

## 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*.

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**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 ( $38.1 \pm 0.5$  SEM) and between 3 and 4 smaller females. Sex was determined by examination of the external genital papilla (Behrens, 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of the pooled, ‘neat’ (1:1) control was used for the serial dilutions. Briefly, 105  $\mu$ l of this sample was transferred to a 1.5 ml microcentrifuge tube and mixed (by vortexing) with 105  $\mu$ l of EIA buffer to create a 1:2 dilution; 105  $\mu$ l 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  $\mu$ l) sample of the goby pooled control was used for quantitative recovery. 100  $\mu$ l of this large sample was pipetted into a tube to constitute the ‘neat’ control. 70  $\mu$ l 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

conducted and, when applicable, post hoc analyses were conducted using Tukey's HSD, which controls for experimentwise error rates. Comparisons were made between females, inexperienced males, and either experienced parenting males or experienced non-parenting males. Paired *t* tests (two tailed) were used to compare individuals sampled twice (experienced males: parenting vs. non-parenting; new males: as female vs. new clutch as male, and female sampled twice, when established male was removed then again when new male had first clutch). Linear regressions were also performed to assess the potential relationship between KT and body size. Logistic regression was performed to assess the influence of both KT concentration and experience on the probability of post-sampling egg consumption. Significance level in all cases was set originally at  $P < 0.05$ . There were three cases in which the same data were used in multiple tests (the two ANOVAs and first paired *t* test described in Results); the *P* values from these analyses were adjusted using the sequential Dunn–Sidak procedure ( $k = 3$ ) to prevent compounding of experimentwise error rates. Adjusted alpha values are reported in Results. Mean values  $\pm 1$  standard error are given. All analyses were carried out using JMP v5.0.1 (SAS Institute Inc.). KT concentrations are represented as pg/sample (= pg/ml \* ml of reconstitution buffer).

## Results

Experienced parenting males showed significantly higher KT concentrations ( $76.51 \pm 6.91$  pg/sample) compared to inexperienced males ( $38.77 \pm 11.77$ ) and females ( $32.83 \pm 12.27$ ) (ANOVA,  $F_{2,46} = 6.41$ ,  $P = 0.0036$ ,  $\alpha_{\text{adj}} = 0.017$ ; post hoc: Tukey's HSD,  $P < 0.05$ ). KT concentrations were not significantly different between experienced non-parenting males, inexperienced males, and females (ANOVA  $F_{2,44} = 2.138$ ,  $P = 0.13$ ;  $\alpha_{\text{adj}} = 0.05$ ). Paired comparisons from individuals sampled twice show that established males ( $n = 23$  pairs) had significantly elevated KT concentrations when parenting compared to when there were no eggs in the nest (Fig. 1; paired *t* test,  $t = 2.64$ ,  $P = 0.015$ ;  $\alpha_{\text{adj}} = 0.025$ ). Individuals who were sampled as females then again as males with their first clutch ( $n = 8$ ) did not show significant differences, but the new males tended to be lower (paired *t* test,  $t = 2.22$ ,  $P = 0.06$ ). Females sampled twice

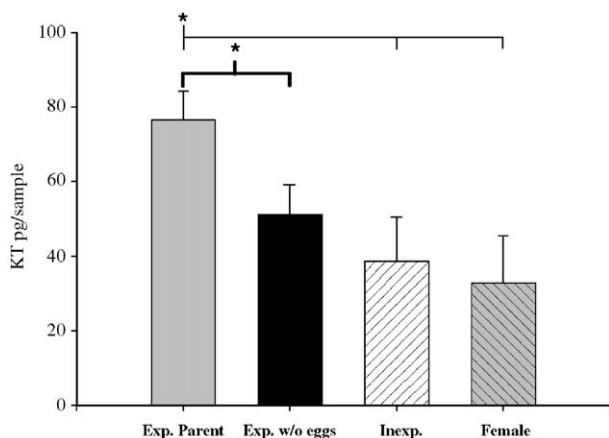


Fig. 1. KT levels of different stages. Thin bars represent significant ANOVA comparisons; the thicker bar represents significant paired *t* test comparisons. Experienced parenting males have significantly higher KT levels than inexperienced males and females. Experienced males without eggs have similar levels of KT to inexperienced males and females. Experienced parenting males ( $n = 23$ ) show significantly higher levels of KT than experienced males without eggs (paired *t* test). Post hoc conducted using Tukey's HSD. Error bars represent standard error.

( $n = 10$ ) showed no differences in KT levels (paired *t* test,  $t = 0.533$ ,  $P = 0.61$ ).

Egg consumption was noted after the "parenting" sampling in 8 of 26 experienced groups and 10 of 11 inexperienced groups. Using a logistic analysis, we examined whether KT concentrations and/or parental experience could predict the probability of egg consumption after sampling. There was not a significant relationship between parenting KT levels and egg consumption (L–R  $\chi^2_1 = 2.61$ ,  $P = 0.11$ ). The relationship between experience and egg consumption was significant, with experienced males being less likely to consume their clutch after sampling (L–R  $\chi^2_1 = 15.07$ ,  $P = 0.0001$ ).

## Discussion

Experienced parenting males had higher KT levels than females or inexperienced parenting males, or those same males when not parenting. We predicted that KT would not decline during parenting, potentially stemming from a male's need to aggressively defend the nest, but we had little reason to predict that KT would increase during parenting in a stable group.

KT levels did not differ between females, inexperienced males (still possessing a large amount of female gonadal tissue), and experienced males (negligible or no female gonadal tissue) that were not parenting. Females who became males had showed no increase in KT, and there was a trend toward a decline in KT. This is somewhat surprising considering the dramatic masculinizing effects of KT on female morphology in this species (Carlisle et al., 2000). However, it has been found that males and females have similar hormone levels in *Gobiodon histrio* (Kroon et al., 2003). Another possible explanation is that KT acts to masculinize the physiology during sex change, but returns to baseline levels once the functional male phenotype has been achieved. While the physiological KT levels recorded in this study are far lower than levels produced by exogenous administration, the role of KT in the expression of sexual phenotype in this species remains unclear.

In *L. dalli*, KT is not incompatible with paternal behavior. In species where a high degree of male parental care is required for successful rearing of offspring, insensitivity to testosterone has been observed (Hunt et al., 1997; Van Duyse et al., 2000; Lynn et al., 2002, 2005). Paternal care is required in *L. dalli* for successful hatching. However, the observed increase in KT while males are actively parenting suggests that KT is positively associated with paternal care in experienced males. While this does not conform to the classic trade-off model of androgens in parenting males, it is consistent with evidence from a number of studies involving fish (Kindler et al., 1991; Ros et al., 2004) and rodents (Trainor and Marler, 2001), where it has been demonstrated that androgens either do not interfere with, or potentially facilitate paternal care (Kramer, 1972; Trainor and Marler, 2002). In cotton-top tamarins, parental experience has differential effects on male testosterone responses to female pregnancy, with more experienced males showing greater elevation of testosterone than less experienced males (Ziegler et al., 2004). Recent work in the bluegill sunfish has shown that

androgens do not interfere with paternal care, and that males in the best condition maintained high androgens throughout the parenting phase (Magee et al., *this issue*).

In many species, territoriality and courting are temporally disassociated from parental care, whereas in *L. dalli* these behavioral suites occur in concert. A positive correlation between androgens and paternal care in species that must simultaneously defend a territory, court females, spawn, and care for offspring has been predicted by Marler et al. (2003), and our findings in *L. dalli* clearly support their prediction.

There are several possibilities for why KT may be elevated during parenting; the first being that KT is positively associated with elevated aggression (Ros et al., 2004) and males may need to raise their level of aggression to adequately defend the nest. While we did not examine rates of aggressive behavior in this study, it is known that *L. dalli* males exclude females, and potentially other males, from the nest regardless of presence of eggs (Rodgers et al., 2005; Black et al., 2005). A male's need to constantly defend the nest should not interfere with his ability to parent appropriately in species that must do both simultaneously. *L. dalli* males continue to court and spawn with females throughout any given brood cycle, and it may be this courting behavior that requires KT to remain high. In many fish species, KT is high when males are courting and during the early stages of parental care (Sikkel, 1993; Knapp et al., 1999; Pall et al., 2002a). It is reasonable to assume that KT may contribute to high levels of courtship behavior or vice versa.

It is not known if KT levels change in *L. dalli* males across clutch development, such as in the plainfin midshipman (Knapp et al., 1999). In our lab, it takes approximately 5 days for the larvae to hatch, whereas it takes up to a month for midshipman eggs to reach the larval stage. Hormonal changes across the parental cycle have yet to be examined in *L. dalli*.

The probability of clutch consumption changes with experience but is not significantly correlated with KT levels. Experienced males tend to be less prone to clutch consumption following sampling disturbance than new males with their first clutch. The relationship, if any, between KT, experience, and clutch consumption is unclear. Experienced males have higher KT when parenting than inexperienced males and consume fewer eggs, but KT does not significantly change consumption probabilities. Other variables are likely to be involved in explaining this phenomenon.

In this study, we examined only levels of KT; we cannot predict the levels of other steroid hormones, such as estradiol or testosterone. Levels of other relevant hormones will be needed to paint a more complete picture of the role of steroid hormones in paternal care, but the present work provides interesting insights into the role of KT in paternal care among male fishes that must simultaneously defend a nest, court females, and parent. Androgen responses and their relationship to parental care have been shown to vary with mating system (Wingfield et al., 1990; Hirschenhauser et al., 2004) and parental necessity (Van Duyse et al., 2000; Lynn et al., 2002, 2005). The hormone profiles in *L. dalli* likely reflect differences in life history in a polygynous species that must continue courtship and territorial defense while engaging in paternal care.

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