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# Paternity, parental behavior and circulating steroid hormone concentrations in nest-tending male bluegill

Bryan D. Neff [a,*], Rosemary Knapp [b]

[a] Department of Biology, University of Western Ontario, 1151 Richmond Street N, London, Ontario, Canada N6A 5B7
[b] Department of Zoology, Graduate Programs in Ecology & Evolutionary Biology and Cellular & Behavioral Neurobiology, University of Oklahoma, 730 Van Vleet Oval, Norman, Oklahoma 73019, USA

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ABSTRACT

Like many teleost fishes, bluegill (*Lepomis macrochirus*) are characterized by sole male parental care of offspring. In addition, bluegill parental males experience cuckoldry by specialized parasitic male morphs. This cuckoldry has previously been shown to influence the expression of parental care behavior. To better understand some of the proximate mechanisms mediating parental behavior, we examined the relationships between circulating steroid hormones, paternity, and parental behavior during the egg and fry stages of care in parentals that spawned during the first third of the breeding season. During the egg stage of care, we found that males with higher paternity had lower levels of testosterone, but there was no relationship between paternity and either 11-ketotestosterone or cortisol. There also was no relationship between the hormones and care behavior comprising fanning of the eggs, nest rim circles, chases of brood predators, or pecking at the eggs (indicative of egg cannibalism), except for a negative relationship between cortisol and pecking behavior. During the fry stage of care, we conversely found that males with higher paternity had higher levels of testosterone and 11-ketotestosterone. There also was a negative relationship between the concentrations of these two androgens and the defensive behavior of males when exposed to a potential brood predator (a pumpkinseed, *Lepomis gibbosus*). We discuss these results in relation to previous work in fishes and other vertebrate taxa. Overall, our data suggest a complex relationship between circulating steroid hormone levels, paternity and parental behavior.



## Introduction

Biologists have long been interested in understanding the evolution of parental behavior (Trivers, 1972; Clutton-Brock, 1991; Westneat and Sherman, 1993). Many related questions center on the costs and benefits of current versus future reproduction. For males, these questions often revolve around how and whether a male's assessment of his paternity affects his current behavior (e.g. Svensson et al., 1998; Neff, 2003a; Magee and Neff, 2006). In addition, the proximate mechanisms that integrate the costs and benefits of paternal care are of interest but are not yet well understood (e.g. Lendvai et al., 2007). Central to understanding proximate mechanisms is the potential role that hormones play in mediating breeding behavior and ultimately in the evolution and maintenance of parental care.

Early behavioral endocrine work focused on the role of hormones in mediating maternal behavior (Young and Insel, 2002; Nelson, 2005). However, more recently, researchers have begun to investigate the role of several hormones in mediating paternal behavior (Wingfield et al., 1990; Schradin and Anzenberger, 1999; Trainor and Marler, 2002; Adkins-Regan, 2005; Storey et al., 2006). Among these hormones, androgens and prolactin have received the most attention. Prolactin has been implicated directly or indirectly in supporting the expression of various forms of parental behavior in several fish species (reviewed in Schradin and Anzenberger, 1999). Androgens have received particular attention because these hormones are important for territory establishment and courtship behavior, and these behaviors are clearly fundamental to producing offspring. In many avian and mammalian species, elevated androgen levels have often been found to interfere with the expression of paternal behavior, and androgen levels tend to be lower during periods of paternal care (Wingfield et al., 1990; Hirschenhauser et al., 2003; Goymann et al., 2007; McGlothlin et al., 2007). However, more recent work in these taxa, and other taxa including fishes, has shown that elevated androgen levels do not always preclude the expression of paternal behavior (Knapp et al., 1999; Wynne-Edwards, 2001; Trainor and Marler, 2002). In many of these cases, androgen levels appear to reflect the potential for future reproduction (e.g. Knapp et al., 1999; Páll et al., 2002a,b; Magee et al., 2006; Rodgers et al., 2006).

In addition to androgens, glucocorticoids (cortisol and corticosterone) may also be important for mediating the expression of parental behavior in vertebrates via their roles as metabolic hormones (Romero and Wikelski, 2001). Although the literature is still rather

* Corresponding author.
*E-mail address:* bneff@uwo.ca (B.D. Neff).

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small and there is considerable variation across individuals (Kotrschal et al., 1998; Knapp et al., 1999; Heath et al., 2003; Magee et al., 2006), plasma glucocorticoid levels are often higher during energetically expensive phases of parental care (Magee et al., 2006) or increase over the period of care when parents do not forage (Kotrschal et al., 1998; Guinet et al., 2004). Thus, increased glucocorticoid levels may be useful as a measure of the energetic and other stresses experienced by the parent. For example, glucocorticoid levels have recently been examined with respect to nest abandonment by males in several species (Love et al., 2004; Magee et al., 2006; Groscolas et al., 2008). These studies have found that mean glucocorticoid levels were higher in parents that abandoned their nest than those that did not abandon their nest. Whether these higher levels reflect reduced paternity or maternity in species with cuckoldry or nest parasitism is currently unclear.

To further explore the relationship between paternity, hormones and paternal behavior, we have been studying bluegill (*Lepomis macrochirus*). This species is endemic to North America (Lee et al., 1980) and many populations are characterized by two distinct life histories termed "parental" and "cuckolder". In Lake Opinicon, Ontario (44°34′N, 76°19′W), parentals mature around 7 years of age, court females and provide sole parental care (Gross and Nowell, 1980). Cuckolders mature at 2 years of age and then steal fertilizations from parentals using one of two tactics termed "sneaker" and "satellite" (Gross, 1982). Sneakers are young cuckolders (age 2–3 years) and they spawn by darting into a parental's nest during a female's egg release. Once sneakers reach about 4 years of age, they change tactics and become satellites (Gross, 1982). Satellites are the size of small females and mimic a female's morphology and behavior (Gross, 1982; Neff and Gross, 2001). Females follow a single life history, maturing at the age of about 4 years.

Bluegill spawning typically occurs between late May and mid-July (Cargnelli and Gross, 1996). Parentals nest in colonies and then spawn synchronously throughout the breeding season in what are termed "bouts." A bout begins when colonies form within the littoral zone of the lake. Females approach a colony in a school and enter parentals' nests to spawn. A female may spawn with more than one parental, and her eggs are sometimes fertilized by cuckolders; about 24% of all eggs spawned in bluegill nests are sired by cuckolders (Neff and Clare, 2008). Spawning in a colony typically lasts a single day.

After spawning, parentals provide sole care for the developing offspring, which lasts from 7–10 days and has two distinct stages. The egg stage typically lasts 2–3 days and parental care involves defense of the brood and fanning of eggs, presumably to actively maintain oxygen levels around the developing young (Tinbergen, 1951, p. 58–59; van Iersel, 1953, p. 38). During this stage, parentals can assess their paternity using an indirect cue, the presence of sneakers around their nest during spawning (Neff, 2003a). After the eggs hatch, the fry are guarded until they leave the nest 4–7 days later. During the fry stage of care, parentals are able to assess their paternity based on a direct, olfactory cue of relatedness released by the newly hatched fry (Neff and Sherman, 2003; 2005). Thus, although during the egg stage of care parentals can only detect cuckoldry by sneakers, during the fry stage they can detect cuckoldry by both sneakers and satellites. The care parentals provide is essential for offspring survival and parentals rarely leave their nest for more than a few seconds.

Parentals have the potential to nest multiple times in a given breeding season (Magee et al., 2006; Magee and Neff, 2006). The factors that influence whether or not a male spawns multiple times are not well understood, but likely are related to the male's ability to handle the energetic costs of care behavior. Males do not actively forage during the care period, and can lose 10% of their body mass during this time (Coleman and Fischer, 1991). Furthermore, parentals known to have spawned a second time during the breeding season were in significantly better body condition and had significantly higher androgen levels during the first spawning bout than males not known to have spawned in a second bout (Magee et al., 2006). In this previous study, however, we did not quantify the care males provided to their first clutches, nor the male's paternity. Thus, the present study was designed to determine whether and how body condition, plasma androgen and cortisol concentrations, and paternity correlate with the quality of care males provide. Based on the literature and previous studies in bluegill, we predicted that quality of care would be positively correlated with all of these variables.

## Methods

### *Parental care behavior*

During the 2005 breeding season, daily surveys were conducted of our study site on Lake Opinicon to locate spawning activity. We selected four colonies in which to study parental care behavior. The colonies spawned between the 5th and 11th of June, which falls within the first third of the breeding season (see Magee and Neff, 2006). The day after spawning, nest position within the colony (central versus peripheral) and egg scores were assigned as a rank between 1 (=eggs covering 20% of nest) and 5 (=eggs covering 100% of nest). The scores have been shown to be highly correlated with the actual number of eggs: score 1, 27–4889 eggs; score 2, 4666–28,806; score 3, 27,072–53,221; score 4, 49,369–86,552; and score 5, 82,063–112,810 ($R=0.96$, $p<0.001$, $n=32$; Claussen, 1991). For two of the colonies, we took behavioral measures during the first day of the egg stage of care ($n=40$ parentals). These measurements comprised the number of (i) egg fanning motions using either the pectoral or caudal fins, (ii) nest rim circles, (iii) chases of potential brood predators, and (iv) pecking motions used to cannibalize eggs (see Coleman and Fischer, 1991, Neff, 2003b for descriptions of the behaviors). Nests were first marked with a numbered tag and behavior of each parental was recorded for 15 min between 1300 and 1600 EST.

Directly after the behavioral observation, the parental male was collected using a dip net and quickly taken to a nearby boat where approximately 500 μL of blood was collected from the caudal peduncle using a heparinized syringe. Blood collection time, from the moment the fish was caught, averaged 84 s (range: 51–159 s; bleed time did not correlate with concentrations of any of the hormones: $p>0.45$ for all). All blood samples were kept on ice until they were returned to the lab (within 2 h), where they were centrifuged to separate the blood plasma. 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 g) and measured for total length (nearest mm). Fulton's condition factor was calculated from mass/length$^3\times10^5$. 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 (Sutton et al., 2000; Neff and Cargnelli, 2004). A small portion of the caudal fin was also collected and preserved in 95% ethanol for later genetic analysis. The fish were then released back into the water and they typically returned to their nest and resumed parental care behavior within a few minutes. Once the eggs hatched a few days later, a subsample of the fry was collected from several locations within the nest and preserved in 95% ethanol for paternity analysis.

For the other two colonies, we quantified behavior during the first day of the fry stage of care ($n=19$ parentals). These measurements comprised brood defense as measured using a predator presentation. The presentation involved a live brood predator (pumpkinseed, *Lepomis gibbosus*) in a plastic bag, which was placed at the edge of each parental's nest (Coleman et al., 1985; Neff, 2003a). The predator (total length of about 160 mm) was presented twice for 30-s intervals with a 30-s interval in between. During the two 30-s intervals, defense was measured by counting the number of lateral displays, opercular flares and bites directed towards the predator. Brood defense was calculated

from an overall index according to 1 × lateral displays + 2 × opercular flares + 3 × bites. The coefficients were selected to reflect the relative intensity (i.e. level of aggression) of each of the three parental defense behaviors (Colgan and Gross, 1977; also see Magee and Neff, 2006).

As with the males from the previous two colonies, immediately after the behavioral observations, the focal male was collected using a dip net and quickly taken to a nearby boat for blood sampling and morphological measurement. An additional 15 males were sampled at that time for hormone levels to ascertain if the hormone profiles were a result of the response to the predator presentation themselves. We did not make behavior observations of these 15 males because naturally occurring predator approaches are relatively infrequent during this stage of care and thus little defense behavior would likely have occurred naturally. Due to logistical considerations, we were unable to include a comparison group that included fish exposed only to the presenter. Blood collection time, from the moment the fish was caught, averaged 78 s (range: 52–191 s; bleed time did not correlate with concentrations of any of the hormones: $p>0.23$ for all). The predator and control groups did not differ significantly in mean blood collection time or levels of any of the three hormones ($p>0.20$ for all).

All procedures conformed to the guidelines outlined by the Canadian Council on Animal Care and were also approved by the University of Oklahoma Animal Care and Use Committee.

### *Hormone assays*

Plasma concentrations of testosterone (T), 11-ketotestosterone (11-KT), and cortisol were determined using radioimmunoassay (RIA) following chromatographic separation. 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 isooctane. Samples were then applied to diatomaceous earth-glycol columns. The steroid hormones were separated from one another via stepwise elution by means of increasing concentrations of ethyl acetate in iso-octane (T: 10% ethyl acetate in isooctane; estradiol: 20% (not assayed); 11-KT: 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 11-KT. The cortisol antibody (F3-314) was purchased from Esoterix Endocrinology (Calabasas Hills, CA). All 74 samples for a given hormone were measured in a single assay. Intra-assay coefficients of variation as calculated from four standard tubes per assay were 3.1% for T, 1.2% for 11-KT, and 14.8% for cortisol.

### *Paternity analysis*

For a subset of the males (19 males from each stage of parental care), paternity was determined using three microsatellite loci and the two-sex paternity model (Neff et al., 2000a; Neff, 2001). This model determines the proportion of offspring sired by the nest-tending parental male. The remaining offspring are assumed to be sired by the specialized cuckolder males. The microsatellite loci comprised RB7, LMAR10, and RB20 (primer sequences are published in: DeWoody et al., 1998; Schable et al., 2002). First, DNA was isolated from the samples using a proteinase K digestion (Neff et al., 2000b). Second, we used a Whatman-Biometra T1 Thermocycler to amplify the microsatellites with the following program: 60 s at 92 °C; 7 cycles of 30 s at 92 °C, 30 s at 54 °C, and 30 s at 72 °C; and 28 cycles of 15 s at 92 °C, 30 s at 54 °C and 20 s at 72 °C. Each 10 μL PCR reaction contained ~75 ng of total DNA, 2 mM $MgCl_2$, 1× PCR buffer (Fisher), 0.4 mM of each deoxynucleotide (Fisher), 0.25 units Taq DNA polymerase (Fisher) and 0.2 μM of each forward and reverse primer (Invitrogen Life Technologies). PCR product was run following the standard protocol for the CEQ 8000 Genetic Analysis System (Beckman Coulter). Genotypes were obtained from a total of 897 offspring (mean: 24 offspring per nest; range: 10–48). The genotypes at these loci did not deviate from Hardy–Weinberg equilibrium. The mean exclusion probability for the parentage analysis was 0.92 (range: 0.85–0.99).

### *Statistical analysis*

Statistical analyses were performed using JMP (v. 4.0.4, SAS Institute Inc.). We used non-parametric statistics because many of the variables deviated from normality. Covariance between variables was examined with Spearman's correlation and means were compared with a Wilcoxon rank-sum test. Morphological variables focused on total length and Fulton's condition factor; we did not also examine body mass because length and mass are highly correlated in bluegill and residual mass is captured by the condition factor (see Neff and Cargnelli, 2004).

## Results

Across all males, T and 11-KT were highly correlated (Spearman's rho = 0.90, $p<0.001$, $N=74$), but there was no relationship between androgen concentrations and cortisol (cortisol and T: Spearman's rho = −0.10, $p=0.42$, $N=74$; cortisol and 11-KT: Spearman's rho = −0.14, $p=0.21$, $N=74$). There was no difference in total length, body mass, Fulton's condition factor or the egg scores between males collected during the egg stage of care and those collected during the fry stage of care (Table 1). However, males collected during the fry stage of care had significantly higher paternity and T levels, and marginally higher 11-KT and cortisol levels than males collected during the egg stage of care (Table 1).

### *Egg stage of care*

There was no difference in the plasma hormone concentrations of T, 11-KT and cortisol between males with central nests and males with nests on the periphery of the colony (Wilcoxon: $p>0.53$ for all). There also was no correlation between male length, Fulton's condition factor or egg score and concentrations of any hormone ($p>0.32$ for all). Paternity was negatively correlated with T (Spearman's rho = −0.48, $p=0.036$, $N=19$), but not with either 11-KT (Spearman's rho = −0.23, $p=0.34$, $N=19$) or cortisol (Spearman's rho = 0.27, $p=0.27$, $N=19$) (Fig. 1). During the 15-min observation period, males performed an average of 14 ± 12 (±SD) fanning behaviors, 27 ± 25

**Table 1**
Mean (±SD) and range of morphological measurements, brood characteristics and plasma hormone concentrations for parental male bluegill (*Lepomis macrochirus*) collected from colonies during the egg or fry stage of parental care.

| Variable | Egg stage | Fry stage | *p*-value |
|---|---|---|---|
| Body length (mm) | 193 ± 7 (180–211) | 191 ± 8 (175–210) | 0.45 |
| Body mass (g) | 133 ± 16 (105–173) | 130 ± 16 (100–165) | 0.61 |
| Fulton's condition factor ($g/mm^3 \times 10^5$) | 1.86 ± 0.12 (1.63–2.18) | 1.86 ± 0.13 (1.62–2.14) | 0.94 |
| Egg score | 1.0 ± 0.5 (1–2) | 1.5 ± 1.1 (1–4) | 0.20 |
| Paternity | 0.59 ± 0.19 (0.23–1.00) | 0.81 ± 0.17 (0.34–1.00) | <0.001 |
| Testosterone (ng/mL) | 1.1 ± 0.8 (0.14–3.4) | 1.7 ± 1.2 (0.05–4.5) | 0.024 |
| 11-Ketotestosterone (ng/mL) | 3.7 ± 3.0 (0.2–11.5) | 6.6 ± 6.4 (0.1–30.5) | 0.055 |
| Cortisol (ng/mL) | 67 ± 69 (2–296) | 133 ± 129 (1–508) | 0.053 |

Values are reported as mean plus or minus one standard deviation with the range in parentheses. Sample size for each variable is $N=40$ for egg stage and $N=34$ for fry stage, except for the paternity measures, which are $N=19$ for both stages. *p*-values are based on Wilcoxon rank-sum tests.

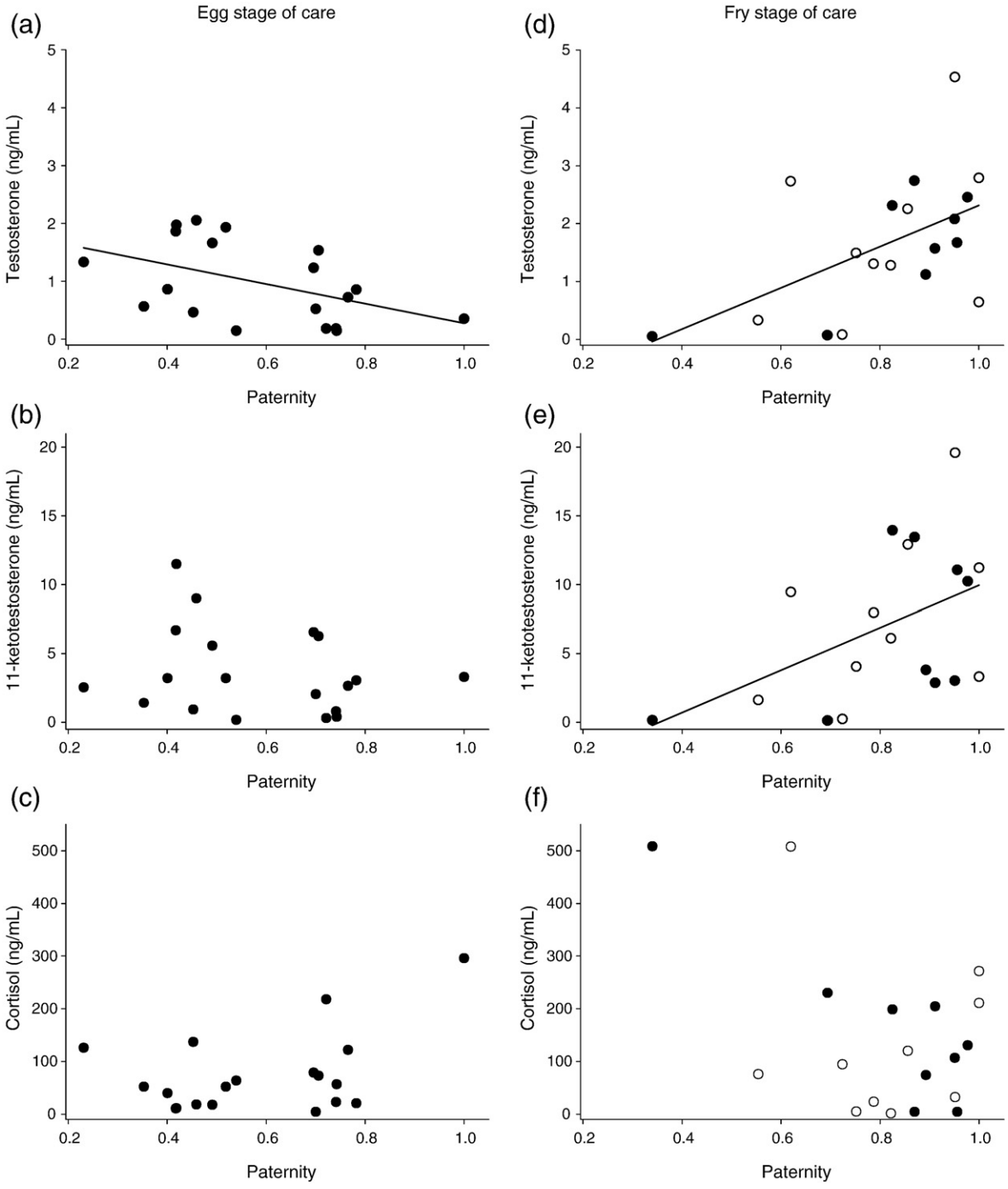


**Fig. 1.** Relationships between circulating concentrations of steroid hormones and paternity in bluegill (*Lepomis macrochirus*). The hormones comprise (a, d) testosterone, (b, e) 11-ketotestosterone, and (c, f) cortisol during the egg or fry stage of care. Regression lines are displayed only for significant relationships, although statistical analyses used non-parametric correlations (see text for details). Filled circles in the fry stage represent males that were challenged with a heterospecific brood predator, open circles represent control males that were not exposed.

rim circles, $3 \pm 3$ pecks, and $15 \pm 12$ chases. There was no relationship between any of the hormone concentrations and these behaviors ($p > 0.08$ for all), except for a negative relationship between cortisol and number of pecks (Spearman's rho $= -0.41$, $p = 0.009$, $N = 40$). There also was no relationship between Fulton's condition factor or paternity and any of the behaviors ($p > 0.14$ for all), except for a negative relationship between paternity and the number of pecks (Spearman's rho $= -0.72$, $p < 0.001$, $N = 19$). Fulton's condition factor was not correlated with paternity (Spearman's rho $= 0.11$, $p = 0.66$, $N = 19$).

*Fry stage of care*

There was no difference in hormone concentrations between males with whom we conducted predator presentations and control males (Wilcoxon: $p > 0.23$ for all). Thus, the presentations themselves did not appear to affect plasma hormone concentrations of T, 11-KT and cortisol, at least over the short time period between predator presentations and blood sample collection. Similar to the egg stage, during the fry stage there was no difference in hormone concentrations between males with central nests and males with nests on the

periphery of the colony (Wilcoxon: $p>0.38$ for all). There also was no correlation between male length, Fulton's condition factor or egg score and hormone concentrations ($p>0.17$ for all), except for a positive correlation between condition factor and T (Spearman's rho = 0.38, $p=0.028$, $N=34$) and a positive trend between condition factor and 11-KT (Spearman's rho = 0.33, $p=0.059$, $N=34$). In contrast to the egg stage, paternity was positively correlated with T (Spearman's rho = 0.53, $p=0.020$, $N=19$) and 11-KT (Spearman's rho = 0.47, $p=0.043$, $N=19$); there was no relationship between paternity and cortisol (Spearman's rho = 0.08, $p=0.74$, $N=19$) (Fig. 1). There were strong negative correlations between the brood defense index and T (Spearman's rho = −0.68, $p=0.001$, $N=19$) and 11-KT (Spearman's rho = −0.72, $p<0.001$, $N=19$), but no relationship between the defense index and cortisol (Spearman's rho = −0.01, $p=0.97$, $N=19$) (Fig. 2). Similar negative relationships were found between T and 11-KT and the individual components of the defense index, although the relationships were significant only for the lateral displays and bites, and not the opercular flares (data not shown). There was no significant relationship between any of the three components of the index and cortisol. Fulton's condition factor was not correlated with paternity or the defense index ($p>17$ for both), and there was no relationship between the defense index and paternity (Spearman's rho = −0.33, $p=0.38$, $N=9$), although the small sample size warrants caution in interpreting this result.

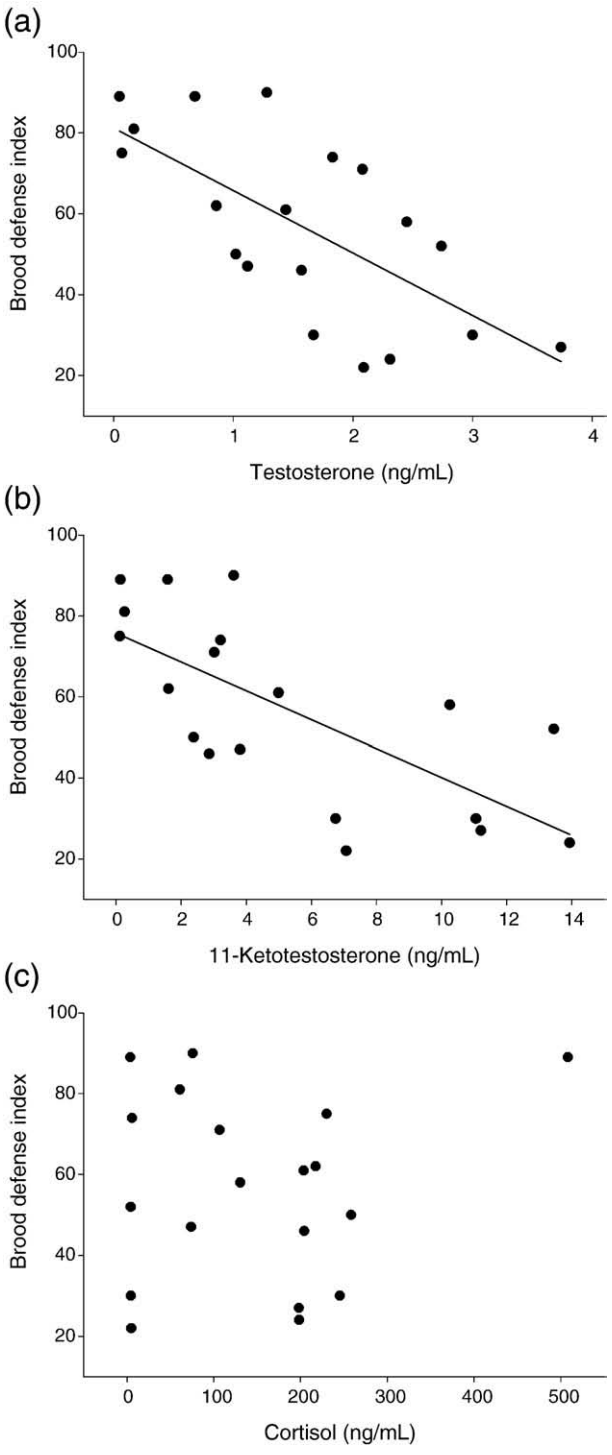


**Fig. 2.** Relationships between brood defense behavior of fry and circulating concentrations of (a) testosterone, (b) 11-ketotestosterone, and (c) cortisol in bluegill (*Lepomis macrochirus*). Regression lines are displayed only for significant relationships, although statistical analyses used non-parametric correlations (see text for details). The brood defense index reflects aggressive behavior of parentals to presentation of a heterospecific brood predator.

## Discussion

To our knowledge, our study is the first to examine the relationship between paternity and steroid hormone levels in any vertebrate. We found associations between paternity and androgen concentrations to be variable during the parental care period in bluegill. During the egg stage of care, we found that paternity was negatively correlated with T but not with 11-KT levels. During spawning, parentals actively chase sneakers away from their nests (Gross, 1982) and increased sneaking reduces a parental's paternity (Fu et al., 2001; Neff and Gross, 2001). Thus, it is possible that the negative correlation between paternity and T in our egg stage data set reflects increased T in those parentals that spent more time aggressively defending their nest from sneakers during spawning. Yet the lack of a correlation with 11-KT suggests that this is not a complete explanation for the relationship.

In contrast, during the fry stage of care, paternity and concentrations of both androgens were positively correlated. It is unlikely that this positive relationship reflects an increased level of aggression in males with high paternity (who may value their brood more and may be more willing to defend it from predators) because we actually found defense behavior to be negatively correlated with both T and 11-KT. Instead, the positive relationship between paternity and T and 11-KT may reflect renesting behavior if males with high paternity are more likely to renest later in the season than males with low paternity. In a previous study, parentals that spawned a second time during the breeding season had significantly higher androgen levels during their first spawning bout than males not known to spawn a second time (Magee et al., 2006). However, nothing is yet known about the direct link between paternity and renesting behavior in bluegill. Furthermore, it remains unclear as to why the relationship between the two androgens and paternity changed from the egg and fry stages.

Androgens have traditionally been considered as inhibitory to paternal behavior, especially in birds and mammals, based on both correlation and manipulation studies (e.g. Wingfield et al., 1990; 1991; Reburn and Wynne-Edwards, 1999; Goymann et al., 2007; but see Trainor and Marler, 2001). Although a few studies of fishes have also found a pattern of declining androgen levels *with the onset* of paternal behavior (e.g. Páll et al., 2002a), several other studies have demonstrated that elevated androgens do not coincide with decreases in paternal care and androgen manipulations often do not have the predicted effects based on the avian and mammalian literature (review by Oliveira and Gonçalves, 2008; see also Páll et al., 2002b; Hirschenhauser et al., 2004; Ros et al., 2004; Rodgers et al., 2006; Desjardins et al., 2008).

In the present study, we found that circulating plasma androgen concentrations in parental male bluegill were more than 50% higher during the fry than egg stage of care. However, during the fry stage of care, androgen levels were significantly negatively correlated with

brood defense. The first finding corroborates our previous results with this species, where androgen concentrations could be as high during the fry stage of care as during establishment of territories and spawning (Magee et al., 2006; also see Kindler et al., 1989, 1991). Based on tracking males through the breeding season in this previous study, we argued that the elevated androgen concentrations relate to renesting potential, with males known to renest having higher androgen concentrations than males not known to renest (Magee et al., 2006; also see Specker and Kishida, 2000). In addition, Magee et al. (2006) found that males in better condition had higher androgen levels and were more likely to renest. In the present study, we similarly found that among males collected during the fry stage of care, individuals in better condition had higher T concentrations.

Generally, the relationships we documented between plasma androgen concentrations and parental care behavior were unexpected based on data from other fish species. During the egg stage of care, we found that the androgen concentrations did not correlate with any of the behaviors, and during the fry stage of care, we found a negative relationship between T and 11-KT and brood defense behavior. In contrast, in the repeatedly spawning, cooperatively breeding cichlid *Neolamprologus brichardi* (now *N. pulcher*), T was positively correlated with a composite parental care score including territory maintenance and territory defense (Desjardins et al., 2008; also see Ros et al., 2004 and Hirschenhauser and Oliveira, 2006). The analyses for this study included subjects both with and without fry in the nest; there were no differences in mean T levels between these two groups. The negative relationship between androgens and brood defense in our study seems at odds with the overall higher levels of this hormone during the fry stage of care as compared to the egg stage of care (this study; Magee et al., 2006), again pointing toward a role for elevated androgens during care to mediate renesting potential rather than paternal behavior per se. Similarly, breeding *N. pulcher* males had higher 11-KT levels during the care period than male helpers (Bender et al., 2008), suggesting that androgen levels are more related to mating behavior than paternal care. Taken together, the currently available literature suggests that, as a generality, androgens do not have as important a role in influencing paternal behavior in fishes as these hormones do in many birds and mammals.

Relative to androgens, the relationship between glucocorticoid concentrations and parental behavior is less well understood, especially in fishes. Generally, data from several species of birds and mammals suggest that parental care is physiologically costly and consequently glucocorticoid concentrations rise during the breeding period from mating through to the end of parental care (Silverin, 1986; Storey et al., 2006). Consistent with these other studies, we previously found that cortisol concentrations in multiply sampled parental bluegill were higher during the fry stage than egg stage of care (Magee et al., 2006). Here, we again found that cortisol levels were higher during the fry stage than the egg stage of care, albeit we did not resample individual males. The higher mean cortisol levels during the fry stage may well reflect the fasting state of the males, who do not actively forage during the care period. Yet there was no relationship between cortisol and body condition, a measure that correlates with non-polar lipid density in bluegill (Neff and Cargnelli, 2004). A further consideration is that the social dynamics among parental males in bluegill colonies also complicates the study of cortisol–behavior relationships. An additional caveat is that the relatively high intra-assay variation for cortisol radioimmunoassay could have obscured weak correlations of cortisol with some of the measured variables, especially body condition. Nevertheless, the lack of relationship between baseline cortisol levels and body condition is similar to what has been found in the black-legged kittiwake (*Rissa tridactyla*) (Chastel et al., 2005). However, it contrasts recent findings that the sensitivity to elevated corticosterone of provisioning nestlings in black-legged kittiwakes and male barn owls (*Tyto alba*) depended on the parents' phenotype (Angelier et al., 2007; Almasi et al., 2008). Clearly, additional work is required to understand the role of glucocorticoids in parental behavior in fishes (see also Bender et al. 2008).

Some caution is warranted when interpreting our results across stages of parental care because the measurements taken during the egg and fry stages were made on different males in different colonies. It is thus possible that the relationships between paternity and the two androgens, and the different direction of relationship between androgens and brood defense, reflect some other aspect of the particular colony and males. For example, the higher mean paternity in the males sampled during the fry stage may well reflect the enrichment of high-paternity males, as those males with low paternity would have been more likely to abandon their clutches upon egg hatching (Neff, 2003a, Magee and Neff, 2006). Alternatively, the higher paternity may reflect selective cannibalization of unrelated fry by parental males (e.g. paternity was negatively correlated with pecking rate in the current study). Nevertheless, our data do indicate that researchers should carefully consider the stage of care when examining androgen concentrations and paternity.

In summary, we have provided the first study examining paternity and circulating hormone concentrations in a vertebrate. We found differing patterns between paternity and androgen concentrations during the egg and fry stages of care, although there was no apparent relationship between paternity and cortisol concentrations during either stage. Perhaps surprisingly, during the fry stage of care we found a negative relationship between androgen concentrations and the defensive behavior of males when exposed to a potential brood predator. Overall, our data help to address the complex relationship between circulating steroid hormone concentrations, paternity and parental behavior.

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## References

Adkins-Regan, E., 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton.

Almasi, B., Roulin, A., Jenni-Eiermann, S., Jenni, L., 2008. Parental investment and its sensitivity to corticosterone is linked to melanin-based coloration in barn owls. Horm. Behav. 54, 217–223.

Angelier, F., Clément-Chastel, C., Gabrielsen, G.W., Chastel, O., 2007. Corticosterone and time-activity budget: an experiment with black-legged kittiwakes. Horm. Behav. 52, 482–491.

Bender, N, Heg-Bachar, Z, Oliveira, RF, Canario, A.V.M., Taborsky, M., 2008. Hormonal control of brood care and social status in a cichlid fish with brood care helpers. Physiol. Behav. 94, 349–358.

Cargnelli, L.M., Gross, M.R., 1996. The temporal dimension in fish recruitment: birth date, body size, and size-dependent survival in a sunfish (bluegill: *Lepomis macrochirus*). Can. J. Fish. Aquat. Sci. 53, 360–367.

Chastel, O., Lacroix, A., Weimerskirch, H., Gabrielson, G.W., 2005. Modulation of prolactin but not corticosterone responses to stress in relation to parental effort in a long-lived bird. Horm. Behav. 47, 459–466.

Claussen, J.E., 1991. Annual Variation in the Reproductive Activity of a Bluegill Population: Effect of Clutch Size and Temperature. M.Sc. thesis. University of Toronto, Toronto.

Clutton-Brock, T.H., 1991. The Evolution of Parental Care. Princeton University Press, Princeton.

Coleman, R.M., Fischer, R.U., 1991. Brood size, male fanning effort and the energetics of a non-shareable parental investment in bluegill sunfish, *Lepomis macrochirus* (Teleostei: Centrarchidae). Ethology 87, 177–188.

Coleman, R.M., Gross, M.R., Sargent, R.C., 1985. Parental investment decision rules: a test in bluegill sunfish. Behav. Ecol. Sociobiol. 18, 59–66.
Colgan, P.W., Gross, M.R., 1977. Dynamics of aggression in male pumpkinseed sunfish (*Lepomis gibbosus*) over reproductive phase. Z. Tierpsychol. 43, 139–151.
Desjardins, J.K., Stiver, K.A., Fitzpatrick, J.L., Milligan, N., van der Kraak, G.J., Balshine, S., 2008. Sex and status in a cooperative breeding fish: behaviour and androgens. Behav. Ecol. Sociobiology 62, 785–794.
DeWoody, J.A., Fletcher, D.E., Wilkins, S.D., Nelson, W.S., Avise, J.C., 1998. Molecular genetic dissection of spawning, parentage, and reproductive tactics in a population of redbreast sunfish, *Lepomis auritus*. Evolution 52, 1802–1810.
Fu, P., Neff, B.D., Gross, M.R., 2001. Tactic-specific success in sperm competition. Proc. R. Soc., Lond., B Biol. Sci. 268, 1105–1112.
Goymann, W., Landys, M.M., Wingfield, J.C., 2007. Distinguishing seasonal androgen responses from male–male androgen responsiveness — revisiting the challenge hypothesis. Horm. Behav. 51, 463–476.
Groscolas, R., Lacroix, A., Robin, J-P., 2008. Spontaneous egg or chick abandonment in energy-depleted king penguins: a role for corticosterone and prolactin? Horm. Behav. 53, 51–60.
Gross, M.R., 1982. Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. Z. Tierpsychol. 60, 1–26.
Gross, M.R., Nowell, W.A., 1980. The reproductive biology of rock bass, *Ambloplites rupestris* (Centrarchidae), in Lake Opinicon, Ontario. Copeia 1980, 482–494.
Guinet, C., Servera, N., Mangin, S., Georges, J.Y., Lacroix, A., 2004. Change in plasma cortisol and metabolites during the attendance period ashore in fasting lactating subantarctic fur seals. Comp. Biochem. Physiol., A-Molec. Integr. Physiol. 137, 523–531.
Heath, J.A., Frederick, P.C., Edwards, T.M., Guillette, L.J., 2003. Reproductive physiology of free-living White Ibises (*Eudocimus albus*) in the Florida Everglades. Gen. Comp. Endocrinol. 133, 118–131.
Hirschenhauser, K., Oliveira, R.F., 2006. Social modulation of androgens in male vertebrates: meta-analyses of the challenge hypothesis. Anim. Behav. 71, 265–277.
Hirschenhauser, K., Winkler, H., Oliveira, R.F., 2003. Comparative analysis of male androgen responsiveness to social environment in birds: the effects of mating system and paternal incubation. Horm. Behav. 43, 508–519.
Hirschenhauser, K., Taborsky, M., Oliveira, T., Canàrio, A.V.M., Oliveira, R.F., 2004. A test of the 'challenge hypothesis' in cichlid fish: simulated partner and territory experiments. Anim. Behav. 68, 741–750.
Kindler, P.M., Philipp, D.P., Gross, M.R., Bahr, J.M., 1989. Serum 11-ketotestosterone and testosterone concentrations associated with reproduction in male bluegill (*Lepomismacrochirus*: Centrachidae). Gen. Comp. Endocrinol. 75, 446–453.
Kindler, P.M., Bahr, J.M., Philipp, D.P., 1991. The effects of exogenous 11-ketotestosterone, testosterone and cyproterone acetate on prespawning and parental care behaviors in male bluegill. Horm. Behav. 25, 410–423.
Knapp, R., Wingfield, J.C., Bass, A.H., 1999. Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*). Horm. Behav. 35, 81–89.
Kotrschal, K., Hirschenhauser, K., Möstl, E., 1998. The relationship between social stress and dominance is seasonal in greylag geese. Anim. Behav. 55, 171–176.
Lee, D.S., Gilbert, C.R., Hocutt, C.H., Jenkins, R.E., McAllister, D.E., Stauffer Jr., J.R., 1980. Atlas of North American Freshwater Fishes. North Carolina State Museum of Natural History, Raleigh.
Lendvai, A.Z., Giraudeau, M., Chastel, O., 2007. Reproduction and modulation of the stress response: an experimental test in the house sparrow. Proc. R. Soc. Lond., B-Biol. Sci. 274, 391–397.
Love, O.P., Breuner, C.W., Vézina, F., Williams, T.D., 2004. Mediation of a corticosterone-induced reproductive conflict. Horm. Behav. 46, 59–65.
Magee, S.E., Neff, B.D., 2006. Temporal variation in decisions about parental care in bluegill, *Lepomis macrochirus*. Ethology 112, 100–107.
Magee, S.E., Neff, B.D., Knapp, R., 2006. Circulating concentrations of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*. Horm. Behav. 49, 598–609.
McGlothlin, J.W., Jawor, J.M., Ketterson, E.D., 2007. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. Am. Nat. 170, 864–875.
Neff, B.D., 2001. Genetic paternity analysis and breeding success in bluegill sunfish (*Lepomis macrochirus*). J. Hered. 92, 111–119.
Neff, B.D., 2003a. Decisions about parental care in response to perceived paternity. Nature 422, 716–719.
Neff, B.D., 2003b. Paternity and condition affect cannibalistic behavior in nest-tending bluegill sunfish. Behav. Ecol. Sociobiol. 54, 377–384.
Neff, B.D., Gross, M.R., 2001. Dynamic adjustment of parental care in response to perceived paternity. Proc. R. Soc. Lond., B-Biol. Sci. 268, 1559–1565.
Neff, B.D., Sherman, P.W., 2003. Nestling recognition via direct cues by parental male bluegill sunfish (*Lepomis macrochirus*). Anim. Cog. 6, 87–92.
Neff, B.D., Cargnelli, L.M., 2004. Relationships between condition factors, parasite load and paternity in bluegill sunfish, *Lepomis macrochirus*. Environ. Biol. Fish. 71, 297–304.
Neff, B.D., Sherman, P.W., 2005. In vitro fertilization reveals offspring recognition via self-referencing in a fish with paternal care and cuckoldry. Ethology 111, 425–438.
Neff, B.D., Clare, E.L., 2008. Temporal variation in cuckoldry and paternity in two sunfish species (*Lepomis* spp.) with alternative reproductive tactics. Can. J. Zool. 86, 92–98.
Neff, B.D., Repka, J., Gross, M.R., 2000a. Parentage analysis with incomplete sampling of candidate parents and offspring. Mol. Ecol. 9, 515–528.
Neff, B.D., Fu, P., Gross, M.R., 2000b. Microsatellite multiplexing in fish. Trans. Am. Fish. Soc. 129, 584–593.
Nelson, R.J., 2005. An Introduction to Behavioral Endocrinology, third ed. Sinauer Associates, Sunderland, Massachusetts, USA.
Oliveira, R.F., Gonçalves, D.M., 2008. Hormones and social behaviour of teleost fish. In: Magnhagen, C., Braithwaite, V.A., Forsgren, E., Kapoor, B.G. (Eds.), Fish Behaviour. InScience Publishers: Enfield, New Hampshire, USA, pp. 61–150.
Páll, M.K., Mayer, I., Borg, B., 2002a. Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus* I. Changes in 11-ketotestosterone levels during the nesting cycle. Horm. Behav. 41, 377–383.
Páll, M.K., Mayer, I., Borg, B., 2002b. Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus* II. Castration and 11-ketoandrostenedione effects on courtship and parental care during the nesting cycle. Horm. Behav. 42, 337–344.
Reburn, C.J., Wynne-Edwards, K.E., 1999. Hormonal changes in males of a naturally biparental and a uniparental mammal. Horm. Behav. 35, 163–176.
Rodgers, E.W., Earley, R.L., Grober, M.S., 2006. Elevated 11-ketotestosterone during paternal behavior in the bluebanded goby (*Lythrypnus dalli*). Horm. Behav. 49, 610–614.
Romero, L.M., Wikelski, M., 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proc. Natl. Acad. Sci. U. S. A. 98, 7366–7370.
Ros, A.F.H., Bruintjes, R., Santos, R.S., Canario, A.V.M., Oliveira, R.F., 2004. The role of androgens in the trade-off between territorial and parental behavior in the Azorean rock-pool blenny, *Parablennius parvicornis*. Horm. Behav. 46, 491–497.
Schable, N.A., Fischer, R.U., Glenn, T.C., 2002. Tetranucleotide microsatellite DNA loci from the dollar sunfish (*Lepomis marginatus*). Mol. Ecol. Notes 2, 509–511.
Schradin, C., Anzenberger, G., 1999. Prolactin, the hormone of paternity. News Physiol. Sci. 14, 223–231.
Silverin, B., 1986. Corticosterone-binding proteins and behavioral effects of high plasma levels of corticosterone during the breeding period in the pied flycatcher. Gen. Comp. Endocrinol. 64, 67–74.
Specker, J.L., Kishida, M., 2000. Mouthbrooding in the black-chinned tilapia, *Sarotherodon melanotheron* (Pisces: Cichlidae): the presence of eggs reduces androgen and estradiol levels during paternal and maternal parental behavior. Horm. Behav. 38, 44–51.
Storey, A.E., Delahunty, K.M., McKay, D.W., Walsh, C.J., Wilhelm, S.I., 2006. Social and hormonal bases of individual differences in the parental behaviour of birds and mammals. Can. J. Exp. Psychol. 60, 237–245.
Sutton, S.G., Bult, T.P., Haedrich, R.L., 2000. Relationships among fat weight, body weight, water weight, and condition factors in wild Atlantic salmon parr. Trans. Am. Fish. Soc. 129, 527–538.
Svensson, O., Magnhagen, C., Forsgren, E., Kvarnemo, C., 1998. Parental behaviour in relation to the occurrence of sneaking in the common goby. Anim. Behav. 56, 175–179.
Tinbergen, N., 1951. The Study of Instinct. Clarendon Press, Oxford.
Trainor, B.C., Marler, C.A., 2001. Paternal behavior, aggression, and testosterone in the California mouse, *Peromyscus californicus*. Horm. Behav. 40, 32–42.
Trainor, B.C., Marler, C.A., 2002. Testosterone promotes paternal behaviour in a monogamous mammal via conversion to oestrogen. Phil. Trans. R. Soc. Lond., B-Biol. Sci. 269, 823–829.
Trivers, R.L., 1972. Parental investment and sexual selection. In: Campbell, B (Ed.), Sexual Selection and the Descent of Man. Aldine Press, Chicago, pp. 136–179.
van Iersel, J.J.A., 1953. An analysis of the parental behaviour of the male threespined stickleback (*Gasterosteus aculeatus* L.). Behaviour 3, 1–159 (Suppl).
Westneat, D.F., Sherman, P.W., 1993. Parentage and the evolution of parental behavior. Behav. Ecol. 4, 66–77.
Wingfield, J.C., Hegner, R.E., Dufty Jr., A.M., Ball, G.F., 1990. The "Challenge Hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136, 829–846.
Wingfield, J.C., Hegner, R.E., Lewis, D., 1991. Circulating levels of luteinizing hormone and steroid hormones in relation to social status in the cooperatively breeding white browed sparrow weaver, *Plocepasse mahali*. J. Zool. 225, 43–58.
Wynne-Edwards, K.E., 2001. Hormonal changes in mammalian fathers. Horm. Behav. 40, 139–145.
Young, L.J., Insel, T.R., 2002. Hormones and parental behavior, In: Becker, JS, Breedlove, SM, Crews, D, McCarthy, MM (Eds.), Behavioral Endocrinology, 2nd ed. MIT Press, Cambridge London, pp. 331–369.