

# Steroid hormones in bluegill, a species with male alternative reproductive tactics including female mimicry

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The proximate mechanisms underlying the evolution and maintenance of within-sex variation in mating behaviour are still poorly understood. Species characterized by alternative reproductive tactics provide ideal opportunities to investigate such mechanisms. Bluegill (*Lepomis macrochirus*) are noteworthy in this regard because they exhibit two distinct cuckold (parasitic) morphs (called sneaker and satellite) in addition to the parental males that court females. Here we confirm previous findings that spawning cuckold and parental males have significantly different levels of testosterone and 11-ketotestosterone. We also report, for the first time, that oestradiol and cortisol levels are higher in cuckolders than in parental males. The two cuckold morphs did not differ in average levels of any of the four hormones. However, among satellite males which mimic females in appearance and behaviour, there was a strong negative relationship between oestradiol levels and body length, a surrogate for age. This finding suggests that for satellite males, oestradiol dependency of mating behaviour decreases with increasing mating experience. Although such decreased hormone dependence of mating behaviour has been reported in other taxa, our data represent the first suggestion of the relationship in fishes.

**Keywords:** alternative reproductive tactics; *Lepomis*; cortisol; estradiol; androgens; female mimicry

## 1. INTRODUCTION

Distinct male alternative reproductive tactics (ARTs) have been described in a variety of taxa including fishes (Taborsky 1994). Species with ARTs typically have two specialized morphs that use either a territorial tactic or a sneaking tactic during breeding. Initial research into ARTs focused on game theory and sperm competition (reviewed in Parker 1990; Gross 1996; Taborsky 1998; see also Fu *et al.* 2001). More recently, the physiological mechanisms contributing to the development and expression of the morphs have received close attention, especially the

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contribution of chemical messengers that could mediate behavioural expression (Moore 1991; Oliveira 2006; Bass & Forlano in press).

Research into endocrine mechanisms underlying male ARTs initially focused on androgen levels and several studies documented morph differences (reviewed by Moore 1991; Brantley *et al.* 1993; Knapp 2003; Oliveira 2006). More recently, contributions of oestrogens have been documented. For example, production of oestrogens from androgens via aromatase in behaviourally relevant brain regions differs between ARTs in plainfin midshipman fish (*Porichthys notatus*; Schlinger *et al.* 1999; Forlano & Bass 2005). However, circulating levels of oestradiol have been investigated only in a few species (e.g. Oliveira 2006). Also, surprisingly, little attention has been given to the role of glucocorticoids despite potentially large differences in the energetic costs of the various tactics (Knapp 2003), and the possibility that glucocorticoids may thus shed light on whether male morphs in particular species represent evolutionarily stable strategies or are instead 'making the best of a bad situation' (Gross 1996; Moore *et al.* 1998).

In the present study, we determined circulating levels of four steroid hormones in spawning male bluegill that adopt one of three ARTs. Males from our study population are characterized by a discrete life history called 'parental' and 'cuckolder' (Gross & Charnov 1980). Parental males mature at about the age of 7 years and compete with one another for nesting sites in colonies, court and spawn with females, and provide sole parental care for the offspring (Gross 1982). Cuckolder males mature precociously at approximately 2 years of age and use a parasitic tactic to steal fertilizations from parental males. When small (approx. 2–3 years of age), cuckold males ('sneakers') use a sneaking tactic to streak spawn, and when large (approx. 4–5 years of age), cuckold males ('satellites') use female mimicry to gain access to nests (Dominey 1980; Gross 1982). Because a previous study (Kindler *et al.* 1989) found no difference in androgen levels between the two cuckold morphs, we hypothesized that oestradiol or cortisol may contribute to the profound differences in spawning behaviour between sneaker and satellite males for the reasons outlined above.

## 2. MATERIAL AND METHODS

Detailed methods are provided in the electronic supplementary material. Briefly, fish were collected from a spawning colony on 14 June 2004. Males were assigned to reproductive tactic based on direct observations of spawning behaviour, then were collected by hand net and immediately brought to a boat where blood sampling occurred. Only data from samples collected within 180 s were used to avoid confounding baseline cortisol levels with a stress response. Fish were then measured for body size.

Plasma hormone levels were measured using radioimmunoassay (see Magee *et al.* 2006 for details). Each sample was assayed in duplicate. For oestradiol, a few samples were considered technically 'non-detectable' because they fell outside the steep portion of the sigmoid standard curve. However, the standard curve equation still allowed estimation of the low oestradiol levels for these samples. Data were analysed using SYSTAT v. 11 (San Jose, CA) as outlined in the electronic supplementary material.

## 3. RESULTS AND DISCUSSION

As documented previously by Kindler *et al.* (1989), courting parental males had significantly higher levels

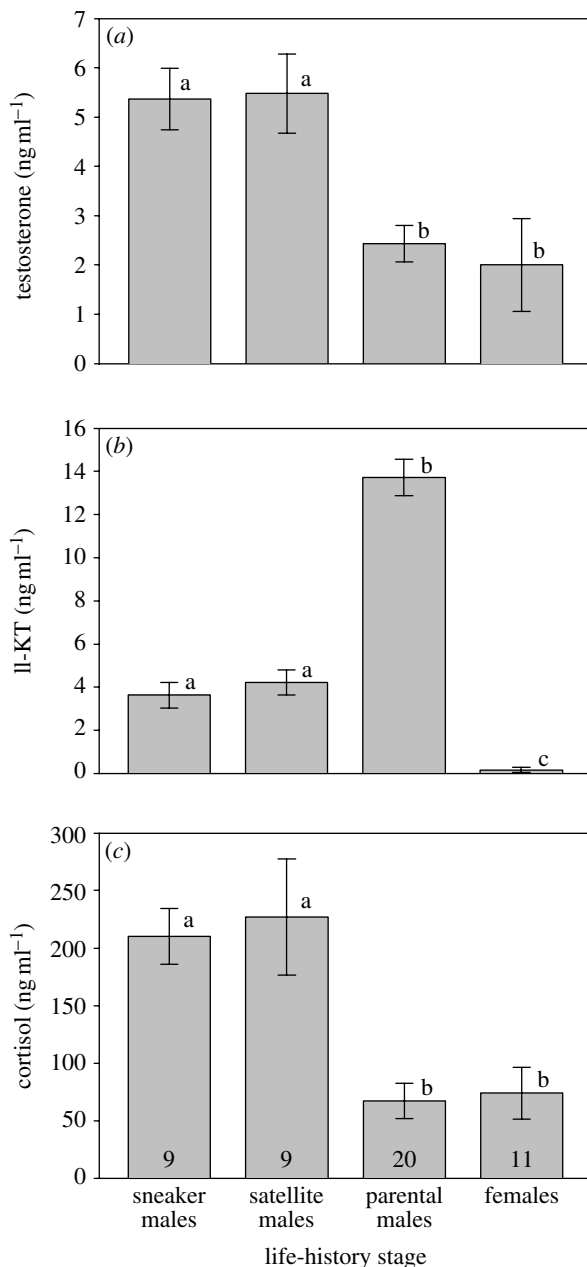


Figure 1. Mean ( $\pm$ s.e.) plasma androgen ((a) testosterone, (b) 11-KT) and (c) cortisol levels for the three male ARTs and females in bluegill on the day of spawning. Groups with different letters differ significantly from each other (*post hoc* Tukey's tests). Numbers in bars represent sample sizes.

of 11-ketotestosterone (11-KT) than cuckold males and females (ANOVA:  $F_{3,45} = 73.28$ ,  $p < 0.001$ ; figure 1). This pattern of 11-KT levels is consistent with other fishes with ARTs (Brantley *et al.* 1993; Oliveira 2006). In contrast, cuckold males had significantly higher testosterone levels than parental males and females ( $F_{3,45} = 7.63$ ,  $p < 0.001$ ; figure 1). This result differs from Kindler *et al.* (1989), who found no significant difference among the male morphs. The reason for the discrepancy between studies is unclear, but may be related to variation in social environment (see Oliveira 2004).

Cuckolder males also had significantly higher cortisol levels than parental males and females ( $F_{3,45} = 10.32$ ,  $p < 0.001$ ; figure 1). This result suggests that cuckold spawning behaviours are energetically

Table 1. Plasma oestradiol levels for the male ARTs and females in bluegill on the day of spawning.

	<i>n</i>	no. samples		median level (ng ml <sup>-1</sup> )
		detectable	non-detectable	
sneaker males	9	6	3	0.18
satellite males	9	8	1	0.25
parental males	20	10	10 <sup>a</sup>	0.05
females	11	11	0	1.98

<sup>a</sup> Significantly different from proportion of cuckold (sneaker + satellite) samples with detectable hormone levels (one-tailed binomial test:  $p = 0.02$ ).

more expensive or otherwise more stressful than courtship and spawning by parental males. However, reproduction by parental males is likely to be more energetically expensive overall when one considers the energetic costs of nest building and parental care. For example, we previously found that parental males' cortisol levels are low on the day of spawning relative to that during nest construction and the week-long care of the young (Magee *et al.* 2006). Parental males can also lose 10% of their body mass over the course of parental care (Coleman & Fischer 1991; Magee *et al.* 2006). Our glucocorticoid data from free-living animals are the first from fishes with ARTs and add only to a few other studies of vertebrates with ARTs (e.g. Mendonça *et al.* 1985; Leary *et al.* 2004). Hormone manipulation studies on tree lizards (*Urosaurus ornatus*) and plainfin midshipman have shown that the parasitic morphs are more sensitive to corticosterone than are the displaying morphs (Knapp & Moore 1997; Remage-Healey & Bass 2007). Thus, although it is premature to draw firm conclusions, it appears that glucocorticoids may play a larger role in parasitic morphs than in displaying or territorial males and thereby could factor into the costs and benefits of the various alternative tactics.

We also found that bluegill males' oestradiol levels were low relative to those of females, but cuckold males had higher levels than parental males (binomial test:  $p = 0.02$ ; table 1). Oestradiol levels are currently known from only five other fish species with two ARTs (Oliveira 2006). In two species, the pattern matches what we report here; in three species, oestradiol levels did not differ significantly between the morphs. Oestradiol's involvement in the expression of ARTs is also supported by neurological responses to oestradiol and morph differences in brain aromatase activity in plainfin midshipman (Schlinger *et al.* 1999; Forlano & Bass 2005; Remage-Healey & Bass 2007; but see Gonçalves *et al.* 2007).

We also explored the hormone dependency of behaviour. In this population of bluegill, as in many fishes, body length is highly correlated with age (Gross & Charnov 1980). We now report a significant negative relationship between body length and oestradiol for satellite males (figure 2). There was no other significant relationship with body length for any other hormones or male morphs (table 2). This finding

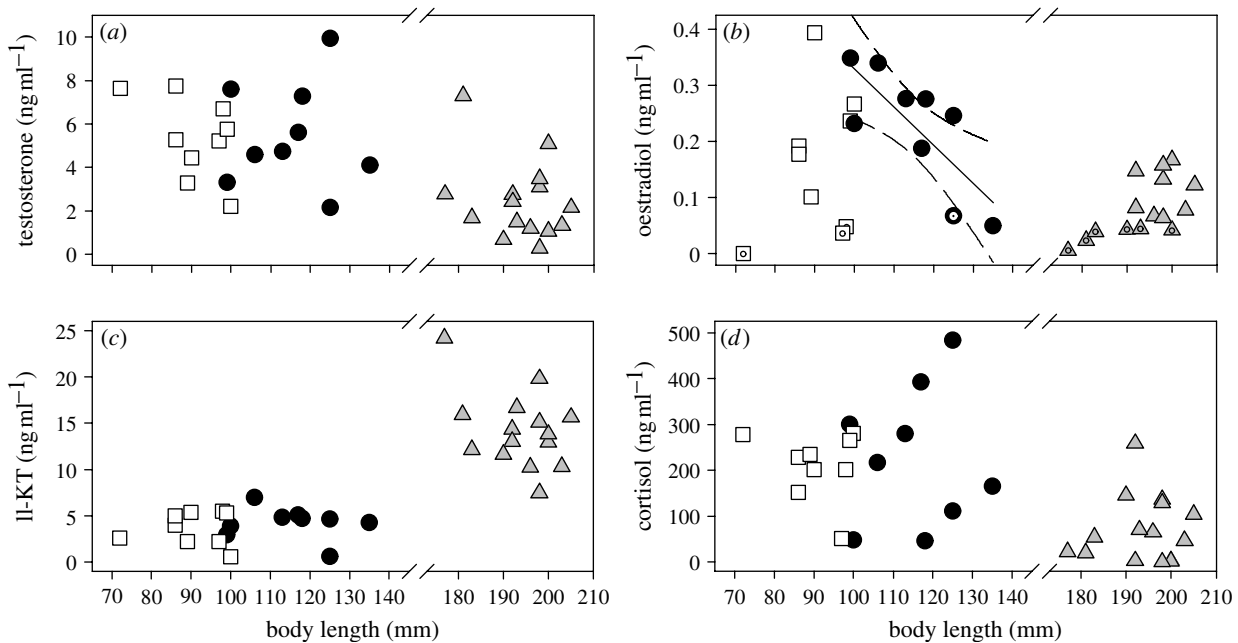


Figure 2. Relationships between body length and plasma hormone levels ( $\text{ng ml}^{-1}$ ) for the male ARTs in bluegill: (a) testosterone, (b) oestradiol, (c) 11-KT and (d) cortisol. Sneakers are represented by squares, satellites are represented by circles and parentals are represented by triangles. The one line (and 95% CI) is from the only significant linear regression (table 2). Dotted data points for oestradiol represent estimates of hormone levels that were below the level of detectability of our assay (see table 2).

Table 2. Results for simple linear regression analyses of body length versus plasma hormone level for the male ARTs in bluegill. (n.d., not determined because half of the parental males had oestradiol levels below the level of detectability in the radioimmunoassay. We did not correct for multiple comparisons because several pairs of hormone levels are connected via steroidogenic enzymes. Thus, each correlation with body length does not represent an independent test as assumed by common corrections (see electronic supplementary material for additional information). Italics indicate relationship where  $p < 0.05$ .)

hormone	sneaker males ( $n=9$ )			satellite males ( $n=9$ )			parental males ( $n=15$ )		
	$R^2$	$F_{1,7}$	$p$	$R^2$	$F_{1,7}$	$p$	$R^2$	$F_{1,13}$	$p$
testosterone	0.245	2.28	0.175	0.000	0.00	0.984	0.092	1.31	0.272
11-ketotestosterone	0.000	0.00	0.955	0.027	0.19	0.674	0.180	2.86	0.114
oestradiol	0.089	0.68	0.435	<i>0.599</i>	<i>10.46</i>	<i>0.014</i>	n.d.		
cortisol	0.042	0.31	0.596	0.010	0.07	0.800	0.006	0.08	0.779

suggests that there is a diminished dependency of female mimicry behaviour on oestradiol with increased mating experience. We cannot, however, rule out that the negative relationship between oestradiol levels and body length (age) instead reflects an increase in oestradiol receptor concentrations and, hence, an increased sensitivity to oestradiol levels in older satellite males. Regardless, our data support that the endocrine mediation of mating behaviour in satellite males changes with age and experience. Because decreases in hormonal dependency of mating behaviour with experience have been reported for males from several mammalian species (Hull *et al.* 2002), our finding opens exciting avenues for future research into the interplay between endocrinology and mating experience in ARTs.

The work conformed to guidelines of the Canadian Council on Animal Care and was approved by the University of Oklahoma Animal Care and Use Committee. We thank Jeff Stoltz, Tim Hain, Sunny Scobell, Elizabeth Adkins-Regan, Frank Phelan, Floyd Connor and Rich

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**Electronic Supplemental Material for Knapp, R. & Neff, B. D. “Steroid hormones in bluegill, a species with male alternative reproductive tactics including female mimicry”**

*Methods.* For the current study, bluegill were collected from a spawning colony on 14 June 2004. Nine to 10 males of each morph were collected between 1000 and 1410 h. Eleven females and an additional 10 parental males were sampled between 1540 and 1700 h. Males were assigned to reproductive tactic based on observations of spawning behaviour, Typically, several spawning observations were taken before individuals were collected. Given the experience of the authors with bluegill, we are confident that our classifications are accurate. Following identification of males to morph type, they then were collected by hand net and immediately brought to a boat where blood sampling occurred. Time to complete blood collection was recorded from the time the net broke the surface of the water until the end of sample collection. Only data from samples collected within 180 sec (mean  $\pm$  SE = 79.1  $\pm$  3.5 sec) were used to avoid confounding baseline cortisol levels with a stress response. The first group of fish were then euthanized using an overdose of clove oil, measured for total length (nearest 1 mm) and body mass (nearest 1 g), and various tissues collected for other studies. The females and the additional 10 parental males were brought back to the lab for use in another study (Stoltz & Neff 2006). Unfortunately, for this second group of fish, body mass and length were obtained from only some individuals due to an oversight.

Blood samples were collected from the caudal blood vessel using heparinized syringes and transferred to microcentrifuge tubes. Tubes were kept on ice or refrigerated for up to 8 hours before being centrifuged, after which the plasma was collected and stored at -20°C until assayed.

Steroid hormones (testosterone [T], 11-ketotestosterone [11KT], estradiol, and cortisol) were measured using radioimmunoassay following step-wise chromatographic separation of the steroids (see Magee *et al.* 2006 for details). Each sample was assayed in duplicate. All samples were run in a single assay, with a few samples that were outside the range of sensitivity of the 11KT assay being reassayed. Intra-assay coefficients of variation as calculated from four standard tubes per assay were 4.3% for T, 3.1 and 3.9% for 11KT, 9.0% for estradiol, and 0.6% for cortisol. Inter-assay variability for 11KT was 2.4%.

**Statistical analyses.** Hormone levels did not differ between parental males collected in the morning and afternoon and so data were combined for analyses (t-tests: all  $P > 0.11$ ).

Preliminary analyses revealed that there was a significant difference among phenotypes in time required to collect blood (ANOVA:  $F_{3,45} = 3.62$ ,  $P = 0.02$ ). However, there was no significant relationship between blood collection time and cortisol levels for any of the reproductive phenotypes (least squares regression: all  $P > 0.19$ ,  $R^2 < 0.23$ ). Nevertheless, to be conservative, initial ANCOVAs for the androgens and cortisol were conducted using time to collect blood as the covariate. In all cases, the covariate was non-significant (all  $P > 0.25$ ); the results we report are thus for ANOVAs without time as a covariate.

For estradiol, a few samples were considered technically “non-detectable” because they fell outside the steep portion of the sigmoid standard curve, which represented 5 pg/tube. However, the standard curve equation still allowed estimation of the low estradiol levels for these samples. Half of the parental males had estradiol levels that were designated non-detectable. To be conservative, we therefore report the median estradiol levels for the reproductive phenotypes. We used a binomial test to determine whether the parental males and

cuckolders (sneakers and satellites) differed significantly in the proportion of males with detectable estradiol levels.

We did not correct for multiple comparisons because several pairs of hormone levels are connected via steroidogenic enzymes and thus each correlation with body length does not represent an independent test as assumed by the correction. For example, estradiol is produced directly from testosterone via the action of aromatase. 11KT is produced from testosterone via two enzymes and an intermediate steroid, 11 $\beta$ -hydroxytestosterone. 11KT and cortisol can be tied to each other because 11 $\beta$ -hydroxytestosterone and cortisol are substrates for 11 $\beta$ -hydroxysteroid dehydrogenase and therefore could compete with each other for access to this enzyme, affecting final levels of both 11KT and cortisol (see Knapp 2003).

To help confirm that the negative relationship between body length and estradiol levels in satellite males is real, we re-ran the regression omitting the largest male. When we do this,  $R^2 = 0.639$ ,  $F_{1,6} = 4.142$ ,  $P = 0.088$ . Removing the second-largest male gives  $R^2 = 0.766$ ,  $F_{1,6} = 8.502$ ,  $P = 0.027$ . Because the two largest males also had the two lowest estradiol levels, we have confidence that the relationship is indeed real. We also calculated  $q$ -values as per Storey and Tibshirani (2003) using the recommended bootstrap option in QVALUE program (available at <http://faculty.washington.edu/~jstorey/qvalue>) to assess the positive False Discovery Rate (pFDR). pFDR is increasingly being suggested and used as a more appropriate alternative to Bonferroni-type corrections when conducting multiple comparisons (e.g., García 2003). We provide the  $q$ -values in Table S1 below for comparison to the  $R^2$  and  $P$  values from Table 2. The number of tests and distribution of  $P$  values yielded an expectation of no false positive tests when a total of 1 test was significant.

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Table S1. Results for simple linear regression analyses of body length versus plasma hormone level for the three male alternative reproductive tactics in bluegill (repeated from Table 2) plus  $q$ -values used to assess the positive false discovery rate.  $q$ -values represent the minimum false discovery rate (the number of false positives relative to the number of significant tests) for all tests considered simultaneously.

Hormone	Sneaker males (n = 9)			Satellite males (n = 9)			Parental males (n = 15)		
	R <sup>2</sup>	P	q	R <sup>2</sup>	P	q	R <sup>2</sup>	P	q
Testosterone	0.245	0.175	0.64	0.000	0.984	0.98	0.092	0.272	0.75
11-Ketotestosterone	0.000	0.955	0.98	0.027	0.674	0.98	0.180	0.114	0.63
Estradiol	0.089	0.435	0.96	<b>0.599</b>	<b>0.014</b>	0.15	ND		
Cortisol	0.042	0.596	0.98	0.010	0.800	0.98	0.006	0.779	0.98

ND = not determined because half of the parental males had estradiol levels below the level of detectability in the radioimmunoassay.