Hormones and Behavior 61 (2012) 631–641

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Rising StARs: Behavioral, hormonal, and molecular responses to social challenge and opportunity

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ARTICLE INFO

Article history:
Received 10 August 2011
Revised 12 February 2012
Accepted 13 February 2012
Available online 21 February 2012

Keywords:
Aggression
Sexual behavior
Social dominance
Plasticity
Sex steroid hormone
Androgen
Luteinizing hormone receptor
Aromatase
Gene expression

ABSTRACT

Across taxa, individuals must respond to a dynamic social environment of challenges and opportunities on multiple biological levels, including behavior, hormone profiles, and gene expression. We investigated the response to a complex social environment including both territorial challenges and reproductive opportunities in the African cichlid fish *Astatotilapia burtoni* (Burton's mouthbrooder), a species well-known for its phenotypic plasticity. Male *A. burtoni* are either socially dominant or subordinate and can transition between the two phenotypes. We used this transition to simultaneously study changes in aggression, reproductive behavior, testosterone and estradiol levels, gonadal histology, and testes expression of three genes involved in testosterone synthesis. We have found that males immediately become aggressive and increase testosterone levels when they become dominant in this paradigm of challenge and opportunity. Reproductive behavior and estradiol increase slightly later but are also up-regulated within 24 h. Increases in steroid hormone levels are accompanied by an increase in expression of steroidogenic acute regulatory protein (StAR), the rate-limiting enzyme during testosterone synthesis, as well as an increase in testis maturation as measured by histological organization. Reproductive behavior was found to correlate with female gravidity, suggesting that males were able to perceive reproductive opportunity. Our study demonstrates the rapid plasticity at multiple levels of biological organization that animals can display in response to changes in their complex social environment.

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 such as those to either social challenges or mating 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 the 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

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doi:10.1016/j.yhbeh.2012.02.016

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 (Borg, 1994; Idler et al., 1960; Kime, 1993; 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; Callard et al., 1990; Cornil et al., 2006). 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 (Burmeister et al., 2005; Francis et al., 1993; Hofmann and Fernald, 2000; Maruska and Fernald, 2010; White et al., 2002). 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 min after being provided with a vacant territory (Burmeister et al., 2005; Maruska and Fernald, 2010). Similarly, within 30 min of becoming dominant, there is an increase in circulating levels of 11-KT (Maruska and Fernald, 2010) as well as an induction of immediate early gene expression 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 h, 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, likely as a consequence of the high energetic cost associated with being dominant: Dominant males grow at a slower rate than subordinates, which allows the latter to eventually gain a size advantage and overtake occupied territories (Hofmann et al., 1999; Hofmann and Fernald, 2001). 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 timepoints 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 (Fig. 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 and used as experimental tanks (Fig. 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), typically resulting in one dominant male and one subordinate male in each compartment. The side compartments each included two terra cotta pots while the center compartment only contained one to ensure that one of the central males established dominance over the other. All males maintained their respective social status in naturalistic communities for at least two weeks before being moved into the

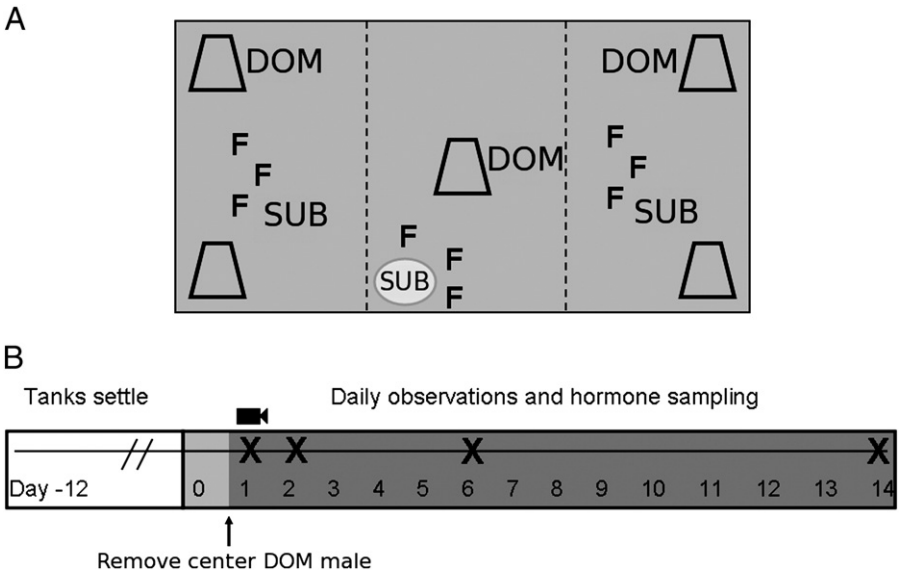

Fig. 1. Experimental design. A) Behavioral paradigm and B) four-week time-line for repeated measures design. Xs represent days on which males were euthanized for Experiment 2. Dom: Dominant male; Sub: Subordinate male; F: Female.

compartmentalized experimental tanks. We then 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 (Francis et al., 1993; Hofmann et al., 1999) before the onset of the experiment.

Experiment 1

Behavioral observations

We observed the subordinate 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 min 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, 25 individuals were observed for ten minutes up to nine times (2 to 9 observations per subject) over two weeks (Day 0: $n=12$; Day 1: $n=13$; Day 2: $n=7$; Day 3: $n=7$; Day 4: $n=13$; Day 5: $n=12$; Day 6: $n=15$; Day 7: $n=5$; Day 8: $n=2$; Day 9: $n=6$; Day 10: $n=8$; Day 11: $n=8$; Day 12: $n=5$; Day 13: $n=10$; Day 14: $n=18$). Behavior patterns were scored based on Fernald and Hirata (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 toward 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 and Hirata, 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 toward dominant males were summed to comprise an aggressive index for that 10-minute observation, and digging, leading, and quivering toward 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). By comparing plasma and water samples on the same ELISA plate, the concentration of free and conjugated (i.e., total) steroid hormones released into the water has been shown to be reflective of circulating levels 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 particulates 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 drawn 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, leaving a residue that was then dissolved in 100 μL assay buffer included in the T ELISA system (Assay Designs; sensitivity = 5.67 pg/mL; maximum known cross-reactivity = 14.64% for 19-hydroxytestosterone and 7.2% for androstenedione), 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. Sensitivity and maximum known cross-reactivity of the E assay were 28.5 pg/mL and 4.64% for estrone and 0.53% for estriol, respectively. Sample sizes ranged from $n=2$ to $n=13$ per day for T and from $n=2$ and $n=4$ per day for E.

Experiment 2

Hormone measurements and tissue collection

For Experiment 2, 38 animals were observed as described for Experiment 1, then approximately three hours post-observation on either Day 1 ($n=9$), 2 ($n=10$), 6 ($n=10$), or 14 ($n=9$), focal males were weighed and measured for standard length following focal observations and collection of water-borne hormone levels (see above). 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 min and then stored at −80 °C for later hormone analysis (see below). We measured both T and E (sample sizes between $n=6$ and $n=9$ per day for both hormones) 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 T and E assay plates ranged from 2% to 7% and were 10.8% across plates for the T assay (only one E assay plate was run). 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 (Fig. S1). 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 (Kidd et al., 2010; Parikh et al., 2006a, 2006b).

Immediately after blood collection, we 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.

Testis 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 (5×) and high (20×) 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 (Fig. 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.

Cloning StAR and LHR

To obtain the *A. burtoni StAR* sequence, we designed degenerate primers (Table S1) 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) to extend the 3′ end of the coding region according to the manufacturer's instructions (Table S1). 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 *Homo sapiens* as an outgroup (*H. sapiens* StAR: CAG46648; *H. sapiens* StARD3: NP_006795; *Rana rugosa* StAR: BAH09112; *Gallus gallus* StAR: AAG28594; *D. rerio* StAR: AAG28593; *Salmo salar* StAR: ABD73012; *Oncorhynchus 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. Fig. 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 S1). 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; *Xenopus 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 Fig. S3B.

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 S2). 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 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 Days 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 χ^2 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 hormone levels for Days 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 investigate possible co-regulation patterns of different variables, which would facilitate the formulation of novel hypotheses for future research. Specifically, we calculated Pearson correlation coefficients to construct a correlation matrix between variables of behavior, hormones, gene expression, testis physiology and size, body size, and female gravidity. Due to the large number of correlations we corrected for multiple hypothesis testing using the Benjamini–Hochberg FDR method (Benjamini and Hochberg, 1995). We then visualized the emerging networks using Cytoscape software (Shannon et al., 2003).

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 (Fig. 2A). Following this initial surge in behavior, aggressive behavior toward other dominant males was significantly dependent on an interaction between day, water-borne T levels, and the number of gravid females present (GEE, Days 1–14: Wald $\chi^2 = 280.5$, $p < 0.001$, $n = 34$); all two-way and main effects of each of these three variables were also significant ($p < 0.001$; Table S3). Reproductive behaviors occurred regularly but at much lower levels than aggressive behaviors (Fig. 2B), and there was a significant three-way interaction of day,

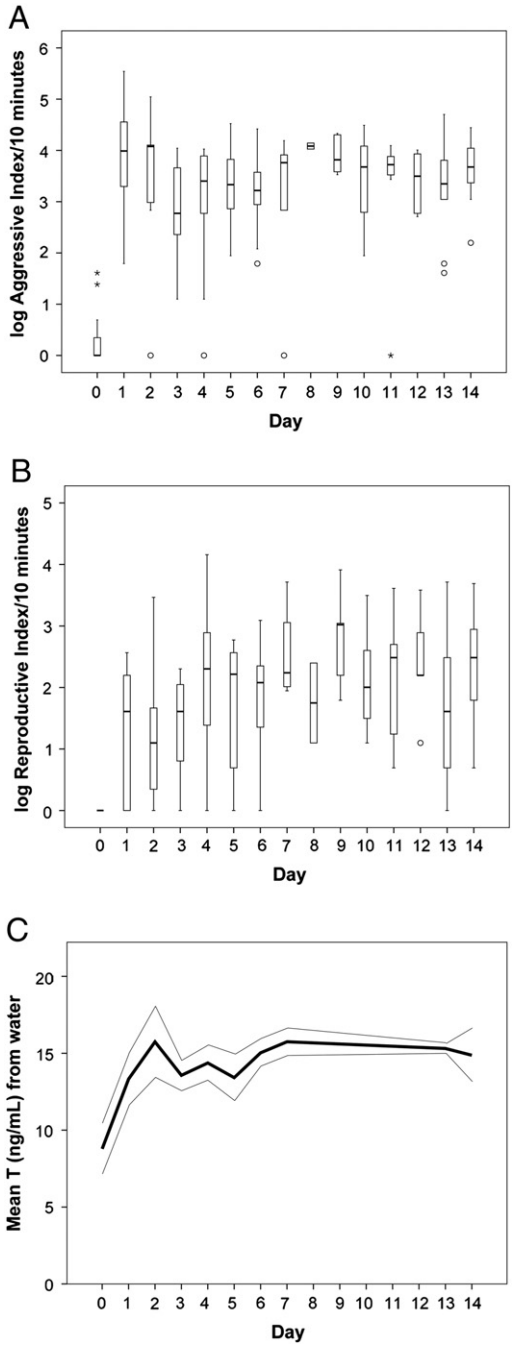

Fig. 2. Responses to social opportunity over the period of two weeks from Experiment 1. A) Box-and-whisker plots of aggressive (sum of chasing males and threat displays) and B) reproductive (sum of leading displays, quivers, and digging) behavioral indices during 10 min focal observations as males transition from subordinate (Day 0) to dominant. As is standard, whiskers represent the minimum and maximum values and filled bars represent the lower and upper quartiles (horizontal lines represent median values). C) Mean levels of testosterone in holding water during the transition (thick line). Thin lines represent standard error.

water-borne T levels, and the number of gravid females present on the amount of reproductive behavior shown by focal males (GEE, Days 1–14: Wald $\chi^2 = 7.186$; $p = 0.007$, $n = 34$), as well as significant main effects of day (Wald $\chi^2 = 9.094$, $p = 0.003$) and T (Wald $\chi^2 = 6.633$, $p = 0.010$). The two-way interactions between T and day (Wald $\chi^2 = 10.518$, $p = 0.001$) and the number of gravid females and day were also significant (Wald $\chi^2 = 6.271$, $p = 0.012$).

Androgen and estradiol responses during transition

We collected water-borne T levels on ten days from Days 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 was a significant interaction effect of both day and the number of gravid females present on water-borne T and E levels from Days 0 to 14 (GEE; Wald $\chi^2 = 49.137$ and $\chi^2 = 15513.8$, respectively; $p < 0.001$; $n = 34$; Fig. 2C) as well as significant main effects of both gravidity and day (Table S3).

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 $U_{22} = 22.0$, $p = 0.015$; 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, $F_{5,40} = 10.26$, $p < 0.001$; Fig. 3A), and the initial increase from Day 0 to Day 1 was significant as well (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, $F_{4,31} = 14.58$, $p < 0.001$), although water measures did not include Day 0 (only 1, 2, 6, and 14). Plasma E levels varied significantly across days as well (ANOVA, $F_{5,36} = 13.846$, $p < 0.001$; Fig. 3B) although, similar to Experiment 1, this was not reflected in water-borne E levels ($F_{2,7} = 1.210$, $p = 0.354$).

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 (Fig. 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 ($r = 0.55$; $p = 0.015$; $n = 10$), but water levels were not ($r = 0.48$, $p = 0.156$, $n = 10$).

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: $F_{3,32} = 4.144$, $p = 0.014$) and increased over the two weeks (t-test Day 1 vs. 14: $t_{14} = -2.425$, $p = 0.029$; Fig. 4A). We then examined the testes histologically and found that early in transition they were less organized than during later stages (Fig. 4B; Pearson's $\chi^2_{15} = 26.234$, $p = 0.035$). 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.

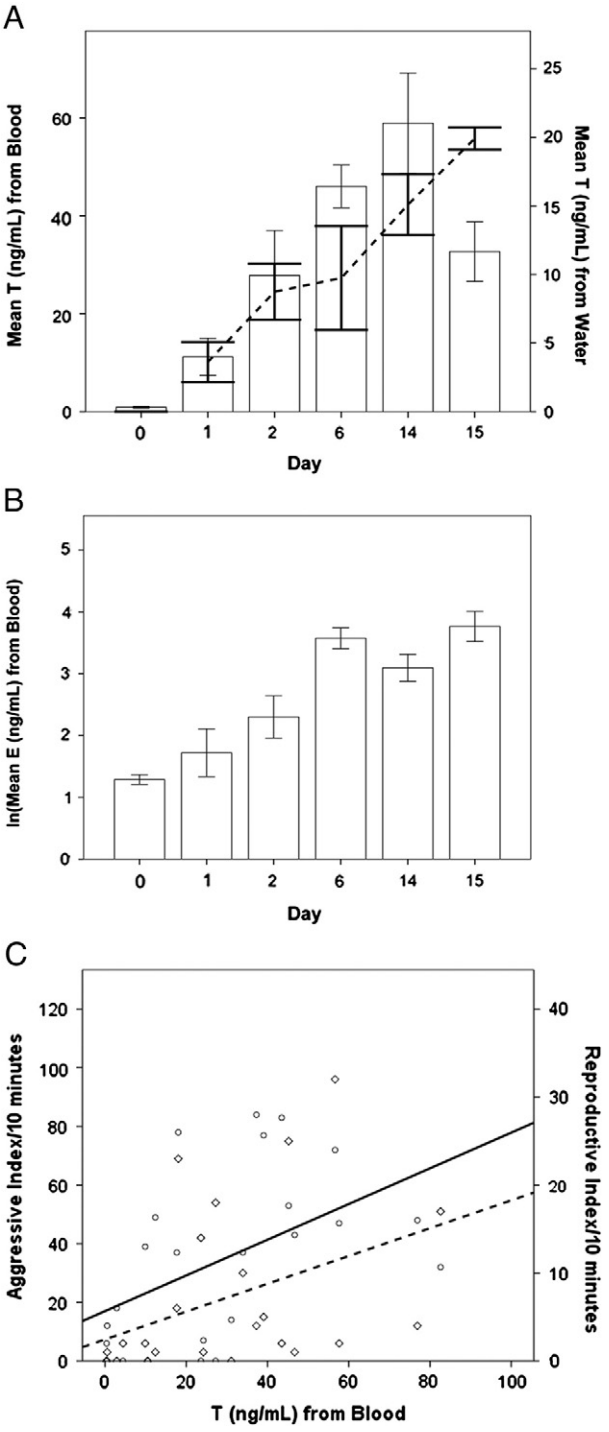

Fig. 3. Hormone measurements and relationships with behavior in Experiment 2. A) Mean values of testosterone in plasma and holding water and B) estradiol in plasma for Days 0/subordinate, 1, 2, 6, 14, and 15/dominant. Error bars represent standard error. Bars represent blood plasma levels (primary y-axis); lines represent water-borne hormone levels (secondary y-axis). C) Linear regression relationships between plasma T and aggressive (circles/solid line/primary y-axis) and reproductive (triangles/dashed line/secondary y-axis) behavioral indices.

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 Fig. 5A, *StAR* mRNA expression changed

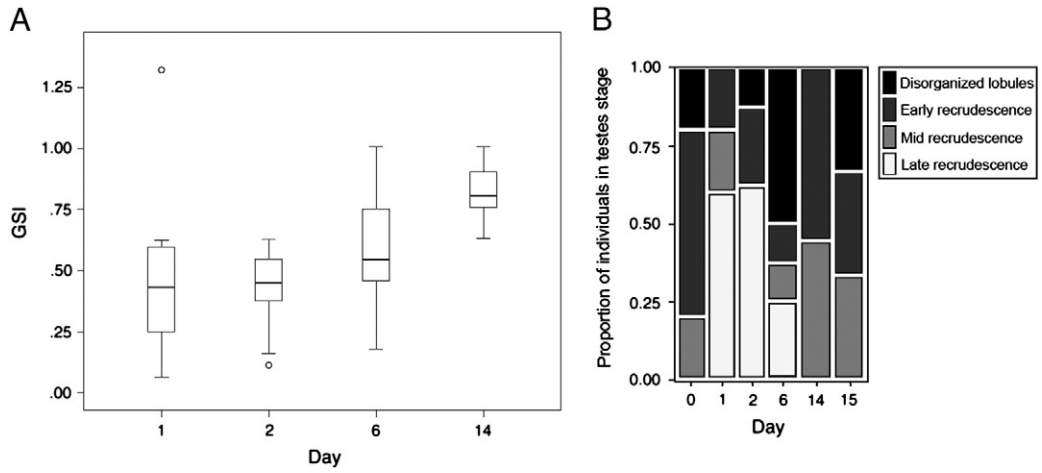

Fig. 4. Gonadosomatic index and testis histology during transition. A) GSI. B) Proportion of individuals in testis stages during phenotypic transition.

significantly throughout transition in *A. burtoni* (Kruskal–Wallis ANOVA: $H_5 = 12.75$, $p = 0.026$). Stable non-territorial males (Day 0) had significantly lower *StAR* expression than males on Day 6 (Mann–Whitney $U_{16} = 56.0$, $p = 0.010$) or Day 14 of transition ($U_{16} = 52.0$, $p = 0.030$). Interestingly, gonadal *LHR* ($H_5 = 4.537$, $p = 0.475$) and *aromatase* ($H_5 = 5.402$, $p = 0.369$) did not change significantly throughout transition (Figs. 5B, C). Nevertheless, *StAR* and *aromatase* expression were positively correlated (Pearson's $r = 0.934$, $p = 0.0001$), as were expression levels of *StAR* and *LHR* ($r = 0.653$, $p = 0.0001$). *Aromatase* and *LHR* expression levels were not correlated ($r = -0.063$, $p = 0.675$).

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 toward the location of gravid females across the two weeks of transition. Indeed, the amount of courting toward each compartment depended on whether any of the females housed in that particular compartment were gravid (GEE: Wald $\chi^2 = 4.172$, $p = 0.041$; Fig. 6).

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 (Table S4), 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 ($r = 0.323$, $p = 0.037$). *StAR* expression was also

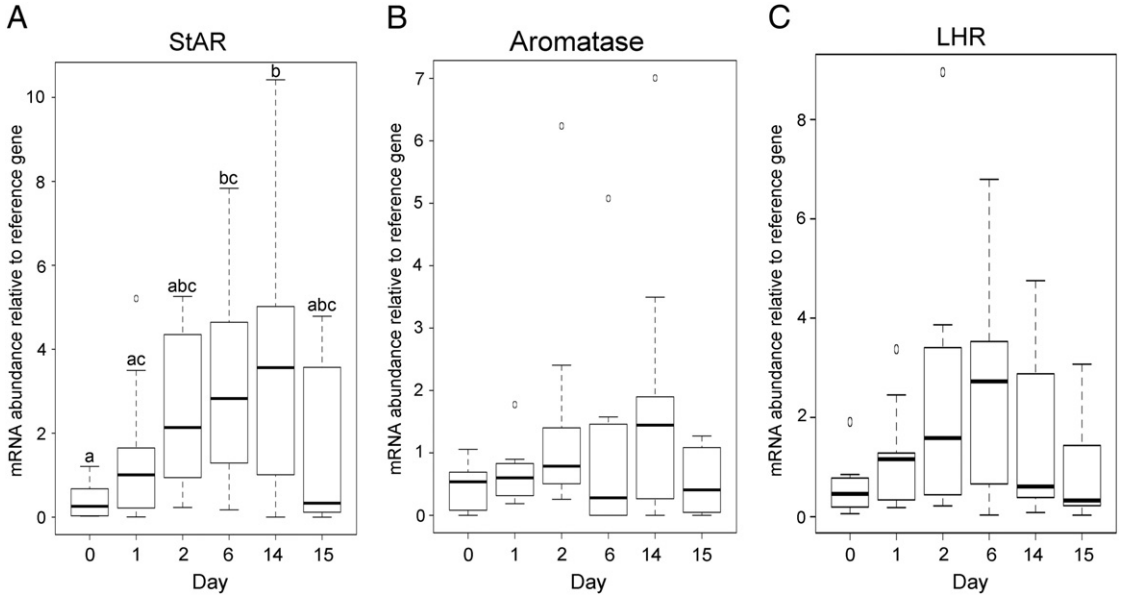

Fig. 5. Gonadal gene expression. Gonadal A) steroidogenic acute regulatory protein (*StAR*), B) *aromatase*, and C) luteinizing hormone receptor (*LHR*) mRNA abundance relative to reference gene are depicted by box and whisker plots for males during transition for Days 1 ($n = 9$), 2 ($n = 10$), 6 ($n = 10$), and 14 ($n = 9$) or from stable communities for Days 0/subordinate ($n = 8$) and 15/dominant ($n = 7$). 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 Days 6 ($p = 0.015$) and 14 ($p = 0.004$) compared to Day 0 and levels are significantly different between Day 1 and Day 14 ($p = 0.047$).

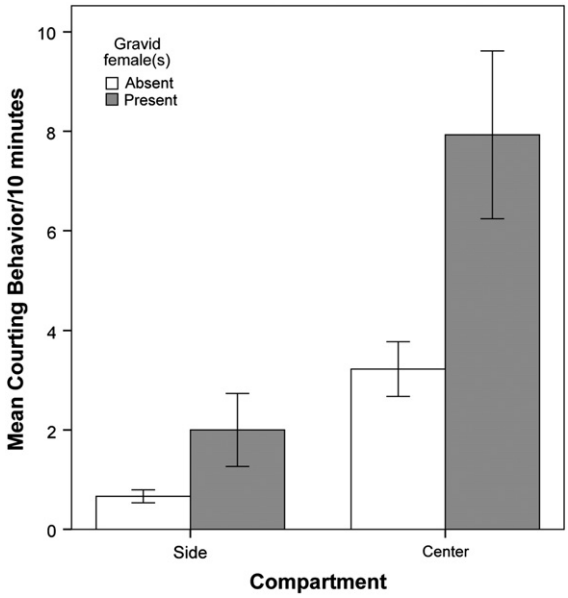

Fig. 6. Compartmental responses to reproductive opportunity. The amount of leading behavior directed toward either the side compartments or center compartment varied based on the presence of gravid females in that compartment.

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.

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 (Fig. 7).

From this model, it is apparent that T, E, body size, aggression, and testis physiology are tightly associated, representing a putative module of co-regulated physiological variables in transitioning males. Expression patterns 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 toward 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

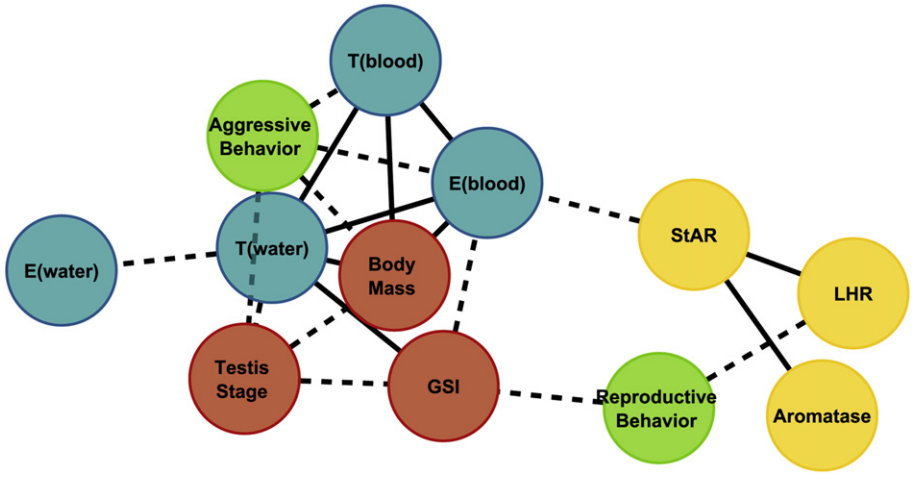

Fig. 7. Integrative model of phenotypic transition. Model illustrates all statistically significant correlations between the variables measured. Variables are colored according to their type: measures of behavioral displays (aggressive, reproductive) are green; hormone levels (T and E in water and plasma) 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.

every 30 days regardless of male behavior (Kidd et al., in review), 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 (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 (cf. "Challenge Hypothesis" by 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 state 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 min 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 h 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 toward 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, these males are under physiological constraints that presumably 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 (Burmeister et al., 2005; Hofmann and Fernald, 2000). 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.

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. It is also interesting to note that plasma hormone levels are more strongly and significantly connected than those extracted from fish holding water. Water-borne hormone assays have made repeated endocrine profiling of small fish possible, 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 integrated over an hour, as it is with water measurements. Therefore, although plasma and water measurements are repeatedly found to correlate in our 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 plasma E levels may not be captured in water measurements.

Our study represents one of the most comprehensive analyses of phenotypic change in terms of both the time points sampled and the number of behavioral, physiological, and molecular variables measured. The rapid yet reversible changes that male *A. burtoni* undergo during social ascent (and descent: see Hofmann and Fernald, 2000; Hofmann et al., 1999) occur on multiple levels of biological organization and inform our understanding of similar dynamic trajectories involving endocrine and behavioral changes across vertebrates, such as puberty (Ebling, 2005; Walker et al., 2009) and reproductive aging (Wu and Gore, 2010). Importantly, our results underscore the dramatic effects the social environment can have on these processes. The approach we (and others: e.g., Burmeister et al., 2005; Maruska and Fernald, 2010) have employed here should prove useful when studying the role of hormones in organizing behavioral development during critical periods in general (Schneirla and Rosenblatt, 1963; Schulz et al., 2009).

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.

Supplementary materials related to this article can be found online at doi:10.1016/j.yhbeh.2012.02.016

Acknowledgments

We thank Celeste Kidd for technical assistance, Peter Dijkstra for advice on statistical methods, Sarah Jane Alger for comments on earlier versions of this manuscript, and members of the Hofmann laboratory for discussions. This work was supported by a University Co-op Undergraduate Research Award (MMM), the Alfred P. Sloan Foundation, the Dwight W. and Blanche Faye Reeder Centennial Fellowship in Systematic and Evolutionary Biology, and the Institute for Cellular and Molecular Biology (HAH).

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