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Sex differences and similarities in the neuroendocrine regulation of social behavior in an African cichlid fish☆,☆☆

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ABSTRACT

An individual's position in a social hierarchy profoundly affects behavior and physiology through interactions with community members, yet little is known about how the brain contributes to status differences between and within the social states or sexes. We aimed to determine sex-specific attributes of social status by comparing circulating sex steroid hormones and neural gene expression of sex steroid receptors in dominant and subordinate male and female *Astatotilapia burtoni*, a highly social African cichlid fish. We found that testosterone and 17β-estradiol levels are higher in males regardless of status and dominant individuals regardless of sex. Progesterone was found to be higher in dominant individuals regardless of sex. Based on pharmacological manipulations in males and females, progesterone appears to be a common mechanism for promoting courtship in dominant individuals. We also examined expression of androgen receptors, estrogen receptor α, and the progesterone receptor in five brain regions that are important for social behavior. Most of the differences in brain sex steroid receptor expression were due to sex rather than status. Our results suggest that the parvocellular preoptic area is a core region for mediating sex differences through androgen and estrogen receptor expression, whereas the progesterone receptor may mediate sex and status behaviors in the putative homologs of the nucleus accumbens and ventromedial hypothalamus. Overall our results suggest sex differences and similarities in the regulation of social dominance by gonadal hormones and their receptors in the brain.

Introduction

In many social species, members of a community form dominance hierarchies where social status profoundly affects an individual's behavior and physiology through interactions with community members (Sapolsky, 2005). The physiological basis of social dominance is often associated with differences in reproductive status and sex steroid hormone levels (Wingfield et al., 1991), which make studying the molecular determinants of social dominance difficult to dissect from reproductive physiology. Moreover, little is known about how these physiological and behavioral differences are integrated within the brain.

In order to disentangle sex-specific physiology from the neuroendocrine mechanisms of social dominance, we utilized a highly social fish that has plastic behavioral phenotypes and readily forms dominance hierarchies within community tanks. Males of the cichlid fish *Astatotilapia burtoni* display phenotypic plasticity in social status, alternating between dominant (DOM) and subordinate (SUB) phenotypes depending on the social environment. DOM males are conspicuously colored, reproductively active, and aggressively defend territories where they court and spawn with females. SUB males are dull in coloration, school with females, and are reproductively inactive. Although neuroendocrine differences between DOM and SUB males have been described in various contexts (Maruska and Fernald, 2010a; O'Connell and Hofmann, 2012), disentangling the neural basis of social dominance from differences in reproductive status is difficult. Female *A. burtoni* provide an excellent opportunity to dissect the mechanisms of social status from reproductive state, as females will also form dominance hierarchies in the absence of males, but both DOM and SUB females are reproductively active (Renn et al., 2012). By comparing DOM and SUB males and females, we have a unique opportunity to determine to which extent the neuroendocrine underpinnings of behavior within a social hierarchy are due to either reproductive state or social status.

In order to function in a social community that is based on a dominance hierarchy, individuals must integrate external social information

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with internal physiology into an appropriate behavioral response to conspecific cues. Steroid hormones and their receptors are crucial in this process, as steroid hormones relay acute social information as well as reproductive status. The influence of social status on steroid hormone levels has been extensively studied in *A. burtoni*, as DOM males have higher levels of androgens and 17β-estradiol compared to SUB males (O'Connell and Hofmann, 2011, 2012, O'Connell and Hofmann, 2012). Furthermore, gonadal hormones seem to mediate distinct components of male social behavior, as manipulation of the estrogen receptors (ERs) alters aggression in both DOM and SUB males, whereas manipulation of the androgen and progestin receptors (AR and PR, respectively) alters courtship behavior in DOM males only (O'Connell and Hofmann, 2012). In SUB males, PR seems to mediate social cognition, as PR antagonists decrease anxiety-related behavior in response to threatening DOM males (O'Connell and Hofmann, 2012). Importantly, hormones can play very dynamic roles, as androgens and estrogens rise in SUBs within 30 min of being given the opportunity to transition to DOM status (Maruska and Fernald, 2010a, 2010b; Huffman et al., 2012; Maruska et al., 2013). Even more striking is that brain gene expression of steroid hormone receptors can change within 30 min of providing *A. burtoni* males with an opportunity to ascend in social status (Maruska and Fernald, 2010b). DOM *A. burtoni* females also have higher androgen levels compared to SUB females (Renn et al., 2012), although this difference is not as pronounced as in males. Despite a great deal of work on hormonal differences between male phenotypes, little is known about where and how sex steroid receptors act to mediate social dominance behavior in the brain. An understanding of how sex steroids mediate social dominance behavior in female *A. burtoni* is also lacking.

Our goal in the design of the present study was to test the hypothesis that sex steroid hormones and their receptors regulate some aspects of social dominance behavior independent of reproductive state and/or sex. Specifically, we hypothesized based on results from our previously published studies described above that androgens would influence social dominance behavior in *A. burtoni* individuals regardless of sex, whereas estrogens and progestins would influence sex-specific behaviors. We quantified mRNA abundance of the androgen receptors (ARα and ARβ), estrogen receptor α (ERα), and the progesterone receptor (PR) using quantitative radioactive in situ hybridization. Our quantification efforts focused on five core brain regions involved in social behavior (O'Connell and Hofmann, 2011; putative mammalian homologs are in parentheses, although note that some of these homologies are tentative and not necessarily one-to-one): the ventral part of the ventral telencephalon (Vv, lateral septum-like), 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/substantia nigra). These regions are important in evaluating the salience of an external stimulus as well as regulating sexual behavior and aggression across vertebrates (Goodson, 2005; O'Connell and Hofmann, 2011).

Methods

Study organism and behavior

A. burtoni males and females descended from a wild-caught stock population were kept in aquaria as described previously (Munchrath and Hofmann, 2010). DOM and SUB males were randomly selected for observation within mixed sex communities containing 8 males and 8 females in sixteen 110 liter tanks. All focal males had been in their respective social states for at least two weeks prior to observation. Sixteen additional 110 liter tanks were populated with 12 females per tank with no males present to stimulate the formation of female dominance hierarchies as previously described (Renn et al., 2012). All focal females were stable in their social status for at least one week prior to observation. Each male in the mixed sex tanks and all females in the female-only tanks were marked with a colored bead attached to a plastic tag inserted just below the dorsal fin, allowing identification of individual animals. Gravel substrate and five terracotta shelters were also placed in each tank to provide the substrate that facilitates the establishment and maintenance of territories. In both mixed sex and female-only communities there were usually two to four DOM individuals, while the remaining animals were of SUB status. DOMs were identified as aggressively defending a territory within the tank and presence of a dark lachrymal stripe (eye bar) across the head, which is characteristic of territory holders. SUBs were identified by absence of a territory, schooling with females in the tank, fleeing from DOMs, dull coloration and lack of an eye bar. There were no observable differences in status within SUB individuals (high-ranking SUBs versus low ranking SUBs), as SUBs rarely display aggressive behavior. Every effort was made to minimize pain or discomfort of the animals and all work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

One DOM (males: $n = 24$; body mass (BM) $= 6.45 \pm 0.92$ g, standard length (SL) $= 5.98 \pm 0.31$ cm; females: $n = 16$; BM: 3.20 ± 0.18 g; SL: 4.91 ± 0.10 cm) and one SUB (males: $n = 24$; BM $= 5.44 \pm 1.01$ g, SL $= 5.7 \pm 0.37$ cm; females: $n = 16$; BM: 2.30 ± 0.10 g; SL: 4.51 ± 0.07 cm) individual from the same community were observed between 09:00 and 11:00 h for 5 min each on three days for one week. As expected, DOM males were larger both in length ($t_{46} = 3.201$, $p = 0.002$) and mass ($t_{46} = 3.637$, $p = 0.003$) compared to SUB males. Similarly, DOM females were larger than SUB females (standard length: $t_{30} = 3.259$, $p = 0.003$; mass: $t_{30} = 4.264$, $p = 1.84 \times 10^{-4}$). A single observer quantified the behavior of DOM and SUB dyads within each community tank. Aggressive (chases, bites, threats, border disputes), sexual (quivers, leads), and fleeing behavior patterns were observed as described in Fernald (1976). On the last day of behavioral observations, weight and length of each focal individual were recorded and blood was drawn from the dorsal aorta for hormone assays. The gonadosomatic index (GSI) was calculated as the ratio of gonad weight to body weight multiplied by 100. Ovaries were fixed in Bouin's solution for ovarian histology (see supplementary methods). Brains were rapidly dissected and fresh frozen for *in situ* hybridization ($n = 16$ male dyads; $n = 8$ female dyads). To avoid the potentially confounding effects of ovarian stage, brain analyses were carried out on a subset of females ($n = 8$ per social status) where the DOM and SUB dyads had similar GSI.

Hormone assays

Free (bioavailable rather than total) circulating testosterone, 17β-estradiol, and progesterone were measured for most individuals using ELISA (Enzo Life Sciences, NY, USA) where inter and intra-assay variations were 5.19% and 3.14%, 3.18% and 4.25%, and 2.94% and 4.41%, respectively. Plasma samples were diluted 1:30 and processed as in Kidd et al. (2010) and according to manufacturer's instructions. As *A. burtoni* are small cichlids, the amount of blood plasma isolated from each individual was sufficient to only measure three hormones in most individuals. We chose to measure testosterone rather than 11-ketotestosterone, as testosterone levels are consistently a magnitude higher than 11-ketotestosterone levels in this species (Trainor and Hofmann, 2006; Maruska et al., 2013; O'Connell et al., 2013). More generally, our recent comparative analyses suggest that testosterone is the active androgen in haplochromine cichlids (Dijkstra et al., 2012).

Radioactive in situ hybridization (ISH)

Brains ($n = 16$ males per social status and $n = 8$ females per social status) were fresh frozen in O.C.T. (Tissue-Tek) and stored at −80 °C until sectioning at 20 μm into four series. For male dyads, one set of brains ($n = 8$ per social status) was used to quantify expression of

ARα, ARβ, and PR, while a series from the remaining brains was used to quantify expression of ERα, which accounts for variation in sample size between males and females. For female dyads, the four series were used to measure expression of ARα, ARβ, ERα, and PR within the same individuals used in the descriptive behavior experiments.

Slides were then taken from −80 °C, fixed in 4% formaldehyde and treated with acetic anhydride as previously described in Munchrath and Hofmann (2010) and Huffman et al. (2013). Slides were incubated at 65 °C for 18 h in 200 μl of hybridization buffer containing 2.0×10^6 cpm of 35-S labeled riboprobe prepared by reverse transcription of templates described previously (Munchrath and Hofmann, 2010). The probes were 646, 516, 788, and 359 base pairs in length for ARα (Genbank AF121257), ARβ (Genbank AY082342), ERα (Genbank AY422089), and PR (Genbank FJ605735), respectively. Probes were purified using NucAway spin columns (Ambion, TX, USA). Control slides were incubated with 2.0×10^6 cpm of sense probe. After hybridization, slides were placed in 65 °C 4× SSC + 1 mM DTT for 5 min to remove coverslips. Slides were then washed once in 65 °C 4× SSC + 1 mM DTT for 1 h, washed twice in 65 °C 50% formamide + 2× SSC + 1 mM DTT for 1 h, washed twice in 65 °C 0.1× SSC + 1 mM DTT for 10 min, and equilibrated to room temperature in 0.1× SSC + 1 mM DTT for 15 min. Next, slides were dehydrated in an ascending ethanol series and air dried overnight. Slides were dipped in Kodak NTM emulsion, dried at 55 °C for 1 h and then maintained in darkness at 4 °C. After 2–3 weeks, emulsion was developed for 4 min in Kodak developer at 15 °C, washed in chilled water for 15 s, fixed in Kodak fixer for 6 min and then washed in distilled water. Sections were counterstained with cresyl violet before dehydration, clearing in xylene and coverslipping in Permount (Fisher Scientific). Control slides showed no binding above background (see Figs. 4 and 5).

Slides were coded and processed by an observer blinded to treatment group. We quantified expression in the anterior tuberal nucleus (aTn; putative homolog of ventromedial hypothalamus); the periventricular part of the posterior tuberculum (TPp; putatively homologous to the mammalian substantia nigra/ventral tegmental area; O'Connell et al., 2013); the dorsal region of the ventral telencephalon (Vd; accumbens-like homolog), the ventral region of the ventral telencephalon (Vv; septal-like region) and the parvocellular, magnocellular, and gigantocellular regions of the preoptic area (pPOA, mPOA, and gPOA, respectively) as neuroanatomically defined in Munchrath and Hofmann (2010) and described in O'Connell and Hofmann (2011) (Fig. S1). POA cell groups were analyzed separately as their neurochemical properties differ between DOM and SUB males (Greenwood et al., 2008).

Images for ISH analysis 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. We modified the protocols of Burmeister et al. (2008) and Hoke et al. (2004). Briefly, 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 100× objective (Fig. S2): 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 tissue with no expression of the target gene (background image) to represent any background level of silver grains that can vary across the slide due to emulsion thickness. We used ImageJ (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. (2004). 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 was determined using ImageJ. 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. We did not obtain measures for some genes in some brain regions of some individuals due to tissue folding during sectioning.

Sex steroid hormone manipulation in females

Twenty-four 110 liter tanks were established as described above with 16 females per tank and no males. One non-gravid female was chosen per tank to serve as focal animals and were tested using a within-subject testing paradigm. Focal females ($n = 8$ per status per drug group) were given an intraperitoneal injection of mineral oil (vehicle) on two consecutive days to establish a baseline level of behavior, and then given a hormone injection for two days. One group of DOM and SUB females served as a control and received vehicle injections all four days. Injection paradigm and concentrations were based on a dose response curve established previously in males (O'Connell and Hofmann, 2012) are as follows: 0.4 μg/gbw (gram body weight) of 17β-estradiol (Steraloids, RI, USA); 0.15 μg/gbw of dihydroxytestosterone (DHT; Sigma); 0.125 μg/gbw of 17α, 20β-dihydroxyprogresterone (17α–20β-P; Sigma). The non-aromatizable DHT has a higher binding affinity to both ARα and ARβ than 11-ketotestosterone (11-KT), an important androgen in some fish species (Sperry and Thomas, 1999; Wells and Van Der Kraak, 2000). Similarly, 17α–20β-dihydroprogesterone (17α–20β-P), which cannot be converted readily into testosterone, has higher binding affinity to the teleost PR than progesterone (Pinter and Thomas, 1997). All steroids were dissolved in mineral oil. Focal animals were injected in the afternoon the day before observation to avoid fast-acting hormone mechanisms (Remage-Healey and Bass, 2006). The animals were immediately put back in their home tank after injection, where they maintained their social status. The behavior of each focal female was scored during five-minute observations between 09:00 and 11:00 h each day by an observer blind to treatment.

Data analysis

All statistical analyses to detect differences in behavior, hormone levels or brain gene expression between social states or sexes were conducted in PASW. Aggressive behavior directed to DOMs by DOMs was summed into a "territorial aggression" variable and included threat displays, border disputes, and carousels, as these three behaviors are only observed between DOM males (Baerends and Baerends-van Roon, 1950). The courtship variable included quivering and leading behavior directed towards females as well as bower digging. All data were log-transformed to satisfy the assumptions of ANOVA. To test for differences in behavior, hormones, and brain gene expression, a two-way ANOVA was used with behavior, hormone levels, or brain gene expression as the dependent variable and sex (male or female) and status (DOM or SUB) as the independent variables. When an interaction effect was significant (sex * status $p < 0.05$), we conducted an ANOVA with a Bonferroni post hoc test to determine between group differences using behavior or gene expression as the dependent variable and group (DOM males, DOM females, SUB males, SUB females) as the independent variable. In cases where we were interested only in comparing status differences within a sex, we used two independent t-tests for males and females with hormone levels or gonadosomatic index as the dependent variable and status as the independent variable. To account for multiple hypothesis testing, a Benjamini–Hochberg false discovery rate correction was applied within each brain region. For the behavioral pharmacology experiment in females, a generalized estimating equation (GEE) model was used, using the behavioral measure as the dependent variable, the drug as an independent variable, and the baseline behavior compared to drug treatment as a within-subject variable. These GEE analyses in DOM and SUB females were conducted separately. To account for multiple hypothesis testing, a Benjamini–Hochberg false discovery rate correction was applied to test for effects of each drug within each behavior. Finally, we tested for correlations using the non-parametric

Spearman statistics for a subset of data followed by a Benjamini–Hochberg false discovery rate correction for multiple hypothesis testing.

Results

Social dominance behavior, physiology, and hormones

We found that the differences between DOM and SUB females in behavior generally mirrored that of DOM and SUB males, although with a lower magnitude (Fig. 1). Chases towards SUBs by DOMs in the community were performed more by DOMs regardless of sex (2-way ANOVA: status: $F(1,76) = 858$, $p = 3.74 \times 10^{-43}$; sex: $F(1,76) = 8.33$, $p = 0.005$; status $*$ sex: $F(1,76) = 20.8$, $p = 1.89 \times 10^{-5}$). Territorial aggression displayed by DOMs to other DOMs were also higher in DOMs regardless of sex (2-way ANOVA: status: $F(1,76) = 147$, $p = 1.80 \times 10^{-19}$; sex: $F(1,76) = 4.11$, $p = 0.046$; status $*$ sex: $F(1,76) = 4.11$, $p = 0.046$). Courtship was only observed in DOM males, not DOM females (2-way ANOVA: status: $F(1,70) = 80.8$, $p = 2.74 \times 10^{-3}$; sex: $F(1,70) = 79.4$, $p = 3.82 \times 10^{-13}$; status $*$ sex: $F(1,70) = 71.2$, $p = 2.88 \times 10^{-12}$). SUB individuals, on the other hand, displayed more fleeing behavior regardless of sex (2-way ANOVA: status: $F(1,76) = 585$, $p = 1.97 \times 10^{-37}$; sex: $F(1,76) = 2.40$, $p = 0.125$; status $*$ sex: $F(1,76) = 5.64$, $p = 0.020$).

In order to better understand how sex steroids contribute to status and sex differences in behavior, we measured circulating levels of testosterone, 17β-estradiol, and progesterone. DOM individuals had higher levels of testosterone than SUBs regardless of sex, while males have higher levels of free testosterone compared to females (Fig. 2A; 2-way ANOVA: status: $F(1,75) = 49.6$, $p = 7.65 \times 10^{-10}$; sex: $F(1,75) = 301$, $p = 5.71 \times 10^{-28}$; status $*$ sex: $F(1,75) = 25.8$, $p = 2.73 \times 10^{-6}$). Specifically, DOM males have higher testosterone compared SUB males ($t_{28} = 8.02$, $p = 9.86 \times 10^{-9}$) and DOM females have higher testosterone compared to SUB females ($t_{29} = 2.39$, $p = 0.023$). We found a similar pattern in circulating 17β-estradiol levels where DOMs had higher 17β-estradiol levels compared to SUBs and males have higher 17β-estradiol levels compared to females (Fig. 2B; 2-way ANOVA: status: $F(1,61) = 36.6$, $p = 9.56 \times 10^{-8}$; sex: $F(1,61) = 337.5$, $p = 1.52 \times 10^{-26}$; status $*$ sex: $F(1,61) = 8.96$, $p = 0.004$). Specifically, DOM males have higher 17β-estradiol levels compared to SUB males ($t_{39} = 8.02$, $p = 8.91 \times 10^{-10}$) although DOM females do not differ from SUB females in 17β-estradiol levels ($t_{22} = 1.73$, $p = 0.098$). Progesterone levels were elevated in DOMs compared to SUBs, although there was no significant sex difference (Fig. 2C; 2-way ANOVA: status: $F(1,54) = 13.5$, $p = 0.001$; sex: $F(1,54) = 2.30$, $p = 0.135$; status $*$ sex: $F(1,54) = 2.13$, $p = 0.150$).

Relative gonad size (gonadosomatic index, GSI) also varied with sex and social status (Fig. 2D; 2-way ANOVA: status: $F(1,76) = 19.9$, $p = 2.82 \times 10^{-5}$; sex: $F(1,76) = 25.9$, $p = 2.57 \times 10^{-6}$; status $*$ sex: $F(1,76) = 4.49$, $p = 0.037$). DOM males had larger GSI than SUB males ($t_{26} = 5.51$, $p = 1.58 \times 10^{-6}$) and DOM females had larger GSI than SUB females ($t_{30} = 2.76$, $p = 0.01$). As we wanted to control for differences in ovarian state in our downstream analyses of gene expression relating to social dominance, we selected female DOM and SUB dyads ($n = 8$) with equivalent GSI ($t_7 = 1.301$, $p = 0.235$) for histological analysis of ovarian stage and neural gene expression. However, when we then examined egg stage in these GSI-matched females, we found the ovaries of DOM females to contain ova that were on average more mature (i.e., of greater diameter) compared to those of SUB females ($t_7 = 2.569$, $p = 0.037$; Fig. S3). Within this subset of females, ovarian stage positively correlated with progesterone (Spearman's rho $= 0.618$, $p = 0.019$), testosterone (Spearman's rho $= 0.593$, $p = 0.020$), and territorial aggression (Spearman's rho $= 0.540$, $p = 0.031$), and there was a negative correlation between ovarian stage and fleeing behavior (Spearman's rho $= -0.687$, $p = 0.003$).

Steroid modulation of social dominance behavior in females

We have previously described the functional role of sex steroid hormone receptors in mediating aggressive and sexual behavior in the context of social dominance in *A. burtoni* males (O'Connell and Hofmann, 2012). In order to determine if sex steroid hormones regulate social dominance in *A. burtoni* females in a similar manner, we used a within-subject design and treated DOM and SUB females with 17β-estradiol, DHT, or 17α–20β-P, and measured changes in aggression, sexual behavior, and fleeing behavior (Fig. 3, Table S1). We only found treatment effects in courtship behavior, which is sometimes displayed by DOM females (although at a much lower rate than seen in males; see Fig. 1C). Courtship behavior in DOM females was increased with 17α–20β-P treatment (GEE, $p = 3.05 \times 10^{-5}$, Wald $\chi^2 = 17.381$), but not with other manipulations (Table S1).

Expression of nuclear sex steroid receptors in the brain

To determine how the behavioral regulation of social status by sex steroid hormones is dependent on sex, we measured expression of androgen receptors (ARα and ARβ), estrogen receptor α (ERα), and the progesterone receptor (PR). We did not measure expression of ERβa

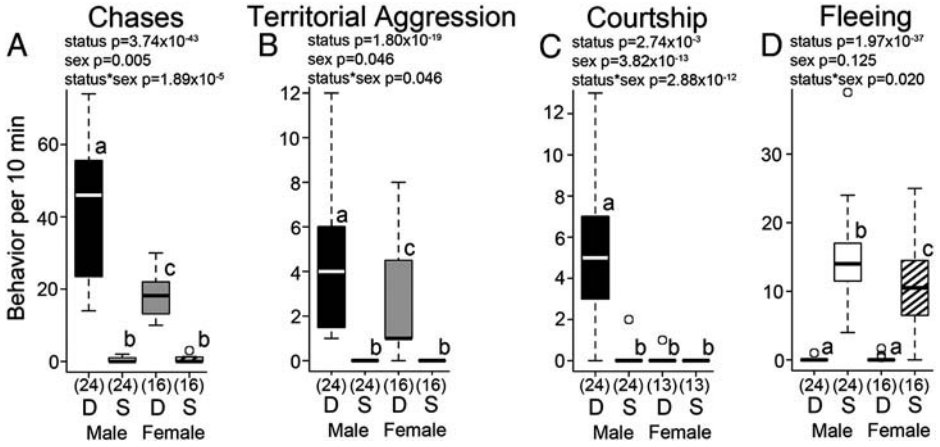

Fig. 1. Social dominance behavior in *A. burtoni* males and females. Box and whisker plots show the first and third quartiles (boxes), the median (line within each box), the minimum and maximum values (whiskers), and outlier values (circles beyond the whiskers) of social behavior between dominant (D) and subordinate (S) males and females. Behavioral measures include (A) chases, (B) territorial aggression, (C) courtship, and (D) fleeing. p-Values from a two-way ANOVA are shown above each graph. A Bonferroni post hoc test was used to determine between group differences if the interaction of sex $*$ status was significant; groups not joined by the same letter are significantly different.

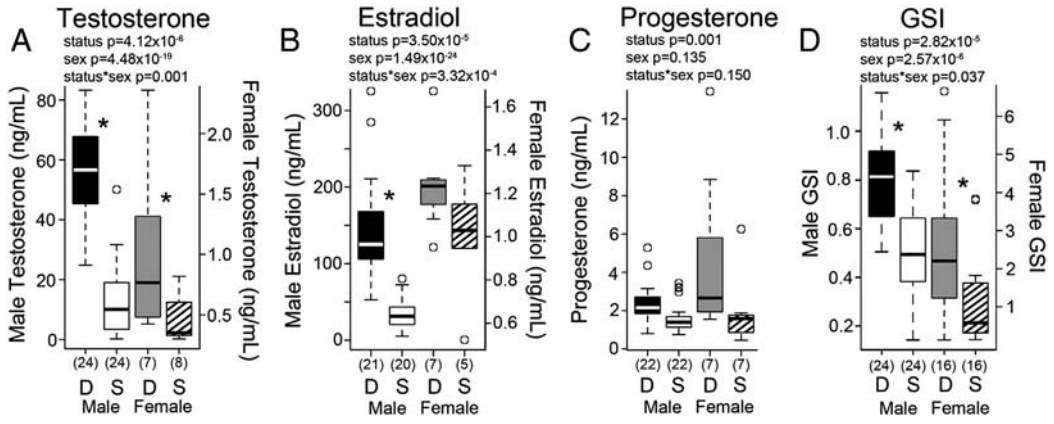

Fig. 2. Sex steroid hormone levels and gonadal physiology. Box and whisker plots show circulating levels of (A) testosterone, (B) 17β-estradiol, (C) progesterone and also (D) gonadosomatic index (GSI) measures between dominant (D) and subordinate (S) males and females. For testosterone, 17β-estradiol, and GSI graphs, values from females are plotted on the secondary axis. p-Values from a two-way ANOVA are shown above each graph. A Bonferroni post hoc test was used to determine between group differences if the interaction of sex * status was significant; groups not joined by the same letter are significantly different.

or ERβb, as tissue was limited and we focused on ERα due to its well-established role in mediating aggression (Tetel and Pfaff, 2010).

Androgen receptors

We found significant differences in ARα expression only in the pPOA (Figs. 4A–E), where males had higher ARα expression compared to females (2-way ANOVA: $F(1,28) = 4.199$, $p = 0.050$), but there was no significant effect of status or interaction of status and sex (2-way ANOVA: status, $F(1,28) = 0$, $p = 0.985$; status * sex, $F(1,28) = 0.176$, $p = 0.678$). ARα pPOA expression was positively correlated with testosterone levels across all individuals (Spearman's rho = 0.396, $p = 0.027$). We did not find differences in sex or status in ARα expression in Vv, Vd, mPOA, gPOA, aTn, or TPp (Table S2).

ARβ expression presented a very different pattern compared to ARα (Figs. 4F–J). Females have higher ARβ expression compared to males in Vd (2-way ANOVA: status, $F(1,26) = 0.939$, $p = 0.006$; sex, $F(1,26) = 4.212$, $p = 0.050$; status * sex, $F(1,26) = 0.007$, $p = 0.933$) and mPOA (2-way ANOVA: status, $F(1,24) = 0.240$, $p = 0.629$; sex, $F(1,24) = 5.838$, $p = 0.024$; status * sex, $F(1,24) = 0.407$, $p = 0.530$). Females also have higher ARβ expression in the pPOA compared to males, while DOMs (regardless of sex) have elevated ARβ pPOA expression compared to SUBs, although there is no interaction effect of sex and status with ARβ pPOA expression (2-way ANOVA: status, $F(1,27) = 4.390$, $p = 0.046$; sex, $F(1,27) = 9.720$, $p = 0.004$; status * sex, $F(1,27) = 2.804$, $p = 0.106$). We found no differences in ARβ expression between sex and status in Vv, aTn, gPOA, or the TPp (Table S2).

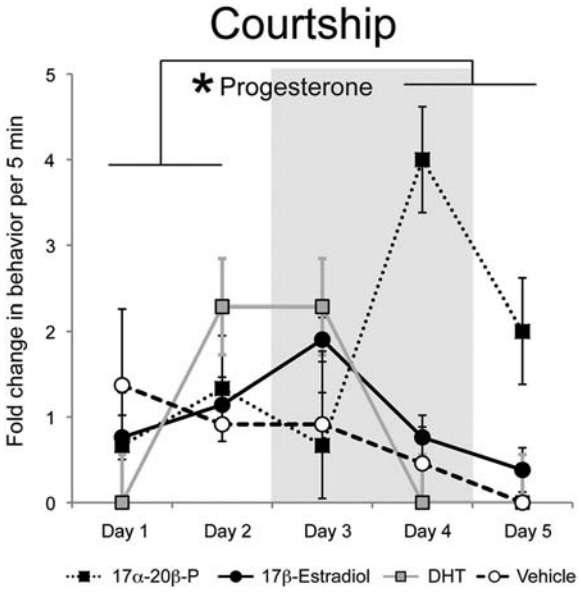

Fig. 3. Progesterone treatment increases courtship behavior in DOM females. Y-axis represents the change in behavior relative to average baseline behavior. Days with hormone manipulation are represented with gray background shading. Dots represent mean fold change in behavior per 5 minute observation period, error bars are ±SE; n = 8 for each group of females; GEE *p < 0.05; ns, no significance.

Estrogen receptor α

We found a sex difference in ERα expression only in the pPOA (Figs. 5A–C) where males had higher ERα expression than females (2-way ANOVA: status, $F(1,27) = 2.268$, $p = 0.144$; sex, $F(1,27) = 4.899$, $p = 0.036$; status * sex, $F(1,27) = 1.202$, $p = 0.283$). We found no other differences between status and sex of ERα expression in Vv, Vd, mPOA, gPOA, aTn, or the TPp (Table S3). Interestingly, ERα pPOA expression in DOM males was correlated positively with territorial aggression (Spearman's rho = 0.901, $p = 0.002$) and negatively with chases (Spearman's rho = −0.781, $p = 0.022$), but no significant correlations between ERα pPOA expression and behavior was found in DOM females.

Progesterone receptor

In contrast to the central role of the POA in ERα and AR expression, PR exhibited a different pattern of sex and status differences (Figs. 5D–G). Females had elevated expression of PR in the aTn compared to males (2-way ANOVA: status, $F(1,27) = 0.004$, $p = 0.949$; sex, $F(1,27) = 6.934$, $p = 0.014$; status * sex, $F(1,27) = 0.914$, $p = 0.347$) (Fig. 5B). We also found an interaction effect in PR expression between sex and status in Vd, although neither sex nor status was significant as main effects (2-way ANOVA: status, $F(1,25) = 0.969$, $p = 0.334$; sex, $F(1,25) = 0.563$, $p = 0.460$; status * sex, $F(1,25) = 5.700$, $p = 0.025$). However, PR expression in Vd was positively correlated with fleeing in SUB females (Spearman's rho = 0.762, $p = 0.028$). PR expression did not differ between status and sex in Vv, pPOA, mPOA, gPOA, or the TPp (Table S4).

Discussion

Disentangling sex and status in social dominance

We found differences and similarities in how social behavior is influenced by sex steroid hormones in DOM and SUB male and female

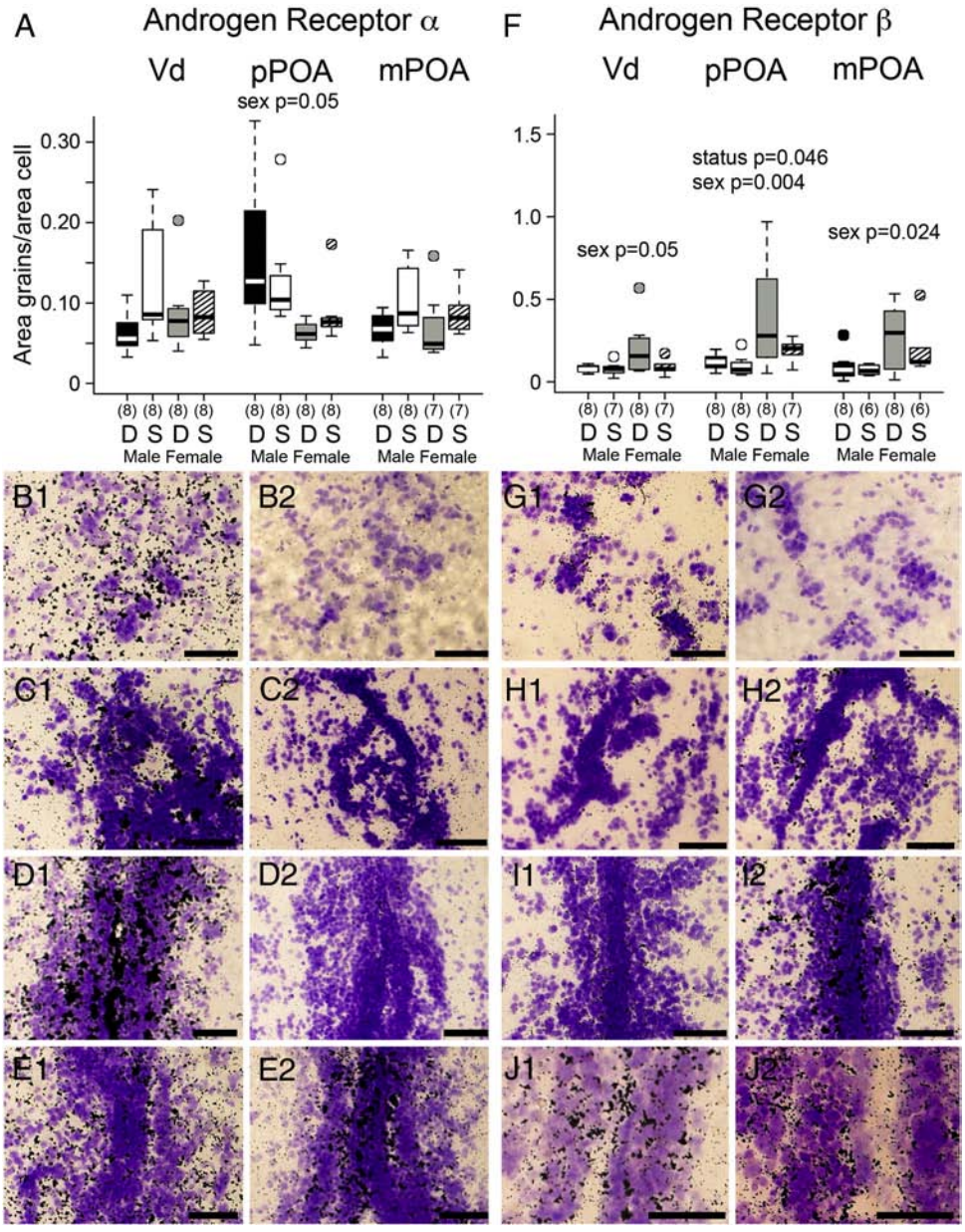

Fig. 4. Differential expression of androgen receptors in the brain. Box and whisker plots indicate androgen receptor α (A, ARα) and androgen receptor β (F, ARβ) expression in dominant (D) and subordinate (S) males and females in the dorsal part of the ventral telencephalon (Vd), parvocellular preoptic area (pPOA), and magnocellular POA (mPOA). Statistics are presented from a 2-way ANOVA if significant ($p < 0.05$). Examples of antisense and sense (control) probes in the anterior tuberal nucleus show probe specificity for ARα (B1, antisense; B2, sense) and ARβ (G1, antisense; G2, sense). For ARα expression, example micrographs are shown for Vd (C1, DOM male; C2, SUB male), pPOA (D1, DOM male; D2, DOM female), and mPOA (E1, DOM male; E2, SUB male). For ARβ expression, example micrographs are shown for Vd (H1, DOM male; H2, DOM female), pPOA (I1, DOM male; I2, DOM female), and mPOA (J1, DOM male; J2, DOM female). Scale bar is 50 μm.

A. burtoni (Fig. 6). DOM and SUB females perform behavior similar to their male counterparts as previously described (Renn et al., 2012) with the exception that DOM females do not frequently display male-typical courtship behavior. Male and females also had similar trends in circulating sex steroid levels, although males have much higher levels of testosterone and 17β-estradiol compared to females, which have also been reported by comparing males with naturally cycling females (Maruska and Fernald, 2010b). Here we show that DOM females also have elevated androgens compared to SUB females, which have previously been reported (Renn et al., 2012), although the magnitude of the status difference in females is lower compared to status differences of hormone levels in males. Moreover, although DOM females have higher androgen levels compared to SUB females, androgen levels in both DOM and SUB females are still relatively low compared to SUB males. Clearly, the relationship between androgen levels and social dominance is complex. Although DOM and SUB males differed in 17β-estradiol and progesterone levels, and despite a significant main effect of social status on hormone levels (Figs. 2 and 6), we did not find significant differences in 17β-estradiol and progesterone levels when comparing DOM and SUB females. Although overall behavioral patterns and hormone levels had similar trends between DOM and SUB status in males and females, there are some distinct differences in how males and females respond to hormone manipulations.

Our work suggests that progesterone regulation of courtship behavior represents a common mechanism of social dominance independent of sex (Fig. 6, O'Connell and Hofmann, 2012). Male-typical sexual behavior is facilitated by progesterone in male mammals, reptiles, and in *A. burtoni* DOM males (Grassman and Crews, 1986; Phelps et al., 1996; O'Connell and Hofmann, 2012). Similarly, we found that treatment with a progesterone receptor agonist also increased

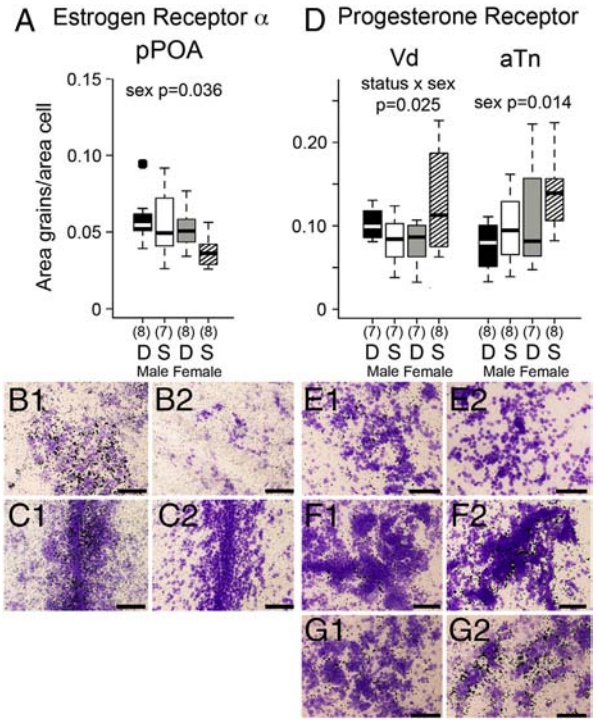

Fig. 5. Differential expression of estrogen receptor α and the progesterone receptor. Box and whisker plots indicate estrogen receptor α (ERα) expression (A) in the parvocellular POA (pPOA) and progesterone receptor (PR) expression (D) in the dorsal part of the ventral telencephalon (Vd) and the anterior tuberal nucleus (aTn) in dominant (D) and subordinate (S) males and females. Statistics are presented from a 2-way ANOVA if significant ($p < 0.05$). Examples of antisense and sense (control) probes in the aTn show probe specificity for ERα (B1, antisense; B2, sense) and PR (E1, antisense; E2, sense). For ERα expression, example micrographs are shown for the pPOA (C1, DOM male; C2, DOM female). For PR expression, example micrographs are shown for Vd (F1, SUB male; F2, SUB female) and the aTn (G1, DOM male; G2, DOM female). Scale bar is 50 μm.

courtship behavior in DOM *A. burtoni* females. In parthenogenetic whiptail lizards (*Cnemidophorus uniparens*), females also display male-typical sexual behavior, although this behavior is most often seen in post-ovulatory individuals when progesterone levels are high (reviewed in Crews, 2005). Similarly, our results indicate that *A. burtoni* females with larger ovaries and more mature ova are more likely to be dominant. This behavioral association with the ovarian cycle suggests a tight link between male-typical sexual behavior displayed by females and circulating hormone levels. Indeed in both whiptail lizards and DOM *A. burtoni* females, administration of progestins alone is sufficient to induce courtship behavior (Grassman and Crews, 1986; present study), suggesting a common mechanism for the display of male-typical courtship behavior by females. However, it is unclear where progesterone may be acting in the brain to facilitate male-typical sexual behavior as we did not observe any status differences in PR expression the brain regions we examined. It is possible that brain regions not examined here could be important sites for PR regulation of male-typical sexual behavior, or that other neuroendocrine gene products serve a co-activator role (Molenda-Figueira et al., 2006).

Unlike courtship behavior, endocrine regulation of aggression is driven by different mechanisms in *A. burtoni* males and females (Fig. 6), as ER mediates aggression in sex-specific ways where treatment with 17β-estradiol increases aggression in DOM and SUB males (O'Connell and Hofmann, 2012), but has no significant effects in females (present study). This sex difference in estrogenic regulation of behavior is paralleled by studies with ERα knockout mice, where aggression compared to wild type animals is reduced in males but elevated in females (Ogawa et al., 1997, 1998). It is important to note, however, that the doses used for female hormone manipulation in this study were determined by a dose–response experiment in males (O'Connell and Hofmann, 2012), and therefore different results may have been seen with different steroid hormone doses in female *A. burtoni*. Nevertheless, our study presents important information regarding sex differences in the steroid-mediated regulation of social dominance.

Sex and status differences in brain sex steroid receptor expression

Expression of sex steroid receptors differed mostly between the sexes rather than between social states (Fig. 6), with the parvocellular POA as a central hub for sex differences in AR and ER expression. Males had elevated preoptic ARα and ERα expression while females had higher PR expression in the aTn as well as higher preoptic and Vd ARβ expression. This general lack of status differences in expression of sex steroid hormone receptors in discrete brain regions mirrors a previous study in which preoptic expression of sex steroid hormone receptors did not vary between DOM and SUB males (O'Connell and Hofmann, 2012). In a study comparing DOM and SUB males, Maruska et al. (2013) also found very few status differences in steroid hormone receptor expression using micropunches and quantitative PCR. However, Burmeister et al. (2007) described status differences in expression of sex steroid receptors in gross brain dissections, where DOM males have higher ARα and ARβ in the telencephalon. This discrepancy between studies highlights that gene expression measures from gross dissections may not reflect gene expression differences in discrete brain regions and that finer measurements are necessary for fully profiling expression differences throughout the brain.

Sex differences in expression of androgen receptors showed different trends depending on which paralog was measured, as males had increased ARα in the pPOA while females had increased ARβ in pPOA, mPOA, and Vd (Fig. 6). The POA is important in regulating sexual behavior in teleosts, as lesions decrease sexual behavior in male bluegill sunfish (*Lepomis macrochirus*, Demski and Knigge, 1971) and male and female sockeye salmon (*Oncorhynchus nerka*, Satou et al., 1984). We have found that manipulation of ARs mediates courtship behavior in DOM male *A. burtoni* (O'Connell and Hofmann, 2012), but not DOM females (present study). It may be the differential expression of the AR paralogs that mediates this sex difference in DOM responses to AR manipulation. Moreover our results suggest a neofunctionalization (i.e., divergence of function after gene duplication; Hahn, 2009) of androgen receptor paralogs in *A. burtoni* (Douard et al., 2008), although whether and how this might impact social behavior is not yet clear, as targeted manipulation of the different paralogs will be required.

The preoptic area is also important in mediating aggression in teleosts, as lesions to this region in male bluegill sunfish inhibit aggression (Demski and Knigge, 1971). Combining insights from our previous study in males (O'Connell and Hofmann, 2012) with the present study, it appears that estrogens increase aggression in *A. burtoni* males, but not females. Regardless of social state, we found that males have higher circulating 17β-estradiol levels as well as higher ERα expression in the pPOA compared to females, suggesting that elevated expression of ERα in this region may facilitate aggression in males. Although tissue limitations prevented us from including ERβa and ERβb in our analysis, Maruska et al. (2013) found little differences in ERβa and ERβb expression between DOM and SUB males. It would be interesting to examine sex differences in expression of these other estrogen receptors as well, as we would predict there would be more robust sex differences than status differences.

Behavioral effects of progesterone are not well understood in teleosts compared to androgens and estrogens. We found sex differences in PR expression in the aTn (putative ventromedial hypothalamus), similar to mammals (Scott et al., 2002; Wagner, 2008) and reptiles (Godwin and Crews, 1995). Studies in female rats utilizing antisense oligonucleotides have shown that PR in the ventromedial hypothalamus is necessary for lordosis behavior (Ogawa et al., 1994), and similarly, this same region is necessary for receptive behavior in female whiptail lizards (Kendrick

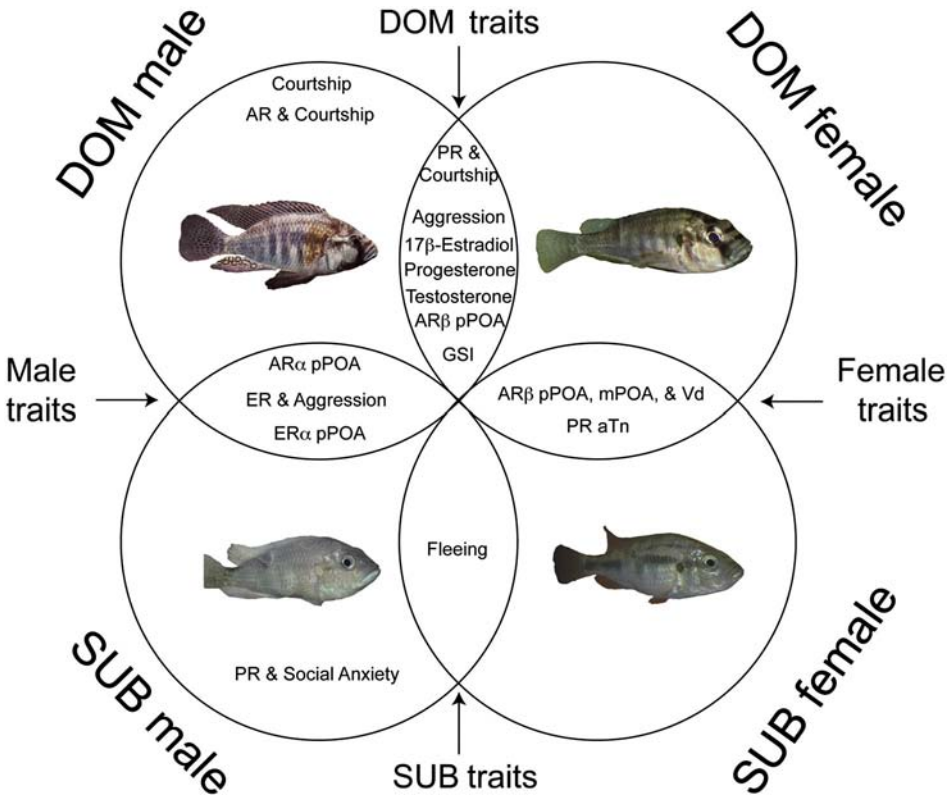

Fig. 6. Disentangling mechanisms of social dominance from sexual dimorphisms in steroid hormone systems. We summarize here patterns of hormonal regulation of social dominance behavior that are sex-specific or social status-specific according to two-way ANOVA. Gene expression is shown if it is highly expressed in that group. Abbreviations: AR, androgen receptor; aTn, anterior tuberal nucleus; ER, estrogen receptor; GSI, gonadosomatic index; mPOA, magnocellular preoptic area; pPOA, parvocellular preoptic area; PR, progesterone receptor; Vd, dorsal part of the ventral telencephalon.

et al., 1995). Given this conserved role of sex differences in ventromedial hypothalamus PR expression as well as a crucial role in female receptivity, we hypothesize that PR in the aTn in female *A. burtoni* contributes more to female reproductive behavior than to social status. We also found that PR expression differed in Vd (putative nucleus accumbens), although the role of the region in mediating social behavior in teleosts is not known. However previous work suggests that PR mediates social cognition in SUBs (O'Connell and Hofmann, 2012) and it would be interesting to determine where in the brain social cognition is mediated by progestins through colocalization of immediate early genes and PR.

In the present study, we have measured many variables (e.g., expression levels of multiple genes in numerous brain regions in several phenotypes), requiring adjustment for false positives to make it less likely for possibly spurious results to be reported as statistically significant. Yet integrative studies such as ours allow us to detect more general patterns across many comparisons that might give rise to novel and testable hypotheses. Specifically, even though ERα and PR expression levels in DOM females appeared to be masculinized, none of these effects were significant after adjusting for multiple hypothesis testing. Interestingly, in female guinea pigs the social environment experienced early in life can indeed result in masculinized behavior as well as brain expression of AR and ERα (Kaiser et al., 2003). Given that we have shown here that behavior can indeed be masculinized in *A. burtoni* females, and considering the remarkable plasticity of social dominance behavior in this system, we have thus a unique opportunity to test in future studies whether the social environment experienced during adulthood can also masculinize (defeminize) brain gene expression patterns.

Conclusions

We have utilized the plastic social dominance phenotypes of male and female *A. burtoni* to disentangle reproductive states from social status in order to learn the neuroendocrine mechanisms of social dominance behavior. Contrary to our predictions, we have found that androgens and estrogens modulate social dominance behavior differently in males and females, while progesterone promotes courtship behavior regardless of sex. Our analyses of sex differences in steroid receptor expression in the brain point to a centralized role for the parvocellular POA in ER and AR expression, while sex differences in PR expression are found in aTn (putative ventromedial hypothalamus) and Vd (putative nucleus accumbens).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2013.07.003.

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