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Molecular Mechanisms of Phenotypic Plasticity in *Astatotilapia burtoni*

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MOLECULAR MECHANISMS OF PHENOTYPIC PLASTICITY IN *ASTATOTILAPIA BURTONI*

by

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Dedication

For Blake and Emma, without whom I would be lost. To God be the glory.

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Molecular Mechanisms of Phenotypic Plasticity in *Astatotilapia burtoni*

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The ability of an animal to respond and adapt to stimuli is necessary for its survival and involves plasticity and coordination of multiple levels of biological organization, including behavior, tissue organization, hormones, and gene expression. Each of these levels of response is complex, and none of them responds to stimuli in isolation. Thus, to understand how each system responds, it is necessary to consider its role in the context of the entire organism. Here, I have used the African cichlid fish *Astatotilapia burtoni* and its extraordinary phenotypic plasticity to investigate how animals respond to a change in social status from subordinate to dominant and attempted to integrate these multiple levels of biological response, as well as the roles of several candidate neuromodulators,. First, I have described how male *A. burtoni* become more aggressive and reproductive during their transition to dominance as well as increasing circulating levels of testosterone and estradiol and the histological organization of their testes. I then mapped the distribution of expression of two behaviorally relevant neuropeptides, arginine vasotocin and isotocin, and their respective receptors, throughout the *A. burtoni* brain, and found that they were highly expressed in several brain areas important for social behavior and decision-making. I then investigated the role of arginine vasotocin in social status and behavior via pharmacological manipulation and qPCR, showing the importance of arginine vasotocin in controlling the transition to

dominance. Lastly, I investigated the role of aromatase, testosterone, and estradiol in male *A. burtoni*, both in stable dominant males and in males as they transition to dominance, using pharmacological manipulation and quantitative radioactive *in situ* hybridization, illustrating that estradiol synthesis during dominance is dependent on aromatase activity and necessary for aggressive behavior.

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Chapter 1: Introduction

Animals must constantly respond to their dynamic environments, making decisions that integrate multiple organ systems and coordinate levels of biological organization, from molecular changes to behavioral output (Nelson, 2005). There are decades of research on how sensory input is received by the brain, the hormones and genes that change in response, the brain areas involved, and the motor outputs that are produced. The majority of this research has been performed using stimulus-response paradigms to isolate the response being studied, which has led to significant insights in the relationship between the body (both as a receiver of signals and a transmitter) and the brain. As informative as these reductionist studies are, it is essential that we take our findings and confirm that they hold true in more complex, naturalistic settings. Here, I will investigate the integrative response of a highly plastic teleost fish, *Astatotilapia burtoni*, to complex social environments using various molecular, histological, and behavioral techniques. Specifically, I will describe the involvement of sex steroid hormones and arginine vasotocin in males as they transition from socially subordinate to dominant, exploring the roles of these neuromodulators at the transcriptional, hormonal, physiological, and behavioral levels.

Teleost fish are excellent model systems for studying dynamic responses to social environments, as individuals are highly plastic and have complex social systems (Barlow, 2002; Helfman et al., 2009). Numerous species of teleosts change sex, social status and/or display multiple reproductive tactics and dramatically different phenotypes within sexes, as well as a variety of mating systems represented in closely related taxa (Gross, 1984; Turner, 1993). Many of these species are amenable to experimentation in the lab, providing opportunities for extensive behavioral experiments, repeated sampling, and

molecular studies. As many of the genes and hormones that are studied in social decision-making are highly conserved across taxa, the neuroendocrinological questions that have been approached reductionistically in other animals can be addressed as part of an integrated system in teleosts. Their plasticity allows for within-species and within-individual comparisons across phenotypes and sexes, making them powerful models for socially relevant neuroendocrine modulators. For example, arginine vasopressin and arginine vasotocin, two neuropeptides that are produced largely in the brain of mammals and non-mammalian vertebrates, respectively, have been studied and synthesized since the 1950's (Katsoyannis and du Vigneaud, 1958). They were originally investigated as osmoregulators and in the late 1970's were identified as potent neuromodulators (Pavel, 1978). For twenty years or so after this discovery, many landmark studies were performed, largely in amphibians, describing the role of arginine vasotocin in simple social behaviors, mostly in a reproductive context (Moore and Miller, 1982; Propper and Dixon, 1997). However, in the last fifteen years, there has been a sudden broadening of our understanding of the role of these neuropeptides on aggressive and reproductive social behavior with studies on various teleost fish, including the weakly electric fish (Bastian et al., 2001), bluehead wrasse (Godwin et al., 2000), plainfin midshipman (Goodson and Bass, 2000), goldfish (Thompson et al., 2008), and convict cichlids (Oldfield and Hofmann, 2011). These studies have taken advantage of the multiple phenotypes and diversity of robust, quantifiable behaviors that these species elicit, allowing us a more complete understanding of the role of these neuropeptides in social behavior.

MODEL SYSTEM

The African cichlid fish *Astatotilapia burtoni* has emerged as a model system for behavior and endocrinology due to its remarkable plasticity and robust social behavior (Fernald, 2002; Hofmann, 2003). Male *A. burtoni* can be either socially dominant or subordinate and switch between statuses every 4-7 weeks (Hofmann et al., 1999). They have a polygynous lek mating system in which dominant males establish contiguously arrayed display territories that they aggressively defend against conspecific intruders and towards which they actively attract females. They are a mouth-brooding species, so the territories serve as breeding grounds for the females to lay their eggs and have them fertilized, at which time they carry them back to the shoal to incubate. Dominant males have a variety of displays they use for aggressive and reproductive interactions, including behavioral displays and color patterns that are under fine neural control. Subordinate males, on the other hand, are cryptically colored, non-aggressive, non-reproductive, and shoal with females. They have lower levels of testosterone than dominant males (Trainor and Hofmann, 2006) and allocate much of their time and energy to foraging. Once they have grown large enough, they descend from the shoal into the breeding grounds and compete for available territories or attempt to overthrow resident dominant males. This transition to dominance is a highly coordinated process that involves changes in gene expression in the brain and gonads, among other tissues, as well as changes in sex steroid hormone levels, gonad physiology, coloration, and behavior. Chapter 2 analyzes this transition in great detail and at high temporal resolution, as these multiple levels of biological organization are integrated to execute a massive (yet reversible!) phenotypic change.

NEUROPEPTIDES AND SOCIAL BEHAVIOR

Arginine vasotocin (AVT) is the oldest member of a group of nine-amino acid neuropeptides that have diverse roles in social behavior, including aggression and reproduction (for a review, see Goodson 2008). This group also includes arginine vasopressin (the mammalian homolog of AVT) and the isotocin/mesotocin/oxytocin family. It seems that all animals studied to date have at least one member each of the AVT family and the isotocin family of neuropeptides, and these have been highly structurally conserved throughout evolution, suggesting functional necessity. Regarding functional conservation, these neuropeptides are consistently involved in regulating social behavior across taxa, but their exact roles appear to be highly variable even between closely related species, phenotypes within species, and social contexts within individuals. Thus, despite the wealth of literature available on neuropeptides, conservation of function is still unclear, and it is difficult to predict their roles in a given model system. It is possible that the multitude of paradigms and behaviors that are examined across animals have simply prevented a functional pattern from emerging; hypotheses that unite this body of literature are an active area of investigation. It has been posited that these neuropeptides are necessary to determine the "valence" of stimuli (positive or negative) and subsequently respond appropriately (Goodson 2008); a more classic school of thought is that the AVT family is for "fight or flight", and the isotocin family serves a "tend and befriend" function (Taylor et al., 2000; Insel, 2010). Alternatively, the AVT family has historically been associated with males and male behavior, while the isotocin family has been associated with females. As "fighting" and "fleeing" are typically ascribed to males, and "tending" and "befriending" are typically associated with females, it is obvious that these hypotheses are not mutually exclusive nor are they comprehensive (as males obviously exhibit affiliative behavior, and females can be aggressive). Because

individual male *A. burtoni* can be both reproductive/aggressive and non-reproductive/submissive at various times in their life, they are an excellent model for elucidating the function of these neuropeptides in social behavior. To more fully understand where these peptides are acting in the brain and in what processes they may serve a role, we first mapped the distribution of these peptides as well as the behaviorally relevant AVT receptor (V1a2) and the isotocin receptor throughout the brain of *A. burtoni*. Chapter 3 describes the protein distributions for the peptides as well as both the mRNA and protein distributions for the receptors. Following this, Chapter 4 describes the functional role of AVT in subordinate and dominant *A. burtoni* males as well as males that are transitioning from subordinate to dominant.

SEX STEROID HORMONES AND SOCIAL BEHAVIOR

In addition to neuropeptides, sex steroid hormones play an essential role in regulating social behavior. Testosterone and estradiol are necessary in both males and females (though in different quantities) for appropriate decision making and behavior. Sex steroid hormones in the periphery are synthesized largely in the adrenals and gonads, and can also be synthesized to act locally in the brain (London et al., 2009); expression of the enzymes necessary for synthesis and the respective receptors are tightly regulated. After testosterone is synthesized, some of it is converted into estradiol via aromatase; thus, the activity of estradiol is largely regulated through the level of testosterone synthesis and the amount of aromatase and estradiol receptor expression. Although testosterone has historically been the primary sex steroid hormone associated with males, particularly with aggressive and reproductive behavior, there is abundant evidence that

estradiol also plays a major role in male behavior (Balthazart and Foidart, 1993; Forlano et al., 2006).

Interestingly, teleost fish express an unusually high amount of aromatase in the brain (Forlano et al., 2006) regardless of sex. In *A. burtoni*, dominant males express more aromatase in whole brain than subordinate males (Renn et al., 2008) although subordinate males express more aromatase in the pre-optic area (O'Connell et al., in prep), which integrates multiple sources of sensory input and relays information all over the brain to coordinate motor output (Newman, 1999). As males become dominant, both their testosterone and estradiol levels increase in the circulation (to levels that surpass baseline levels of *A. burtoni* females, Kidd et al., in prep), suggesting that aromatase expression (and thus estradiol synthesis) may change during the transition (see Chapter 2). To further investigate this, Chapter 5 describes the change in brain aromatase expression as male *A. burtoni* become dominant as well as the behavioral and hormonal effects of pharmacologically manipulating aromatase in dominant males.

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Chapter 2: Phenotypic Plasticity in an African cichlid fish, *Astatotilapia burtoni*

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INTRODUCTION

Across the animal kingdom, individuals encounter social stimuli to which they must respond appropriately on multiple biological levels, including gene expression, protein synthesis, steroid hormone synthesis, and behavior (O'Connell and Hofmann, 2011). These responses must often be rapid and require coordination across levels of biological organization to ensure survival. Although an extensive literature exists for diverse stimuli and taxa describing these responses, only recently has it become possible to examine them in an integrative manner. There is tremendous variation across species as to the specific stimulus conditions and responses, yet they often appear to be functionally equivalent in that we can classify behavioral responses as those to either social challenges or opportunities even across distantly related taxa (O'Connell and Hofmann, 2011; Robinson et al., 2005; Wilson 1975). The classical framework for studying relationships between endocrine responses and behavior has been the "challenge hypothesis" (Wingfield et al., 1990), which focuses on the androgen response of males in relation to aggressive encounters, mating system, and breeding season. This framework has been instrumental in elucidating these relationships and, more recently, has been expanded to include other types of social stimulation (Goymann et al., 2007; Hirschenhauser and Oliveira, 2006). In a recent synthesis, O'Connell and Hofmann (2011) posited that for a truly integrative understanding of social behavior and its evolution, challenges (e.g., defense of offspring, a territory, or some other resource) and opportunities (e.g., reproduction, parental care, or foraging) can serve as functional

metrics shared by all animals that facilitate comparative analyses of the proximate mechanisms.

Testosterone (T) is an androgenic hormone synthesized from cholesterol across vertebrates and is a key regulator of social behavior (Nelson 2005). T synthesis in males occurs mostly in the gonads, though not exclusively (Remage-Healey et al., 2008), and is regulated by binding of the pituitary gonadotropin luteinizing hormone (LH) to its receptor (luteinizing hormone receptor, LHR; Schulz et al., 2001) in the testes. After LHR is activated in the Leydig cells of the testes, cholesterol diffuses across the plasma membrane and is transported to androgen synthesis machinery inside the mitochondria by steroidogenic acute regulatory protein (StAR). StAR is a mitochondrial membrane protein, and its import of cholesterol is known to be the rate-limiting step in T synthesis (Jefcoate et al., 1992). In teleost fishes, T can further be converted to 11-ketotestosterone (11-KT), which appears to be the active androgen in many, though not all, teleosts (Idler et al., 1960; Kime, 1993; Borg, 1994; but see Kidd et al., 2010). Importantly, T can also be converted into estradiol (E) by the enzyme aromatase (Callard et al., 1978) either locally in the testes or in target tissues, as aromatase expression also occurs in the brain and other E target tissues (Balthazart and Ball 1998; Cornil et al., 2006; Callard et al., 1990). Further, teleosts appear to have much higher aromatase expression levels in the brain relative to other vertebrates (Forlano et al., 2001; Pasmanik and Callard, 1985). Although historically, androgens have been associated with male social behavior, E plays a major role in male aggressive and reproductive behavior as well (Cornil et al., 2006). In addition, E is necessary for the renewal of spermatagonial stem cells in male teleost fish (Schulz et al., 2009).

The East African cichlid fish, *Astatotilapia burtoni*, a highly social, polygamous mouthbrooder, has become an important model system in social neuroscience (Hofmann,

2003; Robinson et al., 2008). Males of this species are either dominant or subordinate (Hofmann, 2003). Dominant males are brightly colored, highly aggressive, territorial, and reproductively active. Conversely, subordinate males are cryptically colored similar to females, shoal with females, are non-aggressive, and do not breed. Depending on the social environment, subordinate males can transition to social dominance and dominant males often lose their social status, which indicates a remarkable degree of phenotypic plasticity (Hofmann, 2003; Maruska and Fernald, 2010). This transition from one social status to the other requires rapid and coordinated responses, including dramatic changes in the brain and gonads (Hofmann, 2003), although measurements of gonad size (via gonadosomatic index, GSI) have not been consistently different between the social (reproductive) statuses (Francis et al., 1993; Hofmann and Fernald, 2000; White et al., 2002; Maruska and Fernald, 2010; Burmeister et al., 2005). Thus, because the utility of GSI as a proxy for reproductive maturity is questionable, we investigate here several other avenues of assessing testis maturity.

Previous studies have shown that male *A. burtoni* begin showing aggressive and reproductive behaviors within 15 minutes after being provided with a vacant territory (Maruska and Fernald, 2010; Burmeister et al., 2005). Similarly, within 30 minutes of becoming dominant, circulating levels of 11-KT are found to increase (Maruska and Fernald, 2010) as well as induction of immediate early genes in the preoptic area (Burmeister et al., 2005) and up-regulation of a subunit of LH in the pituitary (Maruska and Fernald, 2011a). After 72 hours, expression of this LH subunit in the pituitary reaches dominant-like levels, and gonadal LHR and aromatase gene expression is up-regulated as well (Maruska and Fernald, 2011b). This extensive transition occurs naturally approximately every 4-7 weeks (Hofmann et al., 1999) and can be reliably replicated in the laboratory. The inherent phenotypic plasticity of this species makes it an

excellent model system for studying integrative responses to complex social environments.

In the present study, we conducted two experiments to investigate the response of subordinate *A. burtoni* males to an opportunity to ascend to dominance in a complex social community that included ongoing aggressive challenges (from neighboring dominant males) and reproductive opportunities (through the presence of gravid females). In Experiment 1, we confirmed and expanded upon the previous work of Burmeister et al. (2005) and Maruska and Fernald (2010, 2011a,b) by examining the behavior of transitioning *A. burtoni* for two weeks and repeatedly sampling their T and E levels using a non-invasive water method. In Experiment 2, we added several variables to the time course by not only quantifying their behavior and circulating androgen levels but also gonadal histology and testes expression of three genes involved in androgen synthesis at four time-points following transition (1, 2, 6, and 14 days). Although evidence for an increase in gonad size as males become dominant is not consistent in the literature, they are undoubtedly an integral part of the transition from being non-reproductive to reproductive and should be investigated more closely as indicators of reproductive capacity. To specifically assess the physiological capacity for androgen production in socially ascending males, we measured testes mRNA levels of StAR, LHR, and gonadal aromatase.

We expected that in this complex social setting, ascending males would rapidly increase circulating T levels as their aggressive behavior increases (Burmeister et al., 2005; Maruska and Fernald, 2010), which would support previous studies in *A. burtoni*. As StAR catalyzes the rate-limiting step in acute production of gonadal T, we expected *StAR* expression in the testes to increase. Because of the role of aromatase in converting T into E and the importance of E to male reproductive physiology and behavior, we

predicted the expression of gonadal *aromatase* as well as circulating E levels would increase and correlate with T levels. Due to the roles of steroid hormones in reproductive behavior and previous studies on transitioning *A. burtoni*, we expected that males would start displaying reproductive behaviors soon after increasing aggressive behavior and steroid hormone levels (Maruska and Fernald, 2010). Finally, we hypothesized that with LH as the functional link between brain and gonads, the expression of *LHR* would change during social transition (Maruska and Fernald, 2011a). By examining all of these levels of biological organization simultaneously, we can attempt to compose an integrative model of the response to a dynamic social environment.

MATERIALS AND METHODS

Animals

All animals used in this study were adult *A. burtoni* males (3.9-6.8 cm in standard length) from a laboratory stock, which was originally derived from a wild population in Lake Tanganyika, Africa (Fernald and Hirata, 1977). Fish were maintained at 28°C on a 12:12 hour light/dark cycle with 10 min dawn and dusk periods to mimic their native tropical environment in 110 liter 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.

Behavioral paradigm

For both Experiments 1 and 2, we used a repeated measures design (Figure 1B) to track individuals as they transitioned from subordinate to dominant, employing a modified paradigm adapted from Burmeister et al. (2005) in which 110 liter aquaria were divided into three compartments using clear, perforated acrylic barriers (Figure 1A). These barriers allowed visual and olfactory communication between compartments while preventing physical contact. Each compartment contained two males and three females (i.e., six males and nine females per tank). The side compartments each included two terra cotta pots while the center compartment only contained one. All males maintained their respective social status for at least two weeks before being moved into experimental tanks. We allowed two weeks for experimental tanks to settle and for the dominant male in the center to sufficiently establish dominance over the subordinate male (the "focal male"). The total of four weeks of social stability ensured complete suppression of the reproductive axis in the subordinate male (Hofmann et al., 1999; Francis et al., 1993) before the onset of the experiment.

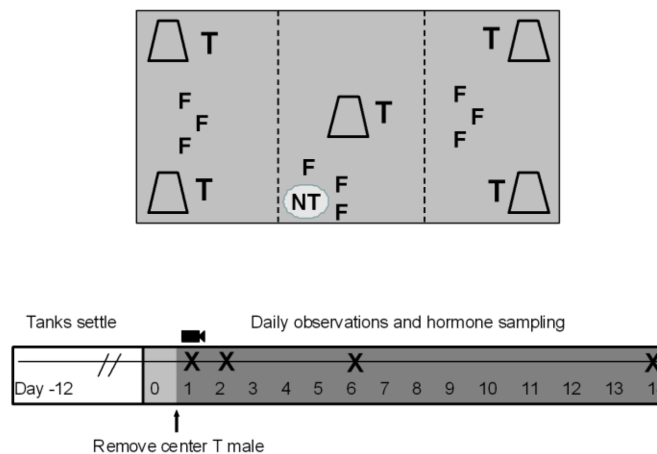

Figure 1: Experimental design. A) Behavioral paradigm and B) four-week time-line for repeated measures design. X's represent days on which males were euthanized for Experiment 2.

Experiment 1

Behavioral observations

We observed the focal male for ten minutes the morning before the experiment began (Day 0) to establish a baseline of behavior. On the first day of the experiment (Day 1) within 30 minutes before light onset, we removed the dominant male from the center compartment. This manipulation provided a social opportunity for the focal male to become dominant when the lights came on. We performed a ten-minute focal observation one hour after light onset. For Experiment 1, each individual was observed for ten minutes up to nine times (2 to 9 observations per subject) over two weeks (Day 1-14). Behavior patterns were scored based on Fernald (1977) and included two aggressive behaviors (attacking and lateral threat displays), one submissive behavior (fleeing), three reproductive behaviors (digging, leading to the spawning site, and quivering), and one neutral behavior (feeding). Attacking was defined as any rapid, directed swim towards an individual and is comparable to chasing and biting in other *A. burtoni* studies. All other behavior patterns (lateral threat displays, fleeing, digging, leading, quivering, and feeding) were scored as described previously (Fernald 1977). It is plausible that behavior patterns that appear identical to the observer might serve different functions depending on the intended target of the display (e.g., attacking a dominant male vs. a subordinate male); thus, we recorded whether the recipient of each behavior was a dominant male (vs. a subordinate male or female). We also recorded the number of gravid females present in the tank at the time of observation. Attacks and displays towards dominant males were summed to comprise an aggressive index for that 10-minute observation, and digging, leading, and quivering towards females were summed to comprise a reproductive index for that 10-minute observation.

Hormone measurements

Throughout the two-week transition, we collected water-borne hormone samples two hours post-observation, following the procedure introduced by Kidd et al. (2010). The concentration of free steroid hormones released into the water has been shown to be reflective of what is present in the circulation for several hormones in *A. burtoni* (Kidd et al., 2010) and thus provides a non-invasive method to assess the endocrine state of the same animal repeatedly, which minimizes stress and maximizes statistical power. Fish were placed in a beaker containing 300 mL of fresh holding water for one hour and then returned to their tanks. We filtered the water to remove particulate before freezing it at -20°C until processing and followed the protocol described in Kidd et al. (2010) for extracting steroid hormones from water samples. Briefly, samples were thawed and immediately filtered through an activated C18 column (Waters Corp.) to bind steroid hormones. Columns were frozen at -20°C until hormones were eluted with 100% ethanol, split into two aliquots, and dried under nitrogen gas. One pellet per sample was dissolved in 100 µL assay buffer included in the T ELISA system (Assay Designs), divided into two aliquots, and frozen at -20°C. Samples were diluted four-fold in assay buffer and ELISAs were run for T and E levels following the manufacturer's instructions.

Experiment 2

Hormone measurements and tissue collection

For Experiment 2, animals were observed as described for Experiment 1, then on either Day 1, 2, 6, or 14, focal males were weighed and measured for standard length following focal observations and collection of water-borne hormone levels (described previously). We obtained blood from focal males through the dorsal aorta using

heparinized 26 gauge butterfly infusion sets (Surflo). The plasma was then separated from the serum by centrifuging the blood at 4000 rpm for 10 minutes and then stored at -80°C for later hormone analysis (see below). We measured both T and E in plasma samples using ELISA (Assay Designs) after diluting the plasma samples 1:30 in assay buffer according to Kidd et al. (2010) and manufacturer's instructions. The coefficients of variation within assay plates ranged from 2% to 7%, and across plates around 10%. For those animals where we had obtained simultaneous plasma and water-borne hormone measures, we used linear regression analysis to confirm that water-borne hormone levels were representative of circulating levels (Supplementary Figure 1). The measures were significantly correlated for T and approached significance for E, where we only had 8 measurements (T: r^2=0.760, p=0.00004, n=22; log(E): r^2=0.663, p=0.073, n=8). We did not measure 11-KT because several studies conducted in this species have shown convincingly that levels of this teleost-specific androgen are highly correlated with T levels and an order of magnitude lower than T (Parikh et al., 2006a; Parikh et al., 2006b; Kidd et al., 2010).

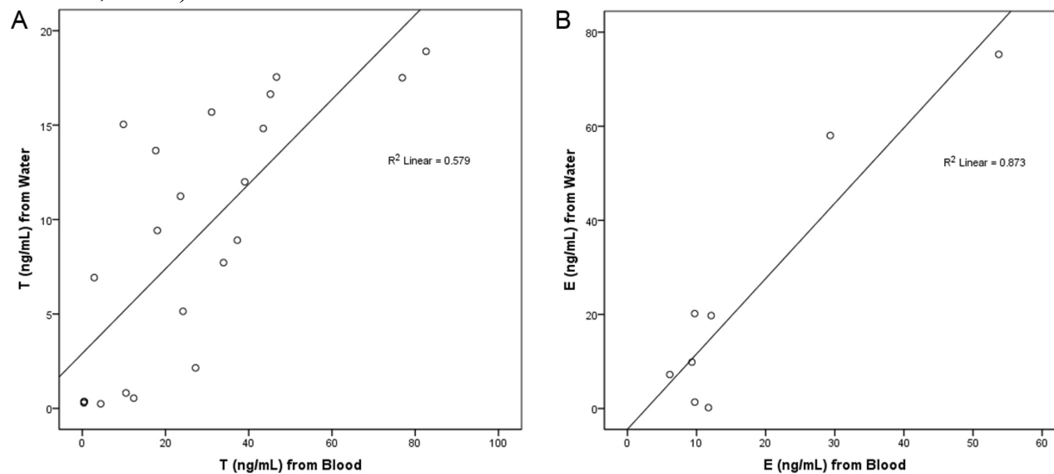

Figure S1: Correlations between waterborne and plasma levels of sex steroid hormones. (A) T and (B) E.

We then euthanized the animals and removed and weighed their testes to determine gonadosomatic index (GSI, calculated as the ratio of testes mass to body mass, multiplied by 100). We stored one testis from each male in RNAlater (Ambion) at -20°C for quantitative PCR analysis and the other in Bouin's fixative at 4°C for histological analysis. For comparison, we also collected plasma and testes from dominant ("Day 15") and subordinate ("Day 0") males in stable communities. Note that the Day 0 males from Experiment 1 refer to subordinate focal animals the day before transition, while Day 0 males from Experiment 2 refer to subordinate males from unmanipulated communities.

Testes histology

Each testis stored in Bouin's fixative was stored at 4°C for 1-3 months then dehydrated with several washes of 0.01% NH_4OH in 70% ethanol, cryoprotected in 30% sucrose in PBS overnight, embedded in OCT (Tissue-Tek), and stored at -80°C until sectioning. Gonads were cryosectioned at 14 μm onto Superfrost Plus slides (Fisher Scientific) and stained with hematoxylin-eosin. We used brightfield optics to visualize the hematoxylin-eosin stain throughout the gonads at low (5x) and high (20x) magnifications. Photographs were taken with a digital camera (AxioCam MRc, Zeiss) attached to a Zeiss Axiomager AX10 microscope (Zeiss) using the Axiovision (Zeiss) image acquisition and processing software. Images were enhanced for brightness and contrast and were compiled in Adobe Photoshop CS3 (San Jose, CA).

We categorized testes based on Grier's (1981) stages of development in teleost testes. However, as this staging scheme was developed for seasonal spawners, we used the following modified categories, which more accurately describe the situation in a tropical, non-seasonal breeder (Figure S2): Stage 1 is characterized by disorganized

lobules; Stage 2 is characterized by organized lobules with the presence of spermatogonia and spermatocytes; Stage 3 is characterized by organized lobules with all stages of sperm development present; and Stage 4 is the same as Stage 3 except that the tubules are filled with dense sperm packets.

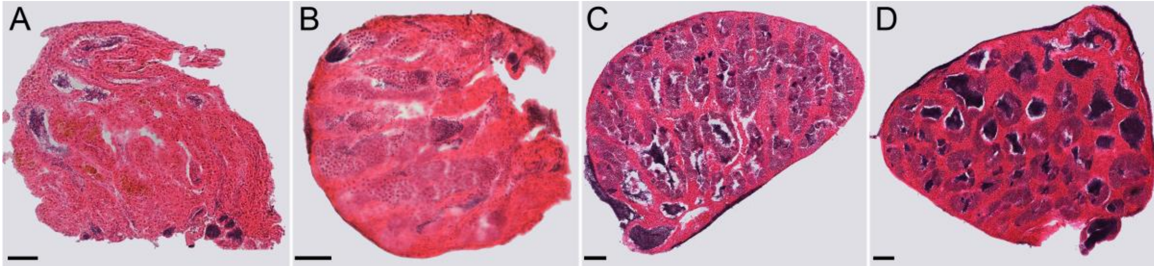

Figure S2: Stages of testis development during phenotypic change. (A) Disorganized lobules; (B) Early recrudescence – organized lobules with spermatogonia and spermatocytes present; (C) Mid recrudescence – organized lobules with all stages of sperm development present; (D) Late recrudescence – organized lobules with all stages of sperm development present; tubules filled with sperm.

Cloning StAR and LHR

To obtain the *A. burtoni StAR* sequence, we designed degenerate primers (Table 1) using CODEHOP (http://blocks.fhcrc.org/codehop.html) based on sequences from *Danio rerio* (GenBank accession numbers: NM_131663), *Acanthopagrus schlegelii* (AY870248), *Micropterus salmoides* (DQ166820), *Sparus aurata* (EF640987), and *Micropogonias undulatus* (DQ646787). Using whole brain cDNA as template, we performed a touchdown PCR reaction, starting with an annealing temperature of 60°C and decreased the annealing temperature by 0.5°C per cycle for 30 cycles. We then continued the PCR for 15 more cycles at an annealing temperature of 45°C. This touchdown approach yielded a 480 bp product, which we cloned into a pCRII-TOPO vector (Invitrogen). We then used RACE (Clontech, Palo Alto, CA, USA)

Gene	Protocol	Forward/Reverse/ Outer/Inner	Sequence (5’-3’)
StAR	Touchdown	Forward	CCGGCACATGCGGAAYATGACNGG
StAR	Touchdown	Reverse	CATCACCATGCAGGTGGGNCCRTKYT
StAR	3’ RACE	Outer	CTGGCAGGTCCAGGCCCCAGT
StAR	3’ RACE	Inner	TCAGCGAGCAGGACGGCTGGA
LHR	Touchdown	Forward	CGCCACCGACTGGCARACNGG
LHR	Touchdown	Reverse	GGCGAAGAAGGAGATGGGNGCCATRCA
LHR	5’ RACE	Outer	CCACAAGAGGGAGCAGGGCAACCAG
LHR	5’ RACE	Inner	CGGGTGGAGTGCTCTGGGTTGTGAA
LHR	5’ RACE	Outer	ATGCACAGGTCGGCAAAGGCCAGAT
LHR	5’ RACE	Inner	TGATCCAGGTGAGACAGCGCAGGAA

Table 1: Oligonucleotide primers used for cloning and RACE of *StAR* and *LHR*.

to extend the 3’ end of the coding region according to the manufacturer’s instructions (Table 1). This approach yielded a 335 bp product, which we also cloned into a pCRII-TOPO vector (Invitrogen). The partial mRNA sequence (total length 773 bp) has been submitted to GenBank (HM153531).

Based on this partial mRNA sequence, we determined the *A. burtoni* StAR amino acid sequence. To assess whether our putative StAR sequence indeed encodes StAR, we compared it to the StAR protein sequences of multiple species as well as a paralog, StAR-related lipid transfer protein 3 (StARD3, isoform 1), from *H. sapiens* as an outgroup (*H. sapiens* StAR: CAG46648; *H. sapiens* StARD3: NP_006795; *R. rugosa* StAR: BAH09112; *G. gallus* StAR: AAG28594; *D. rerio* StAR: AAG28593; *S. salar* StAR: ABD73012; *O. mykiss* StAR: NP_001117674). Using the Mega 4 freeware package (http://www.megasoftware.net/m_con_select.html), we aligned the sequences with ClustalW and generated a bootstrapped nearest neighbor-joining gene trees for StAR. Figure S3A indicates that the obtained sequence indeed encodes the *A. burtoni* StAR protein.

To obtain the *A. burtoni LHR* sequence, we designed degenerate primers using CODEHOP (http://blocks.fhcrc.org/codehop.html) based on sequences from *Acanthopagrus schlegelii* (AY820277), *Rhabdosargus sarba* (DQ522161), *Trimma okinawae* (AB376971), and *Dicentrarchus labrax* (EU282005). Using whole brain cDNA as template, we performed a touchdown PCR reaction that began with an annealing temperature of 68°C and decreased the annealing temperature by 0.5°C per cycle for 22 cycles. We then continued the PCR for 30 more cycles at an annealing temperature of 57°C. This touchdown approach yielded a 450 bp product, which we cloned into a pCRII-TOPO vector (Invitrogen). The 5' end of the coding region was also extended by RACE (Table 1). This approach yielded a 481 bp product, which we also cloned into a pCRII-TOPO vector (Invitrogen) and sequenced. The 5' end of this sequence was further extended by RACE. This RACE approach yielded a 534 bp product, which we also cloned into a pCRII-TOPO vector (Invitrogen) and sequenced. The partial mRNA sequence (total length 867 bp) has been submitted to GenBank (HM153532).

Based on this partial mRNA sequence we determined the *A. burtoni* LHR amino acid sequence. To assess whether our putative LHR sequence indeed encoded LHR, we compared it to the LHR protein sequences of multiple species as well as a human follicle-stimulating hormone receptor (FSHR) as an outgroup (*H. sapiens* LHR: AAA59515; *X. laevis* LHR: ABM68356; *G. gallus* LHR: BAA23736; *A. schlegelii* (ABY56689.1); *R. sarba* (ABI93202.1); *T. okinawae* (BAG56673.1); *H. sapiens* FSHR: CAA43996). A bootstrapped nearest neighbor-joining tree was generated for LHR demonstrating that the obtained sequence indeed encodes the *A. burtoni* LHR protein, as is shown in Figure S3B.

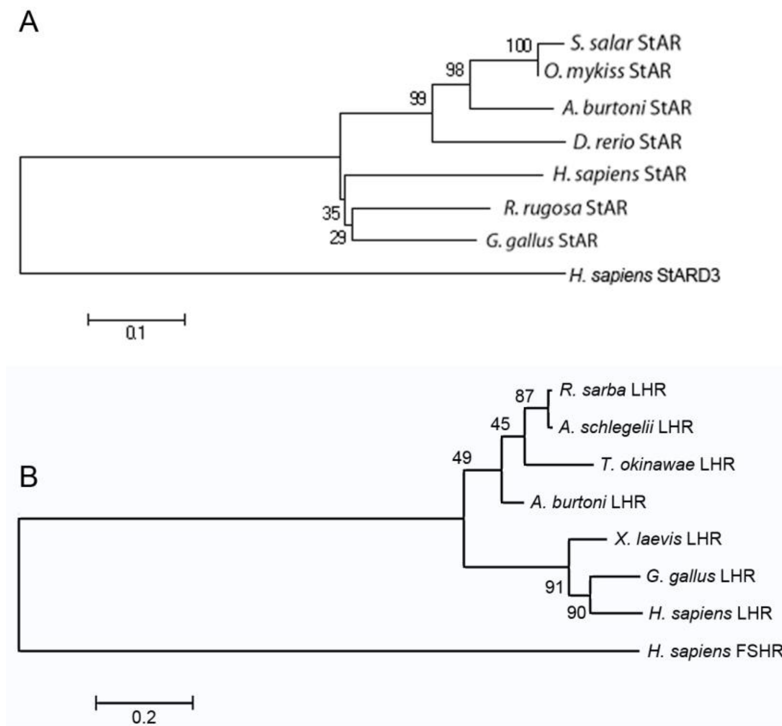

Figure S3: Comparison of *A. burtoni* StAR and LHR amino acid sequences with the orthologs of other vertebrates. A) StAR: a neighbor-joining tree shows that the *A. burtoni* StAR protein sequence is most closely related to StAR of other teleosts (*S. salar* and *O. mykiss*) and vertebrates, and is distinct from StARD3, the most similar member of this gene family; B) LHR: a neighbor-joining tree shows that the *A. burtoni* LHR protein sequence is most closely related to *T. okinawae* (a teleost) LHR and clusters away from *H. sapiens* follicle-stimulating hormone receptor (FSHR).

Quantifying gene expression in testes

We extracted total RNA from each sample using Trizol (Invitrogen) and then treated with DNAse I (Ambion, Austin, TX) according to the manufacturer's instructions. The RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) using gene-specific reverse transcription primers for all four genes (Table 2). Primers for *StAR* and *LHR* were designed using Primer3 (http://frodo.wi.mit.edu/primer3/), and primers for *aromatase* were designed from previously published *A. burtoni* gonadal *aromatase*

sequence (AF114716). Negative controls included testes RNA for which the reverse transcriptase was omitted. Excess primers and salts from the transcription reaction were removed in Microcon YM30 columns (Millipore, Bedford, MA). For each sample, reference gene (*18S*) and target gene abundance were measured in triplicate in an ABI PRISM 7900HT real-time PCR cycler (ABI SDS 2.2.1 software) using SYBR Green (Invitrogen). Standard curves were constructed using known dilutions of cDNA and used to calculate amplification efficiencies. For each individual, median values from the reference and target gene

Gene	**Forward/Reverse/ Reverse Transcription**	**Sequence (5'-3')**
StAR	Forward	GCAGGAATGTCCACTCAGCAC
StAR	Reverse	TCAGCGCAGGGCTTCATAACTA
StAR	Reverse Transcription	GAGCGAGCACTTTGTTTATGATTGT
Aromatase	Forward	CAAGACAGCAGCCCAGGAGT
Aromatase	Reverse	CTGCCGTGAATGTGATGTCG
Aromatase	Reverse Transcription	GTGCGGGTTTTGTTTGAGGA
LHR	Forward	CCTGGAATTTGACTGTCTTAGCAAC
LHR	Reverse	CCGTAATGATCCAGGTGAGACA
LHR	Reverse Transcription	CCATGAAGGCAATTAGGATGAGA

Table 2: Oligonucleotide primers used for Real-Time Quantitative PCR of *StAR*, *aromatase*, and *LHR*.

triplicates were calculated using the standard curve for each gene product, and the median value for each gene was normalized to the abundance of the 18S reference gene. The resulting product lengths were as follows: *StAR* - 99 bp product in the ligand-binding domain; *LHR* - 95 bp product in the ligand-binding domain; gonadal *aromatase* - 108 bp product in the ligand-binding domain. Primers for *18S* are the same as in Burmeister et al. (2007).

Statistical Analyses

All statistical analyses were performed using SPSS software, version 16.0. For data that included repeated measures across two weeks, as in Experiment 1, we used a Generalized Estimating Equations (GEE) model to examine the non-linear changes in behavior from Day 1 to 14. GEE is a non-linear version of a General Linear Model that accounts for missing data points and repeated measures of individuals over time and reports a Wald Chi-Square value. We used this model to examine relationships between individual behaviors, water-borne T levels, day of transition, and the number of gravid females present. Because data were part of a time-series and non-normal count data, we used the AR(1) working correlation matrix and either the Poisson log-linear or the binomial log-link model (depending on fit), respectively. Day of transition and water T level were used as covariates and number of gravid females present in the tank was used as a predictive factor in the GEE models. Models were run for each behavior in an iterative manner such that each model was tested for all two-way and three-way interaction effects. When counts for particular behaviors were too low to model, behavioral indices were analyzed instead (e.g., aggressive behavior, reproductive behavior). Water-borne T levels for Day 0 through 14 also progressed non-linearly so they were modeled using the gamma log-link GEE model (more appropriate for scale data as opposed to count data) and tested for effects of day and the number of gravid females present.

For Experiment 2, all variables were tested for normality by examining Q-Q plots and running the Shapiro-Wilk test. Non-normal variables were natural log-transformed, and non-parametric tests were used when necessary and are indicated here in parentheses. Because Experiment 2 only includes data from terminal days, there are no repeated measures as in Experiment 1, so comparisons across all days (Days 0, 1, 2, 6, 14, and 15)

were made using ANOVA (or Kruskal-Wallis) followed by pair-wise comparisons between days using independent sample t-tests (or Mann-Whitney U post-hoc tests). Pearson's correlation coefficients (or Spearman's rank correlation coefficients) were calculated to look for relationships between variables such as behavior, hormone levels, and gene expression.

To investigate how the spatial distribution of gravid females affected male behavior, we combined individuals from both experiments. Each compartment of an experimental tank (left, center, or right) was coded separately in SPSS, and gravid females were coded as a binary outcome (present or absent). Due to repeated measures and non-normal count data, we again used the GEE model to analyze effects on male behavior.

Finally, all variables were included in a network model to visually investigate the co-regulation of different variables using the software Cytoscape (Shannon et al., 2003). We made a correlation matrix between variables of behavior, hormones, gene expression, testis physiology and size, body size, and female gravidity and corrected for multiple hypothesis testing using the Benjamini-Hochberg FDR method (Benjamini and Hochberg, 1995).

RESULTS

Experiment 1

Behavioral Responses during Transition

On Day 1, within minutes after being presented with an opportunity to ascend in social status in the presence of neighboring territorial males, male *A. burtoni* displayed the suite of aggressive and reproductive behaviors typical for dominant males (Figure

2A). Following this initial surge in behavior, aggressive behavior towards other dominant males was significantly dependent on an interaction between day, water-borne T levels, and the number of gravid females present (Day 1-14, GEE, $p<0.001$, $n=34$); each of these three variables also had a significant main effect on aggression ($p<0.001$). Reproductive behaviors occurred regularly but at much lower levels than aggressive behaviors (Figure

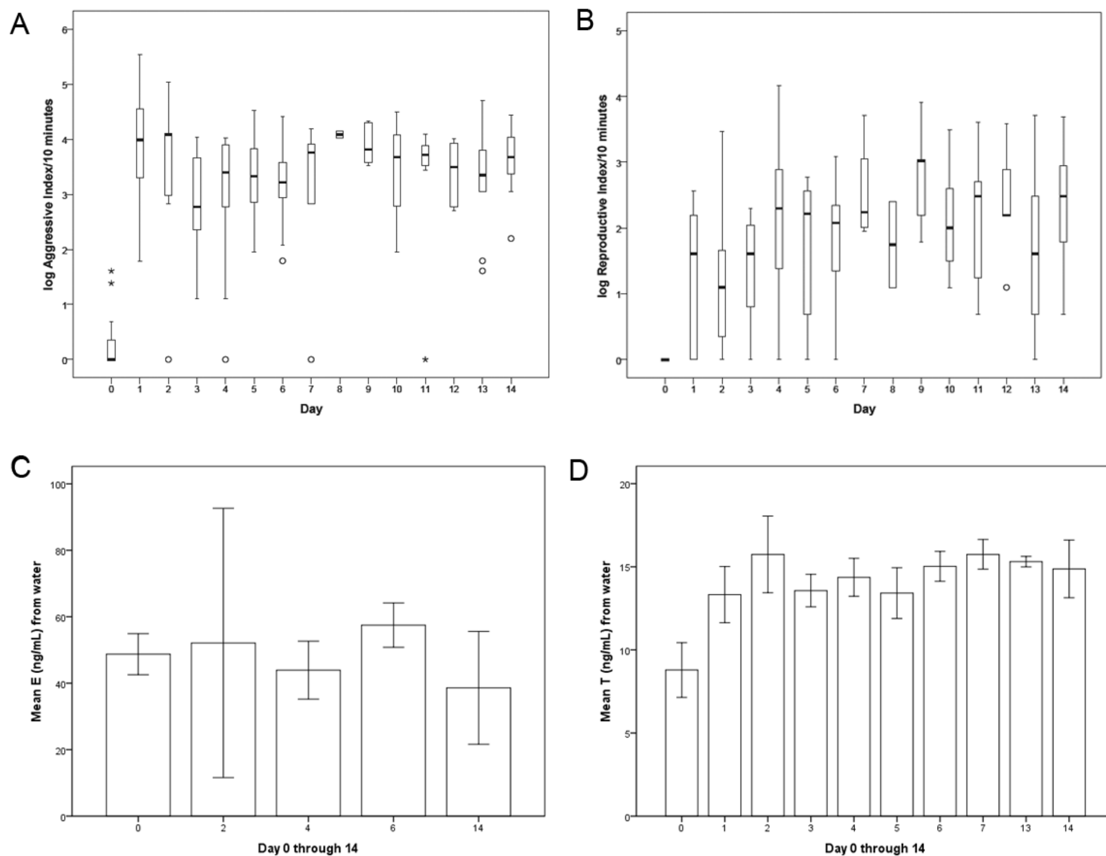

Figure 2: Responses to social opportunity over the period of two weeks from Experiment 1. A) Box-and-whisker plots of aggressive (sum of chasing males and threat displays) and B) reproductive (sum of leading displays, quivers, and digging) behavioral indices during 10 min focal observations. As is standard, t-bars represent the minimum and maximum values and filled bars represent the lower and upper quartiles (horizontal lines represent median values).C) Testosterone and D) estradiol levels in holding water. Error bars represent standard error.

2B), and there was a significant three-way interaction of day, water-borne T levels, and the number of gravid females present on the amount of reproductive behavior shown by focal males (Day 1-14, GEE, p=0.007, n=34), as well as significant main effects of day and T (p=0.003, 0.010, respectively).

Androgen and Estradiol Responses during Transition

We collected water-borne T levels on ten days from Day 0 to 14 and found that this androgen was extremely low on Day 0 (subordinate males, mean ± S.E. = 8.79 ± 1.65 ng/mL), as expected (Parikh et al. 2006a; Trainor and Hofmann, 2006). There were no significant effects on water-borne E, although E was only measured for five days across the transition (Figure 2C). There was a significant interaction effect of both day and the number of gravid females present on water-borne T levels from Day 0 to 14 (GEE, p<0.001, n=34; Figure 2D) as well as significant main effects of both gravidity and day (p<0.001, p=0.022, respectively).

Experiment 2

Androgen and Estradiol Responses during Transition

Behavioral responses of males used in Experiment 2 at Days 1, 2, 6, and 14 were comparable to those seen in Experiment 1 with one exception (on Day 1, males chased other T males slightly less than in Experiment 1; Mann-Whitney U, p=0.015; data not shown). All other behaviors and temporal patterns were similar between the two experiments. Circulating T levels sampled from the plasma significantly varied across days (ANOVA, F=10.26, p<0.001; Figure 3A), and the initial increase from Day 0 to Day 1 was significant (independent sample t-test, t=-2.695, n=15, p=0.031). T levels collected from water reflected the plasma measurements, changing significantly across days

(ANOVA, F=14.58, p<0.001), although water measures did not include Day 0 (only 1, 2, 6, and 14). Plasma E levels varied significantly across days as well (ANOVA, F=13.846, p<0.001; Figure 3B) although, similar to Experiment 1, this was not reflected in the water, which was also log-transformed (p=0.354).

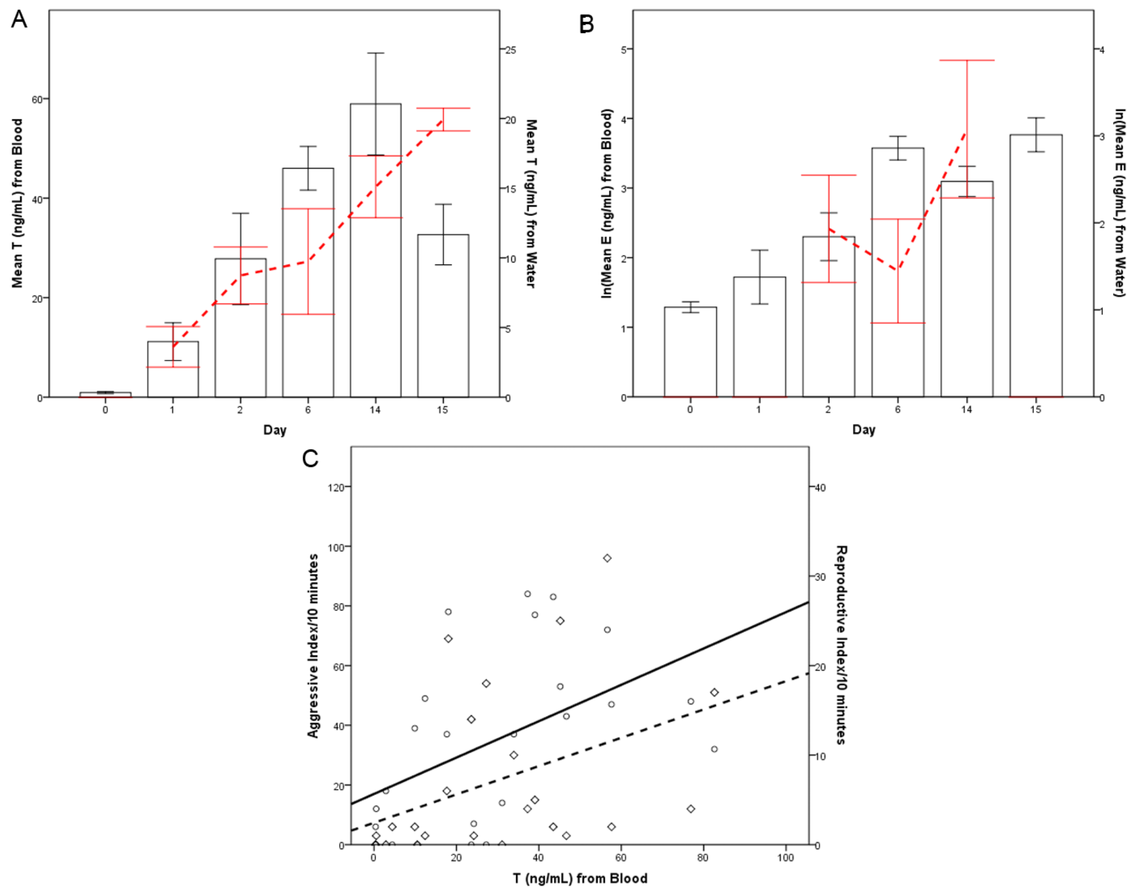

Figure 3: Hormone measurements and relationships with behavior in Experiment 2. A) Mean values of testosterone and B) estradiol in holding water and plasma for days 0 (for water), 1, 2, 6, 14, and 15 (for water and plasma). Error bars represent standard error. Bars represent water levels (primary y-axis); lines represent plasma levels (secondary y-axis). C) Linear regression relationships between testosterone and behavioral indices.

Behavior and Hormones

During the two-week transition to dominance, plasma (Pearson's r=0.49; p=0.015, n=24) and water-borne T (r=0.40, p=0.043, n=26) levels were both positively correlated with aggressive index (Figure 3C). The former also approached a significant correlation with reproductive index (r=0.40, p=0.053, n=24). Interestingly, a strong correlation between reproductive behavior and plasma T levels already appeared on Day 1 (r=0.851, p=0.007, n=8), even though the number of reproductive displays continued to increase throughout the transition (see above). Plasma E levels were also positively correlated with aggressive index (Pearson's r=0.55; p=0.015; n=10), but water levels were not (Pearson's r=0.48, p=0.156, n=10). Further, we examined the ratio between T and E and found that it strongly correlated with reproductive index (Spearman's ρ=0.616, p=0.005, n=19).

Testis Histology and Gene Expression

We then examined the gonads and found that during the transition from Day 1 to Day 14, GSI was significantly variable (ANOVA, F=4.144, p=0.014) and increased over the two weeks (t-test Day 1 vs. 14, p=0.022; Figure 4A). We then examined the testes histologically and found that early in transition they were less organized than during later stages (Figure 4B; Pearson's X^2=26.234, df=15, p=0.035). Specifically, there were more Day 2 individuals with disorganized testes (Stage 1) than expected by chance (adjusted residual: 2.6) and more Day 6 transitioning males with fully developed testes (Stage 4) than expected by chance (adjusted residual: 2.6). On Day 14, there were fewer males with disorganized testes (Stage 1) than expected (adjusted residual: -2.1) and more in Stage 2 than expected (adjusted residual: 2.0). Furthermore, there were no Day 14 transitioning males or stable territorial males with disorganized (Stage 1) testes. Even though these

results are biologically meaningful, they should be regarded with caution, as more than 20% of the cells in the contingency table contained low expected counts.

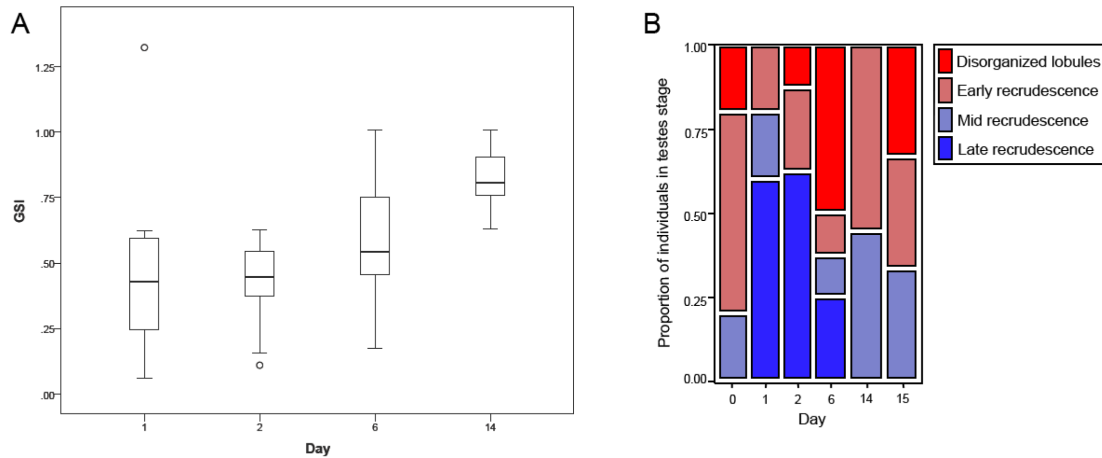

Figure 4: Gonadosomatic index and testes histology during transition. A) GSI. B) Proportion of individuals in testes stages during phenotypic transition.

In order to gain a more robust and detailed understanding of the interaction between testis physiology and social environment, we then analyzed the expression of gonadal genes involved in steroid hormone production. As is shown in Figure 5A, *StAR* mRNA expression changed significantly throughout transition in *A. burtoni* (Kruskal-Wallis X^2=12.057, df=5, p=0.034). Stable non-territorial males (Day 0) had significantly lower *StAR* expression than males on Day 6 (Mann-Whitney *U* post hoc, df=5, p=0.015) or Day 14 of transition (Mann-Whitney *U* post hoc, df=5, p=0.004). Furthermore, Day 1 transitioning males exhibited significantly different *StAR* expression levels from Day 14 transitioning males (Mann-Whitney *U* post hoc, df=5, p=0.047). Interestingly, gonadal *LHR* (Kruskal-Wallis X^2=4.537, df=5, p=0.475) and *aromatase* (Kruskal-Wallis X^2=5.402, df=5, p=0.369) did not change significantly throughout transition (Figure 5B, C). Nevertheless, *StAR* and *aromatase* expression were positively correlated (r=0.934,

p=0.0001), as were expression levels of *StAR* and *LHR* (r=0.653, p=0.0001). *Aromatase* and *LHR* expression levels were not correlated (r=-0.063, p=0.675).

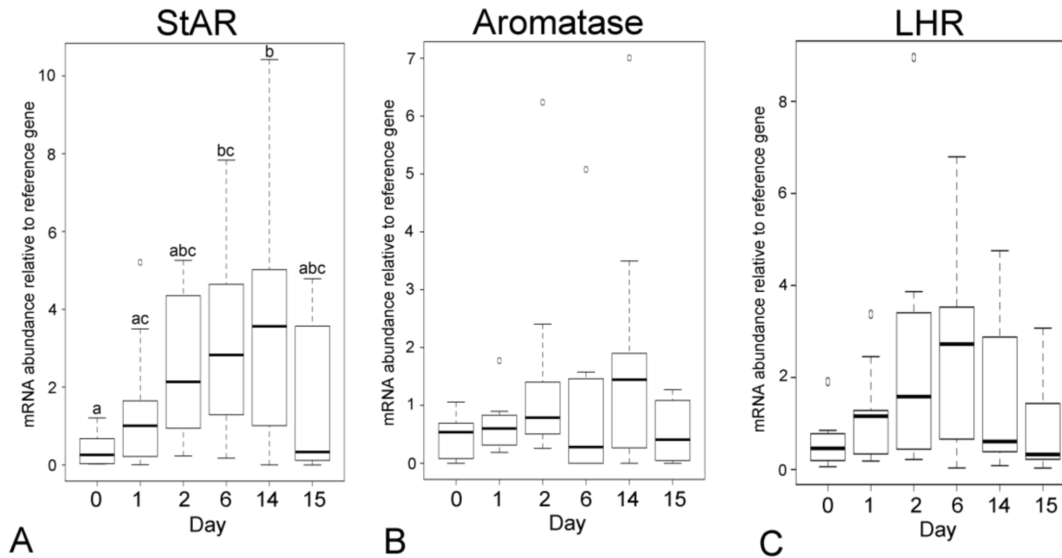

Figure 5: Gonadal gene expression. Gonadal A) *StAR*, B) *aromatase*, and C) *LHR* mRNA abundance relative to reference gene of is depicted by box and whisker plots for males during transition for days 1 (n=9), 2 (n=10) , 6 (n=10), and 14 (n=9) or from stable communities for days 0/NT (n=8) and 15/T (n=7). Error bars represent standard error. Kruskal-Wallis: p<0.05. A) Letters represent significant differences between groups from Mann-Whitney U post-hoc (p=0.034). Relative *StAR* mRNA levels are significantly different on day 6 (p=0.015) and 14 (p=0.004) compared to day 0 and levels are significantly different between day 1 and day 14 (p=0.047).

Reproductive Behavior and Gravid Females

In Experiment 1 we found that the quantity of reproductive behavior exhibited by ascending males was in part dependent on how many gravid females were present. For a subset of animals (n=37) from Experiments 1 and 2, we recorded the location of gravid females (left, center, right) and to which compartment the focal male directed his courting displays. This information enabled us to ask whether males detected and directed their behavioral responses towards the location of gravid females across the two

weeks of transition. Indeed, the amount of courting towards each compartment depended on whether any of the females housed in that particular compartment were gravid (GEE, $p<0.001$, $n=417$; Figure 6).

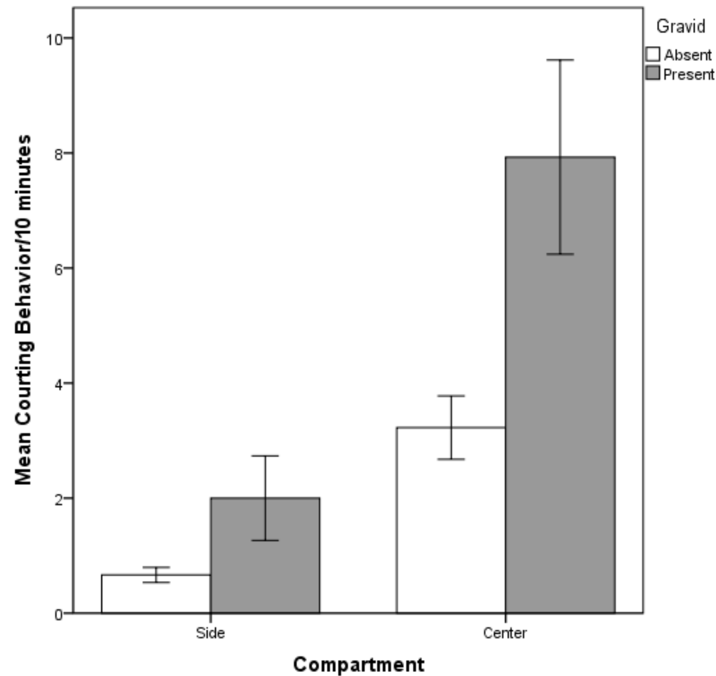

Figure 6: Compartmental responses to reproductive opportunity. The amount of leading behavior directed towards either the side compartments or center compartment varied based on the presence of gravid females in that compartment.

Behavior, Hormones, and Gene Expression

We examined correlations between behavior, hormones, gene expression, testis physiology and size, and body size (a total of 91 comparisons) in an effort to integrate all of these variables for a systems-level analysis. As in Experiment 2, aggressive behavior was positively correlated with T and E as well as testis stage and body size; however, due to the size of the correlation matrix, these did not survive the FDR correction. Reproductive behavior was found to be positively correlated with *LHR* expression and

GSI, although neither of these survived the correction. The only correlation found between any of the three genes and hormone levels, either in the blood or in the water, was between *StAR* expression in the testis and plasma E, although this also did not survive an FDR correction ($r=0.323$, $p=0.037$). However, *StAR* expression was strongly correlated with the ratio of plasma T:E ($\rho=0.551$, $p<0.001$). *StAR* expression was also strongly correlated with both *aromatase* and *LHR* expression ($r=0.936$, $p<0.00001$; $r=0.651$, $p<0.00001$, respectively). T levels were positively correlated with body and testis size and E levels, all of which survived the FDR correction.

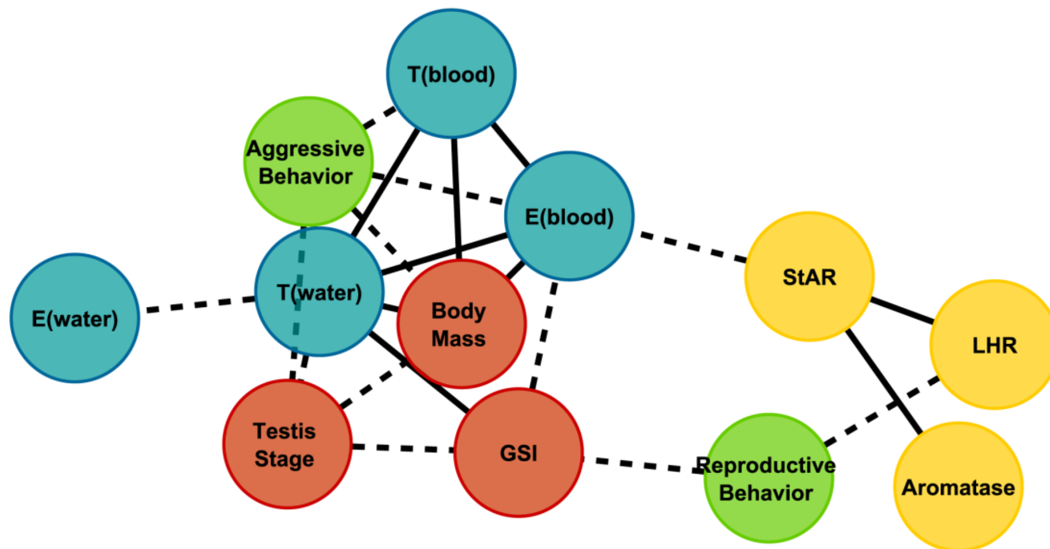

Figure 7: Integrative model of phenotypic transition. Model illustrates all statistically significant correlations between the variables measured. Variables are colored according to their type: measures of behavioral displays (aggressive, reproductive) are green; hormone levels (T and E in water and plasma) are blue; gene expression levels (*StAR, LHR, aromatase*) are orange; physiological measures (body mass, testis stage, GSI) are red. Edge lengths correspond to the inverse Pearson correlation value between nodes (i.e., shorter edges connect variables that are tightly correlated/value close to 1, and longer edges are less correlated/value further from 1); solid edges indicate those that passed a Benjamini-Hochberg FDR correction for multiple hypothesis testing, and dashed edges indicate those that had p-values between 0.05 and the correction threshold.

Even though many of these relationships did not survive multiple hypothesis testing, they suggested several interesting patterns. We therefore used Cytoscape to build a force-weighted network model in which each node represents a different variable, and each edge represents a correlation between two variables (Figure 7).

From this model, it is apparent that T, E, body size, aggression, and testis physiology are tightly correlated, representing a putative module of co-regulated physiological variables in transitioning males. Expression of *LHR, StAR,* and *aromatase* in the testis also form a co-regulated cluster, which is linked to the rest of the network via reproductive behavior. The variable with the most significant connections was circulating T level, suggesting that T plays a central role in regulating multiple aspects of the male phenotype during the transition to social dominance.

DISCUSSION

In the present study we have confirmed that male *A. burtoni* begin behaving aggressively and reproductively within minutes of perceiving an opportunity to transition from subordinate to dominant. This behavioral response is accompanied by a rapid increase in circulating T levels, and we have shown for the first time that these behavioral and endocrine responses are also dependent on the gravidity of the females in the enclosure. When reproductive behavior was investigated more closely, we found that, independent of day, males targeted more reproductive displays towards compartments when they housed gravid females. By extending the previously reported time courses to two weeks, we also found that E levels and reproductive behavior seemed to increase more gradually than T or aggressive behavior. We have described male *A. burtoni* at all stages of social dominance that possess the necessary cellular machinery in their testes to

produce both T and sperm, although cellular organization and amount of sperm within the testes did tend to increase with dominance tenure. Expression of *StAR* increased within one week of males becoming dominant and correlated with *LHR* and *aromatase* expression, although neither of the latter two genes increased expression throughout the transition.

The immediate onset of aggressive behavior and subsequent sustained decrease confirms the findings of Burmeister et al. (2005) and Maruska and Fernald (2010), although our study extended the previous time course by more than a week. Males also showed reproductive behavior on the first day of transition similar to the results found by Maruska and Fernald (2010) as well as a more gradual increase of reproductive behavior relative to aggression. As behavior can vary from day to day, observing the animals for many days allowed us to both capture larger patterns over time and investigate individual variation and some possible mechanisms underlying that variation. For example, circulating T levels and the presence of gravid females also significantly affected levels of behavior. It is not clear from our data what the direction of cause and effect are, but we do know that female *A. burtoni* complete a cycle of gravidity roughly every 30 days regardless of male behavior (Kidd et al., 2011), so it is unlikely that male behavior is driving gravidity in our paradigm.

Although our finding that T levels approximately double within a few hours of transition in males with small, immature testes may seem surprising, there were some subordinate males whose testes were extremely organized. A study by Maruska and Fernald in *A. burtoni* (2011b) also showed that subordinate male testes possessed cells at all stages of sperm production. Thus, it is possible that even subordinate male testes are capable of producing this initial surge in T at the onset of transition. Alternatively, studies on extra-gonadal sources of steroid hormones using songbirds may help to explain this

finding. During the breeding season, circulating T levels are acutely responsive to aggressive interactions (Challenge Hypothesis, Wingfield et al., 1990). Importantly, many species of songbirds are also aggressive outside of the breeding season, when T levels are low. This aggression is not affected by castration (Wingfield, 1994) but is decreased by aromatase inhibitors (Soma et al., 1999), suggesting that non-breeding aggression in songbirds may be mediated by extra-gonadal sources of steroid hormones, particularly E (Schmidt et al., 2008). Similarly, several species of rodents show E-mediated aggression outside of the breeding season, when the reproductive system is regressed (Trainor et al., 2008). Although we saw a rapid increase in T, not E, it is clear that sources other than the gonads are often responsible for surges in sex steroid hormone levels, especially as the subordinate status in non-seasonal breeders may be comparable to the non-breeding season in seasonal breeders. Alternatively, a recent study on androgen responsiveness in songbirds showed that acute stress induced a two-fold increase in T between 15 and 33 minutes of handling (Van Hout et al., 2010). Although all males in our study were handled uniformly such that handling stress was constant between individuals and days, we cannot rule out the possibility that variables such as degree of dominance may affect stress reactivity. In turn, stress reactivity may have immediate or long-term effects on T levels. Thus, it is possible that as males become more territorial, they are more or less reactive to handling stress and hence have transient changes in water-borne T levels during sampling that are not reflective of normal circulating levels. Parikh et al. (2006b) showed that after 24 hours of mimicked territory loss, territorial males had an increased stress response (measured via cortisol) and decreased T. It has also been shown that among subordinate males, those with moderate stress responses to an aggressive video stimulus showed direct aggression in return; subordinate males with high or low stress responses also responded aggressively, but

towards their tank-mates instead of the aggressive fish in the video ("displaced aggression"; Clement et al., 2005). These data suggest an interaction between cortisol, T, aggression, and territoriality, but the exact relationships are not clear (also see Fox et al., 1997).

Subordinate males do not maintain spawning pits and are similar to females in body coloration and behavior; as one might expect, females show no interest in mating with these males. In addition to not having the social opportunity to spawn, it has been assumed that these males are under physiological constraints that limit reproduction, as they have been reported as having significantly smaller testes (Francis et al., 1993; Hofmann and Fernald, 2000) containing largely immature sperm (Fraley and Fernald, 1982), which our comparison of Day 1 and Day 14 males confirmed. However, several other studies did not find a significant difference in GSI between dominant and subordinate males (Hofmann and Fernald, 2000; Burmeister et al., 2005). For example, Francis et al. (1993) demonstrated that after experimentally manipulating social status (in both directions) for four weeks, males had significantly different GSI values when compared to stable males of the initial (unchanged) status. Further, Maruska and Fernald (2010) showed that only five days of territoriality were sufficient to increase GSI. However, five days in the new social status was not found to be enough time for significant changes in GSI (in either direction) according to Hofmann and Fernald (2000), and White et al. (2002) found a significant increase after seven days of territoriality, but not three. In addition, Burmeister et al. (2005) found no significant difference in GSI between stable subordinate and dominant males. Similarly, in our study, stable subordinate males from community tanks did not have significantly smaller GSI than stable dominant males. Changes in gonad mass have been suggested to be due to interstitial cell development (Oslund, 1928; Khanna and Pant, 1966) and not

necessarily associated with changes that reflect reproductive maturity, such as sperm production or maturation. Regardless of these inconsistencies, GSI is often used as a rough indicator of reproductive potential. Histological analysis of the testes, as shown here, provides a more reliable assessment, as one can directly examine the cell types present in the testes and classify them into progressive stages of organization that are reflective of dominance status. Interestingly, a recent report by Maruska and Fernald (2011b) on the histology of *A. burtoni* testes showed that subordinate males contained all spermatogenic stages, and a second study (Kustan et al., 2011) demonstrated that sperm proliferation did not differ between dominant and subordinate males. Further, we have also shown that the gonadal expression of *StAR* (and thus gonadal T synthesis) is indicative of dominance. Future studies would benefit from using these more direct cellular and molecular assays of dominance instead of GSI. Further, these studies taken together suggest that the absence of mating in subordinate males may be an adaptive response, not a necessity due to physiological constraint. Behavioral observations of mixed-sex communities support this hypothesis, as subordinate males that act "inappropriately" (e.g., show aggressive or reproductive displays) are quickly suppressed by the larger, dominant males (unpublished observations). Avoiding these aggressive encounters may provide an advantage to subordinate males, allowing them to grow large enough to defend themselves before attempting to transition to dominance.

Although testes became more organized as subordinate males became dominant, we also found that some subordinate males already possessed testes with all of the major cell types necessary for sperm production and T synthesis, even though their T levels were low and they displayed no reproductive or aggressive behavior. Additionally, not only did some subordinate males appear to be physiologically prepared to produce sperm, but the expression of two genes, *LHR* and *aromatase*, associated with T synthesis did not

differ between social phenotypes. The only transcript that increased with day (as T did) throughout the transition was StAR, which suggests that males prepare to synthesize more T in the testes as they become more dominant, as *StAR* expression was found to gradually ramp up and concurrently increase androgen synthesis. Future studies investigating the relationship between social environment and StAR induction will illuminate these control mechanisms and help us understand how plasticity involves integration of multiple biological levels. Although both T and E also increased during transition, it is not completely surprising that *aromatase* expression did not increase, as it is possible that steroid hormones regulating behavior are synthesized primarily in the brain, whereas synthesis regulating physiology may occur primarily in the gonads. In fact, there is evidence in birds that a large portion of behaviorally relevant aromatization occurs locally in the brain (Schlinger, 1997; Remage-Healey et al., 2010).

We built a network model to facilitate a systems-level understanding of broader patterns, and several clusters of variables stand out visually. Sex steroid hormone levels, aggression, and testis physiology appear to cluster together, possibly forming an "aggression" module of variables that are activated early in transition to establish dominance. We have shown that aggressive behavior and T levels both increased rapidly, as they were extremely responsive to the social opportunity perceived by the male. It is also known that both T and E levels play distinct roles in aggressive behavior as well as the development and regulation of reproductive physiology although the role of T in aggression has been studied in much more detail than that of E. Functional studies manipulating sex steroid hormone production that examine effects on behavior and reproductive capacity will help elucidate this possible module of co-regulated variables. Expression of gonadal genes involved in steroid hormone synthesis and reproductive behavior also cluster together, potentially representing a "reproduction" module. We

investigated *LHR* expression because LHR relays the signal from the pituitary to the gonads to alter synthesis of sex steroid hormones; thus, it is compelling that of the three testis genes examined, LHR was the only one connected to behavior in our model. Functional studies of LHR, StAR, and aromatase in different social states and during transition would help elucidate the roles of these gene products as males initiate and establish their new status. Interestingly, although neither T nor E are part of the putative "reproduction" module, we did find that the ratio of T:E is strongly correlated with both reproductive behavior, suggesting that the relative levels of androgens and estrogens or, possibly, the conversion of androgens to estrogens, is more relevant to reproduction than are absolute levels. The T:E ratio was also strongly correlated with *StAR* expression (also part of the putative "reproduction" module), suggesting that gonadal sex steroid hormone synthesis and the genes involved do affect circulating levels although this was not reflected when analyzing absolute levels. Thus, the relationship between sex steroid hormone synthesis in the brain and the gonads and levels of these hormones in the periphery remains unclear. There are not many other studies that have reported direct measurements of sex steroid hormones in fish, but a study on male zebrafish (Shang et al., 2006) reported approximately two-fold lower levels of T in plasma but similar levels of E at 120 days post-fertilization than the average adult *A. burtoni* in Experiment 2; similarly, the T:E ratio was approximately two-fold lower in zebrafish (1.02 vs. 1.7). A study on male carp reported T levels similar to zebrafish (two-fold lower than adult *A. burtoni*), but almost undetectable E levels (Wu et al., 2003). *Astatotilapia burtoni* may have unusually high levels of sex steroid hormone synthesis relative to other fish, but clearly a comparative study is warranted to verify this and determine the biological significance, if any.

It is also interesting to note that plasma hormone levels are more strongly and significantly connected than those extracted from fish holding water. Water hormone assays have made endocrine profiling of small fish much more amenable, as multiple blood draws on animals of this size are not feasible. However, due to the nature of this technique, in which hormones are collected from holding water over the course of an hour, the measurement being taken is not as "acute" as that of a blood draw. In other words, an acute hormonal response may be captured in a plasma measurement but diluted out when averaged over an hour, as it is with water measurements. Therefore, although plasma and water measurements are repeatedly found to correlate in ours and other studies, plasma measurements may be more reflective of the acute hormonal responses associated with behavioral changes in our study. In fact, the relationship between plasma and water levels of E in our animals was weak and driven by 2 of the 8 data points in the curve, further suggesting that acute changes in E (such as in the plasma) may not be captured in water measurements.

CONCLUSION

We have investigated the responses to social challenge and opportunity as they arise during the transition from social subordinance to dominance in male *A. burtoni* in a complex behavioral paradigm. By simultaneously quantifying the behavioral, endocrine, histological, and transcriptional responses of these males, we have presented a model of phenotypic plasticity at an unprecedented level of biological integration and time resolution.

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Chapter 3: Characterization of Two Neuropeptide Systems in an African cichlid fish, *Astatotilapia burtoni*

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INTRODUCTION

Neuropeptide regulation of social behavior is ubiquitous across vertebrate taxa and can vary by sex, social context, and the neural expression of their respective receptors. Arginine vasotocin (AVT; the non-mammalian homolog of arginine vasopressin) and isotocin (IST; present as oxytocin in mammals and mesotocin in birds, reptiles, and amphibians) are neuropeptides that have been highly conserved throughout vertebrate evolution, consistently influencing aggressive and reproductive behavior, although their specific effects vary widely (for a review, see Goodson 2008). Across diverse taxa, the nonapeptides are consistently found in the preoptic area (POA) and the anterior hypothalamus (AH), suggesting that these cell populations are ancient in the vertebrate lineage. Although the neural distribution of AVT and IST expression and their homologous neuropeptides have been extensively described across vertebrate classes (for a review, see Goodson and Bass, 2001), much less is known about the distribution of their receptors, especially in non-mammalian vertebrates.

The relative expression of neuropeptide receptors across brain regions is exceptionally diverse. While there appears to be only one OXY receptor, three AVP receptors have been described in tetrapods (V1a, V2, V3/V1b; Hasunuma et al., 2007). The V1b receptor is usually associated with the function of ACTH in the pituitary (Jurkevich et al., 2005; Tanoue et al., 2004), whereas the V2 receptor regulates water retention in the kidney via aquaporins (Hayashi et al., 1994). The V1a subtype, on the other hand, is widely distributed throughout the brain and has been shown to regulate sex

and species differences in many social behaviors, in mammals, birds, amphibians, and fish (Insel et al., 1994; Semsar et al., 2001; Goodson and Wang, 2006; Baeyens and Cornett, 2006; Hasunuma et al., 2007)

Surprisingly little is known about the expression and distribution of these receptor genes in the brains of teleost fish. In a recent analysis in the Amargosa pupfish, *Cyprinodon nevadensis amargosae*, Lema (2010) isolated mRNA sequences for three AVT receptor subtypes and identified them by their mRNA tissue distribution and amino acid homologies as V1a1, V1a2 and V2 receptors. Using PCR, this study showed that the two distinct forms of the V1a subtype are expressed in the forebrain, midbrain, cerebellum, and hindbrain. Kline et al. (in prep.), working on the rock hind grouper, *Epinephelus adscensionis,* also used PCR in gross dissections of the brain and found that the V1a2 subtype is more widely distributed in the brain compared with the V1a1 subtype. Furthermore the expression of the V1a2 subtype is more closely associated with sex and reproductive state in rock hind. These authors then used both *in situ* hybridization and immunohistochemistry to describe the distribution of the V1a2 subtype throughout the rock hind brain (Kline et al., 2011). However, no other teleost species have been examined this way, nor are there any published accounts of ITR brain distribution for this vertebrate group.

The African cichlid fish *Astatotilapia burtoni* has become an important model system in social neuroscience due to its extensive suite of complex social behaviors and inducible phenotypic plasticity (Hofmann, 2003; Robinson et al., 2008). Male *A. burtoni* can be either socially dominant or subordinate, and this phenotype is reversible based on social environment. Dominant males display stereotypical patterns of aggression, coloration, and reproductive behavior, while subordinate males are non-reproductive, submissive, behaviorally and morphologically resembling females (Hofmann, 2003). It is

known that preoptic expression levels of AVT differ between dominant and subordinate males (Greenwood et al., 2008) and that expression is largely limited to three nuclei of the POA (gigantocellular, magnocellular, and parvocellular), with a small amount of expression in the anterior tuberal nucleus of the hypothalamus. Interestingly, males showed opposite patterns of differential expression in two of the three POA nuclei, with dominant males having higher AVT expression in the gigantocellular nucleus and subordinate males in the parvocellular nucleus. The physiological functions that are modulated by each nucleus have been investigated, and all three nuclei have projections to the pituitary, but the relationship between each nucleus and social behavior is not known (Greenwood et al., 2008).

The role of IST has not yet been investigated in *A. burtoni*, but work in other teleosts suggests that this neuropeptide is also largely expressed in POA cell populations (Buchholz et al., 1995; Hur et al., 2010). Very little is known, however, about the distribution of the IST receptor throughout the teleost brain (Hausmann et al., 1995). By examining the neural distribution of mRNA and protein for the AVT and IST receptors, we can significantly increase our understanding of how these nonapeptides modulate may phenotypic plasticity in *A. burtoni*.

Based on insights originally obtained from mammals and more recently extended to reptiles, birds, and teleosts, there are two neural networks of fundamental importance to the regulation of social behavior and/or the encoding of stimulus salience. Many studies indicate that the "reward system" (including the midbrain dopaminergic system) is the neural network where the salience of social stimuli is evaluated (Deco and Rolls, 2005; Wickens et al., 2007). The neural substrate of social behaviors has been described by Newman (1999) as the "social behavior network" in mammals and has been expanded to reptiles, birds, and teleosts (Newman, 1999; Crews, 2003; Goodson, 2005). The core

nodes of Newman's network are involved in multiple forms of social behavior, are reciprocally connected, and contain sex steroid hormone receptors. Although the brain regions involved in the dopaminergic reward system and the social behavior network are well studied in mammals and birds, descriptions of the teleost homologs are contentious (Nieuwenhuys et al., 1998; Northcutt, 2008). However, a consensus is emerging from developmental, hodological, neurochemical, and lesion studies that provide support for at least putative partial homologies for relevant areas in the teleost brain (Northcutt, 2006, 2008; Portavella et al., 2002; Rink and Wullimann, 2001, 2002; Wullimann and Mueller, 2004, Bruce and Braford, 2009; O'Connell and Hofmann, 2011).

The main aim of this study was to test the hypothesis that the AVT V1a2 receptor and ITR are widely distributed throughout the brain of a teleost with plastic behavioral phenotypes. Additionally, we describe the distribution of AVT and IST cell bodies and fibers. We also predicted that the neuropeptide receptors would be expressed in brain regions important for the regulation of social behavior and evaluation of stimulus salience in the African cichlid fish, *A. burtoni*.

METHODS

Animals

Astatotilapia burtoni from a wild-caught stock population were kept in aquaria under conditions mimicking their natural environment as in Munchrath and Hofmann (2010). The animals chosen for this study were dominant and subordinate males as described by Fernald (1976), who had been in their respective social states for at least four weeks. Dominant males were identified as aggressively defending a territory within the tank, courting females, and displaying bright color with eye bar. Subordinate males

were identified by absence of a territory, schooling with the females, fleeing from territorial males, and lack of bright body coloration and eye bar. Adult females were also included as described below. All work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

We used the neuroanatomical nomenclature for *A. burtoni* as in Munchrath and Hofmann (2010) and O'Connell et al. (2010).

Nested PCR (outer)	F- 5' AGTACCTGCAGGTGGTGGGNATGTTYGC
Nested PCR (outer)	R- 5' GCAGCAGGAGTTCAGGCAGSCNARNARCAT
Nested PCR (inner)	F- 5' CGGTGCATGGCCATCTGBCARCCNYT
Nested PCR (inner)	R- 5' CATCTGCACGAAGAAGAAGGGNGTCCARCA
3' RACE	Outer- 5' GACTGCTGGGGCGACTTCGTGAAACC
3' RACE	Inner- 5' CGGGAGCAGTGCATAAACCTGACGCCTA

Table 1. Primers for cloning ITR in *A. burtoni*.

Cloning of the *A. burtoni* ITR cDNA

The *A. burtoni* V1a receptor gene sequence was already available in GenBank (accession number AF517936.1). We used nested degenerate primers designed for *Xiphophorus* to initially clone ITR, which gave us a large portion of the highly conserved transmembrane region (See Table 1 for primer details). We then used nested 3' RACE to extend our sequence into 3' UTR, resulting in a final fragment of 751 bp (GenBank accession number: GQ288467.1). To confirm the identity of the sequences, a nearest neighbor tree was assembled in MEGA with pairwise deletion and bootstrap values from 1000 replicates (Figure 1).

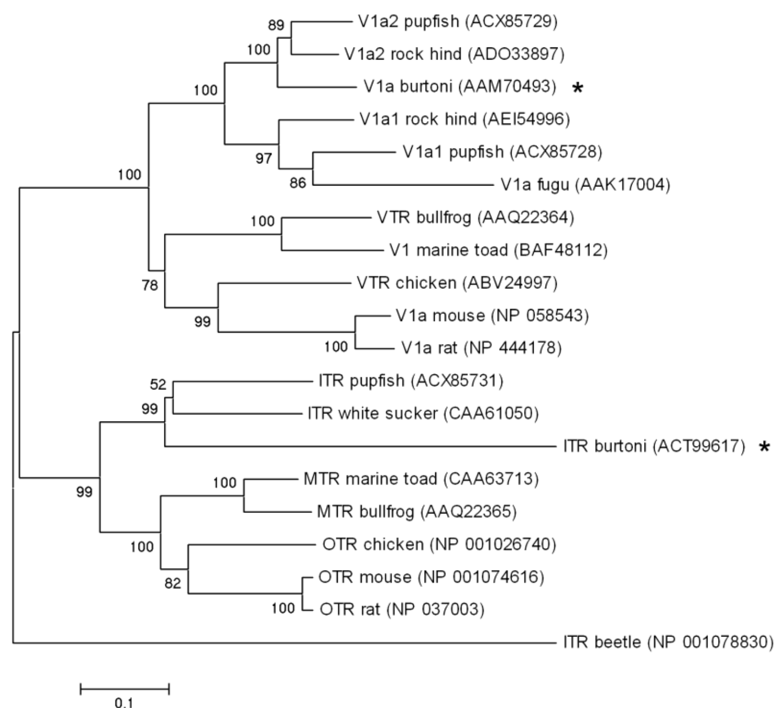

Figure 1. Comparison of *A. burtoni* V1a and ITR receptors with orthologous sequences from other vertebrates. The figure shows a neighbor-joining tree based on the alignment of amino acid sequences for AVT/AVP and IST/OT/MT receptors.

In situ **hybridization (ISH)**

Dominant (n=3) and subordinate (n=3) males and females (n=3) were killed by rapid cervical dissection and their brains were rapidly dissected, fresh frozen in OCT Compound (Tissue-Tek, USA) on a block of dry ice, and stored at -80° C. Brains were then sectioned in four series on a cryostat at 20 µm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) that were stored at -80° C for at least six weeks until processing for ISH as in Munchrath and Hofmann (2010) and O'Connell et al. (2011). Due to regions of high sequence similarity in the coding regions, probes for receptors were designed to exclude the transmembrane region (see Table 2 for primer sequences). The template used to make the V1aR probe was 142 bp in length, and the

Probe	Primer
V1aR	F- 5’ GACAGTAGCCTCCGCAGAAC
	R- 5’ TTAACAGGGAAGGGTGTTCG
ITR	F- 5’ GGCATCTGTTCCAGGATCTTA
	R- 5’ TGTGATGCTCCTCTGACTGC

Table 2. Primers for *in situ* hybridization probes. Forward and reverse primers for V1aR and ITR probes are written 5’ to 3’.

ITR probe was 158 bp in length. Experimental slides were exposed to anti-sense fluorescein-labeled probe, whereas control slides were incubated with sense fluorescein-labeled probe. After the overnight hybridization, slides were processed for detection of mRNA by non-radioactive, non-fluorescent detection. Sections were washed in a series of 0.2x SSC washes at 65° C and equilibrated in 150 mM NaCl/100 mM Tris (pH 7.5) at room temperature before incubation in 1:1000 anti-fluorescein-alkaline phosphatase Fab fragments (Roche) in 0.05% Tween 20/PBS for 2 h at room temperature. Sections were then washed in 150 mM NaCl/100 mM Tris (pH 7.5). Chromogenic product was formed using BM Purple (Roche) at room temperature until desired darkness was achieved and was terminated simultaneously for all slides within a gene group. Slides were then washed, dehydrated in an ethanol series ending in xylene, and cover-slipped with Permount (Fisher Scientific).

Immunohistochemistry (IHC)

Dominant (n=6) and subordinate (n=6) males were killed and their brains rapidly dissected and incubated in 4% paraformaldehyde in 1X PBS; pH 7.4 at 4°C overnight. Brains were then washed in 1X PBS and cryoprotected in 30% sucrose in 1X PBS

overnight at 4°C before embedding in OCT Compound (Tissue-Tek, USA), and then stored at -80°C. Brains were then sectioned in four series on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) that were stored at -80°C until processing for IHC as in Munchrath and Hofmann (2010). Sections were incubated in primary antibody (AVT 1:10000, IST 1:5000; V1a 1:500, ITR 1:500, see Table 3 for antibody details) in PBS with 2% normal goat serum and 0.3% Triton-X at room temperature overnight.

1°	**Antigen**	**Supplier**	**Source**	**IHC dilution**	**Type**
Anti-AVT R-82 antiserum	AVT: CYIQNCPRGA	Custom-made[1]	Synthetic vasotocin	1:10000	Polyclonal
V1a2R	V1a2: IKYKKRKSTAGAANK	Custom-made[2]	Rockhind grouper	1:500	Polyclonal
IST	OT:CYIQNCPLG	Millipore	Synthetic oxytocin	1:5000	Polyclonal
ITR	OTR:3rd intracellular loop of human OTR	MBL	Human	1:500	Polyclonal

Table 3. Antibody information. [1]Antiserum kindly provided by Dr. F. van Leeuwen, Netherlands Institute for Brain Research, Amsterdam. [2]See Kline et al. (2011) for details.

Brightfield visualization for receptors: Sections were rinsed, incubated for 2 hours in a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories), rinsed again and, after treatment with the ABC peroxidase staining kit (Vector Laboratories) according to the manufacturer's instructions, immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) substrate (Vector Labs). Sections were then dehydrated and cover-slipped with Permount (Fisher Scientific, Itasca, IL). For control sections, all procedures were the same except that primary antibody was omitted.

Fluorescence visualization of neuropeptides: Sections were washed twice with 1XPBS, and exposed to both Alexa Fluor 488 goat anti-guinea pig (Invitrogen) and goat anti-rabbit Cy3 (Jackson Immunoresearch) secondary antibodies (1:200, 2% normal goat serum, 0.3% TritonX-100) for 2 hours, and washed again in 1XPBS. The sectioned were then cover-slipped with DAPI hardset fluorescent mounting medium (Vector Laboratories). For control sections, all procedures were the same except that primary antibody was omitted. Additional controls included pre-incubation with either AVT or IST peptides to ensure the specificity of the antibody to the appropriate peptide (Figure 2A,B).

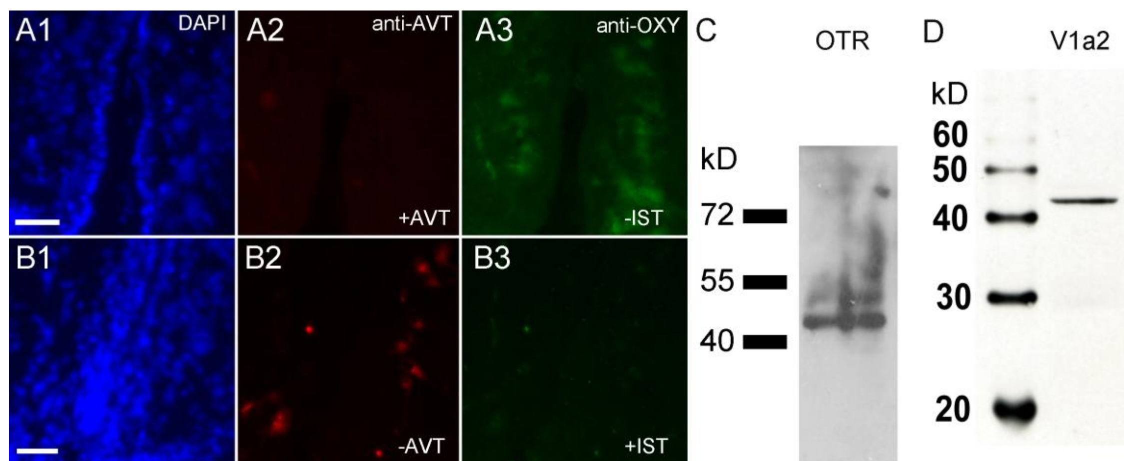

Figure 2. Confirmation of antibody specificity. Immunoprecipitation of the vasotocin (AVT) and oxytocin antibodies with either AVT (A) or isotocin (IST, B) peptides. All scale bars are shown at 20 μm. Western blot of the oxytocin receptor (OTR, C) and V1a2 receptor antibody (D) against *A. burtoni* whole brain protein extract. Ladder units are in kD.

Verification of OTR antibody specificity

We used a commercial OTR antibody (MBL International, Woburn, MA, Cat No. LS-A246) whose antigenic sequence is contained within amino acids 220-270 of the human OTR (Genbank NO_000907.2), corresponding to the 3rd intracellular loop. The *A.*

burtoni ITR sequence that corresponds to this region is YGISFKIWQNFKLKTRREQCINLTPKTTKSNTLARVSSVKL and the amino acids that are identical to human OTR are underlined. To determine whether the OTR antibody would bind specifically to the cichlid antigens, we extracted protein from *A. burtoni* whole brain using a Mammalian Cell Lysis kit (Sigma) according to the manufacturer's instructions. Whole brain protein extract was run on an SDS-PAGE gel and then was transferred onto a nitrocellulose membrane overnight. The membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20 in 1X Tris-buffered saline [TBS]) for 30 minutes and then incubated in primary antibody (1:2000 OTR in 1X TBS and 2% NaN3) for one hour. After incubation, the membrane was washed five times for three min each in wash buffer, and then incubated in goat-anti-rabbit HRP-conjugated antibody (Santa Cruz) in blocking solution for 30 min. After washing five times for three min each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western, Millipore) and exposed to film for ten min. Using the OTR antibody, one band was visualized at the predicted size of 45 kD, putatively representing cichlid ITR (Figure 2C). To predict protein size for ITR, we used the full ITR amino acid sequence of the Amargosa pupfish (*Cyprinodon nevadensis amargosae*; Genbank accession number ACY07774) and the Science Gateway protein molecular weight prediction tool at http://www.sciencegateway.org/tools/proteinmw.htm.

Verification of V1a2 antibody specificity

The V1a2 antibody was raised in rabbit against a 15 AA sequence corresponding to the 3rd intracellular loop of the rock hind V1a2 receptor (Kline et al. 2011). To test the specificity of this antibody in the cichlid brain, we performed a western blot analysis. One male and one female cichlid were killed by an overdose of MS-222 (1 g l-1) and the

whole brain removed. Protein was extracted using the Qproteome Mammalian Protein Prep Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Extracted protein (15 μg) was re-suspended in 1X reducing loading buffer (Pierce, Rockford, IL) and boiled for 10 min, then loaded and run on a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel in duplicate, followed by overnight transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were washed three times for 5 minutes with PBS-T (20 mM phosphate base, 150 mM NaCl, 0.1% Tween-20, pH 7.6) and immersed in blocking buffer (5% normal goat serum and 0.5% porcine gelatin in PBS-T) for one hour at room temperature. Membranes were rinsed in PBS-T and incubated overnight at 4° C with AVTr antibody or antibody pre-absorbed overnight with 1 μg of antigen peptide to 1 μl antibody at a final dilution of 1:1000 in PBS-T. Following primary antibody incubation, membranes were washed 3 X 5 min with PBS-T and incubated with a secondary goat anti-rabbit antibody linked to horseradish peroxidase (AbCam, Cambridge, MA) at a final concentration of 1:5000 in PBS-T with 5% nonfat milk for two hours at room temperature. Membranes were washed 3 X 5 minutes with PBS-T, and immunolabelled band(s) were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL) and ECL hyperfilm (Amersham, Piscataway, NJ). This analysis revealed a single band of ~45 kDa, which corresponds to the expected size of the V1a2 protein (Figure 2D). Results for the male and female samples were identical, though only the male band is shown. Additional controls included a preabsorption of the antibody with the antigen peptide prior to immunohistochemistry, which blocked all signal.

Photomicroscopy

Brightfield optics were used to visualize immunohistochemical staining throughout the brain at low (5X) and high magnification (10X). Photographs 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. Fluorescence signal was detected using a Zeiss AxioImager.A1 AX10 microscope equipped with GFP, rhodamine, and DAPI filters to allow visualization of the fluorescent antibodies and DAPI counter-stain. Photographs were taken in each DAPI, GFP and rhodamine channels as described above, imported into Adobe Photoshop CS3 and assembled.

RESULTS

In the following, we present a distribution map along with photomicrographs of representative brain areas for arginine vasotocin (AVT), the AVT V1a receptor (V1aR), isotocin (IST), and the isotocin receptor (ITR). For each representative section of the map, the nomenclature is displayed on the left side with the peptide distribution while the receptor distribution is presented on the right side. The degree of shading qualitatively represents the density of mRNA expression in that region. The density of dots representing protein indicates qualitatively the density of cells positive for the protein of interest. The general patterns shown here are representative of both dominant and subordinate males for IHC and dominant males, subordinate males, and females for ISH (notwithstanding possible quantitative differences, which we do not investigate here). Overall, the mRNA detection via *in situ* hybridization and protein immunohistochemistry staining for nonapeptide receptors showed high concordance. Control slides that either

omitted antibody for immunohistochemistry or hybridized with sense probes for *in situ* hybridization showed no specific signal. As fiber detection of nonapeptides depends on tissue treatment (e.g., fixation length), we report only fiber distributions that were consistent across individuals. Note that we do not consider these results to comprise the entire extent of nonapeptide innervation of the teleost brain (see Dewan et al., 2009).

Forebrain

Robust expression of V1aR and ITR protein and mRNA is seen throughout the telencephalon, diencephalon, and mesencephalic structures of *A. burtoni*. However, AVT and IST-producing cells are restricted to the preoptic region, and fibers are distributed throughout the hypothalamus. In general, V1aR and ITR show similar patterns of mRNA expression and consistently overlap with protein immunoreactivity.

Telencephalon: Strong signal for V1aR and ITR protein and mRNA is found in discrete parts of the dorsal and ventral telencephalon (Figure 3). There is robust expression of receptor mRNA and protein in the granule cell layer of the olfactory bulb (OB, Figure 3A). Relatively fewer cells are immunoreactive to V1aR and ITR in the glomeruli region, although we did not observe receptor mRNA in this region. In the dorsal telencephalon, there are cells expressing V1aR and ITR including the central, dorsal, lateral, medial, and posterior parts (Dc, Dd, Dl, Dm, and Dp, respectively, Figures 3 and 4). Subdivisions within these regions with heavy staining of both receptors are the granular part and the ventral part of Dl (Dlg and Dlv). V1aR immunoreactivity is nearly absent in the dorsal part of Dl (Dld), Dc-2, and Dm2r while there are more cells positive for ITR protein in these regions. The same is true for mRNA, with the exception of the

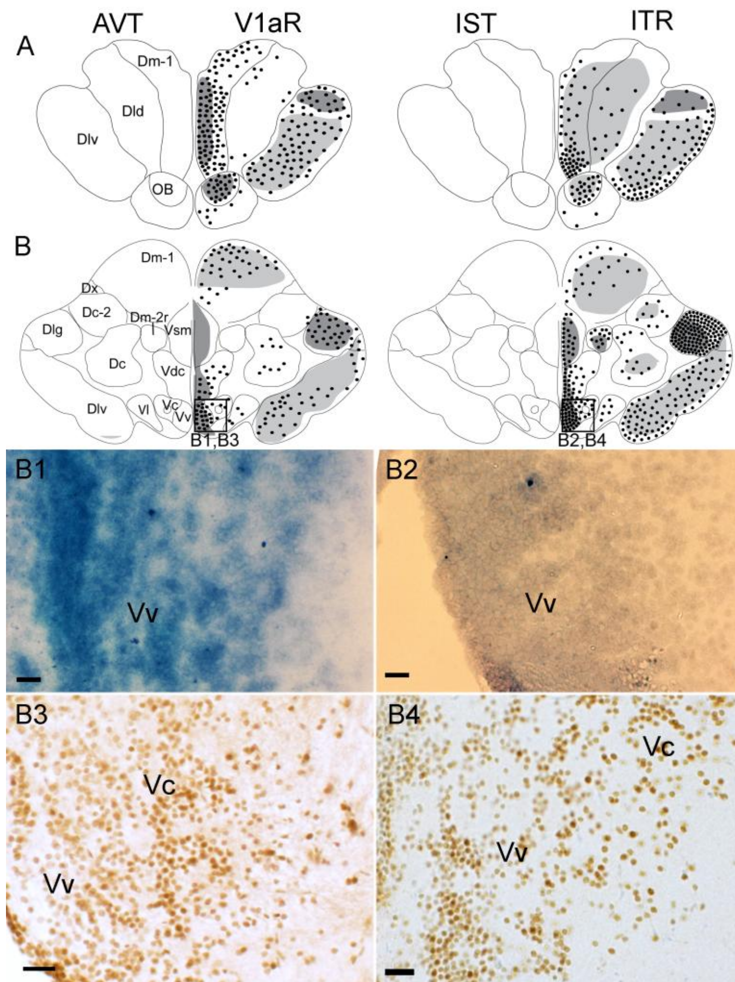

Figure 3. Distribution of neuropeptide systems in the rostral telencephalon of *A. burtoni*. The first panel of representative sections of rostral telencephalon depict the distribution of vasotocin (AVT, left side) and the V1a receptor (V1aR, right side) while the second column depicts the distribution of isotocin (IST, left side) and the isotocin receptor (ITR, right side). mRNA is shown as shading while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the density of expression, while the density of dots indicating protein corresponds to the density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (B1, B2) and in the bottom row show V1aR and ITR protein in the ventral and central part of the ventral telencephalon (B3, B4). All scale bars are shown at 20 μm.

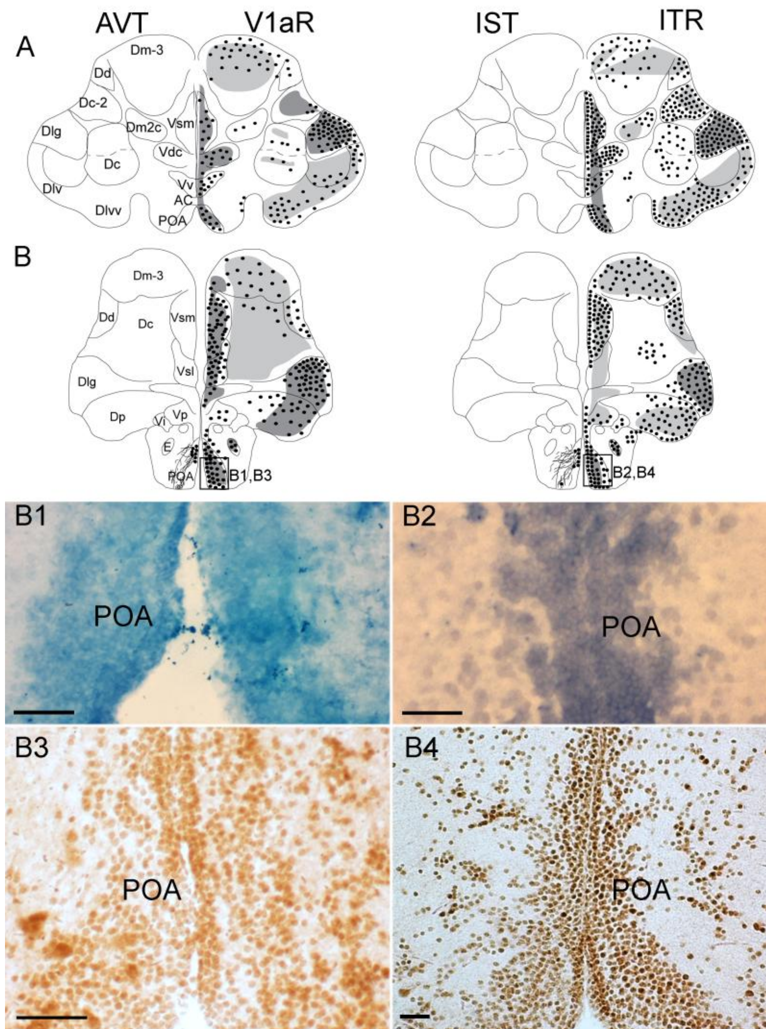

Figure 4. Distribution of neuropeptide systems in the caudal telencephalon of *A. burtoni*. The first panel of representative sections of caudal telencephalon show the distribution of vasotocin (AVT, left side) and the V1a receptor (V1aR, right side) while the second column depicts the distribution of isotocin (IST, left side) and the isotocin receptor (ITR, right side). mRNA is shown as shading while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the density of expression, while the density of dots indicating protein corresponds to the density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (B1, B2) and in the bottom row show V1aR and ITR protein in the preoptic area (POA, B3, B4). All scale bars are 50 μm.

dorsal region of Dc-2, where both V1aR and ITR are well represented. There are two distinct cell groups in Dc that are positive for V1aR and ITR (Figure 4). Overall in the dorsal telencephalon, ITR mRNA is more widely distributed than V1aR.

Within the ventral telencephalon, there is staining of both neuropeptide receptor-immunoreactive cells within the ventral, central, dorsal, lateral, postcommissural and supracommissural parts (Vv, Vc, Vd, Vl, Vp, and Vs, respectively; Figure 3-4). Similar to the dorsal telencephalon, ITR immunoreactive cells are more abundant than V1aR immunoreactive cells. V1aR immunoreactive cells in the medial region of Vs (Vsm) are found in more caudal areas of this region. Both receptor mRNAs are widely distributed throughout Vsm, but ITR protein is not present in the lateral region of Vs (Vsl, Figure 4). Finally, both V1aR and ITR protein and mRNA expression of both receptors are present in the entopeduncular nucleus (E).

The preoptic area (POA) has very heavy staining of V1aR and ITR protein and mRNA as well as AVT and IST peptides (Figure 5). The teleost POA has three cell populations that play distinct roles in modulating behavior (Greenwood et al., 2008):

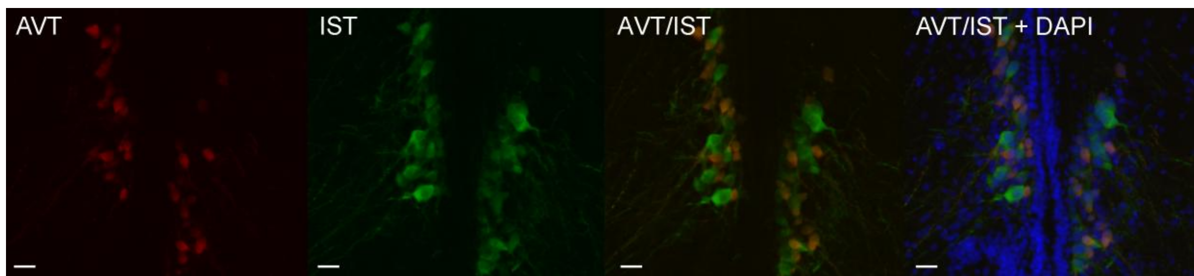

Figure 5. Neuropeptides AVT and IST co-localize in the preoptic area of *A. burtoni*. Arginine vasotocin (AVT, first panel) and isotocin (IST, second panel) are present in the preoptic area (POA). Many of the neuropeptide cells co-localize the in the POA (third panel) indicated by the yellow-orange cells. However, only a subset of POA cells contain neuropeptides as shown with the DAPI counterstain (last panel). All scale bars are shown at 20 µm.

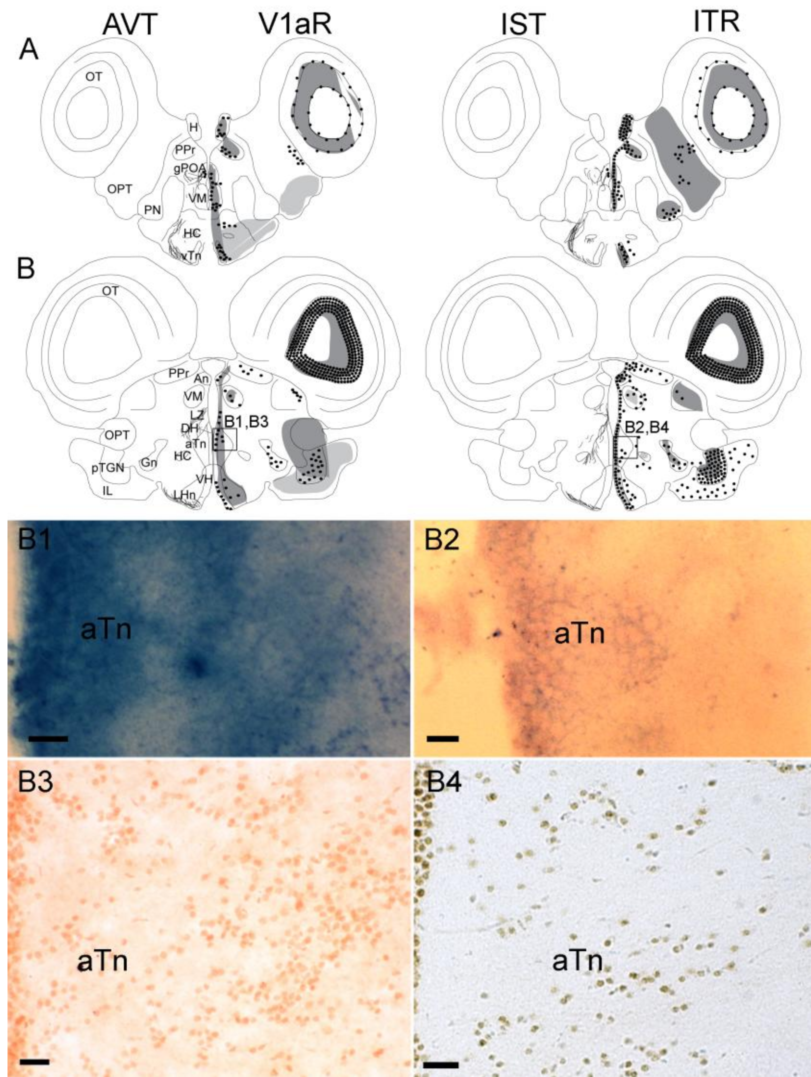

Figure 6. Distribution of neuropeptide systems in the rostral diencephalon of *A. burtoni*. The first panel of representative sections of rostral diencephalon show the distribution of vasotocin (AVT, left side) and the V1a receptor (V1aR, right side) while the second column depicts the distribution of isotocin (IST, left side) and the isotocin receptor (ITR, right side). mRNA is shown as shading while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the density of expression, while the density of dots indicating protein corresponds to the density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (B1, B2) and in the bottom row show V1aR and ITR protein in the anterior tuberal nucleus (aTn, B3, B4). All scale bars are 20 μm.

parvocellular, magnocellular, and gigantocellular neurons. The AVT and IST neuropeptide proteins as well as the V1aR and ITR proteins and mRNA are present in each of these cell types.

Diencephalon: The pattern of both V1aR and ITR expression show extensive overlap, similar to patterns seen in the telencephalon, although the diencephalic patterns of both receptors are more diffuse than those seen in the telencephalon (Figure 6). Caudal to the POA, V1aR and ITR protein and mRNA are found in the habenula (H). mRNA and protein for the receptors are also found in the ventromedial thalamic nucleus (VM). Several periventricular pretectal nuclei also contain V1aR and ITR mRNA and protein including the rostral, dorsal, and ventral regions (PPr, PPd, and vPPn, respectively), with the exception of PPd, which contains only protein. Within the prethalamic nucleus (PN), which lies ventrolateral to VM, we found ITR protein and mRNA but no V1aR. Both V1aR and ITR protein and mRNA are abundant in the ventral tuberal region of the anterior ventral hypothalamic nuclei (vTn; Figure 6). Both receptors are also found in the anterior tuberal region, but only mRNA for V1aR is present (aTn; Figure 6). Protein for both receptors is found in several periventricular hypothalamic regions including the ventral hypothalamus (VH), lateral hypothalamic nucleus (LHn), and the dorsal hypothalamus (DH). There are also fibers immunoreactive to AVT and IST in the vTn, aTn and LHn. mRNA for both receptors is also found in VH, LHn, and DH, but mostly in the more caudal portions. Lateral to these regions, V1aR and ITR protein are found within the inferior lobe including the central (Cn) nucleus; mRNA and protein for both receptors are also found in the diffuse nuclei (Dn) (Figure 7). V1aR and ITR protein and mRNA are also found in the periventricular nucleus of the posterior tuberculum (TPp; Figure 7B), posterior tuberal nucleus (pTn; Figure 8) and the thalamic region, central posterior thalamic nucleus (CP; Figure 7A). Both neuropeptide receptors are also found

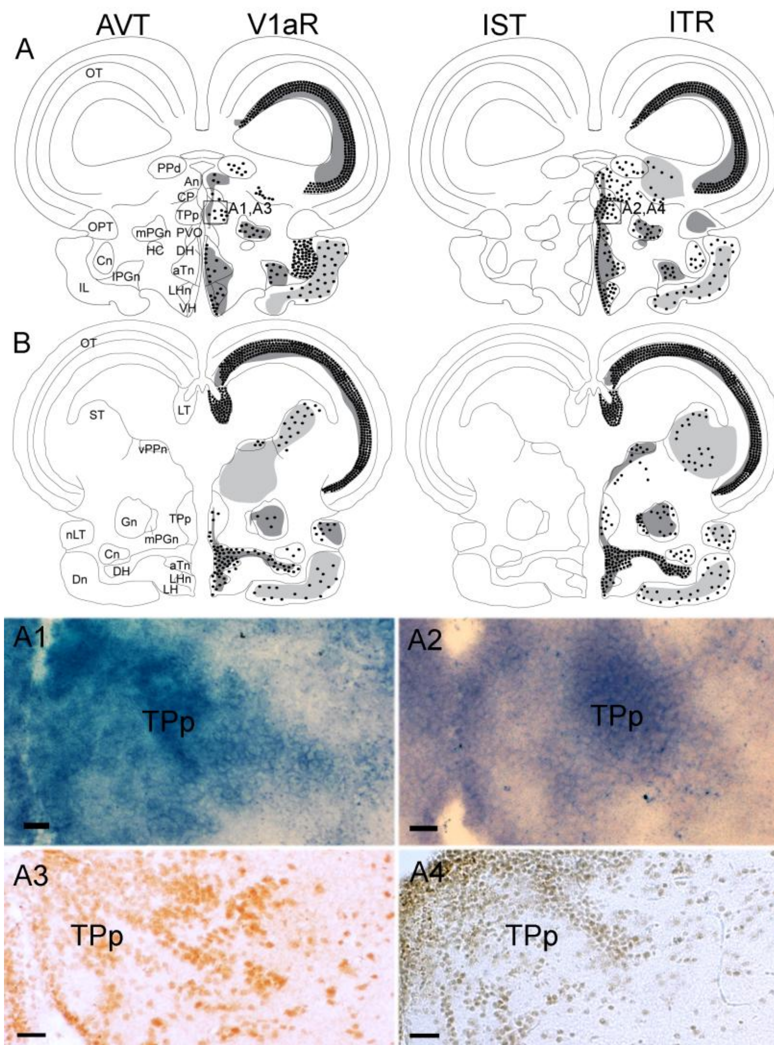

Figure 7. Distribution of neuropeptide systems in the diencephalon of *A. burtoni*. The first panel of representative sections of the diencephalon shows the distribution of vasotocin (AVT, left side) and the V1a receptor (V1aR, right side) while the second column depicts the distribution of isotocin (IST, left side) and the isotocin receptor (ITR, right side). mRNA is shown as shading, while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the density of expression, while the density of dots indicating protein corresponds to the density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (A1, A2) and in the bottom row show V1aR and ITR protein in the posterior tuberculum (TPp, A3, A4). All scale bars are 20 μm.

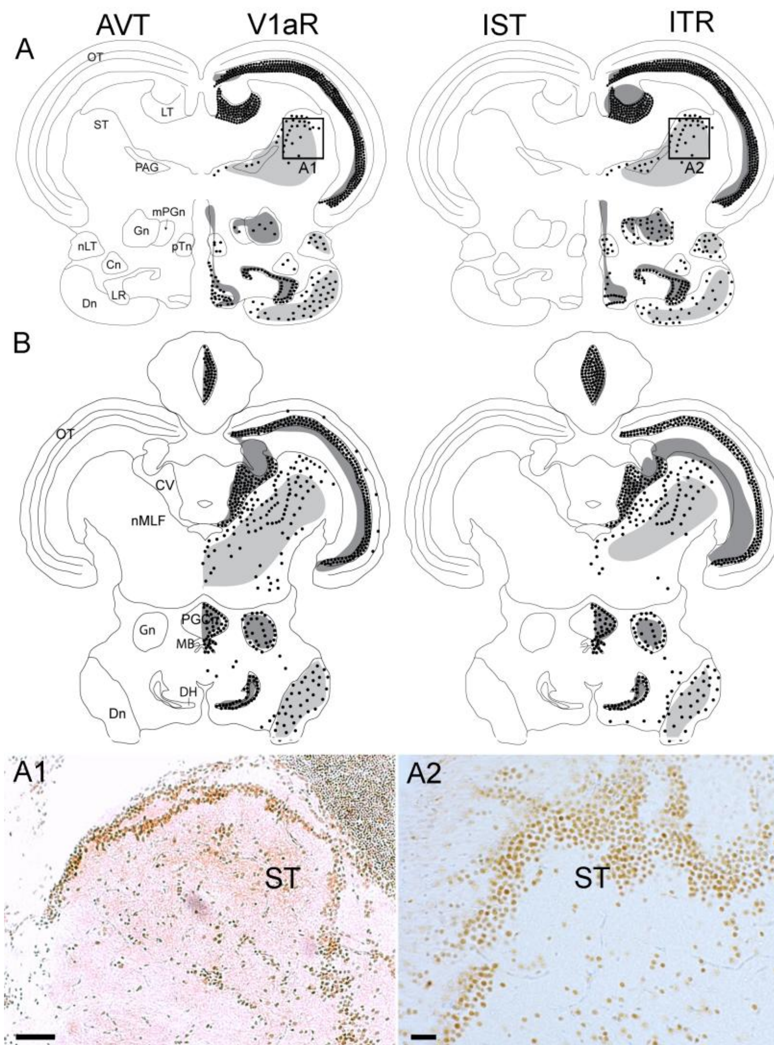

Figure 8. Distribution of neuropeptide systems in the caudal diencephalon and midbrain of *A. burtoni*. The first panel of representative sections of the caudal diencephalon show the distribution of vasotocin (AVT, left side) and the V1a receptor (V1aR, right side) while the second column depicts the distribution of isotocin (IST, left side) and the isotocin receptor (ITR, right side). mRNA is shown as shading while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the density of expression, while the density of dots indicating protein corresponds to the density of cells positive for immunoreactivity. Micrographs show V1aR and ITR protein in the torus semicircularis (ST, A1, A2). All scale bars are 50 μm.

within the medial preglomerular and glomerular nuclei (mPGn and Gn, respectively). Both receptor mRNAs are present in the mPGn and Gn. V1aR and ITR expression and protein are also present within the lateral torus (nLT). Both protein and mRNA of V1aR and ITR are present within the semicircular torus (ST, Figure 8A) and the periaqueductal grey (PAG; Figure 8A). In the caudal diencephalon, the preglomerular commissural nucleus (PGCn) and the mammillary body (MB) also contain both V1aR and ITR protein and mRNA (Figure 8B).

DISCUSSION

We report that nonapeptide producing cells are restricted to the POA, although they project widely, but that nonapeptide receptors are widely distributed throughout the brain of *A. burtoni*, providing an important foundation for understanding how nonapeptides modulate phenotypic plasticity in cichlids. While cells producing these neuropeptides are localized exclusively to the POA, nonapeptide-positive fiber distributions are moderately distributed (vTn, aTn, LHn) throughout the forebrain. Expression and synthesis of the respective receptors, V1aR and ITR, are distributed widely throughout the telencephalon and diencephalon, providing candidate areas for neuropeptidergic regulation of social behavior in teleost fish.

There was extensive overlap between mRNA expression and protein for the receptors, although Dc and PPd contained V1aR-ir and ITR cells but little to no mRNA expression. Cells in the IL were also immunoreactive for ITR but did not indicate mRNA expression. Finding discrepancies between protein immunoreactivity and mRNA expression in receptor distribution was not surprising, as receptor protein may be located on dendrites far from the cell body where the mRNA is located.

Neuropeptide system distribution compared with other teleosts

The distribution of AVT has been extensively studied in teleosts. Most studies report AVT exclusively in the POA (Van den Dungen et al., 1982; Batten et al., 1990; Holmqvist and Ekström, 1991; Dewan et al., 2008), although some studies have found AVT mRNA in tuberal nuclei of the hypothalamus (Godwin et al., 2000; Goodson and Bass, 2000; Greenwood et al., 2008). On the other hand, IST has received relatively less attention. Studies describing IST distribution in teleost fish report IST immunoreactive cells exclusively in the POA (Van den Dungen et al., 1982; Batten et al., 1990; Holmqvist and Ekström, 1991; Goodson et al., 2003). We also report immunoreactive cell bodies containing AVT or IST exclusively in the POA, supporting the high conservation of nonapeptide-producing cell distribution across teleosts.

Although nonapeptide cells are restricted to the POA, fibers are much more widespread. The extent of fiber detection is somewhat dependent on tissue fixation time, and although we report fibers throughout the *A. burtoni* hypothalamus, this is likely not the extent of the fiber distribution throughout the brain. Studies in other teleosts have shown neuropeptide-immunoreactive fibers to be spread extensively throughout the brain. For example, Batten et al. (1990) found AVT-ir and IST-ir fibers throughout the majority of the brain in the green molly, and Goodson et al. (2003) found a relatively wide distribution of IST-ir fibers in the midshipman.

Compared to the extensive literature on nonapeptide distributions in teleosts, it is surprising that detailed descriptions of their receptors are almost completely lacking. Our study is the first to provide a simultaneous description of both the V1a2 and IST receptor distributions in a teleost brain. However, Lema (2010) used PCR to describe nonapeptide receptor expression in gross brain dissections of the Amargosa pupfish and found V1a2 receptor expression in the forebrain, midbrain, cerebellum, and hindbrain. Recently,

Kline et al., (2011) used *in situ* hybridization and immunohistochemistry (using the same custom-made antibody as in the present study) to describe the distribution of the V1a2 receptor in the rockhind grouper, *Epinephelus adscensionis*, and found a distribution pattern almost identical to the one described here.

Functional implications for nonapeptides and their receptors in teleosts

Numerous studies have investigated the behavioral effects of AVT or IST administration in teleosts. Our description of the nonapeptide receptor distribution now provides a mechanistic framework that facilitates hypothesizes as to where neuropeptides may be acting in the brain to regulate social behavior in teleosts. Here, we discuss nonapeptide regulation of aggression and reproduction in teleosts in the context of receptor neuroanatomy.

Nonapeptide regulation of social behavior in teleosts has been most extensively studied in the AVT system. Administration of AVT or a V1a antagonist (Manning compound) in a variety of species supports the role of AVT in modulating both aggression and courtship in males, although the effect directionality appears to vary with species, social state, and context. In the bluehead wrasse and damselfish, AVT increases aggression in males (Semsar et al., 2001; Santangelo and Bass, 2006), whereas AVT inhibits aggression in the brown ghost knife fish (Bastian et al., 2001) and Amargosa River pupfish (Lema and Nevitt, 2004). AVT administration consistently increases teleost male courtship as seen in the bluehead wrasse (Semsar et al., 2001), male white perch, (Salek et al., 2002) and the brown ghost knife fish (Bastian et al., 2001). Lesion and stimulation studies have identified the Vd, Vs, Vv, and POA (putative homologues of the mammalian nucleus accumbens, bed nucleus of the stria terminalis, lateral septum, and POA, respectively; O'Connell and Hofmann, 2011) as potential neural substrates of

aggression and courtship behavior in teleosts (Demski and Knigge, 1971; Macey et al., 1974; Kyle and Peter, 1982; Satou et al., 1984). V1aR is present in all of these regions in *A. burtoni*; thus, the neuropeptides may be acting at one or many of these brain regions to modulate aggression and reproduction in teleosts.

The role of IST in mediating social behavior in teleosts is not well understood, and most work with this nonapeptide comes from studies in goldfish and the plainfin midshipman. IST administration in male goldfish induces social approach to a conspecific while AVT had the opposite effect (Thompson and Walton, 2004). Both IST and AVT inhibit vocal communication in the plainfin midshipman, although IST produces this effect in females and nonterritorial males, and AVT produces this effect in territorial males (Goodson and Bass, 2000a). The vocal-acoustic circuitry that regulates these responses in the plainfin midshipman are well described (Goodson and Bass, 2000b), and we have found ITR and V1aR in each of these brain regions in *A. burtoni*, suggesting that neuropeptides can be modulating equivalent brain regions in the midshipman.

Although nonapeptides are well known for modulating affiliation in monogamous voles (Young and Wang, 2004), surprisingly little is known about the role of these neuropeptides in regulating affiliation in other vertebrates, especially teleosts with monogamous mating systems. Nonapeptide regulation of affiliation has been investigated in the monogamous convict cichlid (Oldfield and Hofmann, 2011). A general V1aR/ITR receptor antagonist inhibited affiliative behavior of males toward potential mates, although this treatment did not prevent pair-bond formation and did not disrupt affiliative behavior in an established pair-bond. Although these effects were not as striking as those seen in monogamous prairie vole males (Winslow et al., 1993), the global administration of a broad antagonist could have diluted the effects. V1aR expression in the lateral

septum in male prairie voles seems to regulate both affiliation and paternal care (Liu et al., 2001), and we have found the V1aR in the homologous Vv region in *A. burtoni*, suggesting that this region may facilitate social behavior in cichlids as well.

Comparison of AVT and IST peptide and receptor distributions to tetrapods

The distribution of AVT, IST and their peptide homologues in other vertebrates are vastly different between vertebrate classes (reviewed in Moore and Lowry, 1998). Strikingly, cell bodies producing AVT or IST are restricted to the POA in teleosts, whereas tetrapods have 19 cell groups or more (Moore and Lowry, 1998). This remarkable neuroanatomical expansion of the neuropeptide system in the transition from water to land has been reviewed extensively (Moore and Lowry, 1998; Goodson and Bass, 2001); thus, we will focus the rest of our discussion on receptor distributions. Compared to our understanding of nonapeptide distributions in a variety of taxa, receptor distribution is not widely studied, especially in non-mammalian vertebrates. Distributions of both AVT and IST receptor mRNA have only been described for two species of birds (Leung et al., 2011) and two amphibians (Acharjee et al., 2004; Hasunuma et al., 2010), and, with the exception of the original study by Kline et al (2011), no published studies (with the exception of the present study) describe distributions of these receptors in reptiles or teleosts.

Nonapeptide receptor distributions in every vertebrate class described thus far are very widespread throughout the fore- and midbrain. As nonapeptides play an important role in modulating social behavior across vertebrates, we focus our comparative discussion on two neural networks that are conserved across mammals and that regulate social behavior and/or the evaluation of stimulus salience. Although the homologues to these mammalian brain regions in other vertebrates have been a contentious debate, a

consensus is emerging that points to homologues in other vertebrate classes (Goodson, 2005; Bruce and Braford, 2009; O'Connell and Hofmann, 2011), suggesting that these networks are ancient in the vertebrate lineage.

The social behavior network, originally proposed for mammals but since expanded to all vertebrate classes (Goodson, 2005), is composed of six forebrain regions (mostly hypothalamic) that regulate reproductive behavior, aggression, and parental care (Newman, 1999). This network includes the lateral septum, bed nucleus of the stria terminalis/medial amygdala, preoptic area, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal grey/central grey. The putative teleost homologues to these regions are Vv, Vs, POA, vTn, aTn, and PAG, respectively (Goodson, 2005; O'Connell and Hofmann, 2011). Mammals (Tribollet et al., 1989; Beery et al., 2008; Campbell et al., 2009), birds (Leung et al., 2011), amphibians (Acharjee et al., 2004), and teleosts (present study) all express nonapeptides receptors in the six nodes of the social behavior network, which points to the important and conserved contribution of these receptors to regulating social behavior across vertebrates.

Nonapeptides have recently received much attention in the context of modulating the evaluation of stimulus salience in concert with the dopaminergic reward system (Young and Wang, 2004). The mesolimbic reward system consists of several fore- and midbrain regions and includes the basolateral amygdala, bed nucleus of the stria terminalis, hippocampus, lateral septum, nucleus accumbens, striatum, ventral pallidum, and ventral tegmental area. Within mammals, the prefrontal cortex is also considered part of the reward system, although we do not include it in our comparative discussion, as its evolutionary antecedents are unclear (Reiner, 1986). The putative teleost homologues to these regions are Dm, Vs, Dl, Vv, Vd, Vc/Vl, - no homologue for the ventral pallidum is known – and the TPp, respectively (O'Connell and Hofmann, 2011). Mammals (Tribollet

et al., 1989; Beery et al., 2008; Campbell et al., 2009), birds (Leung et al., 2011), amphibians (Acharjee et al., 2004), and teleosts (present study) all express the nonapeptide receptors in each of these regions, with the exception that birds do not express the mesotocin receptor in the ventral pallidum, and most mammals to not express the V1a receptor in the striatum (Insel et al., 1994; Lakhdar-Ghazal et al., 1995). This comparison suggests that the nonapeptide system may be working in concert with dopaminergic pathways to evaluate stimulus salience in other vertebrates.

An important observation to note is that quantitative variation in neuropeptide receptor expression has been linked to phenotypic diversity in social behavior in mammals (Insel and Young, 2000). Given the lack of species diversity in nonapeptide distribution descriptions in other vertebrate classes, it would be fruitful to examine quantitative variation in receptor expression in regions that modulate social decision-making across many vertebrate species with diverse forms of sociality in order to elucidate how receptor expression covaries with the evolution of social phenotypes.

CONCLUSIONS

We have shown that while AVT and IST nonapeptide production is restricted to the POA, the V1a and IST receptors are widely distributed throughout the forebrain of *A. burtoni*. Our work provides a functional framework on which to test the nonapeptide modulation of behavior. Furthermore, we have shown that these receptors are present in brain regions important for regulating social decision-making, and analysis across a diverse array of species in the future may help to elucidate how variation in social behavior has contributed to the rapid parallel evolution of cichlids.

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Chapter 4: The Role of Arginine Vasotocin in *Astatotilapia burtoni*

INTRODUCTION

Neuropeptide systems are highly conserved across taxa, but their functional roles in social behavior vary widely. Here, we focus on the effects of arginine vasotocin (AVT), the non-mammalian homolog of arginine vasopressin (AVP), on male social status and behavior. Although this nonapeptide is present (as either AVT or AVP) and involved in the regulation of social behavior in all vertebrates studied thus far, its specific role appears to differ between species, sexes, and social contexts (for a review, see Goodson 2008). Historically, AVT has most often been associated with aggressive behavior in males (Ferris et al., 1997; Goodson, 1998; Delville et al., 2000), but it has also been found to be involved in reproductive (Salek et al., 2002), parental (Wang et al., 1994; O'Connell et al., in prep), pair bonding (Winslow et al., 1993; Oldfield & Hofmann, 2011), stress (Engelmann et al., 2004), and non-reproductive affiliative behavior (Landgraf et al., 2003; Young and Wang, 2004), social status (Ferris et al., 1989; Goodson and Bass, 2001; Semsar et al., 2001) and in females as well as males (Filby et al., 2010). Further, the involvement of AVT in male aggression often seems to be dependent on the context (reproductive, parental, affiliative, etc.) in which the aggression is taking place. Although the role of AVT may be conserved in some manner, it is currently unclear how to resolve this body of literature into a functional consensus although several hypotheses have been posited. Most notably, in a recent review of the AVT system, Goodson (2008) proposed that the role of AVT may be in differentiating "positive" social stimuli from "negative" stimuli, a framework that could potentially account for the numerous paradigms and behavioral contexts examined across taxa.

In tetrapod brains, several neuron populations throughout the fore- and midbrain express AVT/arginine vasopressin (O'Connell and Hofmann, 2011); however, in teleost fish, its expression pattern is restricted to the parvo-, magno-, and gigantocellular nuclei of the preoptic area and the hypothalamic lateral tuberal nucleus (Goodson & Bass 2000, Greenwood et al., 2008), although the projection patterns appear as widespread (Goodson & Bass, 2000; Dewan et al., 2011). Neuropeptide receptors are similarly widely distributed throughout the brain in a manner that is remarkably conserved across major vertebrate lineages (O'Connell & Hofmann, 2011). Importantly, quantitative variation of AVP V1a receptor expression in particular has been associated with variation in mating system in *Microtus* voles (for a review, see Hammock and Young, 2002) and cichlid fishes (Oldfield et al., in prep).

In teleosts, several AVT receptor subtypes have been examined in the Amargosa pupfish, *Cyprinodon nevadensis amargosae*, and classified as V1a1, V1a2, and V2 receptors based on amino acid homologies (Lema, 2010). The two distinct forms of the V1a subtype (probably a result of the teleost-specific whole genome duplication) are expressed in the forebrain, midbrain, cerebellum, and hindbrain (Lema, 2010; Kline et al., in prep). Kline et al. (2011) and Huffman et al. (2011) have recently described the distributions of the V1a2 subtype throughout the brain of the rock hind grouper, *Epinephelus adscensionis*, and the model cichlid *Astatotilapia burtoni*, respectively, and found almost identical patterns. Also, the V1a2 subtype is much more highly expressed in the brain compared with the V1a1 subtype, and the expression of the V1a2 subtype is more closely associated with sex, reproduction, and behavior (Kline et al., in prep).

We investigated the role of AVT in an African cichlid fish, *Astatotilapia burtoni*, due to its simple neural expression pattern and tractable yet complex social system. *Astatotilapia burtoni* is a polygynous species in which the males can be either dominant

or subordinate. Dominant (territorial, T) males are highly aggressive, brightly colored, territorial, and reproductive; subordinate (non-territorial, NT) males are non-aggressive, cryptically colored similar to females, shoal with females, and non-reproductive. This social status is a plastic trait, with males switching status every 4-7 weeks (Hofmann et al., 1999), and can be reproduced reliably in captivity. This remarkable plasticity has been well characterized on the behavioral and hormonal levels (Maruska and Fernald 2010; Huffman et al., in review), but the genetic mechanisms responsible are still unclear. A study by Greenwood et al. (2008) showed that AVT expression in the brain varies with social status, with T males having higher expression than NT males, which is in concordance with other vertebrate studies demonstrating higher AVT levels in dominant than subordinate male phenotypes. More specifically, *A. burtoni* T males were found to have higher expression in the gigantocellular nucleus of the preoptic area, and NT males have higher expression in the parvocellular nucleus of the preoptic area; expression in the magnocellular nucleus does not differ between phenotypes. Although preoptic neurons in general project to the pituitary to regulate secretion of other neuromodulators, and the V1a receptor is present in all three nuclei of the POA (Huffman et al., 2011; see Chapter 3), how this differential expression pattern affects behavior is unclear, as the projection patterns of preoptic AVT neurons from the different nuclei are not currently known. However, these data suggest that AVT plays a role in the regulation of social status and aggression in *A. burtoni*, with different POA nuclei likely subserving different functions depending on an animal's phenotype.

In addition to this suite of behavioral and neuropeptide regulation, T and NT males also differ in their stress axes. Stress is known to suppress the reproductive axis (Pickering et al., 1987), and the social environment that each phenotype is exposed to is drastically different, so this is not surprising. Fox et al. (1997) measured cortisol, a

glucocorticoid stress hormone, in stable pairs and communities of T and NT *A. burtoni* and found that T males had lower cortisol than NT males. Further, males that switched status generally had lower cortisol as a T than an NT. Although this may seem counterintuitive, as the agonistic interactions experienced by a T male appear inherently more stressful than the submissive life of an NT, it is important to note that during the first month of observations, when the community or pair was being established, there was no difference in cortisol levels between phenotypes, suggesting that the harassment received as an NT in a stable community is more stressful than the ritualized dominance displays experienced as a T. The stress axis is also known to be dependent on AVT, as AVT stimulates release of adrenocorticotropic hormone (ACTH), increasing release of corticosteroid hormones such as cortisol (Antonii, 1986). Previous studies in teleost fish have demonstrated that various stressors induce both AVT expression (Gilchriest et al., 2000) and ACTH release (Ruane et al., 1999).

The current study examines the effects of manipulating the AVT system in males and possible differences between social phenotypes. We pharmacologically manipulated the AVT system using an agonist (AVT) and an antagonist to the V1a receptor (Manning compound, MC), the behaviorally relevant AVT receptor (Kline et al., 2011). We manipulated both stable T and NT males to investigate the effects on social status and behavior. The AVT system is often associated with a high degree of plasticity, such as in sex-changing fish, so we also investigated the effects of AVT manipulation on males as they underwent the transition from NT to T. In stable and transitioning males, we examined the relationships between AVT, stress, social status, social behavior, and brain gene expression. Based on the current literature and the finding that AVT is more highly expressed in T males than NT males, we expected AVT to facilitate aggressive behavior

and dominance in these males although the effects in stable males could be dampened due to social reinforcement.

METHODS

Animals

All animals used in this study were adult *A. burtoni* males from a laboratory stock, which was originally derived from a wild population in Lake Tanganyika, Africa (Fernald and Hirata, 1977). Fish were maintained at 28°C on a 12:12 hour light/dark cycle with 10 min dawn and dusk periods to mimic their native tropical environment in 110 liter 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. Tanks were allowed to settle for approximately one week before experiments began. All procedures were in accordance with and approved by Institutional Animal Care and Use Committees at The University of Texas and Harvard University.

Pharmacological manipulations

To investigate the role of AVT in stable males, we tested the effects of AVT and a V1a receptor antagonist (Manning compound, MC) on social status and behavior. We set up communities of ten males and ten females with five terra cotta pots as shelters and allowed them to settle for at least five days. One T male per community was chosen as the focal male, and after being weighed and measured for standard length on Day 1, he was observed for ten minutes on Days 1-3 between the hours of 11:00 and 13:00 to establish a baseline of behavior. Aggressive, reproductive, and neutral behaviors were

recorded as described previously (Fernald and Hirata, 1977) as well as any changes in social status. For each observation, aggressive behaviors (chasing, lateral threat displays, border threats) were summed to comprise an “aggression” score; reproductive behaviors (courting, quivering, digging) were summed to comprise a “reproduction” score. On Days 4-6, all males from the tank were removed to standardize netting stress, and each focal male received an intraperitoneal saline injection using insulin syringes prior to observation to establish any injection effect on behavior. On Days 7-9 prior to observation, each focal male received an injection of either saline (n=11) or AVT (Sigma, 1 μg/gbw; n=10) or MC (Sigma, 3.2 μg/gbw; n=6) dissolved in saline such that each male received only one treatment, for three consecutive days. Doses were based on previous work in bluehead wrasse by Semsar and Godwin (2004). We also tested a range of doses below those previously used (0.5-0.008 μg/gbw, n=6 to 14). Following observation on Day 9, their plasma was collected from the dorsal aorta using heparinized 26G butterfly infusion sets (Surflo) and kept on ice until processing. Following blood collection, the animals were euthanized, and the brains and testes were collected for analysis (see next section).

To test the role of AVT in NT and transitioning males, we set up communities as described previously and chose one NT male per tank as the focal male. After being weighed and measured for standard length on Day 1, he was observed for ten minutes on Days 1-3 at 10:00 to establish a baseline of subordinate behavior. On Day 4, we again netted all of the males in the tank to inject at 8:00 before observing. For the transitioning males, we did not return the T males to their tanks when we removed all of the males to provide NT males with an opportunity to compete for dominance,. This provided open territories for the NT males to compete for on Days 4-6. Focal males were injected with either saline (n=10 NT, 24 transitioning), AVT (n=10 NT, 8 transitioning), or MC (n=9

NT, 10 transitioning) prior to observation at 10:00 such that each male received one treatment, for three consecutive days. Following observation on Day 6, males were weighed, their blood was drawn for cortisol measurement, and they were euthanized for tissue collection.

Hormone measurements and tissue processing

To separate the plasma from the serum, blood samples were centrifuged at 4000 rpm for 10 minutes, and the plasma was stored at -80°C until analysis. Cortisol was measured from plasma samples using ELISA (Assay Designs). Plasma samples were thawed on ice and diluted by a factor of 30 using diluted assay buffer according to Kidd et al. (2010) and manufacturer's instructions before being run in duplicate.After blood collection, animals were euthanized by rapid cervical transection. Their brains were removed and stored in RNAlater (Ambion) at -20°C for qPCR. The testes were removed, weighed to calculate gonadosomatic index (100xgonad mass/body mass), and also stored in RNAlater at -20°C.

Quantitative PCR

Whole brain expression of AVT and the V1a receptor was measured in stable T and NT males as well as in new transitioning T males using quantitative real time PCR, with 18S used as a reference gene. Only saline treated individuals from each pharmacological manipulation experiments were used (stable NT : n=8, NT-T : n=5, Stable T : n=8). The whole brain was dissected, placed in RNA Later (Ambion) and stored at -20C. Total RNA was extracted using Trizol (Invitrogen) and frozen at -80°C. RNA was treated with DNase Amplification Grade I (Invitrogen). RNA content of the

DNAse-treated sample was quantified using Ribogreen quantification (Invitrogen). (Hashimoto et al., 2004). 660 ng of RNA was retrotranscribed in duplicate using Superscript II (Invitrogen) and then pooled for a total of 1320 ng of RNA per fish.

PCR efficiency was verified using a quantitative real time PCR experiment in a RealPlex2 instrument (Eppendorf) for each gene, using a cDNA standard curve made of 7 serial dilutions. The efficiency was calculated using this standard curve with the formula $E = 10^{[-1/\text{slope}]} - 1$, (Pfaffl, 2001) where the slope is calculated from the relationship between the log cDNA quantity of a sample and its quantification cycle (Cq). An efficiency of 1 is optimal. Gene expression was measured by qPCR using a scaled-down version of the manufacturer's protocol: 2 µl of cDNA (diluted 1:10), 12.5µl of SYBR Green PCR Master Mix (Qiagen), 9.5µl of Nuclease Free water (Ambion) and 1 µL of primer pairs (10 µM) in a total volume of 25 µL, in a 96-well plate. All fish were assayed in triplicate on a single plate for a given gene.

Statistical analysis

All statistical tests were run in SPSS software, version 19. We tested for an injection effect on various behaviors in stable T males by comparing Days 4-6 (saline injection) to Days 1-3 (no injection) using t-tests. No significant effect of injection was found in any behavior ($p>0.05$), so for all analyses on stable males, we compared Day 3 (last day of no injection) to Day 9 (last day of drug treatment). Similarly, for NT and transitioning males, we compared Day 3 (last day of no injection) to Day 6 (last day of drug treatment).

Categorical variables such as social status were tested using Fisher's exact tests, which account for small sample sizes. Continuous variables were tested for normality

using the Shapiro-Wilk test; non-normal variables were subsequently log-transformed and retested. Differences in non-repeated normal variables were tested using 1-way ANOVA and subsequent Tukey's HSD post-hoc tests; non-normal variables were tested using Kruskal-Wallis and subsequent Mann-Whitney U pairwise comparisons. To investigate effects of drug treatments on repeated measures (such as behavior), we used a Generalized Estimating Equations (GEE) model and looked for a day*treatment interaction effect. Correlations on normal and non-normal variables were tested using Pearson's and Spearman's correlation coefficients, respectively, and categories such as social status were controlled for using partial correlation coefficients.

RESULTS

AVT in Stable Males

Most of the stable T males treated with AVT (9 of 10) lost their social dominance status, whereas only 2 of 11 saline-treated Ts lost their social status (Fisher's exact test: statistic=0.03, $p=0.002$), but blocking V1aR using MC had no effect on social status relative to saline controls (1/6 vs. 2/11; $p=0.75$; Figure 1A). We next examined aggressive behavior, which is characteristic of the T phenotype, and found a decrease in T male aggression after being treated with AVT (GEE day*treatment effect, $p<0.001$; Figure 1C), but no effect after MC treatment. The status of stable NT males was unaffected by either treatment (Fisher's 1-sided exact test: $p=0.500$, 0.526 for AVT and MC, respectively; Figure 1B). There were no significant effects on aggressive behavior in stable NT males, as it was extremely low in all treatment groups (GEE day*treatment effect, $p=0.525$; Figure 1D).

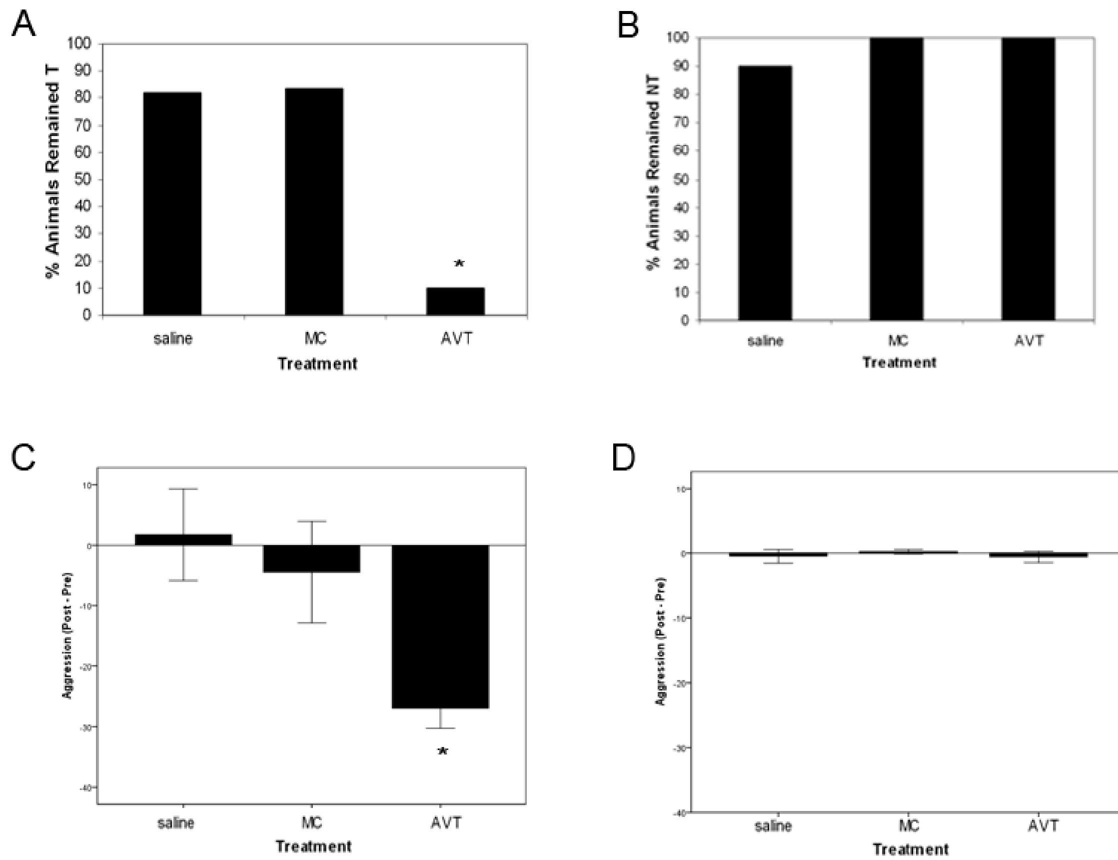

Figure 1. Effects of AVT on social status and behavior in stable males. A) Percentage of T males that remained T following treatment of saline, MC, or AVT. B) Percentage of NT males that became T following treatment of saline, MC, or AVT. C) Change in aggressive behavior in T males following treatment of saline, MC, or AVT. D) Change in aggressive behavior in NT males following treatment of saline, MC, or AVT. Asterisks denote statistically significant differences.

Although the T males treated with AVT lost their dominance status, it was clear from our behavioral observations that they did not become NT. In fact, the treatment left the animals lethargic and unresponsive for up to several hours. In addition, they displayed dark vertical bars along their bodies, which is a typical stress response in *A. burtoni* and other haplochromine cichlids. After AVT treatment, T males also spent significantly less time feeding than saline-treated NT males (Mann-Whitney U_{20}, p=0.040). This

behavioral response was also observed following a range of lower doses, with even the lowest dose (0.008 μg/gbw) causing the majority of males (6/9) to lose their territories. To investigate this apparent stress response further, we measured plasma cortisol levels and found that AVT-treated T males had significantly higher cortisol than either saline- or MC-treated males (Tukey's HSD, $p=3.75*10^{-4}$ and $7.45*10^{-4}$, respectively; Figure 2A). NT male cortisol levels were also significantly increased following AVT treatment relative to controls (Mann-Whitney U, $p=0.004$; Figure 2B).

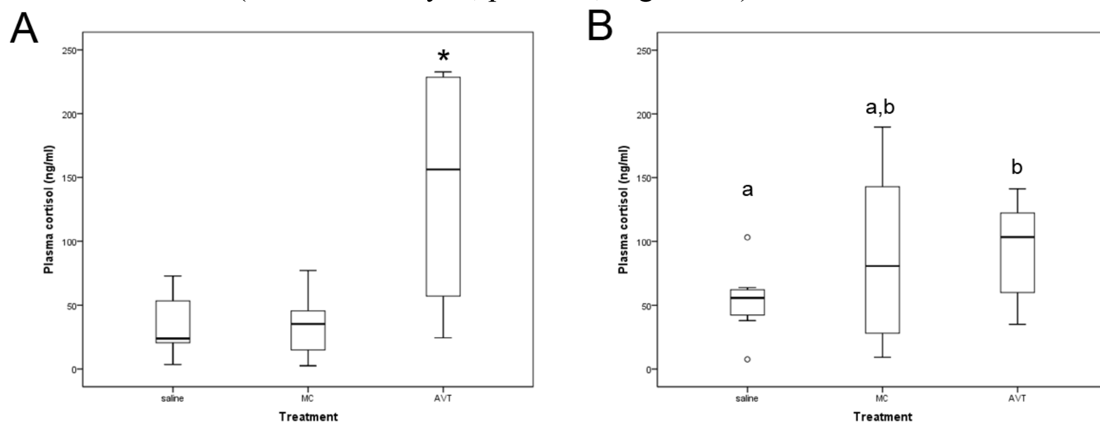

Figure 2. Plasma cortisol levels in stable males following treatment. A) Cortisol levels in T males following treatment of saline, MC, or AVT. Asterisk denotes statistically significant difference. B) Cortisol levels in NT males following treatment of saline, MC, or AVT. Letters denote statistically significant homogeneous subgroups.

AVT and CORT in Transitioning Males

Next, we investigated the role of AVT in NT males given an opportunity to ascend to T status. As expected, these males significantly increased aggression during the three days of social instability (GEE day effect, $p=6.52*10^{-7}$), although ascending control males (saline treatment) did not become as aggressive as stable T control males (Mann-Whitney U, $p=0.012$). As in the stable T males, these transitioning males appeared to have a stress response to AVT treatment, as none of them successfully transitioned to T

(n=8; Figure 3A), and they also showed the characteristic stress coloration and were behaviorally non-responsive. Finally, MC treatment did not have any effect on the likelihood of successfully transitioning: 3 out of 10 MC-treated males and 7 out of 24 saline-treated males became T; Fisher's exact tests p=0.633; Figure 3A). However, when we examined the amount of aggressive behavior displayed by these transitioning males, we found that among individuals that successfully transitioned to T status, those treated

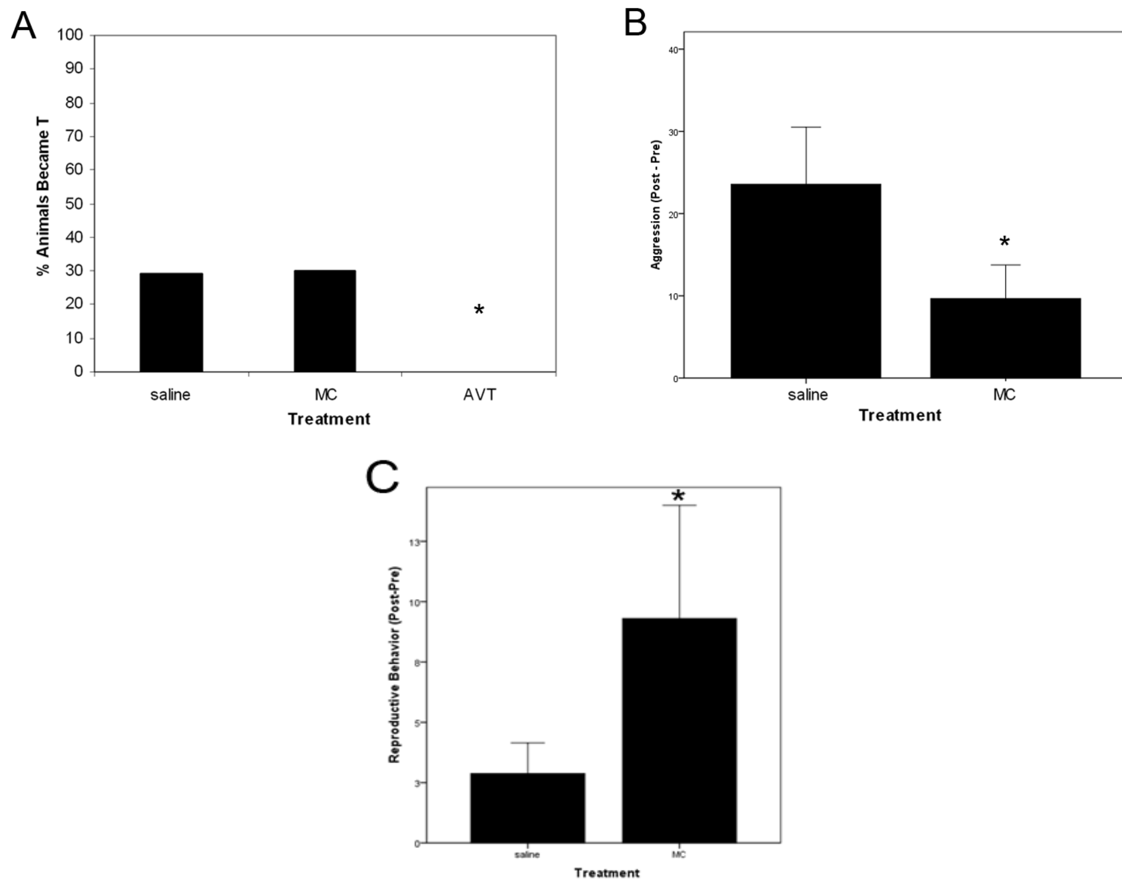

Figure 3. Effects of AVT on social status and behavior in transitioning males. A) Percentage of NT males that successfully transitioned to T following treatment of saline, MC, or AVT. B) Change in aggressive behavior in NT males that successfully transitioned to T following treatment of saline or MC. C) Change in reproductive behavior in NT males that successfully transitioned to T following treatment of saline or MC. Asterisks denote statistically significant differences.

with MC were significantly less aggressive compared with saline-treated males (Mann-Whitney U=19.5, p=0.05; Figure 3B). We also found that among these males, those treated with MC showed more courting behavior (Mann-Whitney U=0; p=0.02; Figure 3C). Note that animals who failed to transition never displayed any courtship behavior.

Because the stress hormone cortisol has been implicated in the regulation of dominance behavior, especially in socially unstable environments (Fox et al., 1997; Sapolsky, 1992), we analyzed plasma cortisol levels in stable NT males, stable T ("established T") males, and males that successfully transitioned to T ("new T"). We found that cortisol levels varied among stable and transitioning males (ANOVA, p=0.031; Figure 4). Cortisol did not differ between established Ts and NTs (Tukey's HSD, p=0.347) but new T males had significantly higher levels than established T males (Tukey's HSD, p=0.027; p=0.245 compared to NT males). Note that cortisol levels did not differ between successful ("new T", n=4) and unsuccessful (n=16) transitioning males (t-test, p=0.563).

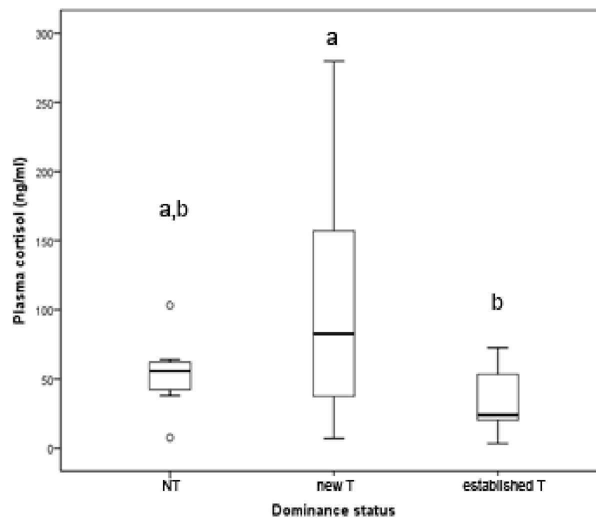

Figure 4. Plasma cortisol levels by social status. Cortisol levels in NT, new T, and established T males following saline treatment. Letters denote statistically significant homogeneous subgroups.

Brain AVT and V1a2R Gene Expression During Social Transition

We then investigated whole brain mRNA levels of AVT and V1aR in the saline-treated controls of NTs, established Ts and new Ts and found that both genes tended to vary in expression between phenotypes (ANOVA, p=0.07, 0.004 for AVT and V1a2R, respectively). To our surprise, and contrary to previous findings (Greenwood et al., 2008; Renn et al., 2008), AVT mRNA levels did not differ between established T and NT males (Tukey's HSD, p=0.892). AVT gene expression was, however, increased in the brains of

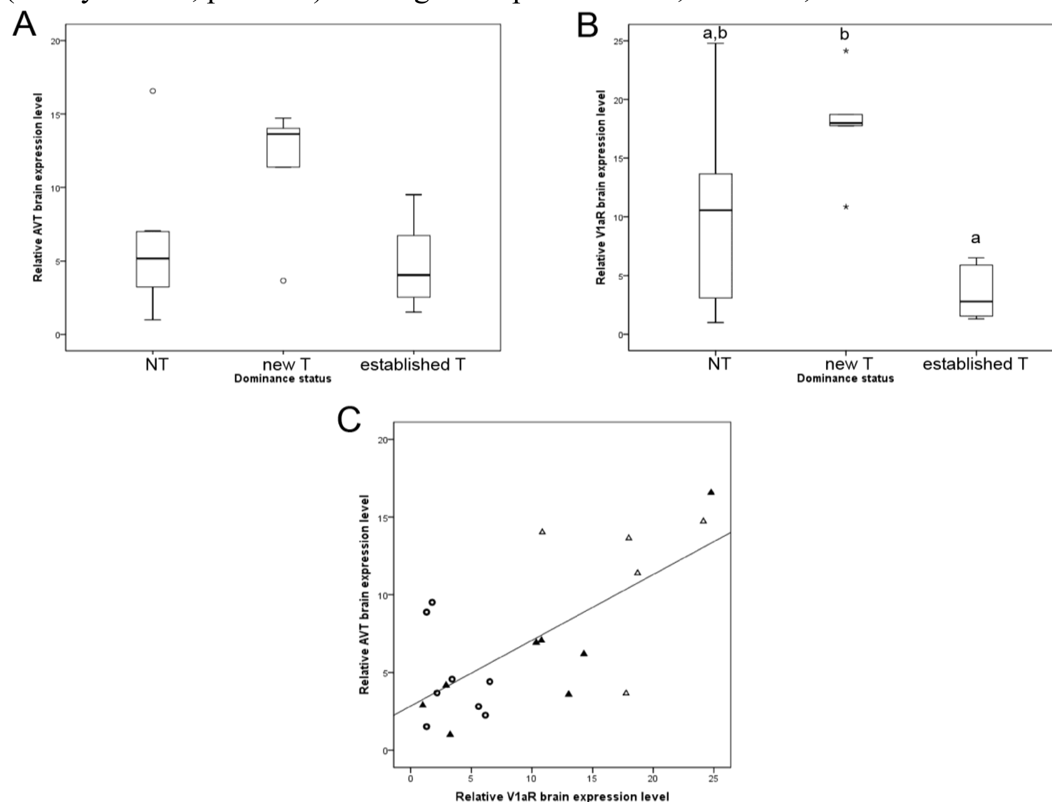

Figure 5. AVT and V1aR expression by social status. A) AVT expression in whole brains of NT, new T, and established T males following saline treatment. B) V1aR expression in whole brains of NT, new T, and established T males following saline treatment. Letters denote statistically significant homogeneous subgroups. C) Linear regression analysis of AVT and V1aR expression levels. Symbols denote different social statuses, with filled triangles (NT), open triangles (new T), and open circles (established T).

new T males compared with established T males, though the difference was not significant (Tukey's HSD, $p=0.07$; Figure 5A). V1a2R mRNA levels were significantly higher in new T males than established T males (Tukey's HSD, $p=0.003$; Figure 5B), with expression levels in NTs in between (Tukey's HSD, $p=0.110$, 0.134 compared to established and new Ts, respectively). Finally, we conducted a partial regression analysis, controlling for initial dominance status (T, NT, or transitioning), and found that AVT and V1a2R expression correlated strongly with each other (Pearson's partial $r^2=0.511$, $p=0.021$, $df=18$; Figure 5C).

DISCUSSION

We have shown here that inhibiting a V1a receptor-like pathway in male *A. burtoni* decreases aggressive behavior and increases reproductive behavior in the first three days of transition from NT to T status but does not affect stable males of either the T or NT phenotype. Administering AVT to male *A. burtoni* elicits a stress response in both behavior and cortisol regardless of phenotype although the increase is strongest in Ts. We have also shown that circulating cortisol and brain expression levels of both AVT and the V1a2 receptor are increased in transitioning males compared to stable males. The effects of the V1a antagonist suggest that endogenous AVT increases aggressive behavior. Although transitioning males had higher AVT and V1a2R expression than stable males, transitioning males were actually less aggressive than stable T males.

Systemic administration of AVT has been used in many other species, including fish, to elicit specific changes in behavior (bluehead wrasse, Semsar et al., 2001; plainfin midshipman, Goodson and Bass, 2000; pupfish, Lema and Nevitt, 2004). However, in *A. burtoni*, a range of doses induced a behavioral stress response. In addition to regulating

social behavior via the V1a receptor, AVT also affects numerous other physiological processes in brain and periphery, such as water balance and osmoregulation via the V2 receptor (Hayashi et al., 1994). It is worth noting that the species of fish that have previously been investigated using systemic administration of exogenous AVT were mostly marine species (with the exception of the pupfish); it is possible that the osmoregulatory system of a freshwater fish such as *A. burtoni* is more sensitive to systemic manipulation of the AVT system. Future studies on freshwater fish may benefit from other methods of administration, such as an intracerebroventricular injection as in Thompson and Walton (2004).

In response to AVT, we found that T males increased circulating levels of cortisol and lost their territories. It was unclear from these results whether the stress response caused the loss of territory, or if losing a territory was inherently stressful. Previous work by Fox et al. (1997) suggested that in *A. burtoni,* plasma cortisol levels increase as a consequence of social defeat, a common phenomenon across vertebrates (Huhman et al., 1991; Overli et al., 1999). When we examined NTs treated with AVT, we found that they also displayed a behavioral stress response and had higher plasma cortisol levels relative to saline controls, suggesting that the stress response was indeed largely due to the AVT treatment. However, the difference in cortisol levels between controls and AVT treated-fish was larger in Ts (~6-fold on average) than NTs (~2-fold), so the experience of territory loss likely contributes to the stress response. Alternatively, it is possible that T males are more sensitive to stressors than NT males, so the same stimulus (AVT injection) would elicit a stronger response in Ts relative to NTs, which is supported by studies in mammals showing a reduced stress response in subordinate individuals (Blanchard et al., 1995).

It has previously been shown that, compared with Ts, NT males have more AVT expression in the parvocellular nucleus of the preoptic area (Greenwood et al., 2008), a brain region associated with social defeat and stress physiology in both mammals (Aubry et al., 1999) and teleosts (Gilchriest et al., 2000). However, NT males did not have higher cortisol levels than T males in our study. Fox et al. (1997) did find a difference in cortisol between T and NT *A. burtoni,* but only after the communities had stabilized for a period of four weeks, not during periods of social instability. The discordance between parvocellular AVT and cortisol in NT males suggests that the vasotocinergic neurons of the parvocellular POA have multiple targets although perhaps a recently defeated male would indeed have more parvocellular AVT expression than a stable NT. The region-specific quantity and distribution of AVT expression in ascending or descending males is not currently known although this information would be helpful in determining the region-specific function(s) of AVT in the brain.

In addition to having unique hormonal and neural expression profiles (Renn et al., 2008), T and NT males also experience vastly different social pressures. For example, NT males are typically non-aggressive, and their subordinate behavior is enforced through constant policing by Ts (Fernald, 1976). Because this strong social reinforcement may severely blunt any potential aggression-increasing effects of pharmacological manipulations in NTs (but see O'Connell & Hofmann, 2011), we also investigated the AVT pathway in NT males given an opportunity to ascend to dominance status ("transitioning males"). By removing the social suppression from these NT males, we have attempted to more clearly elucidate the neuroendocrine basis of social status. AVT has been shown to play in a role in social status across taxa, but we did not find a significant difference among stable males (Ferris et al., 1989; Goodson and Bass, 2001; Semsar et al., 2001). New T males, however, have higher AVT and V1aR expression in

their brains than stable males, indicating a role for AVT in the transition to dominance (as opposed to the maintenance). Normally, transitioning *A. burtoni* males engage mostly in aggressive behavior and less in reproductive behavior (Maruska and Fernald, 2010; Huffman et al., in review). However, when we blocked the V1a receptor in transitioning males, they performed fewer aggressive and more courtship displays than the controls, even though MC did not reduce the overall fraction of males that ascended to dominance successfully. This shift from aggressive to reproductive behavior suggests that AVT might be involved in determining the salience of aggressive and/or sexual stimuli, similar to Goodson's (2008) notion that AVT is necessary to determine which stimuli are "positive" and which are "negative", likely in interaction with dopamine signaling. Clearly, more detailed studies will be necessary to untangle these mechanisms. Alternatively, it is possible that the role of AVT in aggression is specific to transition or, more generally, unstable social environments.

CONCLUSIONS

We have demonstrated that AVT is necessary to successfully become aggressive as males transition from subordinate to dominant and that AVT and the V1a2 receptor are upregulated in males as they transition. By investigating males in both stable and unstable social environments and quantifying behavior, hormones, and gene expression in the brain, we have a greater understanding of the role of this complex neuropeptide in social behavior.

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Chapter 5: Aromatase Modulates Aggressive Behavior in the African Cichlid Fish *Astatotilapia burtoni*

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 have remained elusively complex. Although androgens (principally testosterone) have historically been associated with male aggressive and reproductive behavior, work by Frank Beach (1942) introduced the idea that testosterone may be converted into estradiol before exerting its effects on male sexual behavior. Much later, "the aromatization hypothesis" was formally proposed in 1981 (Mac Lusky and Naftolin), which posits that the effects that androgens exert on male-specific phenotypes are actually mediated by brain-derived estrogens, which are produced by metabolizing testosterone via the enzyme aromatase. Since then, it has become clear that estradiol is as active and important (if not more so) as testosterone in regulating male aggressive (Schlinger and Callard, 1990; Soma et al., 2000; Trainor et al., 2006) and reproductive (Balthazart et al., 2004; Zumpe et al., 1993) 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 (Adkins et al., 1980; Crews et al., 1994; McDonald et al., 1970). Although estradiol clearly has a role in male behavior, the regulation of its synthesis and activity and how this relates to behavior are less well understood.

Aromatase is the product of the *CYP19* gene, a member of the P450 superfamily of cytochrome enzymes. This gene is expressed largely in the brain and gonads, where it converts testosterone into estradiol, but has also been found in other tissues in mammals

(for a review, see Simpson, 2003). Spatial and temporal specificity of expression is attained via variability in tissue- and region-specific promoters and transcription factor binding sites (Forlano et al., 2006; Lephart, 1996). In addition, teleost fish have two isoforms, *CYP19A1* (or *CYP19a*) and *CYP19A2* (or *CYP19b*), which are expressed in the gonads and brain, respectively (Callard and Tchoudakova, 1997), potentially providing an additional mode of local regulation.

Aromatase mRNA expression and activity are unusually high in adult teleost fish (as well as songbirds), especially in the forebrain, where activity has been reported at levels 100-1000 times higher than other vertebrate species (for a review, see Forlano et al., 2006). Thus, the role of aromatase in teleost fish is particularly intriguing, and there have been many studies investigating sex differences in aromatase expression. As estradiol levels are typically higher in females, one might expect aromatase activity to be consistently higher as well, but this pattern is often reversed, depending on the brain region and species in question. For example, aromatase activity in the preoptic area (POA) of the brain is higher in female goldfish (Pasmanik and Callard, 1988) and stickleback (Borg et al., 1987) than males, but the opposite pattern is seen in European sea bass (Gonzalez and Piferrer, 2003) and medaka (Melo and Ramsdell, 2001). To help make sense of these sex differences, teleost fish are especially useful because their extraordinary within-species plasticity allows us to investigate behavioral differences between morphs within a given sex. For example, the plainfin midshipman has two male morphs with very different behavioral phenotypes, including differences in nest-building, courting, reproductive vocal displays, and parental care (for a review, see Bass, 1996). *In situ* hybridization of aromatase in the brain area controlling their reproductive vocal communication (the sonic motor nucleus) has shown that non-courting males have higher mRNA levels that are similar to females than masculinized courting males. As estradiol

does not have an effect on the sonic motor nucleus, it is possible that by metabolizing testosterone at a higher level, aromatase is preventing reproductive “masculinization” of this vocal center in the non-courting males and females (Schlinger et al., 1999). In addition to understanding reproductive behavior, teleost fish have also increased our understanding of the role of estradiol in aggressive behavior (O’Connell et al., 2011). Aromatase has also been implicated in controlling 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 (Goncalves et al., 2008).

Interestingly, in the African cichlid teleost fish *Astatotilapia burtoni*, males have higher circulating estradiol levels than females (Renn et al., 2011), even surpassing the peak seen during egg maturation (Kidd et al., in prep). Male *A. burtoni* display two different phenotypes, a dominant and a subordinate morph; unlike the midshipman, these phenotypes are completely reversible. Males will change social status every 4-7 weeks (Hofmann et al., 1999), which comprises a rapid change in aggression, reproductive status, color, and sex steroid hormones, as subordinate males are phenotypically similar to females (Maruska and Fernald, 2010; Huffman et al., 2011). Although estradiol increases significantly as males become dominant, gonadal aromatase expression does not (Huffman et al., 2011). This is similar to what is seen in songbirds, as high estrogen levels found in males are often not affected by castration, and testicular aromatase expression is low (Schlinger and Arnold, 1991). However, aromatase expression in the brain has been found to be high, and there is evidence that some of the brain-derived estrogen is released into the circulation (Schlinger and Arnold, 1992, 1993). A study by O’Connell et al. (in prep) investigated aromatase expression in *A. burtoni* to determine whether it is differentially regulated between morphs. They quantified expression in five

brain areas important in social decision making (O'Connell & Hofmann, 2011a,b): 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; Moore and Lowry, 1998) regions of the preoptic area (POA). They found that aromatase was differentially expressed only in the gigantocellular portion of the preoptic area (gPOA), with subordinate males expressing more brain aromatase mRNA (CYP19A2) than dominant males (O'Connell et al., in prep), although this pattern is reversed when examined across the whole brain (Renn et al., 2008).

In many vertebrates (for a review, see Balthazart and Ball, 1998), estradiol is known to increase aromatase expression via a positive feedback loop. In teleosts, estradiol and aromatizable androgens have been found to upregulate brain aromatase expression and activity in gonadectomized animals while non-aromatizable androgens have no effect (Callard et al., 2001), but the mechanism by which this occurs is unclear. In birds and mammals, the brain form of aromatase does not appear to contain an upstream estrogen response element (ERE) by which estradiol and binding of its receptor could directly act (Balthazart and Ball, 1998); the promoter region of *CYP19b* in teleosts does contain an ERE (Callard et al., 2001), but the gonadal aromatase also shares this upstream sequence. Further, even though ERα and aromatase have very similar distribution patterns throughout the brain, they do not appear to be extensively co-localized to the same cells. In teleosts, ERα is mostly expressed in neurons of discrete nuclei, and aromatase is primarily (if not exclusively) expressed in radial glia (Diotel et

al., 2010; for a review, see Forlano et al., 2005). Moreover, an RT-PCR study in trout has suggested that glia may express low levels of ERα, and *in vitro* studies using zebrafish have demonstrated the necessity of interactions between ERα and glia for maximum estradiol induction of aromatase expression (Menuet et al., 2005). In addition, the possibility that other estrogen receptor subtypes (teleosts also have two forms of ERβ; Munchrath & Hofmann, 2010) are involved cannot be disregarded. In addition to this feedback loop, ERα also affects aggressive behavior (for a review, see Trainor et al., 2006b). Studies using ERα-knock-out mice have demonstrated that the binding of estradiol to ERα is both necessary and sufficient for aggression in resident-intruder tests (Ogawa et al., 1998; Scordalakes and Rissman, 2003). ERα is also known to be differentially expressed in several brain nuclei involved in male aggression in mammals (such as the bed nucleus of the stria terminalis and the lateral septum; Trainor et al., 2006a). The study by O'Connell et al. (in prep) that investigated aromatase expression in *A. burtoni* also found that, among those five brain regions involved in social decision-making, aggressive dominant males have more ERα-ir cells than non-aggressive subordinate males in only the parvocellular nucleus of the POA (pPOA) and the ventral region of the ventral telencephalon (Vv), the putative homolog of the mammalian lateral septum (O'Connell et al., in prep). Dominant males also have more ERα gene expression in the brain overall (Renn et al., 2008). It is not currently known whether the teleost homolog of the bed nucleus of the stria terminalis (a portion of the supracommissural part of the ventral pallium, Vs) differentially expresses ERα.

Astatotilapia burtoni has been established as an excellent 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 (Renn et al., 2011). Dominant males have high levels of testosterone

(Trainor & Hofmann, 2006) and estradiol (Renn et al., 2011; Huffman et al., 2011) relative to subordinate males and females. They are also extremely aggressive and reproductively active within a community, so we investigated the role of aromatase in behavioral and sex steroid hormone regulation in both stable males and males as they transition from subordinate to dominant. First, we treated dominant males in a naturalistic community with an aromatase inhibitor, fadrozole, to examine the effects on hormone levels and behavior. Second, based on results in this species by O'Connell et al. (in prep) and the importance of these nuclei in social decision-making, we quantified *CYP19A2* expression in the gPOA and *ERα* expression in the pPOA and Vv using radioactive *in situ* hybridization to investigate the effects of fadrozole on transcription and investigated possible relationships with behavior and sex steroid hormone levels. Third, we quantified *CYP19A2* expression in the gPOA and *ERα* expression in the pPOA and Vv of subordinate and dominant males as well as males as they transitioned from subordinate to dominant to create a temporal profile of estradiol regulation. Lastly, we investigated the correlative relationships between gene expression, behavior, testes histology and gene expression, and sex steroid hormone levels in an attempt to integrate these multiple levels of phenotypic change.

METHODS

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 (Fernald and Hirata, 1977). Fish were maintained at 28°C on a 12:12 hour light/dark cycle with 10 min dawn and dusk periods to mimic their native tropical environment in 110 liter 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.

Dose-response curve

To determine the appropriate dose of fadrozole, we set up 30-gallon tanks that were 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 to examine effects on both aggressive and reproductive behavior. Only one male per tank was manipulated at any given time. A stock solution of fadrozole (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 fadrozole was diluted to the appropriate concentration in PBS and stored at 4°C. Our behavioral paradigm consisted of four days (Figure 1A): on Day 1 the focal male was observed at 10:00 for 5 minutes. All aggressive and reproductive behaviors were scored as described previously (Fernald, 1977). 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/gram body weight of fadrozole (n=5 each, volume of 50-150 µL) using an insulin syringe at 9:15 hours. At 10:00 hours, the fish was again observed for 5 minutes to capture any rapid

effects of the drug. On Day 3, the focal male was observed again at 10:00 hours to capture any slower effects of the drug. At 11:00 hours, he was injected again with the same drug. On Day 4, he was observed for a third time at 10:00 hours for 5 minutes to determine if the two consecutive doses from the previous days increased any previously seen effects. The tanks were allowed to rest for two days before the paradigm was repeated using the focal male in the other compartment. The person injecting and observing the animals was blinded to dose/treatment. Behavioral observations showed that aggressive attacks did not change with saline treatment (paired t-test, t=1.513, p=0.205) but decreased after FAD treatment on Day 2, and this difference was greatest at a dose of 10 μg/gram body weight FAD (t=4.765, p=0.009; for 1 μg/gbw, t=2.745, p=0.052).

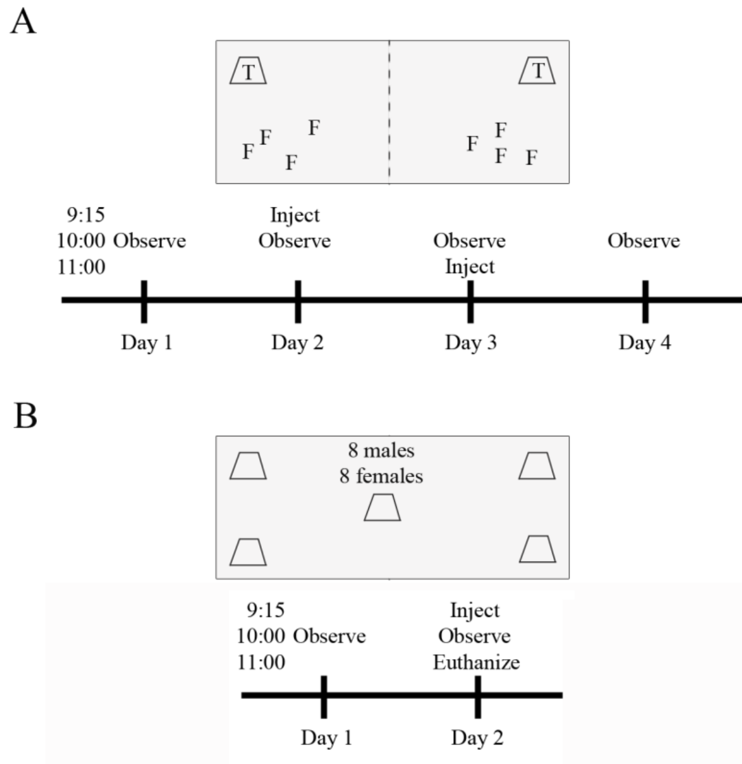

Figure 1: Behavioral paradigm for A) dose-response experiment (T=territorial/dominant male, F=female) and B) main experiment.

Pharmacological manipulations

After an appropriate dose and timeline of efficacy were determined, community tanks were set up containing 5 terra cotta pots, 8 males, and 8 females to test the effects of inhibiting aromatase in a more naturalistic, less restricted paradigm. Only one territorial male per tank was manipulated at any given time. Our final paradigm consisted of only two days (Figure 1B): on Day 1, the focal male was observed at 10:00 for 5 minutes, and behavior was scored as described previously (Fernald, 1977). On Day 2, the focal male was weighed and injected as before at 9:15 with either saline (n=15) or fadrozole (10 µg/gram body weight, n= 14). The male was returned to his tank and observed at 10:00 for 5 minutes, where he remained until approximately 11:00 hours for tissue collection. The tank was allowed to rest for four days before another male was observed, and each tank was used only twice such that the first round of communities contained 8 males, and the second round contained 7 males. Due to the possibility of males changing status in response to competition, the focal male was not replaced. There was no significant effect of round (i.e., number of males) on any behavior.

Tissue collection and hormone analysis

At approximately 11:00 hours, focal males were removed from their tanks to have blood drawn from the dorsal artery using heparinized 26 gauge butterfly infusion sets (Surflo). Blood samples were centrifuged at 4000 g for 10 minutes, and the plasma was transferred to a new tube and frozen at -80°C. We measured both testosterone and estradiol in plasma samples using ELISA (Assay Designs) after diluting the plasma samples 1:30 in assay buffer according to Kidd et al. (2010) and manufacturer's instructions. The coefficients of variation within assay plates ranged from 7 to 14%. Immediately after blood was drawn, males were euthanized via rapid cervical

transsection. Their brains were removed, embedded in OCT (TissueTek), and immediately frozen on dry ice and stored at -80°C until sectioning. The testes were also removed and weighed to calculate gonadosomatic index (testes mass/body mass x 100). Brains and data from stable, untreated males and transitioning males were from a previous study (Huffman et al., 2011; see Chapter 2). Briefly, dominant and subordinate males were taken from stable communities, or subordinate males were given the opportunity to transition to dominance in a semi-natural community containing 2 other males and approximately 8 females. Transitioning males were killed at several time points (3 hours and 1, 5, and 13 days) after behavioral observations, and their plasma, testes, and brains were collected and analyzed for hormone levels, histology, and gene expression to compose an integrative temporal profile of transition.

***In situ* hybridization**

After being embedded in OCT and stored at -80°C, the brains were sectioned into four series at 20 μm, thaw-mounted onto SuperFrost Plus slides (Erie Scientific, Portsmouth, NH), and stored at -80°C. Sections were subsequently thawed, fixed for 10 min in 4% paraformaldehyde, treated with acetic anhydride, dehydrated in a series of ethanol solutions, dried, and stored at -80°C. Probes for *A. burtoni* brain aromatase (*CYP19A2*, GenBank accession number FJ605734; O'Connell et al., in prep) and estrogen receptor alpha (ERα, GenBank accession number AY422089; Munchrath and Hofmann, 2010) were 964 and 788 bp in length, respectively, 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 65ºC for 18 hours in 200 μL of hybridization buffer (Sigma) containing

either 2.0 x 10^6 or 2.5 x 10^6 cpm/slide (for aromatase and ERα, respectively) 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. After hybridization, slides were placed in 65ºC 4X SSC + 1 mM DTT for 5 min to remove cover slips. Slides were then washed at 65ºC in 4X SSC + 1 mM DTT for 1 hr, washed twice at 65ºC in 50% formamide + 2X SSC + 1 mM DTT for 1 hr, washed twice at 65ºC 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 with the aid of a photo-safe red light, dried at 65ºC for 1 hr, and then stored in light-tight boxes at 4ºC. After 3 or 5 days (for aromatase or ERα, respectively), slides were developed for 4 min in Kodak developer at 15ºC, washed in 15°C water for 15 s, fixed in Kodak fixer for 6 min, and then washed in distilled water. Sections were then counterstained with cresyl violet overnight before dehydration in a series of ethanols, clearing in xylene, and cover-slipping in Permount (Fisher).

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.

For quantitative ISH analysis, we followed the protocol described in O'Connell et al. (in prep). For each brain region, we calculated an individual's mean from images taken of one to three sections. For each section, we took three images using the 40X objective: a color image of the black silver grains and purple Nissl bodies (cells 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 and between slides due to emulsion thickness. The cell area was quantified from the cells image 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 Image J (NIH, Bethesda, MD). We then used Image J to convert the grains and background images into black and white images using the "make binary" function. The number of grains was obtained using the "analyze particles" function. For each section, we subtracted the number of background silver grains from the number of silver grains of the area of interest. Silver grain density for each brain region for each individual was calculated as the ratio of the number of silver grains above background to the area covered by cells in the standard-size sampling window.

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 "Attacks" 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

and 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 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 (Benjamini and Hochberg, 1995).

RESULTS

Hormones

Plasma testosterone levels, which were taken approximately two hours after injection, increased significantly following FAD treatment compared with controls (Mann-Whitney U, $p<0.0001$, Figure 2A). Conversely, plasma estradiol levels decreased significantly compared with controls following FAD treatment (Mann-Whitney U, $p<0.0001$; Figure 2B). Interestingly, testosterone and estradiol levels correlated in saline animals (Spearman's rank correlation coefficient, $\rho=0.665$, $p=0.013$, $n=13$) but not in FAD-treated animals ($\rho=0.115$, $p=0.707$, $n=13$). GSI did not differ between treatments (t-test, $t=-0.119$, $p=0.906$). There were no significant correlations between hormone levels or behavior within either treatment.

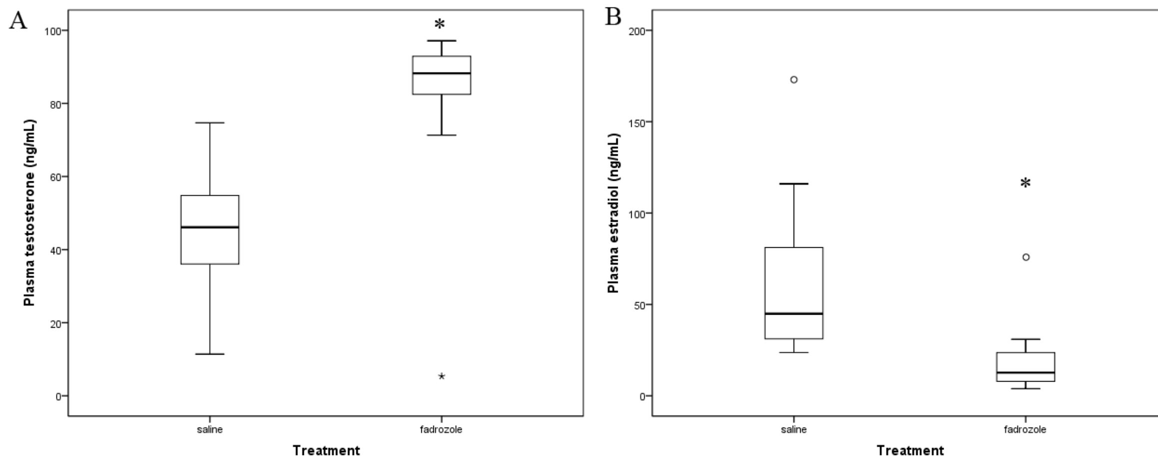

Figure 2: Plasma hormone levels following saline and FAD treatment. A) Testosterone; B) Estradiol. Asterisks indicate statistical significance.

Behavior

When dominant males were injected with FAD, they significantly decreased the number of attacks towards other fish while control animals remained unchanged (saline paired t-test t=-0.994, p=0.337; FAD paired t-test t=2.345, p=0.036; Figure 3A). 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 p=0.018). There was an injection effect on reproductive behavior, as reproductive behavior decreased after saline injection (related-samples Wilcoxon signed-rank test 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; GEE p=0.245; Figure 3B).

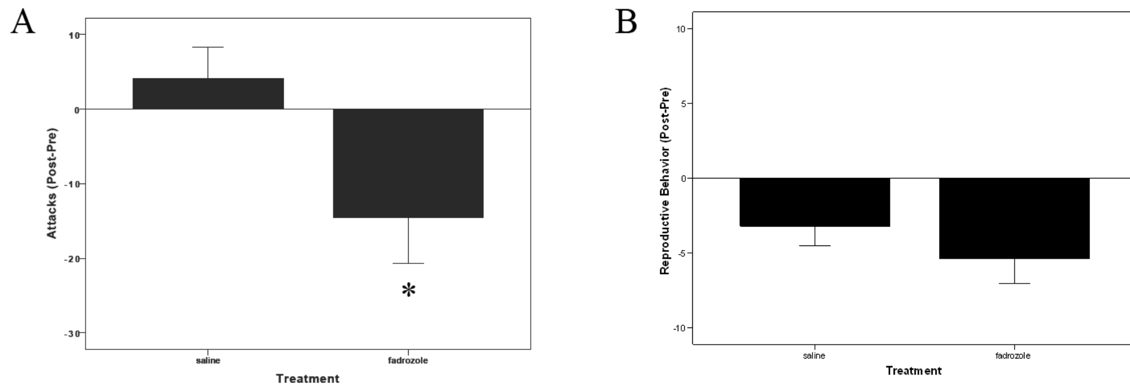

Figure 3. Effects of saline and FAD treatment on behavior. Change in number of A) attacks and B) reproductive behaviors in 10-minute observations. Asterisks indicate statistical significance.

Quantification of *CYP19A2* and *ERα* gene expression in response to saline and FAD treatment

Fadrozole treatment significantly increased *CYP19A2* expression in the gPOA relative to saline-treated males (Figure 4A; t-test, t=-2.485, p=0.026). However, *ERα* expression did not differ between treatments in either the pPOA (Figure 4B; Mann-Whitney U=32.5, p=0.735) or the Vv (Figure 4C; t-test, t=-1.491, p=0.159).

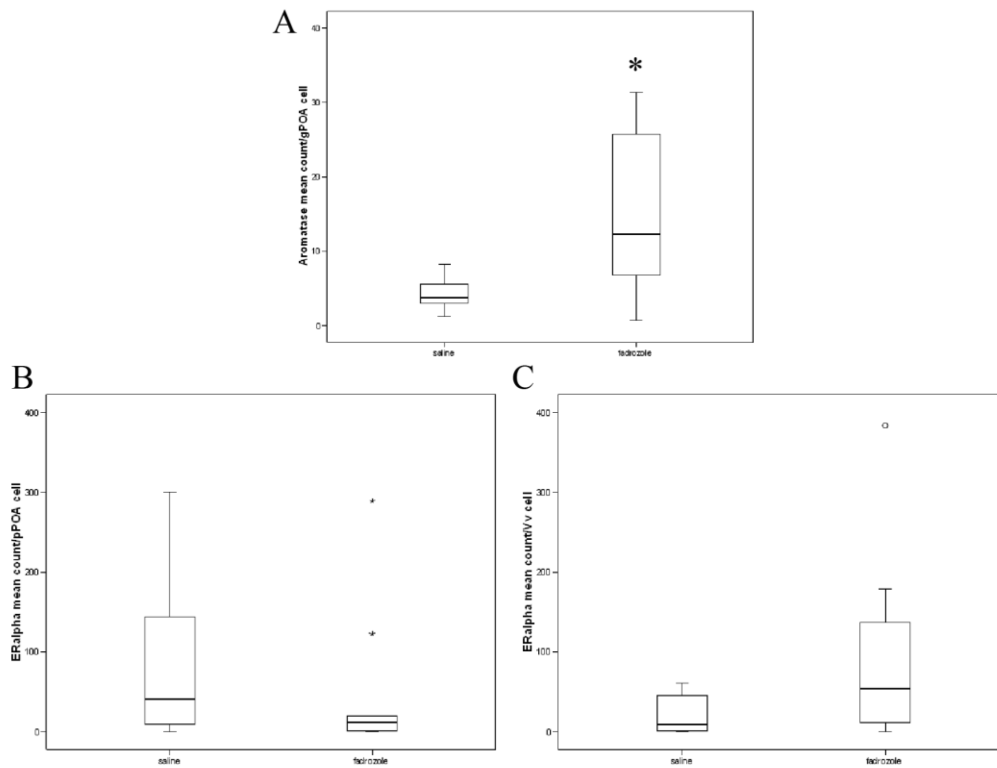

Figure 4. Effects of FAD treatment on gene expression in the brain. A) *CYP19A2* expression in the gPOA. B) *ERα* expression in the pPOA. C) *ERα* expression in the Vv. Asterisks indicate statistical significance.

Quantitative gene expression in transitioning males

CYP19A2 expression in the gPOA did not vary significantly between dominant and subordinate males (t=0.718, p=0.505) nor as males transitioned from subordinate to dominant (Kruskal Wallis X^2=3.405, p=0.333) (Figure 5A). Similarly, *ERα* expression did not differ in either brain region examined, either between dominant and subordinate males (pPOA: t=0.274, p=0.793; Vv: t=-0.998, p=0.364) or as males transitioned (pPOA: Kruskal Wallis X^2=4.563, p=0.207; Vv: ANOVA, F=0.305, p=0.905) (Figure 5B, 5C).

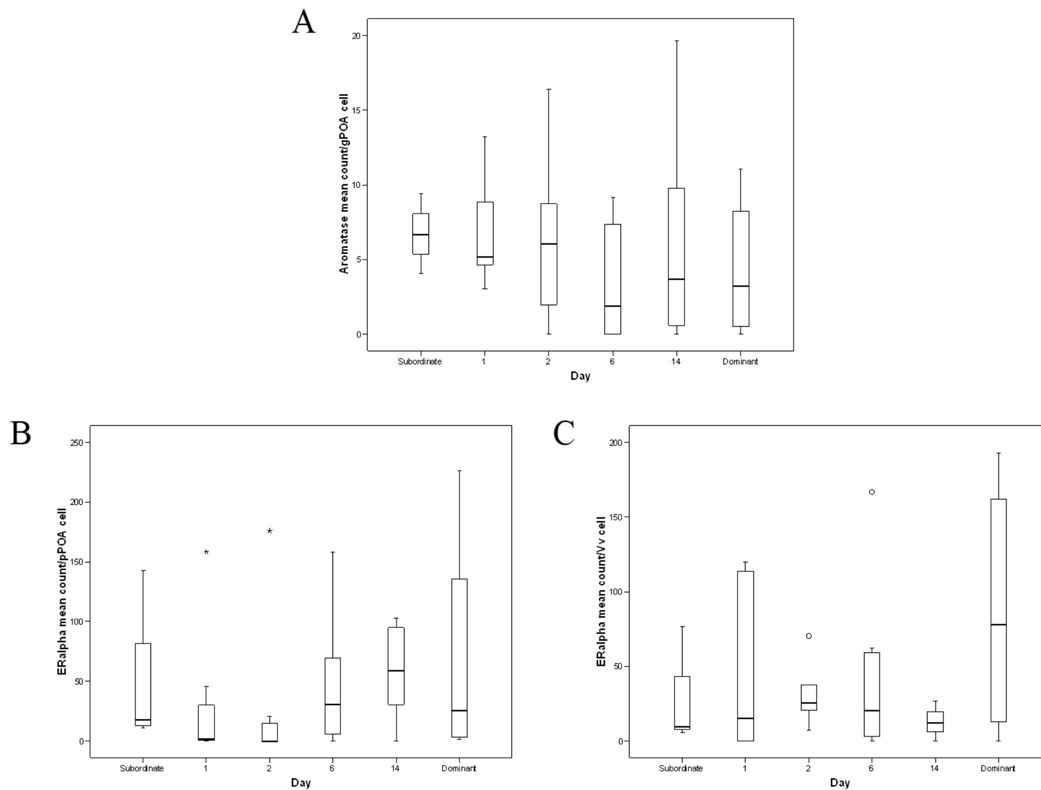

Figure 5. Boxplots of gene expression in males during transition. A) *CYP19A2* expression in the gPOA in subordinate and dominant males and Days 1, 2, 6, and 14 of transition; B) *ERα* expression in the pPOA in subordinate and dominant males and Days 1, 2, 6, and 14 of transition; C) *ERα* expression in the Vv in subordinate and dominant males and Days 1, 2, 6, and 14 of transition.

Connecting central gene expression and peripheral hormone levels in transitioning males

To determine how brain expression of genes involved in sex steroid hormone regulation are related to levels of these hormones in the periphery, we examined the relationships between the expression of *CYP19A2* in the gPOA and *ERα* in the pPOA and Vv and plasma sex steroid hormone levels among subordinate males as they transition to dominance. We found that expression of *CYP19A2* in the gPOA and plasma testosterone levels were negatively correlated (Figure 6A; r=-0.393, p=0.035). When we examined expression of *ERα* in the pPOA and Vv, we found that expression of *ERα* in the pPOA

was positively correlated with expression in the Vv (Figure 6B; r=0.742, p<0.001) and testosterone in both the water (ρ=0.567, p=0.006) and plasma (r=0.477, p=0.012) (Figure 6C).

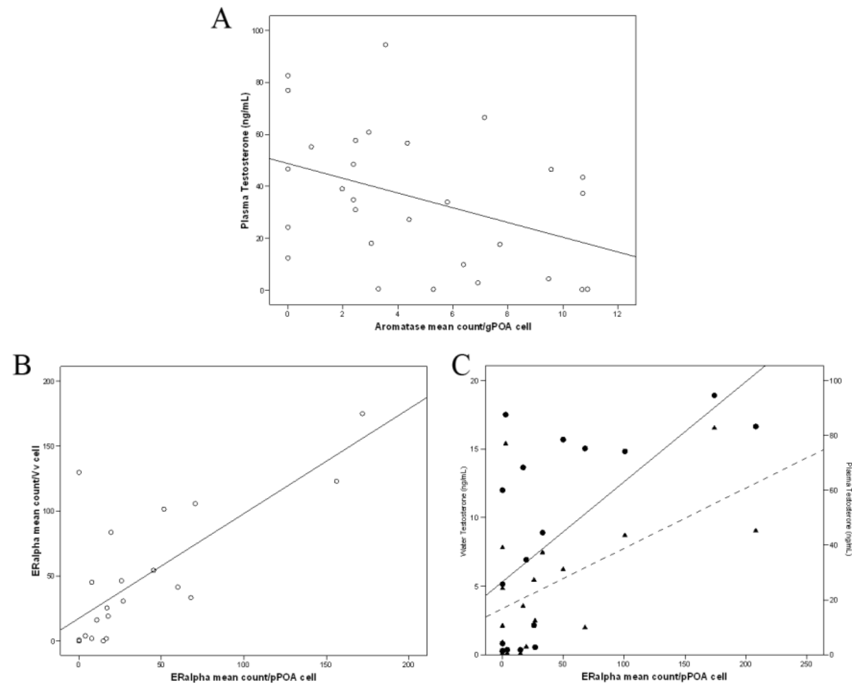

Figure 6. Correlations between gene expression and hormone levels in males during transition. A) *CYP19A2* expression in the gPOA and plasma testosterone levels; B) *ERα* expression in the pPOA and Vv; C) *ERα* expression in the pPOA and water testosterone (circles, solid line) and plasma testosterone (triangles, dashed line).

DISCUSSION

Our data support the hypothesis that in male *A. burtoni* estradiol is necessary for aggressive behavior via the conversion of testosterone by aromatase and is one of the few studies to quantitatively compare aromatase mRNA levels across treatments and phenotypes. 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 our

observed increase in circulating testosterone and decrease in estradiol, similar to what has been reported in *Oncorhynchus kisutch* (Coho salmon; Afonso et al., 1999) and *Pimephales promelas* (fathead minnow; Ankley et al., 2002) 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 (McEwen, 1981), and previous studies of gonadectomized animals have shown that gonadal steroids are not necessary for these sex steroid hormone-dependent behaviors (Balthazart and Foidart, 1993), our results suggest that FAD successfully blocked the action of aromatase in the brain. This conclusion is also supported by the observed increase in *CYP19A2* expression in the gPOA following treatment in an effort to compensate for decreased estradiol synthesis in the brain. Quantitative analysis of mRNA levels suggests that expression levels of aromatase and ERα in the brain are co-regulated, as are ERα and aggressive behavior. Our investigation of males as they transition from subordinate to dominant indicate co-regulation of expression of aromatase and ERα in the brain and sex steroid hormone levels in the periphery.

In saline-treated animals, we found that testosterone and estradiol positively correlated in the circulation, which was expected based on previous studies in *A. burtoni* (Huffman et al., 2011), as more testosterone will be aromatized into estradiol. If we block aromatase using FAD, testosterone and estradiol no long correlate, as testosterone is no longer being metabolized, and estradiol is not being produced. 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 does not affect its substrate (testosterone) and product (estradiol) in a 1:1 manner.

FAD significantly decreased estradiol and the number of aggressive attacks towards other animals in the community. Although estradiol has been well established as

a modulator of aggression, the relationship between estradiol and aggression is not always positive. For example, Trainor et al. (2004) showed that blocking aromatase using FAD increased attack latency in male California mice, *Peromyscus californicus*, and aromatase activity in the whole brain of male bluebanded gobies, *Lythrypnus dalli,* is negatively associated with territorial behavior (Black et al., 2005). 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 in certain species. In our study, circulating T levels increased concurrent with the decrease in aggression; as it is well established that dominant males have higher testosterone than subordinate males (Parikh et al., 2006; Huffman et al., 2011), it is doubtful that this increase in testosterone is responsible for the decrease in aggression.

Estradiol has also been shown to be necessary for male reproductive behavior; however, we did not see an effect of FAD treatment on reproductive behavior. 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 sufficiently dynamic range to detect any differences. As male *A. burtoni* respond behaviorally to the reproductive state of the females in their community (Kidd et al., in prep; Huffman et al., 2011), 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, as well as the effects of estradiol and non-aromatizable androgen treatments.

We examined differential gene expression in three different brain areas, the gPOA, the pPOA, and area Vv (lateral septum homolog) and found that *CYP19A2* (aromatase) expression is up-regulated following FAD treatment, suggesting a

compensatory response to pharmacologically blocking aromatase activity. Nevertheless, the increased expression of *CYP19A2* was not sufficient to prevent at least circulating estradiol levels from decreasing in these males. When we blocked aromatase, estradiol and aggressive behavior decreased; *CYP19A2* expression, which increased, was negatively correlated with ERα expression in the area Vv, suggesting that expression of ERα may be down-regulated in response to lower estradiol levels as a result of blocking aromatase.

To our surprise, we did not find any quantitative changes in expression of either *CYP19A2* or *ERα* in transitioning males. However, we found that over the transition period *CYP19A2* expression in the pPOA negatively correlated with circulating testosterone levels. When aromatase levels are high, testosterone is metabolized into estradiol, causing testosterone levels to decrease; conversely, if aromatase levels are low, testosterone will remain unconverted and will remain present in the circulation. However, in other teleosts, androgens are associated with high aromatase expression in the brain. A study in male plainfin midshipman, *Porichthys notatus*, a seasonally breeding teleost fish, showed that aromatase expression was highest in the POA at the start of the nesting period (Forlano and Bass, 2005), when androgens are also elevated (Sisneros et al., 2004. Although the causal direction is unclear, a relationship between gene expression in the brain and hormone levels in the circulation corroborates the hypothesis that sex steroid hormone synthesis in the brain affects systemic levels, either directly or indirectly. This is further supported by the fact that gonadal aromatase levels do not seem to be related to circulating levels of either testosterone or estradiol (see Chapter 2 or Huffman et al., 2011). Sex steroid hormones produced centrally have been shown to have the potential of being released into the circulation (Schlinger and Arnold, 1992), but we cannot definitively rule out the possible contribution of the gonads and adrenals (or interrenals in

fish), as gonadal steroids have been documented to affect brain aromatase expression in teleost fish (Balthazart and Ball, 1998). We also found that *ERα* mRNA levels in the pPOA and lateral septum were positively correlated, suggesting co-regulation across brain areas. *ERα* in the pPOA was also positively correlated to testosterone levels, both in water and plasma. Huffman et al. (2011) have shown that in transitioning males, testosterone and estradiol levels in the circulation are positively correlated, so it is reasonable to hypothesize that as higher testosterone levels will result in higher estradiol levels, this could be associated with an up-regulation of *ERα* in brain regions that regulate male-typical behavior.

The POA subdivisions as well as the lateral septum are part of the "social behavior network" (Newman, 1999), which comprises a set of hypothalamic and limbic brain areas that regulate aggressive, reproductive, and/or parental behaviors and strongly express neuropeptide and sex steroid hormone receptors. It is known that both the gPOA and the pPOA have projections to the pituitary and regulate different physiological functions. The role of each in social behavior is currently unclear although some studies in other fish species have demonstrated a role for the pPOA in cortisol release during stress response (Gilchriest et al., 2000), which has both physiological and behavioral effects. A few studies have described the distribution of aromatase expression in teleost fish (midshipman, Forlano et al., 2001; trout, Menuet et al., 2003; pejerrey, Strobl-Mazzulla et al., 2005), showing strong 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 differential expression between various phenotypes. O'Connell et al. (in prep) showed that in the gPOA, subordinate males had higher levels of aromatase expression although across the whole brain, dominant males have higher expression (Renn et al., 2008). *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 (Forlano and Bass, 2005). These results suggest that, as in most vertebrates, aromatase expression and male aggressive and reproductive behavior are positively co-regulated; although we did not see differential expression of aromatase between social phenotypes in our study, we did find that in saline-treated males, aromatase mRNA levels and aggressive behavior were correlated. In transitioning males, we did not find any relationships between gene expression and behavior, but we have shown that animals with higher aromatase expression in the gPOA have lower circulating levels of testosterone. If sex steroid hormone synthesis in the brain does indeed affect circulating levels, these results are as expected as testosterone is being metabolized by aromatase into estradiol.

ERα in teleosts has been shown to be expressed in the same regions as aromatase, including the anterior POA and the hypothalamus (Menuet et al., 2003) although it remains to be shown if the low levels of ERα expressed in glial cells, where aromatase is abundant, is sufficient to be the exclusive mechanism by which ERα regulates aromatase expression in teleosts. In male *A. burtoni,* ERα expression is higher in the brains of dominant males overall (Renn et al., 2008), and O'Connell et al. (in prep) found that dominant males had more ERα-ir cells in the pPOA and Vv than subordinate males, supporting a role for estradiol and ERα in positively regulating male-typical social behavior. Although we did not find a difference between social phenotypes in ERα mRNA levels in where, our data do suggest a relationship between ERα, sex steroid hormone synthesis, and social behavior. When males in our study were treated with FAD and aromatase activity was blocked, aromatase expression increased and negatively correlated with *ERα* expression. Although it seems that these two genes are indeed co-

regulated, the directionality of the relationship remains unclear; it is possible that lower aromatase activity and thus lower estradiol synthesis simultaneously causes a compensatory up-regulation in aromatase expression and a down-regulation of ERα.

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. By simultaneously quantifying behavior, sex steroid hormones, and the neural expression of the genes for brain aromatase and ERα, we have increased our understanding of how estradiol synthesis is regulated and modulates social behavior.

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Chapter 6: Conclusion

This dissertation has investigated the molecular mechanisms by which an extremely plastic animal can change social status from subordinate to dominant. There are many levels of biological organization that respond to an opportunity to change in social status, and they must be coordinated within the animal's body and across time. I have attempted to quantify and integrate many of these levels here, including changes in behavior, sex steroid hormones, gonad physiology, and expression of candidate genes such as neuropeptides and those involved in sex steroid hormone regulation.

When a male *Astatotilapia burtoni* perceives an opportunity to become dominant within a community, he becomes extremely aggressive and establishes his territoriality. His testosterone and estradiol levels increase both in his blood and in his micro-environment, as these hormones are released from his body into the surrounding water. Over the next few days, his aggression decreases and he allocates more time to reproductive displays, especially if there are gravid females present. His sex steroid hormone levels continue to increase, and the cells in his testes become increasingly organized, presumably to produce mature sperm. Also in his testes, he increases the expression of a gene necessary for gonadal testosterone synthesis, steroidogenic acute regulatory protein (StAR). Other genes involved in sex steroid hormone regulation, such as luteinizing hormone receptor (LHR) and gonadal aromatase, do not change significantly but are co-regulated with StAR.

In addition to sex steroid hormones, there are many other neuromodulatory substances that are important in regulating social behavior and social status, such as the neuropeptides arginine vasotocin (AVT) and isotocin (IST). These neuropeptides have been established as having roles in controlling aggressive and reproductive behavior, but

their specific functions are complex and extremely species-specific. Their distributions in the brain vary across vertebrates, but are consistently found in nuclei of the preoptic area and in the hypothalamus, areas important in social decision-making and behavior. As teleost fish express AVT and IST in only these conserved areas, they represent an ancestral taxa that can be studied to help elucidate the evolutionary foundations of these neuropeptide systems. I have used immunohistochemistry to confirm that these neuropeptides are only found in the preoptic area and the hypothalamus in *A. burtoni*. Their receptors, on the other hand, are expressed throughout the brain, but still concentrated in areas important for social behavior according to our *in situ* hybridization and immunohistochemistry results. Their pattern of distribution is consistent with what is seen across vertebrates although the quantity within each region can be highly variable between and within species.

AVT is commonly differentially expressed between social statuses within species, with dominant animals usually expressing more than subordinate animals although the opposite pattern has been found. By injecting male *A. burtoni* with an AVT receptor agonist and antagonist, I have shown that while treatment with AVT induces a stress response in dominant and subordinate males, blocking the AVT receptor does not affect males in a stable social environment. However, when subordinate males are given the opportunity to compete for a territory and become dominant, blocking the AVT receptor makes them respond less aggressively and decreases their chances of successfully attaining dominance. Further, they show more reproductive behavior early in their attempt to transition, which may represent an inability to respond appropriately to their social environment, as males typically respond with extreme aggression and only progressively increasing reproductive behavior. Males also upregulate expression of the

genes for both AVT and the AVT receptor during transition, suggesting a coregulation of the AVT system that is important in unstable social environments.

Lastly, I have shown that estradiol is also necessary for aggression in dominant *A. burtoni*. Testosterone is metabolized into estradiol via the enzyme aromatase, and both of these sex steroid hormones are known to be potent regulators of social behavior. However, because testosterone can be aromatized into estradiol, it can be difficult to separate the effects of these sex steroid hormones. To investigate the effects of estradiol, I treated dominant males with fadrozole, an aromatase inhibitor, and found that estradiol did indeed decrease in the circulation. Testosterone also increased, as less was metabolized into estradiol. A decrease in aggressive behavior accompanied these changes in sex steroid hormone levels, and reproductive behavior was unaffected, demonstrating the importance of estradiol, but not testosterone, in social aggression in *A. burtoni*. I have also performed quantitative radioactive *in situ* hybridization in the brains of these animals to quantify the expression of brain aromatase and the estradiol receptor ERα to investigate the transcriptional effects of inhibiting aromatase via a systemic injection, and these data are in the process of being collected. In addition to these brains, I also performed *in situ* hybridization on the brains of stable dominant and subordinate males and the transitioning males from Chapter 2 to investigate the transcriptional regulation of the estradiol system in males as they become dominant.

There are many molecular underpinnings of social status, and I have only investigated a few here. By manipulating and describing the expression of several candidate genes as well as behavioral and neuroendocrinological changes throughout males as they undergo a massive phenotypic change, I have hopefully shed some light on how animals can integrate different levels of biological organization to respond dynamically to their changing social environments.

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