The effects of triclosan exposure in shape changes of sheepshead minnow (*Cyprinodon variegatus*) during early development and metamorphosis

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Introduction

Thyroid hormones are essential for normal development, and for maintenance of normal physiological functions in vertebrates [1, 2]. In fish, thyroid hormones are involved in the control of osmoregulation, metabolism, somatic growth and post-hatching metamorphosis [1, 3, 4]. The regulation of thyroid hormone bioavailability in tissues and cells represents a very complex and unique web of feedback systems [2]. In fish and other vertebrates the thyroid cascade involves two components. First, thyroxine (T4) biosynthesis and secretion are largely under central control by the brain–pituitary–thyroid axis [5]. Second, there is the conversion of T4 to its biologically active form 3,5,3-triiodothyronine (T3) and its metabolism and receptor-mediated actions that seems largely to be under peripheral control in extra-thyroidal tissues [6].

The accumulation in the aquatic environment of anthropogenic chemicals, among which are endocrine disrupting chemicals (EDCs) that alter normal hormonal regulation, is having dramatic consequences for humans and wildlife. Numerous chemicals disrupt thyroid homeostasis affecting thyroid hormone (TH) synthesis and transport, and cellular uptake and metabolism [7, 8].

Triclosan (TCS) is a synthetic chlorinated phenolic compound with a generalized use as an antimicrobial and preservative in many personal care and household products [9-11]. As a result of disposal of TCS through sewage systems and insufficient/variable removal by wastewater treatment plants (WWTP) [9], widespread contamination with TCS has been detected in several countries, particularly in aquatic ecosystems, WWTP influents and effluents; sludges and biosolids; surface or ground water; drinking water; and aquatic sediments [9-11]. TCS and its metabolites have been detected in tissues and body fluids of aquatic organisms including fish, revealing they are accumulating in the food chain [9-11] and TCS has also been detected in human blood, breast milk and urine [9-11].

The structural similarity of TCS with THs [9] suggest it may have adverse effects on the thyroid system. However, little is known about the mechanisms by which TCS disrupts the thyroid axis. TCS effects on fish thyroid axis have not been investigated. It is possible, that the TCS toxic effects reported in fish embryos, larvae and adults [9-11] might be caused, at least in part, through its effect on the thyroid system. We determined how TCS affect ontogenic variations of thyroid hormones in developing sheepshead minnow larvae. Knowing that thyroid hormones are involved in somatic growth and post-hatching metamorphosis, we also tested the hypothesis that TCS alter the development of these larvae. To do this, we used landmark-based geometric morphometric methods. These methods allowed us to analyse the pure shape variations of our developing larvae, regardless orientation, position, and size.

Materials and methods

Animals

Adult sheepshead minnows (*Cyprinodon variegatus*) were purchased from Aquatic Research Organisms (ARO Inc. New Hampshire, USA). Males and females were maintained in 150L glass aquaria at 26°C, at a photoperiod of 14h :10h (L :D) and fed daily with frozen brine shrimp (Artemia nauplii) and flake food. Couples of 2 males and 3 females were paired in spawning boxes for 2 hours [12]. Embryos (1500) were collected from spawning tanks. Embryos were selected under a dissection microscope and randomly assigned to each of four treatment groups: Control, 20 µg/L TCS, 50 µg/L TCS and 100 µg/L TCS and placed in an incubator (BCR-25, Jiangsu Best Electrics Co., Ltd) at 26°C. On day 0, embryos hatched and larvae were transferred to a glass tank (1 litre

working volume, 50 larvae per tank). From 0 day post-hatch (dph) on larvae were fed on cultured brine shrimp, and from 14 dph on they were also fed on flaked fish food. Samples consisting of 10-40 larvae were taken on the time points indicated in the figures. Juvenile fish were netted from tanks, rapidly killed in MS222 (500 mg/L), placed in 1.5 ml microfuge tubes, and wet weights recorded prior to snap-freezing on dry ice and storage at -80°C. All sampling was carried out between 08:00 and 10:00 GMT.

Thyroid hormone extraction and analysis

Larval samples were dried at 60 °C to constant dry weight. Thyroid hormones were extracted as described by Tagawa and Hirano (1987) [13]. Samples (0.01 g larval dry weight) were homogenized in 2.6 ml ice-cold 99:1 (vol./vol.) methanol:ammonia containing 1 mM of the iodothyronine deiodinase inhibitor 6-n-propyl-2-thiouracil (PTU). Homogenate and extraction media were thoroughly mixed for 10 min at 4 °C, and then centrifuged at 2000g (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 containing 0.1% bovine serum albumin. Aliquots of 25 and 50 µl were taken for T4 and T3 analysis, respectively. Total T4 (tT4) and T3 (tT3) concentrations were measured in duplicate with a competitive ELISA (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. Calibrators were prepared in the same barbital buffer matrix as the samples. A 4-parameter calibration curve was calculated with the immunoassay software Gen5.

Geometric morphometrics

Shape changes were studied using landmark-based geometric morphometric methods [14-16]. An extensive introduction to applications of geometric morphometrics in biology is provided by [17] and [18]. A geometric morphometric analysis involves a series of steps, which are briefly described here. The Cartesian coordinates of a configuration of anatomical landmarks first capture the form of an organism. The removal of differences in orientation, position, and size allows pure shape to be analysed. This was achieved in our study by optimally superimposing landmark configurations using a process called generalized Procrustes analysis (GPA), which is based on a least-squares algorithm [19]. During this superimposition, a consensus configuration (average) of landmarks is calculated and will be used as reference. Centroid size (CS) is a measure of the dispersion of landmarks around their centroid and is computed as the square root of the sum of squared distances of all landmarks from the centroid. The new Cartesian coordinates obtained after the superimposition are the Procrustes shape coordinates used for statistical comparisons of individuals. Their Procrustes distance can summarize the shape differences between landmark configurations of two individuals, which is the square root of the sum of squared distances between pairs of corresponding landmarks. The rate of change in the overall body shape was estimated using this Procrustes distance (PD) [15]. This distance was used as a univariate measure of shape difference, but needs to be considered as an overall measure of multivariate shape components.

Statistical tests

All data are presented as means ± standard deviation. Statistics were performed using R (version 3.1.1) [20]. All data were tested for normality. Kruskall–Wallis one-way analysis of variance on ranks was used with nonparametric multiple comparisons for each pair using Wilcoxon methods. Statistical significance was accepted at $p < 0.05$.

Results and discussion:

Each spawning female produced 15 to 30 embryos per spawn; fertilization could be assessed 24 hours after spawning by counting the number of opaque (fertile) and white (nonfertile) embryos. Fertilization rates were low and variable (from 40% to 60%), but the hatching rates of fertilized eggs measured after an incubation time of 6 days were high (86 ± 6 %).

We characterized previously the ontogenic variation of thyroid hormones in embryos and larvae of sheepshead minnows. We observed fluctuations of thyroid hormones level around the 12th and the 15th day post hatching (dph), that may be associated with the transition from larval to juvenile stage during the development of this species. A clear decrease of T4 levels were observed, while T3 levels raised to a peak, as well as an increase in the whole body T3/T4 ratio that is indicative of an increase in outer ring deiodination. These peaks represent the

onset of metamorphosis, the larvae gained considerably in weight and changed from larvae to the juvenile aspect from day 9 dph on. We concluded, that this period could be defined as a critical exposure window to pollutants.

We made the same measurements with Triclosan exposed larvae on these critical dates between 12 and 15 days post hatching. For the control larvae, we can see the same trends, decreasing T4 and peaks in T3 and T3:T4 ratios. The exposed larvae showed a different pattern. Here the T4 levels are rising up to the 15th dph and no fluctuations in T3 levels can be observed (Fig. 1). Fluctuations of thyroid levels during this critical phase may have an effect on development. Therefore we assessed the shape changes during early development and metamorphosis.

The growth of developing sheepshead minnows is highly significant allometric. These results are well supported by the Goodall test (all $P < 0.05$), which means that there is a linear relation between centroid size and shape variables during the whole ontogenic dataset. The significant regression models in shape space account for 30%. The low percentage of variance explained indicates that some variability in shape is possibly due to factors other than size or that the ontogeny could be nonlinear. So, as expected, the larva changes in shape during early development.

Then we wanted to know if the Triclosan exposed larvae differed in shape compared to the control ones. The analysis of the angles between multivariate regression vectors of ontogenetic allometries within- and between exposure groups showed that, the angle between Control and Triclosan exposed larvae is lower than the ranges of the within- exposure group angles. The angles between multivariate regression vectors are not significantly different. Consequently, the null hypothesis of an identical direction to the ontogenetic vectors cannot be rejected. The control larvae and the Triclosan exposed larvae grow thus to the same shape.

But do they attain the shape in the same speed? Control larvae show higher shape change rate values than Triclosan exposed larvae. The procrustes distances between the average shapes at 9 dph and at 21 dph show that the ontogenetic trajectory is always longer in Control larvae than in Triclosan exposed larvae, the former undergoes more shape changes during the ontogeny (Fig. 2). The shape changes are thus stronger and faster in control larvae compared to Triclosan exposed larvae.

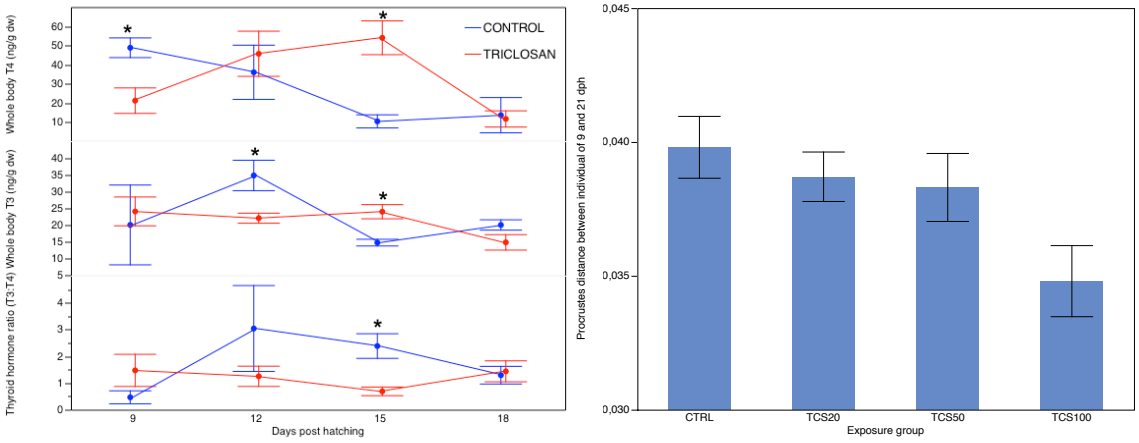

Fig. 1 (left): Whole-body thyroxine, expressed per gram dry body weight (top panel), Whole-body triiodothyronine, expressed per gram dry body weight (middle panel) and Molar ratios of whole body total T3 to total T4 concentrations (expressed in mole per gram dry body weight) (bottom panel), during the development of sheepshead minnows. Control larvae are drawn in blue and Triclosan exposed larvae are drawn in red. Data are expressed as means ± SD. Asterisks(*) indicate significant differences between Control and Triclosan exposed larvae.

In conclusion, we observe that Triclosan alter thyroid hormone levels during the critical period of development. Although the exact mechanisms by which TCS alters the thyroid system functioning remains to be established, a potential effect on outer ring deiodinase can be expected. We saw also that these alterations induce lower shape

changes in triclosan-exposed larvae. That means that these fish stay longer in an larvae shape and take more tme in the larvae-juvenile transition. The importance of the thyroid in basic physiological processes such as metabolism and development means that interference with this axis may have profound consequences for organism health and survival, and the results of the present study highlight the need for more detailed studies of the effects of TCS, which accumulates in sediments and organisms in aquatic environments.

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