

Social regulation of gonadotropin-releasing hormone

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Summary

Behavioral interactions among social animals can regulate both reproductive behavior and fertility. A prime example of socially regulated reproduction occurs in the cichlid fish *Haplochromis burtoni*, in which interactions between males dynamically regulate gonadal function throughout life. This plasticity is mediated by the brain, where neurons that contain the key reproductive regulatory peptide gonadotropin-releasing hormone (GnRH) change size reversibly depending on male social status. To understand how behavior controls the brain, we manipulated the social system of these fish, quantified their behavior and then assessed neural and physiological changes in the reproductive and stress axes. GnRH gene expression was assessed using molecular probes specific for the three GnRH forms in the brain of *H. burtoni*. We found that perception of social opportunity to increase status by a male leads to heightened aggressiveness, to

increased expression of only one of the three GnRH forms and to increases in size of GnRH-containing neurons and of the gonads. The biological changes characteristic of social ascent happen faster than changes following social descent. Interestingly, behavioral changes show the reverse pattern: aggressive behaviors emerge more slowly in ascending animals than they disappear in descending animals. Although the gonads and GnRH neurons undergo similar changes in female *H. burtoni*, regulation occurs *via* endogenous rather than exogenous social signals. Our data show that recognition of social signals by males alters stress levels, which may contribute to the alteration in GnRH gene expression in particular neurons essential for the animal to perform in its new social status.

Key words: behaviour, gonadotropin-releasing hormone, cichlid, *Haplochromis burtoni*, gonad.

Introduction

In vertebrates, gonadotropin-releasing hormone (GnRH) delivered by hypothalamic neurons to the pituitary gland, regulates reproduction through release of pituitary gonadotropins that regulate gonadal function (Cattanach et al., 1977; Mason et al., 1986; Sherwood, 1987). In humans, GnRH secretion during late childhood is essential for puberty (for a review, see Foster and Nagatani, 1999), and levels of GnRH fluctuate cyclically in sexually mature females while less regular oscillations in males maintain fertility (for a review, see Hayes and Crowley, 1998). In many social animals, sexual maturation can be suppressed by the presence of dominant conspecifics (Cardwell and Liley, 1991; Cardwell et al., 1996; Faulkes et al., 1990; Leitz, 1987; McKittrick et al., 1995; Pankhurst and Barnett 1993; Payman and Swanson, 1980; Saltzman et al., 1996; Sapolsky, 1993). Suppressive signals may be passively (Barrett et al., 1990; Gudermuth et al., 1992) or actively delivered during social encounters and include tactile (M. R. Davis and R. D. Fernald, unpublished observations; Sapolsky, 1993), pheromonal (Drickamer, 1989), visual (Barrett et al., 1993; Muske and Fernald, 1987) and/or psychogenic (Sapolsky, 1993) components. Ultimately, such social influences on sexual maturation must be exerted on GnRH-containing neurons in the hypothalamus.

In many fish, social factors regulate growth and reproduction throughout life (Berglund, 1991; Borowsky, 1973; Fraley and Fernald, 1982; Hofmann et al., 1999; Schultz et al., 1991). In the African cichlid *Haplochromis (Astatotilapia) burtoni* (Günther), the species studied here, gonadal maturation is suppressed when juvenile males are reared in the presence of adult males (Davis and Fernald, 1990). Suppressed juveniles have small, unspemiated testes and GnRH-containing neurons in hypothalamo-preoptic area that are, on average, eight times smaller in volume than those of unsuppressed age-mates (Davis and Fernald, 1990; Fraley and Fernald, 1982). In contrast, juvenile females attain sexual maturity irrespective of the presence of adults. This shows that social stimuli produced by adult males suppress sexual maturation in juvenile males *via* preoptic GnRH neurons, but that other cues must regulate sexual maturation in females.

In their natural environment, the shore-pools of Lake Tanganyika, reproductive opportunity for *H. burtoni* males depends upon defense of a territory containing food, which ensures access to females that enter the territory to feed and spawn (Fernald and Hirata, 1977a,b). Territorial males are brightly colored and socially dominant. Since food resources are limited, only a fraction of *H. burtoni* males hold territories

and reproduce at any given time. The remaining males are non-territorial, and their coloration, like that of females, matches the lake bottom. These socially subordinate males postpone sexual maturation until a habitat becomes available, at which time they undergo a transformation to the territorial state.

Mature males can change territorial status in either direction. Switches from territorial (T) and reproductively active to non-territorial (NT) and reproductively inactive (T→NT), or *vice versa* (NT→T), occur in the wild and in aquaria (Hofmann et al., 1999). Switches can be achieved experimentally by moving individuals to new communities where the social constellation influences the direction of the change (Francis et al., 1993). A male introduced to a novel community quickly adopts behaviors and body colors that reflect his new social status. Remarkably, plasticity is also found in the brain, where GnRH-containing neurons within the hypothalamo-preoptic area change size, reversibly, depending on the direction of social change (Francis et al., 1993), with T males having larger preoptic immunoreactive GnRH (irGnRH) neurons than NT males. Preoptic irGnRH neurons in female *H. burtoni* also change size with reproductive state, but these changes are independent of social interactions (White and Fernald, 1993).

The pronounced social control of reproduction in *H. burtoni* males and the apparent lack of it in females provide an opportunity to investigate the mechanisms through which social interactions alter reproductive status *via* the brain. Such mechanisms must ultimately control GnRH delivery to the pituitary. To begin to explore these mechanisms, we tested the hypotheses that increased transcription of GnRH mRNA contributes to reproductive capacity in both males and females, while the signals that drive this upregulation are sexually dimorphic.

In many species, multiple cDNAs code for multiple GnRH peptides (Gestrin et al., 1999; Kasten et al., 1996; Latimer et al., 2000; White et al., 1994), and in *H. burtoni* three genes for GnRH are expressed in distinct neuronal populations (Bond et al., 1991; White et al., 1994, 1995; for nomenclature, see White and Fernald, 1998). One of these genes, GnRH1, is expressed in preoptic neurons that project to the pituitary (Bushnik and Fernald, 1995) and have been shown to change size in response to social change (White et al., 1995). The other two, one localized in the midbrain (GnRH2) and one expressed in cells located along the forebrain terminal nerve (GnRH3), have unknown functions. We used molecular probes specific for each form to test whether social cues alter reproductive capacity *via* gene expression of any GnRH form. We found that social opportunity initiates a cascade of responses in males, including heightened aggressiveness, increased expression of only GnRH1 and enlargement of preoptic GnRH neurons and of gonads. Further, the cortisol levels of ascending males dropped, consistent with idea that social suppression of reproductive capacity in NT males results from stressful behavioral interactions with aggressive T males.

The biological changes in GnRH that occur during social ascent happen faster than those that accompany social descent.

Interestingly, behavioral changes show the reverse pattern: aggressive behaviors emerge more slowly in socially ascending animals than they disappear in socially descending animals. This time course of physiological and molecular change fits well with the life history pattern of male *H. burtoni* in their natural habitat. For comparison, we also measured GnRH and stress indices in female *H. burtoni*, which do not undergo changes in social status but do experience cyclical changes in reproductive state. Although female gonads and preoptic irGnRH neurons exhibit changes that are comparable in magnitude with those in males, in females these changes are regulated *via* nutritional not social cues.

Materials and methods

The goal of these experiments was to investigate the social regulation of reproductive state. Thus, behavioral observations to assess social status were combined with measurements of key physiological variables including body and gonad mass, circulating cortisol levels, mRNA levels for the three GnRH gene forms (GnRH1, GnRH2 and GnRH3) and preoptic irGnRH neuronal soma size. Three different kinds of social manipulations were used to assess the role of social status on physiological changes: males maintained in a *status quo* social situation, males during social ascent and males ascending compared with those descending (Fig. 1).

Subjects

The *Haplochromis (Astatotilapia) burtoni* (Günther) used in this study were bred from wild-caught stock and maintained at Stanford University under laboratory conditions that simulate those of their natural environment in Lake Tanganyika, Africa (Fernald, 1977): pH 7.8–8.2, temperature 29 °C, 12 h:12 h light:dark cycle with full-spectrum illumination (Duralight 30 W; Bob Corey Associates, Merrick, NY, USA). Gravel and terracotta pot-shards provided visual isolation, allowing dominant males to establish and maintain territories, an integral component of their reproductive and social behavior (Fernald, 1977). Fish were fed once daily at 09:00–09:30 h with cichlid formula pellets and flakes (Aquadine, Healdsburg, CA, USA). All work was performed in compliance with the animal care and use guidelines at Stanford University.

Behavioral observations and analysis

To allow behavioral observations of specific individuals, males were tagged near the dorsal fin with a unique combination of colored beads. Focal observations were made as follows. Each male was observed for 3 min between 14:00 and 16:00 h, three times per week (or, where noted, daily), and its social and reproductive state were recorded. Males were classified as T or NT on the basis of their behavior and coloration as follows. NT males are cryptically colored and resemble females. Their behavior consists primarily of schooling and fleeing from attacking T males. T males are either bright blue or yellow and have a dark lachrymal stripe (eye-bar) and orange humeral patches. Their behavior consists of territorial defense,

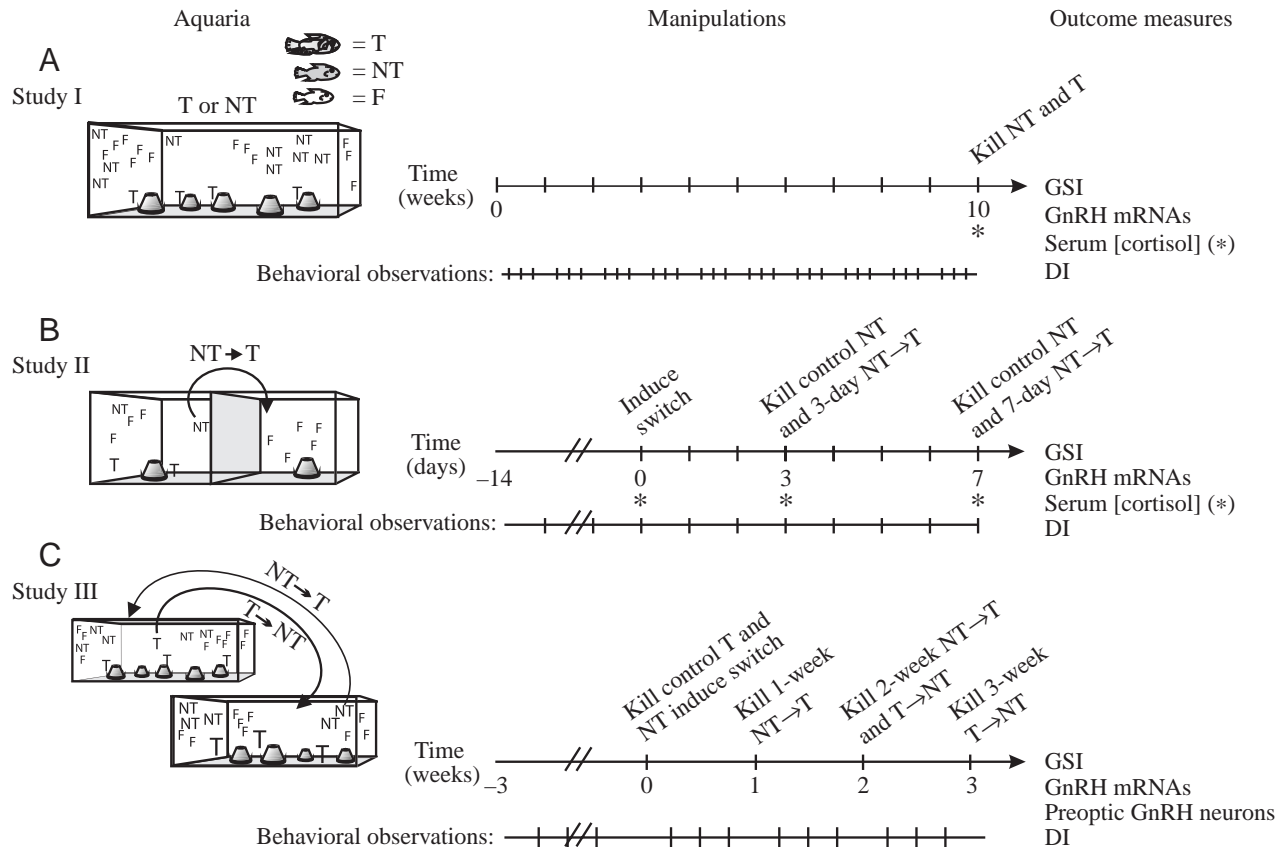


Fig. 1. Timeline of experimental manipulations used for studies I, II and III of *Haplochromis burtoni* males. Three experiments (I, II and III) examined how social opportunity changes behaviors and gonadotropin-releasing hormone (GnRH) expression. These are shown schematically by the three types of aquaria used and the corresponding time courses of behavioral observations and physiological and molecular measures. T, territorial male; NT, non-territorial male; F, female. (A) Study I: undisturbed aquaria were used to identify males that maintained a stable social status of either T or NT for more than 2 weeks. Six NT males and five T males fulfilled this behavioral criterion. After 10 weeks of observation, fish were captured, and blood was sampled for measurement of serum cortisol levels. Gonad and body masses were obtained for calculating the gonosomatic index (GSI; see Materials and methods), used to confirm the behaviorally defined social state. Brains were processed for the detection of GnRH gene expression. DI, index of dominance (see Materials and methods). (B) Study II: to determine how fast GnRH gene expression changes during social ascent (NT→T), half-tanks were prepared with distinct communities on either side of a Plexiglas barrier. A pair of NT males, matched for age and size, were housed in one half of an aquarium containing two T males and a few females. After 2 weeks of baseline behavioral observations, blood samples were obtained, and one member of each pair (NT→T) was then moved to the opposite half of the tank, where there were only females. The other (control NT) was returned to the first side. Behavioral observations continued for 3 or 7 days, when blood was again sampled and NT→T and control NT males were killed for analysis of GSI and GnRH mRNAs. (C) Study III: to examine changes in GnRH expression during both social ascent (NT→T) and descent (T→NT), males were observed for 3 weeks to ascertain their social state. Control males were killed, and experimental males were moved to new tanks containing communities designed to induce a change in their social status. Only males for whom behavioral observations confirmed that the intended social change had taken place were selected for measurement of GSI, GnRH mRNAs and preoptic immunoreactive GnRH neuronal soma sizes at the time points indicated. Asterisks indicate when serum cortisol levels were measured.

solicitation and courtship of females and feeding. Behaviors were scored using established criteria (Fernald, 1977; Fernald and Hirata, 1977b) and were categorized as aggression towards subordinates (chasing or biting females or NT males), aggression towards T males (chasing or biting T males, threat and border displays and fighting), reproductive acts (digging, courting and spawning) or submissive acts (fleeing). The number of instances of each behavior was recorded, and the presence of an eye-bar was noted.

Males were ranked for aggression using an index of dominance (DI). DI was calculated as the sum of the number

of aggressive acts minus the number of submissive acts that occurred during a given observation period. DIs were averaged over the number of days an animal spent in one social setting, as were the number of reproductive displays. These daily mean values were used for comparison.

Experimental design

Three different experimental protocols (I, II and III) were used to examine responses of males to social change: undisturbed animals (study I), males ascending in social status (study II) and males ascending and descending in social status

(study III). These protocols are depicted in Fig. 1 and described below.

Study I: GnRH gene expression levels in H. burtoni males living in undisturbed communities

To measure the effect of ongoing social interactions on GnRH gene expression in mature males, social communities were assembled that had 3–4 T males, 9–12 NT males and 12–16 females (Fig. 1A, study I). Communities were established in three tanks (91 cm×61 cm×46 cm; width×length×height) that were left undisturbed for 4 months except for feeding, behavioral observations and occasional removal of a female for a separate experiment (see below). Behavioral observations were used to select males that had maintained a consistent social status (e.g. T or NT) for a minimum of two (≥ 2) and up to 10 consecutive weeks prior to being killed. Blood samples were obtained immediately prior to killing the fish for measurement of serum cortisol levels. Body and gonad masses were obtained for calculation of the gonosomatic index (GSI, see below). Brains were removed and frozen in liquid nitrogen for subsequent measurement of mRNA levels for the three forms of GnRH using ribonuclease protection assays (RPA; see below).

Study II: changes in GnRH gene expression during social ascent (NT→T)

To determine whether GnRH gene expression changes during an ascent in social status, 13 pairs of young NT males were matched for size and social history and then either induced to change into T males or kept as NT males. To do this, the paired NT males were removed from their home tanks and introduced into one half of a new tank (13 halves, each half 46 cm×46 cm×30 cm; width×length×height) that also contained two larger T males and several females (Fig. 1B, study II). The other half of the tank contained only females. After 2 weeks of daily observations to obtain baseline behavioral values, one member of each pair was chosen at random and moved to the opposite side of the tank where, in the absence of tactile interactions with males, it had the maximum chance of becoming territorial. At the time of transfer, both NT males were caught and blood was taken for subsequent cortisol measurement (see below). One male (NT→T) was then placed in the all-female half of the tank. The other NT (control NT) in each pair was returned to the first side of the tank containing the larger T males. Behavioral observations of both subjects continued daily for 3 or 7 days. Blood samples were then taken, and the experimental males were killed by rapid cervical transection. Body and gonad mass were measured, and the brain tissues were processed for analysis of GnRH mRNA expression levels.

Study III: comparison of the time course for social ascent (NT→T) versus social descent (T→NT)

Although study II provided information about how quickly social change can lead to physiological change during social ascent, we wanted to know the time course of changes in

GnRH expression during social ascent or descent under conditions that more closely paralleled the social situation of animals in their natural habitat (i.e. in the presence of other males, rather than alone with a group of females). To do this, we induced changes in social status within community settings. Groups of males were observed as they established social communities, after which those designated as controls (15 T and 11 NT males) were killed (Fig. 1C, study III). The social status of the remaining T males was then changed from T to NT (T→NT) by moving them to new tanks inhabited by a community of older, larger fish. Conversely, the remaining NT males were induced to become territorial (NT→T) by moving them to tanks containing younger, smaller fish communities. Animals remained in their new tank communities, and behavioral observations continued for a period based on pilot studies (see 3- and 7-day data above; Nguyen, 1996).

Briefly, a pilot study (Nguyen, 1996) was conducted to assess the time course of structural changes in preoptic irGnRH neurons. This study used identical procedures to those used here except that neuronal soma sizes were measured only at 2 weeks following the induced change in social status. These revealed that, in NT males that had ascended in social status, structural changes had already occurred by 2 weeks. At this time, preoptic irGnRH neurons were similar in size to those of control T males. In contrast, in T males that were descending in social state, no change in irGnRH neuron size was observed at 2 weeks. Thus, for the present study, new time points were added to establish when the structural differences accompanying social change happened. Accordingly, NT→T males were killed 1 ($N=8$) or 2 weeks ($N=9$) after entry into the new tank community, while T→NT males were killed after 2 ($N=8$) or 3 ($N=6$) weeks following the social transition. For the 2-week time points, preliminary analysis (see *Statistical analysis*) revealed that data obtained from the pilot study and study II were identical when values were reported as the percentage of control T values for each study. These data were therefore combined and plotted (see Fig. 5). To measure GnRH mRNA expression levels, males were killed 1 ($N=7$) or 3 weeks ($N=5$) after being switched to new communities. Brains were removed and processed either for immunocytochemistry for measurement of preoptic irGnRH neuronal soma size or for the ribonuclease protection assay to quantify GnRH transcript levels.

Study IV: regulation of GnRH gene expression in females

In female *H. burtoni*, preoptic irGnRH neurons change size depending on whether the individual is spawning or brooding fry (White and Fernald, 1993). To determine whether GnRH transcript levels also fluctuate during the reproductive cycle, females were observed until the act of spawning (Fig. 2). Some were caught, blood was drawn and the fish were killed (Sp; $N=6$). For comparison, other spawning females ($N=7$) were allowed to brood their fry for the normal brooding period (2 weeks; Br), at which time blood was drawn and the animals were killed. Body and gonad masses were measured, and brains were processed for comparison of GnRH transcript levels.

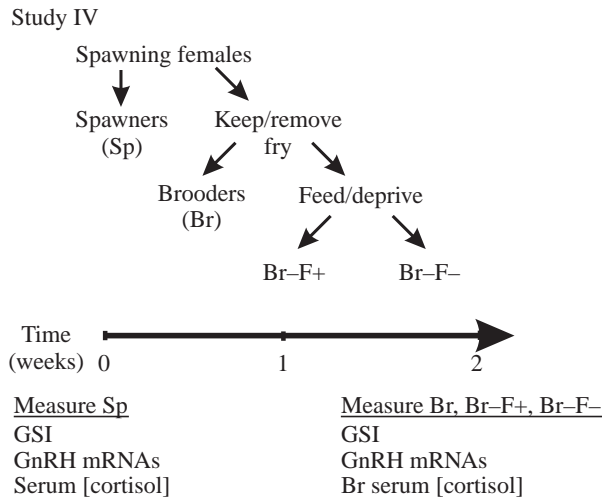


Fig. 2. Experimental timeline used for *Haplochromis burtoni* females. Different reproductive and nutritional states were examined for levels of gonadotropin-releasing hormone (GnRH) expression in females (study IV). Females were observed until the act of spawning. A subset of these (Spawners; Sp) was removed for blood sampling and then killed for determination of body and gonad masses and GnRH mRNA levels. The remainder were divided into three groups. Brooders (Br) were females that brooded fry in their mouths for 2 weeks after spawning. Broodless females (Br-) were females whose eggs were removed immediately following spawning. Of these, half were fed (Br-F+) and the other half were food-deprived (Br-F-). At the end of 2 weeks, brooders were removed for blood sampling, and all three groups were killed for determination of relative gonadal masses and of GnRH mRNA levels. GSI, gonosomatic index (see Materials and methods).

To identify endogenous cues implicated in GnRH production in female *H. burtoni*, the effect of brooding fry on GnRH levels was measured. *H. burtoni* are mouth-brooders, so females do not eat during the 14 days when they maintain their fry in their mouths. To distinguish whether the presence of fry or the lack of food regulates GnRH production, females ($N=38$) in stable communities were observed until the act of spawning. Approximately half were allowed to brood fry for 2 weeks prior to being killed (Br; $N=18$). The remainder were induced to release their eggs and moved to tanks without males. Over the next 2 weeks, some of these 'broodless' females were fed (Br-F+; $N=12$), while the others were not (Br-F-; $N=8$). The fish were killed, and body and gonad masses were measured. Brains were processed to measure preoptic irGnRH neuronal soma size and GnRH mRNA transcript levels. Preliminary analysis revealed that GnRH1 expression in the two groups of brooding females, probed on two different gels, were not significantly different from one another (mean normalized, optical density, gel A 0.62 ± 0.11 , $N=7$; gel B, 0.66 ± 0.05 , $N=6$, Mann-Whitney U -test, $P=0.72$; see *Measurement of mRNA levels*). These data were subsequently pooled for further statistical comparisons.

Cortisol measurement

Serum cortisol levels were used as an index of stress on the

basis of our previous analyses that established control values for serum cortisol levels in animals of different social states (Fox et al., 1997). To compare the magnitude and time course of changes in cortisol levels during the present studies, cortisol levels were measured in serum samples following our established procedures (Fox et al., 1997). Only samples that were obtained within 3 min of approaching a given fish's tank were used to avoid confounding increases in cortisol level due to capture-associated stress.

Body and gonad mass

All animal subjects were killed by rapid cervical transection and then weighed. The brains were removed and frozen in liquid nitrogen. The gonads were removed and weighed, and the gonosomatic index (GSI) was calculated [$GSI = (\text{gonad mass}/\text{body mass}) \times 100$, where mass is in g].

Quantification of preoptic irGnRH neuronal soma size

To compare irGnRH cell sizes, brain tissue was processed and immunostained for detection of preoptic irGnRH neurons using a commercial antibody (anti-LHRH; ImmunoStar; Hudson, WI, USA), as previously described (White and Fernald, 1993). Previous measurements showed that the mean soma sizes of GnRH-containing neurons in the terminal nerve and the diencephalon do not differ in animals of different social status (Davis and Fernald, 1990). Therefore, only the preoptic irGnRH cell sizes were analyzed. Cross-sectional areas were measured on computer-captured video images (NIH Image 1.40 by Wayne Rasband) from a microscope (Zeiss, 600 \times), as described by White and Fernald (1993).

Measurement of mRNA levels

To measure the effects of change in social status on GnRH gene expression, a ribonuclease protection assay (RPA) was developed. Total RNA was isolated from brain tissue using Ultraspec II/RNA tack resin (BioTecx, Houston, TX, USA) following standard protocols. Approximately 30 μg of total RNA was obtained from 30 mg of tissue.

For each of the three different GnRH mRNAs in *H. burtoni*, labeled antisense riboprobes (White et al., 1995) were transcribed from vectors (T7 RNA polymerase; Promega, Madison, WI, USA) incorporating [α - ^{32}P]UTP (NEN, Boston, MA, USA) to a specific activity of approximately 3×10^8 cts $\text{min}^{-1} \mu\text{g}^{-1}$. Probe reactions were size-separated on a 4.8% acrylamide gel, and full-length transcripts were identified by autoradiography. Bands were cut from the region of the gel containing the longest transcripts, and these were eluted either at 37 $^{\circ}\text{C}$ for 3 h or at 4 $^{\circ}\text{C}$ overnight prior to the RPA. To control for loading error, an antisense 18S riboprobe with low specific activity was generated from a T7 vector (Ambion, Austin, TX, USA) using an Ampliscribe transcription kit (Epicentre, Madison, WI, USA). Reactions were run at 42 $^{\circ}\text{C}$, incorporating radiolabeled UTP together with unlabeled ATP, GTP, CTP and UTP nucleotides and typically generated approximately 50 μg of probe.

RPA was performed on samples of total brain RNA (Hybespeed, Ambion) according to the protocol provided. Pilot studies revealed non-specific interactions when RNA samples were simultaneously hybridized with all three GnRH riboprobes and the 18S control. Accordingly, for each experiment, three separate hybridizations were performed from identical pools of total RNA. For detection of GnRH1, 2.5 µg of total RNA from each subject was used. Because the signals for GnRH2 and GnRH3 were weaker, the blots used to detect these transcript levels contained 10 µg of total RNA per subject. All blots were probed with 2×10^4 cts min⁻¹ of the respective labeled probe. A loading control of 500 ng of the 18S riboprobe was used to saturate binding to 10 µg of total RNA. Following size separation on a non-denaturing 4.8% acrylamide gel, the amount of labeled RNA was measured using phosphodetection of the signal (Molecular Dynamics, Sunnyvale, CA, USA) and subsequent quantification of the protected signal optical density (IPLabGel, Signal Analytics, Vienna, VA, USA). Each GnRH optical density (OD) was normalized to that of its corresponding 18S loading control. These individual normalized signals were averaged for each group, and the means \pm S.E.M. are reported.

Pilot studies confirmed that the amounts of GnRH and 18S ribosomal probes used for hybridization reliably saturated endogenous transcripts, thereby allowing quantification of GnRH mRNAs. Samples of total brain RNA (i.e. either 2.5 µg for GnRH1 or 10 µg for the other two GnRH transcripts) as well as twice these amounts were hybridized to 2×10^4 cts min⁻¹ of the respective probes. Following ribonuclease digestion, gel electrophoresis and OD analysis, the intensity of the protected signals reliably reflected the twofold increase (data not shown), indicating that sufficient amounts of each probe had been used. To confirm the consistency of quantitative results across RPAs, sets of samples were processed twice for each of the three GnRH probes. These demonstrated high reliability since correlations of the quantified protected signals between duplicate samples run on separate gels averaged 0.80 (Spearman ρ , $P < 0.005$).

Statistical analysis

Non-parametric tests were used because the data could not be assumed to be normally distributed. The minimum significance level was determined by a one-way χ^2 approximation and set at $P < 0.05$. Two-tailed tests were used throughout. Wilcoxon/Kruskal–Wallace rank sum tests were used for non-parametric comparisons across more than two groups. When warranted, Mann–Whitney U -tests were used to compare behavioral frequencies, GSIs, GnRH mRNA levels, preoptic irGnRH neuronal soma sizes and serum cortisol concentrations between two experimental groups. Repeated measures of serum cortisol levels and behaviors on the same individual were performed using Wilcoxon signed-rank difference tests. Spearman rank tests were used to assess correlations between measures. Values are reported as the mean \pm the standard error of the mean (S.E.M.). All statistical

analyses were performed using JMP IN software (SAS Institute, Inc., Cary, NC, USA).

To obtain the values reported for preoptic irGnRH neuronal soma sizes shown in Fig. 5, mean soma sizes for individual fish were normalized with respect to the mean for the control T fish. This was performed for a pilot study (described in study III, above), in which males underwent a 2-week transition (NT \rightarrow T or T \rightarrow NT) in social status and were compared with control T and NT males, and for the follow-up study (study III) in which controls and males with 2-week transition times were repeated and 1-week and 3-week time points were added. In both the pilot study and study III, identical behavioral manipulations were used except that 1-week and 3-week time points were also sampled in study III. In each study, individual soma size means were normalized to their respective control T mean. Because the normalized values for the 2-week transitions did not differ between studies, these were pooled, converted to percentages of the control T value and plotted in Fig. 5. Statistical comparisons for Fig. 5 are reported in Table 1.

Results

mRNA levels for GnRH1 in T and NT males in stable social settings

Previous studies in *H. burtoni* showed that GnRH-containing neurons within the hypothalamo-preoptic area of T males are larger than those of NT males (Davis and Fernald, 1990). To determine whether GnRH mRNA levels also depend on social status, levels of mRNA encoding the three distinct forms of GnRH in *H. burtoni* brain were analyzed in males of identified social status, living in undisturbed tank communities. The results for study I are shown in Fig. 3 for individual males, while summary data are plotted in Fig. 4 on the right. Focal observations (see Materials and methods, Fig. 3A) allowed males to be designated as either T or NT. Territorial males were more aggressive and displayed eye-bars and reproductive behaviors more often than NT males. Several behavioral patterns were noteworthy during the 10-week period. Of the five males ultimately selected for the T state, initially only one (T5) displayed territorial behaviors (Fig. 3A, right). This male remained territorial for 6 weeks, then exhibited mixed behaviors for 2 weeks, prior to regaining stable territorial behaviors during the last 2 weeks before being killed. Of the remaining four T males, three began the observation period as NT males and the fourth exhibited behaviors characteristic of both social states. All four switched to T status and remained so for the minimum 2 weeks prior to being killed, and T4 just met the criterion. Among the six fish designated as NT (Fig. 3A, left), five were stable, displaying subordinate behaviors during the entire 10-week observation period. The sixth (NT6) was aggressive at the start of the observations and then, 2 weeks prior to being killed, lost his territory and exhibited only subordinate behaviors.

Social status was reflected in brain levels of mRNA

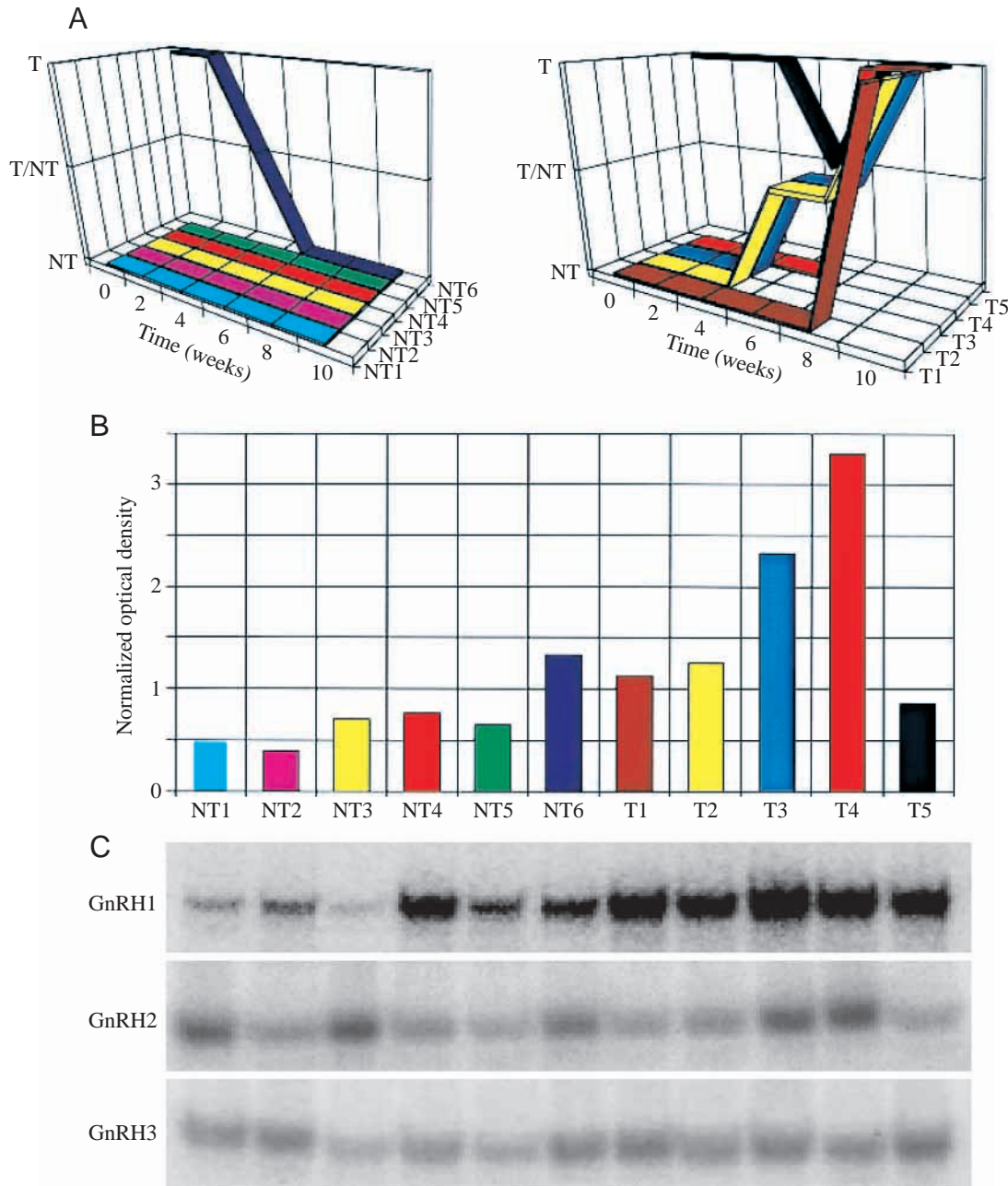


Fig. 3. Study I: gonadotropin-releasing hormone 1 (GnRH1) gene expression differs with social state in undisturbed territorial (T) and non-territorial (NT) males. (A) The behavior of males was examined for 10 weeks to monitor social state. The social status of six males used for the NT group plotted as a function of time is shown on the left; that of T males ($N=5$) is shown on the right. Note that NT6 initially displayed territorial behaviors. NT/T on the vertical axis indicates that the fish exhibited a mixture of aggressive and subordinate behaviors. (B) Preoptic GnRH1 mRNA levels were measured for each individual and are plotted as optical density (OD), described in the Materials and methods and below. Note that NT6 has the highest levels of all NTs. When averaged, T males had higher levels of preoptic GnRH1 mRNA than NT males (see Fig. 4A, and Results). (C) Only GnRH1 exhibits differential expression according to social status. Phosphorimager images show protected bands from three separate ribonuclease protection assays of whole-brain RNA for these 11 males. Each assay was simultaneously probed with an 18S probe (data not shown) as well as a specific probe for GnRH1, GnRH2 or GnRH3. The optical densities of the protected GnRH bands were normalized against the signal for an 18S loading control to obtain the group means reported in the text and plotted in B.

encoding GnRH1. As predicted, T males had higher levels of this transcript than did NT males (OD, T males = 1.77 ± 0.46 , $N=5$, versus NT males = 0.72 ± 0.13 , $N=6$; Mann-Whitney U -test, $P < 0.03$; Figs 3B,C, 4A). Comparison of gonadal size

showed that the mean GSI of T males was greater than that of NT males (GSI, T males = 0.74 ± 0.05 versus NT males = 0.43 ± 0.06 , Mann-Whitney U -test, $P < 0.02$; Fig. 4B). GSIs and levels of GnRH1 mRNA were positively correlated

(Spearman $\rho=0.86$, $P<0.0005$; Fig. 4C). Mean serum cortisol levels in NT males were three times those in T males (NT= 3.19 ± 2.20 ng ml $^{-1}$, $N=4$, versus T= 0.89 ± 0.31 ng ml $^{-1}$, $N=4$, not significant). The high variance in measured cortisol levels, particularly for NT males, and the small number of blood samples successfully obtained rendered this difference not statistically significant.

Stability of social status, as measured by the number of weeks a fish remained dominant, was strongly correlated with GnRH1 mRNA level (Spearman $\rho=0.77$, $P<0.005$) and with GSI (Spearman $\rho=0.86$, $P<0.0005$; Fig. 4C). As shown in Fig. 3, the case of the one NT male (NT6) that was territorial at the beginning of behavioral observations highlights this

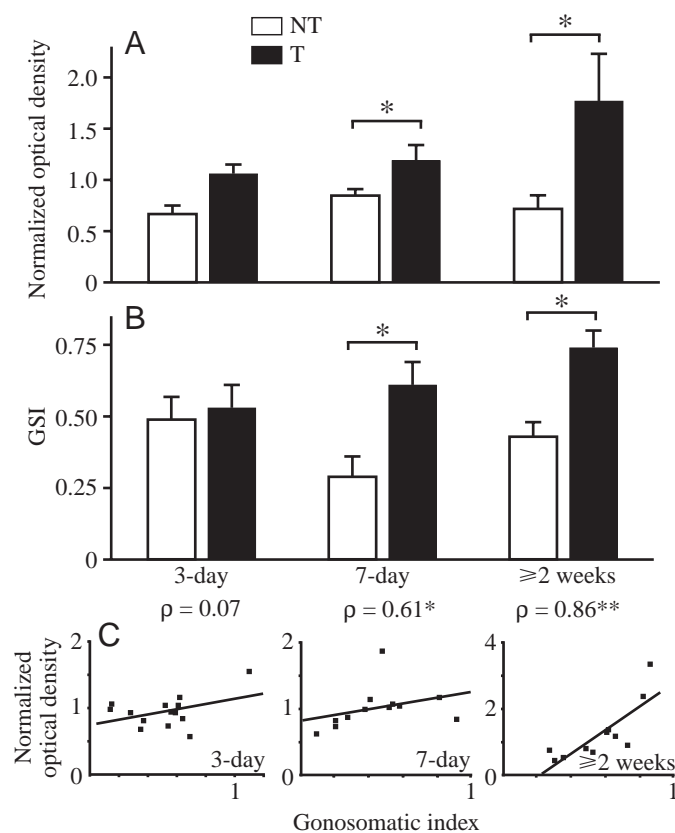


Fig. 4. Study II: gonadotropin-releasing hormone 1 (GnRH1) gene expression increases within 3 days following removal of suppressive tactile cues from dominant conspecifics. Mean GnRH1 mRNA levels (A), plotted as normalized optical density (see Materials and methods), and gonadosomatic indices (GSIs) (B) are plotted for males undergoing a 3- or 7-day transition from non-territorial (NT) to territorial (T) and are compared with those of the NT and T males of stable social status (≥ 2 weeks) from study I. Values are means \pm S.E.M. ($N=6$ NT, 8T for 3- and for 7-day transmissions; $N=6$ NT, 5 T for >2 weeks). Open columns show control NT values. Filled columns show NT \rightarrow T and undisturbed T male values. (C) Gonad size, plotted as the gonadosomatic index (GSI; see Materials and methods) and GnRH1 gene expression (plotted as normalized optical density) are positively correlated. The strength of the correlation (Spearman ρ) increased with the time spent in the T state. Asterisks indicate P -values: * $P<0.05$; ** $P<0.0005$.

trend. This individual had the most GnRH1 mRNA and largest gonads of all NT males. There were no significant correlations between levels of the other GnRH transcripts (GnRH2 and GnRH3) and any other measure.

The onset of aggressive behavior and GnRH1 gene expression during social ascent

To determine how quickly behavioral and physiological changes reflect a change in social status, one member in a pair of NT males was induced to become T (NT \rightarrow T) by moving it to a social setting with only females. In their new social setting, NT males responded by exhibiting color and behavioral patterns characteristic of T males. Behavioral responses, cortisol levels and mRNA levels of all three GnRH forms were compared between NT \rightarrow T males and their NT counterparts 3 days after the move. Prior to experimental manipulation, paired NT males exhibited uniformly low levels of aggressive behavior seen in their mean dominance index scores (pre-switch DI for NT \rightarrow T males= -2.4 ± 0.5 , $N=8$ versus control NT males= -1.4 ± 0.5 , $N=6$, not significant). Moving one NT male to the opposite half of the tank containing only females produced a switch in his social status. Three days after being moved, these NT \rightarrow T males had higher DIs than those of control NT males (post-switch DI for NT \rightarrow T= 4.3 ± 1.4 versus control NT= -1.2 ± 0.4 , Mann-Whitney U -test, $P<0.002$). NT \rightarrow T males also exhibited increased aggressive behavior relative to their own baseline levels as NT males (Wilcoxon signed-rank test, $P<0.01$).

The reproductive measures for study II are shown in Fig. 4B. Three days following the switch, GnRH1 gene expression increased by approximately 20% in NT \rightarrow T males relative to control NT males, but this did not reach significance (OD, NT \rightarrow T= 1.06 ± 0.09 versus NT control= 0.80 ± 0.07 , Mann-Whitney U -test, $P=0.07$; Fig. 4A). Gonad size did not differ between the two groups (GSI, NT \rightarrow T= 0.53 ± 0.10 versus NT control= 0.49 ± 0.08 , not significant, Fig. 4B) nor were GSIs correlated with GnRH1 expression at this time, when all males were included (Spearman $\rho=0.07$, not significant, Fig. 4C). Among NT \rightarrow T males, however, GSI and GnRH1 transcript levels were positively correlated (Spearman $\rho=0.74$, $P<0.05$). There were no differences in expression levels of the other two GnRH forms.

Prior to moving males to the all-female compartment, NT males had similar cortisol levels (NT \rightarrow T prior to switch= 11.3 ± 3.1 ng ml $^{-1}$, $N=7$, versus control NT= 9.7 ± 4.9 ng ml $^{-1}$, $N=4$, not significant). Three days after being moved to the opposite side of the barrier, cortisol levels had decreased in the NT \rightarrow T males relative to their own baseline levels. This shows that the new territorial status was associated with lower stress levels within an animal (NT \rightarrow T post-switch= 2.4 ± 1.3 ng ml $^{-1}$, $N=7$; Wilcoxon signed-rank, $P<0.01$). In addition, there was a non-significant decrease in cortisol levels between NT \rightarrow T and control NT males at 3 days (Mann-Whitney U -test, $P=0.09$). It is worth noting that repeated cortisol measurements within an individual animal provide a more useful analysis of change than measurements made between

individuals because of the high individual variability of this measure (Fox et al., 1997).

To extend the time course for observing changes in GnRH1 transcript levels and cortisol levels, the above experiment was repeated, but fish were killed 7 days following transfer of one male in each pair of NTs to the all-female side of the tank. Baseline behaviors of paired NT males were equivalent until one in each pair was moved. Amidst only females, NT males that were moved quickly adopted T behaviors, as exhibited by elevated DIs relative to their own baseline levels and to matched control NT males (post-switch DI for NT→T males=9.6±1.44, $N=8$, versus pre-switch DI=-2.6±1.4, Wilcoxon signed-rank test, $P<0.001$; versus control NT males=-2.4±0.59, $N=6$; Mann-Whitney U -test $P<0.005$). Seven days after being moved into an all-female setting, NT→T males had elevated GnRH1 mRNA levels relative to control NT values (OD, 1.19±0.15 versus 0.85±0.06, Mann-Whitney U -test, $P<0.03$; Fig. 4A). No differences were detected in expression levels of the other two GnRH forms.

The gonads of NT→T males at 7 days were larger than those of control NT males (GSI, NT→T=0.61±0.08, $N=6$, versus control NT=0.29±0.07, $N=6$; Mann-Whitney U -test, $P<0.03$; Fig. 4B), in contrast to the non-significant difference seen at 3 days (see above). At 7 days, GnRH1 mRNA levels were positively correlated with gonad size in the entire population (Spearman $\rho=0.61$; $P<0.04$; Fig. 4C). Surprisingly, cortisol levels of NT→T males did not differ from their own pre-switch values (pre-switch=4.3±2.5 ng ml⁻¹, $N=5$, versus post-switch=5.7±1.1 ng ml⁻¹, $N=5$; Wilcoxon signed-rank test, $P=0.16$), in contrast to the difference observed after 3 days.

Effects of the direction of social change on the speed of behavioral, cellular and molecular change

To determine the time course of changes in GnRH expression in community settings and during both social ascent and descent, the social status of experimental animals was changed from NT to T, or *vice versa*, by manipulating their social situation (study III, see Materials and methods). Experimentally manipulated animals were remarkably different in both their behavioral and physiological responses to changes in social status. These manipulations resulted in overall significant differences in behavior (Wilcoxon/Kruskal-Wallis rank sum, $P<0.0001$), preoptic irGnRH soma size ($P<0.0001$) and GnRH1 mRNA levels ($P<0.02$) across experimental groups (Fig. 5). Not only were T and NT males significantly different in every comparison but, surprisingly, the time course of the behavioral and physiological changes depended on the direction of status change. All comparisons made between groups are outlined below, and the significance level for each comparison is reported in Table 1.

As expected, control T males were more aggressive than control NT males (DI, T males=9.0±1.8, $N=15$ versus NT males=-1.4±0.2, $N=11$; Mann-Whitney U -test, $P<0.0001$; Fig. 5A). The behaviorally inferred social status of males corresponded to indices of preoptic GnRH expression. As

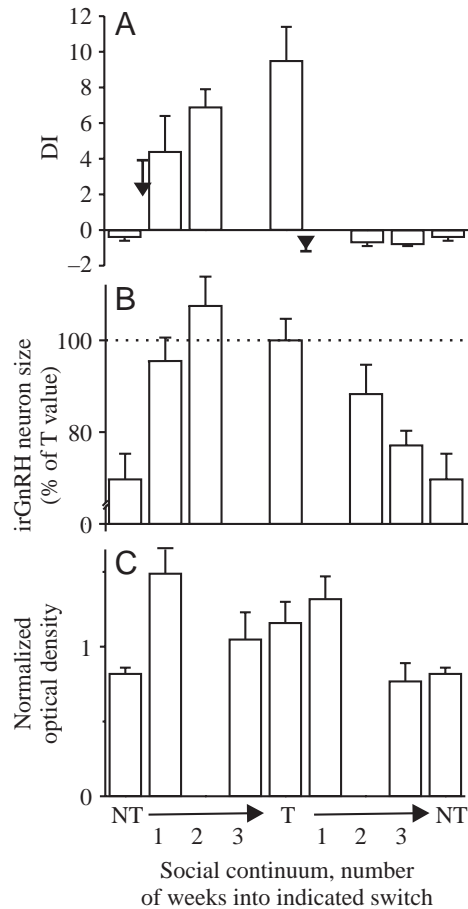


Fig. 5. Study III: asymmetry of behaviors and gonadotropin-releasing hormone (GnRH) expression during social change. Changes in behavior (A, index of dominance, DI), immunoreactive GnRH neuronal soma cross-sectional area (B) and GnRH1 mRNA levels (C) during changes in social status. The x-axis indicates whether males were control territorial (T) or non-territorial (NT) or were undergoing a transition in social status for the indicated number of weeks. In all graphs, data from control NT males, 1- and 2-week NT→T transitions, control T males, and 2- and 3-week T→NT transitions are shown by open columns. Data for control NT males are plotted twice for clarity. Aggressive behaviors (A) were also measured at 1 day (filled triangles) following a transition in social status. (A) Mean aggressive behaviors are plotted as DI scores + s.e.m. ($N=11$ for control NT; 9 for 1 day observations, and 8, 14, 14, 12 and 6 for remaining groups, respectively), which increase slowly during social ascent (NT→T) and disappear within 1 day (filled triangle) during social descent (T→NT). (B) Preoptic immunoreactive GnRH neuronal soma cross-sectional areas are shown as a percentage of control T area, indicated by the dotted line. When NT males become territorial, these neurons grow to T size within 1 week. It takes 3 weeks for neurons to return to NT sizes during social descent. Values are means + s.e.m. ($N=11$ for control NT; $N=8$, 14, 14, 12 and 6 for remaining groups, respectively). (C) GnRH1 transcript levels, normalized for loading, are plotted as optical densities. The time course of changes in GnRH1 levels is also asymmetrical and generally parallels changes in neuron size. Values are means + s.e.m. ($N=7$ for control NT; 4, 3, 7, 3, 2 and 2 for remaining groups, respectively). See Results for statistical comparisons and P -values.

Table 1. P-values for changes in behavior and GnRH levels during social ascent and descent

	NT→T			T→NT		
	NT	1-week	2-week	T	2-week	3-week
DI						
NT		*	*	***	0.74	0.38
1-week	*		0.35	*	*	*
2-week	*	0.34		0.52	0.10	0.10
T	***	*	0.52	***	***	***
2-week	0.74	*	0.10	***		0.42
3-week	0.38	*	0.10	***	0.42	
Preoptic irGnRH neuron size						
NT		**	***	**	*	0.31
1-week	**		0.12	0.06	0.26	*
2-week	***	0.12		0.22	*	**
T	**	0.06	0.22		0.12	**
2-week	*	0.26	*	0.12		0.16
3-week	0.31	*	**	**	0.16	
GnRH1 mRNA level						
NT		**	0.25	*	*	0.78
1-week	**		0.08	0.13	0.48	0.06
3-week	0.25	0.08		0.73	0.28	0.25
T	*	0.13	0.73		0.21	0.14
1-week	*	0.48	0.28	0.21		0.08
3-week	0.78	0.06	0.25	0.14	0.08	

Significance levels are shown for Mann–Whitney *U*-tests, two-tailed comparisons between T and NT individuals. **P*<0.05; ***P*<0.005; ****P*<0.0005.

Nonsignificant *P* values are indicated.

Refer to Fig. 5 for mean values±S.E.M. and *N* values.

GnRH, gonadotropin-releasing hormone; irGnRH, immunoreactive GnRH; DI, index of dominance; T, territorial male; NT, non-territorial male.

anticipated, T males had larger preoptic irGnRH neurons than those of NT males [$T=147\pm 6\mu\text{m}^2$, $N=14$, versus $NT=102\pm 8\mu\text{m}^2$, $N=11$; Mann–Whitney *U*-test, $P<0.002$; normalized values (see Materials and methods) are shown in Fig. 5B] replicating earlier findings (Francis et al., 1993). T males also had larger amounts of GnRH1 transcript (OD, T males 1.16 ± 0.14 , $N=7$, versus NT males 0.82 ± 0.04 , $N=7$; Mann–Whitney *U*-test, $P<0.03$; Fig. 5C) than NT males.

Among NT→T males, the fundamental behavioral patterns characteristic of T males emerged within 1 day (Fig. 5A) but were quantitatively similar to those in control T males only after 2 weeks. That is, within 1 day after being moved to tanks with smaller males, previously NT males began aggressive acts (DI, NT→T at 1 day after move= 2.4 ± 3.8 , $N=9$). However, the level of aggressive behaviors by these newly T males did not match typical T behavior until 2 weeks after the move (DI, NT→T at 2 weeks= 6.9 ± 1.0 versus NT= -1.4 ± 0.02 ; Mann–Whitney *U*-test, $P<0.02$; versus T= 9.0 ± 1.8 , not significant). In contrast to this relatively cautious change in the quantitative behavioral activity, neuronal structure changed rapidly during social ascent. The size of preoptic

irGnRH neurons in NT→T males was equivalent to that of control T males within just 1 week after the change in social status (NT→T at 1 week= $140\pm 9\mu\text{m}^2$, $N=8$, versus T= $147\pm 6\mu\text{m}^2$, not significant; Fig. 5B). During social decline, behavioral changes were relatively fast compared with biological ones. T males descending to NT status assumed subordinate behaviors equivalent to those of control NT males within 1 day (DI, T→NT at 1 day= -0.57 ± 0.57 versus NT, not significant), but their preoptic irGnRH neuronal soma sizes did not match those of control NT males until 3 weeks later (T→NT at 3 weeks= $113\pm 5\mu\text{m}^2$, $N=6$, versus NT, not significant; Fig. 5B).

Changes in GnRH1 mRNA expression associated with social transitions generally paralleled the changes in neuronal soma size (Fig. 5C). During social ascent, the mean GnRH1 mRNA level in NT→T males increased to T levels and beyond that of control NTs within 1 week (OD, NT→T at 1 week= 1.49 ± 0.16 , $N=4$, versus control NT= 0.82 ± 0.04 , $N=7$; Mann–Whitney *U*-test, $P<0.01$). While the mean GnRH1 transcript level of NT→T males at 3 weeks was also equivalent to T levels, it was no longer significantly different from NT values (OD, NT→T at 3 weeks= 1.05 ± 0.18 , $N=3$; versus T or NT, not significant). One week into social descent, no change was evident in GnRH1 expression levels (OD, T→NT at 1 week= 1.32 ± 0.15 , $N=3$, versus control T= 1.16 ± 0.14 , $N=7$, not significant). Although levels of GnRH1 mRNA in T→NT males 3 weeks after being moved to new tanks were less than 70% of those of control T males (OD, T→NT at 3 weeks= 0.77 ± 0.12), the difference was not significant, probably because of the small number of animals (two) that achieved and maintained T status during this part of the study.

As with all other social manipulations tested, the expression levels for GnRH2 and GnRH3 remained the same among animals whatever their social or reproductive state. In summary, during social ascent, GnRH1 indices upregulate more quickly than do aggressive behaviors. In contrast, during social decline, GnRH1 expression persists well after aggressive behaviors have vanished.

Effects of endogenous cues on GnRH I gene expression in females

Female *H. burtoni* have a uniformity in both behavior and color (but see White and Fernald, 1993), yet their preoptic irGnRH neurons undergo cyclical changes in soma size comparable in magnitude with those in males. Spawning females have larger preoptic irGnRH neurons than those of brooding females, similar to the relationship between T and NT males. Internal reproductive signals rather than external social cues are hypothesized to be critical for irGnRH neuronal soma size regulation in females (White and Fernald, 1993). To determine whether these structural changes reflect differences in GnRH mRNA expression, GnRH transcript levels were compared between spawning (Sp) and brooding (Br) females. Reproductive measures for study IV are shown in Fig. 6. Spawning females had twice as much GnRH1 transcript as brooding females (OD, Sp= 1.4 ± 0.18 , $N=6$, versus

Br=0.66±0.06, $N=13$; Mann–Whitney U -test, $P<0.005$; Fig. 6A), while levels of GnRH2 and GnRH3 mRNA did not change between these two reproductive states.

During mouth-brooding of fry, female *H. burtoni* do not eat. Therefore, the source of the signals that produce small preoptic irGnRH neurons and low GnRH1 mRNA levels in brooding females could either be endogenous, as previously hypothesized, and due to nutritional needs, or exogenous due to 'social', e.g. tactile, chemical or other cues provided by fry. To distinguish between these possibilities, indices of GnRH expression were measured in females that had brooded their fry for 2 weeks (Br) and in 'broodless' females, i.e. females that had spawned but whose eggs had been removed from their mouths and were either fed (Br–F+) or fasted (Br–F–; see Fig. 2).

Levels of GnRH1 mRNA varied among female groups when all groups were compared (Wilcoxon/Kruskall–Wallace rank sum, $P<0.002$). To determine which variables

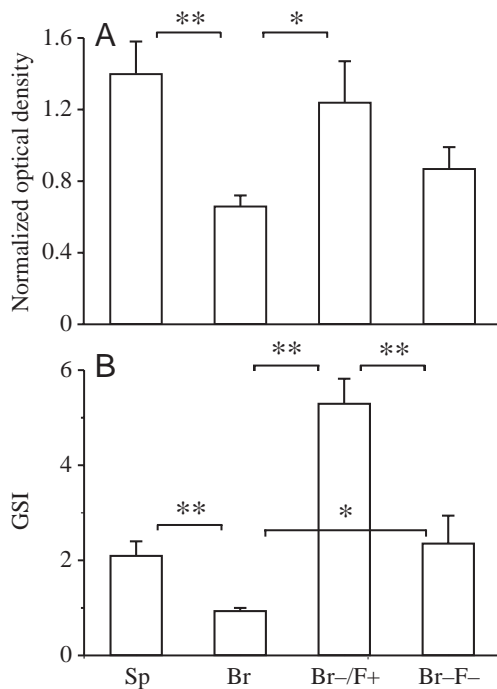


Fig. 6. Study IV: preoptic gonadotropin-releasing hormone 1 (GnRH1) expression is regulated by endogenous cues in females. Spawning (Sp), brooding (Br) and broodless (Br–) females were used to test the relative influence of social *versus* endogenous regulation of GnRH levels. Experimental groups are indicated on the lower x -axis and include two groups of broodless females, either fed (Br–F+) or fasted (Br–F–). (A) Normalized GnRH1 mRNA signals are plotted as their optical density (OD). Values are high in females that eat (Sp and Br–F+) and low in fasted females (Br and Br–F–). Soma sizes from spawning females are from White and Fernald (1993). (B) Brooding females have small ovaries indicated by their small gonosomatic index (GSI). Feeding increases ovary size, as does the absence of broods. The intermediate Sp value reflects egg release during capture while spawning. Values are means \pm S.E.M. ($N=6$ Sp, 13 Br, 8 Br–F+, 6 Br–F–). Asterisks indicate P -values: * $P<0.05$; ** $P<0.005$; *** $P<0.0005$.

contributed to these overall differences, we examined two groups at a time. The presence of broods was not a major contributor to changes in GnRH1 mRNA levels because brooding females and broodless females that were not fed had similarly low GnRH1 levels (OD, Br=0.66±0.06, $N=13$, *versus* Br–F–=0.86±0.12, $N=6$, not significant; Fig. 6A). Thus, irrespective of the presence of broods, GnRH1 mRNA levels in these two groups were low, indicating that fasting contributes to GnRH1 regulation. Indeed, GnRH1 levels of broodless females that were fed were high, similar to those of spawners (OD, Br–F+=1.24±0.23, $N=8$, *versus* Sp=1.4±0.18, $N=6$; not significant; Fig. 6A). Thus, despite distinct reproductive states, these two groups of feeding females had similarly high levels of GnRH1 transcript. However, nutritional state did not completely account for differences in GnRH1 expression in females: the OD value of GnRH1 mRNA tended to be higher in broodless females that were feeding than in their fasting counterparts, but this difference was not significant (OD, Br–F+=1.24±0.23, $N=8$, *versus* Br–F–=0.86±0.12, $N=6$, not significant). No differences were observed in GnRH2 or GnRH3 expression levels between females in the differing reproductive states.

As expected, ovarian mass changed with reproductive state and largely paralleled changes in GnRH1 mRNA levels. Spawning females had large ovaries of variable mass, probably because of the range in number of eggs released during spawning, prior to capture (White and Fernald, 1993). Despite this variability, the ovaries of spawning females were larger than those of brooding females (GSI, Sp=2.1±0.3 *versus* Br=0.94±0.06, Mann–Whitney, $P<0.005$; Fig. 6B), replicating earlier findings (White and Fernald, 1993). Removal of fry and restoration of food to females caused their ovaries to increase in size: broodless females that were fed had larger gonads than those of either brooders or broodless females that were food-deprived (GSI, Br–F+=5.3±0.5 *versus* Br, $P<0.001$; *versus* Br–F–=2.4±0.6, Mann–Whitney U -test, $P<0.005$; Fig. 6B). This suggests that nutritional status plays a role in regulating gonad size in *H. burtoni* females, perhaps *via* regulating GnRH1 expression. However, there was some influence of social cues on GSI. Broodless females that were fasted had significantly larger ovaries than brooding females (GSI, Br–F–=2.4±0.6 *versus* Br=0.94±0.06, Mann–Whitney U -test, $P<0.01$). Thus, ovary size began to increase in females that had had their broods removed compared with females whose broods were intact, even though both these groups were fasting.

Preoptic irGnRH neuronal soma size also differed across brooding and broodless groups (Wilcoxon/Kruskall–Wallace rank sum, $P<0.05$). As previously reported, brooding females in this study had small preoptic irGnRH neurons with a mean cross-sectional area of 94±8 μm^2 ($N=5$), approximately one-third of the size previously reported for spawning females (White and Fernald, 1993). Unfortunately, we were unable to obtain sufficient soma size measurements from the two broodless groups to draw conclusions about the role of nutritional *versus* social cues in regulating preoptic irGnRH

soma size. However, a trend towards increasing soma size following the removal of broods and restoration of food is evident (Br-F+=115±7 μm², N=4, versus Br=94±8 μm², N=5; Mann-Whitney *U*-test, *P*=0.09, not significant).

In contrast to most reproductive measures, cortisol levels did not differ between spawning and brooding females (Sp=10.3±3.1 ng ml⁻¹, N=5, versus Br=7.7±2.1 ng ml⁻¹, N=9; not significant), with the caveat that the comparison was made between groups rather than *via* repeated measures from the same individual in different reproductive states.

Discussion

Our results address the fundamental question of what mechanisms allow an animal to respond to social signals. We have demonstrated that changes in social status triggered by recognition of a new social opportunity regulate GnRH1 gene expression, preoptic irGnRH neuronal size and gonad size in *H. burtoni* males. Specifically, becoming socially dominant upregulates these physiological variables, essential for reproduction, while being socially suppressed has the opposite effect. We have previously shown that males of different social status have different preoptic irGnRH neuronal soma sizes (Francis et al., 1993). We now show that GnRH regulation is a result of social influences on GnRH1 mRNA levels. Hence, even though the brain ultimately controls behavior, it is clear that behavior also regulates the brain.

The profile of change in preoptic GnRH indices was remarkably consistent across the many different social settings examined here. For example, during social ascent in males, changes in both GnRH1 mRNA levels and irGnRH soma sizes occurred within 1 week for both the 3- and 7-day transitions in study II and for the transitions induced within a community in study III. Socially induced decreases in preoptic GnRH levels took longer, as demonstrated by the slow, 3-week decline in mRNA levels in study III and the decrease in neuron size in this same study and a pilot study (see Materials and methods). There is even a hint of this slow decline in study I, in which males of stable social status were compared. There, the one NT male that only became NT 2 weeks before being killed (having been T for 8 weeks previously) had the highest levels of GnRH1 mRNA among NT males (Fig. 3), consistent with a time course of 3 weeks or more for preoptic GnRH downregulation. This consistency in GnRH changes may reflect the fact that the animals have been placed in a new, unambiguously different social milieu, causing an irreversible change from their prior social state. Given this clear social change, it makes sense to change the physiology needed either to start or to stop successful reproduction.

The asymmetries in the responses of behavioral and physiological systems to social change observed in study III were unexpected. During an ascent in social status, the swift increases in GnRH1 expression are in contrast to behavioral changes, which require more than 2 weeks to attain levels equivalent to those of control T males. In contrast, when a previously T male loses his territory, his aggressive behavior

stops immediately (<1 day), while indices of GnRH expression decline more slowly. Thus, somewhat surprisingly, animals in social ascent are relatively slow to display their rise in status despite physiological changes that would allow them to exploit it, while animals in social decline immediately exhibit subordinate behaviors despite the persistence of large GnRH neurons within their preoptic areas for several weeks. Why is the rate of cellular processes oppositely biased to the rate of behavioral change in response to altered social status? Ultimately, both behavioral and neuronal outcomes make sense for *H. burtoni* on the basis of observations of their life in Africa (Fernald and Hirata, 1977b). In their natural habitat, physical conditions change unpredictably as a result of winds and intrusions of large animals, including *Hippopotamus amphibius*, into the shore-pools. This uncertainty probably increases the chances that a male will reproduce since animals exploit these chance occurrences to change social status (Hofmann et al., 1999). Considered in this way, the trajectories of biological change measured here fit well with both field and laboratory data on the rate at which territories become available. For example, the median time for which animals occupy a territory in a fluctuating environment is 3 weeks (Hofmann et al., 1999). Those animals gaining social status and, hence, reproductive access, have an accelerated physiological switch to afford reproduction quickly. Conversely, new opportunities for establishing a territory, either for the first time or after having been 'deposed', occur at an average of 4 weeks (Hofmann et al., 1999). Thus, a male losing his territory and hence reproductive opportunity may maintain a vestige of his prior prowess since a return to that opportunity might soon occur. Covertly, the fish's biology is biased towards reproductive capacity, whereas his overt aggressive behavior is more tentative within new social circumstances. Although neuronal and behavioral strategies differ temporally, together they reflect a delicate balance between pragmatism and optimism needed for survival.

Our data demonstrate that social interactions between male *H. burtoni* change neuronal gene expression and not *vice versa*. To show this, animals in study II were induced to change social status through a manipulation of the social situation. This transition to a higher social status was performed over 3 or 7 days. Pairs of NT males were matched for body size, social history, social setting and level of submissive behavioral acts. One male of each pair was then given the opportunity to ascend in social status (NT→T) by being moved to a tank with only females. Subsequent behavioral observations confirmed that these individuals became dominant rather quickly. GnRH1 mRNA levels began to increase in these NT males within 3 days of the move and were higher at 1 week relative to expression in socially suppressed counterparts. Since the default state for *H. burtoni* males is territorial (Fraley and Fernald, 1982), NT males are socially suppressed by T males, and relief from this suppression triggers the increase in GRH1 indices.

Our data suggest that social suppression of reproductive capacity in NT males results from stressful behavioral

interactions with aggressive T males. Consistent with this interpretation, NT males in study II had higher levels of cortisol than did T males. Moreover, cortisol levels declined within 3 days of the NT being moved to an all-female setting. Stress has been shown in other systems to inhibit reproduction at many levels. For example, in the amphibian *Taricha granulosa*, a rapid and direct suppressive effect of stress on male reproductive behavior is mediated by glucocorticoid receptors on neuronal membranes (Moore and Miller, 1984; Orchinik et al., 1991). Cortisol might mediate similarly rapid behavioral changes in *H. burtoni*, in which dominant behaviors can disappear within minutes and such changes are independent of androgens (Francis et al., 1992). It seems likely that high cortisol levels in NT males act *via* conventional intracellular glucocorticoid receptors to suppress GnRH1 gene expression and keep GnRH1 neurons small and gonads immature. This potential for direct genomic regulation by both cortisol and androgens is supported by the discovery of six putative consensus sequences for glucocorticoid regulation and one for androgens within the GnRH1 gene promoter of *H. burtoni* (White and Fernald, 1998). We have previously shown that differences in cortisol levels between males of high and low social rank arise only after new social groups stabilize (Fox et al., 1997). This supports the hypothesis that social interactions result in a shift in basal cortisol levels, which then alters GnRH1 transcription. Since cortisol levels in NT males were an average of 10 times higher than those of T males, this seems a likely scenario.

In study II, we measured differences in cortisol level between T and NT males that were less than the 10-fold values previously reported. What might account for this apparent difference? One important component of social stress is the nature of the social community (Sapolsky, 1993). In our previous study (Fox et al., 1997), the aquarium was arranged to mimic the natural situation closely. The available space was relatively large for the number of animals, and food was delivered only to territorial areas, similar to the African habitat (Fernald and Hirata, 1977a). In this situation, NT males had to enter defended territories to feed, where they were typically chased by resident T males. In contrast, for the data reported here, animals were kept at a higher density and food was scattered across the surface of the aquarium, possibly reducing the stress of feeding in NT males. Supporting this notion, Fox et al. (1997) also measured cortisol levels in fish kept in conventional tanks and reported that the difference between T and NT levels was only twofold, similar to the present study. From both studies, it is clear that interactions between males produce differences in cortisol levels and that, once a community is stable, T status is associated with low basal cortisol levels. Cortisol could provide the link between social interactions and preoptic GnRH indices, but this study does not reveal whether cortisol also acts *via* social interactions to induce or facilitate changes in social state.

Somewhat surprisingly, in study IV, we found no significant decrease in cortisol level when NT males were moved to all-female settings for 7 days, whereas at 3 days, NT males had

high cortisol levels (approximately 10 ng ml^{-1}), which dropped when they were moved. This is not likely to be due to differences in the social community since this was controlled experimentally. One possibility is that the endocrine profile accompanying social change has distinct phases, reflected in immediate, intermediate and long-term changes. Unfortunately, the frequent blood sampling required to track such profiles creates chronic stress, preventing multiple samples from a single fish and perhaps the resolution to the discrepancy between the 3- and 7-day results.

In contrast to the social regulation of GnRH1 in male *H. burtoni*, results from study IV showed that endogenous cues, including nutritional state, regulate GnRH1 mRNA levels, preoptic irGnRH neuronal soma size and gonad size in females. We have previously reported that spawning females have larger preoptic irGnRH cells than brooding females (White and Fernald, 1993). Here, we show that these differences reflect the amounts of GnRH1 mRNA. Two possible regulatory signals were examined, the presence of a brood and nutritional state. The first is a social cue from fry in the brooders' mouths. The second is a physiological cue reflecting changes in nutritional state associated with the obligate fasting of brooding females. Our data suggest that GnRH1 mRNA level and preoptic neuronal soma size both depend on nutritional cues. Brooding females and Br-F- females, two groups that were fasting, had low GnRH indices, while females that were fed (Sp, Br-F+) had higher GnRH1 mRNA levels and larger preoptic irGnRH neurons (present study; White and Fernald, 1993).

In other systems, nutritional status has been shown to regulate fertility in both sexes (Cunningham et al., 1999; Foster and Nagatani, 1999). Here, fasting females (Br, Br-F-) had smaller ovaries than fed females. However, a complete absence of social cues in regulating GnRH in females cannot be ruled out because Br-F- females had slightly larger ovaries than brooding females. Further, this change may have been triggered by the non-significant increase in GnRH1 mRNA levels following removal of the brood. Although small, this increase resulted in expression levels that were equivalent between Br-F- and Br-F+ females. Thus, the absence of a brood, a social cue, triggers a change in reproductive state in females, but on a smaller scale than in males and of lesser impact than endogenous nutritional cues.

The role of stress as a mediator of GnRH expression is less evident in female *H. burtoni*, since no differences were observed between cortisol levels of brooding *versus* spawning females. This lack of correlation may be real or could be due to our inability to gather repeated cortisol measures from individual females when they were either brooding or spawning. Sampling the blood of spawning or brooding females often ends those activities. In *H. burtoni*, in which cortisol levels in males reflect the stress of social interactions, a lack of correlation between cortisol levels and reproductive state in females underscores the role of endogenous regulators of their GnRH expression. However, the interplay between reproductive and nutritional state in vertebrates is complex and

probably includes the stress axis. For example, leptin, a 16 kDa protein produced by the white adipocytes of vertebrates, has been postulated to link nutritional and reproductive state and is also thought to reduce the stress response (Foster and Nagatani, 1999).

An intriguing result of these experiments is that no changes in mRNA levels were detected for either GnRH2 or GnRH3 despite the diverse set of manipulations performed on the animals. The GnRH peptides encoded by these transcripts have been postulated to play a role in reproduction because of their similarity to GnRH1 and because of the remarkable conservation of all three peptide sequences over evolutionary time (Fernald and White, 1999). However, there are actually few data to support any role for GnRH2 or GnRH3 in reproductive function. For example, although the distribution of GnRH2 is known in several species (Gestrin et al., 1999; Kasten et al., 1996; Latimer et al., 2000; Lescheid et al., 1997; White et al., 1994), the only functional data about its role suggests that it may act as a neurotransmitter and neuromodulator at specific synapses in amphibian sympathetic ganglia (Jan et al., 1979; Jones, 1987; Kuffler and Sejnowski, 1983). For GnRH3, there are no functional studies, but its wide distribution in the terminal nerve area of the forebrain and its projection to the retina of non-mammalian vertebrates (Münz et al., 1982) are consistent with a putative role as a neuromodulator (Oka, 1992).

While release of GnRH from the terminal nerve in the aquatic salamander *Necturus maculosus* appears to modulate odorant sensitivity according to seasonal reproductive cycles, the GnRH form contained in amphibian terminal nerve neurons is GnRH1 (Eisthen et al., 2000). Analysis of mRNA expression levels of a GnRH2 homolog in the musk shrew *Suncus murinus* using both males and females in different developmental and reproductive states failed to find evidence for differences in gene expression (White, 1997). Obviously, failure to detect mRNA regulation in response to changes in reproductive state does not preclude a reproductive role for these peptides. Our sampling protocol may not have been appropriate to detect a change in transcript levels of GnRH2 and GnRH3, or their reproductive regulation might occur at the translational level or *via* their receptors. However, reproductive regulation of GnRH peptides in another teleost fish, the grass rockfish *Sebastes rastrelliger*, is limited to the form found in the pituitary (Collins et al., 2001), suggesting that the lack of transcriptional change in GnRH2 and GnRH3 observed in the present studies is borne out at the peptide level. Perhaps the functions of GnRH peptides have diverged such that GnRH2 and GnRH3 now have non-reproductive roles in the animal. Divergent functions for peptides with similar sequences are well-documented, with oxytocin and vasopressin being prime examples (Mohr et al., 1995; Venkatesh and Brenner, 1995). Unveiling the roles of the other GnRH-like peptides must await identification and mapping of their receptors as well as specific functional tests.

Many features of a functioning organism, such as setting the

circadian clock by the influence of light, are subject to environmental regulation. The pathways by which these influences change gene expression and concomitant protein abundance are becoming understood, although the complexity is daunting. Similarly, there are many reports documenting the effects of social interactions on the behavior of individuals. In contrast, however, much less is known about how these social influences cause changes in the brain through changes in gene expression. This work documents that change in social status directly influences the mRNA levels of GnRH1, which is crucial for reproductive behavior (Mason et al., 1986). Just how social exchanges are transduced into molecular events remains to be determined.

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