Endocrinology

Hormonal and Physiological Profiles of Female *Haplochromis burtoni* as it relates to Affiliative Behavior

The African cichlid fish, Haplochromis burtoni*, has proven to be a useful model for understanding the social control of behavior. In females of this species, there is substantial remodeling of the brain-pituitary-gonadal axis controlling reproduction between gravid (egg bearing) and non-gravid states (White & Fernald, 1993). Moreover, female preference for male phenotype changes during the course of the reproductive cycle (Clement, Grens & Fernald, submitted). To understand what hormonal changes are associated with the behavioral and structural changes, we measured levels of several key hormones and peptides throughout the reproductive cycle of the female as a function of ovarian stage and development. We quantified plasma levels of testosterone and 17ß-estradiol using enzyme-linked immunoassays. The expression of gonadotropin-releasing hormone (GnRH), a hormone produced in the hypothalamus and involved in sexual maturation, was determined using quantitative real time RT PCR. In addition, the expression of two GnRH receptor types in the pituitary was also established using quantitative real time RT PCR. We relate the changes in hormonal profile to female affiliation preference to identify internal cues that might help orchestrate the shift to preference for dominant males.*

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Sexual selection, or mate choice, refers to any behavior in which one sex discriminates among several members of the opposite sex based on some salient criterion, such as size, status, or color. Researchers have documented sexual selection throughout the animal kingdom. Species of insects (Bonduriansky, 2001), spiders (Hebets, 2003), fish (Hughes, Du, Rodd, & Reznick, 1999; Kelley, Graves, Mugarran, 1999; Kodric-Brown, 1993; Clement, Grens, & Fernald, submitted), birds (Karubian, 2002), and mammals (Jurke, Price, & Debeli, 1995) have all been shown to engage in selective behavior. Choice behavior has been described in both females and males, though more frequently in females. Although the cues that may lead a female to distinguish between two possible mates are well described, rarely have the physiological factors determining such choice been examined. The objective of this research is to determine the underlying mechanisms responsible for the variations in female preference across the reproductive cycle.

Mate choice was first described in 1871 by Charles Darwin, who observed that many animals possess traits that appeared useless for survival, such as large plumage or bright colors that attract predators. Darwin postulated that these traits were prized by the opposite sex and thus enhanced the individual's probability of reproducing, which explained why they continued to be passed down from generation to generation. Darwin was also the first to refer specifically to female mate choice: "No doubt this implies powers of discrimination and taste on the part of the female which will at first appear extremely improbable; but I hope to shew (sic) that the females actually have these powers" (Darwin, 1871). Although Darwin's theory of natural selection was widely accepted, it was not until the late twentieth century that Darwin's theories of sexual selection began to gain prominence and recognition. Since then, the topic has been widely investigated in order to determine why sexual selection occurs and what cues animals use to decide among potential mates.

Numerous researchers have used fish to investigate mate choice in simple vertebrates. One common finding is that fish use visual features to distinguish between prospective mates. For example, female guppies prefer brightly colored males to males that are less colorful (Godin & Dugatkin, 1996), while female swordtail fish prefer males who have symmetrical vertical bars to those with asymmetrical ones (Morris & Casey, 1997). It has been suggested that these choice decisions are based on the genetic quality of the mate. In guppies, the male's ability to escape from predators, as well as his boldness towards predators, seem to be indicators of the male's genetic quality. Godin and Dugatkin (1996) established that the brightness of a male correlates positively with boldness towards and escape from predators, demonstrating that bright color may be a reasonable indicator of the quality of the male. Likewise, female swordtail fish may

prefer symmetrical males to asymmetrical males because genetic quality seems to be linked to the symmetry of sexual traits (Morris and Casey, 1997).

Recent research in sexual selection behavior has investigated whether female choice may vary according to the physical state of the female. Clement, Grens, and Fernald (submitted) demonstrated that females' willingness to affiliate with a dominant male changes across the reproductive cycle. They showed that in the African cichlid fish, *Haplochromis burtoni,* the reproductive state of the female determines whether she will show preference for territorial males. Their experiment tested female preference for the two types of *H. burtoni* males – territorial and non-territorial – who differ in size, coloration, status, and reproductive viability. Territorial males are large, aggressive, brightly colored, and control areas of territory and resources. They also have mature gonads capable of reproduction. In contrast, non-territorial males are smaller, less aggressive, camouflage colored, and do not control areas of territory. They only gain access to food when they are mistaken for females, and they are reproductively suppressed with immature gonads. As the female begins her reproductive cycle, she schools with females and non-territorial males. Only after she has become quite gravid (egg bearing and fertile) does she choose to affiliate with territorial males in order to spawn.

Clement, Grens, and Fernald investigated this behavior and found that when given a choice between a territorial male and a non-territorial male, gravid females prefer territorial males, while non-gravid females do not show a preference. This finding suggests that mate choice behavior may be influenced by the hormonal fluctuations during a female's reproductive cycle. However, in order to determine how hormones may affect mate choice either directly or indirectly, we first need to determine the time course of reproductive cycle and corresponding physiological variations. It has been documented that there are physiological changes between gravid and non-gravid females, and that these changes are likely signaled at a hormonal level. We investigated several hormones, hormone receptors, and physiological factors to determine which of these might mediate reproductive behaviors like mate choice.

White, Nguyen, and Fernald (2002) established that gonadotropin-releasing hormone I (GnRH I), a hormone released by the hypothalamus and involved in sexual maturation, varies across reproductive states in *H. burtoni*. We extended this research to look at the expression of GnRH I at several other time points over the entire reproductive cycle. We also examined GnRH I and GnRH III receptor expression in the pituitary. In addition, there are several key hormones known to regulate GnRH, including 17ß-estradiol and testosterone. These two hormones have also been shown to affect both behavior and physiology as they fluctuate during the reproductive cycle. As a result, we determined concentrations of 17ß-estradiol and testosterone in *H. burtoni* plasma. Lastly, we measured the gonadosomatic index (ovary weight/body weight x 100) in order to correlate these changes with the female's reproductive state.

Methods

Housing

We used stock populations of *Haplochromis (Astatilapia) burtoni* that were originally obtained from wild populations that live in Lake Tanganyika, Africa. Males and females were maintained together in aquaria that resemble the conditions of their natural environment: 28° C water temperature, pH 8 and a 12:12-hr light:dark cycle with full spectrum illumination (Fernald & Hirata, 1977).

To ensure that all the females began the reproductive cycle at the same time, we collected females after they had released mature fry (viable offspring). We checked every afternoon for females that were brooding eggs or immature fry and collected the females only when the fry were viable (*H. burtoni* females brood their offspring in their mouths for two weeks prior to release). We collected five to ten females at a time and housed them together in an aquarium with one to two territorial males per five females. The females were kept in these tanks until the day of sacrifice.

Sampling Procedure

Based on our observation that the female reproductive cycle is about 30 days long, we chose data collection points between the first and thirtieth days following the release of the fry. We collected data on days 1, 3, 6, 9, 12, 15, 18, 21, 23, 25, and 29 (Day 1 corresponding to the day that the fry are taken) from August to November of 2003. Sample size ranged from 3-5 subjects per data point. Prior to sacrifice and blood drawing, the fish were weighed and their length was measured. We drew blood from the caudal vein; the blood was centrifuged immediately and the plasma was drawn off and stored at –80˚C. We extracted the brain, pituitary, and ovaries from all of our samples and immediately froze them at –80˚C until analysis. Ovaries were weighed in order to calculate the gonadosomatic index (GSI = 100 x gonad weight/body weight) prior to freezing. Any eggs or immature fry were also immediately frozen and stored at –80˚C.

Determination of Reproductive Hormones and Peptides

We extracted plasma steroids from our plasma samples according to Cayman Chemical's Enzyme-Linked Immunoassay protocol. These extracts were analyzed for 17ß-estradiol and testosterone using enzyme-linked immunoassays (Cayman Chemical, Ann Arbor, Michigan).

We measured levels of GnRH I in the brain and GnRH receptor I and III in the pituitary using real time quantitative Reverse Transcription Polymerase Chain Reaction (RT-Q-RT-PCR), a sensitive assay used to determine levels of gene expression. Levels of GnRH I and GnRH receptor I and III were quantified using a protocol described in White, Nguyen, and Fernald (2002). All of these analyses were carried out with both positive and negative controls. After real time quantitative RT-PCR, we ran out the samples on a 2% agarose-ethidium bromide gel in order to determine the purity of the sample. Results were normalized with G3PDH and are given as percent expressions.

$$\textit{Percent expression} = \frac{\text{rel. exp. of G3PDH}}{\text{rel. exp. of gene of interest}}$$

$$\textit{Relative expression} = \text{Efficiency}^{\text{Average Cycle Threshold}}$$

Results

Gross Anatomy and GSI

As a female becomes gravid (egg bearing), her abdomen increases noticeably in size. The increase in abdominal size is due to the tremendous growth of the ovaries as the female approaches spawning (when she deposits her eggs at the anal fin of the male where they will be fertilized). At the beginning of the cycle, when the female has just released fry, the gonadosomatic index (GSI) is at its lowest point. By twelve days after fry release, most females are gravid again, and the average GSI shows a corresponding increase. Once the females start mouthbrooding their fry around day twenty-five, the GSI drops down to the initial level again (see Figure 1).

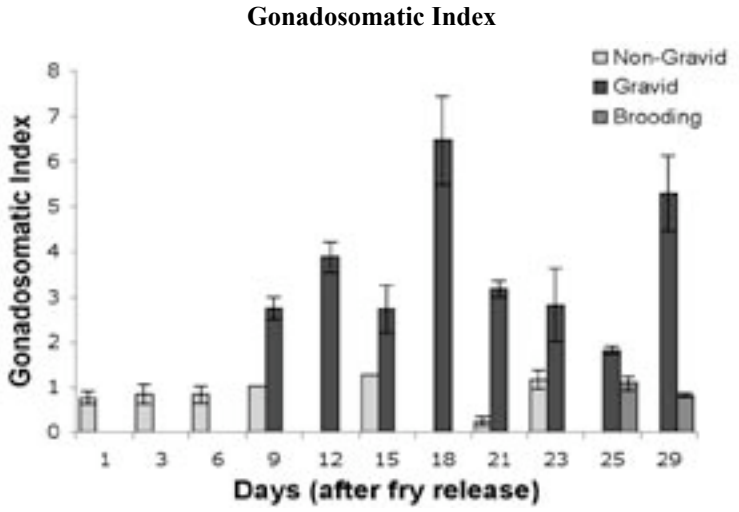

Figure 1. Gonadosomatic Index (GSI) across the female *Haplochromis burtoni* reproductive cycle. GSI = 100 x ovary weight/body weight.

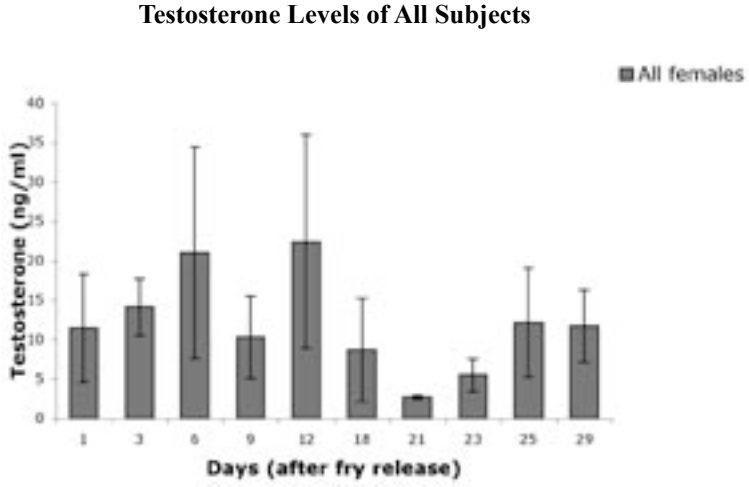

Figure 2. Plasma concentrations of testosterone in all subjects across the female *H. burtoni* reproductive cycle.

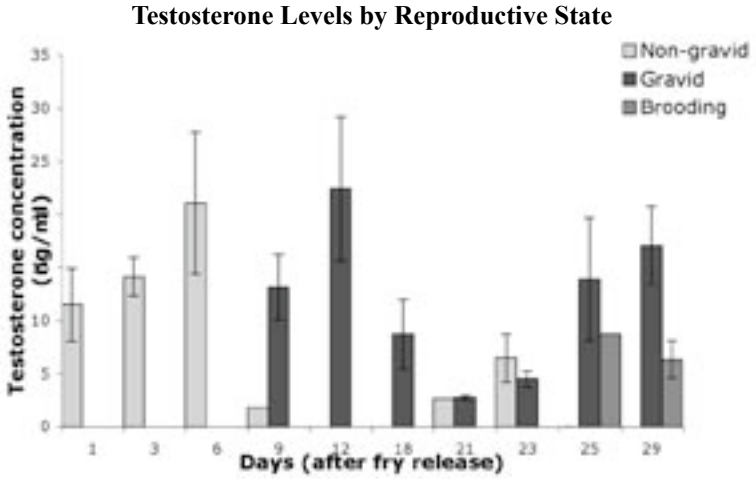

Figure 3. Plasma concentrations of testosterone across the female reproductive cycle, shown by reproductive state (Gravid, Non-Gravid, Brooding).

Hormone Profiles

Circulating plasma levels of testosterone and 17ß-estradiol were measured across the reproductive cycle. When females release their fry, testosterone and 17ß-estradiol concentrations are low. As females near the end of the non-gravid phase, testosterone concentrations increase first, reaching a peak around the sixth day after release (see Figures 2 and 3). They continue to rise as the female becomes gravid. Testosterone levels begin to decrease as the ovaries mature, and the females approach spawning. Testosterone concentrations are lowest during spawning, although they increase slightly during brooding (see Figure 3).

17ß-estradiol levels increase as the female becomes gravid and the ovaries start to mature. The levels peak just prior to ovary maturation, and begin to decrease as the female approaches spawning (see Figure 4). 17ß-estradiol remains low through both spawning and brooding (see Figure 5).

Real Time Quantitative RT-PCR results

Gonadotropin–releasing hormone I (GnRH I) is directly involved in the reproductive cycle of *H. burtoni* and is produced by neurons in the preoptic area of the hypothalamus. We performed real time quantitative RT-PCR in order to measure the amount of GnRH I mRNA transcript produced in the brain across the reproductive cycle. GnRH I transcript shows a peak at the eighteenth day after release, when the females are most gravid. It decreases slightly as the females spawn, and increases again during brooding (see Figure 6).

GnRH receptors I and III show similar patterns of expression across the reproductive cycle (see Figures 7 and 8). Both gradually increase as the female becomes gravid, peaking around the time that the female is most gravid, and then decreasing as she approaches

Estradiol Levels of All Subjects

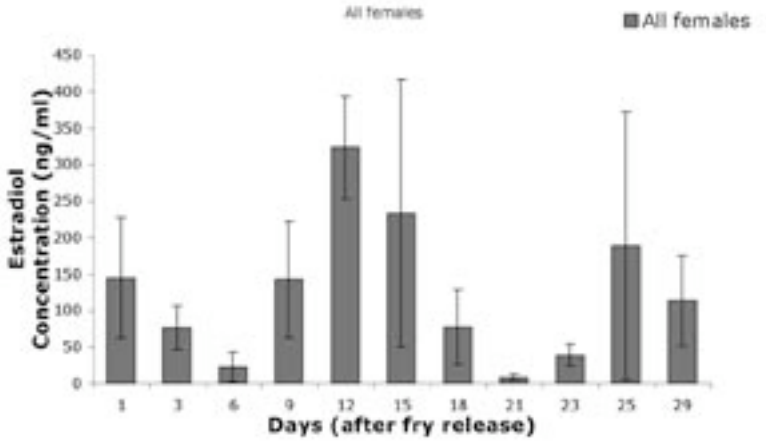

Figure 4. Plasma concentrations of 17ß-estradiol in all subjects across the female *H. burtoni* reproductive cycle.

Estradiol levels by Reproductive State

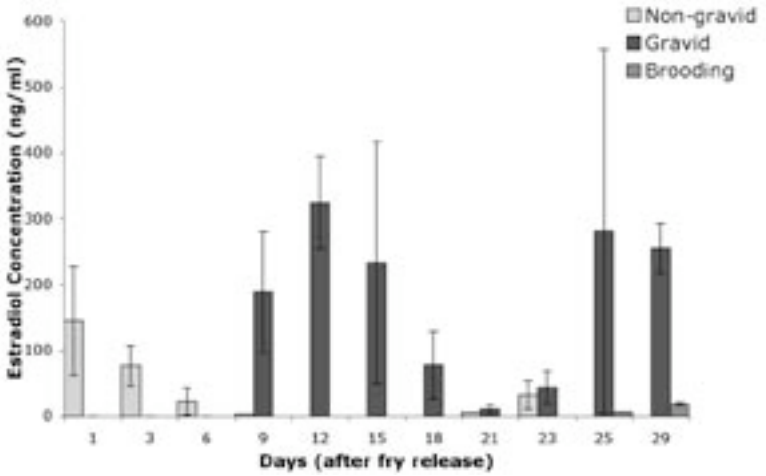

Figure 5. Plasma concentrations of 17ß-estradiol across female reproductive cycle, shown by reproductive state (Gravid, Non-Gravid, Brooding).

GnRH I Expression in the Brain by Reproductive State

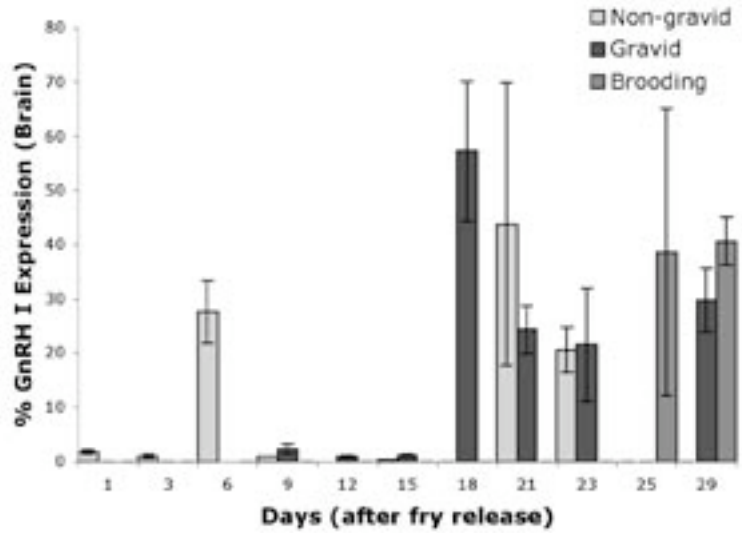

Figure 6. Gonadotropin-releasing hormone I (GnRH I) mRNA expression in the brain of female *H. burtoni*. Shown by reproductive state (Gravid, Non-Gravid, Brooding) across the reproductive cycle.

the end of the cycle. However, GnRH receptor I remains low during brooding while GnRH receptor III expression is high.

Discussion

The primary goal of this study was to identify the hormones involved in the reproductive cycle of female *H. burtoni* in order to correlate these fluctuations with changing behavior. To this end, we examined levels of 17ß-estradiol and testosterone in the plasma, GnRH I transcript in the brain, GnRH receptor I and III transcript in the pituitary, and gonadosomatic index. Our results indicate that when females release their fry, circulating levels of 17ß-estradiol and testosterone are low. The ovaries are regressed, and little GnRH transcript is produced in the brain. As females become gravid, testosterone concentrations increase, followed several days later by 17ß-estradiol. As 17ß-estradiol levels rise, the ovaries mature and increase in size. At the same time, the pituitary upregulates production of GnRH receptor I and III. When the female is very gravid, it appears that GnRH I transcript in the brain increases dramatically. However, additional data points are needed to determine when this hormone is most abundant.

This research extends previous research that indicated that gonadotropin-releasing hormone (GnRH) levels are correlated with the size of GnRH neurons in the preoptic area of the hypothalamus. White and Fernald (1993) demonstrated that GnRH neurons display remarkable plasticity throughout the reproductive cycle, as they are twice as large in females that have never spawned or are in the act of spawning than they are in females who are brooding. They also determined that preoptic GnRH transcript is significantly higher in spawning females than in brooding females (White, Nguyen, & Fernald, 2002), a result replicated here.

Although these physiological factors clearly fluctuate throughout the reproductive cycle, how might they direct reproductive behaviors such as mate choice? One possibility is that gonadal hormones may affect sexual motivation, as they do in primates (Wallen, 2001). Although high levels of gonadal hormones are necessary for reproductive behavior in most mammals, they are not necessary for sexual behavior in humans and primates (Wallen, 2001). For instance, many mammalian males require high levels of gonadal hormones (like testosterone) in order to maintain an erection. In contrast, both rhesus monkeys and human males can still engage in sex if testosterone is eliminated from their bodies, although they will lose sexual motivation. Likewise, levels of ovarian hormones may influence human female sexual motivation, but reproductive behavior is possible during all stages of the female menstrual cycle, and reproductive behavior is often influenced by social and cognitive factors (Wallen, 2001). It is possible that *H. burtoni* females are governed by a similar mechanism. Without the appropriate concentrations of ovarian hormones, female fish simply may not have the motivation to associate prefer-

GnRH receptor III Expression in the Pituitary by Reproductive State

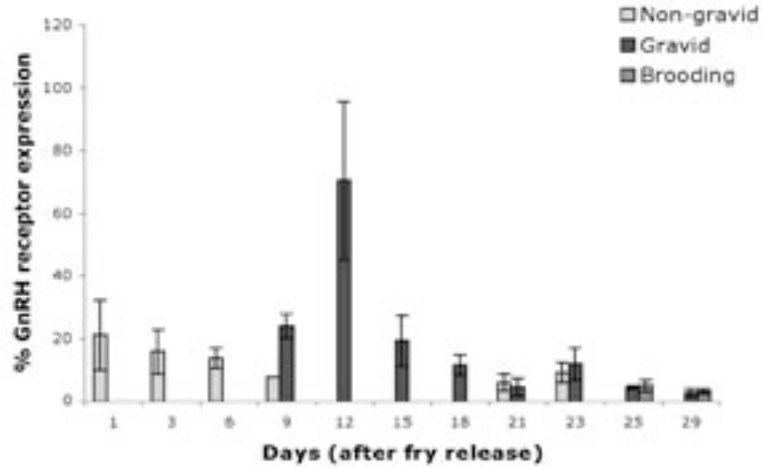

Figure 7. Gonadotropin-releasing hormone receptor I (GnRH receptor I) mRNA expression in the pituitary in female *H. burtoni*. Shown by reproductive state (Gravid, Non-Gravid, Brooding) across the reproductive cycle.

GnRH receptor III Expression in the Pituitary by Reproductive State

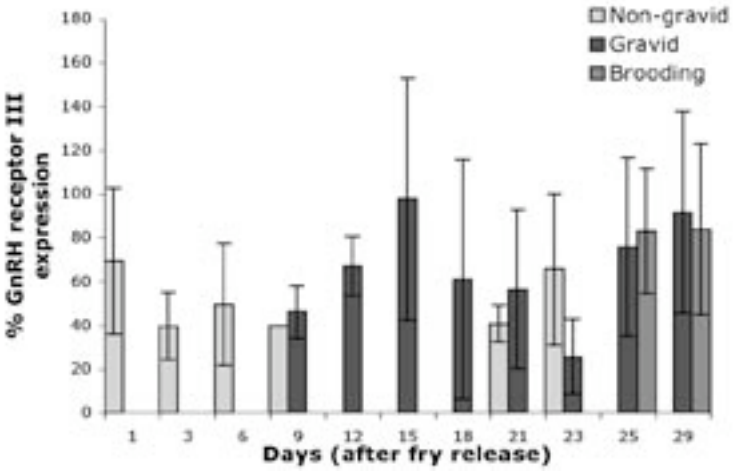

Figure 8. Gonadotropin-releasing hormone receptor III (GnRH receptor III) mRNA expression in the pituitary. Shown by reproductive state (Gravid, Non-Gravid, Brooding) across the reproductive cycle.

entially with territorial males. In particular, estradiol may play a large role in initiating reproductive maturation.

Although we hypothesize that a sex hormone (or perhaps several) mediates the shift in preference to territorial males, it is also possible that hormones may act by controlling behavior through changes in perception, and particularly through vision. The mate choice experimental design prevents any tactile contact between the female and the male, and it also limits the olfactory cues (the fish are separated by a clear plexiglass barrier). Hirata and Fernald (1975) have also determined that *H. burtoni* do not use sound for communication purposes. However, Hoke and Fernald (2002) established that estrogen receptor alpha is found in the retina of *H. burtoni*, so it is possible that either fluctuations in estrogen or estrogen receptors could subtly affect visual acuity or sensitivity. Intriguingly, estrogen, androgen, and progesterone receptors have also been found in several other species, including rats, rabbits, and humans (Wickham, Gao, Toda, Rocha, Ono, & Sullivan, 2000). If results indicate that visual perception changes across the reproductive cycle in *H. burtoni* (leading to changes in mate choice behavior), it is possible that the changing preference for male faces throughout the menstrual cycle in human females (Frost, 1994) may be mediated by a similar mechanism.

Human females also show variations in perception in several other sensory modalities: olfaction (Watanbe, Umezu, & Kurahashi, 2002), hearing (Davis & Ahroon, 1982), nocireception or pain sensitivity (Gaumon, Arsenault, & Marchand, 2002; Kerem, et al., 2002), vision sensitivity (Parlee, 1982), and even taste (Aaron, 1975) have been shown to change throughout the menstrual cycle. It is still unclear how hormones in the female menstrual cycle may influence these changes in perception, and it is likely that this connection will be understood in animal models (such as *H. burtoni*) before it is fully understood in humans.

Even if the *H. burtoni* mate preference shifts are not due to changes in perception, this research does have interesting implications for humans. Somewhat surprisingly, many of the same hormones that are prevalent in human females are also prevalent in *H. burtoni* females. In humans and fish, estradiol and testosterone are important gonadal hormones that govern reproduction and reproductive behaviors. Just as in fish, humans release gonadotropin-releasing hormone from the hypothalamus, which stimulates the release of gonadotropins from the pituitary. Due to this conservation of form and function across species, we can study these hormones and their effects in simpler systems and generate intelligent guesses about how these hormones may function in humans.

References

Aaron, M. (1975). Effect of the menstrual cycle of subjective ratings of sweetness. *Perceptual and Motor Skills*, 40, 974.

Bonduriansky, R. (2001). The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Review*, 76, 305-339.

Clement, T.S., Grens, K.E., & Fernald, R.D. (submitted) Female association preference depends on reproductive state in the African cichild fish, *Haplochromis burtoni*.

Darwin, C. (1871). *The Decent of Man and Selection in Relation to Sex.* New York: D. Appleton and Co.

Davis, M.J. & Ahroon, W.A. (1982). Fluctuations in susceptibility to noise-induced temporary threshold shift as influenced by the menstrual cycle. *The Journal of Auditory Research*, 22, 173-187.

Fernald, R. D. & Hirata, N. R. (1977). Field study of *Haplochromis burtoni*: habitats and co-habitat. *Environmental Biology of Fishes* 2(3), 299-308.

Frost, P. (1994). Preference for darker faces in photographs at different phases of the menstrual cycle: preliminary assessment of evidence for a hormonal relationship. *Perceptual Motor Skills*, 79, 507-514.

Gaumond, I., Arsenault, P., and Marchand, S. (2002). The role of sex hormones on formalin-induced nocireceptive responses. *Brain Research*, 958, 139-145.

Godin, J. J. and Dugatkin, L. A. (1996). Female mating preference for bold males in the guppy, *Poecilia reticulata. Procedings of the National Academy of Science*, 93, 10262-10267.

Hebets, E.A. (2003) Subadult experience influences adult mate choice in an arthropod: Exposed female wolf spiders prefer males of a familiar phenotype. *Proceedings of the National Academy of Sciences*, 100, 13390-13395.

Hirata, N. R. & Fernald, R. D. (1975). Non-intentional sound production in a cichlid fish (*Haplochromis burtoni, Gunther*). *Separatum Experientia*, 31, 299-300.

Hoke, K. L. & Fernald, R. D. (2002). Teleost fish retina contains estrogen signaling pathway. *Society for Neuroscience Abstracts*.

Hughes, K.A., Du, L., Rodd, F. H., and Reznick, D. N. (1999) Familiarity leads to female mate preference for novel males in the guppy, *Poecilia reticulata*, *Animal Behavior*, 58, 907-916.

Jurke, M.H., Pryce, C.R., & Dobeli, M. (1995). An investigation into sexual motivation and behavior in female Goeldi's monkey (*Callimico goeldii*): effect of ovarian state, male familiarity, and mate choice. *Hormones and Behavior*, 29(4), 531-553.

Karubian, J. (2002). Costs and benefits of variable breeding plumage in the red-backed fairy-wren. *Evolution; International Journal of Organic evolution*, 56(8), 1673-1682.
Kelley, J.L., Graves, J.A., and Mugarran, A.E. (1999). Familiarity breeds contempt in guppies, *Nature*, 401, 661.
Kerem, M., Akbayrak, T., Bumin, G., Yigiter, K., Armutlu, K., & Kerimoglu, D. (2002). A correlation between sex hormone levels and pressure pain threshold and tolerance in healthy women. *The Pain Clinic*, 14(1), 43-47.
Kodric-Brown, A. (1993). Female choice of multiple male critieria in guppies: interacting effects of dominance, coloration and courtship. *Behavioral Ecology and Sociobiology*, 32, 415-420.Kurubian
Morris, M. and Casey, K. (1998). Female swordtail fish prefer symmetrical sexual signal. *Animal Behavior*, 55, 33-39.
Parlee, M.B. (1983). Menstrual rhythms in sensory processes: a review of fluctuations in vision, olfaction, audition, taste, and touch. *Psychological Bulletin*, 93(3), 539-548.
Wallen, K. (2001). Sex and context: hormones and primate sexual motivation. *Hormones and Behavior*, 40, 339-357.
Watanabe, K., Umezu, K., and Kurahashi, T. (2002) Human olfactory contrast changes during the menstrual cycle. *Japanese Journal of Physiology*, 52, 353-359.
White, S.A. & Fernald, R.D. (1993). Gonadotropin-releasing hormone-containing neurons change size with reproductive state in female *Haplochromis burtoni*. *The Journal of Neuroscience*, 13(2), 434-441.
White, S.A., Nguyen, T., & Fernald, R.D. (2002). *Social regulation of gonadotropin-releasing hormone. The Journal of Experiemtnal Biology*, 205, 2567-2581.
Wickham, L. A., Gao, J., Toda, I., Rocha, E. M., Ono, M., & Sullivan, D. A. (2000). Identification of androgen, estrogen, and progesterone receptor mRNAs in the eye. *Acta Ophthalmologica Scandinavica*, 78, 146-153.

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