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Social Interactions and Cortisol Treatment Increase the Production of Aggressive Electrocommunication Signals in Male Electric Fish, *Apteronotus leptorhynchus*

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Brown ghost knife fish, *Apteronotus leptorhynchus*, continually emit a weakly electric discharge that serves as a communication signal and is sensitive to sex steroids. Males modulate this signal during bouts of aggression by briefly (~15 ms) increasing the discharge frequency in signals termed "chirps." The present study examined the effects of short-term (1–7 days) and long-term (6–35 days) male–male interaction on the continuous electric organ discharge (EOD), chirping behavior, and plasma levels of cortisol and two androgens, 11-ketotestosterone (11KT) and testosterone. Males housed in isolation or in pairs were tested for short-term and long-term changes in their EOD frequency and chirping rate to standardized sinusoidal electrical stimuli. Within 1 week, chirp rate was significantly higher in paired fish than in isolated fish, but EOD frequency was equivalent in these two groups of fish. Plasma cortisol levels were significantly higher in paired fish than in isolated fish, but there was no difference between groups in plasma 11KT levels. Among paired fish, cortisol levels correlated positively with chirp rate. To determine whether elevated cortisol can cause changes in chirping behavior, isolated fish were implanted with cortisol-filled or empty Silastic tubes and tested for short-term and long-term changes in electrocommunication signals and steroid levels. After 2 weeks, fish that received cortisol implants showed higher chirp rates than blank-implanted fish; there were no difference between groups in EOD frequency. Cortisol implants significantly elevated plasma cortisol levels compared to blank implants but had no effect on plasma 11KT levels. These results suggest that male–male interaction increases chirp rate by elevating levels of plasma cortisol, which, in turn, acts to modify neural activity though an 11KT-independent mechanism. © 2002 Elsevier Science (USA)

Key Words: **electrocommunication; cortisol; 11-ketotestosterone; steroid; glucocorticoids; androgen; electric fish; aggression; *Apteronotus leptorhynchus*; social behavior.**

Most South American knife fish (Order Gymnotiformes) produce weak electric signals that are used both for locating objects in the environment and for social communication. In many species, the electric organ discharge is remarkably constant (Moortgat, Keller, Bullock, and Sejnowski, 1998), but fish can modify the frequency, amplitude, and/or waveform of the discharge in response to changes in the social environment. These modulations of the electric organ discharge (EOD) range from short (~15 ms) transient signals used during agonistic interactions to long-term adjustments (days to months) used in sex recognition during the breeding season. Steroids influence the production of these signals, and, because many of the neural pathways generating these signals are well defined, electric fish are excellent model organisms for examining steroidal regulation of behavior (Zupanc and Maler, 1997; Zakon and Dunlap, 1999).

The brown ghost knife fish, *Apteronotus leptorhynchus,* was used to examine the relationship between social interactions, plasma steroids, and the production of two electrocommunication signals: the continuous EOD and rapid EOD modulations termed "chirps." These signals originate in distinct brain regions, and they are both sexually dimorphic and influenced by androgens. The continuous EOD is generated by the spontaneous firing of the pacemaker

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nucleus in the hindbrain. EOD frequency is higher in males than females and is increased by androgen treatment (Schaefer and Zakon, 1996; Dunlap, Thomas, and Zakon, 1998).

Chirps are produced when the prepacemaker nucleus causes the pacemaker activity to increase in frequency and decrease in amplitude for brief (~15 ms) periods. Although fish chirp occasionally when they are isolated, their chirp rate increases dramatically when occupying the same tank as another conspecific. In these circumstances, chirping is usually accompanied by attacks and bites, indicating that chirps function in aggressive signaling (Hagedorn and Heiligenberg, 1985; Dunlap *et al.*, 1998). Chirping can also be reliably elicited by presenting a fish with an artificial electrical signal that is approximately the same frequency as its EOD (Larimer and Macdonald, 1968; Zupanc and Maler, 1993; Dunlap *et al.*, 1998). Fish often attack the electrodes through which these artificial signals are presented, suggesting that such evoked chirps are also aggressive signals.

Like the continuous EOD frequency, chirping is sexually dimorphic and steroid-sensitive. Compared to females, males chirp at higher rates toward conspecific fish and toward artificial stimuli. Chirp rate in males exposed to other males correlates with endogenous levels of 11-ketotestosterone (11KT) (Dunlap, 2002), and chirp rate toward artificial stimuli increases in females treated with testosterone (T) and dihydrotestosterone (Zupanc and Maler, 1993; Dulka and Maler, 1994; Dunlap *et al.*, 1998).

In many fish, as well as other vertebrates, aggressive interactions can activate the hypothalamic–pituitary–interrenal (adrenal) axis leading to a rise in plasma glucocorticoids (Svare, 1983; Becker, Breedlove, and Crews, 1992). In fish, the principal glucocorticoid is cortisol, and cortisol profiles following agonistic interactions often depend on the long term social consequences of the interactions: subordinate or nonterritorial males often have higher cortisol levels than dominant and territorial males (Ejike and Schreck, 1980; Fox, White, Kao, and Fernald, 1997; Winberg and Lepage, 1998; Overli, Harris, and Winberg, 1999; but see Hannes, 1984; Hannes, Franck, and Liemann, 1984). In addition, experimental elevation of cortisol levels can affect the expression of agonistic behavior (Munro and Pitcher, 1985), indicating that cortisol may cause as well as respond to changes in social interactions. In many species, aggressive behavior is also regulated by androgens (Fernald, 1976; Villars, 1983), although in other species the contribution of androgens is equivocal (e.g., in *Lepomis* sunfish; Smith, 1969; Kramer, 1973; Kindler, Bahr, and Philipp, 1991). The effects of cortisol on agonistic behavior could be direct actions on behaviorally important brain nuclei or indirect effects via the hypothalamo–pituitary–gonadal axis (Carragher, Sumpter, Pottinger, and Pickering, 1989; Orr and Mann, 1992). Thus, long-term social interactions could influence aggressive behavior by altering circulating levels of cortisol, androgens, both, or neither. The following experiments tested whether male interactions modify the production of an aggressive electrocommunication signal and, if so, whether corresponding increases in cortisol or one particular androgen, 11KT, contribute to these behavioral changes.

MATERIALS AND METHODS

All fish were obtained commercially and housed separately in 38-L tanks that were part of a 1235-L circulating system. Water temperature was kept between 26 and 28°C, water conductivity was 500–600 μS/cm, and the light cycle was 12L:12D. Fish were fed frozen brine shrimp and blood worms every other day. All procedures used in this study adhered to ethical standards of animal use specified by the National Institutes of Health (DHEW Publication 80–23).

Measurement of Chirp Rate and EOD Frequency

A chirp testing apparatus similar to that described previously (Dulka and Maler, 1994; Dunlap *et al.*, 1998) was used. This apparatus consisted of a cylindrical PVC tube (length = 17.7 cm, inner diameter = 3.8 cm) covered with nylon mesh at one end and a removable plastic gate at the other. Two silver electrodes for recording the fish's EOD were placed at each end of the tube. These electrodes were connected to an amplifier (Grass Instruments, West Warick, RI; P55) that in turn was connected to an oscilloscope (Tektronix, Beaverton, OR; TDS 210) and audio speaker. Stimuli were presented to the fish through two carbon rods, each 12 cm in length, attached to the sides of the tube. These electrodes were connected to a function generator (Pasco Scientific, Roseville, CA; PI-9587C). The field strength of the stimulus midway between the electrodes was ~1.5 mV/cm during all testing periods.

For each test, the chirp testing apparatus was placed in the fish's home aquarium. To minimize handling stress, the fish was allowed to voluntarily swim into the apparatus or was gently guided into the apparatus

with a net. After a 5-min acclimation period, the EOD was collected through the recording electrodes. EOD frequency was measured by a frequency counter in the oscilloscope and subsequently standardized to a temperature of 28°C using a Q_{10} of 1.62 (Dunlap, Smith, and Yekta, 2000).

To initiate chirps, a fish was stimulated with a sine wave stimulus ~5 Hz below its own frequency (−5-Hz difference frequency (Df)) for 30 s, and chirps were detected from the audio output of the speaker. Chirp rate was quantified as the number of chirps per 30 s.

Social Interaction

Male fish were initially housed in isolation for at least 2 weeks prior to the experiment. Fish were then divided into two groups, one in which individuals remained isolated and another in which they were paired. Both fish in a pair had similar EOD frequencies (within 5–16 Hz), chirp rates (within 2–8 chirps/30 s), and body size (within 1–4 mm) based on prepairing measurements.

Pairs were housed in 38-L aquaria. In previous studies, pairs of males housed with full contact to each other frequently killed each other (personal observation) and so, in this experiment, fish were separated with dividers that partitioned the aquaria into two equal compartments. Each divider consisted of a plastic grid (1 × 1 cm) overlaid with nylon mesh (1 × 1 mm). The divider allowed electrical, chemical, and limited visual communication, but prevented physical contact. Isolated fish were also housed in 38-L aquaria partitioned equally by dividers. Dividers were placed in the aquaria of isolated fish to keep all fish at equal density. At the time of pairing, all fish were moved from their original aquaria to eliminate the possibility that any preexisting territoriality would alter the results.

Three experiments were conducted to examine social interaction and the EOD. Experiment 1 examined behavioral (Fig. 1A) and hormonal changes (Table 1) occurring within 7 days of pairing, experiment 2 examined behavioral changes occurring from 6 to 32 days postpairing (Fig. 2A), and experiment 3 examined hormonal differences between groups at 7 days postpairing (Table 1). In experiment 1, 18 male fish housed in isolation were tested for chirp rate and EOD frequency every 2 days over a 4-day period. At the end of the prepairing period, 12 fish were grouped into six pairs; 6 fish were kept isolated as controls. Fish of differing chirp rate (chirps/30 s), EOD frequency (Hertz), and body size (centimeters) in prepairing measurements were distributed evenly between isolated and paired treatment groups. (For isolated fish, means ± SE were 29 ± 5 chirps/30 s, 959 ± 11 Hz, and 13.2 ± 0.5 cm. For paired fish; means ± SE were 28 ± 6 chirps/30 s, 946 ± 12 Hz, and 14.2 ± 1.0 cm.) The fish were tested as above for chirp rate and EOD frequency at 1 day (24–26 h), 3 days, and 7 days postpairing and bled 6 h after the final chirp test at 7 days postpairing for determination of hormone levels.

In experiment 2, 24 males housed in isolation were tested for chirp rate and EOD frequency every 2–3 days over a 6-day period. Sixteen fish were then grouped into eight pairs; eight fish were kept isolated as controls. Fish of differing chirp rate, EOD frequency, and body size in prepairing measurements were again distributed evenly to isolated and paired treatment groups. (For isolated fish, means ± SE were 70 ± 3 chirps/30 s, 960 ± 12 Hz, and 15.0 ± 0.2 cm. For paired fish; means ± SE were 67 ± 2 chirps/30 s, 962 ± 8 Hz, and 15.2 ± 0.1 cm.) Fish were then chirp-tested every 5–6 days over a 32-day period. These fish were not bled.

In experiment 3, 13 male fish were placed in pairs (n = 8) or kept in isolation (n = 5). Mean body lengths were 13.3 ± 0.6 and 13.8 ± 0.8 cm, respectively. Fish were bled 7 days postpairing for determination of steroid hormone levels; day 7 postpairing corresponded to the day when the behavioral response in the previous experiments was most pronounced. No behavioral data were collected on these fish.

Cortisol Treatment

Male fish housed individually were tested for chirp rate and EOD frequency. Fish were then divided into two groups. One group received Silastic implants (Konigsberg Instruments, Inc., Pasadena, CA; Catalog No. VST025047) containing cortisol (Sigma, St. Louis, MO, hydrocorticosone H-4001) and the other group (controls) received empty Silastic tubes. Again, fish that differed in chirp rate, EOD frequency, and body size in prepairing measurements were distributed evenly between treatment groups. Silastic tubes were sealed at each end with silicone rubber sealant (Dow Corning, Midland, MI, RTV 734). Tubing was 0.63 mm i.d. × 1.19 mm o.d., and implants varied in length depending on body size (3 mm/10 g body weight). Each filled implant contained ~1–2 mg of cortisol. Before implantation, all fish were anesthetized using 2-phenoxyethanol (0.075%; Sigma, P-1126). All im-

plants were placed in the peritoneal cavity through a small hole made with an 18-gauge needle.

Three experiments involving cortisol treatment were conducted. Experiment 4 examined behavioral (Fig. 2A) and hormonal changes (Table 1) within 7 days of implantation, experiment 5 examined behavioral changes from 15 to 35 days postimplantation (Fig. 2B), and experiment 6 examined hormonal differences between groups at 14 days postimplantation (Table 1). In experiment 4, 23 males were chirp-tested twice over a 5-day period. Twelve males were then implanted with cortisol-filled tubes, and 11 were implanted with empty tubes. Mean body lengths were 14.1 ± 0.9 and 13.8 ± 0.8 cm, respectively. Fish were chirp-tested at 1 day (23–26 h), 3 days and 7 days postimplantation and bled 6–8 h after the final chirp test. In experiment 5, 18 males were chirp-tested every 2 days over a 6-day period. Nine fish were then implanted with tubes containing cortisol and 9 were control implanted. Mean body lengths were 14.0 ± 1.1 and 14.3 ± 0.7 cm, respectively. Beginning at day 15 after implantation, fish were chirp-tested every 3–4 days for a total of five times over the next 21-day period. These fish were not bled. In experiment 6, 10 fish were implanted with cortisol ($n = 7$) or empty tubes ($n = 3$). Mean body length was 13.1 ± 0.6 and 13.9 ± 1.3 cm, respectively. Fish were bled 15 days postimplantation, corresponding to the first significant behavioral response from experiment 5. No behavioral data were collected on these fish.

Blood Sampling and Steroid Analysis

To collect blood, fish were first anesthetized as above. Heparinzed syringes and needles (27-gauge) were then used to draw ~75–200 μl blood from the caudal vein of each fish. Blood was collected within 3 min of capture. In other fish species, cortisol does not increase until >4 min after handling (Pottinger and Moran, 1993; Fox *et al.,* 1997), and thus the results in the present study were probably affected minimally by handling-induced secretion of cortisol. Blood was transferred to 1-ml Eppendorf tubes and placed on ice for 1–2 h. Fish were weighed and returned to their home tanks. Blood was then centrifuged for 15 min at 7000 rpm. Plasma was collected and stored at −80°C until radioimmunoassay.

Plasma concentrations of cortisol, 11KT, and T were measured by radioimmunoassay following column chromatography (Knapp, Wingfield, and Bass, 1999). Plasma samples were incubated overnight with 2000 cpm of each radiolabeled steroid to allow recovery calculations for each individual sample. Samples were then extracted with 5 ml dichloromethane. The organic fraction was collected and dried under nitrogen. Samples were resuspended in 10% ethyl acetate in iso-octane and loaded onto diatomacous earth–glycol columns for steroid separation. Increasingly polar mixtures of ethyl acetate in iso-octane were run through the columns under nitrogen to separate the steroids of interest: 4 ml of 0% (discard), 4 ml of 10% (T), 4 ml of 20% (estradiol), 4 ml of 30% (11KT), and 4.5 ml of 52% (cortisol). Each fraction was dried under nitrogen and resuspended in phosphate-buffered saline containing 0.1% gelatin. Each sample was assayed in duplicate. Testosterone antibody (Wien Laboratories, Succasunna, NJ) was used for both androgen assays. Cortisol antibody was purchased from Endocrine Sciences (Agoura Hills, CA). Bound steroid was separated from free steroid using a dextran–charcoal solution in phosphate-buffered saline (without gelatin).

Hormone concentrations were measured in two assays. Intra-assay variation was 6.4–7.9% for T, 19.1–19.6% for 11KT, and 3.9–6.0% for cortisol; inter-assay coefficients of variation were 2.3% for T, 5.5% for 11KT, and 14.0% for cortisol. The recoveries were 72.2–89.9% for T, 84.9–94.4% for 11KT, and 61.5–66.1% for cortisol. The level of detectability for a 50-μl plasma sample was approximately 0.4 ng/ml for T, 0.2 ng/ml for 11KT, and 2.7 ng/ml for cortisol. Assay accuracy was 92.7% for T, 78.8% for 11KT, and 95.5% for cortisol based on quadruplicate standards run simultaneously with the samples. Curves generated by serial dilution of fractions from a pooled plasma sample were parallel to the standard curves for 11KT and cortisol. The pooled sample had too little T for the serial dilution to be within the range of the standard curve. Most fish had undetectable levels of T and so this hormone is not discussed further.

Statistics

One-way repeated-measures ANOVA was used to identify differences between treatment groups. Chirp rate or EOD frequency was the dependent variable, treatment (isolates vs pairs; blank-implanted vs cortisol-implanted) was the independent variable, and time was the repeated measure. A significant treatment x time interaction indicated a treatment effect on chirp rate or EOD frequency. The effect of treatment on steroid levels was determined using nonparametric Mann–Whitney *U* tests because small sample sizes in some treatments made determination of a normal dis-

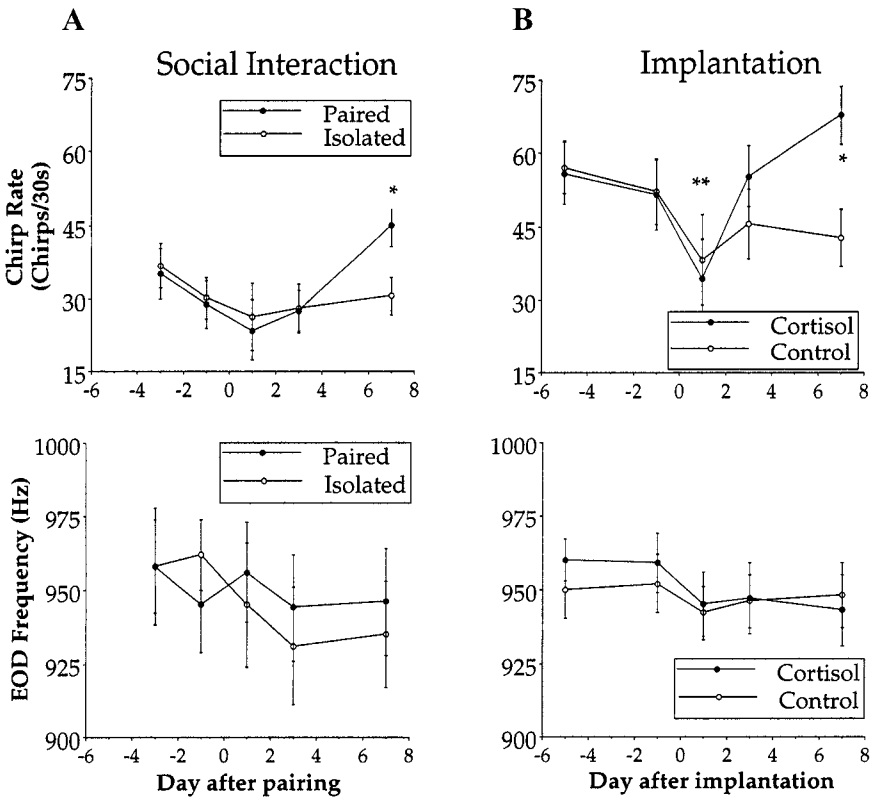

FIG. 1. Short-term (1–7 days) response of electrocommunication signals to social interaction and cortisol implantation in *Apteronotus leptorhynchus*. (A) Mean ± SE rate of evoked chirping and EOD frequency in males before (negative numbers) and after (positive numbers) pairing with another male for 7 days (experiment 1). Control males were kept isolated, but otherwise handled identically to paired fish. n = 7 for isolated; n = 12 for pairs. (B) Mean ± SE chirp rate and EOD frequency in males before and during implantation with empty tubes (control) or tubes containing cortisol (experiment 4). n = 10 for control; n = 12 for cortisol-implanted. An asterisk indicates a significant difference between treatment groups; a double asterisk indicates a time-dependent change in both treatment groups.

tribution difficult. In cases where steroid levels were below the level of detection, the number of undetectable samples between groups was compared using χ^2 tests. To estimate steroid concentrations in treatment groups with undetectable samples, undetectable samples were assigned the minimum level of detectability for the assay. Data are presented as means ± SE. $P < 0.05$ was considered statistically significant.

RESULTS

Social Interaction

Experiment 1: Short-term behavioral and hormonal responses. Paired and isolated fish did not differ in chirp rate or EOD frequency during the prepairing phase of the experiment. Paired fish chirped significantly more toward the sine wave stimulus (Df = −5 Hz) than isolated fish at 7 days postpairing ($F(1, 17) = 4.28$, $P < 0.005$), but the groups did not differ at 1 and 3 days postpairing (Fig. 1A). EOD frequency did not change significantly in either group and the groups did not differ from one another (Fig. 1A).

At 7 days postpairing, paired fish had significantly higher plasma cortisol levels than isolated fish (Table 1; $U = 11.0$; $P < 0.05$). One fish in the isolate group and none in the paired group had undetectable levels of cortisol. Three of 7 isolated fish and 2 of 10 paired fish had undetectable levels of 11KT ($\chi^2 = 0.71$, $df = 1$, $P > 0.05$). There was no difference between groups in estimated plasma 11KT. Chirp rate toward the sine wave stimulus on day 7 correlated significantly with cortisol levels (Fig. 3A; least-squares regression, $r^2 = 0.41$, $P < 0.01$).

Experiment 2: Long-term behavioral responses. During the prepairing phase, both the control and the

TABLE 1

Steroid Concentrations in Male *Apteronotus leptorhychus* as a Function of Social Interaction and Cortisol Treatment[a]

	n	Day of blood sampling	Plasma cortisol (ng/ml)	Plasma 11KT (ng/ml)
Social interaction				
Experiment 1				
Isolated	7	7	4.3 ± 0.1	0.35 ± 0.08[b]
Paired	10	7	10.6 ± 2.6[c]	0.31 ± 0.06[b]
Experiment 3				
Isolated	5	7	1.1 ± 0.5	1.18 ± 0.53
Paired	8	7	14.6 ± 5.4[c]	0.63 ± 0.22
Implantation				
Experiment 4				
Control	10	7	16.4 ± 5.1	0.24 ± 0.03[b]
Cortisol implant	12	7	51.5 ± 15.6[c]	0.26 ± 0.05[b]
Experiment 6				
Control	3	14	4.9 ± 2.9	0.64 ± 0.43
Cortisol implant	7	14	40.8 ± 6.7[c]	0.82 ± 0.40

[a] All data are expressed as means ± SE.
[b] Most samples were below sensitivity of assay. See text.
[c] Statistically different ($P < 0.05$) from the respective control values.

experimental fish maintained a stable chirp rate, and there were no significant differences between the mean chirp rates of the two groups. However, a significant difference between treatment groups emerged within 6 days after pairing (Fig. 2A). When presented with a sine wave stimulus, isolated fish maintained a mean chirp rate of 72 ± 1 chirps/30 s and paired fish increased from 67 ± 3 to 86 ± 3 chirps/30 s ($F(1, 7) = 14.82$, $P < 0.0001$). Paired fish showed a subsequent decrease in chirp rate over the next 5 days, until there was once again no significant difference between the mean chirp rates of the two groups (isolates 73 ± 3 chirps/30 s; paired 79 ± 4 chirps/30 s).

All paired fish either increased or showed no change in their chirp rate; all isolated fish decreased or showed no change in chirp rate. Within each pair, it appeared that both fish showed equivalent increases in chirp rate. All pairs were originally matched for EOD frequency and body size. However, within the present variation, the EOD and body size difference within a pair did not predict the degree of socially induced increase in chirp rate. Mean EOD frequency did not change significantly over time in either group (Fig. 2A).

Experiment 3: Hormonal responses. At 7 days postpairing, paired fish had significantly higher levels of cortisol than isolated fish (Table 1; $U = 3.00$; $P < 0.05$), but there was no difference in plasma 11KT. There was no significant correlation between plasma cortisol or 11KT level and body size for males within a pair.

Cortisol Implantation

Experiment 4: Short-term behavioral and hormonal responses. Before implantation, chirp rate toward the sine wave stimuli and EOD frequency did not change significantly and treatment groups did not differ from one another. At 24–26 h after implantation, chirp rate in both groups decreased significantly (Fig. 1B; $F(1, 22) = 6.3$, $P < 0.005$), perhaps indicating a response to anesthesia and surgery. Mean chirp rates of cortisol-treated fish exceeded those of controls at 3 days postimplantation, reaching statistical significance at 7 days postimplantation (Fig. 1B; $F(1, 22) = 2.93$, $P < 0.05$). EOD frequency neither changed over time nor differed between treatment groups.

At 7 days postimplantation, cortisol-treated fish had significantly higher (approximately threefold higher) plasma cortisol levels than control fish (Table 1; $U = 31.0$, $P < 0.05$). Cortisol levels in control fish were higher than in unmanipulated but similarly housed fish from experiment 1 (Table 1; $U = 41.0$, $P < 0.05$), indicating that, at 7 days postimplantation, sham implantation elevated cortisol levels above basal values. Eight of 10 control and 10 of 12 cortisol-treated fish had undetectable levels of 11KT, and estimated values of 11KT did not differ between treatment groups. 11KT levels were considerably lower in this experiment than in experiment 6 and in other studies of male *A. leptorhynchus* (Dunlap *et al.*, 1998), suggesting that these fish were not in full reproductive condition.

Unlike in experiment 1, chirp rate and cortisol levels were not significantly correlated in either group (Fig. 3B). However, among cortisol-treated fish, a single individual had an unusually high level of chirping for its cortisol level. When this individual was removed from the analysis, the correlation between cortisol levels and chirp rate became highly significant ($r^2 = 0.57$, $P < 0.005$). However, we could find no justification for excluding this individual from the analysis.

Experiment 5: Long-term behavioral responses. Throughout the preimplantation phase, both the control and experimental fish showed a stable mean chirp rate toward the sine wave stimulus, and there was no significant difference in the mean chirp rate of the two groups (Fig. 2B). While the mean chirp rate of control fish did not change throughout the experiment, mean chirp rate of the cortisol-implanted fish increased over

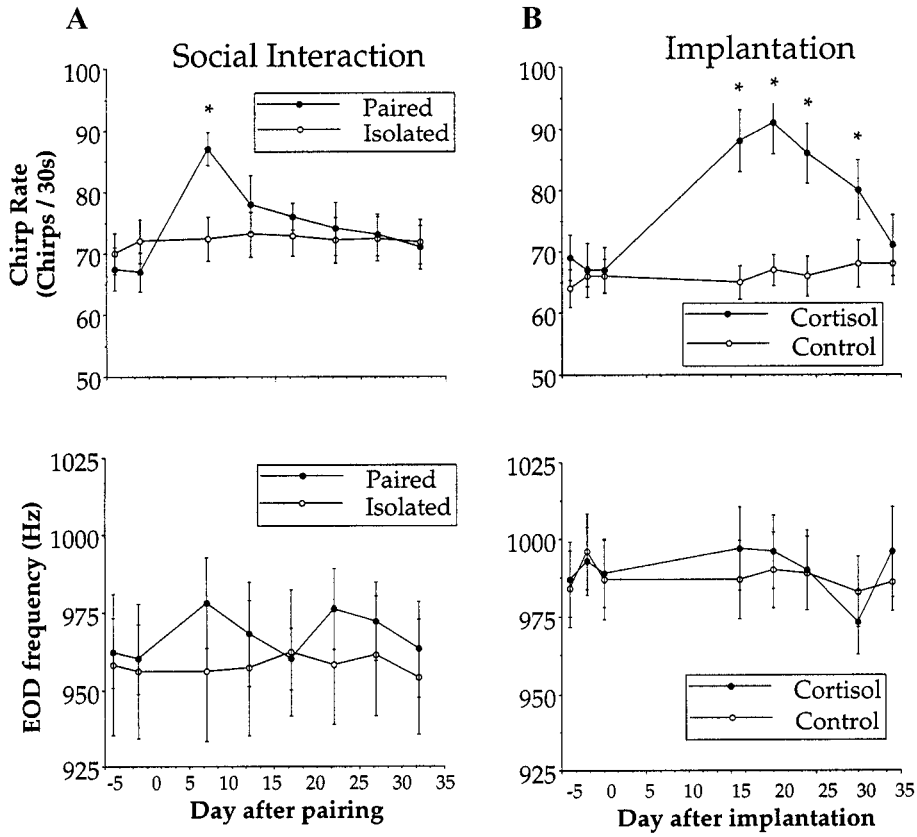

FIG. 2. Long-term (6–35 days) response of electrocommunication signals to social interaction and cortisol implantation in *Apteronotus leptorhynchus*. (A) Mean ± SE rate of evoked chirping and EOD frequency in males before and during pairing with other males for 32 days (experiment 2). $n = 8$ for isolated; $n = 16$ for pairs. (B) Mean ± SE chirp rate and EOD frequency before and during implantation with cortisol or empty tube (control)(experiment 5). $n = 9$ for controls; $n = 9$ for cortisol-implanted fish. An asterisk indicates a significant difference between treatment groups.

time. At 15 days after implantation, there was a significant increase in the mean chirp rate of the cortisol-implanted fish (Fig. 2B; $F(1, 8) = 15.87$, $P < 0.01$), but chirp rate of the control fish did not change. The difference in chirp rate was maximal at 18 days after implantation, increasing to approximately 91 ± 5 chirps/30 s ($F(1, 8) = 16.93$, $P < 0.005$), and then continued to decrease until preimplantation values were reached at 32 days postimplantation.

Mean EOD frequency of both groups never differed from preimplantation values. Cortisol implantation had no significant effect on EOD frequency (Fig. 2B).

Experiment 6: Hormonal responses. At 14 days postimplantation, cortisol implants significantly elevated plasma cortisol levels compared to levels in fish receiving blank implants (Table 1; $U = 11.5$; $P < 0.03$), but had no effect on 11KT. There was no significant correlation between plasma levels of cortisol and 11KT.

DISCUSSION

Social interaction between males increased the chirp rate toward sine wave stimuli and plasma cortisol levels without affecting plasma 11KT levels. Experimental elevation of plasma cortisol caused a similar increase in chirping and, again, 11KT levels were unaffected. These results indicate that cortisol is important in mediating socially induced changes in aggressive signaling, perhaps by acting directly on neural pathways controlling the EOD.

Social Environment, Electrocommunication Signals, and Steroids

Chirping behavior in males paired with other males showed a pattern of sensitization and habituation found commonly in studies of aggressive interactions

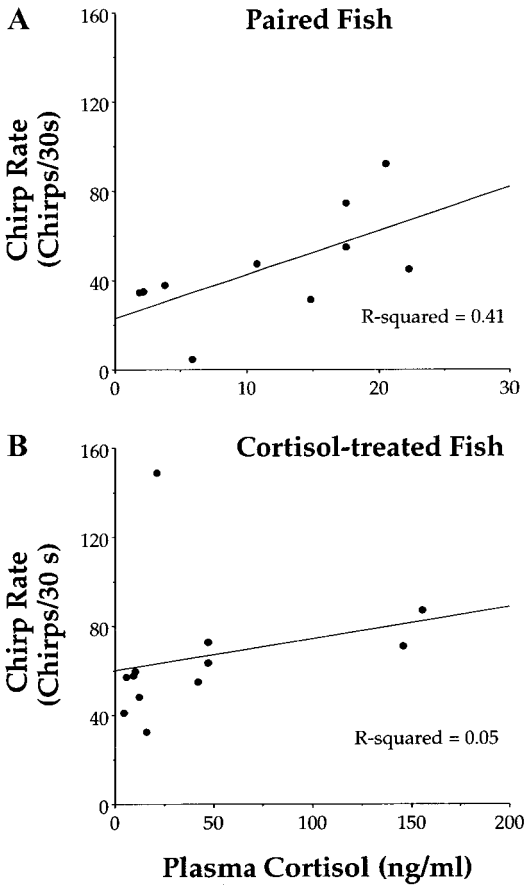

FIG. 3. Correlation between chirp rate and plasma cortisol levels in (A) paired fish (experiment 1) and (B) cortisol-treated fish (experiment 4). All data were collected on day 7 after the beginning of the experiment. Correlation is statistically significant for paired fish ($P < 0.01$) but not cortisol-treated fish.

(Clayton and Hinde, 1968; Peeke, 1983). Chirp rate increased considerably within 6–7 days, decreased for the following 10 days, and returned to prepairing levels within 20 days postpairing. Zupanc and Maler (1993) found that evoked chirping was similar in isolated and socially housed males, but did not specify the precise social environment (sex and number of cohabitants) or the duration of the experimental period. Habituation may have already occurred within these males and/or the specifics of the social environment may be critical in determining socially mediated effects on evoked chirping.

In the present experiments, plasma cortisol levels were elevated in paired males compared to isolated males over the period during which chirp rate increased. Such changes in cortisol have not been documented previously in any electric fish, but increased cortisol in response to changes in social environment have been found in other fish. For example, in the cichlid *Haplochromis burtoni*, males in mixed-sex groups had higher levels of aggressiveness (Heiligenberg and Kramer, 1972) and plasma cortisol (Hannes and Franck, 1983) than males housed in isolation. In many species, the cortisol response to agonistic interaction depends on the time since initiation of interactions (Hannes *et al.*, 1984) or the social status of the male, with subordinate and nonterritorial males showing higher cortisol levels than dominant and territorial males (Fox *et al.*, 1997; Sakakura, Tagawa, and Tsukamoto, 1997; Overli *et al.*, 1999; but see Hannes *et al.*, 1984). Within the limited data set in the present experiment, there was no evidence for differential hormonal or behavioral response based on social status. Fish within a pair had similar levels of cortisol or socially induced changes in chirping, and neither of these measures correlated with EOD frequency or body size, two indicators of dominance in *A. leptorhynchus* (Hagedorn and Heiligenberg, 1985). Definite conclusions on the relationship between cortisol levels, chirping, and social status will require additional experiments using more pairs with known hierarchical relationship.

In contrast to chirp rate, EOD frequency was not affected by long-term social interaction in *A. leptorhynchus*, a species with a wave-type discharge. Several studies on electric fish with a pulse-type EOD have demonstrated that social environment influences the production of a continuous electrocommunication signal. In these fish, brief electrical pulses are emitted continuously every ~10–100 ms. In *Brachyhypopomus pinnicaudatus* and *Brienomyrus brachyistius*, males housed in mixed-sex groups produced EODs with higher amplitudes and broader, more "masculine" wave-forms than males housed in isolation (Carlson and Hopkins, 2000; Franchina, Salazar, Volmar, and Stoddard, 2001). In *Bra. pinnicaudatus*, males were more effective than females in eliciting this response. In *Bri. brachyistius* and *Hypopomus occidentalis*, the magnitude of these socially induced changes in EOD depended on the male's status in the social hierarchy: dominant males showed broader pulses and greater amplitudes than subordinate males (Hagedorn and Zelick, 1989; Carlson and Hopkins, 2000). In *Bri. brachyistius*, dominant males have higher levels of 11KT and in several other pulse-type species, experimental elevation of androgens broadens pulse duration (Bass and Hopkins, 1983; Hagedorn and Carr, 1985; Landsman and Möller, 1988; Herfeld and Moller, 1998).

Concordant with the lack of change in EOD frequency in the present experiment, plasma androgen levels were unaffected by social interaction. However, this conclusion must be qualified by several considerations. The first steroid measurements were made 7 days after social interactions began, and undetected effects could have occurred earlier. Many of the socially induced EOD changes described above occur within minutes to several days after social interaction. In addition, androgen levels in the present study, particularly experiment 1, were lower than those found in other studies of *A. leptorhynchus* (Dunlap *et al.*, 1998), indicating that the fish were not in peak reproductive condition. Social stimulation may affect androgen levels only in fish that already have high circulating androgen. Finally, only 11KT and T were measured, and it is possible that social interaction may have altered levels of other androgens (e.g., androstendione).

In a stable mixed-sex community of *A. leptorhynchus*, the dominant fish is the largest male with the highest EOD frequency (Hagedorn and Heiligenberg, 1985). The high EOD frequency of these dominant males probably results from high levels of endogenous 11KT levels because EOD frequency correlates positively with endogenous 11KT (Dunlap, 2002) and EOD frequency increases in response to exogenous 11KT (Schaefer and Zakon, 1996). Results of the present study suggest that electrical, visual, and olfactory interactions with other males such as those in the present experiment are not important in maintaining this high EOD frequency. Instead, contact interactions with males, interactions with females, or internal factors regulating androgen production must be important in setting EOD frequency in these social contexts.

Cortisol Treatment, Androgens, and Electrocommunication Signals

Implants of cortisol increased plasma cortisol levels approximately three to eightfold over levels in control males. Although resultant levels of cortisol were, on average, about three to five times those found in socially paired males, there was broad overlap in the ranges of both groups. Isolated, cortisol-implanted males chirped to sine wave stimuli at rates similar to those of paired males, while blank-implanted males chirped similarly to those in isolation. Among paired fish, plasma cortisol levels correlated positively with chirp rates. Cortisol may thus participate causally in socially induced changes in chirping behavior. In addition, the lack of effect of social interactions on EOD frequency may be explained by elevated cortisol causing no change in plasma androgens.

Although many studies on fish have shown that cortisol levels commonly increase in response to agonistic behavior, relatively few studies have examined whether cortisol causes changes in behavior during aggression in fish. In the cichlid *Aequidens pulcher*, cortisol treatment selectively increases certain aggressive displays and the response varies according to the stimuli presented and the social rank of the fish (Munro and Pitcher, 1985). It will be interesting to determine whether cortisol increases all expression of aggression in *A. leptorhynchus* or whether it is specific to the production of aggressive electric signals.

It is important to note that the stimuli that fish received in the present study were not identical to stimuli experienced by fish during natural social interactions. Sine wave stimuli were used to evoke chirping during behavioral trials. This procedure exposed all fish to qualitatively and quantitatively equivalent stimuli and thus revealed socially induced changes in the sensitivity or motivation to respond to electrical stimuli. However, both the waveform and noninteractive character of these stimuli differ considerably from those during natural electrocommunication. Additionally, long-term social interaction occurred through a barrier that prevented physical contact. Although the barrier undoubtedly restricted the range of aggressive behaviors, it enabled us to demonstrate that the nearby presence of a male detected through electroreception, vision, or olfaction is sufficient to cause hormonal and behavioral changes. Pairing males together directly would likely cause injury and it would be difficult to distinguish between socially induced and injury-related changes in chirping rate or cortisol levels. Nevertheless, future studies should focus on whether cortisol treatment and social interaction affect chirping when both the behavioral testing regime and the long-term social stimuli more closely mimic natural social interactions.

Possible Mechanisms

How might long-term male–male interactions and cortisol modify chirping behavior? The central posterior/prepacemaker nucleus (CP/PPn) controls the production of chirps and serves as a site of interaction between the electromotor system and the neuroendocrine axis (Wong, 1997; Zupanc and Maler, 1997). This nucleus receives input from multiple brain regions, including the electrosensory thalamus and the hypo-

thalamus, and responds to a large variety of neuroactive compounds, ranging from classical neurotransmitters to neuromodulators.

Four sets of inputs to the CP/PPn are particularly relevant to the present study. First, the CP/PPn contains a low density of fibers and terminals that label positively with antibodies to rat and human corticotropin-releasing factor (CRF) (Zupanc, Jorschke, and Lovejoy, 1999). These fibers likely originate in the preoptic area or anterior hypothalamus and may directly connect the hypothalamo–pituitary–interrenal axis and the CP/PPn. Thus, agonistic interaction may simultaneously modify chirping behavior and increase cortisol levels through efferent CRF fibers from the hypothalamus. The subsequent rise in cortisol may then serve to strengthen the CRF input from the hypothalamus to the CP/PPn. However, if such a positive feedback occurs in *Apteronotus*, it would differ from responses in mammals (e.g., Uht, McKelvy, Harrison, and Bohn, 1988) and another teleost fish (Olivereau and Olivereau, 1988, 1991) in which cortisol treatment decreases hypothalamic CRF immunoreactivity.

Second, neurons in the preoptic area of *A. leptorhynchus* make connections with the prepacemaker region and contain arginine vasotocin (AVT), a peptide known to stimulate chirping (Bastian, Schniederjan, and Nguyenkim, 2001). In trout, AVT-containing neurons of the preoptic area have a high density of immunoactive glucocorticoid receptors (iGR; Teitsma, Anglade, Toutirais, Munoz-Cueto, Saligaut, Decouret, and Kah, 1998). Moreover, stressors that elevate cortisol also up-regulate AVT gene transcripts in the preoptic area (Gilchriest, Tipping, Hake, Levy, and Baker, 2000). Thus, socially induced increases in cortisol may stimulate chirping behavior through AVT-dependent connections between the preoptic area and the CP/PPn.

Third, fibers with substance P-like immunoreactivity (SPl-ir) are found in the CP/PPn (Weld and Maler, 1992) and injection of substance P into this area increases chirping (Weld, Maler, Quirion, and Kar, 1991). SPl-ir fibers are hypothesized to be a locus of androgen actions on chirping because testosterone treatment both increases SPl-ir and stimulates chirping (Dulka and Maler, 1994; Dulka, Maler, and Ellis, 1995; Dulka and Ebling, 1999). An increase in cortisol during long-term male interactions could also promote chirping through similar mechanisms.

Finally, catecholaminergic fibers project to the CP/PPn (Sas, Maler, and Tinner, 1990) and noradrenaline (and possibly dopamine) potently enhances chirping (Maler and Ellis, 1987). Studies of trout show that male–male aggression increases noradrenaline and dopamine in several regions of the brain while increasing cortisol concentrations in the plasma (Overli *et al.*, 1999). Similar changes could occur in paired male *A. leptorhynchus*, with the elevation in cortisol serving to increase the synthesis of noradrenaline and/or adrenergic receptors in the CP/PPn. Such stimulatory effects of glucocorticoids on brain catecholaminergic systems have been documented in rodents (Piazza, Rouge-Pont, Deroche, Maccari, Simon, and Le Moal, 1996).

Determining whether socially induced increases in cortisol act directly on these pathways will require detailed mapping of glucocorticoid receptors in the *Apteronotus* brain and their co-occurrence with these neurotransmitters and peptides. The distribution of iGR has been reported for salmon (Carruth, Jones, and Norris, 2000) and trout (Teitsma *et al.*, 1998), which have iGR in many regions of the telencephalon and diencephalon, with highest densities in the hypothalamus.

Conclusion

Our results demonstrated that males respond behaviorally and hormonally to interaction with conspecific males. These responses are possibly specific to conspecific males, and subsequent studies will determine whether males respond similarly to the presence of females or heterospecific fish. Males are known to chirp toward conspecific females in a reproductive context and to electrical stimuli that mimic females (Hagedorn and Heiligenberg, 1985; Bastian *et al.*, 2001; F. Triefenbach, personal communication). Such chirps have larger frequency modulations and longer durations than those directed toward males. Thus future studies will include a broader range of social manipulations and a detailed analysis of chirp structure.

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