The Rapid Effects of Steroids on Reproductive Processes in Goldfish, Carassius Auratus

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THE RAPID EFFECTS OF STEROIDS ON REPRODUCTIVE PROCESSES IN
GOLDFISH, *CARASSIUS AURATUS*

by
Kimberly Dao

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biology)

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ABSTRACT

It is traditionally understood that sex steroid hormones work through slow genomic pathways. More recently, it has also been discovered that sex steroid hormones also mediate rapid effects through a non-genomic pathway. However, much is still unknown about this mechanism, such as the receptors that mediate these effects, the behavioral consequences, and the contexts in which they occur. Goldfish have been used to investigate these behavioral effects and the receptors used in a sexual social context. To explore these ideas, the Thompson Lab and I tested if sex pheromones that rapidly increase levels of testosterone could also rapidly increase milt and/or courtship behavior, and if any such effects are blocked by the administration of fadrozole (FAD). We also used immunohistochemistry (IHC) to see if we could identify traditional estrogen receptors that might participate in non-genomic regulatory mechanisms. The results from my experiments show that milt increases in the presence of prostaglandin F2 alpha (PGF2α) and 17, 20 beta-dihydroxy-4-pregnen-3-one (17, 20 BP). FAD blocked the 17, 20 BP induced milt response in one experiment, and though it reduced the milt increases stimulated by a combined 17, 20 BP and female exposure in a subsequent experiment, the effect was not significant. In fact, FAD significantly increased milt in the absence of any other social stimuli. This response of increased milt may suggest rapid androgenic effects on these responses. Our IHC experiments demonstrated non-nuclear localization of two estrogen receptors, ERβ and GPR 30, suggesting one or both may mediate estradiol’s rapid effects on physiology and behavior in goldfish.
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Steroid hormones have profound effects on physiological functions extending from immune functions to development of sexual characteristics. These effects act through multiple mechanisms and are mediated by specific intracellular steroid receptors (Marino, 2006). Traditionally, steroid receptors act as transcription factors that work through genomic pathways. Genomic mechanisms initiate when the hormone binds to receptors in the cytoplasm (Appendix A). This process takes hours or days to affect behavior (Steinman & Trainor, 2010). Research suggests that hormones binding to receptors in the cell membrane mediate non-genomic mechanisms (Appendix A) that can lead to rapid changes in behavior within seconds or minutes (Steinman & Trainor, 2010).

Research in the field of steroid hormones has primarily focused on genomic mechanisms, while non-genomic mechanisms have been studied less thoroughly. However, within the past two decades, an increasing number of reports dealing with non-genomic steroid action have emerged (Falkenstein et al., 2000). Yet, the behavioral consequences of such actions, most of which are associated with estradiol (E2), and the social contexts in which they occur have only been demonstrated in a few species. For example, E2 rapidly stimulates female receptivity in rats via actions in the hypothalamus (Dewing et al., 2007); E2 mediates male sexual behavior in Japanese quail (Cornil et al., 2006b); and E2 also facilitates the production of vocalizations related to social signaling in the plainfin midshipman fish via actions in the hindbrain (Remage-Healey and Bass, 2006). It is still undetermined which receptor mechanisms mediate most of these behavioral effects.
Hormone-based sex pheromones in goldfish (*Carassius auratus*) provide an ideal model system to investigate these rapid mechanisms (Sorensen & Stacey, 1999). Male goldfish are exceptionally sensitive to steroid and prostaglandin hormones and the metabolites released into the water by females. The released molecules, known as pheromones, indicate the biological state of the female reflecting her ability to spawn (Wyatt, 2003). Before releasing eggs in the evening, the blood levels of the steroid 17, 20 beta-dihydroxy-4-pregnen-3-one (17, 20 BP) increases and leaks into the water. When females spawn the next morning, males respond to another hormone pheromone released by the female, prostaglandin F2 alpha (PGF2α) (Wyatt, 2003). Specifically, these sexual cues, including pheromonal cues, rapidly modulate physiological responses involving gonadotropin hormone (GTH) and seminal fluid (milt). These cues also affect behavior in male goldfish such as increased following and nudging (Zheng and Stacey, 1996). In addition, the same social stimuli, presumably through their ability to increase GTH, can cause a surge in circulating levels of testosterone (T) (Kobyashi et al., 1986; Stacey et al., 2001).

Testosterone is a precursor to E2, and research has shown that testosterone (T) and estradiol (E2) have rapid effects, occurring within an hour, on both behavior (social approach) and physiology (seminal fluid production) (Lord et al., 2009; Mangiamele & Thompson, 2012). In goldfish, the Lord et al. study showed that T enhances visual approach responses (Appendix B) by demonstrating that males given intraperitoneal injections of T spent significantly increased time in close proximity to female stimuli, which were fashioned behind a partition to prevent olfactory communication. This effect occurred 30-45 minutes after injection and was blocked by a pre-treatment with FAD,
which prevents the conversion of T into E2. In fact, E2 was able to stimulate the same behavior with 10-20 min. Mangiamele and Thompson (2012) also found that at one hour post-injection, males injected with T, E2, or males E2: BSA, an estrogen conjugate that cannot cross neuronal membranes, had more milt than control animals, and again FAD blocked T’s ability to stimulate milt production (Appendix C). Thus, T’s rapid effects on behavior and physiology ultimately depend upon the activation of estrogen receptors (Mangiamele & Thompson, 2012).

Other studies have explored possible estrogen receptors that may mediate the rapid effects of sex steroids (Sorenson, 1989). My experiment focuses on two receptors: estrogen receptor beta, ERβ, and G-protein coupled receptor, GPR 30. The first is the classical receptor ERβ, conventionally thought of as a genomic receptor that acts as transcription factor usually localized in the nucleus of cells. However, it has also been found to localize to cellular membranes (Falkenstein et al., 2000). There is also a novel receptor GPR 30 that binds estrogen and seems to activate many of the responses in cells.

While researchers have recognized these rapid effects of T and E2 on goldfish, they have yet to determine under what context these effects occur and which estrogen receptors mediate those rapid effects. The Thompson Lab and I predict that these rapid effects might occur in a sexual social context, and ERβ and GPR 30 may mediate these effects. In particular, we predict that pheromones exert at least some of their behavioral and/or physiological effects by increasing T, which then activates rapid estrogen signaling mechanisms after the hormones conversion to E2 signaling mechanisms.
CHAPTER 2: METHODS AND MATERIALS

2.1 Goldfish

*Carassius auratus*, goldfish, were purchased from Blackwater Creek Koi Farms, Inc. Goldfish were housed in same-sex aquaria of circulating dechlorinated tap water with a daily cycle of twelve hours of light and twelve hours of dark at 18°C. Fish were fed commercial goldfish pellets once daily. All procedures were in compliance with animal care and use guidelines at Bowdoin College. Males were selected with the criteria that they were sexually mature, expressing milt, and appear physically healthy. Females were selected with the only criteria being that they appear physically healthy.

2.2 Experiment 1: Effects of Isolated Pheromones on Milt

We began by establishing the context for these rapid effects. Initially, we speculated about whether exposure to specific pheromones could artificially produce the rapid effect of increased milt. Two small pilot experiments were conducted with prostaglandin F2 alpha (PGF2α) and 17, 20 beta-dihydroxy-4-pregnene-3-one (17, 20 BP), which were the two most commonly used pheromones in previous research (Sorenson, 1989). Stock concentrations of both pheromones and an ethanol control were made prior to each experiment and added as a single bolus to the tank throughout the experiment, with a final molarity of $10^{-8}$ M (Sorenson, 1989).

Male fish were isolated into four smaller 60 L same-sex tanks at 18°C for a 48-hour acclimation period to ensure stress would not affect results. Fish were divided four
per tank. The experiment ran over a two hour and fifteen minute period. Pheromone concentrations or the ethanol control was added every fifteen minutes starting at t= 15 minutes and ending at t= 120 min, for a total of two hours of exposure.

Pre-weighed capillary tubes were used to weigh milt at t= 0 min and t= 135 min. Milt (seminal fluid) was extracted from the fish by first placing the fish upside down in a rubber cradle lined with a moist paper towel to protect the fish. Then from the ventral abdomen anterior to the gonopore, gentle pressure was applied using a thumb and index finger in a stroking motion while collecting milt into a pre-weighed capillary tube; this process is referred to as “stripping.” If urine or other body fluids were excreted, they were wiped away with a Kimwipe before milt was collected. Males were stripped until milt could no longer be excreted and then the capillary tube was weighed again (Mangiamele & Thompson, 2012).

2.2.1 Experiment 1a: Effects of 17, 20 BP & FAD on milt

I also tested if blocking the synthesis of aromatase could block 17, 20 BP’s ability to increase milt following the same experimental set-up, with an addition of a female fish in the tank. Female goldfish were intramuscularly injected before the experiment with 15 μL of Lutalyse (Lutalyse; UpJohn, MI)—a treatment that stimulates female sexual behavior and release of a prostaglandin spawning pheromone (Sorenson, 1989). Experimental male goldfish were injected intraperitoneally with 480 μg FAD in 0.1% methanol, and control fish were injected with an equivalent volume of saline vehicle at t= 0 min before pheromone exposure to test whether pheromone-induced milt expression was dependent upon aromatase activity (Mangiamele & Thompson, 2012). FAD is an
aromatase inhibitor that blocks conversion of testosterone (T) to estradiol (E2) (Appendix D).

2.3 Experiment 2: Effects of FAD on Milt and Courtship

In the following experiment, I tested if FAD could block both milt and courtship behavior in males. Male fish were isolated into five smaller 60 L same-sex tanks at 18°C for a 36-72 hour acclimation period. Fish were divided two fish per tank. The experiment ran over a 90-minute period. Three treatments were used on male goldfish: 1) FAD-injected males were exposed to the stimuli of a PGF2α-injected female and 17, 20 BP 10^{-9} M bolus; 2) vehicle-injected males were exposed to the same stimuli; 3) vehicle-injected males were not exposed to any female stimuli. At \( t = 0 \) min, male fish were stripped and injected with the designated treatment. At \( t = 30 \) min, the stimuli were added. At \( t = 90 \) min, male fish were stripped again because previous work has shown that milt increases one hour after social contact (Kyle et al., 1985).

In addition, I also measured the courtship behavior in the form of males following and nudging the female, predicting that FAD might also block this sexual response since the Thompson lab has shown that E2 stimulates a rapid approach to females by visual cues. With the visual and olfactory cues working together, I thought similar mechanisms might apply here too. Observations began after an hour and fifteen minutes of exposure to stimuli. Individual fish were observed in two intervals, five minutes at a time, for a total observation time of ten minutes per fish.
2.3.1 Experiment 2a

I then repeated the experiment, with some minor modifications. The experiment again ran over a 90-minute period. Male fish were isolated into six smaller 60 L same-sex tanks at 18°C for a 48-hour acclimation period. Fish were divided 2-3 per tank. At t= 0 min male fish were milted prior to FAD injection. At t= 30 min, I began the treatments. At t= 90 min, male fish were post milted. The same treatments were administered with an additional group of FAD injected males that were not exposed to female stimuli. Observation time was reduced to five minutes per fish and an additional female was added to the stimulus groups.

2.4 Data Analysis

I used IBM SPSS Statistics Version 21 for statistical analysis. Initially for each experiment, I calculated milt difference scores by subtracting the premilt from the postmilt value (postmilt – premilt = difference score). A negative score indicates an inability to regenerate the initial volume of milt, whereas a positive score indicates the fish’s complete replenishing of its initial volume or more. For Experiment 1 and 1a, we performed a square root transformation to normalize the difference scores. After the square root transformation, we conducted between groups t-tests for Experiment 1.

In the following experiments with more than two groups, we ran a Univariate ANOVA (analysis of variance) to determine if there was an overall difference between groups. For Experiment 1a, there were three groups: Control, 17, 20 BP, 17, 20 BP/FAD as fixed factors. I followed up this analysis with LSD (least significant difference) comparisons, a type of post hoc test, to compare the differences between groups.
Experiment 2 had the same three fixed factors. In the final Experiment 2a, there were four groups as fixed factors, and I performed a follow-up planned comparisons test between the groups.

2.5 Immunohistochemistry

Immunohistochemistry (IHC) is a technique that uses the immunoglobulin molecule of an antibody to label where the protein of a specific gene is located. IHC can either be fluorescent or enzymatic. In this experiment, we applied the immunofluorescence method. Using a primary antibody and fluorescently tagged secondary antibody, we were able to visualize with fluorescent microscopy where those primary antibodies bound to, which showed where the protein was localized.

In order to prepare the brains for IHC, we followed a similar protocol to that used by Darden (2012). Brains from male goldfish were extracted, dissected, fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS) (pH 7.4), and stored at 4°C over 48 hours. Following the 48 hours, the brains were moved and kept in 30% sucrose in 0.1 M PBS at 4°C overnight. Using a cryostat (Shanon Cryotome FE and Cryotome E), we then cut the brain tissue into 30 µm sections, mounted the tissue on slides to make four series, and kept it at -20°C until needed.

For the first day of immunofluorescence, slides were initially warmed to room temperature. A hydrophobic barrier was applied around the edges of the slides to prevent the runoff of reagents. Slides were further fixed for 20 minutes at room temperature in chilled 4% PFA. We performed an antigen retrieval step by boiling citric acid in the microwave and adding it to the slides. Slides were incubated for two minutes. This step
was repeated twice with fresh citric acid for five minutes each. Afterward, we washed the slides twice in PBS for five minutes. All the slides were then blocked in 2.5% bovine serum albumin (BSA) + .3% Triton X-100 for 30 minutes. Blocking was performed in order to prevent non-specific binding. A 1:500 dilution of primary rabbit polyclonal antibody solution in 2.5% BSA + .3% Triton X-100 of GPR 30 (Invitrogen, Carlsbad, CA) and a 1:250 dilution of primary rabbit polyclonal antibody solution in 2.5% BSA + .3% Triton X-100 of ERß (Invitrogen) were made and applied to the slides and incubated for twelve hours at 4ºC. Controls with no primary antibody were not performed in this experiment; however, others in the lab conducted those controls. Unfortunately, Invitrogen does not sell the immunizing peptide antigen used to generate the antibody, so pre-incubation controls could not be performed.

For the second day, slides were washed for ten minutes in 0.1M PBS then incubated for two hours in a solution of Alexa Fluor 488-conjugated donkey anti-rabbit secondary (1:1000, Invitrogen) in 2.5% BSA + .3% Triton X-100 at room temperature in the dark. After, they were washed for ten minutes in 0.1 M PBS twice. Finished slides were then mounted with Vectashield plus 4’, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and cover slipped. Once dry, slides were observed using the Olympus CH30 Light Microscope. Images were taken using the QCapture Pro and QICam imaging system.
CHAPTER 3: RESULTS

3.1 Experiment 1: Effects of Isolated Pheromones on Milt

Exposure to PGF2α did not significantly increase milt (see Figure 1), but exposure to 17, 20 BP did, although the effects were marginal (see Figure 2).

Figure 1- Experiment 1: Effects of PGF2α Milt Score
A bar graph (Mean ± SEM) depicting the difference between postmilt and premilt weight (in grams) under control (left; N=8) and PGF2α exposed treatments (right; N=8). Although there was a substantial average difference between the average control vs. PGF2α groups, no statistical difference (p= 0.41) between those treatments was obtained using standard methods.
3.1.1 Experiment 1a: Effects of 17, 20 BP & FAD on milt

There was a significance difference between groups in this experiment (p= 0.018). FAD was able to block 17, 20 BP’s effect. Follow up comparisons indicated significant differences between control vs. 17, 20 BP (p= 0.007) and 17, 20 BP vs. 17, 20 BP + FAD (p= 0.048), but not between control vs. 17, 20 BP + FAD (p= 0.236).

3.2 Experiment 2: Effects of FAD on Milt and Courtship

The main effect on milt was significant between groups (p= 0.003). Exposure to 17, 20 BP significantly increased milt (p= 0.001). When FAD was pre-injected, exposure to 17, 20 BP still elevated milt, although not significantly (see Figure 4). FAD also significantly increased the following behavior (see Figure 5).
Figure 4- **Experiment 2 Milt Scores**
A bar graph (Mean ± SEM) depicting the difference between postmilt and premilt weight (in g) under control (left; N=8); Saline, 17, 20 BP + Female (center; N= 16); and FAD, 17, 20 BP + female treatments (right; N=16). There was a substantial average difference between the average control (Saline, No Female) vs. Saline, 17, 20 BP + Female (p= 0.001); control vs. FAD, 17, 20 BP + Female (p= 0.039); and Saline, 17,20 BP + Female vs. FAD, 17, 20 BP + Female (p= 0.65).

![Graph of Milt Scores](image)

Figure 5- **Experiment 2 Courtship Behavior**
A bar graph (Mean ± SEM) depicting the difference of following behavior between groups: Saline, 17, 20 BP + Female (left; N= 16) vs. FAD, 17, 20 BP + Female (right; N= 16). The Saline 17, 20 BP + Female group had a significantly lower following time than the FAD/17, 20 BP + Female group, where p= 0.049.

![Graph of Courtship Behavior](image)
3.2.1 Experiment 2a

There was a strong trend for a main effect of group on milt, though it was not significant (p= 0.069). We therefore ran planned comparisons to see if we could detect expected differences between the groups. Exposure to 17, 20 BP + Female significantly increased milt (p= 0.01). In both FAD treatments, levels of milt increased (p = 0.046 and p= 0.1) (see Figure 6). FAD also significantly increased following behavior (see Figure 7).
Figure 6- **Experiment 2a Milt Scores**
A bar graph (Mean ± SEM) depicting the difference between postmilt and premilt weight (in g) under control (left; N=9); FAD, No Female (mid-left; N= 7); Saline, 17, 20 BP + Female (mid-right; N= 9); and FAD, 17,20 BP + female treatments (right; N=5). There was a substantial average difference between the average Control (Saline, No Female) vs. FAD, no female (p= 0.046); Control vs. FAD, No Female (p= 0.1); Control vs. Saline, 17, 20 BP + Female (p= 0.01); Saline, 17, 20 BP + Female vs. FAD, 17, 20 BP + Female (p= 0.23).

Figure 7- **Experiment 2a Courtship Behavior**
A bar graph (Mean ± SEM) depicting the difference of following behavior between groups: Saline, 17, 20 BP + Female (left; N= 9) vs. FAD, 17, 20 BP + Female (right; N= 5). The Saline, 17, 20 BP + Female group had a significantly lower following time than the FAD, 17, 20 BP + Female group, where p< 0.001.
3.3 Immunohistochemistry

The control GPR30 brain depicts only non-specific patterns (see Figure 8A). The tagged GPR 30 can be seen as clusters of cells resembling cytoplasmic staining (see Figure 8B). The control ERß brain also depicts non-specific patterns (see Figure 9A). In the experimental ERß slide, we see signaling that is not localized in the nucleus, but is instead fibrous (see Figure 9B).

Figure 8A – Control GPR 30 IHC                Figure 8B – GPR 30 IHC

A 1:500 dilution of primary rabbit polyclonal antibody solution in 2.5% BSA + .3% Triton X-100 of GPR 30 (Invitrogen, Carlsbad, CA). These images depict 30 µm cross-sections of the preoptic area (POA), cut by a cryostat (Shanon Cryotome FE and Cryotome E).
A 1:250 dilution of primary rabbit polyclonal antibody solution in 2.5% BSA + .3% Triton X-100 of ERβ (Invitrogen). These images depict 30 µm cross-sections of most probably the olfactory bulb, cut by a cryostat (Shanon Cryotome FE and Cryotome E).

CHAPTER 4: DISCUSSION

Although PGF2α did not significantly affect milt production, we showed, as have others, that 17, 20 BP can elevate levels of milt (Sorenson, 1989). We extended those findings by showing that FAD, an aromatase inhibitor, seems to block that effect, suggesting that at least part of 17, 20 BP’s effect is mediated by a rapid increase in T which is known to occur because GtH levels elevate in response to this pheromone.

In Experiment 2, the premilt measures were taken after the injection of FAD. When looking back at the data, it appeared as if FAD itself might be increasing the milt volume. Because of the uncertainty of what was affecting the premilt measures, we modified the procedure for Experiment 2a in order to determine what was happening. In
addition, we added another treatment group and an extra female to increase the stimulus strength in effort to generate more robust effects.

Again, we saw an increase in milt in response to contact with females. Although FAD appeared to diminish this response, the effect was not significant. Unexpectedly, FAD, in the absence of any female sexual stimuli, actually increased milt production. This pattern of results suggests that FAD could be working through androgenic and estrogenic mechanisms. FAD injected fish exposed to females had intermediate levels of milt. The difference scores of FAD injected fish exposed to females were less negative than the control fish, but also less positive than the saline, 17, 20 BP injected fish. These in-between results suggest that FAD is blocking part of the effect of exposure to females. In addition, FAD by itself appeared to stimulate more milt. There is supporting evidence that androgens and estrogens can directly affect testes through non-genomic mechanisms (Loomis & Thomas, 2000). Therefore, if androgen and estrogen receptors existed on the testes, then FAD can drive androgen responses and block estrogen responses, which could account for the intermediate effects of FAD observed in my experiments.

4.1 Behavior

In experiment 2 and 2a, the behavioral experiment effects were equivocal as they were robust in the opposite direction in which we hypothesized. We thought because the FAD would inhibit aromatase, the courtship behavior would be lowered in the FAD injected males. One explanation for these results is that blocking T’s conversion to E2 leads to increased T (Lord et al., 2009) and that T may directly stimulate courtship in the contexts in which I tested it (i.e. in a pheromone dominated system).
Another explanation is that when fish were given exposure to actual contact with females, which can activate various mechanisms, there is the possibility that 17, 20 BP and PGF2α could be working together to stimulate the gonads via multiple pathways. The Lord et al. study (2009) has shown that FAD can increase T levels. We also saw a marginal increase of T levels via FAD in this experiment. In other studies, they have found that the behavioral context of visual cues, which are mediated by estrogenic mechanisms, may not be that integral in stimulating a response, but surplus exposure of olfactory stimulation is (Lord et al., 2009). As a result, androgens can directly influence the olfactory systems that can then account for these results.

4.2 Immunohistochemistry

The preoptic area (POA) is an important area for regulation of male sexual behavior in all vertebrates (Lord et al., 2009). In the GPR30 IHC run, the fluorescent signal is located in the POA. Proteins involved in sexual responses and functions are generally localized in this area. The POA is also an area where isotocin cells– proteins that have dramatic effects on social behavior in goldfish– are located (Thompson & Waltson, 2004). Follow up studies in the Thompson lab have confirmed that GPR30 is produced in the same cells that produce isotocin (Appendix F), suggesting that there could be interactions between these rapid steroid effects and the neuropeptide system; GPR30 is likely modulating some responses that isotocin mediates, including social approach, which isotocin stimulates (Thompson and Walton, 2004).

Genomic receptors typically go straight into the cell nuclei and appear as tight, wound spheres inside the cell. The presumed olfactory bulb slide, stained with the ERB
antibody, did not show nuclear signaling, which other studies have seen with another batch of the same antibody. However, this fibrous pattern does look similar to what we have seen in other fish. Although the signal is not nuclear, we cannot distinguish whether it is in the membrane or the cytosol around the membrane. We can interpret that those signals are possibly membrane types of receptors in the olfactory bulb involved in processes that may mediate rapid non-genomic effects. Localization in the olfactory bulb suggests estrogens can influence olfactory processes. It is possible that membrane androgen receptors are there too, or even in the olfactory epithelium where androgens could directly modulate responses to pheromones. These receptors have not been thoroughly reviewed.

4.3 Limitations and Future Studies

4.3.1 Milt Difference Scores

Future studies should consider the variability of milt difference scores. Premilt measures are a function of short and long-term factors that we did not control, while postmilt volumes are more closely correlated with processes that occur within the hour following the stripping procedure. As a result, it is plausible that large increases in levels of expressible milt that may occur within the hour succeeding the stripping would still result in smaller volumes than the premilt volumes, which could have been stored over hours or even days prior to stripping (Mangiamele & Thompson, 2012).

4.3.2 Fadrozole

FAD is a difficult drug to use. If FAD can increase androgens, which can have their own affect, then it will be difficult to completely knock out the effect of estrogens.
While FAD is countering the estrogen effect, it may also be adding an androgen effect. For future studies, researchers should consider other inhibitors to explore these effects.

4.3.3 Behavioral Set-up

To some degree, these behavioral results were not all independent because we had multiple males from the same group in the tank. This test needs to be repeated with individual males or counting as an average across these groups. Limitations of the study include time, space, and keeping the fish from the stress of isolation. We conducted the experiment in a naturalistic way with multiple males in order to address the stress component. Although we did not observe this in our study, multiple males in a tank can also lead to suppression of other males because of competition, another variable to consider for future studies.

4.4 Conclusion

In conclusion, our study provides a contextual framework for exploring these rapid mechanism and the receptors that mediate them. These tests utilized behavioral tests that emphasized olfactory activation. Through this experiment, we have established that in a sexual social context, milt production does increase; FAD can affect courtship behavior; and some preliminary data that shows localization of a novel protein receptor, GPR 30, that could mediate reproductive functions and may do so with interactions with the neuropeptide system. We were unable to confirm that FAD blocks female induced rapid increase in milt, but did by itself increase milt and courtship responses. These results suggest that milt production is perhaps affected both estroganically and androgenically. Although the effects of FAD were observed within an hour, which in
steroid research is relatively proficient, we cannot say it is non-genomic. Research is now being conducted on these interactions.

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APPENDICES

APPENDIX A: Model of genomic and non-genomic steroid hormone action

Genomic mechanisms initiate when the hormone binds receptors in the cytoplasm. The hormone/receptor complex migrates to the nucleus to alter gene expressions (mRNA). This process takes hours or days to affect behavior. Non-genomic mechanisms are usually assumed to be mediated by hormones binding to receptors in the cell membrane. These receptors then activate other cellular signaling pathways (second messengers) that can lead to rapid changes in behavior within seconds or minutes. (Steiner & Trainor, 2010)
APPENDIX B

Mean ± 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; *Within-groups difference across test days, p<0.05, two-tailed; **p<0.01, two-tailed. (Lord et al., 2009)
APPENDIX C

Because the effects of T occur within a time frame (< 1 hr) that is likely too rapid to be mediated by the transcriptional actions of steroid hormones, we tested whether activation of membrane-bound estrogen receptors by E2:BSA could induce an increase in expressed milt via non-genomic mechanisms. We found that at 1 hr post-injection, males injected with E2:BSA had more milt, controlling for baseline differences in milt, than control animals injected with vehicle (F1,27= 1.45, p = 0.02).

Estradiol conjugated to bovine serum albumin (E2:BSA) induces a rapid increase in expressible milt volume. Controls were treated with vehicle (Veh) only. Bars represent estimated marginal means of post-injection milt volume. Error bars indicate standard error. Asterisk indicates a significant difference between groups, p < 0.05. (Mangiamele & Thompson, 2012)
APPENDIX D: Aromatization

General reaction for the conversion of testosterone to estradiol catalyzed by aromatase. Steroids are composed of four fused rings (labeled A-D). Aromatase converts the ring labeled “A” into an aromatic state. (“Aromatase”, 2014)
In order to determine whether T’s effect on milt is dependent on aromatase, males were pre-treated with the aromatase inhibitor Fadrozole (FAD). We compared males injected with vehicle+T, FAD+T, and vehicle+vehicle and found that FAD blocked the T-induced rapid increase in expressible milt volume. Whereas males injected with vehicle+T showed an increase in milt compared to controls (ANCOVA F 2,21 = 12.29, p < 0.001; post hoc t-test, p = 0.002), males injected with FAD+T did not have significantly higher levels of milt 1 hr post-T injection, after controlling for baseline levels, than did control males injected twice with vehicle (post hoc t-test, p=0.54).

Fadrozole (FAD), an aromatase inhibitor, blocks an increase in milt volume when administered before testosterone (T). Control animals were treated twice with vehicle (Veh) only. Bars represent estimated marginal means of post-T injection milt volume. Error bars indicate standard error. Brackets above bars denote pair-wise comparisons. Asterisks indicate significant differences between groups. * indicates p < 0.05, ** indicates p < 0.01. (Mangiamele & Thompson, 2012)
APPENDIX F

Immunohistochemistry of GPR 30 (top left; green) and isotocin (top right; red). The bottom right image shows the complete co-localization of GPR30 within isotocin cells.
BIOGRAPHY OF THE AUTHOR

It was days before Christmas when Kevin and Mai Dao were gifted with their third child on December 21, 1991. Despite being born in Bellflower, CA, Kimberly Dao is a Mainer at heart. Prior to settling in Maine, the Dao Family lived in three other states: California, Virginia, and New Hampshire. Finally in late April of 2000, they found a place to call home in sunny Saco, ME. Although she grew up away from her extended relatives, Kim was able to grow strong bonds with her three siblings and her family. In the fall of 2008, an additional member was added to the family when Vivian, Kim’s cousin, moved to Maine to live with them. In high school, Kim enjoyed participating in Thornton Academy’s field hockey and tennis team, Interact Club, and was student body president; she graduated in 2010. She began her undergraduate education at the University of Maine that fall as a biology major, with a concentration in pre-medical studies and a minor in child development and family relations. As an undergraduate, she participated in various extracurricular activities including All Maine Women, Student Government, Class Council, UMaine Mitchell Scholars, Asian Student Association, Honors College Student Advisory Board, Alternative Breaks, Operation HEARTS, Club Field Hockey, Phi Beta Kappa, Black Bear Mentors, Partners for World Health, the Vagina Monologues, and the International Dance Festival. During the summer of 2013, she studied abroad through Semester at Sea. Upon graduation, Kim will attend Tufts School of Medicine and participate in their Maine Track MD program. Her career goals include becoming a family physician, a public health administrator, and participating in Doctors Without Borders or Physicians for Peace.