Chem. Senses 35: 65–74, 2010

doi:10.1093/chemse/bjp085
Advance Access publication December 4, 2009

Methyltestosterone-Induced Changes in Electro-olfactogram Responses and Courtship Behaviors of Cyprinids

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Accepted October 27, 2009

Abstract

In the tinfoil barb (*Barbonymus schwanenfeldii*; family Cyprinidae), we previously found that increased olfactory sensitivity to a female prostaglandin pheromone could induce sexual behavior display in juvenile fish treated with androgens. Here, we determined if this phenomenon is widespread among cyprinid fishes by adding 17α-methyltestosterone (MT) to aquaria containing juveniles of 4 cyprinid species (tinfoil barbs; redtail sharkminnows, *Epalzeorhynchos bicolor*; goldfish, *Carassius auratus*; zebrafish, *Danio rerio*) and then using electro-olfactogram (EOG) recordings and behavioral assays to determine if androgen treatment enhances pheromone detection and male sex behaviors. In all 4 cyprinids, MT treatment increased the magnitudes and sensitivities of EOG response to prostaglandins and, consistent with our initial study on tinfoil barbs, did not affect EOG responses to the free and conjugated steroid to which each species is most sensitive. In zebrafish, EOG responses to prostaglandins were similar in MT-treated juveniles and adult males, whereas responses of control (ethanol exposed) fish were similar to those of adult females. Finally, as previously observed in tinfoil barbs, MT treatment of juvenile redtail sharkminnows increased courtship behaviors (nuzzling and quivering) with a stimulus fish. We conclude that androgen-induced increase in olfactory responsiveness to pheromonal prostaglandins is common among the family Cyprinidae. This phenomenon will help us unravel the development of sexually dimorphic olfactory-mediated behavior.

Key words: courtship behaviors, Cyprinidae, 17α-methyltestosterone, olfaction, prostaglandins, steroids

Introduction

In vertebrates, gonadal steroids can induce sexually dimorphic olfactory functions in terms of detecting and responding to certain odorants (reviews by Mofatt 2003; Bodo 2008). For example, testosterone (T) treatment increases *fos* immunoreactivity in neurons of the main olfactory bulb glomeruli of castrated ferrets presented with bedding soiled by estrous females (Kelliher et al. 1998), and mice treated with T display increases in neuronal firing in the olfactory bulb in response to female urine odors (Pfaff and Pfaffmann 1969). Given that gonadal steroids induce changes in second-order olfactory centers, potentially affecting perception and motor responses to odorants, it is also possible that gonadal steroids act directly on olfactory sensory neurons (OSNs), bringing about physiological changes in peripheral processing of odorants. To our knowledge, there is no direct physiological evidence that gonadal steroids modulate responses of OSNs in mammals, although indirect evidence is provided by the finding that systemic T or estradiol (E2) increases *fos* immunoreactivity in vomeronasal neurons of gonadectomized mice (Halem et al. 1999, 2001). On the other hand, there is evidence that gonadal steroids affect OSN responses in both fish and amphibia. Treating ovariectomized Japanese fire belly newts (*Cynops pyrrhogaster*) with E2 and juvenile (immature) tinfoil barbs (*Barbonymus schwanenfeldii*) with the androgens 17α-methyltestosterone (MT) and 11-ketotestosterone increased pheromone-induced extracellular field potentials (electro-olfactogram; EOG) recorded from the surface of the olfactory epithelium (OE) (Cardwell et al. 1995; Toyoda and Kikuyama 2000). Although these findings suggest that gonadal steroids act directly on OSNs to influence sensory processing of pheromonal odorants, the phenomenon remains virtually unexplored.

Gonadal steroids are well known to increase the display of male sexual behaviors in a variety of fishes (reviewed by Stacey and Sorensen 2009). Although these behavioral effects likely are mediated by steroid actions at multiple sites within the nervous system, the fact that an intact olfactory system appears to be necessary for the display of male reproductive behaviors (reviewed by Hamdani and Døving 2007; Stacey and Sorensen 2009) suggests that the

olfactory system is one likely target. In particular, seasonal increases in plasma androgens (reviewed by Pankhurst 2008) may act through androgen receptors in the OE (Pottinger and Moore 1997) either to affect the function of existing OSNs or to favor recruitment of particular OSN types, as has recently been suggested for crypt cells in crucian carp, *Carassius carassius* (Hamdani et al. 2007). Consistent with this hypothesis, results from a number of EOG studies show that olfactory responsiveness to putative pheromones increases with sexual development in a variety of fishes (Moore and Scott 1991; Bjerselius and Olsén 1993; Irvine and Sorensen 1993; Cardwell et al. 1995; Moore and Waring 1996) and can be induced by androgen treatment in juveniles (Cardwell et al. 1995; Stacey et al. 2003). A similar phenomenon evidently occurs in mammals, where recent studies have shown that T and E2 affect expression of vomeronasal organ odorant receptor genes in mice (Alekseyenko et al. 2006).

The central objective of this study was to assess the feasibility of using fishes in the family Cyprinidae as models for basic and comparative studies of endocrine effects on development and regulation of sexually dimorphic olfactory function. Cyprinids appear well suited for such studies because they are diverse (more than 2400 species) and include the zebrafish *Danio rerio*, a model vertebrate, and the goldfish, *Carassius auratus*, which has evolved a complex sex pheromone system in which steroids and F-series prostaglandins (PGFs) released by periovulatory females induce a suite of behavioral and endocrine effects in males (Kobayashi et al. 2002; Stacey and Sorensen 2009). Previous cyprinid studies have shown that androgen treatment of juvenile tinfoil barbs (Cardwell et al. 1995) and rainbow sharkminnows (*Epalzeorhynchos frenatus*; Stacey et al. 2003) does not affect EOG responsiveness to steroids but greatly increases response to PGFs, thus mimicking the sexually dimorphic olfactory responses of adults. Perhaps most importantly, because female sex behavior (stereotyped oviposition acts) can be induced within minutes by injecting otherwise untreated juveniles with $PGF_{2\alpha}$ (Cardwell et al. 1995), it is straightforward to conduct behavioral bioassays to determine in juveniles the functional relationship between androgen-induced increase in PGF detection and ability to display male sexual behaviors.

From this perspective, the key specific objective in this study was to determine whether androgen-induced change in olfactory response to pheromones is a widespread phenomenon in cyprinids by conducting EOG recordings on androgen-treated juveniles of phylogenetically diverse species of cyprinids (see Nelson 2006; Wang et al. 2007; Li et al. 2008). Beyond this, we sought to determine if previously reported androgen effects on EOG response to hormonal odorants (Cardwell et al. 1995; Stacey et al. 2003) could be confirmed in tinfoil barbs and extended to 3 additional species: redtail sharkminnow (*Epalzeorhynchos bicolor*), goldfish, and zebrafish.

Materials and methods

Fish

Immature (juvenile) redtail sharkminnows and tinfoil barbs were purchased from Aquatic Imports and maintained in the Aquatics Facility at the University of Alberta (Edmonton, Alberta, Canada); immature goldfish and zebrafish (type AB) were bred and reared in the Aquatics Facility. Fish were initially classified as immature based on size and age. Post EOG and behavioral analysis, this was confirmed by examining the gonads of immature fish. In all cases, thread-like/undeveloped gonads of immature fish were confirmed. All fish were maintained on a 14:10 h light:dark photoperiod in 65 L aquaria with gravel and a flow-through dechlorinated tap water source (27 °C) and were fed brine shrimp (*Artemia* spp.) and commercially available algae (*Spirulina*) flakes.

Steroid treatment

Test fish were treated with MT (Steraloids) added daily to aquaria for 21 consecutive days (methods adapted from Bayley et al. 1999; Stacey et al. 2003); the main route of uptake of lipophilic compounds, such as sex steroids, is most likely via the gills (Randall et al. 1998; Scott and Ellis 2007). On each of the treatment days, water inflow was turned off in the evening and 1 mL of an MT stock solution (0.2 mg/mL dissolved in 95% ethanol) was delivered to each aquarium near the airstone creating an aquarium water concentration of approximately 10^{-8} M; control fish were treated with the same volume of 95% ethanol. The following morning, approximately 12 h after MT addition, water flow was restored. During treatment, juveniles of each of the 4 species were held in 2 control (ethanol treated) and 2 experimental (MT treated) aquaria (5–7 fish per aquarium). Control and MT-treated experimental aquaria for each species began treatment alternately and several days apart, in order that all fish could be used for EOG recording or behavioral testing 21–23 days after beginning treatment.

At the termination of experiments, weight and standard length (mean ± standard deviation) of juveniles of the 4 species were as follows: redtail sharkminnows ($N = 12$; 1.4 ± 0.3 g and 5.2 ± 0.2 cm); tinfoil barbs ($N = 8$; 3.2 ± 0.8 g and 6.7 ± 0.5 cm); goldfish ($N = 10$; 0.9 ± 0.5 g and 4.4 ± 0.6 cm); zebrafish ($N = 8$; 0.2 ± 0.02 g and 2.6 ± 0.1 cm). Weight and standard length of untreated adult zebrafish ($N = 10$) was 0.4 ± 0.1 g and 3.6 ± 0.2 cm. Within each species, weights and lengths of control and MT-treated fish were not significantly different (t-tests, $P > 0.05$).

EOG recording

EOG recording procedures were similar to those described by Cardwell et al. (1995). Immediately prior to recording, fish were anesthetized by immersion in tricaine methanesulfonate (MS-222; 0.4% for redtail sharkminnows, tinfoil barbs, and goldfish and 0.2% for zebrafish), wrapped in

wet tissue, and secured to a stand placed in an electrically grounded water bath. A polyethylene tube was positioned in the mouth so that the gills were continuously perfused with aerated, dechlorinated tap water (~27 °C) containing 0.1–0.2% MS 222 to maintain anesthesia for the duration of the recording. Gelatin-filled (8% in 0.6% NaCl) glass micropipettes were used to bridge the OE to Ag/AgCl electrodes filled with 3 M KCl. A recording electrode was placed over the OE, and the tip of a second reference electrode was placed in the water bath. The OE was constantly perfused with dechlorinated tap water (background water; ~27 °C), and a computer-controlled solenoid delivered 2-s pulses of test odorants by switching between the background water and test solutions (diluted appropriately in background water).

Amplified signals (World Precision Instruments DAM50-H DC amplifier) were digitized (National Instruments Lab-PC A/D converter), and absolute response magnitude (base to peak voltage difference) was recorded for 15 s from the initiation of the odor pulse. Background responses were subtracted from odorant responses and 2 min between each odorant was allowed to avoid adaptation. Recording from anesthetized fish was initiated after confirming an olfactory response to 10^{-5} M L-alanine, a food odor cue (Caprio 1984). If the response to L-alanine was greater than 1 mV and background responses were negligible (response to blank water was 0.00–0.10 mV in all cases), testing with steroid and prostaglandin odorants was initiated. Throughout the course of a recording session, the response to 10^{-5} M L-alanine was monitored frequently to ensure the stability of the recording.

Odorants

For each of the 4 cyprinid species, prostaglandin and steroid test odorants were selected on the basis of previous responses during EOG and olfactory studies. Thus, prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$), 15-keto-$PGF_{2\alpha}$ (15K-$PGF_{2\alpha}$), and 17,20β-dihydroxy-4-pregnen-3-one-sulfate (17,20β-P-s) were used in EOG recordings of redtail sharkminnows (Stacey et al. 2003) and zebrafish (Stacey and Cardwell 1995, 1997; Freidrich and Korsching 1998; Stacey and Sorensen 2009); $PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$, and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were used for goldfish EOG recordings (Sorensen et al. 1987, 1988); and $PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$, and 17,21-dihydroxy-4-pregnen-3-one (17,21-P) were used in recordings of tinfoil barbs (Cardwell et al. 1995).

Stock solutions (10^{-2} M) of L-alanine (Sigma Chemical Company) were prepared in double-distilled, deionized water and stored at 0 °C. L-alanine test solutions (10^{-5} M) were prepared during EOG recordings by diluting stock solution 1:1000 in background water. Stock solutions (10^{-3} M) of PGFs (Cayman Chemical Company) and steroids (Steraloids) were prepared in 100% ethanol and stored at –20 °C. Prostaglandin and steroid working solutions (10^{-6} M) were prepared by diluting stock solution 1:1000 in double-distilled, deionized water, stored at 4 °C prior to use, and used to prepare fresh test solutions during EOG recordings. Prostaglandin and steroid test solutions (10^{-12} to 10^{-8} M) were prepared immediately before each odor presentation by appropriately diluting a 10^{-6} M working solution in 40 mL background water; each test solution was used only once and then discarded. When testing a concentration series, odors were always presented in increasing log molar increments from 10^{-12} M to 10^{-8} M.

Test for male spawning behavior in juvenile redtail sharkminnows

To determine if MT treatment facilitates the display of male courtship behaviors in immature redtail sharkminnows, 40-min behavioral tests were conducted 21–23 days after the start of MT and ethanol treatments using 4 glass tanks (19.5 cm long × 39.5 cm wide × 23.5 cm tall) that contained 18 L of 27 °C dechlorinated water. To facilitate video recording (Panasonic PV-L651) through the tank front, tanks were covered on each side by black plastic and on the back by white plastic. A plastic tube delivering 50 mL/min of 27 °C dechlorinated water was positioned just above the surface at the back right corner of each tank because, in aquaria, redtail sharkminnows are reported to spawn in the current of tank filters or airstones (Naznov 1981; Vajdak 1983). For each behavioral test, 1 MT-treated or ethanol-treated (control) fish was placed with 1 immature stimulus fish of unknown sex and behavioral interactions were recorded continuously for two 20-min test periods. Prior to the first 20-min test, the stimulus fish was injected with teleost saline (and therefore should not have been emitting female ovulatory signals that elicit male courtship); between the first and second 20-min tests, the stimulus fish was injected with $PGF_{2\alpha}$ to induce both the release of pheromonal prostaglandins that elicit male courtship and the display of female spawning behavior (Stacey 1976; Sorensen et al. 1988; Kobayashi et al. 2002). Each MT-treated and control fish was tested only once.

To begin each behavioral test, 1 MT (5.3 ± 0.7 cm; 1.5 ± 0.6 g, $N = 10$) or control (5.1 ± 0.5 cm; 1.4 ± 0.6 g, $N = 10$) fish was added to a test tank together with an immature stimulus fish (5.3 ± 0.7 cm; 1.5 ± 0.7 g, $N = 20$) that could be identified visually by a clipped caudal fin, and the pair was allowed to acclimate to the test tank for 1 h. The stimulus fish was then removed, injected intraperitoneally (i.p.) with 5 μL teleost saline (Hagiwara and Takahashi 1967), and returned to the tank where behavior was videotaped continuously for 20 min. The stimulus fish was then removed a second time, injected i.p. with 5 μL Lutalyse (Pfizer; 25 μg $PGF_{2\alpha}$), and returned to the test tank, and behavior was recorded for a second 20-min period. All behavioral tests were performed in July 2008, between 0900 and 1800 h.

Video recordings of fish interactions were viewed on a Panasonic Color Monitor (BT-S1000N), and time performing courtship-like behaviors was scored. As described by Vajdak (1983), male "courtship" begins with a male chasing a female and progresses to "nuzzling" (male and female

aligned in parallel) and "quivering" (rapid side by side undulations of both fish), at which point oviposition and ejaculation occur, the fish push away from each other, and the male soon resumes chasing. Because redtail sharkminnows exhibit both courtship and agonistic chasing, which appear to be qualitatively similar, we assessed courtship activity by the duration of nuzzling and quivering with the stimulus fish and the occurrence of quivering.

Statistical analysis

EOG data are presented and analyzed as millivolt (±standard error of the mean) responses, rather than being normalized to the L-alanine standard because, in all 4 cyprinid species, the magnitudes of the EOG responses induced by 10^{-5} M L-alanine tended to be greater in control fish than in MT-treated fish (*t*-tests; see Results). For juveniles of each species, differences between control and MT-treated fish in the EOG concentration–response tests were analyzed by 2-way analysis of variance (ANOVA) (factors: treatment and concentration) and Fisher least significant difference (LSD) post hoc tests. Differences in the magnitudes of EOG responses to 10^{-8} M test odorants in adult and juvenile zebrafish were analyzed by 2-way ANOVA (factors: fish type and odor type) and Fisher LSD post hoc test. As in Cardwell et al. (1995), EOG response thresholds for each odorant were defined as the lowest dose where the 95% confidence interval did not include 0.

For the behavioral tests in redtail sharkminnows, the total times spent nuzzling and quivering with the stimulus fish in the first and second 20-min observation periods were compared both within fish (to determine the effect of $PGF_{2\alpha}$ injection in the stimulus fish) and between control and MT treatments by 2-way ANOVA (factors: test fish treatment and stimulus fish treatment) and Fisher LSD post hoc test.

Results

Effects of androgen treatment on EOG responses

Effect of MT on L-alanine responses

In all 4 cyprinid species, the magnitude of EOG responses to the 10^{-5} M L-alanine standard tended to be greater in control than in MT-treated fish, and in redtail sharkminnows, this difference was significant ($P < 0.05$; *t*-test; Figure 1). EOG responses to steroid and prostaglandin test odors are therefore presented as millivolt rather than being normalized as a percentage of the L-alanine standard.

Redtail sharkminnows

MT treatment significantly affected the EOG responses of juvenile redtail sharkminnows to $PGF_{2\alpha}$ but not to 17,20β-P-s (Figure 2A–C). For example, at a concentration of 10^{-8} M, $PGF_{2\alpha}$ induced significantly greater EOG response magnitudes in MT-treated than in control fish (2-way ANOVA with Fisher LSD post hoc test, $F = 189.12$, $P < 0.0001$), although both treatment groups appeared to exhibit similarly extreme sensitivity to this PGF (threshold < 10^{-12} M). In contrast, MT-treated fish were more sensitive to 15K-$PGF_{2\alpha}$ than were control fish (olfactory thresholds of <10^{-12} and 10^{-10}, respectively), even though the magnitudes of 15K-$PGF_{2\alpha}$–induced EOG response did not differ between the groups.

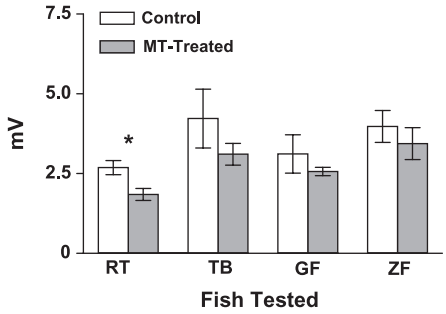

Figure 1 EOG responses (mean ± standard error) to 10^{-5} M L-alanine of ethanol-treated control (white bars) and MT-treated juvenile fish (gray bars). Redtail sharkminnows (RT), $N = 6$ per treatment; tinfoil barbs (TB), $N = 4$ per treatment; goldfish (GF), $N = 5$ per treatment; zebrafish (ZF), $N = 4$ per treatment. * Denotes $P < 0.05$.

Tinfoil barbs

In juvenile tinfoil barbs, MT increased EOG magnitudes in response to 15K-$PGF_{2\alpha}$ but not in response to 17,21-P (Figure 2D–F). For 15K-$PGF_{2\alpha}$, significant increases (2-way ANOVA with Fisher LSD post hoc test, $F = 267.16$) in EOG response magnitudes were observed for concentrations of both 10^{-9} M ($P < 0.01$) and 10^{-8} M ($P < 0.0001$). There were no differences in threshold sensitivity between MT-treated and control fish to both PGFs (10^{-11} M for $PGF_{2\alpha}$ and <10^{-12} M for 15K-$PGF_{2\alpha}$) and 17,21-P (<10^{-12} M).

Goldfish

In juvenile goldfish, MT treatment significantly increased EOG responses to PGFs but not to 17,20β-P (Figure 2G–I). Compared with control goldfish, MT-treated individuals had larger EOG responses to $PGF_{2\alpha}$ at 10^{-8} M (2-way ANOVA with Fisher LSD post hoc test, $F = 146.75$, $P < 0.0001$) and to 15K-$PGF_{2\alpha}$ at 10^{-10} ($P < 0.01$), 10^{-9} ($P < 0.01$), and 10^{-8} M ($P < 0.01$) (2-way ANOVA with Fisher LSD post hoc test, $F = 73.33$). MT treatment also lowered olfactory threshold to PGF (10^{-10} M for MT-treated juvenile fish vs. 10^{-8} M for controls) and 15K-$PGF_{2\alpha}$ (10^{-9} M for MT-treated juvenile fish vs. 10^{-8} M for controls). In contrast, threshold concentration for 17,20β-P was 10^{-11} M for both treated and control goldfish.

Zebrafish

In juvenile zebrafish, MT treatment significantly affected EOG responses to PGFs but not to 17,20β-P-s (Figure

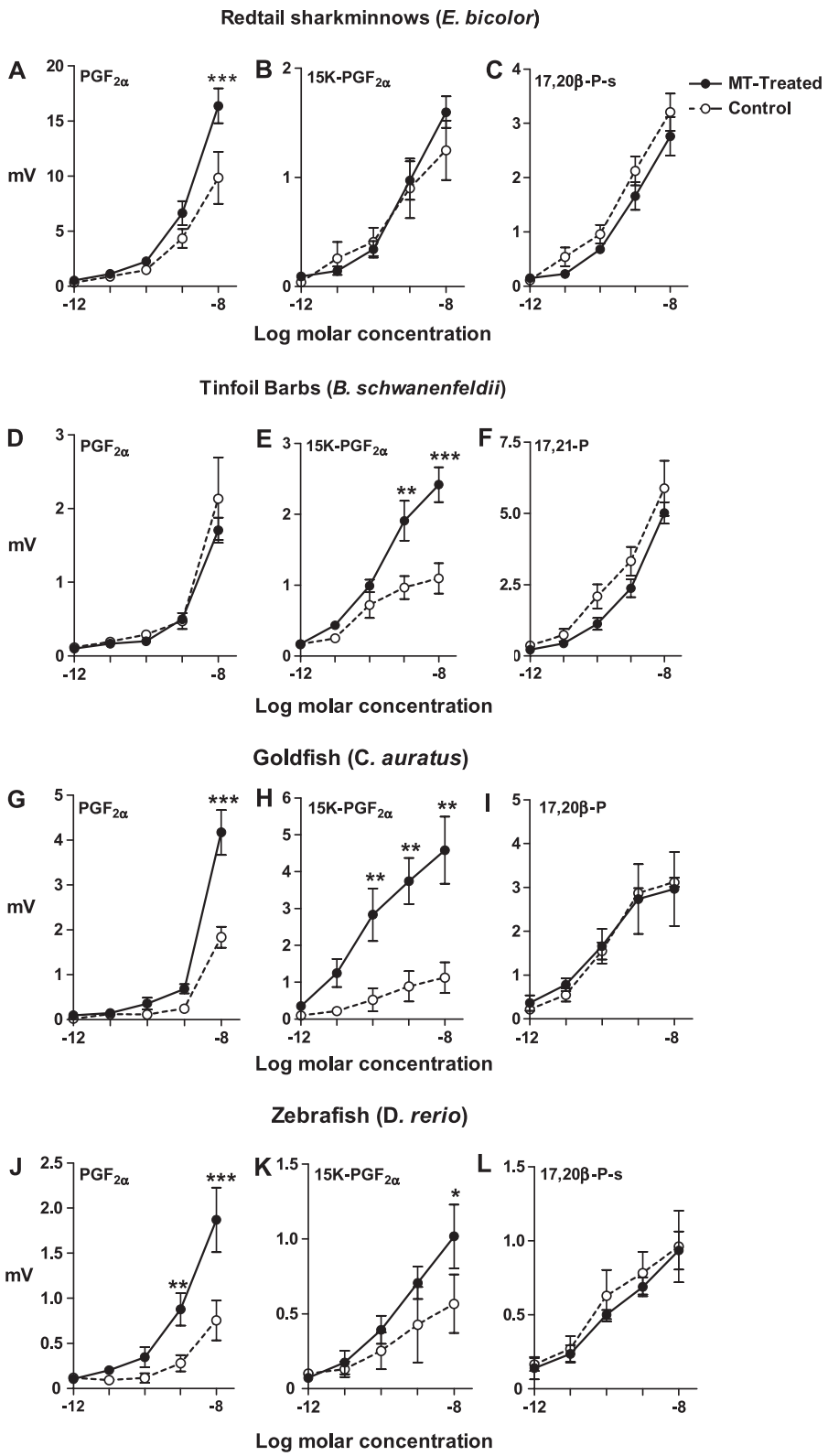

Figure 2 EOG response magnitudes (mean ± standard error) of juveniles of 4 cyprinid species (redtail sharkminnows **[A–C]**, $N = 6$ per treatment; tinfoil barbs **[D–F]**, $N = 4$ per treatment; goldfish **[G–I]**, $N = 5$ per treatment; zebrafish **[J–L]**, $N = 4$ per treatment) following addition of MT (filled circles) or ethanol vehicle (control) juvenile (empty circles) to aquarium water for 21 days. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$ indicate significance of differences between EOG response magnitudes of MT-treated and control fish.

2J–L). Compared with control juveniles, MT-treated fish had larger EOG response magnitudes to both 10^{-9} M ($P < 0.01$) and 10^{-8} M ($P < 0.0001$) $PGF_{2\alpha}$ (2-way ANOVA with Fisher LSD post hoc test, $F = 96.12$) and to 10^{-8} M 15K-$PGF_{2\alpha}$ (2-way ANOVA with Fisher LSD post hoc test, $F = 87.10$). MT-treated fish also had lower olfactory thresholds for both $PGF_{2\alpha}$ (10^{-10} M) and 15K-$PGF_{2\alpha}$ (10^{-9} M) than did control fish (10^{-9} and $>10^{-8}$ M, respectively). In contrast, there was no apparent difference in olfactory threshold to 17,20β-P-s ($<10^{-12}$ M for both treatments).

L-alanine–induced EOG responses of adult zebrafish (adult males 2.5 ± 0.4 mV; adult females 3.0 ± 0.4 mV) and juveniles (MT-treated juveniles 3.4 ± 0.5 mV; control juveniles 4.0 ± 0.5 mV) did not differ (1-way ANOVA, $F = 2.01$, $P > 0.05$). EOG response magnitudes of MT-treated juveniles to 10^{-8} M $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ were similar to those of adult male zebrafish, whereas responses of control fish were similar to those of adult females (2-way ANOVA with Fisher LSD post hoc test; $F = 311.3$, $P < 0.0001$, Figure 3). Overall, there was no difference ($P > 0.05$) in EOG response magnitudes to 17,20β-P-s among the 4 groups of adult and juvenile zebrafish.

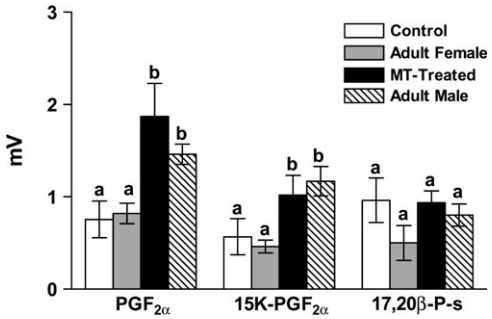

Figure 3 EOG response magnitudes (mean ± standard error) of juvenile (control, $N = 4$; MT treated, $N = 4$) and adult (males, $N = 5$; females, $N = 5$) zebrafish. Treatment groups with the same superscript are not significantly different ($P < 0.05$).

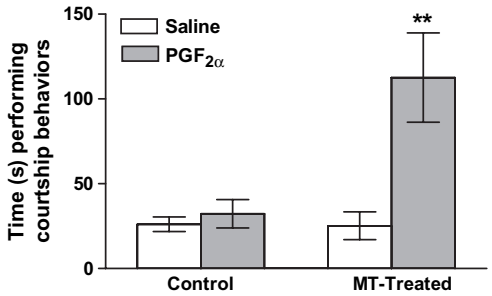

Figure 4 Time (seconds ± standard error) performing courtship behaviors (nuzzling and quivering) by ethanol (control) and MT-treated juvenile redtail sharkminnows ($N = 10$ per treatment) in response to size-matched stimulus fish injected with 5 μL of saline (white bars) and $PGF_{2\alpha}$ (gray bars) (**, $P < 0.001$).

MT-induced changes in courtship behavior

When paired with a saline-injected stimulus fish, both MT-treated and control redtail sharkminnows spent approximately 2% of the first 20-min test period performing apparent courtship behaviors. This level of activity was unchanged in control fish when their stimulus fish were later injected with $PGF_{2\alpha}$; however, MT-treated fish significantly increased courtship duration when exposed to $PGF_{2\alpha}$-injected stimulus fish (2-way ANOVA with Fisher LSD post hoc test; $F = 48.80$, $P < 0.0001$; Figure 4). Importantly, MT-treated fish performed 1.4 ± 0.2 bouts of quivering behavior with $PGF_{2\alpha}$-injected stimulus fish during the second 20-min test period, whereas no quivering events occurred when MT-treated fish were paired with a saline-injected stimulus fish or when control-treated fish were paired with either a saline-injected or a $PGF_{2\alpha}$-injected stimulus fish.

Discussion

This study demonstrates that the androgen MT enhances olfactory responses of 4 cyprinid species to prostaglandin odorants that serve as female sex pheromones in goldfish (Sorensen et al. 1988; Kobayashi et al. 2002) and also are likely to do so in other cyprinids (Stacey et al. 1994; Cardwell et al. 1995). Despite this pervasive effect on olfactory response to PGFs, however, MT did not affect EOG responses to any tested steroid odorant, consistent with earlier studies of androgen-treated juvenile tinfoil barbs and rainbow sharkminnows (Cardwell et al. 1995; Stacey et al. 2003). In addition to our findings that MT increases EOG responsiveness to PGFs, this androgen treatment in juvenile redtail sharkminnows also increases display of behaviors that not only appear indistinguishable from the nuzzling and quivering behaviors described by Vajdak (1983) in spawning adult males but also are directed more toward PGF-injected partners than toward saline-injected partners. Together, these findings indicate that, at least in cyprinid fishes, increased androgen synthesis during sexual maturation and reproductive cycles has the potential to modulate olfactory sensory responsiveness to specific odorants, possibly through the actions of hormonal androgens on nuclear androgen receptors located in the OE (Pottinger and Moore 1997). Androgen receptor activation may then promote differential olfactory receptor expression and/or increases in certain types of OSNs (Alekseyenko et al. 2006; Hamdani et al. 2007), leading to sexually dimorphic olfactory responses.

Our findings that androgen modulates olfactory responsiveness in cyprinids are supported by diverse studies in other vertebrate groups. For example, estrogen feminizes electrosensory and motor systems in the weakly electric *Sternopygus* (Dunlap et al. 1997) and also induces in nonreproductive female plainfin midshipman (*Porichthys notatus*) auditory responses to male vocalizations that are typical of reproductive females (Sisneros et al. 2004). In rats, estrogen treatment changes the sensitivity of vaginal mechanoreceptors

(Komisaruk et al. 1972), and neonatal T treatment increases adult pain thresholds during inflammation (Borzan and Fuchs 2006).

Despite the fact that MT treatment consistently enhanced PGF-induced EOG responses in the 4 cyprinids used in this study, MT had no effect on olfactory responsiveness to any steroid odorant. Although it might seem counterintuitive that MT treatment affects responsiveness to prostaglandins but not to steroids and that the steroid-induced EOG responses of untreated juvenile zebrafish were comparable to those of adults, these findings confirm the previously reported ineffectiveness of MT treatment on steroid-induced EOG responses of juvenile fish (tinfoil barbs, Cardwell et al. 1995; rainbow sharkminnows, Stacey et al. 2003) and are consistent with studies in other fishes. For example, EOG responses to steroid odorants are equivalent in adult males and females of a variety of species including goldfish (Sorensen et al. 1987), crucian carp, *C. auratus* (Bjerselius and Olsén 1993), common carp, *Cyprinus carpio* (Irvine and Sorensen 1993), the round goby, *Neogobius melanostomus* (Murphy et al. 2001), and the cichlid, *Astatotilapia* (=*Haplochromis*) *burtoni* (Cole and Stacey 2006). Although Irvine and Sorensen (1993) found that the pheromonal steroids 17,20β-P and 17,20β-P-s induce larger EOG responses in adult common carp than in juveniles, at least a portion of this reported difference resulted from normalizing steroid-induced responses to those of the L-serine standard, which induced significantly greater EOG responses in juvenile carp than in adults (Irvine and Sorensen 1993); indeed, it is for this reason that we here report our EOG data in millivolts rather than as a proportion of our L-alanine standard. To our knowledge, the only clear evidence for sexually dimorphic EOG responses to steroid odorants in fishes comes from the roach (*Rutilus rutilus*), a cyprinid (Lower et al. 2004). Although EOG thresholds for free and glucuronidated 17,20β-P are similar in male and female roach, suprathreshold concentrations of these steroids induce much larger EOG responses in males than in females. More importantly, Lower et al. (2004) reported that androstenedione is a potent odorant for males (10^{-10} M threshold) but undetectable by females, even at the extremely high concentration of 10^{-6} M (Lower et al. 2004), a sexual dimorphism in EOG response that has not been observed in any other fish. In this regard, it should also be noted that Lastein et al. (2006) report a marked sexual dimorphism in pheromone-induced responses of second-order olfactory bulb neurons of crucian carp, in which 236 of 397 neurons recorded in males responded exclusively to 1 steroid or prostaglandin odorant, whereas only 1 of 96 neurons recorded in females displayed such discrimination. Unfortunately, the relevance of this gender difference to our findings of MT-induced EOG change is unclear.

In fishes with sexually isomorphic EOG responses to steroid odorants, it seems reasonable to hypothesize that male-typical primer and releaser responses to these odorants derive not from differential OSN function but from actions of androgenic hormones on neural sites to which OSNs or higher order olfactory neurons project. Tests of this hypothesis have exploited the sexual bipotentiality of the adult fish brain (Kobayashi et al. 2002) to determine if androgen-treated females exhibit male-typical pheromone-induced responses. For example, in goldfish, where EOG responses to pheromonal 17,20β-P are equivalent in males and females (Sorensen et al. 1987) but luteinizing hormone (LH) responses are gender specific, androgen-treated females exhibit the male-typical LH response when exposed to the pheromone (Kobayashi et al. 1997). An apparently similar situation occurs in the round goby, a perciform, in which a suite of free and conjugated steroid odorants induce sexually isomorphic EOG responses but sexually dimorphic behavioral responses (Murphy et al. 2001); as in goldfish, MT treatment induces female gobies to display male-typical behavioral responses (Murphy and Stacey 2002). These androgen treatments might induce male-typical responses simply by lowering response thresholds to sensory input. In nonreproductive male gobies, for example, the threshold for the estrone-induced ventilation response is 10^{-9} M, whereas in reproductive males, it falls to 10^{-11} M (Belanger et al. 2007), the threshold for estrone-induced EOG response in males and females (Murphy et al. 2001). By demonstrating that EOG responsiveness is androgen independent in a variety of cyprinids that detect distinct steroids and steroid conjugates, our present and earlier studies (Cardwell et al. 1995; Stacey et al. 2003) suggest that cyprinids can be valuable model species for investigating centrally mediated androgen actions on pheromone-induced, sexually dimorphic primer and endocrine responses.

Although EOG studies have shown that PGFs are detected by the olfactory systems of fishes from orders Cypriniformes, Characiformes, Siluriformes, and Salmoniformes (reviewed by Stacey and Sorensen 2009), only in cypriniforms and salmoniforms have PGF-induced EOG responses of males and females been compared. Consistent with the findings of the present study, EOG studies of other cyprinid fishes provide clear evidence that male-typical biological responsiveness to PGF pheromones might be attributable to androgenic actions at the level of the OE. For example, sexually dimorphic EOG responses to PGFs have been reported in adult goldfish (Sorensen and Goetz 1993), tinfoil barbs (Cardwell et al. 1995), *Barilius bendelisis* (Bhatt et al. 2002), rainbow sharkminnows (Stacey et al. 2003), and, in the present study, redtail sharkminnows and zebrafish. These adult gender differences in PGF-induced olfactory response likely are androgen mediated because androgen treatment enhances response to PGFs not only in the 4 cyprinids examined in this study but also in 3 other cyprinids: java barbs (*Barbonymus gonionotus*; Cardwell et al. 1995), *B. bendelisis* (Bhatt et al. 2002), and rainbow sharkminnows (Stacey et al. 2003). In contrast to this consistent trend for sexually dimorphic responses to PGFs in cyprinids, the situation in salmoniform fishes differs in several important respects. First, whereas

EOG studies show that PGFs are detected by all of more than 75 species of cypriniforms representing the breadth of cypriniform diversity, it is clear that some salmonids (e.g., genus *Oncorhynchus*) do not detect these compounds (reviewed by Stacey and Sorensen 2009). Second, reports of sexually dimorphic responses to PGFs in salmoniforms have been inconsistent. For example, Laberge and Hara (2003) report that olfactory thresholds to PGFs are similar in male and female lake whitefish (*Coregonus clupeaformis*) and brown trout (*Salmo trutta*), although males exhibit larger responses than females at suprathreshold concentrations. Moreover, Moore et al. (2002) report that only mature brown trout parr detect PGFs (immature parr had no EOG response even at 10^{-5} M), whereas data from Laberge and Hara (2003) show that PGFs are detected by undifferentiated juveniles, adult females, and bourgeois (parental) males, consistent with findings by Essington and Sorensen (1996) in brown trout and by Sveinsson and Hara (2000) in arctic charr (*Salvelinus alpinus*).

Unexpectedly, exposure to MT also reduced the magnitude of L-alanine–induced EOG responses in all 4 test species, although the decrease was significant only in redtail sharkminnows. The mechanism underlying this effect is not known but could result if MT induced a proliferation of PGF-sensitive OSNs, thereby reducing the density of alanine-sensitive OSNs. Reduced EOG responsiveness to amino acids has also been reported in hungry, but not recently fed, axolotls, *Ambystoma mexicanum* (Park and Eisthen 2003; Mousley et al. 2006), in which both gonadotropin-releasing hormone treatment and neuropeptide Y induce rapid and transient decreases and increases, respectively, in EOG magnitude, suggesting that release of these neuropeptides from the terminal nerve to the OE might normally modulate feeding responses in relation to reproductive condition and hunger. If such short-term neuropeptide effects occur in cyprinids, they are likely distinct from the androgen effects reported here, which evidently represent a sexual dimorphism that is established at puberty and then maintained by continued androgen action throughout reproductive adulthood.

Androgens may exert their effects directly on the OE, causing changes in peripheral sensitivity to PGFs, in several ways. First, androgens may bind receptors found in the OE (Pottinger and Moore 1997), leading to increases in the number of olfactory receptors and/or varying sensitivity of the receptors. Findings by Alekseyenko et al. (2006) support this hypothesis by showing that, in mice, T modulates the expression of vomeronasal receptors, thus altering behavioral responses to pheromones. Second, androgens may increase OSN density because MT treatment increases OE thickness in goldfish (Yamazaki and Watanabe 1979) and OE surface area increases during puberty in platyfish (*Xiphophorus maculatus*) (Schreibman et al. 1984). Increases in OSN cell number may coincide with an increase in putative pheromone detecting OSNs (crypt cells) in the OE of crucian carps (Hamdani et al. 2007). Furthermore, basal olfactory cells differentiate into neurons in approximately 9 days (Graziadei and Monti Graziadei 1979), coinciding with findings by Cardwell et al. (1995) who show that increases in EOG responses to PGFs begin approximately 9 days post-MT treatment. Indeed, androgen-induced changes in peripheral olfactory morphology may be responsible for changes in the sensitivity of the OE to putative PGF pheromones and increases in courtship behaviors observed in this study. Future work examining cellular-level changes in the OE during treatment with gonadal steroids will provide insights on changes in odorant sensory transduction and stimulus response in vertebrates.

In summary, this study shows that androgen treatment of a variety of juvenile cyprinids changes olfactory responses to pheromonal odorants that coincide with induction of the potential for male-typical, olfactory-mediated sex behaviors. Cyprinid fishes should serve as excellent vertebrate model organisms for examining endocrine effects on olfactory system development because they include both the goldfish, in which hormonally derived sex pheromones are well characterized, and the zebrafish, a commonly used vertebrate model organism.

Funding

Natural Sciences and Engineering Research Council of Canada grant to N.E.S.; postdoctoral fellowships from Natural Sciences and Engineering Research Council of Canada; Alberta Ingenuity to R.M.B.

Acknowledgements

We thank the staff in the Aquatics Facility at the University of Alberta for their continued technical support and animal care. Also, we are grateful for the both the adult and juvenile zebrafish supplied by Dr W.T. Allison and A. McCorry.

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