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Serum 11-Ketotestosterone and Testosterone Concentrations Associated with Reproduction in Male Bluegill (*Lepomis macrochirus:* Centrarchidae)[1]

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Male bluegill (*Lepomis macrochirus*) display a complex reproductive behavior involving two alternative life history pathways: delay of sexual maturation to become "parentals" or precocious maturation as "cuckolders." The purpose of our study was to investigate the association of two androgens, 11-ketotestosterone (11KT) and testosterone (T), with reproduction in these two types of males. Radioimmunoassay techniques were used to measure daily levels of the two androgens in the blood serum of parental male bluegill captured during the prespawning, spawning, and nesting periods throughout the reproductive season. Dramatic changes in the levels of 11KT and T were observed among parental males during these periods. Peaks occurred at the onset of spawning activity during each breeding bout. Compared to spawning parental males, spawning cuckolder males had significantly lower serum levels of 11KT. In contrast, the serum levels of T among parental and cuckolder males were not significantly different. These findings suggest that the elevated levels of 11KT are associated with the behaviors displayed by spawning parental males. The levels of T, however, seem to be associated with the occurrence of a phenomenon common to both parental and cuckolder males, such as development of gonads and/or spermiation.

The relationship between hormones and reproductive behavior has attracted considerable attention (Fostier *et al.,* 1983; Liley and Stacey, 1983). Two hormones in particular, 11-ketotestosterone (11KT) and testosterone (T), have been proposed to have important roles in various aspects of male fish reproduction, including maturation of the gonads, development of secondary sexual characters, and induction of reproductive behaviors (Matty, 1985).

A complex facet of fish reproduction is the existence of more than one male life history within a single population (Gross, 1984). For example, it is common for some individuals to have delayed maturity, while others have precocious sexual maturation. Some endocrinological aspects of precocious maturation have been studied previously only among certain salmonids (Crim and Evans, 1978; Stuart-Kregor *et al.,* 1981; Ueda *et al.,* 1983). In general, these studies have shown that during the spawning season "adult" males have higher gonadotropin and 11KT and T levels than precociously mature males. In the present study we investigate the production of 11KT and T associated with the alternative life histories and reproductive behaviors in male bluegill (*Lepomis macrochirus:* Centrarchidae).

Male bluegill have two alternative reproductive strategies (Gross, 1979; Dominey,

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1980; Gross and Charnov, 1980). Males of the "parental" strategy delay maturity until age 7 or 8 years (in Lake Opinicon, Ontario) (Gross, 1982). They construct nests in colonies, court and spawn with females, and guard the eggs and larvae in their nests against predation. These males have large black opercular flaps and dark orange breasts as secondary sexual characters. In contrast, males of the "cuckolder" strategy mature at age 1 or 2 years and may weigh only 15% as much as parental males (Gross, 1982). These precocious males neither build nests nor guard their offspring. Instead, they rapidly enter the nests of parental males during the spawning act, position themselves near the female, and extrude sperm in an attempt to fertilize some of the newly released eggs. If their presence in the nest is detected by the parental male, they are immediately and vigorously chased from the nest. As a result of their behavior, these males are termed "sneakers" (Gross and Charnov, 1980). Sneakers are not aggressive and display light body coloration and small opercular flaps.

As sneakers age and their body size increases, they switch to a satellite tactic, mimicking the behavior and appearance of females by developing a coloration with dark vertical bars (Dominey, 1980; Gross and Charnov, 1980). These males also have relatively small opercular flaps. "Satellite" males slowly enter the nest, usually without provoking aggression from the spawning parental male. Once in the nest, the satellite positions himself between the parental male and the true female to fertilize the eggs extruded by the female. Since parental males apparently cannot distinguish eggs fertilized by themselves from those fertilized by sneaker or satellite males (Gross, 1982), they exert parental care for both their own and the cuckolder males' progeny. Thus, cuckoldry in the bluegill is an evolutionarily viable alternative reproductive strategy (Gross, 1984).

The factors that determine whether a male bluegill will mature into the parental or cuckolder life history strategy are poorly understood. Through a series of *in vitro* crosses, Gross and Philipp (unpublished results) have demonstrated the existence of genetic factors governing the alternative life histories. However, the biochemical factors mediating this genetic influence are still unknown. Furthermore, the functional relationship between physiological factors and various aspects of male sexual behavior remains to be identified.

In the present study, we first present the patterns of variation in serum levels of 11KT and T among parental male bluegill sampled during the prespawning and spawning periods of a single breeding bout. We also present changes in the levels of these steroids among parental males collected from colonies during spawning and postspawning periods through an entire reproductive season. Next, we compare the amounts of 11KT and T that are found in the serum of parental, sneaker, and satellite males during spawning. Finally, we discuss the possible relationships between measured serum androgen levels and the two alternative male bluegill reproductive behaviors.

METHODS

Specimens. All fish were collected from Lake Opinicon (44°34'N,76°19'W), Ontario. Because the Lake Opinicon bluegill population is under intensive study, individual males within a breeding colony can be sampled during each component of the reproductive cycle. Individual fish were captured without disturbing the colony by swimmers using hand nets. Three sets of collections were made. In 1986, parental males were collected from each of the five colonies that formed sequentially at the same breeding site through the entire reproductive season (June–July). In June 1987, both parental and cuckolder males were sampled over a 2-day period from within several colonies in the lake during spawning. Cuckolder males were individually assigned to either the sneaker or satellite category based on the spawning behavior observed by divers during collection. Finally, in 1986, about 200 live adult male and female bluegill were transported to Illinois Natural History Survey (INHS) experimental ponds in Champaign, Illinois. These adults successfully bred during the 1987 reproductive season and were sampled

in May 1988, prior to and during the first breeding bout.

Blood samples. Live fish were bled by heart puncture immediately following capture. The blood samples were held overnight at 4° to allow clotting. Serum was obtained from each sample by centrifugation at 3000*g* for 10 min, after which it was frozen at −20° until assayed. All fish and serum samples were handled similarly during these manipulations.

Synthesis of labeled 11KT. Hydrocortisone (250 μC of [1,2,6,7-^{3}H(N)]) (New England Nuclear, Boston, MA) was first oxidized to 4-androsten-11-ol-3,17-dione using sodium bismuthate, as described by Brooks and Norymberski (1953). The oxidation product was extracted three times with a twofold excess of anesthesia-grade ether and resuspended in acetone. The resulting solution was cooled on ice to 0° and then Jones' reagent (H_2CrO_4/H_2SO_4) was added in excess, as indicated by the solution's orange color. Next, the reaction mixture was warmed to room temperature and stirred for 1 hr. The resulting product, 4-androsten-3,11,17-trione, was extracted with ether as described above, resuspended in potassium phosphate buffer (50 m*M*, pH 7.0) containing 20 m*M* NADH, and reacted with 0.5 IU 17β-hydroxysteroid dehydrogenase (Payne and Talalay, 1985). The final [^{3}H]11KT product was purified on a Sephadex LH-20 column (Isolab Inc., Akron, OH) using methylene chloride–methanol (98:2) as the eluent.

Thin-layer chromatography (TLC) was used to confirm the validity of reactions employed for the synthesis of [^{3}H]11KT. Unlabeled hydrocortisone was treated as described earlier. The product of each reaction, as well as the appropriate steroid standards (Steraloids Inc., Wilton, NH), were applied to silica gel TLC plates (60 F_{254}, Merck, West Germany) and developed in cyclohexane:ethyl acetate (1:1). Upon completion of the chromatographic separation, plates were sprayed with a 20% solution of phosphomolybdic acid in methanol and placed in an oven for 10 min at 100° to allow for the visualization of the steroids. Positions of every reaction product were established and then shown to be identical with positions of the proper steroid standards, as determined by calculation of R_f values.

Steroid radioimmunoassays (RIA). Both androgens (11KT and T) were measured in blood serum according to RIA procedures reported previously (Bahr *et al.*, 1980, 1983). All serum samples obtained from parental male bluegill were assayed individually for 11KT and T. However, since only a very small amount of serum could be obtained from individual sneaker or satellite males, equal amounts of serum from 10 sneaker males or from 6 satellite males were pooled and treated as a single sample. All samples for 11KT and T determinations were extracted with anesthesia-grade ether followed by extraction with a solution of 1 ml hexane and 1 ml 75% methanol. After removal of the hexane phase, methanol was evaporated and samples were reconstituted in PBS-Gel.

The T antibody, generated against testosterone-11-hemisuccinate:BSA in rabbits, cross-reacts <1% with progesterone and estradiol (Bahr *et al.*, 1983). We also established that this T antibody cross-reacts <5% with 11KT and that the 11KT antibody cross-reacts <5% with T and <1% with dihydrotestosterone and adrenosterone.

The assays for 11KT and T in bluegill males were validated by determining parallelism and recovery of unlabeled ligand and comparing values of chromatographed and nonchromatographed samples. When tested for parallelism, pooled serum samples of 25, 50, and 100 μl contained 0.73 ± 0.02, 0.62 ± 0.01, and 0.69 ± 0.01 ng/ml (mean ± SE of two determinations) of 11KT, respectively. Similarly, volumes of 5, 25, and 45 μl of different pooled samples contained 10.30 ± 0.40, 9.86 ± 0.50, and 8.26 ± 0.25 ng/ml of T. The recovery of exogenously added 11KT or T to pooled serum samples resulted in an average recovery of 99 ± 3 and 92 ± 6%, respectively. Finally, the concentrations of 11KT and T in nonchromatographed and chromatographed samples were compared. Samples of 11KT and T were eluted from celite:propylene glycol (1:1) columns using 7 ml of each of the following ratios of isooctane:ethyl acetate: (1) 100:00, (2) 85:15, (3) 70:30, and (4) 60:40 (Abraham *et al.*, 1970, 1971). Values for 11KT and T in chromatographed and nonchromatographed serum were 2.1 ± 0.2 ng/ml vs 2.2 ± 0.1 ng/ml and 3.3 ± 0.3 and 3.2 ± 0.3 ng/ml for 11KT and T, respectively. Interassay and intraassay coefficients of variation were 12 and 3% for 11KT and 16 and 5% for T.

Statistical analysis. Analyses were performed using the Statistical Analysis System (SAS Users Guide, 1985). One and two-way ANOVA were used to analyze the data, and the statistical rejection level for similarity was $P < 0.05$.

RESULTS

Serum concentrations of 11KT and T in parental male bluegill increased from low levels during the prespawning period (Days −19 to −1) to a peak level on the day of spawning (Day 0; Fig. 1). This peak level was significantly higher than levels detected on any other day (one-way ANOVA, $P < 0.001$, followed by Student–Newman–Kuels (SNK) multiple range tests). The levels of T in all cases were

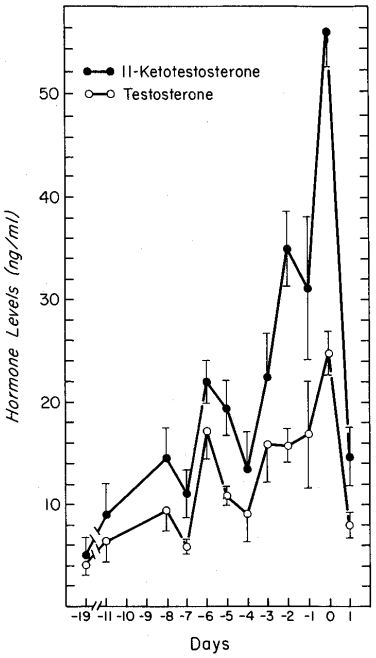

FIG. 1. Serum 11-ketotestosterone and testosterone concentrations ($\bar{x} \pm$ SE) in parental male bluegill during the prespawning and spawning periods of the first breeding bout for the 1988 reproductive season. Individual sample sizes varied from 5 to 12 males ($\bar{x}$ parental male length = 198.6 ± 3.2 mm; $\bar{x}$ weight = 165.6 ± 6.2 g, N = 99). Day 0 is the day of spawning.

lower than levels of 11KT during this period, with statistically significant differences on Days −8, −7, −5, and from Day −2 to Day 1 (two-way ANOVA, $P < 0.001$, followed by paired t tests).

Serum 11KT and T concentrations among nesting parental male bluegill exhibited a marked pattern of variation during each breeding bout (Fig. 2). Five distinct breeding bouts occurred during the 1986 reproductive season, as confirmed by direct daily underwater observations. Except for breeding bout 1, 11KT measured during spawning (Day 0) significantly exceeded 11KT levels detected on the following parental care days (Days 1–8; one-way ANOVA, $P < 0.01$, followed by SNK tests). The profiles for T were in general similar to those of 11KT. However, for each breeding bout, T levels were always significantly lower than those of 11KT when measured on Day 0 (two-way ANOVA, $P < 0.01$, followed by standard t tests).

Serum concentrations of 11KT and T during spawning were compared among parental, sneaker, and satellite males (Fig. 3). Parental males had the highest concentrations of 11KT (13.8 ± 1.7 ng/ml), differing significantly from both sneaker and satellite males (1.2 ± 0.1 and 0.9 ± 0.4 ng/ml, respectively; two-way ANOVA, $P < 0.001$, followed by Tukey's studentized range (HSD) test). Serum T levels, however, were not significantly different among the three types of males (4.4 ± 0.4, 3.5 ± 0.1, and 2.6 ± 0.7 ng/ml, respectively; HSD test, $P > 0.05$). Interestingly, serum levels of 11KT were significantly higher than those of T when compared among parental males (13.8 ± 1.7 ng/ml vs 4.4 ± 0.4 ng/ml; paired t test, $P < 0.0001$). In contrast, levels of 11KT were significantly lower than levels of T detected both in sneaker males (1.2 ± 0.1 ng/ml vs 3.5 ± 0.1 ng/ml; paired t test, $P < 0.0001$) and in satellite males (0.9 ± 0.4 ng/ml vs 2.6 ± 0.7 ng/ml; paired t test, $P < 0.01$).

DISCUSSION

The following findings are reported in our study. (1) In parental male bluegill, 11KT and T levels fluctuate greatly throughout the reproductive season and are highest at the initiation of spawning. (2) During spawning: (a) Levels of 11KT among parental males are significantly higher than those among sneaker or satellite males; (b) levels of T in parental males are not significantly different from those in cuckolder males; and (c) levels of 11KT are significantly

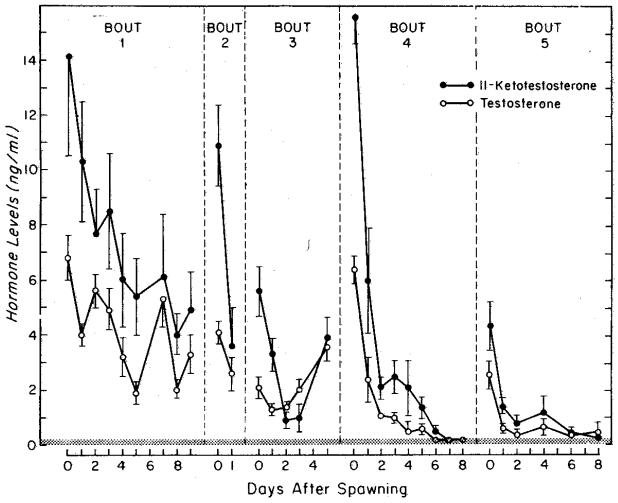

FIG. 2. Serum 11-ketotestosterone and testosterone concentrations ($\bar{x}$ ± SE) in parental male bluegill during the five breeding bouts of the 1986 reproductive season. The shaded area represents undetectable levels of the steroid. Individual sample sizes varied from 7 to 10 males ($\bar{x}$ parental male length = 175.9 ± 0.9 mm; $\bar{x}$ weight = 99.1 ± 1.6 g, N = 278). For each breeding bout, Day 0 is the day of spawning. Except for breeding bout 2, the last day of collection represents the parental care day immediately prior to fry and parental males leaving the colony. In breeding bout 2, few eggs were spawned and all males deserted the colony 2 days later.

higher than levels of T among parental males but the reverse is true among cuckolder males.

In Lake Opinicon, Ontario, 11KT and T concentrations among parental male bluegill rise from very low or undetectable levels during winter and early spring (unpublished results) to a peak at spawning. However, during the bluegill reproductive season there are usually three to five distinct breeding bouts. For any given breeding bout, some as yet unknown environmental or social trigger causes only a subset of the awaiting parental males to initiate colony formation and nest building behavior (J. E. Claussen *et al.*, unpublished results). It is most likely that only the subset of parental males taking part in a given breeding bout experiences these peaks in 11KT and T. This pattern in which 11KT and T reach the peak right at spawning in parental male bluegill differs from that of most other male teleosts (Fostier *et al.*, 1983) in which the peak precedes the spawning, but is similar to that of another repeated spawner, the mummichog, *Fundulus heteroclitus* (Cochran, 1987).

The changing levels of 11KT and T among parental males during the reproductive season suggest that both of these steroids have some role(s) in bluegill reproduction. These results are consistent with conclusions reported by Smith (1969) that nest building, nest defense, and perhaps other prespawning behaviors in centrarchids are closely associated with serum levels of gonadal androgens. These results are also consistent with Smith (1969, 1970) and Kramer (1972) who reported that the postspawning parental care behavior in

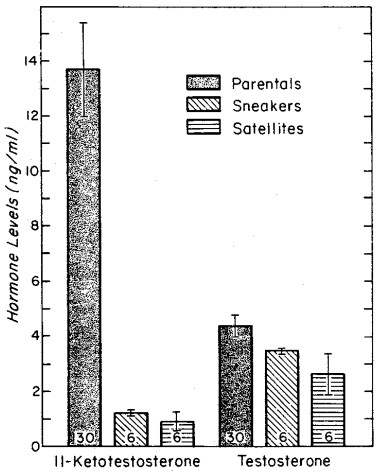

FIG. 3. Serum 11-ketotestosterone and testosterone concentrations in parental, sneaker, and satellite male bluegill during spawning ($\bar{x} \pm$ SE). Individual samples sizes are shown (parental males: $\bar{x}$ length = 175.5 ± 1.4 mm, N = 30 males; sneaker males: $\bar{x}$ length = 73.0 ± 1.7 mm, N = 6; satellite males: $\bar{x}$ length = 105.3 ± 2.0 mm, N = 6).

bluegill is under control of other hormones and/or environmental factors.

However, it also has been reported recently that during spawning activities, pronounced endocrine changes take place as a result of social interactions between conspecific individuals of the goldfish, *Carassius auratus* (Kyle *et al.*, 1985), and rainbow trout, *Salmo gairdneri* (Liley *et al.*, 1986). It is currently unclear whether changing environmental/climatic conditions promote the observed changes in serum androgen levels detected in parental male bluegill or whether these changes are a response to social interactions among conspecifics. It is also unclear as to how direct a role these elevated steroid levels play in governing specific aggressive and/or reproductive behaviors. However, it is clear that there is a strong association between the precipitous rise and fall of 11KT and T and spawning among parental male bluegill.

It is interesting to note the absolute differences in androgen peak heights between parental male bluegill spawning in Lake Opinicon (Figs. 2 and 3) and parental male bluegill moved from Lake Opinicon to spawn in Illinois (Fig. 1). We have no direct explanation for these differences, but speculate that they are associated with climatic, geographic, and/or population density alterations.

Cuckolder males, however, are not involved in nest construction or courtship activities and, therefore, do not exhibit the extreme aggressive behavior of parental males (Gross, 1982). Interestingly, our results showed that during spawning, serum 11KT levels in both sneaker and satellite males were significantly lower than those in parental males. Serum T levels measured among the three types of males, though, were not significantly different. In fact, in contrast to parental males, 11KT levels in cuckolders were significantly lower than levels of T. It would seem most likely, therefore, that the elevated serum levels of 11KT in parental males are associated with the development of secondary sexual characters and parental-specific behaviors such as colony formation, nest construction, and/or courtship of females. The similarities between the serum levels of T among parental and cuckolder male bluegill would seem to indicate that this hormone may be associated with some physiological/behavioral phenomenon common to both male types, such as gonadal development and/or spermiation. In any case, it is clear that the parental and cuckolder male bluegill differ not only behaviorally, but physiologically as well.

The present study provides new insights into the endocrine changes associated with the variety of reproductive behaviors observed among fish. Because male bluegill exhibit both parental and cuckolder reproductive strategies, multidisciplinary investigations of the behavioral, physiological, and genetic bases for these alternative male

life histories present unique opportunities to improve our understanding of the evolution and regulation of fish reproductive behavior.

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