

EVOLUTIONARY RELATIONSHIPS OF THE
TOADS OF THE BUFO PUNCTATUS GROUP

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I hereby recommend that this dissertation prepared under my
direction by J. Homer Ferguson
entitled Evolutionary Relationships of the Toads of
the Bufo punctatus Group
be accepted as fulfilling the dissertation requirement of the
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ABSTRACT

The evolutionary relationships of the Bufo punctatus group of toads were investigated by hybridization experiments, and the analysis of their mating calls, morphology and ecology.

The results of reciprocal hybridization experiments agreed favorably with the data derived from the other methods of analysis. It was demonstrated that the species group clearly consists of the following taxa: Bufo punctatus, B. kelloggi, B. debilis and B. retiformis. The latter three represent a distinct subgroup within the species group.

The entire group occurs today in modern derivative communities of the Madro-Tertiary Geoflora and each species apparently represents a genetic unit which differentiated from an ancestral species dating at least to Miocene time.

As determined primarily from the hybridization experiments, B. punctatus is the most generalized member of the group, and is probably a modern vertical derivation of the Miocene progenitor which may well be represented by the fossil species Bufo suspectus.

Of the three species in the B. debilis subgroup, B. kelloggi is the most similar to B. punctatus. There is no evidence that any of the taxa have differentiated as species during or since Pleistocene events. In fact, B. debilis and B. retiformis probably evolved from the B. kelloggi line during the extensive development of semi-arid grasslands and more arid environments at middle latitudes in western North America during Mio-Pliocene time.

INTRODUCTION

The Bufo punctatus group is comprised of four closely related species, B. debilis, B. kelloggi, B. retiformis and B. punctatus. These toads are found primarily in the southwestern United States and northern Mexico. For more than a century their evolutionary relationships and taxonomic status have been variously interpreted.

Bufo punctatus, sometimes referred to as the red-spotted toad or the desert toad (Stebbins, 1954), was described by Baird and Girard in 1852 (Girard, 1854). This species is widely distributed and has been well known since the time of its description.

B. debilis and B. insidiosus were described by Girard (1854). Many authors subsequent to Girard regarded insidiosus as a synonym of debilis (see Cope, 1886). Taylor (1938) maintained B. debilis and B. insidiosus as distinct species and described B. kelloggi as another species of this group.

Sanders and Smith (1950) reestablished B. insidiosus and B. kelloggi as subspecies of B. debilis and described a fourth member of the group as B. d. retiformis. Savage (1954) elevated B. d. kelloggi and B. d. retiformis to species rank and retained B. debilis debilis and B. debilis insidiosus as subspecies. Bogert (1962) followed Savage's (1954) arrangement.

Investigations related to the phylogeny of North American toads have been primarily carried out in two different ways. One, depending on morphology and particularly osteological morphology, has resulted in

a more logical grouping of species but has not clearly revealed the relationships within a species group. The second and more recent method has involved hybridization between species and subspecies and was first employed for the genus Bufo in the United States by A. P. Blair (1941) who attempted to interpret the interspecific relationships of the Bufo americanus group. Since that time the method has been extensively used in investigations related to various aspects of anuran evolution.

Possible evolutionary relationships between the four species (B. punctatus, B. debilis, B. retiformis and B. kelloggi) were analyzed by hybridization experiments and studies of their morphology, ecology and behavior with special reference to the mating calls.

Toads are nocturnal animals which have well developed calls which function as isolating mechanisms. Their calls have been used by many authors for indication of similarities and differences within species groups. Porter (1964) states that because of its uniformity within a species, the mating call provides a potentially useful but relatively untested tool for the analysis of anuran phylogenies. It is apparent that similar species do have similar calls but because of their utility to the species as an isolating mechanism the results of call analysis must be viewed with caution. In other words, species-specific calls do reflect phylogeny, but can only reflect inter-group evolution when appropriate aspects of the morphologies and ecologies of the species involved have been investigated.

METHODS AND MATERIALS

Hybridization.--Specimens for hybridization were collected from the following localities:

B. debilis.--1.0 miles south of Cochise Junction, Highway 666, Cochise County, Arizona, and Elfrida, Cochise County, Arizona.

B. retiformis.--2.2 miles east of Sells, Pima County, Arizona, and 35 miles east of Ajo, Pima County, Arizona (Tracy's Trading Post).

B. kelloggi.--22.0 miles west, 14.0 miles west and 20.0 miles south of Hermosillo, Sonora, Mexico.

B. punctatus.--Lower Sabino Canyon, Santa Catalina Mountains, Pima County, Arizona, and 1.1 miles west of Dragoon, Dragoon Mountains, Cochise County, Arizona.

All crosses were made during the summer of 1961, 1962 and 1963. The animals were brought into the laboratory and injected with 0.5 cc. Antultrin S manufactured by Parke-Davis and Company. Eggs were stripped from the females approximately six hours after injection.

Testes extracted from the males were macerated with teasing needles in approximately 10 cc. of pond water. Fifty to sixty eggs were then stripped into the sperm suspension and the dish was gently rotated to ensure adequate exposure of eggs to sperm. The eggs were allowed to remain in the sperm suspension for about five minutes before the petri dish was filled with water. After one hour the fertilized eggs were transferred to 18" x 21" white enamel pans, where the embryos were

allowed to progress until hatching. They were then transferred by a large syringe to other enamel pans in lots of approximately forty or eighty individuals.

Laboratory Maintenance of Larvae.--The tadpoles were fed lettuce which had been boiled for approximately thirty minutes to allow it to become soft. Each pan was cleaned daily with a large syringe and fresh water was added when needed.

Water used in raising the tadpoles in 1962 and 1963 was taken from a pond on the University of Arizona campus. In 1961, the animals were raised in "Rainbow Water", a mineral water which was purchased in Tucson.

During the summer of 1962 the tadpoles were maintained in the laboratory at a water temperature which ranged from 24°C to 28°C. Temperature control was more difficult during the summers of 1961 and 1963, and water temperatures varied more widely (ca. 22-30°C).

The embryos were staged daily according to the method of Pollister and Moore (1937). The percentages of fertilization, hatch, and metamorphosis were recorded.

After metamorphosis, the animals were transferred to aquaria which contained about 300 cc. of water; the aquaria were sloped at an angle to allow the recently metamorphosed larvae to move freely to and from the water. After tail absorption, the animals were taken out of aquaria and placed in terraria. The dirt filled bottom of each terrarium was kept moist and a small amount of water was maintained in a petri dish on the floor. Juveniles at this time were fed Drosophila.

Analysis of Mating Calls.--Tape recordings of male calls were made during the summers of 1962 and 1963. All calls of B. kelloggi were recorded on a Nagra III portable tape recorder at a tape speed of 15"/sec. B. debilis calls were recorded on a Midgetape 500 at a tape speed of 7.5"/sec. The calls of B. retiformis and B. punctatus were recorded on both recorders.

Sound spectrograph reproductions were made on a Sonagraph (Kay Electric Co.) which has a scanning width of four inches and two frequency bands (0-6 KC and 6-12 KC). The sound spectrograph was calibrated for frequency with an audio frequency generator, and for time by measuring with a stop watch the time in seconds required for 25 revolutions of the recording drum.

The calls were analyzed for frequency, pulse rate, duration, and structure. All frequencies were determined from sections. Pulse rates were counted from sonograms produced by playing the calls at one fourth or one half normal recorded speed. Durations were determined with a stop watch. Water and air temperatures were taken with each recording (Schultheis thermometer). When possible, the relative humidity was recorded (Bendix psychrometer).

Analysis of Structure.--The following morphological measurements were taken by dial calipers from males of each species:

1. Snout-vent length
2. Length of tibia
3. Length of humerus
4. Length of femur

5. Length of radius
6. Distance from angle of jaw to tip of snout
7. Inter-orbital distance
8. Length of eyelid
9. Length of hind foot
10. Nasal width (distance between external protuberances of nasal bones)
11. Width between outer edges of post-orbital crest
12. Width of parotoid
13. Length of parotoid

Females of each species were measured in the same manner as the males for the first eleven variables mentioned above.

Mean, standard, error, and range were determined for the measurements of each species for both males and females.

Skulls were prepared by the method of Sanders (1953) which involves decapitating the preserved specimens and periodically immersing the head in approximately 5 cc. of Clorox to loosen adhering tissue. Skin, muscle, and connective tissue were then pulled away from the skull with forceps.

A minimum of eight skulls of each species was prepared and the following measurements were made with a stage micrometer fitted to a binocular scope:

1. Width of nasals--greatest distance between lateral edges.
2. Width of frontoparietals--greatest distance between lateral edges of frontoparietals at the posterior

margin of the orbits.

3. Distance between jaw angles--measured from a dorsal position, and consisting of the greater distance between the posterior tips of the quadratojugals.
4. Length of frontoparietals--greatest length of inner margin; anterior limit was taken as angle of greatest curvature of inner margin.
5. Length of nasals--anterior tip of nasals to angle of greatest curvature on the inner margin.
6. Length of parasphenoid.
7. Width of parasphenoid.
8. Total skull length.
9. Interorbital distance--distance between proximal margins of orbits at point of suture between nasals and frontoparietal.

The skulls were also described as to qualitative anatomical characteristics.

The data derived from measurements of females, males, and skulls of males, were processed by digital computer which provided the following statistics:

1. Mean
2. Standard error
3. Student's t
4. Ratios of all combinations of variables per individual

5. Mean ratio
6. Standard error of ratios
7. Student's t for the ratios

Quantitative Relationships.--The positions of B. debilis and B. retiformis for each variable were determined by the use of the following formula from Hubbs et al. (1943):

$$\frac{M_x - M_2}{M_1 - M_2} = \text{position (P)}$$

where M_1 = B. punctatus, M_2 = B. kelloggi and M_x = B. debilis or B. retiformis. The index (I) for B. debilis or B. retiformis was then expressed by 100 (P). The ratios and variables were also analyzed by computing regression lines by the method of least squares (Simpson, Roe and Lewontin, 1960).

RESULTS AND DISCUSSIONS

General Morphology

The toads of the Bufo punctatus group (Figs. 1 and 2) are predominantly small with flattened heads and tuberculate skins. These species are brightly colored dorsally, and have white to cream venters which are frequently punctate. Males are smaller than females and have darkly colored vocal pouches. Pigmentation of the vocal pouch is considerably reduced in B. punctatus.

Ventral body spots are generally located in the pectoral region and are quite variable between different populations of the same species; they are most prominent in B. punctatus and B. kelloggi. The other two species of the group, B. debilis and B. retiformis, show considerable



Fig. 1. Adult female representatives of two of the species of the Bufo punctatus group. B. punctatus (left) and B. kelloggi (right).

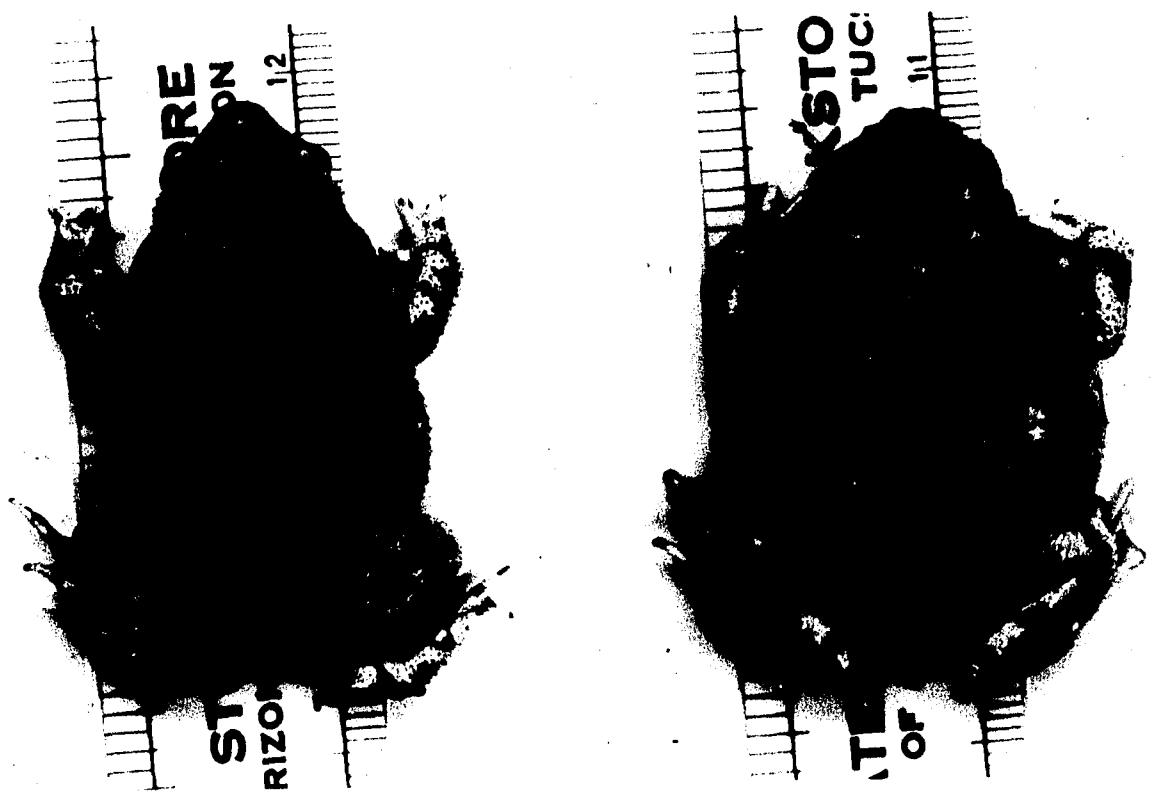


Fig. 2. Adult females of B. debilis (left) and B. retiformis (right). Note elongate parotoids and reticulate patterns.

reduction in punctation in the pectoral region. In fact, this characteristic is generally absent in these two species except for a spot which occurs at the point of junction of the forearm and body.

In B. punctatus the dorsal coloration is reddish brown with small red spots which are usually associated with dermal tubercles. In Bufo kelloggi the dorsal coloration is formed by a brown reticulate pattern on a lighter brown background. The dorsal coloration in B. debilis and B. retiformis is predominantly green and is broken by a black reticulate pattern. This pattern frequently gives the effect of spotting in B. retiformis (Fig. 2).

Coloration and color pattern are good indicators of phylogeny in this group. Savage (1954) states that the color pattern of Bufo kelloggi seems to represent the mode from which the coloration of the rest of the debilis group was derived. It is obvious that the color of a species is not derived from its color pattern. It is certainly true that the striking green coloration in Bufo debilis and B. retiformis is indicative of a close genetic relationship of these species. It also seems probable that the blotched pattern of B. kelloggi resembles more closely the ancestral species of this group than it does the pattern of either B. debilis or B. retiformis. The brown coloration and more blotched pattern of B. kelloggi may also be indicative of a closer relationship to B. punctatus than are the colors and color patterns of the other two species of the group.

Postocular and preocular crests are present, with postoculars extending around the anterior margin of the tympanum. These crests are obvious in B. punctatus and B. kelloggi but are reduced in B. retiformis

and B. debilis. The presence of a distinct cranial crest in B. kelloggi and a reduced crest in B. retiformis and B. debilis prompted Savage (1954) to state that the cranial crests in the debilis group were primitive in nature and were indicative of the relationship of these species to a somewhat more generalized toad related to the present day B. valliceps. It is obvious that cranial crests are indicative of the relationship of B. kelloggi to a B. punctatus-like ancestor, and B. punctatus may indeed be related to B. valliceps (Tihen, 1962a).

The smallness of parotoids also appear to be a primitive characteristic within this group (Savage, 1954). Therefore, on the basis of parotoid size, one should be able to observe the relationship between B. punctatus and the species complex formerly referred to as the B. debilis group (see means of parotoid measurements, Table 1). However, the shape of the round, bead-like parotoid of Bufo punctatus is conspicuous in comparison to the shape of the parotoids of the other species in this group. In other words, the elongate parotoid of B. retiformis, B. debilis and B. kelloggi immediately allows one to recognize their close morphological similarities and this is a trait which has caused many authors to disregard other characteristics of these species which obviously place them in the B. punctatus group. It is of interest that the ratio of width versus length of the parotoid of Bufo kelloggi (Table 2) is smaller than this ratio in either B. debilis or B. retiformis, a fact which further indicates that the parotoid of B. kelloggi is more rounded in shape than the parotoid of B. debilis or B. retiformis and hence more similar in form to the parotoid of B. punctatus (Fig. 3).

Table 1. Means and standard errors of measurements (mm.) taken from males of the species of the Bufo punctatus group.

	<u>Bufo</u> <u>punctatus</u>	<u>Bufo</u> <u>kelloggi</u>	<u>Bufo</u> <u>retiformis</u>	<u>Bufo</u> <u>debilis</u>
N	16	20	20	16
Measurement				
Snout-vent length	49.03 ± 1.10	32.68 ± 0.43	44.46 ± 0.37	36.17 ± 0.36
ulna	12.48 ± 0.35	6.73 ± 0.14	10.11 ± 0.09	8.05 ± 0.10
humerus	12.74 ± 0.34	7.05 ± 0.17	9.71 ± 0.13	8.03 ± 0.14
tibia	19.10 ± 0.62	10.72 ± 0.18	15.52 ± 0.14	12.89 ± 0.17
femur	18.74 ± 0.52	10.76 ± 0.16	16.04 ± 0.17	12.80 ± 0.20
foot	11.23 ± 0.30	6.30 ± 0.11	9.55 ± 0.10	7.82 ± 0.10
parotoid length	4.53 ± 0.17	6.11 ± 0.19	9.61 ± 0.20	8.58 ± 0.20
parotoid width	5.42 ± 0.12	4.47 ± 0.17	5.89 ± 0.24	4.29 ± 0.15
inter orbital distance	6.93 ± 0.15	4.68 ± 0.05	5.89 ± 0.08	5.56 ± 0.09
angle of jaw* to snout	13.92 ± 0.31	8.81 ± 0.12	11.39 ± 0.10	9.87 ± 0.07
post orbital crest-post orbital crest*	16.90 ± 0.39	10.36 ± 0.12	13.68 ± 0.11	11.84 ± 0.10
nasal*	7.51 ± 0.17	5.27 ± 0.68	6.66 ± 0.04	5.94 ± 0.04
orbital length*	6.97 ± 0.12	4.53 ± 0.55	5.85 ± 0.05	4.70 ± 0.02

*N = 11

Table 2. Means of ratios of body measurements of males of the species of the Bufo punctatus group.

	<u>Bufo</u> <u>punctatus</u>		<u>Bufo</u> <u>retiformis</u>		<u>Bufo</u> <u>debilis</u>		<u>Bufo</u> <u>kelloggi</u>	
<u>snout-vent length</u> parotoid width	10.98	0.40	4.66	0.10	4.25	0.09	4.51	0.15
<u>snout-vent length</u> post orbital crest- post orbital crest	2.87	0.04	3.25	0.03	3.05	0.02	3.16	0.03
<u>ulna</u> nasal	1.65	0.03	1.52	0.01	1.36	0.02	1.28	0.03
<u>humerus</u> parotoid length	2.85	0.10	1.02	0.03	0.95	0.03	1.16	0.03
<u>tibia</u> parotoid length	4.26	0.14	1.63	0.03	1.51	0.03	1.77	0.04
<u>tibia</u> nasal	2.56	0.06	2.33	0.02	2.17	0.02	2.03	0.03
<u>foot</u> parotoid width	2.08	0.06	1.67	0.06	1.87	0.07	1.44	0.06
<u>parotoid length</u> parotoid width	0.84	0.02	1.67	0.05	1.65	0.02	1.38	0.05
<u>parotoid length</u> length of eyelid	0.64	0.03	1.64	0.03	1.81	0.04	1.35	0.03
<u>inter orbital distance</u> post orbital crest- post orbital crest	0.42	0.01	0.43	0.01	0.47	0.01	0.45	0.004

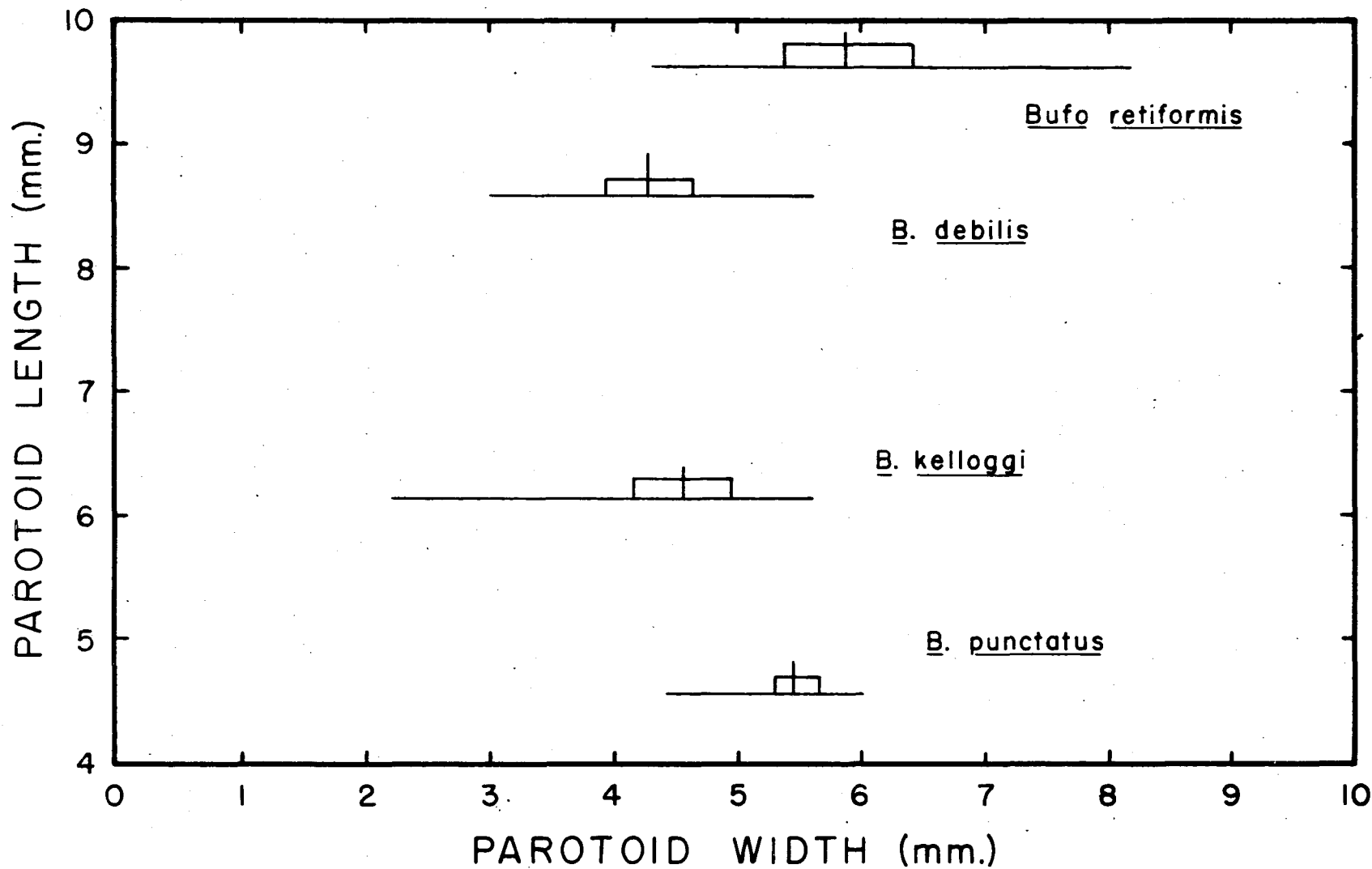


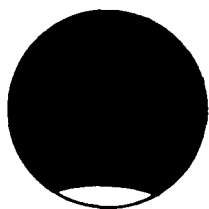
Fig. 3. Comparison of mean parotoid length (Y axis) with parotoid width (frequency distribution, X axis); rectangles represent 95% confidence limits of the mean. Data from Table 1.

Eggs.--The eggs of all of the species are surrounded by two gelatinous membranes. In Bufo punctatus the eggs are laid separately (Wright & Wright, 1949) while those examined of B. kelloggi, B. debilis and B. retiformis are deposited in what at first seems to be a jelly mass. However, close inspection of the eggs of B. debilis, B. kelloggi and B. retiformis reveals the presence of gelatinous strands of eggs which are clumped together due to the agglutinative quality of the outer membrane.

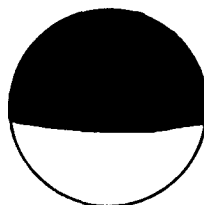
In pigmentation, the eggs of B. punctatus and B. kelloggi differ from the eggs of the other two species by being darkly pigmented from the animal pole to approximately the meridional line (Fig. 4). The eggs of Bufo debilis and B. retiformis are characteristically light cream in coloration with a meridionally located dark stripe (Fig. 4).

Analysis of Structure.--The osteological morphology of the Bufo punctatus group has been examined by Tihen (1962a) who has placed the species of this group in the Caribbean section of the Bufo valliceps group. Blair (1963a) has also made a general review of the external morphology of the North American Bufos and disagrees with the results of Tihen (1962a) on the basis of the presence of a black throat skin in males of B. debilis which is similar to the throat coloration found in the B. americanus group; he failed to note the dark coloration of the throat of the male of B. punctatus, a gular pigmentation that is somewhat more reduced than in other species.

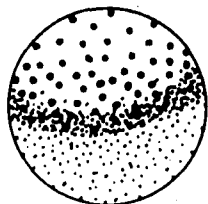
In the present investigation comparisons were made between four species of the Bufo punctatus group by the use of the measurements recorded in Tables 1 and 3. The means of each measured character were



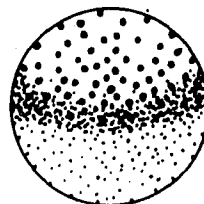
B. punctatus



B. kelloggi



B. debilis



B. retiformis

Fig. 4. Eggs of the species of the B. punctatus group. Note heavy pigmentation at the animal pole in B. punctatus and B. kelloggi, and heavily pigmented meridional area in B. debilis and B. retiformis.

Table 3. Means and standard errors for measurements (mm.) of females of the species of the Bufo punctatus group.

	<u>Bufo</u> <u>punctatus</u>	<u>Bufo</u> <u>kelloggi</u>	<u>Bufo</u> <u>retiformis</u>	<u>Bufo</u> <u>debilis</u>
N	14	19	9	10
Measurement				
snout-vent	61.53 ± 0.97	51.54 ± 0.36	46.97 ± 1.18	43.50 ± 1.02
ulna	14.50 ± 0.17	11.54 ± 0.14	10.03 ± 0.27	8.97 ± 0.25
humerus	14.87 ± 0.36	12.23 ± 0.20	10.14 ± 0.25	8.85 ± 0.37
tibia	21.95 ± 0.30	17.67 ± 0.14	15.58 ± 0.34	13.91 ± 0.38
femur	22.40 ± 0.45	18.24 ± 0.22	15.09 ± 0.33	13.66 ± 0.49
foot	13.31 ± 0.22	11.26 ± 0.13	9.58 ± 0.22	8.81 ± 0.27
inter orbital	8.58 ± 0.16	6.38 ± 0.11	6.48 ± 0.13	5.71 ± 0.18
nasal	9.25 ± 0.15	7.67 ± 0.09	7.14 ± 0.10	6.22 ± 0.11
post orbital crest-post orbital crest	19.77 ± 0.27	15.11 ± 0.11	13.86 ± 0.26	12.44 ± 0.30
angle of jaw- snout	16.71 ± 0.18	12.87 ± 0.06	11.74 ± 0.18	10.68 ± 0.25
orbital length	8.01 ± 0.15	6.32 ± 0.06	5.47 ± 0.09	5.17 ± 0.11

determined for each species and an index for B. retiformis and B. debilis was computed. In these comparisons (Table 4), B. punctatus and B. kelloggi were given the values of 100 and 0.0 respectively.

All possible combinations of ratios were determined from the variables. Indices were computed from ten ratios which were distinct at the 95 percent level of probability (Table 5).

It is obvious that a fairly constant size relationship exists between the species of this group (Figs. 5, 6) and that there should also be some constancy in the hybrid index when applied to the ratios.

From the indices (Table 4), it is obvious that on the basis of general morphological measurements Bufo debilis and B. retiformis are more similar to Bufo kelloggi than they are to B. punctatus. The same relationships are shown by the indices derived from the ratios of the body measurements (Table 5).

When measurements of the males of the four species of the group are plotted on the B. punctatus regression line of tibia on snout-vent length (Fig. 7), the samples of Bufo punctatus and B. retiformis overlap. The same situation occurs in the samples of B. debilis and B. kelloggi. However, no overlap occurs between the samples of B. debilis and B. retiformis. This is taken as strong indication that growth or change in the size of extremities in males of the entire group proceeds arithmetically with snout-vent change. However, this does not necessarily indicate that the relationship between B. punctatus and B. retiformis is any closer than the relationship between B. punctatus and the other members of this group (see also Figs. 8, 9, and 10).

Table 4. Indices determined from the measurements of the males and females of B. debilis and B. retiformis, where B. punctatus = 100.0 and B. kelloggi = 0.0.

<u>Character</u>	<u>Males</u>		% Difference Between Means of <u>B. punctatus</u> and <u>B. kelloggi</u>	<u>Females</u>		% Difference Between Means of <u>B. punctatus</u> and <u>B. kelloggi</u>
	<u>B. debilis</u>	<u>B. retiformis</u>		<u>B. debilis</u>	<u>B. retiformis</u>	
snout-vent	21.30	72.00	33.3	19.23	44.58	29.3
ulna	22.91	58.65	46.0	19.23	46.51	38.1
humerus	17.30	46.72	44.6	21.50	56.16	40.4
tibia	25.83	57.27	43.8	20.74	46.81	36.6
femur	25.53	66.16	42.6	16.35	52.43	39.0
foot	30.80	65.88	47.8	17.07	54.55	33.7
inter orbital distance	39.17	53.80	32.5	26.77	23.32	33.4
angle of jaw to snout	20.77	50.39	36.7	17.66	36.40	36.0
post orbital crest- post orbital crest	22.68	50.75	28.6	19.31	36.35	37.0
nasal width	29.65	61.97	29.8	30.51	47.80	32.7
length of eyelid	8.55	54.01	35.0	10.43	40.65	35.4
parotoid length	-156.05	-221.50	25.8			
parotoid width	- 19.47	148.95	17.5			
mean index	6.84	43.46	36.15	19.89	44.14	32.58

Table 5. Indices of the ratios of body measurements made on the males of B. debilis and B. retiformis where B. punctatus = 100.0 and B. kelloggi = 0.0.

Ratio	<u>B. debilis</u>	<u>B. retiformis</u>	% Difference Between ratios of <u>B. punctatus</u> and <u>B. kelloggi</u>
<u>snout vent length</u> post orbital crest- post orbital crest	34.89	-33.26	9.18
<u>ulna</u> nasal	21.66	65.06	22.9
<u>tibia</u> width of nasal	25.93	56.48	20.3
<u>inter orbital distance</u> post orbital crest- post orbital crest	-47.03	56.45	8.17
<u>snout vent</u> parotoid length	-20.81	-13.50	50.7
<u>humerus</u> parotoid length	-12.83	- 8.50	59.4
<u>tibia</u> parotoid length	-10.31	- 5.80	58.5
<u>foot</u> parotoid width	66.32	35.47	32.5
<u>parotoid length</u> parotoid width	-119.57	-51.87	17.0
<u>parotoid length</u> length of eyelid	-65.89	-42.24	17.7
mean	-12.76	5.83	29.54

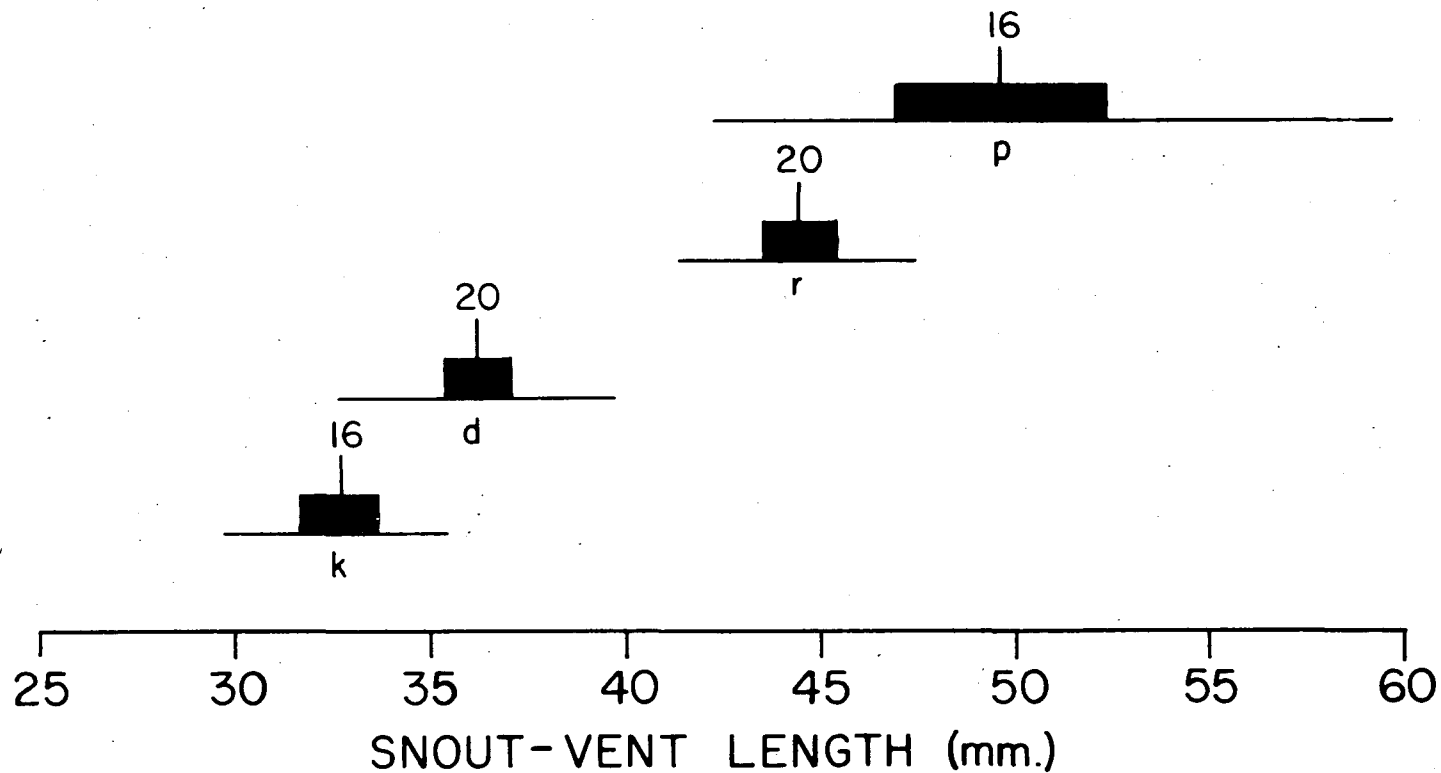


Fig. 5. Frequency distributions of snout-vent measurements of the males of the four species; rectangles represent 95% confidence intervals. Number above the mean indicates sample size. *B. kelloggi*, *B. debilis*, *B. retiformis* and *B. punctatus* are indicated by k, d, r, and p respectively.

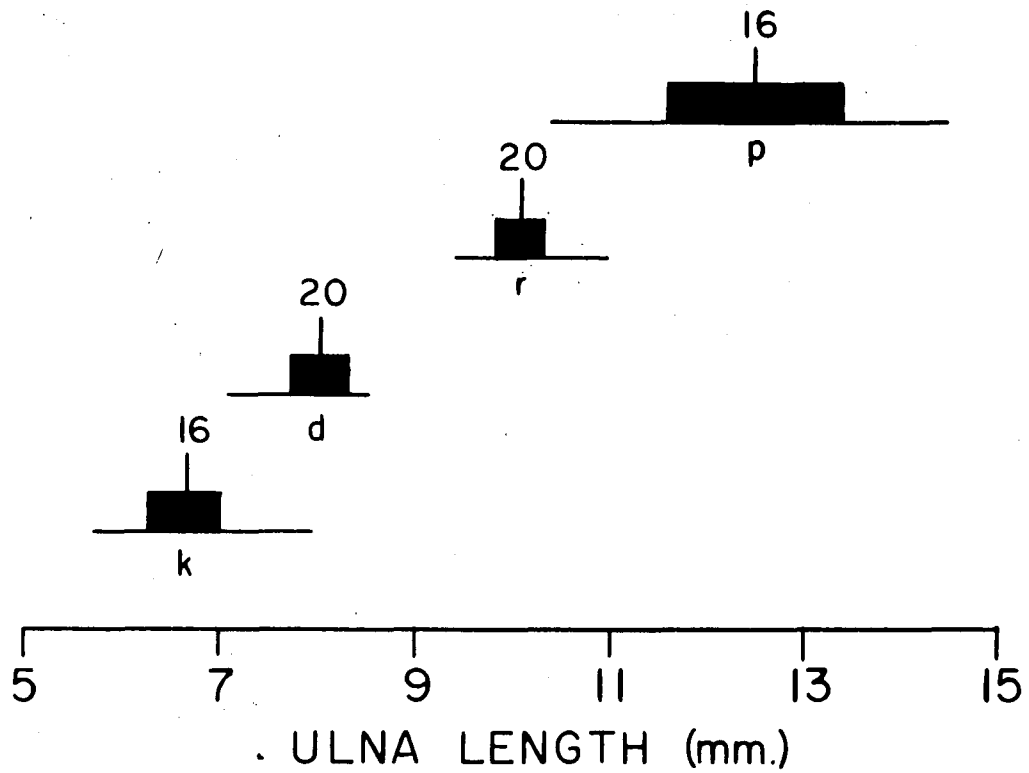


Fig. 6. Frequency distributions of ulna lengths of *B. punctatus* (p), *B. retiformis* (r), *B. debilis* (d), and *B. kelloggi* (k).

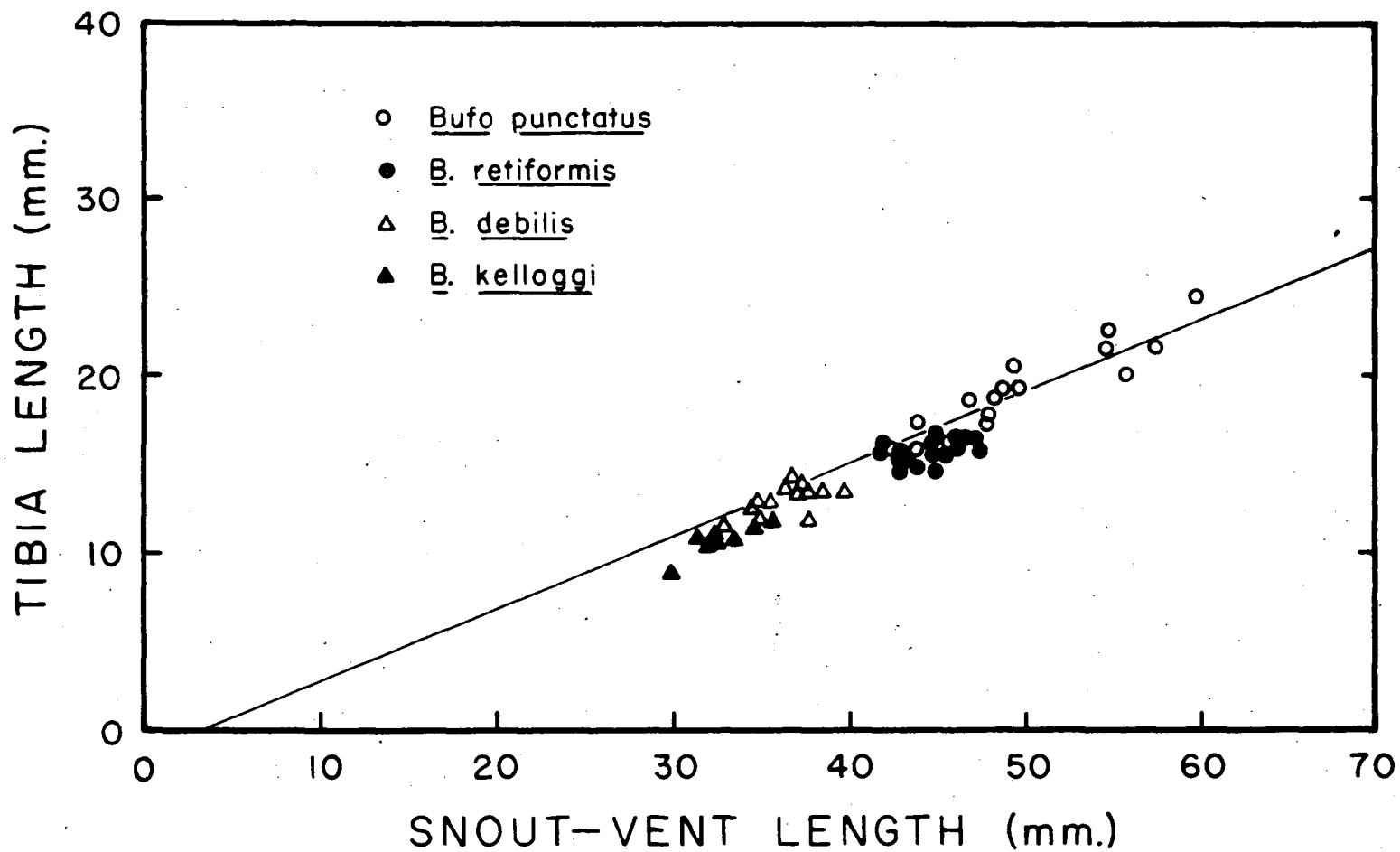


Fig. 7. Comparison of tibia to snout-vent length of individuals of the Bufo punctatus group. Regression line determined from a separate sample of ten specimens of B. punctatus ($Y = -1.40 + 0.41 X$, $r = 0.999$).

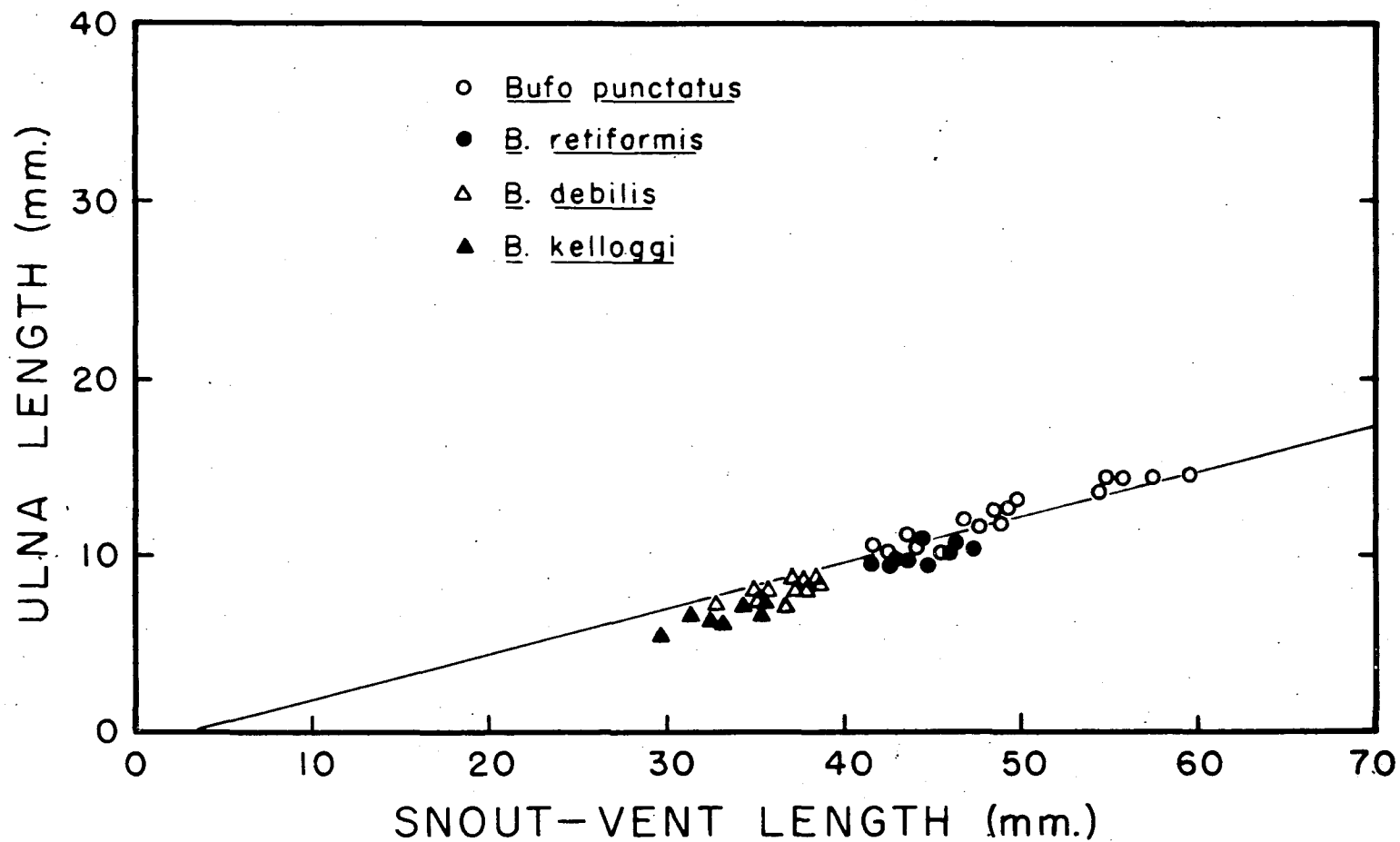


Fig. 3. Comparison of ulna to snout-vent length of individuals of the Bufo punctatus group. Regression line determined from a separate sample of ten specimens of B. punctatus ($Y = -0.6 + 0.258 X$, $r = 0.991$).

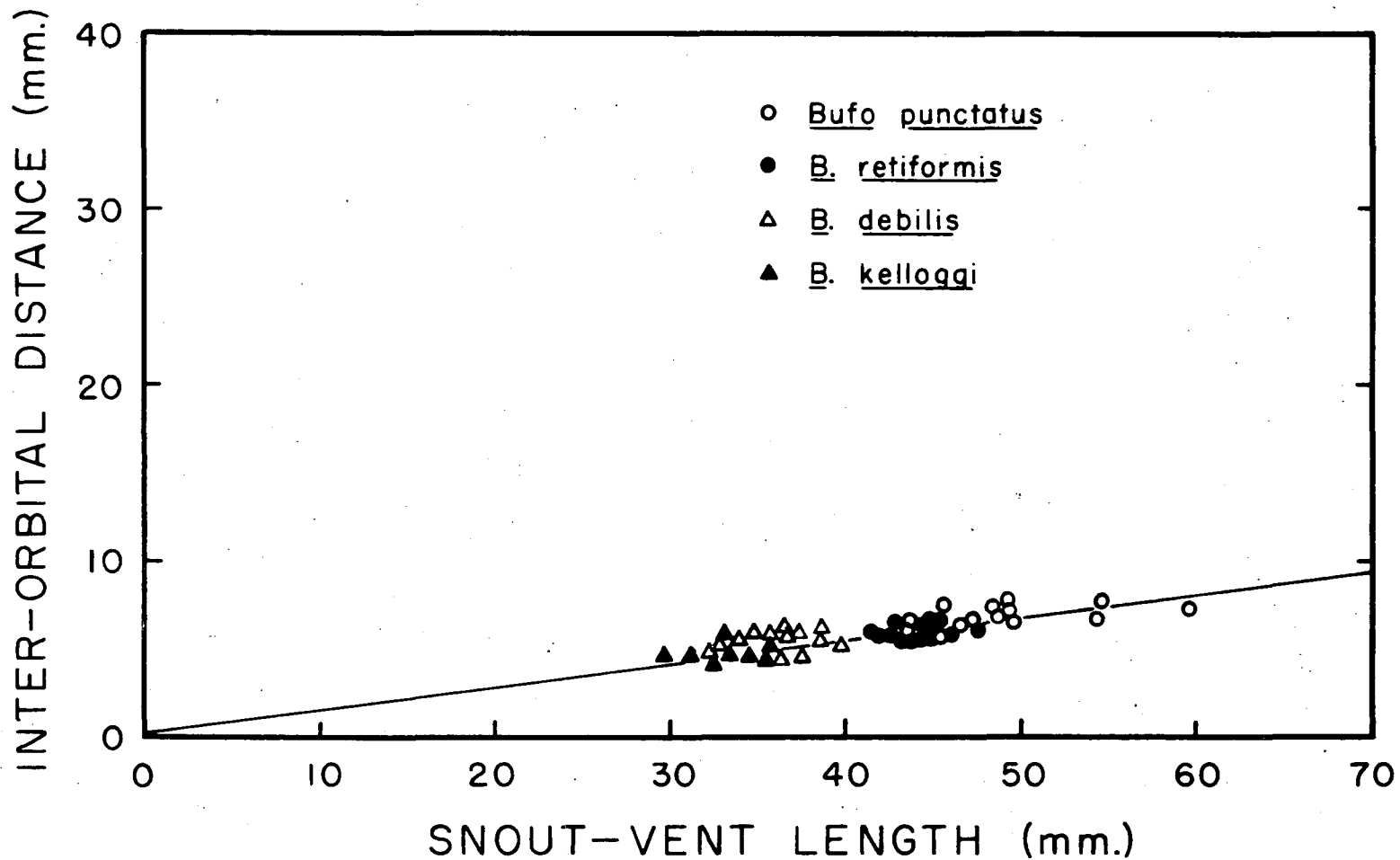
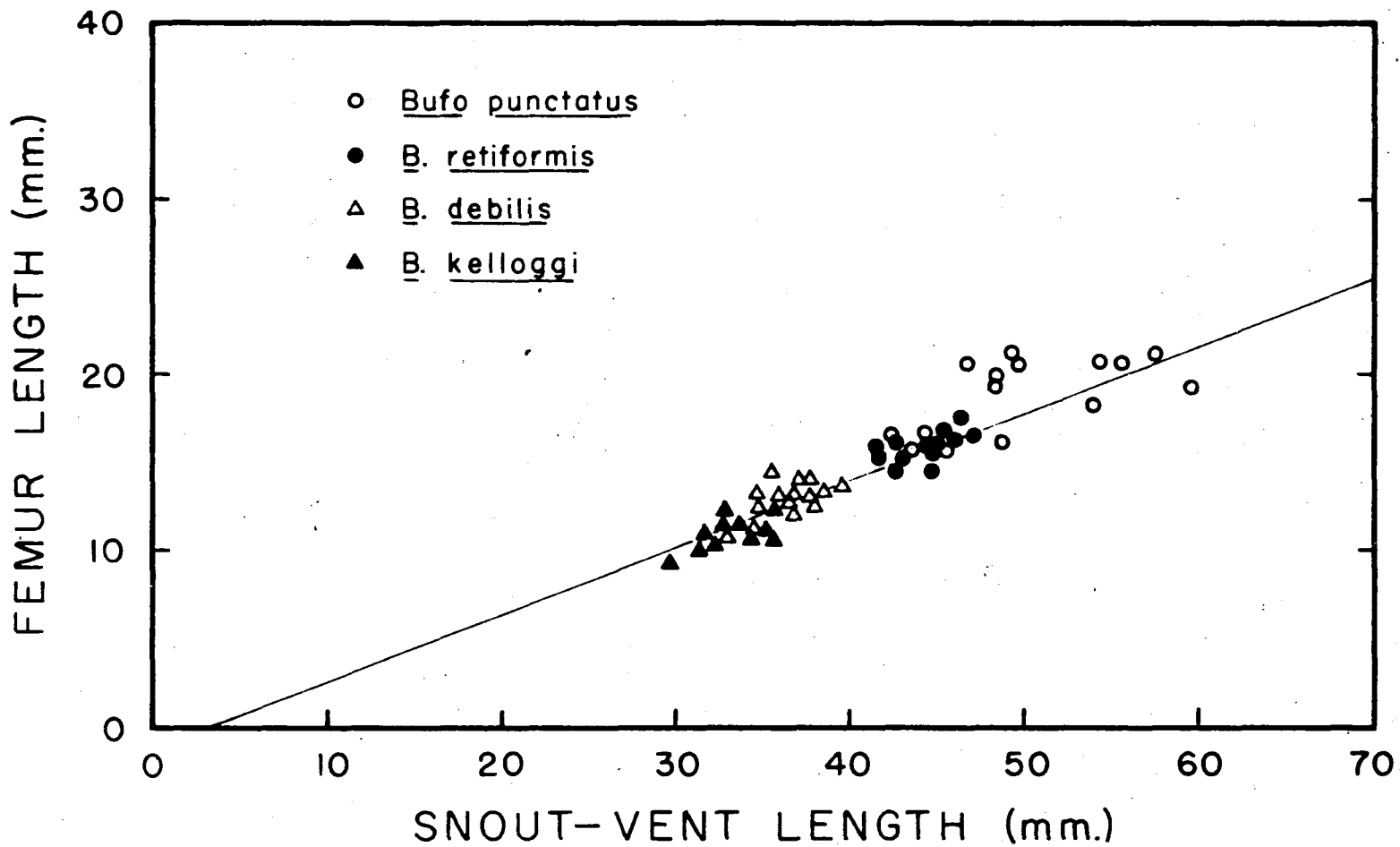


Fig. 9. Comparison of inter-orbital distance to snout-vent length of individuals of the Bufo punctatus group. Regression line determined from a separate sample of ten specimens of B. punctatus ($Y = -0.54 + 0.123 X$, $r = 0.978$).



The ratios represented in Fig. 11 indicate similarity between the body proportions of B. retiformis and B. debilis which is what one would expect of species which were derived from a common ancestor.

On the basis of general morphology and those characteristics not related to size (e.g., eggs, crests, color pattern), it seems apparent that Bufo kelloggi is more closely related to B. punctatus than B. punctatus is to B. debilis or B. retiformis. On the basis of size, one would expect a close relationship between B. punctatus and B. retiformis (Fig. 5). However, size appears to be randomly associated with adaptation during the fortuitous process of speciation rather than a rigid evolutionary trend within the group. In view of the apparent size-habitat relationship it seems more probable that characteristics related to size represent mutational differences which one would expect from species adaptation where environmental pressures can be met with a minimum of major evolutionary change. In other words, ecological conditions which have been necessary for the divergence of these species since the Miocene have been severe, but the physiological changes necessary for their maintenance within their specific environments appear to have been by comparison uncomplicated. Therefore, on the basis of qualitative analysis, it seems probable that B. punctatus is closely related to B. kelloggi and that B. retiformis and B. debilis were derived from a population allied to B. kelloggi.

In the species of this group there is a constant change in limb length with respect to change in body length. In other words, the differences in snout-vent length between individuals of each species are accompanied by constant changes in other body dimensions. This

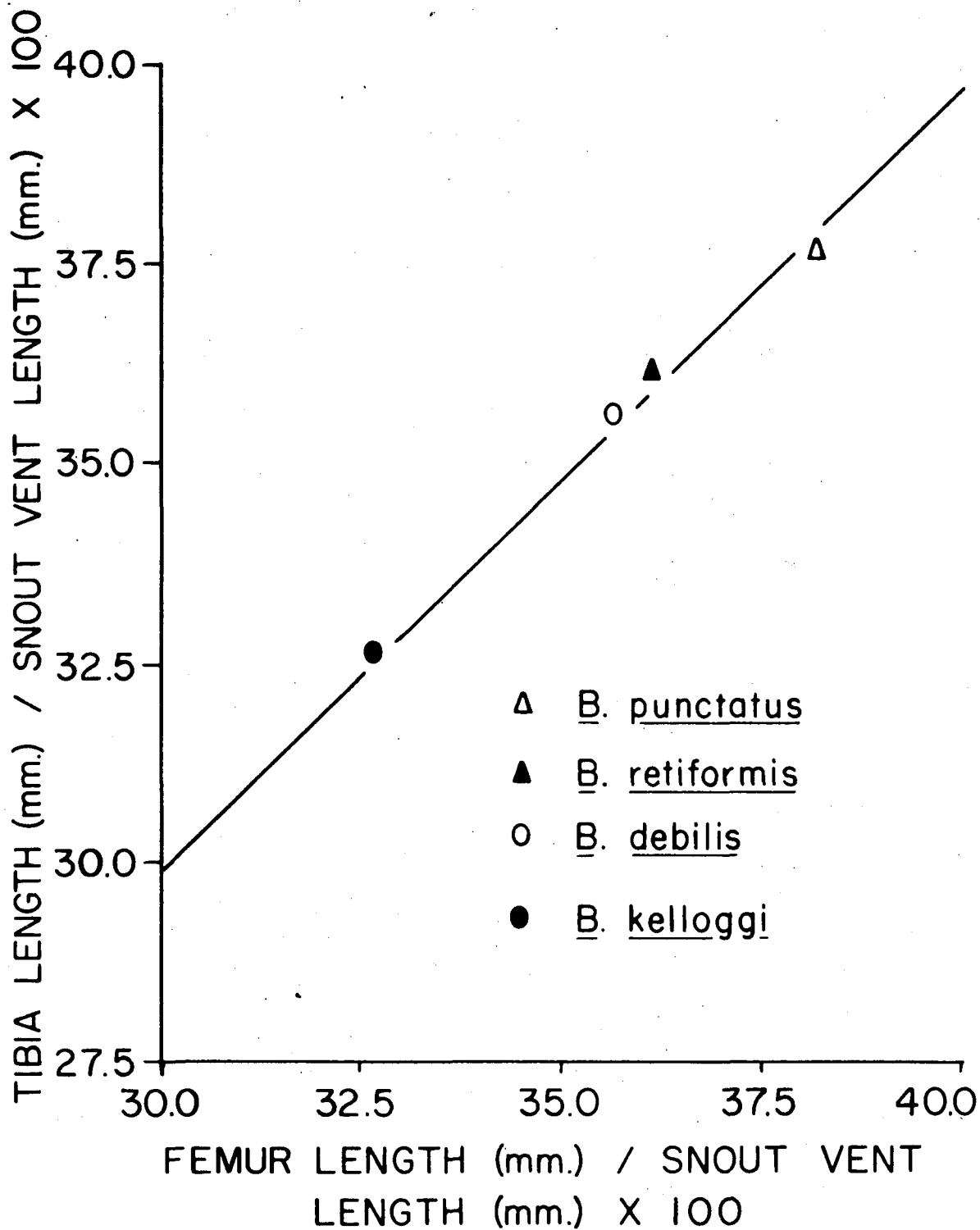


Fig. 11. Regression of tibia and femur length, expressed as a percent of snout-vent length. Regression equation, $Y = -0.04 + 0.983 X$ where $r = 0.996$ and $P = 0.01$.

relationship of body size to limb size is arithmetic and demonstrates that a constant relationship of body size to limb size exists between all the species of the B. punctatus group. It is, therefore, probable that results of analysis dealing with body proportions in this group are to be considered as similarities rather than differences. This would also indicate that size and not body proportion has been that selected for in the males of this species group of anurans and that proportional differences are merely attendant to change in body mass.

A similar situation with respect to body size and proportions exists for the females of this group. In anurans in general, and those occupying arid land in particular, there is a selective advantage for the female to deposit large numbers of eggs. Therefore, an increase in the body size of the female should affect those body dimensions necessary to allow for greater egg production and would therefore vary with the species in a manner dependent upon the selection for particular sizes and numbers of eggs. Selection in this group has been primarily directed toward a larger snout-vent length in females which has resulted in regression coefficients (Table 6) which differ in magnitude from the corresponding coefficients derived from measurements of the males of the group. It is of interest that this selection has been carried to the same degree in all of the species and is probably due to one gene complex for the entire group.

It is obvious that different growth forms exist between the males and females of the B. punctatus group. The indices of body measurements of B. debilis and B. retiformis are listed in Table 4 which clearly indicate that the males of B. retiformis (index of

Table 6. Regression and correlation coefficients for the means of body measurements versus mean snout-vent length of the males and females of each species of the B. punctatus group. N = four in each case.

Measurement	<u>Males</u>			<u>Females</u>		
	<u>b</u>	<u>r</u>	<u>p</u>	<u>b</u>	<u>r</u>	<u>p</u>
length of eyelid	6.53	0.98	0.02	6.11	0.994	0.01
inter orbital distance	7.607	0.95	0.05	5.90	0.95	0.05
width of nasal	7.69	0.99	0.02	6.13	0.99	0.01
length of foot	3.48	0.99	0.01	3.89	0.99	0.02
length of ulna	2.75	0.96	0.05	3.20	0.99	0.02
length of humerus	2.90	0.97	0.05	2.95	0.99	0.02
angle of jaw to snout	3.281	0.97	0.05	2.97	0.9998	0.001
post orbital crest- post orbital crest	2.58	0.97	0.05	2.46	0.99	0.01
length of femur	2.12	0.996	0.01	2.01	0.99	0.01
length of tibia	2.041	0.985	0.02	2.25	0.99	0.01
length of parotoid	0.664	0.205	0.10			
width of parotoid	8.11	0.83	0.10			

snout-vent length = 72.0) are closer in body size to B. punctatus (index = 100) than they are to B. kelloggi. However, the reverse is true in the case of the females of this species. Bufo debilis, on the other hand, has essentially the same index for both males and females.

Indices of this type, where a positive value is obtained, merely reflect positions which are a direct result of size relationships. In no case could these be assumed to reflect more than one type of genetic difference, and that is size alone.

On the other hand, where negative indices are obtained in situations where one would expect positive relationships, as in the case of the parotoid length in both B. debilis and B. retiformis (Table 4) an indication of genetic variation is given which does not strictly reflect variation in size between identical body forms. In other words, the indices for parotoid length clearly indicate a divergence of B. retiformis and B. debilis away from a common ancestor which might also have given rise to B. punctatus and B. kelloggi.

One would expect the indices of the ratios of body parts to result in a clearer indication of genetic affinity. In this investigation, the ratios of paired samples of all possible combinations of thirteen body measurements made on the males were determined. Of the seventy-eight ratios obtained only ten were significantly different from each other in all cases at the ninety-five percent level of confidence (Table 5). This is without doubt strong indication that these species belong to the same species group of anurans, but does little to elucidate interspecific relationships.

The relatively large number of negative indices in Table 6 indicates that B. debilis and B. retiformis have diverged from an ancestral type which was more closely related to B. kelloggi than to B. punctatus, and the indices determined from the ratios of parotoid length with other body measurements would indicate that B. retiformis may have evolved from an ancestral line which eventually resulted in the B. debilis of today.

Some explanation seems necessary for the smaller number of indices which are significantly different at the ninety-five percent level, and for the stressing of the significance of negative indices. A graph of mean snout-vent length against mean body measurement was found to fit a reasonably significant line of regression for the four species of this group (Figs. 12, 13 and Table 6). Obvious exceptions to this are those regressions determined from parotoid length versus snout-vent length and parotoid width versus snout-vent length.

Where statistically significant, these regressions indicate that a constant relationship of snout-vent length to body dimension exists for all the species of the B. punctatus group. In other words, each ratio determined from the points on a regression line from Figs. 12 or 13 should be roughly proportional to the slope of that line.

Also, where the slopes of different regression lines are the same or roughly equal, those body measurements which comprise the points of the different lines do not change in relation to each other with change in snout-vent length. Therefore, one would expect only those ratios of body measurements to be significantly different where the slopes of their regressions with snout-vent length are different.

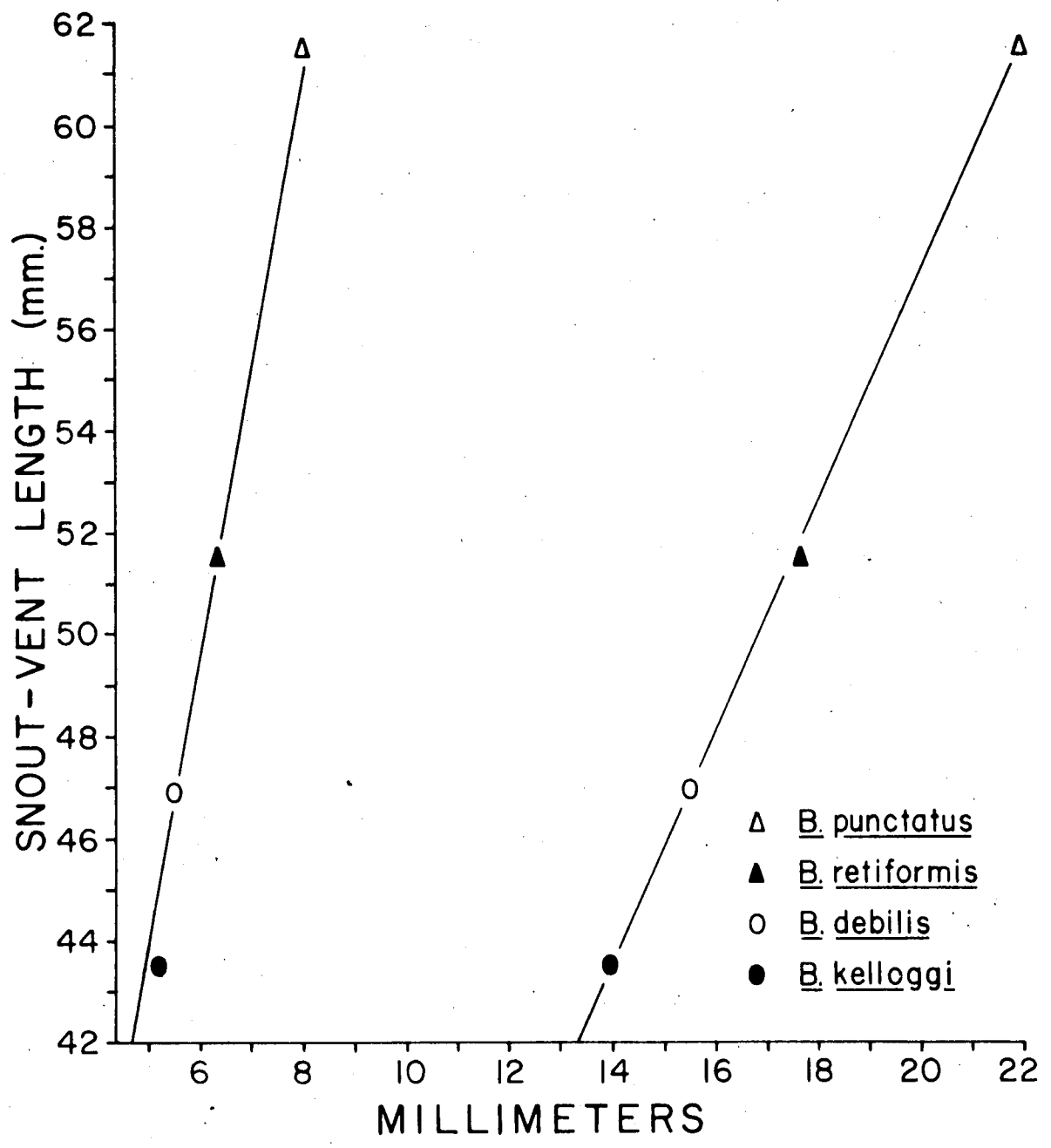


Fig. 12. Regression of mean snout-vent length on mean nasal width (left) and mean femur length (right) of males. Data from Tables 1 and 6.

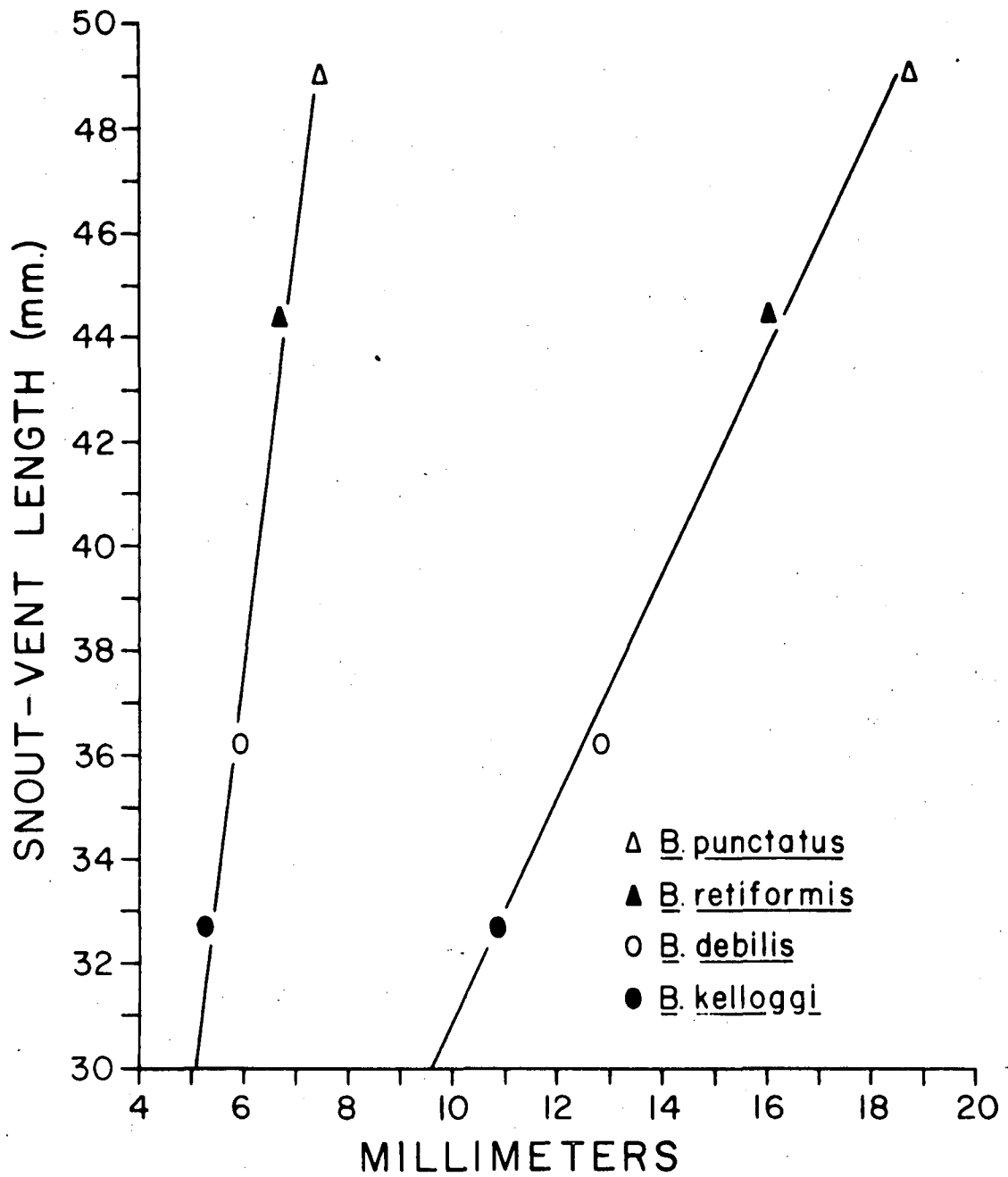


Fig. 13. Regression of mean snout-vent length versus mean length of eyelid (left) and mean tibia length (right) of females. Data from Tables 3 and 6.

The only exceptions to this are those measurements which do not fit a regression with snout-vent length and hence do not change directly with a change in snout-vent length. This would indicate that these values vary independently with the species and are therefore characters which are indicative of a measure of divergence within the group. In other words the evidence for species grouping is based largely on similarities of the species which comprise it (Stone, 1962) but evolutionary relationships within the group can only be estimated through evidence which shows some degree of divergence of the species from a common ancestral type.

Also, where regression coefficients are different the corresponding variables must stand in geometric relationship to each other. In Table 7 it can be determined from the regression coefficient b that at least three classes of values were taken. Ratios determined from any two measurements within one of the first two groups (regression coefficients which vary from 6.53 to 7.69 and from 2.041 to 3.48) would not be expected to be significantly different between different species of the group. On the other hand, the slopes of the regressions indicate that the body dimensions involved in group 1 (Table 6) stand in allometric relationship to those given in group 2. Therefore, one would expect the ratios of body dimensions of different groups to differ between the species of the group. In other words, the greater the difference between the actual sizes of the individuals, the greater the difference between the ratios of allometric body dimensions.

No predictions can be made as to the statistical significance of the ratios which might be derived from the measurements which comprise

Table 7. Means and standard errors (mm.) made on skulls of the species of the Bufo punctatus group.

	<u>Bufo</u> <u>punctatus</u>	<u>Bufo</u> <u>retiformis</u>	<u>Bufo</u> <u>debilis</u>	<u>Bufo</u> <u>kelloggi</u>
N	8	10	10	8
Measurement				
inter orbital width	4.96 ± 0.10	4.59 ± 0.10	4.36 ± 0.09	4.02 ± 0.12
angle of jaw- angle of jaw	19.18 ± 0.46	15.18 ± 0.16	13.18 ± 0.18	12.10 ± 0.35
width of frontoparietal	5.82 ± 0.15	5.31 ± 0.06	5.26 ± 0.08	4.82 ± 0.12
length of frontoparietal	7.75 ± 0.24	7.45 ± 0.12	6.39 ± 0.15	6.70 ± 0.26
length of nasal	3.90 ± 0.18	4.19 ± 0.11	3.77 ± 0.08	3.32 ± 0.14
total skull length	13.19 ± 0.34	12.05 ± 0.18	10.97 ± 0.12	10.87 ± 0.21
width of nasals	8.76 ± 0.16	7.15 ± 0.10	6.70 ± 0.09	6.08 ± 0.20
length of parasphenoid	8.32 ± 0.15	7.15 ± 0.11	6.16 ± 0.09	6.14 ± 0.16
width of parasphenoid	11.02 ± 0.26	8.93 ± 0.15	8.98 ± 0.14	7.99 ± 0.25

the third group, since these measurements do not fit a regression line determined by the method of least squares.

From this one would expect a maximum of forty-one and not less than twenty-seven ratios which are statistically different at the ninety-five percent level of probability (3×7 , 2×3 , and 2×7) for all species. However, this can not be the case as is shown by the positions of the mean snout-vent lengths of the species on the regression lines in Figs. 12 and 13. In other words, as these species approach each other in actual size, the probability of the ratios of their body measurements being different becomes less. This can be readily seen in the case of the females of B. kelloggi and B. debilis, which are very close to each other in actual body size. Of fifty-five ratios determined from body measurements of these two species, only five were significantly different at the ninety-five percent level.

It is to be concluded here, that ratios derived from body measurements of the species of this group are meaningless for showing species divergence except for those values obtained from the width and length of the parotoid. However, the indices of the ratios, regardless of the bias created by size, indicate more clearly than the indices for the body measurements that B. debilis and B. retiformis are very similar in body form to B. kelloggi. These indices also indicate that B. kelloggi is the closest member of this group in body form to B. punctatus.

Qualitative characters which were mentioned earlier should be reemphasized. First, there are numerous characteristics of the group such as flattened heads, reduced cranial crests, very tuberculate skins, and smallness of body size which are quite uncommon to other species

groups of toads occurring in North America. Also, characteristics which are discontinuous within the group allow one to distinguish the species. First of all, the rounded parotoid and absence of egg strands in B. punctatus compared to the elongate parotoids and gelatinous strands of eggs in B. kelloggi, B. debilis and B. retiformis immediately allow one to separate the species group into two subgroups. In the same manner, B. debilis and B. retiformis can be separated from B. kelloggi on the basis of egg pigmentation and body color.

The problem of determining relative points of origin between the species remains. Here much value has been derived from qualitative as well as quantitative data. From these data, it seems probable that an ancestral stock gave rise to two lines which are represented by B. punctatus and B. kelloggi of today. Sometime after these lines were separated, a third line arose from the ancestral stock of B. kelloggi. This third line is represented today by B. debilis and B. retiformis.

Cranial Morphology

In general, the skulls of the species of the B. punctatus group are flattened in the vertical axis with prominent nasals which flare laterally (Figs. 14 and 15). The skulls of B. punctatus and B. kelloggi are usually heavily ornamented with dermal bone which sometimes obscures the lateral edges of the frontoparietals and gives rise to the prominent cranial crests which are present in these species. The parasphenoid of each species is extended anteriorly to the level of junction of the palatine with the sphenethmoid. The lateral wings of the parasphenoid extend almost to the lateral edges of the pterygoid, and are thus usually

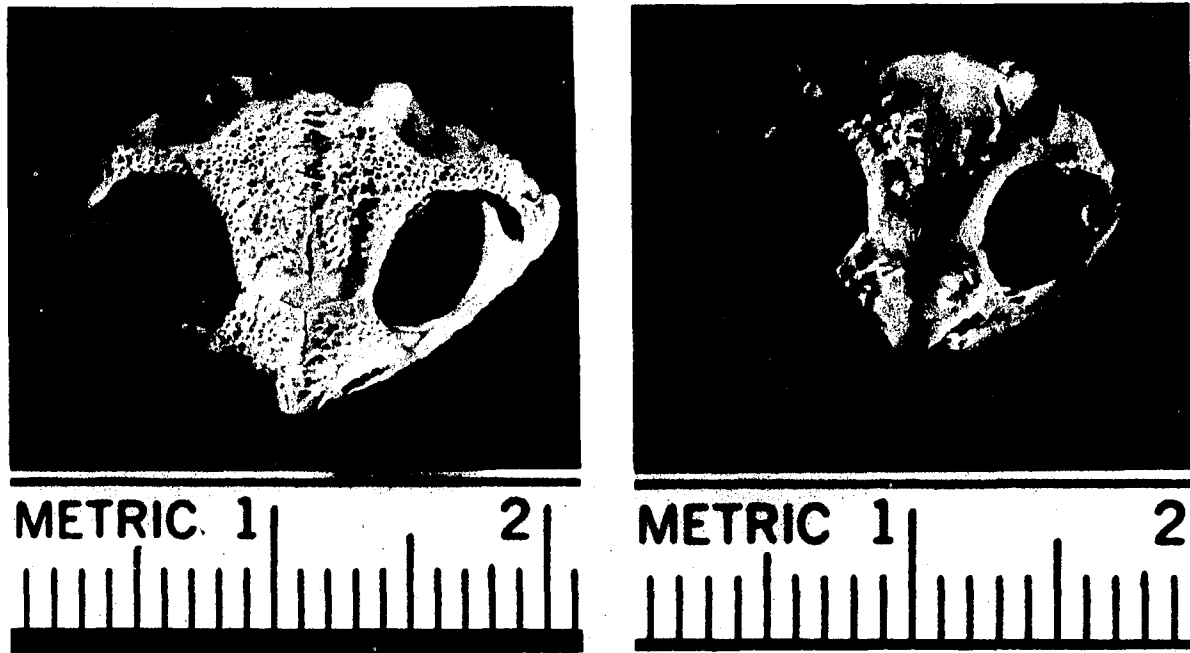


Fig. 14. Dorsal aspect of the skulls of adult males of *B. punctatus* (left) and *B. kelloggi* (right).

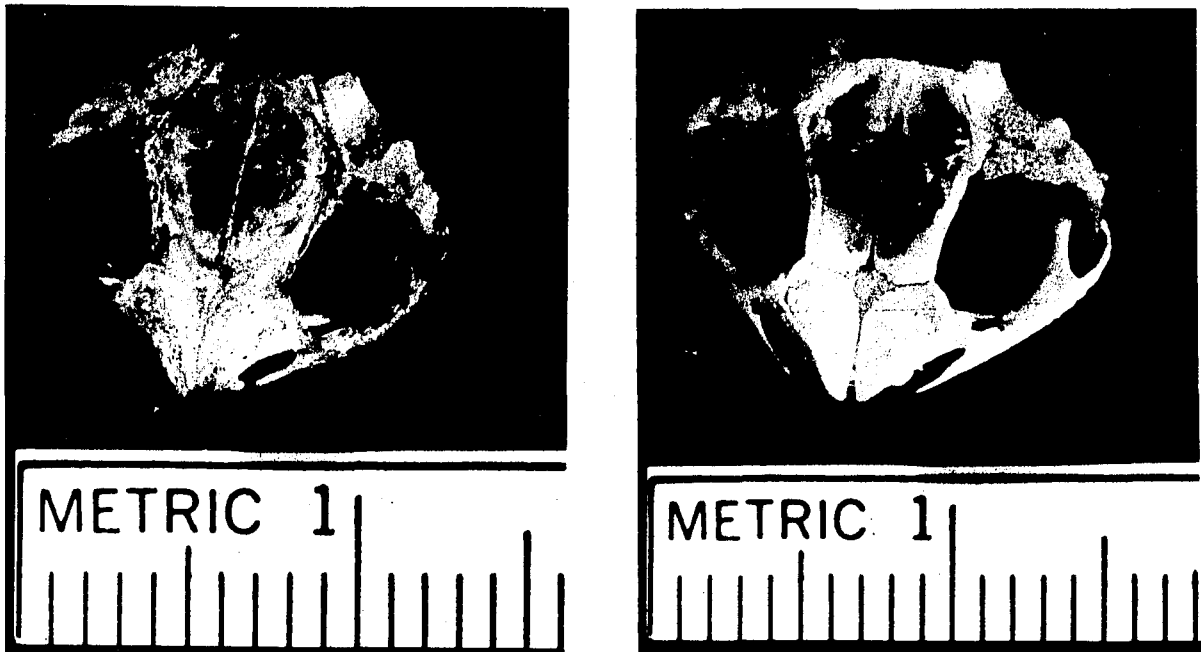


Fig. 15. Dorsal aspect of the skulls of adult males of *B. debilis* (left) and *B. retiformis* (right).

pointed on their anterior edges. The squamosal is rounded at its suture with the prootic in B. debilis and B. retiformis; it is usually straight in B. punctatus and B. kelloggi. B. punctatus usually has an extended phlange on the postero-lateral edges of the maxilla; this is a trait which is sometimes seen in the skulls of B. kelloggi and was not found in the skulls examined of B. debilis and B. retiformis.

Qualitative characteristics of the skulls of the North American Bufos have been investigated by Tihen (1962a). Tihen found the shape of the frontoparietal, a very obvious bone in the skulls of most anurans, to be an extremely reliable characteristic for group allocations of the species of the genus Bufo. However, Tihen could find no evidence on the basis of this or other osteological characteristics for determining phylogenetic relationships within a particular group.

Nine measurements were made on the skulls of each of the four species (Table 7). The ratios of all possible combinations of measurements were also computed. These variables were then statistically compared in the same manner as previously described for other body measurements.

Of the thirty-six ratios computed, only one (total width/total length of the skull) was statistically different at the ninety-five percent level for all species (Table 8). From this ratio it is obvious that the larger species within this group have proportionally wider skulls. This seems to be another growth phenomenon which is characteristic of the entire group rather than of any particular species within the group.

Table 8. Means of the ratios of skull measurements of the species of the Bufo punctatus group.

	<u>B. punctatus</u>		<u>B. retiformis</u>		<u>B. debilis</u>		<u>B. kelloggi</u>	
width of <u>frontoparietal</u> length of frontoparietal	0.753	0.018	0.715	0.018	0.826	0.016	0.723	0.017
<u>total skull width</u> total skull length	1.456	0.021	1.260	0.013	1.201	0.010	1.114	0.024

It is interesting to note that the ratio of frontoparietal width over length, a quantitative determination of a reliable qualitative trait as shown by Tihen (1962a), is statistically unreliable as a character to be used in the separation of B. punctatus from the other species in the B. punctatus group. However, the mean of this ratio is quite distinct between the other species, B. debilis, B. retiformis, and B. kelloggi. Also, it can be seen from Table 8 that B. punctatus is more similar to B. kelloggi with respect to this trait than it is to either B. retiformis or B. debilis.

The absence of the exposure of the sphenethmoid between the frontoparietals and nasals is conspicuous in B. punctatus with respect to the other species in this group (Tihen, 1962a). This seems to be the primary characteristic which separates B. punctatus from the other species in this group. However, from the shape of the frontoparietal it is also clearly apparent that B. punctatus is a generalized member of this species group and could, therefore, be the recent species which most closely approximates the ancestral type.

Of the species discussed here, B. kelloggi seems to be the closest relative of B. punctatus. This can be demonstrated on the basis of dermal ornamentation of the skull, shape of the frontoparietal, and other less obvious morphological characteristics such as shape of the suture line between the prootic and squamosal and the presence of a laterally extended phlange on the posterior border of the maxilla. The close relationship indicated here is strongly supported by experimental data given below (Hybridization).

The skulls of B. retiformis and B. debilis are quite similar in appearance. The skulls of these species have less dermal ornamentation when compared to B. punctatus or B. kelloggi and are quite similar in qualitative characters as previously mentioned. However, the skull of B. retiformis is much larger than that of B. debilis and its sutures are fitted together much more loosely; in other words, more space occurs between the bones (i.e., frontoparietals) and they are much more smooth in appearance, having less dermal ornamentation.

In summary it can be stated that the quantitative morphological characteristics of the skulls of these species are indicative of group affiliation. Those characteristics which are treated qualitatively have probably resulted from species divergence within the group and can therefore be used to show interspecific relationships.

Mating Calls

In structure, the calls of the Bufo punctatus group are very similar (Figs. 16 and 17). Each call consists of two dominant frequency bands and several lesser harmonics. The lower band is referred to as

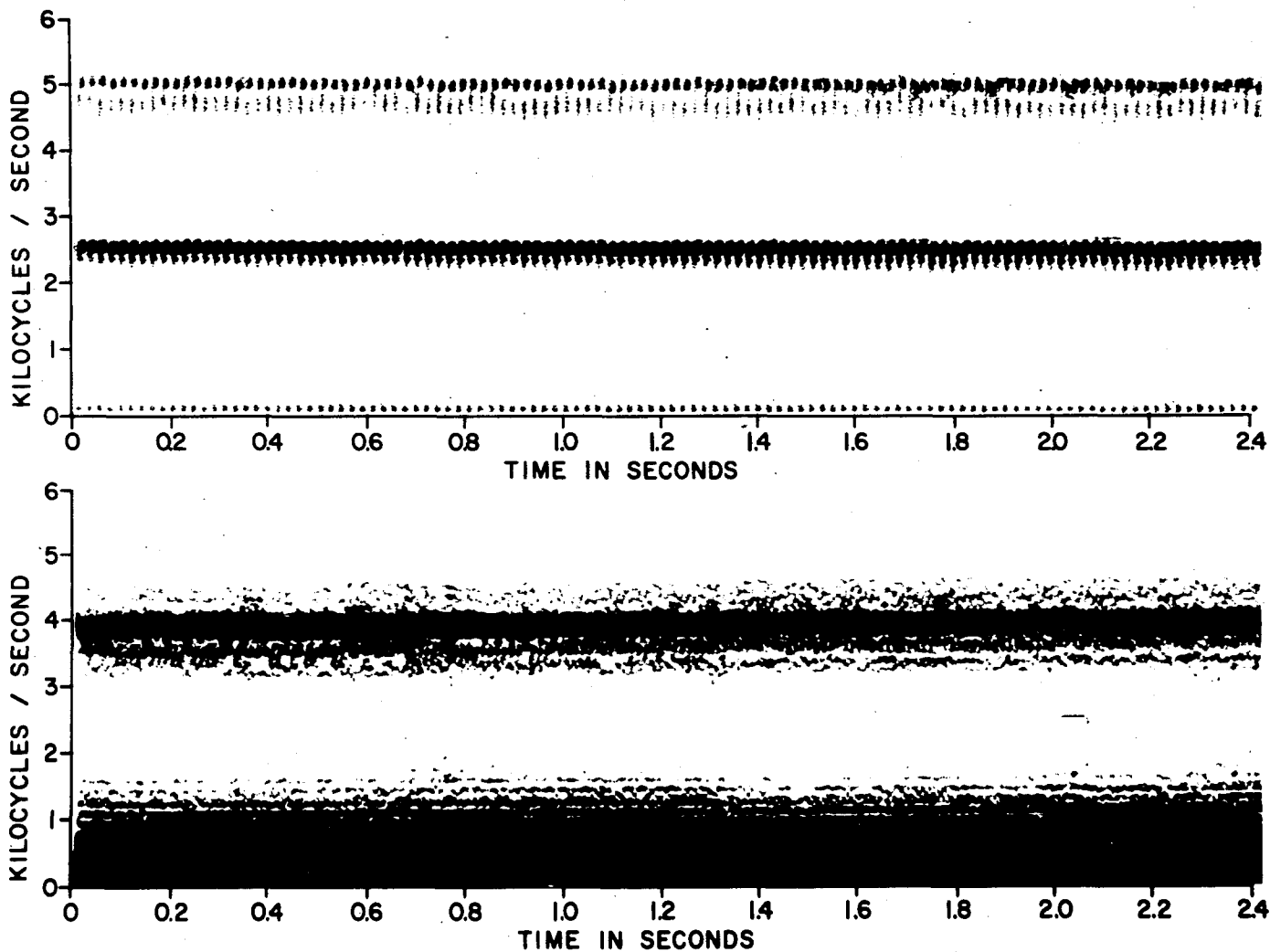


Fig. 16. Sound spectrograms of the male calls of *B. punctatus* (above) and *B. kelloggi* (below). Lowest dark lines in both cases are caused by background noises. In *B. punctatus* both primary and secondary harmonics are visible. The secondary harmonic in the call of *B. kelloggi* occurs above 6 kc and is not shown.

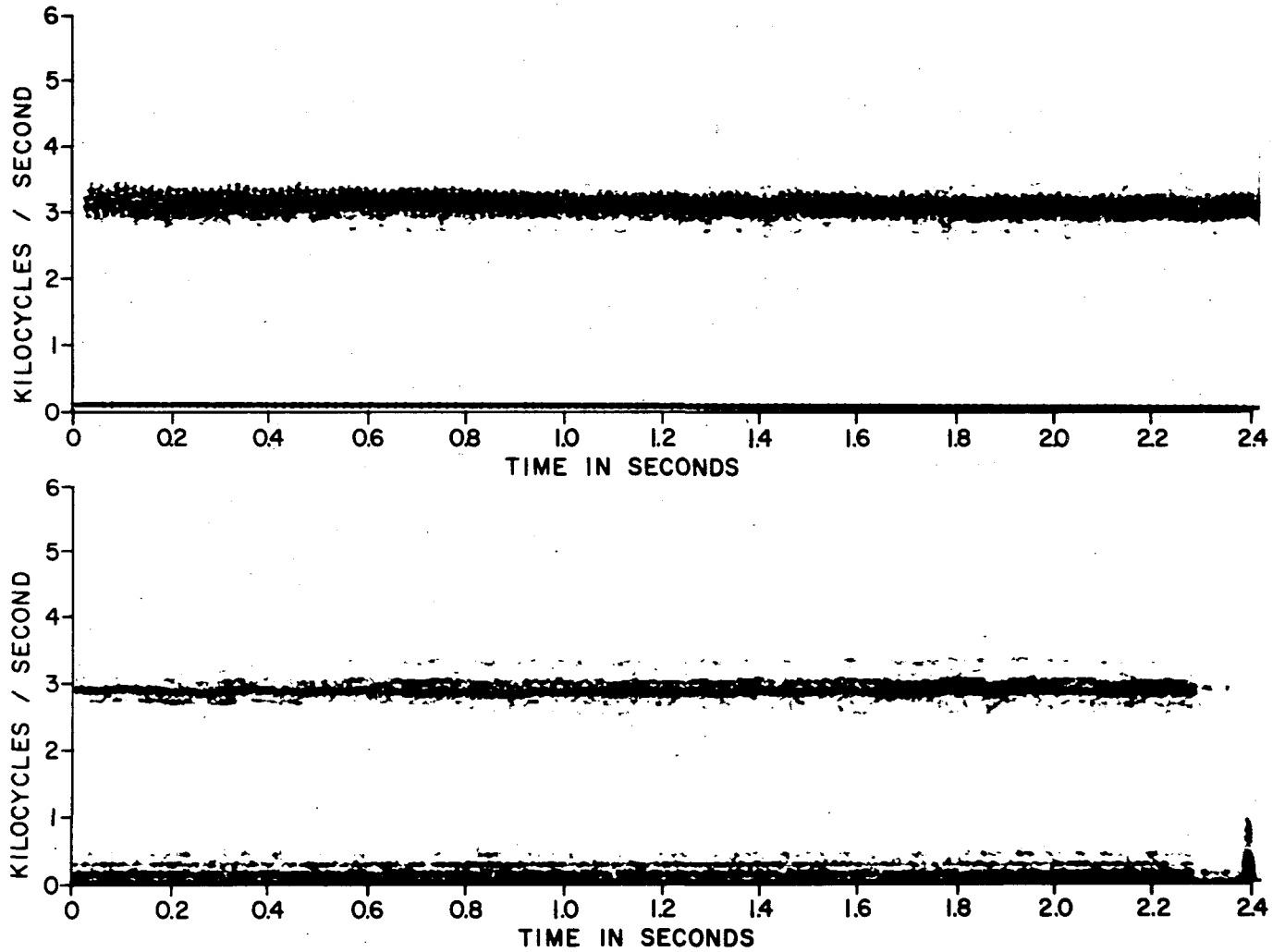


Fig. 17. Sound spectrograms obtained from the male calls of *B. debilis* (above) and *B. retiformis* (below). Primary harmonics are shown for each species. Secondary harmonics in these particular calls occur at frequencies above 6 kc.

the fundamental and from it the emphasized frequencies given in Tables 9 and 10 were determined. The pulses in each species tend to fall into groups of two, with the frequency of the first part of each call slightly lower than the remainder.

There are major differences in the calls of the species of this group that can be noted both in the sonograms and in the field. In B. retiformis the initial frequency increase has the sound of a sharp chirp, which due to its short duration could hardly be termed a "rising crescendo" as described by Savage (1954). The distinctiveness of the call of Bufo punctatus is as obvious to the ear as it is from spectrograph analysis; it has a melodious, somewhat ventriloquistic floating sound which does not seem harsh or sharp like other members of this group when heard at close distances. The calls of B. debilis seem quite similar to B. kelloggi when heard in the field. These lack an initial sharp rise in frequency as in B. retiformis and also lack the melodious slow pulsing observed in the call of B. punctatus. Yet these calls are quite distinct from each other with respect to frequency and duration (Table 10).

Referring to species within the debilis subgroup, Bogert (1962) speaks of "relatively little divergence in their mating calls." His error may be due to the difficulty in distinguishing differences of only one kilocycle by the normal ear. When placed on a logarithmic scale, which reduces the observed frequencies to the same relationship as shown on a musical scale, these frequencies stand in the proportion of 3.51-3.61, or $\log 3,240$ to $\log 4,045$ respectively. This difference lies between two to three notes on a musical scale.

Table 9. Meristic aspects of the mating calls showing variation due to population differences and physical factors of the environment.

	No.	AT	WT	Mean Emphasized Frequency KC	Mean Pulse Rate	Mean Duration and Range
<u>B. punctatus</u>						
Tucson Mts., Pima Co., Ariz.	3	24.6	24.4	2.32	48.7	4.66 (2.0-6.0)
Scottsdale, Ariz.*	1	31.0	28.0	2.42	59.0	3.8 (2.9-7.0)
Austin, Texas*	3	24.0	26.0	2.59	52.3	8.26 (7.2-9.7)
<u>B. debilis</u>						
Cochise, Cochise Co., Ariz.	3	18.4	23.0	3.10	112.0	3.78 (2.65-4.70)
Benson, Ariz.	4	18.2	20.2	2.914	96.5	5.09 (2.4-10.35)
Valentine, Texas*	2	25.0	23.0	3.25	120.0	3.5 (2.6-4.9)
Silver, Texas*	2	23.0	23.0	3.12	122.0	4.0 (3.2-5.4)
Throckmorton, Tex.*	3	14.0	19.0	3.33	112.3	5.73 (4.1-7.3)
<u>B. retiformis</u>						
2.2 mi. E. Sells, Pima Co., Ariz.	4	26.0	25.2ST	2.85	212.0	3.01 (2.15-4.2)
35 mi. E. Ajo, Pima Co., Ariz.	3	26.8	25.4ST	2.81		2.44 (1.6-3.8)
<u>B. kelloggi</u>						
20 mi. S. Hermosillo, Son., Mexico	2	28.0	28.4	4.35(1)	136.0	3.20 (2.4-4.7)
18 mi. W. Hermosillo, Son., Mexico	7	27.2	27.2	4.001	131.7	3.39 (1.4-5.2)

* Calls reported by Blair, 1956

Table 10. Summary of call data. Data graphed in Figs. 18, 19, 20.

	Frequency (KC/sec)	Pulse rate (Pulse/sec)	Duration (sec)
<u>B. punctatus</u> (N)	7	7	7
maximum	2.700	53	9.7
minimum	2.262	46	2.0
mean	2.46 ± 0.06	51.7 ± 0.61	6.08 ± 0.87
<u>B. retiformis</u> (N)	7	4	7
maximum	3.007	220	4.2
minimum	2.714	204	2.2
mean	2.837 ± 0.04	212.0	2.78 ± 0.22
<u>B. debilis</u> (N)	14	14	14
maximum	3.600	128	10.4
minimum	2.795	84	2.6
mean	3.240 ± 0.03	110.2 ± 3.4	4.56 ± 0.4
<u>B. kelloggi</u> (N)	8	9	9
maximum	4.350	144	5.2
minimum	3.818	124	1.4
mean	4.044 ± 0.09	132.7 ± 2.98	3.28 ± 0.35

Blair (1956) describes the call of B. debilis as "a high frequency buzz, which can easily be confused with the call of the narrow-mouthed frogs (Microhyla)". He states that the dominant frequency of the call of B. debilis is higher than the frequency of the call of any other toad in the Southwest. From this it is not certain whether he means that northern Sonora is not in the Southwest or that he is not familiar with the call of B. kelloggi. The data obtained by Blair (1956) from B. debilis at three localities in Texas agree favorably with those presented here for the same species from Cochise County, Arizona. At a water temperature of 23°C. (Table 9) the calls of B. debilis from Cochise County ranged from 3,138 to 3,024 cps while Blair's values for Texas (Valentine and Silver) range from 3,300 to 3,065. However, the calls reported here from Benson, Arizona were recorded at comparable water temperatures to Blair's Throckmorton data (Table 9), yet they differ markedly in frequency and pulse rate. It is rather surprising that in Texas the frequency increased with a decrease in temperature (see Blair 1955, 1956).

The emphasized frequencies as presented by Bogert (1962) for Bufo debilis range from 3,500 to 3,900 cps. These values are almost out of the range shown here for the same species (Fig. 18). While it appears obvious that the calls of individual toads within a breeding population are not extremely variable, there can be marked variation between the calls of animals from different breeding populations.

There is the possibility that wide variation in call frequency within a species is representative of subspecific or other intraspecific variation. This may well be the case in B. punctatus (Table 9). On

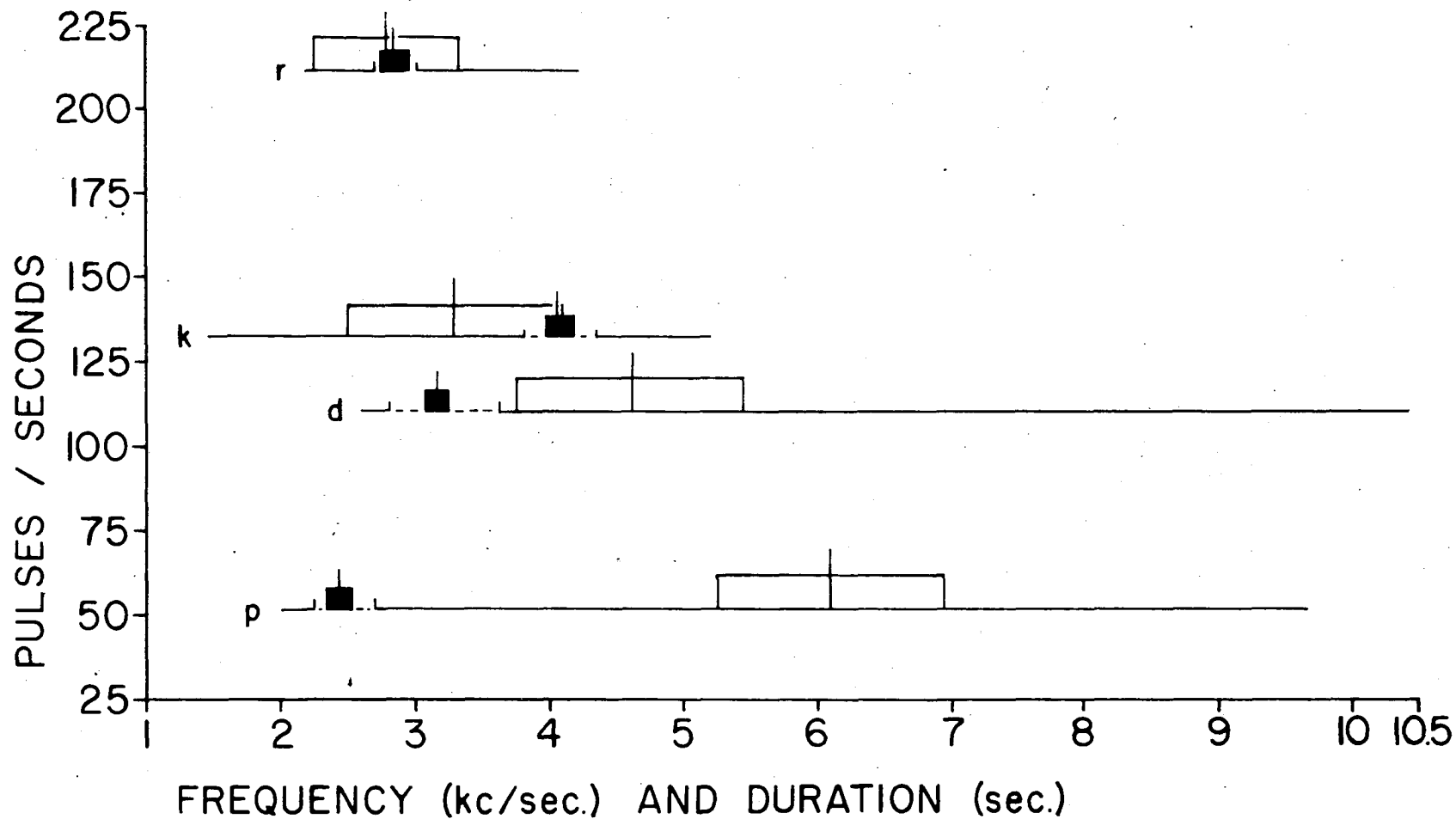


Fig. 18. Comparison of call duration (open rectangles) and call frequency (solid rectangles) with mean pulse rate in *B. punctatus* (p), *B. debilis* (d), *B. kelloggi* (k), and *B. retiformis* (r).

the basis of calls, B. punctatus from the Texas populations is somewhat different from B. punctatus from Arizona, although not of the order of magnitude as seen between the species of the B. punctatus group. The difference in frequency between the calls of B. debilis from Cochise and Benson, Arizona (Table 9) and those of B. debilis calls from Portal, Arizona, reported by Bogert (1962) is certainly not related to subspecific differentiation. A possible explanation is that the frequencies reported by Bogert may not have been determined from enough sections which would indicate that undue personal judgment may have been employed in determining the dominant frequency from the basic harmonic. Some other factors could also have contributed to some degree, such as field temperature and proper sonograph calibrations.

Blair (1956) states his opinion concerning B. punctatus, apparently based on mating call, that "this toad seems to have no close relatives among United States toads." It is obvious from the data given here (Fig. 18) that in important respects, greater variation exists between the calls of the members of the "B. debilis group" (Blair, 1962) than between B. punctatus and members of Blair's group.

In Figure 19, call frequency, pulse rate, and duration are plotted on equal axes. When viewed in this manner, it is clear that all four calls are quite distinct. In other words, were such calls to be confused by the female of another species, at least some overlap would have to result in the characteristics as plotted (Fig. 19), and clearly no overlap is even approached in the Figure for the entire group. Any change in position on one ordiant obligates a change in call structure.

Recent evidence (Martof and Thompson, 1964) indicates that the

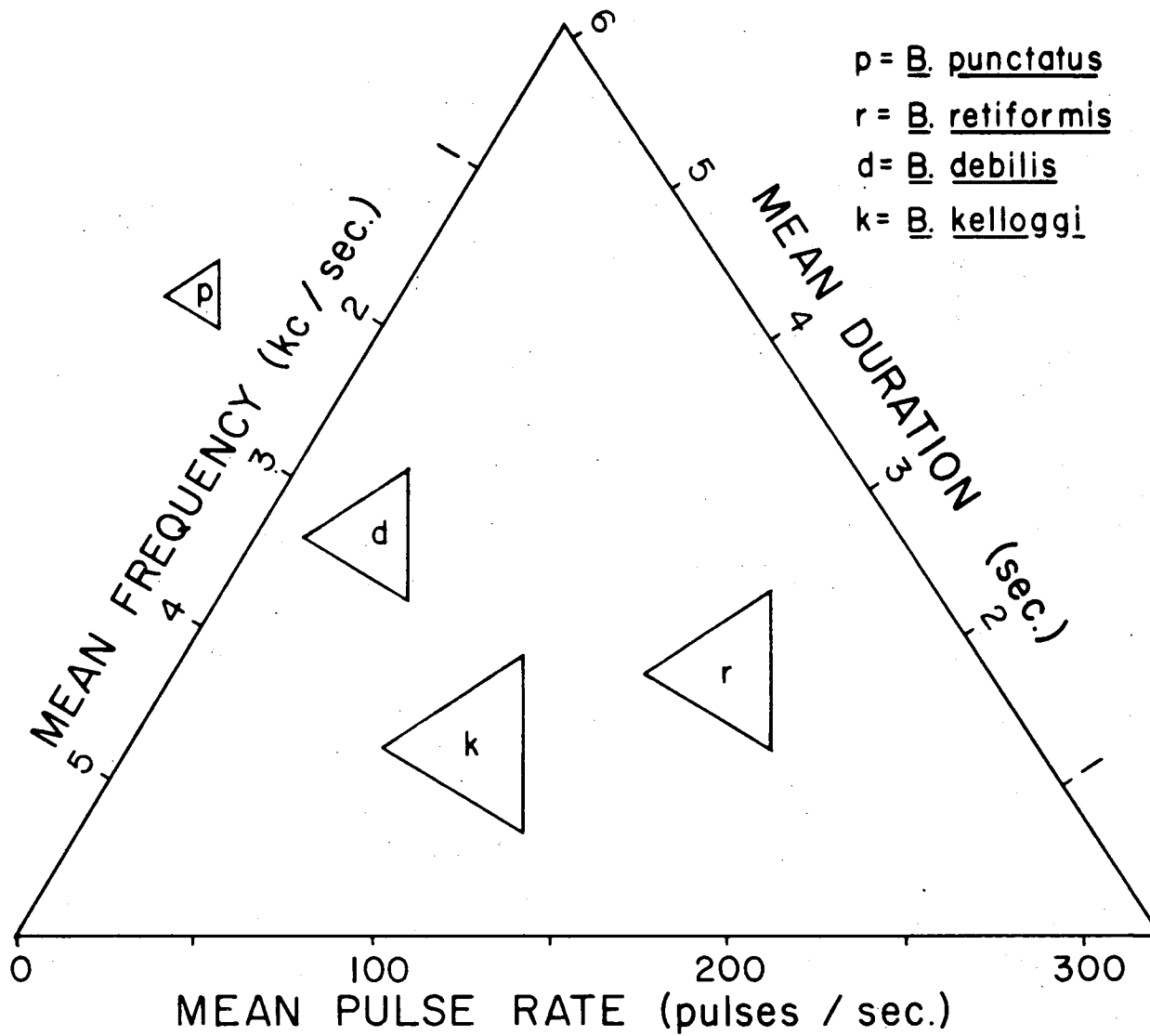


Fig. 19. Two dimensional graph of mean pulse rate plotted against mean duration and mean frequency. Data from Table 10.

female of Pseudocris triseriata does not respond to a species specific pulse rate. In their work the number, spacing and patterns of intensity and frequency of male calls were altered individually and played to the females. A positive response by a female was indicated by her movement toward the source of the calls. Of particular interest is their method of determining the animals' response to pulse rate. The response to a seventeen-note artificial call at 1,000 cps and 0.57 sec. duration was compared to a continuous artificial sound of 0.57 sec. and 1,000 cps. Six out of seven animals responded to the single note call while only four out of eight responded to the artificial call. From these results, the authors concluded that the number of notes per call (pulse rate) and the pattern of intensity and frequency are not essential in evoking a response from the female. However, the authors obtained the same response from the natural call as from the seventeen-note artificial call. Also, it was later demonstrated that the animals responded to a frequency of 1,800 cps and would not respond to 1,000 cps as in the above experiment. Obviously more than one interpretation can be placed on the results they report. Even though less weight can be placed on results which are derived from small sample sizes, it is obvious that one can state that P. triseriata responds to a definite frequency range and quite likely also responds to a species specific pulse rate.

In Figure 18, pulse rate is plotted against frequency and duration. It should be noted that although "relatively little divergent" in Bogert's (1962) statement for his data, the mating calls of B. debilis and B. kelloggi are distinct at the 95 percent level with respect to both frequency and duration. Further, it is to be emphasized that all

species in this group differ in at least two characteristics of the call.

Until more evidence is accumulated, relatively little can be said with assurance about the importance of different characteristics of the call with respect to mate attraction and chorus formation. However, as pointed out by Bogert (1962), pulse rate is probably more important as an isolating mechanism between the species of this group than are other quantifiable characters of call such as duration and frequency (Fig. 18). Nevertheless, frequency can not be discounted. For with a little experience the calls of B. retiformis and B. kelloggi can be distinguished in the field by the human ear, on frequency alone.

In all probability the recognition is dependent on a summation effect of all three measurable properties--frequency, duration and pulse rate. It is also quite possible that frequency and pulse rate might differ in function in that the frequency may be the dominant stimulus at a distance, while pulse may play a greater role in the female's recognition of a mate after the breeding chorus has been reached.

It is shown in Fig. 20 that frequency varies directly with body size ($r = 0.95$). This indicates that the vocal mechanism controlling frequency in this group has changed directly with change in body size during the evolution of the species. This is what one would expect in view of the importance of size as an isolating mechanism in anurans (Lowe, 1954). It is obvious that a nocturnal anuran should have some vocal mechanism which corresponds to size, and especially in a closely related group such as the B. punctatus group where change in body size has been such a conspicuous aspect of its evolution.

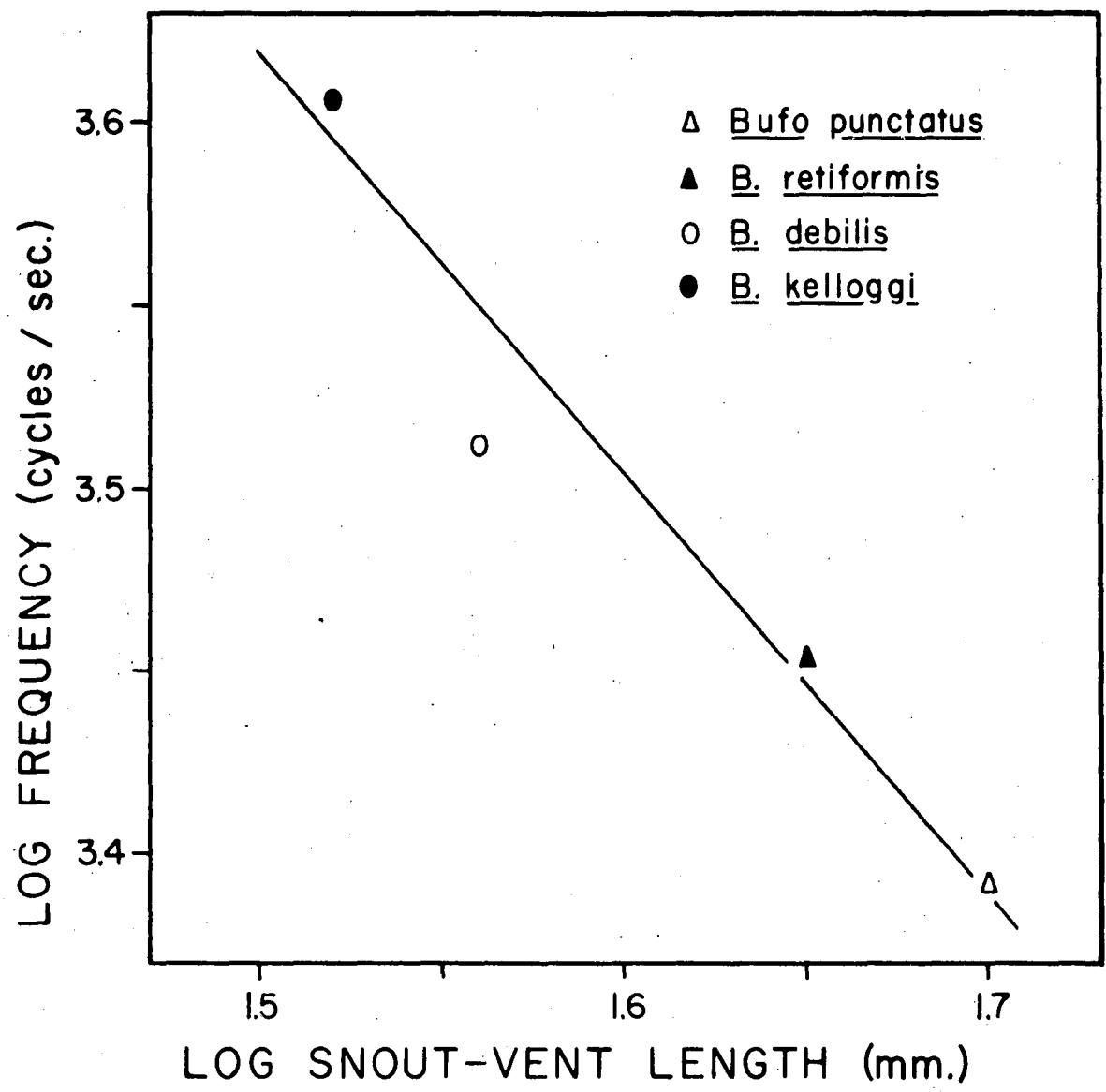


Fig. 20. Regression of log mean call frequency on log mean snout-vent length where $\log Y = 5.23 + (-1.077) \log X$, $r = 0.97$ and $P < 0.05$.

Porter (1964) based the evolutionary relationships of the B. valliceps group primarily on structural morphology and meristic aspects of the mating call. He noted a difference in call-frequency which seemed to be related to body size. However, he noted little quantifiable difference since he did not recognize the logarithmic relationship of the vocal frequency to the body size.

Porter (1964) also states that pulse rate varies inversely with body size. This is not the case in the B. punctatus group (Tables 1 and 10). Pulse rate should be dependent on the tension of the vocal mechanism and the pressure of the air as it is released from the vocal apparatus. Where the call is an important isolating mechanism between two sympatric species such as B. retiformis and B. punctatus, where there is overlap in the effective adult size (Fig. 5) and consequently the call frequency (Fig. 18), a second mechanism such as pulse rate which does not correspond to body size would have unquestioned selective potential.

Call duration, another easily measured portion of the anuran mating call, is probably in part controlled by body size. However, this is also an aspect of the call which one would expect to be controlled by the state of excitement of the individual. Call-duration could be a major factor in the apparently erroneous conclusion drawn by Martof and Thompson (1964), in that their animals could have been responding to states of excitability rather than species specificity. Moreover, in order to draw valid conclusions from the results for call-duration, one should attempt to have constant laboratory conditions which are in effect physiologically ideal for the species involved.

Field Observations.--Bufo kelloggi and Bufo retiformis.--

It is assumed that one function of the anuran call is for spatial isolation, of one degree or another, of sympatric species at the breeding site. The accumulation of individuals of both B. retiformis and B. kelloggi into their separate choruses was studied in the field at localities of sympatry in Sonora, Mexico during the summers of 1961-1963. The principal area of study was north and south of the Kino Road between Hermosillo and 22.5 mi. (rd.) W. Hermosillo where Lowe and Zweifel first discovered B. kelloggi and B. retiformis sympatric, as reported by Bogert (1962). I have been able to verify repeatedly the finding of Lowe and Zweifel that calling and pair-formation (amplexus) in B. retiformis ordinarily takes place on dry, damp, or wet ground, very often at truly remarkable distances from the water's edge (up to ca. 55 ft; 18 m.).

I noted B. kelloggi to be restricted to calling over a much more narrow range of temperature and humidity than B. retiformis, and, further, that it calls predominantly during or immediately after a rain. For example, on July 15, 1963, approximately two days after a heavy rain in the vicinity of Hermosillo, nine B. kelloggi (6 3) and forty B. retiformis were collected at 14.0 mi. (rd.) W. Hermosillo. After reaching the chorus, it was found that the water in a small arroyo had since dried to a small puddle. B. retiformis was situated as usual with the males calling on dry soil where several pairs were in amplexus. None of the six male B. kelloggi were calling and all nine individuals were found either in the water, at its edge, or sitting on the water-saturated mud immediately surrounding it. On the same night, at

22.5 mi. (rd.) W. Hermosillo, another large chorus of B. retiformis was observed. Many of these were also found in amplexus at various distances from the water (3 - 55 ft.). No B. kelloggi were seen. At this time (1:00 a.m.), surface temperature (dry ground) was 30.2°C, air temperature 30.0°C, water temperature 26.6°C, and relative humidity 84 percent.

On July 28, 1961, at 18.0 miles west of Hermosillo, after several days without rain, a shower started at 2:30 p.m. and lasted approximately thirty minutes. After the rain, water in the arroyo breeding site was approximately two feet deep. At this time a large chorus of B. kelloggi was heard and only a few B. retiformis; Scaphiopus couchi, S. hammondi and Microhyla olivacia were also calling. At 3:00 p.m. during the strongest chorus of kelloggi, water temperature was 27.2°C and relative humidity 90.5 percent. By 4:30 p.m. the humidity was much reduced, and the kelloggi chorus had almost stopped, with only occasional calls heard; at this time, water temperature was 27.2°C, air temperature 27.2°C and relative humidity 81.5 percent. At 5:15 p.m. the relative humidity was 82.6 percent, and B. kelloggi was calling more strongly. At dusk (6:15 p.m.) B. kelloggi was again calling strongly; at this time the air temperature was 36.0°C and relative humidity 81.2 percent. In this case, calling in B. kelloggi seemed to be stimulated by two factors: the onset of rain and the approach of dusk.

Bufo debilis.--Bufo debilis was found to call at a somewhat lower temperature than the other species in the group. For example, near Cochise, Arizona, the body temperature of B. debilis was not found to exceed 18.1°C when calling throughout the night of July 30, 1961.

The minimum body temperature obtained for calling males of this species over three summers (1961-1963) was 17.4°C (wet surface, 17.8°C). Air temperature where males of this species were calling ranged from 16 to 20°C during this three year period. This temperature range is much lower than the temperatures recorded for the other species of the group.

At this same locality near Cochise, Arizona, B. debilis was observed to call at the bases of large stands of bunch grass. At Benson, Arizona, it was found calling up to three feet away from the nearest vegetation. This species calls during or immediately after rain on wet ground or in the water.

Bufo punctatus.--Bufo punctatus generally calls out of the water, frequently on boulders or rocks at the water's edge. Although large choruses of this toad have been found, it also frequently calls while alone or in small groups of up to ten males. Bufo punctatus males were found to call where air temperature ranged from 20 to 27°C. This species seldom calls at water temperatures in excess of 26°C.

As seen in Table 9, the frequency of the call of B. debilis at Cochise and Benson, Arizona, increases by 186 cps with a change in water temperature of 2.8°C (20.2-23.0°C). B. retiformis 2.2 mi. E of Sells, Arizona, at a water temperature of 25.2°C has a mean call frequency of 2.85 kc. From this one can see that if sympatric, therefore at comparable body temperatures, the calls of these species would be quite different. All of the species show some overlap with respect to call duration (Fig. 18), which probably varies with the physiological state of the organism as obligated by extrinsic factors.

In summary, it can be said that calls of the members of the Bufo punctatus group are quite similar in structure, but differ in two or more of the three major aspects of the call which are frequency, duration, and pulse rate.

Certain characteristics of the call, such as an initial rise in frequency and overall frequency, can be used after some practice to distinguish the species in the field.

Some overlap occurs between the range of frequencies of the calls of B. debilis and B. retiformis. This overlap is probably due to the differences of breeding temperatures and similarity of body size.

It is shown by the data presented here that the call of B. punctatus is quite similar to that of the other members of this group. Within the group, pulse rate is probably the strongest species specific characteristic. Frequency is correlated with body size, and, due to the importance of body size as an isolating mechanism, frequency has developed as an important secondary isolating mechanism in this group of anurans.

The calling behavior of these species is basically similar, except for the widely sympatric species B. retiformis and B. kelloggi. In these species, B. retiformis tends to call at points away from the edge of the water while B. kelloggi calls from the water or from its edge. B. kelloggi also seems to be restricted to a more narrow range of environmental conditions for reproduction than is B. retiformis, and B. debilis calls at a considerably lower temperature than the other members of this group.

Speculations on Origins.--It certainly appears that the call of B. punctatus or of a Bufo punctatus-like species could well give eventual

rise to those of B. kelloggi, B. debilis and B. retiformis (Figs. 18 and 19). Further, this would seem reasonable in a species existing in the formerly more widespread subtropical scrub of western North America which was evolving toward the Sonoran Desert of today (Axelrod, 1950). In this situation of increasing aridity and seasonal climatic extremes the anuran population might effect a trend toward smaller body size as an adaptation toward efficiency in burrowing habitus. Such a trend in body size would in turn give eventual rise to a species call of higher frequency.

During the development of the grasslands of middle latitudes of North America during Mio-Pliocene time, it appears that a small grassland species evolved in ecological as well as spatial isolation and the resulting call did not diverge greatly from the parental species. For while the call of B. debilis presently found in grassland is distinct--with respect to pulse rate--from the call of B. kelloggi which is found in the subtropical scrub of Sinaloa and southern Sonora, the difference is by no means of the same order of magnitude as the small difference exhibited between B. debilis and the other species in the group.

As a result of progressively increasing aridity, a western portion of this grassland population was later differentiated in western North America in an area of interspersed grassland and subtropical scrub which was destined to become the Sonoran Desert as we know it today (see Axelrod, 1950). B. retiformis probably evolved in this situation.

It appears certain indeed that B. kelloggi evolved in the coastal thornscrub of the Gulf Coast where it remains restricted today in modern derivative communities of the Madro-Tertiary Geoflora,

located directly south of the Sonoran Desert.

Bufo punctatus, which represents a close vertical derivative of the ancestral type (probably represented by the fossil Bufo suspectus Tihen), has become a reasonably successful species well-adapted to arid and semi-arid lands yet more or less restricted to riparian and flood-plain situations throughout its wide range across grasslands and deserts into peripheral communities of woodland and thornscrub. Today Bufo punctatus obviously represents a more ecologically restricted relic of its more moist-adapted and undoubtedly widely spread tropical or subtropical ancestor.

During the sympatric secondary contact of B. kelloggi and B. retiformis which persists today where desertscrub and thornscrub meet in southern Sonora, the much more distinctive call of B. retiformis (as compared to B. debilis which is not greatly different from B. punctatus) and its differentiation from B. kelloggi undoubtedly evolved under selective pressures favoring such a definite character displacement (see Brown and Wilson, 1956).

Hybridization

The first results of hybridization experiments involving members of the genus Bufo in the United States were reported by A. P. Blair in 1941, 1942 and 1943. Blair found that toads of the Bufo americanus group would hybridize both in nature and in the laboratory. The field picture was contributable in part to the destruction of the natural habitats of these species by man thus allowing new intermediate or completely alien habitats to arise and become populated by individuals from

the species of surrounding ecological areas (A. P. Blair, 1941). Temporal and thermal factors are also essential in maintenance of isolation between members of this group. It has been demonstrated that B. fowleri and B. americanus are isolated by temperature and time, although some overlap between the physiological adaptations of both species exists (Cory and Manion, 1955).

The toads of the Bufo punctatus group have been essentially unaffected by man-made ecological disturbance on a large scale where sympatry or approximate sympatry occurs. Of the four species involved, the following sympatric contacts exist:

1. B. punctatus and B. debilis, over much of the range of B. debilis.
2. B. punctatus and B. kelloggi, in the northern part of the range of B. kelloggi.
3. B. punctatus and B. retiformis, over much of the range of B. retiformis.
4. B. kelloggi and B. retiformis, in the southern part of the range of B. retiformis.

In the first three cases, the species remain essentially isolated in the same areas through habitat selection. B. punctatus is largely restricted to rocky, riparian situations, while B. retiformis, B. debilis, and B. kelloggi are restricted for the most part in areas properly termed desertscrub, grassland and thornscrub, respectively (Lowe, 1964). The last case (B. retiformis and B. kelloggi) results in fully sympatric contact on a microhabitat level and isolation here seems to be maintained primarily through behavioral mechanisms.

In view of the wide area of sympatry involved, it is notable that extremely few natural hybrids or intermediates have been reported, these total approximately nine individuals (Smith and Chrapliwy, 1958; Reimer, 1955). In general amphibian hybrids exhibit almost complete intermediacy (Twitty, 1936). The nine specimens noted above have been examined, and of these, only one (collected by Reimer) appears to be intermediate (Fig. 21). The others are without doubt typical of Bufo kelloggi and show no resemblance to B. retiformis except through those morphological characteristics which warrant their allocation to the same species group. The specimen referred to as intermediate (Fig. 21) exhibits an intermediate color pattern, an intermediate head shape more similar to Bufo retiformis, and scoliosis which is characteristic of interspecific hybrids in general (W. F. Blair, 1963a). This is indeed a hybrid and one of the specimens referred to by Bogert (1962) as Bufo kelloggi; it is noted with some surprise that Bogert was unable to distinguish the hybrid of these two distinctive species on which he has recently reported.

The virtually complete absence of natural hybrids in this case is taken as further evidence that genetic isolation between these species is essentially complete and that isolation does not exist merely on some lesser level (e.g., physiological inhibition of hybrid larvae due to temperature stress). Physiological differences between species segregate in the same manner as morphological ones (Volpe, 1952) but may not have the same effect in these populations for the following reasons: (1) no temporal difference between breeding seasons is present due to the necessity of breeding opportunism in arid and semi-arid monsoonal

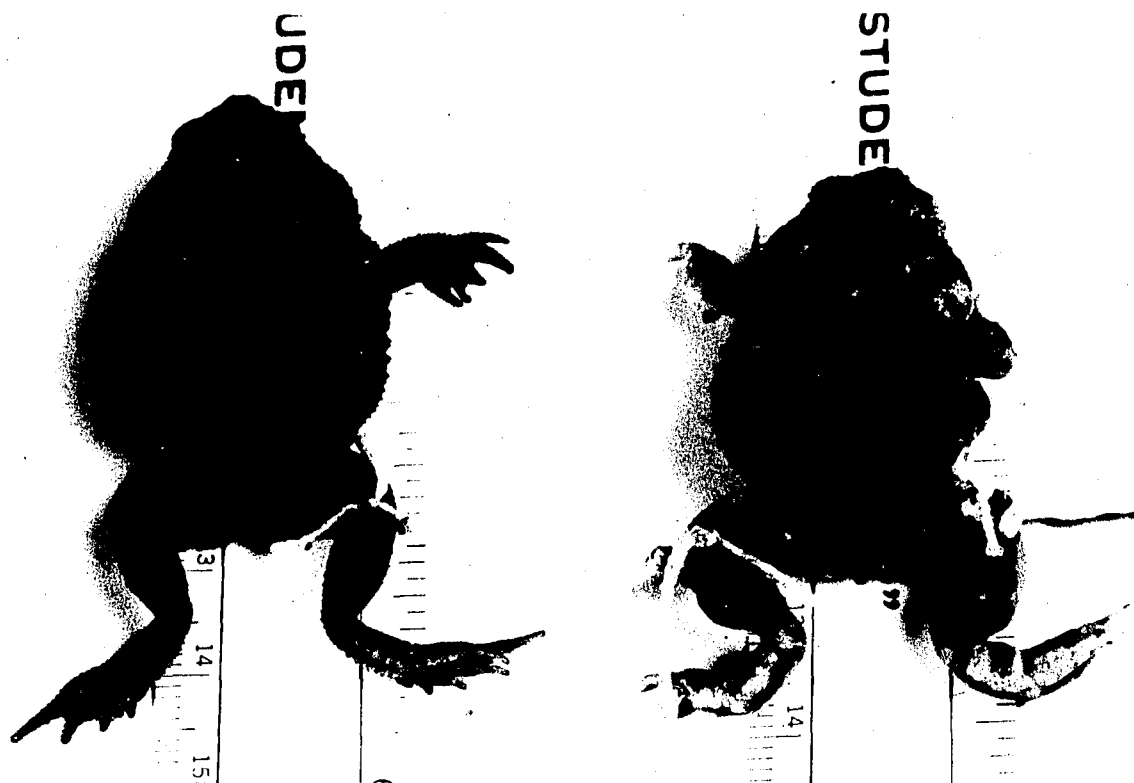


Fig. 21. Left, adult female of Bufo kelloggi (UIMNH 40575), reported as intergrade between B. kelloggi and B. retiformis (Sanders and Smith, 1958). Right, hybrid adult female, B. kelloggi X B. retiformis (MVZ 58793), collected by W. J. Reimer and R. G. Zweifel at 11 mi. NW of Vicam, Sonora, Mexico (Reimer, 1955).

environments; and (2) no temperature differences due to spatial separation in breeding aggregations (of a nature preventing hybrid development) have been observed nor, in fact, appear probable.

Hybrids frequently exhibit morphological anomalies in tissues of the reproductive system. F_1 crosses of Bufo valliceps males and B. fowleri females, exhibit secondary sexual characteristics with atypical testes, the anterior portion of which is composed of unusually large amounts of ovarian tissue somewhat similar to an overdeveloped bidder's organ (Volpe, 1956). The single hybrid (Reimer, 1955) shown in Fig. 21 is obviously a female with well developed eggs which are still inclosed by ovarian tissues. This hybrid indicates that hybrid inviability between B. kelloggi and B. retiformis very likely results in an early interaction which leads to zygotic death rather than gametic inviability.

Whenever natural hybridization is limited, or where more quantifiable data are desired, laboratory hybridization can reasonably be employed as a "crude reflection of total genetic difference and hence of evolutionary affinity" (Blair, 1963a).

Inviability in early stages apparently occurs through varying degrees of restriction and is relative to the particular cytoplasm and sperm involved. Thus, the same points or times of hybrid death do not always occur between reciprocal hybrids of the same species (Volpe, 1955). Hybrid inviability can therefore be related, in many cases, directly to incompatibility between sperm and eggs. It is well known that anuran eggs contain a large amount of cytoplasmic deoxyribonucleic acid (Gregg and Lovtrup, 1955), the amount of which is estimated to be

at least sufficient for all the nuclei up to late blastula (Moore, 1961). Thus it is postulated that during replication, the genetic material of the male pronucleus uses cytoplasmic DNA of the female parent which results in copy errors due to differential sequences between cytoplasmic and nucleic DNA (Moore, 1962).

The toads of the Bufo punctatus group were earlier referred to as the debilis-punctatus group (Camp, 1927) on the basis of skull morphology, and Group II (Baldouf, 1959) on the basis of general osteology. They have been referred to as the Bufo debilis group (Blair, 1963a) and the Caribbean section of the Bufo valliceps group (Tihen, 1962a). These species have been previously investigated through hybridization in a fortuitous way by Blair (1963a) who confused their close relationship as follows: He considered B. debilis, B. retiformis and B. kelloggi to be related to a "northern line" consisting principally of the B. boreas and B. americanus groups, and allocated B. punctatus to a "southern line" consisting principally of the B. valliceps group.

Hybridization as evidenced by results from crosses in this laboratory (Tables 11-17) indicate reciprocal metamorphosis between all members of the B. punctatus group. In general, anuran hybridization data, where reciprocal metamorphosis is evident, is taken as evidence for the allocation of species into species groups.

Previous investigations involving intragroup compatibility between bufonid species have not been extensive except in those species comprising the Bufo americanus species group of toads (Blair, 1963b). As noted in Table 17, reciprocal metamorphosis between each species of the B. punctatus group was obtained whenever laboratory temperature

Table 11. Summary of hybridization data for crosses made during the summer of 1961 using Bufo kelloggi females.

Cross	No. Eggs	% to reach blastula stage	% to reach yolk plug	% to reach neural crest	% to reach tail bud	% hatch
<u>B. retiformis</u> male x						
<u>B. kelloggi</u> female						
a	103	100	53.4	21.4	9.7	-
b	49	100	95.9	61.5	53.1	46.9
c	47	100	70.2	27.6	19.1	-
<u>B. debilis</u> male x						
<u>B. kelloggi</u> female						
a	88	100	79.5	36.1	9.1	-
b	43	100	79.1	60.4	48.8	-
c	45	100	73.3	15.5	11.1	4.6
<u>B. kelloggi</u> male x						
<u>B. kelloggi</u> female						
a	63	100	100.0	11.1	1.6	-
b	59	100	64.4	64.4	45.7	28.8

Table 12. Results of crosses made during the summer of 1961
between Bufo retiformis males and Bufo punctatus females.

Cross	No. Embryos	No. Metamorphosis	% Metamorphosis
1	49	0	-
2	28	2	7.1
3	11	2	18.2
4	10	1	10.0
5	28	1	3.6
6	24	3	12.5
7	25	1	4.0
8	22	2	9.1

Table 13. Results of crosses made during summer of 1962 using Bufo punctatus females.

Cross	No. Embryos	No. Met.	% Met.	Mean and range of metamorphosis time in days
<u>B. punctatus</u> x <u>B. punctatus</u>	71	53	74.5	99.1 ± 5.3 (29-180)
A ₁ <u>B. retiformis</u> x <u>B. punctatus</u>	67	25	37.4	169.6 ± 13.4 (83-306)
A ₃ <u>B. retiformis</u> x <u>B. punctatus</u>	70	18	26.7	133.9 ± 8.6 (101-193)
A ₄ <u>B. retiformis</u> x <u>B. punctatus</u>	66	20	30.3	136.8 ± 9.8 (42-181)
A ₇ <u>B. retiformis</u> x <u>B. punctatus</u>	67	22	32.8	132.1 ± 4.1 (74-216)
A ₉ <u>B. retiformis</u> x <u>B. punctatus</u>	60	23	38.4	153.5 ± 8.3 (87-223)
A ₂ <u>B. kelloggi</u> x <u>B. punctatus</u>	66	58	88	67.4 ± 4.3 (27-142)
A ₅ <u>B. kelloggi</u> x <u>B. punctatus</u>	66	43	65	80.4 ± 6.0 (25-167)
A ₈ <u>B. kelloggi</u> x <u>B. punctatus</u>	70	49	70	52.8 ± 3.2 (24-128)
A ₆ <u>B. debilis</u> x <u>B. punctatus</u>	55	22	40	129.5 ± 7.9 (74-216)
B ₂ <u>B. punctatus</u> x <u>B. punctatus</u>	32	27	84.5	32.1 ± 4.8 (22- 50)
B ₁ <u>B. retiformis</u> x <u>B. punctatus</u>	40	29	72.5	91.6 ± 8.9 (29-175)
B ₃ <u>B. kelloggi</u> x <u>B. punctatus</u>	23	18	78.3	25.8 ± 0.9 (21- 31)
B ₄ <u>B. debilis</u> x <u>B. punctatus</u>	36	33	91.5	74.5 ± 6.1 (27-140)

Table 13 (continued)

Cross	No. Embryos	No. Met.	% Met.	Mean and range of metamorphosis time in days
C ₂ <u>B. retiformis</u> x <u>B. punctatus</u>	29	16	55.2	121.0 ± 9.1 (86-175)
C ₃ <u>B. retiformis</u> x <u>B. punctatus</u>	27	7	26.9	109.4 ± 15.0 (41-163)
C ₁ <u>B. kelloggi</u> x <u>B. punctatus</u>	33	6	18.4	53.2 ± 11.8 (25- 84)

Table 14. Results of crosses made during the summer of 1962
using Bufo debilis females.

Cross	No. Embryos	No. Met.	% Met.	Days to first Met.	Days to last Met.
<u>B. debilis</u> x <u>B. debilis</u>	25	-	-	-	-
<u>B. punctatus</u> x <u>B. debilis</u>	13	12	92.5	19	93
<u>B. retiformis</u> x <u>B. debilis</u>	11	1	.91	66	-
<u>B. kelloggi</u> x <u>B. debilis</u>	6	1	16.7	70	-

Table 15. Results of crosses made during the summer of 1963

using Bufo retiformis females.

Cross	No. Eggs	No. to reach stage 20	% stage 20	No. feeding larvae	% feeding larvae	No. Met.	% Met.	Days Last Met.
<u>B. retiformis</u> x <u>B. retiformis</u>	61	-	-	45	73.8	0.0	-	-
<u>B. punctatus</u> x <u>B. retiformis</u> #3	50	0.0	-	-	-	-	-	-
<u>B. punctatus</u> x <u>B. retiformis</u> #2	56	5	8.95	4	7.15	0.0	-	-
<u>B. punctatus</u> x <u>B. retiformis</u> #1	51	0.0	-	-	-	-	-	-
<u>B. debilis</u> x <u>B. retiformis</u> #2	100	13	13	9	9.0	0.0	-	-
<u>B. debilis</u> x <u>B. retiformis</u> #1	60	18	30.0	5	8.35	1	1.67	47
<u>B. kelloggi</u> x <u>B. retiformis</u> #1	198	17	8.60	14	7.07	0.0	-	-
<u>B. kelloggi</u> x <u>B. retiformis</u> #2	200	0.0	-	-	-	-	-	-

Table 16. Results of crosses made during the summer of 1963

using Bufo kelloggi females.

Cross	No. Eggs	No. to reach stage 20	% stage 20	No. feeding larvae	% feeding larvae	No. Met.	% Met.	Days 1st Met.	Days last Met.
<u>B. kelloggi</u> x <u>B. kelloggi</u>	100	80	80	26	26	0	-	-	-
<u>B. punctatus</u> x <u>B. kelloggi</u> #1	109	9	8.25	6	5.5	6	5.5	29	41
<u>B. punctatus</u> x <u>B. kelloggi</u> #2	79	4	5.06	4	5.06	0	-	-	-
<u>B. retiformis</u> x <u>B. kelloggi</u> #1	203	53	26.1	29	14.3	4	1.97	45	51
<u>B. retiformis</u> x <u>B. kelloggi</u> #2	59	9	15.4	9	15.4	0	-	-	-
<u>B. debilis</u> x <u>B. kelloggi</u> #1	153	40	26.1	35	22.9	1	.09	59	-
<u>B. debilis</u> x <u>B. kelloggi</u> #2	101	1	.99	0	-	-	-	-	-

Table 17. Results of laboratory hybridization during the summers of 1961, 1962 and 1963. Ad = adult, SAd = subadult, M = metamorphosis, L = larvae (feeding).

	<u>punctatus</u>	<u>retiformis</u>	<u>debilis</u>	<u>kelloggi</u>
<u>punctatus</u>		Ad	SAd	SAd
<u>retiformis</u>	L*		M	L*
<u>debilis</u>	SAd	M		M
<u>kelloggi</u>	M	M	M	

* 1963 data, in which laboratory temperature control was probably inadequate and crosses would otherwise have gone to metamorphosis.

conditions permitted. From the data shown here (Tables 13-16), it can be observed that the best results from hybridization experiments occurred in those crosses which utilized B. punctatus as one of the parental forms (sperm or eggs).

It therefore appears obvious enough that the eggs of this species (B. punctatus) contain a general array of cytoplasmic materials essential for development of the other species and hence exhibit a more generalized genetic composition characteristic of a more primitive ancestral species in this particular genetic line. One would assume that an ancestral species would have a larger array of genetic material than a derivative which has evolved into a more restricted habitat, thereby narrowing its genetic assemblage; to this would be added the complication of new genetic additions which would not be complemented in the ancestral stock. Thus, the most recent member of a species group may likely show a greater proportion of newer genetic material which is incompatible with the genetic assemblage of the ancestral stock. Dobzhansky (1951) has stated this proposition as follows: "In the course of evolution, the function of a gene in the development may undergo such changes that a gene may subtend developmental processes other than those with which it was previously concerned." Thus, a greater number of genes in a derivative species which are present in the ancestral species should permit greater development of the hybrid offspring.

Accordingly, due to its certainly more primitive nature, the sperm of B. punctatus is able to complement genetic material of the eggs of B. debilis, B. retiformis, and B. kelloggi. In experimental

crosses which did not involve B. punctatus, it was found that the sperm of B. kelloggi had greater affinity for the eggs of B. debilis than for those of B. retiformis (Tables 14 and 15). In 1961, the reciprocal crosses were made using the sperm of B. debilis and B. retiformis (Table 11). Slightly better results were obtained in the crosses involving males of B. retiformis. When these crosses were attempted again in 1963, better results were obtained with the sperm of B. debilis than with that of B. retiformis (Table 16).

It is noteworthy that, as near as could be determined, fertilization was complete in all crosses. This indicates a closer genetic affinity between the species comprising the Bufo punctatus group than between those comprising the B. americanus group which is an equally close-knit species complex. In the latter, fertilization ranges from low to 100 percent depending upon the particular cross attempted (Blair, 1963b).

In Table 11 the results of crosses between B. retiformis and B. debilis males with the females of B. kelloggi indicate a high mortality between yolk plug and neural crest, but may be partially indicative of experimental temperature stress. These larvae were maintained at room temperature in an air conditioned building for the duration of the experiment. Data for the same period under similar conditions during the summer of 1963 indicates that the water temperature in these samples reached a minimum of at least 18.5°C and did not exceed 22°C.

For the crosses between males of B. retiformis and females of B. punctatus during the summer of 1961 (Table 12), it should be mentioned that there was approximately 90 percent fertility with 35

percent of the embryos reaching a feeding larval stage. The mean temperature for these crosses was approximately 20°C. Metamorphosis in this series of crosses is probably indicative of a tolerance adaptation of B. punctatus to habitats often with colder water than ordinarily experienced by other members of this group which are restricted exclusively to shallow rain pools usually of relatively high temperature; such breeding waters containing eggs and larvae were found to commonly exceed 30°C during the day. In the light of this evidence, it is interesting to note that Blair (1962) has found little evidence to affiliate B. punctatus with his B. debilis group. The water temperature at which his larvae are raised seems to be the most overwhelming evidence for their assumed genetic affinities.

Results of crosses made during the summer of 1962, using females of B. punctatus from the Tucson area, are shown in Table 13. Although the temperatures remained fairly uniform between crosses, three population conditions are represented based on the number of embryos in each pan. Series A represents a situation in which a total of 55 to 71 embryos was placed in single enamel pans at the time of hatching; series B in which there were 23 to 40 embryos per pan; series C represents three crosses with from 25 to 33 larvae placed in smaller pans (7 x 12 in.) which were about one-half the size of the pans used in series A and B. It should be noted that although the percentage of metamorphosis varies with each individual cross, it is fairly uniform for each hybrid combination in each of the three (A, B, C) series. Where the number of individuals per pan differs, as between series, a difference in percent metamorphosis is observed, while in general the same relationship between

crosses is maintained within a given series. In other words, in both Series A and series B, B. retiformis crosses seem to differ more markedly than the crosses involving B. debilis and B. kelloggi from the B. punctatus control. Also, the percent metamorphosis in A₂ (B. kelloggi male X B. punctatus female) is greater than the control, but differs only by approximately 7 percent. In this same series, forty percent of the embryos resulting from a cross between a B. debilis male and a B. punctatus female (A₆) completely metamorphosed. These results are somewhat greater in magnitude than the results of crosses between members of other anuran species groups such as Scaphiopus couchi X Scaphiopus holbrooki (Wasserman, 1963).

The time for metamorphosis varies between crosses and should represent a parameter indicating genetic affinity. From the summary in Table 13 it can be seen that the range of the metamorphosis-time in days is quite short when Bufo punctatus and B. kelloggi males are crossed with Bufo punctatus females, while the crosses between B. debilis and B. retiformis males in comparison demonstrate a greater range of metamorphosis time and a longer time between fertilization and first metamorphosis.

Little crowding of the embryos occurred during the experiments, and the presence or absence of this factor is not important where other conditions are equal. One might also maintain that the high percentage of metamorphosis and the rapid rate of metamorphosis of the B. kelloggi crosses (Table 13) in comparison to the B. retiformis and B. debilis crosses is a result of the smaller size of the B. kelloggi individuals which would give rise to smaller larvae. Although the B. kelloggi X B. punctatus hybrids in some cases appeared to be smaller, they were not

significantly smaller than the other hybrid combinations in this series. The B. kelloggi crosses in each case had a higher percent metamorphosis and shorter metamorphosis time than the debilis crosses even though the latter contained fewer individuals per pan. In fact, A₂ contained 20 percent more individuals than did A₆ while the percent metamorphosis was 110 percent higher. Also, it should be noted that the larger B. punctatus controls had essentially the same percent metamorphosis and the same minimum time for first metamorphosis as the B. kelloggi crosses.

With respect to mean time for metamorphosis and percent metamorphosis, the B. kelloggi crosses are very similar to the B. punctatus control (Fig. 22). When viewed in this manner, the Bufo retiformis and B. debilis crosses in this series are considerably different from the control. The regression line in Fig. 22 represents the means of ten samples and has a regression coefficient of 0.844 with an associated probability value (P) of less than 0.01 (= 0.0058). This clearly demonstrates a close genetic relationship between B. kelloggi and B. punctatus and illustrates the uniform genetic changes which occur between the species of this group.

A high percentage of mortality occurred in the pans which contained embryos resulting from the crosses between B. debilis females and the males of this group (Table 14). Here, death occurred primarily at gastrulation and tailbud. Of the embryos which hatched, only the crosses between B. punctatus males and B. debilis females went to metamorphosis. Of these metamorphosed individuals, almost all went to a subadult stage. It is indicated, therefore, that B. punctatus represents a genetic species that is much closer to the parental stock of

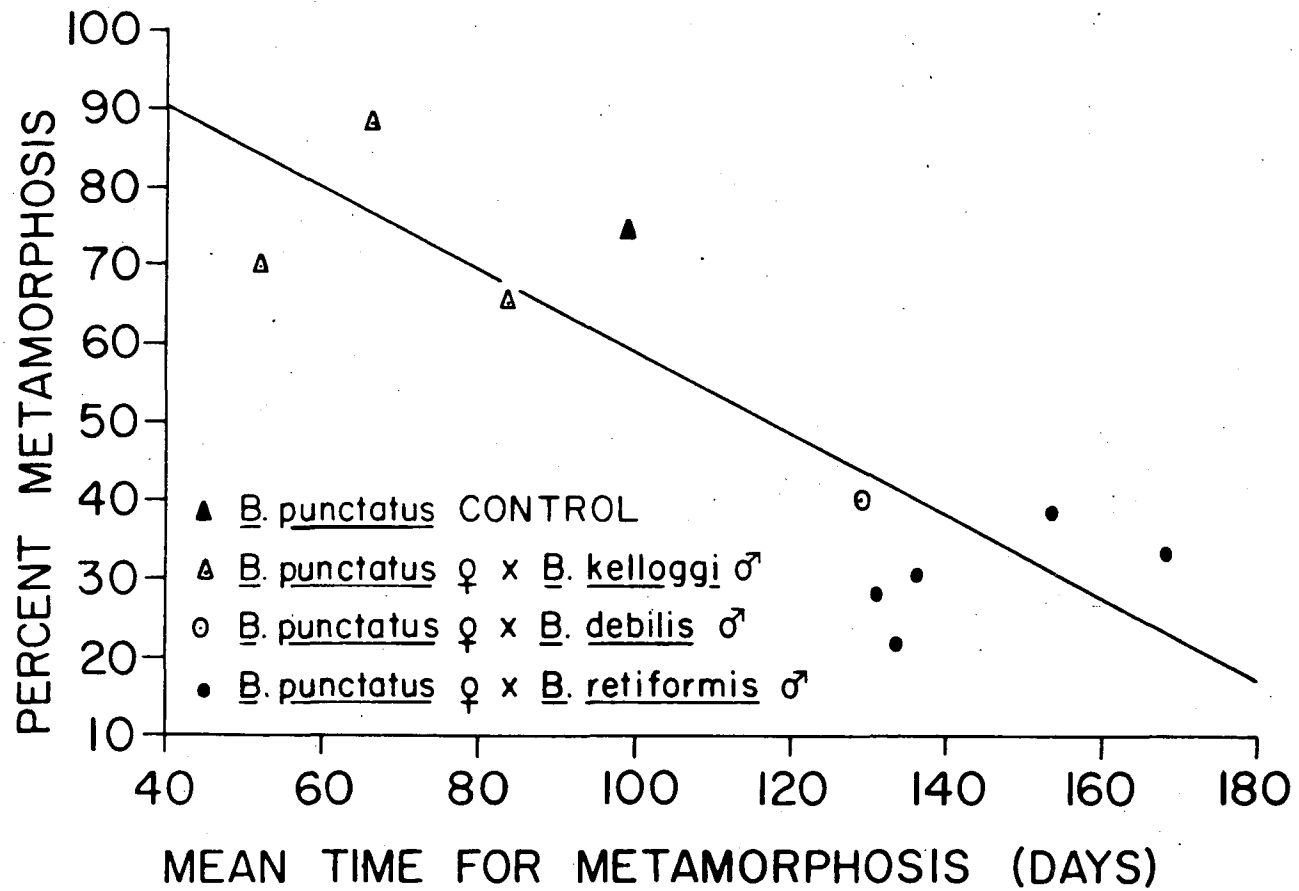


Fig. 22. Regression of percent metamorphosis on mean time for metamorphosis. $Y = 106.53 + (-0.473) X$, $r = 0.844$ and $P < 0.01$.

Bufo debilis than is either B. kelloggi or Bufo retiformis. This would also indicate that genetic isolation between B. debilis and the nearest ancestral species in the group is not as great as it is between B. debilis and the morphologically similar species B. retiformis and B. kelloggi.

In spite of laboratory conditions which were on occasion less than ideal, hybrids resulting from crosses of other species of this group with B. retiformis females were much less viable than the B. retiformis controls. Further, the results as shown here indicate a closer genetic relationship between B. retiformis and B. debilis than between B. retiformis and the other two species (B. kelloggi and B. punctatus).

Crosses between B. kelloggi females and the other members of this group (Table 16) were carried out simultaneously with the crosses represented in Table 15 (interspecific crosses involving B. retiformis females). In this series of crosses, a very low percentage of metamorphosis occurred between all of the hybrid combinations. However, on the basis of staging data, B. punctatus and B. debilis seem to be more genetically compatible with B. kelloggi than is B. retiformis. This is also what one might expect from their ecological relationships and their evolutionary relationships as inferred from the remaining data (above and below).

The colors of the feeding larvae did not differ markedly. Blair (1963a) reports hybrid abnormalities that he refers to as a "hybrid syndrome". Individuals with this syndrome are colorless due to a failure of the melanophores to dilate. Larvae of this type

resulted from a cross in 1961 between a B. debilis male and a B. retiformis female. The tadpoles from the pure species crosses were dark (almost black) with interspersed subcutaneous gold stellate markings. Tadpoles from B. punctatus males X B. debilis females were fairly light in color, approaching amber in some cases. Tadpoles resulting from Bufo retiformis males X B. debilis females (1962) were pale with reddish brown pigmentation located medially and black pigmentation around the eyes.

Metamorphosed Hybrids.---All individuals at metamorphosis were amber to dark brown. This coloration did not differ significantly between hybrids or controls. However, within a short while after metamorphosis, color changes began to appear which resulted in the characteristic brown with red and black spots of Bufo punctatus. By the time the juveniles had attained a snout-vent length of 15 mm., those resulting from crosses between B. kelloggi males and B. punctatus females (Fig. 23) were brown with some of the characteristic brown reticulate pattern of Bufo kelloggi, and all had small red pigment spots characteristic of Bufo punctatus.

Individuals resulting from B. debilis males X B. punctatus females (Fig. 24) were olive with some amber coloration laterally and on the legs, the red punctations of Bufo punctatus were also present and each individual had a particularly abundant accumulation of these on the dorsal surface of the head and on the ventral surface of the pectoral region.

All individuals resulting from crosses between B. retiformis males and Bufo punctatus females (Fig. 23) were identical in coloration,

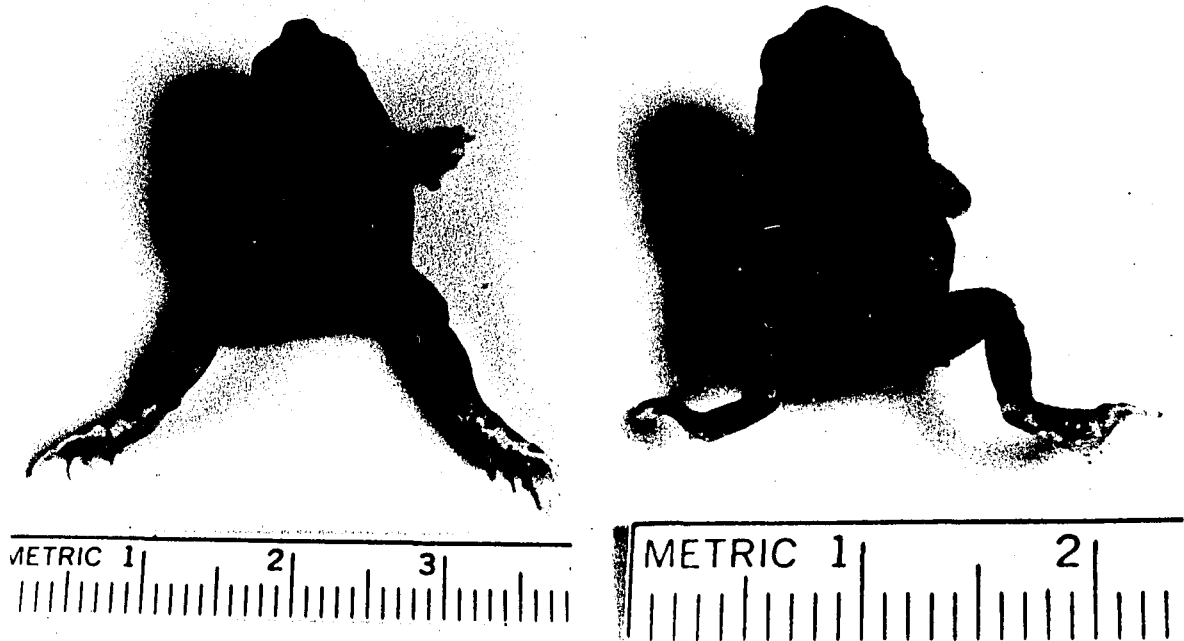


Fig. 23. Hybrids produced experimentally in the laboratory at Tucson during the summer of 1962. Left, Bufo punctatus female X B. retiformis male (UAZ 11941). Right, B. punctatus female X B. kelloggi male (UAZ 11942).

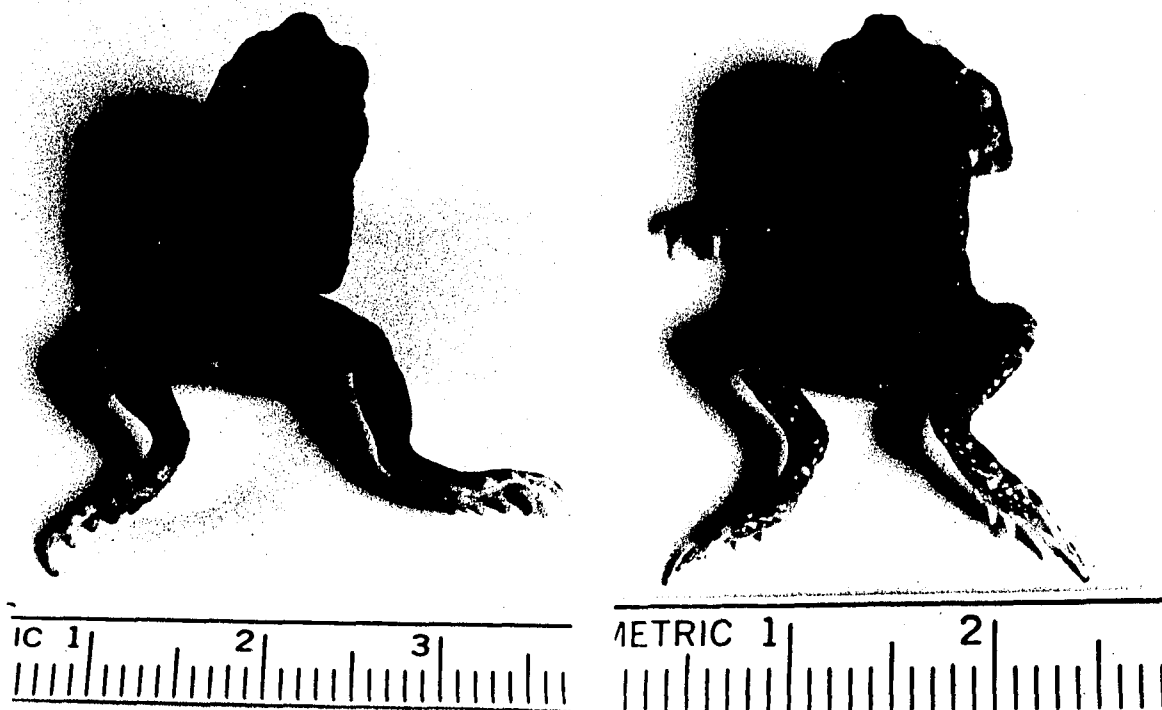


Fig. 24. Hybrids produced experimentally in the laboratory at Tucson during the summer of 1962. Left, B. debilis female X B. punctatus male (UAZ 11943). Right, Bufo punctatus female X B. debilis male (UAZ 11944).

although some were slightly amber in earlier stages. They were olive green with red pigmentation and some black spots in a very similar fashion to those individuals resulting from B. punctatus X B. debilis. The dark pigmentation did not join in a reticulate pattern as in Bufo retiformis.

The hybrids resulting from the cross between B. punctatus males and B. debilis females (Fig. 24) were very similar to their reciprocals, all being green with small black and red punctations. As in the B. retiformis hybrids, no black reticulate pattern was formed.

It is evident from the coloration and the general morphology of the hybrids that diploidy existed in all combinations which survived to a subadult stage. Blair (1963a) states that the existence of haploidy or diploidy in hybrids is not an essential factor and therefore is not critical one way or the other insofar as genetic compatibility is concerned. Indeed the karyotypic state may not be essential when viewed as a "crude" yardstick (Blair, 1963a) but different levels of comparison must certainly exist between cases where haploidy exists on the one hand and diploidy on the other.

New World Establishment.--Blair (1963a) summarizes with some additions the hybridization data presented in some of his earlier papers (Blair, 1959, 1961, 1962) and divides the toads of North America into four phyletic lines. Bufo punctatus appears to him to be derived from a B. melanostictus-like immigrant across the Bering land bridge, and B. debilis from a B. calamita-like immigrant by the same route. This remarkable speculation indicates several things, the least serious of which is an unfortunate disregard for the considerable data derived

from the morphological approach to problems of anuran evolution.

Three entries of the genus Bufo into the new world are postulated by Tihen (1962b). The first, made by a member of the B. calamita group of the Old World, occurred no later than the beginning of the Oligocene and is not represented by extant species in the New World today. The second invasion occurred by early Miocene or before and is represented with one exception by the extant species groups of toads in the new world. The third invasion is represented by the B. boreas group which is the most recent.

Tihen (1962a) also postulates that the B. americanus and B. valliceps groups evolved from the same source in what is now the great plains region. This is a reasonable hypothesis and it is probable that the B. americanus group represents a genetic stock which is close to the original ancestral group. This is what one might expect since B. americanus is still found in the great plains regions, largely in grassland and eastern deciduous forest, while the valliceps group is largely restricted to more southern derivative communities of the Madro-Tertiary Geoflora.

Blair (1963a) states that these species groups represent the results of geologically recent speciation (probably Pleistocene for the most part). It seems obvious that older phylogenetic groups such as species groups, major phyletic lines, etc., within a genus must be older than most of the species (Simpson, 1953). If Blair's eleven species groups represent only the result of Pleistocene speciation, this would indicate that the species in existence today are the result of a most remarkable adaptive speciation of the original eleven. This certainly

does not seem to be the case. Respectable evidence is available which indicates that the Pleistocene--at least since the Illinoian--had little or no effect on much of the herpetofauna of North America with regard to speciation (Holmon, 1959a, 1959b; Tihen, 1954). There is also evidence that little herpetofaunal change occurred from the Wisconsin to the Recent (Brattstrom, 1955; Holmon, 1958). In fact, the fossil record as presently known for the genus Bufo in the New World extends to the Oligocene (on the order of 25 million years B. P.) and it has been demonstrated well enough that many extant forms are certainly pre-Pleistocene in origin. Tihen (1962) has given the following dates of fossil occurrence of extant New World Bufos. B. marinus - Upper Miocene, B. cognatus - Middle Pliocene, B. compactilis - Blancan, B. alvarius - Blancan, B. woodhousei - Blancan.

In essence, the fossil record shows that the major species groups must have been established before or at least during the Miocene (Tihen, 1962b) a time distance of over 20 million years before the Pleistocene. Although the Pleistocene cannot be considered a panacea for anuran speciation and group formation as Blair (1963a) would indicate, it undoubtedly was an influential period for some populations. At least, many extant species do not have pre-Pleistocene fossil records and probably did evolve during that time. It is evident that anuran evolution on the whole has been a gradual process, associated with secular climatic and ecologic events relative to major habitat organization and reorganization such as the formation of modern forests, grasslands, and deserts from more generalized Tertiary Geofloras.

It is also probable that Pleistocene influence may be most clearly

expressed in the genetic divergence of species and subspecies of close physiological and morphological similarity, such as B. boreas and B. canorus (Karlstrom, 1963), and others.

It is interesting to note again at this point that a fossil toad (B. suspectus) is present from Lower Pliocene through Blancan. Specimens of these species have been examined and closely resemble B. punctatus and B. debilis, but cannot be unequivocally allocated to either species (Tihen, 1962b).

It is evident from the results of hybridization in this laboratory that B. punctatus belongs to the same species group as Bufo debilis, B. retiformis, and B. kelloggi and that the genetic affinities of this group fall in the following order: B. punctatus--B. kelloggi--B. debilis--B. retiformis. From fossil evidence it is probable that a Bufo punctatus-like species existed during the Miocene (Tihen, 1962b) which eventually gave rise to B. punctatus of the Recent, and a divergent branch which resulted in a B. kelloggi-like species in the Madro-Tertiary Geoflora of Western North America. From this B. kelloggi-like species, a progenator (of Bufo debilis and B. retiformis) arose in a grassland habitat during Pliocene or Mio-Pliocene time and gave rise to B. debilis which is still in the grassland today; and to B. retiformis which is now almost exclusively restricted to the Sonoran Desert, as a result of the formation of the North American deserts during Plio-Pleistocene time.

SUMMARY AND CONCLUSIONS

Conclusions have been given at appropriate places throughout. In short, it has been demonstrated that a natural species group is

represented by Bufo debilis, B. retiformis, B. kelloggi and B. punctatus, to be known as the Bufo punctatus group. Of the four species, B. punctatus Baird and Girard is the most similar to a common ancestral stock which probably existed during Mio-Pliocene time as the fossil species Bufo suspectus Tihen.

By reciprocally hybridizing individuals of each species, a knowledge of their specific genetic affinities was obtained. From these data it was concluded that (1) more advanced hybrid development occurs when crosses within the group are made using B. punctatus as the maternal or paternal species; (2) the other members of this group (B. debilis, B. retiformis and B. kelloggi) are more strongly isolated genetically from each other than was first assumed from their general appearance and behavior, and from former investigations of their general morphology and the nature of their isolating mechanisms; (3) the genetic compatibilities between members of the group assume the following order of relationship: B. punctatus--B. kelloggi--B. debilis--B. retiformis.

Sound spectrographic analysis has revealed further evidence for including these four species in the same species group. Similarities of the meristic aspects of the calls are shown. Of these, call frequency is dependent upon body size, and the relationship of snout-vent length to call frequency was found to be the same for each species.

The data obtained from structural analysis is found to agree favorably with the results of hybridization. Morphological differences were of a smaller magnitude than previously suspected and species divergence could only be convincingly demonstrated through qualitative characteristics.

The habitat of each species was described and relationships discussed with respect to the ecology of each. The evolution of the group has been associated with the evolution of modern derivative communities of the Madro-Tertiary Geoflora, within which each of the four species occurs today.

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