Effect of the chemical compounds Ioxynil and Diethylstilbestrol, on the thyroid development of *Danio rerio,* after long-term and short-term exposure

Επιδράση των χημικών ενώσεων Ioxynil και Diethylstilbestrol, στην ανάπτυξη του θυροειδούς στο *Danio rerio*, ύστερα απο μακρυπρόθεσμη και βραχυπρόθεσμη έκθεση σε αυτά

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Abstract

Thyroid gland is a key-point for the normal development and function of the organism. The thyroid gland of *Danio rerio* (zebrafish)**,** develops from the endoderm at 24 hours post fertilization (hpf) and just at 32hpf the first thyroid follicle is formed. Several natural and synthetic substances that act like endocrine disruptors have been identified. In this group belong the chemical compounds Ioxynil (Iox) and Diethylstilbestrol (DES). Iox is an herbicide widely used in agriculture, while DES is a synthetic estrogen that was provided to pregnant women as a drug. Both Iox and DES are environmental contaminants, primarily found in groundwater. Their way of action is also connected with disruption of the thyroid gland in teleosts. Specifically Iox and DES have the ability to bind to thyroid hormone binding proteins with high affinity, to affect function and regulation of the thyroid and finally to impair the development of the gland. The aim of the present study was to examine the effects of Iox and DES on the development of the thyroid gland of *Danio rerio* after short-term and long-term exposure. For this reason, zebrafish embryos were exposed for 2 days and 4 days long to Iox (0.1μM, 1μM) and DES (0.1μM, 1μM). Results from whole mount *in situ* hybridization for genes *nk2.1a, pax8* and *tsh* suggest that do not affect the pattern of expression after short-term and long-term treatment. Statistical analysis, from hybridization with *tg* gene, reveals that zebrafish treated with Iox and DES have no significant difference ($P>0.05$) in the expression, comparing with untreated animals regardless to the time of exposure in chemicals. Also expression field of *cmlc2* in the atrium, according to statistics, was not significantly different ($P>0.05$) in Iox and DES-treated animals when they were compared with those reared with no chemicals. Results were similar to both short-term and long-term treatment. Therefore, in the case of short-term treatment in Iox and DES animals seem to recover. As for long-term treatment, in those low dosages, Iox and DES may not affect the thyroid system.

Περίληψη

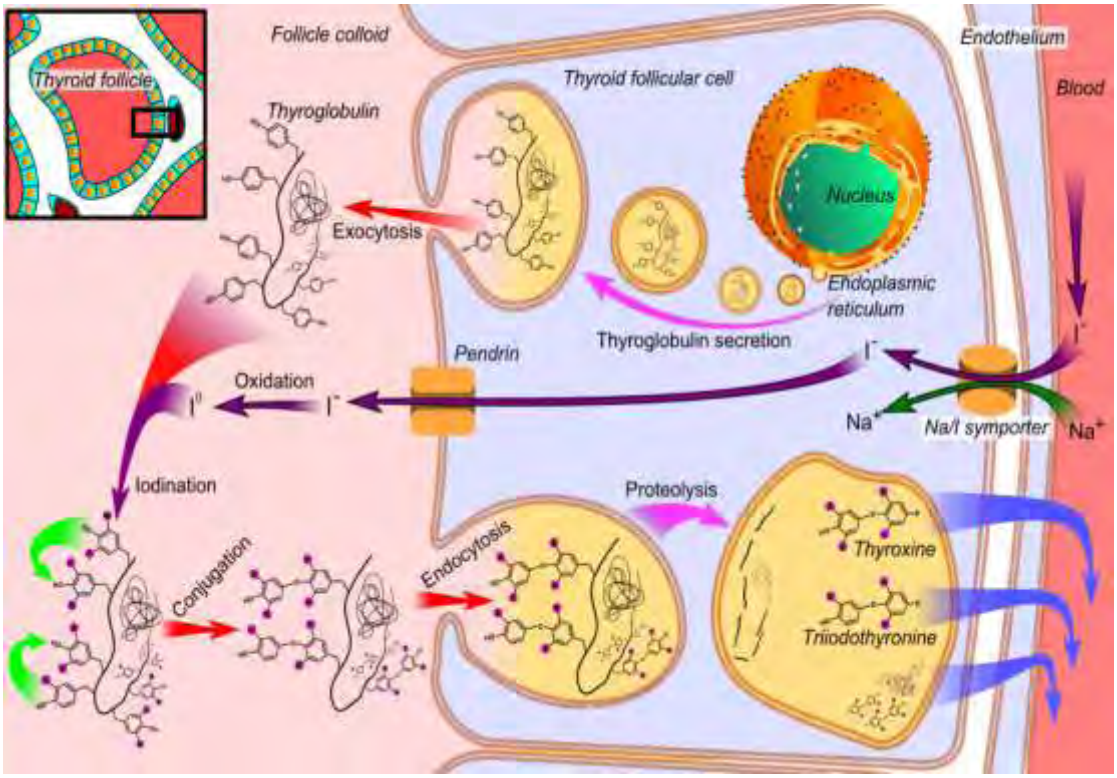
Ο θυρεοειδής αδένας αποτελεί σημείο-κλειδί στην ανάπτυξη και στην φυσιολογική λειτουργία του οργανισμού. Ο θυρεοειδής αδένας στο *Danio rerio* ή ψάρι ζέβρα αναπτύσσεται απο το ενδόδερμα περίπου 24 ώρες μετά την γονιμοποίση (hpf) με το πρώτο θυλακιώδες κύτταρο να σχηματίζεται πλήρως μόλις 32 hpf. Έχουν ταυτοποιηθεί αρκετές ουσίες φυσικές ή τεχνητές που παρεμποδίζουν το ενδοκρινικό σύστημα. Σε αυτές ανήκουν και οι χημικές ενώσεις Ioxynil (Iox) και Diethylstilbestrol (DES). Η Iox είναι ζιζανιοκτόνο ευρέως χρησιμοποιούμενο στις γεωργικές καλλιέργιες, ενώ η DES είναι ένα συνθετικό οιστρογόνο που χορηγούνταν ως συμπλήρωμα σε έγκυες γυναίκες. Και οι δύο αυτές ουσίες αποτελούν περιβαλλοντικούς ρύπους που απαντώνται συχνά στα υπόγεια ύδατα. Η δράση τους, έχει συνδεθεί με παρεμπόδιση της λειτουργίας του θυρεοειδούς στα ψάρια. Συγκεκριμένα οι Iox και DES έχουν τόσο την ικανότητα να προσδένονται με μεγάλη συγγένεια στην επιφάνεια πρωτεϊνών-μεταφορέων των θυρεοειδικών ορμονών και να διαταράσσουν την λειτουργία και ρύθμιση του θυρεοειδούς όσο και να επιδρούν στην ανάπτυξη του αδένα. Στην παρούσα διπλώματική, ο σκοπός ήταν η εξέταση των επιδράσεων των Iox και DES στην ανάπτυξη του θυρεοειδούς αδένα του *Danio rerio,* ύστερα απο βραχυπρόθεσμη και μακρυπρόθεσμη έκθεση στις χημικές αυτές ουσίες. Για αυτό το λόγο, έμβρυα του ψαριού ζέβρα, εκτέθηκαν είτε για 2 ημέρες είτε για 4 ημέρες στα χημικά Iox (0.1μM, 1μM) και DES (0.1μM, 1μM). Αποτελέσματα της *in situ* υβριδοποίησης με *nk2.1a, pax8* και *tsh* υποδεικνύουν ότι οι Iox και DES δεν έχουν κάποια σημαντική επίδραση τόσο στην βραχυπόθεσμη όσο και στη μακρυπρόθεσμη έκθεση. Στατιστική ανάλυση των αποτελεσμάτων της υβριδοποίησης για το γονίδιο *tg* έδειξε ότι στα ψάρια που μεγάλωσαν στα υπο μελέτη χημικά Iox και DES, η έκφραση του γονιδίου, δεν διαφέρει σημαντικά ($P>0.05$) απο τα ψάρια που δεν τους χορηγήθηκαν χημικά, ανεξάρτητα από τον χρόνο έκθεσης. Επιπλέον, η έκφρασης του *cmlc2* στην περιοχή της καρδίας στα ψάρια που χορηγήθηκε Iox και DES, ύστερα από στατιστική μελέτη δεν διέφερε σημαντικά ($P>0.05$) με αυτήν των ψαριών ζέβρα που δεν ήρθαν σε επαφή με χημικές ουσίες. Τα αποτελέσματα ήταν όμοια στην βραχυπρόθεσμη και μακρυπρόθεσμη έκθεση. Φαίνεται, λοιπόν, ότι οι Iox και DES και στις δύο συγκεντρώσεις που χορηγήθηκαν, δεν επιδρούν στον θυρεοειδή των ψαριών που εκτείθονται σε αυτά για μεγάλο χρονικό διάστημα είτε στην περίπτωση της σύντομης έκθεσης μπορούν και ανακάμπτουν.

1. Introduction

1.1 Thyroid Function

Thyroid gland, as part of the endocrine system on vertebrates, has a significant role in maintaining normal function of the organism. Thyroid secretes thyroid hormones (THs) 3,3',5-triiodo-L-thyronine (triiodothyronine) and L-3,5,3',5',-tetraiodothyronine (thyroxine). Thyroid hormones have a great impact in embryogenesis and early life development as much as in metamorphosis, metabolism, Thyroxine and triiodothyronine seem to control homeostasis, protein, lipid, carbohydrate and vitamin metabolism, as they effect development, metabolism and growth of every cell and organ (Liu et al, 2010). Thyroid hormones are α-amino acid residues. Thyroxine is a phenoxyphenyl polyiodinated molecule and it is formed with the molecular addition of four atoms of iodine to amino acid tyrosine, while triiodothyronine contains three atoms of iodine. The functional unit of thyroid gland in vertebrates is thyroid follicles. Thyroid follicles consist of big number of epithelial cells forming a vesicle. Inside the vesicle cells create a hollow, an extracellular space that is called lumen. Inorganic iodide (I^-) from the blood stream, is transported to thyroid epithelial cells through a sodium-iodide (Na^+/I^-) symporter. Iodide crosses the apical side of follicles and moves to the colloid where is oxidizes to I_2 by thyroperoxidases (Eales et al., 1999). Iodide then binds to tyrosine residues of thyroglobulin molecules forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). Thyroglobulin (TG) is a glycoprotein of the globulin family. TG is generated and stored in lumen creating the thyroglobulin colloid. Combination of MIT and DIT particles leads to the production of triiodothyronine, abbreviated as T_3, and thyroxine abbreviated as T_4. Both steps require the presence of H_2O_2 (hydrogen peroxide). The thyroglobulin-T_3/T_4 complex is stored as colloid in the lumen of the follicle. Stimulation of the gland results release of free T_3 and T_4 into the bloodstream. Thyroglobulin colloid enters cytoplasm with endocytosis where the vesicles fuse with lysosomes. Lysosomal proteases proteolyse thyroglobulin and release T_3, T_4, MIT and DIT molecules. Monoiodotyrosine and Diiodotyrosine are deiodinated by a dehalogenase and recycled. Active hormones move to the basal membrane and then through the capillary network that surrounds the gland (Nussey and Whitehead 2001) (Fig. 1).

When THs are released in the circulatory system, because they are lipophilic molecules in an aqueous environment, are transported to other tissues by thyroid binding proteins, transthyretin (TTR), globulin and thyroxine-binding protein (TBP).Thyroxine is the main form of the thyroid hormones that the gland synthesizes, representing approximately the 95% but it works as a precursor of T_3. The biggest part is converted into triiodothyronine. In peripheral tissues, a complex of deiodinase enzymes removes one of the two outer ring iodines, converting L-3,5,3',5',-tetraiodothyronine into 3,5',3'-triiodo-L-thyronine (T_3). This pathway is known as ORD (outer ring deiodination). The process is catalyzed by enzymes iodothyronine deiodinases that belong to superfamily of selenoenzymes, found in endoplasmic reticulum. There are three types of deiodinases, each one with different role and location (Eales et al., 1999). Deiodinase type 1 (D1) and deiodinase type 2 (D2), are present to several peripheral tissues (liver, gill, brain, kidney) and contribute to peripheral and intracellular conversion to T_3. These organs are the extrathyroidal source of the active T_3 (Blanton et al. 2006). The respond of these tissues, depends on the availability of thyroxine in the plasma, except those that contain type 2 deiodinase (intracellular conversion). The third deiodinase (D3) has the ability to turn T_3 and its less potent precursor T_4, to an inactive form of T_3, reverse T_3 (rT_3). (Gereben et al., 2008). Combined, the roles of the deiodinases provide



a homeostatic mechanism that helps the organism adapt, regulating enzymes activity according to iodine availability in the environment.

Figure 1. *In the follicle cell, thyroid hormones synthesis first step is iodine uptake by NIS. Iodine is oxidize and bound in tyrosine residues in the surface of Tg protein. Release of thyroid hormones includes endocytosis and proteolysis of iodinated thyroglobulin. (*Boron, 2003)

1.2 Hypothalamus-Pituitary-Thyroid axis

Function of HPT axis has been analyzed in detail among vertebrates, with teleost fishes (*Danio rerio*, *Solea senegalensis* etc.) being slightly different from mammals (Blanton and Specker, 2007). The primary role of hypothalamus and pituitary is to regulate synthesis and release of the T_4 hormone but not T_3. Production and levels of T_3 in the blood, depend on deiodination pathways in peripheral tissues (Eales et al., 1999).

Thyrotropin realizing hormone (TRH), a tripeptide which is synthesized in the paraventricular nucleus of hypothalamus, is the first step of the negative-feedback system that indirectly regulates the levels of thyroxine in plasma. TRH is transported to the anterior pituitary, also called adenohypophysis, through the portal capillary plexus. There TRH binds to TRH receptors in the pituitary thyrotropes. Thyrotropes is a subpopulation of pituitary cells that produce and secrete thyroid stimulating hormone (TSH). TRH receptors belong to the family of seven-transmembrane spanning receptors, GPCRs. Binding of TRH to the receptor has as a result secretion of TSH and enhanced transcription of TSH mRNA. Except from TRH, Corticotropin- Releasing Hormone seems to play an important role in stimulating TSH secretion in fish (de Groef et al., 2006). Thyroid stimulating hormone is a glycoprotein that consists of α- and β-subunits (Yen, 2001).Thyroid stimulating hormone is regarded as a

major growth factor for the differentiated thyroid in mammals and specifically in adult human (Nussey and Whitehead, 2001). At the early stages of thyroid growth in zebrafish, TSH seems to have no impact in the process (Alt et al., 2006b). In later stages of development TSH binds to TSH receptor which is also a seven-transmembrane spanning receptor, located to the basal side of the thyroid follicular cells. TSH receptor is coupled with a Gs protein. Activation of TSH receptor leads to increased levels of cAMP in the cytoplasm and stimulation of protein kinase A. Thereby TSH triggers signal transduction pathways, promoting expression of several genes that are required for normal thyroid hormones synthesis, like thyroglobulin, thyroperoxidase and NIS (Yen, 2001).Increase of the thyroxine concentration in the bloodstream, acts like negative feedback in the hypothalamus-pituitary axis, controlling secretion of TRH and TSH respectively. This is an example of negative regulation in transcription by thyroid receptors. TRH and the TSHα- and TSHβ- subunit genes have thyroid response elements near to their promoters, where TRs can bind and negatively regulate these genes. However, it is not clear if the suppression of the genes occurs by direct TR bind or by interaction with cofactors. (Oetting and Yen, 2007). Another mechanism that probably controls down regulation of TSH and TRH release is intracellular conversion of T_3 into T_4. As mentioned previously, type 2 deiodinase is the responsible enzyme for intracellular conversion. D2 is present in the hypothalamus and hypophysis (Gereben et al., 2008) (Fig. 2).

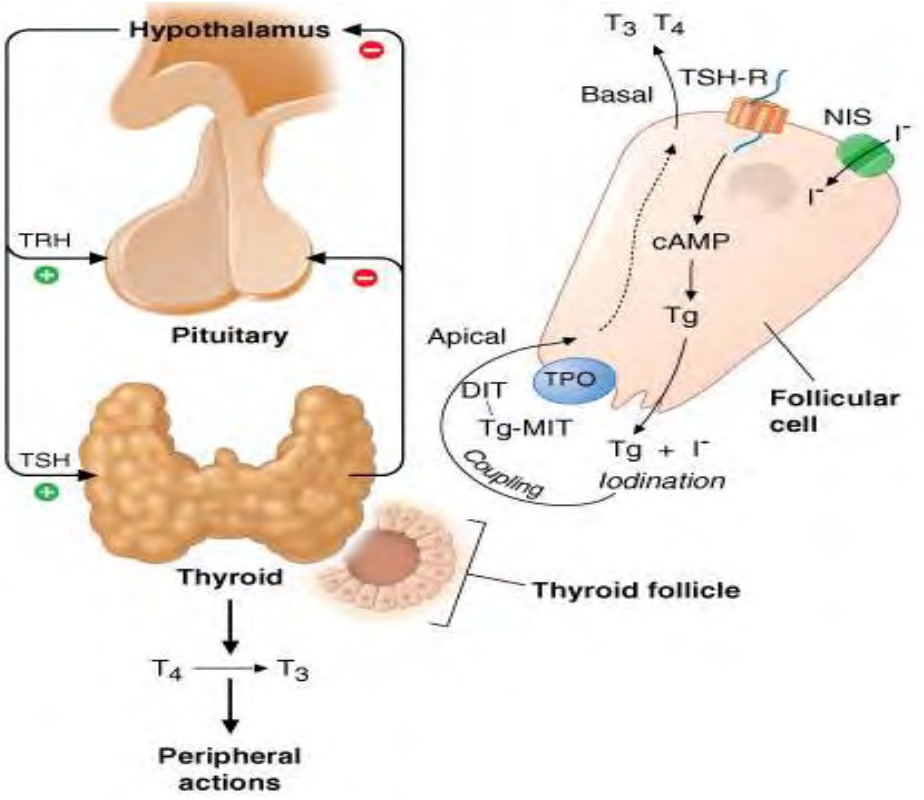
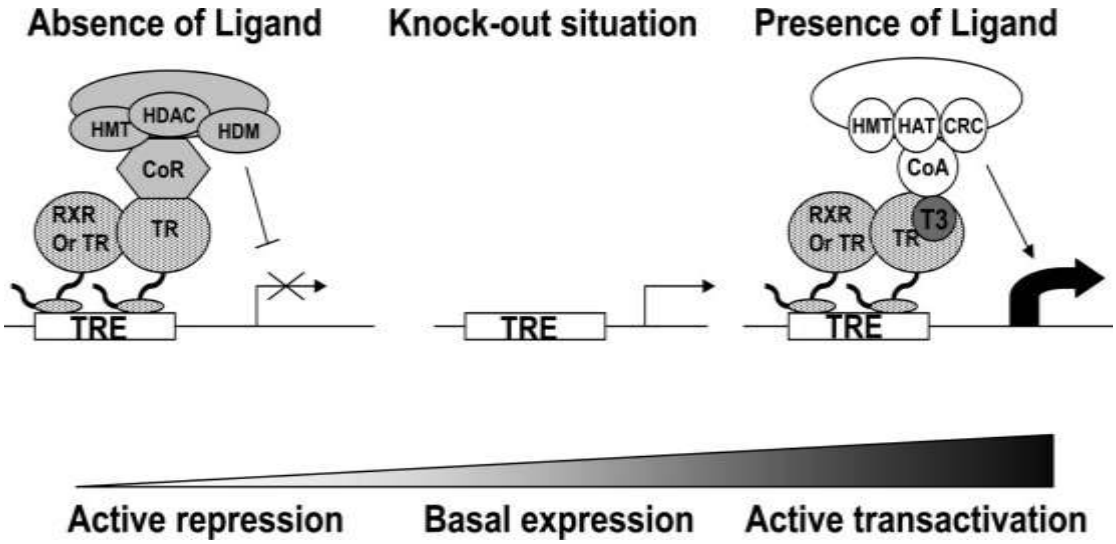


Figure 2. *Thyroid hormones synthesis and secretion is regulated by negative feedback system that includes hypothalamus and pituitary. Increase in THs results negative regulation of hypothalamic TRH and TSH secretion of pituitary. If the levels of THs are decreased TRH induces release of TSH that binds to TSH-R at the thyroid follicles and gives the signal to produce THs (Boron 2003).*

1.3 Thyroid hormone signaling pathway

Thyroid hormones can affect molecular mechanism in two ways, genomic and nongenomic. Genomic effects refer to transcriptional regulation of the target genes that are involved in many pathways such as gluconeogenesis, apoptosis and adenylate cyclase signaling. On the other hand, nongenomic effects do not depend on interaction of thyroid receptor with the nucleus and include non-transcriptional pathways.

The genomic actions of thyroid hormones occur through its binding to thyroid hormone receptors (TRs). TRs are transcriptional factors that regulate gene transcription, positively or negatively, by binding to specific sites of DNA called TREs (Thyroid Response Elements) (Oetting and Yen, 2007). Usually TREs are located in the promoters of target genes. There are two types of thyroid receptors, TRα and TRβ, identified in zebrafish, which belong to the superfamily of nuclear receptors (Darras et al., 2011). The expression of TRs varies among the developmental stages of the organism. The functional TR complex binds to TREs as homodimer or as a heterodimer with retinoic X receptors (RXRs). Receptors have a very similar structure, containing of a central DNA-binding domain and a ligand-binding domain in the carboxyl-terminal site (Oetting and Yen, 2007). TR complex can affect transcription in three ways. In the absence of ligand, RXR/TR complex binds to TRE and induces repression of transcription via co-repressors and recruitment of protein complexes that alter chromatin formation. Knock out situation, refers to the lack of TR where the expression is not affected.



When TR complex is bound with T_3, it attracts other complexes including histone demethylases that remodel and activate transcription (Flamant et al., 2007) (Fig. 3)

Figure 3. *Regulation of transcription by TR. The unliganded TR/RXR heterodimer or TR/TR homodimer binds to TRE in the promoter of target genes, repressing their transcription. The repressive action is mediated by corepressor molecules that, recruit complexes (HDM, histone demethylase; HDAC, histone deacetylase; HMT, histone methyl transferase) that interfere with the basal transcription. In knock out phenomenon TR is absence and does not bind to the TRE without effects in the basal transcription. Liganded TR complex interacts with co-activators and complexes, including histone arginine methyl-transferases (HMT), histone acetyl-transferase (HAT) and chromatin remodeling complex (CRC) that make the target gene accessible to polymerase 2 and activate transcription (Flamant et al., 2007).*

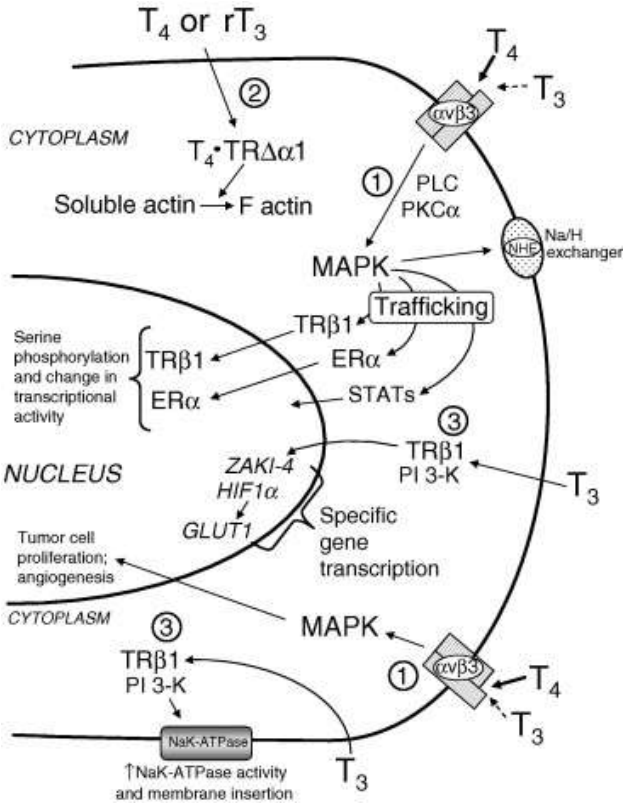


Figure 4. *Overview of non-genomic action of thyroid hormones in the cell (Davis et al., 2008).*

Except from this mechanism that is called genomic, thyroid hormones have non-genomic actions. Non-genomic actions are initiated in the cytoplasm or at the plasma membrane. So there must be a receptor in the plasma membrane and a nuclear receptor in the cytoplasm that recognize and bind thyroid hormones. The role of plasma membrane receptor seems to have integrin receptor αVβ3. Thyroid hormones bind to the cell surface through an Arg-Gly-Asp (RGD) recognition site on integrin receptor (Davis, et al., 2006). This recognition site is also significant for other extracellular proteins. T_3 and T_4 with greater affinity, bind to integrin receptor. The signal transduction pathway starts with phosphorylation and activation of mitogen-activated protein kinase (MAPK, extracellular-regulated kinase 1/2; ERK1/2), by phospholipase C (PLC) and protein kinase Cα (PKCα). Activated MAPK can move to the nucleus and in turn activate, via phosphorylation, thyroid hormone nuclear receptor TRβ1, estrogen receptor ER-α and STAT transcriptional factors. MAPK also can activate NA^+/H^+ exchanger and modulate ER and TR to move from cytoplasm in the nucleus. L-thyroxine bound to membrane receptor integrin αVβ3 induces proliferation and angiogenesis in tumor cells (Davis et al., 2006). Non-genomic actions of iodothyronines that are mediated by integrin receptor are designated "1" in the figure. Reverse T_3 and thyroxine when interact with TR-derived polypeptide TRΔα1, regulate the state of actin cytoskeleton converting soluble actin to F form (Fig. 4, pathway No 2). Triiodothyronine interacts with cytoplasmic TRβ1. Transduction of the T_3 signal includes activation of PI3-kinase (phosphatidylinositol 3-kinase)/Akt pathway. Non-genomic stimulation by T_3 leads to specific gene transcription, such as hypoxia included factor-1α (*HIF-1α)* and *ZAKI-4* an anti-calcineurin factor. HIF-1α is a transcriptional factor that induces expression of genes related to carbohydrate metabolism, for example *GLUT.* In cardiovascular endothelial cells, thyroid hormone can activate PI3-kinase/Akt pathway. In endothelial cells the main isoform of thyroid hormones is TRα1. The liganded receptor interacts with phosphatidylinositol 3-kinase (PI3-kinase), and in turn kinase phosphorylates and activates protein kinase B (also known as Akt) and endothelial nitric oxide synthase (eNOS) (Hiroi et al., 2006). T_3/TRβ1 complex also controls Na, K-ATPase activity and gene expression (fig. 4 part 3). The initial cytoplasmic interaction of thyroid hormone that ends to regulation of transcription is one of the cases of interface between non-genomic and genomic hormone actions (Davis et al., 2008).

1.4 Endocrine Disrupting Chemicals

Endocrine disruptors are man-made or natural chemicals that can disrupt hormone synthesis, action and homeostasis. More than a hundred of chemicals have been identified to be toxic for living organisms. Endocrine disrupting chemicals (EDCs) are routinely found in the environment, as a result of pollution on ecosystem from industrial discharges and other sources. Most of the studies in EDCs examine the estrogenic mechanism of action. Lately a large number of these chemicals seem to interfere with thyroid axis. The term thyroid disrupting chemicals (TDCs) is used to describe substances that are suspected to disrupt the thyroid hormones network, having adverse effects in wildlife and human. TDCs interfere in several steps of the thyroid endocrine cascade such as synthesis of THs, iodide uptake, catabolism and regulation of transcription through TRs (Jugan et al., 2010). For example, perchlorate and nitrate act like inhibitors of NIS of the thyrocytes, isoflavones are potential disrupt of thyroperoxidases, organochlorine pesticides and dioxins probably effect uridine diphosphate glucuronyltranferases (UDPGTS) that glucuronidate T_4 increasing metabolism and polychlorinated biphenyls (PCBs) are found to act as TDCs through interaction with thyroid receptor (Pearce and Braverman., 2009). TDCs can also interfere in transcriptional process directly or indirectly. Transcription can be effected in TR/XRX eterodimerization level, by recruitment/release of transcriptional factors (co-activators and co-repressors), through interaction of the chemicals with ligand-binding site of TR, or directly regulating expression of TR in the cells.

Chemicals compounds diethylstilbestrol and ioxynil are previously reported to disrupt the endocrine system. Diethylstilbestrol (DES) or 4, 4'-(1,2 diethyl-1,2 ethene-diyl) bisphenol is a nonsteroidal synthetic estrogen developed in 1938 (Fig. 5). DES was used to treat to pregnant women, in order to prevent miscarriages or premature births. This kind of treatment had harmful effects in the descendants, causing abnormalities in the reproductive system. In females DES induces abnormalities in uterus, cervix and vagina, decreased fertility. Also, in utero exposure is associated with rare vaginal clear cell adenocarcinoma. Results from toxicological assays in female mice show that DES adverse effect in the development of female reproductive tract and endometrial cancer, is mediated through its ability to disrupt *Wnt* pathway and change *Hox* expression pattern (Ma, 2009; Henley and Korach, 2010). *Wnt* and *Hox* genes have a critical role for normal development and differentiation of the reproductive track. In males, there is no evidence that the drug induces tumor but effects have been observed in their reproductive tracks, such as testicular hypoplasia, cryptorchidism and epididymal cysts. As an estrogenic drug it also causes increase in prostatic cells in male

mice when it is provided in low doses (Saal et al., 1997). DES toxicity is mediated via an ERα-dependent signaling pathway either directly by binding to gene promoters either indirectly through interaction with transcription factors and growth factors (Ma, 2009). Ioxynil (Iox), or 4-hydroxy-3, 5-diiodobenzonitrile, is a herbicide used in agriculture (Fig 5). As a phenolic herbicide, ioxynil prevents photosynthesis in plants, uncoupling oxidate phosphorylation from photosystem II. Exposure in Iox is connected with tumor growth in cells, probably through inhibition of connexin 43 gap junctions (Leithe et al., 2011).

Ioxynil and Diethylstilbestrol in low doses have been lately suggested to act as disruptors of the thyroid system of vertebrates. These chemicals can bind with high affinity to TTR (Morgado et al., 2007) and other THBP in serum and interfere on thyroid system in a species-specific manner (Akiyoshi et al., 2012). Also Iox and DES have an impact in thyroid network affecting pituitary, thyroid gland and deiodinases as treated animals have shown increase in the size of the thyrocytes and reduction in the expression of the TSH mRNA (Morgado et al., 2009). Studies focused in the development and differentiation of the thyroid gland, have shown that Iox and DES decrease the expression of gene markers for thyroid development (Tg and nk2.1a) and their disruption is associated with changes in the heart morphology (Campinho and Power, 2013). Although, the exact mechanism of action in the HPT-axis has not yet identified.

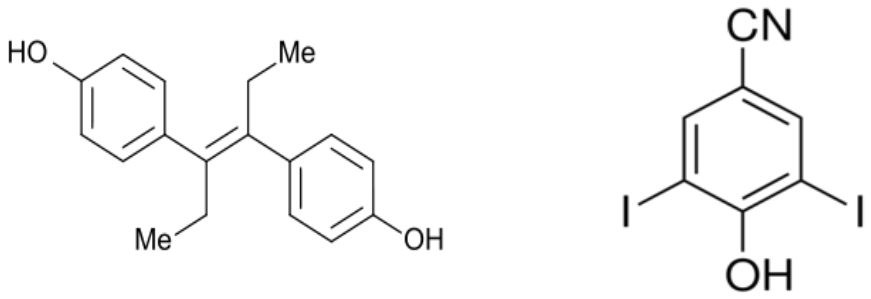


Figure 5. *Chemical structures of synthetic estrogen Diethylstilbestrol (on the right) and of the phenolic herbicide Ioxynil (on the left).*

1.5 Zebrafish as an organism model

The zebrafish, *Danio rerio*, is a small tropical fish of the family (Cyprinidae) of order Cyprinifores which lives in parts of India, Pakistan, Bangladesh Nepal and Burma. Zebrafish is a well-established model organism, important for scientific research. Zebrafish has 25 chromosomes and its genome consists of $1{,}5 \times 10^9$ base pairs. A large number of genes, genetic markers, mutants identified in this organism. All these information about zebrafish genome, genetics, ontology, developmental stages and several publications referring to zebrafish are available in Zebrafish Information Network (ZFIN; http://zfin.org/). Characteristics such as small size, 4-5cm in length the adult fish, the large number of offspring, up to 200 eggs from a single female and rapid embryonic development make zebrafish a perfect tool for science. Also zebrafish embryo is transparent, which allows *in vivo* imaging, and it presents full organogenesis till day 4 of development (Kimmel et al.,

1995). The similarities of this vertebrate in anatomy, physiology and in molecular level with mammals, gives the opportunity to see *in vivo* the effect of several chemicals (D. Raldua et al., 2011). Also, all these knowledge around the thyroid endocrine system make zebrafish ideal for toxicological assays and an excellent animal-model for studying cardiovascular development in vertebrates (Stainier, 1994; Fishman and Chien, 1997).

Zebrafish embryo and larval have a particular pigmentation pattern that is a model to evaluate development (Kimmel et al., 1995). The differences in formation of the pigmentation cells pattern are of interest for identifying diseases and malformations that may impact pigmentation (Nordlund et al., 1998) Pigment cells, known as chromatophores, are derived from multipotential cells of neural crest. There are three types of chromatophores, black melanophores, xanthophores that are responsible for yellow shade in the body and iridescent iridophores (Quigley et al., 2002). The pigmentation pattern in zebrafish is completed by 5 days post fertilization (Kimmel et al., 1995). The pattern of melanophores in zebrafish consists of four stripes: the dorsal stripe, dorsally of the neural tube, the lateral stripe that is localized in the myoseptum, the ventral stripe which extends on the dorsal side of the gut and the stripe in the yolk sac, ventrally of the yolk sac of the zebrafish embryo (Fig. 6) (Kelsh et al., 2000).

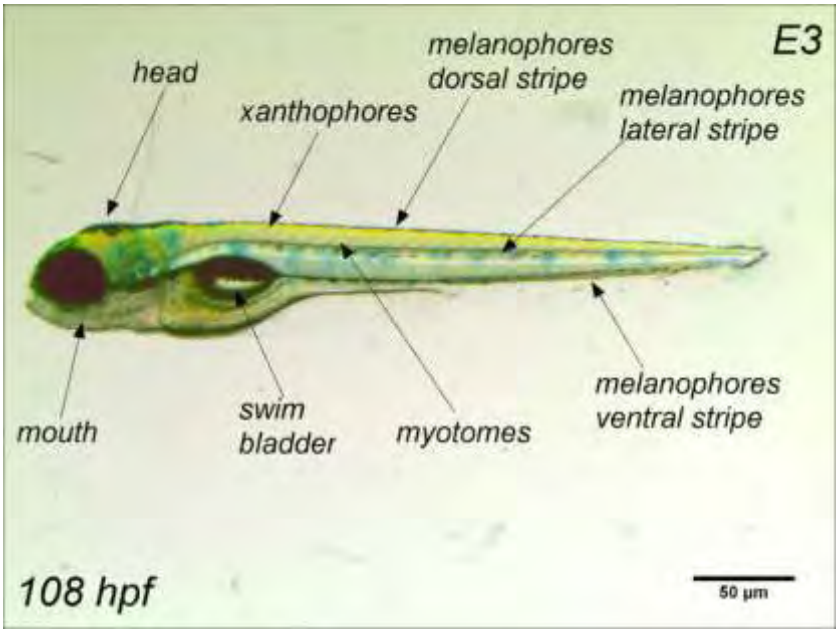


Figure 6. *Anatomical characteristics of larvae 108 hpf, showing stripes of melanophores, xanthophores, head region, mouth, myotomes and swim bladder. The scale bar corresponds to 50µm.*

1.6 Thyroid development and differentiation

The origin and the development of thyroid gland have been examined, in detail, in zebrafish. In zebrafish embryo, follicular thyroid is totally derived from the pharyngeal endoderm (Fig. 7). The differentiated endocrine gland develops from one primordium, the thyroid diverticulum as it is indicated by the expression of *nk2.1a* (Alt et al., 2006b). *Nk2.1a* is a gene-molecular marker of thyroid primordium and plays a significant role in early development of zebrafish (Rohr and Concha, 2000). Zebrafish *nk2.1a* gene, an orthologue of

mouse *Nkx2.1* and human TTF1 is a transcription factor, primarily expressed in thyroid precursor cells at 24hpf onwards. *Nk2.1a* transcripts are also detected at the ventral diencephalon in early developmental stages (at the end of epiboly) in a group of cells, where hypothalamus develops (Rohr et al., 2001). The expression pattern of this gene in the larva and adult zebrafish is localized in hypothalamus and thyrocytes (Wendl et al., 2002).

Even before the thyroid diverticulum has evaginated from the pharyngeal epithelium (Fig. 7), starts the expression of zebrafish *tg*, approximately 32hpf (Alt et al., 2006b). *Tg* expression is a significant marker for thyroid differentiation as it is not expressed in other tissues. During larval development, thyroid follicles are added caudally, along the anterior-posterior axis at pharyngeal midline and increase in size forming the complete functional gland. Gene *pax8* is required for late specification or differentiation of the follicular cells in the zebrafish. (Wendl et al., 2002). *Pax 8* expression begins at about 7-10 somites stage at the midbrain-hindbrain boundary. (Pfeffer et al., 1998). From 28hpf it is also expressed in thyroid gland of zebrafish. In contrast with nk2.1a there are no signs of pax8 expression in the thyroid primordium at 24hpf. Pax 8 is member of the zebrafish pax2/5/8 paralogue group, and except from its role in the thyroid it involves in organogenesis and formation of midbrain hindbrain border. Signs of the endocrinal function of the gland appear at the time embryo hatches, at about 60 hours post fertilization (hpf) and correspond to the most anterior follicles. From day 3 post fertilization, the thyroid is fully differentiated and the hormones synthesis and secretion starts. In the adult fish follicles usually lack contact between cells and vary in diameter from 14μM to 140μM and are found near ventral aorta, from the first gill arch to the bulbus arteriosus (Wendl et al., 2002). At the first 4-6 days post fertilization, the developing embryo relies on maternal deposit of the thyroid hormones, in the yolk sac**.** The expression of *tsh* gene in zebrafish is initiated approximately at 42 hpf, in the adenohypophysis, along with growth hormone (gh) expression (Herzog et al., 2003). Although, in early stages TSH does not seem to induce the production of thyroid hormones.

Several studies have proved the connection between heart formation and the development of the thyrocytes (Alt et al., 2006a; Campinho and Power 2013). Localization of thyroid tissue along the anteroposterior axis of zebrafish is linked to the development of the ventral aorta of the heart (Alt et al., 2006a). Cardiac muscle develops is zebrafish when two bilateral groups of cardiomyocytes fuse to form the heart tube that is called cardiac cone. Looping of the heart tube, trabeculation, valvulogenesis and septation are the next steps of the heart morphogenesis (Bartman and Hove, 2005). In cardiac and other striated muscles, myosin light chain (MLC) plays a significant role at the contraction. Myosin light chain 2 is a regulatory light chain and probably a modulator of contractile activity in heart and skeletal muscle cells. In zebrafish *cmlc2* gene expression was activated at 16 hpf in the bilateral heart field and it is maintained till the formation of the heart tube at 24 hpf when the cardiac muscle eventually begins to beat. At 48 hpf, *cmlc2* transcripts are present at the atrium and the ventricle (Huang et al., 2003). Heart consists of three layers epicardium, myocardium and endocardium. However, *cmlc2* promoter/enhancer occurs only in the myocardial cells. So it is considered as a myocardium-specific marker.

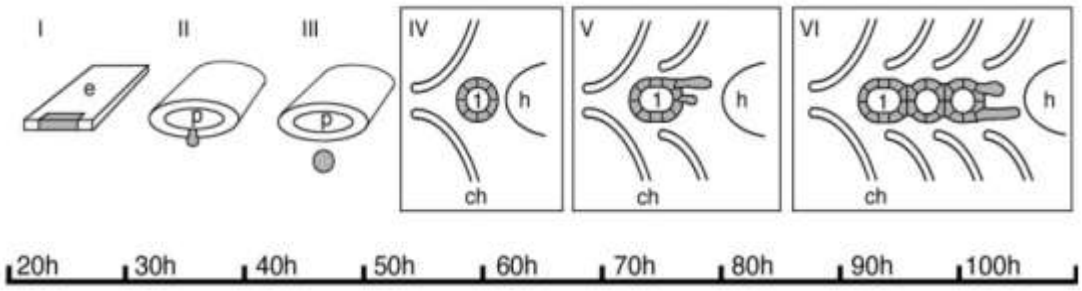


Figure 7. *Overview of the thyroidal morphogenesis at early stages of development. The sketches in the figure show the steps of thyroid development along the time scale of embryonic/larval development in zebrafish. I thyroid primordium in the pharyngeal endoderm, II, III evagination of the thyroid from the pharyngeal epithelium, IV formation of the first follicular cell, V, VI differentiated follicles are added caudally to the midline of the pharynx along. The grey color indicates the thyroid primordium and follicles. Number "1" corresponds to the most anterior follicle that is added first. e, endoderm; p, pharynx; h, heart; ch, cerotohyal (Alt et al, 2006b).*

2. Aim of project

The objective of this experiment was to examine the effects of Ioxynil and Diethylstilbestrol to normal development and function of the thyroid gland in zebrafish larvae. Zebrafish embryos at the developmental stage of 10 somites, were exposed in those chemicals either for 2 days (short-term treatment) or for 4 days (long-term treatment). In short-term treatment zebrafish are exposed in chemicals for 2 days and afterwards are reared in zebrafish medium. On the other hand long-term exposure includes treatment with Iox and DES for 4 days long. In order to visualize the effect of the chemicals in the development, whole-mount in situ hybridization was conducted using animals at the age of 108hpf (5dpf). For WMISH the expression of *nk2.1a*, *TSH*, *pax8*, *Tg* and *cmlc2* genes was analyzed. *Nk2.1a*, *tsh*, *pax8* and *Tg* genes are gene-markers of the thyroid gland development and differentiation. Based on several studies that suggest a connection between thyroid gland and heart*, cmlc2* antisense RNA probe was used. Larvae after 4 days of treatment, at the age of 108hpf, were photographed and image processing and statistical analysis followed (Fig. 8).

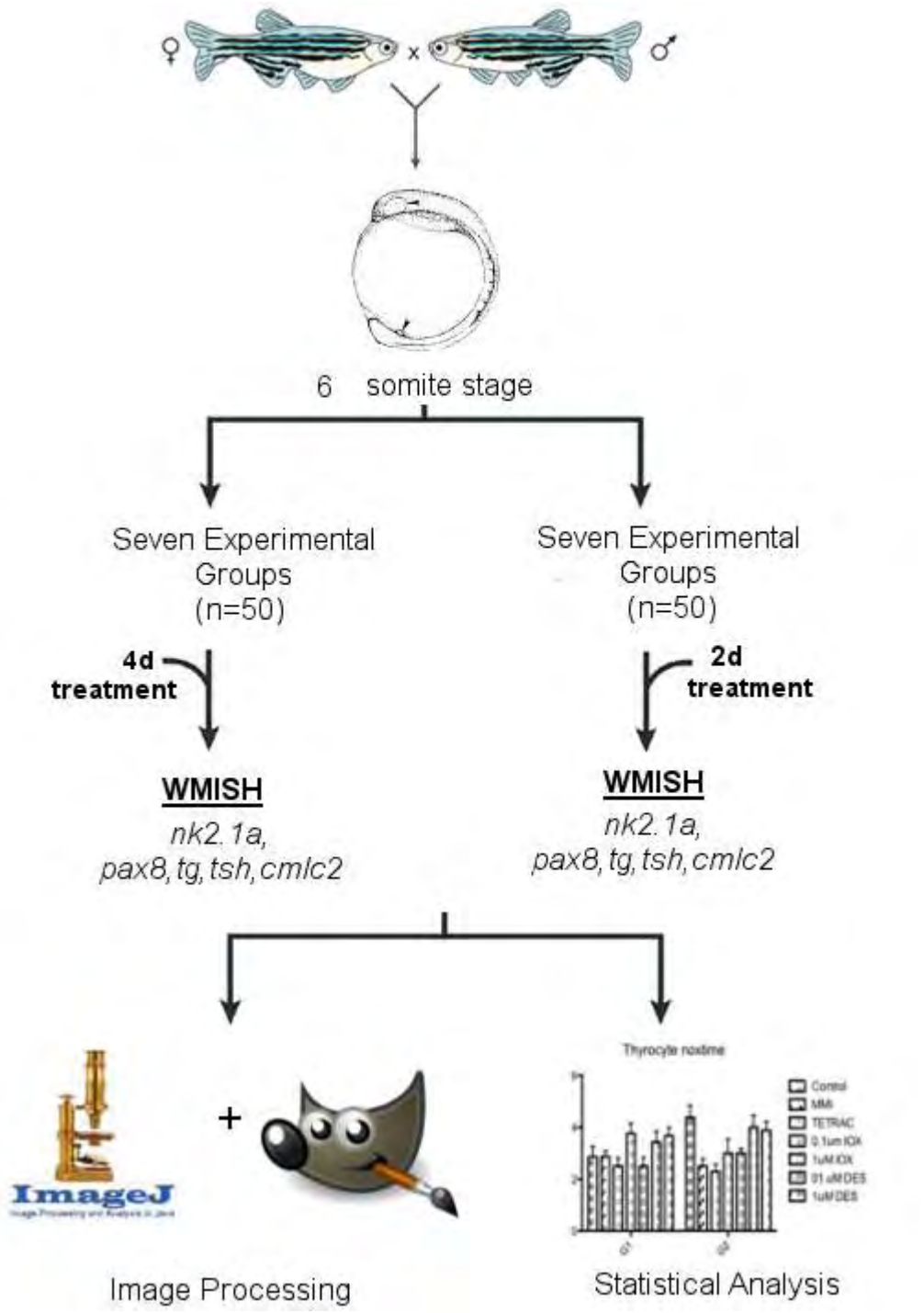


***Figure 8**. Work plan of the present project. Zebrafish were left to mate and eggs collected. At the 10-somite stage (12hpf) embryos were separated to follow two different durations treatment, 2 days and 4 days. Seven experimental groups (n=50) were created for each time-dependent treatment. These groups correspond to six different chemicals: MMI, TETRAC, Iox (0.1µM/1µM) and DES (0.1µM/1µM) and E3 group without chemicals. Zebrafish where then used for WMISH. This method was conducted for five genes (nk2.1a, pax8, tsh, tg and cmlc2). Results were observed with stereoscope and photographed. For further analysis images were processed with Fiji and GIMP. To assess effect of chemicals in animals, statistical analysis was used.*

3. Materials & Methods

3.1 Animals Breeding and Spawning

Artemia salina is a species of brine shrimp (aquatic crustaceans) widely used as food for larvae. Artemias produce dormant eggs, known as cysts that can be stored for long periods. All the zebrafish were fed with Tropical Energy Food (Aquatic Nature), two times a day and once with live *Artemia salina*. Crustacean's eggs were hydrated and led to hatch in tanks containing 90% salty and 10% plain water. After 24hours the artemia mix was enriched with red pepper. At second day artemias were collected. Fishes were maintained in tanks, in a closed circuit (Technoplast) with a biological filter and recirculating aerated fresh water at 28^0C, exposed to a natural photoperiod (14 hours light/10 hours dark).

The larvae used in all experiments came from the same broodstock. A male and a female were placed in spawning chambers of 1lt. Pairs were separated from each other by a Perspex separator overnight (10h) and were left free to mate in the morning, by removing the Perspex separator. The spawning chambers had false bottoms to collect the eggs. The eggs were collected into Petri discs with E3 medium and placed in an incubator (Sanyo, Germany) at $28,5^0$C, an appropriate temperature for zebrafish development. The developmental stage of eggs and larvae was estimated in hours post fertilization (hpf) (Kimmel et al., 1995).

3.2 Chemicals

Control animals with no treatment and positive controls were used to compare with the animals under Iox and DES effect. The assay was developed using as control, animals reared in E3 standard zebrafish embryo medium. Positive controls animals were treated with two chemicals well known for their disruption in the HPT-axis. Methimazole (Sigma M8506) and Tetraiodothyroacetic acid (Sigma T3787). Methimazole (MMI) is an antithyroid drug, which act like goitrogen, reducing TH synthesis by inhibiting thyroperoxidase enzyme function (enzyme necessary for iodination of thyroglobulin). This drug is used for medical treatment in human to decrease thyroid hormones levels in cases of thyroid gland hyperactivity. It contains a thiocarbamide group that is responsible for its goitrogenic activity. The deaminated analogue of L-thyroxin, tetraiodothyroacetic acid (TETRAC), acts as an antagonist of thyroid hormone in the binding site of membrane receptor integrin. As described in TH signaling, thyroid hormones can bind to integrin receptor aVβ3 and then trigger several signaling pathways. All reagents were diluted in NaOH 0.01M with final concentration 300mM for MMI and 200μM for TETRAC and 200μM for both Iox and DES. These solutions were used as stock. By using concentrations of Iox and DES typical of those present in aquatic ecosystem it was possible to assess if they could impact in reality on the development of endocrine systems, in animals and human.

3.3 Treatment

The treatment of all animals begins between 10-12 hpf approximately at 6-somite stage of embryonic development (segmentation period) (Kimmel et al., 1995). Mortality from 6-somite stage and after is minimum, compared with early stages of development. So, any changes in the death rate of the animals will probably be result of the exposure in chemicals. All animals were reared for four days. By day 4 of treatment, meaning 108 hpf, zebrafish larvae morphogenesis is almost completed and it has already began to swim and move actively. Protrusion of the mouth continues to the anterior-posterior axis and next 12 hours fishes start to feed by mouth. Embryos survival was estimated daily, by removing dead animals. Number of hatched zebrafish embryos was also recorded every day. In case that some of them were not hatched till day 4, the chorion was removed by hand with a pair of tweezers.

In order to observe any differences between long-term and short-term exposure and if zebrafish organism has the ability to recover after treatment with chemicals, two way of treatment were followed. In short-term treatment, fishes at 6-somite stage were treated for 2 days and then were moved in E3 till day 4 of treatment. Long-term treatment was provided till the time of collection at day 4. Long-term and short term treatment was carried out for seven experimental groups each: E3, MMI, Tetrac, Iox 0.1 μM, Iox 1μM, DES 0.1 μM and DES 1 μM groups. Each group had total number of animals n=50. E3 group contained untreated animals, reared only in E3 zebrafish medium. These animals are referred as controls. Positive controls were embryos that grew in MMI and Tetrac groups. For that reason two solutions were prepared. One that MMI was diluted in E3 (c_{final}=3mM) and another with Tetrac (c_{final}=3μM). As for the rest of the groups 0.1 μM and 0.1 μM correspond to the concentration of which chemicals had in the solutions. Specifically, Iox 0.1 μM included animals placed in solution with final concentration 0.1 μM Ioxynil and E3 and Iox 1μM describes fishes in Ioxynil diluted in E3 medium with final concentration 1μM. DES 0.1 μM and DES 1 μM groups were created in the same way (Table 1). DES and Iox were both used in two concentrations 0.1μM and 1μM, based on previous studies referring to the mechanism of their action (Campinho and Power, 2013). The concentrations of these solutions were prepared as the table shows below. All reagents were diluted in E3 medium with final volume 25ml. On a daily basis 10% (2.5ml) of the incubating medium was refreshed. Animals and solutions where placed in separated Petri dishes.

The experiment was repeated on three separate occasions to validate the results obtained. The second trial was no efficient, because of the increased mortality that it was observe. Animals in this case were not used to estimate the result of the chemical exposure in endocrine system.

Table 1. *Initial and final concentrations of the solutions used as treatment for zebrafish.*

Solution	$C_{initial}$	C_{final}	$V_{initial}$	V_{final}

E3	-	-	-	50ml
MMI	300mM	3mM	500ml	50ml
Tetrac	200µM	1µM	250ml	50ml
Iox	200µM	1µM	250ml	50ml
Iox	200µM	0.1µM	250ml	50ml
DES	200µM	1µM	250ml	50ml
DES	200µM	0.1µM	250ml	50ml

3.4 Samples Preparation

3.4.1 Fixation

In day 4 all animals were collected and anesthetized in ice. Animals were fixed in 4% (wt/vol) paraformaldehyde (PFA), in 1xPBS overnight at 4 degrees. Next day fixed animals were washed two times for 5 minutes in PBT solution and then 5min in 1xPBS.

3.4.2 Bleaching

For removal of pigmentation samples fixed fishes were incubated at room temperature in a 3% hydrogen peroxide (H_2O_2)/0.5% KOH/1xPBS medium. Progress was checked by observing under stereoscope, until pigmentation has completely disappeared. At this point, fishes were rinsed for 5 min in PBS to remove H_2O_2 and stop bleaching reaction.

Then larvae were progressively dehydrated by washing for 5 min in each of the following solutions: 75% PBS/ 25% methanol, 50% PBS/50% methanol, 25% PBS / 75% methanol, and in the end two times for 5 min in 100% methanol solution. In methanol zebrafish can be stored and maintained for several months at -20 degrees.

3.5 Synthesis of antisense RNA probes

For this experiment five RNA probes, *Nk2.1a*, *Pax8*, thyroid stimulating hormone (*tsh*), thyroglobulin (*tg*) and Cardiac myosin light chain 2 (*cmlc2*), were used to assess the response of the chemicals to the animals. The antisense RNA probes were generated using cDNA templates that were previously amplified by PCR using forward and reverse primers designed to the target genes and available from the NCBI database for zebrafish. In table 1 are the forward and reverse primers of each gene were amplified with PCR. The PCR products were then cloned into pGEM-T Easy vector, which contains RNA polymerase promoters T7 and SP6. Amplification of cDNA templates and cloning procedure was conducted by Marco A. Campinho.

Table 2. *Components and concentrations for the antisense RNA probe preparation*

Components	**$C_{initial}$**	**C_{final}**	**Volume**
SP6 RNA pol buffer (Fermentas)	5x	1x	4µL
SP6 RNA pol	20 U/µL	1 U/µL	1 µL
DIG mix (Roche)	10x	1x	2 µL

M13 DNA probe Tg	43.1 ng/μL	500ng	11.6 μL
RNase guard (Ribolock)	40 U/μL	2 U/μL	1 μL
Sigma water	-	-	0.4μL

Antisense RNA probe for *tg* gene was generated using SP6 RNA polymerase (Fermentas). For the synthesis reaction, the components listed in the table below were placed in an Eppendorf tube with final volume 20μl and were incubated for 3 hours at 37^0C. RQ1 DNase (2μl) was then added in the mix for 30 minutes in 37^0C degrade any DNA left in the solution. To denaturate the enzymes, tubes were placed 5 min in 70 degrees and straight away 5 min in ice. The mix was centrifuged at 10 rpm and RNA was isolated by G-50 Micro-Columns. Synthesis of *TSH* RNA probe was performed following the same steps and components described above. Antisense RNA probes for Nk2.1a, Pax8 and Cmlc2 were provided by Marco A. Campinho.

3.6 Agarose Gel Electrophoresis

Gel electrophoresis is an easy and quick method to measure nucleic acid quantity. Biomolecules can pass through agarose gel pores and channels and separate, based on their length and charge. An electric field is applied DNA and RNA, as they are negatively charged molecules move to the positively-charged anode. In this experiment a standard agarose gel electrophoresis protocol was followed to assess the efficiency of RNA probes amplification. Gel containing 1x agarose was prepared, adding 0.6gr of agarose in 1xTAE RNA buffer. The solution was placed in microwaves for 2 min, for the agarose powder to dissolve. After that the mix was stained with ethidium bromide in concentration 1/1000. This intercalating factor is used to visualize DNA/RNA as it fluoresces under UV light. When the mix temperature decreased it was placed in an electrophoresis casting tray, to stabilize, and then in the electrophoresis tank. Tank was filled with TAE RNA buffer, the same that was used to prepare the gel. All materials were previously cleaned with 1.5% H_2O_2 and DEPC water solution. For electrophoresis assessment 1μL of the RNA probe was used after it became linear, by placing the probe at 65^0C for 2 min. In this volume 6x loading buffer was added in ratio 2:1. Loading buffer includes colored dyes and it is useful to check the progress of the electrophoresis during the procedure. Firstly, 1kb plus Invitrogen DNA ladder was loaded, that provides bands with standard length to compare with the samples. After that RNA probe was also loaded in the wells of the gel. In the end electrophoresis gel was viewed and photographed with an ultraviolet (UV) transilluminator.

3.7 Photometric estimation of nucleic acid concentration

The concentration of DNA-template was calculated with NanoDrop spectrophotometry. To assess the purity of DNA in the sample the ratio of absorbance at 260nm and 280nm is used. First blank is set, using 1μL of sigma water. For DNA concentration measurement 1μL of the solution is pipetted onto the lower measurement pedestal. A ratio higher than 1.5 is accepted as satisfying quantity of DNA, for further probe synthesis. NanoDrop 1000

spectrophotometer (Thermo Scientific) was also used to estimate the integrity of the RNA probes.

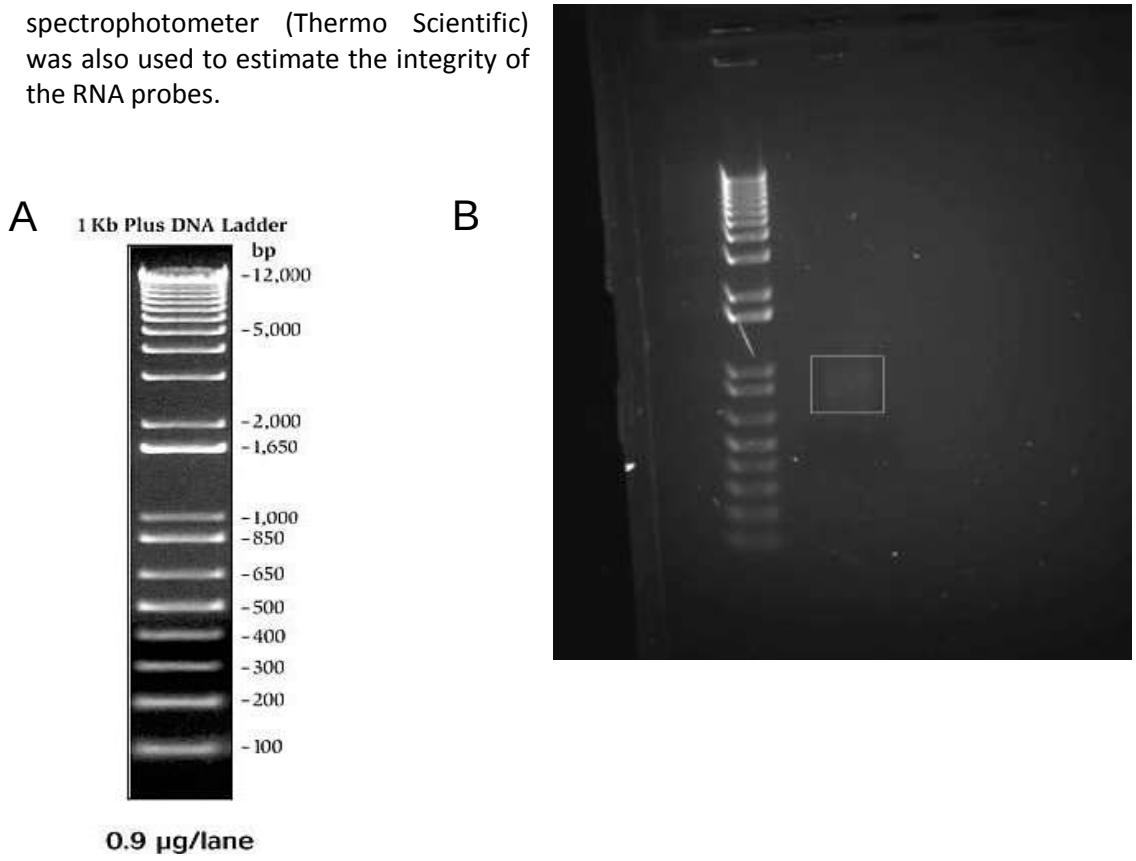


Figure 9. *1 Kb Plus DNA Ladder (Invitrogen) (A), that was used in electrophoresis of tsh antisense RNA probe in 1x agarose gel (B).*

3.8 Whole mount in situ hybridization (WMISH)

In situ Hybridization in whole mount embryos is a technique that localizes and measures the expression of a certain gene in whole organism. It is usually used to optimize the expression of genes during development. The basis of this method is the specific binding between two complementary RNA/RNA strands. Digoxigenin (DIG) labeled RNA probes, specific for the sequence hybridize the target gene. To make the hybridization visible anti-DIG Fab fragments, linked with the enzyme alkaline phosphate (AP). The enzyme stimulates the chromogenic substrate, giving a visible result.

WMISH was performed based on standard hybridization protocol (Christine Thisse & Barnard Thisse, 2007). Whole mount in situ hybridization was conducted for every probe using 10 animals for each experimental group. Gene's expression was examined separately for long term and short term treatment. Animals used for WMISH came from the first and the third trial, five embryos from each one. The samples, placed in sterile Eppendorf tubes, were rehydrated gradually for 5 min using 75% MeOH/25% PBS, 50% MeOH/50% PBS and 25% MeOH/ 75% PBS solutions. Then zebrafish were washed four times with 1xPBS/0.1% Tween20 and were permeabilized by digestion with proteinase K 10 µg/mL in 1xPBS. The time of incubation depends on the age of the fish and in this case the digestion had duration about 30 minutes. To stop the reaction zebrafish were incubated for 20min in 4% PFA in

1xPBS and after that were washed five times, for 5 minutes per wash, to remove residual paraformaldehyde. Prehybridization occurs by incubating the samples for 2 hours in 500μL pre-warmed Hyb-mix (50% Formamide, 5x SSC (pH 6), 200 μg/ml T-RNA, 100 μg/ml Heparin, 1x Denhart's, 5 mM EDTA, 0.1% Tween 20, Sterile Water (DEPC)) at 70^{0}C. For the hybridization step, as the protocol indicates, zebrafish samples were incubated in approximately 200μL Hyb-mix with the DIG-labelled RNA probe in concentration 0.25ng/μL overnight at 70^{0}C. In the morning Hyb-mix is replaced gradually with the following solutions for 5min at 70^{0}C: Hyb-mix (-) buffer (50% Formamide, 5x SSC (pH 6), 0.1% Tween 20, Water DEPC), 75% Hyb-mix (-)/25% SSC, 50% Hyb-mix (-)/50% SSC, 25% Hyb-mix (-)/ 75% 2xSSC and 2xSSC Tw20. Additionally fishes were washed twice, 30 min per wash with 0.1% Tw20 diluted in 0.2xSSC at 70^{0}C. Next step, 0.2xSSC/0.1% Tw20 was discarded and samples were washed for five minutes with different concentrations of Malic acid buffer (MAB) (0.1M malleic acid (pH 7.5), 150 mM NaCl) in 0.2xSSC solution and specifically, in 75% 0.2x SSC Tw / 25% MAB, 50% 0.2x SSC Tw / 50% MAB, 25% 0.2x SSC Tw /75% MAB and finally was added 0.1% Triton X100 diluted in MAB (MAB Tr). For 3h fishes were preincubated in room temperature, in a solution containing MAB Tr / 10% sheep serum / 2% blocking solution, with final volume 500μL. This step is important for saturating nonspecific binding sites for the antibody. Larvae are incubated all night with antibody (Roche) goat anti-DIG-AP in 1/5000 concentration, in MAB Tr/10% sheep serum/2% blocking solution, volume of 200μl, with gentle agitation. The larvae were stored for this reaction at 4 degrees. Next day solution with antibody is removed and samples are washed 30 minutes in MAB Tr (700μL) in room temperature. Washes are repeated 5 more times. MAB Tr solution is replaced with staining buffer (0.1M Tris HCl pH 9.5, 0.1M NaCl, 0.05 $MgCl_2$, 0.1% Tween 20) in the same volume. Animals are transferred to 24-wells plates and washed for 3 times, 5 min each one. To develop the signal of staining is added new staining buffer with NBT/BCIP (6.75μl/ml for NBT and 3.5μl/ml for BCIP). Samples are incubated in room temperature in darkness, for 15 minutes-4 hours till the desired staining intensity is reached. In order to stop the reaction, stop solution (1xPBS pH 5.5, 1mM EDTA, 0.1% Tw 20) was used for three washes. Two washes lasting 15 minutes and the last wash was carried out overnight at 4^{0}C, in the dark. The day after, fishes were moved again in Eppendorf tubes. They were firstly washed for 5 min in PBSx1 pH 3.3 (500μl) and then in 1xPBS pH 7.4. Samples were mounted in 50% glycerol / 50% PBSx1 pH 7.4 solution for about 30 minutes till they move from the top to the bottom of the tube. Finally, larvae were transferred in 400-300μl of 100% glycerol and left in agitation all night. Samples in 100% glycerol can be stored for a long time at 4^{0}C.

3.9 Image Processing

Zebrafish were photographed from 2 days of treatment (60hpf) and forward every 24hours till the end of treatment (108hpf). To become stable and easy to manipulate, they were anesthetized with tricaine. Tricaine methylsulfonate, also called tricaine mesylate or TMS, is an anesthetic agent that causes muscle relaxant and prevents action potentials. A drop of tricaine was added in a Petri dish with E3 medium for immobilization of the fish.

Pictures from all treatment groups were acquired with Olympus SZX7 stereoscope, using an Optikam 0.3 digital camera (Italy). Images were edited with Just Image J (or Fiji; http://fiji.sc/Fiji) and GNU Image Manipulation Program (GIMP; http://www.gimp.org/). Just Image J is a software for image processing and analyzing in Java. This software was used on quantitative analysis, of *tg* expression in thyrocytes. After in situ hybridization intensity of *tg* expression field was estimated using Plot profile analysis tool. Plot profile displays a two-

dimensional graph of the intensities of pixels along the line within the image. The x-axis represents the distance along the line and the y-axis is the pixels intensity. For that reason the type of images used was 8-bit and the line was applied along the thyrocytes expansion. Low "grey value" corresponds to high pixel intensity and high "grey values" correspond to low pixel intensity. Assessment of chemicals disruption on *cmlc2* expression, was also conducted using Just image J. Circularity of the ventricle in zebrafish was calculated through circularity formula 4pi (4π*area/perimeter^2). Circulatory value of 1.0 indicates a perfect circle, while values approaching 0.0 correspond to elongated polygon. Expect from circulatory of ventricle roundness was also measured according to the formula 4*area/ (π*major_axis^2).

3.10 Statistical analysis

Basic statistic values, such as average, were calculated with Microsoft Excel, Microsoft. Statistics for genes expression was carried out with analysis of variance (abbreviated as ANOVA). ANOVA includes statistical models that analyze differences between groups means One-way ANOVA is a technique used to compare means of two or more samples. In this case One-way ANOVA was used to determine the effect that chemicals, E3, MMI, TETRAC, Iox 0.1μM, Iox 1μM, DES 0.1μM and DES 1μM, have on one dependent variable. In this case the dependent variable that was studied was average number of thyroid follicles and intensity of *tg* ISH in animals of all experimental group. Long-term and short-term treated animals were analyzed separately and illustrated in diagrams. To estimate the effect of time of exposure and chemicals in parallel, two-way ANOVA test was used. This tests is an extension of the one-way ANOVA test that examines the influence of different categorical independent variables on one dependent variable. The dependent variable was as in one-way ANOVA average number of thyroid follicles/intensity of *tg* and the two independent factors are time and chemicals. The null hypothesis that is tested in two-way ANOVA is that there is no difference between the groups' means, that there is no effect of either factor and no interaction and a low p-value indicates that the null hypothesis should be rejected. For this experiment, the significance level of the differences that arise in analysis of variance, was determined by *p*-value. For $P < 0.001$ the hypothesis that is examined is probably true and the difference between the data that are compared is highly significant. In contrary $P > 0.05$ theoretically is no significant, as well as intermediate values $P < 0.05$ or $P < 0.01$ indicates significance.

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4. Results

4.1 Zebrafish morphology and Development

Zebrafish were normally hatched, during hatching period between 48-72hpf. In all groups and treatments there was no important rate of death. Late hatching, inability or total inability of hatching is not consistent with late development progress (Kimmel et al., 1995). For that reason, evidence of late hatching were not assigned as side effect of the chemicals. To determine the developmental stage and identify malformation several morphology markers were used such as pigmentation (xanthophores and melanophores) pattern, jaws position, heart formation, swim bladder, yolk extension and others.

4.1.1 1st day of treatment

After 24 hours of treatment animals where at the age of 36hpf. At this stage of development, also called prim 25, embryos had already moved from segmentation period to pharyngula period. Pharyngula period is associated with primordial in the pharyngeal arches. Pigment cells are differentiated and melanophores start to organize resulting a characteristic pattern. In the same period circulatory system is formed and heart beats. At prim 25 circulatory system was extended at about three quarters of the way to the end of the tail. The dorsal stripe, dorsal to the neural tube extends from the head, at the diencephalic region, to the tip of the tail. Also melanophores are present laterally from the trunk to the tail. Embryos of all experimental groups in long-term and short term treatment had no significant differences in the characteristics described previously. Although, body of embryos reared in MMI was fragile and more transparent, with less pigment in the melanophores in comparison with control animals. The yolk sac of MMI embryos was not absorbed in the same level to those from the other experimental groups in both short-term/long-term treatment.

4.1.2 2nd day of treatment

Second day of treatment, animal's age was 60hpf, the pec fin stage according to head-trunk angle (Kimmel et al., 1995). This stage is characterized by the presence of the pectoral fin at the side of the body of the fish. At this point animals have already started to hatch from the chorion. Heart has formed and beats strongly. Melanophores of head and trunk increase in number and xanthophores in the head produce yellow color and some shades are present to the trunk and tail. In short-term exposure, no change in morphology and development was observed to animals of Iox and DES groups of both concentrations (0.1μM and 1μM) comparing with controls. Although, melanophores were less intense in animals

treated with the pattern was from the animals, as there decreased in yolk sac and ventral side of the controls, TETRAC reduction in melanophores in lower level group. Zebrafish treatment had short-time with no changes for Iox/DES differences in (Fig. 10).

Methimazole, and slight different untreated melanocytes were number to the particularly to the trunk. Positive group had melanin and the yolk, but in a compared to MMI from 4 days of morphology with treatment group, in development groups and positive controls

Figure 10. *Images of lateral view of zebrafish at the pec fin stage. The first column contains all experimental groups, E3, MMI, TETRAC, Iox 0.1 µM, Iox 1µM, DES 0.1µM and DES 1µM, from short-term treatment and the second zebrafish from long-term treatment.*

4.1.3 3rd day of treatment

By this day (84 hpf) zebrafish larva had completed most of its organogenesis. The mouth has opened, the most of the yolk has absorbed and myotomes are visible. Xanthophores have progressively increased dorsally and the four stripes of the zebrafish are well distinguished. Experimental groups Iox 0.1μM/1μM and DES 0.1μM/1μM had no difference with E3 groups, both in short-term and long-term treatment. Nevertheless positive controls displayed changes in the morphology. This time TETRAC group had more significant differences in phenotype than MMI relative to E3. Even if the stripes of TETRAC animals had formed, the melanin and also the yellow pigmentation owing xanthophores, were decreased. This observation includes animals from long-term and short term treatment. The mouth in zebrafish reared 2d in Methimazole was not completely but the melanophores were as intense as the control animals. There was difference with long-term exposure, where animals in MMI continued to have low levels of melanin in melanocytes and the mouth was closed (Figure 11, A).

4.1.4 4th day of treatment

The 5th day post fertilization complete organogenesis has occurred. The mouth has started to protrude and the lower jaw gradually develops, bringing jaws together. From that day long, zebrafish starts to feed by mouth. Also the swim bladder has almost formed helping the larva to swim actively. Control group (E3) when compared to Iox and DES groups has no differences in morphology, after 48 and long-term treatment with those chemicals. In MMI group in short term treatment there were no signs of abnormalities and delay in development. After 4d exposure unlikely to short-time exposure, larvae of MMI were delayed in development. More specifically, jaws were not so developed and protruded as the controls, body length was smaller and melanophores less intense. The pattern of pigmentation was also different from control animals in TETRAC experimental group treated for 2d and 4d. The melanocytes did not form the four pigmentation stripes observed in the control group. Also the body shape was curved and the body seemed fragile and transparent. However the characteristics of this developmental stage (swim bladder and growing jaws) were the same with control groups (Figure 11, B).

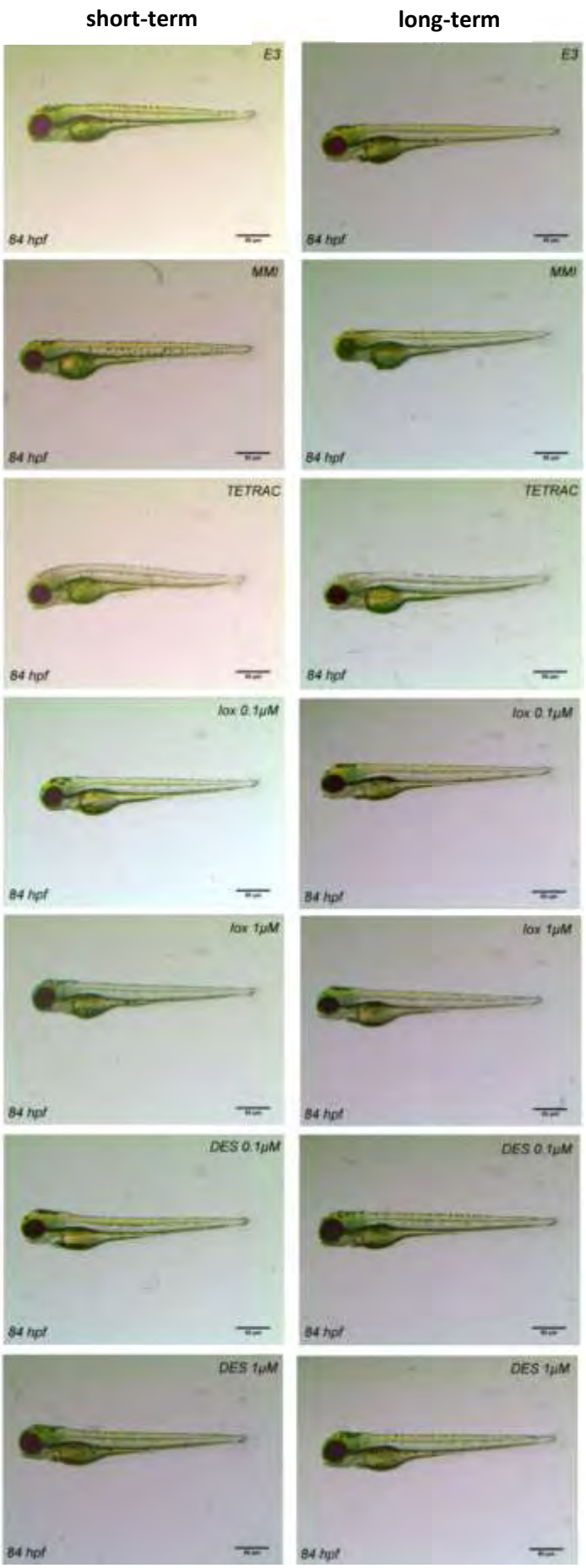
short-term
long-term
E3
84 hpf
MMI
TETRAC
lox 0.1µM
lox 1µM
DES 0.1µM
DES 1µM

Fig.11 (A)

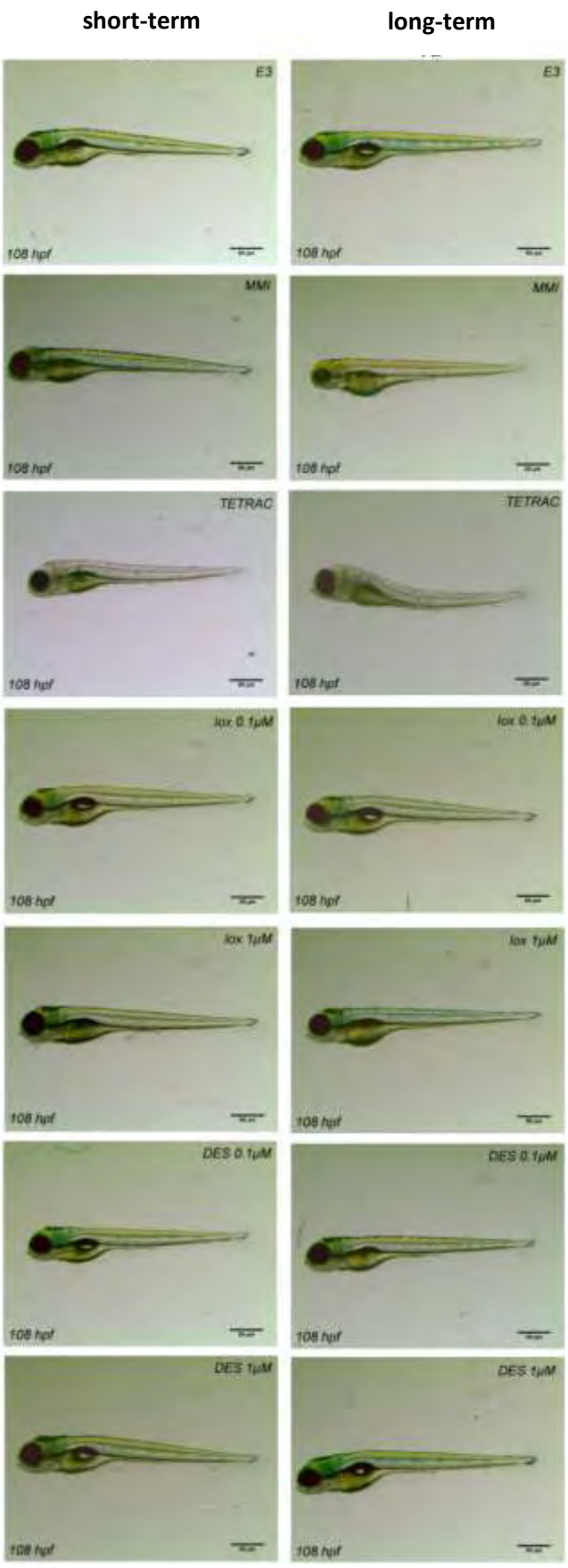

Fig.11 (B)

Figure 11. *(A) Zebrafish at the age of 84 hpf, presented laterally. In the left side row are zebrafish with short-term exposure in chemicals and the right row for long-term. (B) Pictures of zebrafish after 4 days of treatment where the first column indicates short-term treatment and the second 4d.*

4.2 WMISH Results

4.2.1 Nk2.1a

Zebrafish at the age of 108hpf were hybridized with antisense nk2.1a probe. In all treated animals there was no signal of the developmental marker nk2.1a in the thyrocytes. Only zebrafish treated with MMI, 60% of the total number, after short-term treatment were labeled in the thyroid follicles (Fig. 12). These data are not concerned as effect, because in control animals with no treatment thyrocytes were not stained.

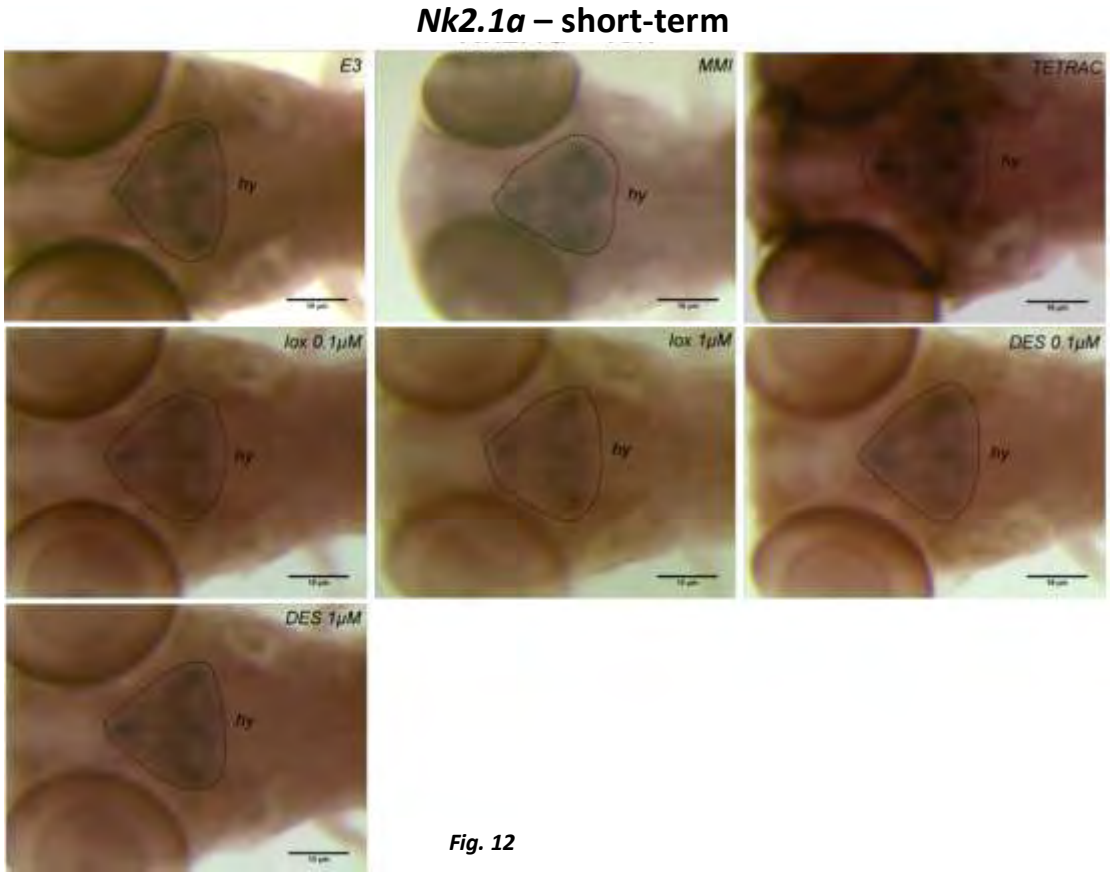

Fig. 12

Figure 12. *Hypothalamic expression of Nk2.1a (dorsal) in zebrafish of all experimental groups, after 2 days of treatment. Dotted line indicates the expression field of nk2.1a and the scale bar corresponds to 10μm (hy; hypothalamus).*

According to expression pattern, Nk2.1a transcripts were found in the hypothalamus of all treated animals. Hypothalamic expression of *nk2.1a* in animals treated with Iox and DES, had no significant difference in both short -term treatments when they were compared with those with non-treated animals. Also there was no significant difference in expression, pattern and intensity, between positive controls (MMI, TETRAC) and Iox/DES groups.

Long-term treatment with chemicals Iox and DES, in concentrations 0.1µM and 1µM, did not seem to disrupt the hypothalamic expression of *nk2.1a*, relatively to E3 group that there was no treatment. TETRAC and MMI group did not appear any difference when they were compared either with controls or with Iox and DES groups (Fig. 13).

_Nk2.1a_ – long-term

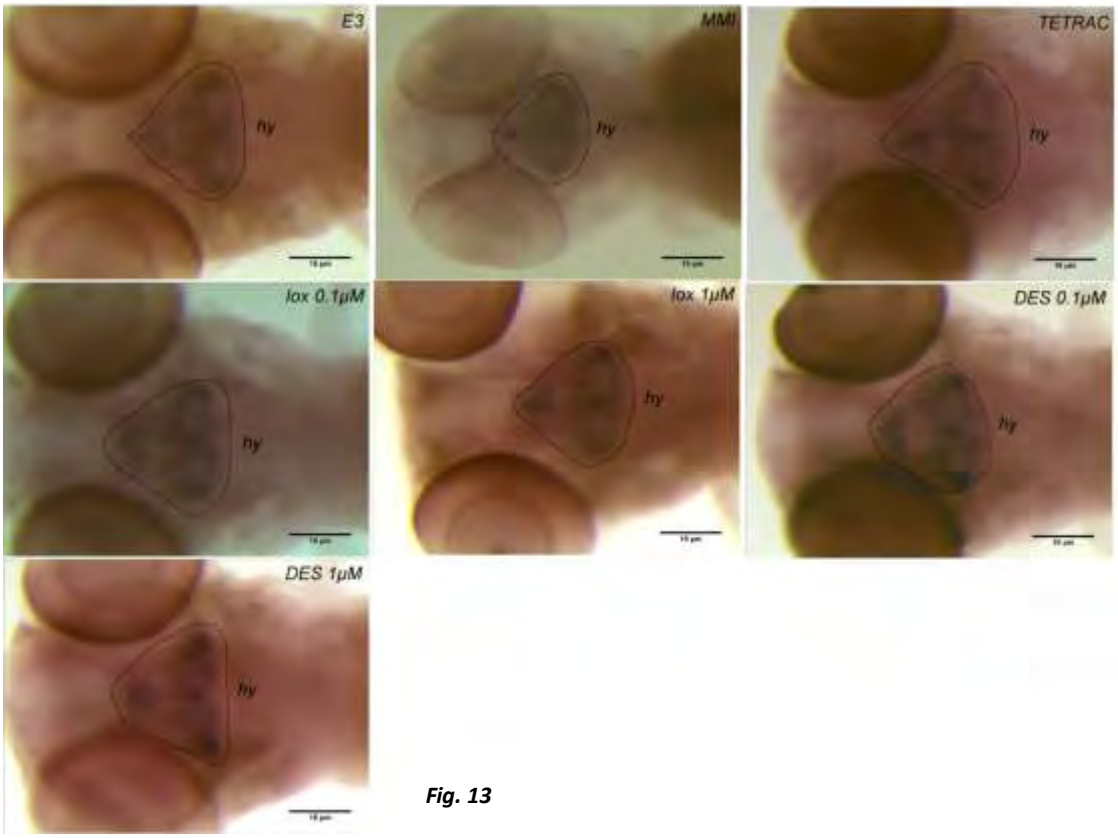

Fig. 13

Figure 13. *Dorsal view of the expression of nk2.1a in the hypothalamus of zebrafish treated for 4 days. The bar responds to 10 µm and the labeled mRNA is lineated with dotted line (hy; hypothalamus).*

4.2.2 Tsh

In order to estimate the effect of Iox and DES in the function of the pituitary, *tsh* expression was detected. After short-term treatment *tsh* expression was detectable in the thyrotropes of the anterior pituitary in all experimental groups (E3, MMI, Iox 0.1μM/1μM and DES 0.1μM/1μM) but not in TETRAC where *tsh* was absent (Fig. 14).

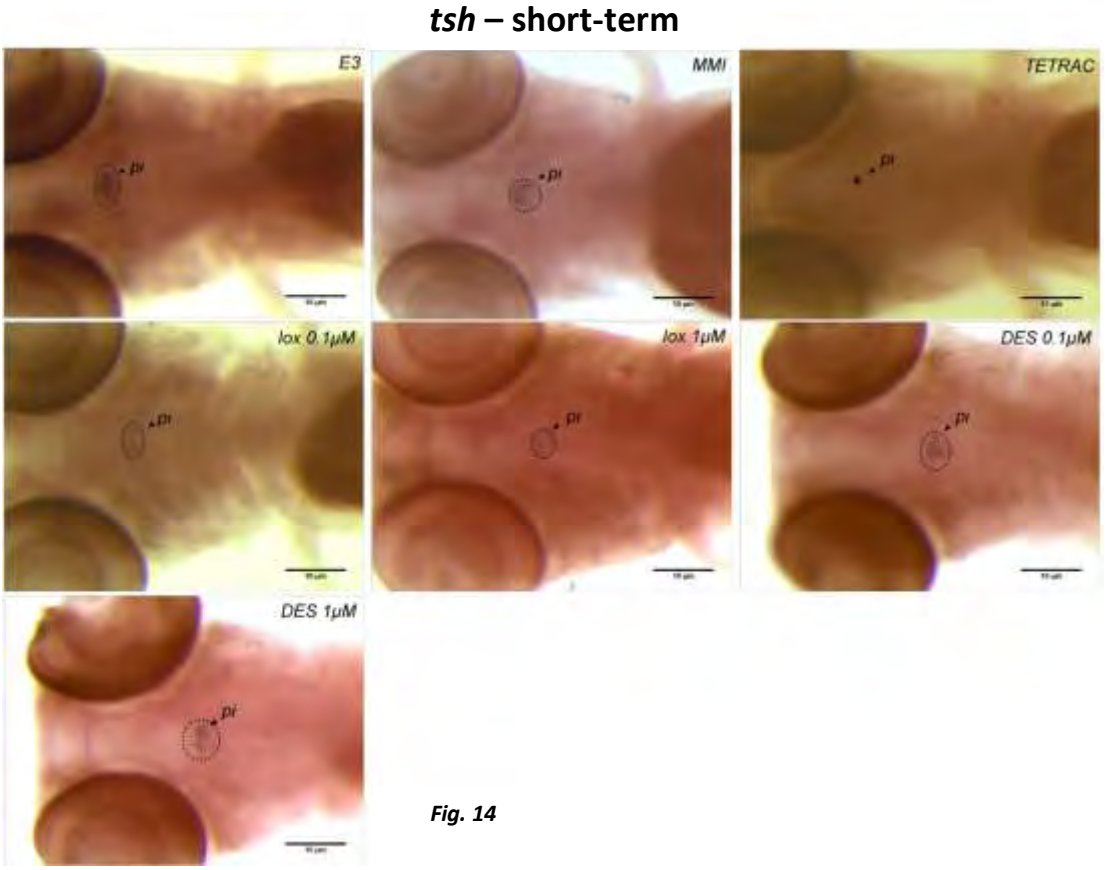

Fig. 14

Figure 14. *WMISH expression of tsh in the thyrotropes of the pituitary in all experimental groups of 2d of exposure. The scale bar corresponds to 10μm, the arrowhead indicates expression field of tsh and the asterisk the lack of signal in the pituitary of the TETRAC group. Animals are presented ventrally.*

In long-term treatment, *tsh* was labeled in experimental groups E3, Iox 0.1μM/1μM and DES 0.1μM/1μM but in positive controls the pattern of expression was different (Fig.15). Animals exposed in TETRAC, as in short-term treatment, had no signal from WMISH with tsh probe. Also in MMI group, expression field of *tsh* was decreased.

tsh – long-term

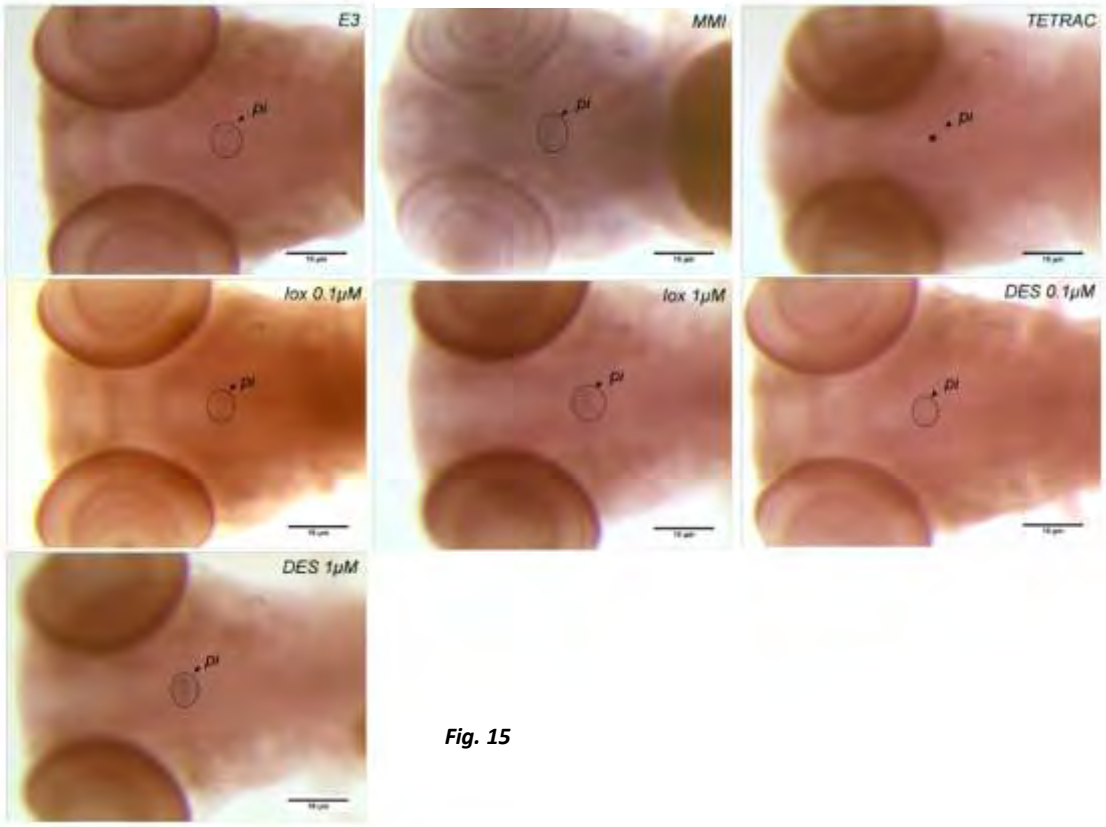

Fig. 15

Figure 15. *Zebrafish treated for 4d, labeled with tsh DIG-antisense probe (ventral view). The dotted circle shows the extension of tsh expression. The arrowhead indicates the field of expression in the pituitary (pi; pituitary) and the bar refers to 10µm length. The asterisk states the lack of signal.*

4.2.3 Tg

In zebrafish from all experimental groups in both cases of treatment, thyrocytes were stained. Iox and DES did not seem affect the *tg* transcription.

4.2.3.1 Tg – Number of thyroid follicles

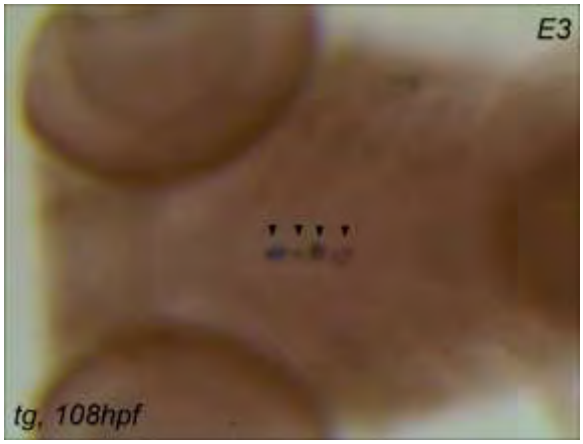

Figure 16. *Ventral view of zebrafish larval at the age of 108hpf, of the control group. The thyrocytes (n=4), that arrowhead indicates, are labeled with antisense probe for thyroglobulin RNA.*

To search in detail if Ioxynil and Diethylstilbestrol compounds impact the expression of thyroglobulin RNA, two techniques of evaluation were followed. Firstly the number of thyroid follicles was measured (Fig. 16) in all groups of short and long term exposure. The average number of follicles present in each group was calculated using Excel, Microsoft Office. To compare the results from every group Newman-Keuls test was used. The Newman-Keuls (also called Student-Newman-Keuls test) compares all pairs of means following one-way ANOVA.

In short-term treatment, no difference was apparent ($P>0.05$) in the average number of thyroid follicles between the larvae treated with Iox groups and the control animals. This observation regards to both concentration (0.1μM, 1μM) that this chemical was used. No significant effect ($P>0.05$) was also observed in animals exposed to 0.1μM and 1μM DES. Additionally, analysis of variation presented no significant change in the number of follicles between groups treated with MMI and TETRAC goitrogens (Fig. 17, table A).

In case of 4-days treatment with chemicals, average follicles number of each group were also compared with one way analysis of variance. Animals reared in Iox 0.1μM and Iox 1μM had no significant difference ($P>0.05$) in thyroid follicles expansion in comparison with control animals, as well as DES 0.1uM and DES 1uM experimental groups ($P>0.05$). However, positive controls (MMI and TETRAC) showed an important difference ($P<0.05$, $P<0.01$ respectively) in average number of follicles compared to control animals. There was also a quite significant difference between TETRAC and DES (0.1uM and 1uM) groups ($P<0.05$) (Fig. 17, table B).

The two-way analysis of variance for number of thyroid follicles (Fig. 17, table C) resulted in three *p*-values, one for each hypothesis. The first hypothesis examined if treatment had the same affect in all values of time (2d-4d of exposure). The second describes how treatment effects the result and the last hypothesis examined refers to the connection between time of exposure and results. The effect of chemicals in this variable seems to be very significant as it accounts for approximately the 19.26% of total variance observed, with P value = 0.0018. This means that there is 0.18% chance that the size of affect is randomly observed. In contrast, the effect of time of exposure in the results, was not considered significant with 0.75% percentage of total variance and $P=0.3433$. Regarding to interaction between the two variables, treatment and time, results from two-way ANOVA showed that the interaction is not quite significant ($P=0.0857$) and the treatment did not affect the time in the same way.

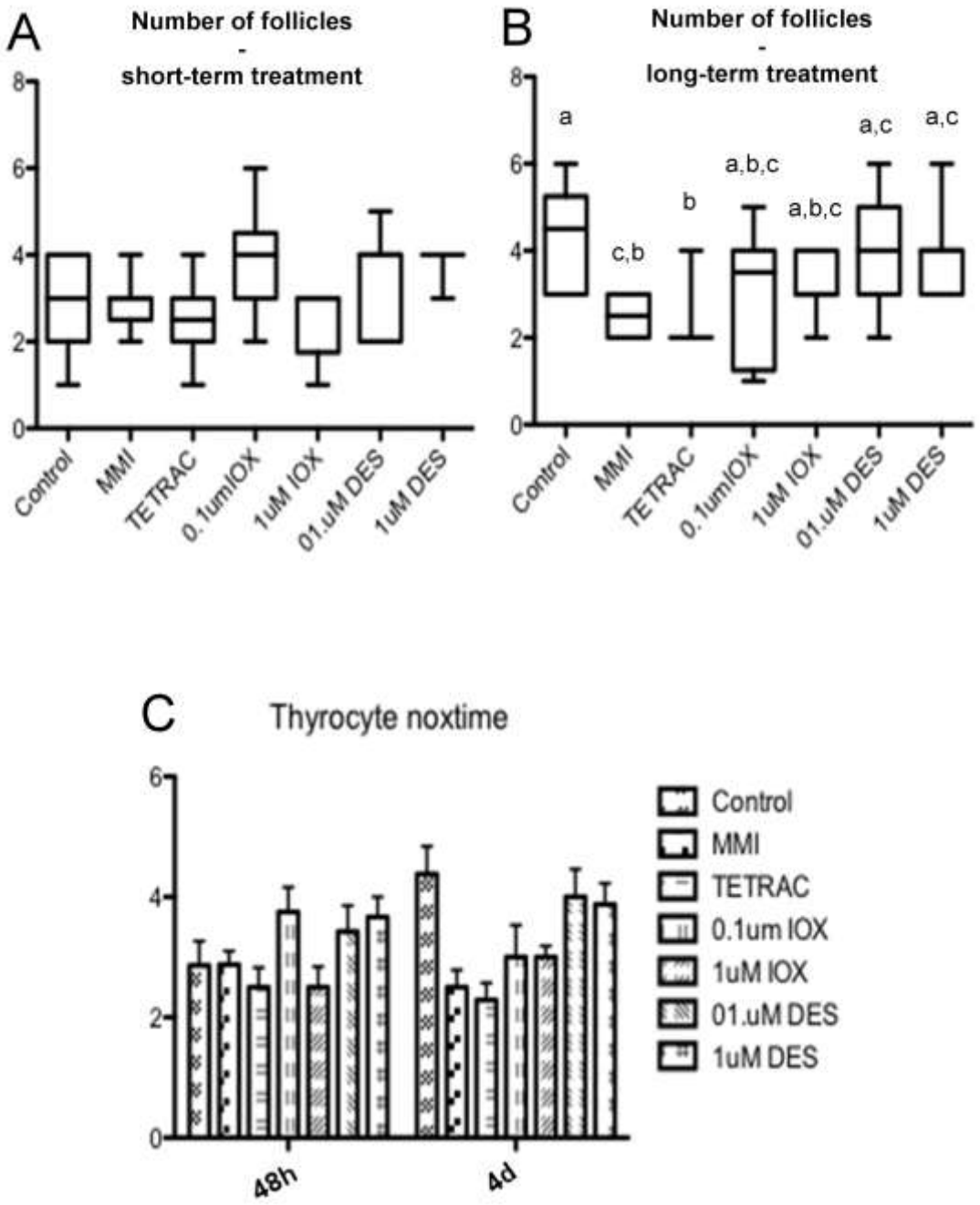

Figure 17. *Graphical representation of one-way ANOVA for the number of thyroid follicles, labeled with tg probe, measurements after 2 days of treatment (A) and 4 days (B). The Newman-Keuls test was used to establish if number of follicles differs significantly (P<0.05). In short-term treatment (A), Iox 0.1μM/1μM and DES 0.1μM/1μM had no significant difference (P>0.05) from the other groups. In long-term treatment (B), Iox groups (0.1μM/1μM) did not differ (P>0.05) from controls. Average number of follicles in DES 0.1μM and DES 1μM differed significantly from TETRAC (P<0.05 for both 0.1μM/1μM) but not from control. The box and whiskers graph below (C), represents results two-way analysis of variance that used to see the interaction between duration of treatment and number of thyrocytes in each experimental group. Bars show minimum and maximum values of the number of thyroid follicles parameter in comparison to the treatment followed in each experimental group. Groups with same letter are not significantly different (B).*

4.2.3.2 Tg - Intensity

Expression level of *Tg* gene in thyrocytes was analyzed measuring the intensity of the signal with Plot Profile by Just Image J program. Means of every experimental group were calculated and compared separately for long-term treatment and short-term treatment, using Newman-Keuls test of one-way ANOVA. Also two-way analysis of variance Bonferroni post hoc test was performed to detect any differences between groups due to long-term and short-term treatment.

Intensity analysis after short-term treatment revealed a significant difference between the MMI and other treatments, but no big differences were observed of intensity in control animals and treated animals ($P>0.05$) (Fig. 18, table A). Intensity of thyroid follicles was reduced ($P<0.001$) in animals of MMI group relatively to the Iox 1uM group which had presented the highest level of intensity of all experimental groups. The difference in intensity of MMI was also significant, but not of this size, when it is compared with TETRAC, Iox 0.1uM DES 0.1uM ($P<0.01$ for each comparison) and DES 1uM groups ($P<0.05$).

Results from one-way analysis in long-term exposure followed the same pattern as in short-term treatment. Intensity of thyroid follicles *tg* hybridization, was affected from MMI. Significant difference ($P<0.001$) was observed in intensity level of MMI and Iox groups of both concentrations (0.1uM and 1uM) as with other kind of treatment but in smaller magnitude ($P<0.05$). The intensity of E3 group was again not significantly different nor with MMI group neither with TETRAC, Iox (0.1uM, 1uM) and DES (0.1uM, 1uM) groups (Fig. 18, table B).

Two-factor ANOVA was used, with treatment and time of exposure as factors were examined in order to observe their effect in intensity of immunostaining. Interaction of these factors did not seem to play an important role ($P=0.0773$) in the thyrocytes intensity observed experimental groups after long-term and short-term. Nevertheless treatment has the greater impact in intensity ($P<0.0001$), as it accounts for approximately 42.48% of total variance. Time effect in intensity is not considered significant ($P=0.5929$) as it accounts only 0.16% of total variance and there is chance 59% that is observed by random (Fig. 18, table C).

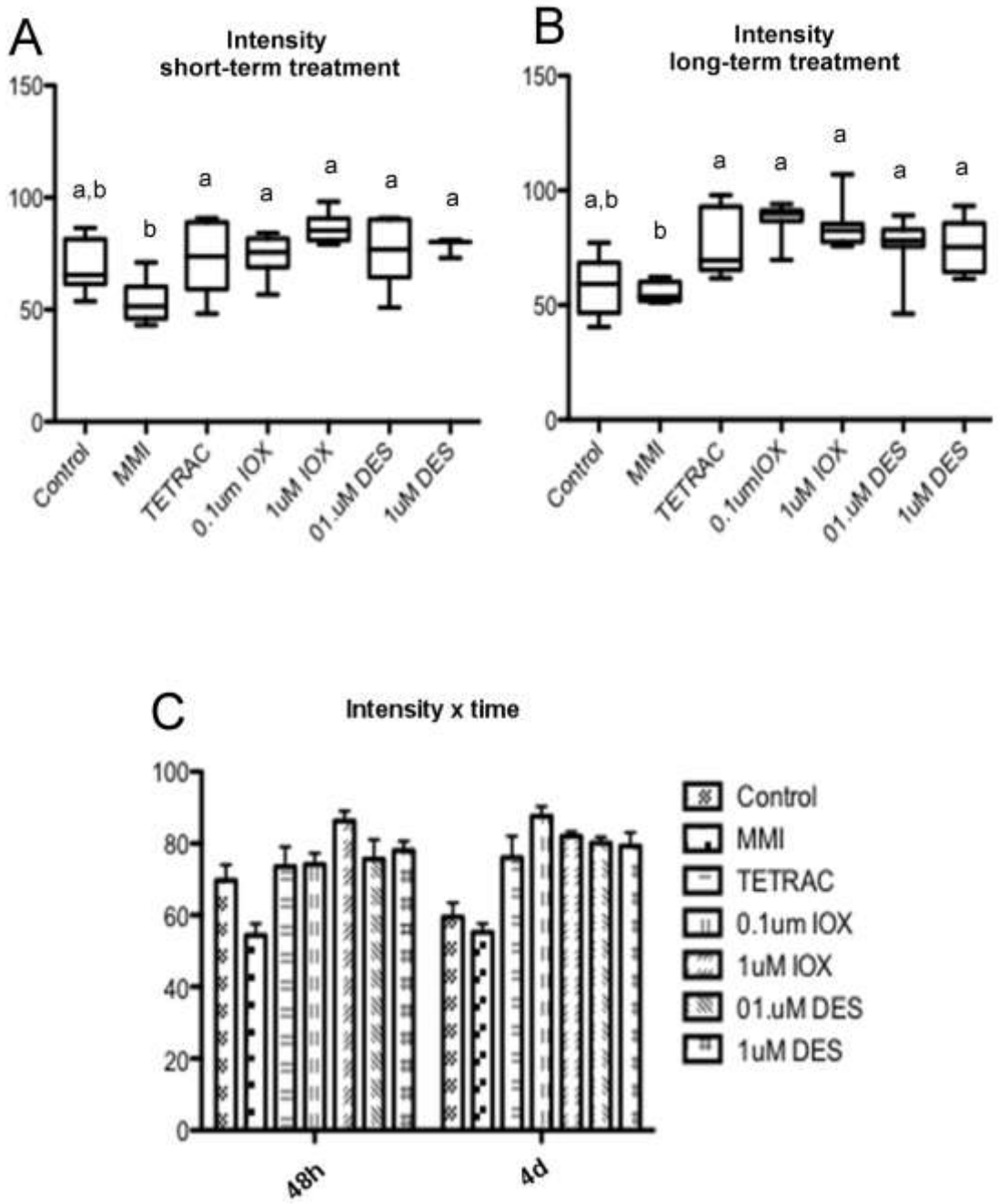

***Figure 18**. Comparison of average intensity of tg expression in thyrocytes, between experimental groups for short-term (A) and long-term treatment (B). One-way ANOVA results for short-term exposure, showed that intensity of Iox 0.1μM, Iox 1μM, DES 0.1μM and DES 1μM had did not differ significantly (P>0.05) from the controls but from MMI group (P<0.01, P<0.001, P<0.01 and P<0.05 respectively) (A). About long-term exposure, Iox 0.1μM, Iox 1μM, DES 0.1μM and DES 1μM had no significant difference (P>0.05) with control group. Just as short-term treatment, these groups were significantly different from MMI: Iox 0.1μM (P<0.001), Iox 1μM (P<0.001), DES 0.1μM (P<0.05) and DES 1μM (P<0.05) (B). Panel C represents two-way ANOVA. A Bonferroni post hoc test was used to establish if intensity in experimental groups, differs significantly in the two time-dependent treatments. Bars show minimum and maximum values of intensity in comparison to the treatment followed in each experimental group. Groups with the same letter do not differ significantly (A)(B).*

4.2.4 Pax8

Animals that were hybridized with antisense *pax* 8 RNA, were stained in the head in the midbrain and hindbrain region. Although there was no signal in the thyroid gland. This expression pattern was similar in all experimental groups. DES/Iox treatment does not appear to impair the expression field in any of groups in comparison with E3 controls it is clearly the same pattern. Results were identical in short term and long term treatment. In both ways of treatment, positive controls, had increased expression of the gene, expanding to the hindbrain region (Fig. 19, 20).

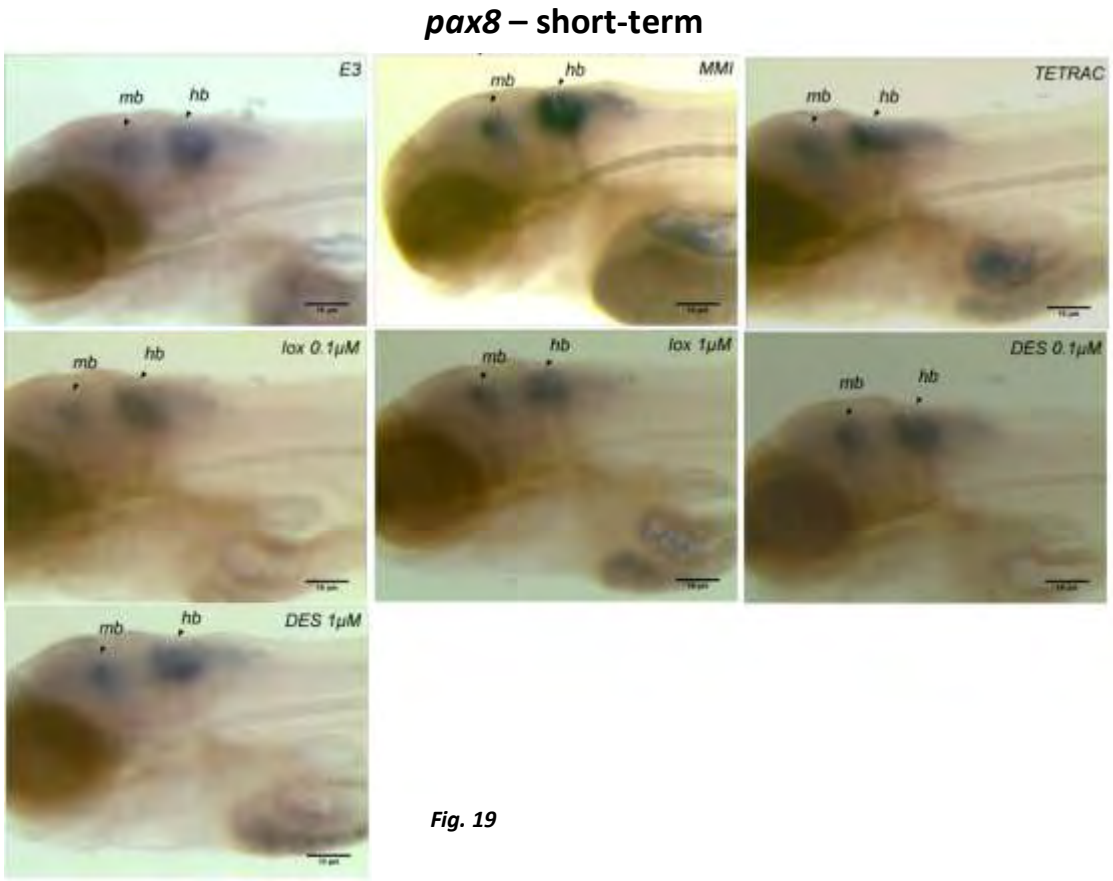

Fig. 19

Figure 19. *Lateral view of zebrafish from 2 days of treatment with chemicals, hybridized with pax8 antisense RNA probe. Arrowheads indicate hindbrain and midbrain regions in the head and the scale bar corresponds to scale bar 10µm (mb; midbrain, hb; hindbrain).*

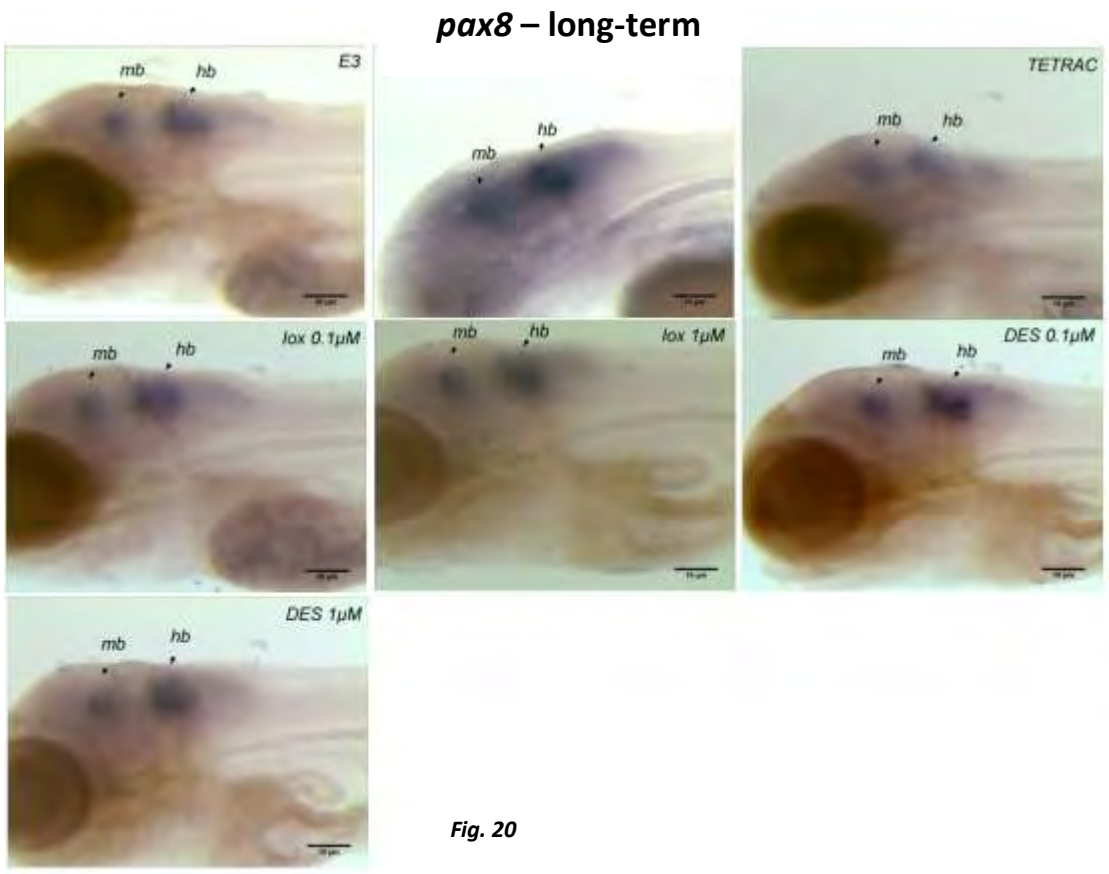

Figure 20. *Expression of pax8 of animals exposed for 4d, as presented from the lateral side. The arrowheads show the labeled hindbrain (hb) and midbrain (mb) and scale bar corresponds to 10µm.*

4.2.5 Cmlc2

Zebrafish of short-term treatment of all experimental groups, were labeled in the ventricle and the atrium of the heart (Fig. 21). Cmlc2 replicated were also present in all experimental groups (E3, MMI, TETRAC, Iox 0.1µM, Iox1µM, DES 0.1µM and DES 1µM).

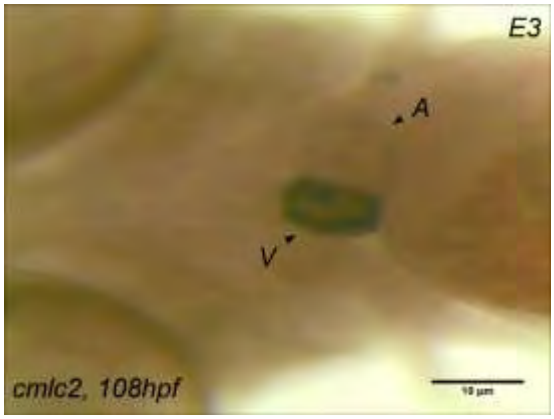

Figure 21. *Ventral view of heart in zebrafish (108hpf) in control group (E3). The ventricle is labeled with cmlc2 DIG antisense RNA probe. The bar corresponds to 10 µm and arrowheads show the ventricle (V) and the atrium (A).*

Normal function of the heart, as in other organs, depends on proper formation of the cardiac chambers ventricle and atrium. Each chamber, till 48hpf, has been shaped with a distinct outer and inner curvature. Formation of the chambers curvature is critical for the formation of the mature heart. In later stages (48-58hpf), only cells of the outer curvature of the ventricle seem to change in morphology as they are flattened and elongated. Abnormalities in the cardiac function are consistent with alterations in the outer curvature of the ventricle (Auman et al., 2007).

For that reason, circularity and roundness of the ventricle were measured using the circumference of the ventricle in Image j software. Data were compared with one-way analysis of variation. Treatment for 2d with chemicals (MMI, TETRAC, Iox and DES) did not seem to affect the circularity of the ventricle ($P>0.05$), comparing with control animals (Fig. 22, graph D). Neither after four days of treatment, the mean circularity differed significantly ($P>0.05$) between treated and untreated zebrafish (Fig. 22, graph E).

The second parameter used to detect deformation of the heart was ventricle's roundness. Results from analysis of variance were similar to the data from circularity measurements. Short-term exposure did not seem affect the roundness in Iox and DES groups ($P>0.05$), when they were compared with the mean roundness of E3 group, as well as positive control groups ($P>0.05$) (Fig.22, graph F). Also, there was no significant difference detected when experimental groups of long-term treatment were compared ($P>0.05$) (Fig.22 graph G).

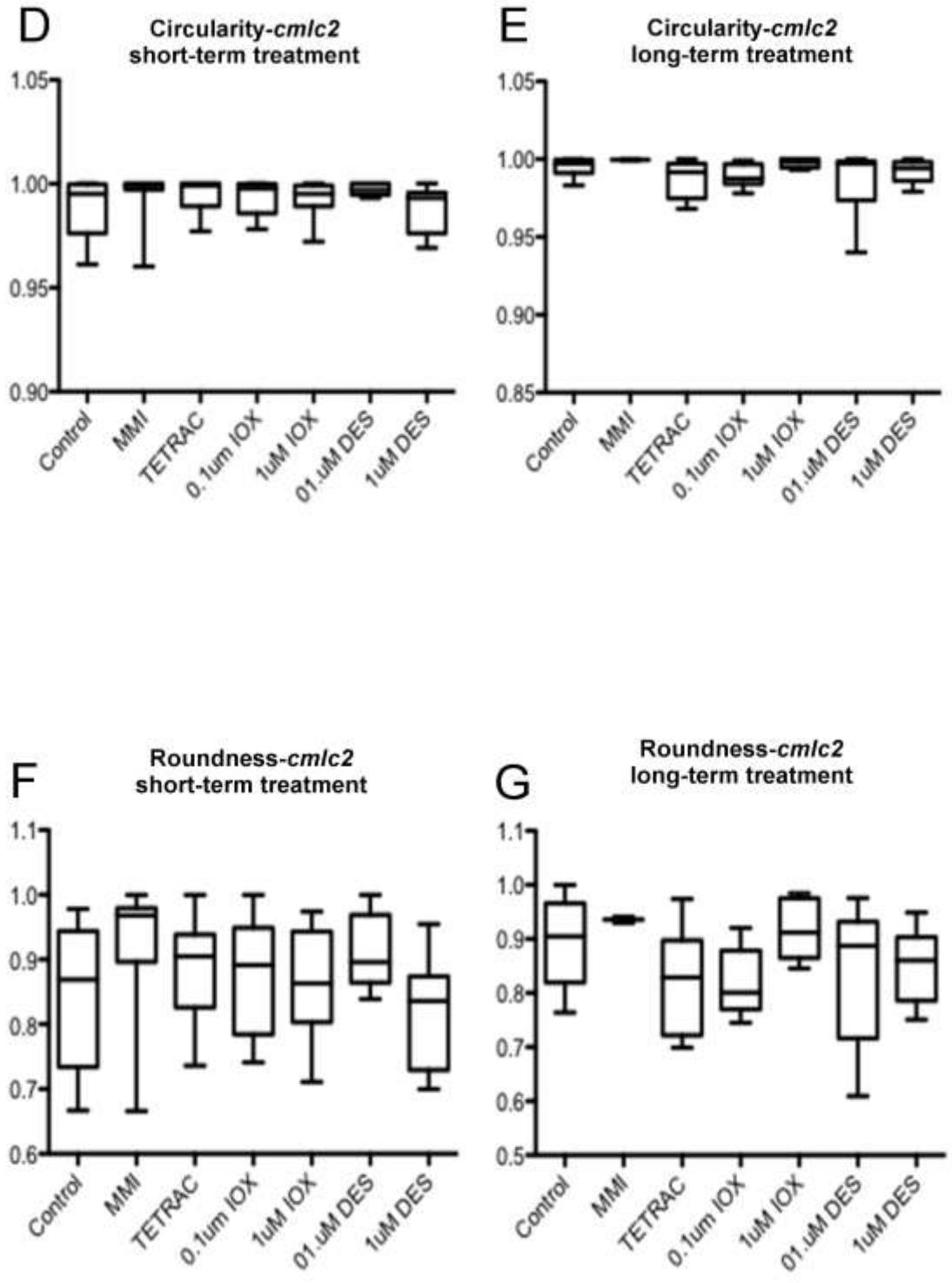

Figure 22. *Graph D and E show results from one-way ANOVA that was conducted to examine differences in circularity of the ventricle of experimental groups. The box and whiskers, in graph D, show any changes in circularity among groups treated for 2d. Iox 0.1μM/1μM and DES 0.1μM/1μM had no significant differences (P>0.05) in circularity, in comparison with controls or any other group (D). Comparison between groups of long-term treatment also showed no significant differences (P>0.05). The panel below presents graphical analysis of one-way ANOVA for roundness of the ventricle. Like circularity, roundness does not seem to differ significant (P>0.05) in groups of 2d (F) and 4d of exposure (G) respectively. Bars show minimum and maximum values of the two parameters (circularity and roundness) in comparison to the treatment followed in each experimental group.*

5. Discussion

Ioxynil and Diethylstilbestrol have been identified as environmental contaminants that affect endocrine system of vertebrates. In the past, several studies have investigated their way of action, focused on the reproductive system (Ma, 2009; Henley and Korach, 2010; Saal et al., 1997). Although, few things are known for the effect of Iox and DES in the thyroid system (Campinho and Power 2013; Morgado et al., 2009; Akiyoshi et al., 2012). The present study was focused in the impact of Iox and DES in the thyroid status of the zebrafish larva (5dpf) and how it depends on the time of exposure. After 2 days and 4 days exposure in these endocrine disruptors, the potential effect in the thyroid system was assessed by WMISH. Developmental genes have been studied and genes-markers of the development/differentiation of the HPT-axis (nk2.1a, pax8, tsh, tg) have been used. Also, effect of chemicals in the heart has been examined by analysis of the *cmlc2* expression. There were no evidence for disruption in general in the zebrafish system and neither specifically in tissue level.

Nk2.1a transcript presence is associated with early stages of development of the thyroid and in the zebrafish (Wendl et al., 2002; Rohr and Concha, 2000; Rohr et al., 2001; De Felice and Di Lauro, 2004). DES and Iox in higher magnitude is suggested to induce decrease of transcription of nk2.1a in the thyroid (Campinho and Power, 2013). However, there was no sign of expression in the thyrocytes. Only animals treated with MMI for 2 days had detectable thyroid follicles which is maybe due to the goistrogenic activity of Methimazole. As for hypothalamic expression there were no difference from in the both short-term and long-term treatment, revealing two things. Firstly, DES and Iox do not affect *nk2.1a*, expression in the hypothalamus, consistent with studies in 48hpf zebrafish exposed in Iox and DES (0.1µM and 1µM) and secondly that time of treatment has no impact in the thyroid.

As a marker for late differentiation of the thyroid follicles *pax8* expression pattern was analyzed (De Felice and Di Lauro, 2004; Wendl et al., 2002). Like nk2.1 hybridization, thyrocytes were not stained in any fish of the experimental groups in long-term/short-term treatment. The lack of sign in thyrocytes for both nk2.1a and pax8 transcripts may be result of experimental mistake. However specific hybridization in the hypothalamus and midbrain/hindbrain region respectively, suggests that these two phenomena are not linked with the technique but probably the signal was not too strong to be visible and detectable with the stereoscope. The expression pattern of pax8 in the head of Iox and DES groups was similar to non-treated animals. There were no evidence for changes in the different duration of treatment, showing again that there is not any relation with time of exposure and effect of the chemicals.

Zebrafish embryos (48hpf) exposure to Iox and DES decreases the expression field of thyroglobulin in thyroid follicles (Campinho and Power, 2013). For that reason *tg* expression field was analyzed measuring the number of thyroid follicles existed in every animal. At the short-term exposure data from ANOVA appeared that the number of follicles was not modified in any of the experimental groups. After long-term exposure there were also no evidence that Iox and DES affect the expression of *tg*. Interestingly in the long-term treatment MMI and TETRAC induced a decrease in the number of follicles in contrast with controls and had significant difference from Iox and DES groups. The fact that in positive controls of long-term exposure in the chemicals effected the expression field, but not in the short-term exposure reveals a recovery mechanism of the thyroid gland. Differences were observed between positive controls and Iox/DES treated animals argue with the suggestion

that these compounds act in a different way from Ioxynil and Diethylstilbestrol. As stated above, time of exposure did not appear to play important role in the results. This is consistent with data coming from two-way ANOVA in the number of thyroid follicles.

The *tg* intensity analysis showed that average intensity of Iox and DES groups (0.1μM/1μM) was significantly different from the MMI group in both short-term and long-term treatment. Although these groups did not differ from the control in the expression. Two-way ANOVA indicates that time has no significant impact in the expression of *Tg* in contrast with treatment that seems to have an extremely significant effect to zebrafish tg replicates.

The release and synthesis of THs is controlled by TSH secretion and in turn THs down-regulate the TSH. To examine the expression thyroid stimulating hormone in the pituitary, hybridization with *tsh* antisense RNA was occurred. In the sea bream, Iox and DES cause decrease in the brain and pituitary TSH (Morgado et al., 2009). In contrast, this study there were no differences detected between Iox/DES groups and E3 group for both 2 days and 4 days treatment. Exception on this findings is the TETRAC experimental group, where animals had no signal in the pituitary. TETRAC is a known antagonist of thyroid hormones which decreases TSH in serum, inhibiting the action of deiodinase type 2 (Lameloise et al., 2001).

Studies based on effect of Iox and Des in the HPT-axis, indicate that these compounds and effect the heart morphology probably in an indirect way (Campinho and Power, 2013). During development heart seems to interact with thyroid gland in the stage of its localization to the pharynx (Alt et al., 2006a,b). ANOVA of heart morphology in experimental groups, revealed that Iox and DES do not have any impact in the two parameters of the heart that were examined, circularity and roundness. Analysis of short- term and long- term exposed animals did not appear any difference.

6. Conclusion

In conclusion, results from morphology, WMISH and analysis of variance show that Iox and DES, in this case, does not disrupt the HPT-axis. The time of exposure does not seem to have an important role, as the results taken from long-term and short-term exposure had no differences. Regarding to short-term treatment it seems possible that the animals recovered from the exposure in Iox and DES in these low dosages, as is indicated from MMI and TETRAC group that rebounded from the initial exposure, referring to the number of follicles and morphological observation of day 4 and 5 post fertilization. The absence of evidence of disruption for long-term treatment suggest that the animals respond and overcome the toxic effect of Iox and DES in low dosages (0.1 and 1μM) or that the treatment is not totally penetrant. Results from two-way ANOVA, concerning to the not significant role of time, suggest that the mechanism of recovery that is observed may be similar. Finally, the differences between MMI and TETRAC with Iox/DES results confirm that Iox and DES do not act in the same way.

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