Published in final edited form as:

Horm Behav. 2009 November ; 56(5): 519–526. doi:10.1016/j.yhbeh.2009.09.002.

Rapid steroid influences on visually guided sexual behavior in male goldfish

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Abstract

The ability of steroid hormones to rapidly influence cell physiology through nongenomic mechanisms raises the possibility that these molecules may play a role in the dynamic regulation of social behavior, particularly in species in which social stimuli can rapidly influence circulating steroid levels. We therefore tested if testosterone (T), which increases in male goldfish in response to sexual stimuli, can rapidly influence approach responses towards females. Injections of T stimulated approach responses towards the visual cues of females 30–45 min after the injection but did not stimulate approach responses towards stimulus males or affect general activity, indicating that the effect is stimulus-specific and not a secondary consequence of increased arousal. Estradiol produced the same effect 30–45 min and even 10–25 min after administration, and treatment with the aromatase inhibitor fadrozole blocked exogenous T's behavioral effect, indicating that T's rapid stimulation of visual approach responses depends on aromatization. We suggest that T surges induced by sexual stimuli, including preovulatory pheromones, rapidly prime males to mate by increasing sensitivity within visual pathways that guide approach responses towards females and/or by increasing the motivation to approach potential mates through actions within traditional limbic circuits.

Keywords

Testosterone; Estradiol; Membrane; Nongenomic; Teleost; Aromatase

Introduction

Social stimuli, particularly aggressive and sexual stimuli, often stimulate rapid increases in sex hormones in vertebrate animals. Such acute changes in sex steroid levels can influence subsequent social encounters, likely through genomic mechanisms that involve changes in gene transcription associated with intracellular steroid receptors (Trainor et al., 2004). Additionally, such steroid elevations may modulate the immediate expression of ongoing behavior through more rapid mechanisms mediated by membrane receptors (reviewed in Balthazart and Ball, 2006; Bass and Remage-Healey, 2008). Thus, in addition to slowly sculpting neural pathways associated with behavioral control, sex steroids are also capable of acting as dynamic regulators of those pathways through rapid, nongenomic mechanisms.

However, with the exception of work in toadfish and plainfin midshipmen showing that sex steroids can rapidly influence hind-brain pattern generators involved in the production of motor output related to social communication (Remage-Healey and Bass, 2006; Remage-Healey and Bass, 2007), very little is known about where and how within the brain sex

steroids act to rapidly influence social behavior. One interesting possibility is that sex steroids may rapidly modulate sensory mechanisms that facilitate the processing of social stimuli. It has been demonstrated that chronic steroid treatments can influence how animals perceive sensory information related to social communication by acting on early stages of sensory detection and processing. For example, chronic androgen treatment alters the tuning of primary electroreceptive sensory afferents in weakly electric fish (Keller et al., 1986) and stingrays (Sisneros and Tricas, 2000) and selectively increases the magnitude and sensitivity of the electroolfactogram response to a putative sex pheromone in a Southeast Asian cyprinid, the tinfoil barb (Cardwell et al., 1995). However, it is not known whether sex steroids can rapidly modulate sensory processes and thus influence social perception in ways that have immediate behavioral consequences.

The importance of olfactory signals for social communication has been well described in many vertebrate species, including goldfish. However, visual cues are also important for social communication in this species, particularly in sexual contexts. Male goldfish follow ovulating females more than nonovulating females, even after ablation of the olfactory tract (Partridge et al., 1976), and males preferentially approach female over male visual stimuli in choice tests during the breeding season (Thompson et al., 2004). That visual processes related to reproduction may be influenced by sex steroids is suggested by the presence of high levels of aromatase, as well as androgen and estrogen receptors, in regions of the brain involved in the detection of (retina) and orientation towards (optic tectum) visual stimuli (Gelinas and Callard, 1993; Gelinas and Callard, 1997). In fact, androgen treatments that masculinize reproductive behavior in female goldfish also induce selective approach responses towards female visual stimuli (Thompson et al., 2004). Interestingly, exposure to preovulatory females causes a T surge in males (Kobayashi et al., 1986) that could rapidly influence those visual responses. We therefore tested if T can rapidly influence male approach responses towards females in an experimental paradigm in which only visual cues were present. To determine the biochemical pathway associated with any such influences, we also tested whether T produces rapid behavioral effects through its conversion by aromatase to estradiol (E₂).

Methods

Subjects

Adult comet goldfish (*Carassius auratus*) 12–16 cm and 25–50 g were kept in same-sex tanks of circulating dechlorinated tap water in controlled environmental conditions. Fish tested in nonbreeding conditions were kept on a 12:12-hr light/dark cycle at 15 °C, and fish tested in reproductive condition were kept on a 16:8-hr light/dark cycle at 20 °C. The animals were fed commercial goldfish pellets once daily. All procedures were in accordance with federal regulations established for the use of vertebrate animals in research and were approved by Bowdoin IRB.

Drugs

For all of our experiments, the control, vehicle injections consisted of 100 µL of a teleost Ringer + 0.1% methanol solution. T was prepared by first making a 30 mg/mL T stock solution by dissolving T powder (Sigma Aldrich) in pure methanol. To prepare the injection mixture, we dissolved the stock in teleost Ringer solution (1:1000) to obtain a final concentration of 0.03 µg/µL T in saline + 0.1% methanol. Fish were intraperitoneally injected with 100 µL of the T injection solution, which resulted in a final T dosage of 3 µg/fish. Previous studies have shown that this dose leads to elevated T concentrations within the physiological range 30–45 min after injection (Huggard et al., 1996; Schreck and Hopwood, 1974). Similarly, a 30 mg/mL solution of estradiol (E₂; Sigma) was dissolved in methanol.

This stock was also dissolved in teleost Ringer (1:1000) to a final concentration of 0.03 $\mu\text{g}/\mu\text{L}$ E_2 in saline + 0.1% methanol in Exp 2a; in Exp 2b a water soluble form of E_2 was dissolved directly into saline, and saline was used for control injections. Fish were injected IP with 100 μL of the E_2 injection solution, again resulting in a dose of 3 $\mu\text{g}/\text{fish}$. We used the same dose of E_2 as T to ensure elevations of local E_2 within the brain that would approach the maximal levels that could have been produced by the local aromatization of our T injections, although we recognized that this would result in supraphysiological levels of peripheral E_2 . Fadrozole (FAD; 3.5 mg/mL; supplied by Novartis) was dissolved in the same vehicle (saline + 0.1% methanol). Fish were injected IP with 100 μL of the FAD solution, resulting in a dosage of 350 $\mu\text{g}/\text{fish}$. This dose, which is approximately 12 mg/kg of fish, was shown in a previous study to rapidly block T actions in teleost fish (Ramage-Healey and Bass, 2007).

Testing apparatus

Experimental test tanks had three separate sections, each divided by acrylic Plexiglas (Polymershapes, Chicago, IL). The middle experimental section was 70 L, and the two side compartments used to hold stimulus fish were 18 L each. Animals were able to see between sections, but the Plexiglas partitions were sealed to prevent water and thus chemical exchange. Dual, full-spectrum light bulbs (Reptisun 5.0, Zoomed, CA) were hung above each tank. Behavioral measurements were recorded with black and white video cameras. No observers were in the experimental room during behavioral testing, except for the motor activity recordings in Experiment 2 (see below). All behavioral testing sessions were carried out in the afternoon.

Experiment 1: Effects of acute T injections on male visual approach responses

Approach responses to female visual stimuli were measured at two times of year. The first test took place in June, when fish were in reproductive condition, as evidenced by the presence of secondary sexual characteristics (tubercles, expressible milt). Because of a high variability in levels of social approach across fish, we used a design in which all fish were tested twice, once after vehicle injections and once after steroid injections, which enabled us to control for that baseline variability (see statistics). However, because steroids may have long-lasting effects, we could not employ a counterbalanced design. Therefore, on the first day of testing, all fish received control IP injections of the vehicle, and on the second test day, 48 hr later, control fish again received vehicle injections, but experimental fish received steroid injections. After all injections fish were placed in the center of the 70 L rectangular test tank described above. After a 15 min habituation, the time the fish spent in proximity (nose within 1 cm) to each Plexiglas barrier was recorded for a 15 min baseline by the software program Limelight®. A stimulus fish was then put in the stimulus tank on the side where the subject had spent the least amount of time during the baseline recording period, which forced the fish to move away from its preferred side to approach the stimulus fish. The time spent in proximity to the Plexiglas partition separating the subject from the stimulus fish was then measured for 15 min. Thus, social approach behavior was recorded 30–45 min after the injections on both test days. Proximity scores were calculated by subtracting the baseline score from the time spent in proximity to the same side when the stimulus fish was present. We then repeated the same test, but used male fish as stimuli instead of females. We also repeated the test later in the summer (August), when fish were no longer in reproductive condition, again using females as stimuli.

We also tested the effects of T injections on motor activity in fish that likewise did not display secondary sexual characteristics indicative of fish in reproductive condition. For that experiment, all fish were injected IP with vehicle on the first test day, as before, and again placed in the center compartment of the test tank. Baseline side preferences were measured

15 min later, and 30 min later, activity was measured for 15 min by counting the number of times the fish's nose crossed each of 2 lines marked on the side of the tank that separated it into thirds. Immediately after the activity test, and thus 45 min after the injections, a stimulus female was added to the least preferred side compartment, and proximity to that side was measured, as described previously, for 15 min. On the second test day, all fish were injected with 3 μ g T, the same dose that effectively stimulated approach responses towards females in the previous experiment. Motor activity tests were again conducted 30–45 min after the injections and thus during the same time interval when we measured T effects on social approach in the previous experiments, and social proximity was again measured 45–60 min after the injections.

Experiment 2: Rapid effects of E₂ on male approach towards females

Two experiments were done to see if E₂, like T, can stimulate social approach responses towards female visual stimuli. Both experiments were done in the same testing apparatus using the same general procedures. The first of these experiments was performed in November when fish were not in reproductive condition. All fish were injected with vehicle on the first test day, and the control group was again injected with vehicle on the second test day, 48 hr later, whereas the experimental group was injected with 3 μ g E₂. We then repeated the experiment in April in fish that were in reproductive condition (expressing milt), but measured behavioral responses 10–25 min after injections to see how quickly E₂ could stimulate social approach responses.

Experiment 3: Effects of an aromatase inhibitor on T's behavioral effects

The goal of this experiment was to test if treatment with the aromatase inhibitor fadrozole (FAD) before a T injection would keep the androgen from stimulating social approach responses towards a female stimulus. The different phases of Experiment 3 were carried out in February and March, all within less than 1 month, and no fish had secondary sexual characteristics at the time of testing. We chose to use fish that were not in reproductive condition and thus would not have maximal levels of endogenous T nor be likely to exhibit socially induced surges in endogenous T so that we could more easily determine if FAD could block the rapid behavioral effects of the exogenous T that we administered. However, attempting to block the behavioral effect of T with FAD required a new testing procedure involving dual injections on each test day. It was therefore necessary to first determine if the addition of an IP vehicle injection 15 min prior to the T injection would interfere with the effect of T on visual approach responses. The experimental procedures employed to do this were identical to the ones described in Experiment 1, except that an additional vehicle injection was given to all fish 45 min before the behavioral task. They were then returned to their holding tanks, captured 15 min later and given a second vehicle injection, placed in the experimental tank, and tested for social approach behavior 30 min later, as described above. The same procedure was followed on the second day of testing, but control fish were injected with vehicle and experimental fish with 3 μ g T 15 min after the initial vehicle injection and thus 30 min before social approach testing.

Although the fish were not in reproductive condition and thus were not expected to have maximum levels of endogenous T or to experience socially induced T surges, we also tested if FAD injections would influence social approach responses in the absence of exogenous T. To do this, all fish were injected with vehicle twice on the first test day, as described above, and the stimulus female was again added 30 min after the second injection. On the second day of testing, control fish were again injected twice with vehicle, whereas experimental fish were injected with FAD first and then the vehicle 15 min later.

Finally, we directly examined whether FAD could block exogenous T's rapid behavioral effects. On the first day of testing, all fish were injected twice with vehicle, as described above, and the stimulus female was again added 30 min after the second vehicle injection. Two days later, control fish were injected with vehicle and then 3 μ g T 15 min later, whereas experimental fish were injected with FAD and then 3 μ g T 15 min later. The stimulus female was added 30 min after the T injections for both groups.

Hormone assays

Blood was collected from all male subjects after the second day of testing, except in Experiment 2B, when no blood was collected. To draw blood, fish were anesthetized by immersing them in 0.1% MS222 and then inserting a 27.5-g heparinized syringe into the caudal vasculature. Approximately 200 μ L of blood was withdrawn from each fish. The samples were centrifuged at 5000 $\times g$ for 15 min, and the plasma was removed and stored at -80°C . Fifty microliters of plasma was then mixed with 50 μ L diethyl ether, then frozen in dry ice. The inorganic phase was removed, and the diethyl ether was allowed to evaporate. Fifty microliters of buffer was then added, and T and E_2 were measured with enzyme-linked immunoassay kits according to the protocols provided with the kits (Cayman Chemical, Michigan) at 1:20 and 1:100 dilutions. The T assay has a sensitivity of 32 pg/mL, and the T antibody has a 2.2% cross-reactivity with 11-ketotestosterone. The estradiol assay has a sensitivity of 129 pg/mL.

Statistics

Mixed-model repeated-measures ANOVAs were used to determine the effects of hormone manipulations on visual approach responses in each experiment, with testing day as a within-subjects factor and hormone manipulation as a between-subjects factor. We focused our analyses on cases where the interaction was significant, which was the most direct statistical test of our hypothesis that steroid manipulations would influence social approach responses on the second day of testing in our experimental groups. We then used follow-up univariate *F* tests (Systat) to compare changes across test days in the control and experimental groups. However, instead of simply comparing proximity scores between the groups on each test day in cases where there was a significant interaction, we ran an additional ANOVA on the second test day, after the steroid manipulation, using the day 1 scores as a covariate. This enabled us to test our specific hypothesis that steroid manipulations would influence approach responses on the second test day in experimental fish while controlling for the large amount of initial variation in social approach. α was initially set 0.05, two-tailed. However, once we observed the initial effects of T on social approach in Experiment 1, we used one-tailed tests, which enabled us to increase statistical power and thus use smaller sample sizes.

To test T effects on general activity, a single-paired *t* test was used to compare the number of line crossings on test day 1, when all fish were injected with vehicle, with the number of crossings on test day 2, when all fish were injected with T. As a positive control, we also used a single, paired *t* test to compare social proximity scores on the tests with stimulus females that were done after the activity tests on both test days within the same group of subjects. Additionally, a Pearson correlation was used to see if changes across test days in motor activity and social proximity (day 2 scores minus day 1 scores for each fish for each variable) were associated within that group of fish.

To ensure that our hormone manipulations elevated levels of circulating steroid, we did between-groups *t* tests on control fish injected with vehicle on the second test day and experimental fish injected with steroid on the second test day in Experiments 1 and 2, and a one-way ANOVA across the different treatment conditions associated with the second day

of testing in the different phases of Experiment 3 (vehicle/vehicle, vehicle/T, FAD/vehicle, FAD/T), followed by univariate F tests comparing each experimental group with the control group (vehicle/vehicle).

Results

Experiment 1

When fish were tested during the breeding season, T injections affected visually guided approach responses towards a stimulus female 30–45 min after the injections, as indicated by a significant interaction between treatment and test day [$F(1,10) = 6.35, p = 0.03$]. Specifically, experimental fish ($n = 7$) spent significantly more time in proximity to the stimulus female after T injections on the second day of testing than after vehicle injections on the first test day [$F(1,10) = 23, p = 0.0007$; Fig. 1A], whereas proximity scores did not change across test days in control fish [$n = 5$; $F(1,16) = 0.6, p = 0.46$]. There was a trend for proximity scores on the second test day to be higher in fish injected with T than in fish injected with vehicle when scores on the first test day, when all fish were injected with vehicle, were used as a covariate, although the effect was not significant [$F(1,9) = 3.62, p = 0.09$].

T injections did not appear to affect approach responses towards stimulus males, as there was no significant interaction between treatment and test day in fish tested with male stimulus animals [controls $n = 5$ and experimental $n = 7$; $F(1,10) = 0.56, p = 0.47$; Fig. 1B], but they again rapidly affected approach responses towards a female visual stimulus in males tested several weeks later after the breeding season was over and fish were no longer in reproductive condition, as indicated by a significant interaction between test day and treatment [$F(1,17) = 3.07, p = 0.002$]. Again, experimental fish ($n = 11$) had significantly higher proximity scores on the second test day, after being injected with T, than they did on the first test day, after being injected with vehicle [$F(1,17) = 11.45, p = 0.004$; Fig. 1C], whereas proximity scores in control fish injected with vehicle on both days ($n = 8$) generally decreased across test days, although not significantly [$F(1,17) = 3.11, p = 0.1$]. Additionally, fish injected with T on the second test day had significantly higher proximity scores than did fish injected with vehicle when scores from the first day of testing, when both groups were injected with vehicle, were used as a covariate [$F(1,16) = 15.25, p = 0.001$].

T injections did not affect motor activity 30–45 min after the injection; in a separate group of fish ($n = 10$) that were injected with vehicle on the first test day and T on the second; there was no significant change in the number of line crossings across test days in response to the T injection [$t(9) = 1.02, p = 0.33$; Fig. 1D]. However, the treatment did stimulate approach responses towards a stimulus female in a social test conducted immediately after the motor activity test and thus 45–60 min after the injection in this group of fish; proximity scores were significantly higher on the second test day, after T injections, than on the first, after vehicle injections [$t(9) = 2.33, p = 0.04$]. Furthermore, there was no significant correlation between changes in motor activity and changes in social approach across test days in response to the T injections ($r^2 = -0.39, p = 0.26$).

Experiment 2

IP E₂ injections administered 30 min prior to the behavioral task also affected approach responses towards females, as indicated by a significant interaction between treatment and test day [$F(1,10) = 6.19, p = 0.04$]. Specifically, experimental fish ($n = 5$) had significantly higher proximity scores on test day 2, after being injected with E₂, than they did on test day 1, after being injected with vehicle [$F(1,8) = 12.84, p = 0.004$, one-tailed; Fig. 2A], whereas proximity scores in control fish ($n = 5$), which were injected with vehicle on both test days,

did not change across test days [$F(1,8) = 0.002$, $p = 0.48$, one-tailed]. Proximity scores were significantly higher on test day 2 in fish injected with E₂ than in fish injected with vehicle when scores on test day 1, when both groups of fish were injected with vehicle, were used as a covariate, although the effect was marginal [$F(1,8) = 3.41$, $p = 0.05$, one-tailed]. Similarly, E₂ affected approach responses towards a stimulus female on tests conducted 10–25 min after the injections, as indicated by a significant interaction between treatment and test day [$F(1,19) = 6.6$, $p = 0.02$]. Again, proximity scores were significantly higher in the experimental group ($n = 12$) on test day 2, after injections of E₂, than on test day 1, after injections of vehicle [$F(1,19) = 17.7$, $p = 0.0003$, one-tailed; Fig. 2B], whereas proximity scores did not change across test days in the control group ($n = 9$; one fish was dropped because it appeared sick on one day of testing and did not move) that was injected with vehicle on both days [$F(1,19) = 0.07$, $p = 0.39$, one-tailed]. Proximity scores on test day 2 were significantly higher in fish injected with E₂ than in fish injected with vehicle when scores on test day 1, when both groups were injected with saline, were used as a covariate [$F(1,18) = 3.91$, $p = 0.03$, one-tailed].

Experiment 3

The addition of a control injection 15 min prior to the T injection did not affect T's ability to rapidly stimulate approach responses towards females; there was a marginal interaction between treatment and test day [$F(1,8) = 5.14$, $p = 0.053$], likely because we used a very small control group ($n = 4$) in this test experiment. However, fish injected with vehicle then T on the second day of testing ($n = 6$) had significantly higher proximity scores than they did after two vehicle injections on the first day of testing [$F(1,8) = 11.04$, $p = 0.005$, one-tailed; Fig. 3A], whereas proximity scores did not change significantly across test days in the fish injected twice with vehicle on both days [$F(1,8) = 0.04$, $p = 0.42$, one-tailed]. There was a strong trend for proximity scores to be higher in fish injected with vehicle then T on the second test day than in fish injected twice with vehicle when scores on test day 1, when both groups were injected twice with vehicle, were used as a covariate [$F(1,7) = 3.38$, $p = 0.055$, one-tailed]. FAD did not affect approach responses towards stimulus females independently of exogenous T, as there was no significant interaction between treatment and test day in Experiment 3B, in which the control group of fish ($n = 5$) was injected twice with vehicle on both test days and the experimental group ($n = 6$) was injected twice with vehicle on the first test day and with FAD then vehicle on the second test day [$F(1,9) = 0.006$, $p = 0.94$; Fig. 3B]. On the other hand, there was a strong trend for the expected interaction between treatment and test day in Experiment 3C, in which one group of fish was injected twice with vehicle on the first test day and with vehicle then T on the second test day and the other group was injected twice with vehicle on the first test day and with FAD then T on the second [$F(1,20) = 3.73$, $p = 0.07$]. We therefore ran our planned comparisons and found that the positive control fish ($n = 10$), as expected, did have significantly higher proximity scores on the second test day, after being injected with vehicle then T, than they did on the first test day, after being injected twice with vehicle [$F(1,20) = 7.12$, $p = 0.005$, one-tailed]. On the other hand, there were no significant differences in proximity scores across the test days in fish injected twice with vehicle on the first test day and with FAD then T on the second [$n = 12$; $F(1,20) = 0.005$, $p = 0.47$, one-tailed]. Also as predicted, proximity scores on the second test day were significantly higher in fish injected with vehicle then T than in fish injected with FAD then T when scores on the first test day, when all fish were injected twice with vehicle, were used as a covariate [$F(1,19) = 3.08$, $p = 0.048$, one-tailed].

Plasma hormone measurements

A cold T-spike extraction experiment indicated that our diethyl ether extractions recovered 90% of initial steroid levels. Therefore, all values reported were increased 10%. The intra-assay variation coefficients for the T assays were 5.1% (Experiments 1A and 1B, run in a

single assay), 10.1% (Experiment 1C), 12.6% (Experiment 4A), 8.2% (Experiment 4B), and 16.1% (Experiment 4C). For Experiment 4, in which data were pooled from two different plates, the inter-assay variation coefficient was 20.7%. The intra-assay variation coefficient for the estradiol assay was 6.1%.

In Experiment 1, male fish in reproductive condition that were treated with T had significantly higher plasma T levels 45 min after injection than did controls [$t(8) = 3.965$, $p = 0.004$; Fig. 4A], as did male fish injected with T in Experiment 1C that were not in reproductive condition [$t(12) = 4.197$, $p = 0.001$]. Fish injected with E₂ in Experiment 2A had significantly higher plasma E₂ levels than controls [$t(6) = 5.084$, $p = 0.002$; mean $\pm$ SEM; control, 2.2 ± 0.75 pg/ μ L; E₂ treated, 10.5 ± 1.71 pg/ μ L]. Although these peripheral levels are supraphysiological for males, our main goal was to produce high local concentrations within brain regions where local aromatase likely produces behaviorally relevant neuroestrogens, which we ensured by using the same dose of E₂ as we did for T. In fact, peripheral levels of E₂ are likely not relevant for behavioral regulation in males (Balthazart and Ball, 2006; Remage-Healey et al., 2008).

In Experiment 3, there was a significant main effect of drug treatments [$F(3,27) = 5.0$, $p = 0.007$]. Fish injected with vehicle then T had significantly higher T than fish injected twice with vehicle ($p = 0.001$; Fig. 4B), as did fish injected with FAD then T ($p = 0.004$). There was a nonsignificant trend for fish injected with FAD then vehicle to also have higher T levels than fish injected twice with vehicle ($p = 0.08$). We did not measure peripheral E₂ in this experiment because aromatase activity is much lower in the periphery than in the brain in male goldfish (Pasmanik and Callard, 1988). Thus, we did not expect FAD injections to rapidly reduce peripheral levels of E₂ following our T injections. On the other hand, we did expect that peripheral T levels would increase if our FAD was working within the brain, as FAD and related aromatase inhibitors have been shown to increase gonadotropin secretion and ultimately T in numerous species, including other teleosts, by blocking E₂ negative feedback (Juniewicz et al., 1988; Lee et al., 2001; Tsai et al., 1994). Although not significant, the trend for increased T in FAD treated fish, despite the very small number of fish from which we were able to get blood in that group ($n = 4$), supports this conclusion.

Discussion

The present results show that T and E₂ rapidly and specifically stimulate approach responses towards the visual cues of females in male goldfish. We suggest that these rapid effects may normally occur in response to the T elevations induced by female sexual stimuli in male goldfish. Specifically, exposure to preovulatory females the night before spawning induces a T surge in male goldfish, most likely as a function of the priming pheromone effects associated with 17,20BP exposure (Kobayashi et al., 1986; Sorensen et al., 1989). Such increases in T might, through the rapid mechanism we have demonstrated, prime male visual systems so that they are better able to detect and orient towards mates and/or stimulate motivational systems that lead to more persistent approach responses once a female is detected.

T injections were able to stimulate approach responses towards females within 30–45 min, and E₂ was able to stimulate approach responses within 10–25 min. This short time course of action suggests that T and E₂ may have influenced approach responses through nongenomic mechanisms. The rapidity of the effects of sex steroids is most striking when observed following systemic administrations, as in the present study, because the hormones must first reach the target tissue and accumulate before they can activate a cellular response and trigger the neuronal circuits involved in the regulation of behavior (Balthazart and Ball,

2006; Cornil et al., 2006). However, studies using transcription inhibitors are necessary to completely rule out rapid genomic mechanisms.

The present study also demonstrates that the aromatization of T is necessary and sufficient for the enhancement of male approach responses towards a female stimulus to occur. E₂ injections had the same behavioral effect as T, and pretreatment with the aromatase inhibitor FAD 15 min prior to T injections kept the androgen from stimulating approach responses. These findings are consistent with studies in Japanese quail showing that T-induced sexual behavior is rapidly blocked by aromatase inhibitors and that E₂ can produce the same rapid behavioral effects that T does (Cornil et al., 2006). However, FAD's lack of effect in the absence of exogenous T in our studies indicates that we have not yet demonstrated the social/environmental context in which this aromatization mechanism normally influences approach responses towards females in goldfish. We did these studies in fish that were not in reproductive condition and thus were unlikely to have fluctuating levels of endogenous T in response to social stimuli so we could more easily isolate FAD effects on the rapid behavioral changes induced by exogenous T. Obviously, future studies need to examine the effects of FAD in fish in full reproductive condition, particularly in males exposed to ovulatory females. It has been demonstrated that exposure to the female pheromone 17,20BP, which is naturally released by females the night before they ovulate (Scott and Sorensen, 1994), can increase T and milt levels and stimulate courtship behavior in males (Poling et al., 2001; Sorensen et al., 1989). Our current results suggest that the activation of courtship-related behaviors induced by pre-exposure to such sexual stimuli may be mediated, at least in part, by rapid effects of E₂ elevations in the brain following the T surges induced by those stimuli. In fact, the levels of T produced by our injections at the time of our tests were within the physiological range of those observed in males exposed to female sexual stimuli, which can get as high as 50 ng/mL (Kobayashi et al., 1986). The trend for an increase in plasma T levels observed in FAD treated fish likely resulted from the inhibition of a negative feedback loop, mediated by E₂, regulating T production. That those elevations did not stimulate social approach further shows the necessity of aromatization for T to produce its behavioral effect.

We do not yet know what kind of molecule mediates E₂'s rapid behavioral effects. Classical intracellular estrogen receptors have been localized in cell membranes, and a membrane version of ER alpha, in particular, appears to mediate some of E₂'s effects on receptivity in rats, although those effects do not appear rapid (Dewing et al., 2007). Another candidate molecule is GPR30, a G-protein coupled receptor that binds E₂ and mediates some of its rapid effects on cell physiology (Kuhn et al., 2008; Pang et al., 2008). Although it appears to localize on endoplasmic reticulum membranes in some cell types (Bologa et al., 2006; Revankar et al., 2005), in teleost fish ovaries, at least, it is clearly localized on external membranes (Pang et al., 2008). Localization on either membrane type could mediate rapid influences of E₂ on cell physiology and thus behavior. We have recently sequenced the GPR30 from cDNA prepared from goldfish brains (unpublished data), so it could mediate E₂'s rapid behavioral effects in this species.

Because most of our behavioral experiments were performed in fish that were not in reproductive condition, it is reasonable to wonder how the exogenous T surge managed to rapidly influence male behavior, as brain aromatase levels are lower in fish that are not in reproductive condition than in fish that are (Pasmanik and Callard, 1988). Although T can induce aromatase transcription in teleosts (Forlano and Bass, 2005), our T injections would have been unlikely to increase expression within the time frame of our behavioral experiments, most of which were completed within 45 min of the injections. Therefore, it is more likely that baseline aromatase levels in male fish remain sufficiently elevated outside of the breeding season to convert the exogenous T into neuroestrogen in the specific neural

pathways mediating sexual approach responses. It is also possible that the exogenous T we administered not only increased levels of locally available substrate but also rapidly increased aromatase kinetics and thus local E₂ concentrations or that the exposure to a stimulus female during testing could have rapidly upregulated aromatase kinetics. In Japanese quail, social interactions can rapidly modulate aromatase kinetics through a calcium-mediated phosphorylation mechanism, although social stimuli appear to decrease, not increase, aromatase kinetics in that species (Balthazart and Ball, 2000; Balthazart et al., 2003).

We hypothesize that acute hormonal fluctuations may modulate visual processes related to reproduction and thus influence a male's ability to detect and/or orient towards potential mates. In goldfish, the retina and the optic tectum, which play important roles in orienting behavioral responses towards visual stimuli, have high levels of aromatase (Gelinas and Callard, 1993; Gelinas and Callard, 1997). Local E₂ synthesis in neural pathways involved in visual processing could therefore sensitize the males to some visual features of females and modulate orientation responses in the context of reproduction, when males do selectively orient towards the visual stimuli of females (Thompson et al., 2004). This hypothesis, however, raises an important question: what specific visual features of females are attractive to the males treated with T, chronically as in our previous study or acutely as in this study? In both studies, T treatments selectively affected responses towards the visual cues of females. However, in contrast to our previous study, none of the females in our current study were induced to ovulate prior to testing, and those in one experiment did not have any eggs. This indicates the cue may not be related to immediate reproductive state. Although there are no obvious sexual dimorphism within the visible spectrum, goldfish can discriminate UV wavelengths (Bowmaker et al., 1991; Neumeyer and Arnold, 1989), and males and females may display distinctive UV emission patterns that could explain the preferential behavioral response to female visual stimuli. UV patterns are thought to be associated with mate assessment in guppies, another teleost (Kodric-Brown and Johnson, 2002; Smith et al., 2002), as well as in several avian species (Bennett et al., 1997; Pearn et al., 2001). Although the Plexiglas partitions in our studies cut off wavelengths below 400 nm, UV cones in goldfish have broad sensitivity to wavelengths that extend above 400 nm (Palacios et al., 1998), so their activation could have played a role in the processing of female visual stimuli in the present study.

It is also possible that T rapidly influences approach responses in males by acting in brain areas known to regulate motivational processes associated with the generation of appetitive behavioral responses. A primary candidate region is the preoptic area, which is known to be involved in the regulation of male sexual behavior in most vertebrate species. Indeed, the preoptic area is characterized by high aromatase levels relative to the rest of the brain, in goldfish (Gelinas and Callard, 1997) as in most other vertebrates, and appears involved in T's rapid regulation of sexual behavior in male Japanese quail (Balthazart et al., 2004). Additionally, because behavioral responses rely on motor commands that are ultimately governed by hindbrain or spinal pattern generators, T could directly act on these circuits to rapidly modulate motor patterns associated with approach responses. In plainfin midshipmen, T does work through such a hindbrain mechanism, rapidly affecting the firing patterns of neurons involved in generating the vocal motor output associated with social communication (Remage-Healey and Bass, 2006). Finally, T could rapidly stimulate approach responses towards females as a secondary consequence of increased arousal. However, T's influences on motor or arousal mechanisms are unlikely to explain its ability to stimulate social approach responses in goldfish because T injections did not rapidly affect motor activity/locomotion when stimulus fish were not present. Future tests will therefore try to dissociate T's influences on sensory and motivational mechanisms.

In conclusion, we have shown that rapid elevations in T or E₂ stimulate male approach responses towards a female stimulus when only visual cues are available and that the aromatization of T is necessary for the androgen to have this effect. We propose a model in which this mechanism allows the socially induced T surges that occur in this species to influence ongoing behavior, potentially by modulating sensory processes related to mate detection. Thus, this study expands on the growing body of literature indicating that sex steroids play dynamic roles in the regulation of vertebrate social behavior.

Acknowledgments

This work was supported by National Science Foundation grant #094692, by a Maine Idea Network of Biomedical Research Excellence (INBRE) predoctoral fellowship, and by a generous donation from the Palters to Bowdoin College.

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Fig. 1.

Mean $\pm$ SEM of corrected time proximity to a stimulus fish 30–45 min after injections on D1, the first day of testing when fish in both groups received control vehicle injections, and D2, the second day of testing, when half the fish were again injected with vehicle and half with T (A, tests in fish in reproductive condition with a stimulus female; B, tests in fish in reproductive condition with a stimulus male; C, tests in fish that were not in reproductive condition with a stimulus female). Mean $\pm$ SEM of the number of line crossings in an activity test 30–45 min after vehicle injections on D1 and T injections on D2 in a separate group of fish (D, left y-axis), as well as the time spent in proximity to a stimulus female for that group of fish in a social test conducted immediately after the activity test (right y-axis).

*Within-groups difference across test days, $p < 0.05$, two-tailed; ** $p < 0.01$, two-tailed.

Fig. 2.

Mean $\pm$ SEM of the time spent near a stimulus female after injections on D1, the first day of testing when all fish were injected with the vehicle, and D2, the second day of testing when half the fish again received vehicle injections and half received E₂ injections (A, 30–45 min after injections; B, 10–25 min after injections). **Within-groups difference across test days, $p < 0.01$, one-tailed.

Fig. 3.

Mean $\pm$ SEM of the time in proximity to a stimulus female 45–60 min after an initial injection and 30–45 after a second injection on D1, the first day of testing, and D2, the second day of testing. In the first experiment (A), all fish were injected twice with vehicle on D1. Control fish again received 2 vehicle injections on D2, whereas experimental fish were injected with vehicle then T. In the second experiment (B), all fish received two vehicle injections on D1, then control fish were again injected twice with vehicle on D2, whereas experimental fish were injected with FAD then vehicle. In the third experiment (C), all fish were injected twice with vehicle on D1, then positive control fish were injected with vehicle followed by T on D2, whereas experimental fish were injected with FAD then T.

**Within-groups difference across test days, $p < 0.01$, one-tailed.

Fig. 4.

Mean $\pm$ SEM of steroid hormone levels in blood collected from males tested during the breeding season and after the breeding season (A). Blood was collected at the end of the second day of testing, when half the fish had been injected with vehicle and half with 3 μ g T. Mean $\pm$ SEM of steroid levels in blood from males in Experiment 4 at the end of the second day of testing when one group had been injected twice with vehicle, one with vehicle then T, one with FAD then vehicle, and one with FAD then T. * p <0.05, two-tailed.