Androgen and Gonadotropin Effects on Male Mate Calls in South African Clawed Frogs, *Xenopus laevis*

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Mate calling is a prominent reproductive behavior of male South African clawed frogs. Calls consist of alternating slow- and fast-amplitude-modulated trills. Each trill is made up of a series of clicks. The effects of administration of exogenous gonadotropin and androgen on mate calling were studied in male *Xenopus laevis*. Males were paired with unreceptive female frogs to elicit maximal calling. The amount of time each animal spent calling during the testing period, the peak fundamental frequency of the calls, the rate of calling, and the interclick interval (ICI, a measure of the temporal patterning of the calls) were measured in intact, castrated, and hormone-replaced frogs. Injection of human chorionic gonadotropin (HCG) into intact frogs increased the amount of time spent calling and the ICI relative to measures taken after water injection. Castrated males did not call even when given HCG. Testosterone and dihydrotestosterone treatment reinstated calling in castrates and increased circulating levels of androgens. When androgen-replaced castrated males were injected with HCG, the amount of time spent calling increased and approached levels of intact. HCG-injected males. The above results suggest that androgens are necessary for the production of calls. Gonadotropins appear to play an important role in mate calling, a role at least partly independent of effects on testicular androgen synthesis.

The reproductive behaviors of vertebrates include sequences of stereotyped, sex-typical responses which are sensitive to endocrine states. Under laboratory conditions, South African clawed frogs display clasping and mate calling (typical of males) and receptive postures and ticking (typical of females). In intact frogs these behaviors are sensitive to gonadotropin (HCG), which increases sexual activity (Russell, 1960; Hutchison and Poynton, 1963; Kelley and Pfaff, 1976; Kelley, 1982).

Amplexus is initiated when a sexually active male grasps a female with his forelegs. Clasping has been found to be modulated by androgens. An intact adult male *Xenopus laevis* displays high levels of clasping in
the laboratory in response to injections of HCG (Russell, Mead, and Hayes, 1954; Hutchison and Poynton, 1963; Kelley and Pfaff, 1976). After castration HCG no longer promotes clasping indicating that its action depends on some factor(s) secreted by the testis (Kelley and Pfaff, 1976). Gonadotropin has been shown to increase blood levels of androgen in male *X. laevis* (Kelley, 1980). Implantation of pellets of testosterone (T) or its metabolite 5-α-dihydrotestosterone (DHT) reinstates clasping in castrated male frogs (Kelley and Pfaff, 1976). Testosterone also stimulates clasping in ovariectomized female *X. laevis*.

The mate call of *X. laevis* is a distinctive metallic sounding trill consisting of a long series of amplitude modulated pulses. Russell (1954) describes the calling as “sawing.” The call is produced by the larynx (Yager, 1982), which is a sexually dimorphic structure (Ridewood, 1897; Hannigan and Kelley, 1981). Little is known of the hormonal control of calling in *Xenopus*, apart from the fact that castration reduces calling (Kelley and Pfaff, 1976). In the present series of experiments we investigated the roles of androgens and gonadotropins in the control of mate calls. The physical parameters of calling that we chose to measure under the different treatments were the overall amount of time spent calling, the rate of calling, the peak fundamental frequency of the clicks (the fundamental unit of calling), and the interclick interval. In the first experiment, we examined the effect of HCG vs distilled water injection on calling in intact sexually mature males. Calling was elicited by pairing the males with unreceptive stimulus females. In the second experiment the effect of castration on calling was examined. In the third and fourth experiments, androgen replacement with different doses of either T or DHT was examined for effects on mate calling. The addition of HCG injection to this regimen enabled us to assess possible androgen–gonadotropin synergisms.

**GENERAL METHODS**

*Subjects.* Laboratory-bred, adult male and female *X. laevis* were obtained from Nasco Corporation, Fort Atkinson, Wisconsin. Mean weight of the males (*n* = 33) when received was 49.0 g (range 33.3 to 68.8 g). The mean weight of females (*n* = 18) when received was 64.4 g (range 47.2 to 79.5 g).

*Maintenance procedures.* Frogs were maintained on a 14-hr on, 10-hr off light/dark cycle. They were fed Nasco Frog Brittle three times per week and were housed in 48 × 25 × 16-cm polycarbonate tanks, three frogs per tank, with approximately 8 liters of aged tap water to which sodium chloride was added (salt concentration, 0.4%). The salt was added to retard bacterial infections, none of which were observed during the course of the experiments. Since 1979 we have routinely maintained frogs in dilute saline solutions. Aside from a reduction in the incidence of “red leg” disease, the behavior of the frogs was not altered from that
observed in the preceding years (1972–1978). Frogs were identified by toe clipping.

**Ovariectomy and castration procedures.** Sexually unreceptive females were produced by ovariectomy. Previous experiments in our laboratory (Kelley, 1982) established that this is a satisfactory procedure for producing a consistent population of unreceptive female frogs to use as stimulus females with the males. Pilot work had indicated that male frogs consistently respond to unreceptive females with high rates of calling. For all castrations and ovariectomies animals were deeply anesthetized by submersion in 0.13% MS-222 (tricane methane sulphonate, Aldrich). Surgical procedures were as previously described (Kelley and Pfaff, 1976). For sham operations the fat bodies were exposed, retracted, and reinserted.

**Surgical and implant procedures.** Hormone and cholesterol pellets were prepared using a Parr pellet press which compresses powdered hormone into uniform-size pellets. Testosterone (17β-hydroxyandrost-4-en-3-one; T), dihydrotestosterone (5α-androstane-3β-17β-diol; DHT), cholesterol (5α-cholesten-3-ol; C), and human chorionic gonadotropin (HCG) were obtained from Sigma Chemical Company. In the case of the lower hormone doses the T- and DHT-pellet concentration was reduced by mixing the hormone with cholesterol; thus, pellets of approximately the same size could be produced (3 mm in diameter and about 1-mm thick). Frogs were anesthetized with ice for hormone-pellet insertion. A small incision (about 4 mm) was made in the skin above the dorsal lymph sac. Pellets were inserted and pushed toward the caudal end of the lymph sac, and the incision was closed with 6-0 surgical silk. This procedure typically results in the formation of a vascularized fibrous capsule around the hormone pellet and a gradual reduction in pellet weight (Kelley and Pfaff, 1976).

**General design.** Baseline measurements of male mate calling \((n = 33)\) in response to unreceptive females were made in the first experiment for both distilled water and HCG. Twenty-six males were then castrated and retested in the second experiment (again with distilled water and HCG). The remaining frogs \((n = 7)\) received a sham operation at this time. Sham-operated frogs were observed in parallel with experimental males for all experimental conditions in order to control the effects of operative procedures and any seasonal variations in sexual activity. The castrated males were then randomly assigned to different testosterone dosage-treatment groups or the control group for the next experiment. Testosterone or cholesterol pellets were in place 3 weeks or more before testing. Shams received cholesterol implants for an equivalent time period. After testing, pellets were removed for 4 weeks. Next males were randomly reassigned to dihydrotestosterone dosage-treatment groups for the DHT experiment. DHT or C pellets were inserted into the dorsal lymph sac 3 weeks before testing. All pellets were removed for 3 months and males
were retested with distilled water and HCG injections 1 year after castration or sham operation. Males were then reimplanted with androgen or cholesterol and blood levels of T and DHT determined by radioimmunoassay. The time intervals between pellet implantation, testing, removal, and reimplantation are described for each experiment.

Testing procedure. In each experiment mate calls were recorded on magnetic tape for 1.5 hr a day on two consecutive days. On Day 1, male frogs were injected with 0.5 ml of distilled water (dH₂O) and placed in a recording tank with stimulus females approximately 6 hr before recording began. Males remained in the recording tank overnight. On Day 2 male frogs were injected with 100 IU of HCG (Sigma) dissolved in 0.5 ml of dH₂O, returned to the recording tank, and observed with a stimulus female after approximately 6 hr. Males were tested in this sequence since previous work from our laboratory had shown that the effects of HCG lasted several days while there is very little difference between frogs injected on Days 1 and 2 with dH₂O. Female frogs made unreceptive by ovariectomy (Kelley, 1982) were used to elicit high rates of calling in males. All tape recordings were made during the first 2 hr of the dark portion of the light/dark cycle.

Recording apparatus. Wilcoxon hydrophones (Model H505) were used to transduce the underwater sounds. Signals were recorded on magnetic tape (Scotch 207) using Akai Model GX-255 tape recorders at a recording speed of 8.25 cm/sec. Recording tanks were 75-liter aquaria lined on the inside with 5-cm-thick closed-cell foam (Airex), thus having interior dimensions of 25 x 50 x 35-cm (deep). Before each recording session tanks were lined with polyethylene bags (Mobil) inside the closed-cell foam and filled with approximately 40 liters of aged, unsalted tap water. Hydrophones were suspended from a Plexiglas top to a height of 10 cm from the tank bottom in the center of the tank. Water temperature was maintained at 18 ± 1°C. In the experiments presented here measurements of absolute sound intensity were not attempted because the frog's position has a large effect on intensity measurements. The position of the frog in the tank (i.e., facing away from or toward the hydrophone) and its distance from the hydrophone were uncontrollable factors.

In obtaining peak fundamental frequency measurements, two possible sources of acoustic error were considered. First, the frequencies detected by the hydrophone might be distorted by the resonant frequency of the tank itself. Free-field measurements of sound frequencies in *Xenopus* (Yager, personal communication) indicate that the principal frequency of the click is approximately 1.8 kHz. This value is very close to values obtained under our experimental conditions. We measured the resonant frequency in the foam-lined tank and found it to be about 4.2 kHz. Since we did not obtain peak frequencies near the resonant frequency of the tank we believe that resonance was not a source of acoustic artifact.
Second, echoes from the glass–foam–air interface could interfere with sound measurement. The loudness of the call together with the attenuation characteristics of the tank lead us to believe that any distortion by echoes was minimal.

Data analysis. Time spent calling was analyzed by listening to tapes and noting the number of 45-sec bins in which calling was present. Frequency characteristics were analyzed using a Princeton Applied Research FFT real time spectrum analyzer (Model 4512) interfaced to a Hewlett-Packard X–Y plotter (model 7035B). Several calls from each animal were randomly selected from the tapes for frequency analysis. Data on interclick intervals was obtained by first filtering the calls (high pass 400 Hz, low pass 15,000 Hz) using a Khronehit (Model 3550) bandpass filter. The signal was then rectified, integrated, and further analyzed with a Frederick Haer window discriminator. The interclick interval (ICI) was finally obtained by analyzing the output of the window discriminator with an Ortex (Model 4620) time histogram analyzer. Rate of calling was determined from oscillograph (Grass 79D) recordings of the filtered and rectified signals. From these oscillographs the number of calls per minute during a 5-min interval of calling was determined. Statistical analyses were performed (as described in each results section) using the statistical computer package BMDP program 2V (Dixon and Brown, 1979).

SPECIFIC METHODS AND RESULTS

Experiment 1: Baseline (dH₂O vs HCG)

Methods. In this experiment the calling of 33 intact male frogs injected with dH₂O on Day 1 and HCG on Day 2 was examined. Male mate calling in response to unreceptive females was observed. The amount of time spent calling during the testing session, as well as the rate of calling, interclick interval (ICI), and mean peak fundamental frequency were studied. This experiment provided baseline measures of calling for each frog to be used in the analysis of subsequent experiments.

Results. *X. laevis* mate calls are a distinctive metallic sounding amplitude modulated trill. The basic unit of the call is the click (Fig. 1), and each click has its own frequency spectrum (Fig. 2), with the fundamental peak frequency usually being approximately 1.8 kHz (±0.03 SEM). The frequency spectrum of a click varies little within a call or a series of calls and is characteristic of a given animal. Clicks are grouped together to form a mate call. A single mate call includes a fast trill and a slow trill portion. An animal rarely makes a single call, but rather chains calls together in long bouts. A sonagram of mate calling from an intact male is shown in Fig. 3. The mate calls are amplitude modulated with the fast portion of the trill louder than the slow portion. Calling bouts can begin with either the fast or slow trill. While alternating fast and slow portions are usually given, occasional fast–fast or slow–slow trill pairs can be emitted by males.
The interval between individual clicks can be measured giving an interclick interval. Figure 4 illustrates the interclick interval distribution of the slow trill portion of the calls of a normal male. The mean interclick interval was 27.8 msec (±0.3 SEM) for dH₂O treated males. The fast trill portion of the call is difficult to consistently measure in all animals and was therefore not used as a dependent variable. The ICI of the fast trill is about 14.0 msec as measured from an oscilloscope tracing. It was also possible to measure the rate of calling (i.e., the number of calls per minute) and the total time spent calling per 1½-hr observation period. In intact males treated with dH₂O the mean rate of calling was 51.8 calls/min. The mean total time spent calling was 13.6 min (±4.8 SEM; see Fig. 5). Of the above three measurements (ICI, rate, and time calling) time calling proved to be the most sensitive to endocrine state. Fifty-

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**Fig. 1.** Oscillograph of an individual click from a mate call of an intact male *X. laevis.*

**Fig. 2.** Frequency spectrum of an individual click from the mate call of an intact male *X. laevis.*
Fig. 3. Sonagram of the male call of an intact male X. leavis. The combination of one fast and one slow trill comprises one male call. The relative intensities of the frequency components of each click are indicated by the darkness of the trace.
eight percent of the frogs injected with dH$_2$O called while 96% of the frogs injected with HCG called.

Intact males treated with HCG spent significantly more time calling during the test period than when treated with dH$_2$O as shown by an analysis of variance (13.6 min ± 4.6 SEM vs 44.0 min ± 60.4 SEM)
The mean ICI of frogs treated with HCG was significantly longer ($\bar{X} = 29.59$ for HCG vs $\bar{X} = 27.84$ for $dH_2O$, $F(1, 45) = 34.31; P < 0.001$) than when treated with $dH_2O$. There were no significant effects of HCG as compared to $dH_2O$ on the fundamental peak frequency of the calls or on the rate of calling.

**Experiment 2: Castration**

**Methods.** In this experiment the calling of castrated and sham-operated frogs was compared to preoperative calling. Twenty-six male frogs were castrated and 9 were sham operated as described above. Frogs were allowed to recover for at least 2 weeks before testing. The testing sequence was as described above. On the first day the frogs were injected with $dH_2O$ and on the second day with HCG. As in Experiment 1, the time spent calling, rate of calling, fundamental peak frequency of calls, and interclick intervals were measured.

After 12 months of testing all pellets were removed from the frogs (see Experiment 3) and they were allowed to recover for at least 2 months. Calling was then recorded as described above. Again, time spent calling, ICI, peak frequency, and rate of calling were measured. This experiment was performed to test for any recrudescence of testicular function over the experimental period.

**Results.** A 2-week period of castration significantly reduces the time spent calling from baseline levels ($F(1, 22) = 44.67; P < 0.0001$, ANOVA) as is illustrated in Fig. 5. Sham-operated animals still called ($\bar{X} = 5.6$ min $\pm 4.7$ SEM with $dH_2O$; $\bar{X} = 49.4$ min $\pm 11.9$ SEM with HCG).

HCG does not have any significant effect on the calling of castrated animals. Since calling fell to negligible levels following castration, no measurements of ICI, peak fundamental frequency, or rate of calling were possible. Castration was also observed to eliminate the secondary sexual characteristic, nuptial pads. After 1 year, castrated frogs did not call at all with $dH_2O$ or HCG. After 1 year, sham-operated animals still called ($\bar{X} = 7.9$ min $\pm 8.1$ SEM with $dH_2O$; $\bar{X} = 47.7$ min $\pm 10.7$ SEM with HCG).

**Experiment 3: Testosterone**

**Methods.** The effects of different dosages of testosterone on calling were examined in castrated *X. laevis* in this experiment. Castrated male frogs (from the previous experiment) were implanted with 1-, 5-, or 10-mg pellets of testosterone. The time interval between implantation and testing was 3–5 weeks. Kelley and Pfaff (1976) found a 10-mg pellet of T or DHT to be effective in reinstating clasping to castrated male *X. laevis* after 4 weeks of treatment. Pellet size was held constant by addition of cholesterol. Testosterone pellets were implanted in eight frogs at each
Hormonal control of calling

Dosage level. Seven sham-operated frogs and two castrated animals were implanted with 10-mg cholesterol pellets. Mate calls were recorded following the procedure outlined above and were analyzed for amount of time spent calling, peak fundamental frequency, rate of calling, and interclick interval. Upon completion of testing, pellets were removed and animals allowed at least 1 month, or until nuptial pads completely regressed, to recover before pellets for the next experiment were implanted.

Results. Replacement of testosterone after castration increases the amount of time spent calling relative to postcastration levels. The 5- and 10-mg doses of T were more effective than the 1-mg dose. There were no significant differences between precastration levels and postcastration levels of calling with testosterone therapy at the 5- and 10-mg dose levels. However there was a significant difference between precastration and testosterone (1-mg dose level) treatment of calling (F(1, 7) = 12.08; P < 0.025). There were no significant differences between amount of calling in sham-operated cholesterol-implanted animals and testosterone-replaced animals. (In shams, $\bar{X} = 0.18 \text{ min} \pm 0.12 \text{ SEM}$ for dH$_2$O; $\bar{X} = 17.3 \text{ min} \pm 7.4 \text{ SEM}$ for HCG; in castrates with 5-mg T pellets, $\bar{X} = 6.25 \text{ min} \pm 2.5 \text{ SEM}$ for dH$_2$O; $\bar{X} = 32.3 \text{ min} \pm 12.6 \text{ SEM}$ for HCG.) Castrates with 10-mg C pellets did not call at all.

Injections of HCG into testosterone-replaced castrates significantly increased time spent calling ($F(1.29) = 25.77; P < 0.0001$) compared to dH$_2$O injections (compare Figs. 6 and 7). There were no significant differences between dosage levels of testosterone with HCG treatment. There were also no significant effects of testosterone (either with or without HCG) on rate of calling, peak fundamental frequency, or ICI compared to precastration values (data not shown).

![Fig. 6](image_url)  
**Fig. 6.** Time spent calling with androgen replacement and distilled-water treatment in castrates. Error bars indicate standard error of the mean. The dotted line indicates the level of calling of intact distilled-water-treated males from the previous experiment (shaded region: standard error of the mean; Fig. 5). T, Testosterone; DHT, dihydrotestosterone.
FIG. 7. Androgen replacement with HCG treatment in castrated frogs. The dotted line indicates the level of calling in intact, HCG-treated male frogs from the previous experiment (shaded region: standard error of the mean; Fig. 5). Abbreviations are as in Figs. 5 and 6.

Experiment 4: Dihydrotestosterone

Methods. The effect of different dosages of DHT on calling in castrated X. laevis was studied. Pellets of DHT in 1-, 5-, and 10-mg dosages were implanted in the dorsal lymph sac of three groups of eight frogs. These were the same frogs used in the previous experiment, randomly reassigned to new DHT-treatment groups. Pellets were implanted 3–5 weeks before testing. Testing procedures were as described above. In addition seven sham-operated cholesterol-treated frogs and two castrated cholesterol-treated frogs were observed. Time spent calling, peak fundamental frequency, ICI, and rate of calling were measured.

Results. The 10-mg DHT treatment increased the amount of calling in castrated males relative to cholesterol-implanted sham operates (dH₂O injection; Tukey test, \( P < 0.01 \)) and resulted in levels of calling that were not significantly different from precastration levels of these same males. The lower doses of DHT produced levels of calling that were not significantly different from cholesterol-implanted sham operates (for the latter, time spent calling was \( \bar{X} = 0.27 \text{ min} \pm 0.28 \text{ SEM dH}_2\text{O}; \bar{X} = 26.5 \text{ min} \pm 3.9 \text{ SEM for HCG} \)). The lower doses of DHT did not restore levels of calling to those obtained precastration; the mean time spent calling under the two conditions was significantly different.
When HCG was injected into DHT-replaced castrates, time spent calling increased over dH2O levels at each dose level of DHT ($F(1, 29) = 13.39; P < 0.001$) (see Fig. 7). DHT at the 10- and 5-mg levels was significantly more effective in increasing time spent calling by HCG-treated animals than DHT at the 1-mg level (Tukey test, $P < 0.05$). Again there were no significant effects on peak frequency, rate of calling, and ICI.

**Experiment 5: Radioimmunoassays**

**Methods.** Following the final behavioral experiment, frogs were reimplemented with androgen or cholesterol pellets and blood levels of testosterone and dihydrotestosterone were assayed. Groups of four castrated male frogs were reimplemented with 1-, 5-, and 10-mg pellets of T or DHT. Five sham-operated and two castrated frogs received 10-mg cholesterol pellets. Four intact, uninjected, experimentally naive male frogs were also bled to obtain baseline androgen values. Pellets were left in place 3 months before sacrifice. Blood was obtained via cardiac puncture from frogs anesthetized in MS-222. Blood samples (1–3 ml) were allowed to clot at 4°C for 1 hr before centrifugation (1200 rpm) for 1/2 hr at 4°C. Serum was rapidly frozen and stored at −70°C for 6 months. Samples were thawed and steroids extracted into hexane:benzene (2:1), dried, and re-dissolved in a Tris buffer. Radioimmunoassay for total androgen was performed on duplicate samples using a commercially available antiserum for testosterone whose cross-reactivity with DHT was 35% (Amersham/Searle). The amount of DHT present in samples was estimated by oxidizing a parallel set of samples, performing the radioimmunoassay, and correcting for antibody cross-reactivity. Values for T levels in samples were obtained by subtracting DHT levels from total androgen (cross-reactivity of the antibody with most steroids is less than 0.05% and with androgens, other than T and DHT, is less than 7%). Assay precision is 5–7% for the total androgen and 7–10% for DHT. Sensitivity is 7–10 pg/sample for total androgen and 15–17 pg/sample for DHT.

**Results.** With increasing dose levels of androgen treatment, higher blood levels of androgen are obtained. The 1- and 5-mg pellets of T, for example, resulted in average T blood levels of 2.7 (± 0.8 SEM) and 53.7 (± 4.4) ng/ml, respectively. The 1- and 5-mg dose levels of DHT resulted in mean blood levels of 9.6 (± 2.9) and 33.1 (± 36.3) ng/ml, respectively. All except one of the 10-mg testosterone and all of the 10-mg dihydrotestosterone pellets produced blood levels of androgen exceeding the range measurable by the assay. The one measurable 10-mg pellet T value gave an apparent blood level of 126 ng/ml. The 1-, 5-, and 10-mg testosterone pellets resulted in DHT blood levels of 1.2 (± 1.6), 5.2 (± 0.9), and 10.6 (± 1.6) ng/ml, respectively.

In cholesterol-implanted castrated males, the blood levels of testosterone averaged 1.1 ng/ml while no detectable DHT was present. The mean
androgen values for sham-operated, cholesterol-implanted frogs were T 0.8 ng/ml (±0.30) and DHT 11.3 ng/ml (±1.6). Mean androgen values for unoperated control males were T 1.9 ng/ml (±0.4) and DHT 0.5 ng/ml (±0.5).

DISCUSSION

Results of these experiments show that the production of mate calls in male South African clawed frogs depends on endocrine state. Human chorionic gonadotropin increases calling in intact frogs but not in castrated frogs. Testosterone and dihydrotestosterone both increase calling in castrated males. T and DHT increased circulating levels of androgens in dose-dependent manner. HCG further increases calling in castrated frogs treated with T or DHT. These results suggest that gonadotropins may play a role in the production of normal calls, a role perhaps not entirely due to stimulation of androgen secretion.

Intact Frogs

In the present study HCG, when compared to dH2O, was found to significantly increase the amount of time spent calling in intact male X. laevis. Increases in male sex behavior (clasping) in response to HCG have been observed by others in X. laevis (Russell et al., 1954; Russell, 1954; Hutchison and Poynton, 1963; Kelley and Pfaff, 1976) as well as in other anuran species (Wada and Gorbman, 1977a; Schmidt, 1966).

When intact males are injected with gonadotropin, the total time spent calling is increased and the ICI’s in the slow part of the trill are increased by 2 msec. In effect, HCG acts to increase the probability that an intact male will call and to slow down the slow trill portion of the call. We should note that this effect of HCG on ICI was not observed in T- or DHT-replaced castrates. Thus the presence of some other testicular factor besides T or DHT may be necessary for HCG to affect the ICI. Gonadotropin could act to change the ICI of calls by acting on the vocal organ or on the CNS pathways which effect vocal behaviors (Wetzel and Kelley, in preparation).

Castration

Castration was found to eliminate mate calling in X. laevis. The effect of castration on male sexual behavior has been well documented in anurans (Steinach, 1894; Dodd, 1960; Russell et al., 1954; Russell, 1954; Schmidt, 1966; Palka and Gorbman, 1973; Kelley and Pfaff, 1976) as well as in most other vertebrate species (Beach, 1948; Young, 1961; Hart, 1974); castration usually dramatically reduces male sex behavior.

Anuran species differ with respect to the reinstatement of clasping in castrated males by testosterone. Palka and Gorbman (1973) were not able to restore clasping in Rana pipiens with either T or DHT administered systemically. In the same species, Wada and Gorbman (1977b) were
unable to reinstate sex behaviors in castrated males with T combined with pituitary implants. Direct implantation of T in the rostral preoptic area does reinstate mating behavior.

The present study found that testosterone and dihydrotestosterone increase calling in castrate *X. laevis*. The 10-mg dose used in the present experiments was the same as that used by Kelley and Pfaff, (1976) to reinstate clasping behavior in male *X. laevis*. Thus, the expression of both prominent behaviors characteristic of sexually active male clawed frogs, clasping and calling, requires androgen.

Does the difference in the effectiveness of different T and DHT doses tell us anything about androgenic metabolites and control of calling? Testosterone is often a prohormone, being aromatized to E or converted to DHT by 5α-reductase (see Mainwaring, 1977, for review). If DHT is the active metabolite controlling calling, then we would expect it to be more active than T at lower doses. However, this was not the case.

Another behaviorally active metabolite of T in vertebrates is estradiol. If T acts to stimulate calling by conversion to E, we would not expect DHT to work since the 5α-reduced metabolite cannot be converted to estradiol. But DHT does increase calling, suggesting that it is not the conversion of T to E which is effecting this increase. Preliminary results from experiments in which E was paired with the different dosages of DHT also suggest that E is not involved.

**Blood Levels of Androgen**

Results of radioimmunoassays indicate that the larger pellets of testosterone or DHT resulted in higher blood levels of androgen than did lower dose levels. We should note, however, that pellet implants of the same size can result in quite different levels of steroid in different frogs. For example, a 1-mg T pellet produced apparent T blood levels ranging from 0.2 to 2.0 ng/ml, a 10-fold range. These differences are presumably due to differences in effectiveness of the implants and may be related to the degree of vascularization of the fibrous capsules which form around the pellet implants. These difficulties in controlling individual blood levels of androgen caution against applying straightforward dose–response relations to the behavioral data obtained in this study.

The RIA data allow us to compare blood levels of androgen in intact males with those of hormone-replaced castrates. In general, the levels recorded from these control males (whose sexual activity we did not measure) is similar to levels obtained by the 1-mg pellets. The 5-mg pellets produced androgen levels 15 to 25 times the control values while the 10-mg pellets produced values exceeding the measurable range of the RIA (in excess of 126 ng/ml).

In a previous study (Kelley, 1980), blood levels of T and DHT were measured in intact, HCG-injected and in castrated, HCG-injected males. Mean values in nanograms/milliliters for these three groups were T 3.2.
DHT 2.5; T 20.9, DHT 22.8; and T 0.8, DHT 3.0, respectively. Castrated, cholesterol-implanted males from the present study have low but measurable T levels but no detectable DHT. Androgen levels of cholesterol-implanted sham operates were similar to the intact values from the previous experiments but somewhat greater than intact, control males. Blood androgen values are increased 10-fold by HCG injection in intact males. Such elevated blood levels fall within the range of the 5-mg pellets we used while the 10-mg pellet produced levels that are clearly greater than any yet measured from intact males.

From the present study, we cannot correlate blood levels of androgen in any individual male frog with the amount of vocal behavior exhibited. However, in the group of castrated frogs, the higher dose levels of androgen often produced significantly greater levels of calling than the lowest dose level. Such larger androgen pellets also produced higher mean levels of blood androgen. No calling was produced in castrates and little calling in castrates receiving 1-mg androgen pellets. It therefore seems reasonable to conclude that androgens are required for vocal behaviors and that higher blood levels of androgen are associated with a greater probability of mate calling in groups of male frogs.

**HCG and Calling**

The behavioral effects of the HCG preparation used may have been due to gonadotropin-like activity or to nongonadotropic actions. The HCG preparation has at least one known attribute of a gonadotropin, the ability to increase blood levels of circulating steroids (Kelley, 1980). Since the steroids, T and DHT, are effective in restoring calling to precastration levels, it appeared reasonable to suppose that HCG effects on intact males are due to increased androgen levels. Two findings from the present experiment argue against this simple interpretation. First, the amount of time spent calling by androgen-replaced castrates never reached the levels displayed by the HCG injected males (even when very high doses of androgen were given). Second, and more dramatically, the calling of androgen-replaced castrates was greatly increased by HCG injection. Since these males were without testes, we cannot invoke testicular androgen secretion to account for behavioral results. The gonadotropin preparation must be acting on a nontesticular structure to produce such a result. Candidate structures include other endocrine organs and neuromodulators for calling. Another feature of the HCG preparation effect is that it is not manifested unless androgens are present. Thus, we must take into account the steroid dependence of the effect when shaping hypotheses of how HCG works.

One way in which the HCG preparation could be acting is by increasing the efficacy of administered androgens. Thus, the gonadotropin preparation could promote androgen entry into CNS targets. The posterior pituitary hormone, vasotocin, when administered together with androgen (Watson
and Capranica, in preparation), promotes calling in *R. pipiens* and also promotes male sexual behaviors in newts under similar circumstances (Moore and Zoeller, 1979), an effect which may be mediated by increased entry of steroid into the CNS. Alternatively, gonadotropins can alter the activity of steroid dehydrogenases (Wiebe, 1970). Alterations in levels of enzymes which convert T or DHT to a more active metabolite, or a decrease in activity of enzymes which degrade T or DHT, could effect the behavioral potency of androgens. Recent evidence indicates that protein hormones, in particular gonadotropins, have and act directly on receptors within the cell (Childs, 1978; McKerns, 1978) and these receptor–gonadotropin units may influence gene expression. HCG could thus directly influence protein and/or enzyme levels in target cells.

In conclusion, we have shown that castration eliminates calling in male *X. laevis* and androgens increase calling in castrated males. The most surprising result was an androgen–gonadotropin synergism. Gonadotropin, when injected in castrated androgen-replaced males, increased calling. Recent work in our laboratory has outlined a neural pathway for calling in *Xenopus* (Wetzel and Kelley, in preparation). Many of the nuclei in this path contain androgen-concentrating neurons (Kelley *et al.*, 1975; Kelley, 1980; Kelley, 1981). The calling effector muscles also accumulate androgen (Kelley *et al.*, 1981; Segil, Silverman, Kelley, and Rainbow, 1983). Gonadotropins may influence calling through interactions with androgen-sensitive neurons or muscles.

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