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Thyroid endocrine disruption and external body morphology of Zebrafish

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ABSTRACT

This study examined the effects thyroid-active compounds during early development on body morphology of Zebrafish (*Danio rerio*). Three-day postfertilization (dpf) larvae were exposed to goitrogen [methimazole (MZ, 0.15 mM)], combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), T4 (2 nM), or control (reconstituted water) treatments until 33 dpf and subsequently maintained in reconstituted water until 45 dpf. Samples were taken at 33 and 45 dpf for multivariate analysis of geometric distances between selected homologous landmarks placed on digital images of fish, and for histological assessment of thyrocytes. Body mass, standard length, and pectoral fin length were separately measured on remaining fish at 45 dpf. Histological analysis confirmed the hypothyroid effect (increased thyrocyte height) of MZ and rescue effect of T4 co-administration. Geometric distance analysis showed that pectoral and pelvic fins shifted backward along the rostrocaudal axis under hypothyroid conditions at 45 dpf and that T4 co-treatment prevented this shift. Pectoral fin length at 45 dpf was reduced by exposure to MZ and rescued by co-administration of T4, but it was not associated with standard length. Methimazole caused a reduction in body mass and length at 45 dpf that could not be rescued by T4 co-administration, and non-thyroidal effects of MZ on body shape were also recognized at 33 and 45 dpf. Alterations in the length and position of paired fins caused by exposure to thyroid-disrupting chemicals during early development, as shown here for Zebrafish, could affect physical aspects of locomotion and consequently other important organismal functions such as foraging, predator avoidance, and ultimately survival and recruitment into the adult population. Results of this study also suggest the need to include rescue treatments in endocrine disruption studies that rely on goitrogens as reference for thyroid-mediated effects.

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1. Introduction

Animal growth and development are associated with changes in external morphology that serve or influence organismal functions such as locomotion, foraging, predator avoidance, and reproduction. Thyroid hormone (TH) [triiodothyronine (T3) and thyroxine (T4)] plays important roles during this process (Power et al., 2001; Blanton and Specker, 2007; Carr and Patiño, 2011). It is well known, for example, that TH is a major endocrine driver of the remarkable morphological changes observed during metamorphosis of flatfishes (Inui and Miwa, 1985; Schreiber and Specker, 1998) and elopomorphs (Kitajima et al., 1967), as well as the relatively less complex and more gradual changes that occur during the larva-to-juvenile transition of other species (Nacario, 1983; Reddy and Lam, 1992; Brown, 1997; Trijuno et al., 2002). Environmental challenges to TH homeostasis during early development could therefore influence external morphology and consequently also affect organismal performance.

While the presence of thyroid-disrupting chemicals in the environment is of concern to wildlife and human health (Pearce and Braverman, 2009; Carr and Patiño, 2011; Patiño and Carr, 2015), few studies have examined the potential impact to fish body form that early exposure to these chemicals may have. The effects of these chemicals can be particularly difficult to study in species where metamorphic change is relatively small, such as Zebrafish (*Danio rerio*), an important animal model for developmental and endocrine disruption studies. Zebrafish show two periods of early posthatch development when disruption of the thyroid endocrine system can influence the adult form: an embryo-to-larva transition (yolk-sac or eleutheroembryo) phase at 3–5 days postfertilization (dpf), and a larva-to-juvenile transition phase (metamorphosis)

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which, depending on rearing conditions, begins ~14 dpf (Brown, 1997; Parichy and Turner, 2003) and ends ~30 dpf (Schilling, 2002; Parichy and Turner, 2003). Substantial craniofacial development, including the formation and forward protrusion of the lower jaw, occurs during the yolk-sac phase and this process is at least partly regulated or influenced by maternal (Mukhi and Patiño, 2007) and endogenous TH (Liu and Chan, 2002; Bohnsack and Kahana, 2013). During metamorphosis, TH-dependent or influenced events include paired fin development and growth, scale formation, pigmentation (Brown, 1997), and differentiation and growth of a number of skeletal structures (Shkil et al., 2012). To our knowledge, however, an integrated assessment of changes in whole-body morphology caused by disruption of thyroid endocrine condition during metamorphosis has not been conducted for Zebrafish or any other teleost exhibiting a gradual pattern of metamorphic change.

The objective of this study is to determine the effects of thyroid manipulation during posthatch development and metamorphosis on the establishment of body shape in Zebrafish. Thyroid endocrine condition was manipulated by treatment with the goitrogen, methimazole (MZ). A "rescue" treatment consisting of MZ and TH was included in the study design to be able to discriminate between specific and non-specific (non-thyroidal) effects of the goitrogen. The primary tool used to examine body shape was landmark-based geometric morphometrics. This technique involves the collection of geometric distances between pairs of homologous landmarks on 2-dimensional digital images of specimens followed by multivariate analysis of these distances (Strauss and Bond, 1990). Results of this study are expected to contribute towards an understanding of the potential impact of thyroid endocrine disruptors to TH-dependent establishment of body morphology in teleosts.

2. Methodology

2.1. General husbandry

Fertilized eggs were obtained by group-spawning (4 males and 8 females per group) of a breeding colony of adult Zebrafish obtained from Aquatic Research Organisms, Inc. (Hampton, NH, USA). Fish from this source are hatchery-bred and not produced for any specific traits, and thus are likely to be natural or "wild-type" (Wilson et al., 2014). Fertilized eggs and posthatch individuals were kept at 28.5 °C and 12 h:12 h light:dark throughout the study. Groups of 50 fertilized eggs, all derived from a single group-spawning batch, were initially placed in glass Petri dishes with 100 ml of reconstituted water containing 310 mg R/O Right, 294 mg $CaCl_2{\cdot}2H_2O$, and 123.3 mg $MgSO_4{\cdot}7H_2O$ per liter of reverse-osmosis water (Ca and Mg salts, CAS 10035-04-8 and CAS 10034-99-8, Sigma–Aldrich (Saint Louis, MO, USA); R/O Right salt mixture, Aquatic Eco-Systems, Inc. (Apopka, FL, USA). At hatching (~72 h postfertilization, hpf), 40 individuals from each Petri dish were placed in respective 2-l beakers containing 800 ml of the appropriate treatment solution. At 21 dpf, larvae were transferred to respective 20-l aquaria containing 15 l of the appropriate treatment solutions, where they were maintained until the end of the experiment (45 dpf).

Larvae were fed *Paramecium* spp. four times daily (8 am, 11 am, 2 pm and 5 pm) from 5 to 9 dpf; *Paramecium* in the morning (8:00 am) and afternoon (5:30 pm) and Zeigler larval diet at noon from 10 to 11 dpf; and *Artemia* nauplii at 8:00 am and 5:30 pm and Ziegler diet at noon from 12 to 45 dpf. Particle sizes of Zeigler diets fed from 10 to 21 dpf and 22 to 60 dpf were 150–250 and 250–450 μm, respectively. Approximately 80 percent of the rearing water was replaced with appropriate pre-heated solutions daily until 20 dpf, and from 21 to 45 dpf two thirds of the water was replaced every other day. Uneaten food and debris were removed 5 min after each feeding. Temperature was recorded every day until 20 dpf; from 21 to 60 dpf, temperature, pH, specific conductance, dissolved oxygen and ammonia were monitored in all tanks every other day.

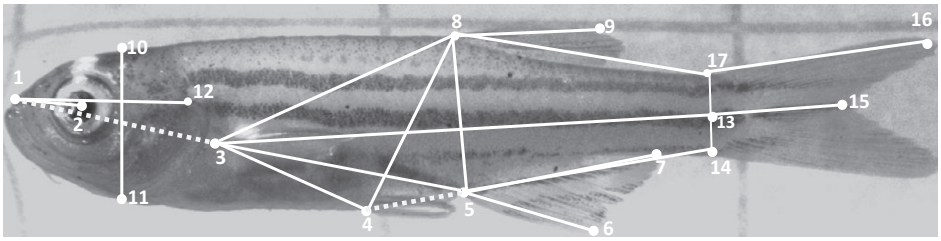
Animal handling and experimental procedures were approved by the Texas Tech University Animal Care and Use Committee (protocol #09028-05C).

2.2. Experimental design

All treatments were prepared in reconstituted water and included a control treatment (reconstituted water), MZ (0.15 mM), rescue treatment consisting of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM). Under this experimental design, a TH-dependent pattern of responses would be one where a morphological change caused by MZ is restored to control or near-control condition by co-treatment with T4.

Thyroxine was initially dissolved in dimethyl sulfoxide (DMSO). To avoid the need for a solvent control, the same volume of DMSO used as carrier solution for T4 was added to all exposure solutions at a final concentration of 0.00025%. The concentration of exogenous T4 used in this study, 2 nM, is within a range of concentrations known to induce hyperthyroid conditions but with limited toxicity to Zebrafish larvae and juveniles (Brown, 1997; Sharma and Patiño, 2013). Although T3 is the physiologically active TH, T4 was used because of its lower toxicity (Brown, 1997). The concentration of MZ used, 0.15 mM, is within a range previously reported to be non-toxic to Zebrafish embryos and larvae (Elsalini and Rohr, 2003) but still effective at inducing hypothyroid responses (Sharma and Patiño, 2013). These concentrations of thyroid-active chemicals are lower than those used in most other studies with Zebrafish. They were deliberately selected to avoid the severe toxicity to Zebrafish larvae typically observed at higher concentrations, a situation which would limit the environmental relevance of this study.

Each treatment consisted of five beakers/tanks with an initial number of 40 individuals per vessel. Exposures started at hatching (~72 hpf) and lasted 30 days, thus covering the entire period of posthatch development through the completion of metamorphosis. Treatment solutions were replaced with reconstituted water at 33 dpf. Samples for analysis were collected at 33 dpf, when the treatment ended, and at 45 dpf, which is generally considered the beginning of the juvenile phase of development (Singleman and Holtzman, 2014). Two fish from each tank (10 per treatment) were sampled at each time for measurements of geometric distances (see next section) and assessments of thyroid histopathology. At sampling, fish were euthanized in MS-222 (tricaine methane sulfonate, 1 g/L) until all movement ceased and placed in 10% buffered formalin. Samples were transferred to 70% ethanol and then water, and images of each individual (Fig. 1) were taken with an Olympus stereoscope (Olympus SZX2-ILLK; Tokyo, Japan) using Image J v. 1.46 image analysis (Rasband, 1997–2012). Samples were subsequently post-fixed in Bouin's fixative (Ricca Chemical Company®, Arlington, TX, USA) for 48 h at 4 °C, washed overnight with running tap water and stored in 70% ethanol until processed for thyroid histological assessments (Section 2.4). Fish remaining at 45 dpf were euthanized and their body mass measured using an electronic balance, and then preserved as already described. These samples were used to "manually" measure (as opposed to digital measurements, see Section 2.3) pectoral fin length and standard length with a stereoscope using an ocular grid calibrated by a stage micrometer. Fish collected at 45 dpf were also processed and used for histological assessment of gonadal sex.



1-2: Eye position
1-3: Snout-to-pectoral fin distance
3-4: Pectoral-to-pelvic fin distance
3-8: Pectoral-to-dorsal fin distance
4-5: Pelvic-to-anal fin distance
5-6: Anal fin length
5-8: Anal-to-dorsal fin distance
8-9: Dorsal fin length
14-17: Caudal peduncle depth
16-17: Caudal fin length
1-12: Head length
10-11: Head depth
3-5: Pectoral-to-anal fin distance
3-13: Pectoral-to-caudal fin distance
4-8: Pelvic-to-dorsal fin distance
5-7: Anal fin base
5-14: Anal-to-caudal fin distance
8-17: Dorsal-to-caudal fin distance
13-15: Caudal peduncle-to-fork distance

Fig. 1. Homologous landmarks and distances used for morphometric analysis of Zebrafish. At 33 days postfertilization, all distances were used except ACD, DCD, CPD, CFD and CFL (could not be accurately determined at this age). Distances were calibrated to a known scale and corrected for individual differences in standard length (landmarks 1–13) by using residuals from regression analysis. Thyroid hormone-dependent distances identified by this study are marked by dashed lines. The age of the individual shown in this picture is 45 days postfertilization.

2.3. Geometric distances

Landmarks were placed on digital images (Fig. 1) using tpsDIG2 v. 2.16 and distances between selected landmark pairs were obtained with tpsUtil v. 1.40 (Rohlf et al., 2005). Landmarks used in this study included those related to the head and fins because these morphological features have been previously found to be influenced by TH (Brown, 1997; Liu and Chan, 2002; Mukhi and Patiño, 2007). Some caudal fin landmarks could not be clearly identified at 33 dpf, and others necessary to assess certain TH-dependent features such as pectoral and pelvic fin length (Brown, 1997) and lower jaw length/morphometry (Liu and Chan, 2002; Mukhi and Patiño, 2007) could not be accurately localized at 33 or 45 dpf. Thus, implementation of a full truss network of landmarks (Strauss and Bond, 1990) was not possible.

2.4. Histology

Sample processing and data collection methods for assessment of thyroid follicle condition were as described by Mukhi et al. (2005). Briefly, serial paraffin sections (6 μm) were prepared longitudinally starting from the ventral side of the trunk and stained with hematoxylin and eosin. Digital images of thyroid follicles were taken with an Olympus digital camera (DP70; Tokyo, Japan) attached to a compound microscope. Thyroid epithelial cell (thyrocyte) height was measured on the images as described by Mukhi et al. (2005) using Image-Pro Express (Media Cybernetics, Silver Spring, MD, USA). Phenotypic sex of individuals collected at 45 dpf was recorded by observation of gonadal tissue as described by Sharma and Patiño (2013). Gonadal sex differentiation is still incomplete at 33 dpf and gender determination by histological examination can yield unreliable results (see Sharma and Patiño, 2013).

2.5. Statistical analysis

Percent survival in each vessel was determined for each sampling period (72 hpf–33 dpf and 34–45 dpf) and data were converted to the arcsine of their square root to meet parametric assumptions. Differences in survival among treatments were analyzed with 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (MCT) (GraphPad Prism, Version 5; GraphPad Software, CA, USA).

Morphometric data manually collected at 45 dpf and thyroid histological measurements at 33 and 45 dpf met parametric assumptions and were not transformed. Pearson's correlation analysis was used to examine the association of pectoral fin length to fish size (SAS, SAS/STAT® 9.2, SAS Institute Inc. Cary, NC, USA). Factorial ANOVAs (sex *x* treatment) were initially applied to pectoral fin length, standard length, body mass, and thyrocyte height to establish if differences exist for these variables between males and females. Male and female data were further analyzed separately if sex or sex-treatment interactions were significant. Differences among treatments were assessed using 1-way nested ANOVA (tanks nested into treatments) followed by Tukey's MCT. When appropriate, differences between sexes within each treatment group were analyzed by linear contrasts of Least Squares means.

Geometric distances were subjected to discriminant function analysis (Strauss and Bond, 1990). This technique is commonly used to discriminate or classify samples into predetermined groups (e.g., treatment groups) based on the simultaneous evaluation of multiple variables (e.g., geometric distances). Geometric distances were $\log_{10}$-transformed prior to analysis because not all distances met criteria for parametric tests. Pearson's correlation analyses between all distances and standard length (geometrically determined) were conducted to examine their association with fish size (GraphPad Prism). If associations were present, distances were regressed against standard length (GraphPad Prism) and residuals were used in the analyses instead of the original measurements. The use of residuals eliminates effects of fish size while avoiding assumptions of isometric relationships (Strauss and Bond, 1990). There are limitations to the number of variables in relation to sample size that can be used in multivariate analysis (McGarigal et al., 2000), and a stepwise-forward approach to the analysis was used to reduce the number of variables (SAS, PROC STEPDISC, PROC DISCRIM). Structure coefficients were used to evaluate the contribution of each variable to significant functions in the resulting model. Only those variables with coefficients > |0.4| are interpreted. Biplots of significant functions were used to graphically examine TH-dependent patterns in data distribution.

3. Results

3.1. Fish survival and thyroid histological condition

Overall survival from 72 hpf to 33 dpf ranged from 60% to 75% (Fig. 2). Survival of larvae/juveniles in the T4 (60%) and rescue (65%) treatments was significantly lower than in the control group (75%) (1-way ANOVA and Tukey's MCT, $F_{(3,16)} = 16.58$, $P < 0.0001$). Most mortality was observed between 10 and 20 dpf regardless of treatment, and no mortality occurred after 21 dpf in any treatment (Fig. 2).

Thyrocyte height did not differ between males and females at 45 dpf ($P > 0.05$). One-way nested ANOVA on pooled data yielded significant models at 33 ($F_{19,39} = 14.43$, $P < 0.0001$) and 45 dpf ($F_{19,39} = 15.1$, $P < 0.0001$), and no tank effects were observed ($P > 0.05$). Mean thyrocyte height was significantly increased in fish from the MZ treatment at 33 and 45 dpf relative to fish in all other groups (Tukey's MCT, $P < 0.05$; Figs. 3 and 4). The rescue treatment (MZ + T4) effectively maintained thyrocyte height at levels similar to those of control fish, and treatment with T4 alone did not influence thyrocyte height. Clusters of hypertrophic, small follicles were observed in some of the fish treated with MZ (Fig. 3) but not in fish from the other treatments. Although these observations confirm the hypothyroid effect of MZ in the present study, basic follicular structure generally remained intact and colloid was still present suggesting that the induction of hypothyroidism was moderate, in an experimental context [for comparison with a higher degree of goitrogen-induced hypothyroidism at the level follicle histology, see Patiño et al. (2003) and Mukhi and Patiño (2007)].

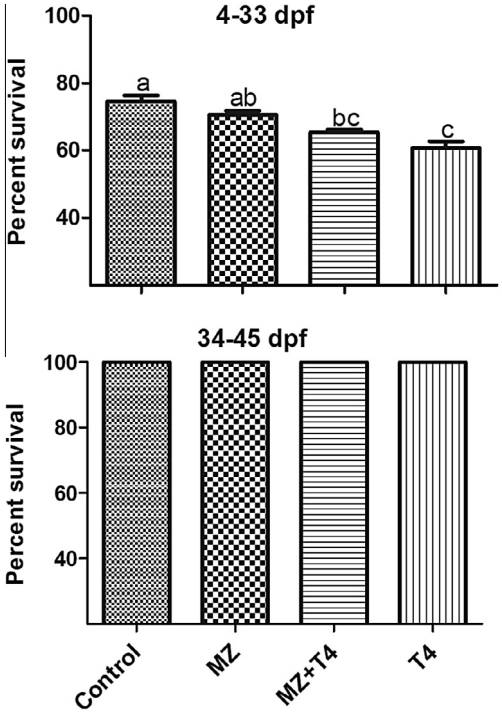


Fig. 2. Effect of methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM) on percent survival (mean + SEM) of Zebrafish at 33 and 45 days postfertilization (dpf). Control fish were raised in reconstituted water. The exposure period began at ~72 h postfertilization and ended at 33 dpf. Bars associated with common letters are not significantly different (1-ANOVA and Turkey's MCT, $P < 0.05$). No mortality occurred in any of the treatments between 34 and 45 dpf.

3.2. Pectoral fin length, standard length and body mass at 45 dpf

Pectoral fin length at 45 dpf was not associated with standard length (Pearson's $r = 0.11$, $P > 0.05$). Pectoral fin length ($P < 0.05$), but not body mass or standard length ($P > 0.05$), differed between males and females. Thus, follow up analyses were conducted separately for males and females only for pectoral fin length.

One-way nested ANOVA yielded significant models for body mass ($F_{19,281} = 5.74$, $P < 0.0001$) and standard length ($F_{19,287} = 3.4$, $P < 0.0001$), and no tank effects were observed ($P > 0.05$). Mean values for both variables were significantly reduced in fish from the MZ and rescue treatments compared to fish from the control and T4 groups (Tukey's MCT, $P < 0.05$; Fig. 5), suggesting that the inhibitory effect of MZ on body mass and length occurred via non-thyroidal pathways. Mean standard length was similar between T4-treated and control fish but mean body mass was significantly higher in T4-treated than in control fish (Fig. 5), indicating that T4 specifically stimulated body mass without affecting length.

One-way nested ANOVA yielded significant models for pectoral fin length in males ($F_{19,\ 152} = 9.17$, $P < 0.0001$) and females ($F_{18,\ 131} = 11.15$, $P < 0.0001$; one T4-treatment replicate had no females among the fish sampled), and tank effects were observed for females ($P < 0.05$) but not males ($P > 0.05$). Significant differences were observed among treatments within each sex (Tukey's MCT, $P < 0.05$). Namely, exposure to MZ led to a suppression in fin length compared to respective control values in males and females, and co-administration of MZ and T4 nullified this effect (Fig. 6). Although the nullification was not complete in rescued females, the trend is clearly evident (Fig. 6). Conversely, T4-treated males and females had pectoral fins longer than those of their respective control fish (Fig. 6). Thus, T4 not only rescued fish from the inhibitory effect of MZ but by itself had a stimulatory effect on pectoral fin length in both sexes. Linear contrasts of Least Squares (LS) means showed no difference ($P > 0.05$) in pectoral fin length between males and females in the control [males = 2.7 ± 0.04 mm (LS mean ± SEM); females = 2.7 ± 0.05 mm] and rescued (male = 2.6 ± 0.04 mm; females = 2.5 ± 0.06 mm) groups, but statistically significant differences ($P < 0.05$) between the sexes were observed in the MZ (males = 2.4 ± 0.06 mm; females 2.2 ± 0.04 mm) and T4 (males = 3.2 ± 0.04; females = 3.0 ± 0.07 mm) groups.

3.3. Geometric distance analysis at 33 dpf

All geometric distances were correlated with geometrically-determined standard length (Pearson's $r \geqslant 0.7$–0.99); therefore, multivariate analysis was conducted using residuals (see also Table 1). Discriminant function analysis yielded a significant model (Wilks' $\lambda = 0.22$; $F_{12,\ 87} = 3.97$; $P = 0.0001$) with two significant functions. Anal fin base (structure coefficient, 0.62), anal-to-dorsal fin distance (0.58) and head depth (0.57) were primary contributors to function 1 whereas anal-to-dorsal fin distance (0.67) was the primary contributor to function 2. A biplot of data on these two functions, however, did not show patterns of distribution that could be specifically associated with MZ-dependent hypothyroidism. Namely, although the graphical distribution of data from the MZ treatment was different than the control, co-administration of MZ and T4 had no apparent rescue effect (Fig. 7).

3.4. Geometric distance analysis at 45 dpf

All geometric distances were correlated with geometrically-determined standard length (Pearson's $r = 0.58$–0.97); therefore, follow up analysis was based on residuals (see also Table 1). Discriminant function analysis yielded a significant model (Wilks' $\lambda = 0.05$; $F_{21,\ 86} = 7.58$; $P = 0.0001$) with three significant functions.

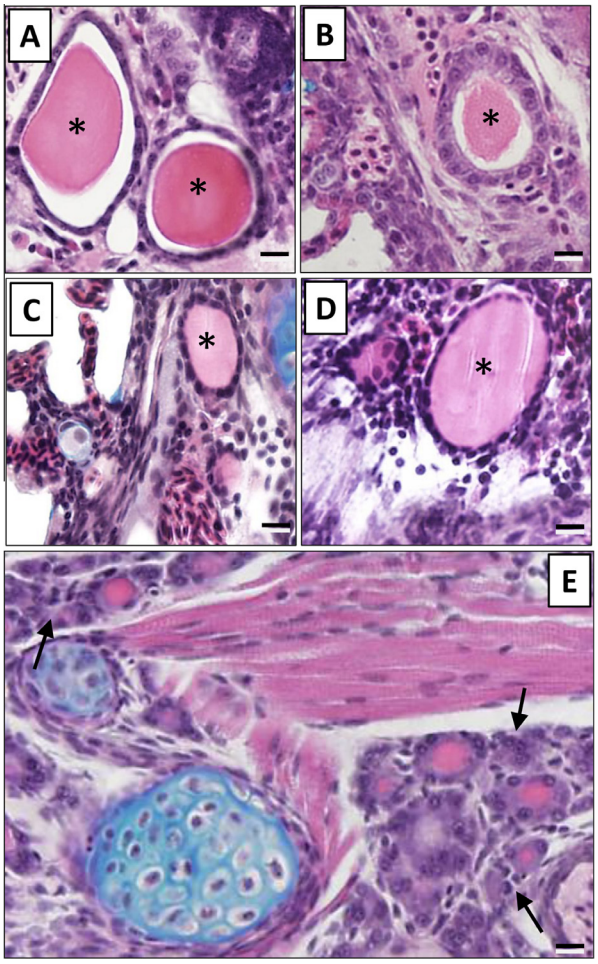


Fig. 3. Photomicrograph of thyroid follicles (asterisks) of Zebrafish at 45 days postfertilization (dpf) exposed to (A) control water, (B and E) MZ (0.15 mM), (C) combination of MZ (0.15 mM) and thyroxine (2 nM), and (D) thyroxine (2 nM) from ~72 h postfertilization to 33 dpf. Note the squamous epithelial cells in thyroid follicles of fish from the control (A) and the two treatments receiving thyroxine (C and D), cuboidal (hypertrophic) cells in fish treated with MZ (B and E), and clusters of hypertrophic small follicles (some very small) associated with or near disorganized follicles or cell aggregates (arrows) in fish treated with MZ (E). Alcian blue, hematoxylin–eosin; scale bars = 10 μm.

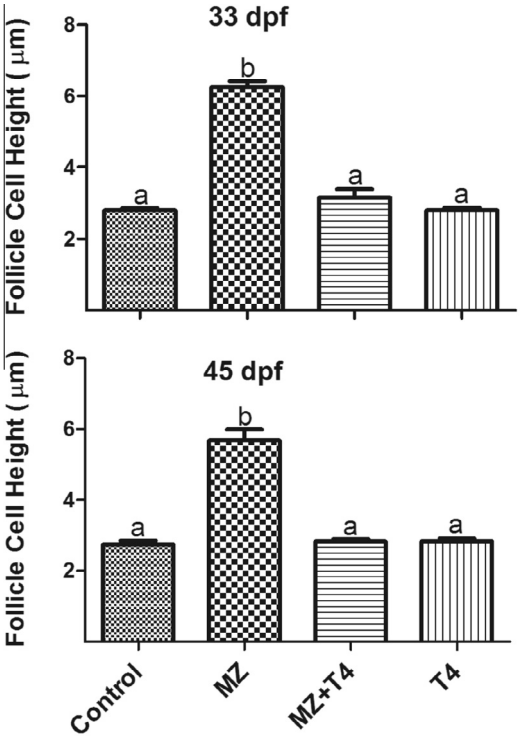


Fig. 4. Effect of methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM) on thyrocyte height (mean + SEM) of Zebrafish at 33 and 45 days postfertilization (dpf). Control fish were raised in reconstituted water. The exposure period began at ~72 h postfertilization and ended at 33 dpf. Bars associated with common letters are not significantly different (1-way nested ANOVA and Turkey's MCT, $P < 0.05$).

Variables contributing to function 1 include caudal peduncle-to-fork distance (structure coefficient, −0.45) and snout-to-pectoral fin distance (0.43), and those contributing to function 2 include snout-to-pectoral fin distance (0.55) and pelvic-to-anal fin distance (−0.43). All variables associated with function 3 had coefficients < |0.4| and this function and its variables were not interpreted. A biplot of data on the first and second functions revealed that variables associated with function 2 separate treatment groups in a manner consistent with thyroid dependency (Fig. 8). Namely, data are largely negative on function 2 for control fish, all positive for MZ-treated fish, evenly divided between positive and negative values for rescued fish (showing a trend toward recovery), and mostly negative for T4-treated fish. The distribution of 95%-confidence ellipses for group centroids confirmed the pattern of thyroid-dependent separation in reference to function 2 (Fig. 8). Data separation on function 1, however, showed a pattern of distribution that cannot be associated with thyroid condition (Fig. 8) and these group differences are considered to be non-specific (non-thyroidal). Snout-to-pectoral fin distance was associated with function 1 at 45 dpf indicating some non-specific influence of MZ on the position of this fin pair, but the strength of the association was higher for function 2 (thyroidal pathway).

4. Discussion

Larval fishes acquire their basic adult form at the completion of metamorphosis and many aspects of this process are driven by TH. In Zebrafish, metamorphosis takes place over a period of about 2+ weeks and is complete by ~30 dpf (Brown, 1997; Schilling, 2002; Parichy and Turner, 2003), and the juvenile phase is generally considered to begin at 45 dpf (Singleman and Holtzman, 2014). Development and growth of pectoral fins are an example of TH-dependent change during metamorphosis in Zebrafish and other species (Nacario, 1983; Reddy and Lam, 1992; Brown, 1997; Trijuno et al., 2002; Shkil et al., 2012). Results of the present study quantitatively confirmed that exposure to goitrogenic compounds can inhibit the development of pectoral fins via thyroidal pathways. Relative to fin length in control fish (2.7 mm in both sexes at 45 dpf), the reduction in fin size caused by exposure to MZ (0.15 mM) was equivalent to 20 and 10 percent in females and males, respectively. Also, in sharp contrast with other landmark distances measured in this study, pectoral fin length was not associated with body size. Thus, pectoral fin length is uniquely independent of body size in Zebrafish cohorts of the same age and more strongly influenced by other factors, such as thyroid condition. (Mean standard length of all fish combined at 45 dpf was 14.6 mm, and the coefficient of variation was 7%.) These observations indicate that pectoral fin length may be a particularly sensitive and useful morphological marker of environmental exposure to thyroid-disrupting chemicals.

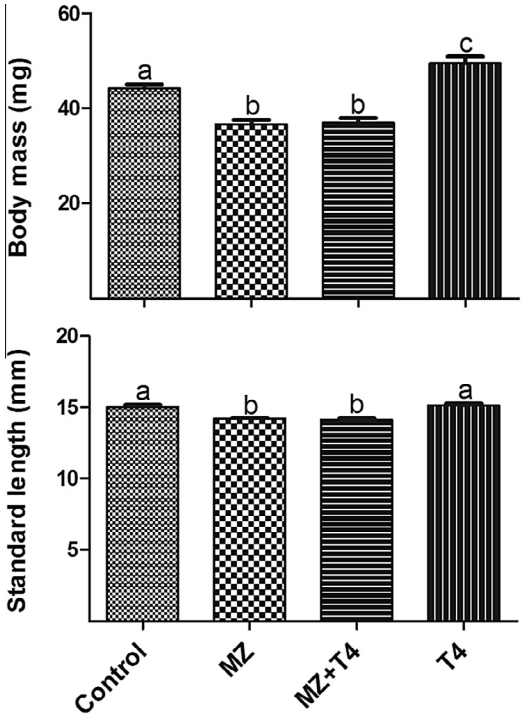


Fig. 5. Effect of methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM) on body mass and standard length (mean + SEM) of Zebrafish at 45 days postfertilization (dpf). Control fish were raised in reconstituted water. The exposure period was from ~72 h postfertilization to 33 dpf. Bars associated with common letters are not significantly different (1-way nested ANOVA and Turkey's MCT, $P < 0.05$). Sample sizes were 81, 75, 67, and 59 for the control, MZ, rescue, and T4 treatments, respectively.

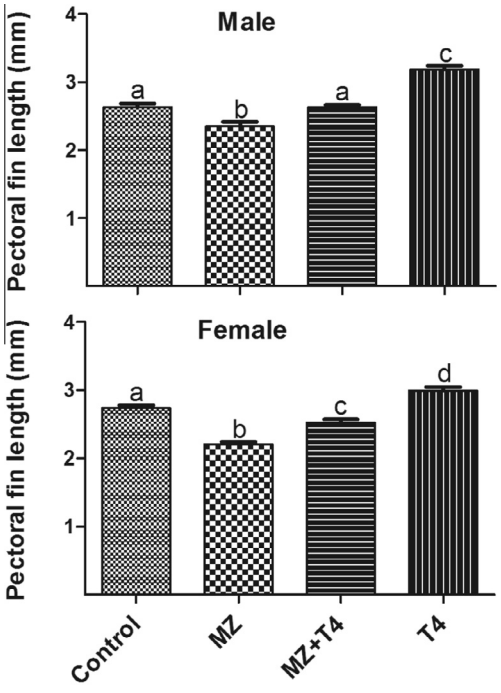


Fig. 6. Effect of methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM) on pectoral fin length (mean + SEM) in male and female Zebrafish at 45 days postfertilization (dpf). Control fish were raised in reconstituted water. The exposure period was from ~72 h postfertilization to 33 dpf. Bars associated with common letters are not significantly different (1-way nested ANOVA and Turkey's MCT, $P < 0.05$). Samples sizes (female/male) were 38/42, 53/23, 25/43, and 16/45 for the control, MZ, rescue and T4 treatments, respectively.

Curiously, pectoral fin length responded differently to thyroid-active chemicals in males and females. While no sex-linked differences in pectoral fin length were observed in the control and rescued fish, males had significantly longer fins than females in the MZ and T4 groups – on average, pectoral fins were ~7–10% longer in males relative to females of these two groups. All females in this study were juveniles (the most advanced stage of oocyte development was perinucleolar), as also were the vast majority of males; only males from the T4-treated group (about half of the male population) showed a more advanced stage (pubertal) of testicular development (Sharma, 2012). It is therefore possible that physiological differences between males and females that are associated with early gonadal development may be at least partly responsible for sex-linked differences in fin length response to MZ and T4.

Results of geometric distance analysis revealed that the relative positions of pectoral fins (snout-to-pectoral fin distance) and pelvic fins (pelvic-to-anal fin distance) at 45 dpf were influenced in TH-dependent fashion, and univariate patterns in data grouped by treatment were consistent with this observation (Table 1). In relation to control fish, snout-to-pectoral fin distance was increased under hypothyroid conditions and this change was prevented in the presence of T4. In terms of actual distance, the estimated median values for snout-to-pectoral fin distance were 4.2 mm and 4.4 mm in the control and MZ treatments, respectively, corresponding to an increase of approximately 5 percent (these values are corrected for a standard length of 15 mm). The opposite pattern was observed for pelvic-to-anal fin distance: it decreased under hypothyroid conditions and no change was evident in the rescue treatment. Because the distance between these fins (pectoral-to-pelvic fin distance) remained constant (landmarks 3–4 in Fig. 1), both fin pairs seem to have shifted backwards along the rostrocaudal axis under hypothyroid conditions. Thyroxine alone did not seem to impact the relative positions of these fins compared to the control group. The underlying mechanism for the displacement of paired fins under hypothyroid conditions is uncertain. However, not all skeletal structures (including those of the axial skeleton) respond in the same manner to changes in thyroid condition during Zebrafish development, a situation that can lead to developmental heterochronies (Shkil et al., 2012) and potentially be the basis for the present observations.

No change in body morphology specifically attributable to thyroid endocrine condition was observed at 33 dpf, the end of the exposure period. This observation suggests that, at the concentrations used, the magnitude of the effects of thyroid-active chemicals is too small to be detected by external morphological assessment at 33 dpf (using the tools of the present study) and that it takes additional growth for the effects to become manifest (i.e., delayed effects at 45 dpf). Alternatively, observations at 45 dpf also could be the result of continued hypothyroid activity of residual MZ that may still have been present in thyroid follicles after treatment termination. Methimazole can accumulate in thyroid follicles (Marchant and Alexander, 1972), and a similarly designed study of Zebrafish reported that it takes an additional 2 weeks (at 60 dpf) for the effects of MZ on thyrocyte condition to completely disappear (Sharma and Patiño, 2013).

In addition to the non-specific (non-thyroidal) effects of MZ exposure on body shape at 33 dpf and several identified at 45 dpf, MZ exposure caused a reduction in body mass and length at 45 dpf via non-thyroidal pathways. The non-thyroidal nature of these MZ activities would have been difficult to recognize if a rescue treatment had not been part of the present experimental design. While the concentration of T4 used in the rescue treatment (2 nM) is low compared to concentrations used by most other studies with Zebrafish embryos or larvae (e.g., Liu and Chan,

Table 1
Residuals (mean ± standard error) of geometric distances regressed against standard length at 33 and 45 days postfertilization (dpf). Only those variables retained in the respective discriminant models are shown.

DPF	Variables	Control	MZ	MZ + T4	T4
33	AFB	−0.034 ± 0.010	−0.008 ± 0.008	0.017 ± 0.007	0.025 ± 0.008
	HD	−0.019 ± 0.007	−0.003 ± 0.005	0.019 ± 0.004	0.004 ± 0.005
	ADD	−0.019 ± 0.009	−0.028 ± 0.012	0.034 ± 0.007	0.014 ± 0.007
	PvAD	−0.008 ± 0.020	−0.013 ± 0.032	0.017 ± 0.021	0.004 ± 0.012
45	***SPD***	***0.011 ± 0.005***	***0.022 ± 0.005***	***−0.024 ± 0.005***	***−0.010 ± 0.006***
	CFD	−0.042 ± 0.010	0.004 ± 0.004	0.022 ± 0.007	0.015 ± 0.009
	PDD	−0.003 ± 0.007	−0.001 ± 0.004	0.010 ± 0.004	−0.006 ± 0.005
	PvAD	***−0.001 ± 0.008***	***−0.020 ± 0.011***	***0.006 ± 0.009***	***0.015 ± 0.007***
	AFL	−0.023 ± 0.010	0.000 ± 0.008	0.023 ± 0.006	−0.001 ± 0.005
	ACD	0.006 ± 0.006	−0.005 ± 0.003	0.002 ± 0.003	−0.004 ± 0.003
	PAD	−0.009 ± 0.004	−0.013 ± 0.005	0.016 ± 0.005	0.006 ± 0.006

Geometric distances were log-transformed prior to regression. Fish were exposed to untreated (control) water, methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM). The exposure period began at ~72 h postfertilization and ended at 33 dpf. Thyroid hormone-influenced distances identified by this study are marked by italic, bold font.
ACD = anal-to-caudal fin distance, ADD = anal-to-dorsal fin distance, AFB = anal fin base, AFL = anal fin length, CFD = caudal peduncle-to-fork distance, HD = head depth, PAD = pectoral-to-anal fin distance, PDD = pectoral-to-dorsal fin distance, PvAD = pelvic-to-anal fin distance, SPD = snout-to-pectoral fin distance.

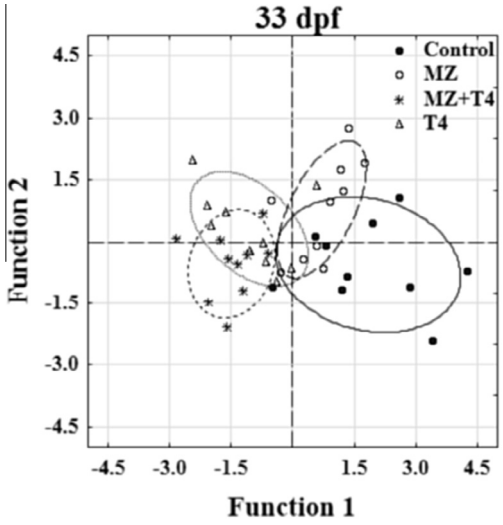


Fig. 7. Discriminant function biplot of geometric distance data obtained from Zebrafish at 33 days postfertilization (dpf). Fish were exposed to untreated (reconstituted) water, methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM). The exposure period was from ~72 h postfertilization to 33 dpf. Ninety-five percent confidence ellipses of the bivariate mean for each group are shown. Patterns of treatment data distribution and their associated confidence ellipses do not indicate TH-dependent group separation on either axis.

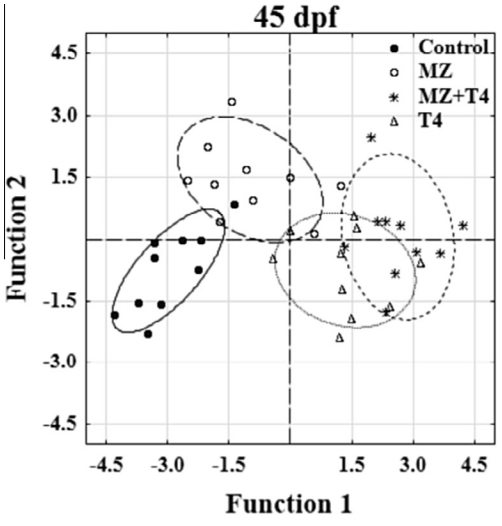


Fig. 8. Discriminant function biplot of morphological data obtained from Zebrafish at 45 days postfertilization (dpf). Fish were exposed to untreated (reconstituted) water, methimazole (MZ, 0.15 mM), combination of MZ (0.15 mM) and thyroxine (T4, 2 nM), and T4 (2 nM). The exposure period was from ~72 h postfertilization to 33 dpf. Ninety-five percent confidence ellipses of the bivariate mean for each group are shown. Patterns of treatment data distribution and their associated confidence ellipses indicate TH-dependent group separation on axis 2 but not axis 1.

2002; Lam et al., 2005; Mukhi et al., 2007; Fetter et al., 2015), it is higher than the concentration of T4 observed in fertilized eggs, embryos, or larvae of this species [0.3–0.7 nM; estimated from data presented in Mukhi and Patiño (2007) and Chang et al. (2012)]. More importantly, T4 at 2 nM was able to nullify the hypothyroid effect of MZ as judged by evaluations of thyrocyte condition and pectoral fin length. Therefore, the existence of non-thyroidal effects of MZ at 0.15 mM – and not insufficient concentration of TH in the rescue treatment – best explains the present observations. Methimazole is a goitrogen commonly used in studies of development and endocrine disruption in teleosts and other vertebrates and often, in aquatic animals, at waterborne concentrations higher than in the present study (e.g., Liu and Chan, 2002; Lam et al., 2005; Thienpont et al., 2011; Jomaa et al., 2014; Fetter et al., 2015). Together with results of a recent study that concluded MZ is toxic to Zebrafish embryos via non-thyroidal mechanisms at concentrations >1 mM (Komoike et al., 2013), results of the present study suggest that the selection of experimental goitrogen concentrations should be made cautiously and that inclusion of rescue treatments in study designs may be necessary to discriminate endpoints associated with thyroidal versus non-thyroidal pathways.

Larval survival was reduced in the presence of T4 at 2 nM. Enhanced mortality of Zebrafish larvae exposed to T4 at 10 nM, but not 1 nM, was previously reported (Mukhi et al., 2007; Sharma and Patiño, 2013). It appears, therefore, that the concentration cut-off for emerging lethal toxicity of T4 in Zebrafish larvae (under continuous exposure) is in the low nanomolar range, between 1 and 2 nM.

In conclusion, this study revealed that the relative position of pectoral and pelvic fins in the Zebrafish body can be specifically influenced by disruption of the thyroid endocrine system. Both fin pairs shifted backward along the rostrocaudal axis under hypothyroid conditions and T4 co-treatment prevented the shift. The present results also confirmed the TH-dependency of pectoral fin length and showed that thyroid endocrine condition is a more

important driver of pectoral fin length than is body length in cohorts of the same age. The shape and relative size of the Zebrafish body and fins normally change during development in a manner that benefits locomotory performance as adults (McHenry and Lauder, 2006), and a recent study provided empirical evidence for an association between swimming speed and natural pelvic-to-anal fin distance in (untreated) adult Zebrafish (Conradsen and McGuigan, 2015). Thus, alterations in the relative position and size of paired fins caused by thyroid endocrine disruption during early development could affect physical aspects of locomotion and, consequently, also impact other important organismal functions and ultimately survival and recruitment into the adult population.

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