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Correlation between Blood Level of Androgens and Sexual Behavior in Male Leopard Frogs, *Rana pipiens*[1]

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Blood levels of androgens in male leopard frogs (*Rana pipiens*) were measured by radioimmunoassay during a period of successive injections of whole pituitary tissue which eventually evoked mating behavior, including calling and clasping. In June, one injection induced a surge from a lower level of plasma testosterone, and this higher level was sustained. The mating calling and clasping increased in frequency and intensity while the pituitary injections were continued. Blood levels of dihydrotestosterone were similar in pattern to those of testosterone. In a second experiment, in October, normal plasma testosterone levels were higher than those associated with experimentally stimulated sex behavior in June, but little or no spontaneous sexual behavior was observed. During a period of pituitary treatment, however, sexual behavior appeared and increased progressively during treatment. Thus, although increased blood levels of androgens, as well as evoked male sex behavior both are consequences of pituitary treatment of frogs, a particular blood androgen level alone is not responsible for initiation of the behavior. Since earlier work with castrates of the same species has shown that testosterone treatment will not evoke male behavior, but implants of testes from pituitary-stimulated frogs will do so, the present data are consistent with a hypothetical second testicular factor which, acting with testosterone, regulates male sex behavior.

Vocalization and clasping are familiar manifestations of male sexual behavior in frogs, but the endocrine control of these phenomena is poorly understood. Castration of frogs shows that a testicular factor is required for sexual behavior (Schmidt, 1966); however, replacement "therapy" with testosterone has consistently failed to restore sexual behavior (Palka and Gorbman, 1973), even though testosterone acts as an androgen with respect to stimulation of other amphibian male secondary sex characters (see Dodd, 1960; Wolf, 1939; Muller *et al.*, 1969; Iwasawa and Kobayashi, 1974). Recently, Palka and Gorbman (1973) tested a variety of other steroids for ability to stimulate male frog sexual behavior, but found all ineffective, although they could demonstrate that testes were the source of an unidentified factor (or factors) that influenced sexual behavior, and that pituitary transplantation always induced sexual behavior in frogs with intact or implanted testes.

Even though there is no direct evidence that an androgenic steroid is involved in manifestation of male sexual behavior in amphibians, such a hormone is still the most rational candidate for this role. If an androgenic steroid does not influence amphibian male sex behavior, this would make the Amphibia unique among the vertebrates insofar as the published literature is concerned. Recently, radioimmunoassays have been developed to measure blood levels of steroids, and use of such a procedure in a study of *Rana esculenta* males has shown

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that testosterone titers rise in October and a relatively higher level is maintained from December to March (D'Istria *et al.*, 1974).

The data we present here represent an attempt to discern a correlation between male sexual behavior evoked by pituitary transplantation and blood levels of androgens. It is to be expected that such information may make possible conclusions concerning androgenic steroid involvement in regulating male sexual behavior in frogs.

MATERIALS AND METHODS

Male leopard frogs (*Rana pipiens*) weighing approximately 30–60 g and measuring 6.5–8.5 cm in snout–vent length, were purchased in February from Bay Biological Supply, Ltd. (Port Credit, Ontario) for Experiment I and were used in June, 1975. Frogs for Experiment II were purchased in September from NASCO Steinhilber (Fort Atkinson, Wisconsin) and were used in an experiment in October. They were kept in covered plastic basins with tap water running continually over the bottoms of the basins and fed weekly. All animals remained in a room with controlled photoperiod (12L/12D) and temperature 16°. When used in the June or October experiments, the frogs were placed in transparent plastic boxes, measuring approximately 31 × 16 × 9 cm, in groups of two or three. During the daily 30-min period of actual observation of sexual behavior, the boxes containing the frogs were taken briefly to another room at approximately 20°. Sexual behavior was noted by the observer concealed behind a shield during a 30-min continuous casette tape playing of mating calls of *Rana pipiens* which was reproduced repetitiously from a phonograph record "Voices of the Night" (Cornell University Records, Cornell University Press). Various studies have shown that taped frog calls may act as evocative stimuli for mating calling in male frogs (Schmidt, 1966; Palka and Gorbman, 1973). During the auditory stimulation period, mating trills, chuckles, and trials of clasping were counted and recorded. After 3 days of pretreatment observation, pituitary transplantations were begun. Two fresh pituitaries from *Rana utricularia* (formerly considered a Southern form of *Rana pipiens*) measuring 9–10 cm in snout–vent length, suspended in frog saline were injected intraperitoneally daily. During the treatment period, observations of sexual behavior were made daily, and four to seven frogs were killed for blood collection on Days 0, 1, 3, 7 (Exp I in June), or Days 0, 1, 3, 5 (Exp II in October) counting the first pituitary transplantation as Day 0. Blood was collected by heart puncture and placed in centrifuge tubes. After clotting overnight in a refrigerator, the blood was centrifuged for 30 min at 3,000 rpm. Sera were collected and stored at −20° until assay. Testes from each frog were fixed in Bouin's fixative, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Serum samples were assayed for 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and testosterone using a radioimmunoassay technique described in detail by Wingfield and Farner (1975). Briefly, samples were extracted in dichloromethane (after prior addition of 2,000 cpm of respective [^{3}H] steroid for recovery determinations) and the extracts were purified on Celite:propylene glycol:ethylene glycol columns, each fitted with a Celite:water glycol trap. Steroid fractions were eluted by increasing concentrations of ethyl acetate in iso-octane as follows: 4.5 ml of 10% for dihydrotestosterone, or 4.0 ml of 20% for testosterone. Dried eluates were assayed in duplicate (a 1/5 aliquot was taken for recovery determination) with the appropriate antibodies, and separation of bound and free counts was achieved by use of dextran-coated charcoal. All results were adjusted for recovery and expressed as ng/ml.

Recoveries of [^{3}H] steroids after extraction and chromatography were 71.8 ± 2.7% (n = 20) for dihydrotestosterone and 60.8 ± 2.4% (n = 20) for testosterone. This is within the range 59.7–93.0% for dihydrotestosterone but slightly below the range 63.5–92.7% for testosterone found in avian plasma by Wingfield and Farner (1975).

With each assay, two solvent blanks (2 × 1 ml of double distilled water) and 0.5 ml of a plasma pool were taken through the entire assay procedure. In all cases solvent blanks assayed below the sensitivity of the standard curves (see Wingfield and Farner, 1975). Determinations on the plasma pool indicated that interassay variations were less than 8%.

RESULTS

Experiment I

Frogs used in June had been purchased in February and kept for 4 months until use in the experiment. In the 3-day pretreatment period none of these frogs responded to auditory stimulation by playing of taped mating calls. After three successive daily pituitary transplantations, three out of eight *R. pipiens* responded to sexual calls, and the number of individuals showing sexual behavior and the number of response calls increased progressively (Fig. 1a). Clasping was observed on Days 6 and 7.

Serum testosterone on Day 0 frogs was

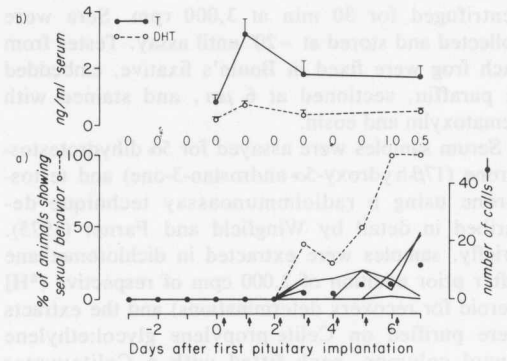

FIG. 1. Appearance of sexual behavior (a) and mean blood levels of androgens (b) after successive pituitary implantation in male leopard frogs in Experiment I (frogs were purchased in February and used in June). Arrows indicate each day of pituitary implantation. Solid lines in (a) represent numbers of calls each individual made, and solid circles represent their means. Numbers across the top of this figure indicate the mean number of attempted or successful efforts at sexual clasping observed per test on each indicated day. Vertical bars in (b) represent standard errors for each mean value.

0.85 ng/ml, and after one pituitary transplantation the mean testosterone level rose to 3.24 ng/ml ($P < 0.01$, compared with previous value, Fig. 1b). On Days 3 and 7 testosterone values decreased but remained higher than the Day 0 value ($0.05 < P < 0.01$). Absolute values of dihydrotestosterone were relatively low during the experiment, but values on Day 1 and on Day 7 were significantly higher than Day 0 ($P < 0.01$).

Histology of testes showed that pituitary transplantation induced prompt release of spermatozoa into the lumen of seminiferous tubules, which gradually expanded in diameter. Interstitial cells of Leydig seemed to increase in number and/or size, or at least they appeared more prominent after several pituitary implantations (Fig. 2).

Experiment II

Frogs purchased in September were used in the experiment in October. At that time a few untreated frogs responded to taped *R. pipiens* calls with several answering trills in a 30-min observation period. After one pituitary transplantation, 25% of the animals showed chuckles, trills, and even clasping. One hundred percent display of calling and clasping was obtained after three successive daily pituitary transplantations, and this level was maintained (Fig. 3).

In Experiment II, the levels of testosterone and dihydrotestosterone in untreated animals were considerably higher than those of Experiment I. Unfortunately, the assays were planned with the expectation that values like those of Experiment I would be obtained, and no dilution of sera was done. Accordingly, minimum values well in excess of those of Experiment I were found, but exact levels of testosterone and dihydrotestosterone could not be determined for most individuals since sera samples contained more hormone than the standard curves permitted estimation for. On Day 0, just before pituitary implantation began, levels of T and DHT were already quite high, comparable to the highest levels in Experiment I obtained by pituitary stimulation. After pituitary implantation, T and DHT appeared to increase further and remained at high levels (Table 1); but again, only minimum values are available, permitting only a conclusion that serum levels of the two measured androgens were increased by the pituitary implantations.

Histology of testes from frogs in Experiment II was also different from that of Experiment I. All frogs had well-developed interstitial components throughout the experiment. Although spermatogenesis apparently was arrested on Day 0 frogs, interstitial cells were well developed. After pituitary transplantation, spermatozoa were released into the lumina of the seminiferous tubules.

DISCUSSION

The results of the first (June) experiment provide a good correlation between increased blood level of testosterone and

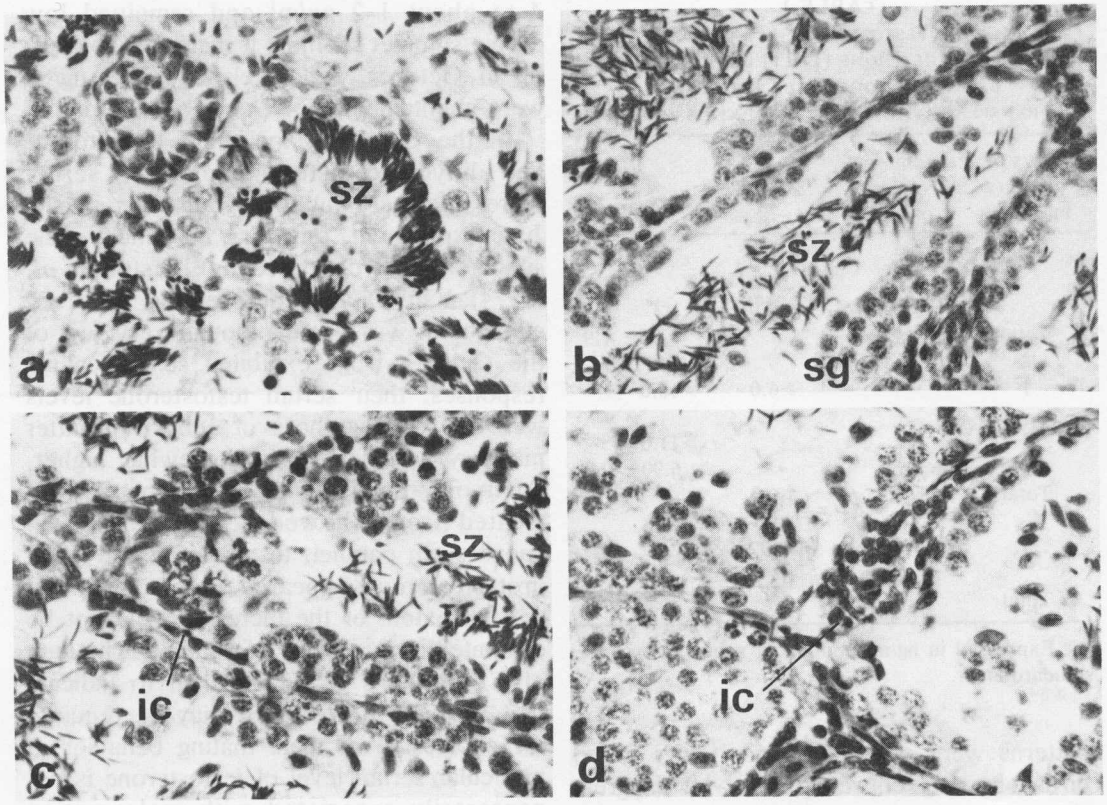

FIG. 2. Photomicrographs of testes from leopard frogs after (a) 0, (b) 1, (c) 3, and (d) 7 successive pituitary implantations in Experiment I. Symbols: ic, interstitial cells; sg, spermatogonia; sz, spermatozoa. Hematoxylin and eosin stain. ×350.

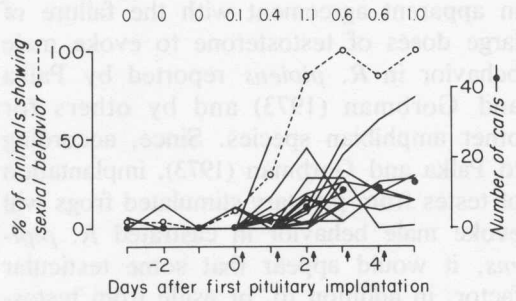

FIG. 3. Incidence of sexual behavior after successive pituitary implantations in male leopard frogs in Experiment II (frogs were purchased in September and used in October). Arrows indicate each pituitary implantation. Other symbols are as in Fig. 1. Numbers across the top of this figure indicate the mean number of attempted or successful efforts at sexual clasping observed per test on each indicated day.

appearance of sexual behavior, as measured in these tests. The initial surge of testosterone was followed by a sustained higher level of serum androgen; sexual behavior, in parallel, increased gradually to a maximum level. A lag time of several days between the rise in blood levels of hormone and the appearance of sexual behavior can be noted by comparing Figs. 1a and b. Correlation of individual values of blood levels of testosterone and sexual behavior indicates in this experiment that frogs which had approximately 2 ng/ml of testosterone in blood responded fairly vigorously in the behavioral test situation.

Recently, blood levels of testosterone in Barbary doves displaying various behavioral

TABLE 1
SERUM TESTOSTERONE (T) AND 5α-DIHYDROTESTOSTERONE (DHT) LEVELS IN MALE LEOPARD FROGS AFTER PITUITARY TRANSPLANTATION (OCTOBER EXPERIMENT) [a]

Days of pituitary injections	Numbers of frogs	T	DHT
0	1	2.44	
	1	5.49	
	5	>6.0	
Total	7		8.80 ± 1.9
1	7	>6.0	>10.0
3	1		>11.0
	6		6.90 ± 0.8
Total	7	>6.0	
5	1	4.97	
	6	>6.0	
Total	7		5.28 ± 0.9

[a] Expressed in ng/ml; individual values or mean ± standard error.

patterns were measured, and it was found birds showing courtship behavior (aggressive and nest-oriented courtship) have high levels of testosterone (Hutchison and Katongole, 1975). In a group showing aggressive courtship, testosterone levels were 0.11 ± 0.02 ng/ml and in another group, showing aggressive and nest-oriented courtship, 1.01 ± 0.66 ng/ml.

In rats, several investigations have estimated blood testosterone levels during sexual maturation. In neonatal young, values were obtained such as 0.21 ng/ml (Resko *et al.*, 1968); 0.52 ng/ml (Miyachi *et al.*, 1973); 0.76 ng/ml (Lee *et al.*, 1975), and in matured 80–90 day-old rats values of 2.04 ng/ml (Resko *et al.*, 1968); 6.5 ng/ml (Miyachi *et al.*, 1973); 2.5 ng/ml (Robel *et al.*, 1973); 2.15 ng/ml (Lee *et al.*, 1973) were obtained.

Recently (D'Istria *et al.*, 1974) in frogs (*Rana esculenta*), seasonal variations of serum testosterone levels were measured by radioimmunoassay. Just after the breeding season, April to June, testosterone fell from 4 to about 1–2 ng/ml and remained low during summer months; it increased to 5 ng/ml in October, and levels of 12–19 ng/ml were maintained from December to March.

In the October experiment testosterone and dihydrotestosterone levels in serum were relatively much higher than in the June experiment, reflecting an annual cycle similar to that described by D'Istria *et al.* (1974). On Day 0, before any pituitary stimulation was begun, though very few of the October frogs exhibited sexual calling responses, their serum testosterone levels were as high as those of June frogs after pituitary stimulation, or somewhat higher. After only one pituitary treatment, 25% of treated frogs showed sexual calling responses. In parallel, testosterone and dihydrotestosterone appeared to increase, but the full extent of the increase could not be estimated in this group of animals. The data of Experiments I and II seem to indicate that although testosterone may be required for expression of male mating behavior, a particular serum level of testosterone is not necessarily associated with male mating behavior in *Rana pipiens*. The same can be inferred in a correlative sense from the seasonal measurements of serum testosterone by D'Istria *et al.* (1974). This finding is in apparent agreement with the failure of large doses of testosterone to evoke male behavior in *R. pipiens* reported by Palka and Gorbman (1973) and by others for other amphibian species. Since, according to Palka and Gorbman (1973), implantation of testes from pituitary-stimulated frogs will evoke male behavior in castrated *R. pipiens*, it would appear that some testicular factor, in addition to, or aside from testosterone, influences the expression of this behavior. Whether such a testicular factor indeed exists, or whether it could evoke the behavior in the absence of testosterone remains for further research to establish.

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