Behavioral/Systems/Cognitive

# Rapid, Hierarchical Modulation of Vocal Patterning by Steroid Hormones

**Luke Remage-Healey and Andrew H. Bass**
Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853

**Vocal control systems have been identified in all major groups of jawed vertebrates. Although steroid hormones are instrumental in the long-term development and maintenance of neural structures underlying vocalization, it is unknown whether steroids rapidly modulate the neural activity of vocal motor systems. The midshipman fish generates advertisement and agonistic calls that mainly differ in duration. A descending midbrain pathway activates a hindbrain–spinal vocal circuit that directly establishes the discharge frequency and duration of the rhythmic vocal motor volley. This vocal motor output, which can be monitored from occipital nerve roots, directly determines the rate and duration of contraction of a pair of sonic muscles and, in turn, the fundamental frequency and duration of vocalizations. Here, we demonstrate that the duration of the vocal motor volley, or fictive vocalization, is rapidly responsive to steroid hormones, including androgens, estrogens, and glucocorticoids. These responses are consistent, in part, with a nongenomic mechanism and are steroid specific at the receptor level, suggesting the possibility of multiple membrane-bound receptor populations. We also show, using intact and semi-intact preparations, that steroids hierarchically modulate fictive vocalizations; whereas the hindbrain–spinal region is both necessary and sufficient for rapid (within 5 min) effects on duration, descending midbrain input is necessary for maintenance (up to 120 min) of these effects. The conserved nature of vertebrate vocal motor systems suggests that the neuroendocrine principles outlined in this study may be a fundamental feature of all vocal vertebrates.**

*Key words:* **motor control; central pattern generator; vocalization; cortisol; testosterone; estradiol**

## Introduction

Steroid hormones fluctuate in plasma over seconds to minutes in response to a variety of stimuli (Wingfield and Wada, 1989; Fox et al., 1997; Romero and Remage-Healey, 2000). The nongenomic, "rapid" effects of steroid hormones on peripheral physiology (e.g., cardiovascular) have received considerable attention (for review, see Losel and Wehling, 2003), yet research on the rapid effects of steroids on the neural basis of behavior is just beginning (Rose et al., 1998; Moore and Evans, 1999; Balthazart et al., 2001; Frye, 2001). The vocal control network of vertebrates may afford a unique opportunity to test the hypothesis that rapid shifts in plasma steroids influence neural activity to produce rapid changes in behavior.

Steroid hormones are associated with the neural control of vocal communication across a broad range of vertebrates. Both seasonal and sex differences in the size and function of vocal control systems have been linked to steroid hormones in birds (Nottebohm and Arnold, 1976; Arnold and Saltiel, 1979; Nottebohm, 1981; for review, see Ball et al., 2002), amphibians (Kelley, 1986; Perez et al., 1996), and fish (Bass, 1995). From this work, it is evident that steroids support the long-term growth and maintenance of neural structures involved in communication. However, the short-term actions of steroid hormones on the neural substrates of vertebrate vocalization are, to our knowledge, unknown.

Available evidence indicates that vocalization behaviors are controlled by discrete networks of vocal control nuclei in fish (Goodson and Bass, 2002), amphibians (Schmidt, 1992), birds (Nottebohm et al., 1976; for review, see Suthers and Margoliash, 2002), and mammals (Jurgens, 2002). The vocal control network of teleost fish presents the simplest example of how a vertebrate generates context-dependent vocalizations (Bass and McKibben, 2003). Here, we consider how steroid hormones alter vocal signals in the hindbrain–spinal and midbrain vocal centers of the midshipman fish (*Porichthys notatus*). Midshipman use vocalizations, which mainly differ in duration, for both mate attraction and agonistic encounters (see Fig. 1*A*,*B*) (Brantley and Bass, 1994; Bass et al., 1999). The output of a hindbrain–spinal pacemaker circuit directly establishes the fundamental frequency and duration of vocalizations (see Fig. 1*C*) (Bass and Baker, 1990). Other forebrain and midbrain nuclei activate the hindbrain–spinal pacemaker (Bass et al., 1994; Goodson and Bass, 2002). Experimentally, this network can be manipulated so that vocal motor output that mimics natural vocalizations (fictive vocalization) can be elicited and monitored in a neurophysiological preparation (Bass and Baker, 1990; Goodson and Bass, 2000a,b). The

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Correspondence should be addressed to Dr. Luke Remage-Healey, Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853. E-mail: lrr4@cornell.edu.

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present experiments assess the influence of steroid hormones on the neural control of vocal patterning in midshipman. We report that the duration of fictive vocalizations is rapidly (within 5 min) increased by androgens, estrogens, and glucocorticoids and that the maintenance of this facilitation after 30 min is steroid specific. We also show that steroids exert independent actions at midbrain and hindbrain–spinal levels. Given the many shared traits in the organization of the vocal motor systems of vertebrates (Bass and Baker, 1997; Goodson and Bass, 2002), we propose that similar patterns of steroid hormone modulation may be present in other vertebrate groups, including birds and mammals.

## Materials and Methods

*Subjects.* Plainfin midshipman (*Porichthys notatus*) have three adult morphotypes: females, type I males, and type II males. Type I males acoustically court females and provide parental care, whereas type II males alternatively sneak or satellite spawn at the nest site (Bass, 1996). All animals used in this study were type I males (14–21 cm), which have the most varied vocal repertoire of the three morphs and emit calls that differ widely in duration (see Fig. 1*A*,*B*) (Bass et al., 1999). Fish were either collected from nest sites or by offshore trawls (Sisneros et al., 2004) in northern California during 2001–2003 and were shipped directly to Cornell University. Fish were maintained in artificial seawater tanks at 15–16°C until the day of experimentation. All neurophysiology procedures, including surgery and extracellular recordings, were similar to those presented previously (Bass and Baker, 1990; Goodson and Bass, 2000a,b). The brain and rostral spinal cord were exposed by dorsal craniotomy under general anesthesia (0.025% benzocaine; Sigma, St. Louis, MO). After surgery, fish were stabilized in a Plexiglas tank and perfused through the mouth with fresh saltwater maintained at 15–16°C with a peltier device. During recording, exposed brain areas were covered with flouroinert (3M, St. Paul, MN), intramuscular injections of pancuronium bromide were used for immobilization (0.5 mg/kg; Astra Pharmaceutical, Westborough, MA), and fentanyl was used for analgesia (1 mg/kg; Sigma). All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

*Recordings.* Intracellular recording and staining studies, together with transneuronal tract tracing studies (Bass and Baker, 1990; Bass et al., 1994), have shown that the hindbrain–spinal vocal circuit of midshipman fish includes (Fig. 1*C*): (1) paired sonic motor nuclei along the midline that begin at caudal levels of the vagal motor complex and extend into the rostral spinal cord; (2) a more diffuse column of pacemaker-like neurons along the ventrolateral margins of the sonic motor nuclei that bilaterally innervate the motor nuclei and establish its rhythmic firing rate; and (3) paired ventral medullary nuclei that are immediately rostral to the pacemaker–motor neuron complex and provide extensive coupling across the midline. The vocal motor volley resulting from the activity of the pacemaker circuit is easily monitored with external electrodes placed on ventral occipital nerve roots that exit the brain at the level of the sonic motor nucleus and innervate each of the sonic muscles attached to the swimbladder (Bass and Baker, 1990).

Insulated tungsten electrodes (125 $\mu$m diameter; 20 $\mu$m exposed tips) were used to deliver brief (25–30 msec) trains of stimuli (0.1 msec duration; 300 Hz) to midbrain and hindbrain sites to evoke vocal motor volleys. The midbrain site used here and in previous neurophysiological studies (Bass and Baker, 1990; Goodson and Bass 2000a,b) projects to the hindbrain–spinal vocal circuit (Fig. 1*C*) (Goodson and Bass, 2002). The hindbrain stimulation site lies either within or close to the ventral medullary nucleus that projects to the pacemaker–motor neuron circuit (Bass et al., 1994; Goodson and Bass, 2002). Output from an occipital nerve root was monitored unilaterally with an extracellular electrode (Teflon-coated silver wire with exposed ball tip; 50–100 $\mu$m diameter). Because both sides of the brain fire in phase, unilateral monitoring reflects bilateral synchrony of the descending vocal motor volley (Bass and Baker, 1990). Evoked motor volleys occurred as discrete bursts, and three parameters were assessed from the recordings (Goodson and Bass, 2000a,b): burst latency (interval between stimulus train offset and burst onset), burst duration, and discharge frequency of the burst. Recordings consisted of 15 trains of stimuli at 1 sec intervals. Recordings were digitized using a Power Macintosh 8100 with IGOR Pro software (Wavemetrics, Lake Oswego, OR; software customized by R. Wyttenbach, Cornell University, Ithaca, NY).

Once a stable output was established (defined as a stereotyped, repeatable response to stimulus), the tungsten electrode was removed from the stimulation site to avoid damage resulting from possible experimenter-related movement of the animal during hormone injection. A 23 gauge butterfly needle (infusion set SV23BLK; Terumo, Tokyo, Japan) attached to a 1 cc syringe filled with hormone or vehicle solution was inserted into the dorsal epaxial muscle. The stimulation electrode was reinserted, and two baseline recordings were obtained, separated by a 5 min interval. The baseline recording with the least variance (SD) for all measures (duration, latency, and frequency) was used to standardize all subsequent recordings for that experiment (relative to baseline output, 100%).

We studied the influence of four steroid hormones on the vocal motor system: cortisol, 11-ketotestosterone (11KT), 17$\beta$-estradiol, and testosterone. All of these steroids are naturally occurring in wild populations of midshipman fish (Brantley et al., 1993; Knapp et al., 2001; Sisneros et al., 2004). 11KT is a teleost-specific androgen that, like dihydrotestosterone in other vertebrates, is not converted to estrogens by aromatization (Bentley, 1998). To assess steroid modulation of vocal motor output, cortisol (1.0 mg/kg; 0.05 mg/kg), 11KT (2.0 mg/kg; 0.2 mg/kg; 0.04 mg/kg), 17$\beta$-estradiol (1.0 mg/kg; 0.02 mg/kg), testosterone (0.02 mg/kg; 0.002 mg/kg), or oil vehicle (peanut oil; equivalent volume) was injected intramuscularly through the pre-inserted butterfly needle (all steroids from Sigma). Only one steroid hormone was delivered per experiment. Hormone doses were based on previous studies in which steroid levels were experimentally manipulated to achieve short-term changes in circulating plasma levels of cortisol (Veillette et al., 1995; Remage-Healey and Romero, 2001; Wood et al., 2001), 11KT (Townsend et al., 1991; Bar et al., 1996; Watanuki et al., 2002), 17$\beta$-estradiol (Salbert et al., 1993; Olsson and Kling, 1995), and testosterone (Townsend et al., 1991; Joubert and Tobin, 1995; Bar et al., 1996; McAllan et al., 1998). After hormone or vehicle injection, a subsequent series of vocal motor recordings were obtained at 5, 15, 30, 45, 60, 90, and 120 min. Blood samples were obtained from a subset of animals in each injection group after the 60 min recording to verify steroid delivery and monitor plasma levels. Whole blood was separated under centrifugation, and plasma was frozen until analysis.

*Receptor specificity experiments.* Results of the above experiments suggested that steroid hormones interact specifically with different membrane receptor populations. To directly investigate the possibility of steroid specificity, we used two steroid receptor antagonists, cyproterone acetate (CA; an anti-androgen) and mifepristone (RU486; an anti-glucocorticoid). CA selectively interferes with androgens binding to the androgen receptor in goldfish brain (Wells and Van Der Kraak, 2000) and has been shown to be an effective androgen antagonist in many fish species (Billard, 1982; Kindler et al., 1991; Singh and Joy, 1998; Kiparissis et al., 2003). A more recently developed androgen receptor antagonist, flutamide, does not bind to androgen receptors in teleost brain (Wells and Van Der Kraak, 2000), necessitating the use of CA. RU486 is a commonly used glucocorticoid receptor antagonist (it is also an antiprogesterone) and has been shown to interfere with rapid, nongenomic effects of glucocorticoids in other systems (Liu et al., 1995; Roozendaal et al., 2002; He et al., 2003). For the receptor antagonist experiments, fish were pretreated with intramuscular injections of either CA (0.25 mg/kg; $n = 8$) or RU486 (0.25 mg/kg; $n = 8$) for 30 min, and then baseline measurements were obtained as above before intramuscular injection of cortisol (0.05 mg/kg), 11KT (0.2 mg/kg), or no-injection (control; receptor antagonist alone). After injection, a subsequent series of vocal motor recordings were obtained at 5, 15, 30, 45, 60, 90, and 120 min. In a separate set of experiments also examining steroid specificity, we tested for stereospecificity of the effects of 17$\beta$-estradiol by injecting a similar dose (1.0 mg/kg) of 17$\alpha$-estradiol (Sigma), a biologically inactive stereoisomer. After injection of 17$\alpha$-estradiol alone, a subsequent series of vocal motor recordings were obtained at 5, 15, 30, 45, 60, 90, and 120 min ($n = 2$).

*Hormone assays.* Plasma was analyzed for steroid hormones using RIA

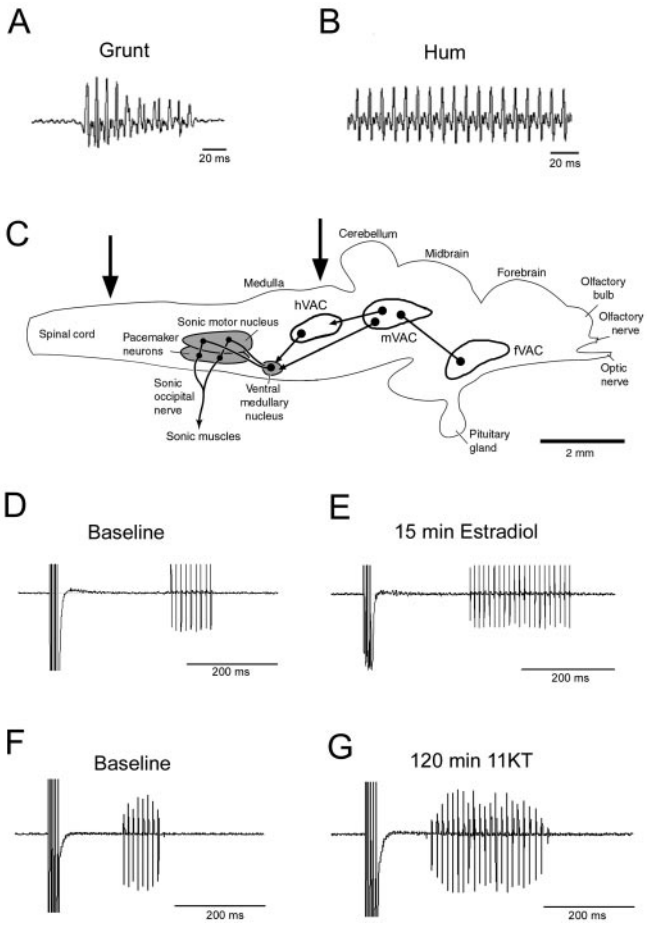


**Figure 1.** *A*, *B*, Sounds recorded from type I male midshipman in their natural habitat. Males emit short-duration grunts (*A*) during aggressive encounters and long-duration hums (*B*) to attract females. *C*, Sagittal view of the midshipman brain, showing forebrain, midbrain, and hindbrain–spinal vocal motor regions. Arrows indicate rostral and caudal transection sites used in the hindbrain–spinal isolation experiments; fVAC, mVAC, hVAC are forebrain, midbrain, and hindbrain vocal acoustic complexes, respectively (Goodson and Bass, 2002). *D–G*, Oscillograms of fictive vocalizations evoked by electrical stimulation in two experiments on type I male midshipman. Stimulus artifact is presented for reference on left side of each trace. Recordings shown are under baseline conditions (*D*, *F*) and either 15 min after 17$\beta$-estradiol injection (*E*; 0.02 mg/kg) or 120 min after 11KT injection (*G*; 0.2 mg/kg). Note in each case the rapid change in burst duration but neither discharge frequency nor latency after steroid administration.

and enzyme immunoassay (EIA). Cortisol and testosterone were analyzed with RIA at the Diagnostic Laboratory, New York State College of Veterinary Medicine at Cornell University. 17$\beta$-Estradiol and 11KT were analyzed with EIA (Cayman Chemical, Ann Arbor, MI), using techniques previously optimized for fish (Cuisset et al., 1994). A subset of samples was assayed for 11KT using both EIA and RIA to verify that assay results were linearly correlated (data not shown; correlation coefficient $R^2 = 0.93$). On an absolute scale, EIA-reported steroid hormone levels are higher than RIA for the same plasma sample. For reference, high physiological levels of circulating steroids (75%) as determined by RIA are as follows: cortisol, 429.6 ng/ml; 11KT, 34.82 ng/ml; 17$\beta$-estradiol, 13.88 ng/ml; testosterone, 7.21 ng/ml (Sisneros et al., 2004). To verify that our method of steroid delivery produced rapid changes in plasma hormone levels, one animal was serially blood sampled from the gill arch after injection of 11KT (0.04 mg/kg) into the dorsal epaxial muscle. Plasma 11KT levels in this individual rapidly increased after intramuscular injection (baseline, 2.19 ng/ml; 5 min after injection, 3.14 ng/ml; 30 min after injection, 9.98 ng/ml).

*Isolated hindbrain–spinal circuit.* The majority of studies were performed using only the midbrain stimulation site to activate a vocal motor volley. However, a subset of studies aimed to demonstrate that the hindbrain–spinal pacemaker circuitry is both necessary and sufficient for the rapid effects of steroid hormones. Surgical isolation of the hindbrain–spinal pacemaker circuit in the closely related toadfish has shown that this brain region is both necessary and sufficient to produce a rhythmic vocal motor output as monitored from the sonic occipital nerve roots (Weiser et al., 1986). Similar brainstem transections were performed on a subset of animals in an attempt to localize the effect of steroid hormones to the hindbrain–spinal pacemaker circuit. Dorsal craniotomy exposed all brain structures as above. Using fine forceps, the rostral hindbrain was separated from the midbrain by a transverse crush immediately caudal to the cerebellum (Fig. 1*C*, right vertical arrow). This transection isolates all nuclei in the hindbrain–spinal pacemaker circuit (Fig. 1*C*) from rostral portions of the vocal motor network.

In all cases ($n = 11$) immediately after transection, the isolated hindbrain–spinal circuit produced a series of long-duration, spontaneous motor volleys. This spontaneous activity subsided after 15–30 min, after which experiments could proceed. Brief, low-amplitude trains of stimuli (parameters as above) were delivered to the ventral medullary region to elicit fictive vocalizations from the isolated hindbrain–spinal circuit. After baseline recordings, oil vehicle, 11KT (0.04 mg/kg), or cortisol (0.05 mg/kg) was delivered intramuscularly (as above), and subsequent recordings were obtained at 5, 15, 30, 45, 60, and 90 min after injection.

To verify that steroid hormones modulate the completely isolated hindbrain–spinal circuit, one animal was also transected at the rostral spinal cord (caudal to the sonic motor nucleus) (Fig. 1*C*, left vertical arrow) and given 0.04 mg/kg 11KT. This preparation therefore contained the completely isolated vocal pacemaker circuit. Results from this preparation were indistinguishable from those obtained in the single-transection experiment (data not shown), thus supporting the hypothesis that all of the circuitry both necessary and sufficient to evoke and modulate rhythmic vocal motor output is within the region containing the hindbrain–spinal circuit shown in Figure 1*C*.

*Intact stimulation of hindbrain–spinal and midbrain circuitry.* As reported below, the transection experiments revealed a separation between the rapid and sustained effects of steroids on vocal motor activity. To further investigate the site dependency of these temporal events, another series of experiments included a subset of nontransected (i.e., intact) animals stimulated in the hindbrain (ventral medullary) and midbrain sequentially. These experiments were designed to test whether steroid hormones differentially interact with hindbrain–spinal versus midbrain vocal centers. After insertion of the intramuscular needle (for steroid delivery, as above), baseline output from the midbrain site was established, and this site was identified for future stimulation by using a combination of visual surface markings and depth measurements on the micromanipulator (Goodson and Bass, 2000a). The stimulating electrode was then moved to the hindbrain ventral medullary site, and baseline output was recorded. Either 11KT (0.04 mg/kg) or 17$\beta$-estradiol (0.019 mg/kg) was then delivered intramuscularly, and a series of recordings were made after stimulation at the hindbrain site (5, 15, 30, and 45 min). In the 11KT experiment, for each of the 45, 60, 90, and 120 min recordings, stimuli were also delivered to the established midbrain site, and recordings were made for comparison of hindbrain- and midbrain-evoked output.

*Analysis.* All recording data (including burst latency, burst duration, and discharge frequency) were standardized according to baseline measurements (relative to 100%) to adjust for between-experiment variation. Results were analyzed with statistical analysis software (SAS, Cary, NC) on a within-subject basis with repeated-measures ANOVA, followed by Tukey's multiple comparison tests for differences among sampling times.

## Results

Representative records of fictive vocalizations that depict a typical change in output duration after steroid hormone administration are shown in Figure 1*D–G*. For all treatment groups, discharge frequency and burst latency were not significantly altered at all time points after steroid hormone administration (e.g., 30 min data shown in Table 1) ($p > 0.05$ for all comparisons).

**Table 1. Discharge parameters of vocal bursts 30 min after low-dose injection**

| Treatment | Latency (msec) | Frequency (Hz) |
|---|---|---|
| Oil | 88.05 ± 7.71 | 102.19 ± 3.68 |
| 11KT | 94.08 ± 6.54 | 101.55 ± 1.04 |
| Cortisol | 96.43 ± 6.23 | 99.55 ± 1.22 |
| 17β-Estradiol | 104.26 ± 19.29 | 96.53 ± 1.64 |
| Testosterone | 104.62 ± 10.50 | 103.69 ± 3.19 |

Data are mean ± SEM.

**Table 2. Circulating plasma levels (nanograms per milliliter) of steroid hormones after short-term steroid injection**

| Treatment | Sample size (*n*) | Plasma steroid (mean ± SEM) |
|---|---|---|
| Cortisol | | |
| 1.0 mg/kg | 8 | 478.53 ± 89.37 |
| 0.05 mg/kg | 4 | 259.86 ± 59.97 |
| 11KT | | |
| 2.0 mg/kg | 5 | 263.25 ± 45.81 |
| 0.2 mg/kg | 5 | 99.54 ± 11.38 |
| 0.04 mg/kg | 5 | 21.38 ± 2.43 |
| 17β-Estradiol | | |
| 1.0 mg/kg | 3 | 115.43 ± 22.68 |
| 0.02 mg/kg | 5 | 53.71 ± 11.09 |
| Testosterone | | |
| 0.02 mg/kg | 5 | 48.41 ± 12.24 |
| 0.0024 mg/kg | 3 | 4.036 ± 1.43 |
| Oil control (equivalent volume) | 6 | |
| Cortisol | | 77.47 ± 18.6 |
| 11KT | | 1.71 ± 0.51 |
| 17β-Estradiol | | 0.59 ± 0.10 |

Presented below are results for burst duration after midbrain stimulation, unless noted otherwise. Steroids were administered at multiple doses (see figure legends for the number of animals in each group), and plasma levels were monitored for verification (Table 2) to confirm that injected steroids reached physiological levels for this species. Although measured plasma levels sometimes exceeded known physiological levels, each experiment included doses within the naturally occurring range for midshipman (75% percentile) (Table 2) (Brantley et al., 1993; Sisneros et al., 2004); the one exception to this was the 17β-estradiol treatment group (but see Discussion for physiological relevance).

### Oil injection

Oil vehicle (peanut oil) was used as an injection control for all hormone treatments ($n = 6$). Another subset of animals ($n = 2$) received no injection but underwent the same series of neurophysiology recordings as above. This no-injection group was to control for the presence of neuroactive compounds in the oil solution. All parameters measured were indistinguishable between oil injection and no injection; consequently, these data were combined with the oil controls for statistical analysis (all figures show data from oil controls only). The duration of fictive vocalization was unchanged after oil injection ($F = 0.67$, $p = 0.17$).

### Cortisol

Two doses of cortisol (1.0 and 0.02 mg/kg) were used to mimic stress-induced release of glucocorticoids over a rapid time course. The duration of fictive vocalization was facilitated by cortisol compared with baseline (Fig. 2*A*) ($F = 2.87$; $p = 0.02$). This effect was significant within 5 min after injection ($p < 0.05$), and burst duration remained significantly elevated (up to 140% of baseline) through the 60 min sampling time ($p < 0.05$). Burst duration was not significantly different from baseline by 90 min after injection ($p > 0.14$).

### 11-Ketotestosterone

11KT was administered in three doses (2.0, 0.2, and 0.04 mg/kg) (Table 2) to mimic the rapid release of androgens during courtship and territorial encounters. The duration of fictive vocalization was significantly facilitated by 11KT (Fig. 2*B*) ($F = 4.12$; $p = 0.004$). This effect was apparent within 5 min after injection ($p < 0.05$), and burst duration remained elevated (up to 180% of baseline) through the 120 min sampling time ($p < 0.05$ for all time points). The response to the medium dose of 11KT (0.2 mg/kg) was most elevated, whereas the low and high doses produced similar patterns; together, this indicated an inverse-U response relationship with a dose commonly observed in studies of steroid effects on behavior (Breuner and Wingfield, 2000). In addition, the response to the highest (supraphysiological) dose of 11KT (2.0 mg/kg) indicated an early response ramp through 15 min that then subsided, followed by a steady increase in the sustained portion of the response (>30 min). This first suggested to us that the rapid effects and sustained effects of 11KT could be differentiated with additional experimentation (see below).

### 17β-Estradiol

The sonic motor nucleus and adjacent column of pacemaker neurons of midshipman contains high levels of aromatase, the enzyme that converts testosterone to 17β-estradiol (Schlinger et al., 1999; Forlano et al., 2001). 17β-Estradiol was administered in two doses (1.0 and 0.02 mg/kg). The duration of fictive vocalization was facilitated by 17β-estradiol (Fig. 2*C*) ($F = 6.47$; $p = 0.0004$). This effect was significant within 5 min after injection ($p < 0.05$), and burst duration remained elevated (up to 139% of baseline) through the 15 and 30 min time points ($p < 0.05$). Fictive vocalization duration was not significantly different from baseline at each of the time points thereafter ($p > 0.05$ through 120 min).

### Testosterone

Testosterone is the synthesis precursor to both 11KT and 17β-estradiol in teleost fishes (Bentley, 1998). Testosterone was administered at two doses (0.02 and 0.002 mg/kg). The duration of fictive vocalization was unchanged after testosterone administration (Fig. 2*D*) ($F = 0.11$; $p = 0.75$). Alhough testosterone is converted to 17β-estradiol by the enzyme aromatase in midshipman (Schlinger et al., 1999), the lack of an effect of testosterone seen here could be attributable to seasonal expression of aromatase. The testosterone experiments were conducted in the months of October and April, when aromatase expression assayed with midshipman-specific antibodies and oligonucleotide probes is reduced to nearly nondetectable, compared with levels of expression during the breeding season (Forlano and Bass, 2003). Similarly, we suspect the levels of 11β-hydroxysteroid dehydrogenase the enzyme that converts testosterone to 11KT may be depressed during the months when these experiments were conducted. All other steroid treatments, including 11KT, 17β-estradiol, and cortisol, occurred in animals in both breeding and non-breeding condition, and no systematic differences were noted in those experiments with respect to season.

### Receptor specificity experiments

A subset of experiments was designed to directly test for response specificity at the steroid receptor level. Vocal activity remained

unchanged after treatments of either the anti-androgen CA or CA plus 11KT (Fig. 3*A*) ( $p > 0.05$). However, the duration of fictive vocalization was facilitated by cortisol in the presence of CA (Fig. 3*A*) ($F = 4.61$; $p = 0.001$). This effect of cortisol was significant within 5 min after injection ( $p < 0.05$), and burst duration remained elevated (up to 149% of baseline) through the 60 min time point ( $p < 0.05$ for all time points). Fictive vocalization duration was not significantly different from baseline at each of the time points thereafter ( $p > 0.05$ through 120 min). Thus, CA interfered with the rapid effects of 11KT but not cortisol on vocal motor duration.

Vocal activity remained unchanged after treatments of either the anti-glucocorticoid RU486 or RU486 plus cortisol (Fig. 3*B*) ( $p > 0.05$). However, the duration of fictive vocalization was facilitated by 11KT in the presence of RU486 (Fig. 3*B*) ($F = 3.92$; $p = 0.005$). This effect of 11KT was significant within 5 min after injection ( $p < 0.05$), and burst duration remained elevated (up to 159% of baseline) through the 120 min time point ( $p < 0.05$ for all time points). Thus, RU486 interfered with the rapid effects of cortisol but not 11KT on vocal motor duration. In addition, vocal activity remained unchanged in response to administration of the biologically inactive stereoisomer 17$\alpha$-estradiol (data not shown; $p > 0.05$), indicating that the response to 17$\beta$-estradiol is stereospecific, similar to rapid estrogen signaling in hypothalamic neurons in guinea pigs (Qiu et al., 2003).

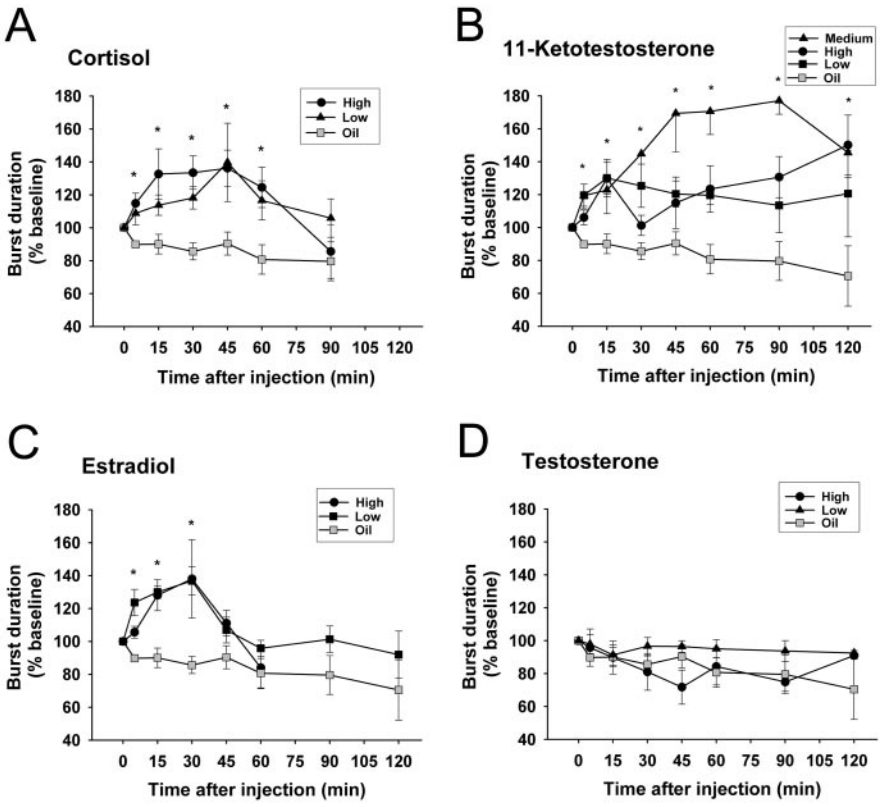


**Figure 2.** Rapid and sustained steroid modulation of vocal motor output during midbrain stimulation. Data are presented as mean ± SEM for Figures 2–4. Control experiments (oil injection; $n = 6$ animals) are plotted in each figure for comparison. *A*, Cortisol, the major circulating teleost glucocorticoid, rapidly increases vocalization duration after intramuscular injection. This effect is both rapid (within 5 min) and sustained (up to 60 min) for both low ($n = 4$) and high ($n = 8$) doses. *B*, 11KT, the major circulating teleost androgen, rapidly increases vocalization duration after intramuscular injection. This effect is both rapid (within 5 min) and sustained (up to 120 min) for low ($n = 5$), medium ($n = 5$), and high ($n = 5$) doses. *C*, 17$\beta$-Estradiol, a neuroactive steroid hormone, rapidly increases vocalization duration after intramuscular injection. This effect is rapid (within 5 min) but not sustained beyond 30 min for both low ($n = 5$) and high ($n = 3$) doses. *D*, Testosterone, the synthesis precursor to both 17$\beta$-estradiol and 11KT, causes no change in vocalization duration after intramuscular injection at either low ($n = 3$) or high ($n = 5$) dose.

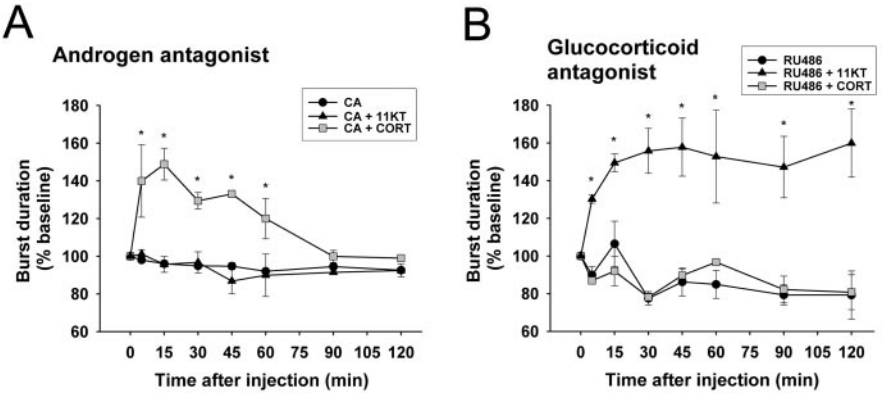


**Figure 3.** The effects of 11KT and cortisol on vocal motor activity are eliminated in the presence of specific steroid receptor antagonists. *A*, The anti-androgen CA blocks the effects of the androgen 11KT ($n = 3$), whereas the response to cortisol (CORT) remains unchanged in the presence of CA ($n = 3$). *B*, The anti-glucocorticoid mifepristone (RU486) blocks the effects of CORT ($n = 3$), whereas the response to 11KT remains unchanged in the presence of RU486 ($n = 3$). Either CA ($n = 2$) or RU486 ($n = 2$) alone did not significantly affect vocal motor activity.

### Isolated hindbrain–spinal circuit

In an attempt to localize the effect of steroids to the hindbrain, a subset of animals underwent a brainstem transection and received an intramuscular injection of 11KT (0.04 mg/kg), cortisol (0.05 mg/kg), or oil (equivalent volume). The duration of fictive vocalization evoked from the isolated hindbrain was facilitated by 11KT (Fig. 4*A*) ($F = 2.83$; $p < 0.05$) and cortisol (Fig. 4*A*) ($F = 2.763$; $p = 0.05$). These effects were apparent within 5 min of injection ( $p < 0.05$), and burst duration was elevated (up to 164% of baseline) through the 15 and 30 min sampling times ( $p < 0.05$ for both 11KT and cortisol). Fictive vocalization duration was not significantly different from baseline at each of the time points thereafter for either hormone ( $p > 0.05$ through 120 min). In vehicle controls, there was no significant change from baseline duration at any sampling time after oil injection ($F = 1.65$; $p = 0.13$).

### Intact stimulation of hindbrain and midbrain

The isolated hindbrain–spinal experiments revealed effects of steroids that were rapid (i.e., within 5 min) but not sustained (>30 min), as occurred in intact preparations. This suggested that the

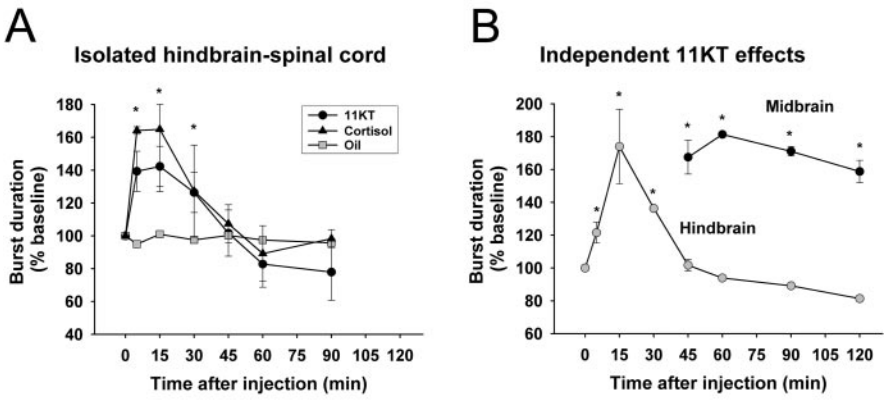


**Figure 4.** The hindbrain–spinal vocal center is both necessary and sufficient for rapid steroid modulation. After hindbrain–midbrain transection (*A*), both 11KT (0.04 mg/kg) and cortisol (0.05 mg/kg) rapidly increase the duration of vocal bursts after intramuscular injection. These effects are rapid (5, 15, and 30 min) but not sustained for both 11KT ($n = 5$) and cortisol ($n = 3$). Oil injection ($n = 3$) produced no change in vocal motor activity. *B*, 11KT hierarchically modulates the duration of vocal bursts elicited from the midbrain and hindbrain in the same intact preparation ($n = 4$). After injection, 11KT rapidly increases burst duration in the hindbrain (5, 15, and 30 min), but this effect is not sustained beyond 30 min. In contrast, parallel stimulation in the midbrain evokes responses to 11KT that are sustained through 45, 60, 90, and 120 min after injection.

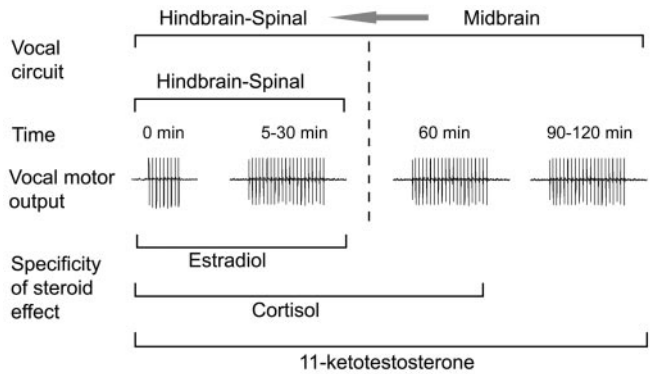


**Figure 5.** Summary figure depicting rapid, hierarchical modulation of midbrain and hindbrain–spinal vocal motor activity by steroid hormones. The hindbrain–spinal vocal circuit is both necessary and sufficient for the rapid effect of steroids (up to 30 min), whereas descending input from the midbrain is necessary for sustained effects of steroids (>30 min). The rapid and sustained effects of steroid hormones on vocal motor circuitry are represented by neurophysiological recordings shown in the center of the figure. Also shown is the temporal specificity that different steroid hormones exert on the midbrain– hindbrain vocal motor system, suggesting the presence of multiple receptor populations in the vocal motor system of midshipman.

midbrain might be a source of longer-lasting elevations in burst duration in intact preparations. To investigate this possibility, a subset of animals was stimulated at both midbrain and hindbrain sites after intramuscular injection of 11KT (0.04 mg/kg), the steroid that showed the most sustained effects in intact preparations (up to 120 min) (Fig. 2*B*). In addition, a subset of animals was stimulated in the hindbrain after intramuscular injection of 17$\beta$-estradiol (0.019 mg/kg), the steroid that showed the least sustained effect in intact preparations (<45 min) (Fig. 2*C*).

Baseline duration was not significantly different between the hindbrain and midbrain stimulation sites ($p = 0.18$). However, burst latency was significantly decreased during hindbrain stimulation (hindbrain, 2.50 msec; midbrain, 7.89 msec; $p < 0.03$). The duration of fictive vocalization evoked from the intact hindbrain was rapidly increased by 11KT (Fig. 4*B*) ($F = 12.32$; $p < 0.0001$). These effects were apparent within 5 min of injection ($p < 0.05$), and burst duration was elevated (up to 174% of baseline) through the 15 and 30 min sampling times ($p < 0.05$). However, burst duration was not significantly elevated with hindbrain stimulation at all time points from 45–120 min ($p > 0.05$). Surprisingly, in the same preparation, 11KT facilitated the duration of fictive vocalization after midbrain stimulation beginning at 45 min (Fig. 4*B*) ($F = 49.76$; $p < 0.0001$), and this effect was sustained for all time points through 120 min ($p < 0.05$). Consistent with earlier midbrain-only stimulation experiments, 17$\beta$-estradiol also rapidly facilitated the duration of fictive vocalization evoked from the hindbrain alone (data not shown; $F = 8.19$; $p < 0.001$). These effects were apparent within 5 min of injection ($p < 0.05$), and burst duration remained elevated (up to 163% of baseline) through the 15 and 30 min sampling times. Burst duration was not significantly elevated thereafter ($p > 0.05$ for 45, 60, and 90 min) for hindbrain stimulation.

As summarized in Figure 5, both intact and isolation experiments together showed that the hindbrain–spinal region is both necessary and sufficient for the rapid effects of steroids (5–30 min), whereas the midbrain is necessary for more sustained effects over the range of 45–120 min.

## Discussion

Despite extensive study of descending vocal motor pathways, little is known about how hormones interact with these pathways to alter ongoing vocal behavior. The results of this study are significant in this regard for several reasons (Fig. 5). First, they show that steroid hormones act independently at midbrain and hindbrain–spinal levels to regulate vocal patterning. Second, steroids rapidly modulate the neural basis for vocalization, most likely through a nongenomic mechanism. Third, the observed effects are steroid specific, suggesting the presence of multiple membrane-bound steroid receptors in the vocal circuit.

### Hierarchical modulation of vocal patterning

Vocalization in birds and mammals is controlled by a network of nuclei that are hierarchically organized (see above). This study demonstrates that steroids can hierarchically modulate the output of vocal nuclei by having site-specific effects at hindbrain–spinal and midbrain levels to produce, respectively, rapid and sustained effects on vocal signaling.

Our experiments with the isolated hindbrain–spinal region confirm that it contains the circuitry both necessary and sufficient for rapid steroid modulation of vocal motor output. Previous studies delineated a hindbrain–spinal circuit of vocal nuclei (Fig. 1*C*), the output of which sets the duration and fundamental frequency of natural sounds (Bass and Baker, 1990). 11KT, cortisol, and 17$\beta$-estradiol each rapidly facilitates vocal motor activity in the hindbrain, and this pattern is similar to the effects with midbrain stimulation up to 30 min. However, whereas vocal motor activity remains elevated through 120 min after injection with midbrain stimulation (Fig. 2), activity in the transected hindbrain–spinal preparation returns to baseline after 30 min post injection (Fig. 4*A*). Indeed, parallel stimulation reveals that the rapid effects (5–30 min) of 11KT are evident with hindbrain–spinal stimulation, whereas sustained effects (45–120 min)

are only present with an intact descending midbrain pathway (Fig. 4*B*).

The results suggest that steroids can independently regulate midbrain and hindbrain–spinal nuclei in the descending vocal pathway. This dual action of steroids could be an example of metamodulation (Katz and Edwards, 1999), whereby steroids interact with different downstream neurochemicals at each midbrain and hindbrain–spinal site to achieve hierarchical modulation. The hierarchical nature of rapid steroid effects observed here may exist in other steroid-sensitive motor systems (Zakon et al., 1999; Frye, 2001).

**Rapid steroid action**

We report that steroids facilitate vocal motor activity in both a rapid and sustained manner. The rapid (5 min) effects on vocal patterning are consistent with a nongenomic mechanism (Falkenstein et al., 2000; Makara and Haller, 2001), possibly mediated through a membrane-bound steroid receptor (Orchinik et al., 1991).

Cortisol, the major circulating glucocorticoid in teleosts, produced rapid and sustained increases in vocal activity at circulating levels comparable with those after acute stress in midshipman and the closely related toadfish (Table 2) (Wood et al., 2001; Sisneros et al., 2004). The teleost-specific androgen 11KT produced similar, although longer-lasting, effects at physiologically relevant levels, consistent with evidence that 11KT is elevated in humming versus non-humming type I males (Knapp et al., 2001). The present experiments provide direct evidence that 11KT regulates the motor signals underlying vocalization in this species.

Previous studies documented rich expression of the enzyme aromatase, which converts testosterone to 17$\beta$-estradiol, throughout the midshipman brain (Schlinger et al., 1999; Forlano et al., 2001) and predicted an effect of 17$\beta$-estradiol on the sonic hindbrain–spinal region. The present results support this prediction. Although it was unfeasible to inject a dose of 17$\beta$-estradiol that yielded plasma levels in the physiological range ($<$1.0 ng/ml) (Sisneros et al., 2004), Schlinger and Arnold (1992) reported that estrogens measured in the brain of male zebra finches were elevated threefold over those in plasma. Thus, local concentrations of 17$\beta$-estradiol in midshipman brain may be elevated compared with plasma levels, so that our injections produced physiological 17$\beta$-estradiol levels in the brain.

Although testosterone is a synthesis precursor to both 17$\beta$-estradiol and 11KT, testosterone did not produce changes in fictive vocalizations, whereas both 11KT and 17$\beta$-estradiol were potent modulators. Testosterone might yet be effective (via conversion to 17$\beta$-estradiol) in breeding males when hindbrain–spinal aromatase levels are elevated (Forlano and Bass, 2003). However, other evidence suggests that the pattern observed here is specific to type I males. Although the predominant circulating androgen is 11KT in type I males, testosterone predominates in type II males, the alternative reproductive morph that does not acoustically court females (Brantley et al., 1993). Interestingly, the fictive vocalization duration of type II males is responsive to testosterone but not 11KT (our unpublished observations), an opposite pattern to that observed here for type I males. This divergence between male morphs is consistent with other neuromodulatory traits (Goodson and Bass, 2000a). Because testosterone and 11KT may interact with two distinct androgen receptors (Sperry and Thomas, 2000), the above results may be attributable to differences in brain androgen receptor expression in type I versus type II males.

**Steroid specificity**

Together, the results show that the magnitude and duration of the effects on vocal signaling are steroid specific, suggesting that these effects are mediated through separate steroid receptors (Fig. 5). In other systems, the rapid effects of steroids are mediated through membrane-bound receptors that exhibit high specificity (Orchinik et al., 1991; Moore and Orchinik, 1994; Loomis and Thomas, 2000). Our receptor antagonist experiments indicate specificity for the effects of both 11KT and cortisol (Fig. 3). The pharmacological inhibition by cytosolic receptor antagonists also supports the notion that the putative membrane receptors that mediate rapid effects are structurally similar to cytosolic steroid hormone receptors (Hua and Chen, 1989). We also showed the stereospecificity of the 17$\beta$-estradiol response by using the biologically inactive estrogen 17$\alpha$-estradiol. Given the dense GABAergic input to the sonic motor nucleus (Marchaterre et al., 1989), future studies could investigate the potential interaction between steroids and GABA transmission in this system, as done recently for hypothalamic neurons (Qiu et al., 2003).

**Behavioral significance**

Steroid hormones affect three behaviorally relevant, temporal parameters of vocal output in type I male midshipman fish: rapidity, increased duration, and maintenance of the duration increase. As for rapidity, our findings suggest that glucocorticoids, androgens, and estrogens regulate similar changes in communication behavior over a rapid time course (within 5 min). Although chronically elevated glucocorticoids tend to suppress androgen levels (Matsumoto et al., 1970; Sapolsky, 1985; Jain et al., 1996; Consten et al., 2001), these two hormone classes may function synergistically over the short term. Plasma glucocorticoids and androgens can be released simultaneously during acute stress (Barbaccia et al., 1996; Oberbeck et al., 1998; Zinder and Dar, 1999) and territorial interactions (Hannes and Franck, 1983; Woodley et al., 2000), including those that involve vocalizations (Burmeister and Wilczynski, 2000). In Gulf toadfish (*Opsanus beta*), a close relative of midshipman, field experiments demonstrate that plasma 11KT and cortisol levels are concomitantly elevated during advertisement calling (Remage-Healey and Bass, 2003).

The present study shows that steroid hormones can change the duration of vocal motor output, but are these changes important to midshipman in their natural environment (e.g., during courtship or territorial interactions)? Playback studies show the importance of duration for acoustic recognition; female midshipman discriminate differences in signal duration of at least 250 msec (for review, see Bass and McKibben, 2003). The increase in vocal pattern duration and its sustainability likely relates to the type I male's production of long duration signals such as hums (Fig. 1*B*). The longest duration vocal outputs evoked here with cortisol (242 msec), 11KT (348 msec), and 17$\beta$-estradiol (296 msec) approach the minimum duration recorded for hums (370 msec) (Brantley and Bass, 1994). Also, during some aggressive encounters, type I males emit an extended train of grunts (at one to two grunts per second), and the duration of individual grunts increases twofold over the course of a grunt train (Bass et al., 1999; M. Marchaterre and A. Bass, unpublished observations). The present results suggest that steroids may also mediate rapid changes in the duration of individual grunts emitted during a grunt train.

Similarly, vocalization duration in *Opsanus beta* is a key parameter for male–male interactions. Playbacks of tone stimuli that mimic the duration of naturally occurring advertisement

calls in that species can readily escalate the rate of advertisement calling by males, apparently simulating a vocal "challenge" (Wingfield et al., 1990) by a rival male (our unpublished observations). Such simulated male–male interactions cause concomitant, rapid (within 20 min) increases in both plasma androgens and the duration of advertisement calls. Importantly, males show up to twofold increases in call-duration, which is very similar to the magnitude of steroid-induced increases in fictive calling observed in the present study (11KT and cortisol also cause increases in fictive vocalization duration in toadfish) (Remage-Healey and Bass, 2003). We expect that elevations in plasma androgens are associated with rapid shifts in territorial vocal signaling in both toadfish and midshipman. We further expect that the synergistic actions of steroids and other neurochemicals will shape the final vocal motor output of these signals. To this end, possible molecular interactions with neuropeptides are likely, especially given that neuropeptides and steroids perform opposing, rapid actions on motor patterning in midshipman (the present study; Goodson and Bass, 2000a,b) and roughskin newts (Rose et al., 1995).

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