Hormones and Behavior 60 (2011) 706–712

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Functionally similar acoustic signals in the corncrake (*Crex crex*) transmit information about different states of the sender during aggressive interactions

Paweł Ręk *, Tomasz S. Osiejuk, Michał Budka

Department of Behavioural Ecology, Institute of Environmental Biology, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland

ARTICLE INFO

Article history:
Received 20 June 2011
Revised 13 September 2011
Accepted 14 September 2011
Available online 1 October 2011

Keywords:
Signaling
Aggressiveness
Testosterone
Information content
Signal meaning
Corncrake
Crex crex

ABSTRACT

We combined playback experiments with hormonal manipulations to study the information content of acoustic signals during aggressive interactions between male corncrakes. During territorial conflicts, fights are uncommon, but the intensity of signaling usually increases. Such signals can be temporally and contextually associated with many aggressive behaviors and most likely function as threats or as indicators of the sender's quality or motivation. However, such correlational data are unsatisfactory for the proper interpretation of the function and information content of signals. Experimental tests are required to determine whether signals and aggressive behaviors are controlled by common or independent mechanisms. In our experiment, we assigned subjects to four groups: testosterone-implanted birds, flutamide-implanted birds, birds with empty implants, and non-captured control birds. Males produced two types of calls (quiet soft calls and loud broadcast calls), both of which are known to be reliable predictors of aggressive escalation. When testosterone action was blocked with flutamide, males significantly limited the amount of time spent close to the playback speaker and stopped responding to playback with soft calls. Broadcast calling was unaffected by the blockage of testosterone. Conversely, increased levels of testosterone neither affected calling nor the time spent near the speaker, indicating a permissive, rather than a graded effect of androgens. We concluded that, despite the seemingly similar function, both signals may transmit information about different states of the sender; soft calls seem to imply a threat of force, while broadcast calls appear to be more similar to an announcement, which is only indirectly associated with a male's aggressive behavior.

Introduction

Animals use a broad spectrum of behavioral traits when expressing or communicating aggression (Bradbury and Vehrencamp, 1998; Nelson, 2006; Searcy and Nowicki, 2005). However, we do not know much about the information content of such signals except that they are associated with aggression (Seyfarth et al., 2010). In most communication systems, this information is encoded through a correlation between signal form and the quality or state of the sender (Bradbury and Vehrencamp, 1998). In extreme cases, such relationships can be causal (indexes of quality) (Maynard Smith and Harper, 2003) or arbitrary (conventional signals) (Guilford and Dawkins, 1995), neither of which rules out the reliability of the information transmitted by the signal (Searcy and Nowicki, 2005). If this relationship is not causal, then ascribing specific information to the signal based only on behavioral observations can be subjective and biased (Searcy and Beecher, 2009). For example, a signal preceding attack may be interpreted as a predictor (signal) of attack. Nevertheless, attacks are usually preceded and followed by many different signals, making the signals very difficult to accurately interpret. To understand the existence and stability of behavioral correlations, an important issue is whether the behaviors are governed by common or independent mechanisms (Sih et al., 2004).

A majority of behavioral traits used during aggressive interactions are facultative expressions of an individual's internal state (Houston and McNamara, 1999), which means that aggressiveness is necessary for behaving aggressively but does not have to always result in aggressive behavior. The same principle applies to signals used during interactions, with the main difference that only a receiver who knows the code is able to understand the information being transferred and associate it with a specific internal state of the sender. In order for an unfamiliar observer (e.g., researcher) to understand the information content of a signal, an experiment manipulating a state affecting one behavior (e.g., pecking) and observing how it changes the characteristics of another behavior (e.g., singing) would be required (Bell, 2007; Coppens et al., 2010). A change in a ritualized signal that results in a change in an associated action (e.g., attacking) indicates that the signal contains information about that internal state of the sender if both signal and action are conditional upon the same internal state. Additionally, the more self-evident behavioral traits we can identify and associate with an individual's internal state in a given context, the better we can approximate the

* Corresponding author.
E-mail address: rek@amu.edu.pl (P. Ręk).

0018-506X/$ – see front matter
doi:10.1016/j.yhbeh.2011.09.011

information transferred through signals. Recognizing the mechanistic basis of aggressive behaviors is therefore helpful for precisely determining their function and to identify the information transmitted during these interactions.

The aim of this study was to link behavioral observations with the analysis of proximate mechanisms of signaling in order to better understand the information transmitted by signals. It was assumed that if signals and behaviors mirror the current internal state of an individual, a subsequent change in this state should affect all behaviors dependent on that state. To verify this assumption, we tested the influence of testosterone (T) on acoustic signaling and aggressive behaviors of male corncrakes (*Crex crex*), a territorial non-passerine bird (Rallidae: Gruiformes), during territorial conflicts. It was shown that T is associated with the intensity of acoustic signaling in birds (Chandler et al., 1994; Enstrom et al., 1997; Hunt et al., 1997; Ritschard et al., 2011; Silverin, 1980; Van Duyse et al., 2000) and that it affects both the activation and the increase of territorial aggression and modifies the communication of aggressiveness (i.e., countersinging, singing to rivals, or threatening and attacking them) (Ball et al., 2003; Balthazart, 1983; Harding, 1983; Moore and Marler, 1987; Mougeot et al., 2005; Wingfield et al., 1999). Male corncrakes are predominantly active during the night and produce two non-learnt acoustic signals (broadcast calls and soft calls) that are reliable predictors of aggressive escalation (Fig. 1) (Ręk and Osiejuk, 2010; Ręk and Osiejuk, 2011b). Broadcast calls (syn. cracking, rasping calls) are loud (approximately 96 dB SPL at 1 m) and produced almost nonstop throughout the night, both at short and long distances from a receiver. They consist of a long series of syllables and intervals (Fig. 1b, c) that create specific syntactic patterns (i.e., rhythms; see Fig. 1, examples and definition). Variation in rhythm constitutes a graded aggressive signal with syllables given at less regular intervals (higher/more intermittent rhythm) by males behaving more aggressively (Ręk and Osiejuk, 2010). Soft calls (syn. growling–mewing calls) are quiet (approximately 70 dB SPL at 1 m), typically produced near a receiver, and given at higher rates by males behaving more aggressively (see Fig. 1d, an example) (Ręk and Osiejuk, 2011b). During the breeding season, the rhythm of broadcast calls is positively correlated with body mass but is not correlated with T levels in the plasma (Osiejuk et al., 2004). However, males can vary their rhythm over short timeframes, and both the level of T and male aggression can fluctuate noticeably during the reproductive period (Osiejuk et al., 2004; Ręk and Osiejuk, 2010). It is therefore possible that the use of both types of vocal signals is associated with short-term fluctuations in T level. Because the influence of hormones on target tissues is usually not graded but instead follows a threshold effect (Leary, 2009), and the level of T in the plasma during the reproductive period in birds is higher than that required for the activation of behaviors (Ball and Balthazart, 2008; Schwabl et al., 2005; Silverin et al., 2004), it is also possible that the influence of T on the activation of aggressive behaviors in the corncrake is only permissive. In order to study the influence of T on behavior, we increased T levels (by implanting exogenous T) or blocked androgen receptors (flutamide implantation) in different groups of males.

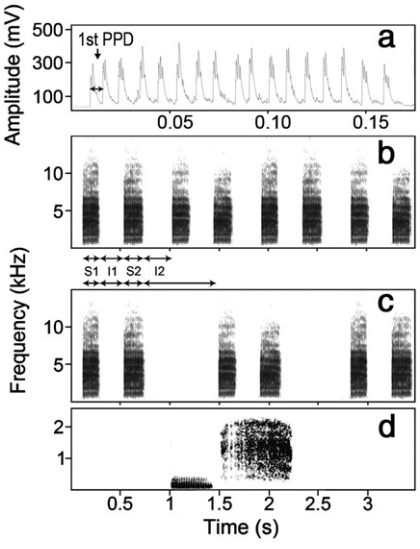

Fig. 1. Sonograms of corncrake calls: a — an envelope of a broadcast call syllable showing pulse-to-pulse duration (PPD — pulse to pulse duration), a feature used for discriminating between individuals (lengths of consecutive PPDs are individually distinctive and stable); b — broadcast call with a monotonous rhythm; c — broadcast call with an intermittent rhythm; d — soft call. The rhythm of the broadcast calls was defined as the ratio of the length of the second interval (I2) to the sum of the lengths of the first syllable (S1), first interval (I1), and second syllable (S2). One broadcast call contains a single sequence of S1, I1, S2, and I2. Note that rhythm is not a discrete but graded signal.

We expect that if aggression is proportionally dependent on T level in corncrakes, we will observe an increased level of aggression (including aggressive signaling) among males with an increased level of T or decreased aggression among males with blocked androgen receptors. Secondly, depending on the influence of hormonal treatments on both types of calls, we should be able determine whether they provide information regarding similar or different states of the sender. Finally, depending on whether the signals are conditional upon the same or different internal states as the observed actions of males, we should be able to link information content of signals with specific behaviors and states of senders.

Methods

Study site and subjects

To induce aggressive behavior, we carried out playback experiments ($N = 78$) in Kampinoski National Park (center of the study area: 20°23′E i 52°19′N; ca 80 km^2) between the 19th of May (sunset/sunrise — 1931/0337) and the 12th of June (sunset/sunrise — 1958/0315) 2010 (between 2200 and 0215 h local time) on a sample of $N = 39$ territorial males (distance to the nearest neighbor: mean ± SE = 363.08 ± 73.52 m).

Preparation of playback samples and course of experiments

We used 78 five-minute-long samples of natural broadcast calls recorded from different males in 2008 about 250 km NE (Ręk and Osiejuk, 2011b). All calls were digitally prepared to match a 96 ± 5 dB signal pressure level (SPL at 1 m) (natural amplitude) and the average rhythm (mean ± SD = 0.85 ± 0.02, within a sample). For the playback experiments, we used a Creative ZEN player (quality of files: PCM, 48,000 Hz, 16 bits) with a wireless SEKAKU WA-320 (Taichung, ROC Taiwan) loudspeaker with 20 W amplifier and 50–15,000 Hz frequency range. For recordings, we used one omni-directional microphone (Sennheiser K6/ME 62) recording to an Edirol R9 portable recorder. Experiments were carried out from the smallest distance possible, which means that we approached the male as long as its calling did not seem to be interrupted by our presence or until we could assess relatively well its position. In the second case, we did not approach to less than 5 m from the male (5–12 m). Given that soft calls are quiet and that their frequency is extremely low (Fig. 1c), except for recording, we counted the number of soft calls detected by ear and synchronized both data sources to avoid omissions.

Call stimuli were broadcast interactively (Dabelsteen and McGregor, 1996) by stopping playback if the male had stopped calling. Before each experiment, the loudspeaker was placed <0.5 m above the ground within the subject male's territory (5–10 m from a calling male). We started experiments only if the focal male was calling and continued

the playback for 5 min unless the male stopped calling for more than 10 s, in which case we would stop the playback and restart (after about 2 s) as soon as the male resumed calling. We did not stop the playback if the male stopped but resumed calling within 10 s.

Playbacks did not contain soft calls because they were not intended to threaten a subject but only to imitate the presence of an intruder within the subject's territory. Our intention was to test responses of males (both acoustic and behavioral) to the presence of an intruder, not to acoustic signals. Note that in the corncrake, aggressive motivation is signaled by the rhythm of broadcast calls, not by the presence or number of broadcast calls (Ręk and Osiejuk, 2010); however, males produce broadcast calls almost continually to signal their presence (during the night). Hence, all males received playbacks with the same average rhythm of broadcast calls to limit the effect of the rhythm itself but to provide males with sufficient information regarding the position of the intruder.

Experiments and treatments

Males were randomly assigned to four experimental treatment groups: 1) T-implanted birds (group T, $N=10$); 2) flutamide-implanted birds (group F, $N=10$); 3) placebo-implanted birds, where the implant device was identical but did not contain any active agent (group P, $N=9$); and 4) control birds (birds not captured but subjected to playback experiments) (group C, $N=10$). Each bird was tested two times at a two-day interval. During the first night we performed the experiment with playback (E1) and then caught the male (except group C) using a mist net. Males were ringed, blood sampled, and implanted. Blood samples (100–200 μl) were taken within 5 min of capture from the wing vein into heparinized tubes. Silastic implants were inserted subcutaneously via a small puncture along the wing before the birds were released. These implants (RX-50 Medical Grade Tubing, Dow Corning; length = 15 mm; outer diameter = 0.7 mm; inner diameter = 0.4 mm), either filled with T (Sigma-Aldrich, cat. no. T1500) or flutamide (Sigma-Aldrich, cat. no. T9397) or left empty, were sealed to 1 mm on both ends with silicone medical adhesive (NuSil, MED-1000). On the third night, we repeated the playback experiment (E2 — with different call samples) and recaptured a male after the playback. Because males were very timid during E2, we recaptured a total of only 12 males: 4 T males, 4 F males, and 4 P males. Blood samples were obtained from recaptured birds. The implants caused no infections or wounds, and captured and recaptured birds were calling within a few minutes after being released. We both attracted males into the mist net with playback and drove them into the net from behind. Consequently, we sampled for hormone levels approaching and non-approaching males. Males were ringed after E1 to be sure that we would blood sample the same birds after E2; however, all males (including C males) were identified from recordings with the use of the inner syllable structure of broadcast calls, which is individually distinctive and stable for long periods (Fig. 1a) (May, 1998; Peake et al., 1998; Ręk and Osiejuk, 2011a). We held all the necessary permissions (Director of Kampinoski NP and Polish Ministry of Environment) required for conducting the described procedures.

Hormonal measurements

Blood samples were centrifuged within four hours after the blood was collected, and plasma was stored at −20 °C until analysis. Testosterone concentrations in the plasma were analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) (DRG Testosterone ELISA, EIA-1559). This assay has been developed and validated for determining testosterone levels in small volume avian plasma samples (Bortolotti et al., 2009; Díaz-Ruiz et al., 2010; Mougeot et al., 2009; Washburn et al., 2007), and the results of this assay are in line with testosterone concentrations found in the corncrake in 2002 in the same area, with the use of standard radioimmunoassay test (Orion Diagnostica SPECTRIA testosterone RIA test) (Osiejuk et al., 2004) (2002: mean ± SE = 7.20 ± 0.76; 2010: mean ± SE = 7.46 ± 0.85; Mann–Whitney U test: U = 1030, $P=0.249$). This test has a high sensitivity to T (0.083 ng/ml), an intra-assay variation of less than 10% (here it was 4.54%), and a high specificity for T (relative specificity to T, 100%; to 5a-dihydrotestosterone, 0.8%; to androstenedione, 0.9%). All samples from this study were evaluated in a single assay, thus eliminating any interassay variation. The analysis was carried out according to the manufacturer protocols. The absorbance readings of the assay were determined with a Sunrise Absorbance (TECAN) microtiter plate reader. The concentration of T was determined with a standard curve that was calculated using a five-parameter logistic curve fit (SigmaPlot 11.0). Because the range of the assay is 0.083–16 ng/ml (according to the producer) and we calculated one measurement as 17.166 ng/ml, we fixed this (only this) value at 16 ng/ml.

Statistics

To compare T concentrations in the plasma after E1 and E2 (within subject factor) in males from the T, F, and P groups (between subject factor), we used generalized estimating equations (GEE), and then we performed pairwise post-hoc comparisons of mean T levels in 6 groups (E1 and E2 means in three groups). In analogous analyses, we compared the behaviors of males from 4 treatment groups (T, F, P, and C) before and after the treatment (E1 and E2). We used GEE and performed pairwise post-hoc comparisons of means in eight groups (eight means: E1 and E2 experiments in four groups). We examined three behaviors independently: rhythm of broadcast calls (measured before the playback), the number of soft calls (measured during the playback), and the amount of time that the focal male spent within 3 m from the speaker during the playback (as a score of aggressiveness). Rhythm values used in the analysis were mean rhythms calculated from 10 consecutive calls (Fig. 1). The rhythm variable was fitted using a normal distribution (log link function), the number of soft calls variable was fitted using a negative binomial distribution (log link function), and the time spent near the speaker variable was fitted using a binomial distribution (logit link function). We used the SPSS 19 software for all statistical analyses. All P values were two-tailed.

Results

Variability of T within and between experiments

We implanted birds with testosterone (T group, $N=10$), flutamide (F group, $N=10$), and placebo (P group, $N=9$) after the first experiment and expected to see an increase of plasma T in T-implanted birds and no change of plasma T in the remaining two groups after the second experiment. During the initial measurements (i.e., after E1 and before males had been implanted), the birds had higher T levels than during the second measurements (i.e., after males had been implanted and after E2) (E1: mean ± SE = 7.42 ± 0.81; E2: mean ± SE = 4.45 ± 1.2; Table 1), and the effect of experiment was modified by the effect of treatment (Table 1). Post hoc analysis

Table 1
Factors associated with T levels.

	Wald χ^2	df	P
Intercept	98.83	1	<0.001
Experiment	5.74	1	0.017
Treatment	4.38	2	0.112
Experiment*treatment	22.93	2	<0.001

GEE model including experiments (E1, E2) and playback treatments (T, F, P).

Table 2
Pairwise comparisons of T levels in plasma in three groups of males caught after two playback experiments.

	Experiment–treatment						*N*	Mean ± SE [ng/ml]
	E1-T	E1-F	E1-P	E2-T	E2-F	E2-P		
E1-T		0.392	0.567	0.011	0.009	0.087	10	7.59 ± 1.28
E1-F	0.392		0.197	0.128	<0.001	0.251	10	6.09 ± 1.21
E1-P	0.567	0.197		0.988	0.006	0.002	9	8.83 ± 1.75
E2-T	0.011	0.128	0.988		0.001	0.030	4	8.80 ± 1.31
E2-F	0.009	<0.001	0.006	0.001		0.991	4	3.18 ± 1.10
E2-P	0.087	0.251	0.002	0.030	0.991		4	3.15 ± 2.26

Fisher's LSD test.

(Fisher's LSD test) indicated that the initial measurements of T did not differ significantly among treatment groups (Table 2) and that during the second measurements, T males had higher T levels than males from the F and P groups (Table 2). At the same time, T levels increased in the T group and decreased in the F and P groups (Table 2).

Behaviors of males before and after implantation

To see how the individual behavioral data are related to the hormone data we correlated the rhythm of the broadcast calls, the number of soft call, and the time spent within 3 m of the speaker during E1 with T levels of 29 males blood sampled after E1 (from T, F, and P groups). T level was not correlated significantly with any behavioral variable (Spearman's r; T/rhythm: $r = -0.262$, $P = 0.170$; T/soft calls: $r = -0.098$, $P = 0.612$; T/time: $r = 0.086$, $P = 0.659$).

To test the influence of different treatments on the behavior of males, we compared the data from E1 and E2 in the four experimental groups (T, F, P, and C). The rhythm of the broadcast calls did not differ between E1 and E2 for a given male, nor did it differ among experimental groups (Table 3, Fig. 2).

Males produced more soft calls during E1 than during E2 (E1: mean ± SE = 1.55 ± 0.34; E2: mean ± SE = 0.65 ± 0.18; Table 3); however, this difference was caused chiefly by the significant decrease in soft calling among the F-treated birds (Fisher's LSD test: $P = 0.006$; Fig. 2), whereas changes in other groups were not significant (C E1–E2: $P = 0.83$; P E1–E2: $P = 1$; T E1–E2: $P = 0.26$). Contrary to the C, P, and T males, all soft calling F males during E1 decreased the frequency of soft calls during E2 (6 out of 7 males down to 0 and 1 male from 5 to 2; Fig. 2). Consequently, the F males had the lowest mean frequency of soft calls during E2 (Fig. 2; Fisher's LSD test; F–T: $P = 0.040$; F–P: $P = 0.224$; F–C: $P = 0.113$). It should be noted that the difference in the F group was not due to the highest initial frequency of soft calls in this group (Fig. 2) because this initial surplus stemmed from the behavior of a single extremely communicative male (13 soft calls vs. 1.66 on average). The removal of this male from the analysis did not affect the results, and the difference between E1 and E2 among the F-treated males was still significant (Fisher's LSD test: $P = 0.014$).

Similarly, males spent significantly more time within 3 m of the speaker during E1 than during E2 (E1: mean ± SE = 44.63 ± 8.53 s; E2: mean ± SE = 24.15 ± 6.28; Table 3); this difference was also due to the significant difference among the F-treated males (LSD test; F: $P = 0.007$; C: $P = 0.87$; P: $P = 0.84$; T: $P = 0.66$; Fig. 2). During E1, males from different treatment groups spent comparable times in the vicinity of the speaker (Fisher's LSD test; all $P \geq 0.44$), whereas during E2, the F-treated males spent less time within 3 m of the speaker than the males from other groups (Fig. 2; Fisher's LSD test; F–T: $P = 0.032$; F–P: $P = 0.015$; F–C: $P = 0.079$).

Based on these results, we conclude that as rhythm was independent of T level and an androgen receptor mediated action, the number of soft calls and the time spent near the speaker were both independent of graded T levels but were significantly limited in males with blocked androgen receptors.

Discussion

We used playback experiments, manipulated the T concentration in plasma, and blocked androgen receptors to study the mechanistic basis of acoustic signaling and aggression between male corncrakes. In earlier work, we have shown that corncrakes produce two structurally different but functionally similar acoustic signals during territorial interactions: rhythm of the broadcast calls and soft calls. These were determined to be aggressive signals because males calling with a higher (more intermittent) rhythm and soft calling males behaved more aggressively after producing the signal than males calling with a lower (more monotonous) rhythm and those males that did not soft call, and because both signals elicited aggressive responses when presented in on-territory playback (Ręk and Osiejuk, 2010; Ręk and Osiejuk, 2011b). Our results demonstrated that soft calls are controlled by an androgen receptor mediated action. Aggressive behavior (time spent close to the speaker) was controlled by an analogous permissive mechanism, confirming the mechanistic link between soft calling and aggression of males. There was only one male that still soft called and spent time near the speaker during E2 after it had been flutamide implanted (2 soft calls and 40 s near the speaker during E2 vs. 5 soft calls and 215 s near the speaker during E1). It is unclear why this male was still aggressive during E2; however, we do not know whether the flutamide dose we administered and the time between experiments were sufficient to trigger behavioral changes. Therefore, we still believe the results support that flutamide negatively affects soft calling and aggression by males (Fig. 2). Conversely, we did not find an effect of T or inhibition of androgen receptors on the rhythm of broadcast calls. It is possible that, in this system, testosterone levels have more of an effect on male–female interactions than on male–male interactions.

Individuals may produce numerous signals and take many actions in a short period of time during an interaction. Theoretically, one or more signals might be linked with one or more internal states, which can be of no small importance for the correct interpretation of information encoded by signals. The production of several signal types during an aggressive interaction may provide support for the 'backup signal' or redundancy hypothesis (Johnstone, 1996). In this case, the sender produces multiple signals to facilitate the transfer of a single message. Because each signal contains some error or chance for misinterpretation (Johnstone, 1996; Møller and Pomianowski, 1993), it might be beneficial to use different channels (e.g., visual

Table 3
Factors associated with male signaling and aggression.

	Rhythm			Soft calling			Time close speaker		
	Wald χ^2	df	*P*	Wald χ^2	df	*P*	Wald χ^2	df	*P*
Intercept	155.06	1	<0.001	0.001	1	0.976	287.21	1	<0.001
Experiment	0.26	1	0.613	9.46	1	0.002	9.78	1	0.002
Treatment	0.89	3	0.829	4.75	3	0.191	2.62	3	0.453
Experiment*treatment	3.36	3	0.339	12.15	3	0.007	23.65	3	<0.001

GEE models including experiments (E1, E2) and playback treatments (T, F, P, and C).

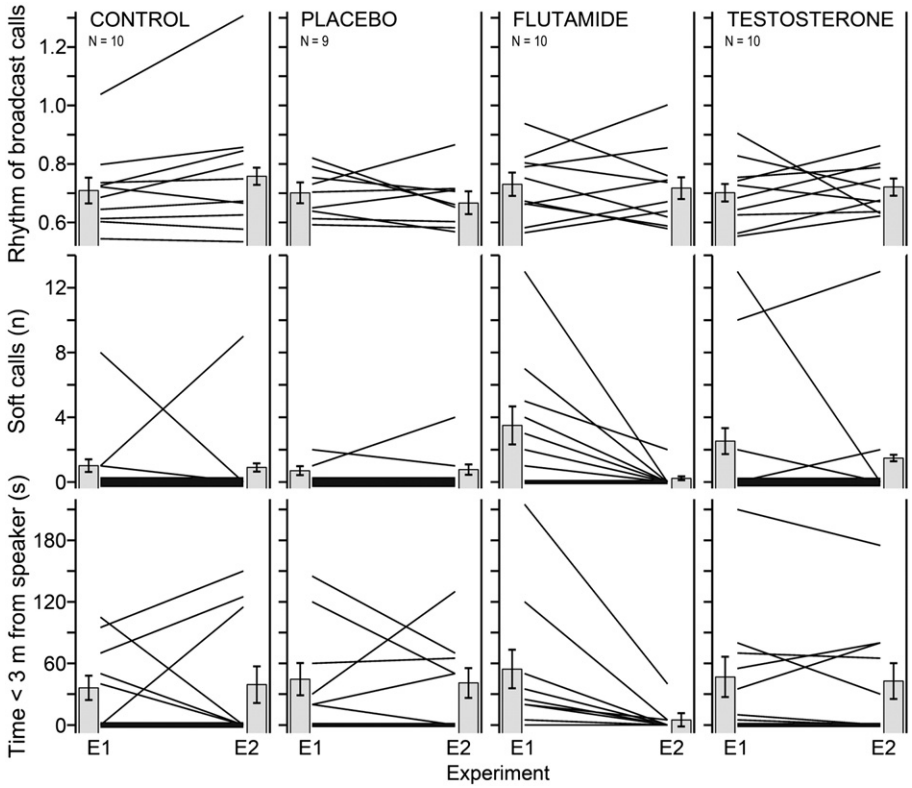

Fig. 2. Changes in signaling (rhythm of broadcast calls and the number of soft calls) and time spent <3 m from the speaker between two experiments (E1, E2) in four treatment groups: C — control, P — placebo, F — flutamide, T — testosterone. The graph shows changes in an individual's behaviors (lines, thickness of lines corresponds with the number of individuals) and mean reactions (±SE). Rhythm is in its own units (see Fig. 1).

and acoustic) to broadcast each of the redundant signals (Ballentine et al., 2008), whereas there may be no advantage in using several similar signals to transmit the same information. Alternatively, each of the signals produced during an interaction may transmit information about different aspects of the quality or state of the sender (the 'multiple message' hypothesis) (Johnstone, 1995; Johnstone, 1996; Møller and Pomianowski, 1993; Ord et al., 2001). Contrary to redundant signals, those transmitting individual messages are likely to be difficult to coordinate mechanistically because they might be activated independently, which could enable communication at different spatial and temporal scales. Although the results do not allow determination of which mechanism is associated with changes in rhythm, they show that changes in rhythm and soft calling are associated with different mechanisms. This is important, given the functional similarity between both signals, because it implies that both signals transmit information about different states of the sender (Johnstone, 1996). Because soft calls are strong predictors of aggressive behaviors (Ręk and Osiejuk, 2011b) and are mechanistically similar to an aggressive action such as time spent near the speaker (Vehrencamp, 2001), we think that information transferred by soft calls is directed at a specific receiver and signals how far the sender is willing to escalate the conflict if the receiver does not retreat (Waas, 1991). In contrast, it seems that rhythm does not convey information about the sender's aggressiveness per se, but rather it provides information about subtle aspects of the motivational state of the sender.

Despite insignificant correlations between T level and individual behavioral data, and the lack of noticeable influence of T supplementation on each corncrake's behavior, it is worthy to note that the average T level decreased significantly between experiments (Table 1). However, this is based on a limited amount of data from a small number of recaptured males, and the cause of the decrease of T between experiments could be an effect of the low sample size at E2. A comparison of mean T levels from both measurements in the treatment groups indicated there was a tendency for T to decrease, except in the T group, which showed an expected increase due to the artificial supplementation of T by the T implants. However, the increase among the T males was small relative to the decrease among the P and F males. Therefore, we noted the decrease only in the P and F groups, although the lack of a difference between the F and P males was expected because flutamide blocks androgen receptors, but it does not affect T production (Alonso-Alvarez et al., 2007; Hegner and Wingfield, 1987; Schwabl and Kriner, 1991; Van Roo, 2004). Based on these results, the cause of the decrease of T between experiments could be due to the experimentation on or the capture of males due to the inherent stress involved. One characteristic of physiological response to stress is the activation of the hypothalamo-pituitary-adrenal axis that results in increased plasma glucocorticoids (cortisol or corticosterone) (Norris, 2007). Elevated levels of glucocorticoids have also been discovered in individuals that have recently lost a fight (Øverli et al., 2004), and levels of glucocorticoids often correlate with the amount of aggression the loser received during the fight (Sloman et al., 2001). At the same time, there is some evidence that cortisol can inhibit androgen production in fish (Consten et al., 2002) and that stress can directly inhibit the production of reproductive hormones in mammals (James et al., 2008) and birds (Deviche et al., 2010).

Furthermore, despite similar T levels in the P and F males during both measurements, the P males, contrary to the F males, were characterized by relatively stable aggressiveness and intensity of signaling during both experiments. This means either that the level of T necessary for soft calling and aggressive actions was lower than the levels from the second measurements or that it was not T but something like DHEA (dehydroepiandrosterone), an inert sex steroid precursor that binds with low affinity to androgen receptors (Mo et al., 2004), that was responsible for the aggressive behaviors of males during territorial

conflicts (Hau et al., 2004). In fact, the relationship between T and aggressiveness is not as obvious as is commonly assumed (Adkins-Regan, 2005; Wingfield, 2005). Experimental research indicated that T influences aggression only during the reproductive period when its concentration is high and social relationships are unstable (Wingfield et al., 1999). At the same time, some evidence suggests that non-breeding aggression can be independent from circulating T levels (Canoine and Gwinner, 2002; Soma et al., 2008) and can be regulated by non-gonadal steroids in some species (Wingfield and Soma, 2002). It is also not without significance that despite the same neural circuitry, hormonal activation of aggression in one context may not be appropriate in another (Wingfield et al., 2001a; Wingfield et al., 2001b). Some studies have shown that territorial aggression during the breeding season can be mediated by aromatization of T into estradiol within the brain (Wingfield et al., 2006). The results regarding the effects of flutamide on aggression and signaling in the corncrake show that they are mediated by androgen and not by estrogen receptor pathways. Although we are far from reaching definitive conclusions, this research is a major step in identifying mechanistic pathways of aggressiveness and signaling in this primitive group of birds.

Conclusion

The blockage of testosterone with flutamide significantly limited the amount of time spent by focal males close to the playback speaker. Similarly, except for one male, all flutamide implanted males stopped responding to playback with soft calls; however, flutamide did not affect broadcast calling. By contrast, increased levels of testosterone neither affected soft and broadcast calling nor the time spent near the speaker, which suggests that the influence of T on the activation of aggressive behaviors in the corncrake is only permissive. These results thereby provide support for the hypothesis that soft and broadcast calls transmit information regarding different states of the sender. At the same time, because soft calls and time spent close to the playback speaker (aggressive behavior) were inhibited by the same mechanism, our results show the existence of the mechanistic link between soft calling and aggression of males, which is an experimental confirmation of the information content of soft calls.

Acknowledgments

We thank M. Dabert and U. Baraniak for help in hormonal analyses; E. Briefier, S. Dale, S.R. de Kort, and M.L. Hall for valuable comments on the manuscript; and B. Olech for organizational help. This work was supported by Ministry of Science and Higher Education (grant no. NN303 423836).

References

Adkins-Regan, E., 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton.
Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O., Sorci, G., 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. Proc. R. Soc. Lond. B. 274, 819–825.
Ball, G.F., Balthazart, J., 2008. Individual variation and the endocrine regulation of behaviour and physiology in birds: a cellular/molecular perspective. Phil. Trans. R. Soc. Lond. B. 363, 1699–1710.
Ball, G.F., Castelino, C.B., Maney, D.L., Appeltants, D., Balthazart, J., 2003. The activation of birdsong by testosterone. Multiple sites of action and role of ascending catecholamine projections. Ann. N. Y. Acad. Sci. 1007, 211–231.
Ballentine, B., Searcy, W.A., Nowicki, S., 2008. Reliable aggressive signalling in swamp sparrows. Anim. Behav. 75, 693–703.
Balthazart, J., 1983. Hormonal correlates of behavior. In: Farner, D.S., et al. (Ed.), Avian Biology. Academic Press, New York, pp. 221–365.
Bell, A.M., 2007. Future directions in behavioural syndromes research. Proc. R. Soc. Lond. B. 274, 755–761.
Bortolotti, G.R., Mougeot, F., Martinez-Padilla, J., Webster, L.M., Piertney, S.B., 2009. Physiological stress mediates the honesty of social signals. PLoS One 4, 1–8.
Bradbury, J.W., Vehrencamp, S.L., 1998. Principles of Animal Communication. Sinauer Associates, Sunderland, MA.
Canoine, V., Gwinner, E., 2002. Seasonal differences in the hormonal control of territorial aggression in free-living European stonechats. Horm. Behav. 41, 1–8.
Chandler, C.R., Ketterson, E.D., Nolan, V., Ziegenfus, C., 1994. Effects of testosterone on spatial activity in free-ranging male dark-eyed juncos, *Junco hyemalis*. Anim. Behav. 47, 1445–1455.
Consten, D., Lambert, J.G.D., Komen, H., Th., G., H.J., 2002. Corticosteroids affect the testicular androgen production in male common carp (*Cyprinus carpio* L.). Biol. Reprod. 66, 106–111.
Coppens, C.M., De Boer, S.F., Koolhaas, J.M., 2010. Coping styles and behavioural flexibility: towards underlying mechanisms. Phil. Trans. R. Soc. Lond. B. 365, 4021–4028.
Dabelsteen, T., McGregor, P.K., 1996. Dynamic acoustic communication and interactive playback. In: Kroodsma, D.E., Miller, E.H. (Eds.), Ecology and Evolution of Acoustic Communication in Birds. Cornell University Press, Ithaca, pp. 398–408.
Deviche, P.J., Hurley, L.L., Fokidis, H.B., Lerbour, B., Silverin, B., Silverin, B., Sabo, J., Sharp, P. J., 2010. Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: potential site of action and mechanism. Gen. Comp. Endocrinol. 169, 82–90.
Díaz-Ruiz, F., García, J.T., Pérez-Rodríguez, L., Ferreras, P., 2010. Experimental evaluation of live cage-traps for black-billed magpies *Pica pica* management in Spain. Eur. J. Wildl. Res. 56, 239–248.
Enstrom, D.A., Ketterson, E.D., Nolan, V., 1997. Testosterone and mate choice in the dark-eyed junco. Anim. Behav. 54, 1135–1146.
Guilford, T., Dawkins, M.S., 1995. What are conventional signals? Anim. Behav. 49, 1689–1695.
Harding, C.F., 1983. Hormonal influences on avian aggressive behavior. In: Svare, B. (Ed.), Hormones and Aggressive Behavior. Plenum Press, New York, pp. 435–467.
Hau, M., Stoddard, S.T., Soma, K.K., 2004. Territorial aggression and hormones during the non-breeding season in a tropical bird. Horm. Behav. 45, 40–49.
Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. Auk 104, 462–469.
Houston, A.I., McNamara, J.M., 1999. Models of Adaptive Behaviour. Cambridge University Press, Cambridge.
Hunt, K.E., Hahn, T.P., Wingfield, J.C., 1997. Testosterone implants increase song but not aggression in male Lapland longspurs. Anim. Behav. 54, 1177–1192.
James, P., Rivier, C., Lee, S., 2008. Presence of corticotrophin-releasing factor and/or tyrosine hydroxylase in cells of a neural brain-testicular pathway that are labelled by a transganglionic tracer. J. Neuroendocrinol. 20, 173–181.
Johnstone, R.A., 1995. Honest advertisement of multiple qualities using multiple signals. J. Theor. Biol. 177, 87–94.
Johnstone, R.A., 1996. Multiple displays in animal communication: 'backup signals' and 'multiple messages'. Phil. Trans. R. Soc. Lond. B. 351, 329–338.
Leary, C.J., 2009. Hormones and acoustic communication in anuran amphibians. Integr. Comp. Biol. 49, 452–470.
May, L., 1998. Individually distinctive corncrake *Crex crex* calls: a further study. Bioacoustics 9, 135–148.
Maynard Smith, J., Harper, D., 2003. Animal Signals. Oxford University Press, New York.
Mo, Q., Lu, S., Hu, S., Simon, N.G., 2004. DHEA and DHEA sulfate differentially regulate neural androgen receptor and its transcriptional activity. Mol. Brain Res. 126, 165–172.
Møller, A.P., Pomianowski, A., 1993. Why have birds got multiple sexual ornaments? Behav. Ecol. Sociobiol. 32, 167–176.
Moore, M.C., Marler, C.A., 1987. Effects of testosterone manipulations on nonbreading season territorial aggression in free-living lizards, *Sceloporus jarrovi*. Gen. Comp. Endocrinol. 65, 225–232.
Mougeot, F., Dawson, A., Redpath, S.M., Leckie, F., 2005. Testosterone and autumn territorial behavior in male red grouse *Lagopus lagopus scoticus*. Horm. Behav. 47, 576–584.
Mougeot, F., Martínez-Padilla, J., Webster, L.M., Blount, J.D., Pérez-Rodríguez, L., Piertney, S. B., 2009. Honest sexual signalling mediated by parasite and testosterone effects on oxidative balance. Proc. R. Soc. Lond. B. 276, 1093–1100.
Nelson, R.J. (Ed.), 2006. Biology of Aggression. Oxford University Press, New York.
Norris, D.O., 2007. Vertebrate Endocrinology. Academic Press, Amsterdam.
Ord, T.J., Blumstein, D.T., Evans, C.S., 2001. Intrasexual selection predicts the evolution of signal complexity in lizards. Proc. R. Soc. Lond. B. 268, 737–744.
Osiejuk, T.S., Olech, B., Ratyńska, K., Owsiński, A., Gromadzka-Ostrowska, J., 2004. Effects of season, plasma testosterone and body size on corncrake (*Crex crex*) call rhythm. Ann. Zool. Fenn. 41, 647–659.
Øverli, Ø., Korzan, W.J., Höglund, E., Winberg, S., Bollig, H., Watt, M., Forster, G.L., Barton, B.A., Øverli, E., Renner, K.J., Summers, C.H., 2004. Stress coping style predicts aggression and social dominance in rainbow trout. Horm. Behav. 45, 235–241.
Peake, T.M., McGregor, P.K., Smith, K.W., Tyler, G., Gilbert, G., Green, R.E., 1998. Individuality in corncrake *Crex crex* vocalizations. Ibis 140, 120–127.
Ręk, P., Osiejuk, T.S., 2010. Sophistication and simplicity: conventional communication in a rudimentary system. Behav. Ecol. 21, 1203–1210.
Ręk, P., Osiejuk, T.S., 2011a. No male identity information loss during call propagation through dense vegetation: the case of the corncrake. Behav. Process. 86, 323–328.
Ręk, P., Osiejuk, T.S., 2011b. Nonpasserine bird produces soft calls and pays retaliation cost. Behav. Ecol. 22, 657–662.
Ritschard, M., Laucht, S., Dale, J., Brumm, H., 2011. Enhanced testosterone levels affect singing motivation but not song structure and amplitude in Bengalese finches. Physiol. Behav. 102, 30–35.
Schwabl, H., Kriner, E., 1991. Territorial aggression and song of male European robins (*Erithacus rubecula*) in autumn and spring: effects of antiandrogen treatment. Horm. Behav. 25, 180–194.
Schwabl, H., Flinksb, H., Gwinner, E., 2005. Testosterone, reproductive stage, and territorial behavior of male and female European stonechats *Saxicola torquata*. Horm. Behav. 47, 503–512.

Searcy, W.A., Beecher, M.D., 2009. Song as an aggressive signal in songbirds. Anim. Behav. 78, 1281–1292.
Searcy, W.A., Nowicki, S., 2005. The Evolution of Animal Communication: Reliability and Deception in Signaling Systems. Princeton University Press, Princeton, NJ.
Seyfarth, R.M., Cheney, D.L., Bergman, T., Fischer, J., Zuberbühler, K., Hammerschmidt, K., 2010. The central importance of information in studies of animal communication. Anim. Behav. 80, 3–8.
Sih, A., Bell, A.M., Johnson, J.C., Ziemba, R.E., 2004. Behavioral syndromes: an integrative overview. Q. Rev. Biol. 79, 241–277.
Silverin, B., 1980. Effects of long-lasting testosterone treatment on free-living pied flycatchers, *Ficedula hypoleuca*, during the breeding period. Anim. Behav. 28, 906–912.
Silverin, B., Baillien, M., Balthazart, J., 2004. Territorial aggression, circulating levels of testosterone, and brain aromatase activity in free-living pied flycatchers. Horm. Behav. 45, 225–234.
Sloman, K.A., Metcalfe, N.B., Taylor, A.C., Gilmour, K.M., 2001. Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. Physiol. Biochem. Zool. 74, 383–389.
Soma, K.K., Scotti, M.L., Newman, A.E.M., Charlier, T.D., Demas, G.E., 2008. Novel mechanisms for neuroendocrine regulation of aggression. Front. Neuroendocrinol. 29, 476–489.
Van Duyse, E., Pinxten, R., Eens, M., 2000. Does testosterone affect the trade-off between investment in sexual/territorial behaviour and parental care in male great tits? Behaviour 137, 1503–1515.
Van Roo, B.L., 2004. Exogenous testosterone inhibits several forms of male parental behavior and stimulates song in a monogamous songbird: the blue-headed vireo (*Vireo solitarius*). Horm. Behav. 46, 678–683.
Vehrencamp, S.L., 2001. Is song-type matching a conventional signal of aggressive intentions? Proc. R. Soc. Lond. B. 268, 1637–1642.
Waas, J.R., 1991. The risks and benefits of signalling aggressive motivation: a study of cave-dwelling little blue penguins. Behav. Ecol. Sociobiol. 29, 139–146.
Washburn, B.E., Millspaugh, J.J., Morris, D.L., Schulz, J.H., Faaborg, J., 2007. Using a commercially available enzyme immunoassay to quantify testosterone in avian plasma. Condor 109, 181–186.
Wingfield, J.C., 2005. A continuing saga: the role of testosterone in aggression. Horm. Behav. 48, 253–255.
Wingfield, J.C., Soma, K.K., 2002. Spring and autumn territoriality in song sparrows: same behavior, different mechanisms? Integr. Comp. Biol. 42, 11–20.
Wingfield, J.C., Jacobs, J.D., Soma, K., Maney, D.L., Hunt, K., Wisti-Peterson, D., Meddle, S., Ramenofsky, M., Sullivan, K., 1999. Testosterone, aggression, and communication: ecological bases of endocrine phenomena. In: Hauser, M.D., Konishi, M. (Eds.), The Design of Animal Communication. The MIT Press, Cambridge, Massachusetts, pp. 255–283.
Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001a. Avoiding the "costs" of testosterone: ecological bases of hormone–behavior interactions. Brain. Behav. Evol. 57, 239–251.
Wingfield, J.C., Soma, K.K., Wikelski, M., Meddle, S.L., Hau, M., 2001b. Life cycles, behavioral traits and endocrine mechanisms. In: Dawson, A., Chaturvedi, C.M. (Eds.), Avian Endocrinology. Narosa, New Delhi, India, pp. 3–17.
Wingfield, J.C., Moore, I.T., Goymann, W., Wacker, D.W., Sperry, T., 2006. Contexts and ethology of vertebrate aggression: implications for the evolution of hormone–behavior interactions. In: Nelson, R.J. (Ed.), Biology of Aggression. Oxford University Press, New York, pp. 179–210.