



# Developmental Toxicity of Thyroid-Active Compounds in a Zebrafish Embryotoxicity Test

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## Summary

*Zebrafish embryos were exposed to concentration ranges of selected thyroid-active model compounds in order to assess the applicability of zebrafish-based developmental scoring systems within an alternative testing strategy to detect the developmental toxicity of thyroid-active compounds. Model compounds tested included triiodothyronine (T3), propylthiouracil (PTU), methimazole (MMI), sodium perchlorate (NaClO<sub>4</sub>) and amiodarone hydrochloride (AMI), selected to represent different modes of action affecting thyroid activity. Tested time windows included 48-120 hours post fertilization (hpf), 0-72 hpf and 0-120 hpf. All tested compounds resulted in developmental changes, with T3 being the most potent. The developmental parameters affected included reflective iridophores, beat and glide swimming, inflated swim bladders, as well as resorbed yolk sacs. These effects are only evident by 120 hpf and therefore an existing General Morphology Score (GMS) system was extended to create a General Developmental Score (GDS) that extends beyond the 72 hpf scoring limit of GMS and includes additional parameters that are affected by exposure to model thyroid-active compounds. Moreover, the GDS is cumulative as it includes not only the scoring of developmental morphologies but also integrates developmental dysmorphologies. Exposures from 48-120 hpf did not provide additional information to exposures from 0-120 hpf. The results indicate that the zebrafish GDS can detect the developmental toxicity of thyroid toxicants and may be of use in an integrated testing strategy to reduce, refine and, in certain cases, replace animal testing.*

**Keywords:** thyroid, zebrafish, developmental toxicity, morphological scoring, *in vitro* alternatives

## 1 Introduction

The EU Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) requires all substances marketed or manufactured in quantities above 10 tons per annum to be tested for developmental toxicity (OECD 421 or OECD 422). Substances that are marketed or manufactured in quantities above 100 tons per annum are required to be tested for pre-natal developmental toxicity (OECD 414) (REACH, 2006). Based on data on chemical production from 1991 to 1994, the EU estimates that 2.6 million animals will be needed for all REACH testing or a total of 9 million when offspring produced during reproductive and developmental toxicity test-

ing are taken into account. While some studies have suggested much higher numbers, the EU is sticking to its initial predictions (ECHA, 2009; Hartung and Rovida, 2009; van der Jagt et al., 2004; Pedersen et al., 2003). The issues associated with these tests include ethical acceptability considerations, high costs and long duration. This has prompted research into the development of suitable high-throughput alternative tests that, once validated, could reduce animal testing within an integrated testing strategy (ITS).

In a previous study we investigated to what extent thyroid (TSH-screen) and pituitary (T-screen) cell proliferation assays could be used as *in vitro* screens for detecting the effects of thyroid hormone-disrupting compounds on thyroid and pituitary

Received February 1, 2014; accepted in revised form April 7, 2014; Epub April 10, 2014;

<http://dx.doi.org/10.14573/altex.1402011>

**Abbreviations:** AMI, amiodarone hydrochloride; dpf, days post-fertilization; hpf, hours post-fertilization; EC<sub>50</sub>, effective concentration 50%; GDS, General Development Score; GMS, General Morphology Score; HPT axis, hypothalamus-pituitary-thyroid axis; IC<sub>50</sub>, inhibitory concentration 50%; IHC, immunohistochemistry; ITS, integrated testing strategy; MMI, methimazole; PTU, propylthiouracil; T3, triiodothyronine; T4, tetraiodothyronine; TH, thyroid hormone



weights used as endpoints in *in vivo* assays (Jomaa et al., 2013). While the effect of estrogen-mimicking compounds on cell proliferation in the *in vitro* E-screen was found to correlate with their effects on *in vivo* uterine weight ( $r^2=0.85$ ) (Wang et al., 2012), the effects of thyroid-active compounds on thyroid (TSH-screen) and pituitary (T-screen) cell proliferation was found to have poor correlation with their effects on the weight of these respective organs *in vivo*. The results of the study indicated that a complex interplay between factors within the hypothalamus-pituitary-thyroid (HPT) axis may underlie the effects of thyroid-active compounds on thyroid and pituitary organ weight endpoints *in vivo*. Therefore, it was proposed that the development of future alternative tests, aiming at the refinement, reduction, or replacement of animal studies, should include as an initial screen a broad battery of *in vitro* tests that cover the various modes of action of thyroid-active compounds and should in addition be based on more complex model systems that better integrate the various processes involved in the HPT axis. The vertebrate *Danio rerio* (zebrafish) may represent such a complex model system that includes an intact HPT axis. Given that alterations in the thyroid system can have severe effects on development (Boyages and Halpern, 1993; Haddow et al., 1999; Utiger, 1999), the aim of the present study was to investigate whether a zebrafish-based developmental toxicity assay would be able to detect thyroid hormone-active compounds.

Hermesen et al. (2011) developed a zebrafish-based quantitative scoring system for developmental and teratogenic endpoints, with the former called the general morphology score (GMS). The developmental endpoints include abnormalities related to the completion of gastrulation, formation of somites, development of the eyes, spontaneous movement, blood circulation, pigmentation, edemata, malformations of the chorda structure, spinal cord (scoliosis, rachitis), head, sacculi/otoliths, tail, heart, yolk sac, growth retardation, and tail length (Nagel, 2002). The GMS is meant to be semi-quantitative and hence more efficient than more complex scoring systems while maintaining the same relevance in terms of output (Hermesen et al., 2011). The GMS scoring system uses the 0-72 hpf time window and was vali-

dated using eight glycol ethers and six 1,2,4-triazole antifungals (Hermesen et al., 2011). While alternative methods for developmental toxicity testing are unlikely to replace testing on rodents, they can potentially be used in pre-screening in order to reduce the need for consecutive *in vivo* testing (Piersma, 2006).

In order to investigate whether a zebrafish-based developmental toxicity assay would be able to detect thyroid hormone-active compounds, the effects in the zebrafish-based bioassay of a series of model thyroid-active compounds representing different modes of thyroid action *in vivo* (Tab. 1) was investigated. The selected compounds were triiodothyronine (T3), sodium perchlorate ( $\text{NaClO}_4$ ), propylthiouracil (PTU), methimazole (MMI) and amiodarone (AMI). All selected compounds are known to affect thyroid hormone levels *in vivo* albeit by different modes of action (Grover et al., 2007; Hood et al., 1999; de Sandro et al., 1991; Stoker et al., 2006; Yamasaki et al., 2002).

## 2 Materials and methods

### Compounds

All compounds were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and were of high purity ( $\geq 95\%$ ). All test chemicals were added from 500-fold concentrated stock solutions in dimethylsulfoxide (DMSO, Acros Organics, Geel, Belgium) to Dutch Standard Water (DSW; demineralised water supplemented with  $\text{NaHCO}_3$  (100 mg/l),  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$  (200 mg/l),  $\text{KHCO}_3$  (20 mg/l), and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (180 mg/l)). The exposure medium was then aerated for 24 h at 27°C, the pH was adjusted to 7.4-8.4 and  $\text{O}_2$  concentration was above 6.5 mg/l. The concentration range that was selected for the model compounds was based on reported effective concentrations for inducing reduction in T4 levels in zebrafish (Raldúa and Babin, 2009).

### Zebrafish embryos

Zebrafish, obtained commercially (Ruinemans Aquarium BV, Montfoort, The Netherlands), were maintained and bred at RIVM (Bilthoven, The Netherlands) for more than five years.

**Tab. 1: Selected model thyroid-active compounds**

List of compounds used in this study with their main mode of action on the thyroid hormone system based on rodent studies, and concentration ranges used in the experiments. The logarithm of the octanol/water partition coefficient (logP), which is a measure of lipophilicity, was predicted based on the chemical structure of the test compound using chemaxon software.

Compound	Abbreviation	CAS No.	Description	logP	Mode of Action	Concentration Range ( $\mu\text{M}$ )
Triiodothyronine	T3	6893-02-3	Endogenous ligand	2.80	Thyroid hormone receptor agonist	0.00005 - 0.5
Propylthiouracil	PTU	51-52-5	Antithyroid drug	1.2	Inhibits thyroid peroxidase and deiodinase type 1	25 - 400
Methimazole	MMI	60-56-0	Antithyroid drug	0.75	Inhibits thyroid peroxidase	125 - 2,000
Sodium perchlorate monohydrate	$\text{NaClO}_4 \cdot \text{H}_2\text{O}$	7791-07-3	Environmental contaminant	-0.10	Inhibits iodide uptake	25 - 400
Amiodarone hydrochloride	AMI	19774-82-4	Antiarrhythmic drug	7.64	Increases iodine load	0.001 - 10

Their daily diet consisted of two servings of dry flakes (Special Diet Services, Tecnilab-BMI BV, The Netherlands) and one serving of *Artemia* (Landman BV, The Netherlands). Constant filtering and permanent water flow-through kept contaminants to a minimum. Male and female adult zebrafish free from externally visible diseases were housed together in 7.5 l ZebTEC aquaria at  $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with a light/dark (LD) cycle of 14-hour light/10-hour dark. Three days prior to mating, males and females were placed in spawning chambers within separate tanks. Mating involved placing two males with two females in the spawning chambers with reduced water flow and keeping them in the dark overnight after which turning on the lights induced mating, spawning and fertilization within 30 minutes. The spawning chambers have a perforated bottom surface that allows the eggs to fall through, thus preventing the predation of eggs by adult zebrafish.

#### Exposure

Spawned eggs were rinsed in DSW and unfertilized eggs, less than 10% of total, were removed. Embryos were selected at the 4 to 32-cell stage and one embryo was added per well of a 24-well plate containing 2 ml of test medium per well. Alternatively, the transfer to the 24-well plates containing 2 ml of the test medium was performed at 48 hpf. The 24-well plates were kept in an incubator at  $26.5^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with a light/dark (LD) cycle of 14-hour light/10-hour dark. Exposure was performed at the start of the light cycle.

#### General morphology score (GMS)

Scoring of the developmental morphology was performed using a Leica Labovert FS microscope as outlined by Hermesen et al. (2011). Briefly, an incremental binary scoring system was used to assign a 0 to a non-event and a 1 to each normal morphologic developmental event that is easy to visualize under a regular stereo microscope. Sets of developmental events included detachment of tail, somite formation, eye development, movement, heartbeat, blood circulation, pigmentation of the head and body, pigmentation of the tail, pectoral fin development, protruding mouth, and hatching. The subtotals of all sets of developmental events were added towards a general morphology score (GMS), which in normal non-exposed embryos amounts to 15 at the 72 hour time point. In a separate binary scoring system, teratogenicity was scored, whereby normal embryos were assigned a 1 and malformed embryos were given a 0 for every malformation studied. These malformations included pericardial edema, yolk sac edema, eye edema, malformation of the head, absence/malformation of sacculi/otoliths, malformation of tail, malformation of heart, modified chorda structure, scoliosis, rachischisis and yolk deformation. Testing was considered valid when no more than 10% of control embryos were coagulated, underdeveloped or malformed.

#### Immunohistochemistry

Whole-mount immunohistochemistry of T4 was performed as previously described by Raldúa and Babin (2009), with slight modifications. Briefly, zebrafish embryos at 5 dpf were sacri-

ficed by incubation on ice for 5 min and then fixed overnight in 4% paraformaldehyde at  $4^{\circ}\text{C}$ . They were then washed repeatedly with PBS, dehydrated with a methanol row (30%, 60%, 100%) and stored overnight or longer at  $-20^{\circ}\text{C}$ . The embryos were then rehydrated with a methanol row (60%, 30%) and washed several times with PBS containing 1% Triton X-100. Permeabilization was achieved by 15 minute incubation at room temperature with 0.1% collagenase in PBS. Immediately after the incubation, the embryos were washed several times with PBS containing 1% Triton X-100 and blocked in blocking buffer containing 4% goat serum, 1% BSA, 1% DMSO, 0.8% Triton X-100 and 0.1% Tween-20 in PBS for 2 h. This was followed by two washes in 1% BSA in PBS and an overnight incubation in a solution consisting of a polyclonal antibody against T4 (MP Biochemicals, Amsterdam, The Netherlands) diluted 1:4,000 in blocking buffer containing 0.02% sodium azide. The following day, the embryos were washed several times in 1% BSA in PBS and incubated on a gentle rotation device for 2 h in a solution containing a 1:300 dilution of the secondary antibody conjugated to the fluorescent label Alexa Fluor 488 (Life Technologies, Breda, The Netherlands). Right after the incubation, the embryos were either stored in glycerol or embedded in 1% gelatine for imaging on an a Zeiss Axiovert 100 M confocal microscope (Zeiss Netherlands bv, Weesp, The Netherlands).

#### Data analysis

Non-linear curve fitting was done using the Hill equation with the help of GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). For the *in vivo* data that were collected from literature, a dose of 10 ppm MMI, equivalent to 10 mg/kg, was converted to 0.5 mg/kg bw/day based on the assumption that rats consume 5% of their body weight of food each day.

## 3 Results

### 3.1 Zebrafish thyroid development within selected exposure period

A limit of exposure was set at 120 hpf (the end of the embryonic phase), which is the regulatory limit to *in vitro* experimentation as defined by Directive 86/609/EEC (European Council, 1986). To place the exposure regimen into perspective, an analysis of zebrafish, rat and human developmental staging was performed (Fig. 1a). This comparison was based on O'Rahilly and Müller's developmental stages of human embryos, Witschi's development of the rat, and Kimmel's stages of embryonic development of the zebrafish (Kimmel et al., 1995; O'Rahilly and Müller, 1987; Witschi, 1962). Carnegie stage 24 is the last stage of embryonic development and occurs at 33 days for humans, the equivalent of 16 days for rat and 72 h for zebrafish. Additional stages are added as indicated in the figure. The results obtained reveal many parallels between the different species. Thyroid hormone production starts after 71 days in man, 20 days in rat and 2.5 days (~ 60 hpf) in zebrafish (Bohsack et al., 2011; Contempré et al., 1993; Strum et al., 1971).

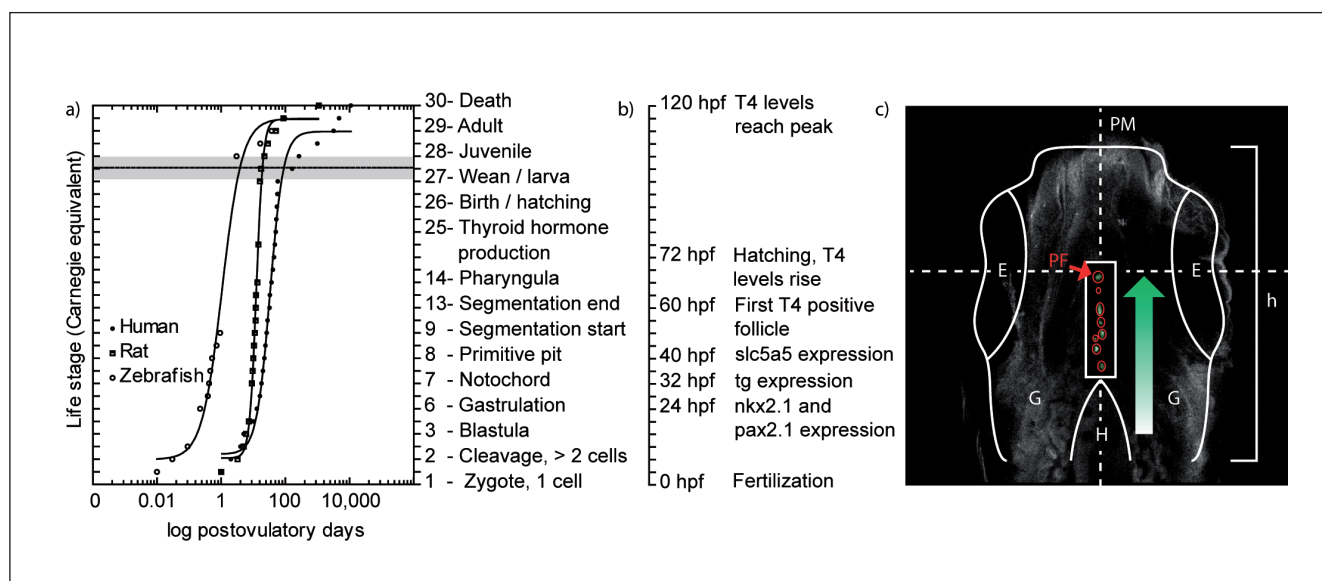
Figure 1b provides a schematic overview of landmarks associated with the development of the thyroid and the production of T4 in zebrafish from 0 until 120 hpf. Briefly, the zebrafish thyroid gland develops from endodermal cells through a process that is in large part evolutionarily conserved (Wendl et al., 2002). The two critical transcription factors *nkx2.1* and *pax2.1* (functionally similar to human *pax 8*) are already expressed at 24 hpf (Antonica et al., 2012; Wendl et al., 2002). A fate mapping study showed that the thyroid precursor cells are located near cardiac lateral plate mesoderm (Wendl et al., 2007). Thyroglobulin gene expression starts around 32 hpf and the expression of the sodium iodide symporter (*slc5a5*) at 40 hpf. A first follicle is positive for the thyroglobulin protein by 55 hpf and for T4 by 60 hpf and expands anteriorly away from the heart, leaving a trail of thyroid follicles along the pharyngeal midline (Alt et al., 2006; Wendl et al., 2007). By 120 hpf, 6–10 thyroid follicles span along this axis from the gill region until they reach an axis that intersects the middle of the eyes. This is a time of peak T4 production, with the rise starting at around the time of hatching (72 hpf) (Chang et al., 2012). Live imaging using a transgenic zebrafish line expressing fluorescent thyroglobulin has recently provided further insight into the development of the thyroid, suggesting a role for the hypobranchial artery in guiding late thyroid expansion (Opitz et al., 2012). Figure 1c provides a schematic overview of thyroid tissue development with T4 immunohistochemistry (IHC) at 120 hpf providing a visual reference. The first follicle to be formed (primary follicle)

cle) is formed near the heart during early development and expands anteriorly along the pharyngeal midline until it reaches the mid-region of the eyes, leaving behind a trail of follicular cells that later form additional follicles.

Based on this analysis, two time windows of exposure, namely 48 hpf to 120 hpf and 0 to 120 hpf were chosen, with the prior avoiding the potential effect of compounds on morphogenesis of the first thyroid follicle and the second one including such effects. These thyroid-specific time windows were further compared with the exposure time window of 0–72 hpf, which was used by Hermesen et al. (2011) in order to assess the GMS of developmental toxicants. Exposures were conducted with 12 replicates per compound concentration for all time windows in one experiment. In addition, for the 0–120 hpf time window, an additional experiment with 8 replicates was performed resulting in a total of 20 replicates per compound concentration for the 0–120 hpf time window.

### 3.2 Morphological effects of thyroid-active model compounds

Figure 2 shows an overview of the altered morphologies of the zebrafish exposed from 0 to 120 hpf to the highest tested concentrations of the test substances. The highest tested concentration of T3 (500  $\mu$ M) affected zebrafish embryos dramatically with an observed reduction in melanophore density and size, abnormal iridophores, deflated swim bladder, accumulation of biliverdin around the liver, and an indication of



**Fig.1: Selection of exposure period relevant to thyroid disruption**

a) Human, rat and zebrafish developmental stages (Carnegie equivalent) plotted against time in postovulatory days. Carnegie stage 24 is the last stage of embryonic development and occurs at 33 days for humans, the equivalent of 16 days for rat and 72 h for zebrafish. b) Developmental landmarks of the thyroid hormone system in zebrafish from 0 to 120 hpf, including the expression of the differentiation markers *nkx2.1* and *pax2.1*, expression of thyroglobulin (*tg*), the sodium iodide symporter (*slc5a5*), the first follicle that can be detected by T4 IHC, as well as the rise and peak of T4 levels. c) Schematic representation of thyroid follicle development. PF stands for primary follicle (additional follicles are circled in red); H for heart; PM for pharyngeal midline (represented by a dotted line); E for eyes; G for gills; h for head region. The green arrow indicates the direction of PF development from 0 to 120 hpf. In the background, T4 IHC of a 120 hpf zebrafish provides a visual reference.



an adrenergic response – mainly tachycardia, which is a well documented effect of T3 – as well as dyskinesia observed as a lack of swimming behavior. In terms of teratogenicity, an observation was made for kyphosis (hunchback), however since the teratogenicity parameters used only included scoliosis (oblique bending), it was scored as such. Most effects on morphology could already be seen at 50 nM T3. The effects were similar in the 48-120 hpf exposure window while for the 0-72 hpf time window only abnormal pigmentation, kyphosis and a mean percentage failed or delayed hatching of  $41.7 \pm 1.2\%$  (mean  $\pm$  SEM) could be observed. Intestinal convolution could be clearly observed with histological slices (Fig. 2b) but could not be clearly viewed under a stereo microscope and hence was not included as a parameter in the scoring system. Lower jaw protrusion was also clearly visible, especially with Alcian blue staining (Fig. 2c).

At doses of 250  $\mu$ M MMI and higher, failure to hatch was observed for the exposure periods 0-72 hpf and 0-120 hpf. For the exposure period from 48-120 hpf, only the highest tested concentration of 2 mM led to the failed hatching of  $16.7 \pm 0.9\%$  of the zebrafish embryos, compared to  $100.0 \pm 0.0\%$  failed hatching at the highest tested concentration for 0-72 hpf and  $84.2 \pm 0.4\%$  for 0-120 hpf. Unhatched embryos had deflated swim bladders, unresorbed yolk sacs and, as can be expected from unhatched embryos, a lack of swimming behavior.

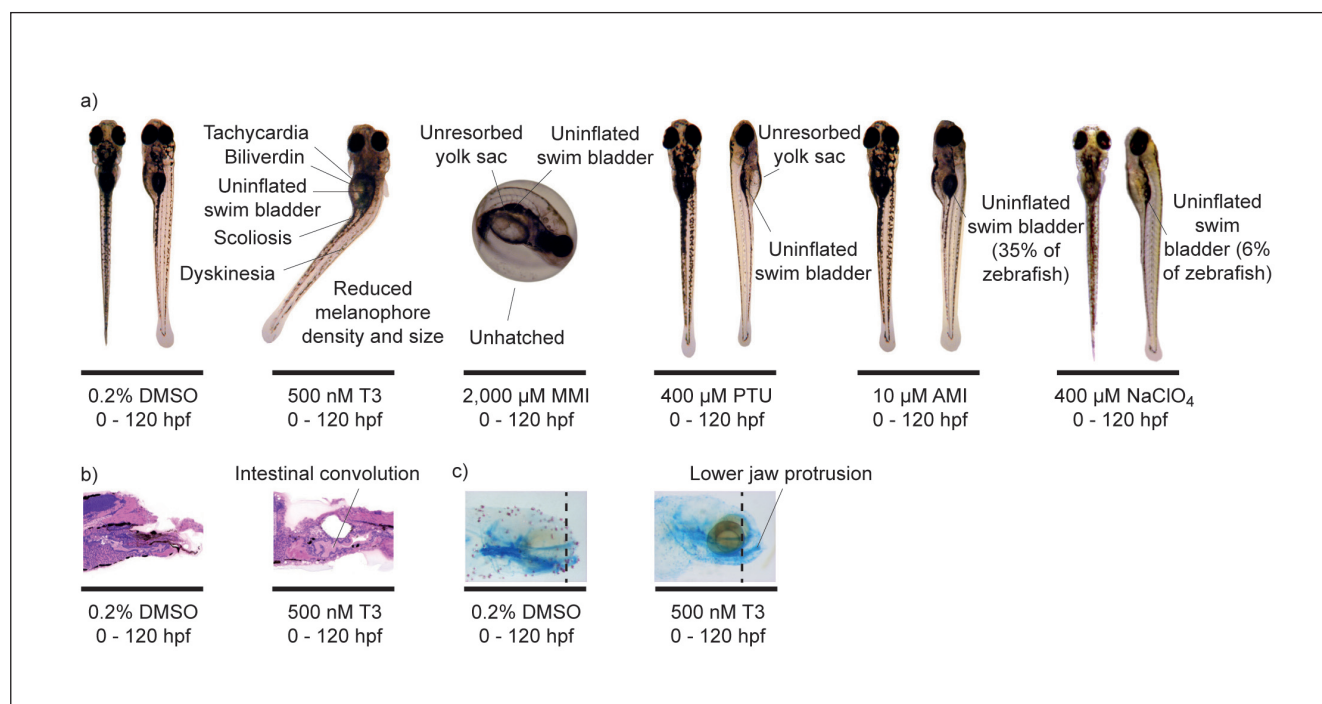
PTU also led to failed hatching at the highest tested concentration of 400  $\mu$ M at a rate of  $27.3 \pm 1.2\%$  for exposures from

0-72 hpf,  $11.1 \pm 0.5\%$  from 0-120 hpf while no failed hatching was observed for the exposure period from 48-120 hpf.

Upon exposure to NaClO<sub>4</sub> from 0-120 hpf, deflated swim bladders were observed starting at 25  $\mu$ M up to the highest tested concentration of 400  $\mu$ M in an average of  $12.0 \pm 0.4\%$  of embryos, while exposure to AMI at the highest tested concentration of 10  $\mu$ M led to deflated swim bladders in  $35.3 \pm 0.7\%$  of embryos exposed during the same time period. No significant effects could be observed during exposures to either compound using the other time windows tested (0-72 hpf or 48-120 hpf) suggesting exposure duration to influence the sensitivity of the assay and 0-120 hpf to be the preferred time window for exposure.

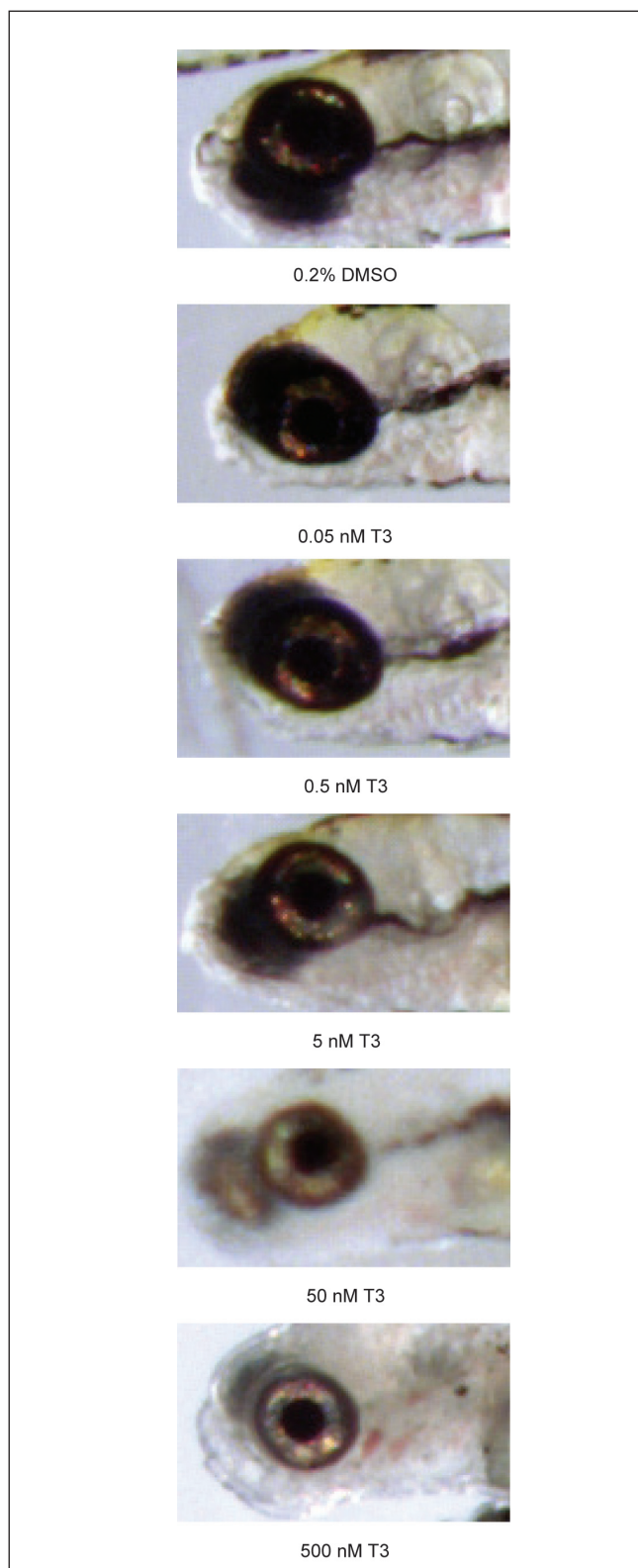
### 3.3 Adjusted morphological scoring: general development score

The GMS scoring system was developed to look at the effect of chemicals on zebrafish development at 72 hpf (Hermesen et al., 2011). Based on the results obtained here with the selected thyroid modulating compounds, four main adjustments were made to the GMS scoring system. First, 120 hpf-specific developmental endpoints affected by thyroid-active compounds were added to the GMS. These endpoints included reflective iridophores (Fig. 3), beat and glide swimming, inflation of swim bladder and resorption of yolk sac (Fig. 2). Since these endpoints are not included in the GMS for 0-72 hpf, they were incorporated in a new scoring system denoted general devel-



**Fig. 2: Changes in morphology following exposure to thyroid-active compounds**

a) Zebrafish morphology upon exposure to solvent control (0.2% DMSO), 500 nM T3, 2,000  $\mu$ M MMI, 400  $\mu$ M PTU, 10  $\mu$ M AMI and 400  $\mu$ M ClO<sub>4</sub>. b) Sagittal section of zebrafish exposed to 500 nM T3 stained with hematoxylin and eosin (H&E) reveals a high degree of intestinal convolution that is absent in the solvent control. c) Alcian blue staining of the cartilage revealing the anterior-dorsal protrusion of the lower jaw following exposure to 500 nM T3 from 0-120 hpf.



**Fig. 3: Dose-dependent increase in zebrafish iridophore pigmentation following exposure to T3**

Lateral view of 120 hpf zebrafish showing iridophore pigmentation (reflective pigments) increasing dose-dependently upon exposure to T3.

opment score (GDS). Secondly, a refinement was made in the newly defined GDS by combining both morphology and teratogenicity endpoints in a similar way to the total morphological score (TMS) applied in the method of Piersma et al. (2004) for the rat whole embryo culture (WEC) which combines teratogenicity and developmental toxicity (Fig. 4). Thirdly, a refined scoring approach was also implemented similar to that used by Teixidó et al. (2013) whereby developmental abnormalities for an endpoint are treated as a lack of development and therefore result in a score of 0 for that developmental endpoint compared to a score of 1 per event for normal development. Fourthly, the terms used for developmental abnormalities are compliant with major databases with the use of the Entity Quality Ontology (EQO) convention suggested by the Open Biomedical Ontologies (OBO) Consortium, which involves describing an entity (e.g. tail vasculature) and a quality (e.g., edema) (Balhoff et al., 2011; Dahdul et al., 2010). This leaves room for the standardized annotation of additional abnormalities based on anatomical location or feature (such as movement). Database compliance also enables computable phenotypes as well as the development of iterative models that improve the accuracy of future chemoinformatic predictions. Examples of commonly observed abnormalities are included in Figure 4 for reference purposes. Minor adjustments to the highlighted abnormalities include the addition of visible biliverdin or bilirubin as well as the changing of the term scoliosis to spinal curvature in order to encompass all forms of a bent spine, which include kyphosis and lordosis in addition to scoliosis (Fig. 4).












### 3.4 GMS compared to GDS

The GMS for the highest concentration of T3 (500  $\mu\text{M}$ ) decreased to  $12.9 \pm 0.7$  ( $n=12$ ) for the exposure period from 0-72 hpf,  $11.3 \pm 0.5$  ( $n=12$ ) from 48-120 hpf and  $11.1 \pm 2.6$  from 0-120 hpf ( $n=20$ ), as compared to a value of  $15.0 \pm 0.0$  for the unexposed control. The GDS for the highest concentration of T3 was  $12.2 \pm 3.7$  ( $n=20$ ) from 0-120 hpf as compared to  $18.8 \pm 0.7$  for the control. For better comparison, normal growth was set as a common denominator with a baseline value of 1, and the fold change relative to the controls was also calculated. The fold change relative to the controls for the two highest concentrations of T3 (1-120 hpf,  $n=20$ ) went from 0.9 and 0.7 in the GMS to 0.8 and 0.6 in the GDS, respectively, thereby increasing the statistical significance from  $p \leq 0.01$  to  $p \leq 0.0001$ .

The GMS at the highest tested concentration of MMI (2,000  $\mu\text{M}$ ) was  $13.1 \pm 0.3$  as an average of all exposure periods, which represents a fold change of 0.9 relative to the control (GMS =  $15.0 \pm 0.3$ ). The GDS at the highest tested concentration of MMI was  $12.8 \pm 2.7$  (0-120 hpf), which represents a fold change of 0.7 relative to the control (GDS =  $18.4 \pm 1.6$ ;  $p \leq 0.0001$ ). Moreover, statistical significance could be reached at the lower concentration of 1,000  $\mu\text{M}$  ( $p \leq 0.05$ ) with the GDS but not the GMS.

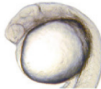

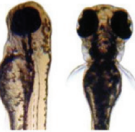



Figure 5 gives an overview of the effects of T3, MMI, PTU, and CIO4 on zebrafish development, in both GMS and GDS, represented as fold change relative to the controls. These tested compounds led to developmental abnormalities at the highest tested concentration and the GDS was better able to reflect these abnormalities than the GMS.

### General Development Score - Part 1 of 3




	Abnormality	12 hpf	24 hpf	48 hpf	72 hpf	120 hpf
Detachment of tail	<u>All tail malformations including:</u> <ul style="list-style-type: none"> <li>tail, malformation</li> <li>tail vasculature, edema</li> <li>vertebral column, curvature (scoliosis, kyphosis, lordosis)</li> <li>neural tube, malformation (rachischisis, anencephaly)</li> <li>notochord, malformation</li> </ul>	 0	 	 	 	 
		 18hpf				
Somite formation	<u>All somite malformations including:</u> <ul style="list-style-type: none"> <li>somite border, incomplete</li> </ul>	0	1	2	3	3
Eye development	<u>All eye malformations including:</u> <ul style="list-style-type: none"> <li>eye, single (cyclopia)</li> <li>eye, absent</li> <li>eye, edema</li> </ul>	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
		 0	 1	 2	 2 + 1 for pigment	 3 + 1 for reflective iridophores
Movement	<u>All movement abnormalities including:</u> <ul style="list-style-type: none"> <li>organismal movement, abnormal (spasms)</li> </ul>	0	No = 0	Yes = 1	Yes = 1	Yes = 1
						Yes = 1 + 1 for beat and glide swimming



## General Development Score - Part 2 of 3

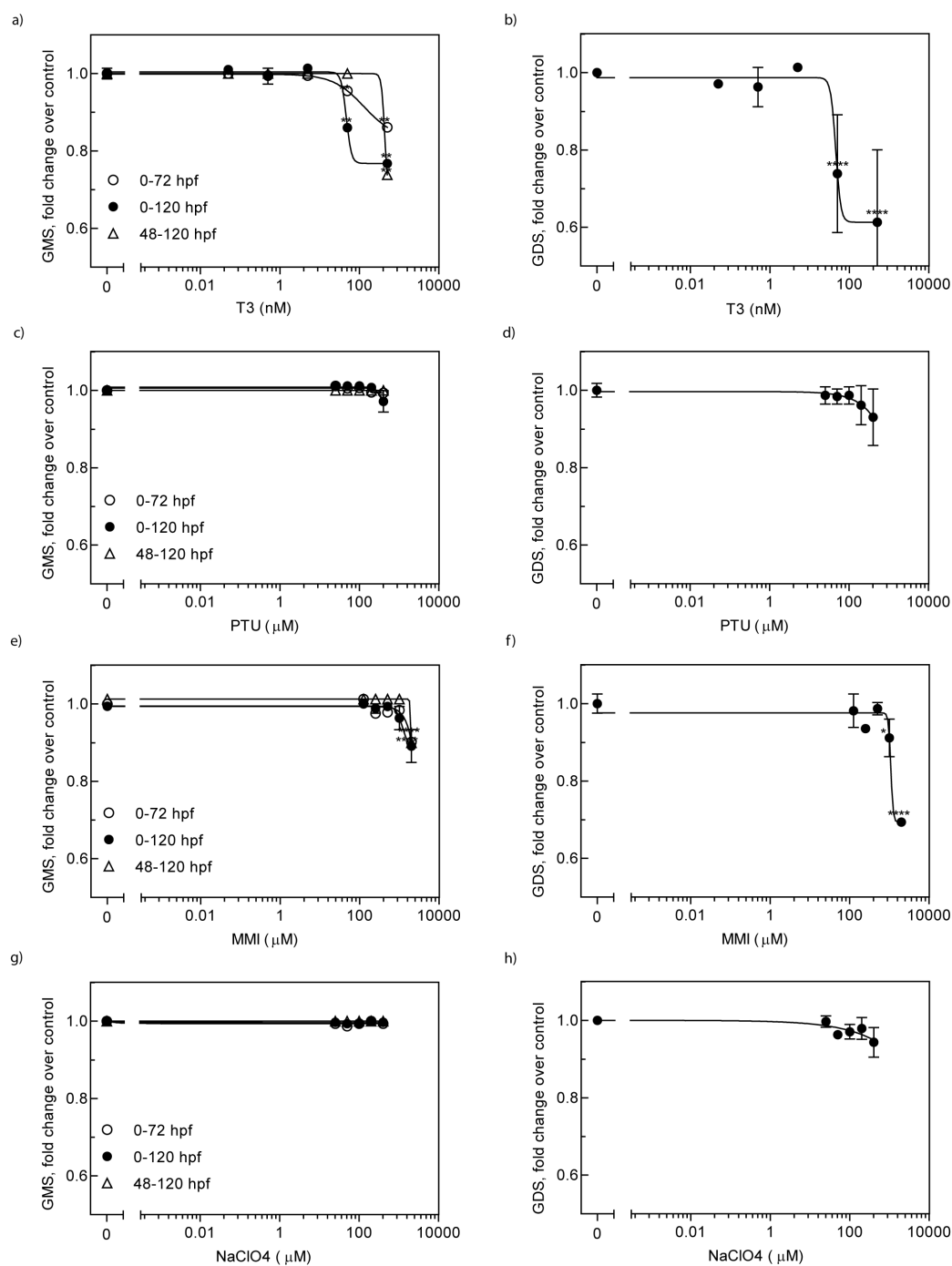
	Abnormality	12 hpf	24 hpf	48 hpf	72 hpf	120 hpf
Heartbeat	<u>All heart malformations including:</u> <input type="checkbox"/> heart, malformation <input type="checkbox"/> pericardial sac, edema 0	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
Blood circulation	<u>All circulation abnormalities including:</u> <input type="checkbox"/> blood coagulation, present <input type="checkbox"/> bilirubin oxidase activity, increased in magnitude 0	No = 0	No = 0	Yes = 1	Yes = 1	Yes = 1
Pigmentation head - body	<u>All pigmentation abnormalities in head body region (except eyes) including:</u> <input type="checkbox"/> pigmentation, irregular spacial pattern <input type="checkbox"/> pigmentation, decreased <input type="checkbox"/> pigmentation, increased 0	0	 0	 1	 1	1
Pigmentation tail	<u>All pigmentation abnormalities in tail including:</u> <input type="checkbox"/> pigmentation, irregular spacial pattern <input type="checkbox"/> pigmentation, decreased <input type="checkbox"/> pigmentation, increased 0	0	 0	 1	 1	1



General Development Score - Part 3 of 3						
	Abnormality	12 hpf	24 hpf	48 hpf	72 hpf	120 hpf
Pectoral fin						
	0	0	0	0	1	1
Head: Protruding mouth	<u>All head malformations (except for eyes) including:</u>  <input type="checkbox"/> head, malformation <input type="checkbox"/> head, edema <input type="checkbox"/> saccule / otolith, malformation <input type="checkbox"/> brain, small volume					
	0	0	0	0	1	1
Hatching						
	0	No = 0	No = 0	No = 0	Yes = 1	Yes = 1
Inflated swim bladder	<u>All swim bladder malformations</u>					
	0	No = 0	No = 0	No = 0	No = 0	Yes = 1
Yolk sac resorbed	<u>All yolk malformations including:</u>  <input type="checkbox"/> yolk, malformation <input type="checkbox"/> yolk extension, malformation					
	0	No = 0	No = 0	No = 0	No = 0	Yes = 1
<b>GDS</b>	<b>0</b>	<b>1</b>	<b>7</b>	<b>12</b>	<b>15</b>	<b>19</b>

**Fig. 4: The GDS system for the scoring of general development**

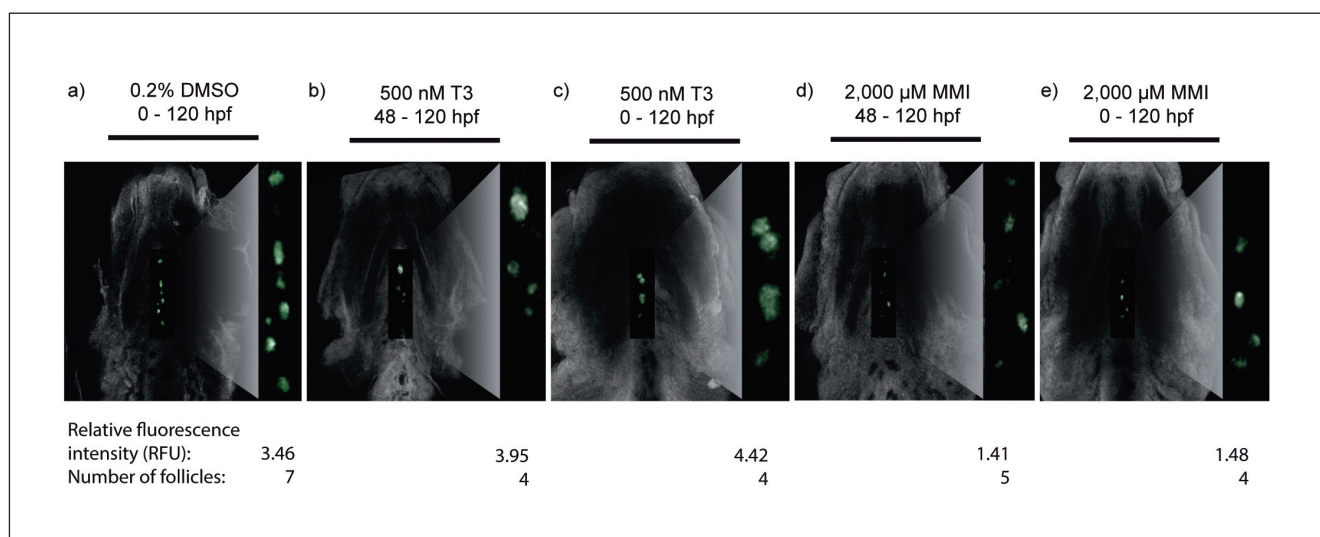
GDS system for the assessment of general developmental toxicity at 120 hpf. The system takes into account morphology, dysmorphology as well as non-morphological parameters such as swimming. Additions made to the GMS scoring system (Hermesen et al., 2011) in order to better accommodate effects of thyroid-active compounds are highlighted with a bold outline.



**Fig. 5: T3, PTU, MMI and NaClO<sub>4</sub>-induced alterations in development assessed by both the GMS and GDS**

Effect of T3 exposure on a) GMS and b) GDS; PTU exposure on c) GMS and d) GDS; MMI exposure on e) GMS and f) GDS; ClO<sub>4</sub> exposure on g) GMS and h) GDS. Results are based on 12 replicates per concentration (n=12) and since the total scores are different, these are expressed as fold decrease relative to the full score (normal development). A top down approach was used to minimize the number of embryos used and the 0-120 hpf exposure window, which was found to be the most appropriate time window for the thyroid-related parameters studied, was selected for an additional independent experiment at a separate laboratory with 8 replicates per concentration (n=8; a total of n=20 for 0-120 hpf experiments). The error bars are shown for only this time window as the standard error of the mean of these two experiments (N=2). The calculation of statistical significance was done by pooling all replicates from each experiment for each concentration and comparing to the 0.2% DMSO solvent controls in a two-tailed student's t-test.

\*P≤0.05; \*\*P≤0.01; \*\*\*P≤0.001; \*\*\*\*P≤0.0001.



**Fig. 6: Effect of T3 and MMI on T4 immunofluorescence in zebrafish thyroid follicles**

T4 whole mount immunohistochemistry upon exposure of zebrafish to a) 0.2% DMSO as the solvent control from 0-120 hpf; 500 nM T3 from b) 48-120 hpf and c) 0-120 hpf; and 2,000  $\mu$ M MMI from d) 48-120 hpf and e) 0-120 hpf. Thyroid follicles were tested for the signal intensity of T4 immunofluorescence and expressed as relative fluorescence units (RFU). The number of follicles was counted based on the images taken with the confocal laser scanning microscope (the region of interest is enlarged and presented at the right side of each panel). T3 exposure at the highest tested concentration and both time points resulted in a decrease in the number of follicles. MMI exposure at the highest concentration resulted in both lower T4 signal intensity and lower number of follicles.

A comparison of the exposure time windows of 0-72 hpf and 0-120 hpf, reveals that the most significant results were achieved with exposures from 0-120 hpf (Fig. 5). The time window of 48-120 hpf did not add any additional information or significance compared to the other time windows. In the GDS, T3 and MMI exposure resulted in a no observed adverse effect level (NOAEL) of 5 nM and 500  $\mu$ M, respectively. The GDS for PTU, amiodarone and ClO<sub>4</sub> did not change significantly and therefore a NOAEL could not be established for these compounds.

### 3.5 T4 immunohistochemistry

The model compounds tested were selected based on their known ability to alter thyroid homeostasis and only those that significantly affected the GDS score, namely T3 and MMI, were further tested for thyroid status by T4 immunohistochemistry (IHC) in order to confirm that the developmental toxicity detected by the GDS for these compounds was accompanied by an effect on the thyroid system (Fig. 6). Under control conditions, the thyroid follicles are well spaced out anteriorly by 120 hpf. In zebrafish exposed to T3 both number of follicles and their spread were affected compared to the control (Fig. 6a), indicating potential thyroid dysmorphogenesis, while MMI exposure led to a clear drop in signal intensity and in the number of follicles compared to the control (Fig. 6b,c), confirming a drop in follicular T4 levels.

## 4 Discussion

Critical to the proper development of the vertebrate embryo, thyroid homeostasis is prone to disruption by man-made chemi-

cals with an ability to act through very diverse modes of action and within multiple organs (Hartoft-Nielsen et al., 2011; Jomaa, 2014; Tan et al., 2007). Given that alterations in the thyroid system can have severe effects on development (Boyages and Halpern, 1993; Haddow et al., 1999; Utiger, 1999), the aim of the present study was to investigate whether a zebrafish-based developmental toxicity assay would be able to detect thyroid hormone-active compounds.

The pathways involved in human thyroid development are conserved in the zebrafish and so are its tissue architecture, function and feedback regulation by the HPT axis, making it a promising model for the study of thyroid-active compounds (Bourque and Houvras, 2011; Jomaa et al., 2013; Raldúa and Babin, 2009).

T3, acting through its nuclear receptor, is a morphogenic agent in certain animal species, controlling the metamorphosis of tadpoles and frogs (Shi et al., 1996). In the present study, T3 exposed zebrafish clearly showed significant developmental toxicity evident as scoliosis, abnormal iridophores, reduced melanophore density and size as well as a lack of an inflated swim bladder (Fig. 4). Moreover, T3 exposed zebrafish showed signs of acute toxicity such as excess biliverdin that is visible under a stereo microscope, tachycardia and dyskinesia. The latter two are symptomatic of an adrenergic response and are likely a result of T3 increasing beta-adrenergic receptor density (Mooradian and Scarpace, 1993). A high rate of 42% failed hatching occurred only at the 0-72 hpf exposure window. Increased sensitivity for hatching failure is expected at 72 hpf since that is around the time hatching occurs. However, as can be seen in Figure 5, overall sensitivity to thyroid-active com-



pounds is higher in the GDS at 0-120 hpf as it includes additional parameters that are specific to that time window.

PTU and MMI have a direct effect on thyroglobulin iodination by thyroid peroxidase, thereby inhibiting thyroid hormone synthesis (Taurog, 1976). These compounds are used medically to treat hyperthyroidism. A decrease in the circulating T3 levels can affect various developmental endpoints including intestines, brain and reproductive capacity. Raldúa and Babin (2009) reported a complete lack of T4 immunoreactivity beyond 0.75 mM and 0.18 mM in zebrafish for MMI and PTU, respectively. This reduction in thyroid hormone levels was accompanied by a reduction in the GDS (Fig. 5). Moreover, the hatching rate was strikingly low in the MMI-treated group and this was deemed significant, not only statistically, but also physiologically. This result is in line with the observation that chicken embryos have been reported to have delayed hatching upon exposure to MMI (Haba et al., 2011). Interestingly, another compound shown to affect zebrafish hatching rate is perfluorooctanoic acid (PFOA), an established thyroid-active compound (Lopez-Espinosa et al., 2012; Melzer et al., 2010; Zheng et al., 2011). Hence, the change in zebrafish hatching rate that was induced by thyroid active compounds was accurately reflected in the GDS developmental toxicity assay (Fig. 5). Moreover, MMI exposure resulted in a NOAEL of 500  $\mu$ M in the GDS, which is several orders of magnitude higher than the T3 NOAEL of 5 nM, and this relative potency compares well with the NOAEL based on changes in thyroid stimulating hormone (TSH) levels derived from rodent studies (a NOAEL of 0.5 mg/kg bw/day for MMI and a NOAEL of less than 10  $\mu$ g/kg bw/day for T3) (Grover et al., 2007; Hood et al., 1999).

NaClO<sub>4</sub> inhibits sodium iodide symporter (NIS) transport of iodide into the thyroid follicular cells, thereby resulting in a decline in thyroid hormones and a rise in thyrotropin (Stoker et al., 2006). The morphological changes observed in the developing zebrafish embryo included uninflated swim bladder and incomplete resorption of the yolk sac. While these were consistent observations that could only be made by including 120 hpf-specific parameters, they did not have enough weight within either the GMS or GDS system to significantly change the overall score. This corroborates *in vivo* data on altered thyroid hormone levels in rodent studies showing that NaClO<sub>4</sub> is less potent than either PTU or MMI (Hood et al., 1999; Stoker et al., 2006; Yamasaki et al., 2002). Moreover, a human epidemiological study failed to find an association between perchlorate levels in drinking water and congenital defects of the thyroid (Crump et al., 2000). In another study with volunteers given perchlorate in drinking water the administered dose was negatively correlated with thyroidal iodide uptake but even a 70% decrease in iodide transport did not result in altered T4 levels (Greer et al., 2002; Strawson et al., 2004). The relatively low developmental toxicity of perchlorate is supported by animal studies showing that pregnant rats and rabbits exposed to a perchlorate dose as high as 30 mg/kg bw/day do not give birth to offspring with signs of developmental toxicity and changes in thyroid hormone levels were seen as adaptive and reversible (York et al., 2003, 2005).

Amiodarone is an anti-arrhythmic agent (heart), beta blocker

and calcium blocker. Its chemical structure resembles that of T4 and the compound alters thyroid hormone levels *in vivo* (de Sandro et al., 1991), likely due to its high iodide content (Pitsiavas, 1999). In zebrafish, amiodarone was reported to lead to a significant reduction in T4 immunoreactivity at 1  $\mu$ M (Raldúa and Babin, 2009). However, while morphological changes were observed in the present study upon exposure to amiodarone, such as the lack of an inflated swim bladder in some animals, which is included in the GDS, statistical significance could not be reached in both the GMS and GDS (data not shown). Indeed, human studies on amiodarone treated mothers indicate that transient hypothyroidism in the newborn may occur but there is no adverse effect on development (Matsumura et al., 1992; Valensise et al., 1992).

The thyroid-active compound associated phenotypes observed in 120 hpf zebrafish include 1) hatching (discussed in the previous section), 2) yolk sac resorption, 3) iridiophore pigmentation, 4) melanophore pigmentation, 5) swim bladder inflation, 6) bent spines, 7) beat and glide swimming, 8) lower jaw cartilage protrusion and 9) maturation of the gastrointestinal system, with the last two being better suited for staining techniques or high resolution microscopy for accurate assessment. These developments occur concurrently with a surge in thyroid hormone levels, which reach a peak at 120 hpf (Chang et al., 2012). The overall results have indicated that the time window of 0-120 hpf is better able to reflect the effect of thyroid-active compounds on zebrafish development than the other time windows studied (Fig. 5) and support the suggestion by Liu and Chan that there is a distinct thyroid hormone-mediated embryonic to larval transition in zebrafish that is analogous to the thyroid hormone-mediated larval to juvenile transition originally proposed by Brown (Brown, 1997; Liu and Chan, 2002).

The period from the time the zebrafish embryo has hatched (72 hpf) until the time it is free swimming (120 hpf) is termed the yolk-sac larva (Balon, 1990). The resorption of the yolk sac and hence the maternal food supply is only evident at around 120 hpf, with total resorption evident by around 168 hpf (Jardine and Litvak, 2003; Kimmel et al., 1995). The role of thyroid hormones in this developmental process has been previously documented whereby MMI co-administered with AMI resulted in the retardation of yolk sac resorption and this was rescued with T4 (Liu and Chan, 2002).

Overall, the results obtained in this paper show the importance of the 0-120 hpf time window of zebrafish exposure in the assessment of the developmental toxicity of thyroid-active compounds. The newly developed GDS scoring system takes into account parameters that occur after 72 hpf. The defined GDS can successfully detect zebrafish developmental toxicity induced by thyroid-active compounds and can benefit from the model organism's potential for high-throughput, which, for screening purposes, is a key advantage over rodent assays. The parameters affected, which include reflective iridophores, beat and glide swimming, inflated swim bladders, and resorbed yolk sacs, are shown to be sensitive towards toxicity induced by TH disrupting compounds. One could envisage that other compounds causing developmental toxicity by different modes of



action may also affect these parameters. Since this opens the way to false positives if the assay were to be used in isolation to detect TH activity, we envision that the GDS could be part of an ITS that includes specific biomarkers of thyroid hormone disruptor activity. Although the GDS will detect developmental toxicity resulting from various modes of action, it is shown in the present study that the GDS can be used to flag compounds as potential thyroid disruptors. Due to the presence of an intact thyroid hormone feedback loop, the zebrafish represents an experimental *in vitro* platform that is vastly more comprehensive than individual cell lines. Moreover, the zebrafish embryo up to 120 hpf is more socially-acceptable as a test organism than higher vertebrates. When applied in combination with other *in vitro* tests within an ITS, the zebrafish GDS holds promise to reduce, refine and, in certain cases, replace animal testing.

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### Acknowledgements

This project was financially supported by The Netherlands Genomics Initiative (Netherlands Toxicogenomics Centre, grant number 6162500134).

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