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Effects of 17β-trenbolone on Eastern and Western mosquitofish (*Gambusia holbrooki* and *G. affinis*) anal fin growth and gene expression patterns

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ABSTRACT

The Eastern and Western mosquitofish (*Gambusia holbrooki* and *G. affinis*) are potential bioindicator organisms for endocrine disruptors. Male mosquitofish have an elongated anal fin (gonopodium) used for internal fertilization whose formation is driven by androgens. Normal female mosquitofish have a normal, rounded anal fin which undergoes elongation into a gonopodium structure when female mosquitofish are exposed to androgenic chemicals. Significant issues with using mosquitofish as a bioindicator include the lack of knowledge on how anal fin growth in females corresponds to endpoints relevant to biological integrity and the lack of information on the molecular pathways that regulate anal fin growth. The objectives of this study were to understand how androgen-induced anal fin elongation relates to changes in endpoints related to the female reproductive system and to understand how anal fin elongation occurs in androgen-exposed female mosquitofish. To achieve these objectives, adult female *G. holbrooki* were exposed to a vehicle control or one of three doses of the androgen 17β-trenbolone (TB) at nominal concentrations of 0.1, 1 or 10 μg TB/L. Anal fin measurements were taken and livers were used for quantitative polymerase chain reaction analysis of *vitellogenin* (*vtg*) mRNA expression at multiple time points. 10 μg TB/L induced anal fin elongation after 7 days of treatment (one-way ANOVA, $p < 0.05$) as did 0.1 and 1 μg TB/L at later time points (one-way ANOVA, $p < 0.05$). 10 μg TB/L significantly reduced hepatic *vtg* gene expression at all time points assessed (one-way ANOVA, $p < 0.05$). There was no correlation between anal fin elongation levels and *vtg* gene expression (Spearman's ρ, $p > 0.05$). In a separate experiment, female *G. holbrooki* and *G. affinis* were exposed to the vehicle control or 1 μg TB/L. Anal fins were used for qualitative gene expression analysis of the genes *sonic hedgehog* (*shh*), *muscle segment homeobox C* (*msxC*), and *fibroblast growth factor receptor 1* (*fgfr1*) by *in situ* hybridization. *Shh* was expressed in the distal tip of the gonopodium while *msxC* and *fgfr1* were more widely expressed along the same anal fin rays during androgen exposure. These data provide insight into the molecular pathways involved in anal fin elongation and pave the way for future work toward developing the mosquitofish into a bioindicator organism for endocrine disruptors.

1. Introduction

Endocrine disrupting compounds (EDCs) can negatively impact the normal function of the endocrine system. Wild animal populations exposed to EDCs exhibit a range of conditions including abnormal thyroid function, impaired immune responses, formation of abnormal secondary sexual characteristics, decreased hatching success, and population declines (Colborn et al., 1993; Marty et al., 2011). While there is data on the occurrence of EDCs in the environment, a complete evaluation of their impacts on ecosystems has yet to be conducted. One method for evaluating the biological effects of pollutants in the environment is by the use of a bioindicator, an organism with quantifiable biological endpoints that indicate exposure to or the effects of chemicals in the environment. These bioindicator organisms can then be used to evaluate the biological integrity of environments impacted by pollutants (US EPA, 2011).

The Eastern and Western mosquitofish (*Gambusia holbrooki* and *G. affinis*) are candidate bioindicator organisms for evaluating the impacts of EDCs in the environment. Mosquitofish are globally distributed in freshwater systems and are small in size (Pyke, 2005), making them ideal for long-term and widespread sampling efforts. In addition, the dimorphic secondary sexual characteristics of mosquitofish make them an attractive bioindicator organism candidate specifically for EDCs. Upon reaching sexual maturity, male mosquitofish develop an extension of the 3rd, 4th, and 5th anal fin rays with specialized hooks and serrae at the tip. This structure is called the gonopodium and is used by males for internal fertilization of female mosquitofish (Turner, 1941). Normal female

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mosquitofish do not have any anal fin elongation but when exposed to androgens they will exhibit anal fin growth (Angus et al., 2001; Ogino et al., 2004; Sone et al., 2005; Stanko and Angus, 2007; Turner, 1941). This led to the hypothesis that the androgens produced by the male testis are responsible for initiating anal fin growth. Female mosquitofish with elongated anal fins have also been found downstream of three pulp and paper mills in Florida (Bortone and Cody, 1999; Bortone and Drysdale, 1981; Howell et al., 1980) and more recently in China (Hou et al., 2011) but the causative chemicals and ecosystem-level implications of this abnormal physiological masculinization have yet to be elucidated.

Previous studies determined that the androgens 11-ketotestosterone (11-KT), methyl testosterone (MT), androstenedione (ADE), and 17β-trenbolone (TB) could induce anal fin elongation in adult female Western mosquitofish (*G. affinis*) (Angus et al., 2001; Sone et al., 2005; Stanko, 2005; Stanko and Angus, 2007). However, the impacts of androgen exposure on female reproductive health have not been thoroughly evaluated for this species. Understanding if there is a quantifiable relationship between androgen-induced anal fin growth and the deleterious impacts of androgens on the female reproductive system would provide insight into whether this organism can be used as a predictive bioindicator for the higher-level effects of EDC exposure such as population declines. While it is known that androgens cause anal fin elongation, the molecular pathway of anal fin elongation is not known, and such information on the basic biology of anal fin growth is crucial for future bioindicator development and usage.

Prior studies within the family *Poeciliidae* determined the expression patterns of three genes in anal fins after androgen treatment. *Sonic hedgehog* (*shh*) was up-regulated in the basal epithelium of the distal portion of the gonopodial rays (the 3rd, 4th, and 5th anal fin rays) in *G. affinis* fry after androgen treatment (Ogino et al., 2004). There was also an increase in the expression of the genes *muscle segment homeobox C* (*msxC*) and *fibroblast growth factor receptor 1* (*fgfr1*) in the gonopodial rays of juvenile green swordtail (*Xiphophorus hellerii*) and southern platyfish (*X. maculatus*) during androgen exposure (Offen et al., 2008; Zauner et al., 2003). Developing molecular tools and biomarkers is important for evaluating the impacts of chemical exposure as part of the *Toxicity testing in the 21st Century* report (National Research Council, 2007). These predictive biomarkers and bioindicators can also become an important part of ecological risk assessments in the future by incorporation of molecular data into an Adverse Outcome Pathways (AOP) framework (Ankley et al., 2010).

With the long-term goal of developing the mosquitofish into a bioindicator organism for EDCs, the objective of this study was to determine the impacts of androgen exposure on mosquitofish gene expression patterns. The two working hypotheses were 1) 17β-trenbolone (TB) exposure would decrease *vtg* gene expression while inducing anal fin elongation due to its androgenic and anti-estrogenic activities, and 2) TB exposure would induce expression of the genes *shh*, *msxC*, and *fgfr1* in the anal fin. To evaluate these hypotheses, adult female mosquitofish (*G. affinis* and *G. holbrooki*) were exposed to TB. Anal fin elongation measurements and mRNA expression of *vtg* were quantified across multiple time points and doses of TB, and spatial gene expression of *shh*, *msxC*, and *fgfr1* in the anal fin was determined using *in situ* hybridization at multiple time points with a single dose of TB.

2. Materials and methods

2.1. Animals and chemical treatments

To evaluate the two hypotheses, two separate exposures were conducted using sexually mature (as determined by the presence

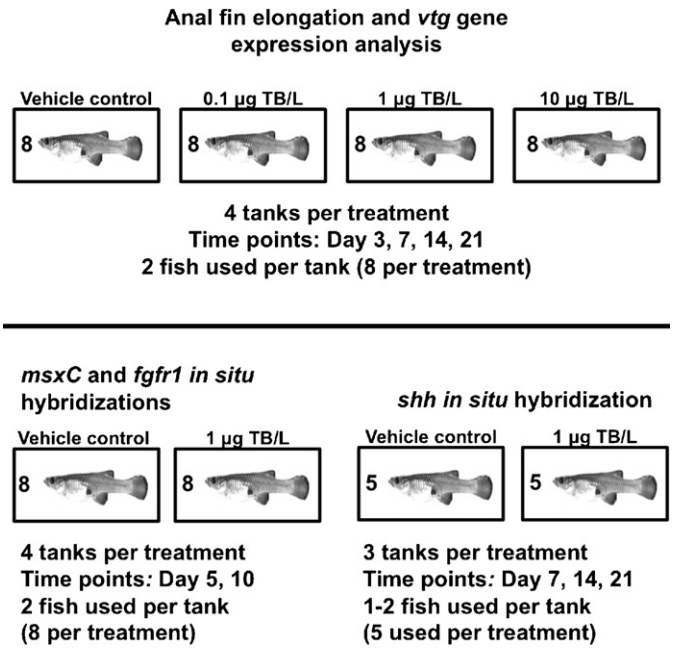

Fig. 1. Experimental schematic for the mosquitofish exposures conducted for evaluating the two hypotheses. For the anal fin elongation and *vtg* gene expression experiment, four tanks per treatment were utilized for a total of 128 female *G. holbrooki* to evaluate the first hypothesis. Two *G. holbrooki* were sampled from each tank in all four of the treatment groups at days 3, 7, 14, and 21. For the *msxC* and *fgfr1 in situ* hybridizations experiment, four tanks per treatment were utilized for a total of 32 female *G. holbrooki* to evaluate the second hypothesis. Two *G. holbrooki* were sampled from each tank in both of the treatment groups at days 5 and 10. For the evaluation of *shh* also as part of the second hypothesis, the experiment was conducted using *G. affinis* with five females housed in one tank with three tanks per treatment. Two to three *G. affinis* were sampled from each tank in both of the treatment groups at days 7, 14, and 21.

of the gravid spot near the vent) adult female mosquitofish (*G. holbrooki* or *G. affinis*) >3 months of age; see Fig. 1 for an outline of the experimental design and numbers of fish utilized. *G. affinis* was utilized for *shh* gene expression evaluation because this work was conducted at the Okazaki Institute for Integrative Bioscience in Japan where only this species of mosquitofish is present. For analyzing anal fin elongation and *vtg* gene expression, *G. holbrooki* from laboratory stocks were transferred to aerated 7 L glass tanks and exposed *via* semi-static renewal (60% water change daily) to either the vehicle control (ethanol, 0.0014% final concentration) or one of three doses (0.1, 1 or 10 μg/L) of the potent androgen receptor (AR) agonist TB (17β-hydroxyestra-4,9,11-trien-3-one) (Steraloids, Newport, USA). These doses were chosen based on a previous study with *G. affinis* (Sone et al., 2005) while covering the range of waterborne levels for the isomer 17α-trenbolone (0.12 μg/L) albeit not of the 17β form (20 ng/L) (Durhan et al., 2006) from concentrated animal feedlot operations. Water temperatures were monitored every day and 100 mL water samples were collected from two TB tanks per dose every four days and stored at 4 °C until sample preparation for high-performance liquid chromatography (HPLC) analysis. To determine how TB influenced bone segment formation and anal fin differentiation (formation of hooks and serrae structures at the tip), the aforementioned experiment was repeated by housing individual *G. holbrooki* in 1.2 L of water and exposing these fish *via* semi-static-renewal methods (with a 100% water change daily) to the vehicle control, 1 μg TB/L, or 10 μg TB/L for 21 days. For evaluating anal fin gene expression, *G. holbrooki* (for *fgfr1* and *msxC*) or *G. affinis* (for *shh*) were exposed using the aforementioned methods but only with the vehicle control and 1 μg TB/L treatments and no HPLC samples were collected. For the *shh in situ* hybridizations, adult female *G. affinis* were housed in 1 L plastic tanks with 5 fish

per tank and 3 tanks per treatment and a 100% water change and re-dosing was completed every other day. Fish were fed brine shrimp flake food once daily (Brine Shrimp Direct, Ogden, USA) and all fish were treated in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Sample preparation and chemical analysis

Mosquitofish were anesthetized using Tricaine-S (Western Chemical, Ferndale, USA) and sacrificed *via* spinal transection. Body length, anal fin ray 4 and ray 6 lengths, and oocyte developmental stage (as determined using the stages described by Haynes, 1995) were assessed upon dissection of the *G. holbrooki* samples that were used to evaluate *vtg* gene expression and anal fin growth. Livers were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. For the bone segment evaluation and anal fin *in situ* hybridization gene expression analysis, the posterior portion of the fish (from gonad to caudal fin) was removed and fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Fixed samples were washed in PBS with 0.1% tween (PBT) and dehydrated using increasing concentrations of methanol in PBT (25/50/75/100%) before storing in 100% methanol at −20 °C until bone segment evaluation or *in situ* hybridization.

Water samples collected for HPLC analysis were filtered using Whatman paper to remove any detritus. Samples were concentrated with 6 mL sampliQ C18 columns (Agilent, Santa Clara, USA) after being conditioned with 5 mL of methanol and 5 mL of milli-Q water using a vacuum manifold system. Water samples were added to the column to maintain a flow rate of 1–2 mL/min before they were eluted with 5 mL of methanol, evaporated to dryness with nitrogen, reconstituted in 250 μL methanol, and stored at 4 °C. TB concentrations were determined by HPLC with fluorescent and UV light detection using the Series 200 HPLC and LC240 fluorescence detector (PerkinElmer, Massachusetts, USA). In brief, 100 μL of the sample was injected into a 150-mm long and 4.6 mm diameter C-12 column with 4 μm particle size (Phenomenex, Torrance, USA) by an isocratic method with an acetonitrile/methanol/water mixture (60/8/32). The HPLC run time was 6 min with a 1.5 min wash step between each sample. For UV detection, the excitation and emission wavelengths were 359 and 458 nm respectively. A standard curve of serially-diluted TB in methanol (made using 8 standards ranging from 10 ppb to 10 ppm) was analyzed during each run. Chromatographic analysis of the standards and samples was conducted using TotalChrom software (PerkinElmer, Massachusetts, USA). Under the aforementioned conditions, the TB peak was visible at 2.9 min. The detection limit was 10 ppb and the lowest TB dose (0.1 μg TB/L) was concentrated to 40 ppb using the aforementioned method.

2.3. RNA isolation and real-time quantitative PCR

RNA was isolated from frozen liver samples using TRIzol reagent (Invitrogen, Grand Island, USA) following the manufacturer's protocol. In brief, livers were homogenized in 1 mL TRIzol and after a 5 min incubation at room temperature, 200 μL chloroform was added and the upper aqueous layer containing the RNA was separated from the organic layer *via* high-speed centrifugation. The RNA was precipitated out of the aqueous layer using isopropanol and washed twice with ethanol. After drying, RNA was rehydrated using RNAsecure Reagent (Ambion, Grand Island, USA) and quality and quantity of the resulting RNA sample was determined spectrophotometrically by the Nanodrop (ThermoScientific, Waltham, USA). Only samples with a 260/280 ratio greater than 1.80 were used for subsequent analyses. Any remaining genomic DNA was removed by DNase treatment (Ambion, Grand Island, USA). 2 μg of the DNase-treated RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Fitchburg, USA) and stored at −20 °C until gene expression analysis.

Real-time quantitative polymerase chain reaction (qPCR) analysis of *vtg* and *L8* mRNA expression was conducted using the myiQ single color real-time PCR detection system and iQ5 software (Bio-Rad, Hercules, USA). *L8* is a protein of the large ribosomal subunit and has been demonstrated to be an effective and consistently expressed housekeeping gene for fish exposed to EDCs (Filby and Tyler, 2007). The average variability seen in the *L8* signal was 8.19% (range 3.96%-13.05%). 2 ng of reverse transcribed RNA was PCR-amplified in 96-well plates with SYBR Green Supermix (Bio-Rad, Hercules, USA) using qPCR primers designed with the Primer3 software (Rozen and Skaletsky, 2000) (see table S1 in supplemental materials for sequences of primers). Gene copy number was determined by the absolute standard curve method using serially-diluted plasmids containing a cloned partial sequence of the genes that were analyzed on each qPCR plate. A negative template control (water only) was analyzed on each plate to determine whether non-specific products formed during the PCR amplification. All samples, standards, and negative controls were analyzed in duplicate using a two-step protocol with an initial denaturation at 95 °C for 3 min and 40 cycles of 95 °C for 10 s followed by a 1 min annealing step at 58 °C for *vtg* and at 60 °C for *L8*. Standard curve efficiencies and slopes ranged from 86.4% to 103.6% and from −3.70 to −3.24 respectively. A final melting curve with a gradually increasing temperature gradient for 30 s intervals from 55 to 95 °C was analyzed to verify that a single PCR product was amplified on each plate. Amplification data was analyzed using iQ software (BioRad, Hercules, USA). The signal threshold was placed within the logarithmic area of the amplification plots and these data were used to calculate a sample copy number based on the values of the standards.

2.4. In situ hybridization

A modified protocol by Thisse et al. (1993) was utilized for the whole-mount *in situ* hybridization analysis. In brief, samples were re-hydrated by washing with decreasing concentrations of methanol in PBT before treatment with 50 μg/mL of proteinase-K for 45 min. The proteinase-K reaction was stopped using 4% PFA and then samples were washed with PBT. After incubation with pre-hybridization buffer (50% formamide, 5× saline sodium citrate (SSC), 0.1% tween-20, and 0.05 mg/mL heparin, and 0.5 mg/mL tRNA), an anti-sense digoxigenin-labeled RNA probe (1 μg/mL for *shh* and 2 μg/mL for *msxC* and *fgfr1*; see table S1 in supplemental materials for GenBank IDs of probe sequences) synthesized by Sp6/T7 *in vitro* transcription (Roche, Penzberg, Germany) was added to each sample and incubated overnight at 60 °C. After a series of washes with tRNA-free hybridization buffer/SSC and blocking buffer (2 mg/mL bovine serum albumin and 2% fetal bovine serum), samples were incubated with 1/2000 (v/v) anti-digoxigenin alkaline phosphatase antibody (Roche, Penzberg, Germany) in 1× maleic acid buffer, 2% blocking reagent (Roche, Penzberg, Germany), and 5% fetal bovine serum (ClonTech, Mountain View, USA) overnight at 4 °C in the dark. Samples were washed in PBT and alkaline phosphate buffer (NTMT) before incubation with the BM purple AP substrate (Roche, Penzberg, Germany). Once color developed, the alkaline phosphatase reaction was stopped using PBT and PFA. Microscopy photos were taken using the MZ FLIII microscope (Leica Camera, Solms, Germany) and visualized using the Leica Application Suite software v 3.6 (Leica Microsystems, Wetzlar, Germany) after sample preservation.

2.5. Statistical analysis

Anal fin growth was measured as the ratio of the length of the 4th anal fin ray divided by the length of the 6th anal fin ray, which

corresponds to the termination of the gonopodium in males and the tip of the gonopodium respectively. These data were not normally distributed and because log-transformation still did not result in a normal distribution, a Kruskal–Wallis one-way analysis of variance (ANOVA) was utilized for the log-transformed anal fin ratio data. For anal fin elongation ANOVA results with $p < 0.05$, the Dunn's follow-up test was utilized to determine treatments that were significantly different from the time point controls. For qPCR data, the *vtg* copy number was divided by the *L8* copy number for each sample and then multiplied by the average *L8* value for each time point, resulting in the final *vtg* copy number. All of these data were log-transformed to achieve a normal distribution before conducting a one-way ANOVA. For *vtg* qPCR ANOVA results with $p < 0.05$, the Bonferroni follow-up test was utilized to determine treatments that were significantly different from the time point controls. To determine if there was a correlation between *vtg* gene expression and anal fin elongation, non-log transformed data were analyzed using the Spearman's rank correlation coefficient test (ρ). All statistical results were considered significant at a p-value < 0.05.

3. Results

3.1. Experimental parameters

Average water temperatures during the 21 day *G. holbrooki* TB exposure (±standard deviation) were 22.7 ± 1.6 °C (control), 22.8 ± 1.5 °C (0.1 μg TB/L), 22.0 ± 1.4 °C (1 μg TB/L), and 21.9 ± 1.4 °C (10 μg TB/L). There was a significant difference between the temperature of the control tanks and the 10 μg TB/L tanks (one-way ANOVA with Bonferroni correction, $p = 0.019$). Mortality rates for the 0.1 and 10 μg TB/L treated fish were significantly greater than for the controls in the first experiment (χ^2 test, $p < 0.05$). It was noted on day 5 of the 21 day *G. holbrooki* TB exposure that the sides of the tank became filmy and the water cloudy while fish from one of the four tanks of the 0.1 μg TB/L and the 10 μg TB/L treatments exhibited signs of stress. After these early mortalities, a change in the tank cleaning procedure was introduced with scrubbing conducted during each water change and a 100% water change conducted every 5 days. This allayed the negative effects that initially arose from housing the fish in a semi-static system. The final number of mosquitofish sampled at each time point and treatment is indicated within the bar graphs in Figs. 2 and 4. In the bone segmentation and *in situ* hybridization exposures, which included the aforementioned protocol modifications, there was only one mortality in each of the treatment groups. The average concentrations of TB (±standard deviation) as determined by HPLC were 0.13 ± 0.04 μg TB/L, 1.17 ± 0.34 μg TB/L, and 16.38 ± 3.67 μg TB/L; concentrations throughout the remainder of the article are reported as the nominal concentrations (0.1, 1, or 10 μg TB/L). Spike-in studies indicated that the recovery of TB from water samples using the methods described was 77%.

3.2. Anal fin growth

Administration of TB *via* waterborne semi-static renewal induced anal fin elongation, which was monitored as the ratio of the length of the 4th anal fin ray divided by the length of the 6th anal fin ray (Fig. 2). The anal fin ratio of the mosquitofish exposed to the high TB dose (10 μg TB/L) was significantly greater than the controls at day 7 ($p < 0.05$), the middle dose (1 μg TB/L) was significantly greater than the controls at days 14 and 21 ($p < 0.05$), and the lowest dose (0.1 μg TB/L) was significantly greater than the controls at day 14 ($p < 0.05$). Anal fin growth was also evaluated by determining the number of bone segments in the 3rd ray of the anal fin and the presence of hooks and serrae were also monitored. As

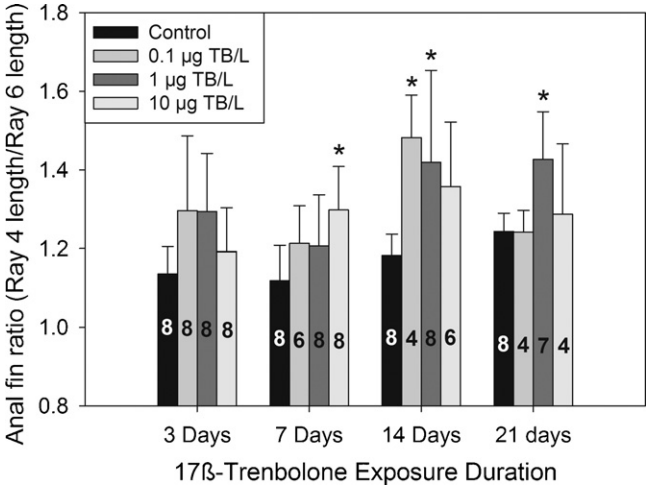

Fig. 2. Anal fin ratios during 17β-trenbolone (TB) exposure. Female Eastern mosquitofish (*G. holbrooki*) were exposed to either one of three doses of TB (0.1, 1, and 10 μg TB/L) or the vehicle control (ethanol) for 3, 7, 14, or 21 days. Anal fin measurements were taken at each time point and the anal fin ratio was calculated as the total fin length (ray 4) divided by the base of the anal fin (ray 6, where the gonopodium structure ends in males). Each bar represents the mean of each group and error bars represent standard deviation. An asterisk indicates statistical significance from the time point control and numbers inside each bar represent the number of female mosquitofish sampled at each time point.

demonstrated in Fig. 3, the highest dose (10 μg TB/L) caused a significant increase in the number of bone segments compared to the controls after 21 days of exposure ($p = 0.032$). No hooks or serrae were observed after 21 days of exposure.

3.3. Vitellogenin gene expression

Hepatic *vtg* gene expression was decreased during TB exposure (Fig. 4). Treatment with 10 μg TB/L decreased hepatic *vtg* gene expression at all time points measured ($p < 0.05$) and 1 μg TB/L decreased hepatic *vtg* gene expression at day 14 ($p < 0.05$); this was also the same time point that anal fin elongation was significantly increased over the controls at this dose (Fig. 2). When these data

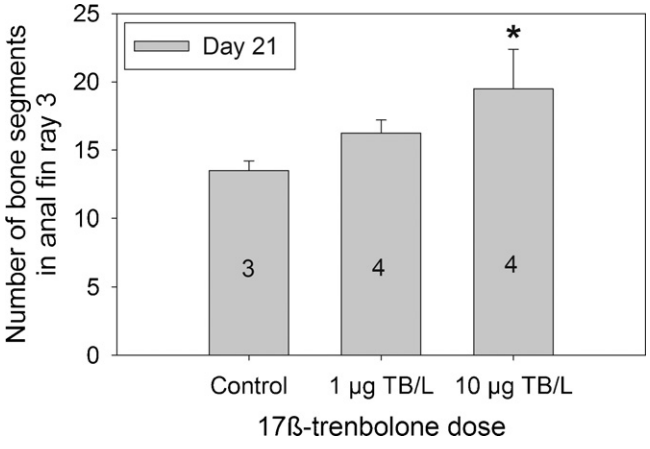

Fig. 3. Number of bone segments in the 3rd ray of the anal fin during 17β-trenbolone (TB) exposure. Female Eastern mosquitofish (*G. holbrooki*) were exposed to either 1 μg TB/L, 10 μg TB/L, or the vehicle control (ethanol) for 21 days. Anal fins were removed upon dissection and preserved overnight in 4% paraformaldehyde in phosphate buffered saline and bone segments were counted using microscopy on the following day. Each bar represents the mean of each group and error bars represent standard deviation. An asterisk indicates statistical significance from the control samples and the number inside each bar represents the number of female mosquitofish sampled.

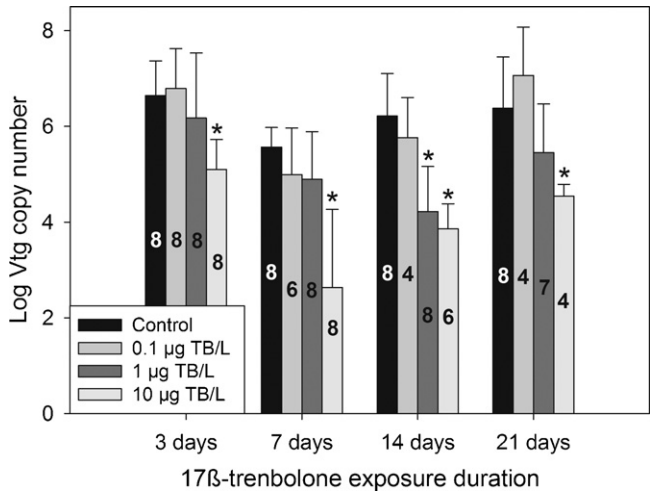

Fig. 4. *Vitellogenin* (*vtg*) gene expression during 17β-trenbolone (TB) exposure. Female Eastern mosquitofish (*G. holbrooki*) were exposed to either one of three doses of TB (0.1, 1, and 10 μg TB/L) or the vehicle control (ethanol) for 3, 7, 14, or 21 days. Hepatic *vtg* gene expression was quantified using real-time quantitative PCR (qPCR). Data are presented as log-transformed hepatic *vtg* expression normalized by the hepatic expression of the housekeeping gene *L8*. Each bar represents the mean of each group and error bars represent standard deviation. An asterisk indicates statistical significance from the time point control and numbers within each bar represent the N for each time point and treatment.

were analyzed *post hoc* by separating samples by oocyte development stage, a similar trend can be seen (Fig. S1 in supplemental materials), albeit with a reduction in the comparisons with statistical significance as a result of a reduced number of samples for each comparison, since not all stages of oocyte development were well-represented at each time point.

While TB was able to induce anal fin elongation and decrease *vtg* gene expression, a quantitative correlation between the anal fin ratio and *vtg* gene expression was not observed (Spearman's ρ test, $p > 0.05$). Spearman's rank correlation tests were conducted using all of the data combined, data separated by either time point or dosage, and data separated by both time point and dosage. The only significant correlations that were found were in the positive direction and were for data that were separated by both time point and TB dose: 1 μg TB/L at day 7 (Spearman's $\rho = 0.874$, $p < 0.001$) and 1 μg TB/L at day 14 (Spearman's $\rho = 0.898$, $p < 0.001$).

3.4. *shh, msxC, and fgfr1 gene expression*

Exposure of female mosquitofish to 1 μg TB/L induced the expression of *shh*, *msxC* and *fgfr1* in the distal portion of the gonopodial structure (Fig. 5). *Shh* gene expression was visible in the distal tip of the 3rd, 4th, and 5th rays of the anal fin (the rays that comprise the gonopodial structure in males) of the TB-treated samples after 7 and 14 days of exposure. Expression was also visible at day 21 of TB exposure but with a reduction in the strength of the signal (data not shown). A wider spatial range of *msxC* and *fgfr1* expression was seen along the 3rd, 4th, and 5th anal fin rays, with stronger expression at day 10 as compared to day 5. For all three genes, there was a lack of expression in the samples treated with the vehicle control.

4. Discussion

EDC-related effects can still be found in wildlife residing in paper mill-impacted areas (Hewitt et al., 2008), including the presence of masculinized female mosquitofish downstream of paper mills (Bortone and Cody, 1999; Bortone and Drysdale, 1981; Brockmeier, unpublished results; Hou et al., 2011; Howell et al., 1980). The biological impacts of paper mill effluent exposure on mosquitofish in Florida were previously evaluated (Jenkins et al., 2001; Noggle et al., 2004; Orlando et al., 2002, 2007; Parks et al., 2001; Toft et al., 2004); but it was not determined if the masculinized mosquitofish exhibited any reproductive impairment due to the hypothesized androgen exposure. This may be due to the use of the mosquitofish as a bioindicator organism without a complete understanding of how abnormal anal fin elongation occurs and how androgen-induced anal fin growth correlates with other endpoints related to reproduction that can be used to evaluate biological integrity.

In this study, we demonstrate TB's ability to induce anal fin elongation in *G. holbrooki* in a similar manner as what was reported in *G. affinis* (Sone et al., 2005). The anal fin ratios obtained during this study (Fig. 2) are also comparable to other androgen exposures of female *G. affinis* to ADE (Stanko and Angus, 2007), 11-KT (Angus et al., 2001), and MT (Stanko, 2005). The role of the dose of androgen on inducing the formation of bone segments, hooks, and serrae in the anal fin (Turner, 1947) was also evaluated. An increase in the number of bone segments was found in the 3rd anal fin ray after 21 days of 10 μg TB/L exposure (Fig. 3) but no hooks or serrae were observed after 21 days of this exposure. These results were similar to what was observed in *G. affinis* fry during ethyl testosterone (ET) treatment, with high doses of ET resulting in less elongation (Ogino, unpublished results). While there were no strong differences in the number of bone segments in the 3rd anal fin ray compared to *G. affinis* fry exposed to lower doses of ET, distal bone segments at higher doses were shorter in length compared to a low dose exposure (Ogino, unpublished results). Exposure to high doses of androgens seems to result in the formation of shorter, thicker bone segments, and in the case of *G. holbrooki* the addition of a greater number of bone segments; a longer exposure would likely result in the formation of the hook and serrae structures (Angus et al., 2001). A dual approach of anal fin elongation measurements, looking at both anal fin length and bone segmentation, appears to be the most appropriate for evaluating androgen exposure in mosquitofish in the field.

Fig. 4 demonstrates TB's ability to reduce *vtg* at the level of gene expression during treatment with 1 μg TB/L or 10 μg TB/L. When samples were analyzed separately by oocyte developmental stage, this trend was still present (Fig. S1). The egg yolk precursor protein VTG is synthesized in the liver in response to estrogen signals from the gonad and is incorporated into developing oocytes to provide nutrients to developing fry.

TB exposure of fathead minnows has been demonstrated to have numerous impacts on the female reproductive system, including decreased levels of the female sex hormone 17β-estradiol (E2), VTG protein levels, and a significant decrease in the cumulative number of eggs spawned in doses as low as 0.05 μg TB/L (Ankley et al., 2003). TB has also been demonstrated to reduce *vtg* at the level of gene expression in fathead minnows with a lowest effect dose of 0.5 μg TB/L (Miracle et al., 2006) and fathead minnow population models have demonstrated a correlation between VTG levels in females and potential population declines (Miller et al., 2007). In other species, TB caused male sex reversal in zebrafish (*Danio rerio*) exposed for 60 days to 15.5 ng/L (Morthorst et al., 2010), resulted in decreased cumulative egg counts in medaka (*Oryzias latipes*) exposed to 2 μg TB/L for 32 h (Zhang et al., 2008), and decreased the fertility rates, daily egg production rates, and *vtg* protein levels of sheepshead minnows (*Cyprinodon variegatus*) exposed to 0.87 μg TB/L (Cripe et al., 2010). While extrapolation from previous studies on other fish species to mosquitofish may be possible, the unique reproductive biology of the family *Poeciliidae* and their responses to chemical contaminants (Vinson et al., 1963, Raut et al., 2011) warranted direct experimentation of this species' response to androgen exposure. From the results of this and other studies, it appears that the mosquitofish is less sensitive to TB exposure than fathead minnows and is similarly as sensitive as medaka and sheepshead minnows.

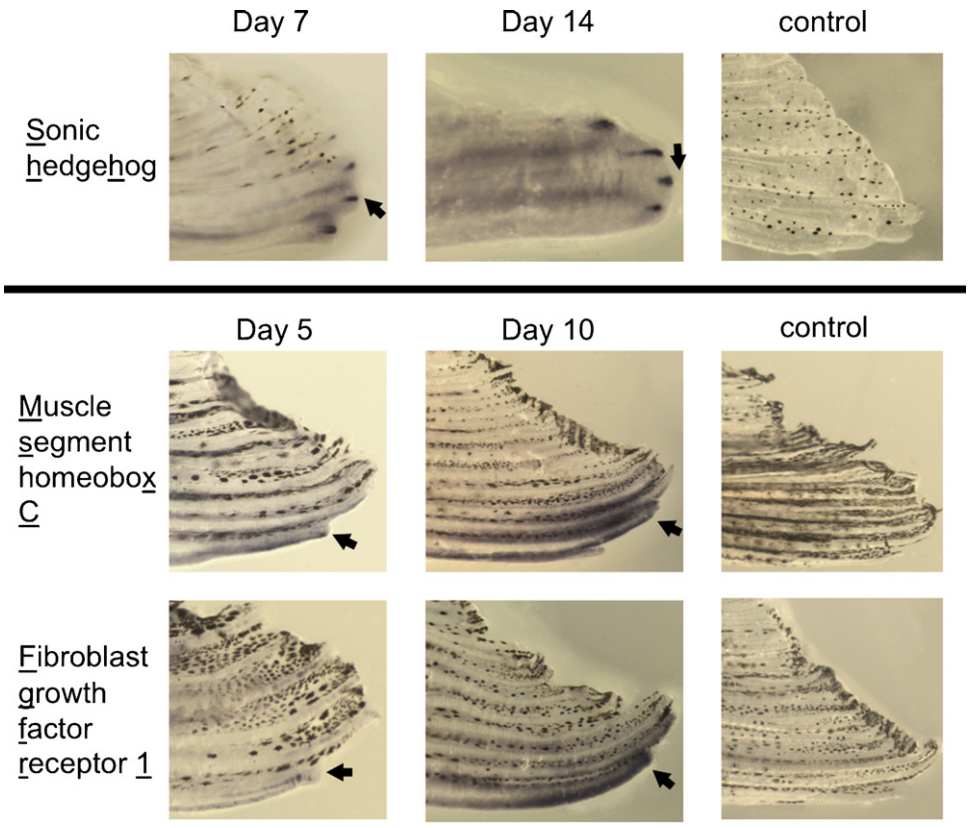

Fig. 5. *Sonic hedgehog* (*shh*), *muscle segment homeobox C* (*msxC*), and *fibroblast growth factor receptor 1* (*fgfr1*) gene expression during 17β-trenbolone (TB) exposure. Female Western mosquitofish (*G. affinis*) were exposed to either 1 μg TB/L or the vehicle control (ethanol) for 7 or 14 days to evaluate *shh* gene expression and female Eastern mosquitofish (*G. holbrooki*) were exposed to either 1 μg TB/L or the vehicle control (ethanol) for 5 or 10 days to evaluate *msxC* and *fgfr1* gene expression. Anal fins were removed upon dissection and preserved overnight in 4% paraformaldehyde in phosphate buffered saline. *In situ* hybridization was used to determine gene expression location in preserved mosquitofish anal fin tissues. Dark staining as indicated by the black arrows is a result of the alkaline phosphatase reaction and is indicative of sequence-specific binding of the probe. These photographs are a representative sample of the fins that were analyzed ($N = 4$ per treatment and time point); all photographs are shown as 10× objective except for *shh* at day 14 which is shown as 12×.

The mechanism for TB's anti-estrogenic effects are still unknown, but it is hypothesized that endpoints such as *vtg* decrease do not occur *via* estrogen receptor antagonism but are rather due to a decrease in E2 from reduced T levels, the abnormal expression or activity levels of steroid metabolizing enzymes, or feedback inhibition within the hypothalamus-pituitary-gonad (HPG) axis (Yarrow et al., 2010). In the fathead minnow, exposure to 0.5 or 5 μg TB/L resulted in significant decreases in E2 concomitant with a decrease in VTG protein levels (Ankley et al., 2003) which could be an explanatory factor in the *vtg* decreases demonstrated in this manuscript. These anti-estrogenic effects do not appear to be due to binding of the AR, as co-treatments of fathead minnows with TB and the anti-androgen flutamide prevents the formation of nuptial tubercles but fails to prevent VTG declines (Ankley et al., 2004).

Determining relationships between different levels of biological organization, such as between anal fin elongation and potential effects on the reproductive system, can be utilized for predicting effects relevant to ecological risk assessments as described in the AOP framework (Ankley et al., 2010). In this paper we evaluated if there was a relationship between anal fin growth and potential effects on reproduction after TB exposure as determined by *vtg* mRNA expression. When samples were separated by both day and treatment, significant correlations were found between these two endpoints, but the comparison was not consistent enough for anal fin elongation to be recommended as a strong predictor of effects on *vtg*. Because the mechanisms of androgen-induced anal fin elongation and the impacts on *vtg* are different, using anal fin elongation to predict higher-level effects linked to the female reproductive system is not biologically supported. This lack of predictability is also supported by other studies, as Stanko and Angus (2007) found that high doses of ADE could induce anal fin elongation but did not reduce VTG protein levels while MT was able to reduce VTG protein levels and induce anal fin growth. In addition, since not all androgenic chemicals act the same *in vivo* due to differences in aromatization abilities and in affinities for the AR (Katsu et al., 2007), the use of the androgen-driven anal fin elongation measurement to predict effects on the HPG axis may only be utilized if anal fin elongation could be linked to other endpoints that are correlated to reproductive success (*i.e.* decreased mating attempts by male mosquitofish). This study focused on *vtg* mRNA expression because of the increased sensitivity of this endpoint to lower doses of androgenic chemicals, as *vtg* mRNA has a short half life compared to VTG protein and therefore responds more quickly to a change in transcriptional control (Denslow et al., 2005; Ekman et al., 2011), which includes the lower doses of TB (less than 1 μg TB/L) that can lead to negative impacts on the ability to reproduce (Ankley et al., 2003; Miracle et al., 2006). Anal fin elongation can still serve as a useful metric for determining if exposure to an androgenic chemical has occurred and based on previous studies and the results presented in this paper, the mosquitofish anal fin appears to be more sensitive to androgen exposure than the HPG axis.

Gonopodium development occurs in all members of the subfamily *Poeciliinae*, which includes the genera *Xiphophorus*, *Poecilia*, and *Gambusia* (Zauner et al., 2003). Understanding what genes regulate gonopodium development is a crucial first step toward elucidating the mechanisms of androgen-induced anal fin elongation and can also lead to the development of a molecular biomarker of androgen exposure. Currently, the glue protein Spiggin produced

by the three-spined stickleback (*Gasterosteus aculeatus*) has been evaluated as an androgenic biomarker (Katsiadakia et al., 2002), but because this species has a distribution limited to colder waters, additional research of androgen biomarkers is warranted. Previous studies on genes expressed in the anal fin of *G. affinis* found no difference in *ARα* and *ARß* mRNA expression between untreated male and female whole anal fins or between the male anal fin and other fins belonging to the same fish (Sone et al., 2005), which led to the hypothesis that genes other than the ARs were involved in anal fin outgrowth. In this study, expression of the genes *shh*, *msxC*, and *fgfr1* were induced in the anal fins of adult female mosquitofish by 1 μg TB/L exposure. *Shh* was expressed after 7 and 14 days of androgen treatment (Fig. 5); this signal was present but weaker after 21 days of exposure (data not shown). This may potentially be due to the fin reaching the end of the elongation phase and the beginning of the differentiation phase. *MsxC* and *fgfr1* were expressed after 5 and 10 days of androgen treatment, with stronger expression at day 10 (Fig. 5) prior to the significant anal fin elongation which was seen at day 14.

In studies of regeneration in zebrafish (*Danio rerio*), *shh* is expressed on both contralateral sides of the proximal regions of the early blastema (a region of undifferentiated cells at the proximal tip of the regenerating fin) where the bone forming matrix is synthesized by scleroblasts (cells that will differentiate into bone). *Shh* signals are transduced from the epithelial region to the mesenchymal region where they activate bone morphogen proteins (BMPs) that determine downstream scleroblast fate (Laforest et al., 1998; Marí-Beffa and Murciano, 2010). In androgen-treated *G. affinis* fry, *shh* is expressed in the lateral basal epithelium of the anal fin as early as two days after androgen exposure. Co-treatment of androgen and flutamide resulted in decreased *shh* expression, along with a decrease in other anal fin growth-related endpoints (Ogino et al., 2004). While not a direct androgen target itself, investigations on the usability of *shh* as a molecular biomarker of androgen exposure is an important field of research for the future. However, it is crucial to correlate molecular biomarker results with more integrative endpoints in order to develop robust AOPs (Ankley et al., 2010).

Msx genes are considered markers of mesenchyme, the loose connective tissue that can migrate easily and that contains unspecialized cells (Poss et al., 2003). In regeneration studies, *msxA* and *msxD* are found in the wound epithelium and *msxB* and *msxC* in the distal tip of the blastema mesenchyme during regeneration. *MsxC* is thought to regulate blastema proliferation rates, as prolonged expression of this gene results in rapid cell proliferation (Offen et al., 2008). *Fgfr1* is a receptor tyrosine kinase that binds to fibroblast growth factors to trigger mitosis and cell proliferation. *Fgfr1* is crucial in regeneration for the development of the blastema, and a loss of *fgfr1* function results in the lack of blastema formation, a reduction in cell proliferation, and a lack of regenerative outgrowth in fin tissues (Poss et al., 2000). *MsxC* and *fgfr1* were also expressed in the anal fins of androgen-treated swordtails and platyfish (Offen et al., 2008). In this study, we observed *msxC* and *fgfr1* expression along the 3rd, 4th, and 5th anal fin rays near the distal tip of the elongating anal fin in adult female mosquitofish during TB exposure. Because of their weaker expression patterns at earlier time points, these two genes may not serve as strong androgen biomarker candidates but additional experimentation to determine their roles in anal fin elongation is warranted.

5. Conclusions

We have demonstrated that TB can induce anal fin elongation, decrease hepatic *vtg* gene expression, and induce *shh*, *msxC*, and *fgfr1* gene expression in the anal fins of adult female mosquitofish. There was no quantitative correlation between anal fin elongation and *vtg* gene expression. Future work on how *shh*, *msxC*, and *fgfr1* can potentially be developed into molecular biomarkers of androgen exposure will be evaluated during additional laboratory exposures to androgens and in field work at paper mill-impacted sites. With further insights into the molecular pathways involved in anal fin elongation and the understanding of the relationship between gene expression patterns, anal fin elongation, and the reproductive system, the mosquitofish can become a robust bioindicator organism for endocrine disrupting chemicals in the environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2012.12.007.

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