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Plasma levels of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*

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Abstract

Like many teleosts, male bluegill sunfish (*Lepomis macrochirus*) provide sole parental care. To understand some of the proximate costs of parental care, we measured body condition and plasma levels of testosterone (T), 11-ketotestosterone (11KT) and cortisol in nesting bluegill males during pre-spawning, spawning and parental care stages. T and 11KT were at their highest mean levels during the pre-spawning period and decreased to lower levels early during the parental care period before rising again when the eggs hatched. Cortisol levels fluctuated across the breeding stages, but there was a noticeable increase from low levels on the day of spawning during the first 2 days of parental care when egg fanning is most intense. Levels of all hormones varied considerably among males, with androgen levels often correlating positively with a male's body condition. We also demonstrate, using a brood reduction experiment and repeated sampling of known individuals, that the presence of eggs affects hormone levels shortly after eggs hatch. Parental males in better body condition had higher levels of androgens during parental care. Males that were known to renest later in the season also had higher androgen levels and were in better body condition during the first nesting bout than males only known to have nested once. However, circulating levels of cortisol did not differ significantly between these groups. We discuss our findings in the context of proximate and ultimate costs of parental care and propose several reasons why elevated androgen levels may not be as incompatible with the expression of paternal care in male teleosts, as compared with avian and mammalian fathers.

Keywords: Testosterone; 11-Ketotestosterone; Cortisol; Reproduction; Parental care; Bluegill; Body condition

Introduction

Many studies investigate parental behavior and emphasize functional or adaptive explanations of why parents provide care to offspring (e.g., Buchan et al., 2003; Dixon et al., 1994; Neff and Gross, 2001). In parallel to this work, endocrinology is increasingly being used as a tool for understanding proximate mechanisms underlying parental behavior. Most of this work has focused on androgens because of their role in mediating aggressive and courtship behavior (see Wingfield et al., 1990) and the potentially detrimental effects of elevated androgen levels on other systems critical for survival, such as immune function (Folstad and Karter, 1992; Wingfield et al., 2001; Oliveira, 2004). Research has generally supported the hypothesis that decreased androgen levels during periods of paternal care reflect the general incompatibility of androgens and the expression of paternal behavior (because high circulating levels of testosterone (T) facilitate the expression of male–male aggression in the contexts of territory establishment and courtship). Most support for this "challenge hypothesis" has come from studies of birds (e.g., Wingfield et al., 2001; but see Hirshenhauser et al., 2003), but additional support has come from other taxa including mammals (e.g., Reburn and Wynne-Edwards, 1999) and teleosts (Páll et al., 2002; Pankhurst, 1990; Sikkel, 1993; Specker and Kishida, 2000). However, conflicting results from teleosts (e.g., Hirschenhauser et al., 2004; Knapp et al., 1999) suggest that androgens may not be responsible for

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regulating parental care. Rather, androgens may decrease during the care period due to other factors such as termination of physiological processes required for the production of sperm or associated secretions (e.g., Páll et al., 2005). In addition, high levels of the major teleost androgen 11-ketotestosterone (11KT) are being found not to inhibit (Ros et al., 2004) or correlate with (Rodgers et al., 2006) reduced parental behavior. Thus, the role of androgens in mediating paternal behavior in teleosts is still unclear.

Glucocorticoids (GC) are also likely important in regulating reproductive behavior because they play a central role in the vertebrate stress response (Romero, 2002). However, little work has focused on the role of GCs during parental behavior even though these hormones play a central role in mobilizing the energy reserves that support energetically expensive behavior such as nest defense (Nelson, 2000) or that are needed during periods of food shortage (Romero and Wikelski, 2001). Parents commonly face stressful physiological conditions due to reduced or halted foraging or an increased level of agonistic behaviors associated with male–male competition or defense of immobile young. Thus, increased GC levels may be useful as an indicator of the level of energetic stress; i.e., a proximate cost that parents experience during the care period. Yet despite many studies of the general effects of stress on reproduction, few have attempted to correlate GC levels with specific paternal behaviors, and there is currently no consistent pattern of GC changes with parental care (Heath et al., 2003; Knapp et al., 1999; Kotrschal et al., 1998).

The present study investigated endocrine correlates of parental care in bluegill sunfish (*Lepomis macrochirus*) and begins to assess the magnitude of costs associated with parental care. Parental male bluegill provide all care for the offspring, the care is energetically expensive both with respect to behaviors displayed and because males do not forage during this period, and males may breed multiple times during a single breeding season. Males do not court females while providing parental care. Kindler et al. (1989) found that, as in many bird species, mean plasma T and 11KT levels gradually increased during the pre-spawning stage (during nest construction), peaked on the day of spawning (courtship) and then gradually declined throughout the parental care stage. There are currently no published data on cortisol, the primary GC in teleosts, in relation to parental care in bluegill. In the present study, we first describe steroid hormone profiles during the pre-spawning, spawning and parental care periods. Second, we test the hypothesis that offspring number influences a parental male's hormone levels, body condition and probability of renesting later in the season. We did this by repeatedly sampling known individuals over the care period, experimentally manipulating brood size, and by preventing some males from abandoning their nests even in the face of reduced brood size. We predicted that parental males caring for full broods would have decreased androgen levels, increased cortisol levels and greater weight loss over the period of parental care. We also predicted that these proximate costs would translate into ultimate costs: males that invested more in their first brood of the season would have lower future reproductive success (i.e., renesting potential). Our results support some, but not all, of these predictions.

Methods

Animals and study site

Bluegill sunfish are endemic to North America with populations ranging from Northern Mexico to Southern Quebec and Ontario, Canada (Scott and Crossman, 1973). Our study population is found along the northern shore of Lake Opinicon, Ontario (44°34′N, 76°19′W), a shallow lake that measures approximately 900 ha, which is adjacent to the Queen's University Biological Station (Gross and Nowell, 1980). In this lake, parental males nest in colonies and spawn synchronously in what are termed "bouts" during the May–July breeding season. A bout begins when colonies form within the littoral zone of the lake and ends when the last of the fry become free swimming and leave the nest. Colonies contain anywhere from a few nests to several hundred nests, and competition occurs among parental males for preferred nest sites within the center of the colony (Cargnelli, 1995; Gross and MacMillan, 1981). Gravid females approach a colony as a group and deposit eggs in the nests of selected parental males. Females may spawn with more than one parental male, and their eggs are sometimes fertilized by cuckolders, an alternative male phenotype (about one quarter of all eggs are fertilized by cuckolders; Neff, 2001). Spawning at any given colony typically lasts a single day, and once completed, only parental males remain to provide care for the offspring. The care period lasts from 7 to 10 days and has two distinct phases (egg phase and fry phase). The egg phase lasts approximately 3 days, and parental care involves defense of the brood and fanning of eggs to maintain oxygen levels around the developing young. After the eggs hatch, the fry are guarded until they leave the nest some 4–7 days later. Parental males rarely leave the nest area and do not actively forage during the care period, losing an average of 11% of their body mass during this time (Coleman and Fischer, 1991). Once the fry leave the nest, parental males return to deeper water to forage and replenish their energy reserves before potentially nesting again.

Breeding hormone profiles

The 2004 breeding season began on 2 June. The formation of each new colony was identified by swimmers, and all spawning activity was noted. On 5 June, a large colony was identified so that a subset of about 10 males could be sampled each day over the entire parental care period. Two days pre-spawning, the day of spawning and on each subsequent day throughout the nesting period, between 5 and 11 different parental males were captured one at a time using a dip net and quickly brought to a nearby boat where approximately 500 μl of blood were immediately collected from the caudal peduncle using a heparinized syringe. All blood samples were collected between 1200 and 1900 h EST and were kept on ice until they were returned to the laboratory (within 2 h), where they were centrifuged to separate the blood plasma. The plasma was then stored at −20°C until the hormone assays were conducted. After blood sampling, males were weighed using a portable electronic balance (to the nearest gram) and measured for total length (nearest millimeter). Fulton's condition factor was calculated from mass/length^3 × 10^5. This factor is an estimate of each individual's energetic state and is positively correlated with stores of non-polar lipids, the main energy source used during periods of starvation (Neff and Cargnelli, 2004; Sutton et al., 2000). Following Claussen (1991), egg scores were assigned to each parental male's nest as a rank between 1 and 5. The scores are highly correlated with the actual number of eggs: score 1, 1–4900 eggs; score 2, 4600–29,000; score 3, 27,000–53,000; score 4, 49,000–87,000; and score 5, 82,000–113,000 (Claussen, 1991). After measurements were taken and before the male was returned to the water, a 2-cm^2 portion of a male's upper caudal fin was removed to prevent resampling males on subsequent days. Fin clipping in this location does not affect the male's ability to fan the eggs.

Egg manipulation study

From 6 June to 17 June, 5 colonies were identified for the egg manipulation study, whose purpose was to investigate some of the proximate costs of male

parental care. Parental males were haphazardly assigned to one of four treatment groups (see below), and their nests were individually marked with a numbered tile placed at the nest edge. On the first day after spawning ("egg" stage), the study began by first collecting a parental male using a dip net and taking him to a nearby boat where 200 μl of blood was collected as described above. A PIT tag (12 mm × 2.1 mm, 132.4 kHz ISO, Biomark, Boise, ID) was then inserted into his body cavity ventrally at the base of the pectoral fins using a syringe. The tags were used to track these males through the remainder of the breeding season to assess renesting behavior. Parental males were weighed, measured (total body length), assigned an egg score and nest location (central or peripheral), and a 2-cm^2 portion of their upper caudal fin was removed to facilitate subsequent visual identification of the males.

Egg and cage manipulations were performed, while males were being processed on the boat. Four treatment groups comprised the experimental design:

Caged egg reduction—a cage was placed over the nest and parental male, and we removed about three-quarters of the eggs;
Uncaged egg reduction—no cage was used, and we removed about three-quarters of the eggs;
Caged control—a cage was placed over the nest and parental male, but no eggs were removed;
Uncaged control—no cage was used, and no eggs were removed.

The cages were plastic, mesh laundry baskets that were cylindrical in shape, white in color and measured 45 cm in diameter by 54 cm in height. The mesh size measured 3 cm × 3.5 cm and allowed active exchange of water while preventing the parental male from abandoning his nest. All removed eggs were disposed of away from the colony. Eggs in control nests were not handled to avoid any potential effect of handling on the parental male's subsequent behavior. Following handling and egg and cage manipulation, parental males were returned to their nest where they usually immediately returned to active care.

All males remaining on their nest were recollected 1 day post-egg hatching (3 or 4 days post-spawning; "hatch") and again at the end of the care period prior to fry swim-up (golden-eye fry stage, 6 or 7 days post-spawning; "fry"). On these days, each male's body mass was measured, and another 200 μl blood sample was collected before returning the male to his nest. Weight loss for each male was calculated as the difference between the first and last mass measurements. Caged males were released from their cages on the day that uncaged males from that colony left their nests after fry swim-up.

After the initial spawning bout, tagged parental males were tracked to subsequent spawning attempts within the study area. Renesting males were collected 1 day post-spawning (egg stage) and again on the final day of nesting before fry swim-up (fry stage). Each time, 200 μl of blood was collected, and the renesting male's body mass was recorded as described above before returning the male to his nest. All blood samples were processed as described above and were collected between 1000 and 1800 h.

Hormone assays

Plasma levels of T, 11KT, estradiol and cortisol were determined using radioimmunoassay (RIA) following chromatographic separation as described in Sisneros et al. (2004). Briefly, approximately 2000 cpm of tritiated steroid was added to each sample for subsequent correction for losses during the ether extraction and chromatography. After overnight incubation at 4°C, samples were extracted using two 2 ml diethyl ether washes and resuspended in 10% ethyl acetate in iso-octane. Samples were then applied to diatomaceous earth-glycol columns. The steroid hormones were separated from one another via step-wise elution by means of increasing levels of ethyl acetate in iso-octane (T: 10% ethyl acetate in iso-octane; estradiol: 20%; 11KT: 30%; cortisol: 52%). Each hormone fraction was dried under nitrogen and then resuspended overnight in phosphate-buffered saline containing 0.1% gelatin. Each sample was assayed in duplicate, and a charcoal-dextran solution was used to separate unbound steroid from steroid bound to the antibody. A T antibody (Wien T-3003) from Research Diagnostics (Flanders, NJ) was used to assay both androgens because it has a high level of cross-reactivity for 11KT. The cortisol antibody (F3–314) was purchased from Esoterix Endocrinology (Calabasas Hills, CA). The estradiol antibody (7010–2650) came from Biogenesis (Kingston, NH).

Plasma samples from the breeding hormone profiles were assayed in two batches, and samples from the manipulation study were assayed in four batches. For the breeding hormone profiles, samples from different days were divided between the two batches. For the manipulation study, all plasma samples from a given male were run together in the same batch to avoid introducing inter-assay variation into the repeated measures analyses. Samples from the four treatment groups were distributed across the four batches as evenly as possible. Estradiol was found to be non-detectable in the parental males in the first batch of samples and therefore was not assayed in the remaining samples. Intra-assay coefficients of variation as calculated from 2–4 standard tubes per assay for T ranged from 4.1 to 8.3%, for 11KT from 9.3 to 16.6% and for cortisol from 6.2 to 11.2%. Inter-assay coefficients of variation were 11.5%, 14.9% and 14.0% for T, 11KT and cortisol, respectively.

Statistical analysis

Any blood sample that took more than 200 s to obtain from the point of capture of the parental male was a priori discarded in order to avoid including any hormone levels that may have been elevated due to handling stress ($n = 6$ for breeding hormone profiles, $n = 3$ for egg manipulation study). Before analysis, data were examined to make sure they fit all test assumptions. In cases where assumptions of distributions were not met, the appropriate transformations were conducted (Quinn and Keough, 2002). Testosterone was square-root transformed, whereas 11KT and cortisol were cube-root transformed. Although some analyses were run on transformed data, all figures and tables present the untransformed data as means ±1 standard error.

Breeding hormone profiles were analyzed using analysis of covariance with body length, Fulton's condition factor and egg score as covariates. We did not use body mass as a covariate because body length and mass are highly correlated, and the residual body mass is captured in the Fulton's index (Neff and Cargnelli, 2004). Covariates were removed from the final models when they were non-significant and not part of a significant interaction. For the manipulation study, all caged and uncaged males were initially analyzed together in a fully factorial repeated measures analysis of variance (RMANOVA) model. However, being caged had a significant effect on T ($F_{1,50} = 15.6$, $P < 0.001$), 11KT ($F_{1,50} = 13.0$, $P < 0.001$) and cortisol ($F_{1,50} = 6.7$, $P < 0.013$) levels. Therefore, we report analyses of caged and uncaged males separately to more clearly highlight the effect of egg manipulation on hormone levels. Initially, stepwise analyses of T, 11KT and cortisol levels were conducted with three covariates: body length, Fulton's condition factor and egg score. As above, non-significant covariates were removed from the final models. For all ANOVAs, any significant interactions were further investigated using simple linear regressions.

For renesting males, comparisons of hormone levels between corresponding stages of care from the first and second nesting bouts were made using paired t tests. Hormone levels at each stage of the first nesting bout were compared between males that only had one nesting bout and males that nested twice within the study area using independent t tests. RMANOVA tests were run using the statistical program JMP (v. 4; SAS Institute, Cary, NC). All other analyses were run using SPSS (v. 12.0; SPSS Inc., Chicago, IL).

Results

Breeding hormone profiles

Parental males were sampled from 2 days pre-spawning (males already have built nests) to the last day fry were in the nest (day 7). There was a significant difference in handling time to collect a blood sample among days ($F_{8,71} = 3.52$, $P = 0.002$). A Tukey's post hoc test revealed the difference to lie between days 0 and 6, 0 and 7, and 4 and 6. Handling time was longest on day 0 and shortest on days 6 and 7. However, a simple linear regression

revealed no significant effect of handling time on T ($R^2 = 0.011$; $t_{77} = 0.93$, $P = 0.36$), 11KT ($R^2 = 0.010$; $t_{60} = 0.80$, $P = 0.43$) or cortisol ($R^2 = 0.008$; $t_{77} = 0.78$, $P = 0.44$) levels. Estradiol levels were non-detectable in the first batch of samples run and so were not assayed in subsequent batches; thus, estradiol data were not subjected to any statistical analyses. There was no significant effect of sampling order within a day on T ($R^2 = 0.002$; $t_{77} = 0.39$, $P = 0.70$), 11KT ($R^2 = 0.021$; $t_{61} = 1.14$, $P = 0.26$) or cortisol ($R^2 = 0.000$; $t_{77} = 0.001$, $P = 0.999$) levels. Thus, handling time and order were not included in subsequent analyses. Among sampling days, there was no significant difference in male body length ($F_{8,72} = 0.83$, $P = 0.58$), body mass ($F_{8,72} = 0.34$, $P = 0.95$) or body condition ($F_{8,72} = 1.44$, $P = 0.20$).

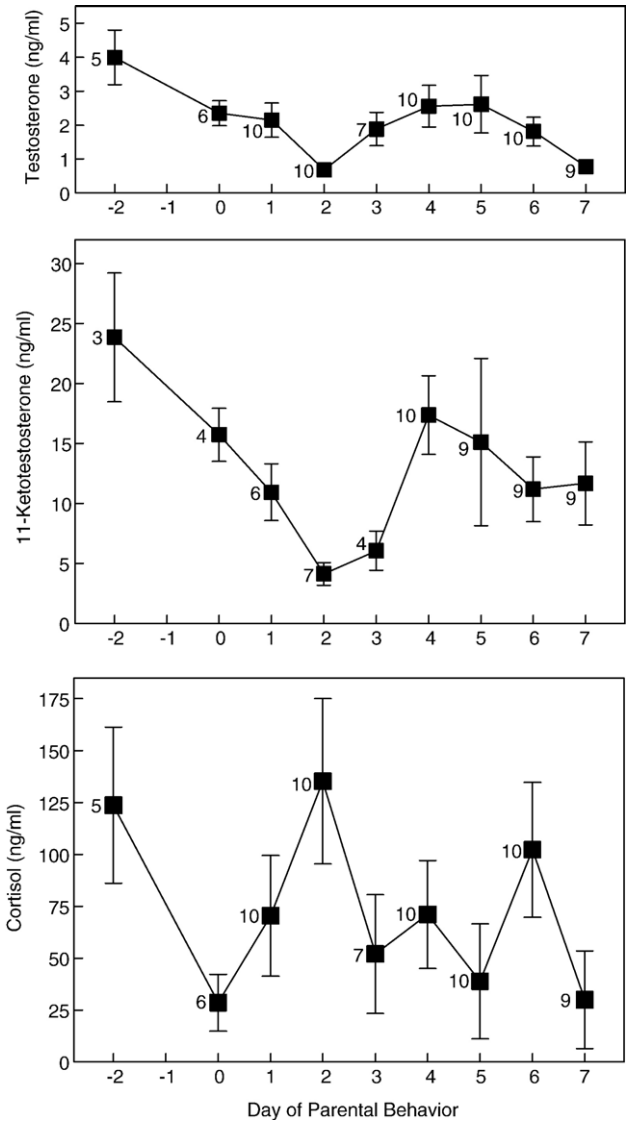

Fig. 1. Changes in mean (±SEM) breeding hormone profiles of parental male bluegill (*Lepomis macrochirus*) across the nesting cycle from 2 days before spawning to 7 days post-spawning. The zero denotes the day of spawning and eggs hatched on days 3–4. Numbers next to bars denote sample sizes. Lower sample sizes for 11-ketotestosterone are due to an assay failure and inability to reassay all samples.

Table 1
Summary of analysis of covariance results for plasma levels of testosterone, 11-ketotestosterone (11KT) and cortisol in sub-samples of parental male bluegill (*Lepomis macrochirus*) sampled across the nesting cycle from 2 days before spawning to 7 days post-spawning

Hormone	Variable	*df*	*F*	*P*
Testosterone	Day of nesting cycle	8,62	2.78	0.011
	Fulton's condition factor (covariate)	8,62	3.16	0.080
	Day of cycle × Fulton's condition factor	8,62	2.85	0.009
11KT[a]	Day of nesting cycle	8,53	1.95	0.072
	Fulton's condition factor (covariate)	8,53	5.87	0.019
Cortisol	Day of nesting cycle	8,71	1.67	0.120

[a] 11KT levels were not obtained for all males due to small blood plasma samples for some males which precluded reassay when an assay failed.

Both T and 11KT were at their highest mean levels before spawning and steadily decreased to their lowest mean levels 2 days after spawning, with a second smaller peak at 4 to 5 days after spawning (Fig. 1). There was a significant difference in mean T levels among days of the nesting cycle, a trend for a main effect of body condition and a significant interaction between day of the nesting cycle and body condition (Table 1). There was a significant positive relationship between body condition and T concentration on day 5 ($R^2 = 0.593$; $t_9 = 3.42$, $P = 0.009$) and a positive trend between these two variables on day 6 ($R^2 = 0.360$; $t_9 = 2.12$, $P = 0.067$).

11KT showed a similar pattern to T. There was a trend for a difference in mean 11KT concentration among days of the nesting cycle and a significant main effect of body condition (Table 1). A positive relationship between body condition and 11KT concentration was found on day 1 ($R^2 = 0.872$; $t_5 = 5.22$, $P = 0.006$) and on day 6 ($R^2 = 0.474$; $t_8 = 2.51$, $P = 0.040$), and a trend for a negative relationship was found on day 4, the first day with fry in the nest ($R^2 = 0.318$; $t_9 = 1.93$, $P = 0.090$). Across all days of the nesting cycle, levels of T and 11KT were positively correlated ($r = 0.737$; $P < 0.001$). Although mean 11KT levels generally declined after spawning, individual males exhibited androgen levels similar to those of spawning or pre-spawning males on all days except days 2 and 3 during the period of parental care.

Mean cortisol levels were highly variable across days and males, and thus, there was no significant effect of the day of the nesting cycle on cortisol levels (Fig. 1; Table 1). Mean cortisol concentration was high 2 days before spawning and then dropped to a low on the day of spawning before increasing to a second peak 2 days after spawning. Cortisol levels then declined again and continued to fluctuate until the end of the parental care period (Fig. 1). Across all days of the nesting cycle, cortisol was not correlated with T or 11KT ($P > 0.22$ for both) or with body condition ($P = 0.15$).

Egg manipulation study

Descriptive statistics

On the day of nest manipulation, treatment groups did not differ in mean nest location, body length, initial body mass,

Fulton's condition factor, handling time to collect the blood sample or plasma levels of T, 11KT or cortisol (all $P > 0.2$). Treatment groups did differ in egg score ($F_{3,86} = 1.63$, $P = 0.001$), with *Caged controls* having significantly more eggs in their nests. There was no significant relationship between body condition and egg score ($R^2 = 0.021$, $P = 0.29$). There was a significant difference in handling time until blood sample collection among the 3 stages of the nesting cycle for caged and uncaged males combined ($F_{2,111} = 5.06$, $P = 0.008$). Handling times were longest for the earliest samples compared to later samples. However, there was no significant difference in handling time across treatment groups ($F_{3,86} = 0.37$, $P = 0.78$), and simple linear regressions for all treatments combined revealed no effect of handling time on plasma levels of T ($R^2 = 0.004$; $t_{208} = 0.92$, $P = 0.36$), 11KT ($R^2 = 0.011$; $t_{208} = 1.49$, $P = 0.14$) or cortisol ($R^2 = 0.007$; $t_{208} = 1.20$, $P = 0.23$). The mean change in male body mass from the start to the end of the nesting bout differed significantly among treatment groups ($F_{3,50} = 8.41$, $P < 0.001$) with the significant differences being between caged and uncaged groups (*Uncaged control* (mean ± SEM): −2.7 ± 1.1 g; *Uncaged egg reduction*: −3.9 ± 1.2 g; *Caged control*: −7.8 ± 0.6 g; *Caged egg reduction*: −9.4 ± 1.2 g; see Fig. 2).

Of the 35 males that initially made up the *Uncaged control* group, 13 (37.1%) abandoned their nests before the end of the first nesting bout. Of the 26 males that initially made up the *Uncaged egg reduction* group, 19 (73.1%) abandoned their nests before the end of the first nesting bout. These two values suggest higher abandonment in the latter group ($\chi^2 = 3.67$,

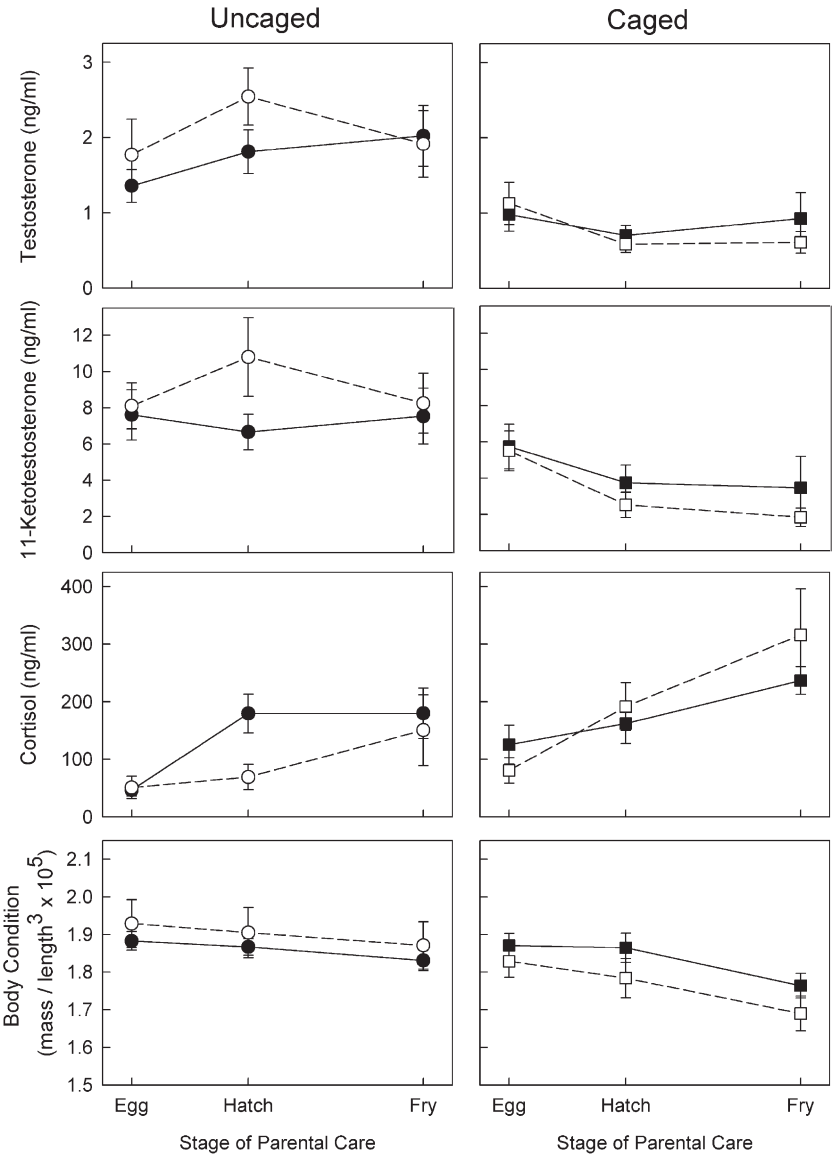

Fig. 2. Changes in levels (mean ± SEM) of androgens, cortisol and body condition for parental male bluegill (*Lepomis macrochirus*) sampled across three stages of the nesting cycle; (1) eggs, 1 day post-spawning; (2) hatch, 1 day post-egg hatching; and (3) fry, 1 day before fry swim-up. *Control* males are denoted by filled circles and *Egg reduction* males are denoted by open circles. Sample sizes: *Uncaged controls* = 22 (21 for body condition at hatch), *Uncaged egg reduction* = 7, *Caged controls* = 14, *Uncaged controls* = 11.

df = 1, *P* = 0.055). None of the caged males were able to abandon their nest.

Uncaged males

Because some males abandoned their nests before the end of the nesting bout, we included data from only those males for whom we had all three plasma samples for overall comparisons (RMANOVA) between *Control* and *Egg reduction* groups. Mean T levels did not differ significantly among males across the nesting cycle and there was no effect of egg treatment (*Uncaged control* versus *Uncaged egg reduction*; Fig. 2; Table 2). There was no significant interaction between nesting stage and egg treatment. However, pair-wise tests between egg treatments for each of the 3 days sampled revealed a trend for T levels to be higher at the hatch stage in *Egg reduction* males (t_{22} = 1.76, *P* = 0.092; Fig. 2). There was a significant positive relationship between Fulton's condition factor and T levels (R^2 = 0.105; t_{126} = 14.60; *P* < 0.001; Table 2).

The general pattern of 11KT levels mimicked that of T (Fig. 2). Mean 11KT levels did not differ significantly among nesting stages or between egg treatment groups (Table 2). There was no significant interaction between nesting stage and egg treatment. However, pair-wise tests between egg treatments for each day of the nesting cycle revealed that *Egg reduction* males had significantly higher 11KT levels than *Control* males at the hatch stage (t_{19} = 2.19, *P* = 0.041). There was a significant positive relationship between Fulton's condition factor and 11KT levels (R^2 = 0.095; t_{126} = 13.10; *P* < 0.001; Table 2).

Cortisol levels differed significantly among nesting stages (Fig. 2; Table 2). There also was a significant effect of body length on cortisol levels and a significant interaction between length and the repeated measure (Table 2). Specifically, across all nesting stages, there was a negative relationship between cortisol levels and body length (R^2 = 0.035; t_{126} = −2.15; *P* = 0.033). The interaction was driven by a significant negative relationship between body length and cortisol levels at the fry stage (R^2 = 0.159; t_{28} = −2.26; *P* = 0.032) and at the hatch stage (R^2 = 0.101; t_{37} = −2.01; *P* = 0.052). There was no relationship between body length and cortisol levels at the egg stage (R^2 = 0.001; t_{60} = 0.25; *P* = 0.81). Mean cortisol levels did not differ significantly between egg treatments, and there was no significant interaction between egg treatment and nesting stage. However, pair-wise tests between egg treatments for each day of the nesting cycle found that *Control* males had significantly higher mean cortisol levels at the hatch stage than did *Egg reduction* males (t_{27} = 2.65, *P* = 0.013; Fig. 2). There was no correlation between cortisol and T or 11KT levels within a given treatment group and stage of parental care (all *P* > 0.50).

We also investigated whether males that abandoned the nest at different stages differed in various characteristics at the egg and hatch phases (Table 3). There was no difference among *Uncaged control* males that abandoned or stayed in nest location, egg score, body length or Fulton's condition factor at both the egg and hatch stages. Similarly, there was no difference between males that abandoned or stayed in initial (egg stage) T or 11KT levels. However, mean levels for both androgens at the hatch stage were higher in males that completed the nesting bout than in males that abandoned after the eggs hatched. In contrast to androgen levels at the egg stage, mean cortisol levels did differ significantly among *Uncaged control* males based on when they abandoned the nest. Parental males that abandoned after the egg or hatch stages had significantly higher cortisol levels at the egg stage than those males that stayed through the fry stage and thus completed the nesting bout (Tukey's post hoc test: *P* < 0.02 for both). However, there was no difference in mean cortisol levels at the hatch stage for males that abandoned after hatching compared to males that completed the nesting bout.

Among *Uncaged egg reduction* males, there was no significant difference in body length or Fulton's condition factor at the egg and hatch stages (Table 3). However, egg score prior to manipulation differed significantly among males that abandoned during different stages of the nesting cycle; parental males that abandoned after the egg or hatch stages had lower initial egg scores than those males that completed the nesting bout (Tukey's post hoc test: *P* < 0.001 for both). There also was a significant difference in nest location among males that abandoned during different stages of the nesting cycle. Parental males that abandoned after the egg stage were found more often in peripheral nests than those males that stayed (Tukey's post hoc test: *P* = 0.016). There was no difference among males that abandoned or stayed in initial (egg stage) levels of T, 11KT or

Table 2
Summary of repeated measures ANOVAs for plasma steroid hormone levels in parental male bluegill (*Lepomis macrochirus*) sampled at three points during the nesting cycle

Cage treatment	Variable [a]	Testosterone			11-Ketotestosterone			Cortisol		
		df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Uncaged	Nesting stage (repeated measure)	2,52	0.50	0.11	2,52	0.42	0.66	2,51	5.49	0.007
	Treatment (*Control* vs. *Egg reduction*)	1,27	0.75	0.39	1,27	0.93	0.34	1,27	0.94	0.34
	Nesting stage × Treatment	2,52	0.50	0.61	2,52	1.02	0.37	2,51	0.79	0.46
	Covariate [b]	1,52	7.71	0.008	1,52	8.91	0.004	1,51	5.22	0.027
	Nesting stage × Covariate [b]	–	–	–	–	–	–	2,51	3.11	0.053
Caged	Nesting stage (repeated measure)	2,48	8.03	0.001	2,46	17.97	<0.001	2,48	7.49	0.002
	Treatment (*Control* vs. *Egg reduction*)	1,27	0.17	0.69	1,27	0.36	0.56	1,27	0.01	0.92
	Nesting stage × Treatment	2,48	0.42	0.66	2,46	2.19	0.12	2,48	0.73	0.49

Analyses were performed on males nesting during their first breeding bout and for whom hormone samples were available for all three time points.
[a] Only significant covariates were included in the final models (see text).
[b] The covariate for androgens is Fulton's condition factor; for cortisol, the covariate is body length.

Table 3
Summary of multivariate analysis of variance results for *Uncaged* parental male bluegill (*Lepomis macrochirus*) based on whether males abandoned at the egg stage, abandoned at the hatch stage, or completed the nesting bout

Variable	Abandoned at egg stage	Abandoned at hatch stage	Completed nesting bout	*df*	*F*	*P*
Uncaged control						
Sample size	7	6	22			
Nest location[a]	1.1 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	2,32	0.41	0.67
Egg score (prior to manipulation)	1.7 ± 0.6	1.2 ± 0.2	1.6 ± 0.2	2,32	0.71	0.50
Body length (mm)	190.4 ± 3.3	192.0 ± 2.0	189.7 ± 2.1	2,32	0.16	0.85
Fulton's condition factor ($g/mm^3 \times 10^5$), egg stage	1.93 ± 0.10	1.90 ± 0.08	1.88 ± 0.03	2,32	0.21	0.81
Fulton's condition factor ($g/mm^3 \times 10^5$), hatch stage	–	1.83 ± 0.09	1.87 ± 0.02	1,25	0.39	0.54
Testosterone (ng/ml), egg stage	1.2 ± 0.3	0.6 ± 0.1	1.4 ± 0.2	2,32	1.44	0.25
Testosterone (ng/ml), hatch stage	–	0.7 ± 0.4	1.8 ± 0.3	1,26	5.49	0.027
11-Ketotestosterone (ng/ml), egg stage	8.3 ± 2.9	4.2 ± 1.5	7.6 ± 1.4	2,32	0.84	0.44
11-Ketotestosterone (ng/ml), hatch stage	–	3.9 ± 2.9	6.7 ± 1.0	1,26	4.42	0.045
Cortisol (ng/ml), egg stage	145.1 ± 34.1	120.7 ± 16.3	46.9 ± 10.5	2,32	6.90	0.003
Cortisol (ng/ml), hatch stage	–	213.2 ± 62.1	179.7 ± 33.7	1,26	0.43	0.52
Uncaged egg reduction						
Sample size	16	3	7			
Nest location[a]	1.8 ± 0.1	1.3 ± 0.3	1.1 ± 0.1	2,23	4.93	0.017
Egg score (prior to manipulation)	1.0 ± 0.1	1.0 ± 0.0	2.1 ± 0.1	2,23	35.10	<0.001
Body length (mm)	192.5 ± 2.3	190.3 ± 2.4	190.9 ± 1.7	2,23	0.16	0.85
Fulton's condition factor ($g/mm^3 \times 10^5$), egg stage	1.84 ± 0.04	1.77 ± 0.03	1.91 ± 0.06	2,23	1.41	0.27
Fulton's condition factor ($g/mm^3 \times 10^5$), hatch stage	–	1.79 ± 0.04	1.90 ± 0.07	1,8	1.07	0.33
Testosterone (ng/ml), egg stage	1.7 ± 0.4	0.7 ± 0.1	1.8 ± 0.5	2,23	0.74	0.49
Testosterone (ng/ml), hatch stage	–	1.1 ± 0.4	2.5 ± 0.4	1,8	8.06	0.022
11-Ketotestosterone (ng/ml), egg stage	6.8 ± 1.5	5.5 ± 1.9	8.1 ± 1.3	2,23	0.56	0.58
11-Ketotestosterone (ng/ml), hatch stage	–	8.3 ± 4.8	10.8 ± 2.2	1,8	0.91	0.37
Cortisol (ng/ml), egg stage	144.7 ± 45.0	80.3 ± 51.4	51.0 ± 19.5	2,23	0.86	0.44
Cortisol (ng/ml), hatch stage	–	62.7 ± 22.0	69.2 ± 22.1	1,8	0.01	0.91

Data for the fry stage are presented in Fig. 2.
[a] Nest locations within a colony were either central, scored as 1, or peripheral, scored as 2.

cortisol, or in mean 11KT or cortisol levels at the hatch stage. However, mean T concentration at the hatch stage was higher for males that went on to complete the nesting bout than for males that abandoned the nest after the eggs hatched.

Caged males

The results for this group differed in some important ways from the *Uncaged* males. Mean T levels did not differ between males from the different egg treatments, and there was no significant interaction between nesting stage and egg treatment (Fig. 2, Table 2). However, there was a significant main effect of nesting stage on T levels. *Caged control* and *Caged egg reduction* males had overlapping T levels at the egg, hatch and fry stages, with the highest levels occurring at the egg stage ($P = 0.001$ for both treatments; Fig. 2).

Mean 11KT levels also differed significantly across the nesting stages (Table 2). Mean 11KT levels were significantly lower at the hatch and fry stages compared to the egg stage (both $P < 0.03$; Fig. 2). There was no significant difference in 11KT levels between egg treatments and no interaction between egg treatment and nesting stage (Table 2).

Mean cortisol levels differed among *Caged* males at the different nesting stages (Table 2). Cortisol levels were lowest at the egg stage and increased in each subsequent stage of the nesting cycle (all $P < 0.05$; Fig. 2). There was no main effect of egg treatment and no interaction between nesting stage and egg treatment on cortisol levels (Table 2). The elevated levels of cortisol in the hatch and fry stages likely account for the lower androgen levels at these stages, especially when compared to the *Uncaged* males.

Renesting males

We were able locate a total of 8 fish that renested: 4 *Uncaged control* males (11.4% of the initial sample) and 4 *Uncaged egg reduction* males (15.4% of the initial sample). Six of the 8 males renested in the same colony site as their first nesting bout.

Despite the relatively small number of renesters, some interesting patterns of hormone levels and body condition emerge across the two nesting bouts (Fig. 3). Testosterone levels of these males were significantly higher during the egg stage of their first nesting bout compared to the egg stage of their second nesting bout ($t_5 = 4.94$, $P = 0.004$), but there was no difference between bouts at the fry stage ($t_2 = 2.10$, $P = 0.17$). Similarly, 11KT levels were significantly higher during the egg stage of the first nesting bout compared to the egg stage of the second bout ($t_5 = 7.60$, $P = 0.001$; Fig. 3) but again did not differ during the fry stage of the two bouts ($t_2 = 2.47$, $P = 0.13$). Cortisol levels did not differ significantly at the egg or fry stage of the two nesting bouts ($P > 0.15$ for both). Fulton's condition factor was significantly higher during the egg stage of the first nesting bout compared to the egg stage of the second bout ($t_6 = 3.86$, $P = 0.008$; Fig. 3) but did not differ during the fry stage of the two bouts ($t_2 = 2.35$, $P = 0.84$). We did not sample males during the hatch stage of the second bout.

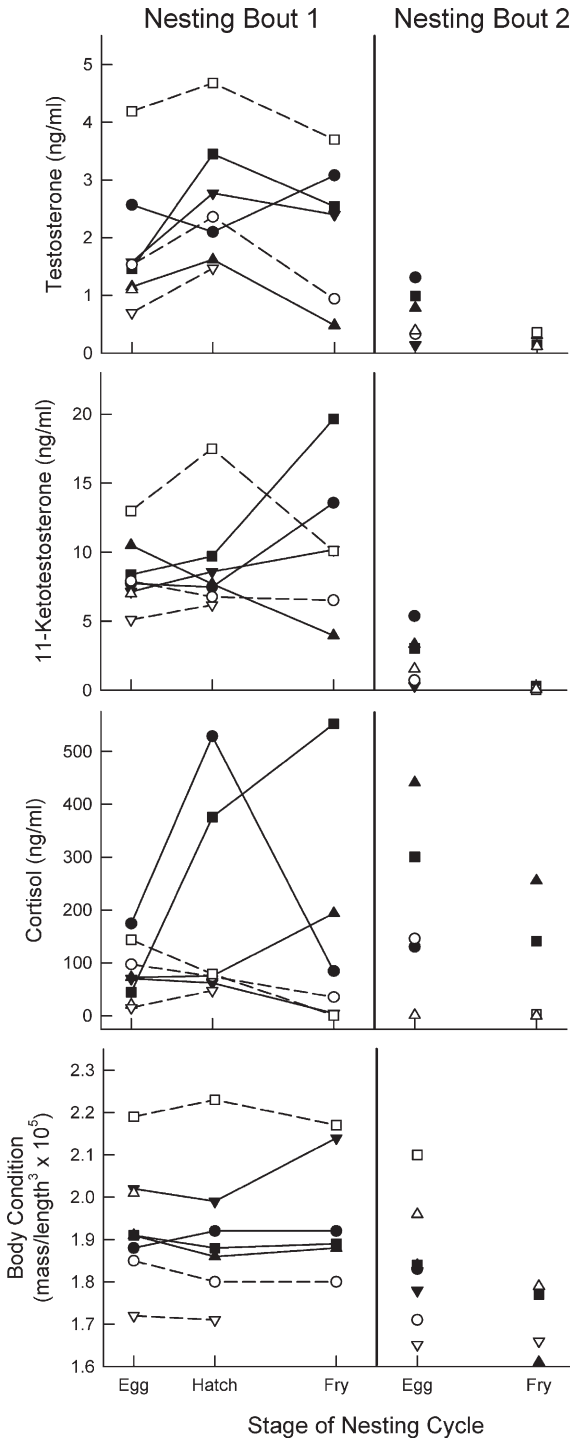

Fig. 3. Plasma androgen levels, cortisol levels and body condition for renesting *Uncaged* parental male bluegill sampled across three stages (egg, hatch and fry) and both nesting bouts. Each symbol represents a different renesting parental male. Solid symbols and lines indicate males from the *Control* treatment ($n = 4$). Open symbols and dashed lines indicate males from the *Egg reduction* treatment ($n = 4$). Some individual data points are missing for some males. Males were not sampled on the day after eggs hatched for nesting bout 2. Mean values for these males for nesting bout 1 are shown in Fig. 2. Notice the difference in androgen levels at the egg stage between the two bouts.

We also compared various measures across the first nesting bout for males known to have nested only once versus males known to have nested a second time (Fig. 4). There were no significant differences in nest location, egg score or body length between these two groups of males (all $P > 0.38$). However, T levels were significantly higher for renesters than for one-time nesters at the hatch stage ($P = 0.003$) but not at the egg ($P = 0.12$) or fry ($P = 0.43$) stage. 11KT levels were significantly higher for renesters than for one-time nesters at all three nesting stages (egg: $P = 0.014$, hatch: $P = 0.014$, fry: $P = 0.033$). In contrast to androgen levels, cortisol levels did not differ between these groups of males at any of the three sampling times (all $P > 0.32$). Finally, males that nested twice were also in significantly better body condition when sampled at the fry stage ($P = 0.007$) than males known to have nested only once. However, there was no difference in body condition

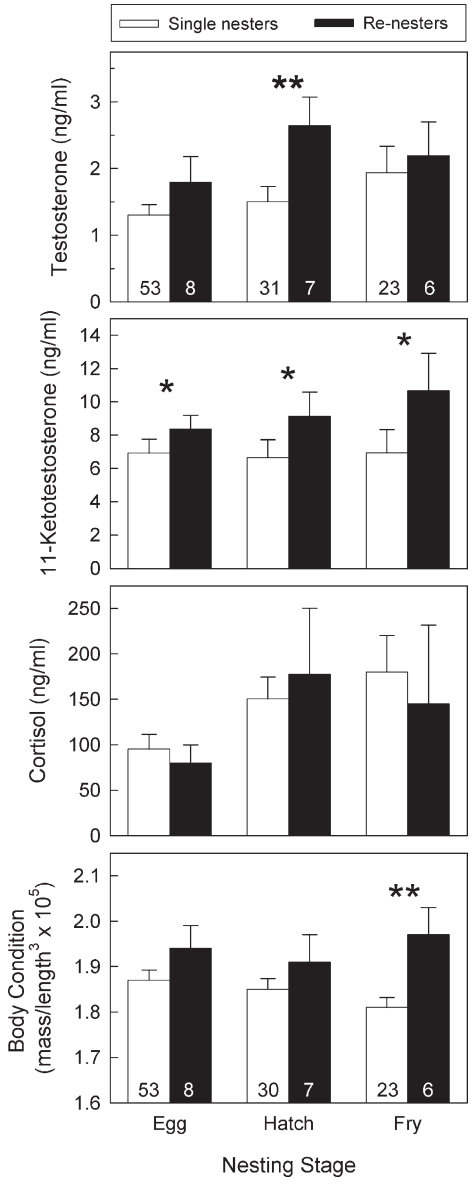

Fig. 4. Mean (±SEM) plasma steroid levels and body condition across the first nesting bout for *Uncaged* parental male bluegill known to have nested only once (Single nesters) and for those known to have renested (Renesters) later in the breeding season. *Control* and *Egg reduction* treatments are combined. Numbers inside the bars represent samples sizes; sample sizes for testosterone apply to all hormones. $^{**}P < 0.01$; $^{*}P < 0.05$ when compared to single nesters.

between these groups of males at the egg or hatch stage (both $P = 0.27$).

Discussion

Our results shed light on some of the proximate factors mediating paternal and nesting behavior in parental male bluegill. As has been shown previously (Kindler et al., 1989), we found that mean androgen levels were high during courtship and spawning and then decreased at the initiation of the paternal care period. However, mean androgen levels then returned to spawning levels for the remainder of the parental care period in the breeding hormone profile data set, a pattern seen in only one of the four breeding bouts documented by Kindler et al. (1989). There also was considerable individual variation in both of our data sets, with some males maintaining spawning or pre-spawning levels of T and 11KT well into the period of parental care. This maintenance of elevated androgen levels during the period of parental care stands in sharp contrast to what has been described for many species of birds (see Introduction and below). Parental males with high androgen levels were also often in better body condition throughout their first reproductive cycle than were males with lower androgen levels, and males that renested maintained higher androgen levels throughout their first nesting attempt than did males that were known to have nested only once. Sustained elevated androgen levels in bluegill may therefore be an indicator of renesting potential, although our present data set is too small to determine whether or how nest location and associated male–male interactions also contribute to the observed variation. We also found that cortisol levels were highly variable within and among males, but a trend for an increase in cortisol following the egg stage for parental males with full broods suggests a physiological cost of providing that care.

The challenge hypothesis suggests that androgens mediate a tradeoff between parental behavior and aggressive, territorial behaviors by males (Wingfield et al., 1990). Several studies of teleosts also support this hypothesis (see Introduction). In the present study, androgens were also at their highest mean levels before spawning when parental males aggressively defend their nest from other males and court females and were low during the early stages of the parental care period. However, androgen levels then increased again in the later stages of parental care. In the egg manipulation study, *Uncaged control* males had lower androgen levels than *Uncaged egg reduction* males after the egg fanning period (i.e., when eggs hatched). Parental males are known to adjust their fanning effort based on brood size, with larger broods being fanned more than smaller broods (Coleman and Fischer, 1991). Thus, *Uncaged control* males likely provided more fanning than *Uncaged egg reduction* males, and this may be reflected in their lower androgen levels, perhaps as an effect of elevated cortisol levels (see below). Collectively, our studies present conflicting results relevant to the predictions of the challenge hypothesis. Our data from early in the parental care period are consistent with a tradeoff between androgens and parental care as proposed in the challenge hypothesis. However, once the eggs hatch, mean androgen levels rise once again, often to levels seen at spawning.

Previous research on bluegill and other teleosts has demonstrated that exogenous application of androgens does not necessarily suppress paternal behavior (Kindler et al., 1991; Ros et al., 2004). Ros et al. (2004) suggested that time budget tradeoffs between territorial and paternal behavior, rather than direct inhibition of paternal behavior, may explain the general observation that androgen implants reduce paternal behavior, especially in free-living animals. Therefore, androgens must play some other role than suppressant during the parental care stage. Our results suggest a correlative relationship between androgens and parental care rather than a causative relationship. They also demonstrate considerable variability in androgen levels among bluegill parental males; parental males in better condition maintain higher androgen levels during the parental care period than males in poorer condition. These data thus suggest that endogenous androgens do not directly suppress paternal behavior.

Elevated androgen levels during the parental care period may instead reflect renesting potential. Elevated androgens are associated with pre-spawning behaviors and parental males may maintain higher levels of 11KT and T throughout a breeding attempt to allow for a quicker ramp-up of physiological processes when attempting to renest. Indeed, we found that renesting parental males maintained higher androgen levels throughout their initial nesting attempt than males that were not known to renest. We also found body condition to be positively related to androgen levels and parental males that nested twice were in better condition than were males known to have nested only once. The latter finding is consistent with previous research (Cargnelli, 1995; Cargnelli and Gross, 1997). Similar findings were reported in the black-chinned tilapia, where androgen levels were found to be elevated before spawning, dropped at the initiation of mouthbrooding but then subsequently returned to pre-spawning levels towards the end of mouthbrooding (Specker and Kishida, 2000). The authors hypothesized that initiation of mouthbrooding triggered a drop in androgen levels early on in parental care in order to delay the initiation of the next brood, which is caused by the eventual rise of androgens at the end of the parental care stage. Similar patterns of T change have been observed in male demoiselles (Pankhurst, 1990; Pankhurst and Peter, 2002).

Additional data are accumulating that elevated levels of 11KT are not always incompatible with the expression of paternal behavior in teleosts (e.g., Knapp et al., 1999; Rodgers et al., 2006; Sikkel, 1993) as elevated T levels seem to generally be in birds and mammals (but see Lynn et al., 2005; Reburn and Wynne-Edwards, 1999; Trainor and Marler, 2001, 2002). At least three non-mutually exclusive hypotheses could explain these observations. First, androgen levels are elevated during parental care because such levels are required to support coincident courtship behavior (e.g., Knapp et al., 1999). For bluegill, we can reject this hypothesis a priori because parental males do not engage in courtship during the period of care. Second, as the main androgen in many teleosts, 11KT may not

carry as high a cost in terms of immunosuppression and energetic costs as does T in birds and mammals (e.g., Saha et al., 2004; Slater et al., 1995). Third, teleosts may in general provide qualitatively different types of paternal care than mammals or birds, and these paternal behaviors may be less susceptible to inhibitory effects of elevated androgen levels. For example, male teleosts generally do not feed the young, as is the case for many avian fathers, but rather paternal care in teleosts generally consists mainly of defense against predators, fanning the eggs and nest cleaning. Elevated androgen levels might, in fact, support the expression of these behaviors, as they involve aggressive movements and/or the use of major muscle groups that could benefit from elevated levels of an anabolic hormone. We are currently obtaining quantitative behavioral data to determine whether or not either of the latter two hypotheses is supported in bluegill.

Relative to androgens, the relationship between GC levels and parental behavior during reproduction is poorly understood. In free-ranging bi-parental Greylag Geese and White Ibis, GC levels are higher during the primary care periods compared to the pre-mating courtship period (Kotrschal et al., 1998; Heath et al., 2003). This increase in GC levels suggests that providing care for offspring is physiologically costly for parents. In the present study, we found that cortisol levels increased over the 2 days of intense fanning in the breeding hormone profile data set. Also, *Uncaged control* males had higher mean cortisol levels at the hatch stage of care than *Uncaged egg reduction* males. This period of care immediately follows energetically costly egg fanning, and the lower mean cortisol levels in the *Egg reduction* group may reflect the reduced physiological cost of providing this care to a smaller brood. A similar relationship between cortisol levels and brood size was reported in the Pied Flycatcher where both males and females with experimentally increased clutch sizes exhibited increased levels of corticosterone, likely due to increased rates of feeding required to nourish all of the chicks (Silverin, 1986). Silverin (1986) also artificially increased corticosterone levels using implants and found that males with implants produced less than half as many fledglings as control males. Thus, GC levels elevated beyond a certain threshold may inhibit parental behavior via excessive physiological cost to the parent. Furthermore, we found a negative relationship between males' body length and cortisol levels at the end of the care period (fry stage). This suggests that larger males pay a lower physiological cost for providing care. Thus, providing care for offspring appears to be physiologically costly, but these costs are not equivalent for all individuals and may depend in part on the individual male's phenotype.

Stress is known to suppress the reproductive axis and to cause a reduction in gonadal steroids in several species of fish (Pankhurst and Van Der Kraak, 1997; Pottinger, 1999). In our study, caged males had significantly depressed androgen levels relative to uncaged males, regardless of brood size. Furthermore, caged males exhibited a three-fold increase in cortisol levels from the day after spawning to the end of the nesting period and simultaneously exhibited a 6.5% reduction in body condition. In comparison, uncaged males showed a less than two-fold increase in cortisol levels and only a 1.6% reduction in body condition during this time. These cortisol results are similar to those for tilapia, where males confined for 1 or 24 h had significantly higher circulating cortisol levels than unconfined, control males (Auperin et al., 1997). Similarly, mature male brown trout subjected to acute or chronic confinement suffered from increased plasma cortisol levels compared to unconfined, control males during the reproductive period (Pickering et al., 1987). Thus, our confining parental males in cages appeared to be a stressor that suppressed the reproductive axis and caused changes in body condition that likely precluded the males' ability to nest a second time that breeding season. Additional potential explanations exist for the differences in hormone levels between caged and uncaged treatments. First, caged males' lower androgen levels may reflect decreased aggression by the parental males directed towards nest predators given that the caged nests were physically protected from predation. Second, caging may have resulted in overall lower activity levels as a result of lower predation pressure, and this could be reflected in circulating steroid levels. However, other things being equal, such changes in activity would predict higher cortisol levels in uncaged males compared to caged males. This is opposite to the pattern we observed.

Taken together, our data indicate the relationship among androgens, glucocorticoids, body condition and paternal behavior in bluegill is complex. Our repeated sampling of known individuals has helped make sense of the large variation in hormone levels we observed among singly sampled males at any one time point in parental care. We are exploring the exact nature of the relationship among these variables, initially by correlating quantifications of parental behavior with circulating hormone levels and body condition. The importance of energetics in understanding hormone–behavior relationships in the context of male reproductive behavior has been previously highlighted (Knapp, 2003). Similar methodology in other species should also prove valuable for understanding the relationship of androgens and paternal behavior in species where the results of androgen manipulation studies seem to be at odds with interpretations of mean endogenous androgen levels.

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