

From social behavior to neural circuitry: Steroid hormones rapidly modulate advertisement calling via a vocal pattern generator

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Received 17 March 2006; revised 22 May 2006; accepted 24 May 2006

Abstract

Across vertebrates, androgens are rapidly elevated within minutes in response to aggressive or reproductive stimuli, yet it is unclear what the causal relationship is between fast androgen elevation and the ongoing (minute-by-minute) expression of behavior. This study tested the hypothesis that rapid increases in plasma steroid levels induce similarly rapid increases in both vocal behavior and the neurophysiological output of a central pattern generator that governs vocal behavior. In Gulf toadfish (*Opsanus beta*), males call to attract females to their nesting sites, and both males and females vocalize in aggressive interactions. Previous field experiments with males showed that simulated territorial challenges produce rapid and concurrent elevations in ongoing calling behavior and circulating levels of the teleost-specific androgen 11-ketotestosterone (11kT), but not the glucocorticoid cortisol. The current field experiments showed that non-invasive (food) delivery of 11kT, but not cortisol, induced an elevation within 10 min in the ongoing calling behavior of males. Electrophysiological experiments revealed that intramuscular injections of either 11kT or cortisol, but neither testosterone nor 17- β -estradiol, induced increases within 5 min in the output of the vocal pattern generator in males, whereas only cortisol had similarly fast effects in females. The field behavioral results support predictions generated by the challenge hypothesis and also parallel the 11kT-dependent modulation of the vocal pattern generator in males. The cortisol effect on the vocal pattern generator in both sexes predicts that glucocorticoids regulate vocalizations in non-advertisement contexts. Together, these experiments provide strong support for the hypothesis that surges in circulating steroid levels play a causal role in shaping rapid changes in social behavior (vocalizations) through non-genomic-like actions on neural (vocal motor) circuits that directly encode behavioral patterning.

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Keywords: Non-genomic; Membrane receptors; Song; Testosterone; Challenge hypothesis

Introduction

The challenge hypothesis, first proposed to explain variation in androgen responsiveness in birds (Wingfield et al., 1990) and then extended to teleost fish (Oliveira et al., 2002), states that baseline plasma androgens are elevated for reproduction, and that acute increases above this baseline are involved in the regulation of territorial and/or aggressive behavior. This hypothesis originally proposed that androgen responsiveness was a function of monogamous vs. polygamous mating systems, but a recent, vertebrate-wide meta-analysis indicates that aggressive interactions are associated with acute elevation in plasma androgens, independent of mating system (Hirschen-

hauser and Oliveira, 2006). The widely observed fast increases (within minutes) in circulating levels of plasma androgens in response to either aggressive or reproductive stimuli have been proposed to prepare individuals for future encounters, in both terrestrial and non-terrestrial vertebrates (Harding, 1981; Oliveira et al., 2001; Ross et al., 2004; Wingfield, 2005). In empirical tests of this hypothesis, androgen surges have been shown to reinforce the ‘winner effect’ by priming winners of aggressive interactions for future contests (i.e., within days; Trainor et al., 2004; Oyegbile and Marler, 2005). Furthermore, in male vertebrates, hormones derived from the pituitary and gonads are released within minutes in response to exposure to female cues (Coquelin and Bronson, 1979; O’Connell et al., 1981; Kyle et al., 1985; Liley et al., 1986) and these hormonal changes can promote the expression of courtship, territoriality and/or mate-guarding within days-to-weeks (Harding, 1981;

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Kindler et al., 1991; Liley et al., 1993; Barnett and Pankhurst, 1994).

On a rapid time scale, in captive conditions, surges in plasma steroid hormones are known to rapidly modify the expression of

behavior within minutes (Orchinik et al., 2002; Mikics et al., 2005), likely via non-classical ('non-genomic') steroid actions in the brain (e.g., Pfaff and Pfaffman, 1969; Orchinik et al., 1994; Frye, 2001). This raises the question: do fast elevations in

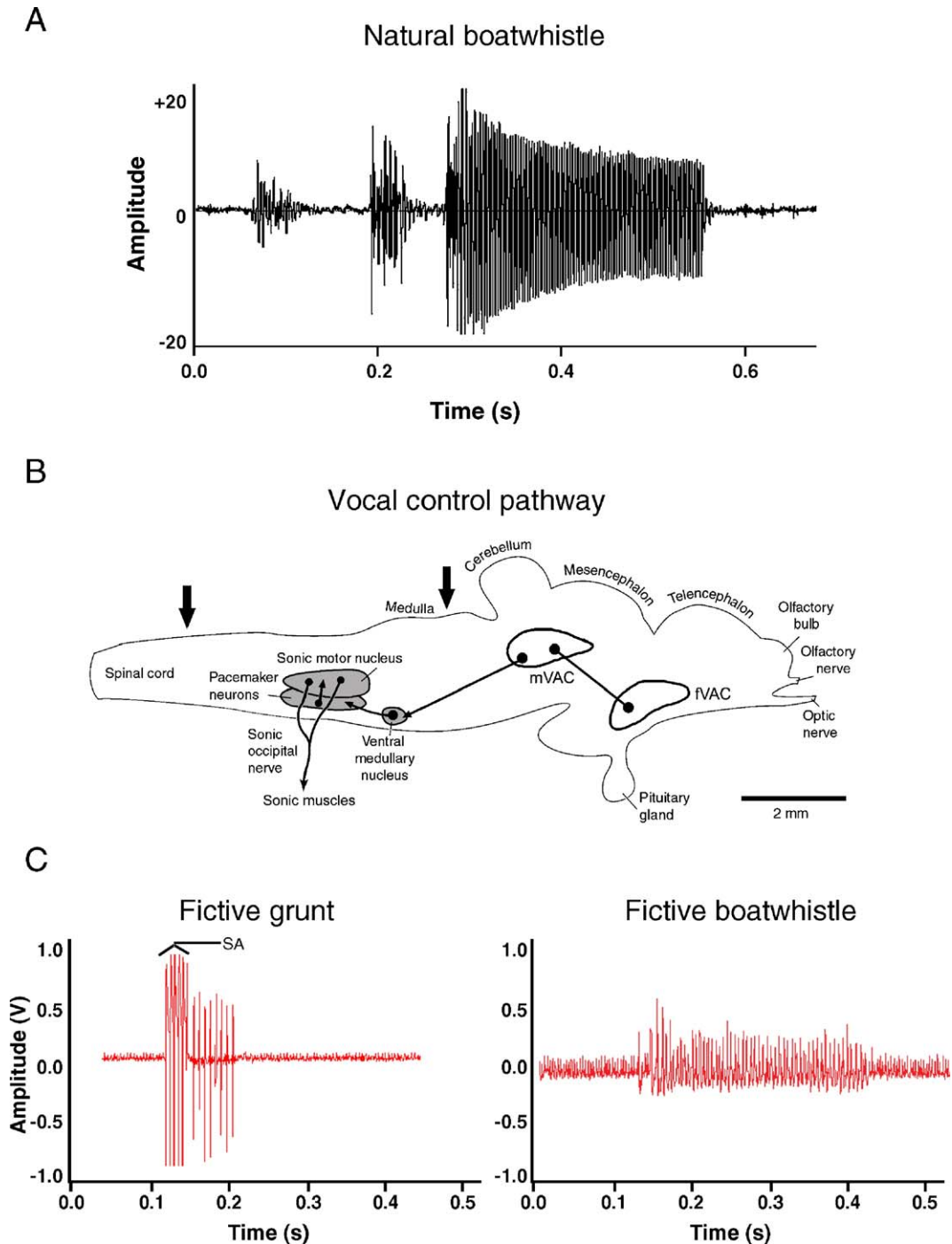


Fig. 1. (A) Field recording of a male toadfish advertisement call, or 'boatwhistle' recorded in May 2002 at 28.6°C. A boatwhistle consists of one or more introductory, short-duration grunts, followed by a long-duration tonal 'hoot.' The oscillogram amplitude scale is relative. (B) The vocal control pathway in Gulf toadfish contains forebrain (f) and midbrain (m) vocal-acoustic integration centers (VAC) and a vocal hindbrain-spinal pattern generator (VPG) that includes a pacemaker-sonic motor neuron circuit and a ventral medullary nucleus that bilaterally couples the pacemaker circuitry (see text for details; modified from Bass and McKibben, 2003). Electrical stimulation in the mVAC elicits fictive grunts (C, left) recorded from a ventral occipital nerve root in a neurophysiology preparation. A stimulus artifact (SA) immediately precedes the fictive grunt (C, left). Surgical isolation (indicated by arrows) of the region containing the VPG from mVAC/fVAC input leads to immediate spontaneous vocal activity, including fictive 'boatwhistles' (C, right). No stimulus artifact is present in the fictive boatwhistle trace, as these patterns are spontaneous. After spontaneous activity subsides, fictive grunts like those in the intact preparation, can be elicited from the isolated VPG via electrical stimulation in the ventral medullary region (B).

androgens regulate minute-by-minute changes in ongoing behavior in vertebrates? It has been difficult to experimentally test this idea in free-living animals, in part because field studies typically employ methods (e.g., surgical implants) that elevate plasma steroid levels over extended time periods (days to weeks). Thus, these experimental approaches have established that long-term elevations in basal androgen levels increase the expression of aggressive and advertisement behaviors, especially in response to multiple encounters (Ketterson et al., 1992; Marler and Moore, 1988; Ros et al., 2004; Wingfield, 1984).

This study employs behavioral and neurophysiological methods to test whether rapid surges in plasma steroid hormones promote equally fast changes in vocal behavior and the activity of the central pattern generator that determines temporal features (duration, repetition rate and fundamental frequency) of that behavior. The study species, Gulf toadfish (*Opsanus beta*), breed in the shallow habitat of the Gulf of Mexico. Although both male and female toadfishes vocalize in agonistic encounters, only males excavate and defend nests, and then broadcast vocalizations ('boatwhistles' Fig. 1A) to attract females and interact with other territorial males (Gray and Winn, 1961; Remage-Healey and Bass, 2005a). In support of the challenge hypothesis (Wingfield et al., 1990; Oliveira et al., 2002), field experiments show that increases in circulating androgen (and cortisol) levels are associated with the transition from a non-calling to a calling state in male toadfish (Remage-Healey and Bass, 2005a). Furthermore, simulated territorial challenges (via acoustic playbacks) cause rapid (within 10 min) elevations in both ongoing calling behavior and plasma androgens (but not cortisol) in males (Remage-Healey and Bass, 2005a). Therefore, rapid elevation in androgens during territorial challenges may cause similarly rapid changes in calling behavior in toadfish, but this possibility remains untested.

The temporal patterning of vocalizations among toadfishes is determined by a central vocal motor network that receives input from the auditory system. This network includes forebrain and midbrain vocal-acoustic centers that can modulate the output of a hindbrain-spinal vocal pattern generator (VPG), which in turn includes a pacemaker-motor neuron circuit that innervates paired sonic muscles attached to the walls of the swim bladder (Fig. 1B; Bass and Baker, 1990; Bass et al., 1994; Goodson and Bass, 2002; Kittelberger et al., 2006). The rhythmic output of the hindbrain-spinal VPG directly determines the simultaneous contraction rate of the sonic muscles and thereby determines temporal features of vocalizations, including duration and fundamental frequency (Bass and Baker, 1990, 1991; Remage-Healey and Bass, 2004). The patterned motor volley generated by the VPG in the absence of muscle contractions, referred to as a fictive vocalization, is predictive of a natural vocalization's temporal features and can be readily elicited in a neurophysiology preparation (Fig. 1C). In a closely related species, the plainfin midshipman, the duration of male fictive vocalizations (VPG activity) is rapidly regulated by androgens, estrogens and glucocorticoids (Remage-Healey and Bass, 2004). While these results suggest that rapid modulation of the VPG can induce rapid shifts in vocal behavior in nature, this possibility also remains untested.

Here, we test the general hypothesis that rapid changes in circulating steroid levels cause fast changes in ongoing social behavior (toadfish vocalizations) that depend upon rapid modulation of the activity of a neural circuit which determines the temporal patterning of that behavior (toadfish VPG). Our previous field studies (Remage-Healey and Bass, 2005a, see above) directed us to focus on the Gulf toadfish.

Materials and methods

Subjects

Adult male and female toadfish were collected by trawl and from nests near the Florida State University Marine Laboratory (FSUML) in St. Teresa, FL (29°54.9'N; 84°30'W; collection permit 03SR-688 to LRH from Florida Division of Marine Fisheries). For neurophysiology experiments, fish were shipped overnight to Cornell University and maintained in artificial seawater tanks until the day of experimentation on a diet of frozen scallops and small goldfish (average interval between arrival date and experimental date was approximately 23 days). Male and female toadfish were in full breeding condition at the time of collection (April–August) and maintained mature gonad sizes through the duration of laboratory housing (on two occasions fish spawned and reared a brood of embryos in laboratory housing conditions). For field experiments, fish were kept in flow-through seawater tanks at FSUML and fed thawed (quick-frozen) scallops. Within 24 h of capture, males were returned to a natural calling site in the field for experimentation (see below). All fish were identified as male or female using methylene blue dye applied to the urogenital papilla (see Remage-Healey and Bass, 2005a). All procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Field behavioral experiments

Procedures for recording the vocal activity of identified male toadfish in the field under semi-natural conditions followed those of Remage-Healey and Bass (2005a). Briefly, non-calling males were placed individually in wire enclosures (70 × 70 × 10 cm) at an underwater field site where calling male toadfish are naturally found; males began calling within 48 h under these conditions. Each enclosure contained a section of PVC tubing and one male per experiment.

To avoid handling stress related to hormone implants or injections, steroids were delivered non-invasively by inserting crystalline steroid pellets into food items and offering these to focal individuals (after Breuner et al., 1998; Øverli et al., 2002). This method was first verified in the laboratory with male toadfish fed thawed scallops (quick-frozen) implanted with either 11kT (approximately 10 µg crystalline steroid per scallop) or nothing (control). For males ($n=4$) fed one 11kT scallop each, 11kT levels were significantly and rapidly elevated (15.11 ± 0.30 ng/ml at 10 min; 41.57 ± 6.95 ng/ml at 20 min) compared to 11kT levels in males ($n=3$) fed one control scallop (4.55 ± 0.20 ng/ml at 10 min; 3.86 ± 1.80 ng/ml at 20 min; $p < 0.001$ for hormone treatment effect). Beyond 20 min, plasma 11kT levels in animals fed 11kT scallops in this pilot experiment were suprphysiological (e.g., 65.25 ± 4.72 ng/ml at 60 min) but returned to physiological range after 80 min (29.98 ± 3.24 ng/ml). There was no significant change in circulating cortisol in animals fed 11kT vs. control scallops (data not shown, $p > 0.05$).

A pilot study also verified that male toadfish in the field fed scallops implanted with 11kT experienced a significant increase in plasma 11kT within 10 min (16.37 ± 4.56 ng/ml for 11kT scallops, $n=4$; 3.98 ± 1.56 ng/ml for control scallops, $n=3$; $p < 0.001$ for hormone treatment effect). These rapid surges in plasma 11kT were within the physiological range for male toadfish in their natural environment (normal range = 0.65–39.25 ng/ml; Remage-Healey and Bass, 2005a).

Advertisement calls (boatwhistles) of identified, individual male toadfish in nest enclosures were recorded through a hydrophone (5 cm adjacent to the nesting male within each enclosure) connected to a Sony laptop computer via a switchbox (see Remage-Healey and Bass, 2005a). Vocal activity for each male was recorded for a 10-min period prior to scallop feeding ("Pre") and then males in enclosures were offered a scallop positioned on the end of a thin metal rod (which allowed remote feeding through the wire enclosures and reduced

experimenter-induced disturbance of ongoing behavior). In experiment 1, the scallop contained either 10 µg crystalline 11kT ($n=9$) or nothing (control, $n=7$). In experiment 2, the scallop contained either 10 µg crystalline cortisol ($n=10$) or nothing (control, $n=5$). After a 10-min intervening period, the vocal activity for each male was sampled again for 10 min ("Post"). In a previous study, rapid changes in call rate and duration in response to playback challenges were observed during (5 min) and following (5 min) playback (Remage-Healey and Bass, 2005a), and therefore, a 10-min sampling period was chosen for behavioral sampling in the current study. Immediately after the 10-min post-recording period, nest enclosures were individually brought to the surface for an immediate plasma sample (mean sample time from initial disturbance=3.56 and 4.57 min for experiments 1 and 2, respectively) to compare hormone levels between treatment groups. Thus, blood samples were obtained about 25 min after feeding. All vocal activity was standardized to 100% of baseline (Pre) activity.

Evoked activity of the vocal pattern generator (VPG)

Neurophysiology procedures closely followed those outlined in earlier studies with the closely related plainfin midshipman fish, *Porichthys notatus*, and oyster toadfish, *Opsanus tau* (Bass and Baker, 1990, 1991; Goodson and Bass, 2000; Remage-Healey and Bass, 2004). The brain and rostral spinal cord were exposed by dorsal craniotomy under general anesthesia (0.025% benzocaine; Sigma) and long-lasting analgesia (0.25% bupivacaine (Abbott Laboratories, Chicago, IL) with 0.01 mg/mg epinephrine (International Medication Systems, El Monte, CA)). Following surgery, fish were stabilized in a plexiglass tank and perfused through the mouth with fresh saltwater. Exposed brain areas were covered with Fluorinert (3 M Corp., St. Paul, MN), and intramuscular injections of pancuronium bromide (0.5 mg/kg; Astra Pharmaceutical Products, Westborough MA) were used for immobilization. Brief electrical stimuli (pulse width=0.1 ms, frequency=350 Hz, train duration=30 ms) were delivered via an insulated tungsten electrode exposed at the tip to established midbrain vocal sites (mVAC, Fig. 1B), and evoked motor volleys were recorded with an extracellular electrode (Teflon-coated silver wire with exposed ball tip) placed on a ventral occipital nerve root that carries sonic motor axons to the ipsilateral sonic muscle (Fig. 1B). Rhythmic vocal motor volleys occurred as discrete bursts ("fictive vocalizations") and were recorded as 15 trains of stimuli at 1-s intervals. Recordings were digitized (Power Macintosh 8100 using IGOR Pro software; Wavemetrics, Inc., Lake Oswego, OR). The duration of each fictive vocalization was measured from the recordings and the mean burst duration was computed for each time point and normalized to 100% of baseline output.

Two baseline recordings were obtained, and then either male ($n=25$) or female ($n=15$) toadfish were administered an intramuscular injection (dorsal trunk muscle) via a pre-inserted 23-G butterfly needle of either 11kT (0.04 mg/kg; $n=9$ males, 3 females), testosterone (0.0024 mg/kg; $n=3$ males, 3 females), 17β-estradiol (E_2 ; 0.02 mg/kg; $n=5$ males, 5 females), cortisol (0.05 mg/kg; $n=4$ males, 4 females), or vehicle (peanut oil of equivalent volume, $n=4$ males). Only one treatment was administered per experiment, and hormone doses were based on a previous study in the closely related midshipman fish (see Remage-Healey and Bass, 2004). Immediately following the injection, a subsequent series of vocal motor recordings were obtained at multiple time points (5, 15, 30, 45, 60, 90, 120 min) and standardized to 100% of the baseline output. For the analysis, data from the two baseline recordings (separated by 5 min) were combined to provide a stable basis for comparison of all subsequent data. Means for these pooled baseline data are presented in all figures for fictive vocalizations at time=0 min (for individual means for baseline 1 vs. baseline 2 see Results). Blood samples were obtained to verify that steroid injections achieved physiological levels observed in field-caught Gulf toadfish (see Remage-Healey and Bass, 2005a). Plasma was analyzed for testosterone and cortisol via radioimmunoassay (Diagnostic Laboratory, New York State College of Veterinary Medicine at Cornell University), and for E_2 and 11kT using enzyme immunoassay (Cayman Chemical, Ann Arbor, MI; techniques reported previously in Remage-Healey and Bass, 2004, 2005a).

Isolation of hindbrain-spinal vocal pattern generator (VPG)

To assess whether rapid steroid actions occur directly on the VPG region, a second set of toadfish underwent craniotomy as described above followed by surgical isolation of the region containing the VPG (after Weiser et al., 1986; Remage-Healey and Bass, 2004). For ten males, fine forceps were used to

transect the rostral hindbrain immediately caudal to the cerebellum (anterior vertical arrow, Fig. 1B). Spontaneous, vocal activity patterns (Fig. 1C right) similar to natural boatwhistles in both fundamental frequency and duration occurred immediately following isolation. Spontaneous vocal activity subsided after 20–35 min, after which brief electrical stimuli (parameters as above) delivered to the ventral medullary region of the VPG (Fig. 1B) elicited fictive vocalizations very similar to those observed in the intact preparation (Fig. 1C left). As in the intact preparation (see above), baseline recordings were obtained, and then males were administered the same intramuscular dose of either 11kT ($n=4$), cortisol ($n=3$), or oil vehicle ($n=3$), followed by recordings. Complete isolation of the VPG via caudal spinal and anterior medullary transections (posterior and anterior vertical arrows respectively, Fig. 1B) was performed in four other males using the same transection method (see above). Results for 11kT treatments from the completely isolated VPG were indistinguishable from the anterior alone transections ($p>0.05$).

Analysis

Results were analyzed with Statview version 4.57 and SAS V8. Wherever possible, a within-subject, repeated-measures-over-time experimental design was used to reduce the influence of individual variation on neurophysiological and behavioral measures. Neurophysiological data were analyzed using repeated measures ANOVA followed by Tukey's post hoc tests that compared within-group changes over time following hormone treatments. Field behavioral data were analyzed using paired *t*-tests for within-subject changes in calling behavior (comparing pre- and post-treatment calling behavior). Field hormonal data were analyzed using unpaired *t*-tests for between-subject differences in plasma steroid levels (the design did not allow sampling of hormone levels prior to treatment since blood sampling may have reduced or eliminated calling behavior).

Results

Behavioral field experiments

11kT increases calling behavior

Two-tailed, paired *t*-tests revealed that 11kT-implanted scallops fed to calling males produced a significant increase in call rate (10-min Post period vs. 10-min Pre period;

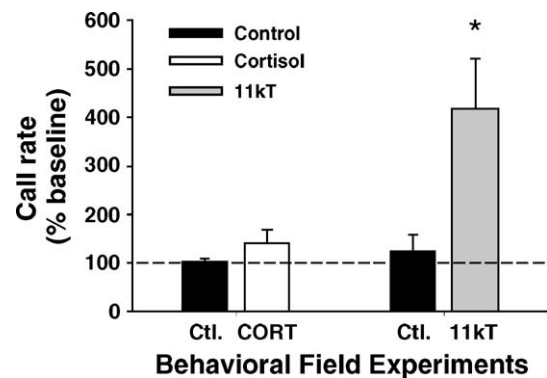


Fig. 2. Acute, non-invasive androgen, but not cortisol, treatment (steroid-implanted scallops) produces rapid changes in vocal behavior in male toadfish in their natural environment. Responses are standardized to pre-treatment calling behavior (dashed line). Scallops were fed to male toadfish guarding nests and emitting advertisement boatwhistles. In the first experiment scallops were implanted with either 11kT ($n=9$) or nothing (control, Ctl.; $n=7$). In the second experiment scallops were implanted with either cortisol (CORT, $n=10$) or nothing (control, Ctl.; $n=5$). Relative to pre-treatment baseline calling behavior, only the androgen 11kT causes significant increases in call rate (number of boatwhistles/10 min) within 10–20 min of administration. * $p<0.05$ for paired *t*-test (pre-treatment vs. post-treatment). Mean baseline call rate was 10.64.

Fig. 2; $p < 0.014$), while there was no significant change in call rate for the equivalent periods following treatment with control scallops (Fig. 2; $p = 0.53$). There was no significant effect of 11kT on call duration (data not shown; $p = 0.21$). There was a significant difference ($p < 0.05$) in plasma 11kT levels immediately following the 10-min Post period between animals fed 11kT (41.83 ± 6.16 ng/ml) vs. control (1.96 ± 0.51 ng/ml) scallops. There was no significant difference in cortisol levels between males fed either 11kT (8.38 ± 4.14 ng/ml) or control (8.11 ± 2.78 ng/ml) scallops.

Cortisol does not change calling behavior

Two-tailed, paired *t*-tests revealed that there were no significant changes in call rate following the feeding of either cortisol-implanted (Fig. 2, $p = 0.19$) or control (Fig. 2; $p = 0.44$) scallops to calling males. There was also no significant effect of cortisol on call duration (data not shown; $p = 0.22$). There was a significant difference ($p < 0.05$) in plasma cortisol levels immediately following the 10-min Post period between animals fed cortisol (69.9 ± 11.55 ng/ml) vs. control (4.75 ± 3.24 ng/ml) scallops (elevated levels were in the high physiological range for this species, see Remage-Healey and Bass, 2005a; Wood et al., 2001; Sloman et al., 2005). There was no significant difference in plasma 11kT levels between males fed either cortisol (2.55 ± 0.42 ng/ml) or control (3.98 ± 1.56 ng/ml) scallops.

Evoked activity of the vocal pattern generator (VPG)

Steroids rapidly regulate VPG activity in intact preparations

Repeated measures ANOVA revealed strong overall treatment effects of steroid hormones on vocal burst duration in both males (Fig. 3A; $F = 6.47$; $df = 4, 140$; $p < 0.002$) and females (Fig. 3B; $F = 6.94$; $df = 3, 77$; $p < 0.007$), indicating that steroids rapidly modified fictive vocalizations in Gulf toadfish. Females

vocalize during agonistic interactions (see Introduction) and were included in this study to establish sex-specific rapid modulation by steroid hormones. There was no effect ($p > 0.05$ for all comparisons) of steroids on the fictive call discharge frequency (the number of fictive sound pulses/seconds within each burst, which directly establishes the fundamental frequency of natural vocalizations, see Bass and Baker, 1990).

Male vocal burst duration was significantly and rapidly increased following treatment with either 11kT ($F = 4.95$; $df = 7, 56$; $p < 0.0003$) or cortisol ($F = 2.91$; $df = 7, 14$; $p < 0.042$) (Fig. 3A). Tukey's post hoc analyses revealed significant ($p < 0.05$) effects of treatment-over-time at 5, 15, 30, 45, 60, 90 and 120 min for both 11kT and cortisol. There were no significant changes following treatment with testosterone, E_2 , or oil vehicle ($p > 0.05$ for each treatment). Plasma steroid levels were within the physiological range for each hormone treatment: 23.61 ± 3.14 ng/ml (11kT), 298.22 ± 15.29 ng/ml (cortisol), 13.43 ± 8.16 ng/ml (testosterone), and 19.64 ± 7.45 ng/ml (E_2). Baseline recordings were highly stable prior to all steroid treatments and did not differ by more than 8% (means for baseline 1 vs. baseline 2 durations, respectively, for each experiment were 11kT, 14.74 vs. 15.11 ms; cortisol, 16.26 vs. 15.88 ms; testosterone, 17.14 vs. 16.94 ms; E_2 , 11.93 vs. 12.84 ms; oil, 15.53 vs. 15.28 ms).

To investigate the sex specificity of the steroid effects on fictive calling, we also tested the effects of 11kT on fictive calling in females. There were no significant changes in vocal burst duration following treatment with 11kT, testosterone, or E_2 in females ($p > 0.05$ for each treatment, respectively; Fig. 3B). By contrast, female vocal burst duration was significantly and rapidly increased following treatment with cortisol ($F = 3.03$; $df = 2, 14$; $p < 0.04$) (Fig. 3B). Tukey's post hoc analysis revealed significant ($p < 0.05$) effects of treatment-over-time at 5, 15, 30, 45, 60, 90, and 120 min for the cortisol treatment. Plasma steroid levels were within the physiological

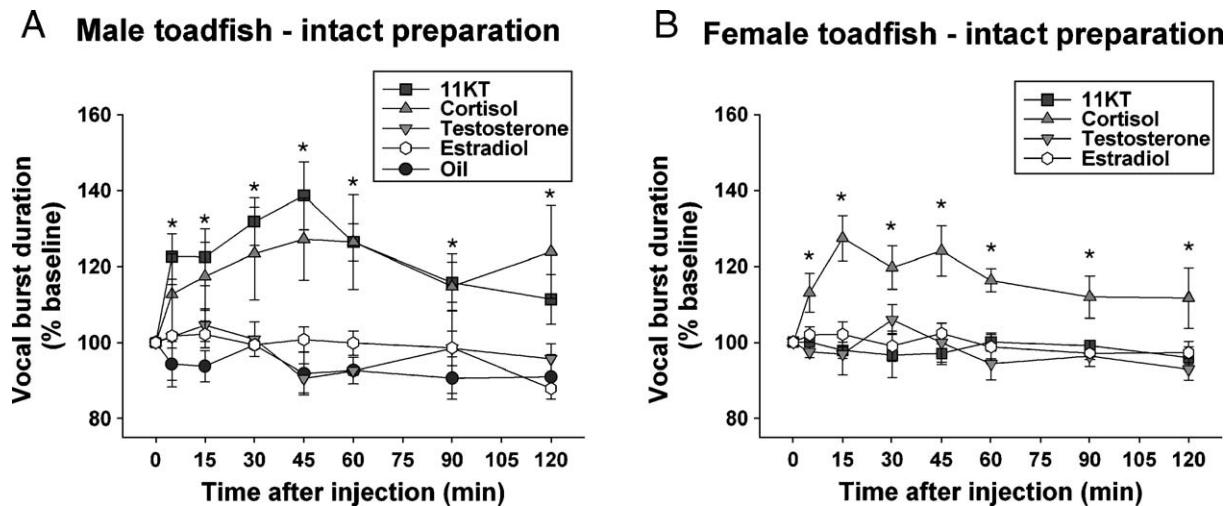


Fig. 3. Rapid actions of steroid hormones on vocal burst duration in male (A) and female (B) toadfish. (A) Both the predominant teleost androgen 11kT ($n = 9$) and the glucocorticoid cortisol ($n = 4$) cause rapid and sustained increases in fictive vocalization duration, whereas testosterone ($n = 3$), estradiol ($n = 5$), or oil vehicle ($n = 4$) do not significantly alter vocal patterning in males. Mean baseline fictive call duration for males was 13.99 ms. (B) In female toadfish, as in males, cortisol ($n = 4$) causes rapid and sustained increases in fictive vocalization duration, while 11kT ($n = 3$), testosterone ($n = 3$), or estradiol ($n = 5$) each do not significantly alter vocal patterning. Mean baseline fictive call duration for females was 17.71 ms. * $p < 0.05$ for within-subject treatment effects.

range for each hormone treatment: 32.58 ± 5.66 ng/ml (11kT), 234.27 ± 6.16 ng/ml (cortisol), 18.99 ± 4.92 ng/ml (testosterone), and 14.85 ± 2.76 ng/ml (E_2). Baseline recordings were highly stable prior to all steroid treatments and did not differ by more than 11% (means for baseline 1 vs. baseline 2 durations, respectively, for each experiment were 11kT, 15.45 vs. 15.83 ms; cortisol, 15.86 vs. 16.34 ms; testosterone, 18.93 vs. 20.01 ms; E_2 , 18.03 vs. 20.25 ms). The sex-specific response to 11kT could depend on baseline differences in fictive vocalizations between male and female toadfish. However, no significant differences ($p > 0.05$ for all comparisons) were detected between the baseline duration of fictive calls elicited from males (mean = 13.99 ms) and females (17.71 ms) at baseline.

Steroids rapidly regulate VPG activity in isolated preparations

For males with a surgically isolated VPG region, repeated measures ANOVA revealed a significant overall effect of the same-dose hormone treatments as in the intact preparation ($F = 9.21$; $df = 2,42$; $p < 0.038$) (Fig. 4A). Vocal burst duration was significantly and rapidly increased following treatment with either 11kT ($F = 3.14$; $df = 7,21$; $p < 0.02$) or cortisol ($F = 3.30$; $df = 7,14$; $p = 0.027$) and Tukey's post hoc analysis revealed significant ($p < 0.05$) effects of treatment-over-time at 5, 15, 30, 45, and 60 min for 11kT and at 5, 15, 30, and 45 min for cortisol. There were no significant changes following treatment with oil vehicle in the isolated VPG ($F = 0.49$; $df = 2,14$; $p = 0.82$). Baseline recordings were highly stable prior to all steroid treatments and did not differ by more than 2% (means for baseline 1 vs. baseline 2 durations, respectively, for each experiment were: 11kT, 24.15 vs. 24.99 ms; cortisol, 30.85 vs. 30.94 ms; oil, 42.08 vs. 42.52 ms).

In the isolated VPG, vocal bursts were not elicited in every case by each stimulus at each time point. Unlike intact preparations (where average burst probability is = 99.5%) vocal responses in isolated preparations were highly variable

in terms of burst probability (i.e., ranged from 15 bursts/15 stimuli to 5 bursts/15 stimuli at 5 min). Therefore, these results were analyzed at each time point for burst probability (number of bursts/total number of stimuli) for each treatment. There was a significant effect of hormone treatment on burst probability (Fig. 4B, $F = 8.02$; $df = 2,42$; $p < 0.01$) and a significant interaction of treatment-over-time ($F = 2.95$; $df = 12,42$; $p < 0.005$). 11kT produced a significant increase in burst probability ($F = 11.22$; $df = 6,18$; $p < 0.0001$) and Tukey's post hoc analysis revealed that burst probability was significantly elevated following 11kT treatment at 5, 15, 30, and 45 min after injection ($p < 0.05$). There were no significant changes in burst probability in preparations treated with either cortisol ($F = 0.48$; $df = 6,12$; $p = 0.81$) or oil injection ($F = 1.94$; $df = 6,18$; $p = 0.13$). Baseline recordings prior to all steroid treatments and did not differ by more than 6% (means for baseline 1 vs. baseline 2 burst probabilities, respectively, for each experiment were 11kT, 0.84 vs. 0.87 ms; cortisol, 0.70 vs. 0.71 ms; oil, 0.87 vs. 0.82 ms). Therefore, in addition to its rapid effects on burst duration in the isolated VPG, 11kT produced a large and significant increase in burst probability in male toadfish (range of mean burst probabilities = 0.84–0.99 following 11kT injection; 0.32–0.84 following cortisol injection; 0.46–0.88 following oil injection).

Discussion

Empirical evidence strongly supports the hypothesis that elevations in plasma androgens in both mating and aggressive contexts prepare individuals for future physiological and behavioral challenges (see Introduction). This phenomenon is widespread in vertebrates, and there is reason to expect that it occurs in humans (e.g., Bernhardt et al., 1998; Schultheiss and Rohde, 2002). Despite this, it is unknown whether rapid elevations in circulating androgen levels cause similarly fast changes in social behavior in vertebrates. Here, we show that

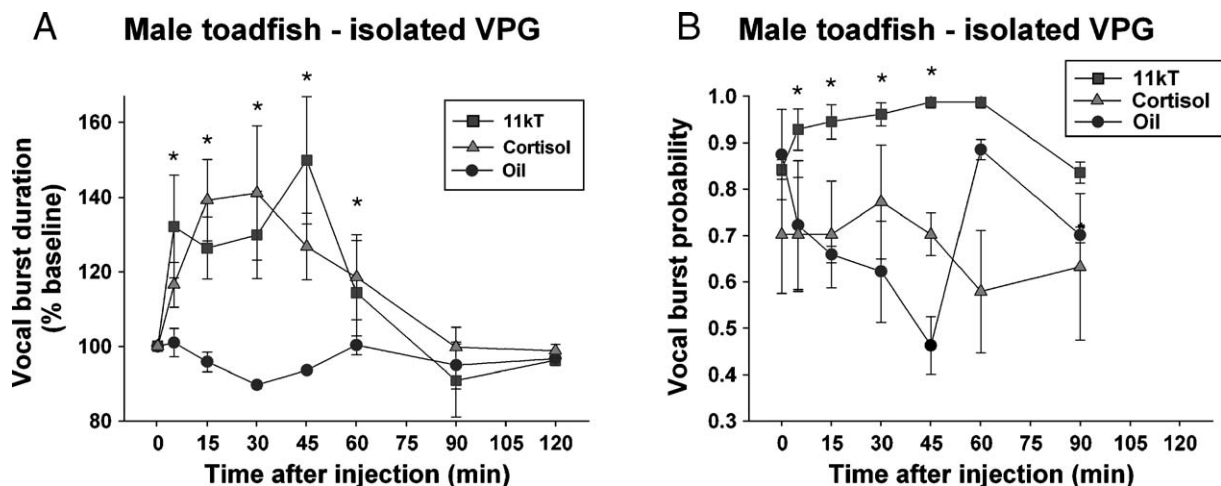


Fig. 4. Rapid androgen actions occur directly on the hindbrain-spinal region containing the VPG in male toadfish. (A) When the VPG is surgically isolated, 11kT ($n = 4$) and cortisol ($n = 3$) each rapidly alters vocal burst duration, while oil vehicle has no effect ($n = 3$). The sustained effects of 11kT on fictive vocalizations beyond 60 min appear to require intact input from the mVAC/fVAC (as in Fig. 3A). Mean baseline fictive call duration was 31.21 ms. (B) Androgens also have rapid actions on the probability evoking a fictive vocalization in the isolated VPG circuit. When the VPG is surgically isolated, 11kT ($n = 4$) rapidly increases vocal burst probability, while neither cortisol ($n = 3$) nor oil vehicle ($n = 3$) have significant effects on burst probability. * $p < 0.05$ for within-subject treatment effects.

experimentally induced surges in androgens regulate moment-by-moment changes in a social behavior, vocalization, via interactions with a central vocal pattern generator. The time course of these events is similar to that observed during acoustic playback challenges, when ongoing calling and plasma androgens are simultaneously elevated (Remage-Healey and Bass, 2005a). We therefore propose that plasma androgens, in addition to their well-established long-term effects on future behavioral interactions (see Introduction), exert causative influences on ongoing social behavior via rapid, non-genomic actions on the central nervous system.

Behavioral mechanisms

11-Ketotestosterone (11kT) is the predominant circulating androgen in male teleosts (Bentley, 1998), including toadfishes (Modesto and Canario, 2003; Sisneros et al., 2004; Fine et al., 2004; Remage-Healey and Bass, 2005a). In the absence of other experimenter-manipulated cues, non-invasive treatment with 11kT caused rapid changes in calling behavior in this study. However, the perception of a social encounter likely plays an important role in shaping the full complement of behavioral and hormonal responses to conspecific cues (see Oliveira et al., 2005). Acoustic playbacks with male Gulf toadfish evoke rapid increases in plasma 11kT levels, ongoing call rate and call duration (Remage-Healey and Bass, 2005a), whereas oral 11kT administration in the absence of playbacks causes rapid increases only in call rate (this study). The lack of an 11kT effect on natural call duration suggests that it is the perception of acoustic cues that leads to call duration changes in response to territorial challenges. Moreover, we observe that 11kT has rapid effects on fictive call duration, but no observable effects on natural call duration in advertising males (see Results). Since fictive calls most closely resemble natural, short-duration grunts (10–50 ms; Figs. 1A, C), we therefore hypothesize that 11kT regulates the transition from making brief grunts in the non-breeding season to long-duration boatwhistles in reproductive contexts (Gray and Winn, 1961) which would be concurrent with increasing plasma 11kT levels (see Remage-Healey and Bass, 2005a). Further duration increases during territorial challenges may be mediated by other hormonal mechanisms, such as progestins (Davis and Marler, 2003) and/or neuropeptides (Goodson and Bass, 2000).

Neurophysiological/behavioral mechanisms

The male-specific modulation of fictive calling by 11kT is related to three observations in Gulf toadfish. First, males have 10–100× higher circulating 11kT levels than females (Remage-Healey and Bass, 2005a; unpublished observations). Second, 11kT is elevated during acoustic challenges that also increase advertisement calling (Remage-Healey and Bass, 2005a), a male-specific behavior associated with dimorphisms in sonic muscle morphology and metabolic capacity (Walsh et al., 1995). Third, as shown here, 11kT rapidly induces increases in advertisement calling in males. While the mechanisms for rapid steroid action are as yet unknown for toadfishes, sex differences

in these effects may be mediated by differences in the expression of membrane or nuclear androgen receptors (ARs) in the vocal control pathway. Although we currently have no direct information on toadfish ARs, a nuclear AR in the closely related midshipman has recently been cloned and is localized in the hindbrain-spinal VPG (Forlano et al., 2005). In addition, a nuclear AR that binds 11kT with high affinity was recently characterized in sticklebacks (Olsson et al., 2005) and a membrane AR that is rapidly regulated by 11kT has also recently been documented (Thomas et al., 2006). Future experiments will therefore address the hypothesis that differential 11kT action observed in the current study is related to differential expression of ARs. Alternative mechanisms for sex differences in rapid androgen actions may include the differential regulation of dopamine (Becker, 1999), gamma-aminobutyric acid (GABA, Qiu et al., 2003) or second-messenger systems (Abraham and Herbison, 2005). In contrast to androgens, the rapid effects of cortisol in the current study were not dimorphic, suggesting a common role in modulating vocal output during acute stress (see below).

Detectable levels of both testosterone and E₂ are found in the plasma of male and female toadfishes (see earlier references), but rapid changes in vocal motor patterning were not observed in response to either steroid in either males or females. The present study demonstrates that the non-aromatizable androgen 11kT alone rapidly regulates male vocalizations, in both field and laboratory settings. This is consistent with the finding that, unlike 11kT, neither E₂ nor testosterone is elevated during the transition from a non-calling state to advertisement calling in males (Remage-Healey and Bass, 2005a). Similar vocal mechanisms are likely operative in the closely related plainfin midshipman fish (see Bass and McKibben, 2003). Nest-guarding midshipman males show seasonally related increases in 11kT levels as they transition from a non-calling to a calling state (Brantley et al., 1993; Knapp et al., 2001; Sisneros et al., 2004) which includes acoustic courtship and territorial challenges (Brantley and Bass, 1994). It seems likely that 11kT surges will also play a causal role in modulating ongoing vocal behaviors in midshipman. However, the current study also reveals an unexpected species difference in rapid steroid mechanisms. In midshipman, testosterone and E₂ (as well as 11kT and cortisol), each exert rapid but distinct actions on VPG activity in males (Remage-Healey and Bass, 2004; Remage-Healey and Bass, 2005b). The behavioral significance of E₂-dependent modulation of the midshipman VPG is unknown, but may further relate to the abundance of aromatase, the enzyme that converts testosterone to E₂, in the VPG region (Schlinger et al., 1999; Forlano et al., 2001) and more generally to the neuroendocrine mechanisms that influence the presence and performance of alternative male reproductive tactics in this species (Bass, 1996; Forlano and Bass, 2005; Remage-Healey and Bass, 2005b).

Surgical isolation of the vocal VPG resulted in spontaneous vocal activity that closely resembled naturally occurring boatwhistles. Thus, the VPG region alone can account for temporal attributes of the advertisement call, consistent with findings from the closely related midshipman fish (Remage-Healey and Bass, 2004). Furthermore, 11kT's actions ranging from 5 to 45 min were localized to the VPG region, suggesting

that the more sustained effects up to 120 min observed in the intact preparation reflect a dependence on descending midbrain input, as demonstrated in midshipman (Ramage-Healey and Bass, 2004). The isolated VPG preparation in toadfish also revealed a previously unobserved effect of 11kT on fictive call probability, and we propose that this effect is linked to the increase in natural call rate observed following 11kT treatment. A connection between the two observations has inherent logic, since as call rate increases, the probability of call initiation over time also increases, indicating a proximate link between increased natural call rate and fictive call probability. This conclusion is consistent with previous observations that male toadfish in full breeding condition (presumably with elevated 11kT; see Fine et al., 2004; Ramage-Healey and Bass, 2005a) are more likely to produce sounds following disturbance than males in non-breeding condition (Gray and Winn, 1961). Also, brain stimulation studies in free-swimming toadfish originally showed that the probability of evoking natural calls is increased following increased midbrain stimulation (Demski and Gerald, 1974). Subsequent neurophysiological studies have demonstrated that midbrain-evoked calling is directly dependent on activation of the hindbrain-spinal VPG (Bass and Baker, 1990; Bass et al., 1993) and that the probability of eliciting fictive calls depends on the amount of activity in the midbrain-VPG pathway (Kittleberger et al., 2006). Thus, while a specific region of the midbrain (the periaqueductal gray) plays an essential role in call initiation (Kittleberger et al., 2006), the current study shows that activity within the VPG itself also contributes to call initiation. Together, the results of the current study support the hypothesis that 11kT causes rapid changes in naturally occurring vocal behavior via rapid modulation of VPG activity. A similar link between the initiation of in vivo walking behavior and pattern generator activity (increased fictive locomotion) by noradrenergic agonists has been observed in cats (Grillner, 1986).

Cortisol did not produce rapid changes in fictive call probability (see Results), consistent with a lack of an effect on natural call rate, and a lack of significant elevation in plasma cortisol following playback treatments (Ramage-Healey and Bass, 2005a). However, while cortisol rapidly increases fictive call duration, it does not produce effects on natural call duration in advertising males (see Results). Since cortisol has neurophysiological effects in both males and female toadfish, it likely exerts rapid actions on vocal behavior in contexts outside of male advertisement calling. For example, cortisol could regulate the expression of grunts when toadfishes are engaged in escalated physical interactions (Gray and Winn, 1961; Brantley and Bass, 1994) or during encounters with predators (unpublished observations). Similarly, the relationship between cortisol levels and aggressive behavior is highly dependent on environmental context in African cichlids (Clement et al., 2005).

Concluding comments

Together, the behavioral and neurophysiological studies reported here support the general hypothesis that aggression and reproductive behavior in territorial male teleosts are predominantly regulated by rapid, androgen-dependent mechanisms.

Recent studies in the bluebanded goby (*Lythrypnus dalli*) show that increased aggression is correlated with rapid decreases in brain aromatase activity, which likely leads to an increase in the androgen:estrogen ratio (Black et al., 2005). Thus, rapid mechanisms for modulation of aggressive behavior in teleosts may be particularly associated with the androgen 11kT, which is non-aromatizable. These observations are in contrast with findings in many tetrapod vertebrates, in which aggression and reproductive behavior are regulated, in part, via brain aromatization of androgens into E₂ (Clark and Nowell, 1979; Hutchison and Steimer, 1985; Schlinger and Callard, 1990; Soma et al., 2000; Cornil et al., 2006).

Finally, this study represents a unique integration of field studies of behavior and neurophysiology experiments to reveal neuroendocrine mechanisms for fast changes in natural behavior. Observations of rapid steroid regulation of moment-by-moment changes in reproductive behavior are becoming widespread in vertebrates (e.g. mammals: Cross and Roselli, 1999; Frye, 2001; Xiao et al., 2003; birds: Cornil et al., 2006; amphibians: Orchinik et al., 2002). We propose that the rapid regulation of vocalizations by steroids is similarly prevalent, particularly in light of the conserved nature of vertebrate vocal motor systems (see Bass and Baker, 1997; Goodson and Bass, 2002).

Acknowledgments

We thank Andrew Tuccillo and Rebecca Calisi for intrepid field assistance; Bobby Henderson, Barbara Shoplock and staff at FSUML for logistical support; and Anthony Hay for technical support. Elizabeth Adkins-Regan, Christiane Meyer and two anonymous reviewers provided helpful comments on an earlier version of the manuscript. This work was supported by NSF DDIG IBN-0407802, NSF predoctoral fellowship, NIMH training fellowship, and Cornell University research grant (to LRH) and NSF IBN-9987341 and IBN-0516748 (to AHB).

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