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REPRODUCTION IN THE MEXICAN LEAF FROG (*PACHYMEDUSA DACNICOLOR*)

I. BEHAVIORAL AND MORPHOLOGICAL ASPECTS

By

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The reproduction, courtship, and breeding patterns of amphibians are highly variable (Duellman and Trueb, 1986). Among one of the divergent anuran patterns is the placement of eggs on vegetation above water, into which hatchling tadpoles drop. This mode of reproduction is characteristic of hylid frogs of the subfamily Phyllomedusinae. One of these species, *Pachymedusa dacnicolor*, has been studied in the field by Pyburn (1970) and Wiewandt (1971), who provided some excellent information about breeding activities.

More details concerning the reproduction of *P. dacnicolor* were derived from observations of captive frogs made in an artificial environment that partially duplicated prevailing natural conditions (Bagnara, 1978). This artificial environment was available in greenhouses in Tucson, Arizona, just a few hundred miles from the northernmost distribution of *P. dacnicolor* (Duellman, 1970). In the Tucson greenhouses, annual cycles of light, temperature and rainfall did not differ markedly from those in Mexico. The diet of the captive frogs resembled their diet in nature, for they fed on insects attracted to the greenhouse. The frogs exhibited a profile of activities that seemed normal for the species, grew rapidly, and remained in good health. The captive colony successfully had ten generations, with one breeding season per year.

Because of long experience gained from working with these captive frogs, their availability in large numbers, and their surprising tameness, we made a broad investigation between 1975 and 1985 of reproduction in *P. dacnicolor*. This paper on behavioral and morphological aspects lays the basis for two dealing with the endocrinology of the testis (Rastogi, *et al.*,

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1986a) and ovary (Iela, *et al.*, 1986), and another with spermatogenesis (Rastogi, *et al.*, 1986b).

METHODS

Most observations were made in greenhouses designed to duplicate the natural conditions near Los Mochis, Sinloa, Mexico, the source of the initial stock of adult frogs. The majority of observations were made in a small greenhouse (6' × 8' × 6') located at the home of JTB in the foothills of the Santa Catalina Mountains near Tucson, Arizona. Additional observations were made at a slightly larger greenhouse, 8' × 12' × 6', maintained at the University of Arizona Farm in the valley of the Rillito River at the base of the Santa Catalina Mountains. The principal water source in each greenhouse was provided by a plastic container, 2' × 3' × 1', that was sunk into the ground to mimic a natural pool. Numerous potted house plants and bushes including hibiscus, rose, bougainvillia, and fig provided shade and hiding places for the frogs during the day. Because the frogs are nocturnal, it was possible during warm periods from February to November to utilize naturally occurring insects, notably moths, for food. Insects were attracted by black lights placed at a window in each greenhouse. The original plastic window was replaced by a wire screen of $\frac{3}{4}$ inch mesh. The frogs took positions near the window and fed *ad libitum*. As moths were more abundant in the foothills, it was not necessary to supply extra food even during the cooler months of early spring and late fall. However, crickets were added to supplement the food supply in the farm greenhouse during cooler periods when few moths were available.

Because near-freezing conditions often obtained during the winter, the frogs were moved indoors and kept in aquaria and environmental chambers of various types and sizes. They were fed crickets during this time. In April, when the danger of freezing was past, the frogs were returned to the greenhouses. As summer approached (late May and June), greenhouse temperatures were very high during the day (often over 45°C). The frogs apparently had no problem withstanding either this daily high temperature or the sharp drop in temperature at night when the minimum often dropped below 20°C. No records of relative humidity were kept during this time, although humidity seemed to be low judging by how rapidly the potted plants dried out. During the summer rainy season high humidity in the greenhouse was more easily maintained by daily waterings. Very likely watering alone during early June (when the relative humidity in southern Arizona is very low) was only marginally sufficient in providing humidity high enough for breeding. As shown in Table 1, it was not until July and August that a high frequency of breeding occurred. This coincided with the annual summer rainy period when the relative humidity was high.

The breeding activities described in this paper are the result of both planned and fortuitous observations. The original intent of establishing the greenhouse colonies was to obtain eggs and embryos and *P. dacnicolor*;

TABLE 1. A Summary of Bimonthly Breeding Records 1975-1984. Average number of eggs per clutch (E/C) and number of clutches (C) are indicated for each bimonthly period.

Month	June			July			August			September		
	E/C	C	E/C	E/C	C	E/C	E/C	C	E/C	C	E/C	C
1975				824	4	365	1					
1976	875	2	954	13	14	725	6	700	10	713	8	
1977			1125	4	1	450	1	200	3			
1978*			2000	1								
1979*						400	1	400	1			
1980			400	1	600	2	490	13	544	13	675	8
1981**	300	2	330	5	216	16	329	28	351	27	290	12
1982**	500	2	450	4	335	8	358	13	267	9	280	5
1983	290	5	200	4	350	3	277	5	343	3	200	1
1984	100	1	442	6	4	295	11	445	10	300	1	
Total												
C's	9	17		53	82	66	63	35	3			
Average												
E/C	339	382	565	511	390	448	471	412				

*Complete records are not available.

** In addition, one clutch was laid at the end of May 1982 and two clutches were laid during the beginning of October 1981.

thus, no particular regimen of observations was planned at the outset. The greenhouses were each checked for eggs daily during the breeding season. At the foothills greenhouse this was done almost every morning about 5:30 a.m. during the months of June, July, and August. If breeding was occurring, observations were made for as long as five hours. Prolonged periods of observation were made less frequently at the farm greenhouse. Altogether, as many as 25 hours per week were spent in monitoring breeding activities during the height of the season. There was no indication that the presence of an observer or observers had any effect on behavioral activities, especially during mating. It was possible to film with close-up cinematography the entire mating process with no apparent effect on sexual behavior. In some cases individual frogs and pairs were identified on the basis of various morphological characteristics including size, color, shape, and skin markings. In Autumn 1980, 65 frogs were cold-branded on the mid-dorsal surface with implements made from copper wire shaped into numbers. The implements were cooled in dry ice and when placed on the skin left clear lesions which lasted as long as a year, but eventually faded so that they were difficult to see during the 1981 season. In late March 1984, 24 frogs were cold-branded on their backs and on their thighs, and thus it was possible to follow these individual frogs for that season. In at least two cases, marked pairs were followed during the oviposition of an entire clutch.

SEXUAL DIMORPHISM

In *Pachymedusa dacnicolor* there is obvious sexual dimorphism, particularly in body size. Sexually mature adult males in our colony ranged in snout-vent length from just over 60 mm to a little more than 80 mm and correspondingly, ranged in weight from 20 to 45 g (Fig. 1). Males are markedly smaller than females; the latter ranged in length from 70 to more than 100 mm with corresponding weights from 30 to almost 90 g (Fig. 2). Both sexes showed a linear relation between length and weight, as did developing immature males (Fig. 3) and females (Fig. 4).

Males become sexually mature at an earlier age than females. They are usually about 60 mm when they become sexually active, although we observed a unique male of 42 mm that contained testes full of sperm bundles. Males can become sexually active as early as four months after metamorphosis; we observed such males mating successfully. Females less than one year of age are also capable of breeding. Females raised from eggs obtained in the summer became sexually mature during winter and spring, and bred early the next summer. While no exact data on the average breeding age of females are available, endocrine studies (Iela, *et al.*, 1986) suggested that females are capable of breeding 9-10 months after metamorphosis.

Several other morphological differences between the sexes merit attention. As noted by Duellman (1970), the face of the female is blunt while that of the male is longer and pointed (Fig. 5). The rostral end is truncate in profile; this portion of the chin usually rests on the dorsum of

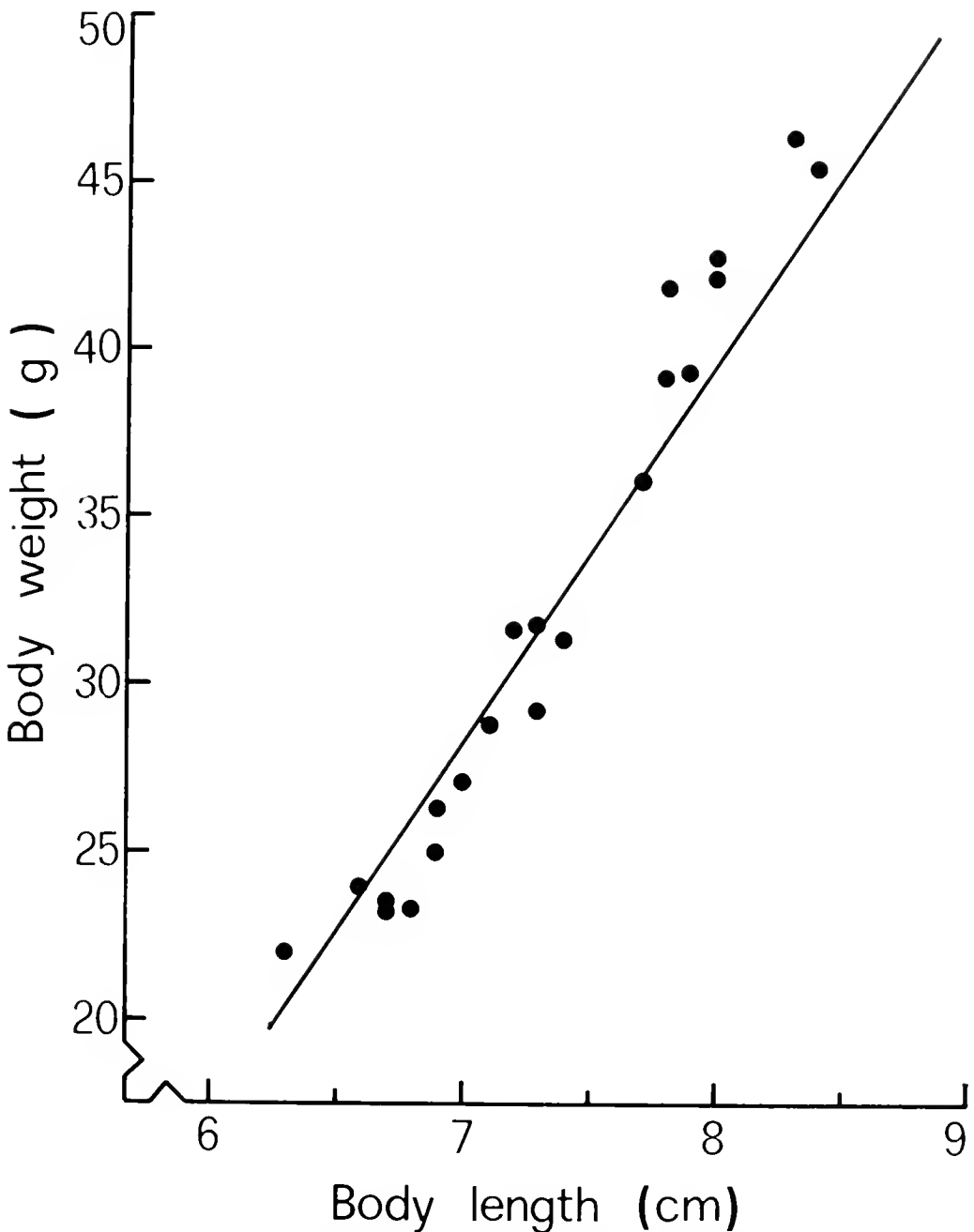


FIGURE 1. Relationship between body weight and body length in adult males. Body weight (y) is regressed on body length (x). Coefficient of regression. $r = 0.979$.

the female during amplexus. The female is more robust than the male, but possesses forelimbs that are relatively smaller than those of the male.

DESCRIPTION OF THE MATING PROCESS

The onset of hot weather in spring had a profound stimulatory effect on the gonads of both sexes (Rastogi, *et al.*, 1986a; Iela *et al.*, 1986). There were no external secondary sexual features exhibited by the female at that time; however, the male developed black triangular callosities at the base of the thumb that were rough to the touch. The forelimbs enlarged, the

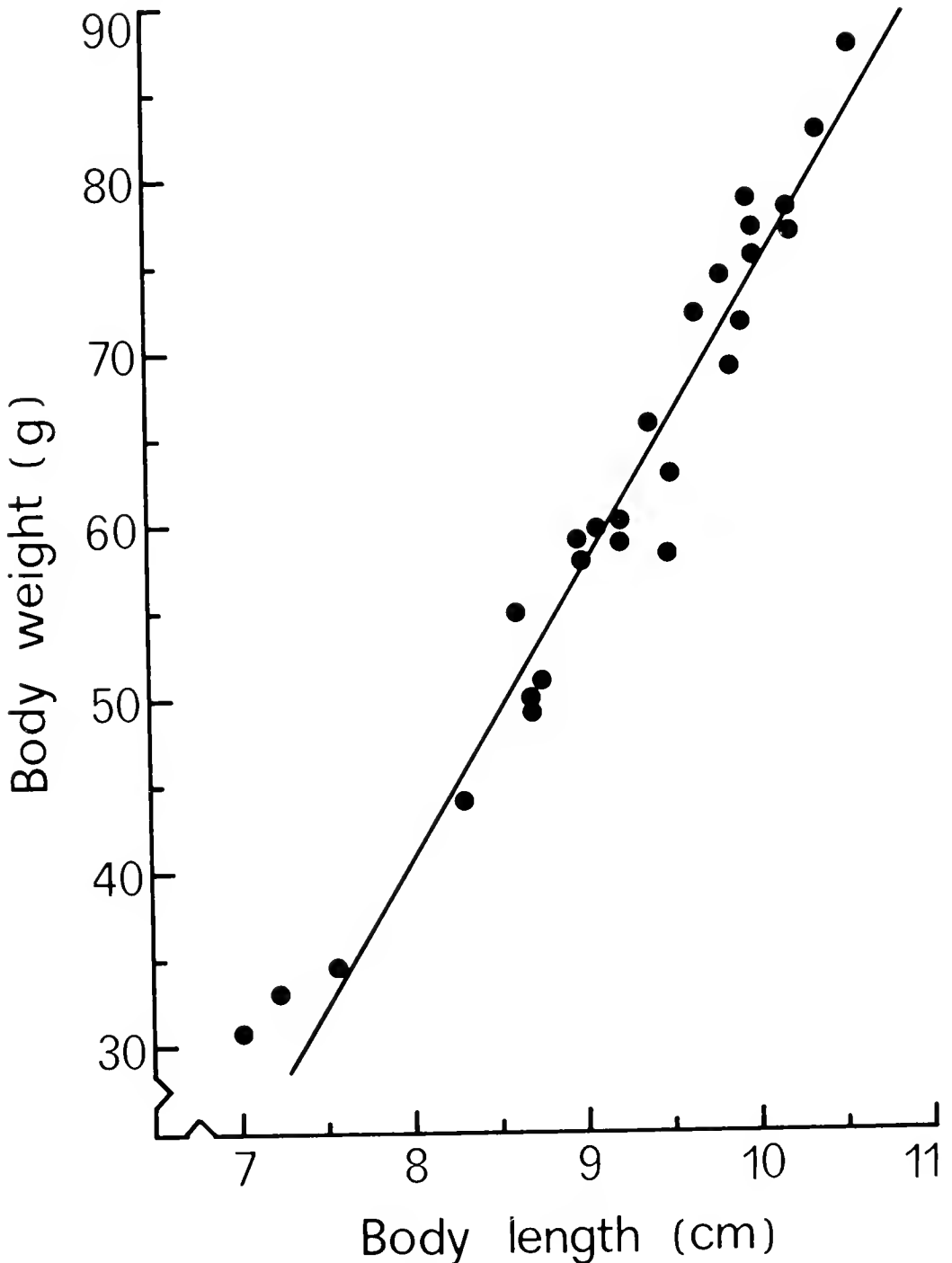


FIGURE 2. Relationship between body weight and body length in adult females. Body weight (y) is regressed upon body length (x). Coefficient of regression, $r = 0.949$.

cloaca seemed swollen, the vocal sacs were well developed, and calling began.

At this time, males established territories in the greenhouses and responded aggressively to nearby calling males by grappling with them. If females were available, whether gravid or not, they might clasped by males. Spawning could involve a pair that had been in amplexus for days, or pairs that had been in amplexus only since the previous evening. Early in the season, females not yet prepared to spawn offered only slight

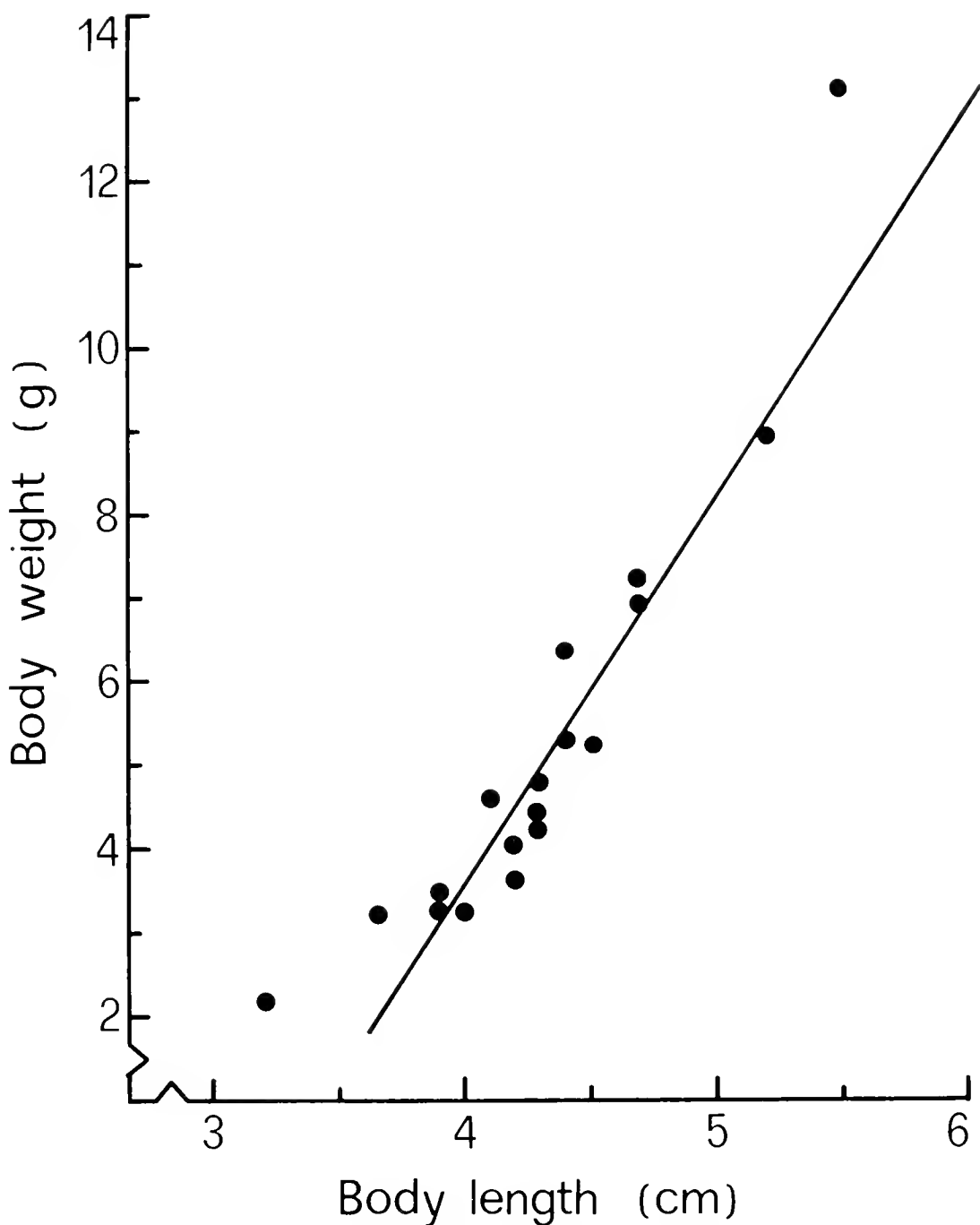


FIGURE 3. Relationship between body weight and body length in postmetamorphic males up to the attainment of sexual maturity. Coefficient of regression, $r = 0.959$.

objection to clasping males by issuing a short release call accompanied by vibrations of the body wall. However, this often was insufficient to cause the male to leave, and he might remain clasped to the female for many days.

From the summer of 1975 through 1984, we observed more than 500 spawnings. Records were kept on 331 of these, and we followed the complete sequence of events for many spawnings. Although complex, the entire breeding process was remarkably consistent among pairs. Except

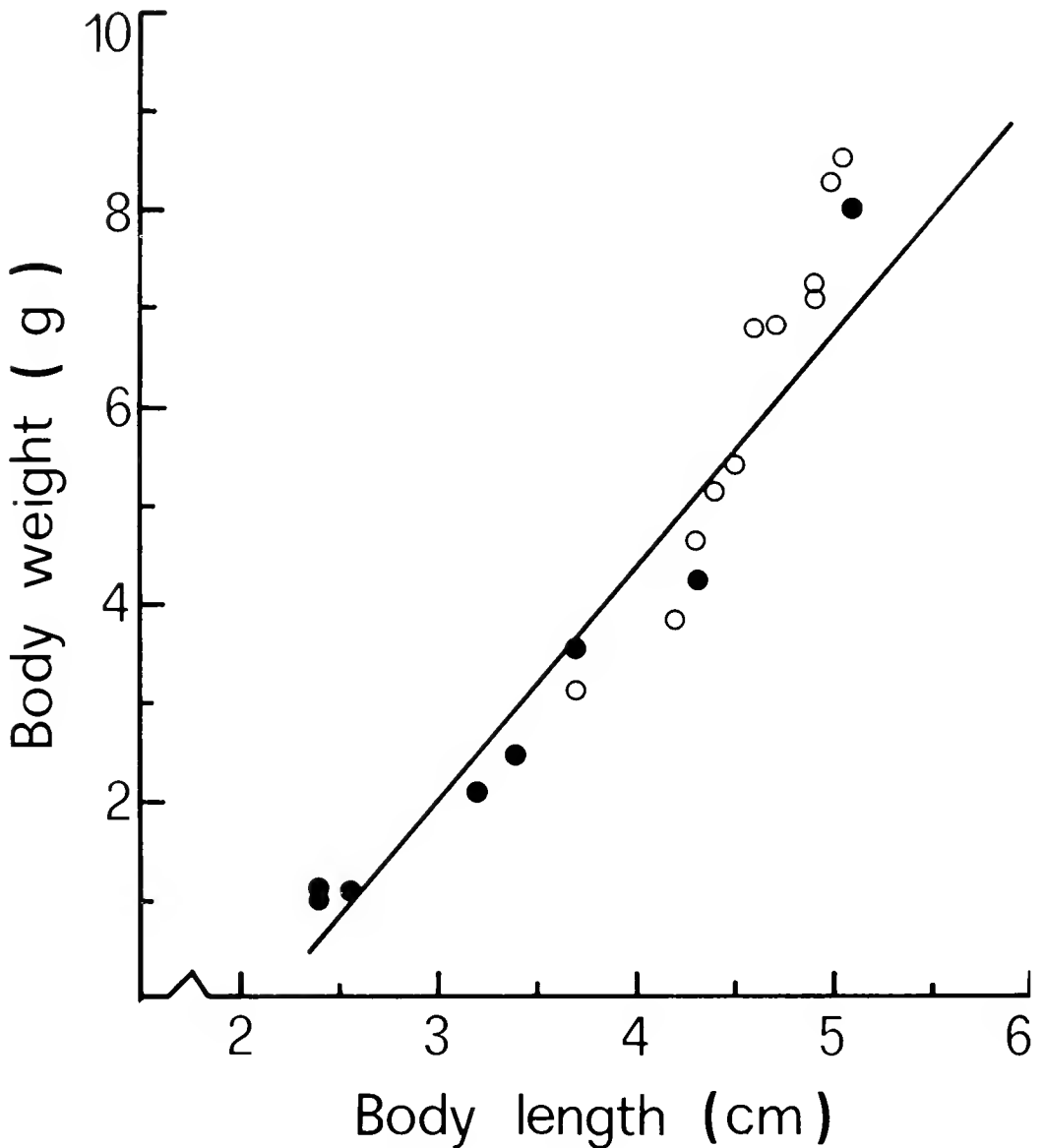


FIGURE 4. Relationship between body weight and body length in postmetamorphic, developing females. Black circles represent animals raised in captivity, whereas open circles represent those collected in the field. Coefficient of regression, $r = 0.958$.

for being more detailed, the following description resembled previous field observations by Pyburn (1970) and Wiewandt (1971).

In this nocturnal frog, active spawning began just past midnight, but more often occurred near dawn. In rare cases, it started as late as mid-morning. Depending on the final clutch size, the entire process of oviposition might last up to six hours. The onset of spawning was announced by a series of trills and squawks from the amplexant male, augmented by vocal contributions from other males that were either in amplexus with other females or seeking females. The female made no sound at this time, even when she and her mate were harrassed by other males who clambered over them and often impeded her from reaching the site she had selected for oviposition. As the pair proceeded to the site of



FIGURE 5. Water uptake by the female prior to oviposition.

egg deposition, her rotund form (Fig. 5) showed that she had taken up a considerable amount of water into her bladder (Pyburn, 1970). Every few minutes during this short journey the male issued a series of calls. Unattached males might return the call and approach the amplexant couple. The mounted male answered this challenge by issuing strong calls followed by a "vibratory purr" (Wiewandt, 1971). This vocal response became more insistent if an intruding male contacted the pair. We assumed that it was a release call because it sounded very much like that issued by unwilling females. The vocalizations of the amplexant male usually were sufficient to discourage the intruder; however, a persistent intruder might have attempted to clasp the female. The original male usually was so firmly mounted that the intruder was content with clasping either the male or the flank of the female. This rather unsatisfactory "ménage à trois" (Fig. 6) often did not persist for long, and the original pair finally proceeded with spawning. The female might arrive by a rather circuitous route to the breeding site. As she made her way through a tangle of branches she might pass through a space large enough for herself alone. The male might be badly scraped in the process (this accounted for the rather battered appearance of males at the end of the breeding season). In one case, a projecting branch snagged the male partner and he was pulled off as the female continued forward. Even though he was in the midst of spawning, he moved away from the female and began to call on an adjacent branch. The female returned to him and he remounted her. Unfortunately,

his position was somewhat askew and he again left the female and moved at least two feet away where he again began to call. The female returned to him and he remounted in a more customary position, and they were finally able to return to the site of egg deposition.

Spawning often occurred near the end of branches that were suspended either over the water or near the water's edge (Fig. 7). In the greenhouse, clutches often were deposited on the sides of containers used as planters (Fig. 8). The egg masses usually were about two feet above the surface, but in some cases their lower edge hung just a few centimeters from the water. Broad-leaved grasses at the water's edge often were chosen for egg deposition. When the female was ready, she assumed a secure position either by grasping the vegetation or the sides of the container, and was suspended in the form of an arc (Fig. 7). The lower end of the arc was marked by her vent. The male was suspended in even a more precarious manner such that his body at times hung horizontally. The male often called while the female was taking her position, but during oviposition he remained silent unless harassed by other males.

Because of the disparity in size (Figs. 5, 7), the cloacas of the pair were not in contact. Nevertheless, a fluid that we assumed was diluted urine containing spermatozoa "leaked" out on the female's back and reached the extruding eggs by flowing downward. Barely perceptible vibrations of the female's lower back signaled the quick extrusion of about 6 to 10 eggs; this might be accompanied by an ooze of urine from the male. After a brief pause of perhaps a minute another small group of eggs

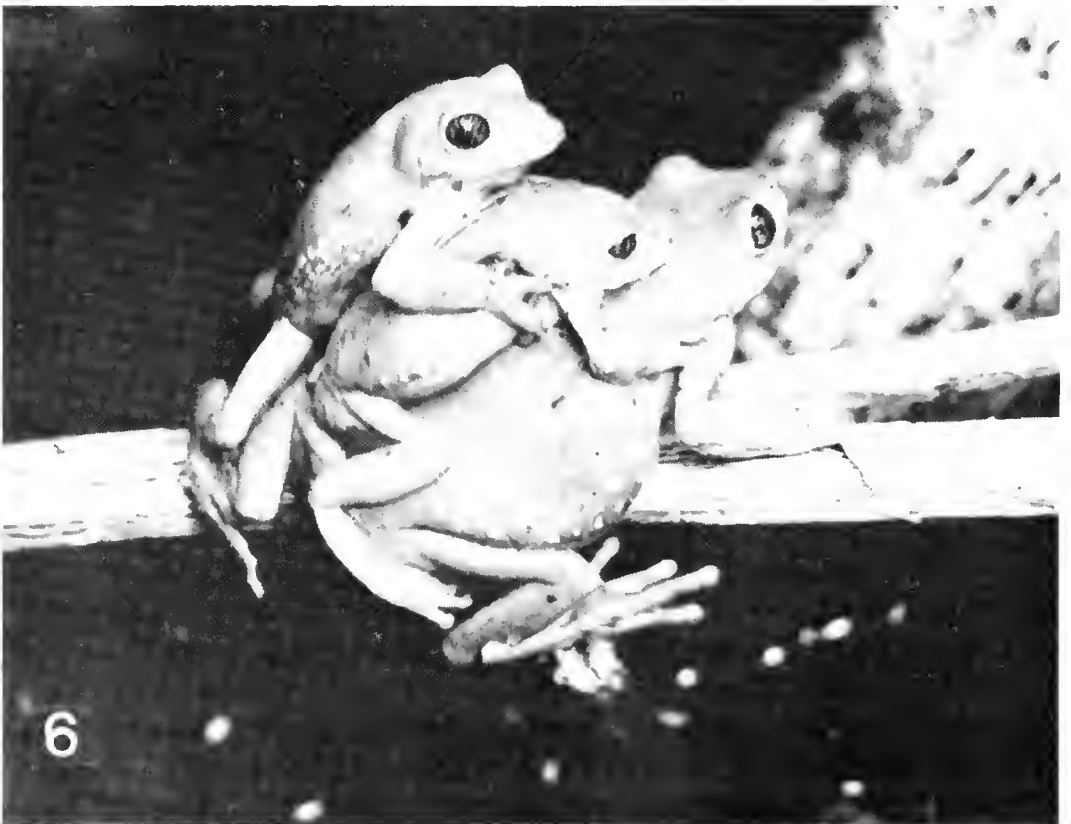


FIGURE 6. Attempt by intruding male to dislodge principal male.



FIGURE 7. Typical act of oviposition.

appeared. Females might move their hind legs during this process but there seemed to be no pattern to the movement, and there was no indication that they were trying to shape the egg mass. Similar leg movements of the male might take place in some cases, but they were usually slight and might have been designed for a better flow of urine. It is possible that they aided passively, in the extrusion of eggs from the female; however, females were fully capable of ovipositing in the absence of an amplexant male, as is described later. Often, males performed what seemed to be a wiping of the female; we interpreted this as a pushing of the spermatozoa-laden urine toward the eggs. As these small groups of eggs emerged from the cloaca, a small wet mass of eggs began to form. The jelly around the eggs was already somewhat swollen as it left the female and swelling continued by the addition of fluid that flowed out with each extrusion of eggs. Spawning continued until some 100 to 150 eggs were deposited over a period of about 15 minutes.

After the female withdrew from the eggs, she moved a short distance away and rested for several minutes. The male called briefly at this time, although he had remained silent throughout oviposition. The female, with the amplexant male still attached, then descended to a site for water uptake. We observed such a female striding down a log and walking over a male that had come to intercept her. When she had reached her watering place, she might flatten herself out on a wet spot in the soil for a period of several minutes or she might immediately immerse her posterior end into water (Fig. 5). At any rate, invariably after the deposition of the first group (or aliquot) of eggs, she chose standing or slow-flowing water for refilling her bladder. During this period, the couple remained very quiet for about 45 minutes. When the female was ready, she left the water to the accompaniment of calls from the amplexant male and retraced her route back to the original spawning site. The final approach to the earlier deposit of eggs was more difficult because the female almost always tried to establish herself immediately over the earlier deposit (Fig. 7). In this way an elongate egg mass was formed (Fig. 8), and the excess fluid that was released with the last aliquot flowed over the earlier deposits, giving them additional moisture. In locating the egg mass, the female somehow sensed its position despite a minimum of physical contact with it. Sometimes when it was impossible to continue the vertical build up of the egg mass in the same substrate, she might deposit an aliquot on nearby leaves. As the clutch of eggs built up it extended out far enough to contact the earlier deposit; as the jelly masses swelled they fused, thus continuing the formation of a vertical egg mass. If it was impossible to oviposit above the previous egg deposition, the female would deposit alongside it. After the second aliquot had been released, the pair usually returned to the watering place used before, and again some 45 minutes or more were spent during bladder refilling. The pair returned for a third egg deposition, and required about 15 minutes to lay about the same number of eggs. The process was repeated until the entire clutch was deposited. The average clutch size was 467 eggs (s.d. = 251) but clutches of over 1000 eggs were not uncommon.

Egg deposition required about five or six hours during which five or six aliquots were deposited. Events associated with the deposition of each aliquot were almost always the same with respect to both temporal factors and location. On a few occasions we observed a female change watering sites and quite frequently we observed that between ovipositions, the female might choose to spend most of the intervening period on wet soil rather than in water. For this and other reasons we suspected that the long interval between successive egg depositions might be necessitated by more than the need for water uptake alone. Possibly the time period was necessary for continued ovulation and passage of recently ovulated eggs down the oviducts. This suggestion was supported by the fact that two females sacrificed during oviposition contained eggs in their oviducts and body cavity, and unovulated eggs in their ovaries (Fig. 9).

Termination of spawning was an event that seems to involve some form of communication between male and female. When the female had



FIGURE 8. Vertical buildup of egg mass resulting from each egg aliquot having been deposited upon the previous one.



FIGURE 9. A female sacrificed during oviposition. Eggs were present in the oviduct, body cavity and in the ovary which was removed for fixation prior to photography.

finished depositing the last of her eggs, the male suddenly released the grasp that he had retained so firmly and patiently, walked over the female to take a position a foot or more above or in front of her, and often began to call. He would continue this calling until he clasped another female or retired for the day. Concurrently, the female remained with the eggs for an additional three or four minutes during which time she continued to release fluid on the most recent deposit. This spread over the entire mass giving the eggs additional moisture. Deposition of the last aliquot required about five minutes longer than the earlier ones; this might be the cue that leads the male to leave.

At the height of the breeding season, it was not uncommon for several pairs to spawn at the same time. For example, during July 1977, of 12 mature females in one greenhouse, ten spawned within two weeks. Five of these spawnings took place within three days, and three occurred simultaneously. Because conditions were presumed to be the same throughout this

period, it seemed possible that simultaneous spawning might be a product of mutual reinforcement. Because the greenhouse was small and spawning sites were restricted, it was not surprising to find two females depositing in close proximity. In one such situation wherein the two egg masses were being deposited about 10 cm apart on branches of a tomato plant, we observed confusion by one of the females. The laying cycle of the two females was such that while one female was depositing, the other was taking up water. Upon her return from water, one of the females returned to the wrong egg mass and, instead of depositing on the mass where she had left her last aliquot, she chose that of the other female. We were neither able to observe if the second female "returned the favor," nor if this mistake was repeated during the entire spawning. We observed this phenomenon only one time and do not know whether this occurs in nature.

When humidity remained high five or six days after deposition, hatching took place in a most spectacular manner. Apparently many hatchlings fell from the mass into the water or onto the adjacent bank, but more frequently they seemed to "explode" from their respective perivitelline spaces. As a result of vigorous prehatching movements, they burst the vitelline membranes and literally flew out into space. Apparently, they were launched by a flipping action that both Pyburn (1970) and Wiewandt (1971) hypothesized to be a mechanism for getting newly hatched larvae from the bank to the water. We suspected that this action might help insure that some larvae from egg masses that were laid over land or over the edge of a rain pool that had receded would make it to the water. This suggestion was supported by the fact that during embryonic development, each egg with its surrounding perivitelline space and membrane moved to the periphery of the jelly mass. Thus, when the vitelline membrane burst at hatching, the larvae were on the outside of the mass and were expelled freely, unimpeded by a viscous coat of jelly.

SEXUAL ACTIVITIES OF THE MALE

An early indication of sexual readiness of male frogs is the onset of calling. The call of *Pachymedusa dacnicolor* "can be described as a rubbery squawk" (Wiewandt, 1971) that is usually uttered singly but may be repeated a few times in succession. In the greenhouse, calling first occurred with the onset of hot weather in mid-May. The first few males to call often did so a few days in advance of the others; some were easily recognized because they called from the same location for several days and seemed to establish a territory. Their nuptial callosities were black and rough to the touch (Figs. 10, 11). Examination of other males, most of which did not seem to have started to call, revealed a progressive series in the development of callosities. In winter, the site of the base of the thumb were callosities would later form was smooth and white. The first indication of their development was an indistinct gray triangular patch. This patch became progressively darker and rougher due to the development of prominent papillae that were heavily keratinized, especially at

their distal ends which were capped in black (Fig. 11). About two weeks were required between the first suggestion of the presence of callosities and their final appearance as black rough patches. While no attempt was made to correlate the degree of callosity development with the onset or frequency of calling, the most aggressive males with respect to calling or territorial behavior were those with fully developed black callosities. Similarly, no attempt was made to evaluate the degree of forelimb hypertrophy in the male or to correlate this with the development of callosities. A final secondary sexual characteristic concerned changes in the cloacal area such that sexually active males possessed swollen cloacal lips. The pattern of swelling caused the cloaca to tilt downward and gave it an appearance reminiscent of a suction cup (Fig. 12). Sections of the integument in this area revealed the presence of numerous skin glands, both serous and mucous (Fig. 13); however, no studies were made on their distribution or seasonal variation.

Although the greenhouses and environmental chambers in which these frogs were maintained are relatively small, and despite a certain degree of crowding in comparison to their natural habitat, adult male leaf frogs exhibited strong territoriality. One manifestation of this was the persistence of calling by a given male from the same location night after night. Often the calling site was in an open place near the water. The calling male sometimes was challenged by another male that responded to the call by moving directly and quickly to him. The intruding male



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FIGURE 10. Thumb pad callosity of sexually active male.



FIGURE 11. Section through the maximally developed callosity of a sexually active male showing well developed skin glands.



FIGURE 12. Cloacal swelling of a sexually active male.

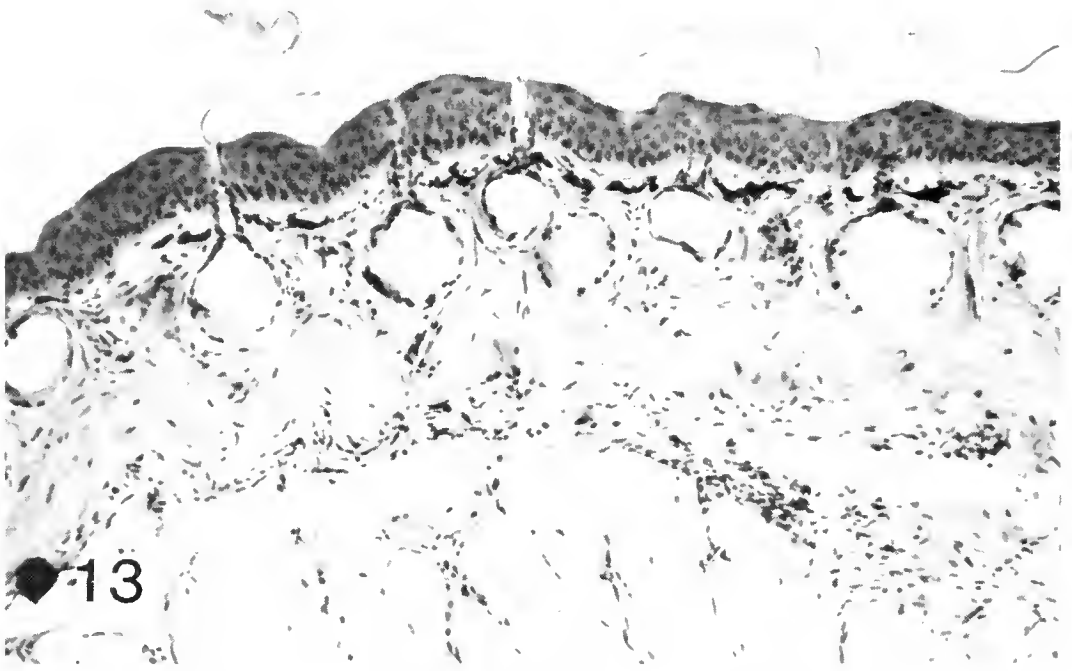


FIGURE 13. Section through the thickened cloacal region of a sexually active male showing well developed skin glands (X 200).

attempted to clasp the caller; the latter reacted with a rapid chatter not unlike the release call of the female. Most likely, the clasping that occurred at this time was not amplexus, but rather a tactic of aggression. The two males actually wrestled with one another (Fig. 14), and the stronger of the two flattened his antagonist to the ground pinned either on the back or ventrum. When the defeated male retreated, his opponent often gave chase. We had never seen more than two males engaged in such contests. Another form of aggression displayed by calling males involved the chasing of intruders that strayed too close. These observations of aggressive territoriality are only suggestive, for they were made in an artificial situation; however, they indicated that such behavior may be an important element in the reproductive biology of the leaf frog.

Although male leaf frogs became sexually ready long before females, calling males did not necessarily go into amplexus. We did not know whether large adult males could recognize the gravid state of females, but early in the season such males were seen in amplexus much less frequently than were smaller males. At this time, most females were not yet ready to breed; nevertheless, these smaller males would stay in amplexus for long periods, often a week or more. It should be pointed out that at this time of year, mid-May through June, the humidity, even in the greenhouse, was relatively low. This seemed to indicate that relative humidity might not be a factor in initiating breeding behavior in the male. Nevertheless, moisture might play a role in inducing calling in the male. During this dry period of the year, when water was sprayed into the greenhouse, even late in the morning when it was already hot and males had begun their daily period of inactivity, they responded by issuing a few calls.

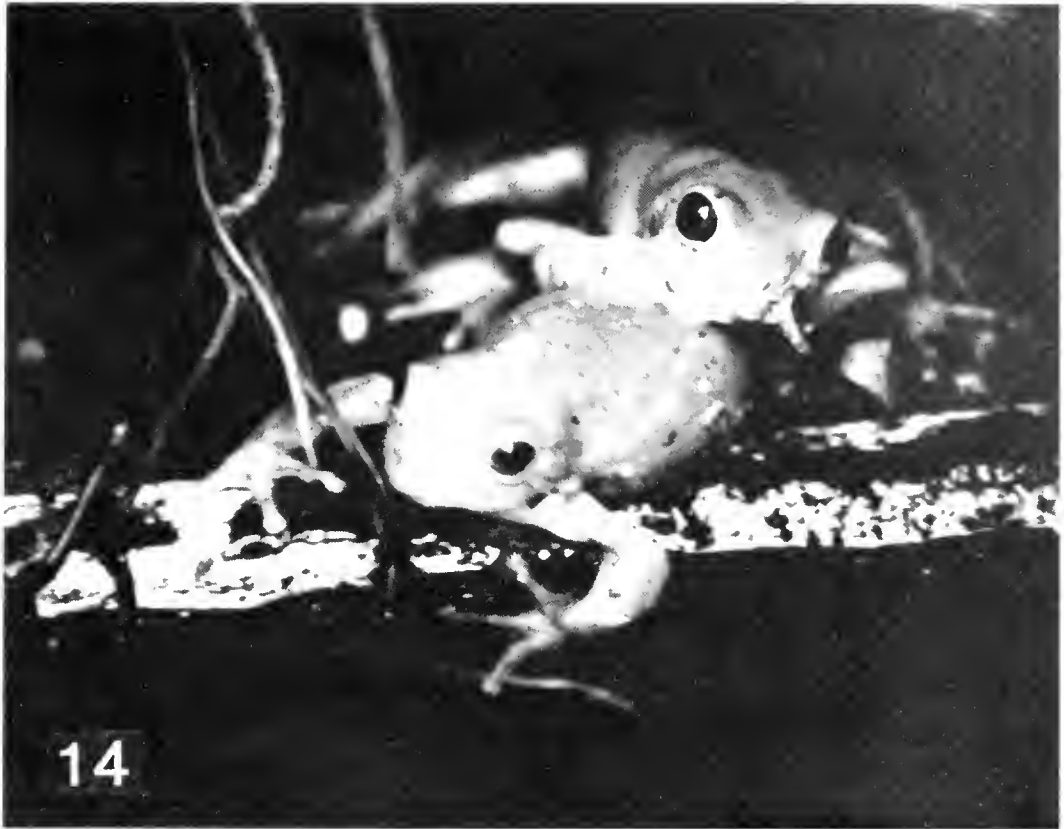


FIGURE 14. Male—male grappling in a territorial fight.

Amplexus in *P. dacnicolor* was like that of many other anurans. The large forelimbs of the smaller male allowed him to grasp firmly females larger than himself. The thumbs were hooked in deeply under the axillae and the remaining digits were folded over the top of the forelimb with the callosities fully pressed against the skin of the anterior portion of the trunk just behind the shoulder (Figs. 5, 6, 7). The strength of the forelimbs and the friction provided by the rough surface of the callosities made it difficult to dislodge a persistent male. The amplexant male was arched over the female with the flat pointed tip of his chin resting on her back just behind the head. The other end of the arc was formed by the flattening of the swollen cloacal lips against the female's back at some distance (depending upon the disparity in size), in front of her cloaca. We presumed that the wide area of contact made by the cloacal area of the male allowed for a broader spreading of spermatozoa released during oviposition. Spermatozoa were present in the urine of the male only during actual breeding, while that of males in amplexus with non-breeding females did not contain spermatozoa. Apparently the mobilization of stored spermatozoa occurred quickly following perception of the female's readiness to oviposit.

The perception that a female was ready to oviposit apparently was not restricted to the amplexant male. Unattached sexually ready males were more attentive to an amplexant pair about to oviposit than to non-breeding amplexants. After the period of water uptake, when the amplexant breeding pair moved toward the oviposition site, they were frequently

accosted by several other males. As the female moved through the vegetation these males attempt to clasp the amplexant couple in any way they could, but the strong female was undeterred and continued forward. By the time the oviposition site was reached, some of the extra males had been left behind and often there was only one extra male present. Despite the efforts of this male to dislodge the amplexant male he seldom was successful, because the original male was securely attached. The presence of competing males might serve as a serious impediment to the female who was trying to retain her balance as well as position herself precisely on rather fragile branches. In some cases, an extra male might successfully clasp the female alongside the original male, who had stopped issuing release calls (Fig. 15). Thus, typical of the male during actual oviposition, no calling was elicited. Sometimes, two extra males were present and contributed to the mating. These observations confirmed those of Pyburn (1970) and Wiewandt (1971), and supported the view that polyandry occurs in the reproduction of the Mexican leaf frog. The opportunistic males involved in such polyandry might contribute to more than just one aliquot of a clutch. When this occurred, these males did not accompany the female when she descended for water uptake. Only the original male retained his amplexant position during this period and the additional male or males remained near the site of oviposition until the original pair returned, at which time they remounted.

We observed variation in the expression of polyandry. In one case, an amplexant pair with a very small male was accosted by a large male that unsuccessfully attempted to dislodge the original male before the female was ready to oviposit. The large male, from his lateral position might have been a secondary contributor in the fertilization of this aliquot. However, by the time of the next aliquot, he had displaced as amplexant the small male, who then assumed the secondary role. The small male did not contribute to later aliquots of this clutch. Because the first aliquot was fathered largely by the small male, and because the whole clutch was fertile, it was clear that both males contributed to the gene pool of the embryos produced. Small male body size might not always be an impediment as was demonstrated in another example of polyandry, in which a very small male displaced a large male. The small male was not capable of pulling the large male off of the female, but he was small enough to squeeze under the larger male and thus inserted himself between the larger male and female. The larger male then released his grasp of the female and the small male replaced him as the primary male. On subsequent ovipositions the large male displaced the small male and regained his position as principal male. Again, the entire clutch was fertile. These examples of polyandry probably were due to the presence of an excess of males at the breeding site, together with the fact that males could breed many times during the season. While females also could breed several times during the season, a longer interim between successive breedings usually held. Thus, both in the field and in the greenhouses, when the sex-ratio of a population was about even or biased toward males,



FIGURE 15. An example of polyandry.

the chance of having more sexually ready males than female at the breeding site was high and polyandry was likely. This situation was not obvious to us in our early greenhouse observations when the population had an excess of females. For example, during 1976 fifty-three spawnings took place from 22 June to 12 September among the population of five males and no more than 20 females. No polyandry was observed. During this relatively short period, had all frogs been equally sexually active, each male would have bred on an average of more than ten times and each female between two and three times. This breeding activity was consistent with the fact that the testis of the male by the start of the breeding season had undergone a profound growth that was much greater than that known to characterize other anuran species (Rastogi, *et al.*, 1986a).

SEXUAL ACTIVITIES OF THE FEMALE

The onset of the breeding season for the female was not marked by any

overt changes in behavior or external form. However, internally a rapid progression of vitellogenesis occurred during late May and early June (Iela *et al.*, 1986), apparently cued by the persistence of higher ambient temperatures. When vitellogenesis had proceeded to a point where the ovary might contain as many as 2000 postvitellogenic follicles, the female had the rotund external form typical of the gravid female. Actual breeding began when the proper combination of temperature and humidity existed—daily minimal temperature consistently above 25°C and very high relative humidity. Apparently, the choices of when and where to breed were made by the female. Although we never observed a female respond positively to a calling male, except for the displaced male mentioned earlier, we assumed that when a female was ready, she either moved to such a male or indicated in some way other than vocalization that she was willing to breed. It was possible that the subsequent amplexus provided a cue for the onset of ovulation, the search for a site of oviposition, and the filling of the bladder prior to ovulation. Once the process began the female proceeded with oviposition of the entire clutch in the cyclical pattern described earlier. She may have even withstood considerable adversity until oviposition had been accomplished completely. One problem might have been an inadequate site for oviposition, necessitating the deposition of aliquots in separate places. Thus, on occasion what appear to be individual small clutches of eggs may in fact have been separately deposited aliquots of the same clutch. This was easily verified because each clutch of eggs had a distinctive green color, depending upon the relative content of green and yellow egg pigments (Marinetti and Bagnara, 1983). Color could thus serve as an identifying marker.

Once a female had completed oviposition she usually moved away from the egg mass and might rest for a few minutes before departing. Most often, she then proceeded to the watering site and appeared to take up water either from the moist ground or directly from the water. As the day heated up she disappeared into the vegetation to pass her inactive period in shade. The next few weeks were a period of vigorous feeding and vitellogenesis. During this time, females were much more vigorous in their rejection of the sexual advances of males. They might run from pursuing males and, if clasped, they might issue such a steady and intense stream of release chatter that even the most ardent males desisted. It was difficult to explain this change in behavior toward the male, but it may have been related to the need of the female to feed efficiently, unencumbered by an amplexant male during this period of rapid vitellogenesis.

One of the more striking aspects of the reproductive biology of the female was a remarkable capacity for vitellogenesis, allowing the production of many large eggs (usually slightly over 2.5 mm in diameter) in a very short period of time. Unlike many ranid frogs which undergo vitellogenesis in the late summer and fall, and store postvitellogenic eggs over the winter, the ovary of *P. dacnicolor* persisted in a previtellogenic state over the winter until late spring when vitellogenesis occurred rapidly (Iela, *et al.*, 1986). Records kept over ten years, 1975 to 1984, indicated that breeding can begin as early as May, but that the peak periods were in

July and August (Table 1). Part of the frequency of breeding during this time related to the fact that females were capable of breeding several times during the season. This conclusion was not derived from prolonged observations of individually marked females, because the procedure was utilized for only part of 1984. However, other means provided data that indicated that females not only bred several times during the season, but that they were capable of producing individual clutches containing more than 2000 eggs and successive clutches totalling even more. As pointed out earlier, during the 1976 season, 20 females produced 53 clutches, and if it was assumed that each bred, the average frequency of breeding per female was 2.6. Many of the clutches produced at this time were large; one had about 2000 eggs, and three others each contained 1000. These values were consistent with those derived from determinations of "potential clutch size." Potential clutch size equaled the sum of the number of ovarian postvitellogenic follicles and the number of eggs in the body cavity, oviducts, and the aliquots. In an investigation of the relationship of body weight to breeding capacity of *P. dacnicolor* females, body weight was plotted against potential clutch size (Fig. 16) for 15 sexually ready females which were yet to breed, were breeding, or had just bred. A strong correlation existed between female size and the number of eggs available for a clutch. This correlation was maintained even to the extent of compensatory hypertrophy. As is indicated by the arrows in Figure 16, females hemiovariectomized on May 31–June 1 (mainly at the onset of vitellogenesis) achieved in the remaining ovary a potential clutch size equal to that of intact females of comparable weight. The potential of large females to produce over 2000 eggs might have explained the large clutches obtained in 1976. Examining the ten year record (Table 1), it was striking that large clusters of more than 1500 eggs occurred only in 1976 and 1977, while the ten year average was near 467 eggs per cluster. Yet large breeding females were present every year. A possible explanation for this disparity was derived from an analysis of the 1976 spawning which took place in two different greenhouses. The smaller of the two (foothills) was the site of the large egg clutches and the average number of eggs in the 29 clutches laid there was 1067 (S.d. = 452). In the larger greenhouse (farm); the average clutch size for 24 spawnings was 644 (S.d. = 130) eggs and the largest clutch contained 900 eggs. It is tempting to hypothesize that this highly significant difference in egg number was related to nutritional differences; however, this does not seem likely because there were always moths flying and the frogs could feed *ad libitum*. On the basis of its construction and location, it was likely that the proper conditions of high humidity were present less frequently in the foothills greenhouse. Thus, females in the foothills greenhouse might have retained postvitellogenic oocytes for a longer period before they were able to oviposit. In this way, more than the usual number of ovulatable eggs were available when the female oviposited and the actual "potential clutch size" was realized. If this reasoning was correct, the potential clutch present in the ovary could be released under proper conditions of humidity as two or more separate clutches. This interpretation was supported from breeding records of

specific females. For example, on 1 Aug. 1984, a female weighing 66 g was sacrificed immediately after ovipositing a clutch of about 500 eggs. She still had some 50 eggs in her oviducts and about 50 to 60 large follicles. She had previously bred on 14 July and deposited 600 to 700 eggs. The total of these eggs or oocytes approximated 1200, the potential clutch size estimated for a female of her weight (Fig. 16).

The potential clutch size probably did not represent the total number of eggs to be laid by a female in one season. When potential clutch size was

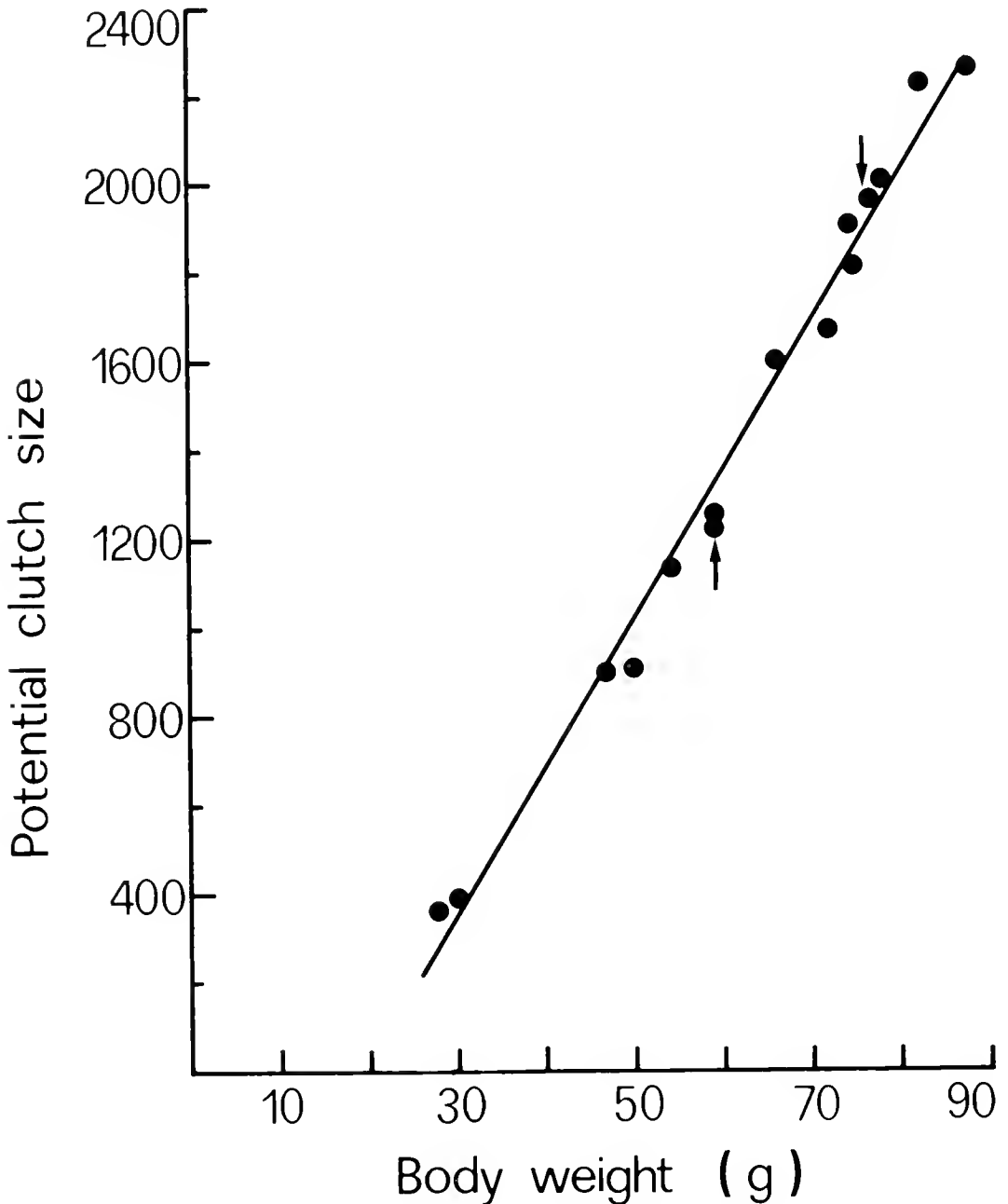


FIGURE 16. Relationship between potential clutch size and body weight. Clutch size (y) is regressed on body weight (x). Coefficient of regression, $r = 0.994$. Potential clutch size was determined by counting ovarian postvitellogenic follicles (where available), oviductal eggs, and the number of eggs in the just laid clutch. Arrows indicate hemiovariectomized females.

determined for the 15 females indicated in Figure 16, the large number of ovarian follicles in the early and mid stages of vitellogenesis were not counted. Because ovaries in the winter contained neither vitellogenic follicles nor signs of vast atresia, it was concluded that these follicles subsequently completed vitellogenesis and were ovulated before the end of the season. Through the good fortune of the emergence in our colony of a mutation that led to the production of yellow eggs (Bagnara, unpublished), we followed the breeding of a single female carrying the mutation through the 1982 season. From July 7 to September 23 she laid 7 times and produced about 2,250 eggs. It seemed reasonable to conclude that an average-sized female would breed several times during the season and would produce a clutch size of about 450 eggs. Females sacrificed during breeding had eggs in the oviduct and body cavity, and their ovaries contained oocytes not yet ovulated (Fig. 9). In ranid frogs all mature eggs are ovulated and passed to the uterus before mating begins. By contrast, in the leaf frog ovulation and egg passage occurred during mating. Such a strategy had obvious adaptive benefits, for ovulation could thereby be better coordinated with the perception of high humidity. In addition, the progression of ovulation coincided with the oviposition of smaller aliquots, each needing to be provided with sufficient water. An interesting question that remained to be answered concerned the proximal cue for ovulation. Did the female's perception of the proper humidity lead her to ovulate, or did it render her capable of ovulating in response to the previously unregarded advances of males? In any case, once the female had started to ovulate, it seemed that she continued until oviposition was completed. An excellent example of such persistence was provided by one female travelling to deposit an aliquot, probably the third in a series. She and her partner were so harrassed by other males as they approached the oviposition site that the male released the female and moved off into the vegetation and was lost from our sight. Even in the absence of an amplexant male, this female oviposited an aliquot in the usual way and all of the eggs of this aliquot were fertile. We presumed that fertilization was due to the presence of sperm that had remained on her back following oviposition of the earlier aliquots, and that these sperm spread downward to fertilize the eggs of this last aliquot.

EXPERIMENTS

The descriptive aspects of this study have emphasized that two specific environmental cues seem to provide the primary basis for seasonal breeding in the leaf frog: high temperature is responsible for inducing breeding readiness, and high humidity induces oviposition. Thus, sexual activities commence in the early summer and close in late September when the relative humidity begins to decrease and the daily minimum temperature drops consistently to below 15°C (Table 1). Accordingly, it should be possible to induce breeding in the winter through the appropriate manipulation of temperature and humidity. Such an experiment was first

performed in an environmental chamber (approximately 2.5m × 1m × 1.5m), starting in October 1976 and continuing to June 1977. Into the chamber were placed 10 to 12 mature females, three mature males, and six young frogs that had metamorphosed late in the summer. The frogs flourished at the 27°C temperature on an ample diet of crickets. They also were provided with hiding places, plants and a water source very much as in the greenhouse habitat. Although relative humidity could not be controlled with precision, a fairly high humidity was maintained through the use of many open pans of water and flats of soil moistened daily. At the outset, a light cycle of 12L:12D was maintained. In late January the light cycle was changed to 14L:10D and the maximum daily temperature was elevated to 37°C; when the lights were off at night, the temperature dropped to 21°C. In a few weeks, it was noticed that males had begun to develop their typical sexual callosities. At this time, an ordinary bedroom humidifier was added to the chamber, which seemed to be drying too rapidly through the system of ventilation. In a few days, several pairs were seen in amplexus and males had begun to call. Unfortunately, the pairs separated in a few days, possibly because the night time temperature of 21°C was too low. After appropriate manipulation, a program was adapted such that when the lights were on, the temperature rose to about 37°C whereas in the dark it dropped to near 28°C. This temperature and humidity regime led to the resumption of sexual activity, and seven spawnings took place from March 20 to May 17. These were much like those described above. The mean clutch size was 800 eggs. An interesting feature about clutches found in this experiment is that they were often deposited on flat vertical surfaces, even when large amounts of vegetation were provided in places overhanging the water. The chamber walls were frequently a site of oviposition even if the pair had difficulty adhering to the surface. In Figure 17 such a pair are breeding on a varnished wall. Because of the difficulty the female had in maintaining her grip, she had to move somewhat above the previous aliquot to oviposit. The problem was even extended to the male who had to grasp the female farther back than usual. A probable explanation for the use of such surfaces relates to the fact that they are not absorbant. Among all the clutches we have obtained, we have never seen an egg mass deposited on a surface that might possibly absorb water from the mass. For instance, dirt, uncoated wood, or bark are never sites of oviposition.

In the experiment in the spring of 1977, not all of the mature females bred during the almost two month period studied. This emphasizes that manipulating temperature and humidity alone cannot duplicate the conditions that normally induce breeding in nature. This is not only indicated by the failure of some gravid leaf frogs to breed in the environmental chamber, but by the fact that after transferral to one of the greenhouses, breeding often takes place after a few days. For example, in spring 1984, a group of young frogs derived from summer 1983 breeding, were induced to become sexually ready in an environmental chamber. These young males began to call and show callosities in late May, and amplexus first began in early June. Pairs were in and out of amplexus, often for bouts as

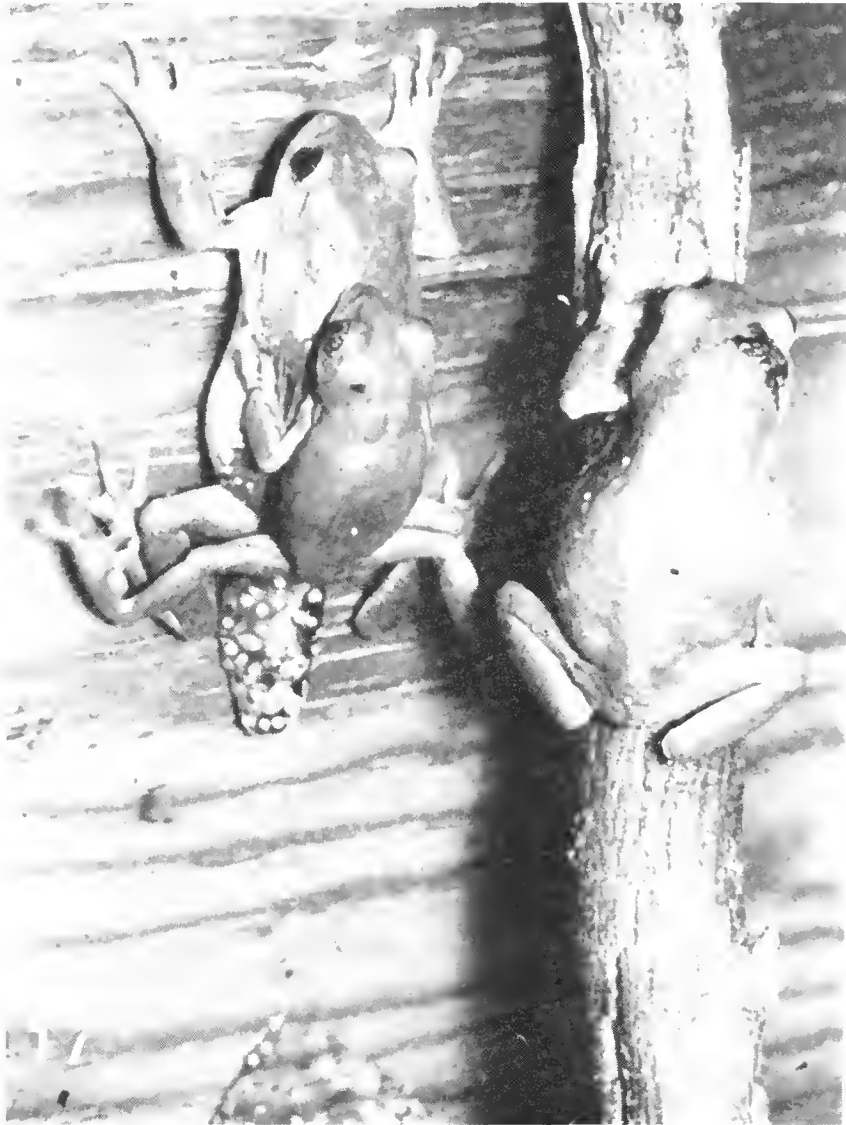


FIGURE 17. Induction of breeding in an environmental chamber in the winter by means of simulating summer conditions of native habitat.

long as six days, but no egg laying took place. In mid-July, eight of the females were moved to a greenhouse, and the next day one bred. Three others bred during the next few weeks. We were curious about the sexual state of the remaining 17 young animals which failed to breed in the environmental chamber. Accordingly, three rather robust females were sacrificed. An examination of their ovaries (Iela, *et al.*, 1986) revealed that more than 55% of postvitellogenic follicles were atretic and that ovarian weight was reduced. Similarly, it was noted that some of the males had stopped calling and had lost their callosities. Four such males were sacrificed: sections of their testes revealed a lack of spermatogenesis, empty tubules, and a histological picture like that of males after the summer breeding season (Rastogi, *et al.*, 1986a). Whether these males had shed their sperm during an abortive earlier amplexus or whether sperm had instead been resorbed is not known. These observations suggest that without a completely favorable environment for breeding, both males and

females will cease sexual activities and retreat toward the winter non-breeding state. There is, as yet, no knowledge of what factors from the environment may be missing in the chamber experiments, but their effect may not be mediated by the usual hormones. The administration of CG or LHRH (Calbiochem) to gravid females failed to induce ovulation. The same was true for the implantation of *R. pipiens* pars distalis. The administration of LHRH to males induced spermiation and a brief period of calling, but no amplexus with successful breeding followed. The failure of males to continue to be sexually active for long periods probably is a function of their being seasonal breeders. We have observed that in mid-winter, males can be stimulated to sexual activity within a few weeks after being placed in an environmental chamber under appropriate temperature conditions, but in the early fall males will not respond. This observation is consistent with the histological picture of the testis which reveals the presence of empty seminiferous tubules in the fall. Whether a similar refractory period holds for the female is not known.

DISCUSSION

The availability of the colony of captive *P. dacnicolor* used in this study provided a unique opportunity to study in detail the spectrum of reproductive biology of the Mexican leaf frog, including the endocrinological information provided by other papers in this series (Rastogi, *et al.*, 1986a; Iela, *et al.*, 1986) and the behavioral and morphological observations presented here. It was possible to obtain a diversity of data about specific behavioral events. Behavioral observations compiled over a ten year period with some 331 spawnings permitted the development of a consistent picture of the annual cycle as well as of specific events in reproduction. The fact that this species is subtropical provides an added value to these observations, for our understanding of amphibian reproductive biology has been previously derived largely from temperate species. We found that the reproduction of the Mexican leaf frog depends on two environmental cues—high temperature and high humidity; both are needed for spawning to occur. Alterations in temperature are a fundamental environmental cue in inducing sexual activity in amphibians (Rastogi, 1976; Rastogi, *et al.*, 1978a; Rastogi, 1980). However, that increasing humidity is a specific cue for oviposition in *P. dacnicolor* is a new observation that demands further study not only for this species, but for many others that oviposit out of water. Perception of proper humidity for breeding seems to be a function of the female. Our observations indicate that she chooses not only when to breed, but also where to breed. The chosen site must mechanically support her and her mate during oviposition, should allow a vertical buildup of an egg mass with the deposition of successive aliquots, and should allow hatching larvae quick access to water.

The induction of reproductive activity following the perception of ambient climatic changes indicates that *P. dacnicolor* is a seasonal breeder, in keeping with its highly seasonal natural environment. Never-

theless, its seasonal reproduction may seem a little surprising. Being a neotropical species, one might expect it to be a continuous breeder, as is often the case for other tropical species (Rastogi, 1976; Saidapur, 1983). Because breeding success depends largely on the existence of a summer rainy season, it is likely that humidity played a major role in the evolution of a seasonal breeding cycle in *P. dacnicolor*. Unlike other seasonal breeders such as various temperate frogs that "anticipate" the breeding season far in advance, this frog does not initiate development of morphological changes attendant to reproduction until just before the season begins. Thus, in response to the rapid onset of high temperature in the spring, male leaf frogs quickly begin spermatogenesis and the manifestation of secondary sexual characters (Rastogi, *et al.*, 1986a). This means that the testis is in a quiescent state for a long time (from about October to April). The same seems to be true of the female, which undergoes rapid vitellogenesis in response to the high temperature of spring, after a long period of winter quiescence with minimal feeding. In concordance with increasing levels of estradiol and testosterone in the spring, females can undergo complete vitellogenesis in about a month (Iela, *et al.*, 1986). This rapid and substantial vitellogenesis seems explicable on the basis of the substantial fat bodies present in this species. Preliminary data on changes in the fat bodies of *P. dacnicolor* during breeding, indicate that fat body weights of the female are very high at the outset of oogenesis, but are quite low at the end of the summer breeding season (Iela, *et al.*, 1986). It is likely that rapid vitellogenesis is accomplished by the mobilization of fat body reserves together with nutrition obtained from prodigious summer feeding. Apparently a rapid recrudescence of fat bodies takes place in the early fall when abundant food is available. A detailed study of cyclic changes in fat body weight and composition would add much to our understanding of the reproductive biology of the female in this species.

The capacity of female leaf frogs to undergo rapid vitellogenesis at the start of the breeding season apparently continues well into the summer, thus a female may breed several times during the season and may produce more than 2000 large eggs by the end of the summer. The number of eggs per clutch and the number of spawnings per female seem to be related to the size of the female. The availability of appropriate ambient conditions also seems to influence clutch size. Apparently this is based upon the perception of proper breeding conditions by the female. She does not ovulate until conditions are right, and thus ovulation and egg transport commence abruptly and continue during the oviposition of early aliquots of a clutch. The relationship of ovulation and oviposition to the supply of moisture to individual aliquots needs study.

The morphological and behavioral manifestations of sexual readiness on the part of the male leaf frog also offer challenges for continuing investigation. The seasonality of reproductive activity of the male is important from several standpoints. One of these is that such activity, from spermatogenesis to the manifestation of secondary sexual characteristics, is expressed rapidly following exposure to the cue of high temperature. An examination of pituitary gonadotropic activity would be enlightening. The

apparent refractory period that follows the termination of breeding in early fall also deserves further study. The length of this refractory period is not known. It is not known if it pertains to both spermatogenesis and the expression of secondary sexual characteristics. The male secondary sexual characteristics are prominent and also in need of study. The range of vocalizations from calling to the various kinds of chatter that are emitted in male-male interaction need to be analyzed with respect to both quality and function. Cloacal swelling in the male *P. dacnicolor* seems to be a unique characteristic that needs investigation. The degree of swelling has not been accurately ascertained nor has there been an analysis of the integumental changes that occur in that area. Of considerable interest is the obvious territoriality displayed by the male. This behavior needs to be considered in a more natural setting than the greenhouses used in the present study.

Just as with the female, the ability of the male to breed many times during the approximately three month breeding season is of considerable interest. The male becomes sexually ready before the female and at the onset of readiness, spermatogenesis for the season has been completed and the seminiferous tubules are full of spermatozoa (Rastogi, *et al.*, 1986a). The urine of early males in amplexus with non-breeding females does not contain spermatozoa, but that of males in amplexus with ovipositing females contains spermatozoa that must be released during the greater part of a long session of oviposition by the female. It would seem likely that if a relatively efficient means for sperm storage and release were not in operation, excessive number of sperm might be released and wasted, rendering the male incapable of fertilizing clutches later in the season. Apparently this happens from time to time, for we have observed pairs that have produced clutches containing only a few cleaved eggs. A possible means by which males can control sperm release is a sperm reservoir in the bladder. Whether sperm-containing pockets in the bladders of the few males we examined is significant in this regard is an interesting possibility that warrants investigation.

The events of reproduction in the Mexican leaf frog are interesting and important not only in their own right, but because they offer subjects for investigations that have broad implications in several areas of biology ranging from behavior to cell biology. The ready availability of all stages in the development of this frog, together with its ease of maintenance, makes it an outstanding candidate for future study.

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