# Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part 2 : zebrafish

# Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole – Part II: zebrafish

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**Abstract**

Disruption of the thyroid hormone (TH) system, an important mode of action, can lead to ecologically relevant adverse outcomes, especially during embryonic development. The present study characterizes the effects of disruption of TH synthesis on swim bladder inflation during zebrafish early-life stages using 2-mercaptobenzothiazole (MBT), a thyroid peroxidase (TPO) inhibitor. Zebrafish were exposed to different MBT concentrations until 120/168 hours post fertilization (hpf) and 32 days post fertilization (dpf), in two sets of experiments, to investigate the effects of TPO inhibition on posterior and anterior swim bladder inflation respectively, as well as whole body thyroid hormone concentrations (triiodothyronine (T3) and its prohormone, thyroxine (T4)). At 120 hpf, MBT did not directly impair posterior chamber inflation or size, while anterior chamber inflation and size was impaired at 32 dpf. As previously shown in amphibians and mammals, we confirmed that MBT inhibits TPO in fish. Whole-body T4 decreased after MBT exposure at both time points, while T3 levels were unaltered. There was a significant relationship between T4 levels and the anterior chamber surface at 32 dpf. The absence of effects on posterior chamber inflation can possibly be explained by maternal transfer of T4 into the eggs. These maternally derived THs are depleted at 32 dpf and cannot offset TPO inhibition, resulting in impaired anterior chamber inflation. Therefore, we hypothesize that TPO inhibition only inhibits swim bladder inflation during late development, after depletion of maternally derived T4. In a previous study, we showed that iodothyronine deiodinase (ID) knockdown impaired posterior chamber inflation during early development. Our findings, in parallel with similar effects observed in fathead minnow (see part I, this issue) suggest that thyroid disruption impacts swim bladder inflation, and imply an important distinction among specific subtypes of TH disrupting chemicals. However, the existence of another – yet unknown – mode of action of MBT impacting swim bladder inflation cannot be excluded. These results can be helpful for delineating adverse outcome pathways (AOPs) linking TPO inhibition, ID inhibition and other TH related molecular initiating events, to impaired swim bladder inflation in fish during early life stages. Such AOPs can support the use of *in vitro* enzyme inhibition assays for predicting reduced survival due to impaired posterior and anterior chamber inflation.

**Keywords**: thyroid disruption, 2-mercaptobenzothiazole, zebrafish embryo, swim bladder inflation, fish early-life stage, adverse outcome pathway

## 1. Introduction

The presence of certain chemicals in the aquatic environment can pose severe risks for both environmental and human health. The vast number of chemicals that are used in industry, in combination with the growing desire to reduce animal testing, has generated a strong focus on the development of new test methods for exposure assessment and hazard identification for use in ecological risk assessments. Today, the fish early-life stage (FELS) test (OECD TG 210; OECD, 2013) is one of the most commonly used assays to estimate the chronic toxicity of chemicals in fish. However, important limitations of this test are being recognized, including the large number of animals used, the relatively low-throughput, and the lack of mechanistic information, because observations are limited to a few apical endpoints such as mortality and growth. The development of alternative testing strategies is currently being explored by the scientific community, often focusing on *in silico*, *in chemico* and *in vitro* methods that are predictive of perturbation of specific toxicity pathways or modes of action (MoA), potentially resulting in apical effects relevant for population impact. Adverse outcome pathways (AOPs) have been proposed as a framework to assist in the identification of processes at specific levels of biological organisation (termed key events, KEs) that are essential in a given toxicity pathway (Ankley et al., 2010). Information contained within such AOPs can then be used to design assays for hazard identification and risk prediction.

Disruption of the thyroid hormone (TH) system is increasingly being recognized as an important MoA that can lead to adverse outcomes, especially during embryonic development. In fish, many different adverse effects during early development resulting from disruption of the TH endocrine system have been reported (e.g., effects on otolith formation, otic vesicle length, head-to-trunk angle, pigmentation index, swim bladder inflation, hatching time, body and eye size, somite formation, heartbeat, tail and pericardial oedema, escape response and photoreceptor development)(Bagci et al., 2015; Heijlen et al., 2014; Jomaa et al., 2014). The present study was designed to further characterize the effects of disruption of TH metabolism on swim bladder inflation during fish early-life stages using an environmentally relevant TH disrupting compound. Effects on swim bladder inflation can alter swimming performance and buoyancy and therefore affect essential endpoints such as feeding behaviour and predator avoidance, resulting in lower survival probability (Czesny et al., 2005; Woolley and Qin, 2010).

2-Mercaptobenzothiazole (MBT) was chosen as a test compound because it can disrupt thyroid function by inhibiting thyroid peroxidase (TPO) and because of its presence in environmental water

bodies (Paul et al., 2014, 2013; Tietge et al., 2013). TPO is essential for the synthesis of thyroid hormones [triiodothyronine (T3) and thyroxine (T4)] in the lumen of thyroid follicles, and compounds that alter TPO activity can critically affect biological processes. TPO inhibiting chemicals have been demonstrated to decrease TH concentrations in a wide-variety of vertebrates, including amphibians (Paul et al., 2013), birds (Rosebrough et al., 2006) and rodents (Zoeller and Crofton, 2005). MBT is primarily used as a rubber vulcanizing agent. It is an intermediate for the synthesis of other benzothiazole derivatives, and also has been used as a fungicide, microbiocide and bacteriostat (US EPA, 1994). MBT is also the main metabolite of 2-(thiocyanomethylthiol)benzothiazole (TCMBT) which is used for preservation of partially processed leather and wood preservation (Rodriguez et al., 2004). There is a large potential for this chemical to be released into the aquatic environment because of its high production volume (Tietge et al., 2013; US EPA, 1994). MBT levels ranging from few µg/L up to 1.3 mg/L have been found in industrial wastewater, in the effluents of wastewater treatment plants and in different natural waters (Fiehn et al., 1994; Rennie, 1988; Rodriguez et al., 2004). For example, Fiehn et al. (1994) found MBT levels of 650-690 µg/L in tannery wastewater and 36 µg/L remained after wastewater treatment. Tietge et al. (2013) showed that MBT retarded metamorphic development in *Xenopus laevis*, which is consistent with disruption of thyroid function. A recent study confirmed the TPO inhibitory potential of MBT both *in vitro* and *ex vivo*, and showed *in vivo X. laevis* responses indicative of T4 synthesis inhibition, including induction of sodium iodide symporter mRNA and decreases in glandular and circulating thyroid hormones (Hornung et al., 2015). However, very few studies have focused on the effects of MBT in fish (Choi et al., 2007; Sollmann, 1949; Stephensen et al., 2005), with only one study linking MBT to thyroid hormone levels (Li et al., 2012).

In the present study, zebrafish (*Danio rerio*) were used as a model organism. In zebrafish, the transition from embryo to larva takes place around 3-5 days post fertilization (dpf). As in amphibians, the transition between the different developmental phases, including maturation and inflation of the swim bladder, have been shown to be mediated by THs (Brown, 1997; Liu and Chan, 2002; Terrien and Prunet, 2013). Chang et al. (2012) established a baseline for TH levels during zebrafish development and linked peaks in whole-body T3 content at 5 and 10 dpf, and at 21 dpf for T4 to specific developmental processes and transitions. The swim bladder of zebrafish consists of a posterior chamber, which inflates around 96 hours post fertilization (hpf), and an anterior chamber, which inflates around 21 dpf. Both time points coincide with the specific TH peaks observed by Chang et al. (2012). Both swim bladder chambers operate as a hydrostatic organ, adjusting the volume of gas in the adult swim bladder to regulate body density and buoyancy. The anterior

chamber has an additional role as acoustic resonator, as Weberian ossicles connect the anterior chamber to the otic apparatus (Dumbarton et al., 2010; Roberston et al., 2007). Several studies already suggested the involvement of THs in posterior swim bladder inflation (Bagci et al., 2015; Heijlen et al., 2014; Jomaa et al., 2014), but the role of THs in inflation of the anterior chamber has not yet been described. Some studies demonstrated effects on anterior chamber inflation in fish after exposure to various metals (Witeska et al., 2014). However, the amount of data available on inflation of the anterior chamber is very limited. The exact relationship between inflation of the posterior and anterior chamber therefore remains unclear.

In this paper, zebrafish were exposed to different MBT concentrations until 120/168 hpf and 32 dpf, in two sets of experiments, to document the effects of TPO inhibition on posterior and anterior swim bladder inflation respectively. Furthermore, whole-body T3 and T4 levels were measured at 120 hpf and 32 dpf. A similar study has been carried out on fathead minnow (Nelson et al., this issue). The results can be helpful for delineating AOPs relating thyroid hormone disrupting mechanisms to fish early-life stage toxicity.

## 2. Materials and Methods

### Ethics statement

According to EU Directive 2010/63/EU and the Commission Implementing Decision 2012/707/EU, fish are non-protected animals until the stage of free feeding. This limit was set at 120 hpf for zebrafish. All experiments of this study exceeding 120 hpf were approved by the Ethical Committee for Animals of the University of Antwerp (project number 2014-29). Fish husbandry and all experiments were carried out in strict accordance with the EU Directive on the protection of animals used for scientific purposes (2010/63/EU).

### 2.1 Egg production

Adult zebrafish (in house wild type zebrafish line), designated for breeding, were used for egg production and were accommodated in a ZebTEC standalone system (Tecniplast, Buguggiate, Italy). Non-exposed adult zebrafish as well as embryos used in all experiments were kept in reconstituted freshwater with adjusted pH (using $NaHCO_3$, 7.5 ± 0.3) and conductivity (using Instant Ocean® Sea Salt, Blacksburg, VA,USA, 500 ± 15 µS/cm), a constant temperature of 28 ± 0.2°C and a 14/10 h

light/dark cycle. Water quality was monitored twice a week using Tetratest kits (Tetra Werke, Melle, Germany) and values for ammonium, nitrite and nitrate remained below 0.25, 0.3 and 12.5 mg/L respectively. Adult zebrafish were fed four times a day, twice with granulated food (1.5% of their average wet weight, Biogran medium, Prodac International, Cittadella, Italy) and twice with frozen Chironomidae larvae, *Artemia sp.* nauplii, Chaoboridae larvae and *Daphnia sp*. (Aquaria Antwerp bvba, Aartselaar, Belgium) alternately. Breeding pairs of two females and one male were separated in breeding tanks with a perforated bottom. The divider was removed the next morning when lights were switched on and eggs were collected, rinsed and transferred to clean reconstituted water 30 minutes after fertilization. All eggs were monitored using a stereo microscope (Leica S8APO, Leica Microsystems GmbH, Germany) and unhealthy, non-fertilized eggs, eggs with anomalies or damaged membranes or dead embryos were discarded.

**2.2 Chemical exposures**

A stock solution of 28 mg/L MBT (CAS 149-30-4, Sigma-Aldrich, Saint Louis, USA, ≥96.5% purity) was prepared by dissolving 28 mg of MBT in 10 mL reconstituted freshwater and 100 µL 10M NaOH, by stirring at room temperature, in order to prepare a solvent-free solution. Once in solution, reconstituted freshwater was added up to 1 L and pH was adjusted to 7.5 using 1N HCl. The stock solution was stored in the dark, at room temperature. Every 24 h, exposure solutions were freshly made and pH (7.5 ± 0.2) and conductivity (500 µS ± 50) was checked. 24-well plates (sterile tissue culture plates, Greiner Bio-One, Frickenhausen, Germany) were saturated by filling the plates with test solutions one day in advance of egg collection and renewing the medium before exposure. Exposure of fertilized eggs started within 0.5 to 1 hpf (at least two 24-well plates/condition; n=40/condition; 2 mL/well; one embryo/well). The remaining four wells per plate were used as an internal negative control (i.e., at least eight controls per condition). A test was considered valid if ≥80% of the controls successfully hatched and ≥90% survived until the end of the 120 h exposure (OECD TG 236, OECD 2013b). 3.4-Dichloroaniline (CAS 95-76-1, purity of 98%, Sigma-Aldrich, nominal concentrations 0.5, 1, 2 and 4 mg/L, 12 embryos per concentration) was used as positive control (OECD TG 236, OECD 2013b). Plates were sealed with parafilm (Parafilm®, Bemis Europe, Soignies, Belgium) and stored in an incubator (MIR-254-PE, Panasonic, TCPS, Rotselaar, Belgium) with a day/night cycle of 14/10 h and a constant temperature of 28.5°C ± 0.2°C. Test solutions were renewed daily to avoid degradation. Every 24 h, mortality (coagulation, absence of heart beat, absence of somite formation, no tail detachment; OECD TG 236), hatching and swim bladder

inflation were evaluated using a stereomicroscope (Leica S8APO, Leica Microsystems GmbH, Germany). Dead embryos and the remains of the chorion after hatching were removed every 24 h.

**2.3 Zebrafish embryo acute toxicity test (ZFET)**

We performed extended fish embryo acute toxicity tests (OECD TG 236, OECD 2013b). In a first experiment, zebrafish embryos were exposed until 120 hpf to nine concentrations of MBT (nominal concentrations 0, 0.1, 0.35, 0.56, 0.7, 0.88, 1.75, 7 and 14 mg/L; n=40 for each concentration). Concentrations were selected based on preliminary range finding experiments (data not shown). In this experiment, we found an effect on hatching (see Figure 1A), impacting inflation of the posterior swim bladder chamber since non-hatched larvae are unable to reach the water surface. In a second experiment, designed only to monitor hatching, 120 embryos were exposed to 0.7 mg/L MBT until 13 dpf. We established that the hatching effect was transient (i.e., delayed but not impaired hatching, see Figure A.1), indicating that dechorionating embryos was necessary to properly study swim bladder inflation. In a third experiment, zebrafish embryos were exposed until 168 hpf to 10 concentrations of MBT (the same nine concentrations as in the first experiment plus 3.5 mg/L; n=40 for each concentration, except for the four highest concentrations: n=80). All embryos of this experiment were manually dechorionated at 48 hpf using a forceps (#5 Dumont, Fine Science Tools, Berlin) to exclude the confounding effect of hatching on swim bladder inflation. All ZFET data reported in this paper originate from the third experiment unless stated otherwise. A fourth experiment was carried out to measure T3/T4 concentrations. Larvae were exposed to 0, 0.1, 0.35 and 0.7 mg/L MBT until 120 hpf. Subsequently, larvae were euthanized using an overdose of 1g/L MS-222 adjusted to pH 7.5, transferred to a nylon 100 µm filter (BD Biosciences, Durham, NC, USA), rinsed with reconstituted water, transferred to clean reconstituted water (30 mL) three times, and finally pooled in cryovials. Each sample consisted of 70 to 80 larvae, resulting in four biological replicates per condition. Samples were kept at -80°C until T3/T4 measurements were performed.

*2.3.1 Swimming behaviour analysis*

At 120 hpf, swimming activity of all larvae of the first and third ZFET experiment was determined in the 24-well plates using a Zebrabox 3.0 video tracking device (ViewPoint, Lyon, France). The swimming capacity was recorded during 40 minutes in light. Data were analysed using the ZebraLab software version 3.20.5.104. The total distance travelled was calculated using the recorded route of

each embryo. The sum of all swimming movements (mm) during 40 min was compared among all conditions. For statistical analysis, non-hatched and dead larvae were excluded.

*2.3.2 Morphological assessment*

After behaviour analysis at 120 hpf, all larvae of the first ZFET experiment and larvae with an inflated posterior chamber in the third ZFET experiment were anaesthetized using 100 mg/L MS-222 (tricaine methanesulphonate, Sigma–Aldrich) adjusted to pH 7.5 using $NaHCO_3$. Non-hatched larvae of the first experiment were dechorionated before morphological assessment. Morphological deviations were scored, including failure to hatch, impaired swim bladder inflation (posterior chamber), tail malformations (curvature and elbow), oedemas (pericardia, yolk and head), blood accumulations (heart, yolk (extension), tail), absent or disturbed blood circulation in the tail, abnormal pigmentation and malformations of fins (absence, curved), yolk, mouth, eyes and otoliths. Furthermore, the lateral view of the larvae was photographed together with a calibrator, using a camera (Canon EOS 600D, 18 megapixels) mounted on a Leica S8APO stereomicroscope. Larvae were positioned in 2.5% methylcellulose to ensure a lateral position. Digital images were analysed using the ImageJ software (available at http://rsbweb.nih.gov/ij/) to determine larval length and the surface of the swim bladder and the eye. Surface measurements were performed by marking the actual circumference of the posterior chamber and the eye. Relative surfaces were calculated according to Hagenaars et al. (2014) by dividing the measured surface by the expected surface based on larval length. The expected surface was calculated using the following formula:

$$S_e = aL_o{}^b \qquad \text{(Eq. 1)}$$

where $S_e$ is the expected surface, $L_o$ the observed length, and $a$ and $b$ are parameters of the length-swim bladder surface or length-eye surface relationship of control larvae (Figure A.2). These power regressions were based on control larvae of different ages (24, 48, 72, 96, 120 and 144 hpf), all with a significant length-surface correlation ($p < 0.0001$, Pearson r values available in figure A.2). A relative value larger/smaller than 1 indicates that the biological parameter in question is larger/smaller than to be expected for larvae of that length.

**2.4 Fish early-life stage toxicity test (FELS)**

A fish early-life stage toxicity test (OECD TG 210, OECD 2013) was performed, including a control (n=80), 0.1 mg/L (n=120) and 0.35 mg/L (n=140) nominal MBT concentrations, until 32 dpf. These two concentrations were selected based on the ZFET experiment (see 2.3). They both resulted in

100% survival and hatching. 0.35 mg/L was the NOEC for impaired hatching. From 5 dpf, larvae were transferred from the 24-well plates and equally distributed into four replicate test chambers (960 mL plastic beakers containing 200, 400, and 740 mL at 5-10, 10-20, and 20-32 dpf, respectively). Exposure solutions were renewed daily. Fish were fed three times a day with paramecia (5-20 dpf), *Artemia sp.* nauplii (10-32 dpf), and Special Diets Services (SDS)-100 dry food (20-32 dpf).

Swimming behaviour of a set of randomly picked larvae (n=24/condition) was determined in six-well plates on 26, 29, and 32 dpf (see 2.3.1). In addition to daily observations of mortality, hatching and posterior chamber inflation, anterior chamber inflation was assessed daily from 20 until 32 dpf. At 32 dpf, larvae were photographed using a camera and graph paper, and larval total length and posterior and anterior chamber surface were determined (see 2.3.2). Subsequently, larvae were euthanized, rinsed and pooled in cryovials as previously described (see 2.3). Cryovials containing pooled samples were weighed (fresh weight). The condition factor was calculated following a similar logic as given in equation 1, i.e., dividing the measured weight by the expected weight based on the mean larval length of the pooled larvae (Figure A.2). Each sample consisted of two to five larvae, resulting in 12, 19, 17 and 7 biological replicates for the controls, 0.1 and 0.35 mg/L MBT treatments with inflated anterior chamber, and 0.35 mg/L MBT without inflated anterior chamber, respectively. Samples were kept at -80°C until T3/T4 or dose measurements were performed.

**2.5 T3 and T4 measurements**

Triiodothyronine (T3) and its prohormone, thyroxine (T4), were measured in ZFET controls and samples exposed to 0.1, 0.35 and 0.7 mg/L MBT, and in FELS controls and samples exposed to 0.1 and 0.35 mg/L MBT. T3 and T4 measurements were performed using four biological replicates per condition, with exception of FELS larvae exposed to 0.35 mg/L MBT that did not inflate their anterior chamber (three biological replicates). Each ZFET replicate consisted of 70 to 80 pooled larvae while each FELS replicate consisted of three to five pooled larvae. Whole-body T3 and T4 were extracted following a modified published procedure (Crane et al., 2004) with detailed modifications previously described (Nelson et al., this issue). Briefly, sample processing involved analyte extraction in ethanol, hexane defatting, and solid-phase extraction (SPE) cleanup. Thyroid hormones were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS), as detailed by Hornung et al. (2015). Method blanks (n=2), control larval fish (n=4), and spiked larval fish (n=4, spiked with 0.25 ng T3 and T4) were analysed with each sample batch to document accurate quantification of analytes. Spike concentrations were calculated by subtracting endogenous hormone concentration determined in

control larval fish from that in spiked larval fish. Method blanks were below limits of quantification for both T3 and T4. Control larval fish replicates exhibited acceptable precision with a coefficient of variation (CV) of 2.6% and 13.9% for T3 and T4, respectively. Spiked larval fish demonstrated good accuracy at 103 ± 4% and 94 ± 6% of spiked amounts for T3 and T4, respectively. To facilitate statistical analysis, non-detections of T3 and T4 were given a value of one-half the limit of quantitation (0.005 ng) before calculating final concentration on a ng/g basis.

**2.6 Analytical measurements**

During the FELS experiment, three independent replicate medium samples of exposure solutions (500 µL) were taken before and after medium renewal at 5, 18 and 30 dpf. Additionally, medium samples were taken for 0.35 mg/L MBT at 8, 14 and 25 dpf. Additional exposure experiments were performed without embryos with 0.35 mg/L MBT in order to investigate stability of MBT during a 14/10 h light/dark cycle and during 24 h dark. Furthermore, *Artemia sp.* Nauplii and paramecia (including the solution used to culture the paramecia; Liquifry) was added to the medium to investigate effects of nutrition on MBT stability. Three independent replicate medium samples of each condition (500 µL) were taken at 0 h and after 24 h. Samples were stored in 1.5 mL glass vials (5182-0716, Agilent Technologies, Palo Alto, CA, USA) at -20°C until analysis. MBT was measured using liquid chromatography (LC, Agilent Technologies 1200 series) equipped with a binary pump and coupled to a triple quadrupole mass spectrometer (MS, Agilent Technologies 6410). MS parameters can be found in the Supplementary Materials (Table A.1). To facilitate statistical analysis, non-detections of MBT in medium were given a value of one-half the limit of quantitation (0.0025 mg/L).

Dose measurements in larvae were performed using four biological replicates per condition, with the exception of larvae exposed to 0.35 mg/L MBT without anterior chamber inflation (two biological replicates). For extraction of the larvae, 100 µL methanol/water (1:1) and 100 µL internal standard (benzotriazole) were added to the larval samples. Subsequently, samples were ultra-sonicated until larvae were dissolved (typically 10 min) and vortexed for 1 min. The mixture was centrifuged for 1 minute at 9000 rcf and the supernatant was injected into the LC-MS/MS. The calibration curves used for quantification were prepared as follows. Various concentrations of MBT standard and the same concentration of internal standard, both prepared in 100% methanol, were mixed and then evaporated under nitrogen gas. To correct for matrix effects, the calibration extracts were reconstituted in 100 µL of extract obtained from non-exposed larvae, prepared in

methanol/water (1:1). For statistical analysis, non-detections MBT in larvae were given a value of one-half the limit of quantitation (0.4 ng) before calculating final dose on a µg/g basis.

**2.7 Data analysis**

Data were considered significantly different when p-values were <0.05. Percentages of the occurrence of mortality and sublethal morphological effects were analysed as a function of logarithmic exposure concentrations using a nonlinear regression estimate (variable slope, bottom and top constrained at 0 and 100), and $LC_{50}$- and $EC_{50}$-values were calculated. A sum-of-squares F test was performed using the log$EC_{50}$ and HillSlope to determine significant differences between dose-response curves. A Log-rank (Mantel-Cox) test was performed on posterior chamber inflation over time. Data for survival, relative swim bladder surfaces, relative eye surface, distance travelled, larval length, condition factor, dose measurements and $T_3$ and $T_4$ measurements were analysed using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test. Data for medium measurements were analysed using a two-way ANOVA with a Tukey's post hoc test. Data normality was assessed using the Shapiro-Wilk normality test. Data were log-transformed if they were not normally distributed. The percentage of anterior chamber inflation was compared among conditions and time points using a chi-squared contingency table test, with a Tukey's multiple comparisons test. A Pearson correlation coefficient was calculated between T4 levels and the anterior chamber surface. All statistical analyses were performed using GraphPad Prism version 6.00 (Graphpad Software, LA Jolla California, USA).

**3. Results**

**3.1 Zebrafish embryo acute toxicity test (ZFET)**

*3.1.1 Effects on posterior chamber inflation*

MBT exposure significantly delayed hatching, which is required for the posterior chamber to inflate, at a concentration ($EC_{50}$=0.6 mg/L, 95% CI: 0.57-0.63 mg/L) significantly lower than the MBT $LC_{50}$ (6.9 mg/L, 95% CI: 6.3-7.5 mg/L) (Figure 1A). Control larvae all hatched at 72 hpf, while 100% hatching was only observed at 264 hpf for 0.7 mg/L MBT exposed embryos (Figure A.2). Dechorionating the embryos at 48 hpf eliminated this confounding effect: the posterior chamber inflated normally although slightly delayed, except for the 1.75 and 3.5 mg/L MBT treatment groups (Figure 1B). At 3.5

mg/L, where 19% mortality was observed, as well as severe malformations that could interfere with posterior chamber inflation, such as oedema and curvature and kinks of the tail, 50% of the embryos had inflated posterior chambers at 168 hpf. The $EC_{50}$ value of posterior chamber inflation (3.2 mg/L, 95% CI: 2.7-3.8 mg/L) was not significantly different from the $LC_{50}$ value (4.2 mg/L, 95% CI: 3.5-5 mg/L) at 168 hpf (Figure 1C). Furthermore, the posterior chamber surface was not affected by MBT exposure in dechorionated embryos (Figure 1D).

*3.1.2 Other observed effects at 120 hpf*

MBT caused a concentration-dependent increase in mortality ($LC_{50}$: 6.9 mg/L, 95% CI: 6.3-7.5 mg/L), and concentration-dependent sublethal effects (Figure 2D), including reduced pigmentation of the eye ($EC_{50}$: 0.66 mg/L, 95% CI: 0.66-0.67 mg/L) and body ($EC_{50}$: 0.56 mg/L, 95% CI: 0.56-0.57 mg/L) and the occurrence of a malformed mouth ($EC_{50}$: 0.61 mg/L, 95% CI: 0.60-0.61 mg/L). The $EC_{50}$ values of these parameters were significantly different from the $LC_{50}$ value ($p < 0.0001$). Additionally, distance travelled was reduced compared to control larvae after exposure to 0.56 mg/L MBT or higher (Figure 2E). Finally, MBT exposure reduced eye surface (Figure 2F) and larval growth (Figure 2G) compared to control larvae after exposure to 0.7 mg/L MBT or higher.

**3.2 Fish early-life stage toxicity test (FELS)**

*3.2.1 Effects on anterior chamber inflation*

During the first five days of the 32 dpf FELS experiment, slightly delayed hatching was observed with 5.6% non-hatched larvae at 120 hpf after exposure to 0.35 mg/L MBT. All hatched larvae had inflated posterior chambers at 5 dpf. However, the anterior chamber did not inflate properly at 0.35 mg/L MBT. At 21 dpf, 91% of the control larvae had an inflated anterior chamber, while only 34% of the larvae exposed to 0.35 mg/L MBT had inflated anterior chambers (Figure 3). At 32 dpf, 22% of larvae exposed to the highest concentration had non-inflated anterior chambers.

The relative surface of the posterior chamber was significantly larger for fish from the 0.1 mg/L and 0.35 mg/L MBT treatment groups compared to control larvae when the anterior chamber was inflated, and even significantly larger within the same test condition (0.35 mg/L MBT) when the anterior chamber was not inflated (Figure 4D). The relative surface of the anterior chamber was significantly smaller for the highest concentration (Figure 4E). The sum of both surfaces,

representing the total amount of gas in both chambers, was larger when larvae were exposed to 0.1 mg/L. However, no effect on total surface was observed after exposure to 0.35 mg/L MBT (Figure 4F). The ratio of the anterior and posterior chamber surface was significantly smaller for the highest concentration compared to controls (Figure 4G), confirming that the anterior chamber is smaller than expected and the posterior chamber is larger than expected after MBT exposure.

*3.2.2 Other observed effects*

MBT exposure did not affect survival of zebrafish larvae (one-way ANOVA with Tukey's multiple comparison test, p=0.6117). Although exposure to 0.35 mg/L MBT decreased swimming distance, impaired inflation of the anterior chamber did not further reduce swimming activity when compared to exposed juveniles with inflated anterior chamber (Figure 5A). Furthermore, both length (Figure 5B) and the condition factor (Figure 5C) were reduced in all exposure conditions.

*3.2.3 Analytical measurements*

During the FELS experiment, the mean measured MBT concentrations in fresh medium were within 90 to 100% of the nominal concentrations (0.1 and 0.35 mg/L; 0.1 ± 0, 0.09 ± 0.01 and 0.1 ± 0 mg/L on days 5, 18 and 30 respectively, and 0.34 ± 0.031, 0.35 ± 0.025, 0.35 ± 0.026, 0.33 ± 0.017, 0.33 ± 0.015 and 0.34 ± 0.015 mg/L on days 5, 8, 14, 18, 25 and 30 respectively). After 24 h, measured concentrations did not differ significantly from fresh medium at five dpf, for both 0.1 and 0.35 mg/L. At 18 and 30 dpf, exposure concentrations dropped by 94-95% for 0.1 mg/L and at 8, 14, 18, 25 and 30 dpf, exposure concentrations dropped by 53-69% for 0.35 mg/L (Figure 6A and B). Internal doses in larvae were 1.9 ± 0.9 and 4.73 ± 1.4 µg MBT/g (wet weight) for larvae exposed to 0.1 and 0.35 mg/L MBT, respectively (Figure 6C). Additional experiments without embryos and analyses by LC-MS/MS showed that MBT concentrations remained stable for 24 h, consisting of a 14 h light and 10 h dark cycle (Figure 6D). After adding *Artemia sp.* Nauplii to the medium, no significant differences were observed. However, when paramecia (including the solution used to culture the paramecia; Liquifry) was added to the medium, concentrations dropped by 79%.

**3.3 T3 and T4 measurements**

All tested MBT exposure concentrations higher than 0.1 mg/L significantly decreased whole body T4 concentrations at 120 hpf and 32 dpf ($p < 0.001$ at 120 hpf and $p=0.01$ at 32 dpf; Figures 7A and 7C). There was no effect of MBT exposure on T3 concentrations, although exposure for 32 d to 0.35 mg/L

MBT did increase the amount of variation observed in T3 concentrations (Figures 7B and 7D). Neither T3 or T4 concentrations differed significantly between 32 dpf larvae exposed to 0.35 mg/L MBT with non-inflated versus inflated swim bladders ($p=0.91$ for T4 and $p=0.79$ for T3). There was a strong correlation ($r=0.9083$; $p < 0.0001$) between whole body T4 concentration and the surface of the anterior chamber of the swim bladder at 32 dpf, calculated over all tested exposure concentrations (Figure 7E).

## 4. Discussion

### 4.1 Analytical measurements

The mean measured concentrations in fresh medium were within 90 to 100% of the nominal concentration and remained stable at least until 5 dpf. However, measured concentrations dropped significantly for both tested MBT concentrations after 5 dpf (Figure 6A and B). Although, additional experiments without embryos suggest that the measured concentration of 0.35 mg/L MBT remains stable for 24 h, the addition of paramecia and/or the solution used to culture the paramecia significantly affected the MBT concentration (Figure 6D), suggesting depletion of the MBT. This is in agreement with the stability of MBT until 5 dpf, since no nutrition was needed up to this time point. However, the exposure medium was refreshed daily and internal doses of 1.9 ± 0.9 µg and 4.73 ± 1.4 µg MBT/g for larvae exposed to 0.1 and 0.35 mg/L MBT, respectively (Figure 6C), were observed, indicating MBT uptake in MBT exposed larvae. Furthermore, direct effects of MBT on anterior inflation and size, distance travelled, length and weight were observed, indicating sufficient uptake of MBT to study these effects.

### 4.2 Effects of MBT on swim bladder inflation

#### *4.2.1 MBT does not impair posterior chamber inflation*

The process of early swim bladder inflation, during which the posterior chamber is formed and inflates around 96 hpf, has already been described in detail (Winata et al., 2009). By transferring gas through the pneumatic duct, a connection between the posterior chamber and the foregut, zebrafish can inflate or deflate the swim bladder to regulate buoyancy (Dumbarton et al., 2010; Finney et al., 2006). The inflation process of the swim bladder has been suggested to be mediated by

thyroid hormones in different teleost species (Brown, 1997; Winata et al., 2009). In this study, we observed that MBT exposure did not directly impair posterior chamber inflation, nor did it affect the surface of the posterior chamber (Figure 1). These results are consistent with similar findings after MBT exposure in fathead minnow (Nelson et al., this issue), and have also been observed after methimazole (MMI) exposure, a pharmaceutical specifically designed to inhibit TPO activity (Elsalini and Rohr, 2003; Liu and Chan, 2002).

The lack of effect on posterior inflation can be explained by assuming maternal transfer of T4 into the eggs, which was demonstrated by Nelson et al. (this issue) for fathead minnow. Maternal transfer of THs has been shown to be important for growth and development before the onset of endogenous TH production (Reider and Connaughton, 2014). Reider and Connaughton (2014) suggested that TH-inhibiting compounds only start exerting their effect in zebrafish embryos after the embryonic thyroid gland becomes functional around 60-72 hpf, suggesting a potential insensitivity during early development until depletion of maternally transferred T4 (Alt et al., 2006). Although MBT exposure causes other effects such as delayed hatching (Figure 1A), improper mouth closure (Figure 2D), reduced body pigmentation (Figure 2D), reduced eye pigmentation and size (Figure 2D and 2F) and compromised growth (Figure 2G), our results indicate that embryonic TPO activity is not essential for inflation of the posterior chamber of the swim bladder.

Our results, however, do show a reduction in whole body T4 concentration at 120 hpf (i.e., after the embryonic thyroid gland becomes active at 60-72 hpf, and after the posterior chamber of the swim bladder inflates at 96 hpf), after exposure to 0.35 mg/L MBT or higher. We did not observe a concurrent reduction in T3 levels. Similar results for both T3 and T4 were found in fathead minnow at 6 dpf after exposure to 1 mg/L MBT (Nelson et al., this issue). As previously shown for amphibians and mammals, this confirms that MBT is a potent TPO inhibitor in fish, leading to reduced levels of T4, the hormone produced directly by TPO. T3 present at 120 hpf could therefore be derived from maternally derived T4. In addition, it could be produced from further depletion of any T4 still produced by the thyroid gland (as TPO may not have been fully inhibited at the tested exposure concentrations).

Deiodinase (ID) type 1 (ID1) and type 2 (ID2) are essential to activate T4 (including maternally derived T4) into its biologically active form, T3. If the inflation process of the posterior swim bladder chamber is indeed mediated by thyroid hormones, but maternal T4 transfer is sufficient to compensate for TPO inhibition, we can assume that TPO inhibitors do not impair posterior chamber

inflation, while ID inhibitors do. Indeed, in previous studies (Bagci et al., 2015; Heijlen et al., 2014), we reported that knockdown of ID1+ID2 in zebrafish resulted in impaired posterior chamber inflation. Heijlen et al. (2013) and Dong et al. (2013) used whole mount *in situ* hybridization to show that ID1 and ID2 mRNA is present in zebrafish swim bladder tissue at, but not before, 96 hpf, suggesting a tissue-specific role of T3 in the inflation process of the posterior chamber. This hypothesis is further supported by previously described effects of propylthiouracil (PTU) and methimazole (MMI), two drugs specifically designed to inhibit TPO (Thienpont et al., 2011). These studies report that while MMI shows no direct impact on posterior inflation as expected, PTU does impair posterior inflation. This is in line with studies showing that PTU, but not MMI, inhibits ID1 in addition to TPO (Jomaa et al., 2014; Körhle, 2002; Roy and Mugesh, 2006).

*4.2.2 MBT impairs anterior chamber inflation*

We observed that MBT exposure impairs anterior chamber inflation (Figure 3). At 21 dpf, 91% of control larvae successfully accomplished anterior chamber inflation, while only 34% of larvae exposed to 0.35 mg/L MBT could achieve anterior chamber inflation. More than 20% of the larvae failed to inflate the anterior chamber even at 32 dpf. Therefore, we hypothesize that while TPO inhibitors do not impair posterior swim bladder inflation during early development, they impair anterior chamber inflation at later developmental stages because at later stages the maternally derived THs present during early development are depleted and cannot offset TPO inhibition. On the other hand, since IDs are essential to convert T4 into T3 already during early development, and based on our previous work showing impaired posterior chamber inflation after ID knockdown (Bagci et al., 2015; Heijlen et al., 2014), we hypothesize that ID inhibitors impair posterior chamber inflation and subsequently also anterior chamber inflation since that chamber evaginates from the posterior chamber (See 4.2.1).

After exposure to 0.35 mg/L MBT, T4 levels were significantly deceased at 32 dpf, again showing the inhibitory effect of MBT on TPO. However, there was no effect on T3 concentrations. Since T3 is the biologically active hormone, the unchanged T3 levels could suggest that inflation of the anterior chamber is not dependent on thyroid hormones (and thus, suggesting a potential additional, unknown, mode of action of MBT). First, the significant relationship between T4 levels and the surface of the anterior swim bladder chamber (Fig. 7E) strongly suggests that inflation of that chamber is affected by thyroid hormones. Second, it should be emphasized that whole body T3 was measured. It is widely recognized that local conversion of T4 into its biologically active form T3 by

tissue-specific expression of ID1 and ID2 is the main driver of many thyroid-mediated processes (Bianco and Kim, 2006; Bianco and Silva, 1987; Dentice et al., 2013). Since it is the activity of deiodinases (in addition to the transport of T4 and T3 into the cell) that determines tissue and cellular thyroid levels and not serum thyroid levels, whole body T3 concentrations do not necessarily reflect tissue thyroid levels under different physiological and toxicological conditions. The increased biological variation we observed in T3 concentrations illustrates that at the whole body level, the relationship between T4 and T3 is difficult to predict. Third, in this study T3 levels were measured at the end of the experiment (32 dpf) and were not different from controls at that time point. The anterior chamber of zebrafish inflates around 21 dpf. Nelson et al. (this issue) also observed effects of MBT exposure on anterior chamber inflation in fathead minnow, and they did observe decreased T3 levels after exposure to 1 mg/L at 14 dpf, which corresponds to the time of anterior chamber inflation for this species. This further increases the weight of evidence suggesting that a hypothyroid state is indeed related to the failure of the anterior chamber to inflate. In line with our findings at 32 dpf, they found that at 21 dpf (which is well after anterior chamber inflation for fathead minnow) T3 levels had recovered to control levels. They observed a delay (all anterior chambers eventually inflated), but all inflated swim bladders had a smaller size. However, they observed a compensatory T4 response at 21 dpf (7 d past the normal inflation period in fathead minnow) which was not found in zebrafish at 32 dpf, possibly explaining why not all anterior chambers were inflated in our study.

Several underlying mechanisms could potentially be involved in the effects on anterior chamber inflation and size (Villeneuve et al., 2014). First, gas exchange between the posterior and the anterior chamber could be impaired. The anterior chamber evaginates from the posterior chamber around 20 dpf and inflates with gas from the posterior chamber through the communicating duct, around 21 dpf (Lindsey et al., 2010; Roberston et al., 2007). In the present study, inflated anterior chambers were significantly smaller for the highest tested concentration (consistent with effects in fathead minnow; Nelson et al., this issue), while posterior chambers were significantly larger for both tested concentrations in cases where the anterior chamber was inflated, and even larger when the anterior chamber was not inflated at 0.35 mg/L (Figure 4D and E). The ratio of the anterior and the posterior chamber surface was smaller and the total amount of gas in both chambers did not change after exposure to the highest tested concentration of MBT (Figure 4F and G). These results suggest an altered gas distribution between both chambers, with gas retention in the posterior chamber. Although several aspects of swim bladder function have been studied in various cyprinid species (Smith and Croll, 2011; Zheng et al., 2011), the mechanisms of gas exchange between both chambers are poorly understood. Possibly, TPO inhibition impairs smooth muscle fiber development

in the region of the ductus communicans, as well as in both chambers, which has been suggested to play a role in regulating gas distribution (Lindsey et al., 2011; Winata et al., 2009). Furthermore, a delay in anterior inflation could impair inflation completely, as the ductus communicans progressively narrows and eventually closes (Roberston et al., 2007).

A second potential mechanism involves disruption of the anterior chamber formation itself. The bud of the anterior chamber is already formed during early development, around 64 hpf (Winata et al., 2009). Yin et al. (2011) and Yin et al. (2012) reported that anterior bud formation was affected by a deficiency in hedgehog and Wnt signalling, two pathways that play a critical role in fish swim bladder development. Both pathways have been related to THs in amphibian and rodent species (Kress et al., 2009; Plateroti et al., 2006; Stolow and Shi, 1995), indicating that MBT could alter these pathways leading to inhibited anterior bud formation. MBT could also impair the evagination of the anterior bud, occurring around 20 dpf during which the anterior chamber forms a spherical extension of the posterior chamber (Lindsey et al., 2010; Roberston et al., 2007), resulting in compromised anterior chamber inflation at 21 dpf.

**4.3 Additional effects associated with MBT exposure**

In this study, MBT exposure affected general growth during early and late development (Figure 2G, 5B and 5C), in line with other studies documenting TH balance disruption (Bagci et al., 2015; Brown, 1997). Additionally, disruption of the TH balance can affect endpoints associated with the morphological changes during zebrafish transitions. In this respect, MBT exposure delayed hatching (Figure 1A and A.1) and affected mouth maturation (Figure 2D), two developmental processes in which THs have been shown to play an important role in several species (De Groef et al., 2013; Liu and Chan, 2002; Reddy and Lam, 1991; Shepherdley et al., 2002). It has been reported that exogenous T3 accelerates hatching, while excessive T4 impaired mouth protrusion, resulting in improper mouth closure in zebrafish (Liu and Chan, 2002; Walpita et al., 2007). Furthermore, delayed hatching has been reported in zebrafish as well as in chicken embryos upon MMI and perfluorooctanoic acid exposure, two compounds that have been suggested to be TPO-inhibitors compounds as well (Haba et al., 2011; Jomaa et al., 2014).

In our study, MBT exposure also resulted in severe inhibition of body and retina pigmentation (Figure 2D), as well as in impaired eye development, characterized by a smaller eye surface (Figure 2F). These results are in line with previously described effects of MBT in zebrafish (Choi et al., 2007;

Li et al., 2012), indicating that pigmentation and eye development are TPO/TH-mediated processes. Furthermore, Choi et al. (2007) reported that MBT can also directly inhibit tyrosinase, the key enzyme in melanin synthesis, and affect genes related to pigmentation in the zebrafish retina. Additionally, Walpita et al. (2009) reported a downregulation of tyrosinase mRNA expression in D2 knockdown zebrafish embryos suggesting tyrosinase gene expression may be regulated by THs. It remains to be elucidated which specific mechanisms underlie the effects of THs, and in particular TPO inhibition, on pigmentation in fish. The results of Li et al. (2012) indicate that alterations in this pigmentation pathway do not explain the reduced eye size. They attributed this effect to a tighter packing of the cells in the retina and a smaller lens caused by TPO inhibition in the proximity of the eye region.

**4.4 Ecological relevance of swim bladder inflation impairment**

The volume of the adult swim bladder can be altered to adjust buoyancy, with an additional auditory role for the anterior chamber (Roberston et al., 2007). Therefore, it is likely that impaired inflation of the posterior chamber (and consequently the absence of the anterior chamber) has a severe impact on swimming performance, as has been suggested previously (Bagci et al., 2015; Hagenaars et al., 2014). Although swimming performance is not that critical in a laboratory setting, in a natural environment, it can impact feeding behaviour and predator avoidance, resulting in a lower survival probability. The impact of impaired anterior chamber inflation and size on swimming activity however is less clear in this study. Swimming activity was indeed reduced during early and late development in our study (Figure 2E and 5A). However, our results show that impaired anterior chamber inflation did not account for the observed reduction in swimming activity since, although swimming capacity was reduced after exposure to 0.35 mg/L MBT at 32 dpf, there was no difference between fish with and without inflated anterior chamber (Figure 5A). On the other hand, it has been reported that larvae that fail to inflate their swim bladder use additional energy to maintain buoyancy (Lindsey et al., 2010), possibly contributing to growth reduction and reduced swimming activity. Robertson et al. (2007) suggested that the swim bladder only starts regulating buoyancy actively from 32 dpf onward, indicating that impaired swim bladder inflation possibly affects swimming activity at ages beyond the scope of our study. The observed effects on swimming activity may be a direct consequence of the other observed effects upon MBT exposure, such as eye size or length. They may also be caused by a general disturbance of TH balance or by another yet unknown mechanism of MBT. In the context of AOP development, the relevance of impaired anterior chamber

inflation for survival should be investigated beyond the timeframe of the FELS test. This includes potential impacts on swimming performance, energy budget, and hearing.

### 4.5 Conclusion

In conclusion, MBT does not impair inflation of the posterior swim bladder chamber, but it does affect anterior chamber inflation and size. MBT exposure decreases whole body T4 concentrations but does not affect whole body T3 levels, both within an exposure timeframe corresponding to posterior chamber as well as to anterior chamber inflation. Furthermore, a significant correlation between T4 levels and the anterior chamber surface was observed at 32 dpf. Although the existence of another – yet unknown – mode of action of MBT impacting swim bladder inflation cannot be excluded, our findings suggest that thyroid disruptors impact swim bladder inflation, with an important distinction among specific subtypes of TH disrupting compounds. TPO inhibition only appears to be relevant during late development with respect to swim bladder development, after depletion of maternally derived T4. In contrast, ID inhibition, as shown by our previous knockdown studies, already impairs posterior chamber inflation during early development and, therefore also prevents anterior chamber inflation at later age.

These results can aid in describing AOPs leading from TPO inhibition, ID inhibition and other TH related molecular initiating events to impaired swim bladder inflation in fish during early life stages. Once described, the key events of these AOPs can be used to develop fast and animal-free screening tools for identifying chemicals potentially impacting population status via disruption of the thyroid hormone balance. Potential test systems include *in vitro* enzyme inhibition assays (e.g. TPO and/or ID inhibition assays [Freyberger and Ahr, 2006; Paul et al., 2014]) and *in vivo* zebrafish assays up to 120 hpf. These assays can be useful to predict reduced survival due to impaired posterior chamber inflation, as well as anterior chamber inflation, the latter occurring in a FELS timeframe and therefore not observed in a ZFET test.

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Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Figure 1: Effect on hatching, posterior chamber inflation and surface after MBT exposure. (A) Dose-response curve of hatching (grey line) and mortality (black line) (%) at 120 hpf as a function of the logarithm of the nominal MBT concentration (µg/L). The grey area illustrates the difference between $EC_{50}$ and $LC_{50}$ values ($p < 0.001$). Data originating from the first ZFET experiment. (B) Percentage of successful posterior chamber inflation upon MBT exposure as a function of time (hpf). All larvae were dechorionated at 48 hpf. (C) Dose-response curve of posterior chamber inflation (grey line) and mortality (black line) (%) at 168 hpf as a function of the logarithm of the nominal MBT concentration (µg/L). The grey area illustrates the difference between $EC_{50}$ and $LC_{50}$ values ($p=0.15$). (D) Mean relative posterior chamber surface at 120 hpf (see 2.3.2). Error bars indicate standard deviations. Graph represents data of surviving larvae with inflated posterior chamber at 120 hpf (n is given in parentheses). N/A: 'n' was too small for statistical analysis because of mortality and delayed posterior chamber inflation. Different letters indicate significant differences.

Figure 2: Effects observed after MBT exposure at 120 hpf. All figures are represented as a function of (logarithmic) nominal MBT concentrations. (A) Control larva. (B) Larva exposed to 1.75 mg/L MBT with reduced pigmentation of the eye (*) and body (**) and improper mouth closure (arrow). (C) Larvae exposed to 0, 0.1, 0.35, 0.56, 0.7, 0.88, 1.75 and 3.5 mg/L nominal MBT concentrations with increasing effect on pigmentation of the body and eyes from left to right. (D) Dose-response curve of mortality (black), reduced body and eye pigmentation and improper mouth closure (grey). (E) Mean distance travelled of surviving, hatched larvae. Per condition, two 24-well plates were measured. (F) Mean relative eye surface (see 2.3.2) without dechorionation. Data originating from the first ZFET experiment (red cross: 3.5 mg/L was not included during the first ZFET experiment). (G) Mean larval length of surviving, hatched larvae with inflated posterior chamber at 120 hpf. For the two highest concentrations, 12 larvae were selected at random since the posterior chamber was not yet inflated at this time point. Error bars indicate standard deviations. Graphs represent data of surviving larvae (n is given in parentheses). N/A: no data because of a high percentage of mortality, severe malformations and/or a lack of pigmentation of the eye. Different letters indicate significant differences.

Figure 3: Effects on anterior chamber inflation. Percentage of non-inflated anterior chambers as a function of nominal MBT concentration and days post fertilization. Different letters indicate significant differences in time within a tested condition, as well as among exposure concentrations. Photographs of 32 dpf larvae with and without anterior chamber inflation are represented in figure 4A and B.

Figure 4: Posterior and anterior swim bladder surface of MBT exposed zebrafish larvae at 32 dpf. All measurements are expressed as relative surfaces (see 2.4). Photographs represent 32 dpf larvae. (A) Control larva with inflated posterior and anterior chamber (100% inflation at 32 dpf). (B) Larva exposed to 0.35 mg/L MBT with inflated posterior chamber and impaired anterior chamber inflation (78% inflation at 32 dpf, white arrow). (C) Larva exposed to 0.35 mg/L with overinflated posterior and partly inflated anterior chamber (white arrow). (D) Mean relative surface of the posterior chamber. (E) Mean relative surface of the anterior chamber. (F) Sum of the relative surface of both chambers. (G) Ratio of the relative surface of the anterior and posterior

chamber. Error bars indicate standard deviations. Graphs represent data of surviving larvae (n is given in parentheses). Different letters indicate significant differences.

Figure 5: Other observed effects after MBT exposure during 32 dpf. Larvae with and without anterior chamber inflation were analysed separately. (A) Mean distance travelled at 26, 29 and 30 dpf. (B) Mean total length. (C) Mean condition factor. Error bars indicate standard deviations. Graphs represent data of surviving larvae (n is given in parentheses). Different letters indicate significant differences.

Figure 6: MBT measurements in both medium and larvae. Mean measured MBT concentrations in medium (A and B) are represented as a function of time (dpf). Right y-axes show percentages of nominal MBT concentrations, with 0.1 mg/L (A) and 0.35 mg/L (B) as 100% (dotted line). Black bars represent fresh medium, grey bars represent samples taken before renewal after 24 h. (C) Dose measurements of pooled larvae. Replicate values are given with mean ± SD (black line). (D) Stability of MBT during 24 h in light (14/10 h light/dark) and dark (24 h) without the presence of embryos. The nominal concentration is indicated by the dotted line (0.35 mg/L MBT, 100%). Bars represent mean MBT concentrations after 24 h. Error bars indicate standard deviations. Different letters indicate significant differences (n is given in parentheses).

Figure 7: Whole body T3 and T4 concentrations after 120 hours (A, B) and 32 days (C, D) of exposure to MBT. Since T3 and T4 concentrations did not differ between 32 dpf larvae exposed to 0.35 mg/L MBT that did, or did not have inflated swim bladders, this exposure concentration is depicted as a single group. Replicate values are given with mean ± SD (black line). Different letters indicate significant differences (n is given in parentheses). (E) Correlation between T4 concentration and the surface of the anterior chamber of the swim bladder at 32 dpf.

Figure 1

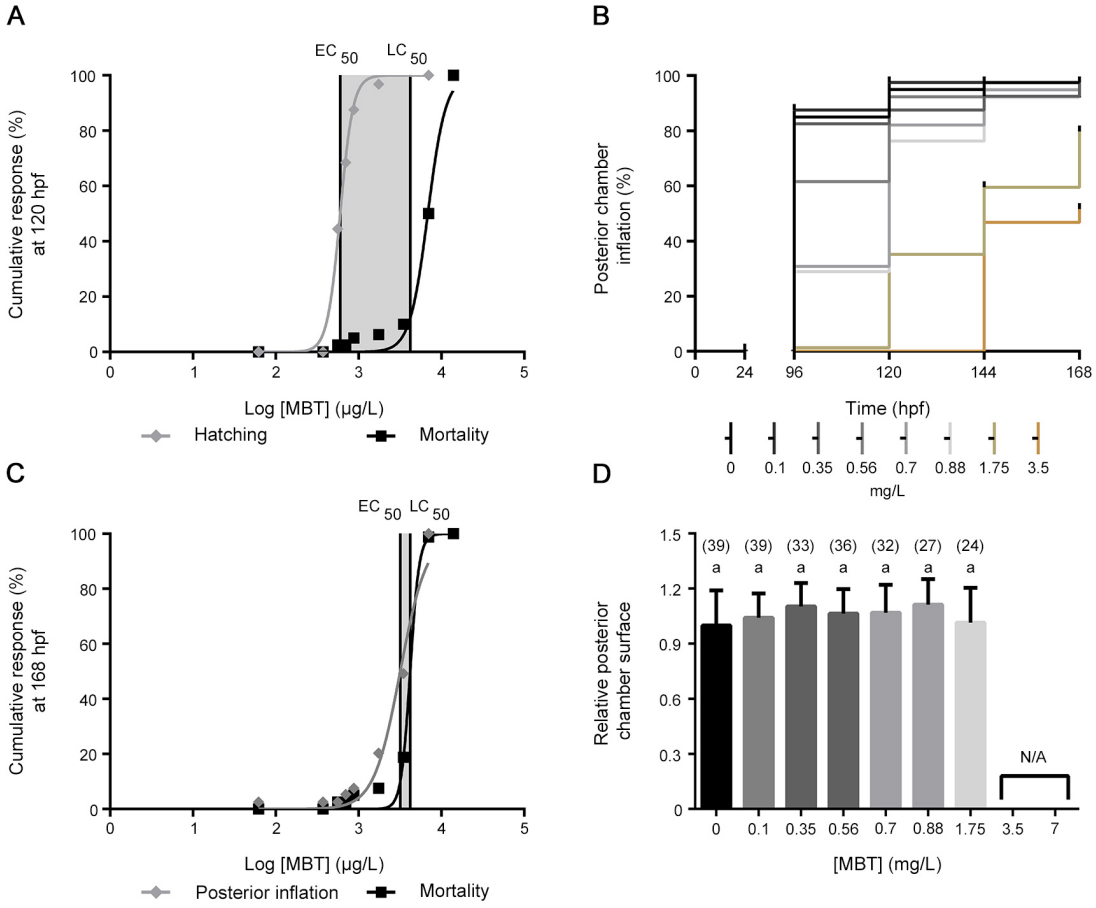

A
EC 50
LC 50
Cumulative response (%) at 120 hpf
Log [MBT] (µg/L)
Hatching
Mortality
B
Posterior chamber inflation (%)
Time (hpf)
0
0.1
0.35
0.56
0.7
0.88
1.75
3.5
mg/L
C
EC 50
LC 50
Cumulative response (%) at 168 hpf
Log [MBT] (µg/L)
Posterior inflation
Mortality
D
Relative posterior chamber surface
(39) (39) (33) (36) (32) (27) (24)
a a a a a a a
N/A
[MBT] (mg/L)

Figure 2

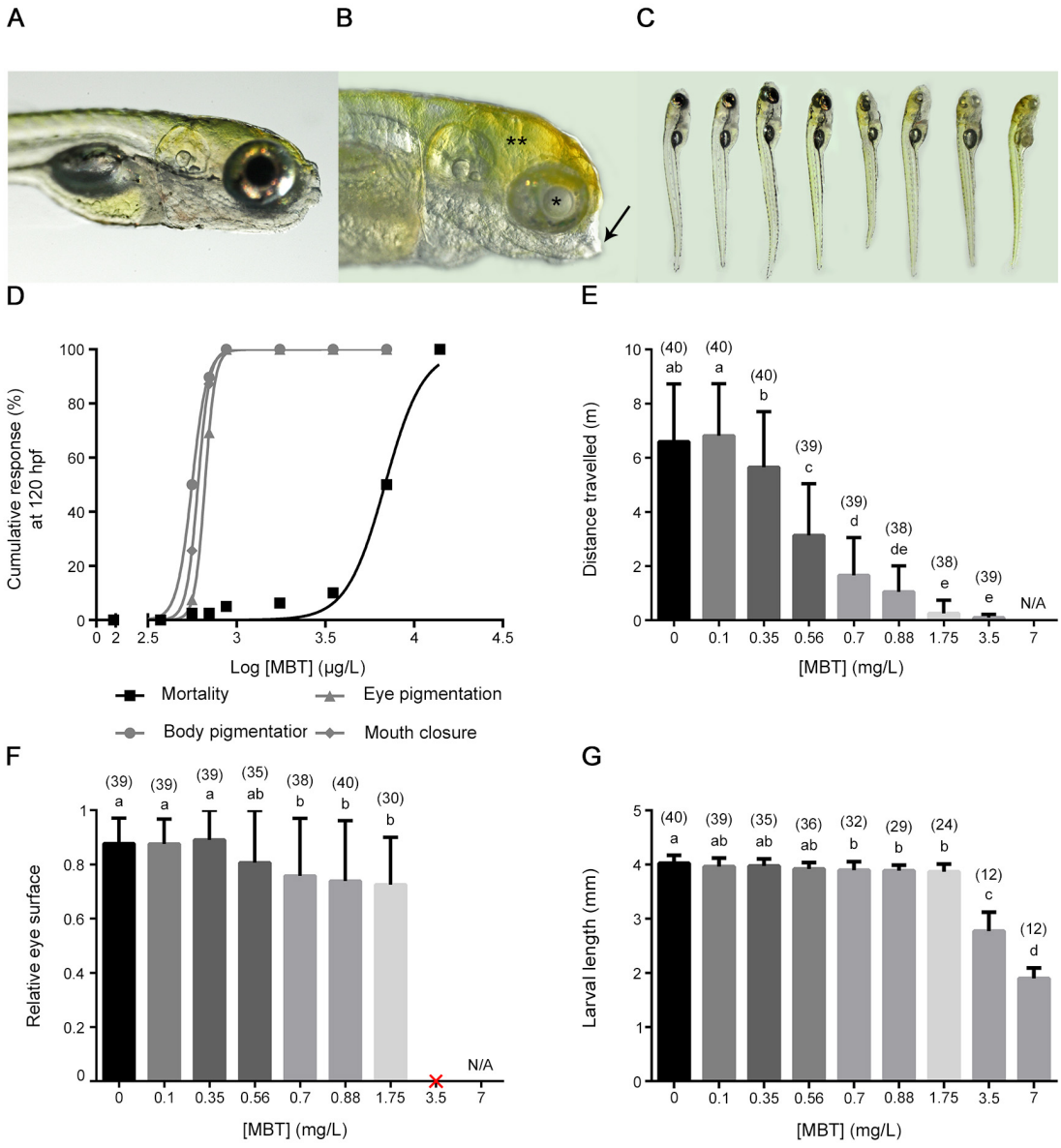

A
B
C
**
*
D
Cumulative response (%) at 120 hpf
Log [MBT] (µg/L)
Mortality
Eye pigmentation
Body pigmentatior
Mouth closure
E
Distance travelled (m)
[MBT] (mg/L)
F
Relative eye surface
[MBT] (mg/L)
N/A
G
Larval length (mm)
[MBT] (mg/L)

Figure 3

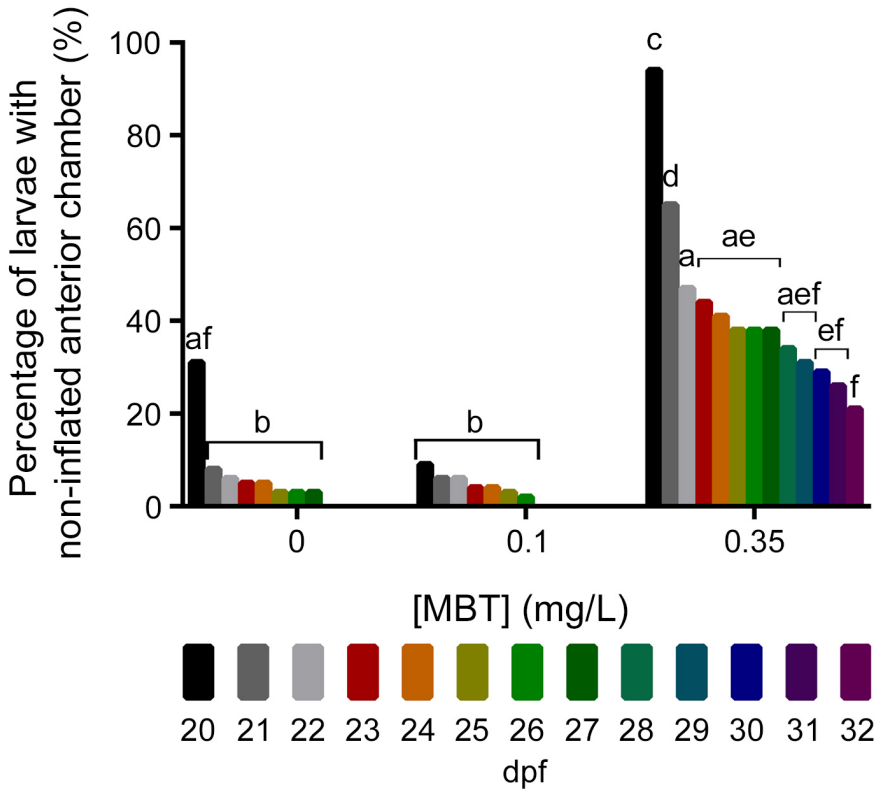

Percentage of larvae with non-inflated anterior chamber (%)
100
80
60
40
20
0
af
b
b
c
d
a
ae
aef
ef
f
0
0.1
0.35
[MBT] (mg/L)
20 21 22 23 24 25 26 27 28 29 30 31 32
dpf

Figure 4

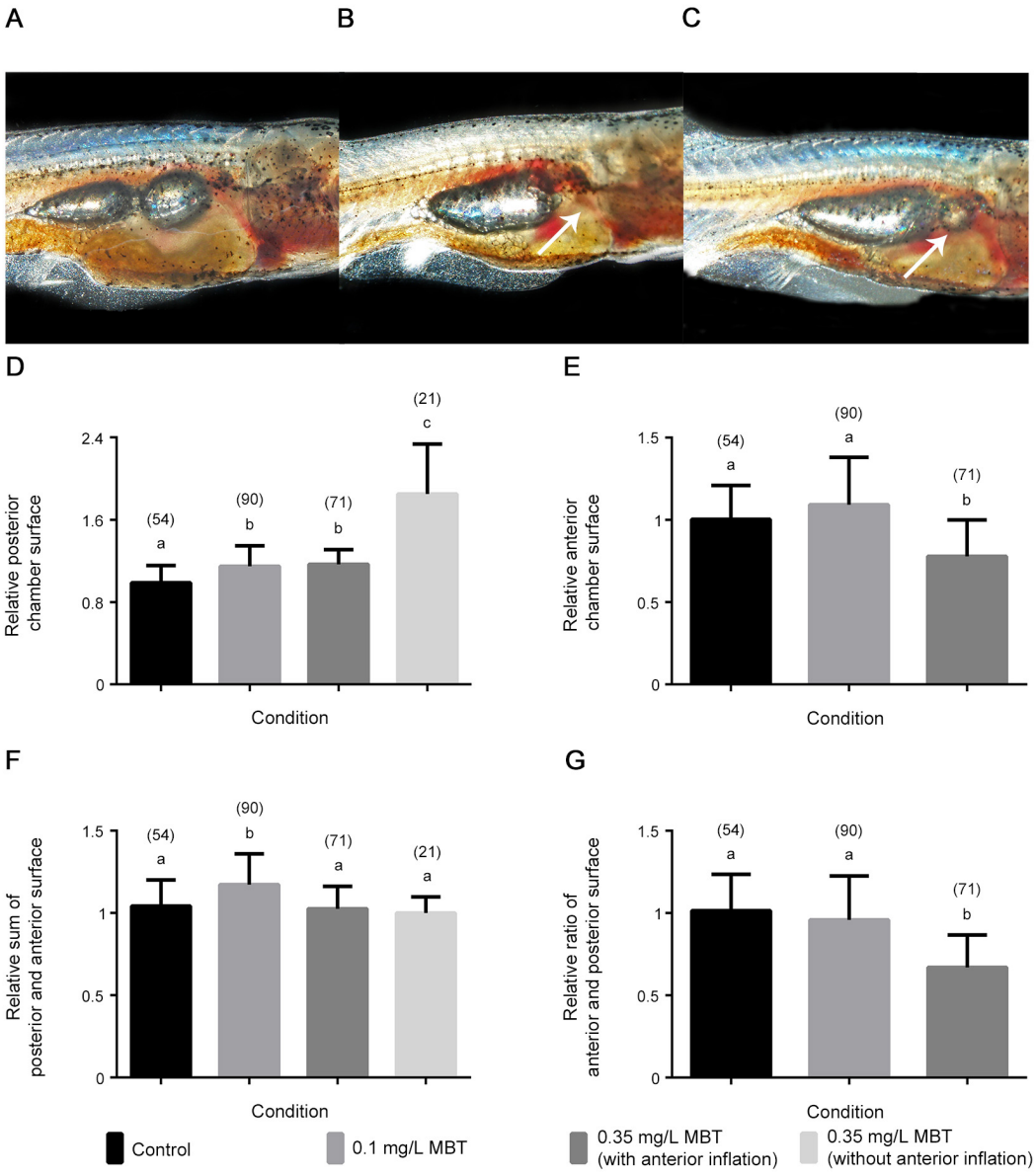

A
B
C
D
Relative posterior chamber surface
2.4
1.6
0.8
0
(54) a
(90) b
(71) b
(21) c
Condition
E
Relative anterior chamber surface
1.5
1
0.5
0
(54) a
(90) a
(71) b
Condition
F
Relative sum of posterior and anterior surface
1.5
1
0.5
0
(54) a
(90) b
(71) a
(21) a
Condition
Control
0.1 mg/L MBT
G
Relative ratio of anterior and posterior surface
1.5
1
0.5
0
(54) a
(90) a
(71) b
Condition
0.35 mg/L MBT (with anterior inflation)
0.35 mg/L MBT (without anterior inflation)

Figure 5

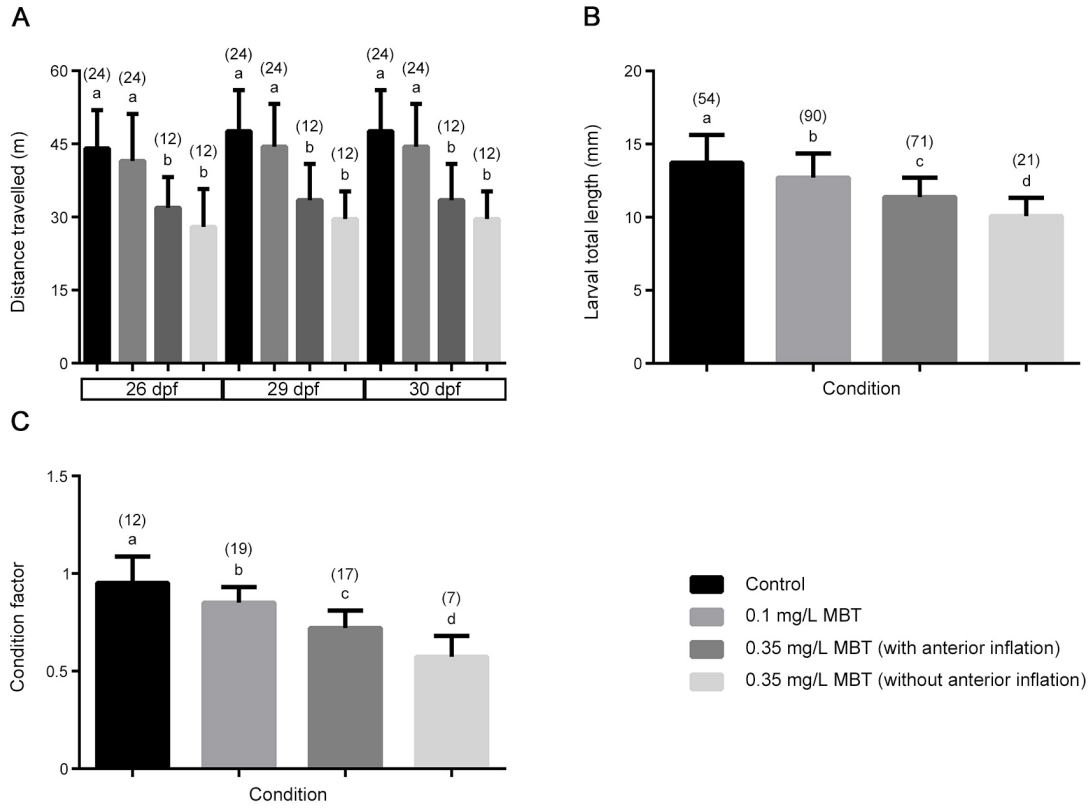

A
Distance travelled (m)
60
45
30
15
0
(24) a
(24) a
(12) b
(12) b
(24) a
(24) a
(12) b
(12) b
(24) a
(24) a
(12) b
(12) b
26 dpf
29 dpf
30 dpf
B
Larval total length (mm)
20
15
10
5
0
(54) a
(90) b
(71) c
(21) d
Condition
C
Condition factor
1.5
1
0.5
0
(12) a
(19) b
(17) c
(7) d
Condition
Control
0.1 mg/L MBT
0.35 mg/L MBT (with anterior inflation)
0.35 mg/L MBT (without anterior inflation)

Figure 6

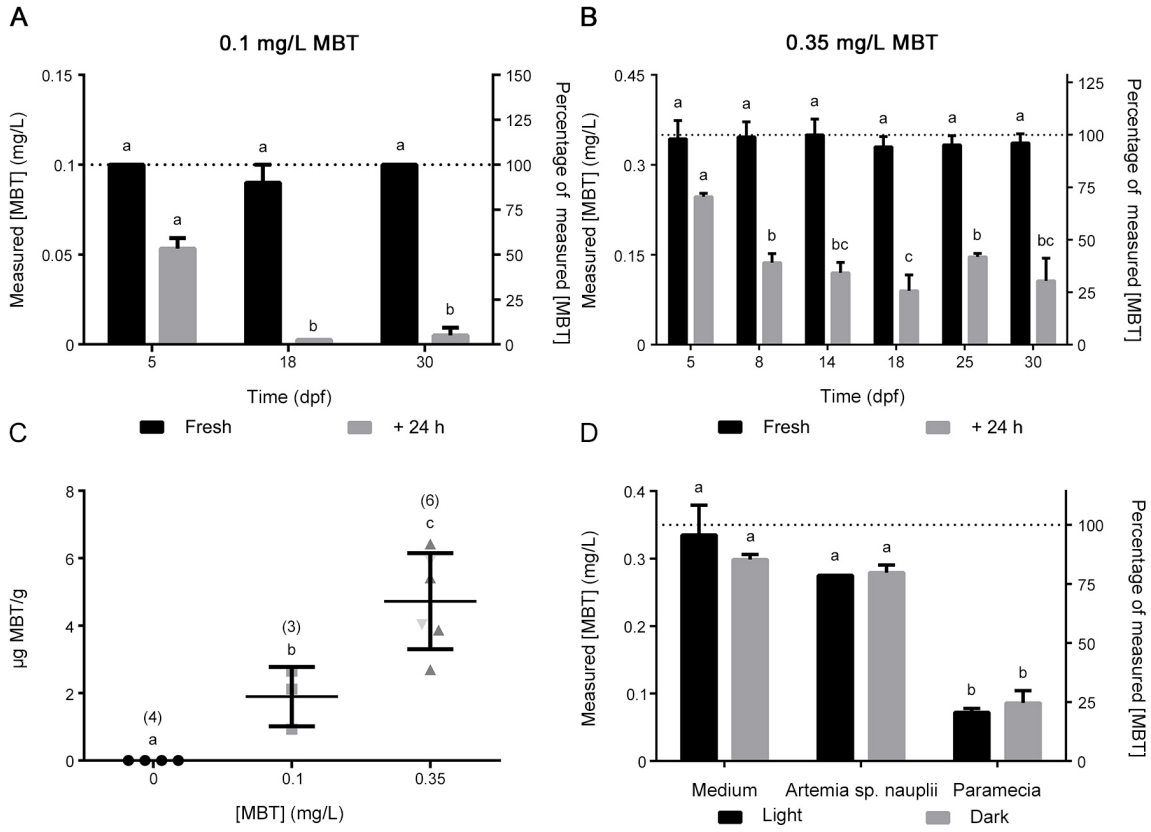
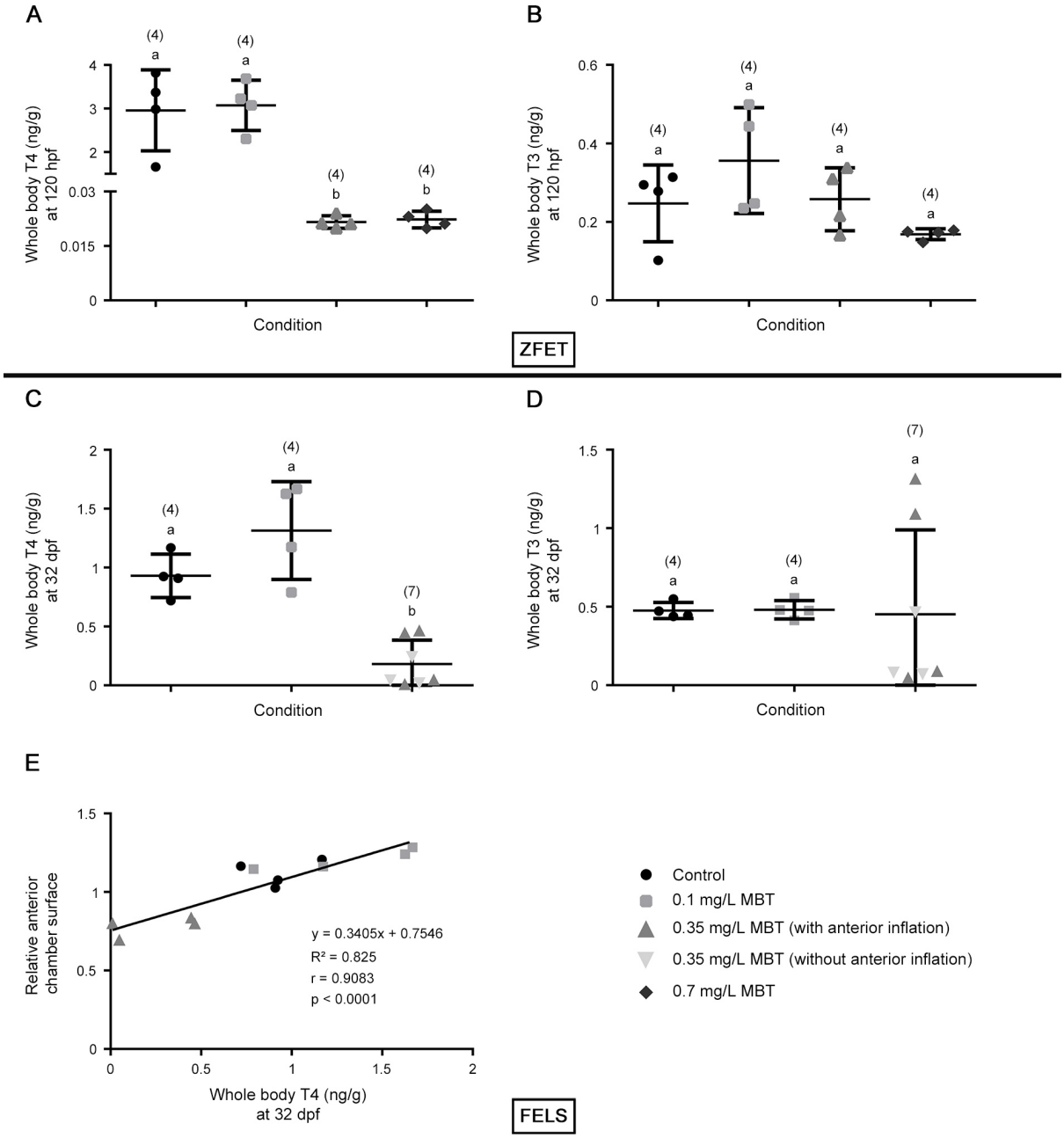

A
0.1 mg/L MBT
Measured [MBT] (mg/L)
Percentage of measured [MBT]
Time (dpf)
Fresh
+ 24 h
B
0.35 mg/L MBT
Measured [MBT] (mg/L)
Percentage of measured [MBT]
Time (dpf)
Fresh
+ 24 h
C
µg MBT/g
[MBT] (mg/L)
D
Measured [MBT] (mg/L)
Percentage of measured [MBT]
Medium
Artemia sp. nauplii
Paramecia
Light
Dark

Figure 7


A
Whole body T4 (ng/g) at 120 hpf
Condition
B
Whole body T3 (ng/g) at 120 hpf
Condition
ZFET
C
Whole body T4 (ng/g) at 32 dpf
Condition
D
Whole body T3 (ng/g) at 32 dpf
Condition
E
Relative anterior chamber surface
Whole body T4 (ng/g) at 32 dpf
y = 0.3405x + 0.7546
R² = 0.825
r = 0.9083
p < 0.0001
Control
0.1 mg/L MBT
0.35 mg/L MBT (with anterior inflation)
0.35 mg/L MBT (without anterior inflation)
0.7 mg/L MBT
FELS