

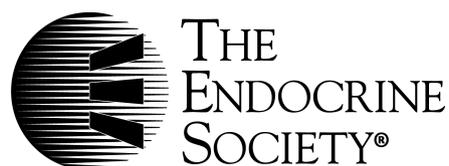
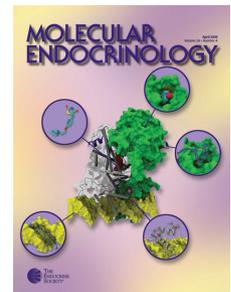
Endocrinology

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Endocrinology 2011 152:281-290 originally published online Nov 10, 2010; , doi: 10.1210/en.2010-0875

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Plasticity of the Reproductive Axis Caused by Social Status Change in an African Cichlid Fish: I. Pituitary Gonadotropins

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Social position in a dominance hierarchy is often tightly coupled with fertility. Consequently, an animal that can recognize and rapidly take advantage of an opportunity to rise in rank will have a reproductive advantage. Reproduction in all vertebrates is controlled by the brain-pituitary-gonad axis, and in males of the African cichlid fish *Astatotilapia burtoni*, GnRH1 neurons at the apex of this axis are under social control. However, little is known about how quickly social information is transformed into functional reproductive change, or about how socially controlled changes in GnRH1 neurons influence downstream actions of the brain-pituitary-gonad axis. We created an opportunity for reproductively suppressed males to ascend in status and then measured how quickly the perception of this opportunity caused changes in mRNA and protein levels of the pituitary gonadotropins. mRNA levels of the β -subunits of LH and FSH rose rapidly in the pituitary 30 min after suppressed males perceived an opportunity to ascend. In contrast, mRNA levels of GnRH receptor-1 remained unchanged during social transition but were higher in stable dominant compared with subordinate males. In the circulation, levels of both LH and FSH were also quickly elevated. There was a positive correlation between mRNA in the pituitary and circulating protein levels for LH and FSH, and both gonadotropins were positively correlated with plasma 11-ketotestosterone. Our results show that the pituitary is stimulated extremely rapidly after perception of social opportunity, probably to allow suppressed males to quickly achieve reproductive success in a dynamic social environment. (*Endocrinology* 152: 281–290, 2011)

The social environment plays an important role in regulating reproductive physiology and fitness across taxa, but little is known about how changes in social status control the reproductive axis at multiple levels within a single species. Dominant individuals within a population often suppress the reproductive function of subordinates through social interactions, which ultimately represses fertility via actions on the brain-pituitary-gonad (BPG) axis (1–3). GnRH1 neurons in the hypothalamus and preoptic area of the brain sit at the apex of the BPG axis and stimulate the pituitary to synthesize and release the gonadotropin hormones FSH and LH to the bloodstream. In males, circulating FSH and LH then target their receptors in the testes to promote spermat-

ogenesis and steroid production. Although several studies have examined changes in gonadotropin synthesis, release, and action along the BPG axis in seasonally breeding or sex-changing animals (4–6), plasticity within elements of the gonadotropin system in response to a dynamic social environment is not well understood.

In the African cichlid fish *Astatotilapia burtoni*, reproductive capacity is tightly coupled to social status (7), which makes it an excellent model for examining how the social environment regulates different components of the BPG axis. Males of this species have evolved two distinct, reversible phenotypes as an adaptation to their dynamic social environment: 1) dominant males are brightly col-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

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doi: 10.1210/en.2010-0875 Received July 30, 2010. Accepted October 4, 2010.
First Published Online November 10, 2010

Abbreviations: BPG, Brain-pituitary-gonad; CT, cycle number at threshold; GnRH-R1, GnRH receptor type 1; 11-KT, 11-ketotestosterone; qRT-PCR, quantitative RT-PCR; GSI, gonadosomatic index; RACE, rapid amplification of cDNA ends.

For companion see page 291

ored, defend a spawning territory, and display aggressive and courtship behaviors; and 2) subordinate males have cryptic coloration similar to females, lack a spawning territory, display submissive behaviors, and do not court females (8). In addition to these behavioral and coloration differences, dominant and subordinate males also differ in several key reproductive physiological traits. For example, dominant males have larger GnRH1 somata (9, 10), higher GnRH1 gene expression in the brain (11), higher GnRH receptor type 1 (GnRH-R1) gene expression in the pituitary (12), higher circulating androgen levels (13), higher steroid receptor expression in the brain (14), and larger testes (15) compared with subordinate individuals. When a subordinate male perceives an opportunity to ascend in social status and become dominant, he displays territorial and reproductive behaviors within minutes (16, 17) and shows rapidly increased immediate early gene mRNA expression (*egr-1*) within the preoptic area and in GnRH1 neurons (16). This rapid activation of the GnRH1 neurons may be an early trigger in the molecular cascade that culminates in physiological changes associated with reproductive competence, but it is not known whether this immediate early gene activation is transformed into functional activation of the pituitary and gonadotropin release.

The pituitary gonadotropins are critical components of the BPG axis in all vertebrates that relay information from the brain and pituitary to the testes and can be used as a proxy measurement for GnRH1 functional activation of the reproductive axis. This proxy is necessary both because GnRH1 in fishes is released directly from nerve endings that innervate the pituitary and because GnRH1 binds quickly to its receptors and is rapidly catabolized after activation, often rendering it undetectable. Despite the previous studies on social control of reproduction in *A. burtoni*, nothing is known about how social status and the ascension to full reproductive capacity influences pituitary FSH and LH gene expression and release of mature hormone into the bloodstream. Understanding whether and how quickly the gonadotropin system is also influenced by social status is critical for interpreting how the entire BPG axis, and hence reproductive capacity, is rapidly and reversibly switched between dominant and subordinate male phenotypes.

This is the first of two complementary studies designed to understand how quickly the reproductive axis is up-regulated in *A. burtoni* upon ascent to social dominance. Here we examine the temporal expression patterns of physiological traits at the pituitary gonadotropin level, whereas the companion paper addresses the temporal expression pattern at the level of the testes (18). The goal of this study was to determine the temporal expression pat-

terns of FSH β and LH β mRNA levels in the pituitary as well as the circulating gonadotropin levels in the blood at different time points after social ascent. Our results show rapid up-regulation of the BPG axis at the level of the pituitary gonadotropins and demonstrate that this level of the reproductive axis is also influenced by the social environment.

Materials and Methods

Animals

Laboratory-bred male cichlid fish *A. burtoni*, derived from wild-caught stock in Lake Tanganyika, Africa, were maintained in aquaria under environmental conditions that mimic their natural equatorial habitat (28 C, pH 8.0, 12 h light and 12 h dark with full spectrum illumination, and constant aeration) and fed cichlid pellets and flakes (AquaDine, Healdsburg, CA) each morning. Aquaria contained gravel-covered bottoms with half terra cotta pots that served as spawning territories. All experimental procedures were approved by the Stanford Administrative Panel for Laboratory Animal Care.

Social manipulation

We created an opportunity for social ascent using an experimental paradigm identical to that described previously (17), which was modified from that originally described by Burmeister *et al.* (16). Briefly, dominant males (5.5–7.5 cm standard length) from community tanks were placed into aquaria for 4–5 wk with several larger dominant suppressor males, females, and subordinate males. At the end of the suppression period, subjects were moved into the central compartment of an experimental tank that contained one larger resident dominant male and four females. This central compartment was isolated from community tanks on either side that contained multiple dominant males, subordinate males, and females with transparent acrylic barriers so that fish could interact visually but not physically with community animals. All dominant males in adjacent community tanks were smaller in size than the suppressed subject male to ensure his ascent upon presentation of social opportunity.

Subject males remained in the experimental tank for 2 d during which time we confirmed with behavioral observations that they remained suppressed by the larger resident male. On the following morning (day of ascension), the resident suppressor male was removed with a net in the dark 1 h before light onset using infrared night vision goggles (Bushnell night vision, model 26-1020). Behavior in the experimental aquarium was recorded for 45 min for later quantification beginning at light onset in the morning (digital video camera, Sony HDR) on the day before, day of, and for each of 5 d after social ascent (data are described in Ref. 17). Time of ascent was defined as the time between light onset and the time when males performed dominance behaviors at a rate of three behaviors per minute (as described in Ref. 17).

Stable dominant and stable subordinate males were also used as control comparisons to males ascending in social status. Stable subordinate males were suppressed in community tanks for 4–5 wk and transferred to the experimental tank as described above. On the day of ascension, removal of the suppressor dominant male was simulated by dipping a net into the tank before light onset. The dominant resident was not removed, however, which kept the subject male in subordinate status. Stable dominants

were dominant males that maintained their status in community tanks for 4–5 wk and were then placed in the experimental tank with four females but no larger resident male, which maintains their dominance status. On the stimulus day, a net was dipped into the water before light onset to simulate resident male removal. Stable dominant animals were all killed on the simulated stimulus day at 30 min after they displayed dominance behaviors as described above, whereas stable subordinates were killed at a time point after light onset that was equivalent to the kill time of stable dominants.

Sequencing of LH β and FSH β

At the start of the study, the sequences for LH β and FSH β in *A. burtoni* were unknown. To identify a partial cDNA sequence for these transcripts, a combination of PCR and rapid amplification of cDNA ends (RACE) PCR (Clontech Laboratories, Inc., Mountain View, CA) were used. The hormone-specific β -subunits of the heterodimeric glycoprotein gonadotropins were chosen for measurement rather than the common α -subunit because they confer the biological activity of each hormone (19).

cDNA for PCR was prepared by first isolating RNA from the pituitary (RNeasy micro kit; QIAGEN, Valencia, CA) of a dominant male, followed by first-strand cDNA synthesis for preparation of 5'- and 3'-RACE-ready cDNA according to the manufacturer's instructions (SMART-RACE protocol; Clontech). PCR primers were designed based on the sequences of tilapia (*Oreochromis niloticus*) LH β and FSH β . A fragment from each gene was amplified on a thermal cycler using a touchdown protocol: 1 min at 95 C, 10 cycles of decreasing annealing temperatures (1 C increments from 65 to 55 C) for 30 sec and 72 C extensions for 3 min, followed by 28 cycles of 1 min at 95 C, 30 sec at 55 C, and 3 min at 72 C, with a final extension for 5 min at 72 C. The reaction products were then visualized and purified by running on a 0.8% agarose Sybr Green CloneWell gel (Invitrogen, Carlsbad, CA), and the bands were collected and sequenced (Sequetech, Mountain View, CA.). BLAST analysis confirmed each sequence had high similarity ($\geq 93\%$) to its respective transcript in other perciform fishes, including closely related cichlids (E values $\leq e^{-100}$ for *O. mossambicus* and *O. niloticus*). The sequences were then used to generate *A. burtoni*-specific primers for RACE reactions to isolate the 3' and 5' ends of the cDNA and to design primers for quantitative RT-PCR (qRT-PCR). To verify sequence identity, phylogenetic and molecular evolutionary analyses were conducted on the translated amino acid sequence using MEGA version 4.1 (20). The percentage of replicate trees in which *A. burtoni* clustered together with *O. niloticus* in the bootstrap test (1000 replicates; neighbor-joining method) was 100% for FSH β and 88% for LH β .

Tissue preparation

All stable dominant, subordinate, and ascending males were anesthetized in an ice-cold tank water, measured for standard length (± 1 mm) and weighed (± 0.001 g), and blood samples were collected by caudal severance within 2 min of capture into 100- μ l capillary tubes. Ascending males were sampled at 30 min and 6, 24, 72, and 120 h after social ascent. These time points were chosen to encompass the rapid behavioral and brain activation changes (16, 17) as well as the longer-term morphological changes in GnRH1 neuron size and testis mass that occur within approximately 4–7 d (7, 11). Blood was centrifuged for 10 min at 8000 rpm, and the plasma was removed and stored at -80 C.

All fish used in this study were the same as those used in a previous study to examine the temporal changes in behavior and circulating 11-ketotestosterone levels during social ascent (17).

Pituitaries were rapidly removed, flash frozen, and stored at -80 C until use. Testes were also removed and weighed to calculate the gonadosomatic index [GSI = (gonad mass/body mass) $\times 100$]. A 2- to 5-mg portion of the central right testis was removed for qRT-PCR, and the remainder of the testis was fixed in 4% buffered formalin for histological processing [see companion paper (18) for these data].

Pituitary tissue was homogenized and RNA extracted following standard kit protocols (RNeasy micro kit; QIAGEN). RNA was treated with deoxyribonuclease (ribonuclease-free deoxyribonuclease set; QIAGEN) during the isolation procedure according to kit instructions to remove contaminating genomic DNA. RNA quality and concentration was estimated from spectrophotometric absorbance (260 and 280 nm) for all samples. Approximately 0.5 μ g of total RNA was reverse transcribed to cDNA (iScript cDNA synthesis kit; Bio-Rad, Hercules, CA) and diluted before use as a template for qRT-PCR.

Quantitative RT PCR

Quantitative RT-PCR was used to measure mRNA expression of LH β , FSH β , and GnRH-R1 from the pituitary. The GnRH receptor subtype GnRH-R1 was chosen for two reasons. First, previous studies in *A. burtoni* showed that GnRH-R1 was expressed in the ventral-anterior and posterior pituitary region where the gonadotrope cells are located, suggesting it may be important for LH and FSH synthesis and release (21, 22). Second, pituitary mRNA levels of GnRH-R1, but not GnRH-R2, were higher in dominant compared with subordinate males (12). Thus, GnRH-R1 may be an important regulatory component for socially induced reproductive plasticity. The iQ Sybr Green supermix (Bio-Rad) was used for qRT-PCR with gene-specific primers. Primers for LH β and FSH β were designed with PrimerQuest (IDT Technologies, Inc., San Diego, CA) from the sequences for each cDNA obtained from RACE reactions described above and synthesized commercially as follows: LH β forward 5'-TGT CAG CTC ATC AAC CAG ACA GTG-3' and reverse 5'-GCA CAC ATG CTG GTA CAC ATT GCT-3' (147-bp product) (GenBank HQ147565) and FSH β forward 5'-CGA CAC CAC CAT TTG TGA AGG ACA-3' and reverse 5'-CCC GTG CAC ATT GCT CTG TGT ATT-3' (134-bp product) (GenBank HQ147566). Primers for GnRH-R1 and the reference genes, 18s rRNA, and glyceraldehyde 3-phosphodehydrogenase (*G3PDH*), were also commercially synthesized and identical to those used in previous studies (12, 14, 23). Each primer pair produced a single melting curve peak in the presence of cDNA template and showed no amplification when water was used as a template in the reaction mix or when reverse transcriptase was omitted from the cDNA synthesis reaction (negative controls). PCR was performed on an iCycler (Bio-Rad), and the reaction progress in 30- μ l volumes was monitored by fluorescence detection at 490 nm during each annealing step. Reaction parameters were 3 min at 95 C followed by 45 cycles of 95 C, 60 C, and 72 C for 30 sec each and followed by a melting curve analysis over the temperature range of 95–55 C (decrease by 0.5 C increments each cycle). All reactions were performed in duplicate, and several reaction products per gene were verified by DNA sequencing (Sequetech).

Fluorescence thresholds for each sample were automatically measured (MyiQ software; Bio-Rad), and then PCR Miner software (23) was used to calculate reaction efficiencies and cycle

thresholds from the fluorescence readings of individual wells during the reaction. This curve-fitting real-time PCR algorithm objectively calculates reaction efficiency and the fractional cycle number at threshold (CT) of the amplification curve for more accurate computation of mRNA levels. By using the kinetics of individual reactions, estimates of efficiency and CT are independent of the specific equipment used to perform PCR, and data can be more reliably compared across plates. The relative amount of target gene mRNA was then normalized to the geometric mean of two housekeeping genes (*18s* and *G3PDH*) that were also measured in each sample, as previously described (12, 14, 24). Normalization to multiple reference genes, rather than a single gene, provides a more accurate quantification of mRNA levels (25, 26). Mean CT values for *18s* and *G3PDH* did not differ among subordinate, ascending, and dominant pituitary samples ($P > 0.05$), demonstrating they are appropriate reference genes for this study.

LH and FSH plasma assays

Frozen plasma samples (50 μ l) were lyophilized and shipped to Prof. B. Levavi-Sivan (The Hebrew University of Jerusalem, Jerusalem, Israel) for measurement of circulating levels of LH and FSH. The large volume of plasma required for these LH and FSH assays precluded measurement of circulating steroids in the same samples, with the exception of 11-ketotestosterone (11-KT), which was reported previously (17) and in Fig. 1. A specific and homologous ELISA was developed for the related cichlid *O. niloticus* (27) and validated for use in *A. burtoni* (see below). Competitive ELISAs were performed using specific primary antibodies against tilapia LH or tilapia FSH β and recombinant tilapia LH $\beta\alpha$ (28) or recombinant tilapia FSH $\beta\alpha$ (29) for the standard curves. The wells were coated with recombinant tilapia LH β or FSH β , and the antibodies were used at a final concentration of 1:5000 (LH) or 1:50,000 (FSH). The intraassay and interassay coefficients of variation, respectively, were 7.2 and 14.8% for LH and 8.0 and 12.5% for FSH. The sensitivities of the assays were 0.65 ng ml $^{-1}$ for LH and 0.55 ng ml $^{-1}$ for FSH (see Ref. 27 for further details on the ELISA procedure).

It was shown previously that the ELISA used in the current study can be used to measure both LH and FSH of other cichlid species like *Tilapia zillii* and the Malawi cichlid Electric blue Hap (*Haplochromis abli*) (27). To validate the ELISA for *A. burtoni*, serial dilutions of *A. burtoni* plasma were measured in the same assay together with the standards. These dilution curves were found to be parallel to that of the tilapia LH and FSH standards, indicating that *A. burtoni* LH and FSH are immunologically similar to that of tilapia and can be measured by the same ELISA. When the data of the standard curves were transformed (LOGIT) to a linear plot, the correlation coefficients of the lines were 0.977 ± 0.002 and 0.976 ± 0.005 for *A. burtoni* plasma LH and FSH, respectively ($n = 3$ for each curve). ANOVA of the slopes for the different regression lines showed that the slopes did not differ ($P > 0.05$).

Statistical analyses

Data sets that were normally distributed (Shapiro-Wilk test) with equal variances (Levene median test) were analyzed with one-way ANOVA with *post hoc* Student Newman Keuls tests for multiple comparisons, whereas data that did not meet the assumptions of parametric statistics were compared with Kruskal-Wallis tests with *post hoc* Dunn's tests. Correlations were assessed with either Pearson product moment tests (parametric) or

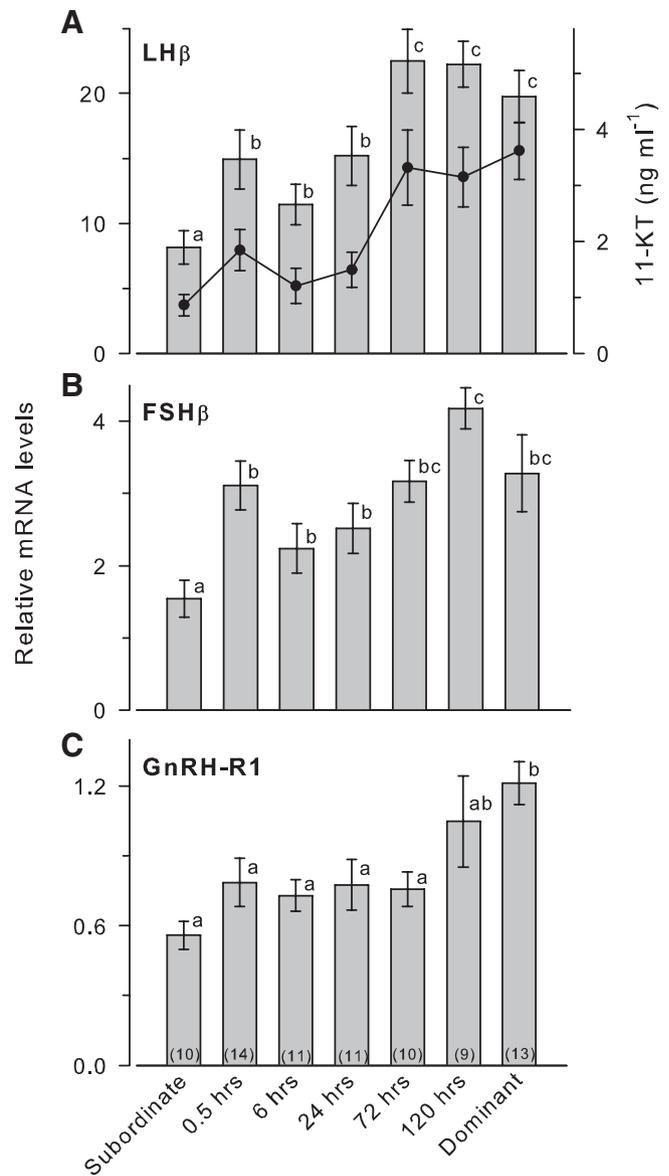


FIG. 1. Levels of LH β , FSH β , and GnRH-R1 mRNA in the pituitary of male African cichlid fish *A. burtoni* during social ascent. A, Levels of LH β (bars) were rapidly elevated above stable subordinate levels at 30 min after perception of social opportunity and further increased to reach stable dominant levels by 72 h. Serum 11-KT levels (●) (data from Ref. 17) showed a similar pattern of increase at 30 min, were relatively stable through 24 h, and then further increased to stable dominant levels by 72 h. B, Levels of FSH β were also rapidly elevated at 30 min after ascent and reached a maximum at 120 h after perception of social opportunity. C, GnRH-R1 levels did not change during social transition but were approximately 2-fold higher in stable dominant males compared with stable subordinates. Data are plotted as relative mRNA levels (mean \pm SE) referenced to the geometric mean of two housekeeping genes (*18s* and *G3PDH*). Bars with different letters represent significant differences ($P < 0.05$), and sample sizes are indicated within each bar on the bottom graph.

Spearman rank tests (nonparametric). For consistency, however, all data are plotted as mean \pm SE with appropriate statistical test values reported in the text. Statistical comparisons were performed with SigmaPlot version 11.0 (Systat Software, Inc., San Jose, CA).

Results

Temporal expression of pituitary mRNA levels

Pituitary LH β mRNA levels were rapidly elevated in fish sampled 30 min after ascent, remained relatively stable for 24 h, and then increased to stable dominant male levels by 72 h [ANOVA, $F_{(6,72)} = 5.89$, $P < 0.001$; Student-Newman-Keuls, $P < 0.05$] (Fig. 1A). Pituitary FSH β levels were also rapidly elevated at 30 min after ascent to a level that was equivalent to that of stable dominant males [ANOVA, $F_{(6,72)} = 4.35$, $P < 0.001$; Student-Newman-Keuls, $P < 0.05$] and then showed a further increase at 120 h after ascent (Fig. 1B). Pituitary GnRH-R1 mRNA levels were 2-fold higher in stable dominant compared with stable subordinate males [ANOVA, $F_{(6,72)} = 4.216$, $P = 0.001$; Student-Newman-Keuls, $P < 0.05$], and although there appeared to be higher levels at 120 h after ascent, the high variation among individuals at this time point precluded detection of statistical significance (Fig. 1C).

Temporal expression of circulating gonadotropin levels

Circulating levels of LH ranged from 14–116 ng ml $^{-1}$ in stable subordinate males and 100–350 ng ml $^{-1}$ in stable dominant males. Plasma LH levels were rapidly elevated in fish sampled 30 min after ascent to a level that was equivalent to stable dominant males [ANOVA, $F_{(6,72)} = 4.420$, $P < 0.001$; Student-Newman-Keuls, $P < 0.05$] (Fig. 2). LH levels were then lower from 6 h through 72 h and then showed a 2-fold increase at 120 h to a level similar to that of stable dominant males (Fig. 2).

Circulating levels of FSH were on average much lower than serum LH levels and ranged from 1–29 ng ml $^{-1}$ in stable subordinate males and 19–44 ng ml $^{-1}$ in stable dominant males (Fig. 2). Similar to LH, plasma FSH levels were also rapidly elevated by 30 min after ascent to a level that was similar to stable dominant males [ANOVA, $F_{(6,72)} = 6.658$, $P < 0.001$; Student-Newman-Keuls, $P < 0.05$]. FSH levels were relatively stable in fish sampled from 6 h through 120 h, and stable dominant males had higher levels than ascending males only at the 6- and 24-h time points.

Relationships between pituitary mRNA levels, circulating gonadotropin levels, and GSI

Pituitary mRNA levels were positively correlated with circulating plasma levels for both LH and FSH (Fig. 3). GSI was also positively correlated with plasma levels of LH and FSH (Fig. 4). Pituitary mRNA levels of FSH β and LH β were positively correlated with GSI (Fig. 4). Plasma levels of the fish androgen 11-KT (measured in these same animals as part of a previous study) (17) were also positively

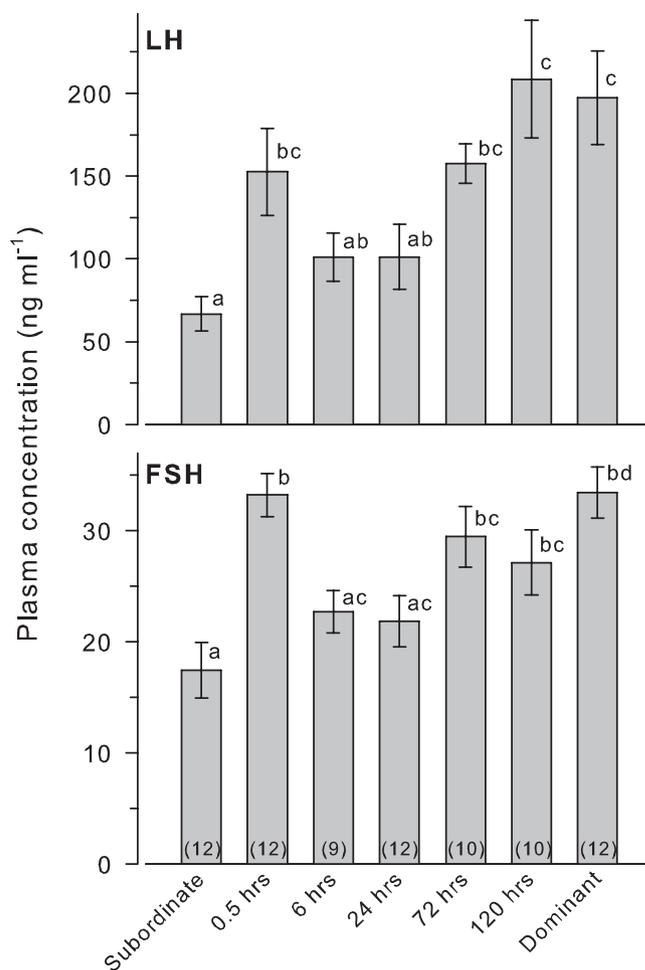


FIG. 2. Circulating plasma levels of LH and FSH in male *A. burtoni* during social ascent. Plasma levels of both LH and FSH were 2-fold higher in stable dominant males compared with stable subordinate males. Furthermore, LH and FSH were rapidly elevated at 30 min after a suppressed subordinate male was given an opportunity to ascend in social status. Data are plotted as mean \pm SE. Bars with different letters represent significant differences ($P < 0.05$), and sample sizes are indicated within each bar on the bottom graph.

correlated with mRNA levels of both LH β ($r = 0.38$; $P = 0.002$) and FSH β ($r = 0.39$; $P = 0.002$).

Discussion

Our results show rapid up-regulation of pituitary gonadotropin hormones during social ascent in an African cichlid fish (Fig. 5). The lower but measurable levels of both LH and FSH in stable subordinate males suggests that the BPG axis is not arrested in these socially suppressed individuals but, rather, maintained at a subthreshold level in anticipation of the chance to gain a territory and become reproductively active. We also show that the reproductive axis is stimulated extremely quickly after perception of social opportunity, presumably as an adaptation for suppressed males to rapidly achieve higher reproductive suc-

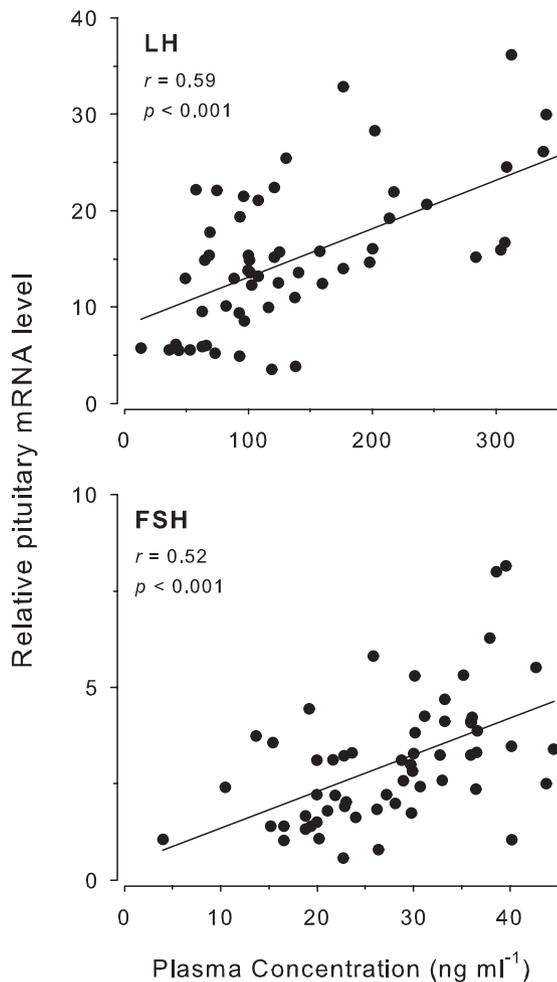


FIG. 3. Pituitary gonadotropin mRNA levels are correlated with circulating plasma concentrations in male *A. burtoni*. There was a positive correlation between pituitary mRNA levels of LH β and FSH β and circulating plasma levels of LH and FSH. Transcript expression is plotted as relative mRNA levels referenced to the geometric mean of two housekeeping genes (*18s* and *G3PDH*). Correlation coefficients (r) and P values are shown.

cess in a dynamic social environment. Thus, the ascension to dominance can be viewed as a reactivation of the BPG axis akin to puberty in other vertebrates (30).

In ascending male *A. burtoni*, LH and FSH were rapidly elevated at the mRNA level within the pituitary as well as at the functional protein level in the circulation. One possible explanation is that perception of social opportunity triggers rapid GnRH1 release from its terminals within the proximal pars distalis of the pituitary, which then has the dual effect of simultaneously releasing stored LH and FSH to the bloodstream as well as increasing transcription or mRNA stability of LH β and FSH β within the gonadotrope cells. Because both of these effects require GnRH ligand binding to its receptor, the apparent slower change in GnRH-R1 mRNA expression during social transition suggests that receptors are kept at a sufficient level in subordinate animals to accommodate the transient ligand-receptor binding interactions required for activation.

Thus, up-regulation of GnRH receptor mRNA may not be necessary during initial stages of transition.

In mammals, GnRH pulsatility is required for maximal GnRH receptor mRNA expression (31), whereas in tilapia, a single GnRH analog injection was enough to increase GnRH-R1 mRNA expression (32). Because GnRH-R1 has relatively low affinity for GnRH1 peptide, it was previously suggested that high concentrations of GnRH1 would be required in the pituitary or that another yet undiscovered GnRH receptor with higher GnRH1 affinity exists in *A. burtoni* (22). Other possibilities are that changes in cell surface receptor concentrations, posttranscriptional or posttranslational modifications of GnRH receptors, or changes in signal transduction pathways, rather than changes in GnRH-R1 mRNA levels, are more important regulatory mechanisms during early stages of social ascent.

In *A. burtoni*, circulating LH and FSH levels were elevated by 30 min after social ascent, suggesting that the perception of social opportunity rapidly signals GnRH neurons to release peptide to the pituitary gonadotropes. There is evidence that GnRH acts as a secretagogue for both LH and FSH from the pituitary in mammals (33, 34) as well as in fishes (27, 35–38). In the tilapia *O. niloticus*, injections of GnRH analog increased plasma LH and FSH levels within 6 h (29), and in the catfish *Clarias gariepinus*, ip injections of GnRH2 increased plasma levels of LH within 30 min (the earliest time point measured) (36). Irvine and Alexander (34) also measured levels of GnRH, LH, and FSH in pituitary venous blood of female mares *in vivo* and showed mean delays between GnRH maxima and gonadotropin peaks to be less than 1 min (FSH, 0.18 min; LH, 0.62 min), which highlights the rapidity with which the pituitary gonadotropes can be activated by the GnRH signal. Given that teleosts lack a hypothalamo-hypophyseal blood portal system but instead have GnRH1 neurons that directly innervate the pituitary, it is possible that the delay between GnRH action and gonadotropin release is negligible in fishes. We also know that the immediate early gene, *egr-1*, is expressed in GnRH1 neurons and up-regulated in the preoptic area of *A. burtoni* by 20 min after social opportunity (16), which supports the rapid timeline of the gonadotropin response seen here. Although the BPG axis of subordinate male *A. burtoni* is suppressed, they still possess a functional GnRH1-gonadotrope system to serve as a substrate for rapid phenotypic change during social transition.

In addition to rapid elevations of circulating gonadotropins, ascending *A. burtoni* also showed increased mRNA levels of both LH β and FSH β in the pituitary at 30 min after ascent. In mammals, variations in LH β and FSH β gene expression are regulated by changes in the fre-

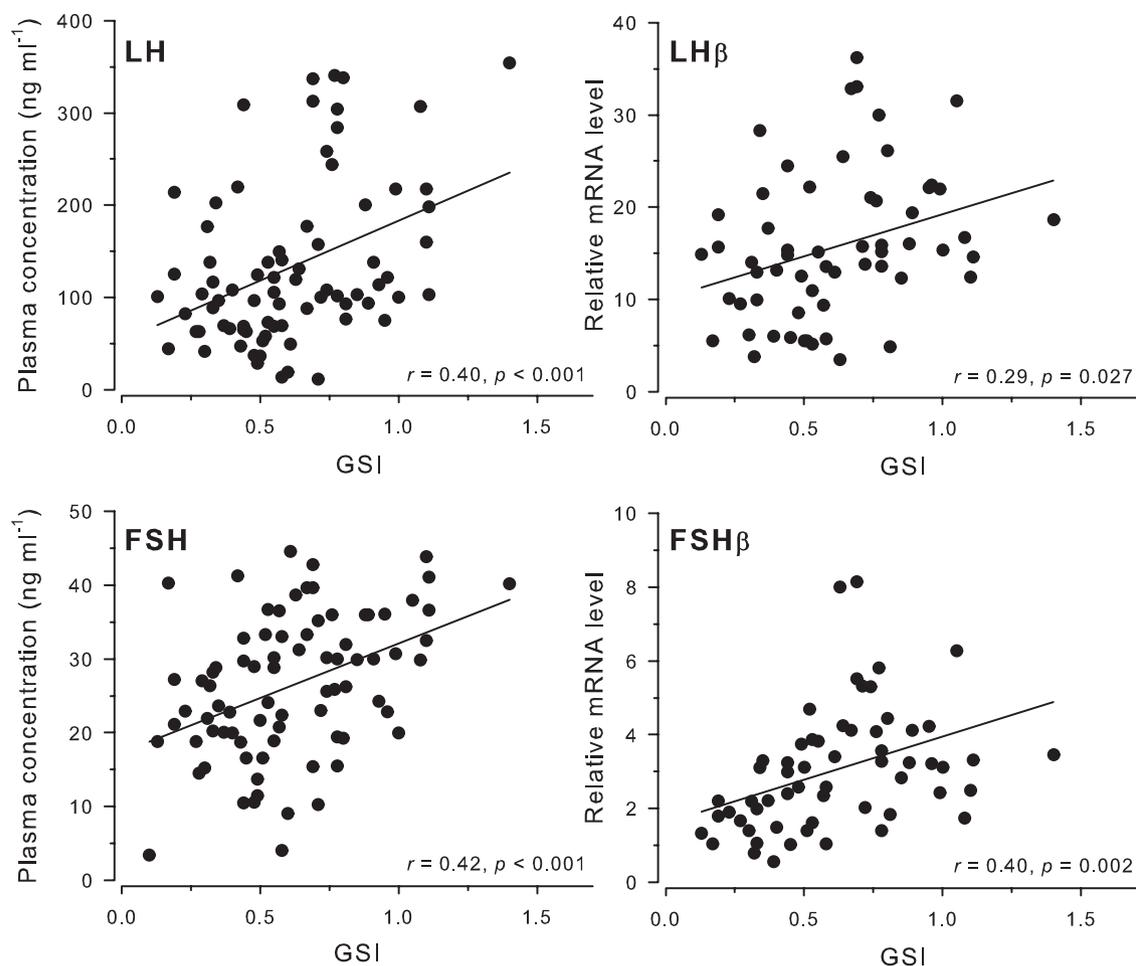


FIG. 4. GSI is correlated with pituitary mRNA and circulating plasma levels of LH and FSH in male *A. burtoni*. GSI was positively correlated with both circulating LH and FSH and pituitary mRNA levels of LH β and FSH β . Correlation coefficients (r) and P values are shown.

quency of pulsatile GnRH delivery to the gonadotropes, which allows selective regulation of different gonadotropin subunits by the same ligand. For example, increasing GnRH pulse frequency stimulates LH β gene expression, and lowering it results in a decrease in LH β but an increase in FSH β mRNA expression (39). Importantly, increased transcription rates of LH β and FSH β are detected within 1 h of GnRH exposure and often decline by 4–6 h later (39). This timeline is similar to that observed here in *A. burtoni*. Experiments in goldfish *Carassius auratus* also demonstrate increased LH β mRNA at 6 h after GnRH application (the earliest time point measured) (40, 41). Although pulsatile secretion of GnRH is thought to be present in all vertebrates (42), there is no direct evidence for this pulsatility in fishes. Pulsatile administration of GnRH, however, is more effective at stimulating LH β gene expression from the goldfish pituitary than is continuous application (40), providing at least some indirect evidence for GnRH pulsatility in fishes. The desensitization of gonadotropes after continuous GnRH exposure that occurs in mammals is evident in some fishes (40) but absent in others (43–45). The discrepancy, however, may

be due in part to the change in GnRH sensitivity of the gonadotropes with sex and reproductive stage of the fish (41, 44). It is also important to recognize that the rapidly increased mRNA levels of LH β and FSH β observed here in *A. burtoni* may not be due to changes in transcription rate but, rather, due to posttranscriptional mechanisms such as changes in mRNA stability or regulation by small RNAs (46). Puberty in mammals is characterized by structural, molecular, and functional changes in the GnRH1 neurons themselves (30, 47), and the next step is to test for similar plasticity in the GnRH1 cells of *A. burtoni* during social transition.

In *A. burtoni*, there was a positive correlation between mRNA levels of each β -subunit in the pituitary and circulating levels of LH and FSH. There are relatively few fish species where both pituitary mRNA and circulating gonadotropin levels in the same individuals are known, primarily due to the lack of plasma gonadotropin assays (especially FSH) available for fishes (44). Pituitary FSH β mRNA levels were well correlated with plasma FSH levels in salmonids and other fishes with group synchronous gonad development (19, 48). In contrast, mRNA levels of

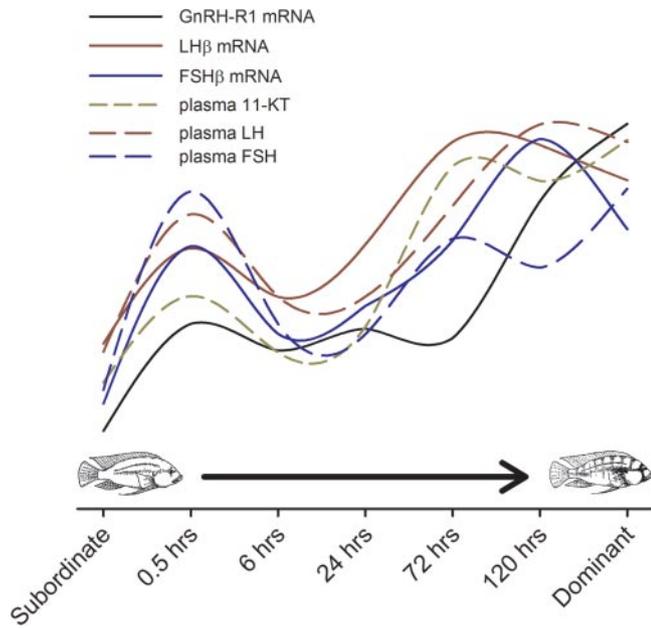


FIG. 5. Summary of physiological changes in the pituitary and circulation during social ascent in male *A. burtoni*. Coincident physiological elevations occur rapidly at 30 min after social opportunity in both the pituitary and the circulation, followed by further increases at later time points to reach stable dominant male levels. Data are expressed as arbitrary values to illustrate overall patterns of temporal changes among measures. 11-KT data are from Ref. 17.

pituitary LH β do not always reflect plasma LH levels, but this appears to be highly dependent on species, sex, stage of maturity, and spawning cyclicality (19). In male *A. burtoni*, however, LH β and FSH β transcript levels in the pituitary do appear to be a good predictor of both circulating gonadotropin levels and GnRH-induced activation of the reproductive axis.

Plasma FSH levels were elevated rapidly in ascending male *A. burtoni* and remained relatively high throughout the 5-d sampling, a time period also characterized by high testicular growth and spermatogenesis (18). Plasma LH levels were also elevated rapidly but then showed a second rise at 120 h after ascent, which was coincident with higher GSI and greater percentages of later (meiotic) spermatogenic stages (18). In male teleosts, FSH is thought to play a major role in early spermatogenesis, whereas LH is more involved in final stages of testicular growth and maturation (37, 44). This generality stems from the fact that during sexual maturation, and in many seasonally breeding fishes, pituitary mRNA levels of FSH β are often elevated during early spermatogenesis and testicular growth, whereas LH β levels peak closer to spermiation and spawning (49–51). This also seems to be the case in *A. burtoni*, albeit on a much shorter time scale, and associated with social transition rather than seasonality or ontogenetic sexual maturity. Higher serum levels of both FSH and LH may then be maintained in stable dominant males to

support both high androgen levels required for territory defense and courtship as well as high testicular activity needed to constantly replenish sperm stores that are used during frequent spawning, which is characteristic of the dominant male phenotype.

Plasma levels of the potent fish androgen 11-KT measured in the same ascending males used in this study (17) also showed a very similar pattern to that of plasma LH and FSH during social transition (Fig. 5). In contrast to mammals where LH is the primary stimulator of steroidogenesis, both gonadotropins are equipotent in causing steroid production in many teleosts. For example, both LH and FSH increase steroid production in salmon (52), tilapia (28, 29), and catfish (53) testis; purified FSH from sea bass pituitary stimulates testosterone and 11-KT release from testicular tissue (54); and through the use of bionutralizing FSH β antisera, Aizen *et al.* (29) confirmed that FSH is indeed involved in androgen secretion from mature tilapia (*O. niloticus*) testis. In the zebrafish *Danio rerio*, FSH was 20-fold more potent than LH in stimulating androgen production from the testis (55). Steroidogenic Leydig cells in fishes also contain both LH and FSH receptors, indicating that both gonadotropins can contribute to androgen synthesis and release (53, 55). In addition to stimulating spermatogenesis, the rapid increase of both LH and FSH at 30 min after ascent in *A. burtoni* may also promote steroid production and release as an anticipatory mechanism to prepare ascending fish for future challenges they are likely to encounter to maintain their new dominance status (56). This rapid steroid production may also function to further increase sperm production in the testes via paracrine mechanisms or alter sperm motility and quality (57, 58).

In summary, our data show rapid activation of the reproductive axis at the level of the pituitary gonadotropin hormones in male *A. burtoni* that perceive an opportunity to ascend in social status and gain full reproductive capacity (Fig. 5). For male *A. burtoni*, it makes evolutionary sense to maintain some level of reproductive competence during social suppression to be prepared for often unpredictable opportunities that may arise in their dynamic environment. Our results also highlight the profound physiological changes that can occur as a result of social perception and the rapidity with which these changes can be detected at multiple levels of the reproductive axis. This rapid reactivation of the BPG axis (*e.g.* after initial sexual maturity) associated with reversible social transitions can be considered a reoccurrence of puberty, which makes *A. burtoni* an attractive model for understanding the common processes underlying puberty and pubertal disorders among vertebrates.

Acknowledgments

We thank the reviewers for their insightful comments that improved the manuscript.

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This research was funded by National Institutes of Health (NIH) F32NS061431 to K.P.M., NIH NS 034950 to R.D.F., and United States-Israel Binational Science Foundation Grant 2005096 to B.L.S. and R.D.F.

Disclosure Summary: The authors have nothing to disclose.

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