1	*CG4757-like*	17950_t	-10,481	2,34E-09
55	c45487_g1	*CG5590-like*	NW_013581593.1.5	-10,480	2,34E-09
56	c47712_g1	*astray*	NW_013581245.1.45	-10,475	2,41E-09
57	c52931_g1	*CG9747-like*	NW_013581266.1.30	-10,454	2,80E-09
58	c49237_g1	*CG4266-like*	NW_013581233.1.39	-10,379	4,33E-09
59	c34297_g1	*CG7185-like*	NW_013581249.1.17	-10,372	4,61E-09
60	c57747_g1	*CG1024-like*	NW_013581280.1.32	-10,353	5,07E-09
61	c56234_g1	*split ends*	NW_013581242.1.94	-10,352	5,24E-09
62	c53020_g1	*CG8243-like*	NW_013581233.1.54	-10,341	5,41E-09
63	c56726_g1	*PTEN-induced putative kinase 1*	NW_013581231.1.28	-10,275	8,33E-09
64	c57660_g1		not predicted	-10,269	8,62E-09
65	c50703_g1	*CG40160-like*	NW_013582263.1.2	-10,253	9,55E-09
66	c50574_g1	*CG12121-like*	NW_013582327.1.2	-10,229	1,10E-08
67	c54908_g1	*CG8858-like*	NW_013581229.1.3	-10,222	1,13E-08
68	c55183_g1	*CG16935-like*	NW_013582071.1.6	-10,211	1,22E-08
69	c55990_g1	*ance-4-ra*	NW_013583092.1.3	-10,197	1,35E-08
70	c56768_g6	*CG1090-like*	NW_013582064.1.1	-10,193	1,35E-08
71	c46700_g1	*Hexosaminidase 1*	NW_013581275.1.12	-10,187	1,40E-08
72	c34700_g1	*Smad anchor for receptor activation*	NW_013581209.1.20	-10,171	1,56E-08
73	c56128_g2	*moody*	NW_013582266.1.2	-10,162	1,62E-08
74	c54701_g1	*strawberry notch*	NW_013581414.1.4	-10,148	1,81E-08
75	c47569_g1	*CG11414-like*	NW_013581215.1.108	-10,135	1,94E-08
76	c55175_g1	*Vacuolar protein sorting 24*	NW_013581374.1.3	-10,133	1,94E-08
77	c52369_g1	*CG8888-like*	NW_013581233.1.61	-10,114	2,17E-08
78	c48551_g1	*Nucleoporin 214kD*	NW_013581215.1.31	-10,098	2,43E-08
79	c71420_g1	*Phosphoenolpyruvate carboxykinase*	NW_013581568.1.4	-10,082	7,02E-09
80	c53306_g2	*ldlCp-related protein*	NW_013582073.1.8	-10,079	7,02E-09
81	c51656_g1	*CG5390-like*	NW_013581211.1.51	-10,069	7,02E-09
82	c37891_g1	*Heat shock protein cognate 20*	NW_013581285.1.42	-10,067	7,58E-09
83	c55308_g1	*odd skipped*	NW_013581667.1.1	-10,065	7,58E-09
84	c54308_g1	*CG1208-like*	NW_013581589.1.12	-10,054	8,20E-09
85	c46027_g1	*CG8417-like*	NW_013583228.1.2	-10,047	8,52E-09
86	c53094_g7	*Acid phosphatase 1*	NW_013581380.1.16	-10,046	8,52E-09
87	c56461_g1	*elbow B*	NW_013583577.1.1	-10,013	1,04E-08
88	c47034_g1	*CG5254-like*	NW_013581478.1.4	-10,009	1,08E-08
89	c56555_g3	*GluClα*	NW_013581276.1.14	-10,008	1,08E-08
90	c56397_g3	*Ryanodine receptor*	NW_013581420.1.2	-10,007	1,08E-08
91	c57800_g1	*heixuedian*	NW_013581242.1.70	-10,002	1,13E-08
92	c10628_g1	*CG4332-like*	NW_013583288.1.2	-9,996	1,17E-08
93	c36907_g1	*CG16727-like*	NW_013581259.1.32	-9,994	1,08E-08
94	c49121_g2	*Microcephalin*	NW_013581448.1.9	-9,994	1,17E-08
95	c55037_g1		not predicted	-9,987	1,22E-08
96	c43052_g1	*CG7011-like*	NW_013581241.1.24	-9,972	1,33E-08
97	c51676_g1	*SP2637-RC like*	NW_013581226.1.57	-9,972	1,33E-08
98	c46198_g1	*CG4603-like*	NW_013581228.1.2	-9,971	1,33E-08
99	c23655_g1	*CG17221-like*	NW_013581662.1.6	-9,968	1,38E-08
100	c55935_g1	*CG4896-like*	NW_013581251.1.13	-9,959	1,44E-08

6.7 The annotated list of genes from the transcriptomic analysis of female lower reproductive tract (Table 6.6)

B. oleae transcriptome					
Tissue: female lower reproductive track					
N	transcript_id	Annotation name	gene_id	logFC	PValue
1	c52845_g1	*neprilysin 2*	NW_013581321.1.34	-15,445	1,41E-14
2	c55628_g1	*Neural Lazarillo*	NW_013581373.1.1	-15,097	1,48E-13
3	c42759_g2	*troponin-C*	NW_013581215.1.48	-14,991	3,01E-13
4	c51837_g1	*cryptocephal*	NW_013583142.1.1	-14,886	6,09E-13
5	c53966_g8	*midlin fascelin*	NW_013581231.1.21	-14,761	1,05E-12
6	c44387_g1	*CG34189-like*	NW_013583061.1.3	-14,744	1,18E-12
7	c30526_g1	*Ribosomal protein S16*	NW_013581356.1.32	-14,608	2,90E-12
8	c53346_g1	*yolk protein 2*	NW_013581230.1.18	-14,608	2,90E-12
9	c47567_g2	*rudimentary-like*	NW_013581298.1.19	-14,424	9,85E-12
10	c71357_g1	*CG6426-like*	NW_013581234.1.7	-14,331	1,84E-11
11	c53746_g2	*Imaginal disc growth factor 3*	NW_013581222.1.28	-14,301	2,24E-11
12	c53092_g2	*Ornithine decarboxylase antizyme*	NW_013581383.1.6	-14,298	2,28E-11
13	c54618_g1	*starvin*	NW_013581477.1.2	-14,271	2,72E-11
14	c47917_g2	*Cyp6g1*	NW_013581325.1.20	-14,195	3,31E-11
15	c53040_g1	*Ecdysone-inducible gene L3*	NW_013582894.1.2	-14,188	3,48E-11
16	c54829_g2	*serpin42da*	NW_013581585.1.8	-14,176	3,77E-11
17	c57143_g2	*Vacuolar H+-ATPase 55kD subunit*	NW_013581338.1.3	-14,107	5,92E-11
18	c55918_g1	*CG14210-like*	NW_013581232.1.22	-14,060	8,10E-11
19	c52436_g1	*scylla*	NW_013581315.1.11	-14,005	1,16E-10
20	c21198_g1	*CG9676-like*	6587_t	-13,942	1,74E-10
21	c53599_g1	*Secreted protein, acidic, cysteine-rich*	NW_013581490.1.3	-13,940	1,76E-10
22	c51323_g1		not predicted	-13,871	2,76E-10
23	c27262_g1	*ERp60*	NW_013581448.1.6	-13,871	2,76E-10
24	c10647_g1	*ribosomal protein S17*	NW_013582719.1.1	-13,869	2,80E-10
25	c56768_g3	*CG6770-like*	3377_t	-13,867	2,85E-10
26	c45452_g1	*thin*	NW_013581209.1.155	-13,780	3,67E-10
27	c29117_g1	*defensin*	NW_013581584.1.4	-13,750	4,48E-10
28	c44594_g1	*CG7265-like*	NW_013581239.1.34	-13,725	5,25E-10
29	c58085_g1	*megalin*	NW_013581232.1.2	-13,692	6,54E-10
30	c52934_g1		not predicted	-13,678	7,10E-10
31	c57634_g1	*CG10178-like*	NW_013581223.1.78	-13,660	8,00E-10
32	c58253_g1	*cathD*	NW_013581226.1.24	-13,590	1,26E-09
33	c51907_g4	*ribosomal protein L21*	NW_013581210.1.127	-13,565	1,47E-09
34	c32492_g1	*ribosomal protein L23*	NW_013581313.1.21	-13,555	1,57E-09
35	c52931_g1	*CG9747-like*	NW_013581266.1.30	-13,521	1,96E-09
36	c23076_g1	*NADH dehydrogenase 13 kDa B subunit*	NW_013582233.1.1	-13,469	1,99E-09
37	c46559_g1	*knockdown*	NW_013581243.1.12	-13,425	2,64E-09
38	c42417_g1	*prip*	NW_013581220.1.123	-13,420	2,73E-09
39	c44954_g1	*Cuticular protein 57A*	NW_013583188.1.8	-13,352	4,22E-09
40	c44953_g1	*CG2852-like*	NW_013593435.1.1	-13,338	4,60E-09
41	c43875_g1	*Vacuolar H+-ATPase SFD subunit*	NW_013581318.1.19	-13,334	4,69E-09
42	c49340_g1	*Gelsolin*	NW_013581416.1.11	-13,307	5,58E-09
43	c54856_g2	*Ubiquinol-cytochrome c reductase core*	NW_013581287.1.15	-13,299	5,89E-09
44	c51944_g2	*refractory to sigma P*	NW_013586868.1.2	-13,296	5,99E-09
45	c50798_g2	*ADP ribosylation factor at 79F*	NW_013581336.1.12	-13,294	6,10E-09
46	c50481_g1	*Cytochrome P450-4g1*	NW_013581572.1.3	-13,239	8,61E-09
47	c10600_g2	*Ribosomal protein S14a*	NW_013581258.1.15	-13,238	8,69E-09
48	c96905_g1	*tryptophanyl-tRNA synthetase*	21294_t	-13,218	9,80E-09
49	c50073_g1	*translation elongation factor 1 alpha 2*	NW_013581377.1.9	-13,195	1,14E-08
50	c54629_g1	*Histone H4 replacement*	NW_013581368.1.23	-13,181	1,24E-08

51	c55864_g1		not predicted	-13,156	1,05E-08
52	c57594_g1	*CG42768-like*	NW_013581916.1.3	-13,155	1,06E-08
53	c54659_g1		not predicted	-13,148	1,10E-08
54	c58082_g2	*atlastin*	NW_013581457.1.10	-13,142	1,15E-08
55	c48434_g1	*Rab1*	NW_013581293.1.12	-13,126	1,27E-08
56	c84110_g1	*Heat shock protein cognate 5-RA*	NW_013581313.1.14	-13,116	1,36E-08
57	c53327_g2	*N-m-D-a receptor-associated protein*	NW_013581247.1.40	-13,059	1,94E-08
58	c56913_g5	*Protein kinase regulatory subunit type 1*	NW_013581359.1.26	-13,048	2,09E-08
59	c49653_g2	*Rho1*	NW_013581209.1.264	-13,046	2,11E-08
60	c56580_g1	*CG4276-like*	NW_013583226.1.1	-13,019	2,49E-08
61	c46189_g1	*purity of essence*	NW_013581503.1.2	-13,006	2,72E-08
62	c49765_g1	*CG9572-like*	NW_013581212.1.180	-12,984	3,12E-08
63	c55509_g7	*Argonaute 2*	NW_013581786.1.1	-12,971	3,37E-08
64	c58054_g1	*CG8177-like*	NW_013581302.1.29	-12,955	3,72E-08
65	c56851_g2	*CG13776-like*	NW_013581210.1.73	-12,929	4,35E-08
66	c46581_g1	*coro*	NW_013581849.1.8	-12,925	4,50E-08
67	c53123_g1	*Mitochondrial assembly regulatory factor*	NW_013581256.1.7	-12,895	5,39E-08
68	c55319_g1	*CG12702-like*	NW_013581246.1.53	-12,889	5,58E-08
69	c46085_g1	*CG12605-like*	NW_013581221.1.123	-12,887	5,65E-08
70	c57012_g1	*calnexin 99a*	NW_013581348.1.10	-12,879	5,91E-08
71	c57266_g3	*CG7115-like*	NW_013581251.1.23	-12,874	6,12E-08
72	c27268_g1	*Adenylosuccinate Synthetase*	NW_013581262.1.41	-12,864	4,68E-08
73	c49662_g2	*Neural conserved at 73EF*	NW_013581423.1.20	-12,851	5,08E-08
74	c52754_g1	*Gelsolin-isoform B*	NW_013581310.1.24	-12,810	6,61E-08
75	c42687_g1	*CG10576-like*	NW_013581296.1.14	-12,796	7,11E-08
76	c42956_g1	*Sterile20-like kinase*	NW_013581314.1.26	-12,793	7,29E-08
77	c27318_g1	*CG6236-like*	NW_013581321.1.8	-12,768	8,45E-08
78	c43468_g1		not predicted	-12,740	1,01E-07
79	c50627_g1	*Imaginal disc growth factor 1*	NW_013581222.1.30	-12,716	1,17E-07
80	c48875_g2	*Glutamine synthetase 2*	NW_013581268.1.16	-12,708	1,24E-07
81	c47214_g1	*Nimrod B2*	NW_013584023.1.3	-12,704	1,27E-07
82	c12311_g1	*Tudor staphylococcal nuclease*	NW_013581296.1.10	-12,693	1,35E-07
83	c54043_g1	*mushroom-body expressed*	NW_013581465.1.2	-12,667	1,58E-07
84	c55085_g1		not predicted	-12,666	1,58E-07
85	c53746_g1	*Imaginal disc growth factor 3*	NW_013581222.1.28	-12,659	1,67E-07
86	c23095_g1	*intronic protein 259*	NW_013581346.1.1	-12,645	1,81E-07
87	c46280_g1	*vacuolar H[+] ATPase PPA1 subunit 1*	NW_013581485.1.9	-12,645	1,81E-07
88	c51868_g1	*tetracycline resistance*	NW_013581225.1.87	-12,625	2,04E-07
89	c47782_g1	*sugarless*	NW_013581551.1.6	-12,617	2,16E-07
90	c54301_g1	*prolyl-4-hydroxylase-alpha SG1*	NW_013583024.1.4	-12,594	1,77E-07
91	c42523_g1	*odorant-binding protein 99c*	NW_013581311.1.25	-12,594	1,77E-07
92	c50891_g1	*Rab7*	NW_013581264.1.50	-12,590	1,82E-07
93	c57564_g1	*Hexokinase C*	NW_013581215.1.93	-12,563	2,12E-07
94	c57765_g3	*multiple ankyrin repeats single KH domain*	NW_013581297.1.9	-12,553	2,25E-07
95	c52177_g1	*CG33203-like*	NW_013581264.1.62	-12,552	2,28E-07
96	c50839_g1	*kayak*	NW_013581264.1.69	-12,536	2,52E-07
97	c52840_g1	*Akt1*	NW_013581333.1.4	-12,518	2,79E-07
98	c36177_g1	*Novel nucleolar protein 1*	NW_013581226.1.40	-12,452	4,16E-07
99	c54520_g1	*lipophorin receptor 1*	NW_013581259.1.9	-12,444	4,35E-07
100	c57577_g2	*CG8839-like*	NW_013581458.1.13	-12,417	5,15E-07

6.8 Housekeeping genes

Housekeeping genes				
	primers	sequences	Tm	Product size
male tissues	RPL19	5'-CTTCACGTACTTTATGCCTTC-3' 5'-GCAAGGGTAATGTGTTCAA-3'	55	126
female tissues	GAPDH	5'-ATGAAGGTCGTATCTAATGC-3' 5'-TAGTTGCGTGAACAGTAGTC-3'	55	115

Table 6.7: Housekeeping primers used for the qRT-PCR. The RPL19 was used for the normalization of the values obtained from qRT-PCR for male tissues and GAPDH for the normalization of the values obtained from qRT-PCR for female tissues.

7. Publications

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Olive fly transcriptomics analysis implicates energy metabolism genes in spinosad resistance

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Abstract

Background: The olive fly, *Bactrocera oleae*, is the most devastating pest of cultivated olives. Its control has been traditionally based on insecticides, mainly organophosphates and pyrethroids. In recent years, the naturalyte spinosad is used against the olive fly. As with other insecticides, spinosad is subject to selection pressures that have led to resistance development. Mutations in the α6 subunit of the nicotinic acetylcholine receptor (nAChR) have been implicated in spinosad resistance in several species (e.g., *Drosophila melanogaster*) but excluded in others (e.g., *Musca domestica*). Yet, additional mechanisms involving enhanced metabolism of detoxification enzymes (such as P450 monooxygenases or mixed function oxidases) have also been reported. In order to clarify the spinosad resistance mechanisms in the olive fly, we searched for mutations in the α6-subunit of the nAChR and for up-regulated genes in the entire transcriptome of spinosad resistant olive flies.

Results: The olive fly α6-subunit of the nAChR was cloned from the laboratory sensitive strain and a spinosad selected resistant line. The differences reflected silent nucleotide substitutions or conserved amino acid changes. Additionally, whole transcriptome analysis was performed in the two strains in order to reveal any underlying resistance mechanisms. Comparison of over 13,000 genes showed that in spinosad resistant flies nine genes were significantly over-expressed, whereas ~40 were under-expressed. Further functional analyses of the nine over-expressed and eleven under-expressed loci were performed. Four of these loci (Yolk protein 2, ATP Synthase FO subunit 6, Low affinity cationic amino acid transporter 2 and Serine protease 6) showed consistently higher expression both in the spinosad resistant strain and in wild flies from a resistant California population. On the other side, two storage protein genes (HexL1 and Lsp1) and two heat-shock protein genes (Hsp70 and Hsp23) were unfailingly under-expressed in resistant flies.

Conclusion: The observed nucleotide differences in the nAChR-α6 subunit between the sensitive and spinosad resistant olive fly strains did not advocate for the involvement of receptor mutations in spinosad resistance. Instead, the transcriptome comparison between the two strains indicated that several immune system loci as well as elevated energy requirements of the resistant flies might be necessary to lever the detoxification process.

Keywords: Insecticide tolerance, Spinosyns, Next generation sequencing, Expression analysis

Background

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera, Tephritidae) is the most important pest of cultivated olives. The female insect deposits its eggs in the olive fruit mesocarp where the developing larvae feed and grow. Furthermore, oviposition provides entry points for bacteria and fungi, increasing the consequences of damage. As a result olives either drop before maturity and become inedible or oil quality is affected [1]. More than 30% annual olive crop losses are attributed to the olive fly [2], which accounts to an economic impact of more than 800 million dollars [3].

During the last fifty years, the control of the fly has been traditionally based on chemical insecticides, mainly organophosphates (OPs) and, more recently, pyrethroids. Apart from the harmful effects of pesticides in the environment, insecticide exposure has led to the selection of resistant alleles in natural populations and the development of widespread insecticide resistance, mainly to organophosphates [4] but also to pyrethroids [5]. The mechanism of resistance to OPs has been extensively studied and has

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been attributed to target site mutations in the acetylcholinesterase (AChE). Two of these are point mutations that reside in the catalytic gorge of the enzyme [6] and a third one is a small deletion located in the carboxyl-terminal of the enzyme [7,8].

Replacement of organophosphates with other environmentally friendlier products such as spinosad, has been a trend in recent years. Spinosad belongs to the naturalyte class [9] and has demonstrated particular efficiency against the Tephritid family of insects [10]. It is derived from the bacterium *Saccharopolyspora spinosa*, and is composed of a mixture of two macrocyclic lactones, spinosyn A (50-95%) and spinosyn D (5-50%) [9]. This insecticide acts by two main routes. Firstly, by activating the nicotinic acetylcholine receptor, but at a different site from that used by nicotine and imidacloprid [11], and secondarily through the GABA receptor, but at a distinct site from that used by abamectin [12,13]. Spinosad may enter the organism by contact or through ingestion. The symptoms are limp paralysis, tremors and finally insect death [14].

Despite the relatively short history of spinosad in the marketplace, spinosad-associated resistance has been reported in many insects [15]. The first reports of spinosad resistance in the field were for the beet armyworm, *Spodoptera exigua* [16,17]. Spinosad resistance has also been reported in several other species, such as the melon fly, *Bactrocera cucurbitae* [18], the Colorado potato beetle, *Leptinotarsa decemlineata* [19], the housefly, *Musca domestica* [20] and the tobacco budworm, *Heliothis virescens* [21]. The molecular mechanism of resistance to spinosad has not been fully clarified. There is evidence that resistance is a result of either enhanced metabolism of detoxification enzymes or a consequence of changes in a target site. The most noticeable target site of spinosad resistance is the nicotinic acetylcholine receptor (nAChR). In the case of *Drosophila melanogaster*, mutations in the α6 subunit of the nAChR (Dα6) confer high-fold resistance to spinosad, clearly implicating the Dα6 subunit in resistance [22,23]. The α6 subunit of nAChR has been associated in spinosad resistance in other insects as well. For example, mis-spliced or truncated nAChR-α6 transcripts in the diamondback moth, *Plutella xylostella* [24,25], truncated *Bdα6* transcripts of *Bactrocera dorsalis* [26], or a point mutation (*G275E*) in the transmembrane domain of the nAChR-α6 subunit in the western flower thrips, *Frankliniella occidentalis* [27], all confer high levels of resistance to spinosad. In contrast, spinosad resistance in *Musca domestica* does not seem to be related with the α6 subunit of nAChR. Instead, it correlates with a recessive factor on chromosome I [20], rather than the three nicotinic acetylcholine subunits (α5, α6, β3) that reside on the same chromosome [28].

In other cases, however, enhanced metabolism of detoxification enzymes have been implicated in spinosad resistance. For example, the microsomal-O-demethylase as well as monooxygenases were shown to be involved in resistance in *Spodoptera exigua* from China [29], an increase in cytochrome P450 monooxygenase was associated in cotton bollworm, *Helicoverpa armigera* [30], while enhanced activity of detoxifying mixed-function oxidases were connected with resistance in the Chilean populations of *Tuta absoluta* [31].

Until now, the most frequently used approach for isolating genes from an organism with few genetic and molecular tools was through PCR amplification with heterologous primers of the respective genes of closely related species. This approach, however, is greatly biased and excludes the possibility of identifying either genes that do not have homology in other organisms, or loci responsible for mechanisms that have not been studied in relative species. A transcriptomics approach, instead, may assess the differences of all expressed genes at the same time between sensitive and resistant individuals, without any preconceived ideas about specific genes, and thus reveal novel mechanisms that might be involved in resistance.

In the present study, we determined the sequence of the α6 subunit of nAChR of both a sensitive and a spinosad resistant olive fly strain, in order to explore possible presence of resistance mutations. In addition, we compared the entire transcriptomes of these two strains, in search of unknown loci that might be involved in spinosad resistance. Differential expression observed in several genes was validated both in laboratory colonies and field collected flies.

Results

Cloning of the *B. oleae* nAChR subunit α6 gene

A total of 2,367 bp of the *Bactrocera oleae* nAChR α6 subunit (Boα6) cDNA sequence was obtained from a susceptible laboratory (LAB) and a spinosad-selected (SPIN) strain. Initially, the *B. dorsalis*-based primers Bdα6F and Bdα6R amplified a partial ~1,800 bp coding fragment and subsequent 5′- and 3′-RACE reactions unraveled a 5′-UTR region of 300 bp upstream the start codon and a 3′-UTR of 600 bp that ended in a poly-A tail.

The beginning of the coding sequence was determined by the presence of a methionine residue at the expected place, as compared with known sequences from *Drosophila melanogaster* and *Bactrocera dorsalis*. Upstream of that residue there was no significant ORF. The next downstream Met residue was after 467 bp that would result in a substantially truncated product. An open reading frame of 1,467 bp encodes a putative 489 amino acid protein. The putative protein has 97% identity to the reciprocal *B. dorsalis* (AFN88980.1) protein. The Boα6 has all typical nAChR α subunit characteristics (Figure 1). The mature

MDPSLLVVLIFLVIIKESCQGPHEKRLLNHLLSTYNTLERPVANESEPLEVKFGLTLQQIIDV

DEKNQLLITNLWLSLEWNDYNLRWNESEYGGVKDLRITPNKLWKPDVLMYNSADEGFDGTYHT

NIVVKHGGSCLYVPPAIFKSTCKMDITWFPFDDQHCEMKFGSWTYDGNQLDLVLSSEDGGDLS

DFITNGEWYLLAMPGKNTIYACCPEPYVDVTFTIQIRRRTLYY***FFNLIVPCVLISSMALL***GFT

LPPDSGEKLT***LGVTILLSLTVFLNLVA***ETLPQVSDAIPLIGTYFN***CIMFMVASSVVLTVVVL***N

YHHRTADIHEMPPWIKSVFLQWLPWILRMGGPGRKITRKTILLSNRMKELELKERSSKSLLAN

VLDIDDDFRHTISGSQTAIGSSASFGRPTTVEEHHNTIGCNHKDLHLILKELQFITSRMRKSD

DEAELISDWK***FAAMVVDRFCLIVFTLFTIIATVTVLLSAPH***IIVQ

Figure 1 Basic characteristics of the *Bactrocera oleae* nAChR α6 subunit. N-terminal site is presented in dashed line and it is consisted of 20 amino acids. There are four transmembrane domains (TM1-4) (bold italic letters) and three glycosylation sites (blue boxes). The YxCC motif of alpha subunits is shown in orange box and the Cystein residues in green ovals. Six ligand binding loops are underlined. The three mutations are indicated by vertical arrows.

protein has a calculated molecular weight of 55.57 kDa and an isoelectric point of 4.49. It has all the characteristics of neurotransmitter-gated ion channels, with a signature of two cysteines separated by 13 amino acids [32] and four hydrophobic transmembrane domains (TM1-4) of conserved nAChR [33]. The Boα6 protein also possesses six loops and the alpha subunit character of YxCC motif [34].

Alignment of the two cDNA sequences obtained from the LAB and SPIN strains showed 13 point mutations (Additional file 1: Table S1). Ten of them were silent substitutions, while the remaining three led to homologous missense alterations: an Alanine (A) to Glycine (G) substitution at position 142 and two Lysine (K) to Arginine (R) substitutions at positions 145 and 149. The mutations are located in the N-terminal site and cause no changes on chain polarity or charge. In fact, the protein structure prediction server [35] indicated that the molecular structure of the receptor between the sensitive and the resistant strains remained unaffected. It is also known that nAChR α6 transcript undergoes RNA editing [36-38], although this process has not thus far been related to spinosad resistance. None of the 13 point mutations of Boα6 coincided with the recognized RNA editing sites of *Drosophila melanogaster* or *Bombyx mori*.

Solid ABI sequencing and reads assembly

In order to explore possible mechanisms and pathways involved in spinosad resistance in *Bactrocera oleae*, the entire transcriptomes of the LAB and SPIN strains were compared. For transcriptome assembly, four libraries were sequenced and used. The sample names for the libraries are LAB, SPIN, MALE and FEMALE. Each library was sequenced with paired-end sequences, where each sequence pair consists of a 35 nt and a 50 nt fragment with a variable length insert between these fragments. Sequencing obtained a total of 122,623,894 read pairs. The reads of the libraries were pooled to construct a reference transcriptome assembly of 69,359 contigs using the SOAPdenovo assembler [39] (Table 1).

Sequence annotation

Annotation of the assembled sequences was obtained by aligning the 69,359 assembled *B. oleae* sequences against the NCBI non-redundant (Nr) protein database using blastx and collecting the annotations with the BLAST2GO tool [40]. Using an E-value threshold of ≤1e^{-6},

Table 1 Sequencing and assembly statistics

Sequencing and assembly summary		
Total number of paired-end reads		122,623,894
Total number of bases		10,423,030,990
LAB sample	number of paired-end reads	26,713,286
	number of bases	2,270,629,310
SPIN sample	number of paired-end reads	36,252,803
	number of bases	3,081,488,255
FEMALE sample	number of paired-end reads	36,962,061
	number of bases	3,141,775,185
MALE sample	number of paired-end reads	22,695,744
	number of bases	1,929,138,240
Large contigs (≥500 bp)		
Number of contigs		1,573
Number of bases		1,035,345
Average contig size		658
N50*		633
Largest contig size		2,301
All contigs (≥100 bp)		
Number of contigs		69,359
Number of bases		12,709,410

*Contig length, where all contigs of that length or longer sum up to at least half of the total of the lengths of all contigs.

20207 (29.13%) of the contigs were aligned. The top 19 species in these alignments are diptera. Of the 69,359 contigs, 23,042 (33.22%) have almost exact hits in the *B. oleae* transcriptome of Pavlidi et al. [41] (E-value ≤1e^{-6}).

Only synonymous SNPs in detox genes

The presence of significant SNPs or truncations in known detoxification loci was assayed in the SPIN transcriptome. One hundred and fifty five genes involved in detoxification were analyzed. SNP calling was performed with the mpileup tool [42]. There are 9 SNPs in the sensitive strain (LAB) that are not in the resistant strain (SPIN), of which only 2 have more than 10 reads and were found to be synonymous. There are 19 SNPs in SPIN that are not in the LAB, of which only 2 have more than 10 reads and were found to be synonymous.

Differentially expressed genes

The Cuffdiff [43] tool was used in order to reveal the differentially expressed genes between the spinosad resistant and the laboratory flies, a stringent cutoff (p value adjusted for multiple testing, called q value <0.05) was used. This resulted in 46 differentially expressed transcripts in the LAB vs. SPIN strain comparison.

Twelve of these transcripts were up-regulated in SPIN resistant *B. oleae* flies than in sensitive (LAB) strain. More careful analysis revealed that three of these transcripts coincided with others and, therefore, nine distinct genes of the initial set of twelve were chosen for further functional analysis by quantitative real time PCR. These genes are listed in Figure 2 and Additional file 1: Table S2. Additionally, Cytochrome *P450 6α23-like* gene, a gene belonging in a group of known detoxification genes often involved in insecticide resistance, was considered. This gene was highly over-expressed, albeit not statistically significantly, falling below the stated criteria (p value = 0.000388, q value = 0.11).

An M-A plot was also constructed for comparison of the genes for resistant vs sensitive strain with q value < 0.05. In Figure 3 the 12 up-regulated and 40 down-regulated genes in the resistant strain are depicted in red.

Finally, functional annotation was made for the assembled sequences of the significantly differential up- and down-regulated genes mentioned in Additional file 1: Table S2, based on gene ontology (GO) categorization obtained using BLAST2GO. The GO analysis performed for two main categories, molecular function and biological process, is shown in Figure 2.

Gene name	GO analysis (Biological Process)	GO analysis (Molecular Function)	InterPro (IP)
ATP synthase	ATP synthesis coupled proton transport	Hydrogen ion transmembrane transporter activity	
Antigen 5 precursor (CAP domain)			Cysteine-rich secretory protein, allergen V5/Tpx-1
Yolk protein (Vgs)	Lipid metabolic process	Catalytic activity	
Replicase			RNA virus methyltranferase
4-nitro-phenyl-phosphatase (pNPPase)	Dephosphorylation	Phosphatase activity	
Low affinity cationic amino acid transporter-2 [CAT-2]	Amino acid transmembrane transport	Amino acid transmembrane transporter activity	
Elongation factor-1 alpha		Binding, Catalytic activity	
Salivary Cys-rich secreted peptide (vWf)			von Willebrand factor domain
Serine protease 6	Proteolysis		
Cytochrome P450 6a23-like (Cyp6a23)	Oxidation-reduction process		Cytochrome P450
Polyprotein			Hypothetical protein
Protease/RNA-dependent RNA polymerase		Hydrolase activity	
Cell division cycle-associated protein 7			HAD-superfamily hydrolase
Microtubule-actin cross-linking factor 1			Viral-Sacbrood virus
Hsp 70	Response to stress	ATP binding	
LSP1 gamma		Nutrient reservoir activity	
Hsp 23	Response to stress		
Oxidase/peroxidase	Response to oxidative stress	Peroxidase activity, heme binding	
Cht5	Chitin catabolic process		
Macrophage mannose receptor 1 (MMR)		Carbohydrate binding	
Hexamerin L1		Nutrient reservoir activity	

Figure 2 Functional annotation of differentially expressed genes. Gene expression levels of the differentially expressed genes (Log2, fold change), as resulted from the RNA-seq analysis, is shown at the left part of the Figure. Gene Ontology (GO) classification of the same genes for the ontologies: Biological Process (BP), Molecular Function (MF), and Interpro (IP) protein domains, are listed at the right part of the Figure. In crimson red are the up-regulated genes. The non-statistically significantly up-regulated *Cytochrome P450 6a23-like (Cyp6a23)* is shown in lighter color. In green are the down-regulated genes.

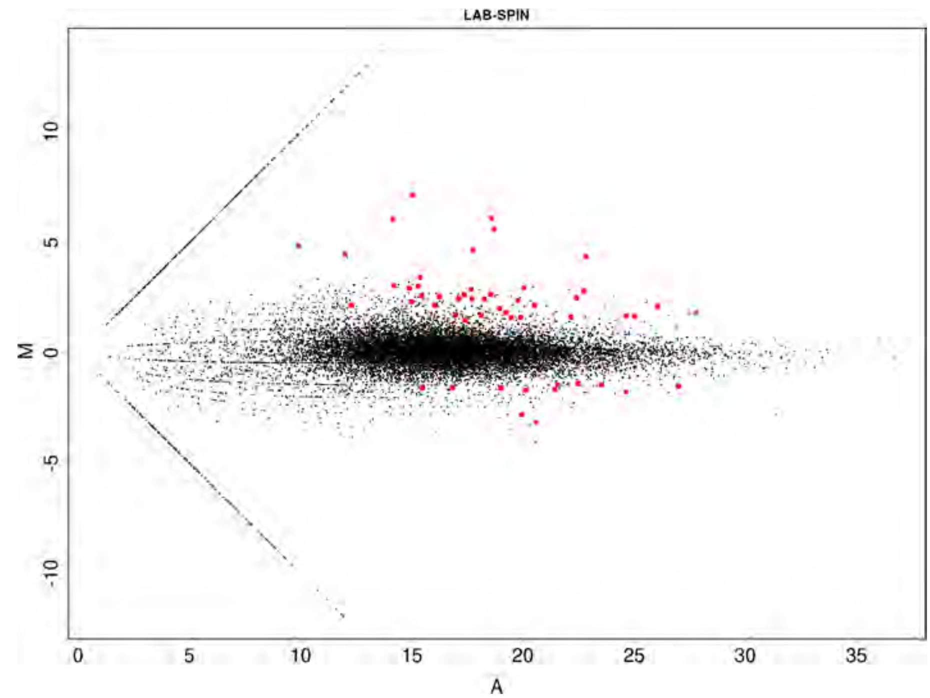

Figure 3 M-A plot of the differential expression between sensitive and resistant flies. The red color spots are the statistical significant differentially expressed genes with q-value < 0.05.

Functional analysis of genes that are putatively involved in spinosad resistance

In order to find the most suitable reference gene for the functional analyses of gene expression in the *B. oleae* head tissue, nine candidate genes were tested with NormFinder [44] and Bestkeeper [45] analysis. The nine genes were: *RPL19* (*ribosome protein L19*), *tbp* (*TATA-binding protein*), *ubx* (*ultrabithorax*), *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenease*), *α-TUB* (*α-tubulin*), *β-TUB* (*β-tubulin*), *14-3-3zeta*, *RPE* (*RNA polymerase II*) and *actin3*. Normfinder analysis showed that the best housekeeping gene is the *14-3-3 zeta* with stability value 0.027 and the best combination of two genes is *tbp* and *14-3-3 zeta* with a stability value 0.031. From most stable (lowest stability value) to least stable (highest stability value) the candidate reference genes are ranged as: *14-3-3 zeta* < *ubx* < *tbp* < *β-TUB* < *GAPDH* < *actin3* < *RPE* < *RPL19* < *α-TUB*. These results were also confirmed by the Bestkeeper software since standard deviation and coefficient of variance values of *14-3-3 zeta* and *tbp* fell within the accepted range.

Functional analysis of all significantly over- or under-expressed aforementioned genetic loci was performed in conjunction with the best combination of the two housekeeping genes (*tbp* and *14-3-3 zeta*). Separately, the expression of all the target genes was calculated by normalization with *tbp* and *14-3-3 zeta*. The final expression value for each target gene was calculated as the geometric mean of its relative expression to the two housekeeping genes. Geometric mean values, range and standard error of expression are shown in Additional file 1: Table S3. More specifically.

Up-regulated genes

Yolk protein 2 gene *(Yp2)* showed no relative expression in the sensitive flies (LAB, w-GR), while the expression in the resistant flies varied between 0.0075-5.656 and 3.265-17.178 fold for the SPIN and the w-CAL, respectively. As expected, the higher expression of spinosad resistance is observed only in female individuals, as *Yp2* is not expressed in males (Figures 4A and 5). Likewise, the relative expression of *ATP synthase F_O subunit 6* in the sensitive flies of LAB and w-GR is approximately at the same range, nearby zero. The expression values in the two resistant groups (w-CAL, SPIN) were higher (Figure 4B), while a single male individual of the SPIN strain presented a remarkably high expression value (12.124-fold). Expression of *Low affinity cationic amino acid transporter 2* was 0.399-fold and 0.328-fold in w-GR and LAB, respectively, while expression in the resistant group was significantly elevated, 2.222-fold and 1.428-fold for w-CAL and SPIN (Figure 4C). *Serine Protease 6 (SP6)* was also significantly over-expressed in SPIN (2.763-fold) compared to the LAB (0.016-fold), while the expression of the wild flies was relatively low (0.838-fold for w-GR and 0.519-fold for w-CAL) (Figure 4D). The expression of *4-nitrophenylphosphatase*

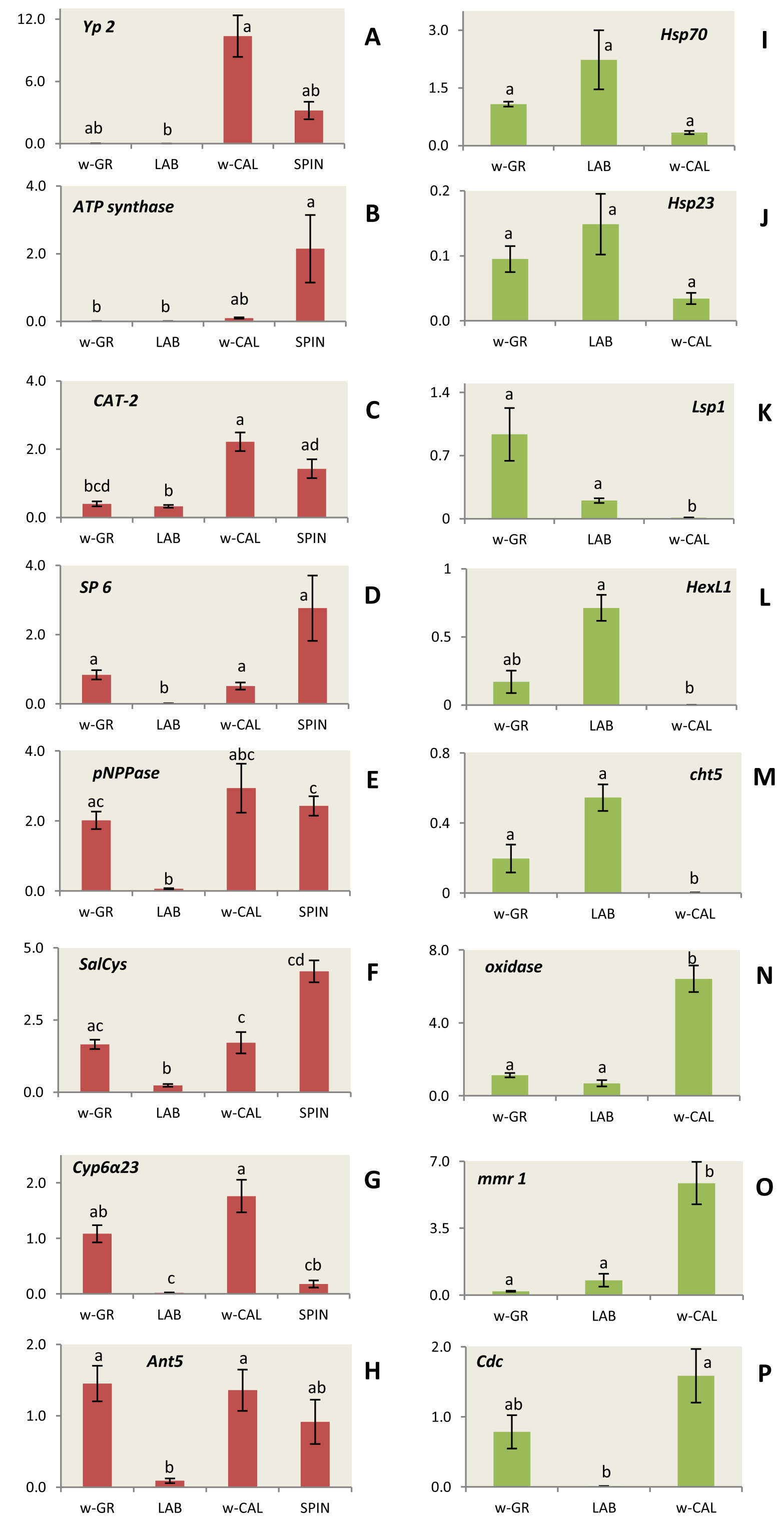

Figure 4 (See legend on next page.)

(See figure on previous page.)

Figure 4 Relative expression profiles of genes potentially associated with spinosad resistance. The red color bars represent the up-regulated genes, *Yolk protein 2* (*Yp2*, Panel **A**), *ATP synthase* F_O *subunit 6* (*ATP synthase,* Panel **B**), *Low affinity cationic amino acid transporter 2* (*CAT-2,* Panel **C**), *Serine protease 6* (*SP6*, Panel **D**), *4-nitrophenylphophatase* (*pNPPase,* Panel **E**), *Salivary Cys-rich secreted peptide*-vWF (*SalCys*, Panel **F**), *Cytochrome P450 6a23-like* (*Cyp6a23,* Panel **G**) and *Antigen 5 precursor* (*Ant5*, Panel **H**), for the mean of three male and three female individual flies, after functional analysis by qRT-PCR. Only for the *Yolk protein* the evaluation was based on female expression, since males show zero expression values. The green color bars represent the down-regulated genes *Heat-shock protein 70* (*Hsp70,* Panel **I**), *Heat-shock protein 23* (*Hsp23,* Panel **J**), Larval serum protein 1 (*LSP1,* Panel **K**), *Hexamerin L1* (*HexL1*, Panel **L**), *Chitinase 5* (*Cht5,* Panel **M**), *Oxidase/peroxidase* (*oxidase*, Panel **N**), *Macrophage mannose receptor 1* (*mmr1*, Panel **O**), *Cell division cycle-associated protein 7* (*Cdc*, Panel **P**), for the mean of three male and three female individual flies, after functional analysis by qRT-PCR. The five RNA viral genes are not included. Standard error is also depicted in the bars. Small letters next to the error bars indicate significantly different mean values estimated by pairwise comparisons (either Tukey's or Kruskal-Wallis tests). All comparisons were performed on Ln transformed data except for *macrophage mannose receptor 1*.

(pNPPase) was significantly higher in w-CAL as compared to LAB (2.937 vs 0.064), while that of w-GR was intermediate (2.016) but not significantly different from w-CAL (Figure 4E). The same pattern holds true for *Salivary Cys-rich secreted peptide* (vWF domain) and *antigen 5 precursor* (Figures 4F and 4H). Finally, for *cytochrome P450 6a23-like (Cyp6α23)* while the expression of SPIN was higher than LAB (0.179 vs 0.019) and w-CAL was higher than w-GR (1.762 vs 1.083), the differences were not statistically significant (Figure 4G).

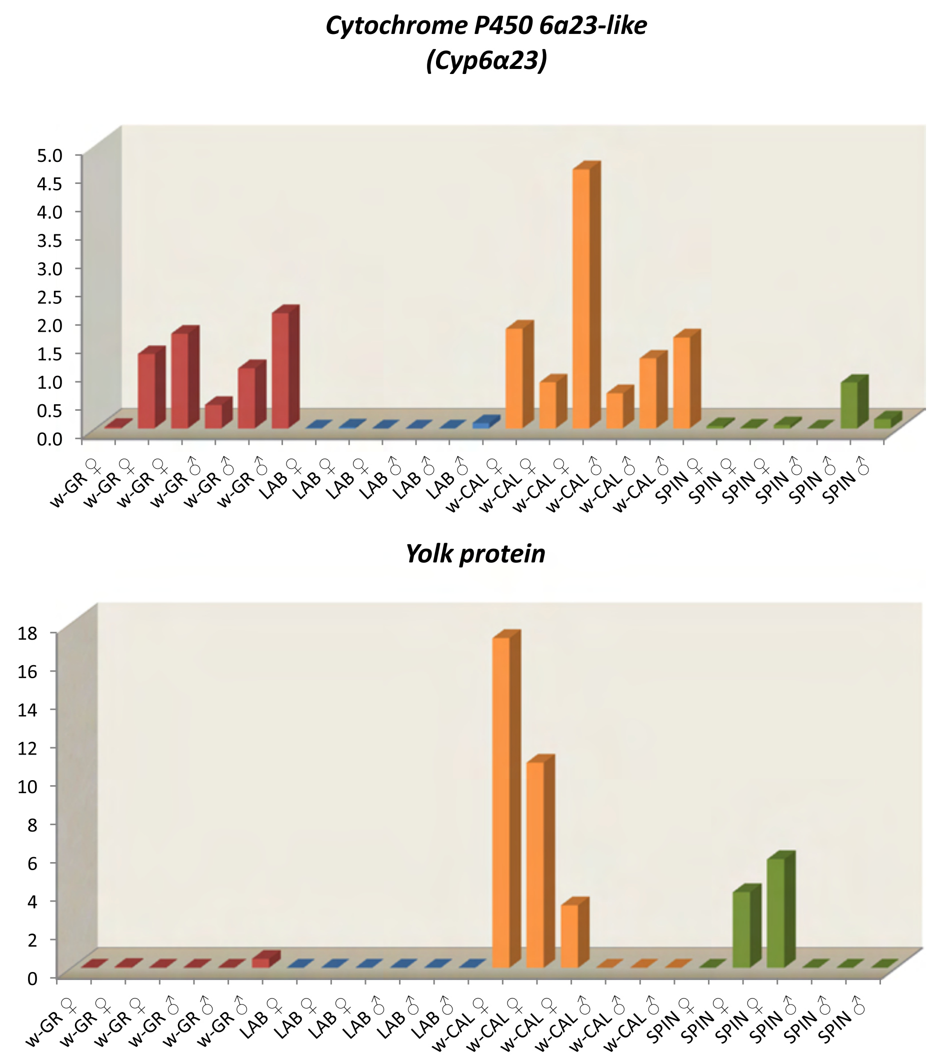

Figure 5 Relative expression of *Cyp6α23* and *Yolk protein* in individual flies. Relative expression of *Cyp6α23* **(Panel A)** and *Yolk protein 2* **(Panel B)** gene loci in the heads of individual olive flies of the w-GR (brown color bars), LAB (blue bars), w-CAL (orange bars) and SPIN (green bars) populations after functional analysis by qRT-PCR.

Down-regulated genes

Functional analysis for the down-regulated genes was performed for LAB, w-GR and w-CAL populations since our SPIN colony was no longer available. Relative expression of both *Hsp* genes was not significantly different in the various groups of flies. *Hsp70* expression was 1.082-, 2.236- and 0.337-fold for w-GR, LAB and w-CAL, respectively, while *Hsp23* expression was 0.095-, 0.149- and 0.034-fold (Figure 4I and 4J). *Larval serum protein 1 (Lsp1),* on the other hand, was significantly under-expressed in w-CAL flies as compared to both w-GR and LAB (0.012 vs 0.937 and 0.203) (Figure 4K). Similarly, *Hexamerin L1* showed higher expression in the sensitive flies (LAB: 0.713, w-GR: 0.17), while for the resistant w-CAL the expression range was 0.001 (Figure 4L). Under-expression was even more pronounced in the *chitinase 5* locus of the resistant w-CAL (0.002) compared to both w-GR (0.197) and LAB (0.545) (Figure 4M). The expression pattern of *oxidase/peroxidase* did not confirm the RNA-seq results, since expression of the resistant w-CAL was higher than that observed in the sensitive w-GR and LAB (6.148 vs 1.129 and 0.685) (Figure 4N). The same reverse pattern was observed for *Macrophage mannose receptor 1 (MMR)* (5.856 vs 0.196 and 0.776) and *cell division cycle associated protein7 (Cdc)* (1.585 vs 0.784 and 0.0102) (Figures 4O and P).

Discussion

Spinosad is a relatively new and very promising insecticide used against a variety of insect pests. As is the case with any other chemical, resistance has already developed in several natural and greenhouse populations of insects. In several cases of resistance, mutations in the α6 subunit of the nAChR were shown to be responsible, while in others this locus was shown to not be involved. Yet, general detoxifying systems have also been implicated. In order to understand the mechanism of spinosad resistance in the olive fly, we both looked for mutations in the *Boα6* nAChR as well as searched the entire transcriptome for potential new loci involved in resistance.

Firstly, the *Boα6* cDNA from the olive fly *Bactrocera oleae* was identified and characterized. The deduced amino acid sequence presented very high similarity with α6 subunits of other diptera and retained typical subunit characteristics with the nAChR homologs. Comparison of the Boα6 between the laboratory sensitive (LAB) and spinosad-resistant (SPIN) strains yielded three homologous amino acid substitutions. This fact most likely excludes the involvement of Boα6 nAChR in resistance, at least under the conditions of the experiment. Indeed, it should be pointed out that all published reports that implicate α6 nAChR subunit in spinosad resistance, the resistance level of the organism is considerably high: ~1200-fold in *D. melanogaster* [22], >2000-fold in *B. dorsalis* [26], >350,000-fold in *F. occidentalis* [27], 1070-fold in *H. virescens* [21], 18,600-fold in *P. xylostella* [24,25]. On the contrary, lower levels of resistance are associated with mechanisms that do not involve target site resistance but, rather, more generalized detoxification systems. This is the case, for example, of *M. domestica* (~150-fold; [20]), *H. armigera* (20-fold; [30]), *S. exigua* (~350-fold; [29]), *T. absoluta* (1.8 to 4.6-fold; [31]) and *B. oleae* (~35-fold; this study).

Secondly, in our efforts to shed light on other possible mechanisms of resistance, we compared the entire transcriptomes of a laboratory sensitive (LAB) and a spinosad resistant (SPIN) strain through RNAseq. During the course of our study, Pavlidi and co-workers produced a basic transcriptome dataset for *B. oleae* using 454 pyrosequencing [41]. Due to the different sequencing technology used in the present study, our reference transcriptome has fewer long contigs but a significantly higher number of total contigs and contigs with alignments (Table 1), which is more relevant for the purpose of detecting differentially expressed genes. Our comparative LAB vs SPIN analysis yielded several over- and under-expressed loci that are discussed below. Two caveats should be added at this point. Firstly, since LAB and SPIN transcriptomes were sequenced only once, we ought to validate the observed differences through qRT-PCR in multiple samples. In order to ascertain that the observed differences reflected differences that would hold true not only under laboratory but also under natural conditions, we decided to extend our validation experiments in natural spinosad-sensitive and spinosad-resistant populations. As such, wild flies were collected from a presumably untreated orchard in the surroundings of the city of Volos (Greece) (w-GR), where there is no documented use of spinosad and from a site in Sonoma County in California (w-CAL) with the highest documented naturally observed spinosad-resistance ratio (see Methods). However, resistance bioassays showed that while the resistance ratio (RR) of the SPIN strain was ~35, w-CAL and w-GR had RRs 12.96 and 3.14, respectively. Therefore, w-GR cannot be considered as a source of truly spinosad sensitive flies. Indeed, the expression of various genes was shown to be intermediate between LAB and SPIN values. Secondly, the presence of different resistance mechanisms in the laboratory or naturally selected flies cannot be excluded. While this is plausible, we doubt it for three main reasons: One, the genetic background of the SPIN strain and the w-CAL is similar since the SPIN strain was enriched by w-CAL; two, the selective force used in the laboratory was the same as the one used in California (i.e., spinosad); and, three, the difference between w-CAL RR and SPIN RR (~13 vs ~35) is not that large to indicate the presence of a different mechanism. As stated earlier, usually high

RR levels are associated with target-site resistance while lower RR levels are associated with more generalized detox mechanisms. Be that as it may, and even if more resistance mechanisms are at play, our analysis should point towards their underlying common ground. And since spinosad selection is common between SPIN and w-CAL flies, any transcriptome differences with LAB (and partly w-GR) should indicate events involved in spinosad resistance.

Potential interactions between the up- and down-regulated genes were examined through STRING (Search Tool for the Retrieval of Interacting Genes). STRING makes available precomputed results in predicted functional linkages among proteins by comparative genomics and text-mining [46]. STRING analysis using the MCL clustering algorithm yielded several links between the examined differentially expressed genes (Figure 6). The generated network supports the hypothesis of non-randomness in the results and rather reflects a regulation feature by both activator genes (the up-regulated expression) and maybe also repressor genes (down-regulated expression). However, for the characterization of the transcriptional regulatory network and the understanding of these genes dynamic association and their possible involvement in insecticide resistance, we should first consider their function and their well-documented role.

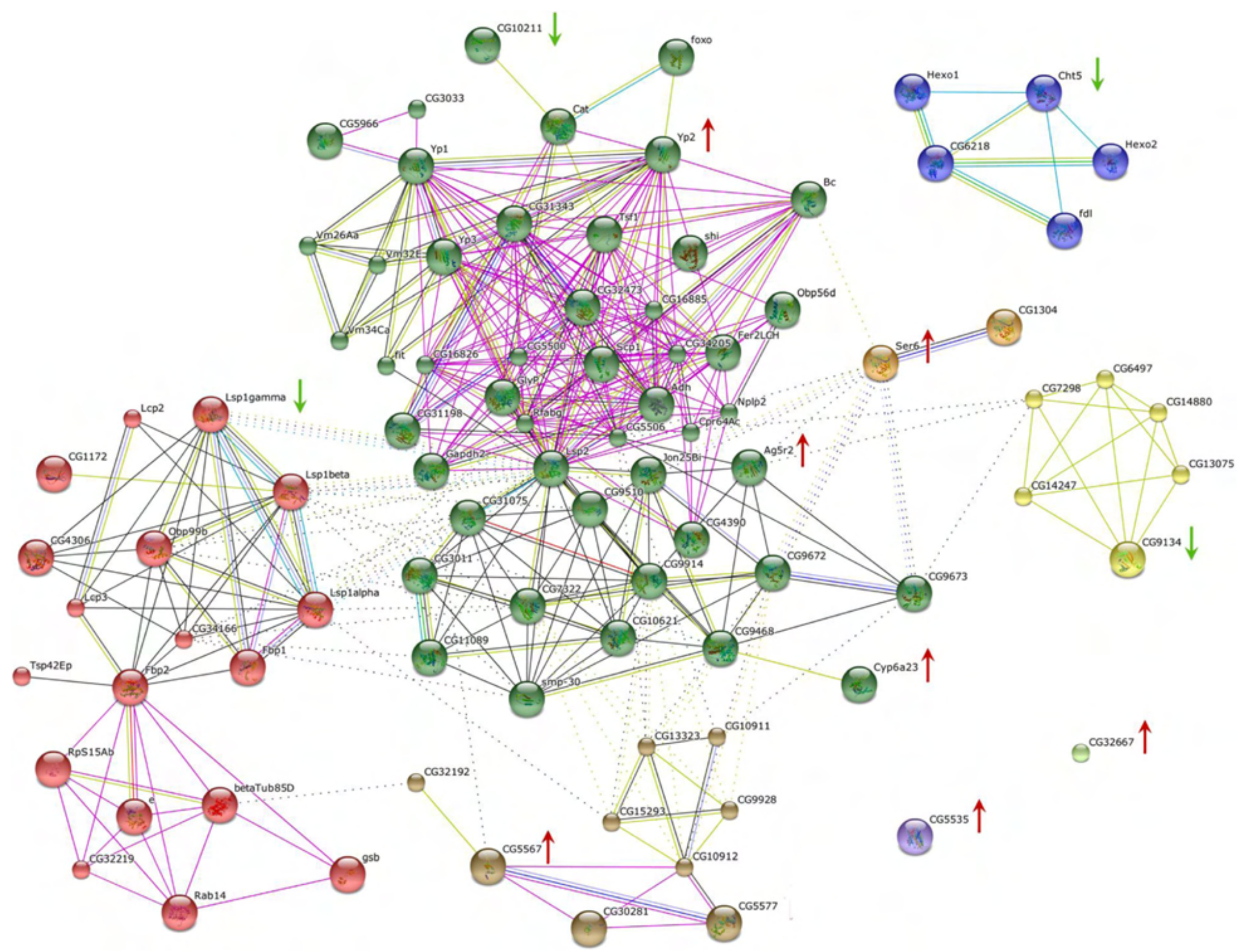

Figure 6 STRING analysis. The network displays the predictions of protein interaction and association with experimentally determined interactions plus those from the literature of the selected gene list of up-regulated as well as down-regulated gene-products. The input gene list included the following genes: *CAT-2* (CG5535), *Serine protease 6* (CG2071), *Yolk protein 2* (*Yp2*, CG2979), *pNPPase* (CG5567), *Lsp1* (CG6821), *oxidase* (CG10211), *MMR* (CG9134), *Cytochrome P450 6a23-like* (*Cyp6a23*, CG10242), vWF domain (CG32667) and *chitinase* (CG9307). Network was enlarged based on Drosophila protein interactions. The *ATP synthase gene* (CG8189) and the *Hsps* were withdrawn from the list, because the resulting network was very dense and uninterpretable. Interestingly, the gene *Ag5r2* (*Antigen 5*) even if it was also absent from the input list, it appears to be correlated with the other genes, supporting our hypothesis of interacting pathways. STRING Version 9.O was used for this analysis. Different colored edges indicate the types of evidence used in predicting the associations[1]. Up-regulated genes are indicated by red arrows, whereas down-regulated ones by green arrows. [1]A red line indicates the presence of fusion evidence; a green line - neighborhood evidence; a blue line - coocurrence evidence; a purple line - experimental evidence; a yellow line - textmining evidence; a light blue line - database evidence; a black line - coexpression evidence.

Increased energy and metabolism demands

ATP synthase is an important enzyme that provides energy for the cell to use through the synthesis of ATP. Located within the mitochondria, ATP synthase consists of 2 regions: the F_O portion is embedded in the mitochondrial membrane and functions as a proton pore; and the F_1 portion is inside the matrix of the mitochondria and is associated with the ATP synthase activity. Through differential proteome analysis and enzyme activity assays, increased expression of ATP synthase was found in the midgut of pyrethroid resistant populations of *Helicoverpa armigera* [47]. Since more energy related proteins (such as vacuolar-type ATPase A/B and arginine kinase) were up-regulated, the authors suggested that increased energy metabolism may be a general prerequisite for compensating the costs of energy-consuming detoxification processes. As a matter of fact, inhibitors of mitochondrial ATP synthase, such as Diafenthiuron, are known insecticides for aphids, whiteflies and hoppers [48]. Significantly elevated levels of *ATP synthase* F_O *subunit 6* were observed in SPIN flies as compared with LAB and w-GR, while w-CAL levels were intermediate (Figure 4B). This constitutes an indication of elevated energy requirements of the resistant flies so as to lever the detoxification process.

The Low affinity cationic amino acid transporter-2 (CAT-2) belongs to a large group of solute carrier proteins, a group of over 300 membrane transport proteins organized into 52 families [49]. These transporters utilize the energy of ATP hydrolysis to transport various substrates across cellular membranes. Several functions are controlled such as protein synthesis, hormone metabolism, catalytic functions, nerve transmission, regulation of cell growth, production of metabolic energy, synthesis of purines and pyrimidines, nitrogen metabolism and biosynthesis of urea. In addition, in the mammalian cells, the uptake of amino acids is mediated by energy-dependent and passive transporters with overlapping substrate specificities. Most energy-dependent transporters are grouped either to the co-transport of Na^+ or Cl^- or to the counter-transport of K^+. As reported for system y^+, the CAT proteins catalyze the Na^+-independent uptake of arginine, lysine and ornithine and the Na^+-dependent uptake of some neutral amino acids [50]. Both SPIN and w-CAL olive flies showed significantly higher *CAT-2* levels compared to LAB and w-GR (Figure 4C). While there are no data in the literature suggesting that *CAT-2* may be involved in transport or extrusion of spinosad from the cell, we think that the up-regulation of this locus is related with the up-regulation of *ATP synthase* and reflects the increased energy and metabolic needs of the resistant flies.

Egg and larval development proteins

Vitellogenins (Vgs) are precursors of the major egg storage protein, vitellin, in many oviparous animals. In higher Diptera, Vgs are called Yolk proteins (Yps) and are produced by both the fat body and the ovary in the majority of the species. Three main factors regulate vitellogenesis in *D. melanogaster*: a brain factor, an ovarian factor that stimulates fat bodies Yp synthesis (further recognized as ecdysone) and a thoracic factor (Juvenile Hormone, JH) involved in the Yp uptake by ovaries. JH regulates the Yp synthesis and uptake, while ecdysone is involved only in Yp synthesis [51,52]. In Culex mosquitoes the head factor is released 4–8 minutes after the beginning of feeding [53]. The vitellogenic phase is initiated after feeding on non-autogenous species or after the adult emergence of autogenous species, when the *corpora cardiaca* stimulating factor (CCSF) is released from the ovary [54,55]. Insect Vgs are synthesized in the fat body in a process that involves substantial structural modifications (e.g., glycosylation, lipidation, phosphorylation, de-phosphorylation, proteolytic cleavage, etc.) of the nascent protein prior to its secretion and transport to the ovaries (for a review see [56,57]). *4-nitro-phenyl-phosphatase (pNPPase)* catalyzes the hydrolysis of nitrophenyl phosphates to nitrophenols. At acid pH it is probably acid phosphatase; at alkaline pH it is probably alkaline phosphatase. In the kissing bug *Rhodnius prolixus*, acid phosphatase activation follows oocyte fertilization and *pNPPase* seems to be involved in vitellin dephosphorylation [58]. Taken together, *pNPPase* should follow elevated levels in *Yp* expression since it is involved in its modification during transport to the ovaries (Figure 4E). In the case of spinosad resistant flies, the elevated levels of *Yp2* and, to a lesser extent of *pNPPase*, observed in the resistant flies is most likely related to events taking place in the fat body surrounding the heads of the insects rather than their brain and probably associated with feeding rather than processes associated to egg development. Furthermore, it has been reported that there is a physiological link between vitellogenin activity, oxidative damage and mortality, suggesting an antioxidant role of vitellogenin. RNAi experiments in bees demonstrated that vitellogenin expression was linked to the bees' level of resistance to oxidative stress [59]. In the same study, excess mortality of vg^- bees was shown to be linked to cellular damage that included a severe oxidative insult to the fat body, after exposure to paraquat. This elevated expression of *Yp2* gene in spinosad-resistant flies is somewhat analogous to the observance of persistent production of a vitellogenin-like protein in insecticide-resistant mosquitofish *Gambusia affinis*. Normally, in the mosquitofish Vg is produced during reproductive season. However, insecticide-resistant mosquitofish produce a vitellogenin-like protein year around [60]. The authors suggest that xenobiotics may induce the formation of a vitellogenin-like protein in order to bind and transport insecticides. Finally, we questioned whether the observed up-regulation is female-specific only.

In fact, as expected, functional analysis in three female and three male flies of SPIN, w-CAL, LAB and w-GR showed elevated *Yp* expression in female SPIN and w-CAL heads only (Figure 5). Interestingly, the within population variability was very high. While values for w-GR and LAB were close to zero (0.0016-0.0548 and 0.00036-0.00079, respectively), values for w-CAL ranged from 3.265 to 17.178 and for SPIN 0.0075 to 5.656. Considering that all SPIN flies fed on constant spinosad diet, the low *Yp* expression observed in a SPIN female (0.0075) suggests that high *Yp2* expression may be protective but not necessary for spinosad resistance in female flies.

By contrast, two storage proteins, *hexamerin larval protein 1 (HexL1)* and *larval serum protein 1 (Lsp1)*, showed a tendency of down-regulation. In holometabolous insects, which go through distinct stages, essential nutrients obtained in one stage but required in another are sequestered in storage proteins and carried across stages until they are utilized. In that sense, if an insect does not feed or restricts its diet during a specialized stage, its activities should be supported by nutrient intake during a previous feeding stage. Egg development in mosquitoes, for example, heavily depends on a protein-rich blood feed. Nectar feeders, on the other hand, should obtain most protein destined for eggs during the larval stage and stored until synthesis of yolk proteins. This storage takes place through various structurally similar hexamerins (for a review, see [61-63]). Storage proteins are not only produced during larval development. Adult females of the diamondback moth, *Plutella xylostella*, synthesize hexamerins within hours post eclosion to resequester amino acids that have been utilized until then [64]. Hexamerins are also implicated in JH regulation. In termites, hexamerins are involved in nutrient storage and nutritional signaling and are also known to bind JH [65]. It is thought that by binding to it hexamerins sequester JH, thus preventing it from eliciting downstream effects on developmental gene expression [66]. Indeed, RNAi-based hexamerin silencing affected 15 out of 17 morphogenesis-associated genes that are members of a JH-responsive genomic network [67].

So, why are storage protein transcripts down-regulated in spinosad resistant flies? It is plausible that the resistant w-CAL flies (and to a lesser extent the less resistant w-GR flies) have developed the ability to store sufficient amounts of the necessary amino acids for their adult lives during their larval stages and to not require additional replenishments during adulthood. Such nutrient availability may be necessary for overcoming the elevated demands in energy and metabolism in the 'toxic' environment of the resistant flies. Instead, under 'normal' conditions, when the flies have the luxury of acquiring and store amino acids later in their adult lives, they can activate their storage proteins after a meal. In order to prove this claim, however, further detailed experiments should be performed to assess the expression of storage and related genes during the larval, pupal and adult stages, under different nutritional conditions.

Immunity, detoxification and stress related loci

Six genes that fall in this category have raised our interest.

Serine protease 6 (SP6). While the role of other detoxification enzymes in insecticide resistance is well understood, the involvement of proteases/serine proteases is not. Proteases are involved in protein digestion outside the cells and also in the expression and regulation of cellular proteins [68]. Cellular proteases function to create biologically active molecules or destroy biologically active proteins and peptides [69,70]. Additionally, the signalling transduction system/pathways that are controlled by G protein coupled receptors (GPCRs), protein kinase/phosphatases and proteases are involved in the regulation of *P450s* genes [71]. Very interestingly, elevated levels of all cytoplasmic and lysosomal proteases were detected in spinosad-resistant *M. domestica* flies 48 hours after exposure to spinosad at LD_{50} dose level [72], indicating involvement of proteases in the development of spinosad resistance to the housefly. Two serine protease genes (*trypsin* and *chymotrypsin*) were also shown to have threefold higher expression in deltamethrin-resistant *Culex pipiens pallens* mosquitoes [73]. These two enzymes were further shown to hydrolyze deltamethrin [74]. Moreover, up-regulation of serine proteases was also documented in permethrin resistant *Culex quinquefasciatus* mosquitoes [75]. Finally, in the mosquito *Aedes aegypti*, *serine proteases* are also expressed in the salivary glands and thought to have a defense role against bacterial growth ingested with saliva during sugar meals [76,77]. In the olive fly, the level of *serine protease 6* in the resistant SPIN and w-CAL flies strain is significantly elevated compared to LAB (Figure 4D), while w-GR has also considerable expression. Apparently, *serine proteases* are required not only for the digestion of more complex nutrients of the wild flies, compared to the standardized laboratory diet, but may also participate in the defense against bacterial pathogens during feeding.

An oxidase/peroxidase family protein was found down-regulated in the transcriptome of the SPIN strain. However, further comparisons between LAB, w-GR and w-CAL reversed the trend and showed higher level of expression in w-CAL flies. While such proteins present protein-protein binding properties and are known to be involved in defense mechanisms (such as intracellular phagocytosis of apoptotic cells or foreign material) [78], the gene was not further evaluated.

A *macrophage mannose receptor (MMR)* was also found to be down-regulated in the SPIN strain. The MMR is a C-type lectin receptor, a family of surface carbohydrate-binding receptors that require calcium for binding. In humans they are known to recognize microbial carbohydrate moieties, also sense products from dying cells and transduce inflammatory signals that modulate the immune system [79]. In crustaceans, on the other hand, they are thought to be involved in the regulation of the exoskeleton calcification [80]. Its expression displayed molt cycle-related differential profile. In the same study, members of the serine protease superfamily also varied their expression during different molting stages. In insects, secretory C-type lectins are thought to play roles in cellular interactions during development [81]. In addition, they are considered important in the immune system, including the detection and neutralization of pathogenic and non-self materials in several insect species [82]. In the mosquito *Aedes aegypti* and the flesh fly *Sarcophaga peregrina*, C-type lectins are expressed in the salivary gland and are considered to control bacterial pathogens from ingested meals [76,77,83,84]. In the olive fly transcriptome a *macrophage mannose receptor* was found to be down-regulated in the SPIN strain but the trend was reversed in the functional analysis of the LAB, w-GR and w-CAL strains and, therefore, cannot be evaluated before further analyses are performed.

A von Willebrand factor domain within a Salivary cys-rich peptide was also up-regulated. The majority of vWF-containing proteins are extracellular. The oldest ones in eukaryotes, however, are parts of intracellular proteins involved in transcription, DNA repair, ribosomal and membrane transport and the proteasome. vWF tends to bind to other proteins and thus it appears to be involved in multiprotein complexes. In insects, huge vWF-containing proteins, such as hemolectin in *D. melanogaster* and hemocytin in *B. mori*, are thought to function in the hemolymph coagulation or hemocyte aggregation processes, such as nodule formation [85,86]. Such processes are fundamental responses of insect innate immunity in order to clear microorganisms from the hemocoel. A similar role might be envisaged in SPIN flies of *B. oleae*. This up-regulation is concordant with the up-regulation of the previously described defense loci. Functional analyses on LAB, w-GR and w-CAL flies confirmed a significant under-expression in the LAB strain (Figure 4F).

Cytochrome P450 6a23-like (Cyp6α23)

This gene belongs to a superfamily of monooxygenases that catalyze the oxidation of organic substances. They are involved in drug metabolism and bioactivation of about 75% of all the different metabolic reactions [87]. *P450s* have been implicated in insecticide resistance against various substances (for reviews see [88-90]). Their role in spinosad detoxification has been hypothesized at least in *Helicoverpa armigera* [91], *Musca domestica* [92] and *Bombus huntii* [93], whereas it has been disputed in *Drosophila melanogaster* [94]. *Cyp6α23* was highly over-expressed in the RNAseq of the olive fly SPIN strain (232,692-fold), albeit not statistically significantly, falling below the stated criteria (p value = 0.0003877, q value = 0.109514). Functional analysis in three female and three male flies of SPIN, LAB, w-CAL and w-GR showed, on average, elevated levels of expression in SPIN and w-CAL compared to LAB (Figure 5). However, w-GR had intermediate levels of expression. Two things should be mentioned at this point. Firstly, the large variability of *Cyp6α23* levels. In some SPIN individuals the *Cyp6α23* level was lower than that of some LAB individuals. However, since the RNA for the RNAseq was obtained from a pool of 40 female and 40 male flies, the RNAseq result should reflect the average expression in the population. In addition, *P450s* expression levels vary throughout the life cycle of the insect [93] and the observed variability in *Cyp6α23* expression in olive fly individuals may reflect the asynchrony of their life stage. Secondly, w-GR flies had, on average, intermediate levels of *Cyp6α23* expression. As mentioned in the Methods section, even though these flies were obtained from a presumably untreated orchard in Greece, their resistance ratio was three times higher than that of the LAB flies and, therefore, w-GR cannot be considered as a source of truly spinosad sensitive flies.

Heat shock proteins

Two heat shock proteins, Hsp70 and Hsp23, were found to be down-regulated in the SPIN transcriptome, a fact that was not confirmed after functional analyses. Hsp70 proteins are very conserved and ubiquitously expressed in virtually all living organisms, being very important in folding and unfolding of proteins, detoxification of pesticides and heavy metals. Hsp23 belongs to a lens alpha crystalline-related superfamily, also found in the salivary gland cells of *D. melanogaster* [95]. In all reported cases of stress and detoxification where Hsp were involved, their transcripts were strongly up-regulated. In order to clarify their role in spinosad resistance in the olive fly, further experiments should be performed.

Antigen 5 precursor (Ant5)

This gene product shows similarity to Drosophila's *Antigen 5-related 2 gene (Agr2). Agr2* proteins belong to the CAP family of proteins, which include the mammalian Cysteine-rich secretory proteins, wasp venom Antigen 5 proteins, and plant group 1 Pathogenesis-related proteins. The gene product of the *Drosophila melanogaster* ortholog *Agr2* is suggested to function either as a novel type of protease inhibitor or as an antimicrobial protein [96]. In our study, *Ant5* was over-expressed in the SPIN transcriptome. However, further functional analysis showed over-expression in both the w-GR and w-CAL populations (Figure 4H).

Chitinase 5 (Cht5)

In insects, chitin is known as a scaffold material, providing both exo- and endo-support to the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium [97]. The midgut *chitinases* seem to be involved in the formation, perforation and degradation of the midgut peritrophic matrix, which protects the gut epithelium from damaging factors, toxins and pathogens [98-100]. *Chitinases* have also been proposed as biopesticides, as transgenic plants expressing chitinolytic enzymes potentiate the efficacy of other biological toxins (e.g. Bt or fungal toxins) [101,102]. In the olive fly, *Cht5* was under-expressed in the SPIN transcriptome and was found down-regulated in the w-CAL populations (Figures 2 and 4M). Given the aforementioned role of *chitinases*, we can hypothesize that by under-expressing *chitinase* genes the resistant flies decrease spinosad penetrance, thus increasing resistance.

Cell division cycle-associated protein 7 (Cdc)

This gene belongs to the HAD-superfamily hydrolase, according to Interpro [103]. RNAseq analysis showed that *Cdc* was under-expressed in the SPIN transcriptome. However, after functional analysis the RNAseq result was not confirmed, since both the resistant w-CAL population and the sensitive w-GR were up-regulated compared to the sensitive LAB flies (Figure 4P). Therefore, further analysis is required in order to clarify *Cdc's* role in spinosad resistance.

RNA viral genes

Five more genetic loci were of curious origin. Two of them were up-regulated: a *replicase-like protein* was identified as having considerable similarity with a dimethyl transferase domain of an RNA virus; and an elongation factor had similarity with a viral helicase domain. Three of them were down-regulated (*hypothetical B. oleae polyprotein; RNA-dependent RNA polymerase*; *microtubule-actin cross-linking factor 1*), but they are also implicated with viral functions as homology searches matched sacbrood virus sequences. Finding similarities with viral sequences is not surprising. In fact, the presence of viral sequences has been reported in previous both smaller and larger transcriptome sequencing efforts [41,104,105]. Obviously, such genes reflect the presence of RNA virus infections in different laboratory or wild populations. The impact of such infections has not been studied and cannot be assessed at this point whether this might have been among the causes of our SPIN colony collapse.

Conclusion

Adaptation and survival of the flies in the altered environment caused by insecticide stress appears to be a consequence of changes in multiple genes' expression, affecting both biological and physiological pathways. Our perception about the development of insecticide resistance in insects, traditionally attributed to either a target site alteration or the up-regulation of various detoxification genes (such as *P450s, esterases* and *GSTs*), is recently changing due to our ability to address such questions in a more holistic way through transcriptomic analysis. This gives us the opportunity to consider diverse regulatory networks of interacting genes via complex mechanisms. In the present study, we conducted whole transcriptome comparative analyses between spinosad resistant and susceptible olive flies, in order to investigate and identify genetic loci and molecular mechanisms that are most likely to be involved in spinosad resistance. The observed changes at the RNA level as well as the functional analyses and bioassays, point towards a multi-level impact of the insecticide to the insect's physiology. Our results indicate that the organism's response to this novel environmental stressor mainly affects energy metabolism pathways, immunity defense pathways and detoxification. The oxidative, xenobiotic, and innate immune stress response pathways appear to be coordinated, leading to the regulation of numerous cellular and biological/physiological processes. Further studies are required to determine the molecular mechanisms and significance of this cross-regulation.

Methods

Ethics statement

The study was carried out on laboratory reared olive flies and wild olive flies collected from the area around the city of Volos, Greece, and the Sonoma County in California. No specific permissions are required for these experiments or collections, since these studies did not involve endangered or protected species.

Fly culture and stocks

Laboratory strain

The laboratory strain of the olive fly (LAB) is part from the original stock from the Department of Biology, 'Demokritos' Nuclear Research Centre, Athens, Greece, and has been reared in our laboratory for over 15 years. The flies are reared at 25°C with a 12 h light/12 h dark photoperiod in $30 \times 30 \times 30 cm^3$ cages, as described by [106-108].

Development of a spinosad-resistance colony

A spinosad resistant strain (SPIN) was also developed in our laboratory. Starting material for this colony was the aforementioned LAB colony that was supplemented with ~ 1000 wild flies from Argalasti (Pelion, Greece). Increasing amounts of spinosad were gradually introduced into the colony's feeding water that reached 0.04 g/ml after 10 generations. The colony was maintained for about 22 generations (~2 years) under constant 0.04 g/ml spinosad selection. This amount of spinosad corresponds to approximately 2× the recommended amount for field applications that would result in 100% mortality. It also corresponds to 125× the LC50 of the susceptible LAB strain. In order to increase the resistance to spinosad, the colony was refreshed a second time with wild flies from Sonoma County (CA, USA), since this area was shown to have the highest spinosad resistance level [109]. Six months later the colony practically crashed and was recovered by a single female, under no selection. Progeny of that female were put under gradually increasing amounts of spinosad. The colony recovered previous levels of resistance (0.04 g/ml) after only 4 generations. After a total of 46 generations, a more precise estimation of the resistance ratio (RR) was obtained by ingestion bioassays, as described in Kakani et al. [109], showing that resistance level had reached 35×. This is the stage from where all spinosad resistant laboratory flies (referred to as SPIN throughout the text) were collected, both for the isolation of the nAChR and the RNAseq analysis. Finally, during the fall of 2012, entirely unexpectedly and without any obvious changes in the insectary environment, the spinosad resistant colony crashed. Initially it was noted that females did not oviposit in the offered waxed cone, while both male and female adult numbers started to decline. During that time, new wild material arrived from California, which was intended to enrich the laboratory colony with new alleles. Nonetheless, after about 3 months of continuous efforts the last adult flies died and no progeny emerged.

Field-collected flies

Wild flies were collected from two geographical locations, one from an untreated orchard in Greece [Agria, Pelion (w-GR)] and another from a different site in Sonoma County [CA, USA (w-CAL)] that was the source of flies used to refresh the SPIN strain, but where flies had also shown highest levels of spinosad resistance in the Kakani et al. study [109]. Contact bioassays were performed on these flies according to Kakani et al. [109], using seven doses of spinosad ranging between 1/2× to 1/128×, plus a blank control of acetone. LD_{50} values and 95% confidence intervals were calculated by probit analysis using SPSS v.13 (SPSS Inc, Chicago, IL). The calculated resistance ratio (RR) of the w-CAL was 12.96 (11.62-14-28) whereas that of the w-GR was 3.14 (2.25-4.2). Infected olives were brought into the laboratory and emerged flies were put in $30 \times 30 \times 30cm^3$ cages and fed on the standard yeast hydrolysate diet [107]. Female flies were allowed to oviposit in fresh olives, since wild olive flies do not oviposit on artificial substrates. Flies from this F1 generation were used for the functional analysis experiments described in the Results.

Extraction of RNA, cDNA synthesis, cloning of nAchR Boα6 and sequencing

Total RNA was isolated from pools of four heads of adult flies from the LAB and SPIN strains with the use of TRIzol® Reagent (Ambion-Invitrogen). One to five micrograms of total RNA was used for first strand synthesis of poly(A) of cDNA using the MMLV high performance Reverse Transcriptase (GeneOn) and random primers (GeneOn) according to the manufacturer’s instructions.

Partial cDNA of the LAB acetylcholine nicotinic receptor α6 gene of *B. oleae* was amplified by PCR using primers Bdα6-F (ACATGGTTCCCATTCGATGACC) and Bdα6-R (GCGACCATGAACATGATGCAATT) designed on conserved regions of the published nAChRα6 cDNA sequence of *Bactrocera dorsalis* (Bdα6-JN560169.1) [26]. The PCR amplification reaction consisted of 2 μl of the first strand cDNA reaction mix as a template, 0.7 μl of 10 mM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1unit Taq DNA Polymerase (GeneOn) in a 20 μl reaction. Cycling conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30s, 49°C for 2 min and 72°C for 1.5 min and a final extension at 72°C for 10 min in a thermal cycler (MJ Mini Biorad). The amplified PCR product was then separated in a 1% agarose gel, stained with ethidium bromide. The amplified PCR product was isolated by the GF-1 Gel recovery kit (Vivantis) and subcloned into the pBluescriptII SK(+) plasmid vector and sequenced. Based on the obtained sequence, four gene specific primers were designed to amplify the full-length cDNA: two reverse primers for 5′-RACE PCR (5GSP1: 5′- GTCCTTAGAT TTCAGCTACC-3′ for the first round reaction and 5GSP2: 5′-GGGCGGGTGGGTATAAGTAT-3′ for the nested reaction) and two forward primers for 3′-RACE PCR (3GSP1: 5′- CACAACGGTGGAGGAGCATC-3′ for the first round reaction and 3GSP2: 5′-GGGCGGGTGGG TATAAGTAT-3′ for the nested reaction). A poly-A tail was added to the 3′-end of the resulting strand of 5′-RACE by terminal deoxynucleotidyl transferase (TdT, Biolabs). Thermal cycling conditions for the 5′- and 3′-RACE were: pre-denaturation 5 min at 94°C, 30 cycles of 94°C for 30 sec, 49/52°C (first/second round) for 45 sec and 72°C for 2 min (according to the size of the expected fragment) with a final extension of 15 min at 72°C. The resulting PCR products of 5′-RACE and 3′-RACE were subcloned into pBluescriptII SK (+) vector and sequenced. Each time plasmids were sequenced, three different isolates were used and no variation was observed.

Sequence comparison between sensitive and resistant *Bactrocera oleae* nAChRα6 subunits

For comparison of the *Boα6* transcripts, total RNA was extracted from a pool of 4 adult heads from the two strains (LAB and SPIN), as described above. The specific primer pair Boα6-F (5′-AGATTAGTGACAGCATAACC

G-3′) and Boα6-R (5′-TCTATCCACAACCATTGCCG C-3′) was used for the amplification of the full-length open reading frame of BoAChR-α6 gene. The PCR products were sequenced directly with the use of Boα6-F, Boα6-R and two more internal primers (Boα6F1: 5′-AT GAATCGGAATATGGAG-3′ and Boα6R1: 5′-AACGGA TTTAATCCAAGG-3′). No multiple peaks were observed in the obtained sequences, indicating the absence of sequence polymorphism in the pools.

Nucleotide sequence similarity searches were performed using BLAST [110]. Multiple sequence alignments [111] with other insect nAChR subunits were performed with ClustalW2 [112]. The calculated molecular weight and isoelectric point of the putative protein encoded by *Boα6* were predicated by Compute pI/Mw tool in Expasy Server [113]. Phosphorylation sites and N-linked glycosylation sites were identified by the PROSITE database [114].

RNA isolation for library preparation and functional analysis

Total RNA was isolated from fly heads with the use of TRIzol® Reagent (Ambion-Invitrogen) following the instructions of the manufacturer with minor modifications. More specifically, RNA was extracted from forty male and forty female heads from the laboratory colony (LAB) and from an equal number of spinosad resistant fly heads (SPIN). For more complete sequence assembly, two more libraries were constructed and sequenced: a FEMALE library made of female accessory glands and spermathecae of ~300 female flies and a MALE library made of testes of ~150 male flies [115]. RNA extraction was followed by an additional DNA removal using the TURBO DNA-free Kit (Ambion-Invitrogen), according to manufacturer's instructions. The integrity of RNA was assessed by 1% agarose gel electrophoresis and the purity of all RNA samples was evaluated at Fleming Institute (Greece) with the use of (Agilent 2100 Bioanalyzer) and NanoDrop (2000).

For functional analysis, RNA was extracted as described above from three different individual male and female heads from the LAB strain, the SPIN resistant strain, the Sonoma County wild population (w-CAL) and the Agria (w-GR) wild population.

Whole transcriptome library preparation for next-generation sequencing with the SOLiD 4 Sequencing System

RNA transcripts expressed in the head of the spinosad-sensitive (LAB) and spinosad-resistant (SPIN) olive fly strains were used to construct cDNA library for high throughput sequencing analysis on the SOLiD 4 Sequencing System. More specifically, polyadenylated RNA (polyA-RNA) was isolated from 5 μg of total RNA using the Dynabeads Oligo(dT) kit (Ambion, Life Technologies Corporation). The isolated polyA-RNA was randomly fragmented by chemical hydrolysis at 94°C for 5 minutes and was then treated with antarctic phosphatase to remove phosphate groups from the fragments' ends, followed by treatment with T4 polynucleotide kinase to add a Pi at the 5′ end of each fragment. The resulting RNA fragments were hybridized and ligated to the P1 and P2 adaptor sequences specifically designed for sequencing with the SOLiD system (SOLiD Total RNA-Seq Kit, Life Technologies Corporation). The RNA produced was reverse transcribed to cDNA which was then amplified in a 15-cycle PCR. At this step, the use of different barcoded 3′ PCR primers from the selection included in the SOLiD barcoding kit allowed the preparation of cDNA libraries for multiplex sequencing. From the cDNA produced, only fragments of average size 200–300 bp were selected with two rounds of magnetic bead purification (Agencourt AMPure XP Reagent, Beckman Coulter).

The quality and size of the purified cDNA library was assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc.) and with quantitative PCR using the Library Quant Kit ABI Solid (KAPA Biosystems). A multiplex library mix (500pM) was used to prepare a full-slide for analysis on the SOliD 4 Sequencing System (Applied Biosystems) with 35 + 50 bp PE –chemistry.

Bioinformatics analysis

The reads of the libraries were assembled to construct the reference transcriptome using the SOAPdenovo assembler [39] with a word size of 25 nt and using all paired and unpaired reads. Annotation of the assembled sequences was obtained by aligning against the NCBI non-redundant (Nr) protein database using blastx [116] and collecting the annotations with the BLAST2GO tool [40]. TopHat [117] was used to generate a spliced alignment to the reference transcriptome. Transcripts were assembled using Cufflinks and Cuffdiff [43] was used in order to reveal differentially expressed genes. SNP calling was performed with the mpileup tool and converted to the vcf fomat using the vcfutils, both from the SAMTOOLS package [42]. The SNP loci were intersected with the gene coordinates using the intersectBed tools from the BEDtools suite [118].

Expression stability of candidate reference genes in *B. oleae* head

In order to find the most suitable reference gene for gene expression analyses in *B. oleae* head tissue, nine different housekeeping genes commonly used in other dipteran species were analyzed. The nine genes were: *RPL19* (*ribosome protein L19*), *tbp* (*TATA-binding protein*), *ubx* (*ultrabithorax*), *GAPDH* (*glyceraldehyde 3-*

phosphate dehydrogenease), *α-TUB* (*α-tubulin*), *β-TUB* (*β-tubulin*), *14-3-3zeta*, *RPE* (*RNA polymerase II*) and *actin3.* To determine the expression stability of the selected genes in *B. oleae* head, the expression of the reference genes was measured in 24 heads (6 individuals from each of the LAB, SPIN, w-GR and w-CAL populations, i.e., 24 biological replicates) in duplicate reactions (two technical replicates). The amplification efficiency of the reactions was calculated by the CFX Manager™ software (Bio-Rad) (Additional file 1: Table S4). Using the comparative Cq method with a procedure of specific PCR efficiency correction, all the Cq values were converted to relative quantities and transformed to an input file format with raw data for subsequent analysis by the Normfinder Excel applications.

Normfinder [119] is an algorithm for identifying the optimal normalization gene among a set of candidate genes. This software is based on a mathematical model of gene expression that enables estimation not only of the overall variation of the candidate normalization genes but also of the variation between samples subgroups of the sample set [44].

BestKeeper determines the most stably expressed genes based on the coefficient of correlation to the BestKeeper Index, which is the geometric mean of the candidate reference gene Cq values. Additionally, it calculates the standard deviation (SD) and the coefficient of variation (CV) based on the Cq values of all candidate reference genes [45]. Reference genes are identified as the most stable genes, i.e. those that exhibit the lowest coefficient of variance and standard deviation [120].

Additional file 1: Table S5 presents the data on the ranking of the tested reference genes.

Functional analysis of spinosad-resistance differentially expressed genes

Specific primers for the amplification of the differentially expressed genes revealed by the transcriptome analysis were designed by Primer-BLAST [121] (Additional file 1: Table S4).

For the functional analysis experiments, RNA was extracted from the heads of six individual flies (equal number of males and females) of all different strains and populations described previously. Subsequently, one microgram of each DNA-free total RNA was converted into cDNA using 300 ng Random hexamer primers (equimolar mix of N_5A, N_5G, N_5C and N_5T), 200 units MMLV Reverse Transcriptase (Geneon), 5X reaction buffer, 40 mM dNTP mix and 40 units RNase Inhibitor (GeneOn) according to the manufacturer's instructions.

Relative quantitation was used to analyze changes in expression levels of the selected genes using a Real-time PCR approach. Expression values were calculated as the geometric mean of the relative expression of each target gene against the expression of each one of the reference genes *tbp* and *14-3-3 zeta* gene. The qRT-PCR conditions were: polymerase activation and DNA denaturation step at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing/extension and plate read at 56°C for 30 s and finally, a step of melting curve analysis at a gradual increase of temperature over the range 55°C to 95°C. In this step, the detection of one gene specific peak and the absence of primer dimer peaks was assured. Each reaction was performed in a total volume of 15 μl, containing 5 μl from a dilution 1:10 of the cDNA template, 1X iTaq Universal SYBR Green Supermix (Bio-Rad) and 400 nM of each primer. The reactions were carried out on Bio-Rad Real-Time thermal cycler CFX96 (Bio-Rad, Hercules, CA, USA) and data analysed using the CFX Manager™ software. All assays were performed three times (three technical replicates), contained six different individuals (six biological replicates) and three negative controls. A standard curve was generated for each gene using 5-fold serial dilutions of pooled cDNA from the flies head. The PCR efficiency (E) and the correlation coefficient (R^2) characterizing each standard curve are given in Additional file 1: Table S4. Efficiencies for all tested genes varied between 93.3% to 109.2%. The $2^{-\Delta\Delta Ct}$ method was used for the analysis of relative gene expression [122].

String analysis

In order to investigate the potential interactions between the up- and down-regulated genes, we queried the resource STRING (Search Tool for the Retrieval of Interacting Genes) which makes available precomputed results in predicted functional linkages among proteins by comparative genomics and text-mining [46]. Specifically, the gene IDs of the *Drosophila melanogaster* orthologs of our genes were used as input in the online database resource STRING in order to be placed in a biological context according to a large number of computational predicted and experimentally determined functional associations and protein-protein interactions. Results were graphically displayed and scored using a STRING specific scoring scheme that correlates with validated protein-protein functional associations.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 [123] after normalization of raw Cq values. The normality for all genes was based on the Kolmogorov-Smirnov and Dallal-Wilkinson-Lillie tests (alpha = 0.05). For the genes that passed the normality test, one-way ANOVA and the Tukey's multiple comparison tests were performed. Genes that did not pass the normality test were analyzed by the non-parametric Kruskal-Wallis test with $P < 0.05$.

Availability of supporting data

All data have been deposited at the Sequence Read Archive (SRA) of NCBI. All reads for each sample are summarized at the BioProject page: http://www.ncbi.nlm.nih.gov/bioproject/PRJNA231981.

Additional file

Additional file 1: Table S1. Polymorphic sites in the nAChR α6-subunit sequences in the olive fly LAB and SPIN strains **Table S2.** Up- and down-regulated genes in spinosad resistant olive fly heads. **Table S3.** Basic statistics of relative expression of the up- and down-regulated genes. **Table S4.** Primer sequences used for q-PCR. **Table S5.** Normfinder and Bestkeeper analysis results.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ES maintained the laboratory strains, participated in the construction of the transcriptome libraries, performed the functional and statistical analyses and part of the bioinformatics analysis; MG cloned and analysed the Boα6 nAChR; MR performed most of the bioinformatic analysis of the transcriptome; KT performed the network analysis and part of the bioinformatic analysis; NZ reared the spinosad resistant strain; KS constructed the transcriptome libraries and analysed the sequencing data; FGZ participated in the design of the study and organized the California samples; JR directed the bioinformatics analysis; KDM designed and coordinated the study. All authors participated in drafting the manuscript and read and approved the final document.

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The molecular biology of the olive fly comes of age

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Abstract

Background: Olive cultivation blends with the history of the Mediterranean countries since ancient times. Even today, activities around the olive tree constitute major engagements of several people in the countryside of both sides of the Mediterranean basin. The olive fly is, beyond doubt, the most destructive pest of cultivated olives. The female fly leaves its eggs in the olive fruit. Upon emergence, the larvae feed on the olive sap, thus destroying the fruit. If untreated, practically all olives get infected. The use of chemical insecticides constitutes the principal olive fly control approach. The Sterile Insect Technique (SIT), an environmentally friendly alternative control method, had been tried in pilot field applications in the 1970's, albeit with no practical success. This was mainly attributed to the low, non-antagonistic quality of the mixed-sex released insects. Many years of experience from successful SIT applications in related species, primarily the Mediterranean fruit fly, *Ceratitis capitata*, demonstrated that efficient SIT protocols require the availability of fundamental genetic and molecular information.

Results: Among the primary systems whose understanding can contribute towards novel SIT approaches (or its recently developed alternative RIDL: Release of Insects carrying a Dominant Lethal) is the reproductive, since the ability to manipulate the reproductive system would directly affect the insect's fertility. In addition, the analysis of early embryonic promoters and apoptotic genes would provide tools that confer dominant early-embryonic lethality during mass-rearing. Here we report the identification of several genes involved in these systems through whole transcriptome analysis of female accessory glands (FAGs) and spermathecae, as well as male testes. Indeed, analysis of differentially expressed genes in these tissues revealed higher metabolic activity in testes than in FAGs/spermathecae. Furthermore, at least five olfactory-related genes were shown to be differentially expressed in the female and male reproductive systems analyzed. Finally, the expression profile of the embryonic *serendipity-α* locus and the pre-apoptotic *head involution defective* gene were analyzed during embryonic developmental stages.

Conclusions: Several years of molecular studies on the olive fly can now be combined with new information from whole transcriptome analyses and lead to a deep understanding of the biology of this notorious insect pest. This is a prerequisite for the development of novel embryonic lethality female sexing strains for successful SIT efforts which, combined with improved mass-reared conditions, give new hope for efficient SIT applications for the olive fly.

Background

When Athena, the goddess of peace and wisdom, offered an olive tree to the people of Attica to sway them into choosing her name for their city - and not that of her brother's Poseidon - neither she nor the people of Attica were aware of the 'worm' that could destroy the precious fruit of that tree. That was described much later in the 3rd century AD, by the botanist Theophrastus who, in his works *"Enquiry into Plants"* and *"Causes of Plants"* [1], talked about the 'worm underneath the skin of the olive that destroys the fruit'. Indeed, the female olive fly (*Bactrocera oleae*, Rossi) lays her eggs in an olive fruit and the resulting larva feeds on the olive sap, opening channels inside it, thus destroying it. In this way, a female fly can damage more than 300 olives in her lifetime. Given the

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fact that during the summer and fall months about five generations of these flies are born, one can imagine the cumulative damage that can take place in an olive orchard. If untreated, practically every single olive will get infested. It is estimated that due to olive fly infestation olive oil production is reduced by more than 30% annually [2].

Control of these flies is traditionally based on cover or bait sprays with chemical insecticides. During the last 40-50 years, organophosphate insecticides have been extensively used against the olive fly, mainly dimethoate and fenthion. More recently, pyrethroids as well as the naturalyte spinosad have been added in the arsenal against the olive fly. The use of chemical pesticides, however, entails many known hazards. Among these are ecological disturbances, the development and spread of insecticide resistance, harmful toxicological effects on human health [3]. Many of these risks are apparent not only to scientists but also to growers and consumers who require a cleaner and safer environment as well as products of high quality. Alternative, environmentally friendly control methods against insect pests, such as the Sterile Insect Technique (SIT) have been experimented in the past with considerable success [4]. The SIT involves the mass production, sterilization and subsequent release of the sterilized insects [5]. The sterilized males will mate with wild females, whose unfertilized eggs will never hatch, thus reducing the numbers of the following generation. In theory, if continued releases are performed over several consecutive generations, the population will progressively be reduced and, eventually, a total eradication could occur.

Given the substantial economic burden of the olive fly in olive producing countries and the concerns raised about the heavy use of insecticides to control the flies, the SIT was proposed [6] and implemented in two pilot efforts. In the early 1970s, about 150,000 laboratory-reared male and female flies were sterilized by gamma-irradiation and subsequently released in the environment [7]. Although initially the releases seemed to contribute to low infestation levels, by the end of the season olives were as highly infested as in the two nearby control plantations. The sterilized flies were proven ineffective to reduce infestation. Similar results were obtained in a second pilot SIT effort that took place in the late '70s in a small Greek island. These unsuccessful pilot experiments led to funding suspension and the eventual abandonment of the program [8-10]. Apart from the high cost and labor-intensive rearing of the olive fly, extensive research that followed these first pilot efforts revealed several key issues of olive fly biology that should have been sorted out before a successful SIT could be implemented. The first issue regarded assortative mating of the released and wild populations. Laboratory-reared flies mated several hours before scotophase whereas wild flies mated at the end of the photophase [11]. Apparently, mass-laboratory rearing caused substantial alterations in the genetic makeup of the flies due to selective pressures in the artificial laboratory environment [12,13]. The second issue regarded the quality of the radiation-sterilized mass-reared flies. Radiation did not leave the vigor of the flies unaffected [14]. Another factor that probably exacerbated the low fitness of the laboratory reared flies was the use of antibiotics in the flies' diet that destroyed the endosymbiotic bacteria that are now known to play a very important role in the organism's fitness [15-19]. Finally, but equally importantly, extensive stinging of the olive fruits from the released females led to further fungal infestation [7].

Since those early years, several molecular and genetic studies have changed *B. oleae*'s research landscape. First, the development of microsatellite markers [20] and the analysis of the mitochondrial genome [21] have offered tools for a fairly detailed analysis of population structure and dynamics in the Mediterranean basin [22-26]. Second, cytogenetic analysis, including *in situ* hybridization of several molecular markers, established the details of the chromosomal complement [27-31]. Third, isolation and characterization of various genes has shed light on important processes such as insecticide resistance [32-35], female germline differentiation and morphogenesis of epidermal cells [36], enzyme catalytic mechanisms [37], sex-determining cascades [38,39]. Fourth, an initial assessment of the genome of the olive fly was gained by an accurate estimate of its size [40] and the characterization and analysis of centromeric repeats [41] and several EST loci [42]. This was followed by a whole transcriptome analysis with 454 pyrosequencing [43]. Fifth, *B. oleae* was successfully transformed with the use of a *Minos*-based transposon [44]. Transformation efforts recently led to the development of *piggyBac*-based conditional female-lethal olive fly strains that provide highly penetrant female specific lethality, dominant fluorescent marking and genetic sterility [45]. Sixth, *B. oleae* was recently trans-infected with a cherry fly *Wolbachia* strain and shown to induce complete cytoplasmic incompatibility in the fly [46]. Finally, the experience gained during the first two pilot SIT efforts and the relevant research that followed, underlined a few key requirements for the maintenance of high quality and well-fit mass-reared olive flies (reviewed in [47]). Among them were changes in larval and adult diets (eg removal of antibiotics) that would preserve the endosymbiotic flora (that is now known to improve fitness) and occasional enrichments of the long-term laboratory colonies with wild individuals (that provide natural vigor). These achievements have renewed the interest in using SIT for olive fly control. In fact, there is a large international effort led by the Joint Division of the Food and Agricultural Organization and the International Atomic Energy Agency

(FAO/IAEA) to develop a vigorous laboratory olive fly strain that could be used in such new SIT efforts.

Further scientific and technological developments, in addition to successful SIT applications in other insects, point to the direction olive fly research could go. Indeed, SIT has proven particularly effective in the medfly, the prototype Tephritid species where most genetic and molecular tools have been developed. One of the most active medfly research areas in recent years has been the development of the RIDL technology. RIDL (Release of Insects carrying a Dominant Lethal; [48,49]) is a variant of the conventional SIT, in which sterilization of the released insects is induced not by irradiation but by homozygocity for a dominant lethal gene. Mating with wild individuals results in offspring that are heterozygous for the lethal gene leading to the death of all progeny [50,51]. This dominant lethal gene can be placed under the control of an inducible early embryonic female promoter [51,52] that could achieve genetic sexing at a very early developmental stage. In this way, both genetic sexing and sterilization can be accomplished by the same construct. One other active research area regards the analysis of biological systems with relevance to SIT. Of particular interest are those that regard reproduction and olfaction. The first one is involved in successful mating and egg development, while the second in food and mate localization. A possible manipulation of either or both of these systems would severely affect the destructive ability of the flies. In that sense, transgenic flies could be developed in which genes regulating food and mate recognition or fertility are knocked-down, over-expressed or mis-expressed (depending on the case). Such flies would be safer and more efficient to be released in control programs in an SIT context.

The falling prices of next generation sequencing make it now possible to sequence the entire transcriptome of non-model organisms under different settings and identify differentially expressed genes relevant to the chosen conditions. Subsequently, these genes can be manipulated *in vitro* and re-introduced into the genome of the organism through well-established transgenic technologies. In a first attempt to explore the relevant-to-SIT transcriptome of the olive fly, we present differences observed in female and male reproductive systems and we examine the differential expression of olfactory genes in the same tissues. Finally, we assess the developmental expression of two of the most commonly used early embryonic genes.

Results and discussion

1. Sequencing and annotation

1.1. Solid ABI sequencing and reads assembly

In order to explore differentially expressed genes in the transcriptome of reproductive organs of the olive fly that could be useful in SIT development, the entire transcriptomes from female accessory glands and spermathecae were compared to male testes. For transcriptome assembly, the sequences from these two libraries (FEMALE and MALE) were combined with two more obtained from heads of spinosad-sensitive (LAB) and spinosad-resistant (SPIN) olive flies [53]. Paired-end sequencing with 35nt and 50nt read sizes was performed for each library and a total of 122,623,894 read pairs was obtained. All reads of the libraries were pooled to obtain a reference transcriptome assembly using SOAPdenovo assembler [54].

1.2. Sequence annotation

Annotation of the assembled sequences was obtained by aligning the 69,359 assembled *B. oleae* sequences against the NCBI non-redundant (Nr) protein database using blastx and collecting the annotations with the BLAST2GO tool [55]. Using an E-value threshold of $\leq 1e^{-6}$, 20207 (29.13 %) of the contigs were aligned. Of the 69,359 contigs, 23,042 (33.22%) have almost exact hits in the *B. oleae* transcriptome of Pavlidi et al [43] (E-value $\leq 1e^{-6}$).

2. Female vs male differential expression

The Cuffdiff [56] tool was used in order to reveal the differentially expressed genes between the reproductive systems of female and male flies, a stringent cutoff (p value adjusted for multiple testing, called q value <0.05) was used. This resulted in 1568 differentially expressed transcripts in the FEMALE vs. MALE comparison. Three hundred and thirty of these transcripts were up-regulated in FEMALE, while 1238 were up-regulated in MALE *B. oleae* flies. The top 40 up-regulated genes in each category are listed in Table S1. The entire lists of all significantly (q<0.05) up-regulated genes in FEMALE and MALE are given in Tables S3 and S4, respectively.

An M-A plot was constructed for comparison of the genes for FEMALE vs MALE flies with q value < 0.05. In Figure 1 the de-regulated genes are depicted in red.

Functional annotation was made for the assembled sequences of the significantly differentially expressed female- and male- specific genes mentioned in Table S1, based on gene ontology (GO) categorization obtained using BLAST2GO. The FEMALE and MALE GO analysis performed for biological process of the top 40 female and male expressed genes is shown in Figure 2. In general, more GO terms appear in female tissues than in male (16 vs 12), a point that holds even in deeper GO-term analysis. This can be attributed to the fact that the FEMALE library was comprised of both FAGs and spermathecae, while the MALE from testes only. Furthermore, there were more male- than female-specific genes involved in metabolism and development, a fact that can be attributed to sperm activity in the MALE tissue. Finally, the presence of three immune system process genes in the female list should be noted. In fact, increased

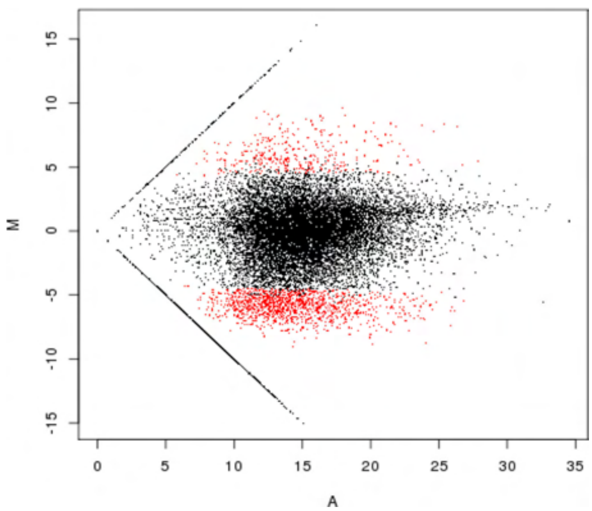


Figure 1 M-A plot of gene expression for female and male flies. Genes up-regulated in females have positive M values. Significantly differentially expressed genes (q-value < 0.05) are shown in red.

levels of immune response genes have been found in transcriptome analyses of insect female reproductive systems, particularly after mating [57,58]. Upregulation of these genes may assist females to combat pathogens introduced during copulation. Alternatively, it could be a result of female's perception of sperm as non-self molecules.

A more direct comparison between FEMALE-only and MALE-only GO-term distribution is shown in Figure 3. Interestingly, numbers of GO-terms for biological process appear different in the two datasets, suggesting a different complexity of the studied female and male reproductive tissues. In most terms, there are more male- than female-specific transcripts that are differentially expressed. Many of these terms (cell cycle, intracellular organelle part, primary metabolic process, organic substance metabolic process, macromolecule metabolic process, cellular metabolic process, multicellular organismal development) refer to higher metabolic processes. This could be attributed to higher metabolic and cellular activity that takes place in the testes before mating.

3. Genes that might be implicated in sexual differentiation in *B. oleae*

In order to validate the differential expression of various genes observed after the RNAseq analysis of reproductive tissues of female and male olive flies, further functional analysis was performed for twelve genes that were differentially expressed in female accessory glands and spermathecae, on one hand, and male testes, on the other (Figure 4). These genes were selected on the basis of known involvement in sexual differentiation in other insects. Seven of them were selected from the 1238 significantly up-regulated in MALE (Table S4): *kl2 (male fertility factor kl2), kl3* (*male fertility factor kl3*), *kl5*

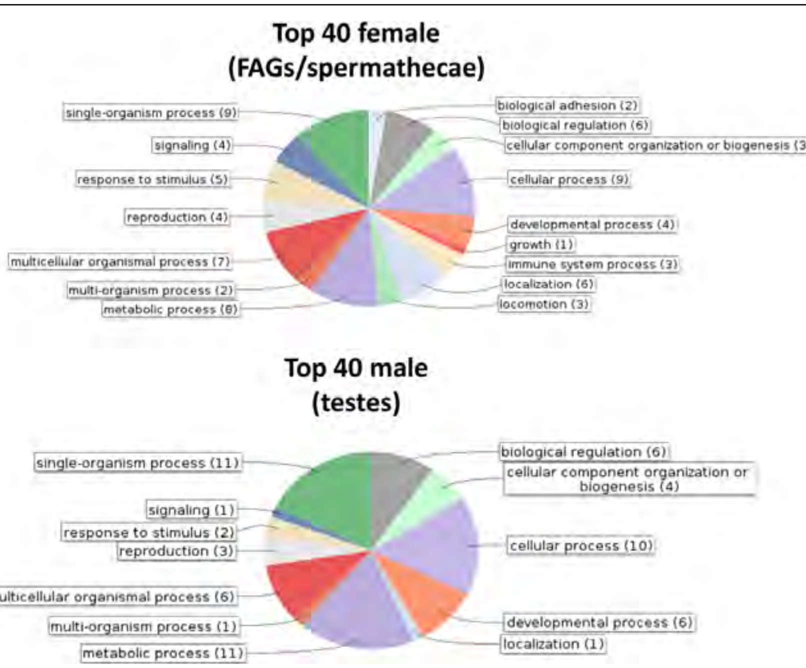


Figure 2 GO Term associations for the top 40 genes expressed in the female and male tissues. Associations were identified with BLAST2GO, using terms at the second level of the GO hierarchy.

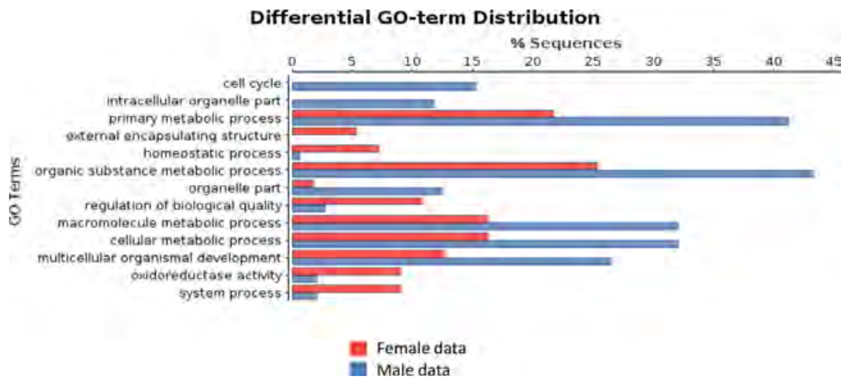


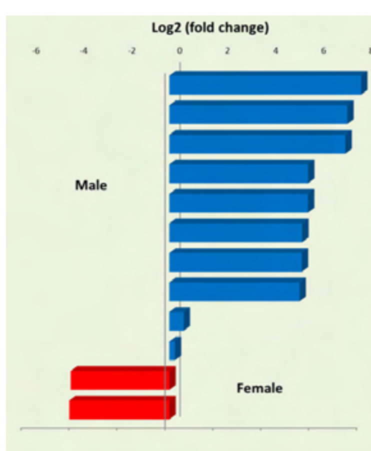
Figure 3 GO-terms (GO-Slim) differentially distributed between male and female transcriptomes. Contigs expressed only in female tissue are used as the test set (red bars) and contigs expressed only in male tissues as the reference set (blue bars).

(*male fertility factor kl5*), *ory* (*occludin-related Y protein*), *fem-1* (*sex-determining protein fem-1*), *gas8* (*growth arrest specific protein 8*) and *lobo* (*lost boys*). Three more genes that were up-regulated in MALE [*ix* (*intersex*), *pbl* (*pebble*) and *hcf* (*host cell factor C1*)] and two that were up-regulated in FEMALE [*sox and pcp* (*pupal cuticle protein 78E*)], albeit with lower statistical power (i.e., q>0.05) were also selected for further validation.

3.1. Drosophila Y-linked genes kl3, kl5 and ory

Quantitative RT-PCR confirmed the elevated expression of *kl2, kl3, kl5* and *ory* in male testes of the olive fly (Figure 5). In *Drosophila melanogaster, kl3* and *kl5* (along with *kl2*) are known Y-linked fertility factors. The lack of *kl3* or *kl5* causes the loss of the outer arm of the sperm tail axoneme [59], a structure known to contain the molecular motor protein dynein in other organisms [60]. Indeed, Goldstein et al. showed in 1982 that sperm from mutant *kl3*⁻ and *kl5*⁻ males lack three discrete high molecular weight proteins with mobility similar to dynein heavy chains of *Chlamydomonas reinhardtii* and proposed that these fertility factors are the structural genes of three different dynein heavy chain proteins [61]. In 1993, Gepner and Hays sequenced part of *kl5* and showed that it encodes an axonemal β-dynein heavy chain that is expressed in the testes [62].

ory is also Y-linked in *D. melanogaster*, although details on this gene are scarce. *kl3, kl5* and *ory* are Y-linked in 12 different sequenced Drosophila genomes [63]. In Drosophila, the closest paralogs of *kl2, kl3*, and *kl5* are autosomal and not X-linked, suggesting that the evolution of the Drosophila Y chromosome has been driven by an accumulation of male-related genes arising *de novo* from the autosomes [64]. While the most likely function of the



Gene name	GO analysis (Biological Process)	GO analysis (Molecular Function)	InterPro (IP)
pbl	single-organism developmental process; biological regulation; cellular component organization or biogenesis	semaphorin receptor binding; GTPase activator activity	Dbl homology (DH) domain; BRCT domain
ory	Unknown		
kl5	microtubule-based movement	microtubule motor activity; ATP binding	AAA+ ATPase domain
kl2	microtubule-based movement	microtubule motor activity; ATP binding	AAA+ ATPase domain
lobo	sperm storage; sperm motility		Transglutaminase-like
kl3	microtubule-based movement	microtubule motor activity; ATP binding	AAA+ ATPase domain
fem-1	male somatic sex determination		Ankyrin repeat-containing domain
gas8	sperm motility		
ix	sex determination; sex differentiation; regulation of transcription	transcription factor binding	Mediator complex, subunit Med29, metazoa
hcf	chromatin remodeling; histone H3-H4 acetylation; histone H3-K4 methylation.	sequence-specific DNA binding transcription factor activity; histone acetyltransferase activity	Fibronectin, type III
pcp	chitin-based cuticle development		Insect cuticle protein
sox	regulation of transcription, DNA-templated	RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity	High mobility group (HMG) box domain

Figure 4 Functional annotation of differentially expressed sex-differentiation genes. In the left part of the figure, the gene expression levels of the differentially expressed sex-differentiation genes (Log2, fold change) are shown, as resulted from the RNA-seq analysis. The up-regulated genes in males are depicted in blue bars and the up-regulated genes in females in red bars. At the right part of the figure, the Gene Ontology (GO) classification of the same genes for the ontologies: Biological Process (BP), Molecular Function (MF), and Interpro (IP) protein domains is listed.

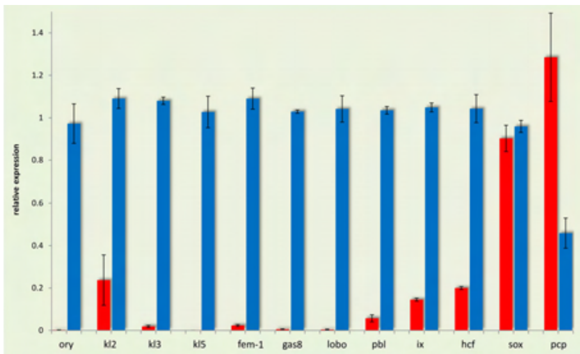


Figure 5 Validation profiles of differentially expressed sex-differentiation genes. Differentially expressed sex-differentiation genes of Figure 4 were further validated by qRT-PCR. Expression in male testes is depicted in blue color columns and expression in female accessory glands and spermathecae in red. Standard error of the mean of the two biological replicates is shown in bars. In all genes, except *sox* and *pcp*, expression in FEMALE and MALE was significantly different, as determined by t-test ($p < 0.05$).

three genes in the olive fly might be similar to that of Drosophila, we have no indication with regard to their chromosomal localization in the olive fly. Such information could shed some light to the evolutionary origin of the olive fly's Y chromosome.

3.2. Spermatogenesis and sperm motility genes

One spermatogenesis and two sperm motility genes were shown to be differentially over-expressed in male olive fly tissues both in the transcriptome analysis and after q-RT PCR (Figure 4 and 5). The first locus, ***sex-determining protein fem-1 (fem-1)***, encodes an essential spermatogenesis product in *Caenorhabditis elegans*. Three *fem* genes, *fem-1, fem-2*, and *fem-3*, have been shown to be essential for male development [65]. Loss-of-function mutations in any one of the *fem* genes prevent all aspects of male development and transform the animals that are genetically males into females [66,67]. The predicted product of the *fem-1* gene is an intracellular protein that contains ankyrin repeats, which in many other proteins mediate specific protein-protein interaction [67]. In *D. melanogaster*, a *fem-1* homolog with similar structure has been found [68]. The second locus, ***growth arrest-specific protein 8 (Gas8)*** is a microtubule-binding protein localized to regions of dynein regulation in mammalian cells. In mouse, Gas8 is predominantly a testicular protein, whose expression is developmentally regulated during puberty and spermatogenesis. In humans, it is absent in infertile males who lack the ability to generate gametes [69]. Gas8 has not been studied in insects. Finally, ***lost boys (lobo)***, has been shown to affect sperm entry movement into the female seminal receptacle and does not affect sperm exit movement from the seminal vesicle of *D. melanogaster* [70]. Given a similar function of these two loci in the olive fly, over-expression in male testes is expected.

3.3. Sex determination genes

In *D. melanogaster*, ***intersex (ix)*** controls somatic sexual differentiation only in females, acting near the end of the sex determination hierarchy. Its product does not have a known DNA-binding domain and, therefore, it is thought to act as a transcriptional co-factor for the female variant of Doublesex protein (DSX^F), a key gene of the sexual determination cascade in *D. melanogaster* [71]. Minimal differences were observed in *ix* expression between the two sexes of the olive flies.

Transcriptome analysis also showed a four-fold over-expression of ***sox*** in female tissues, a result that was not confirmed after validation. The *sox* gene family is a group of related transcription factors that play critical roles in embryonic development. This family was originally identified in mammals based on sequence similarity to SRY, the sex-determining region Y chromosome [72]. In the honeybee, as SOX proteins play key roles in gonad differentiation, the SoxE group orthologues were up-regulated in the drone testes [73]. In Drosophila SoxN is a new group B Sox gene expressed in the developing CNS and is one of the earliest transcription factors to be expressed in a pan-neuroectodermal manner [74].

3.4. Other genes

The ***Pebble (pbl)*** gene belongs to a family of GTP exchange factors that are essential for the construction of a contractile ring and the initiation of cytokinesis during the embryonic division cycles of the somatic cells in *D. melanogaster* [75,76]. Its role in spermatogenesis has not been elucidated yet. Expression of *pbl* in *D. melanogaster* testes is low [68]. On the other hand, expression in olive fly testes was found elevated in comparison to its expression in female accessory glands/spermathecae (Figure 4 and 5).

Host cell factor C1 (Hcf) is involved in a wide variety of cellular functions, including regulation of transcription, cytokinesis, cell cycle progression and chromatin remodeling [77]. The protein is essential for cellular viability and demonstrates similar activity among a broad range of species. A single *hcf* homolog is also present in *Drosophila* (called dHCF) and is expressed in all tissues, although at relatively low levels [68]. The transcriptome analysis in the olive fly tissues showed a ~0,2-fold higher expression in the male tissues. This result was confirmed after qRT-PCR in the same tissues, where higher levels of expression in testes were observed in comparison with female accessory glands/spermathecae (Figure 4 and 5).

Quantitation by RT-PCR confirmed the over-expression of ***pupal cuticle protein (pcp)*** in female accessory glands/spermathecae as compared to male testes. Cuticle proteins, along with chitin, are the two components of insect cuticle. The cuticular proteins seem to be specific to the type of cuticle that occurs at stages of the insect development. Flexible proteins are found in the flexible cuticle of larva and pupa, but can also be found in the soft endocuticle of adult insects [78].

Female insects require the steroid hormone 20-hydroxyecdysone (20E) in order to activate vitellogenesis, a process required for egg development. In *Anopheles gambiae* mosquitoes, large amounts of 20E are produced and stored in male accessory glands and subsequently delivered to female mosquitoes during mating [79]. Pupal cuticle proteins, on the other hand, are known to accumulate in response to a pulse of 20E [80]. However, given that FAGs/spermathecae collected were from unmated females, we cannot offer a plausible explanation for the over-expression of *pcp*s.

4. Validation of olfactory gene differential expression

Insects possess very sensitive chemosensory systems that can detect and discriminate among a diverse array of odors. These systems play a crucial role in insect survival and reproductive success, mediating responses to food detection, mating and oviposition. Odor recognition is a coordinated process requiring the combined specificities contributed by odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) as well as odorant receptors (ORs) (Reviewed in [81]). Insect odorant-binding proteins (OBPs) are soluble proteins surrounding the extracellular lymph of olfactory neurons [82]. OBPs are capable of binding and solubilizing small hydrophobic molecules from the environment and therefore transport them to the underlying ORs, which are expressed on peripheral olfactory receptor neurons. Insect ORs are either ionotropic receptors (IRs) or seven-transmembrane proteins (ORs) with an inverse topology compared to GPCRs, that form heterodimers of a ligand-binding OR and an ubiquitous highly conserved co-receptor named Orco [83]. These complexes are suggested to constitute ligand-gated nonselective cation channels triggering the olfactory signaling [81].

While OR expression in olfactory tissues is obvious and well-established, the distribution of ORs beyond the olfactory system has also been documented in different mammalian species [84-86], suggesting that ORs may play an important role in the ectopic expression of non-chemosensory tissues. Interestingly, OR expression has been documented in human and mouse germ cells [87-91] and recently in mosquitoes [92]. Similarly, other non-olfactory functions have been reported for OBP-like proteins including the B proteins of *Tenebrio molitor* accessory glands [93], the male specific serum proteins of *Ceratitis capitata* [94], and the heme-binding protein of *Rhodnius prolixus* [95]. These demonstrate that OBPs are not restricted to olfaction and are likely to be involved in broader physiological functions, suggesting that their roles may be restricted to general carrier capabilities with broad specificity for lipophilic compounds [96].

With that in mind, we opted to explore the expression of various olfactory-related genes in the reproductive systems under investigation. Twelve olfactory-related genes were present in the annotated list that resulted from the transcriptome assembly of the FEMALE and MALE olive fly tissues (Figure 6), nine of which presented various levels of over-expression in MALE, whereas the remaining three in FEMALE. In order to get a deeper insight, the relative expression of five of these genes was further analyzed in female FAGs/spermathecae, male testes and male accessory glands (MAGs), before and after mating.

obp83a, obp8a and *obp19a* genes are over-expressed in MALE tissue (Figure 6). qRT-PCR revealed that these genes share the same expression pattern in MAGs. *obp83a and obp8a* are over-expressed before mating in testes while *obp83a* and *obp19a* are over-expressed after mating in FAGs/spermathecae (Figure 7). All three genes are characterized by a GOBP (general odorant binding protein) domain that is also found in their orthologues in *Drosophila melanogaster*. This structural domain is found in pheromone binding proteins, which exist in extracellular fluid surrounding odorant receptors [97]. The presence of these OBPs in the reproductive tissues implicates their interaction with other substrates except the olfactory system as transporters in the post-mating events in the male reproductive system. In fact, *D.* melanogaster's o*bp8a* shows the highest levels of expression in male accessory glands [98,99] and has been associated with non-olfactory functions such as RNA transcription [100].

os-d is over-expressed in MALE tissue (Figure 6) while qRT-PCR showed similar expression patterns in mature FAGs/spermathecae, MAGs and testes, but no expression in MAGs before mating (Figure 7). Os-D is a chemosensory protein (CSP) that encodes the antennal protein 10 in *D. melanogaster*. CSPs are secreted in the sensillum lymph of insect chemosensory sensilla and some OS-D-like proteins bind short to medium chain length fatty acid derivatives with low specificity [101,102]. Their specific function remains uncertain [103], suggesting a more general physiological function relating to the transport/solubility of hydrophobic ligands in various tissues.

or10 showed expression in male tissues (Figure 6) while qRT-PCR detected same transcriptional profiles in all three tissues before and after mating (Figure 7). *or10* encodes an olfactory receptor protein and has a G-protein coupled receptor activity. The expression of ORs in testes has been reported for a number of species [90,104]. ORs' function in mammalian sperm is thought to regulate motility in response to exogenous signals derived from the existence of sperm-egg chemotaxis in invertebrates. The small peptides, speract and resact, are secreted by sea urchin eggs and attract spermatozoa in a species-specific

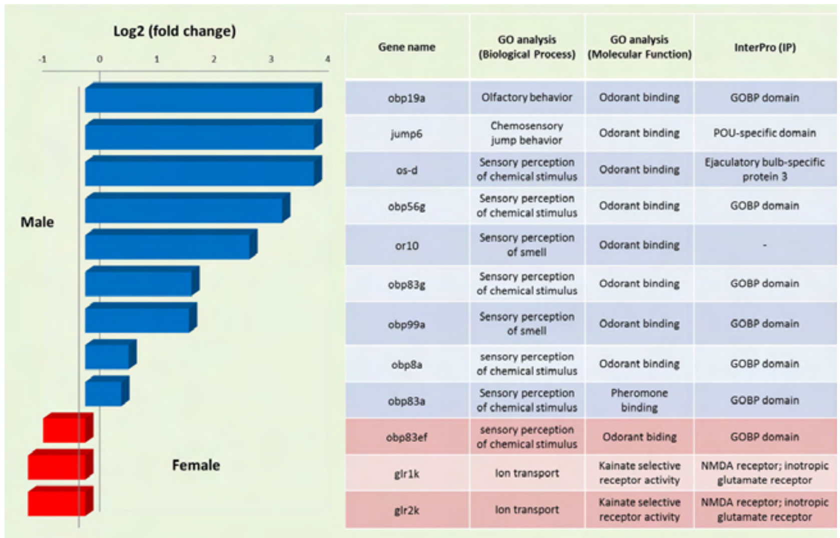


Figure 6 Functional annotation of differentially expressed olfactory genes. At the left part of the figure, the expression levels of the differentially expressed olfactory genes (Log2, fold change) are shown, as resulted from the RNA-seq analysis. The up-regulated genes in males are depicted in blue bars and the up-regulated genes in females in red bars. At the right part of the figure, the Gene Ontology (GO) classification of the same genes for the ontologies: Biological Process (BP), Molecular Function (MF) and Interpro (IP) protein domains is listed. Gene names are based on the nomenclature of the *Drosophila melanogaster* homologues [68].

manner by stimulating sperm motility and respiration [105,106]. The presence of a similar chemoreceptor may be essential in female spermatheca in order to establish a concentration gradient of a putative chemo-attractant. Since female accessory glands and spermatheca were dissected together, we are not able at this point to establish which exact tissue is the source of the observed expression of *or10*.

5. Early embryonic gene expression in the olive fly

As mentioned in the Background, promoters of early embryonic genes in combination with pro-apoptotic cell death genes are very important tools in inducing dominant early-embryonic lethality during insect transgenesis [107]. In that regard, the *serendipity-α* (*sry-α*) and *head involution defective* (*hid*) genes were selected for expression evaluation during embryonic development in the olive fly.

The embryonic developmental progress begins with the egg maturation and formation of the zygote, then enters the stage of blastoderm formation and gastrulation and ultimately ends with the organogenesis. Accordingly, three stages of embryogenesis have been also designated in *B. oleae*, whose average duration is 65-70h at 25 ± 1°C under standard laboratory conditions [108]. Microscopy morphological observations in living embryos report that cellularization of the blastoderm begins 6h after oviposition and lasts until 10h. During the third stage of organogenesis, the ventral furrow formation starts by 22h and the head and abdominal lobe masses become visible by 46h. Gut and mouth hook formation can be identified by 52h, whereas the development of other systems are distinct by 60h.

In *Drosophila melanogaster, sry-α* gene is specifically transcribed at the blastoderm stage in all somatic nuclei, from nuclear cycle 11 to the onset of gastrulation [109]. The gene product is required for the complete reorganization of the microfilaments at the onset of membrane invagination [110]. *sry-α* is fast evolving even within the Drosophilidae [111] and extensive divergence of many developmental genes within dipterans has also been reported [112-114]. This was most likely the reason for the unsuccessful efforts in *C. capitata* to obtain *sry-α* by degenerate PCR on the basis of sequence similarity with the homologous *D. melanogaster* [115]. Given the availability of both *D. melanogaster* and *C. capitata sry-α* sequences in the NCBI database, a homology search in the *B. oleae* transcriptome identified the relevant *B. oleae sry-α* gene homologue.

Based on this sequence, *B. oleae*-specific primers were designed and the expression profiles of *sry-α* mRNA were studied by qRT-PCR analysis at different stages of *B. oleae* embryonic development. Eggs were collected throughout embryogenesis from the time of egg laying to larval hatching. The selected time points represented embryos at 0h, 4h, 8h, 9h, 10h, 11h, 12h, 15h and 18h after oviposition (Figure 8, panel A). This analysis revealed that *sry-α* mRNA is developmentally regulated during the second major event in the first stage of embryogenesis. It is initially present in large amounts just after oviposition (0h embryos), following a reduction in 4h embryos. The larger

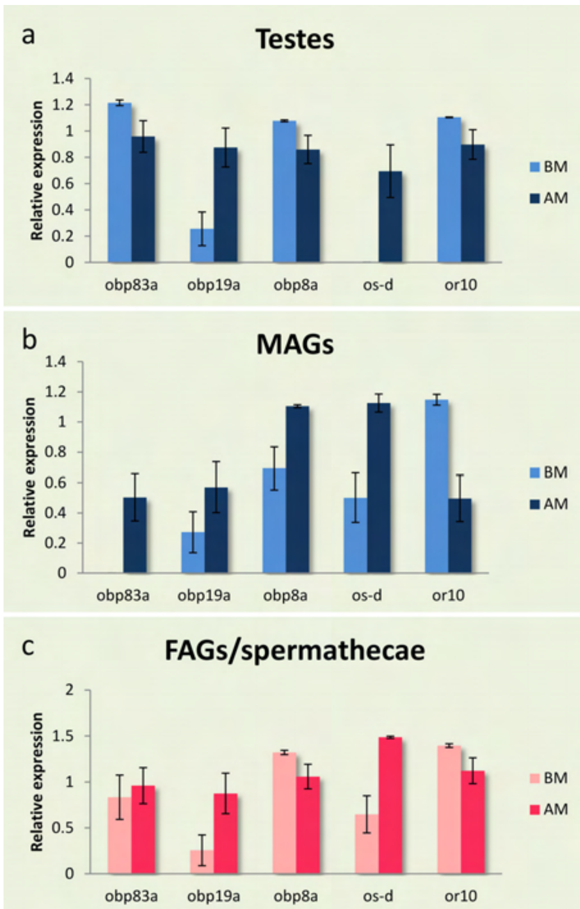


Figure 7 Relative expression profiles of differentially expressed olfactory genes. Expression profiles of five olfactory genes [odorant binding proteins *obp83a, obp19a, obp8a*, chemosensory protein, *os-d*, and odorant receptor 10, *or10*] as determined by qRT-PCR in three different tissues: Testes (a), MAGs (b) and FAGs/spermatheca (c) before (BM) and after (AM) mating. Standard error of the mean of five biological replicates is depicted in bars. No significant difference (for P < 0.05) was detected.

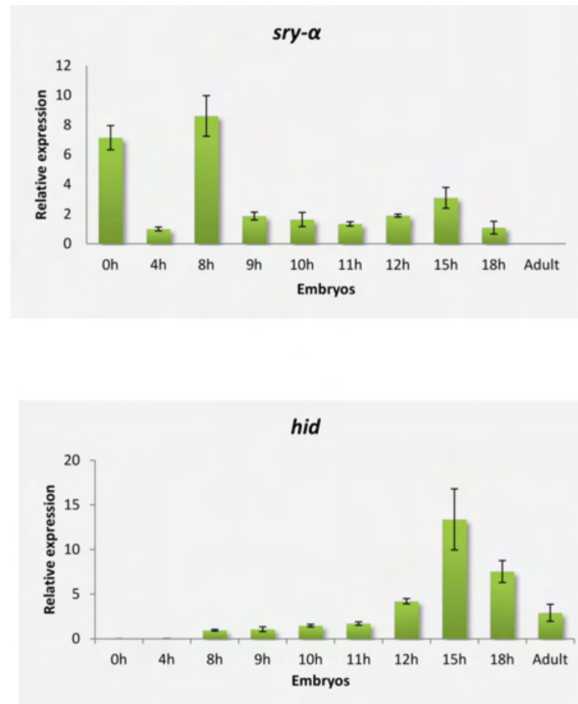


Figure 8 Expression profile analysis during the early stages of embryogenesis. Expression levels of A) *Bosry-a* and B) *Bohid* in individual eggs collected at different time points during embryonic development, as determined by qRT-PCR. Standard error of the mean of two biological replicates per time point is depicted in bars.

amounts of the transcripts among all time points examined were detected in 8h embryos. This suggests the presence of maternal mature transcripts which in turn are eliminated probably in the first event of maternal-to-zygotic transition (MZT). The subsequent wave of 'zygotic' activity requires zygotically synthesized transcripts [116]. In *D. melanogaster* as well as in *C. capitata*, *sry-α* is expressed only in the zygote [117]. However the retrieved *B. oleae* transcript shared greater amino acid similarity to the *D. melanogaster* CG8247 gene than to *sry-α*, as was also reported for the *Ccsry-α like* gene [118]. The orthologous CG8247 in *D. melanogaster* is characterized as a *sry-α*-like gene being also involved in cellular blastoderm formation. However, it is maternally inherited in contrast to *sry-α*, demonstrating a different mechanism of molecular control of transcription. In our case *Bosry-α like* gene seems to be maternally supplied in the embryos as mature transcripts. Previous studies have designated that the cellular blastoderm formation in *C. capitata* occurs within 9 h and 11 h after oviposition [115]. In accordance with *C. capitata*, a relative Tephritid species, we suggest that the cellurarization process in *B. oleae* during embryogenesis also occurs at 8h, since the *sry-α* transcripts were detected at higher levels during this time.

6. Apoptotic gene expression

At the same time, *head involution defective* (*hid*), known to have a central role in apoptosis pathway, was also selected for further study. Apoptosis is a genetically controlled mechanism of cytological events that results in programmed cell death. During development, programmed cell death plays a key role by eliminating unwanted cells from a variety of tissues, such as, for example, larval tissues during insect metamorphosis (Reviewed in [119]). A series of caspases, a family of cysteine proteases, play a central role during apoptosis. Once activated, caspases can cleave more than 100 different cell target proteins, bringing about ultimately the cell death [120]. Regulators of caspase activation may either promote apoptosis (pro-apoptotic) or inhibit apoptosis (anti-apoptotic). Drosophila Hid belongs to a family of pro-apoptotic proteins which act as antagonists of IAPs (Inhibitor of Apoptosis Proteins), thus resulting in caspase activation and apoptosis [119,121,122]. Such pro-apoptotic genes have been used in transgenic control systems for pest insects. In tetracycline-suppressible systems for female-specific lethality and conditional embryonic expression of a Drosophila *hid*-containing transgene, for example, 100% lethality was observed in Drosophila [123], as well as in the Tephritid flies *Ceratitis capitata* [117] and *Anastrepha suspensa* [124].

The developmental regulation of *Bohid* was explored by determining the transcript levels during embryogenesis. A qRT-PCR approach with species-specific primers was used to evaluate the expression pattern of *hid* in embryos at 0 h, 4 h, 8 h, 9 h, 10 h, 11 h, 12 h, 15 h, 18 h after oviposition. Based on *D. melanogaster hid* expression pattern, no expression was expected in embryos prior to formation of the syncytial blastoderm [125]. Indeed, until 8h no transcripts were detected. *hid* expression was first detected at 12h and peaked during 15h (Figure 8, panel B).

It is noteworthy that most developmental programmed cell death occurs during the gastrulation process of *D. melanogaster* embryonic development [126], suggesting that the onset of this period in *B. oleae* could be defined approximately at 12h, occurring mainly within 15-18h.

However, further examination of the pro-apoptotic function of *hid* gene is required in order to explore its ability of inducing apoptosis in *B. oleae* cells. Specific lethal embryonic phenotypes need to be obtained to characterize its role in the cell-death pathway. Ongoing analysis for the isolation of the complete gene will provide the essential tools for the generation of an endogenous effective lethal effector system.

Conclusions

In serious agricultural pests (like the olive fly) which are not model experimental organisms (unlike the medfly), the major focus of most scientific research is, in the end, directed towards control of the pest. Old and new environmental concerns and sensibilities, that regard mostly insecticide use, drive science to the quest of alternative, environmentally friendlier methods of pest control. Time and again it has been shown that such methods go through thorough understanding of the biology and ecology of the target organism. Since the initial unsuccessful SIT efforts, molecular and genetic studies in the olive fly have focused on genetic analyses of natural populations, cytogenetics, isolation and characterization of genes that control important biological processes, as well as the identification and mapping of several microsatellite loci. Just a few years ago, *B. oleae* was successfully transformed, an achievement that gave new perspective towards the efficient use of the SIT. Lately, this is being coupled with genomics studies and transcriptomics analyses of various important systems, as well as efforts in advancing olive fly mass-rearing, that are setting the ground for the application of modern control approaches through the genetic manipulation of the insect.

Methods

Ethics statement

The study was carried out on laboratory reared olive flies. No specific permissions are required for these experiments, since these studies did not involve endangered or protected species.

Fly culture and stocks

Laboratory strain

The laboratory strain of the olive fly (LAB) is part from the original stock from the Department of Biology, 'Demokritos' Nuclear Research Centre, Athens, Greece, and has been reared in our laboratory for over 15 years. The flies are reared at 25°C with a 12h light/12h dark photoperiod in 30x30x30cm^3 cages, as described by [127-129].

Egg collection

For embryo analysis, eggs were collected from 10-day old mated females maintained in our laboratory, which were fed with artificial adult diet to ensure high oviposition rates and embryo viability. Adults were exposed to paraffin oviposition domes for 10 minutes and the eggs were obtained with a 0.3% propionic acid solution, assigning this as the start time point. Eggs were maintained in an incubator according to the standard rearing conditions.

RNA isolation for library preparation and functional analysis

Total RNA was isolated from female accessory glands (FAGs) and spermathecae of ~300 female flies and from testes of ~150 male flies. Four-day old sexually immature unmated insects were used. For RNA isolation, the TRIzol® Reagent (Ambion-Invitrogen) was used, following the instructions of the manufacturer with minor modifications. RNA extraction was followed by an additional DNA removal using the TURBO DNA-free Kit (Ambion-Invitrogen), according to manufacturer's instructions. The integrity of RNA was assessed by 1% agarose gel electrophoresis and the purity of all RNA samples was evaluated at Fleming Institute (Greece) with the use of (Agilent 2100 Bioanalyzer) and NanoDrop (2000).

Whole transcriptome library preparation for next-generation sequencing with the SOLiD 4 Sequencing System

RNA transcripts from olive fly FAGs/spermathecae (FEMALE) and testes (MALE) were used to construct two cDNA libraries for sequencing analysis on the SOLiD 4 Sequencing System. More specifically, polyadenylated RNA (polyA-RNA) was isolated from 5 μg of total RNA using the Dynabeads Oligo(dT) kit (Ambion, Life Technologies Corporation). The isolated polyA-RNA was randomly fragmented by chemical hydrolysis at 94°C for 5 minutes and was then treated with antarctic phosphatase to remove phosphate groups from the fragments' ends, followed by treatment with T4 polynucleotide kinase to add a Pi at the 5' end of each fragment. The resulting RNA fragments were hybridized and ligated to the P1 and P2 adaptor sequences specifically designed for sequencing with the SOLiD system (SOLiD Total RNA-Seq Kit, Life Technologies Corporation). The RNA produced was reverse transcribed to cDNA which was then amplified in a 15-cycle PCR. At this step, the use of different barcoded 3' PCR primers from the selection included in the SOLiD barcoding kit allowed the preparation of cDNA libraries for multiplex sequencing. From the cDNA produced, only fragments of average size 200-300 bp were selected with two rounds of magnetic bead purification (Agencourt AMPure XP Reagent, Beckman Coulter).

The quality and size of the purified cDNA library was assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc.) and with quantitative PCR using the Library Quant Kit ABI Solid (KAPA Biosystems). A multiplex library mix (500pM) was used to prepare a full-slide for analysis on the SOliD 4 Sequencing System (Applied Biosystems) with 35+50 bp PE-chemistry.

RNA isolation and expression analysis of selected genes

RNA extraction for expression analysis of sexually differentially expressed genes. For the validation of the differential expression of sexually differentially expressed genes, RNA was extracted from two pools of 40 pairs of spermathecae/FAGs and 40 pairs of testes (two biological pool replicates), dissected from an equivalent number of female and male adult laboratory flies, respectively.

RNA extraction for expression analysis of olfactory and early embryonic developmental genes. For the validation of the olfactory genes expression, RNA was extracted from five female and five male individual insects (five biological replicates, respectively) before and after mating of the aforementioned laboratory strain. Two groups of insects were considered. Firstly, unmated insects, i.e., sexually mature 7-day old unmated insects (before mating, BM). Secondly, mated insects, i.e., sexually mature 7-day old insects that were allowed to mate on the seventh day and were dissected 12 hours after mating (after mating, AM). For the validation of the sexually differentially expressed genes, the RNA isolated for the construction of the two libraries was used. RNA was extracted using TriZol reagent according to manufacturer's protocol.

For the validation of the early embryonic genes, eggs were removed from the incubator at different time intervals throughout embryonic development and total RNA was extracted from each egg using TriZol reagent according to the manufacturer's protocol. Two individual eggs (two biological replicates) from the various time points during the embryonic developmental stages were used for the extractions.

Following extraction, the RNA was treated with 1.0 unit of DNase I (Invitrogen) according to manufacturer's instructions. In all of the above cases, the total amount of DNA-free RNA obtained from each tissue (between 400 to 700 ng) was converted into cDNA using 300ng Random hexamer primers (equimolar mix of N_5A, N_5G, N_5C and N_5T), 200 units MMLV Reverse Transcriptase (Geneon), 5X reaction buffer, 40mM dNTP mix and 40 units RNase Inhibitor (GeneOn) according to the manufacturer's instructions. Reverse transcription was conducted at 42°C for 50 min and 70°C for 15 min. The resulting cDNA was used in the subsequent qPCR reactions.

Specific primers for the amplification of selected differentially expressed genes revealed by the transcriptome analysis were designed by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Table S2). To identify sequences with homology to the genes *sry-α* and *hid*, the orthologous genes of *C. capitata* and *An. suspensa* were used as queries to search for *B. oleae* transcripts using tBLASTX in the TSA Database. Species-specific Blast hits for each of the query sequences were retrieved (Genbank: GAKB01005111.1, GAKB01003654.1) and used to design primers (Table S2) for the subsequent amplification of gene-specific sequences by quantitative real-time PCR (qRT-PCR).

Relative quantitation was used to analyze changes in expression levels of the selected genes using a Real-time PCR approach. Expression values were calculated relatively to the housekeeping *rpl19* gene. *Rpl19* and *14-3-3z* genes were used as reference in MAGs and testes while *actin3* and *a-tubulin* in FAGs/spermathecae. The qRT-PCR conditions were: polymerase activation and DNA denaturation step at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing/extension and plate read at 56 °C for 30 s and finally, a step of melting curve analysis at a gradual increase of temperature over the range 55 °C → 95 °C. In this step, the detection of one gene specific peak and the absence of primer dimer peaks was assured. Each reaction was performed in a total volume of 15 μl, containing 5 μl from a dilution 1:10 of the cDNA template, 1X iTaq Universal SYBR Green Supermix (Biorad, Gaithesburg, MD) and 400nM of each primer. The reactions were carried out on Bio-Rad Real-Time thermal cycler CFX96 (Bio-Rad, Hercules, CA, USA) and data analysed using the CFX Manager™ software. All qRT-PCRs were performed in triplicate (i.e., three technical replicates).

Bioinformatics analysis

All paired and unpaired reads of the libraries were assembled to construct the reference transcriptome using the SOAPdenovo assembler [54] with a word size of 25 nt. Annotation of the assembled sequences was obtained by comparing to the NCBI non-redundant (Nr) protein database (May 7th, 2014 version) using blastx [130] and collecting the annotations with the BLAST2GO tool [55]. TopHat [131] was used to generate a spliced alignment to the reference transcriptome. Transcripts were assembled using Cufflinks and differentially expressed genes were identified using Cuffdiff [56]. GO-term enrichment between male and female transcriptomes was analyzed using the using the GOSSIP [132] application embedded in BLAST2GO.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files. Additional File 1, Additional File 2, Additional File 3 and Additional File 4

Additional material

Additional File 1:
Additional File 1:
Additional File 1:
Additional File 1:

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ES was involved in the transcriptome library construction and performed the analysis of the sex-determination genes; MR performed the bioinformatics analysis of the transcriptome; VH constructed the transcriptome libraries and analysed the sequencing data; KTT and AMM analyzed the embryonic and apoptotic genes; MEG, ST and KA analysed the olfactory genes; JR directed the bioinformatics analysis; KDM designed and coordinated the study. All authors participated in drafting the manuscript and read and approved the final document.

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Housekeeping in Tephritid insects: the best gene choice for expression analyses in the medfly and the olive fly

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Real-time quantitative-PCR has been a priceless tool for gene expression analyses. The reaction, however, needs proper normalization with the use of housekeeping genes (HKGs), whose expression remains stable throughout the experimental conditions. Often, the combination of several genes is required for accurate normalization. Most importantly, there are no universal HKGs which can be used since their expression varies among different organisms, tissues or experimental conditions. In the present study, nine common HKGs (*RPL19*, *tbp*, *ubx*, *GAPDH*, *α-TUB*, *β-TUB*, *14-3-3zeta*, *RPE* and *actin3*) are evaluated in thirteen different body parts, developmental stages and reproductive and olfactory tissues of two insects of agricultural importance, the medfly and the olive fly. Three software programs based on different algorithms were used (*geNorm*, *NormFinder* and *BestKeeper*) and gave different ranking of HKG stabilities. This confirms once again that the stability of common HKGs should not be taken for granted and demonstrates the caution that is needed in the choice of the appropriate HKGs. Finally, by estimating the average of a standard score of the stability values resulted by the three programs we were able to provide a useful consensus key for the choice of the best HKG combination in various tissues of the two insects.

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and the olive fruit fly, *Bactrocera oleae* (Rossi), belong to the Tephritidae family of insects. As typical fruit flies, females lay their eggs in fruits or vegetables and the emerging larvae feed in the fruit sap, thus destroying the fruit. The medfly is one of the most devastating insects, easily adapting to new environments and hosts, infecting more than 260 species of fruits and vegetables worldwide[1,2], and causing great economic losses in fruit production and quarantine costs. The olive fruit fly, on the other hand, is a monophagous species, the most important enemy of olive cultivations[3,4]. Whole genome sequencing of both species has been completed[5,6], offering a holistic view of the entire genomes, allowing the study of any desired gene and thus leading to a profound understanding of the biology of these species. Such understanding is a prerequisite for novel, alternative to insecticides, control approaches.

The study of any gene inevitably goes through detailed and thorough scrutiny of its expression profile in various tissues and under different conditions. An invaluable tool for such expression analysis is RT-qPCR. The same way PCR revolutionized modern day molecular biology, RT-qPCR gave tremendous impetus to studies of gene expression, quantitative genotyping, genetic variation, disease diagnosis, forensics and many more. Due to the simplicity of the reaction, data can be easily collected and published in high impact journals without, necessarily, following good practices of RT-qPCR[7]. One of the most important parameters that should be addressed in order to standardize the reaction and perform a valid RT-qPCR analysis is the selection of suitable reference housekeeping genes. Since the reaction has several limitations as a result of the quality and quantity of starting RNA and the efficiency of its reverse transcription, housekeeping genes are used for the systematic normalization of gene expression data in order to improve the fidelity and accuracy of RT-qPCR[8–10]. Time and again, it has been demonstrated that the use of an unsuitable reference gene can lead to false results of the qPCR data and, consequently,

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to erroneous interpretations[11–14]. Most frequently, indeed, more than one housekeeping genes are required for proper normalization of the data[15,16].

In insects, many articles have been published on the identification and selection of the best reference gene in specific tissues and under different conditions. In the Tephritidae family there are two studies on the oriental fruit fly, *Bactrocera dorsalis*[17,18] and one in the West Indian fruit fly *Anastrepha obliqua*[19]. Among other dipteran species, there are three studies on *Drosophila melanogaster*[15,20,21] and a single one on each of *D. suzukii*[22], *Musca domestica*[23], *Lucilia cuprina*[24] and the Calliphoridae family[25]. Interestingly, there are no studies published on any mosquito species. In many mosquito publications, normalization of RT-qPCR is at best performed using a housekeeping gene (HKG) that demonstrates stable expression in microarray or RNAseq results[26,27]. This strategy may seem biologically reasonable, but there is a potential technical artifact considering that microarrays, RNAseq and RT-qPCR constitute quite different methods, with different limitations, requiring different standardization each. Most frequently, however, there is no specific justification regarding the selection of the utilized HKGs[28–34] except, at most, that it may have been used previously in the same[35,36] or related species[37,38]. Furthermore, with regard to published HKG studies on Diptera, the number of HKGs tested varies from as low as six[18,21,25] to over 20[15]. Unfortunately, neither the same genes nor the same tissues and conditions are studied, a fact that makes any effort to compare results practically impossible. Very importantly, these studies hardly ever indicate the use of the same housekeeping gene or gene combination in different tissues of the same insect or in the same tissue of different insects. Since, as mentioned above, the use of improper housekeeping genes for the normalization of the RT-qPCR can lead to erroneous results, this variability necessitates each time, for every organism and every tissue, the search for the proper housekeeping genes. Additionally, given the fact that the available software (such as *geNorm*[9], *NormFinder*[39], *BestKeeper*[40] *and* the web-based *RefFinder* platform[41]) are based on different statistical algorithms, they do not result in the same HKG suggestions for a particular tissue[42,43].

Here we present the most extensive study on HKGs, at least in the dipteran order of insects. The study validates nine candidate reference genes in thirteen different tissues of the model tephritid fly, the Mediterranean fruit fly, *C. capitata,* and the olive fruit fly, *B. oleae*. The genes are: *RPL19* (*ribosome protein L19*), *tbp* (*TATA-binding protein*), *ubx* (*ultrabithorax*), *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenease*), *α-TUB* (*α-tubulin*), *β-TUB* (*β-tubulin*), *14-3-3zeta*, *RPE* (*RNA polymerase II*) and *actin3.* The tissues selected for the analysis were mostly tissues from either the reproductive [testes, ovaries, male and female accessory glands (MAGs and FAGs, respectively), ovipositors] or the olfactory (maxillary palps and antennae) systems of the flies. In addition, we analyzed three developmental stages (egg, larva, pupa) and the three sections of the insect body (head, thorax, abdomen), as they are often convenient controls for comparison with other tissues.

Results

In the present study, the best choice for reference genes for RT-qPCR in thirteen tissues of two insects of the Tephritidae family, the Mediterranean fruit fly, *Ceratitis capitata* and the olive fruit fly, *Bactrocera oleae*, was examined. Three available software programs were used for the analysis and, since each program is based on a different algorithm, an effort was put to generate a consensus of the three programs.

Gene choice and amplification performance. Nine different housekeeping genes, commonly used in other dipteran species, were chosen for the analysis. The genes considered were: *RPL19* (*ribosome protein L19*), *tbp* (*TATA-binding protein*), *ubx* (*ultrabithorax*), *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenease*), *α-TUB* (*α-tubulin*), *β-TUB* (*β-tubulin*), *14-3-3zeta*, *RPE* (*RNA polymerase II*) and *actin3*. Gene names and IDs for the two species are presented in Supplementary Table S6.

In all instances, primers were designed by Primer-BLAST[44] in order to get amplicons ranging from 82 to 150 bp, as shown in Supplementary Table S7. Reaction conditions described in the Methods section resulted in one gene-specific peak and the absence of primer dimers peaks (data not shown). The PCR efficiency (E) and the correlation coefficient (R^2) characterizing each standard curve are also given in Supplementary Table S7. Efficiencies for all tested genes varied between 90.1% and 106.4%.

All reactions were done in triplicate (three technical replicates). The expression of the reference genes was measured in 8 or 10 biological replicates, as indicated in Table 1. Three negative controls were also used.

Expression stability by *geNorm*. *geNorm* is a Visual Basic Application (VBA) for Microsoft Excel that automatically calculates two parameters: the gene-stability measure M and the pairwise variation V. The lower the gene-stability M value indicates the more stably expressed gene. Values of M higher than 1.5 are not considered stable across measurements. The pairwise variation V, on the other hand, indicates the least number of the most stably expressed genes that should be combined for optimal normalization. Additionally, V should be below the cut-off value of 0.15, otherwise, the lowest V should be considered. Using this algorithm, we ranked the nine housekeeping genes in the thirteen tissues tested according to their expression stability (Fig. 1). For *B. oleae* egg, for example, under the cut-off value of 0.150 is V4/5 (0.136, Fig. 1-D) and, therefore, the four most stable genes for the eggs (*ubx* with M = 0.508*, 14-3-3zeta* with M = 0.556*, tbp* with M = 0.601 and *RPE* with M = 0.658) should be combined in order to obtain optimal normalization. For the other tissues, the lowest pairwise variation value and the suggested combination of HKGs are presented in Supplementary Table S1. In most cases, *geNorm* suggests the combination of 2–3 HKGs for optimal normalization. In one case (FAGs of *B. oleae*) it suggests the combination of six; and in one other (ovipositor of *B. oleae*) it suggests the combination of seven. For *C. capitata*, *α*- and *β-tubulin* are most frequently among the suggested HKGs, while for *B. oleae 14-3-3zeta* is the winner. *RPE* and *ubx* are never among the suggested HKGs in *C. capitata*.

Expression stability by *NormFinder*. *NormFinder* algorithm identifies the optimal normalization gene among a set of candidate genes, providing a stability value for each gene. This value is the estimated expression

Tested tissues		Biological replicates
Developmental Stages	Egg	10 individuals
	Larva	10 individuals
	Pupa	10 individuals
Body parts	Head	10 individual parts (5 male and 5 female)
	Thorax	10 individual parts (5 male and 5 female)
	Abdomen	10 individual parts (5 male and 5 female)
Reproductive System	MAGs	10 pairs (1 pair of MAGs/fly)
	Testes	10 pairs (1 pair of testes/fly)
	FAGs	10 pairs (1 pair of FAGs/fly)
	Ovaries	10 sets (1 set of ovaries/fly)
	Ovipositors	8 pools (4 flies/pool)
Olfactory System	Maxillary palps	8 pools (4 flies/pool)
	Antennae	8 pools (4 flies/pool)

Table 1. The thirteen tested tissues of *C. capitata* and *B. oleae*.

variation if a given gene is used for normalization. Therefore, the candidate genes can be ranked according to their expression stability in the different tissues or experimental conditions[45]. The calculated stability values for each HKG and the according ranking in the thirteen tissues are shown in Supplementary Tables S2A and S2B for *C. capitata* and *B. oleae*, respectively. For *C. capitata*, *GADPH* ranks first in four tissues, *14-3-3zeta* in three, while *ubx* and *actin3* never rank first. For *B. oleae*, *RPE* and *14-3-3zeta* rank first in three tissues each, while *ubx* and *GADPH* never rank first.

Expression stability by *BestKeeper*. *BestKeeper* software estimates standard deviation (SD) of the Ct values of all candidate genes. Since the expression levels of suitable HKGs should be highly correlated, the lower the SD the more stable the gene[40]. The disadvantage of *BestKeeper* is that it does not provide a combination of reference genes required for an experiment. The calculated SD values and CV (coefficient of variation) for each HKG in the thirteen tissues are shown in Supplementary Tables S3A and S3B for *C. capitata* and *B. oleae*, respectively. According to *BestKeeper*, *α-TUB*, *GADPH* and *RPL19* have the least SD values in three different tissues of *C. capitata* each, while *tbp* and *ubx* in none. For *B. oleae*, *α-TUB* and *RPE* have the least SD in four different tissues each, while *β-TUB*, *ubx*, *GADPH* and *actin3* in none.

Seeking consensus. Since the different software programs use different algorithms to estimate gene expression stability, they rarely reach the same ranking. *RefFinder* software theoretically integrates the results of the previous analyses (by *geNorm*, *Normfinder* and *BestKeeper*). It then assigns an appropriate weight to an individual HKG and calculates the geometric mean of their weights for an overall final ranking[41]. We ran this user-friendly web-based tool as well. However, since the values that *RefFinder* calculated for, e.g., *geNorm* differed from those estimated by *geNorm* itself, we considered *RefFinder* unreliable and we did not use it any further. *RefFinder* results are presented in Supplementary Tables S4A and S4B.

In order to propose a combination of the most stable HKGs that a researcher can use for normalization of gene expression in *C. capitata* and *B. oleae*, we took a different route. We first estimated the average of a standard score (z-score) of the stability values resulted by all three software packages for every single gene and then ranked them according to this new average score. Complete results of this ranking are presented in Supplementary Tables 5A and 5B. The first three genes of this consensus ranking are presented in Table 2. In the medfly, *RPL19* is the HKG that is most often found in the best three ranking genes, followed by *β-TUB*, while *ubx* is never among the top three. Similarly, in the olive fly *14-3-3zeta* is the HKG that is most often found in the best three ranking genes, followed by *GADPH*, while *α-TUB* is not found at all. To our experience, the combination of at least two HKGs and at most the number of genes suggested by *geNorm*, provides an excellent internal control in all RT-qPCRs.

Discussion

Several times in the recent years it has been documented that the choice of the right reference gene/s for the standardization of RT-qPCRs is of paramount importance and the possible use of the inappropriate HKGs can lead to incorrect results[11–14]. Common housekeeping genes, that are supposed to be constitutively expressed in order to maintain basic cellular functions, may not have constant and stable expression throughout an experiment. This may be due to the special characteristics of the organism or tissue analyzed or the particular conditions of the experimental design. Good practice of an RT-qPCR experiment requires the establishment of the appropriate HKGs for its standardization[46], even though good practice is not always observed.

We set out to address the above question for the medfly, *C. capitata*, and the olive fly, *B. oleae*, both very important agricultural pests. Particularly, the medfly is a cosmopolitan pest and due to its great importance in the cultivation and export of more than 260 fruits and vegetables[1,2], it has turned out to be a model organism in the Tephritidae family of insects and beyond, for studies ranging from classical genetics to genomics[5,47–52], as well as area-wide control practices[53,54]. The olive fly, on the other hand, is a strictly monophagous cousin of the medfly,

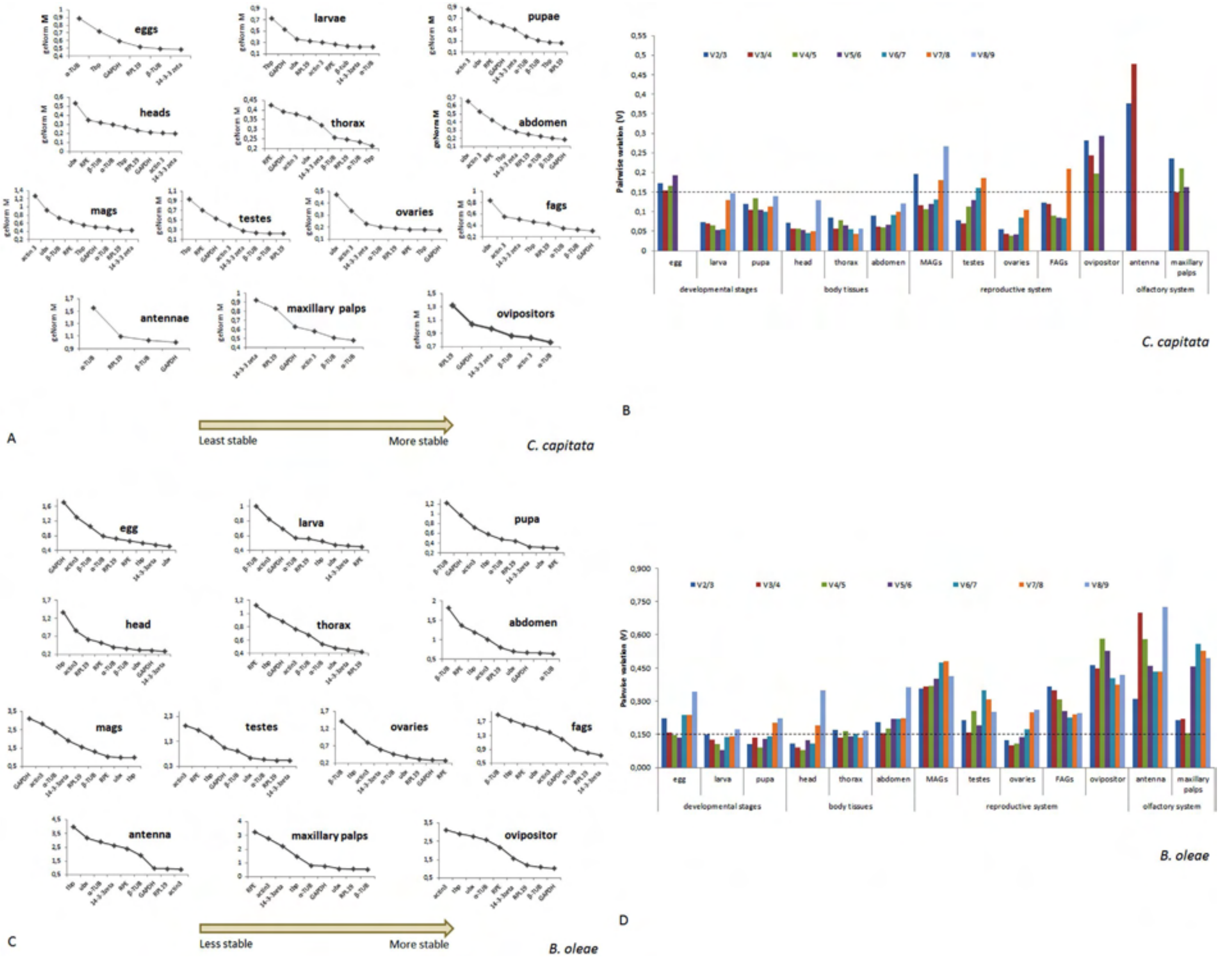

Figure 1. (**A**) Stability values of the reference genes in the 13 *C. capitata* tissues under study as generated by the *geNorm* algorithm. The average expression stability values from least stable (left) to most stable (right) for the egg, larva, pupa, head, thorax, abdomen, MAGs, testes, ovaries, FAGs, antennae, maxillary palps and ovipositor of the Mediterranean fruit fly. (**B**) Pairwise variation (V) of the housekeeping genes computed by *geNorm* in *C. capitata*. The pairwise variation (V_n/V_{n+1}) analysis determines the optimal number of reference genes for all of the tissues under study. (**C**) Stability values of the reference genes in the 13 *B. oleae* tissues as generated by the *geNorm* algorithm. The average expression stability values from least stable (left) to most stable (right) for the egg, larva, pupa, head, thorax, abdomen, MAGs, testes, ovaries, FAGs, antennae, maxillary palps and ovipositor of the olive fruit fly. (**D**) Pairwise variation (V) of the housekeeping genes computed by *geNorm* in *B. oleae*. The pairwise variation (V_n/V_{n+1}) analysis determines the optimal number of reference genes for all of the tissues under study.

of particular interest in the olive producing areas of the world[4]. Recent development of molecular and genomics tools have made it focus of active research, with renewed interest in its control[55,56]. The tissues selected for the analysis were mostly tissues from either the reproductive (testes, ovaries, male and female accessory glands, ovipositors) or the olfactory (maxillary palps and antennae) systems of the flies. The reproductive system is involved in the successful mating and egg development while the olfactory system plays a crucial role in insect survival and reproductive success, mediating responses to food, mates and oviposition. Beyond their general interest, such systems can serve as targets for alternative control approaches, such as the Sterile Insect Technique and its alternatives[52,57–59] and, therefore, are currently under scrutiny in the scientific community. In addition, we analyzed three developmental stages (egg, larva, pupa), as they are useful in order to obtain the expression profile throughout the life cycle of an insect. Finally, we included the three sections of the insect body (head, thorax, abdomen), as they are often convenient controls for comparison with other tissues.

In order to determine the best combination of HKGs, we performed our analyses with the three most popular software programs, *geNorm*[9], *NormFinder*[39] and *BestKeeper*[40]. As anticipated, results were largely inconsistent among them, as they are based on different algorithms. A fourth user-friendly web-based software, *RefFinder*[41], that is supposed to integrate the results of the previous three programs, gave inconsistent results with the programs themselves and so it was deemed untrustworthy. Instead, we decided to take a different route: we first transformed the raw scores of the three programs into standard scores, then calculated the average of the standard scores and finally ranked them. The use of the average of scores is based on the underlying idea of producing a

Tested tissues		The best ranking reference genes in *C. capitata*			The best ranking reference genes in *B. oleae*		
Developmental stages	Egg	***14-3-3 zeta***	***RPL19***	*β-TUB*	*RPE*	***14-3-3zeta***	***RPL19***
	Larva	***RPE***	*actin3*	*RPL19*	*14-3-3zeta*	***RPE***	*GAPDH*
	Pupa	*tbp*	***RPL19***	*β-TUB*	***RPL19***	*14-3-3zeta*	*RPE*
Body tissues	Head	***14-3-3zeta***	***RPL19***	***actin3***	***14-3-3zeta***	***RPL19***	***actin3***
	Thorax	*RPL19*	***GAPDH***	***14-3-3zeta***	***14-3-3zeta***	***GAPDH***	*β-TUB*
	Abdomen	*α-TUB*	***GAPDH***	*β-TUB*	*14-3-3zeta*	***GAPDH***	*ubx*
Reproductive system	Testes	***actin3***	*RPL19*	*α-TUB*	*14-3-3 zeta*	***actin3***	*RPE*
	MAGs	***RPL19***	*α-TUB*	***GAPDH***	***RPL19***	*actin3*	***GAPDH***
	Ovaries	***GAPDH***	*α-TUB*	*RPE*	*actin3*	***GAPDH***	*RPL19*
	FAGs	*β-TUB*	*tbp*	***RPE***	*GAPDH*	***RPE***	*actin3*
	Ovipositor	*β-TUB*	*α-TUB*	***14-3-3 zeta***	*tbp*	***14-3-3zeta***	*RPL19*
Olfactory system	Antennae	***14-3-3zeta***	*β-TUB*	***GAPDH***	***14-3-3zeta***	*actin3*	***GAPDH***
	Maxillary palps	*α-TUB*	*RPL19*	*β-TUB*	*ubx*	*GAPDH*	*actin3*

Table 2. Consensus ranking of tested *Ceratitis capitata* and *Bactrocera oleae* housekeeping genes according to the mean of the z-scores of their stability values obtained by *geNorm*, *NormFinder* and *BestKeeper*. Only the first three genes are indicated, listed from the most stable (left) to the least stable (right) gene order. Genes in bold contain highly ranked HKGs that are common in both *C. capitata* and *B. oleae*.

composite score using a linear combination of the individual score values. This practice is common in Statistics, e.g., Principal Components Analysis, Factor Analysis and other multivariate[60–62] methods. In all these methods the individual variables do not have to be similar in derivation nor do they have to measure the same quantity. Instead, they measure different facets of the same concept, in many instances using different measurement tools. This is the case in our work. Averaging is the simplest form of linear combination (all scores have the same coefficient).The major issue in this case is not how the individual scores are derived, but if their values are in a similar range. When this is not true the score with the larger values would dominate the composite score. We resolved this problem by score standardization.

The aforementioned approach resulted in a useful consensus key (Table 2) for the choice of the best HKG combination in various tissues of the medfly and the olive fly. A few qualified comments based on Table 2 are worth making. First, the most common genes found in the top three choices for both the medfly and the olive fly (i.e., found five times or more in both organisms in Table 2) are *14-3-3zeta*, *RPL19* and *GAPDH*, while the least common (two times or less) are, *tbp* and *ubx*. Curiously, *α*- and *β-tubulins* are quite frequently found in the medfly (6 and 7 times, respectively), while only *β-tubulin* is found only once in the olive fly. Secondly, in quite a few occasions (indicated by the genes in bold in Table 2) the same HKGs are found in the top three genes in the same tissue of both insects. For example, in eggs *14-3-3zeta* and *RPL19* are ranked in the top three HKGs in both the medfly and the olive fly. All things being equal, the probability of finding one particular gene out of the nine tested HKGs among three selected genes is $^{1}/_{3}$; the probability of finding two particular genes out of nine tested HKGs among three selected genes is $^{1}/_{12}$; while the probability of finding three particular genes out of nine tested HKGs among three selected genes is $^{1}/_{84}$ (calculation based on hypergeometric probabilities). Furthermore, the probability of finding the *same two* of the nine tested HKGs among three genes of both organisms (independent selections from a probability point of view) acquires the statistically significant value of 0.0069 ($^{1}/_{12} \times ^{1}/_{12}$), while finding the *same three* of the nine tested HKGs among three genes of both organisms acquires the statistically significant value of 0.00014 ($^{1}/_{84} \times ^{1}/_{84}$). This observation may suggest a biological explanation for the stability of *14-3-3zeta*, *RPL19* and *actin3* in heads or the stability of *14-3-3zeta* and *RPL19* in eggs of both species. A similar situation is detected in thoraces, MAGs and antennae, but not in FAGs or maxillary palps. Therefore, one can imagine that similar patterns of neuronal development in heads or embryonic development in eggs of both species would require similar expression of HKGs; on the contrary, differences in the female reproductive system (FAGs) or different diets (perceived by the maxillary palps) between the two insects would be reflected in the expression of different HKGs. More analyses are needed to substantiate such claims that are beyond the scope of this article.

Closing, we should iterate once again that the stability of common HKGs should not be taken for granted and that a lot of caution is needed in the choice of the appropriate HKGs. In fact, there is a need to validate the use of the proper HKG more often than practically encountered in recent literature. Even though we consider that our analysis offers a useful tool in the medfly and olive fly research community, we do encourage researchers to check these HKGs on their own subjects before use in a particular expression study.

Methods

Fly strains. The 'Benakeion' medfly and the 'Demokritos' olive fly strains were used in the experiments. The 'Benakeion' strain was originally established at the Benakeion Institute of Phytopathology, Athens, Greece, and has been kindly provided by Prof Nikos Papadopoulos at the Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Greece. The 'Demokritus' strain originally comes from the Nuclear Research Centre in Athens, Greece, and has been reared in our laboratory for over 15 years. Both strains are maintained in wooden, nylon-screened, holding cages (30 × 30 × 30 cm) under an LD 14:10 h photocycle at 25 ± 1 °C and 60 ± 10% relative humidity. Olive fly rearing conditions are described in refs 63–65, while medfly conditions are described by Boller[66].

RNA isolation from specific tissues of *Ceratitis capitata* and *Bactrocera oleae*. Thirteen specific tissues at different developmental stages were used, as shown in Table 1. Eggs were collected from adult females 15 minutes after being laid. Larvae were 2nd stage and pupae were harvested 12 hours after pupation. All dissected tissues (heads, thoraces and abdomens, as well as reproductive and olfactory) were from 5 day-old adult male and female insects.

Total RNA was isolated with the use of TRIsure™ (Bioline) following the instructions of the manufacturer with minor modifications. RNA extraction was followed by an additional DNA removal using the TURBO DNA-free Kit (Ambion-Invitrogen), according to manufacturer's instructions. The integrity of RNA was assessed in a 1% agarose gel electrophoresis and quantified by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific).

The RNA extracted from: a single larva, a single pupa, a single head, a single thorax, a single abdomen and a set of ovaries from a single female, was quantified by Qubit and the amount of 2 μg was used for cDNA preparation. The entire RNA amount extracted from: a single egg, one pair of MAGs, one pair of testes, one pair of FAGs, and maxillary palps, antennae or ovipositors from a pool of 4 individual flies, was used for cDNA preparation, since the amount of RNA was undetectable.

DNA-free total RNA was converted into cDNA using 300 ng Random hexamer primers (equimolar mix of N_5A, N_5G, N_5C and N_5T), 200 units MMLV Reverse Transcriptase (Bioline), 10× reaction buffer, 40 mM dNTP mix and 40 units RNase Inhibitor (Bioline) according to the manufacturer's instructions.

Expression stability of candidate reference genes in *C. capitata* and *B. oleae*. 82 to 150 bp amplicons from nine different housekeeping genes commonly used in other dipteran species were analyzed. The genes considered were: *RPL19* (*ribosome protein L19*), *tbp* (*TATA-binding protein*), *ubx* (*ultrabithorax*), *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenease*), *α-TUB* (*α-tubulin*), *β-TUB* (*β-tubulin*), *14-3-3zeta*, *RPE* (*RNA polymerase II*) and *actin3* (Supplementary Table S6). For the medfly, primers were based on sequences retrieved in the NCBI database. For the olive fly, primers were based on the sequences obtained during the transcriptome analysis of *B. oleae*[55,67]. Specific primers for the amplification of these HKGs were designed by Primer-BLAST[44] (Supplementary Table S7). Each primer was also evaluated using OligoAnalyzer 3.1 tool[68] in order to avoid hairpin formation and self-/hetero-dimerization of the oligonucleotides.

Relative quantitation was used to analyze changes in expression levels of the selected genes using a quantitative real-time PCR approach. The RT-qPCR conditions were: polymerase activation and DNA denaturation step at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing/extension and plate read at 56 °C (for all the tested housekeeping genes) and 60 °C (only for the reference genes *RPE* and *actin3*) for 30 s and finally, a step of melting curve analysis at a gradual increase of temperature over the range 55 °C → 95 °C. In this step, the detection of one gene specific peak and the absence of primer dimer peaks were assured. Each reaction was performed in a total volume of 15 μl, containing 5 μl from a 1:10 dilution of the cDNA template, 1 × iTaq Universal SYBR Green Supermix (Bio-Rad) and 400 nM of each primer. The reactions were carried out on Bio-Rad Real-Time thermal cycler CFX96 (Bio-Rad, Hercules, CA, USA) and data analyzed using the CFX Manager™ software. The expression of the reference genes was measured in 8 or 10 biological replicates, as indicated in Table 1. Three negative controls were also used. All reactions were done in triplicate (three technical replicates). The amplification efficiency of the reactions was calculated by the CFX Manager™ software (Bio-Rad). The PCR efficiency (E) and the correlation coefficient (R^2) characterizing each standard curve are given in Supplementary Table S7. Efficiencies for all tested genes varied from 90.1% to 106.4%. The $2^{-\Delta\Delta Ct}$ method was used for the analysis of relative gene expression[69].

geNorm analysis. The expression stability of the nine reference genes was assessed using the *geNorm* software. This algorithm is based on the principle that the logarithmically transformed expression ratio between two genes should be constant if both genes are stably expressed in a given sample set. The candidate reference genes were ranked by *geNorm* based on the expression stability value M, which is calculated for all genes under study. The lower the M value, the higher the gene's expression stability. Furthermore, *geNorm* performs a stepwise calculation of the pairwise variation (V_n/V_{n+1}) between sequential normalization factors (NF_n and NF_{n+1}) to determine the optimal number of reference genes required for accurate normalization[9]. Results are presented in Fig. 1 and Supplementary Table S1.

Normfinder[39] is an algorithm for identifying the optimal normalization gene among a set of candidate genes. This software is based on a mathematical model of gene expression that enables estimation not only of the overall variation of the candidate normalization genes but also of the variation between samples subgroups of the sample set[39]. Results are presented in Supplementary Table S2A and S2B for *C. capitata* and *B. oleae*, respectively.

BestKeeper determines the most stably expressed genes based on the coefficient of correlation (r) to the *BestKeeper* Index (BI), which is the geometric mean of the candidate reference gene Cq values. Additionally, it calculates the standard deviation (SD) and the coefficient of variation (CV) based on the Cq values of all candidate reference genes[40]. Reference genes are identified as the most stable genes, i.e. those that exhibit the lowest coefficient of variance and standard deviation[70]. Results are presented in Supplementary Table S3A and S3B for *C. capitata* and *B. oleae*, respectively.

The *RefFinder* tool ranks all the potential reference genes according to the gene expression stability based on the rankings from *geNorm*, *Normfinder*, *BestKeeper* and the comparative ΔΔCt method programs. Also, this program assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking[71]. Results are presented in Supplementary Table S4A and S4B for *C. capitata* and *B. oleae*, respectively.

Statistical Analysis. Four different types of Microsoft Excel-based software, *geNorm*[9], *NormFinder*[39], *BestKeeper*[40] and *RefFinder*[41] were used to rank the expression stability of reference genes for all the experimental sets in the specific tissues of the medfly and the olive fruit fly. Relative quantities were used for *geNorm* and *NormFinder*, while *BestKeeper* analyses and the web-based program *refFinder* were based on untransformed Cq values. All four software packages were used according to the manufacturer's instructions.

The consensus rank of the reference genes was estimated by the combination of the stability measurements obtained by *geNorm*, *Normfinder* and *BestKeeper*. More specifically, the raw scores calculated by these three software (M value by *geNorm*, stability value by *Normfinder* and SD by *BestKeeper*) were transformed into standard scores (z-score) for each housekeeping gene separately. The average of the three z-scores was subsequently calculated and the final rank was computed using the RANK function in Excel software. The above measurements were produced for every single reference gene in each one of the insect tissues under study. Thus, a consensus ranking of all nine genes was estimated for each one of the 13 tissues separately. Results are presented in Supplementary Table S5A and S5B for *C. capitata* and *B. oleae*, respectively.

Ethics statement. The study was carried out on laboratory reared olive flies and medflies. No specific permissions are required for these experiments or collections, since these studies did not involve endangered or protected species.

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Author Contributions

E.S. maintained the laboratory strains, isolated the tissues egg, larva, pupa, head, thorax and abdomen, performed the functional analyses for *Bactrocera oleae* and the bioinformatics analysis and designed part of the study; P.K. isolated all the tissues for the medfly, performed the functional analyses and the bioinformatics analysis for *Ceratitis capitata*; M.G. isolated the tissues MAGs, FAGs, testes, ovaries and ovipositor for *Bactrocera oleae*; K.T. isolated the tissues antennae and maxillary palps for *Bactrocera oleae*; Y.C.B. guided the calculation of the consensus ranking of the three software programs used; K.D.M. designed and coordinated the study. All authors participated in drafting the manuscript and read and approved the final document.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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