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Triclosan exposure results in alterations of thyroid hormone status and retarded early development and metamorphosis in *Cyprinodon variegatus*

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ABSTRACT

Thyroid hormones are critically involved in somatic growth, development and metamorphosis of vertebrates. The structural similarity between thyroid hormones and triclosan, an antimicrobial compound widely employed in consumer personal care products, suggests triclosan can have adverse effects on the thyroid system. The sheepshead minnow, *Cyprinodon variegatus*, is now used in ecotoxicological studies that have recently begun to focus on potential disruption of the thyroid axis by endocrine disrupting compounds. Here, we investigate the *in vivo* effects of exposure to triclosan (20, 50, and 100 $\mu g\,L^{-1}$) on the thyroid system and the embryonic and larval development of *C. variegatus*. Triclosan exposure did not affect hatching success, but delayed hatching time by 6–13 h compared to control embryos. Triclosan exposure affected the ontogenetic variations of whole body thyroid hormone concentrations during the larval phase. The T3 peak around 12–15 dph, described to be indicative for the metamorphosis climax in *C. variegatus*, was absent in triclosan-exposed larvae. Triclosan exposure did not produce any deformity or allometric repatterning, but a delayed development of 18–32 h was observed. We conclude that the triclosan-induced disruption of the thyroid system delays *in vivo* the start of metamorphosis in our experimental model. We observed a global developmental delay of 24–45 h, equivalent to 4–7% prolongation of the developmental time in *C. variegatus*. The costs of delayed metamorphosis can lead to reduction of juvenile fitness and could be a determining factor in the outcome of competitive interactions.

1. Introduction

Numerous chemicals present in aquatic environments affect thyroid hormone synthesis, transport, cellular uptake and metabolism and can ultimately disrupt thyroid homeostasis (Boas et al., 2006; Brown et al., 2004). Among these is triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), a synthetic chlorinated phenoxyphenolic compound with a generalized use as an antimicrobial and preservative in many personal care and household products (Bedoux et al., 2011; Dann and Hontela, 2011; Fang et al., 2010). Currently, widespread contamination with triclosan has been detected in aquatic ecosystems of several countries (Bedoux et al., 2011; Dann and Hontela, 2011; Fang et al., 2010). Triclosan is commonly measured in various environments and it is detected in the majority of US rivers, making this one of the most frequently encountered organic contaminants (Kolpin et al., 2002). The structural similarity between triclosan and thyroid hormones (Dann and Hontela, 2011) suggests it can have adverse effects on the thyroid system. Toxic effects of triclosan have been reported in aquatic organisms (Bedoux et al., 2011; Dann and Hontela, 2011; Fang et al., 2010). However, little is known about the mechanisms by which triclosan disrupts the thyroid axis.

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In teleost fishes, thyroid hormones (THs) are involved in many physiological processes such as growth, pigmentation and the development of cardiovascular, digestive and muscular systems (Blanton and Specker, 2007; Power et al., 2001). Metamorphosis, *i.e.* the transition from larval to juvenile stage, is also regulated by THs (McMenamin and Parichy, 2013; Paris and Laudet, 2008; Power et al., 2001) and involves morphological, physiological and behavioural changes (Dufour et al., 2012) The mechanisms and genes involved have still not yet been fully elucidated but it has been hypothesized that a disruption of thyroid system influences the fish metamorphosis, and thus the viability of larvae (Power et al., 2001). These early developmental stages might be the most sensitive to environmental toxicants that target the thyroid axis.

Landmark-based geometric morphometric methods (Bookstein, 1997; Rohlf and Marcus, 1993; Zelditch et al., 2012) provide valuable tools to analyse variations of shape and size during ontogeny. Conceptually, the pattern of ontogenetic shape changes can be described by the trajectory of an organism in the shape space plus the rate at which it proceeds along the trajectory (Klingenberg and McIntyre, 1998) (Fig. 1). In an ecotoxicological context, some pollutants can induce developmental and morphological divergence among organisms as a result from the directional change in the trajectories of shape changes (*i.e.* ontogenetic repatterning), in the duration or in the rate of development (Webster and Zelditch, 2005) (Fig. 1).

The sheepshead minnow *Cyprinodon variegatus* is widely used in ecotoxicological studies that have only recently begun to focus on potential disruption of the thyroid axis by xenobiotics and endocrine disrupting compounds. The present study aims to quantify the effects of exposure to triclosan on *C. variegatus* during embryonic and larval development. First, we investigated several parameters of embryonic development such as fertility ratio, hatch ratio, and timing of hatching. Secondly, we determined if and how triclosan affects ontogenetic variation of thyroid hormones in developing larvae. Thirdly, knowing that thyroid hormones are involved in somatic growth and metamorphosis, we tested the hypothesis that triclosan alters the pattern of ontogenetic shape changes (*i.e.* ontogenetic trajectory, rate of shape variation) using geometric morphometrics. Using these combined approaches, we illustrate in *C. variegatus* that triclosan exposure alters THs concentrations, and induces a delay of development and metamorphosis without ontogenetic repatterning.

2. Materials and methods

2.1. Animals, fertility ratio, hatch ratio and hatching delays

Adult *C. variegatus* were purchased from Aquatic Research Organisms (ARO Inc. New Hampshire, USA) in April 2013. Males and females were maintained in 150-L glass aquaria in aerated, filtered seawater at a salinity of 20 ± 1, temperature at $26 \pm 1\,^{\circ}C$, at a photoperiod of 14 h:10 h (L:D) and fed daily with brine shrimp (*Artemia* nauplii) and flake food (Sera Vipan).

Groups of 2 males and 3 females were paired in 5 spawning tanks with dimensions (length × width × height) of the main body measuring 350 mm × 250 mm × 200 mm for 2 h (Cripe et al., 2009) during 10 breeding sessions. Embryos were collected from spawning tanks. Approximately 2000 undamaged embryos were selected using a dissection microscope and randomly assigned to each of four treatment groups: Control, 20, 50 and 100 $\mu g\,L^{-1}$ triclosan (Irgasan > 97%, Sigma-Aldrich). Among these, three breeding sessions including 522 eggs in total were especially selected to evaluate hatching rhythms and the influence of the experimental treatments. These embryos were placed in 24-well plates with 1 mL volume of test solution to follow the individual evolution of each embryo (3 replicate breeding sessions, with two replicate well plates per treatment). The plates were placed in an incubator (BCR-25, Jiangsu Best Electrics Co., Ltd) at 26 °C and the test solutions were refreshed daily to avoid oxygen deficiency. The number of newly hatched larvae was registered every hour from 91 h post-fertilization (hpf) until 163 hpf. At the end of the experiment, the total number of hatched larvae was recorded in order to calculate the hatching ratio. The other eggs (ca. 1450 from 7 breeding sessions) were placed in petri dishes with 50 mL working volume per and incubated in groups of 50 embryos in an incubator (BCR-25, Jiangsu Best Electrics Co., Ltd) at 26 °C. Incubation media were changed every 24 h and the number of dead embryos and live fry were recorded in order to calculate the fertility and hatch ratios. After 6 days, the embryos hatched and larvae were transferred to a glass tank (1 L working volume, 50 larvae per tank).

Larval density and feeding rate were controlled and identical for each exposure group. From day 0 post-hatch (dph), larvae were fed with cultured brine shrimp. We refreshed 100% of the medium every 3 days for all exposure conditions in order to maintain a constant exposure load and to avoid water quality degradation by excess food and excrements. The concentration of triclosan in water was analysed using a commercial Abraxis Triclosan Assay kit purchased from Abraxis LLC (Warminster, USA). The Abraxis Triclosan Assay is an immunosorbent enzyme-linked assay (ELISA) designed for the quantification of triclosan and methyl triclosan. The triclosan concentrations in our experimental media were analysed when freshly prepared and immediately prior to renewal at five occasions during the experimental period (Supplementary material Table 1). The measured concentrations remained within 80–120% of the nominal concentrations, so that the effect concentration can be expressed relative to the nominal concentration according to the OECD Guidelines for testing chemicals (OECD, 2013).

Juvenile fish were netted, and rapidly euthanized in overdose of MS222 (500 $mg\,L^{-1}$) at 9, 12, 15, 18 and 21 dph and randomly assigned either to thyroid hormone (round 1200 individuals from five replicate breeding sessions) or to geometric morphometric analysis (250 individuals from three replicate breeding sessions).

2.2. Thyroid hormone extraction and analysis

Whole body concentrations of thyroid hormones show a typical pattern during the metamorphosis of teleost fishes, indicating that the ontogeny of thyroid hormones is related to specific morphological characteristics that represent early development (Chang et al., 2012; Crane et al., 2004; de Jesus and Hirano, 1992; Johns et al., 2009; Kawakami et al., 2003; Klaren et al., 2008; Shiao et al., 2008; Szisch et al., 2005; Yamano, 2005). In *C. variegatus*, metamorphosis is associated with increases in thyroid hormone levels around 9 and 18 days post hatching (Schnitzler et al., 2015). Samples of 10–20 larvae were placed in preweighed 1.5-mL microfuge tubes, and wet weights recorded prior to snap-freezing on dry ice and storage at −80 °C. Larval samples were dried at 60 °C to constant dry weight (d.w.). Thyroid hormones were extracted as described by Tagawa and Hirano (1987). Samples (0.01 g larval d.w.) were homogenized in 2.6 mL ice-cold 99:1 (vol./vol.) methanol:ammonia containing 1 mM of the iodothyronine deiodinase type-1 inhibitor 6-*n*-propyl-2-thiouracil (PTU). Homogenate and extraction medium were thoroughly mixed for 10 min at 4 °C, and then centrifuged at 2000 x *g* (15 min, 4 °C). This procedure was repeated twice, supernatants were pooled and lyophilized. The residue was resuspended in 875 µL of a 6:1 vol./vol. mixture of chloroform and 99:1 methanol:ammonia including 1 mM PTU, and 125 µL barbital buffer (50 mM sodium barbitone in distilled water, at pH 8.6). Samples were mixed for 10 min at room temperature. The upper phase was aspirated and lyophilized at 45 °C. Residues were redissolved in 60 µL barbital buffer contain-