

## Changes in Hormone Levels in Relation to Breeding Behavior in Male Bullfrogs (*Rana catesbeiana*) at the Individual and Population Levels

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Fluctuations in plasma androgen (testosterone and 5 $\alpha$ -dihydrotestosterone), corticosterone (B), and luteinizing hormone (LH) of male bullfrogs in central California were measured during the spring mating season. Androgen and LH levels generally increased in the population prior to the initiation of chorusing and establishment of territories, whereas plasma B peaked in a 2-week period at the start of heavy chorusing; this coincided with a transitory, 1 week decline in androgen. Individual males showed fluctuations in plasma androgen and LH levels throughout the breeding season, often within 1 day, but there was no clear correlation between changes in the two hormones. No time of day effect was apparent on any of the hormones. B and androgen levels were significantly but weakly correlated, ( $r = 0.35$ ) but LH and androgen were not. Although the seasonal trend of increasing androgen corresponded with the start of intense chorusing (and presumably sexual activity) by the population, behavior of individuals and their circulating androgen levels did not correlate. After acquiring territories, males showed no overall trend of increased plasma androgen. Moreover, males that showed no vocal or territorial behavior had significantly higher androgen and lower B levels than calling males. Increased B levels suggest that territorial behavior and especially direct agonistic encounters represent stresses that could have an inhibitory effect on androgen secretion. © 1985 Academic Press, Inc.

There are few data on the relationship between reproductive behavior and natural variations in circulating levels of pituitary and gonadal hormones in amphibians. Licht *et al.* (1983) reported seasonal changes in the levels of circulating gonadal and interrenal steroids and pituitary gonadotropins in the bullfrog, *Rana catesbeiana*, with emphasis on the relation between changes in hormone levels and gross gonadal morphology and breeding activity. The additional discovery of profound postcapture stress effects on the endocrine system of the bullfrog indicated that further study on normal seasonal fluctuations and their effect on reproductive behavior might be more effectively conducted in the field (Licht *et al.*, 1983). Also, because frogs

were sampled from many different localities at relatively long intervals (usually monthly) only a general seasonal pattern was described; a more detailed study is needed to discern how hormones might affect reproductive behavior.

Increases in androgen levels have been implicated in initiating reproductive behavior in other animals, but their effect in amphibians remains unclear (Greenberg and Crews, 1982; Moore, 1983). There have been numerous explanations for this ambiguity, but since these experimental studies have been conducted in the laboratory, the stress of holding animals in captivity may have contributed to the lack of correlation between androgen and reproductive behavior.

As an extension of our previous study, we now focus on the hormonal changes in a single field population of male bullfrogs

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before and during the breeding season with an emphasis on the hormone fluctuations in individuals and how they relate to the different components of reproductive behavior. Attention was focused on gonadal androgens, corticosterone, and pituitary gonadotropin.

### *Bullfrog Reproductive Behavior*

The most extensive behavioral studies on bullfrogs have been conducted in the northern portion of the species range (Emlen, 1969; Weiwandt, 1969; Howard, 1978; Ryan, 1980). Male bullfrogs in these areas migrate to the breeding pond from surrounding marsh areas and begin sporadic chorusing. As the breeding season progresses, sustained choruses begin at dusk and can continue until dawn. Morphological changes also take place in male bullfrogs at this time. We observed that the thumbs and forearms become enlarged and the throat color changes from a pale to a deeply yellow color (see also Holmes, 1906).

When chorusing begins, males become territorial and defend areas ranging 2–6 m in length (Ryan, 1980). Males have two types of calls: the familiar “mating” call and a “territorial” call (a “bonk” sound) (Capranica, 1968; Weiwandt, 1969). Territorial males assume a “high” posture (floating in the water, lungs inflated, yellow gular sac extended—Ryan, 1980). Intrusion of a male in this position into another male’s territory, may result in either (1) an “encounter”—the two may approach one another while calling until one breaks away by submerging or assuming a “low” posture (eyes and snout barely above water—Emlen, 1968); or (2) a “fight”—an aggressive interaction in which frogs lock forearms and attempt to hold one another underwater. Most such agonistic encounters are brief, but they can last up to an hour (Howard, 1978). In contrast, if a male assumes a “low” posture, it can approach a territorial male without being attacked.

Howard (1978) referred to males that maintained these low postures (not calling or defending territories) near territorial males presumably to “steal” copulations as “parasitic” males. In the present study, we attempted to obtain blood samples from identified individuals displaying these behaviors.

### METHODS

*Study area.* The study was conducted at Ball’s Fish Camp, Friant, Fresno County, California. The study pond was one of a series of neighboring ponds that were used (50 years previously) as quarry pits. Ponds were located ~1 km from the San Joaquin River, the major drainage for California’s Central Valley. The pond was approximately rectangular, ca. 150 × 60 m. It had little emergent vegetation but its eastern and western shoreline had thick clumps of willows.

The pond was used extensively by bullfrogs, both before and during the breeding season. As many as 30 adult bullfrogs were caught in a night and the adult population was estimated to be three times that number. Females and males were caught in equal numbers before the breeding season, but as calling began the population became heavily skewed toward males. The presence of large tadpoles indicated that the pond had been used as a breeding site in the previous year.

*Sampling and behavioral observation.* Frogs were observed and captured from dusk to dawn (2000–0500 hr) although most collections ended at 0200 hr. There were nine sample periods, ranging from 2 to 5 consecutive nights each for a total of 23 nights (107 hr). Sampling extended from 8 March to 31 May 1983. Unfortunately, we were unable to gain access to the site after May and therefore lack data for the end of the breeding season.

We patrolled the pond periodically throughout the night. Animals were captured by hand and bled immediately by cardiac puncture with heparinized syringes (~0.5 ml of blood was taken). Blood samples were kept on wet ice in the field, centrifuged after the night’s collecting, and the plasma frozen on dry ice and stored at -70°. The location and behavior of each individual before capture (e.g., with reference to calling, posture, fighting, mating, proximity to another frog) was recorded. Frogs were toe-clipped, their snout-vent length measured, and a color-coded tag was affixed to their backs. They were then released near the point of capture. This entire procedure normally took less than 5 min; the actual blood collection usually took about a minute.

*Hormone analyses.* Corticosterone (B) and gonadal androgen—testosterone(T)+ 5 $\alpha$ -dihydrotestosterone (DHT)—were measured by RIA on ether extracted,

nonchromatographed plasma samples by techniques previously described for this species (Licht *et al.*, 1983). The antiserum was cospecific for the two androgens, but a previous study (Licht *et al.*, 1983) demonstrated a very high correlation ( $r = 0.94$ ) between them: DHT/T ratios are near unity in winter (when they are at minimal levels) and range from 2 to 4 during the period encompassed by this study. Since levels of plasma follicle-stimulating hormone closely parallel those in plasma luteinizing hormone (LH), but at lower levels (Licht *et al.*, 1983), only LH was measured here. A homologous LH-RIA as described by McCreery *et al.* (1982) was employed.

Statistical analyses (descriptive statistics, paired  $t$  tests, correlation coefficients, ANOVA) were performed with the Microstat program (Ecosoft, Inc., 1982). Differences among means were compared using Duncan's multiple range test. All significance levels were set at the 0.05 level unless otherwise noted.

## RESULTS

Sampling began about a month before the start of calling and then included the period of sporadic calling (April 6–8), the start of heavy chorusing (April 14–18), and its subsequent plateau (remaining sample periods). During this time, 469 blood samples were taken from 165 tagged males. Forty-three percent of the animals were sampled in only one period and 12% of them in at least 5 different periods. Figure 1 presents

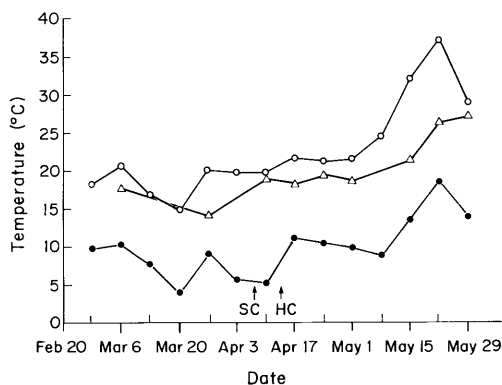


FIG. 1. Changes in weekly mean minimum and maximum air temperatures (circles) recorded at Fresno meteorological station (NOAA, 1983) and mean shallow water temperatures (triangles) recorded during sampling periods at the study site in spring, 1983. SC, Initiation of sporadic chorusing; HC, initiation of heavy chorusing.

climatic information for the study period and indicates the initiation of sporadic and heavy chorusing. Mean weekly air temperatures remained relatively stable during the observation period. Water temperatures taken during sampling were relatively stable and approximated mean weekly maximum air temperatures. Air temperatures fell as much as  $10^{\circ}$  in a night but the water never cooled more than  $2^{\circ}$ . Initiation of chorusing in mid-April coincided with a rise in water temperature, but the greatest change in mean temperatures took place toward the end of our study (well into the breeding season).

### Time of Day

There was no significant correlation between time of night and either plasma androgen ( $r = 0.016$ ;  $P = 0.74$ ,  $N = 421$ ) or B ( $r = 0.09$ ;  $P = 0.09$ ,  $N = 421$ ). There was a low correlation between time of night and LH levels ( $r = -0.14$ ;  $P = 0.01$ ,  $N = 421$ ).

### Body Weight

Body weight of tagged animals at first capture averaged 314 g (SE = 5.8;  $N = 136$ ). However, the mean weight of males first captured in the latter part of the sampling period (May 1–31) was significantly less than that of those first captured at the start of the season (March 8–April 8). Also, in two of the last four sample periods, the mean weight of males at first capture was significantly less than that of the males recaptured at the same time (May 1–3:  $t = -2.16$ ,  $df = 24$ ,  $P = 0.02$ ; May 10–15:  $t = -2.39$ ,  $df = 69$ ,  $P = 0.01$ ). Thus males captured for the first time late in the season weighed significantly less than those recaptured after they had been in the pond over 2 weeks, even though these long-term males had lost weight ( $\chi^2 = 7.56$ ;  $df = 2$ ,  $N = 27$ ). These data suggest that smaller males migrated to the pond later in the breeding season.

### Population Hormone Fluctuations

Figure 2 summarizes the changes in mean hormone levels for males throughout the study, both at the time of first capture and at recapture. Plasma androgen averaged 25 ng/ml in March and rose steadily for both newly and later sampled animals until peaking in mid-April. There was a transient drop in androgen levels at the next sample period, and levels then plateaued in May (Fig. 2a). In two of the last three sample

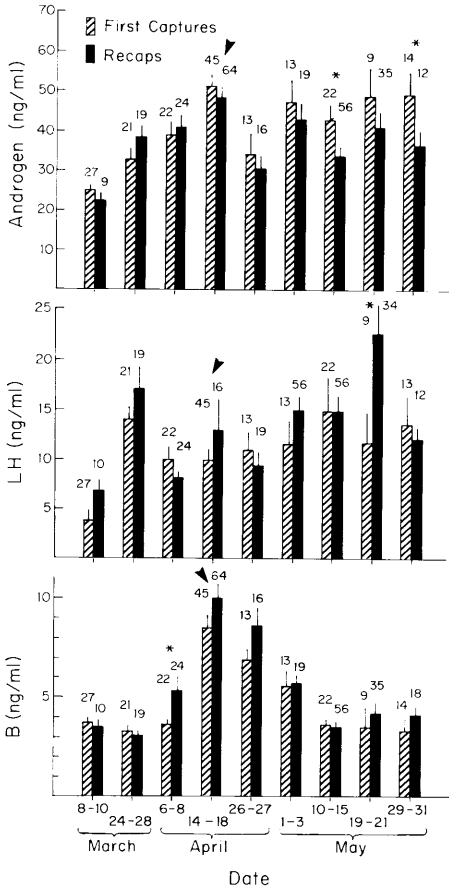


FIG. 2. Changes in mean plasma androgen, luteinizing hormone (LH) and corticosterone (B) (a, b, and c, respectively) for male bullfrogs sampled at initial capture and at recapture. Bars = mean; vertical lines = SEM; numbers above bars = sample size. Recaps = sum of all recaptures. Asterisks represents a significant difference between mean values for first capture and recapture. Arrows indicate the period when active chorusing commenced (cf. "HC" in Fig. 1).

periods, androgen levels were significantly higher in animals captured for the first time as compared to those recaptured on the same nights. Mean androgen levels peaked at the time of the initiation of heavy chorusing.

LH did not exhibit a discernible pattern except that the first sample period levels were significantly lower than all others (Fig. 2b). First capture and recapture levels of LH differed significantly at only one sample period (May 19–21). LH was significantly but only weakly correlated with androgen ( $r = 0.18$ ;  $P = 0.02$ ,  $N = 159$ ) for males when first captured, and not correlated when levels of recaptured males were used ( $r = 0.084$ ,  $P = 0.23$ ,  $N = 256$ ). Plasma LH levels and B levels did not covary (all captures:  $r = -0.043$ ,  $P = 0.38$ ,  $N = 422$ ).

B levels peaked in mid-April in both first captured and recaptured males (Fig. 2c). There were no significant differences among B values from early and late in the study period but these periods were significantly lower than the maxima achieved in the second half of April (April 14–18 and April 26–27). The timing of the peak mean B values corresponded with the peak mean androgen levels, which was also the time when heavy chorusing began. The two hormones were significantly correlated overall ( $r = 0.35$ ;  $P = 0.0001$ ,  $N = 373$ ) but mean B levels were still high at the April 26–27 sample period when mean androgen values had fallen to precalling levels.

### Individual Hormone Fluctuations

The levels of all measured hormones fluctuated greatly over time. Figure 3 illustrates changes in hormone levels in four males recaptured over several sample periods. Overall, androgen levels tended to rise in mid-April but did not remain constant. Androgen sometimes fluctuated 60 ng/ml in a day with no corresponding observed change in LH. Individual LH levels also fluctuated greatly, following no consis-

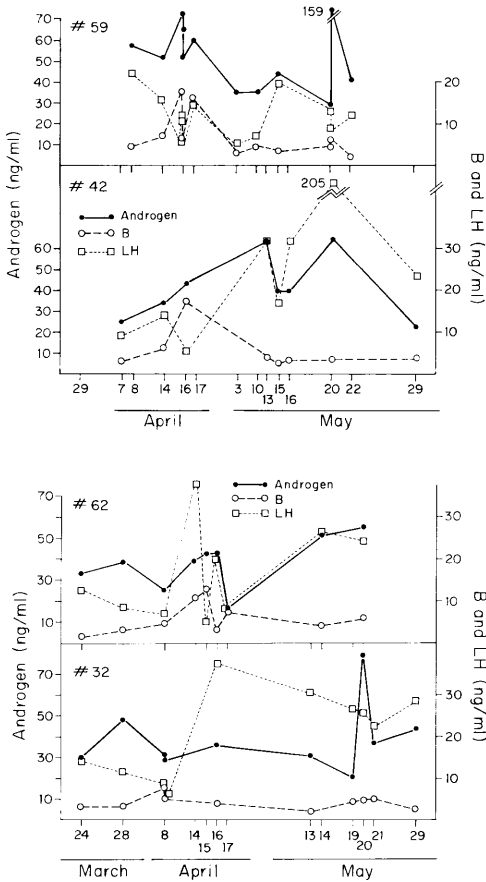


FIG. 3. Fluctuations in plasma androgen, luteinizing hormone (LH), and corticosterone (B) in four individual male bullfrogs sampled repetitively throughout the study period.

tent pattern (see Fig. 3). No behavior was observed, at the time of capture, that might account for these fluctuations. Individual B levels followed the population pattern of peaking in mid-April and being low and constant during the rest of the study period.

Behavioral changes of individual males were not clearly reflected in their hormone levels. Males first recorded as noncalling and having pale-colored throats and then later observed as calling in a high posture with bright yellow throats had significantly higher androgen levels as a group at the latter sampling ( $t = 2.61$ ,  $df = 24$ ,  $P = 0.008$ ). However, the *pattern* of change was not significantly different from random

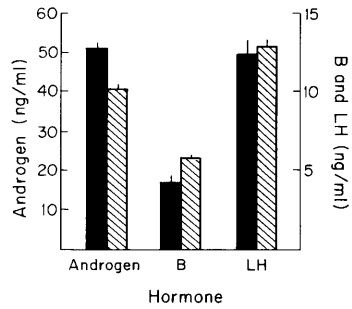


FIG. 4. Mean plasma androgen, corticosterone (B), and luteinizing hormone (LH) levels for calling, territorial males (hatched bars;  $n = 416$ ), and for males demonstrating no recognizable "reproductive" behavior (dark bars;  $n = 13$ ). Bars = mean; vertical lines = SEM.

(i.e., levels showed an equal probability of increasing, decreasing, or remaining the same;  $\chi^2 = 2.48$ ,  $df = 2$ ,  $N = 25$ ).

A subset of males ( $N = 13$ ) in the pond weighed significantly less ( $\chi^2 = 270$  vs 322 g;  $t = 3.21$ ;  $df = 232$ ;  $P = 0.007$ ) than calling males, had pale yellow throats, were never seen to call, never observed in the high position, and when observed on more than one night, were always in a different part of the pond (i.e., nonterritorial). These noncalling males had significantly higher mean androgen levels ( $t' = 1.74$ ,  $df = 416$ ,  $P = 0.04$ ) and significantly lower mean B levels ( $t' = -2.71$ ,  $df = 417$ ,  $P = 0.008$ ) than calling males. There was no significant difference between the mean LH levels of the two groups (Fig. 4).

Only one fight, six encounters, and two incidents of amplexus were witnessed in 107 hr of observation. Table 1 summarizes the hormone levels of these study area males and includes values for six other males from adjacent areas displaying these behaviors. Fighting males had B levels that were higher or equivalent to the highest levels seen at any time during this study in calling males (e.g., the Apr 14–18 sample period:  $\bar{X} = 9.38 \pm 0.55$ , range = 2.58–28.3). The B levels of males involved in encounters were more typical of the mean B values for calling males. Both the androgen

and LH levels of agonistic males were typical of values recorded for calling males. The two individuals involved in amplexus had very high levels of LH as observed previously (Licht *et al.*, 1983), but B and androgen levels were indistinguishable from those of nonamplexing males.

## DISCUSSION

### *Population and Individual Hormone Fluctuations*

The seasonal pattern in plasma androgen of male bullfrogs was generally similar to that described previously (Licht *et al.*, 1983). Both studies show that androgen and LH were already elevated above seasonal (winter) minima by early March (e.g., 20–25 ng/ml vs less than 2 ng/ml in January), peaked in early April, and then underwent a transitory decrease. Fluctuations in plasma androgen during the breeding season were observed in the previous study (1980–1981), but the second peak occurred later (in June; Licht *et al.*, 1983). Sixty-six percent of long-term recaptured males that were sampled over a sufficient time span in the present study also showed a bimodal pattern similar to that for the overall population. Thus the second rise in androgen levels during the breeding period is not only due to the influx of new males (who had significantly higher androgen levels) into the pond during the later months.

*LH vs androgen.* Another similarity between this and the previous study was the generally weak correlation between LH and androgen both at the individual and population level. The only parallelism in pattern seen between the two hormones in the 1983 study was that both were low at the beginning of the season and then rose up to the time when active chorusing began. A lack of correlation was most striking in individuals resampled within a few hours or days. Increases in plasma androgen levels were not preceded by in-

creases in LH levels within the period of sampling.

The apparent temporal dissociation between LH and androgen levels in the field study is contrary to all experimental results for bullfrogs using either exogenous LH or GnRH treatment (Muller, 1977; McCreery *et al.*, 1982; McCreery and Licht, 1983). However, the lack of correlation is not unprecedented; although circulating LH and androgen have shown the expected close correspondence throughout the annual cycle in some mammals (e.g., Lincoln, 1981), there are examples in mammals and birds in which the two hormones appear temporally dissociated in a manner reminiscent of that seen here (e.g., Balthazart *et al.*, 1981; Donham *et al.*, 1982; Soares and Hoffman, 1981). It is noteworthy that the concentrations of circulating LH resulting from experimental manipulations in the bullfrog usually greatly exceeded the mean values observed in field captured animals. Thus the possibility exists that the close concordance between rising and falling LH and androgen may represent a "pharmacological" response rather than a natural phenomenon. It remains to be demonstrated whether changes in circulating LH are the primary proximate causes of changes in androgen levels under normal conditions. Much more frequent sampling may be required to resolve this issue, especially if either hormone shows a pulsatile release pattern.

*Corticosterone.* The seasonal pattern of plasma B with a peak in mid to late April (Fig. 2c) was not apparent in our earlier study (Licht *et al.*, 1983). In view of the transient nature of the changes in B and androgen, the discrepancy between this and the earlier study was likely due to the use of mixed populations in the former. The correlation between androgen and B levels was primarily due to the coincidence of mean peak values of the two hormones in mid-April. While B levels were again significantly higher than both March and May

TABLE 1  
 BODY WEIGHT AND PLASMA HORMONE LEVELS IN INDIVIDUAL MALE BULLFROGS OBSERVED IN VARIOUS  
 BEHAVIORAL INTERACTIONS WITH OTHER FROGS

Behavior	Date	Body wt (g)	Androgen (ng/ml)	B (ng/ml)	LH (ng/ml)
Fighting <sup>a</sup>	4/26	360	26.1	34.7	28.4
	4/26	360	35.1	23.8	39.7
	6/10 <sup>b</sup>	300	62.1	13.8	NA <sup>c</sup>
	6/10 <sup>b</sup>	380	71.2	21.9	NA
	6/10 <sup>b</sup>	400	42.0	7.2	NA
	6/10 <sup>b</sup>	360	37.4	8.4	NA
Encountering	4/14	330	36.3	3.7	5.3
	4/18	440	24.7	7.2	7.6
	5/2	295	69.5	6.3	32.3
	5/2	310	52.4	6.3	25.0
	5/13	305	64.0	3.9	31.8
	5/15	300	62.1	3.8	12.7
Amplexing	4/17	390	49.8	18.8	260.0
	5/21	440	82.2	19.3	172.0
	5/5 <sup>b</sup>	NA <sup>c</sup>	30.2	6.6	112.0
	5/5 <sup>b</sup>	NA	53.1	6.9	119.0

<sup>a</sup> Fighting occurred between successive pairs listed.

<sup>b</sup> Individuals captured outside the study area.

<sup>c</sup> NA, Data not available.

values at the next sample period (25–26 April), androgen values had then dropped to prebreeding season levels. When plasma androgen levels were again elevated, plasma B levels were minimal. The seasonal B pattern observed here is similar, though over a much briefer time period (2 weeks vs 2 months), to that reported for *Rana esculenta*, in which a peak in plasma B corresponded with the breeding season (LeBoulenger *et al.*, 1979). Mean peak B values for *R. esculenta* (both sexes) were twice those observed in calling male *R. catesbeiana*, but this difference may relate to other behavioral correlates (see below).

In addition to seasonal fluctuations, LeBoulenger *et al.* (1982) reported a pronounced diurnal variation in B in male and female *R. esculenta* captured in June. Such time of day effects were not observed within the more limited sampling employed here even though our sampling times encompassed the period (0000–0400 hr) when the maximum values occurred in *R. esculenta*. LeBoulenger *et al.* (1982) suggested

that this increase in B in *R. esculenta* was associated with reproductive behavioral “components” (e.g., mating calls, territorial defense, quest for partner). In this regard, it is noteworthy that their maximum values (24–28 ng/ml) were equivalent to those observed in fighting male *R. catesbeiana*.

Although B levels are highly labile and sensitive to disturbance in the bullfrog (Licht *et al.*, 1983), capture and bleeding per se were not judged to be the main causes of these changes since of the 15 individuals bled twice in the same night, plasma B levels rose slightly in 5 (~2-fold), fell in 5 and remained unchanged in 5; the same lack of pattern was found in individuals bled within a 3-day period. Overall, mean plasma B values for recaptured animals tended to be higher than for animals at first capture but the increase was significant only in the April 6–8 sample period. Also, if repeated recapture itself was stressful, one might have expected that plasma B levels would have increased pro-

gressively throughout the season instead of showing the distinct mid-season peak that was observed.

### *Hormones and Behavior*

In the Ball Pond population, the peak mean B plasma levels coincided with the initiation of heavy chorusing and the mean peak androgen levels. The only period when recaptures had significantly higher B plasma levels was when sporadic chorusing first began. The initiation of chorusing in bullfrogs is when territories are established and most fights and encounters occur. It may be that the occupation of a territory and its associated behavior constitute a stress that is reflected in higher plasma B levels. Fighting and amplexus were events that raised B levels while encounters did not seem to have the same effect (Table 1). Sustained territorial behavior could account for the peak in B plasma levels in mid-late April.

The androgen levels of male bullfrogs engaged in encounters or fights were not significantly different from those of other calling males. Lack of precise information on the timing or duration of these encounters hinders interpretation of these data, but results of studies on such a relation between androgens and aggression in other types of animals have frequently been equivocal (Lumia, 1972; Balthazart, 1979; Harding and Follett, 1979; Tsutsui and Ishii, 1981; Rowher and Wingfield, 1981).

The rapid increases in B levels following postcapture stress were also associated with a marked decline of gonadal steroids; androgens fell to minimal levels after 8 hrs and did not recover for at least 4 days (Licht *et al.*, 1983). It may be that the transient drop in androgen levels observed in the April 26–27 sample period was an effect of a prolonged elevation in B (stress?) related to territorial activities. The physiological basis of such a response is still unknown. However, a direct inhibitory effect of adrenal glucocorticoids on steroidogen-

esis as shown in rats (e.g., Bambino and Hsueh, 1981) may explain why androgens are depressed without a drop in LH.

While the above data suggest that some behaviors (e.g., amplexus and fighting) caused rapid changes in hormone levels, it is not clear that differences in hormone levels mediate differences in behavioral response. The role of androgen in amphibians is especially problematic. Kelley and Pfaff (1976) reported that androgen injections induced clasping in male *Xenopus laevis* but other studies on amphibians, especially ranids, have generally found that androgen levels alone are not sufficient indicators of whether an animal will exhibit a reproductive behavior (e.g., amplexus—see Moore, 1983 for review; calling—Wada *et al.*, 1976; Wetzel and Kelley, 1983) and the method of administering the steroid may be critical (see review by Greenberg and Crews, 1982). Androgens do appear to have a maintenance effect on sex behavior but it is not clear whether they are the primary stimulatory agents or whether other factors (e.g., prolactin, gonadotropins, and neuropeptides have all been implicated) are the actual causative agents. Also, low levels of gonadal steroid may be sufficient to activate the necessary brain regions at the start of the season and fluctuations throughout the breeding season may not further influence behavior (DaMassa *et al.*, 1977).

Corticosterone has been shown to have inhibitory effects on amplexus in the rough-skinned newt, *Taricha*, but it was not indicated whether this inhibition was due to direct effects of interrenal steroids on testicular secretion (Moore and Miller, 1984). The pituitary–adrenal axis has been implicated in intermale agonistic behavior in several mammals, but dose responses are complex and possibly bimodal (reviewed by Leshner, 1982).

The overall seasonal increase in plasma androgen in the male bullfrog corresponded to the initiation of heavy chorusing, but so did a mean seasonal increase in water tem-



perature. Not all of the frogs that apparently became territorial (i.e., calling, remaining in one area) had significantly elevated androgen levels. Moreover, males that appeared not to be territorial at all (neither morphologically nor behaviorally) had significantly higher androgen levels than males that had been seen calling (Fig. 4). These males may have been equivalent to the "parasitic" males described by Howard (1978). If so, our results indicate that it is clearly *not* the lack of high androgen levels that is responsible for these males adopting another (nonaggressive) behavior. Thus, elevated androgen levels may be necessary but certainly are not sufficient to induce characteristic male reproductive behavior in the bullfrog.

We emphasize that bullfrog reproductive behavior is known to vary among populations (Ryan, 1980) and that our results pertain to a population and not the species. The more northern bullfrog populations, with a shorter breeding season, had many more aggressive interactions and matings than we recorded during part of the longer California breeding season (Emlen, 1968; Howard, 1978; Ryan, 1980). It is possible that in a shorter, more intense breeding season gonadal steroids would correlate more closely with behavior but the above cautions must certainly be taken into account.

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