Female preference for males depends on reproductive physiology in the African cichlid fish Astatotilapia burtoni

Michael R. Kidd a,b,1,2, Lauren A. O’Connell a,c,1, Celeste E. Kidd a, Christine W. Chen a, Miles R. Fontenot a, Sidney J. Williams a, Hans A. Hofmann a,b,c,*

a Section of Integrative Biology, The University of Texas at Austin, Austin, TX 78712, USA
b Institute for Neuroscience, The University of Texas at Austin, Austin, TX 78712, USA
c Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA

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A B S T R A C T

Mate choice is fundamental to sexual selection, yet little is known about underlying physiological mechanisms that influence female mating decisions. We investigated the endocrine underpinnings of female mate choice in the African cichlid Astatotilapia burtoni, a non-seasonal breeder. In addition to profiling behavioral and hormonal changes across the female reproductive cycle, we tested two hypotheses regarding possible factors influencing female mate choice. We first asked whether female mate choice is influenced by male visual and/or chemical cues. A. burtoni females were housed for one full reproductive cycle in the center of a dichotomous choice apparatus with a large (attractive) or small (unattractive) conspecific male on either side. Females associated mostly with small, less attractive males, but on the day of spawning reversed their preference to large, attractive males, with whom they mated almost exclusively, although this choice depended on the relative amount of androgens released into the water by small males. We next asked whether male behavior or androgen levels change in relation to the stimulus females’ reproductive state. We found that stimulus male aggression decreased and reproductive displays increased as the day of spawning approached. Moreover male testosterone levels changed throughout the females’ reproductive cycle, with larger males releasing more testosterone into the water than small males. Our data suggest that female association in a dichotomous choice assay is only indicative of the actual mate choice on the day of spawning. Furthermore, we show that male behavior and hormone levels are dependent on the reproductive state of conspecific females.

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1. Introduction

Female mate choice provides an important mechanism for the evolution of sexually dimorphic traits that contribute to speciation [49,51]. Although our understanding of the ultimate mechanisms governing female mate choice has greatly increased in the past few decades [6,21], studying the physiological and endocrine underpinnings is difficult, as female choosiness changes with reproductive status [19], across the life span [40], and with experience [20]. Hormonal influences on female receptivity have been well studied [1,14,38], yet the role of both gonadal and other hormones in mediating female mate choice is not well understood.

The African cichlid, Astatotilapia burtoni, is an important model system in social neuroscience [24,48] and has recently been used to study female mate choice [7,15]. Cichlids of the great African lakes have undergone rapid and repeated adaptive radiations that provide a unique opportunity to study how female mate choice and male–male competition have contributed to this diversity [31,51]. Since many molecular and genomic resources have been developed for A. burtoni as a model cichlid [45,50], we now have an exceptional opportunity to study the proximate mechanisms of sexual selection. In their native Lake Tanganyika, socially dominant males occupy display territories that are contiguously arrayed throughout the habitat. Females visit territories within such a lek to spawn [12], directly coupling female mate choice to sexual behavior, before incubating the developing young in their mouths for up to two weeks. While much progress has been made in understanding male–male competition and male behavioral plasticity in this species by measuring hormone levels and brain gene expression [24,36], relatively little is known about female mate choice [7]. Although some work has been done on hormonal differences between female phenotypes and reproductive stages in A. burtoni...
sured levels of gonadal steroids (17β-estradiol, testosterone, and progestins) across the A. burtoni female reproductive cycle, as steroid hormones are important in modulating female reproductive physiology and behavior across vertebrates [1,18,58,60]. Additionally, we measured levels of the lipid hormone prostaglandin F2α (PGF2) across the ovarian cycle, as PGF2 plays an important role in the reproductive physiology of female vertebrates, both at the level of ovarian maturation and behavior [reviewed in 41]. More generally, many studies in teleosts have profiled steroid hormone levels in seasonal breeders [5,9,25,32,35], whereas the hormonal profiles of species that breed year-round are comparatively understudied [44].

In addition to profiling hormonal changes across the A. burtoni female reproductive cycle, we tested two hypotheses of possible mechanisms affecting female mate choice. We first asked if female mate choice is influenced by males size and hormone levels by examining the females’ choice of mate (large or small male) on the day of spawning and how this choice was influenced by testosterone release into the water by the stimulus males. Secondly, we asked if behavior of androgen release by stimulus males is linked to female behavioral or hormonal changes throughout the ovulation cycle.

2. Materials and methods

2.1. Animals

Mixed sex groups of adult A. burtoni from a wild-caught stock population were maintained in community tanks: pH 8.0, 28 °C water temperature, and 12:12 h light:dark cycle with 10 min ea. dusk and dawn periods. Gravel and terracotta shelters provided the substrate that facilitates the establishment and maintenance of territories necessary for reproduction. Fish were fed every day with cichlid flakes (Arcata Pet Supplies, Arcata, CA) after 10:00. All work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

2.2. Female reproductive behavior

After spawning within a community setting, eggs were removed from the females’ mouths and females (n = 84) were transferred to the central compartment of a 200-l rectangular observational arena divided into three sections by transparent and perforated divider panels into three sections by transparent and perforated divider panels. Each male compartment included one half of a clay saucer (bower) with the other half extending into the female compartment. This bower served as the focal point for reproductive behavior. Behavior was recorded daily for 10 min each at 9:00, 12:00, 15:00, and 19:00 h using a multichannel digital video surveillance system (Video Insight, Inc., Houston, TX). The spawning location of all females (n = 84) were recorded. For a subset of females (n = 8) association with a male (scored as the amount of time spent in the “association zone” (within 15.5 cm of the male’s partition) and in the bower (proceptive sexual behavior) was quantified for 10 min at 10:00 h on days 1, 2, 3, 7, 8, 13, and 14 post spawning and 8, 7, 6, 5, 4, 3, 2, 1 days prior to spawning and on the day of spawning. On a subset of these days (1, 2, 3, 4, 5, 15, and 27 days until spawning) and at the same time of day we also quantified male aggressive and reproductive displays as described Fernald and Hirata [17]. Aggressive behavior was bites directed toward the female displayed by the stimulus males. Reproductive displays included quivers and leading behavior characterized by the male directing the female towards the bower.

2.3. Hormone assays

Both males and females were removed from the testing arena after the 10:00 h observation period on days 2, 7, 14, 21, 23, 25, 26, 27, and every subsequent day until spawning. Each animal was individually confined in 300 ml of fresh aquarium water for 60 min. Holding water was filtered (Whatman, Fisher Scientific, Pittsburgh, PA) to remove particulates, and hormones were extracted from the water with solid phase extraction (SPE) cartridges (Sep-pak® Plus C18, Waters Ltd., UK) as previously described [26]. Enzyme-linked immunosorbutant assays (ELISA) were used to determine at each time point waterborne hormone levels for testosterone, progestins, 17β-estradiol, and prostaglandin F2α (Enzo Life Sciences, Farmingdale, NY; catalog numbers ADI-900-006, ADI-900-008, ADI-900-011, ADI-900-069, respectively) for females. In fishes, the major bioactive progestins are progesterone derivatives, such as 17α,20β-P [43]. We previously validated the cross-reactivity of the commercial progesterone assay system as well as all other hormone ELISAs described here (for details see 26). For stimulus males we only measured testosterone. 11-Ketotestosterone levels are very low in this species [26] and thus were not measured. The cross-reactivity of the testosterone ELISA is <0.001% for dihydrotestosterone and <0.5% for 11-ketotestosterone [26]. Inter- and intra-assay variation were 1.53% and 1.88% for testosterone, 5.71% and 1.74% for progestins, 8.40% and 2.27% for 17β-estradiol, and 2.10% and 1.59% for the prostaglandin F2α ELISA, respectively. Inter-assay variation was determined by calculating the standard deviation of the means of the duplicates of standard samples on different plates divided by the grand mean of the duplicates multiplied by 100. Intra-assay variation was determined by calculating the mean of the standard deviations of 10 randomly selected sample duplicates divided by the grand mean of the duplicates multiplied by 100. We have previously shown that this non-invasive hormone sampling provides a reliable representation of circulating hormone levels [26]. Not all hormones were measured from the same individual each day due to insufficient sample collection.
2.4. Ovarian histology and stage determination

Ovaries from females at select time points throughout the reproductive cycle (7, 14, 25, and 29 days after the last spawn; n = 8 per time point except day 29, where n = 6) were fixed in Bouin's solution overnight and washed in 70% ethanol for one day to remove the Bouin's fixative. Ovaries were cryoprotected in 30% sucrose overnight and then in ascending OCT (Tissue-Tek)/sucrose solutions for 3 days before embedding in 100% OCT and stored at −80°C for two to six weeks until sectioning. Ovaries were sectioned at 16 μm, mounted onto gelatin-coated slides (Superfrost/Plus; Fisher Scientific), and stained with hematoxylin and eosin for histological analysis. Reproductive state was determined based on an oocyte maturation staging protocol developed for another cichlid fish, the redbelly tilapia Tilapia zillii [13].

2.5. Statistical analysis

Statistical tests were conducted in PASW (IBM, Somers, NY). Significance is considered as p < 0.05. In statistical analyses where multiple hypotheses were tested, a Benjamini-Hochberg false discovery rate correction was applied [4].

2.5.1. Female behavior across the reproductive cycle

Fisher's Exact test for goodness of fit was used to test if the observed choice of the larger or smaller male was different than the expected frequency (expected 1:1 ratio). Six (of 84) females that either spawned with both males or spawned by themselves in their shelter (n = 6) were excluded from this analysis. Total time females spent in the bower was analyzed using a Generalized Estimating Equations (GEE; Hardin and Hilbe, [22]) model to account for non-independence in repeated measures across time points and the non-normal distribution of data. We analyzed behavior on the day of spawning, the eight days prior to spawning, as well as select days throughout the cycle. The model included day of cycle and time of day as within-subject variables and aggressive (bites) or reproductive (sum of leads and lateral displays) behavior as the dependent variable. To examine differences on each day, association time and time spent in the bower (of either the small or large male) as the dependent variable, and size of male as the independent variable, and time of day as a covariate.

2.5.2. Stimulus male behavior across the reproductive cycle of focal females

Behavioral displays were divided by the time in the association zone. To examine if male behavior varied across the female reproductive cycle, we selected separate General Estimating Equations (GEE) models for small and large males with day of cycle and time of day as within-subject variables and aggressive (bites) or reproductive (sum of leads and lateral displays) behavior as the dependent variable. To examine differences between large and small males, we considered waterborne hormone measurements as reliable estimates of circulating levels [26], and thus we normalized the data to standard length (cm) of each individual to account for variation in focal female size. We did not, however, normalize to standard length waterborne androgen measurements obtained from males, as we wanted to test for differences in androgen release into the water that might be detected by females. The males for which we measured testosterone levels were not the same males used for behavior quantification. As hormone data throughout the focal female reproductive cycle were based on a repeated measures design and were not normally distributed, a GEE approach was used with day of cycle as the within-subject independent variable and hormone level as the dependent variable. To examine differences in testosterone release between stimulus males on each sampling day, a GEE model was used for each day with testosterone as the independent variable, and size of male as the dependent variable. When examining differences between large and small male waterborne testosterone levels on the day of spawning, we used a t-test.

3. Results

3.1. Female behavioral profiles throughout the reproductive cycle

To first establish a framework in which to study the neuroendocrine mechanisms of female mate choice in A. burtoni, we examined female behavioral preferences when presented with attractive (large) and less attractive (small) males throughout the reproductive cycle (see Fig. 1) and noted with which male mating occurred. Females overwhelmingly prefer to spawn with the larger male over the small male (Fig. 2A; Fisher's Exact Test: χ² = 40.21, p < 0.001). Out of 84 observed spawns, 67 occurred in the bower of the large male, while 11 occurred in the bower of the small male, one female deposited eggs in the bowers of both males, and five females deposited eggs in the shelter in the center of her compartment. When we examined male testosterone levels in a subset of these spawning events, we found that when females chose to spawn with the larger male (Fig. 2B), the larger males released more waterborne testosterone compared to the smaller males (t14.761 = 4.675, p < 0.001). However, in cases where the female chose to spawn with the smaller male (Fig. 2C), small males released just as much testosterone into the water as large males (t10.653 = 0.480, p = 0.646).

The time females spent in the bower varied significantly (GEE: Wald χ² = 29.339, df = 7, p = 0.001) over the course of the female reproductive cycle (Fig. 3). Importantly, female time spent in association or in the bower did not reflect the final mate choice until the day of spawning itself. To our surprise, females associated significantly more with the small male throughout the reproductive cycle (ANOVA; p < 0.05, see Table S1 for statistical details) with the exception of day 14 (no significant difference: p = 0.129) and the day of spawning (see below). Females also spent significantly more time in the small male's bower (ANOVA; p < 0.05, see Table S1 for statistical details) with the exception of Days 2 (t = 0.172) and -7 (t = 0.82), and again the day of spawning (Fig. 3). On the day of spawning, female bower preference (ANOVA: F8,1 = 9.248, p = 0.003) reversed dramatically, and they also showed a trend for increased association time with the larger male (ANOVA: F8,1 = 3.485, p = 0.065).

3.2. Female hormone profiles throughout the reproductive cycle

To better understand which reproductive hormones may be associated with changes in preference in the days prior to mate choice, we noninvasively measured 17β-estradiol, testosterone, progesterins, and PGF2 at several time points, including the days...
immediately preceding spawning (Fig. 4), as these hormones play important roles in mediating female reproductive behavior across vertebrates [2,18]. 17b-Estradiol peaked six days prior to spawning (GEE: Wald $\chi^2 = 1751.920$, df = 15, $p < 0.001$), followed by a simultaneous rise in progestins and testosterone four days prior to spawning (GEE; progestins: Wald $\chi^2 = 26.279$, df = 11, $p = 0.006$; testosterone: Wald $\chi^2 = 63.792$, df = 13, $p < 0.001$). Finally, PGF2 levels peaked three days prior to spawning (GEE: Wald $\chi^2 = 37.676$, df = 10, $p < 0.001$).

3.3. Gonadal physiology across the ovarian cycle

To examine the relationship between gonadal physiology, female behavior, and hormone profiles across the reproductive cycle, we quantified oocyte stage at 7, 14, 25, and 29 days after spawning (Fig. 5). As expected, we found that the average oocyte stage changes throughout the reproductive cycle (ANOVA, $F_{30,3} = 5.828$, $p = 0.003$). Oocyte stage increased between 7 and 14 days post spawning (Tukey's HSD $p = 0.018$) and then remained in a mature state until day 29 (Tukey's HSD $p < 0.338$).

3.4. Stimulus male behavior and hormone levels vary with the female reproductive cycle

To determine if male behavior changes throughout the female reproductive cycle, we also quantified male aggressive and reproductive displays. Both the large and small males varied in aggression (Fig. 6A; GEE: large males: Wald $\chi^2 = 373.62$, df = 8, $p < 0.001$; small males: Wald $\chi^2 = 23.195$, df = 8, $p = 0.003$) and reproductive displays (Fig. 6B; GEE: large males: Wald $\chi^2 = 373.62$, df = 8, $p < 0.001$; small males: Wald $\chi^2 = 23.195$, df = 8, $p = 0.003$).
v = 93.944, df = 8, p < 0.001; small males: Wald χ² = 57.615, df = 8, p < 0.001) across the female reproductive cycle. Large males performed higher levels of both aggressive and reproductive displays compared to smaller males (aggression days -5 to -3: GEE, p < 0.001; reproductive displays: GEE, p < 0.001) until the day immediately prior to and the day of spawning (see Table S2 for detailed statistics).

As aggression and courtship behavior of males change throughout the female reproductive cycle, we measured waterborne testosterone levels (Fig. 6C). We found that male testosterone release into the water varies with female reproductive state regardless of male size (GEE: Wald χ² = 14.170, df = 4, p = 0.007). Moreover, larger males generally released more testosterone into the water compared to smaller males (GEE: Wald χ² = 46.997, df = 1, p < 0.001). This difference in testosterone release between large and small males was significant on most days prior to spawning (GEE, df = 1; day -28 (Wald χ² = 19.5, p < 0.001), day -15 (Wald χ² = 12.4, p < 0.001), day -1 (Wald χ² = 16.2, p < 0.001), except three days prior to spawning where we found only a marginally significant difference in testosterone release (Wald χ² = 3.461, df = 1, p = 0.063). The difference was also highly significant on the day of spawning (Wald χ² = 12.99, p < 0.001).

4. Discussion

4.1. Female preference for larger males

In teleosts, experimental measures of female mate preference often use female association time with a male as the response variable without confirming the actual mating choice [but see 27, 55]. However, our results clearly show that in A. burtoni association time is predictive of the final choice of mate only on the day of spawning when females spent more time with the larger, more attractive male. This is consistent with a previous study in A. burtoni, which compared gravid and non-gravid females given a choice between a dominant and subordinate male [7], where female association times differ with reproductive state; however, we have presented here a more detailed analysis and observed the actual choice of mate. Furthermore, female preference for larger males has been previously described in many other teleosts, including those with lek-based mating systems [11] and in species that display paternal defense of offspring [10]. However, our experimental paradigm cannot distinguish between attraction to small males or avoidance of large males. Larger dominant males are typically very aggressive, and thus a female may try to avoid risking injury unless she is receptive and ready to spawn. This is similar to females of some mammalian species that avoid males until they enter into breeding condition [16].
4.2. Sex steroids across the reproductive cycle

Even though the average cycle period for *A. burtoni* females is 28 ± 3 days, the peaks of the various reproductive hormones were compressed into the final week before spawning. In teleosts, most studies on female reproductive endocrinology have focused on seasonal spawners, in which plasma estradiol levels peak many weeks or months before spawning, followed by one or two testosterone peaks immediately prior to ovulation and spawning [3,23,28,29,35,47,52]. In the trout (*Salmo trutta*), both 17β-estradiol and testosterone peak 30 days prior to ovulation [44]. Progestins peak on the day prior to spawning or on the day of spawning in many teleosts [30,44,56]. In female tetrapods, progestins increase as levels of estradiol and testosterone decrease [39,42]. In female *A. burtoni*, however, progestins and testosterone peak at the same time (4 days prior to spawning).

4.3. PGF2 and spawning

The role of PGF2 in female sexual behavior has been well described in teleosts [reviewed in 18,41]. However, in our experimental paradigm, the PGF2 peak in *A. burtoni* females occurs three days prior to the actual spawning event (Fig. 4), and male reproductive displays peak shortly thereafter (Fig. 6), as one might expect based on work by Cole and Stacey [8], who showed in the Black Acara cichlid, *Cichlasoma bimaculatum*, that exogenous PGF2 can induce spawning behavior at any time throughout the reproductive cycle. The relationship between PGF2 production and ovarian physiology has not yet been examined in *A. burtoni*, although given its role in ovulation it is doubtful that the PGF2 peak would normally occur days before spawning in this fish. It seems thus more likely that the experimental constraints imposed by our choice apparatus caused this dissociation. Specifically, a female’s sensory experience was restricted to visual and chemical cues in our paradigm, even though auditory and tactile cues are likely also important in this context [37,59]. Finally, a female’s choice is also limited to only two individuals in our paradigm, where normally an entire lek is available [17].

4.4. Changes in gonadal physiology

We observed a sharp increase in oocyte maturation between days 7 and 14 post-spawning, whereas days 14 through 29 remained relatively consistent. This is similar to the patterns of oocyte stage progression described by Coward and Bromage [13] in the redbelly tilapia (*T. zillii*), where mature oocytes (stage 6/7 characterized by yolk incorporation) appear quickly (3–4 days) after spawning, and the majority of oocytes have incorporated yolk.
within seven days after spawning. We have found that oocyte maturation post-spawning in *A. burtoni* females follows a similar pattern as in *T. zillii* females, where the majority of oocytes in the ovary are mature from day 14 post-spawn onwards. However, in *T. zillii*, the increase in mature oocytes coincides with an increase in 17β-estradiol and testosterone levels, whereas the rise in these hormones and oocyte maturation in *A. burtoni* females appears to be decoupled.

4.5. Male behavior and hormone release is dependent on female behavior and/or physiology

Relatively large males, although more attractive to females, are generally more aggressive than smaller, less attractive males [11]. It is important to note, however, that in our choice paradigm both the large and small males were dominant, reproductively active individuals; and some females still spawned with the smaller male, indicating smaller males are still regarded as a potential mate rather than a non-reproductive member of a school. The differences in aggression between large and small males disappear the day prior to and the day of spawning, with a concurrent increase in the frequency of reproductive displays. Moreover, aggressive displays by both large and small males appear to briefly increase three days prior to spawning before they then again decrease as the day of spawning approaches, suggesting that both large and small males alter their behavior in response to female cues. From our study it is difficult to determine whether female behavior or chemical cues (or both) alter male behavior to decrease aggression and increase courtship in large males. Although PGF2 is released into the water by female goldfish [33] where it acts as a pheromone [53], it is unlikely that this alters behavior in *A. burtoni* males of our study, as in this species the olfactory epithelium cannot detect PGF2 [8]. However, we cannot exclude the possibility that *A. burtoni* males can detect PGF2 metabolites. We therefore suggest that female behavior itself or other chemical cues, such as steroidal pheromones [54], alter male behavior, rather than the PGF2 directly changing behavior in males.

We also found that the amount of testosterone males release into the water changes with the female reproductive cycle. Moreover, larger males generally release more androgens into the water compared to small males. We do not know whether *A. burtoni* can detect the free testosterone we have measured here. However, conjugated androgens are reliably detected and are likely co-released along with free steroids [8,54]. In fact, our results suggest that a female's choice of a male may at least in part depend on androgens released into the water, as females consistently chose to spawn with the large males except in those cases when the small males released similarly high levels of testosterone into the water as large males.

4.6. Conclusions

We have presented here a detailed analysis of the behavior and physiology of female *A. burtoni* throughout the reproductive cycle and final choice of mate. Furthermore we have shown that male change their aggressive and reproductive behavior as well as release of testosterone into the water in response to female physiology and/or behavior. Future work will seek to disentangle the contributions of female hormone release and behavior to changes in male behavior and physiology as well as determine the molecular and neural mechanisms of female mate choice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.ygenec.2012.10.014](http://dx.doi.org/10.1016/j.ygenec.2012.10.014).

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