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ORIGINAL PAPER

# Acute effects of sex steroids on visual processing in male goldfish

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**Abstract** Elevations of sex steroids induced by social cues can rapidly modulate social behavior, but we know little about where they act within the nervous system to produce such effects. In male goldfish, testosterone (T) rapidly increases approach responses to the visual cues of females through its conversion to estradiol. Because aromatase is expressed in the retina, we tested if T can acutely influence retina responses to visual stimuli, and investigated the receptor mechanisms that may mediate such effects. Specifically, we measured FOS protein immunoreactivity to determine if T affects cellular responses to visual stimuli that include females, and used electrophysiology to investigate whether T can generally affect light sensitivity. We found that T acutely increased FOS responses to the simultaneous onset of light and the presence of female visual stimuli, both of which would normally be associated with early morning spawning, and increased electrophysiological responses to low intensity light pulses. Both effects were blocked by an estrogen receptor beta (ERβ) antagonist, indicating that T is likely being converted to estradiol (E2) and acting through an ERβ mediated mechanism to acutely modulate visual processing. Changes in sensory processing could subsequently influence approach behavior to increase reproductive success in competitive mating environments.

**Keywords** Non-genomic · Courtship · Androgen · Estrogen · Social

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**Abbreviations**

| | |
|---|---|
| BSA | Bovine serum albumin |
| E2 | Estradiol |
| ERα | Estrogen receptor alpha |
| ERβ | Estrogen receptor beta |
| FAD | Fadrozole |
| FOS-ir | FOS-immunoreactive |
| GCL | Ganglion cell layer |
| GPR30 | G protein-coupled receptor 30 |
| INL | Inner nuclear layer |
| MS-222 | 0.1% Tricaine methanesulfonate |
| PBS | Phosphate buffered saline |
| PFA | Paraformaldehyde |
| T | Testosterone |
| UV | Ultraviolet |

## Introduction

Sex steroid hormones play a dynamic role in the modulation of social behavior through their ability to influence cell physiology rapidly via non-genomic mechanisms. These mechanisms are activated by elevations of sex steroid hormones, peripherally or in the brain, that occur in response to social cues (Cornil et al. 2012). The rapid behavioral effects of sex steroid hormones are particularly salient in reproductive contexts, in which they affect numerous behavioral and physiological responses in a wide range of species from diverse vertebrate groups (Remage-Healey and Bass 2005, 2007; Cornil et al. 2006; Huddleston et al. 2007; Lord et al. 2009; Mangiamele and Thompson 2012; Seredynski et al. 2015). Socially induced fluctuations in steroid levels in the brain and periphery may not be necessary for the expression of reproductive behaviors, but they may have acute effects on responses to sexual stimuli that increase

reproductive success, particularly in competitive mating contexts (Thompson and Mangiamele 2013).

Much is still unknown about where steroids act within the nervous system to induce acute effects on behavior. In addition to influences on brain processes involved in motivation and motor output, they may also rapidly affect sensory processing, perhaps even at early, peripheral stages. Classical sex steroid receptors are present in primary sensory neurons and/or in structures associated with early stages of sensory detection in some species, including the vomeronasal sensory neurons of mice (Cherian et al. 2014), the inner ear of plainfin midshipman (Forlano et al. 2005, 2010; Fergus and Bass 2013) and zebra finches (Noirot et al. 2009), the retina of rats (Cascio et al. 2007), and the olfactory bulbs of African cichlid fish (Maruska and Fernald 2010). Indeed, sex steroids can modulate early stages of sensory processing; androgens influence electrosensory processing in stingrays (Sisneros and Tricas 2000), and in cyprinids they increase electro-olfactogram responses of olfactory sensory neurons to prostaglandins (Belanger et al. 2010). Both testosterone (T) and estradiol (E2) affect auditory processing in plainfin midshipman (Sisneros et al. 2004). However, most of these influences have been observed after chronic steroid treatments, and it is unknown if the classical receptors found in those areas can be trafficked to membranes, as they can in other tissues. It is therefore not yet clear if sex steroids can rapidly modulate early stages of sensory processes in ways that might dynamically alter an animal's perception of the social environment. Estradiol was recently shown to rapidly decrease responses evoked by non-social odorants in the olfactory receptor neurons of mice, possibly by binding to G protein-coupled receptor 30 (GPR30), which has been located in these neurons (Kanageswaran et al. 2016). The social significance of these findings, if any, is difficult to determine, though it is possible that E2 could sharpen responses to pheromones by decreasing responses to non-social odorants.

In goldfish, sex steroid hormones have a variety of influences on reproductive processes, including rapid influences on behavioral and physiological responses to sexual stimuli. Injections of T increase approach responses towards the visual cues of females within 45–60 min, an effect blocked by treatment with fadrozole (FAD), an aromatase inhibitor, and mimicked by E2, indicating that T rapidly modulates behavioral responses to female visual stimuli via estrogen receptor activation (Lord et al. 2009). Sex steroid hormones also rapidly affect physiological processes that likely enhance reproductive success. Male goldfish injected with T show increased milt volume and sperm density within just 1 h, an effect that is also blocked by FAD (Mangiamele and Thompson 2012). This increase is also seen with E2:BSA, which binds to membrane, but not intracellular, receptors; moreover, the effect is blocked by both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) antagonists, suggesting that membrane versions of both receptors are necessary to produce these physiological effects. All of these effects are likely induced by social stimuli in natural contexts; previous research has shown that T rapidly increases in male goldfish in response to female spawning stimuli (Kobayashi et al. 1986). During spawning, multiple males typically follow and court ovulating females, so these T surges could activate non-genomic estrogen receptor mechanisms that prime males to approach females, and once spawning begins, release maximal amounts of sperm. Both these effects likely serve to increase mating success in a competitive mating context.

The rapid behavioral effects may be associated with influences on visual processing, even at early, peripheral stages. Although pheromones are critical for goldfish mating, visual cues also play a role; anosmic males spend more time courting ovulating females than non-ovulating females, potentially a function of specific visual cues associated with ovulating females (Partridge et al. 1976). Additionally, females treated with androgens, like males in reproductive condition, preferentially orient towards the visual cues of females (Thompson et al. 2004). Aromatase and estrogen receptors are located not only in visual processing regions in the brain, most notably the optic tectum, but also peripherally in the retina, where they are found in horizontal, bipolar, and amacrine cells in the inner nuclear layer (INL), as well as in retinal ganglion cells (Gelinas and Callard 1993; Callard et al. 1995). G protein-coupled receptor 30 mRNA has also been identified in the retina and optic tract (Mangiamele et al. 2017). However, it is still unknown if and how T, through its conversion to E2, regulates visual processing in goldfish.

We hypothesized that T may influence early stages of visual processing that could ultimately enhance behavioral responsiveness to female visual stimuli in male goldfish. To determine if T has relatively rapid effects on retina responses to visual stimuli that would typically be associated with spawning, which usually begins in the early morning as light is increasing, we tested the effects of acute T administration on FOS responses to illumination plus females. In an initial attempt to dissociate influences on general light sensitivity from those on stimulus features associated with females, we also tested the effects of T on FOS responses to just the light stimulus used to illuminate the females. To further explore potential influences of T on general retina sensitivity, we also developed an electrophysiological protocol, which might be a more sensitive measure of light sensitivity than FOS immunohistochemistry. That method also allowed us to measure responses more quickly after T administration. Finally, we tested whether an ERβ antagonist can block the acute effects of T on retina responses to visual stimuli.

## Methods

### Animals

Sexually mature male and female *Carassius auratus*, 12.5–16 cm long, 25–50 g, were purchased from Black Water Creek Fisheries in spring and early summer (Eustis, FL) and housed in same-sex tanks at 20 °C on a 14:10 h light:dark cycle. Experimental males were tested during the spring or early summer. Breeding condition was determined by the presence or absence of milt. In all experiments, animals were anesthetized in 0.1% tricaine methanesulfonate (MS-222) in tank water buffered in sodium bicarbonate prior to killing.

### Acute steroid effects on retina responses to semi-naturalistic visual stimuli

*Exp 1* To determine if FOS protein immunoreactivity (FOS-ir) could be used as a marker of cellular responses to illumination plus female visual stimuli in goldfish, a preliminary experiment was conducted in which males were individually dark-adapted in a small, aerated tank (28 × 15 cm) overnight. In the morning, control fish ($n = 4$) were killed immediately. Room lights and full-spectrum lights (Reptisun 5.0, Zoomed, CA) directly over the tanks were then turned on to full brightness, and one female fish was placed into each of four small Plexiglas chambers (26 × 9 cm), two of which allowed ultraviolet (UV) wavelengths to pass, surrounding the experimental fish. In this tank configuration, females were always in the visual fields of both eyes of the male, at similar distances, no matter where the male turned in the center compartment. After 90 min, the male "light fish" ($n = 4$) were killed and the females were returned to their holding tanks. Because breeding typically occurs in the early morning as light increases and after males have been in dim/dark conditions overnight, we tested responses to female visual stimuli in dark-adapted male goldfish. However, it is important to note that we cannot distinguish between FOS responses to a sudden change in illumination and responses to the female stimuli. Additionally, we did not expose fish to slowly increasing levels of illumination, and the illumination was likely brighter than what reaches fish in pond water in typical early morning spawning conditions.

*Exp 2* To determine if T can acutely influence FOS responses to illumination and/or female visual stimuli, male fish were housed individually overnight in the same test chambers. For this experiment, "dark"-adaptation took place in dim light (approximately 0.002 µW) so that we would not need to turn the lights on for injections the next morning. In the morning, males were intraperitoneally injected (50 µl) with 2.5 µg T in 0.1% EtOH ($n = 8$) or vehicle ($n = 7$). Testosterone doses in this range result in increased plasma levels of T that are within physiological range for goldfish (Lord et al. 2009). Forty-five minutes later, the room lights and full spectrum lights immediately above the tanks were, as in Exp 1, turned on to full brightness, and one female fish was placed into each of the four Plexiglas chambers. After 90 min, the male fish were killed and the females were returned to their holding tanks. Thus, the FOS activation would primarily reflect visual responses that occurred 60–90 min earlier and thus 45–75 min after T injections, consistent with the time course for T's acute effects on behavioral responses to female visual stimuli in our previous studies (Lord et al. 2009).

*Exp 3* To determine the receptor mechanisms that mediate T's acute effects on retina responses, we followed the same general procedures, but an additional group of fish injected with T and an ERβ antagonist was included. Males were again kept in dim light overnight, and injected with either vehicle (100 µl/fish; $n = 8$), T (5 µg/fish; $n = 8$), or T + PHTPP (5 µg of each/fish; $n = 8$) in the morning. We increased the dose of T to 5 µg/fish for consistency with the dose used in the electrophysiology experiment (see below). Forty-five minutes later, the room lights were turned on, full spectrum lights were turned on to 75% brightness, and one female fish was placed into each of the four Plexiglas chambers. After 90 min, the male fish were killed and the females were returned to their holding tanks. Overhead illumination from full-spectrum lights was reduced in this experiment to decrease the likelihood that FOS activation was maximum in the control condition, which could have made it difficult to detect further increases in response to T in Exp 2.

*Exp 4* To determine if T was generally enhancing FOS responses to a change in illumination independent of the stimulus females, we repeated the experiment but did not add females when the lights were turned on. The same procedures were otherwise followed. Fish were habituated overnight in dim light and injected in the morning with vehicle ($n = 9$) or T ($n = 7$); 45 min later the room lights and full spectrum lights over the tanks (75% brightness) were turned on. Fish were anesthetized and killed 90 min later.

### Tissue processing/immunohistochemistry

Eyes were removed and the lens was taken out. Tissue was immediately fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) for 45 min, and then sunk in 30% sucrose in PBS overnight. The tissue was embedded in Shandon™ Cryochrome™ embedding resin and stored at − 80 °C until sectioning. Eyes were sectioned at − 20 °C into 20 µm slices using a Cryotome E cryostat (ThermoFisher), before being mounted onto positively charged slides (Fisher Scientific). All slides were stored at − 80 °C.

For immunohistochemistry, slides were first warmed to room temperature and then fixed in chilled 4% PFA solution

before being washed twice with PBS at room temperature. All slides were then blocked in 300 µl of a blocking buffer, which consisted of 2% bovine serum albumin (BSA) and 0.3% Triton X-100 (Sigma Aldrich) in PBS, for 1 h at room temperature.

Slides were incubated in 300 µl of polyclonal, rabbit-raised anti c-Fos (1:1000; Santa Cruz Biotechnology Lot #A2313, Santa Cruz, CA) in blocking buffer overnight at 4 °C in a moist hybridization chamber. After overnight incubation, primary antibodies were washed off twice with PBS at room temperature for 10 min each. Then slides were blocked with 10% horse serum in PBS for 20 min. Slides were incubated in 300 µl of secondary antibodies in PBS containing 10% horse serum for 2 h at room temperature in a moist hybridization chamber. Secondary antibodies used were Alexa Fluor 488-conjugated donkey anti-rabbit (1:500; Invitrogen Lot #853503) or Dylight 488 horse anti-rabbit (1:500, Vector Laboratories DI-1088; the same secondary was used in each individual experiment).

Secondary antibodies were washed off after 2 h, twice with PBS for 10 min each and once with deionized water for 5 min. Then, slides were coverslipped using 2–3 drops of Vectashield Hardset media with DAPI (Vector Labs), stored at room temperature for 15 min and then at 4 °C until visualization.

### Imaging and quantification of cFos-positive cells

Immuno-labeled slices were viewed with a BX-51 microscope (Olympus) and images were captured with the QCapture imaging software (QImaging). Twenty pictures were taken using a 100× objective from one eye of each male goldfish tested. This included ten images from the lateral and ten images from the medial retina. For each eye, imaging started with the section adjacent to the section where the optic nerve entered the retina. Five pictures were taken from this section. These five images were spaced out evenly around the section, and did not cover the entire section. The next three sections were skipped, and another five pictures were taken from the next section. These ten pictures constituted the "medial retina". Ten sections were then skipped, and the procedure was repeated for images of the lateral retina. The number of cells producing nuclear FOS were counted in each image using ImageJ. The area of tissue included in each image was also measured, and counts were converted to density. The experimenter was blind to condition while taking pictures and counting cells.

### Acute steroid effects on light sensitivity

*Exp 5* To determine whether T affects general light sensitivity, we used an electrophysiological method that is likely more sensitive to light than FOS immunohistochemistry. We used a similar experimental design, but measured rapid effects of T on the amplitude of the electrical responses of the retina to pulses of light; specifically, we measured the amplitude of b-wave responses in an electroretinogram.

For these experiments, male goldfish were dark adapted overnight. Under dim light conditions, the fish was intramuscularly injected with 50 µL of 0.2% flaxedil in teleost saline. The fish was placed onto half of a Petri dish (100 mm dia) and held in place with modeling clay; it was brought to the recording cage with its eye covered to ensure dark adaption. Throughout the experiment, the gills were gravity perfused with a solution of 150 mg/l MS-222, aerated and buffered with 0.015% sodium bicarbonate; perfusion fluid entered the mouth and exited through the gills. A butterfly needle attached to a syringe was inserted into the dorsal musculature. Intramuscular injections were used in this experiment because the needle was more stable throughout the procedure when inserted into muscle than into the body cavity.

Using a sapphire blade knife, an incision was made at the edge of the pupil. Two cuts, one on each side of the initial cut, were made using microscissors to open up a slit. An electrode was inserted into the slit and lowered a few millimeters beneath the surface into the vitreous chamber. A silver–silver chloride ground wire was inserted into the tail, but did not touch the MS-222 in the dish. All surgical procedures were done in very dim light conditions; once completed, the fish was left to acclimate for 10 min before recordings began.

The electrode used for recording was a glass micropipette (1 mm diameter), with a ~ 100 µm opening, filled with teleost saline. An electrode holder with a silver silver-chloride wire was used to couple the electrode to an AM Systems Differential AC Amplifier (Model 1700), with the low cutoff filter set at 0.1 Hz, high cutoff at 500 Hz, and amplification at 1000×. Data were acquired using a Cambridge Electronic Design A-D converter (CED micro 1401 mkII). Electroretinogram recordings were sampled at a rate of 5000 Hz using the program Spike2 (Version 7; CED).

The light stimulus was controlled by an AM Systems Isolated PulseStimulator (Model 2100), which triggered light pulses 500 ms in duration; each light pulse was a single beam that mixed the outputs of three individual LED bulbs, with wavelengths of 455, 530, and 625 nm, which correspond to maximal sensitivities for the visible spectrum cones in goldfish (Palacios et al. 1998). We did not use a UV-producing LED, although goldfish have UV sensitive cones, because we found that repeated presentations bleached responses to all wavelengths.

For each fish, the electroretinogram elicited by four different light intensities (0.1, 0.5, 2.5, and 12.5% of the maximum intensity of the light source) was recorded. Light intensity at the level of the goldfish retina was measured using a Newport Photodetector, series 818. The intensities

recorded were determined to be 0.052, 0.088, 0.322, and 1.670 μW/cm$^2$. The retinas were exposed to light starting at the low, and increasing to the high light intensity, with ~1 min between each measurement to allow recovery of the retina. Five pulses at each intensity were delivered. After one sweep through all intensities was completed, the fish was injected intramuscularly, through the attached syringe, with 50 μl of either T (5 μg/fish in 0.1% EtOH), T + PHTPP (5 μg of each/fish) or teleost saline; the fish was left to recover for 10 min before another sweep through the light intensities was performed. Recordings were typically completed within 90 min from when the fish were first anesthetized. The time from injections to stimulus presentation was shorter in this experiment than in the FOS experiments because longer intervals led to reduced responses across time in control fish in pilot experiments. At the end of each experiment, the fish was removed from the setup and the heartbeat was verified to make sure the fish was still alive. The sex and reproductive condition of the fish was determined by checking for expressible milt. All fish were milting males.

### Data analysis

Data were analyzed using IBM SPSS Statistics Packages. All cell counts from the FOS experiments were divided by tissue area before analysis, and separate analyses were done for cells in the inner nuclear layer (INL) and in the ganglion cell layer (GCL). For FOS experiments in which data were not normally distributed and/or in which there was an inequality of variance across groups (Exp's 1 and 4), we used Mann–Whitney *U* tests to compare the numbers of FOS immunoreactive (FOS-ir) cells between groups. For the FOS experiments for which data were normally distributed and there was equality of variance across groups (Exp's 2, 3), mixed model, repeated measures ANOVAs were performed with location within the retina (medial vs lateral) as a within subjects factor and drug condition as a between subjects factor. Tukey post-hoc, pairwise comparisons were used to test the specific predictions that T would increase FOS responses relative to vehicle and that the antagonist would prevent T from having that effect and/or decrease responses relative to controls in Exp 3.

Electrophysiological data were not normally distributed and lacked equality of variance across treatment groups, so only non-parametric tests were used. The goldfish electroretinogram consists of three parts, an initial negative-going a-wave, followed by a positive b-wave and a much slower positive-going c-wave. The amplitude of the b-wave was used to quantify our results, measured from the nadir of the a-wave to the peak of the b-wave. The value of the b-wave amplitude used for each fish was the average of the responses to five replicates at each light intensity. Wilcoxon signed ranks tests were used to compare b-wave amplitude between saline and post-injection recordings at each intensity in each group. We then calculated the percent change from baseline to post-injection recordings for each intensity and compared percent change across the three groups with Kruskal–Wallis tests. To test the specific predictions that T would increase responses and the antagonist would block those increases and/or decrease responses relative to controls, pairwise comparisons between groups were conducted at intensities where the main effect was marginal ($p = 0.06$) or significant, and at intensities at which one or more of the groups showed significant changes across recording periods.

For data analyzed with non-parametric statistics, medians and interquartile ranges are shown. For data analyzed with parametric statistics, mean values and SEMs are shown.

## Results

### Acute steroid effects on FOS responses to semi-naturalistic visual stimuli

#### *Exp 1. Effects of visual stimuli on FOS responses in the retina*

Visually stimulated retinas contained significantly more FOS-ir cells than did dark-adapted retinas in the INL, averaged across medial and lateral regions, [mean + SEM/0.01 mm$^2$; dark ($n = 4$): 0.870 + 0.118; light ($n = 4$): 3.83 + 0.309; $U = 0.000$, $p = 0.029$; see Fig. 1 for an image of FOS-ir cells in dark and light fish]. The number of FOS-ir cells in the GCL from visually stimulated fish tended to be higher than from dark-adapted retinas, but the difference was not significant [mean + SEM/0.01 mm$^2$; dark ($n = 4$): 0.576 + 0.097; light ($n = 4$): 1.60 + 0.134 ; $U = 1.00$, $p = 0.057$]. This preliminary experiment indicated that we could use FOS as a marker of visually induced cellular activity in the goldfish retina, particularly in the INL.

#### *Exp 2. Effects of T on FOS responses in the retina*

In the INL, T significantly increased FOS responses to light plus female visual stimuli; there were significantly more FOS-ir cells in the retina of fish that had been injected with T than in the retina of fish injected with vehicle [main effect of condition: $F(1,13) = 5.870$, $p = 0.03$; Fig. 2]. There were no significant differences across groups in the GCL [$F(1,13) = 0.01$, $p = 0.76$]. There were no significant differences in the numbers of FOS-ir cells in different retina locations in the INL or the GCL, nor was there a significant interaction between condition and location for either cell group.

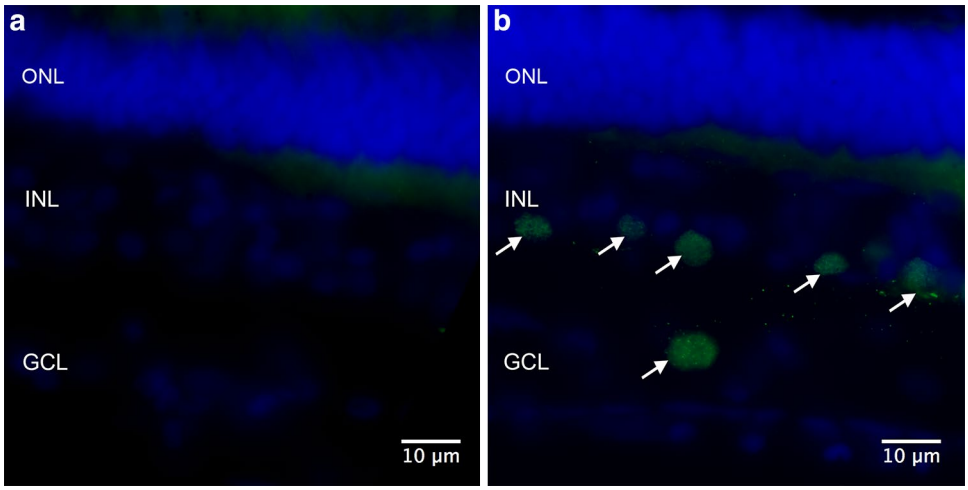


**Fig. 1** FOS immunostaining in **a** dark and **b** visually stimulated retinas. FOS signal appears in green, as indicated by arrows, in both the INL and GCL. Pictures taken with 100× objective. Background was subtracted from each picture

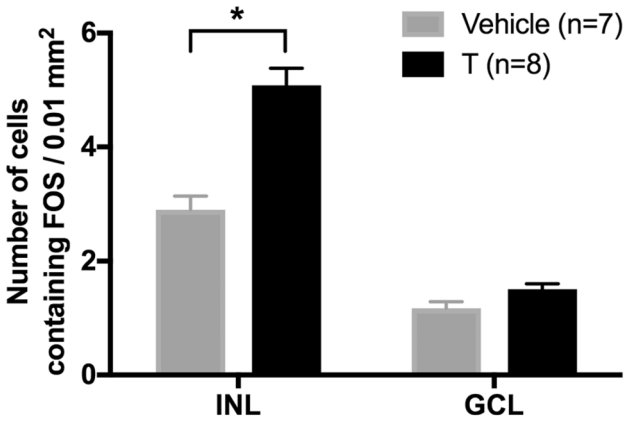


**Fig. 2** Mean (+SEM) number of cells containing FOS in the INL and the GCL in retinas of males injected with T and vehicle. Retinas from males injected with T contained significantly more FOS-ir cells than did retinas from males injected with vehicle in the INL ($p = 0.03$)

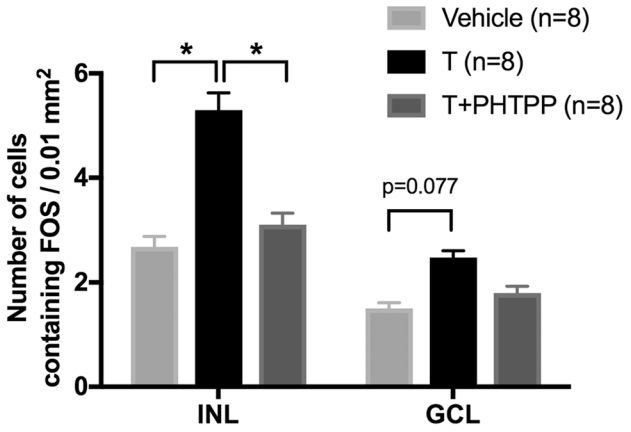


**Fig. 3** Mean (+SEM) number of cells containing FOS in the INL and the GCL in retinas of males injected with T+PHTPP, T, and vehicle. Retinas from males injected with T contained significantly more FOS-ir cells than did retinas from males injected with T+PHTPP ($p = 0.01$) and vehicle ($p = 0.003$) in the INL

*Exp 3. Effects of an ERβ antagonist on FOS responses in the retina*

There was a main effect of condition on the number of FOS-ir cells in the INL of the retina [$F(2, 21) = 7.98$, $p = 0.003$]. Pairwise comparisons showed that fish injected with T had significantly more FOS-ir cells than fish injected with T+PHTPP ($p = 0.01$; Fig. 3), as well as significantly more than fish injected with vehicle ($p = 0.003$). The number of FOS-ir cells in fish injected with T+PHTPP and fish injected with vehicle were not significantly different ($p = 0.80$). There was no significant main effect of condition in the GCL [$F(2,21) = 2.78$, $p = 0.09$]. There was a non-significant trend for higher numbers of FOS-ir cells in the GCL from fish injected with T than vehicle ($p = 0.077$), but not for differences between T and T+PHTPP ($p = 0.27$), nor between vehicle and PHTPP treated fish ($p = 0.76$). There were significantly more FOS-ir cells in the medial than in the lateral divisions of the GCL ($p = 0.02$), but no significant interactions between drug condition and location in the retina. Although two fish were not milting, their exclusion did not change the results.

*Exp 4. Effects of T on FOS responses to constant illumination*

Testosterone did not affect FOS responses to constant illumination; there were no significant differences between vehicle and T groups for the number of FOS-ir cells in the INL [averaged across medial and lateral areas; mean+SEM/0.01 mm$^2$; vehicle ($n = 8$): 3.48+1.72; T ($n = 6$): 1.1+0.57; $U = 15$, $p = 0.25$] or in the GCL [mean+SEM/0.01 mm$^2$; vehicle ($n = 8$): 1.89+0.52; T ($n = 6$): 1.15+0.42; $U = 14$, $p = 0.2$].

Data could not be collected from one fish in each group for histological reasons.

### Acute effects of sex steroid hormones on b-wave responses to light pulses

All the light intensities used induced clear electroretinogram responses, with measurable b-waves, indicating that our lowest light intensity was above threshold. Over the range of intensities used (a 32-fold range), b-waves increased with increasing intensity (Fig. 4 for a representative recording). Recordings using higher intensities indicated that the highest intensity used in our analysis (12.5%) elicited maximal responses.

*Exp 5. Effects of T on b-wave responses to light pulses*

There were no significant differences in b-wave amplitude between baseline and post-injection recordings at any light intensity after injection of vehicle. After injection of T, responses tended to increase at all intensities, but the difference was significant only for responses to the lowest intensity ($Z = -2.37$; $p = 0.02$; see Fig. 5b). In the T + PHTPP group, responses decreased significantly from baseline in response to stimulation at 2.5% light intensity (0.322 µW/cm$^2$: $Z = -1.99$, $p = 0.046$; see Fig. 5c) and tended to be lower at the highest intensity (12.5%), though the change was not significant (1.67 µW/cm$^2$; $Z = -1.79$, $p = 0.075$).

Consistent with the possibility that T was selectively influencing responses to the lowest intensity, there was a marginal effect only at that intensity in the between groups comparison of differences in the percent change from baseline ($Chi^2 = 5.87$, $p = 0.06$). Pairwise comparisons between the groups at that intensity confirmed that the percent

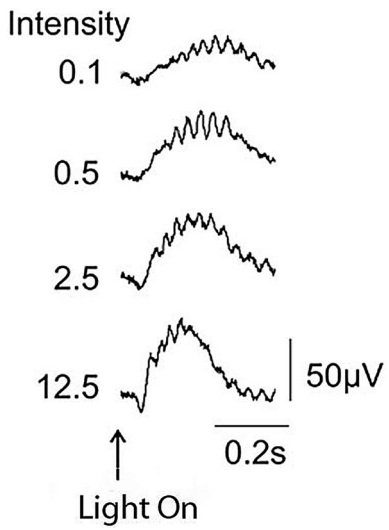


**Fig. 4** Electoretinogram of b-wave responses to each of the four intensities (0.1, 0.5, 2.5, and 12.5% of the maximum intensity of the light source, corresponding to 0.052, 0.088, 0.322, and 1.670 µW/cm$^2$), measured from a representative fish in baseline conditions. Arrow indicates light onset, which lasted 500 ms

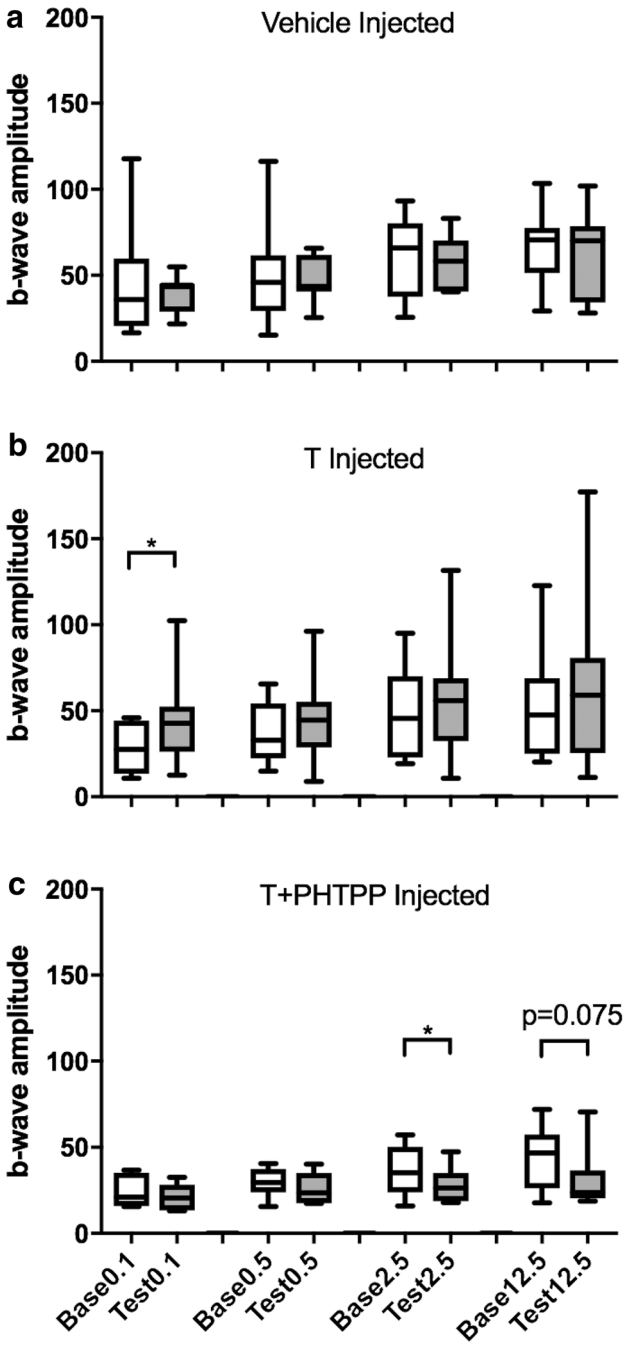


**Fig. 5** Median and interquartile range of b-wave amplitudes to pulses of light at each intensity during baseline recordings and again 10–30 min after injections of **a** vehicle, **b** T, and **c** T + PHTPP. Asterisk indicates significant changes from baseline to post-injection, which occurred at the lowest intensity, 0.1% of the maximum intensity (0.052 µW/cm$^2$) in T injected fish and at 2.5% of the maximum intensity (0.322 µW/cm$^2$) for the T + PHTPP injected fish

change was significantly higher in T-injected animals than in vehicle injected animals ($U = 8$, $p = 0.04$; Fig. 6a), and as predicted, tended to be higher in T-treated animals than in T + PHTPP injected animals, though the difference was not significant ($U = 8$, $p = 0.06$; Fig. 6a). Consistent with the declines observed in the T + PHTPP injected group at the higher intensities, there was a significant difference between the T and T + PHTPP groups at the highest intensity ($U = 6$; $p = 0.03$; Fig. 6d).

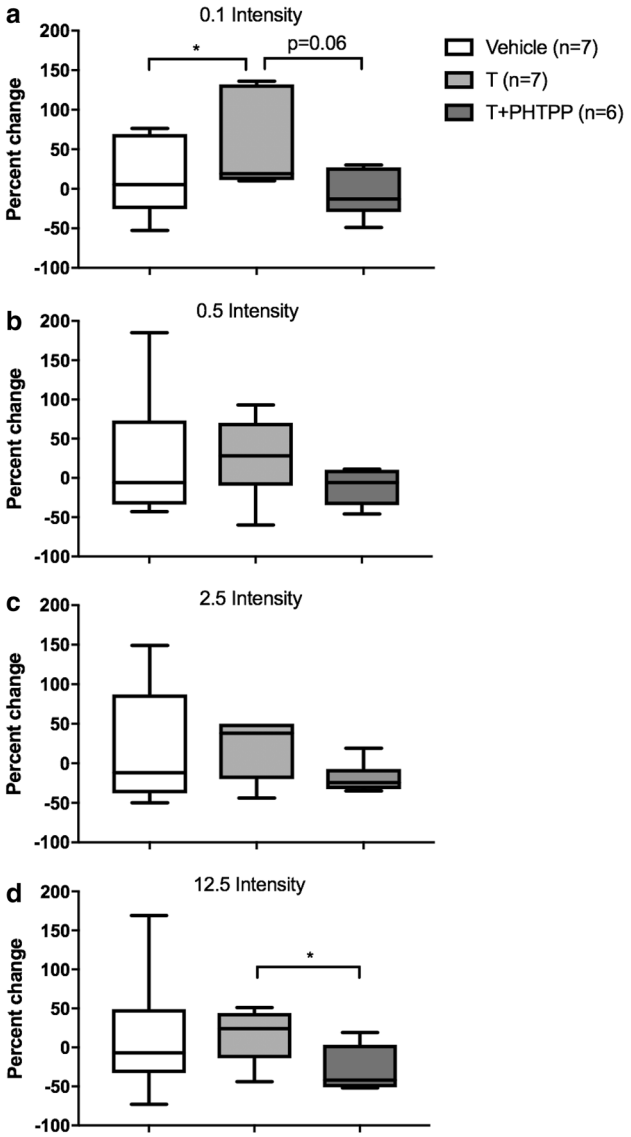


**Fig. 6** Median and interquartile range for the % change from baseline to test at each intensity, **a** 0.1%, **b** 0.5%, **c** 2.5%, **d** 12.5%, of the maximum intensity, for vehicle, T, and T + PHTPP injected fish. At the lowest intensity (0.1%, 0.052 μW/cm$^2$), T-injected fish showed a significant increase in % change from baseline to test relative to vehicle-injected fish. At the highest intensity (12.5%, 1.670 μW/cm$^2$), T + PHTPP injected fish showed a significant decrease in % change from baseline to test relative to T-injected fish

## Discussion

Courtship in goldfish typically occurs in early morning, when dark-adapted males, primed overnight by pre-ovulatory pheromones that elevate T, encounter increasing light and female visual stimuli. Although we cannot conclusively determine the critical stimulus features affected by T from these experiments, they demonstrate that T acutely increases cellular responses to visual stimuli that include females in the INL of the retina 45–75 min after T injections, and that an ERβ antagonist can block this relatively rapid effect. Testosterone did not enhance FOS responses to just the illumination, suggesting it may selectively influence the processing of a stimulus feature associated with females, though interpretations based on negative data should always be treated cautiously. Indeed, our electrophysiology results, albeit preliminary, suggest that T can increase general retina sensitivity to low levels of visible light in the absence of female visual cues, and that it does so within 10–30 min. The ERβ antagonist also tended to reduce this effect at lower light intensities and to depress responses significantly at higher intensities. Together, these results suggest that T, after being converted to E2 and activating ERβ receptors, acutely enhances retina responses to visual input, which may increase the ability of males to detect and track females during courtship.

Previous work in our lab demonstrated that T, also through an estrogen receptor mechanism, rapidly increased approach responses towards the visual cues of females (Lord et al. 2009). Whether the influences of T on retina responses to visual stimuli identified by the present experiments are part of the mechanism through which T induced those behavioral effects remains to be determined, particularly in light of our inability to detect influences on responses in the GCL, which relays visual input to the brain (see further discussion below). Additionally, our previous studies were conducted in bright light conditions in which stimulus detection was unlikely difficult. Nonetheless, our current data suggest that T surges, which are typically elicited by female primer pheromones (Kobayashi et al. 1986), enhance the earliest stages of visual processing and, as a result, could promote behavioral responses to salient visual stimuli. To explore the behavioral significance of such influences more fully, future studies should determine if T alters visual detection thresholds and, as a result, promotes approach responses to females in extremely low levels of light and/or across distances at which males would not otherwise approach them.

### Receptor mechanisms that mediate acute effects of T in the retina

Testosterone's acute enhancement of responses to visual stimuli appeared dependent on ERβ, as a selective ERβ

antagonist blocked T's effects. Elevations of FOS responses to visual stimuli likely reflect influences of T that occurred 45–75 min after administration (60–90 min prior to killing). Although that is a relatively rapid effect for a steroid, it is not fast enough to attribute conclusively to a non-genomic receptor mechanism. However, T's electrophysiological effects were evident 10–30 min after administration, which is consistent with a non-genomic mechanism, and those effects also tended to be blocked by the ERβ antagonist. At least one previous study has shown that ERβ also mediates non-genomic estrogenic influences on sexual motivation in male quail (Seredynski et al. 2015). However, while our results indicate that ERβ is necessary for T to induce acute effects on retina processes, they do not preclude the possibility that other receptors also play some role. G protein-coupled receptor 30 is expressed in goldfish retina (Mangiamele et al. 2017), and though we do not yet know if ERα is present in the retina, membrane versions of ERα, like ERβ, also mediate rapid effects of estrogens on behavior in other species (Dominguez-Ordonez et al. 2016). Indeed, rapid effects of steroids may depend on the simultaneous activation of several receptor types, which may even work together as membrane complexes. In some cell types, there is evidence that GPR30 may help transmit membrane ERα-generated signals to downstream signaling cascades (Vivacqua et al. 2006) and in others, ERs exist as homodimers and heterodimers in the plasma membrane (Razandi et al. 2004; Guo et al. 2005). Pre-treatment with an ERβ antagonist and an ERα antagonist each blocked rapid, T-induced increases in milt volume and sperm cell density in goldfish (Mangiamele and Thompson 2012), indicating that both receptors may mediate acute influences of T on physiological processes related to reproduction. We attempted an experiment with a selective ERα antagonist, but that experiment was done later in the breeding season and T did not enhance FOS responses to female visual stimuli, so we were unable to draw any conclusions about ERα's role in mediating T's effects. The number of FOS labelled cells in the INL, where T had its most pronounced influence in the current experiments, were substantially lower in the control condition in that experiment, suggesting either that retina responses had generally changed as a function of time of year or that a methodological error occurred. Clearly, that experiment needs to be repeated earlier in the breeding season, along with tests of GPR30 antagonists.

### Selectivity of T's effects for female visual stimuli

Testosterone affected FOS responses to illumination plus female stimuli, but not to just the illumination stimuli used in at least one of those experiments. This suggests that T's effects on FOS were selective to the female stimuli, though a single experiment in which the ability of T to influence responses to just illumination and illumination plus female stimuli will be necessary to make that determination conclusively. Although retina *c-fos* responses to simple illumination have been demonstrated in other species (Koistinaho and Sagar 1995; Yoshida et al. 1998; Hannibal et al. 2002; Bertolesi et al. 2014), we have not yet verified that the illumination used in our experiments, by itself, increases FOS-ir in the retina of goldfish that are in reproductive condition (it does not in fish tested in winter that are not in reproductive condition; unpublished data). If it does, but T does not enhance that response, it would indicate that T specifically influences the processing of a stimulus feature associated with the females. Such specificity would be consistent with T's selective enhancement of approach responses to female, but not male, visual cues (Lord et al. 2009). Males can discriminate sex based on visual stimuli (Thompson et al. 2004), though we do not yet know what specific cues they use. Goldfish do have UV photoreceptors (Bowmaker et al. 1991), which could, as in other fish, be important for sexual discrimination (Kodric-Brown and Johnson 2002; Macías Garcia and de Perera 2002). This raises the possibility that T could selectively enhance responses to a UV cue specific to females. However, we have observed that males appear to reflect UV more than females (unpublished observations), and T influenced responses to visible spectrum wavelengths in our electrophysiology experiment, both of which suggest that T is unlikely to selectively promote approach responses to female visual stimuli by enhancing UV responsiveness. It also seems unlikely that T would selectively influence responses to a particular visible wavelength, as there is a variation in goldfish coloration and males and females are not dimorphic in the visible range, or to a specific female shape, which is unlikely represented in retina coding processes.

However, it is possible T affected how the retina processed a stimulus feature associated with females, but not unique to them, like their movement and/or the changes in illumination that resulted from that movement. Indeed, our preliminary electrophysiological finding that T can rapidly increase b-wave amplitudes to low intensity pulses of light, likely a much more sensitive measurement of light sensitivity than FOS immunohistochemistry responses to constant illumination, is consistent with that possibility. If so, T's ability to stimulate responses to females plus illumination in the FOS experiments, but not just to illumination, may have been associated with the amplification of FOS responses to the movement of the females and/or the changes in illumination associated with that movement. Such a generalized mechanism would not diminish its potential importance in the detection and tracking of potential mates, particularly in dim light conditions and in coordination with responses to ovulatory pheromones critical for determining which female to court. Testosterone's selective enhancement of approach responses to female visual cues may therefore depend on

effects in higher brain regions. Aromatase is found not only in cells in retina, but also in the optic tectum, which mediates orientation responses to visual stimuli, and in the preoptic area, where visual input is likely integrated into behavioral responses related to reproduction (Gelinas and Callard 1993; Callard et al. 1995). Parallel actions in the retina and in these areas, where T may more specifically influence responses to visual stimuli unique to females, may thus help males detect potential mates and selectively orient towards them, respectively. It also remains possible that T can generally increase light sensitivity in some retina cells and selectively enhance responses to some visual stimulus feature associated with females in others. Aromatase is present in multiple cell types within the retina (Gelinas and Callard 1993; Callard et al. 1995), so locally produced estrogens could influence multiple visual processes. Clearly, additional studies are necessary to resolve the critical stimulus features necessary for T to exert its effects on retina responses to visual input.

**Localization of acute T effects in the retina**

We only observed effects of T on FOS responses in the INL, which contains bipolar, amacrine, and horizontal cells. Visually induced *c-fos* expression has been observed in rod bipolar cells in the INL in mice (Yoshida et al. 1998), in bipolar and amacrine cells in *Xenopus* tadpoles (Bertolesi et al. 2014), and in amacrine cells in rabbits (Koistinaho and Sagar 1995; Bertolesi et al. 2014), though we were unable to determine the cell type(s) in which FOS responses were elevated by T in the goldfish INL in the current study. There is no evidence, to our knowledge, that light induces *c-fos* in horizontal cells, though that does not preclude the possibility that T influences on horizontal cells could ultimately affect FOS production in other cell types. The activity of ON bipolar cells is believed to be the major contributor to the b-wave of the electroretinogram (Dong and Hare 2002), whose amplitude in response to light was increased in males injected with T. This result, combined with the finding that FOS protein immunoreactivity increased in the INL of fish injected with T, suggests that E2 produced locally by aromatase may modulate the activity of bipolar cells, directly or indirectly, leading to changes in visual sensitivity. However, other studies have found that amacrine cells and Muller cells also contribute to the b-wave (Awatramani et al. 2001), raising the possibility that estrogens generated from T could also affect visual responses, including b-wave amplitude, via direct actions in those cells. Estrogenic effects in any or all of these cells types are possible, as aromatase has been found in bipolar, amacrine, and horizontal cells of the INL, as well as in fibrous processes in these layers (Gelinas and Callard 1993). Aromatase could thus be located at synaptic terminals, where it could convert T to E2 that acts upon membrane estrogen receptors within the synapses. Previous studies have shown that synaptic aromatase activity is upregulated in the forebrain of zebra finches during singing behavior (Remage-Healey et al. 2009), and that neuronal estrogen production can be controlled by depolarization-induced calcium influx within presynaptic terminals (Remage-Healey et al. 2011). Thus, aromatase can rapidly produce estrogen at synapses during social interactions, though similar mechanisms have not yet been documented at early stages of sensory processing, such as in the retina, or in goldfish. It is also possible that estrogens produced inside of cells can influence intracellular domains of membrane receptors. Estrogen receptor beta mRNA has been localized in the goldfish retina (Tchoudakova et al. 1999), but it is not known if ERα is also located in the retina, nor whether membrane versions of either receptor are present. G protein-coupled receptor 30, a membrane estrogen receptor, has also been found in the goldfish retina, though it is not known in which cells and if it is found on post-synaptic membranes (Mangiamele et al. 2017). In light of the ability of an ERβ antagonist to block T's effects on retinal responses, it will be particularly important to investigate the potential localization of these receptors on cellular processes in the retina.

Surprisingly, we were unable to detect T effects on FOS responses in the GCL, which is responsible for transmitting information from the retina to the brain. If T effects on retina responses do not result in altered signals to the brain, then its influences on INL processing may not directly affect ongoing behavior. Alternatively, T effects in ganglion cells may be gated by the concurrence of other stimuli associated with mating, such as the presence of ovulatory pheromones and/or female tactile cues. There are direct projections from the olfactory bulbs to the retina, particularly from cells associated with the terminal nerve (Münz et al. 1982), that could play such a role. Such input could filter sensory information, perhaps allowing T-amplified visual input to reach the brain only in contexts in which mating is possible, i.e., when there is an ovulating female releasing pheromones and with which the male can physically interact. Input from the olfactory bulbs does decrease thresholds for ganglion cell firing (Huang et al. 2005), so such a mechanism is possible. If so, then T's selective stimulation of approach responses to female visual cues in our previous study were unlikely associated with influences on retina processes, because olfactory cues were blocked and males could not physically interact with females in that study. Of course, our inability to detect T influences on FOS responses in the GCL may have simply been the result of methodological issues and/or reflect a lack of statistical power. FOS may not be as sensitive a measure of GCL activity in goldfish as it is of INL activity, as suggested by significant effects of visual stimulation on FOS responses in the INL in our first experiment, despite the small sample size, but only marginal effects in the GCL. That possibility is also consistent with a previous study

demonstrating that a smaller percent of cells in the GCL than in the INL exhibit visually induced c-fos responses (Bertolesi et al. 2014). Additionally, even if FOS production is not induced as easily in the GCL as in the INL, if the levels that were induced by visual stimuli in the light conditions we used were at or near their maximum, then it would have been difficult to detect additional elevations induced by T on those responses. Consistent with that possibility, when we reduced overall light levels in Exp 3 to try to reduce background activation in control conditions, we observed similar, though still non-significant, increases in the GCL in fish injected with T relative to those injected with vehicle to the increases induced by T in the INL in Exps 2 and 3. Reducing background illumination further to more closely mimic the light typical of early morning mating conditions might therefore reveal T effects on FOS responses in the GCL that reflect behaviorally relevant responses to female visual stimuli.

### Role of endogenous T in retina processing

Since our fish were tested during the breeding season and most were expressing milt, males should have had seasonally elevated levels of T, though not the maximal levels that are induced by exposure to female mating stimuli (Kobayashi et al. 1986). Thus, it is reasonable to have not only expected that acute injections that mimicked female-induced surges would increase retina responses, but also that the ERβ antagonist, even if delivered together with exogenous T, might decrease visual responsiveness relative to controls, at least to the degree to which ERβ mediates endogenous T's effects. Consistent with that expectation, only fish injected with the antagonist exhibited decreased retina responses to high intensity light pulses in the electrophysiology experiment relative to an initial baseline response. Thus, while baseline, endogenous T may acutely modulate visual responsiveness through an ERβ mechanism, that modulation could only be detected, through inhibition with the ERβ antagonist, in conditions in which visually evoked responses were otherwise near maximal. On the other hand, FOS responses were not diminished, relative to controls injected with vehicle, in fish injected with the antagonist concurrent with exogenous T. It is possible that endogenous T activates multiple receptor mechanisms in the retina that can compensate for inhibition caused by the ERβ antagonist on FOS responses. It is also possible that elevations of T typical of those that occur in natural breeding contexts upon exposure to spawning stimuli, as mimicked by our T injections, must exceed some threshold above the seasonal baseline to induce acute elevations of FOS-ir. We did not measure hormone levels in this experiment, but we previously found that a dose of 3 μg/fish, which is approximately 0.05–0.1 μg/g in fish the size used in that as well as in the present study, resulted in elevations near, but below, those induced by female spawning stimuli. We used a slightly higher dose in some of the current studies because we had not elevated T in our previous study to the maximum levels induced by female stimuli and because of the likelihood that maintaining fish under anesthesia in our electrophysiology experiment would reduce circulation and therefore the amount of T that would reach the retina. Unfortunately, we were unable to process the blood from those experiments, so we cannot conclusively say that the increase from 3 to 5 μg/fish did not lead to levels that were supraphysiological. However, we did observe similar effects of T on FOS responses in Exp 2, in which we used a lower dose (2.5 μg/fish), to those induced by the higher dose in Exp 3.

### Sensory processing or a secondary consequence?

Future behavioral studies should confirm that T's relatively rapid effects on FOS responses reflect direct influences on sensory processing, and not a secondary consequence of increased visual input that could result from changes in behavior. We attempted to address this issue by constraining the males in a small space surrounded on all sides by female fish, which resulted in similar visual input no matter where the male was in the test tank. Nonetheless, it remains possible that small differences in proximity to the female visual stimuli could have resulted in more retina stimulation. However, it is doubtful that T had profound behavioral effects in what was likely a stressful context (housed alone overnight in a small tank); moreover, the results from our electrophysiology experiment, in which fish were immobile, are consistent with direct T effects on sensory processing.

## Conclusions

In competitive group mating contexts, endogenous steroid surges elicited by olfactory stimuli may serve to amplify visual cues that help males detect and maintain proximity to potential mates. While a great deal of work needs to be done to clarify how T affects visual processes and to determine the role that such influences may play in the regulation of courtship behavior; these results indicate that T surges can acutely enhance early stages of visual processing by acting through ERβ. Such mechanisms may rapidly change males' perceptions of the social environment, leading to subsequent changes in behavioral output that facilitate reproductive success.

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