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Elevated 11-ketotestosterone during paternal behavior in the Bluebanded goby (*Lythrypnus dalli*)

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Abstract

The relationship between androgens and paternal behavior is not straightforward, potentially because of the diversity of tasks a male must undertake to maximize reproductive success, notably alternating between courtship, aggression, and offspring care. In some species, these events are separated in time, but in others they are coincident. The endocrine profiles of species that simultaneously court, parent, and defend a nest, such as male bluebanded gobies (*Lythrypnus dalli*), are not well understood. We sampled a potent fish androgen, 11-ketotestosterone (KT), at different life history stages (experienced parenting males, experienced males not actively parenting, inexperienced males with their first clutch, and females), to examine this relationship. We found that experienced parenting *L. dalli* males have the highest KT levels of any group, while none of the other groups differed significantly. Males showed elevated KT levels when they had eggs compared to when they did not. Our data suggest that KT facilitates at least some aspects of parental care in *L. dalli*.

Keywords: 11-KT; Parental experience; Sex changing fish; Challenge hypothesis

Introduction

In many species, androgens are elevated during some facets of male reproductive behavior, namely courtship and aggressive behavior, and are depressed during others, such as paternal care (Wingfield, 1984; Wingfield et al., 1997; Oliveira et al., 2002). Although an inverse relationship between androgens and paternal care is present in many species (Ketterson and Nolan, 1994; Wynne-Edwards, 2001; Van Roo, 2004), it is not evident in all paternal species (Ziegler and Snowdon, 2000; Ziegler et al., 2004; Trainor and Marler, 2001, 2002; Ros et al., 2004).

One common component of paternal care that may complicate the androgen relationship is aggression (Marler et al., 2003). Males of many species must actively defend a nest or territory while providing parental care. This necessity for aggressive behavior may preclude a decline in androgen levels during paternal care. Fish provide a unique opportunity to test these ideas, because many species exhibit high levels of paternal care that co-occur with aggression.

11-Ketotestosterone (KT), a potent androgenic steroid in fishes, activates a suite of male typical behavioral and morphological traits in most fish species, including sex changing species and species exhibiting alternative male reproductive tactics (Oliveira, 2004). Interestingly, the territorial male phenotype in species exhibiting alternative reproductive tactics often provides exclusive care of offspring and as a rule have higher levels of KT than sneaker or satellite males (Brantley et al., 1993; Oliveira et al., 2001). KT has been shown to be the androgen that mediates the challenge response in fishes (Hirschenhauser et al., 2004). The role of androgens in paternal care in fishes has generally been addressed in two ways: by sampling endogenous androgens over the course of the mating cycle, or administering exogenous steroid hormones to parenting males. These studies have yielded mixed results. Many studies of endogenous androgens document a decline when a male fish is parenting, most often following an initial period of high androgens (Pankhurst, 1990; Sikkel, 1993; Knapp et al., 1999; Specker and Kishida, 2000; Oliveira et al.,

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2002; Pall et al., 2002a, 2005), while exogenous administration of androgens does not appear to inhibit paternal care to a substantial degree (Kindler et al., 1991; Pall et al., 2002b; Ros et al., 2004) and can facilitate parenting in the blue gourami (Kramer, 1972).

An important question then is how androgens might mediate a trade-off between aggression and paternal care in species that simultaneously defend a territory and care for offspring. The sex changing fish *Lythrypnus dalli* is an excellent model system in which to examine how KT might impact behavior in different life history stages. Females do not provide any parental care and will consume eggs if allowed. Dominant *L. dalli* females change sex following male removal, and males exhibit parental care that often co-occurs with, but at times is dissociated from (e.g., when no clutches are present) nest defense. Males in this species are territorial, parental, and polygynous, mating with all females in their harem. Like many marine teleosts, *L. dalli* have a pelagic larval phase that begins immediately following hatching (Steele, 1997), and thus male parental care ends when eggs hatch. *L. dalli* also allows for comparisons of non-parenting females and inexperienced males to parenting males, and examination of whether KT levels are altered as a non-parenting female changes sex to a functional male that provides exclusive parental care.

We hypothesize that females will have the lowest KT levels, with inexperienced and experienced males having higher levels, respectively. Because *L. dalli* males must continue to defend their nest aggressively and court additional females while parenting, we predict that KT will not decline when they are providing parental care.

Methods

Subjects

Twenty-six groups of 4–5 individuals were established and allowed to co-habitate and reproduce for at least 2 months. Groups consisted of 1 large male (38.1 ± 0.5 SEM) and between 3 and 4 smaller females. Sex was determined by examination of the external genital papilla (Behrents, 1983). *L. dalli* females have a papilla length-to-width ratio of approximately 1.0 whereas males have a ratio of 1.6 or greater (St Mary, 1994). Each group in the study was determined to be reproductively and parentally competent, as measured by successful rearing of eggs by the male to the eyed larvae stage. Groups were kept at 18.3°C, with 12 h light:12 h dark photoperiod, and fed frozen brine shrimp twice daily. Animals were housed in 38 L aquaria, each with an individual filter system (Marineland). Fish were collected (California Fish and Game permit # 803034-01) off the coast of Santa Catalina Island, California, using an anesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets. The fish then were transported back to the laboratory at Georgia State University, Atlanta. This research was carried out in accordance with the IACUC standards for use of animals in research at Georgia State University.

Experimental design

Parentally experienced males were sampled for water borne KT at two time points: when they were parenting (24 h after the appearance of a new clutch), and when they were between clutches (24 h after hatching a clutch, when no eggs were present). Egg status (presence or absence) was recorded each day for all groups. Samples for water-borne KT were collected by removing the male and the nest tube from the home tank, and placing the male in 100 ml of freshly mixed seawater (DI water with Instant Ocean™, ~1.022 specific gravity) for 1 h. To prevent the remaining females from consuming eggs while the male was being sampled, the nest tube was placed in a separate container filled with water from the home tank. At the conclusion of sampling, the male and the tube were returned to the tank. Sampling order was balanced, with half the males being sampled first while parenting and half the males sampled first between clutches. There was no effect of sampling order on KT levels (paired *t* test, $t_{21} = 0.339$, $P = 0.737$). In addition, there was no correlation between body size and KT ($R^2 = 0.002$, $F = 0.064$, $P = 0.8$). Hormone sampling was conducted at the same time each day, between 1000 and 1100 h. We noted that in several cases ($n = 8$) eggs were consumed within 24 h after the parenting sample. Twenty-four of 26 males were sampled for both time points. The other two males never were without eggs in the 3 weeks of the study.

In the second part of the study, we collected hormone samples from females and new "parentally inexperienced" males. To do this, we removed the two largest females in 12 of the group tanks. We placed them in individual beakers for hormone collection as described above. When the samples were completed, we returned the females to their respective tanks and removed the existing male. This will induce the largest female to change sex into male. The same two fish (now the new male and female) were then sampled again at the first appearance of eggs, following the procedure described above.

Hormone assays

Steroids were extracted from 100 ml of water using Lichrolut C18 columns (Carlisle et al., 2000) and the hormone was eluted from the column with 4 ml of methanol. The methanol was then evaporated in a vacuum centrifuge at 40°C and re-suspended in 110 µl of assay buffer (Greenwood et al., 2001). KT levels were assessed using commercially available KT EIA kits (Cayman Chemicals Inc.). All samples were run in duplicate, and all three 96-well assays were conducted on the same day. Intra- and interassay coefficients of variation were derived from two *L. dalli* pooled water extract samples (see below) included in each assay. Intra-assay coefficients of variation were 2.13%, 1.74%, and 7.9%; the interassay coefficient of variation was 7.2%. Samples were excluded from analysis if they exceeded the uppermost values on the serial dilution and standard curves.

The kit was validated for *L. dalli* by assessing parallelism of a serial dilution curve with the standard curve and quantitative recovery. Hormones were obtained and extracted from 48 non-experimental fish (males and females) using a method similar to that described above (collection period of 8 h). The evaporated samples then were re-suspended in 60 µl 0.1 M phosphate buffer and combined into a pool of 2.9 ml. The pool was kept either at 1:1 (for serial dilutions) or diluted 1:16 in EIA buffer aliquoted and frozen; the aliquots were used for dilutions and quantitative recovery.

210 µl of the pooled, 'neat' (1:1) control was used for the serial dilutions. Briefly, 105 µl of this sample was transferred to a 1.5 ml microcentrifuge tube and mixed (by vortexing) with 105 µl of EIA buffer to create a 1:2 dilution; 105 µl of 1:2 dilution was mixed with an equal volume of EIA buffer to create a 1:4 dilution, and so on until 1:64. The serial dilutions were run in duplicate. The log-logit transformed dilution curve was constructed using average %B and pg/ml for the seven samples. The dilution curve was parallel to the standard curve (comparison of slopes: $t_{11} = 0.001$, $P = 0.99$; Zar, 1996, p. 355).

A large (560 µl) sample of the goby pooled control was used for quantitative recovery. 100 µl of this large sample was pipetted into a tube to constitute the 'neat' control. 70 µl of the large sample was then pipetted into 8 additional tubes and mixed with an equal volume of one of the following standards (obtained from the Cayman Chemicals, Inc. KT EIA kit): 0.78, 1.57, 3.13, 6.25, 12.5, 25, 50, 100 pg/ml. Expected recovery concentrations were based on the known amount of KT in the *L. dalli* control sample (e.g., known *L. dalli* concentration + 25 pg/ml divided by 2). Minimum observed recovery was 92.6%. The slope of the observed vs. expected curve was 1.029, indicating a highly linear relationship between observed and expected recovery ($F_{1,7} = 832.4$, $P < 0.0001$, $R^2 = 0.99$).

Statistics

The data were normally distributed, and were analyzed using parametric statistics. For independent samples, analysis of variance (ANOVA) was