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Aromatase regulates aggression in the African cichlid fish *Astatotilapia burtoni*

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HIGHLIGHTS

- We measured brain aromatase expression in subordinate and dominant male African cichlid fish.
- We inhibited aromatase in dominant males and quantified behavioral and hormonal changes.
- Subordinate males have higher aromatase expression in the preoptic area.
- Inhibiting aromatase decreases aggression and circulating estradiol and increases testosterone.

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ABSTRACT

The roles of estrogen and androgens in male social behavior are well studied, but little is known about how these hormones contribute to behavior in a social hierarchy. Here we test the role of aromatase, the enzyme that converts testosterone into estradiol, in mediating aggression and reproductive behavior in male *Astatotilapia burtoni*, an African cichlid fish that displays remarkable plasticity in social behavior. We first measured aromatase expression in subordinate and dominant males in brain regions that regulate social behavior and found that subordinate males have higher aromatase expression than dominant males in the magnocellular and gigantocellular regions of the preoptic area. Next, we functionally tested the role of aromatase in regulating behavior by intraperitoneally injecting dominant males with either saline or fadrozole (FAD), an aromatase inhibitor, and found that FAD treatment decreases aggressive, but not reproductive, behaviors compared to saline controls. To determine the underlying physiological and molecular consequences of FAD treatment, we measured estradiol and testosterone levels from plasma and brain aromatase expression in FAD and saline treated dominant males. We found that estradiol levels decreased and testosterone levels increased in response to FAD treatment. Moreover, FAD treated males had increased aromatase expression in the gigantocellular portion of the POA, possibly a compensatory response. Overall, our results suggest aromatase is a key enzyme that promotes aggression in *A. burtoni* males through actions in the preoptic area.

1. Introduction

Across vertebrates, sex steroid hormones are key regulators of social behavior. Despite the remarkable conservation of steroid pathways across taxa, their specific roles in different species are complex. Although androgens (principally testosterone) have historically been associated with male aggressive and reproductive behavior, the aromatization of androgens to estrogens by the enzyme aromatase also influences male-specific phenotypes. In the past few decades, it has become clear that estradiol is as active and important as testosterone in regulating male aggressive [33,38,42,44,45] and reproductive [3,47] behavior. There have been numerous studies comparing the organizational and activating effects of non-aromatizable androgens (such as dihydroxytestosterone, or DHT), testosterone, and estradiol that support this hypothesis [1,8,24]. Clearly, estradiol plays a fundamental role in the control of male-typical behavior, yet how its synthesis and activity are regulated in relation to behavior is much less known.

Aromatase is the product of the *CYP19* gene, a member of the P450 superfamily of cytochrome enzymes, is expressed largely in the brain and gonads [40]. Teleost fish have two isoforms, *CYP19A1* (or *CYP19a*) and *CYP19A2* (or *CYP19b*), which are expressed in the gonads and brain, respectively [22]. Intriguingly, aromatase mRNA expression and activity are unusually high in the brains of teleost fish (as well as songbirds), especially in the forebrain, where activity has been localized mostly to glia cells and reported at levels 100–1000 times higher than in other vertebrate taxa [14]. Much work has been done on sex-differences in aromatase expression in teleosts [7,9,17,26,35], but less is known about the contributions of aromatase to behavioral variation within a sex. Male territorial and sneaker

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phenotypes in the plainfin midshipman (*Porichthys notatus*) have differential expression of aromatase in brain regions influencing vocal communication [13]. Aromatase has also been implicated in influencing phenotypic transitions, as in the peacock blenny (*Salario pavo*) where aromatase activity in the brain increases as males transition from the small sneaker phenotype to the large nesting phenotype [16].

The African cichlid fish *Astatotilapia burtoni* has been established as a model system for understanding the neuroendocrine mechanisms of social behavior, with many studies taking advantage of the extraordinary phenotypic plasticity and dynamic social behavior of this species [20,48–50]. Dominant males aggressively defend territories where they court and spawn with females while subordinate males are socially and reproductively suppressed. Dominant males have high levels of testosterone [51] and estradiol [33,49] relative to subordinate males and females. Importantly, these social phenotypes are reversible, as males repeatedly switch from subordinate to dominant and back again depending on the immediate social environment [52]. Previous work manipulating steroid hormone receptors has shown that androgens regulate sexual behavior in dominant males while estradiol via the estrogen receptor regulates aggression in males regardless of social status [33]. Furthermore, both circulating estradiol and testosterone levels increase as *A. burtoni* males ascend from subordinate to dominant status, although gonadal aromatase expression does not [20]. We therefore wanted to test the role of aromatase in regulating aggression or reproductive behavior in *A. burtoni* males.

To better understand the role of aromatase in regulating social behavior in *A. burtoni*, we compared brain gene expression of aromatase in dominant and subordinate males. Our hypothesis was that aromatase mRNA levels would be higher in dominant compared with subordinate males. We focused on quantifying expression in five brain areas important in social decision making [30–32]: the anterior tuberal nucleus (aTn; putative homolog of ventromedial hypothalamus); the periventricular part of the posterior tuberculum (TPp; ventral tegmental area); the dorsal region of the ventral telencephalon (Vd; nucleus accumbens); the ventral region of the ventral telencephalon (Vv; lateral septum); and the parvocellular (putative homolog of the mammalian paraventricular nucleus of the preoptic area), magnocellular, and gigantocellular (putative homologs of the mammalian supraoptic nucleus of the preoptic area; [28]) regions of the preoptic area (POA). We then functionally tested the role of aromatase regulation of behavior by treating dominant males with fadrozole (FAD), an aromatase inhibitor, and subsequently measuring behavior, hormone levels, and brain aromatase expression compared to control males. We specifically wanted to examine the role of aromatase in regulating behavior rather than specific steroid hormone receptors, which we have done previously [33]. We predicted that FAD would reduce aggressive and/or reproductive behavior, possibly associated with a decrease in estradiol levels, while androgen levels would remain unchanged.

2. Materials and Methods

2.1. Animals

All animals used in this study were adult *A. burtoni* males from a laboratory stock originally derived from a wild population in Lake Tanganyika, Africa [11]. Fish were maintained at 28 °C on a 12:12 h light/dark cycle with 10 min dawn and dusk periods to mimic their native tropical environment in 110 L aquaria that were integrated into a re-circulating life support system. All tanks contained gravel substrate to facilitate digging behavior and terra cotta pot shards, which served as territorial shelters. Prior to introduction into the experimental tanks, we observed all male fish in communities consisting of approximately eight males and eight females for two weeks to determine their social status. All procedures were in accordance with and approved by The University of Texas Institutional Animal Care and Use Committee.

2.2. Dose–response curve

We chose to use FAD to inhibit aromatase, an effective blocker of aromatase activity in teleost fish and other vertebrates [15,46]. In order to determine an appropriate dose of FAD for *A. burtoni*, we used 115 L aquaria that were part of a re-circulating system (ca. four water changes per hour) and bisected with clear, perforated acrylic dividers to allow visual and olfactory communication between the two halves of the tank while preventing the animals from physically interacting. Each half contained one terra cotta pot as a territorial shelter, one dominant male, and four female fish. One male per tank was manipulated at any given time. A stock solution of FAD (Sigma) was prepared by dissolving the powder in 100% ethanol at a concentration of 10 μg/μL (Dr. David Crews, personal communication) and storing it at −20 °C. The day before the experiment, the FAD was diluted to the appropriate concentration in PBS and stored at 4 °C. The experiment was carried out over the course of four days: on Day 1 the focal male was observed at 10:00 for 5 min (this observation period is more than sufficient for obtaining a representative behavioral profile; see [33,51]). All aggressive and reproductive behaviors were scored as described previously [10], and the person injecting and observing the animals was blinded to dose/treatment. On Day 2, the focal male was weighed to calculate the appropriate injection volume and intraperitoneally injected with either saline or 1, 10, or 100 μg/g body weight (b.w.) of FAD ($n = 5$ males each, volume of 50–150 μL) using an insulin syringe (VWR) at 9:15 h. At 10:00 h, the fish was again observed for 5 minutes to capture any rapid effects of the drug. On Day 3, the focal male was observed at 10:00 h to capture any slower effects of the drug, and at 11:00 h he was injected again with the same drug dose. Finally, on Day 4, he was observed for a third time at 10:00 h for 5 min to determine if the second dose augmented any effects observed after the first treatment. The tanks were allowed to rest for two days before the paradigm was repeated using the focal male in the other compartment. Behavioral observations showed that on Day 2 aggressive attacks did not change with saline treatment (paired t-test, $t = 1.513$, $p = 0.205$) but decreased after FAD treatment, and this difference was greatest at a dose of 10 μg/g b.w. FAD ($t = 4.765$, $p = 0.009$; for 1 μg/g b.w., $t = 2.745$, $p = 0.052$). Four of the five animals treated with 100 μg/g b.w. FAD died, suggesting that this dose was too high. We did not observe any (slower-acting) drug effects on Days 3 or 4, and therefore settled on an experimental paradigm consisting of a baseline observation on Day 1 and treatment of 10 μg/g on Day 2.

2.3. Pharmacological manipulations

To test the effects of aromatase inhibition in a naturalistic setting, we established 15 communities in 115 L tanks with eight males, eight females and five terra cotta pots each. Only one territorial male per tank was manipulated at any given time. Our experimental paradigm consisted of two days: on Day 1, the focal male was observed at 10:00 for 5 min, and aggressive and sexual displays were scored as described previously [10]. On Day 2, the focal male was weighed and injected between 9:15 and 10:00 h with either saline ($n = 14$) or FAD (10 μg/g b.w., $n = 14$). The male was then returned to his tank and 45 min later observed for 5 min, as this was the time period at which we had observed the greatest behavioral effects in the pilot study (see above). All observations of up to eight tanks were completed between 10:00 and 10:50 h. Experimental tanks were then allowed to rest for four days before a different male in the community was observed and manipulated in the manner described. Each tank was used only twice such that the first round of communities contained eight males each,

and the second round contained seven males each. Due to the possibility of males changing status in response to novel competitors, the focal male was not replaced. There was no significant effect of round (i.e., number of males remaining in tank) on any behavior.

2.4. Tissue collection and hormone analysis

Because up to eight focal males were treated on a given day, focal males were removed from their tanks, 11:00 and 11:40 h in the same order the injections and observations were carried out. Blood was drawn from the dorsal artery using a heparinized 26 gauge butterfly infusion set (Sur-flo Plastics, Warren, MI). Blood samples (n = 13 each for FAD-treated and control animals) were centrifuged at 4000 *g* for 10 min, and the plasma was stored at −80 °C until hormone analysis. We measured both testosterone (intra-assay coefficient of variation (CV) = 7%) and 17β-estradiol (intra-assay CV = 14%) using ELISA (Enzo Life Sciences, Farmingdale, NY) after diluting the plasma samples 1:30 in assay buffer according to Kidd et al. [21] and manufacturer's instructions. Immediately after blood was drawn, males were euthanized via rapid cervical transsection. Brains were removed, embedded in OCT (Tissue-Tek, Fisher Scientific) on dry ice and stored at −80 °C until sectioning. The testes were also removed and weighed to calculate gonadosomatic index (GSI: testes mass/body mass × 100).

2.5. In situ hybridization

After being embedded in OCT and stored at −80 °C, the brains obtained from the animals used in the pharmacology experiment were sectioned into four series at 20 μm, thaw-mounted onto SuperFrost Plus slides (Erie Scientific, Portsmouth, NH), and stored at −80 °C. An additional series of sections was obtained from the brains of established dominant and subordinate males from a prior study to determine whether aromatase expression varied according to social status. Both series were processed separately but in the exact same manner. Sections were subsequently thawed and fixed for *in situ* hybridization as described previously in Munchrath and Hofmann [29]. The probe for *A. burtoni* brain aromatase (*CYP19A2*, GenBank accession number FJ605734) was 964 bp in length and reverse-transcribed using the MEGAscript kit (Ambion). Probes were subsequently purified using NucAway spin columns (Ambion) and quantified for radioactivity on a scintillation counter. Slides were incubated at 65 °C for 18 hours in 200 μL of hybridization buffer (Sigma) containing either 2.0×10^6 cpm/slide of S^{35}-labeled riboprobe and 1 mM DTT. Control slides were incubated with an equal amount of sense probe in place of the antisense probe and showed no signal above background. After hybridization, slides were placed in 65 °C 4X SSC + 1 mM DTT for 5 min to remove cover slips. Slides were then washed at 65 °C in 4X SSC + 1 mM DTT for 1 h, washed twice at 65 °C in 50% formamide + 2X SSC + 1 mM DTT for 1 h, washed twice at 65 °C in 0.1X SSC + 1 mM DTT for 30 min, then equilibrated to room temperature in 0.1X SSC + 1 mM DTT for 15 min. Slides were then dehydrated in an ascending ethanol series and air dried. Slides were dipped in Kodak NTM emulsion (VWR, Cat No. IB8895666) with the aid of a photo-safe red light, dried at 65 °C for 1 h, and then stored in light-tight boxes at 4 °C. After 3 or 5 days (for aromatase or ERα, respectively), slides were developed for 4 min in Kodak developer (VWR, Cat No. 100488–596) at 15 °C, washed in 15 °C water for 15 s, fixed in Kodak fixer (VWR, Cat No. 100488–602) for 6 min, and then washed in distilled water. Sections were then counterstained with cresyl violet overnight before dehydration in an ethanol series, clearing in xylene, and cover-slipping in Permount (Fisher Scientific).

2.6. Microscopy and quantification

Images for ISH were taken with a digital camera (AxioCam MRc, Zeiss) attached to a Zeiss AxioImager.A1 AX10 microscope (Zeiss) using the AxioVision (Zeiss) image acquisition and processing software. Images were compiled and brightness- and contrast-enhanced in Adobe Photoshop CS3. Our initial quantification of aromatase between dominant and subordinate males focused on five core brain regions involved in social decision-making ([31,32]; putative mammalian homologues are in parentheses): the ventral part of the ventral telencephalon (Vv, lateral septum), the dorsal part of the ventral telencephalon (Vd, putative nucleus accumbens); POA, the anterior tuberal nucleus (aTn, putative ventromedial hypothalamus), and the periventricular part of the posterior tuberculum (TPp, putative ventral tegmental area). To quantify the ISH signal we modified the protocols of Burmeister et al. [53] and Hoke et al. [19]. Specifically, for each brain region, we captured two random non-overlapping images from each of three to four sections. For each section, we took three images using the 100X objective: a color image of the black silver grains and purple Nissl bodies (cell image), a blue-filtered image of the silver grains in the same field of view (grains image), and a blue-filtered image on a nearby area of the slide containing no tissue (background image) to represent any background level of silver grains, which can vary across the slide due to emulsion thickness. We used Image J (NIH, MD, USA) to convert the grains and background image into black and white images using the "make binary" function. The area of the grains was obtained using the "analyze particles" function. For each section we subtracted the area of background silver grains from the area of the silver grains of interest. The cell area was quantified using an automated counting procedure in Adobe Photoshop as described in Hoke et al. [19]. Purple Nissl bodies were isolated using the "select color" function, thresholds were set individually for each image, and the remainder of the image was erased. The area covered by Nissl bodies were determined using Image J. Silver grain density for each brain region for each individual was calculated as the ratio of the area of silver grains above background to the area covered by cells in the standard-size sampling window. An experimenter blinded to treatment group did the quantification.

2.7. Statistical analysis

All statistical analyses were performed using SPSS software, version 19.0. We tested all variables for normality using the Shapiro–Wilk test. To investigate changes in aggressive behavior, we summed the number of attacks towards other dominant males, subordinate males, and females to comprise an "Attack" score. We used a paired t-test to compare post-drug to pre-drug levels for each treatment and a General Linear Model (GLM) to confirm a day x treatment interaction. To investigate reproductive behavior, we summed leading, quivering, and digging to comprise a "Reproductive Behavior" score, which was not normally distributed, so we compared post-drug to pre-drug levels for each treatment using the related-samples Wilcoxon Signed-Rank test. We then used a Generalized Estimating Equations (GEE) model [23] to detect a day-by-treatment interaction. To compare normal continuous variables such as gene expression between treatments, we used a t-test; for non-normal continuous variables such as hormone levels, we used a Mann–Whitney U test. To examine relationships between continuous variables such as behavior, hormones, and gene expression, we used either Pearson's correlation coefficient or Spearman's rank correlation coefficient as appropriate. Statistical significance was considered as having a p-value less than 0.05; when multiple correlations were investigated, we used the Benjamini–Hochberg method of correcting for multiple hypothesis testing [5].

3. Results

3.1. Status differences in aromatase expression

As 17β-estradiol may be important for male status differences in aggressive behavior [33], we quantified aromatase expression in stable dominant and subordinate males within regions important for aggression

and sexual behavior [32]. Note that one control (saline-treated) animal was excluded from the analysis as an outlier since its aromatase mRNA levels were more than three standard deviations greater than the mean for this experimental group. Surprisingly, subordinate males have higher aromatase expression in the mPOA ($t_{11} = -4.100$, $p = 0.002$) and gPOA ($t_{10} = -2.317$, $p = 0.043$) compared to dominant males (Fig. 1). We did not find aromatase expression differences between DOM and SUB males in Vv ($t_{11} = -0.577$, $p = 0.575$),Vd ($t_{11} = 0.454$, $p = 0.659$), pPOA ($t_{11} = 0.754$, $p = 0.467$), aTn ($t_{11} = -1.205$, $p = 0.254$), or TPp ($t_{11} = -2.159$, $p = 0.054$).

3.2. Aromatase regulation of behavior

As 17β-estradiol treatment increases aggression in *A. burtoni* males ([33]), we next asked if aromatase plays a role in facilitating aggressive displays by treating dominant males with FAD, an aromatase inhibitor. We did not inhibit aromatase in subordinate males as they rarely display aggression. FAD-treated dominant males significantly decreased the number of attacks towards other fish while control animals remained unchanged (paired t-test: saline, $t_{14} = -0.994$, $p = 0.337$; FAD, $t_{14} = -2.313$, $p = 0.036$; Fig. 2A). This difference was confirmed using a general linear model, where we examined the effects of day (post-drug vs. pre-drug) and treatment (FAD vs. saline) and found a significant day-by-treatment interaction effect (GLM: $F = 6.407$, $p = 0.018$). There was an injection effect on reproductive behavior, as reproductive behavior decreased after saline injection (Wilcoxon signed-rank test: $X = -2.135$, $p = 0.033$). Reproductive behavior also decreased after FAD treatment, but this decrease was not significantly different from the injection effect (i.e., no treatment effect; GZLM: Wald $\chi^2 = 1.353$, $p = 0.245$, $n = 58$; Fig. 2B).

3.3. Hormonal response to saline and FAD treatment

To determine if aromatase was indeed inhibited by FAD, we measured circulating testosterone and 17β-estradiol in control and FAD-treated males. Plasma testosterone levels, which were taken approximately two hours after injection, increased approximately two-fold following FAD treatment compared with controls (Mann–Whitney $U_{12,12} = 167.00$, $p < 0.0001$, Fig. 3A). Conversely, 17β-estradiol levels decreased almost two-fold compared with controls following FAD treatment ($U_{12,12} = 14.00$, $p < 0.0001$; Fig. 3B). Interestingly, testosterone and 17β-estradiol levels correlated significantly in saline animals (Spearman $\rho = 0.665$, $p = 0.013$, $n = 13$) but not in FAD-treated animals ($\rho = 0.115$, $p = 0.707$, $n = 13$). GSI did not differ between treatments

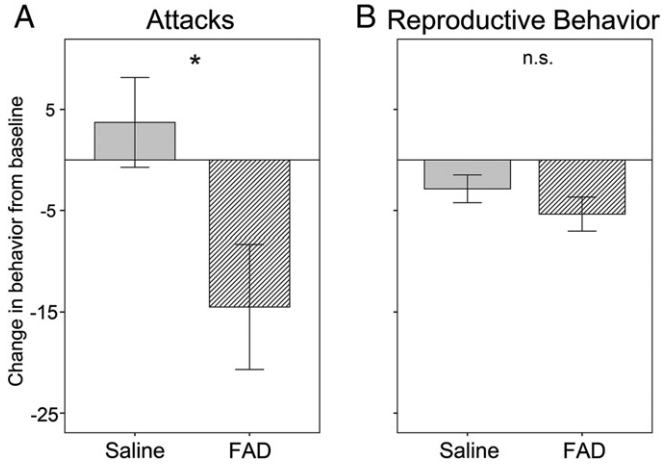

Fig. 2. Effects of FAD treatment on behavior of dominant males. Change in number of (A) attacks and (B) reproductive behavior displayed after treatment with either saline of fadrozole (FAD) compared to baseline behavior. Data are represented as the mean change in behavior (baseline-treatment) and error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$) comparing the effects of saline vs. FAD treatment using either a General Linear Model (aggression; $F = 6.407$, $p = 0.018$) or Generalized Linear Model (reproduction; Wald $\chi^2 = 1.353$, $p = 0.245$, $n = 58$).

(t-test, $t_{27} = -0.119$, $p = 0.906$). There were no significant correlations between hormone levels or behavior within either treatment.

3.4. Aromatase expression in response to saline and FAD treatment

As aromatase seems to promote aggression through production of 17β-estradiol, we next asked how the changes in aromatase activity and resulting changes in hormone availability altered brain gene expression in dominant males. We measured aromatase expression in the gPOA, as we had observed differential expression between dominant and subordinate males (see above). Importantly, FAD treatment significantly increased brain aromatase expression in the gPOA relative to saline-treated males (Fig. 4; t-test, $t_{14} = -2.485$, $p = 0.026$).

4. Discussion

Our study and is one of very few to quantitatively compare aromatase mRNA levels across both pharmacological treatments and behavioral phenotypes. We have previously examined the behavioral effects of pharmacologically manipulating ER and AR in *A. burtoni* males [33]. The present study considerably extends these findings as it has allowed

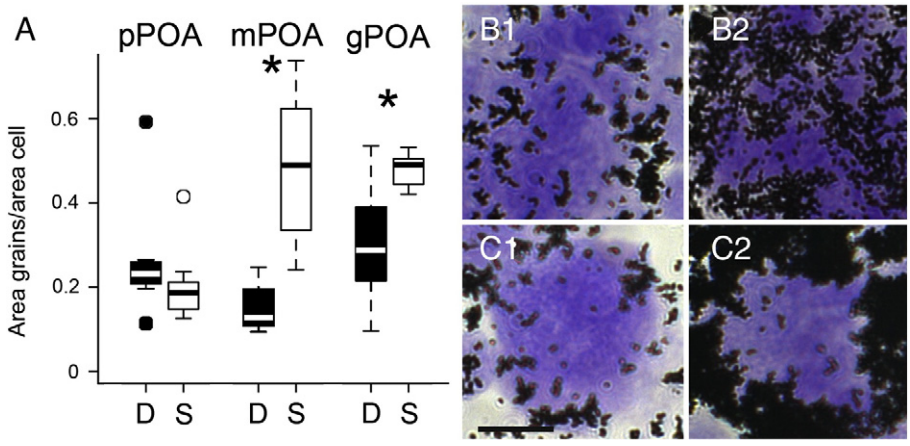

Fig. 1. Expression of aromatase in the male brain. Box and whisker plots indicate aromatase expression in dominant (D) and subordinate (S) males in the parvocellular, magnocellular, and gigantocellular preoptic area (pPOA, mPOA, gPOA, respectively) (A). Boxes indicate the first and third quartiles, the line within the box represents the mean, the whiskers represent the minimum and maximum values, and the circles represent outliers. Statistics are presented from a t-test if significant ($p < 0.05$). Micrographs show representative aromatase expression in the mPOA (B) and gPOA (C) of dominant (B1, C1) and subordinate (B2, C2) males. Scale bar is 10 μm.

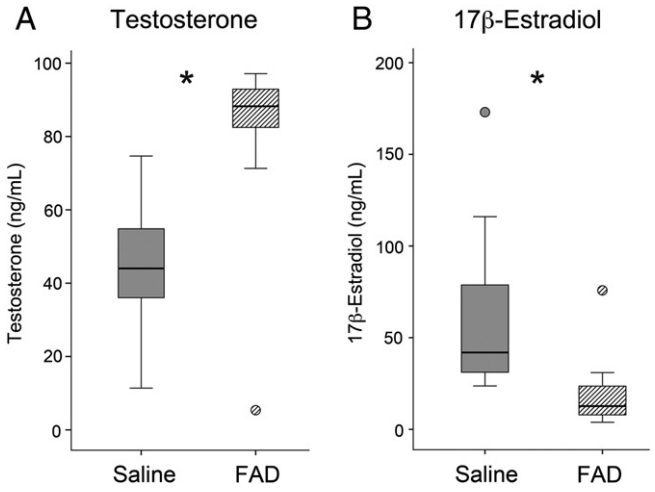

Fig. 3. Plasma hormone levels in dominant males following saline and FAD treatment. Circulating free (A) Testosterone and (B) 17β-Estradiol were measured after saline (grey) or FAD (hatched) treatment. Asterisks indicate statistical significance ($p < 0.0001$) according to Mann–Whitney U tests.

us to determine the role of aromatase and neurosteroid synthesis in regulating social behavior. The results presented here support the hypothesis that in male *A. burtoni* estradiol is necessary for aggressive behavior via the conversion of testosterone by aromatase. We found that pharmacologically blocking aromatase decreased aggressive behavior but did not affect reproductive behavior. By blocking aromatase using FAD, less testosterone should be metabolized into estradiol, which was confirmed by the increase in circulating testosterone and corresponding decrease in estradiol. Similar responses were observed in Coho salmon (*Oncorhynchus kisutch*; [2]) and fathead minnow (*Pimephales promelas*; [54]) following IP injection of FAD, although these studies reported both testosterone and estradiol for only females. As aromatase is expressed in the brain to locally produce estradiol and regulate behavior [25], and because previous studies of gonadectomized *A. burtoni* males have shown that gonadal steroids are not necessary for these sex steroid hormone-dependent behaviors [41], our results suggest that FAD successfully blocked the action of aromatase in the brain.

In saline-treated animals, we found that circulating testosterone and estradiol levels positively correlated in the circulation, which was expected based on previous studies in *A. burtoni* [20]. However, when we blocked aromatase activity with FAD, testosterone and estradiol no long correlated, presumably because testosterone was no longer metabolized into estradiol and estradiol preproduction more or less ceased as a result. It is interesting to note that there is a lack of correlation as opposed to a negative correlation between testosterone and estradiol in FAD treated animals. This suggests that the inhibition of aromatase either does not affect its substrate (testosterone) and product (estradiol) in a 1:1 manner or not all of the enzyme molecules were pharmacologically inhibited. However, we cannot rule out the possibility that testosterone that is no longer aromatized to estradiol due to FAD treatment may be shunted to other metabolic pathways [18].

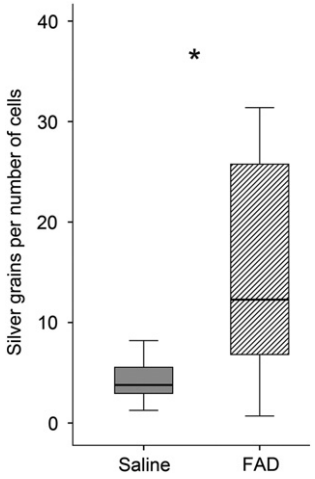

Fig. 4. Effects of FAD treatment on aromatase gene expression. *CYP19A2* expression significantly increased in the gigantocellular POA of dominant males after FAD treatment compared with saline-treated controls. Asterisk indicates statistical significance at $p < 0.05$.

In *A. burtoni*, FAD significantly decreased estradiol and the number of aggressive attacks towards other animals in the community. Our findings are consistent with those in male California mice (*Peromyscus californicus*), where attack latency was decreased by fadrozole treatment [55]. However, other studies have shown a negative relationship between aromatase activity and aggression. For example, in male bluebanded gobies (*Lythrypnus dalli*) territorial behavior is negatively associated with aromatase activity [6]. Although the majority of evidence across vertebrates suggests a positive relationship between estradiol and male aggression, it is unclear at this time how to resolve this discrepancy other than by invoking species-specific differences. In our study, circulating testosterone levels increased concurrently with the decrease in aggression; as it is well established that dominant males have higher testosterone than subordinate males [20,34], it is doubtful that this increase in testosterone is responsible for the decrease in aggression, as manipulations of the androgen receptor in male *A. burtoni* influence courtship behavior, but not aggression [33].

Estradiol has also been shown to be necessary for male reproductive behavior [44,45]; however, we did not see an effect of FAD treatment on reproductive behavior. Previous application of 17β-estradiol or an ER antagonist in *A. burtoni* males had no effect on reproductive behavior [33]. It is possible that the injection effect masked any specific behavioral effects of reduced aromatase activity on reproduction or that because reproductive behaviors are usually expressed at lower frequencies than aggressive behaviors, we did not have a sufficient dynamic range to detect any differences. As *A. burtoni* males respond behaviorally to the reproductive state of the females in their community [20], it is also possible that there were no gravid females present, which would contribute to a low level of reproductive activity. It would be interesting to examine the effects of FAD in the presence of gravid females in future studies.

We found that FAD treatment increased aromatase expression in the gPOA. If expression of aromatase is androgen-dependent in cichlids, as is the case in mammals [37], higher testosterone levels as a consequence of blocking aromatase likely led to increased gPOA aromatase levels. Yet, increased aromatase expression in the gPOA was not sufficient to prevent circulating estradiol levels from decreasing in these males, possibly because FAD may also have inhibited aromatase activity in other organ systems that express different isoforms of this enzyme, particularly in the gonads [20,22]. It is, however, important to take into consideration the timeline of sampling, as we collected brains of focal males less than two hours after treatment with either saline or FAD. Although we found a difference in aromatase gene expression, we did not determine any changes in aromatase protein abundance or enzymatic activity. Interestingly, brain aromatase activity can change rapidly in the avian brain [4]. It therefore seems quite likely that aromatase activity could be decoupled from gene expression in *A. burtoni* as well, a research avenue that should be investigated in the future.

The POA is an important neuroendocrine regulator necessary for aggression and sexual behavior [56,57]. We found that subordinate males had higher aromatase expression in the mPOA and gPOA compared to dominant males, possibly indicating a compensatory

response to low sex steroid levels. How each POA cell group contributes to physiology and behavior is currently unclear, although a previous study in *A. burtoni* showed that arginine vasotocin expression in particular POA cell populations differs between dominant and subordinate males [58]. Importantly, a previous microarray study showed that dominant males have higher whole brain aromatase mRNA levels compared to subordinate males [36]. However, whole brain gene expression measurements may obscure opposing gene expression variation in specific brain regions or cell types, as appears to be the case with aromatase in the gPOA. Thus, it is likely that the brain region(s) contributing to the high whole-brain aromatase expression in dominant males compared with subordinate males do not include the preoptic area and were not examined in the present study. Future studies will need to conduct a broader analysis of the *A. burtoni* brain to further identify regions with status differences in aromatase expression. A few studies have described the distribution of aromatase expression in teleost fish (midshipman, [12]; trout, [27]; pejerrey, [43]), showing elevated expression in both the pPOA and the magnocellular portion of the POA (mPOA, which is often considered to include the gPOA), but these studies did not examine expression differences between various phenotypes (but see [39]). *In situ* hybridization in the plainfin midshipman showed differential aromatase expression in the pPOA based on reproductive status, with reproductive males having higher aromatase expression than non-reproductive males [13]. These results suggest that, as in most vertebrates, aromatase expression and male aggressive and reproductive behavior are positively co-regulated; similarly, we did find that in saline-treated males, aromatase mRNA levels and aggressive behavior were correlated.

5. Conclusion

We have shown that by blocking aromatase, we can increase testosterone and aromatase expression and decrease estradiol and aggression in a highly social, dominant male cichlid fish. However, aromatase manipulation did not affect reproductive behavior. By simultaneously quantifying behavior, sex steroid hormones, and the neural expression of the genes for brain aromatase, we have increased our understanding of how estradiol synthesis is regulated and modulates social behavior.

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