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ISOLATING MECHANISMS IN THREE SYMPATRIC TREEFROGS IN THE CANAL ZONE[1]

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Three species of small treefrogs, *Hyla microcephala* Cope, *H. phlebodes* Stejneger, and *H. ebraccata* Cope, are sympatric in the Canal Zone. They are rather similar in morphological appearance, and have similar breeding calls, and commonly call and breed in the same areas during the same periods of time. The question arises: how are these three frogs able to maintain their identities as discrete species under these conditions?

Investigations were carried out in the Canal Zone during the period from April 1955 to November 1956, while stationed there with the U. S. Air Force, and during the summer months of 1958. The area studied was in the lowlands of the Pacific drainage of the Canal Zone, chiefly within a four-mile radius of the town of Pedro Miguel.

All three species are of rather small body size (mean snout-vent length, millimeters—*ebraccata:* males 24.6, females 31.2; *microcephala:* males 22.8, females 27.1; *phlebodes:* males 22.2, females 27.4), and are quite similar in form and proportions. The basic color of all three, when active in the field (at night) is generally a rather vivid yellow with brown markings. The chief noticeable difference is in pattern, and in this characteristic *ebraccata* is the most distinctive. More detailed comparisons of the morphology will be presented elsewhere. Suffice it to say that the data from this source suggest that the species are quite closely related.

The scheme of classification used as a guide in checking the possible types of

isolating mechanisms is presented below. It is designed basically after the concept of Muller (1942) and represents an attempt to combine the best ideas of other classifications to apply to the problems of anuran speciation.

I. Anti-mating isolating mechanisms
    A. Geographic isolation (allopatry) (Spatial Isolation)
    _____
        (Reproductive Isolation)
    B. Habitat isolation (difference in breeding site)
    C. Seasonal isolation (difference in breeding season)
    D. Temporal isolation (difference in time of day breeding occurs)
    E. Psychological or Climatic isolation (difference in response to climatic and similar physical environmental factors)

II. Courtship isolating mechanisms
    F. Ethological isolation (difference in courtship behavior)
    G. Mechanical isolation (structural difference preventing interbreeding)

III. Post-mating isolating mechanisms
    H. Gametic isolation (incompatibility of gametes)
    I. Hybrid inviability
    J. Hybrid sterility
    K. Hybrid selective inferiority.

Anti-mating mechanisms, a category more or less equivalent to Muller's "bars to crossing," are factors which do not involve a wastage of gametes or other waste of reproductive energy resulting from attempts to breed with the wrong species. The term "anti-mating" is used in preference to Muller's term only because it combines well with "mechanisms," and is

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preferred to "pre-mating" (Littlejohn, 1957, 1959; Mecham, in press) because the latter term carries an implication that mating will occur.

Courtship mechanisms, an intermediate grouping, may sometimes allow a delay in breeding with the correct species, or even wastage of gametes, but at other times may not. These are all mechanisms which involve reactions between individuals, whereas the first category includes mechanisms which involve reactions between an individual and the physical environment, and the division following includes mechanisms which involve reactions between gametes (or various stages of resulting zygotes).

Post-mating mechanisms are those which are characterized by wastage of gametes. This is equivalent to Muller's "incapacitation of hybrids," and is used in the same way that Littlejohn and Mecham have used the term.

The simpler divisions are much the same as in Gulick's (1890) original classification, as modified, chiefly in terminology, by Romanes (1906), Robson (1928), Robson and Richards (1936), Dobzhansky (1937) and others.

ANTI-MATING MECHANISMS

The treefrogs were located by tracing the sound of their breeding choruses. The general ecology of these calling stations was noted with reference to (1) type of vegetation; (2) distribution of calling frogs in relation to water and vegetation, as well as in relation to other treefrogs of the same and other species; (3) temperature of the air and water; (4) weather conditions; and (5) local time.

Habitat isolation. The three species appear to differ somewhat in their preference for breeding sites, but their tolerances overlap to a large extent. *H. microcephala* apparently breeds in any area of standing water which has grass or sedge growing in it. If the grass is tall males generally call from a position just above the water, or just above the ground near the water. These frogs commonly call in roadside ditches, boggy areas of second growth where grasses are abundant, and grassy fields in low spots where water is standing. *H. phlebodes* apparently prefers a breeding site of dense, rather high grasses, usually in bogs or swamps, but may also breed in roadside ditches. *H. ebraccata* also seems to prefer high vegetation and rather permanent standing water. Males frequently call from roadside ditches, generally from a tangle of vines and woody vegetation, or from thick clumps of high grass somewhat up and away from the ditch proper. In contrast, when *microcephala* calls from a ditch it usually does so from grass growing in the water, while *phlebodes* may call from either position but is usually up away from the water, and prefers the high grass to the vines and woody vegetation.

Because of the overlap of breeding-site tolerances, such isolation as is effected by the differences in preference breaks down in many places, and all three species are commonly found calling at the same location. I have often observed individuals of all three species calling within a few inches of one another. Sometimes there was a concentration of individuals of the same species in one part of the area and another species in another part, and at other times there was apparently random intermingling of the species. More often, one of the species was dominant in number of calling individuals and was dispersed throughout the area, while the other two were intermingled with the dominant species, but more or less concentrated in different parts of the area.

A rather careful study of calling stations was made at a swamp about seven miles north of Miraflores Locks, where the three species commonly call together. All three generally call from the tall scandent grass which grows in the water, and no significant difference in calling station could be detected. Another aspect of the breeding habitat is the egg-deposition site, and a difference was found in this factor for two of the species. A

pair of *microcephala* was observed spawning, and the eggs were deposited in small masses on the surface of the water. A small mass of *ebraccata* eggs was found clinging to a blade of grass about 20 inches above the water. Taylor (1951) also reported that *ebraccata* lays its eggs on vegetation above the water. This utilization of different parts of the habitat for oviposition may support reproductive isolation to some extent. The ovipositional habits of *phlebodes* are unknown.

Seasonal isolation. The three species seem to call strongly throughout the rainy season, which is generally from mid-May to late December or early January. During the dry season, *microcephala* apparently migrates to permanent lakes and sloughs formed by waters backed up from the Canal, and continues to call from floating vegetation in the water. There is apparently no breeding in the dry season, although this has not been thoroughly investigated. Periods during which one of the species forms large choruses to the extent that it is the dominant species calling in many localities are from about May to November for *ebraccata,* May to January for *microcephala,* and May to September for *phlebodes.* Breeding peaks (based primarily on presence of amplexing pairs, and secondarily on increased calling activity) are apparently June–July and possibly November for *ebraccata,* May–July and October for *microcephala,* and May–September for *phlebodes.* Thus the breeding season of these three species overlaps completely, although there may be some isolation due to differences in the actual breeding peaks.

Temporal isolation. Little difference was found in the time of day during which mating occurs in the the three species. The dominant calling period is about the same in all three, from about sunset until shortly after midnight. The earliest that *ebraccata* and *phlebodes* were heard calling strongly was 7 P.M., and *microcephala* as early as 6:45 P.M. The latest that *ebraccata* and *phlebodes* were

heard calling strongly was midnight, and *microcephala* as late as 1:45 A.M. Occasional calls of all three species were heard beyond this period, both earlier and later, but they were not calling *strongly,* i.e., repeating their calls at regular intervals and calling in choruses rather than as scattered individuals.

The earliest that actual breeding was observed was about the same for all, between 8 and 9 P.M. The latest time amplexing pairs of *microcephala* were observed (1:30 A.M.) is significantly later than for the others (9:30 for *phlebodes,* 10:20 for *ebraccata*), but probably only because of the much greater abundance of *microcephala,* making observation of amplexing pairs of that species more likely at all periods. Thus there is probably no daily temporal factor of any significance contributing to the reproductive isolation of these species.

Psychological or climatic isolation. Temperature data indicate that there is no correlation between this factor and breeding activity of any of the species. All were found calling strongly and breeding throughout the entire range of nocturnal air temperatures measured during the breeding season (22°–28° C). It probably does not normally get too cool or too warm for any of the species to call and breed during the breeding season, which is more or less to be expected in a tropical area. Also the light of the moon apparently had no effect on the breeding and calling activity of the three forms. The number of individuals calling and clasping apparently was independent of whether the night was dark and moonless or brightly lit by a full moon, or any intermediate condition.

There is no apparent correlation of rainfall with the activities of *ebraccata;* however, there seems to be a tendency for *microcephala* to increase its activity immediately after heavy rains, whereas *phlebodes* seems to show increased activity after a few days of little or no precipitation. This is based on generalized observations and cannot be supported by

definite data. Evidence that this tendency is real seems to be supported by a decrease in activity of *phlebodes* in October and November, when the rains are heaviest and most frequent, and by a breeding peak in *microcephala* during October. Thus among the psychological factors checked, only a possible subtle difference in response to rainfall between two of the species may have some bearing in supporting reproductive isolation.

Courtship Mechanisms

Ethological isolation. The most significant factor which may serve to isolate species of frogs appears to be the breeding call. It is generally accepted that the breeding call of male frogs serves at least two important functions. First of all, it is the sexual "display" which attracts the female. Several workers have reported observing females of various species approaching calling males of their own species. Among frogs of the family Hylidae (Blair, 1958; Littlejohn, 1958; Mecham, in press), the male usually continues calling, apparently oblivious to the presence of the female until she makes physical contact, at which time the male stops calling and amplexus occurs. In addition, Martof and Thompson (1958) recently demonstrated experimentally that female *Pseudacris triseriata feriarum* are attracted to the call of the male of that species.

The other important function generally attributed to the call is that it is the primary method of species recognition. Until recently, most workers tended to accept this hypothesis with no experimental evidence to support it. However, Littlejohn and Michaud (1959) demonstrated that female *Pseudacris streckeri* respond to the call of the male of that species, but not to the call of the sympatric species *P. clarki,* thus presenting conclusive experimental evidence that the female frogs can discriminate between the two calls. This establishes the fact that difference in breeding call may act as an isolating mechanism in frogs. Blair and Littlejohn (1960) further showed that female *Pseudacris streckeri* effectively discriminate in favor of the call of male *streckeri* when given the choice between this and the call of the closely related allopatric *P. ornata,* which has a call that apparently differs from *streckeri* only by about a 20% average difference in dominant frequency. The two calls are so similar that it is very unlikely that a human ear could distinguish them, so that the discriminatory ability of some frogs may be extremely well developed.

The breeding calls of individual male treefrogs of all three species were recorded in the Canal Zone using a Magnemite (Model W610EV) battery-operated tape recorder, at a tape-speed of 15 inches per second, and a Shure dynamic microphone (Model 55S). The calls were later analyzed in the laboratory at the University of Texas by means of a Kay Electric Co. Sona-Graph. Blair and Pettus (1954), Blair (1955), and Fouquette (in press) have described most phases of this method in detail. Sample audio-spectrograms (sonagrams) of normal and abnormal calls of all three species are shown in figures 1 and 2. The frogs were usually collected and preserved so as to permit correlation of any given call with the individual that produced it. Temperatures were recorded but it was found that there was so little variation in this factor here that it could be disregarded in comparing the calls.

The breeding calls of the three frogs are rather similar, and it is believed that this is a reflection of their close relationship. However, they are readily distinguished by the human ear, with a little practice, so it is assumed that they are distinguishable to the frog's ear. Data will be presented to demonstrate good quantitative differences in the three calls, thus providing a basis for ethological isolation.

The calls of the three species consist of a series of short buzzes or squeaks. Each of these short bursts of sound is
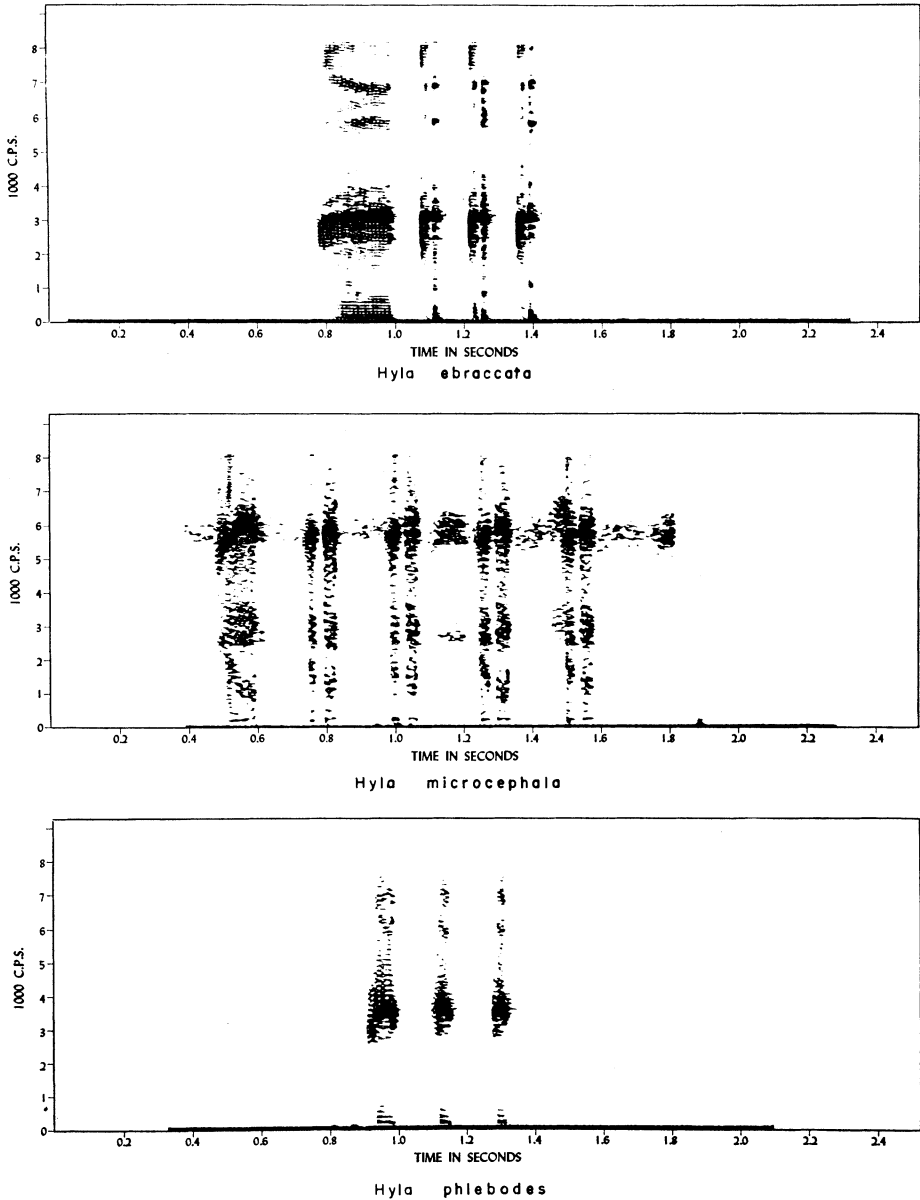
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FIG. 1. Sonagrams of normal calls of three species of *Hyla,* recorded in the Canal Zone. For each species is shown a representative call-group composed of a single primary note followed by several secondary notes.

termed a *note.* Characteristically, the call pattern consists of a group of several of these notes repeated in rapid succession. This is followed by a longer pause, which is followed in turn by another group of notes, and so on. Each of these groups of notes is referred to as a *call-group.* The first note of a call-group is longer in duration than those that follow it and usually differs somewhat in frequency characteristics. Consequently, it is distinguished from others as the *pri-*

mary note, while the others are called *secondary notes.* A call-group may consist of one note (always a primary note) or, in the sample studied, up to 29 notes (one primary and 28 secondaries). Call-groups with more than one note are referred to as *compound* call-groups.
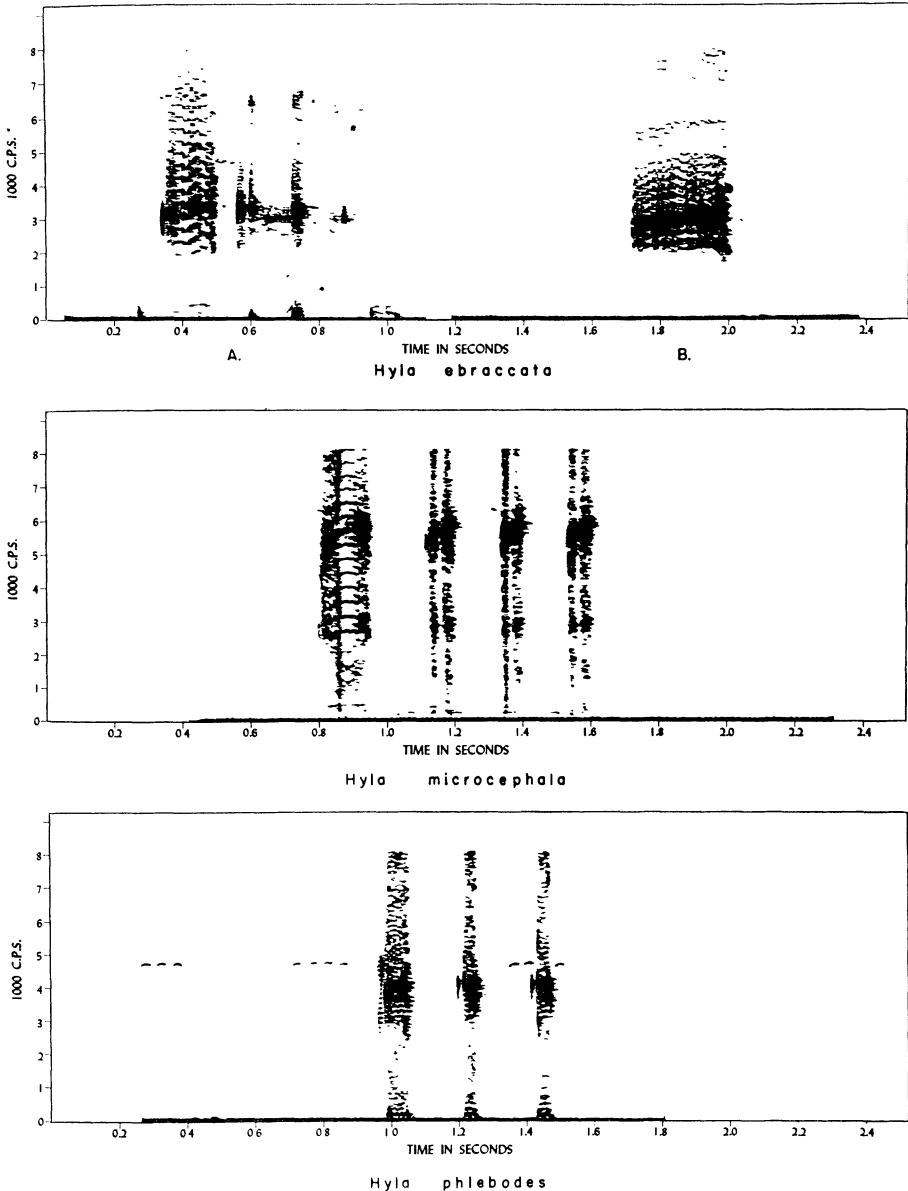
The *fundamental* frequency is the fre-

FIG. 2. Sonagrams of abnormal calls of three species of *Hyla.* *H. ebraccata:* Call-group to the left composed of a *high primary* note, and a normal *paired secondary* note and an *unpaired secondary* note. Call-group to the right consists of a single *high primary* note. *H. microcephala:* Call-group composed of a *high primary* note and three normal paired secondary notes. *H. phlebodes:* Call-group in which each note has a short auxiliary component preceding it. Such notes are considered as *paired* (see text).

quency of vibration of the vocal cords and is very important in determining the way a call "sounds." This characteristic was measured to the nearest cycle per second, but the accuracy is probably not this great. The *dominant* frequency is the frequency resonated by the vocal sac of the frog. It is chiefly a function of the size of the vocal sac and is not correlated with the fundamental. The fundamental may be drastically changed without causing any significant change in the dominant frequency. However, when such a change in the fundamental occurs, there is a very noticeable change in the "sound" of the call, in both the pitch and quality. Probably it is the fundamental and dominant together which are chiefly responsible for the "sound" of a call.

In a simple call, the dominant frequency is a harmonic of the fundamental. However, in the calls of the three *Hyla* species studied here, a dominant frequency band cuts across the harmonics of the fundamental, obscuring the harmonic pattern, and shifting upward in freqency throughout the duration of the note. Arbitrarily, I have selected the mid-point of this band measured at the terminal border of the note, and measured this for each note to the nearest 50 cps. This will be referred to henceforth as the *mid-point of the dominant* or simply as the *dominant*.

The *duration* of each note was measured to the nearest .01 second, and an average of three or more notes per individual rounded off to the nearest .005 second. The *note-repetition rate* was also measured. This is the rate, in notes per minute, at which the notes of a call-group are produced. This is applicable only to compound groups, of course. Other characteristics of the calls were also noted and all of these are included in table 1. Among these other characteristics are the approximate areas of the emphasized harmonics other than the dominant and fundamental. These are merely estimates based on one or two measurements and are presented merely to help fill in the

structure of the call. The number of notes in each call-group recorded was counted, and the mean number computed for each individual. The over-all range in this number is given for each species, as well as a mean calculated from the individual means.

H. ebraccata has the lowest pitched call of the three and to my ear the loudest. The low pitch is attributable to the fact that it has the lowest fundamental and dominant frequencies. Because the fundamental is of such a low frequency it imparts to the call an almost "trilled" quality. That is, the human ear can almost discriminate the individual modulations. The rather long primary note is usually followed by one to five very short secondary notes. The secondary notes have a slightly higher fundamental, and a somewhat lower dominant frequency than the primary note. The note-repetition rate is generally a little faster than that of the other species. The secondary note is actually composed of two notes which are so closely spaced that it is not usually noticeable to the human ear. This type of note will be referred to as a *paired secondary*, as opposed to an *unpaired secondary* which has a single component. Over half of the individuals recorded would occasionally emit a primary note which sounded high-pitched and squeaky. This type of note is referred to as a *high primary*. Upon analysis it is found that the only major change is a drastice rise in the fundamental frequency (figure 2). The dominant is scarcely affected, although it is usually slightly lower than that of the normal primary.

The call of *microcephala* is very similar in over-all pattern to that of *ebraccata*. The primary note is generally of shorter duration and the secondary note of longer duration than the comparable notes of *ebraccata*. Of the 40 individuals recorded, only one had all unpaired secondary notes, and only two others had an occasional unpaired note. This species has the highest fundamental, the highest dominant frequency, the slowest note-

TABLE 1. *Characteristics of the breeding calls. P(n) = normal primary note, P(hi) = high primary, S = secondary note. Standard deviation is given, rather than standard error, since the dispersion is of more interest than the level of significance in regard to call as an isolating mechanism*

|	ebraccata			microcephala			phlebodes	
	P(n)	P(hi)	S	P(n)	P(hi)	S	P(n)	S
Mid-point dominant frequency (cps) — Mean±std. dev.	3,064±58.9	3,060±208.2	3,014±181.9	5,636±193.8	5,570±175.6	5,540±148.9	3,580±256.7	3,557±220.7
Absolute range	2,500–3,450	2,600–3,300	2,500–3,250	4,850–6,000	5,300–5,800	4,850–5,900	2,450–4,100	2,400–4,100
Rng. indiv. avgs.	2,630–3,367	2,600–3,300	2,583–3,225	5,150–5,962	5,300–5,800	5,262–5,886	2,800–4,067	2,900–4,050
Sample size	19	10	19	40	11	39	26	24
Fundamental frequency (cps) — Mean±std. dev.	87.9±6.0	357.4±64.6	98.8±9.7	206.9±13.0	385.1±30.8	224.5±13.3	149.2±6.7	154.9±4.7
Absolute range	75–92	251–436	78–114	184–238	315–419	200–254	122–158	145–162
Sample size	19	10	18	38	11	21	26	23
Areas of other emphasized harmonies (approx.)	175	—	as P(n)	2,400	—	as P(n)	300	as P(n)
	6,000			8,500			7,000	
	9,000			11,000			10,500	
	12,000			13,500			14,000	
Duration (seconds) — Mean±std. dev.	.210±.025	.180±.046	.068±.012	.133±.010	.143±.020	.106±.003	.112±.018	.082±.013
Absolute range	.16–.29	.15–.30	.04–.10	.08–.17	.11–.18	.08–.14	.07–.16	.05–.12
Rng. indiv. avgs.	.18–.275	.15–.30	.04–.09	.10–.155	.11–.18	.09–.135	.085–.14	.055–.11
Sample size	19	10	19	40	11	38	26	24
Notes per group — Mean indiv. avgs.		1.9			3.4		2.3	
Absolute range		1–6			1–19		1–29	
% groups compound (combining all calls of all individuals recorded)		53.9%			62.0%		32.0%	
Note repetition rate (compound groups) — Mean		351.6			257.8		290.1	
Absolute range		217–429 (notes per minute)			192–311		206–348	
% Primary notes with high fundamental (all calls combined)		15.0%			3.5%		0.00%	
% individuals recorded with high fundamental		52.6%			27.5%		0.00%	
% Primary notes with high fundamental in individuals that demonstrate it		21.8%			14.6%		—	
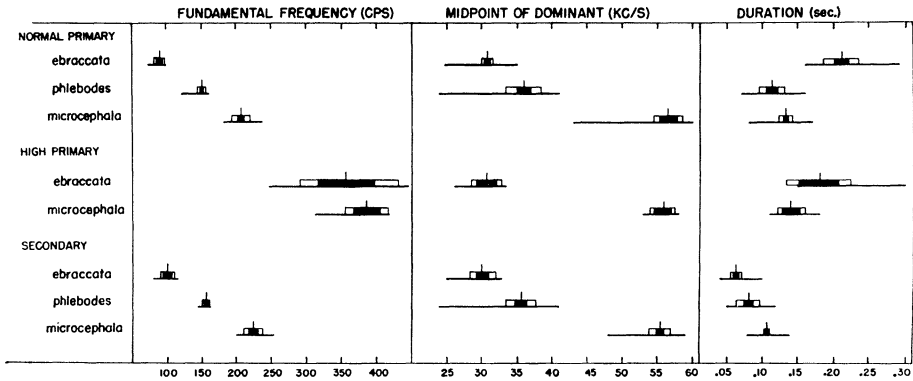
FIG. 3.   Comparative call structure.   The bar graphs follow the
method of Hubbs and Hubbs (1953).

repetition rate, and generally the most notes per call-group of the three species. As in *ebraccata* some of the *microcephala* occasionally emit a high primary note (fig. 2). In both species the primary is the only note which undergoes this phenomenon. The occurrence of this high primary note is not as common in *microcephala* as it is in *ebraccata,* since only 27.5% of the individuals recorded demonstrated it.

The call of *phlebodes* is intermediate between the calls of the other species in most characteristics, including the fundamental and dominant frequencies. Only about one-third of the call-groups are compound, but when the group is composed of more than one note, the number of notes per group is often very high, as many as 29 in one individual. The secondary notes differ from the primary in that they are of shorter duration, but in other characteristics the two are essentially similar, although the secondaries generally tend to have a slightly higher fundamental and a lower dominant frequency than the primary. In *phlebodes* the secondary note is almost always unpaired, although in three individuals there is an auxiliary component with some of the notes, including the primaries, so that it gives the same impression audibly as the paired notes of the other species. This is not structurally the same type of double-note found in the other species

(fig. 2), but since it sounds similar to my ear it is considered as a paired note for the purposes of comparisons with the other species in figure 4. A high primary note is not known in this species.

The differences in fundamental frequency among the three species are highly significant (fig. 3), with no overlap in either normal primary or secondary notes. The high primary notes of *microcephala* and *ebraccata* have very erratic fundamental frequencies, and the two overlap broadly. The difference between the means ($\pm$ standard error) of the normal primary notes of *ebraccata* and *phlebodes* is 61.3 ± 1.91 cps, between those of *phlebodes* and *microcephala* it is 57.7 ± 2.47 cps, and between those of *ebraccata* and *microcephala* it is 119.0 ± 2.52 cps. The differences in the secondary notes are of the same magnitudes. The difference between the means of the high primary notes of *ebraccata* and *microcephala* is 27.7 ± 22.43 cps, which is not significant.

The dominant frequencies are significantly different in all three species, although there is considerable overlap in this character between *ebraccata* and *phlebodes* (fig. 3). The difference between the means of the normal primary notes is 516 ± 51.2 cps. There is no overlap in the range of dominant frequencies between *microcephala* and the other species. The difference between the means of *microcephala* and *phlebodes* is

2,056 ± 59.0 cps, and between those of *microcephala* and *ebraccata* it is 2,572 ± 33.4 cps. The differences between the means of the dominant frequencies of both the high-primary and secondary notes are of the same magnitude.

The durations of normal primary and secondary notes overlap considerably, although the means differ significantly (fig. 3). The difference between the means of the normal primary notes of *phlebodes* and *microcephala* is .021 ± .0037 seconds, between those of *microcephala* and *ebraccata* is .077 ± .0065 seconds, and between those of *phlebodes* and *ebraccata* is .098 ± .0042 seconds. The difference between the means of the durations of the high primary notes of *ebraccata* and *microcephala* is .037 ± .0157 seconds, which is of rather low significance. The difference between the means of the secondary notes of *ebraccata* and *phlebodes* is .01 ± .0039 seconds, between those of *phlebodes* and *microcephala* is .024 ± .0025 seconds, and between those of *ebraccata* and *microcephala* is .038 ± .0030 seconds.

In using the various measurements of call structure as taxonomic characters we observe that there are highly significant differences in most of those structural characteristics analyzed. However, the effectiveness of call as an isolating mechanism depends upon virtual complete lack of overlap in the characteristics which the females discriminate. There is a great deal of actual overlap in most of the structural characteristics of the calls among these species (fundamental being a possibly important exception), although the sum total of these characteristics yields a sound which may always be distinguished from that produced by either of the other species. Thus, there may be no single characteristic by which a frog distinguishes calls of its own species from others, but it may be the combination of all characteristics of a call which gives it its distinctive sound, which the female frogs discriminate. Three characteristics judged to be among the most important are employed in figure 4—fundamental, dominant, and paired or unpaired condition of secondary notes. It is seen that the symbols representing individuals of each species fall into rather closely-packed groups, quite well separated from the other species (one exception is a *phlebodes,* field no. 1158, whose frequency characteristics fall between those of *phlebodes* and *ebraccata;* morphologically this specimen cannot be distinguished from typical *phlebodes,* and the pattern of the call is that of *phlebodes*). This
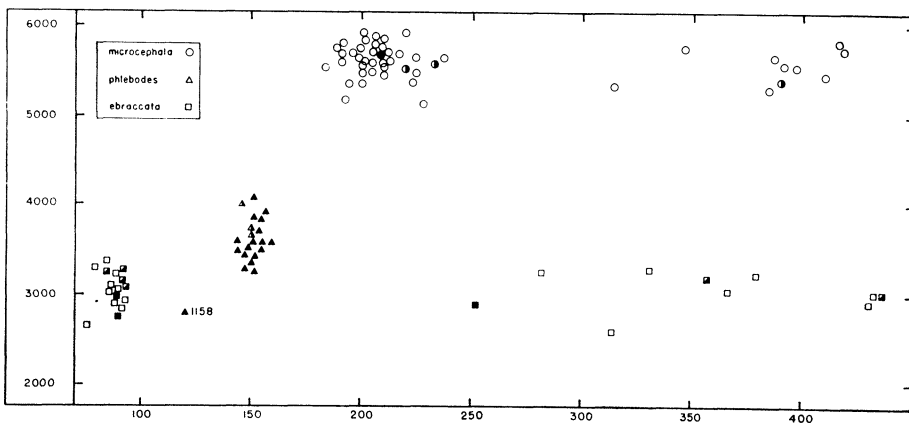

FIG. 4. Scatter diagram employing three important characteristics of the calls: fundamental frequency, indicated on horizontal axis; dominant frequency, indicated on vertical axis; and components of the secondary note, indicated as paired by clear symbols, unpaired by blackened symbols, or both types in one individual by half-blackened symbols.

degree of difference probably indicates an important ethological isolating mechanism.

The call difference may fail to isolate two species if the female does not discriminate between the two calls, and if the male does not discriminate in his choice of females. Littlejohn and Michaud (1958) demonstrated that it is unlikely that the female will fail to discriminate between the calls. They also found (personal communication) that the male shows no discrimination, attempting amplexus with any frog which contacts or hops near them. Thus it is probable that if a female enroute to a calling male should accidentally contact a male of another species, amplexus would occur. Additional ethological mechanisms could then be of importance in causing the male to release the foreign female. No experiments were performed with the species in the Canal Zone to test their amplectic compatibility, so nothing further can be added concerning ethological isolation.

Mechanical isolation. This is reproductive isolation resulting from the physical non-conformity of the pair of frogs involved. It is felt that probably the only true mechanical isolation existing in most frogs is due to a size difference so great as to make amplexus a physical impossibility. Because of the rather small differences in size, there is probably no effective mechanical isolation among the three Panamanian *Hyla*.

POST-MATING MECHANISMS

Among the hundreds of frogs of these three species which I examined in the laboratory and observed in the field, no morphologically intermediate individuals were seen. Occasional specimens of *microcephala* showed a breaking-up of the lines of the dorsal pattern, tending toward the *phlebodes* pattern, but these were found to be good *microcephala* in all other respects, including call. Furthermore, of the amplexing pairs noted, no interspecific pairs were seen, although I

have taken several amplexing pairs of all three species in situations where one or both of the other species were calling, sometimes only a few inches away. Thus, natural hybridization among these frogs is probably quite rare, if it ever occurs. In order to determine whether hybridization was biologically possible, artificial crosses were made in all possible combinations, using methods based on those of Rugh (1948). A few crosses were made in 1956, but at this time no suitable food had been discovered for the larvae, and numbers surviving at various stages were merely estimated. In 1958 more careful counts of survivors were made and a suitable food had been found so that the larvae were carried to more advanced stages. However, it was necessary to return to Texas prior to metamorphosis of the larvae, and all of them died in the laboratory at the University of Texas (including controls). Their death is now attributed to ammonia-poisoning (Hubbs, Littlejohn, and Littlejohn, 1960). In spite of the small number of crosses from which the data presented are derived (table 2), it is felt that the results give a good indication of the interfertility of the parental forms.

Gametic isolation. Fertilization occurred in all crosses except those between female *ebraccata* and males of the other two species. In the latter crosses, none of the eggs rotated or underwent any cleavage, so it is assumed that fertilization did not occur. Thus, in the event that all anti-mating and courtship isolation breaks down, gametic isolation appears to be 100% effective in preventing hybridization between female *ebraccata* and males of the other species. The relatively low percentage of eggs undergoing cleavage in the crosses ♀ *microcephala* × ♂ *phlebodes* might suggest some degree of lowered fertility, but it is felt that this is probably not true. Actually, in one of the two trials, 39 eggs of a total of 41 underwent cleavage, where as in the second trial only 9 of 73 cleaved. This low percentage in the second cross is thought

TABLE 2. *Results of crosses.* E = ebraccata, M = microcephala, P = phlebodes

Cross	Trials	No. eggs	Fertilized (cleavage)	No. hatched	Survival: normal +abnormal (total)			% hatched surviving 7 days	Rate of development
					72 hours	7 days	14 days		
♀E × ♂E	2	70	63	63	63+0 (63)	63+0 (63)	63+0 (63)	100.0	control
♀E × ♂M	2	93	0	—	—	—	—	—	—
♀E × ♂P	2	97	0	—	—	—	—	—	—
♀M × ♂M	2	206	179	66	66+0 (66)	57+2 (59)	54+1 (55)	89.4	control
♀M × ♂E	2	98	82	59	26+20 (46)	1+3 (4)	0	6.8	retarded
♀M × ♂P	2	114	48	42	35+2 (37)	29+1 (30)	29+0 (29)	71.4	accelerated
♀P × ♂P	1	*e50	*e50	*e50	*e 50+0	*e 50+0	1+0 (1)**	100.0	control
♀P × ♂E	1	*e50	*e45	*e45	*e 45+0	0+2 (2)	0**	*e4.0	retarded
♀P × ♂M	1	*e50	*e50	*e50	*e 50+0	*e 45+0	*e 20+0	*e90.0	accelerated

* e = estimated; cross made in 1956, actual counts not made.
** = poor laboratory conditions and lack of proper food probably responsible for high mortality.

to be the result of faulty technique, namely over-crowding of the eggs.

Hybrid inviability. It is believed that in most crosses development proceeded far enough so that one might predict the degree of success of the cross, even though none of the larvae were carried to metamorphosis. In a number of preliminary feeding experiments with *microcephala* larvae, nearly all survived past the seventh day without feeding, so any deaths during the first seven days can probably be attributed to genetic causes. Thus it is felt that the figures for percentage of hatched larvae surviving seven days are reasonably reliable for all crosses, and may be used as an index of genetic viability.

Crosses between male *ebraccata* and females of the other species produced a large percentage of hybrids which were obviously deformed or abnormal, and many died in every stage of larval development. The rate of development (which was estimated by comparing the time it took for each cross to reach various embryonic stages, with that for the controls, as well as by comparing the general rate of gain in physical size) in the most "normal-appearing" of these hybrids was noticeably slower than that of either control. Less than 7 per cent of the hybrids that hatched were alive after seven days, and all were dead at the end of two weeks. It seems doubtful that any hybrid with *ebraccata* as one of the parents would survive to metamorphosis.

Reciprocal crosses between *microcephala* and *phlebodes* showed no more abnormal-appearing larvae than did the controls, and the percentage surviving past the seventh day are not much lower than those of the controls. The rate of development is faster than that of either control, especially in the earlier stages. The acceleration of development, plus the apparently higher survival rate of the hybrids after 14 days, suggest the possibility of heterosis. Thus hybrid inviability is probably not an isolating mechanism of importance between these two species.

Further hybrid breakdown was not tested because of the aforementioned death of the experimental stock.

DISCUSSION AND SUMMARY

The breeding call seems to be the primary isolating mechanism operating to prevent interbreeding of the three species studied. All three species have calls which are demonstrated to be qualitatively and quantitatively different, and it is assumed that the females discriminate and respond only to the call of their own species.

In some areas differences in breeding habitat may support isolation, although there is complete intermixing in some areas. Two of the frogs (*microcephala* and *ebraccata*) differ in the site of oviposition, and this may lend additional support to habitat isolation. There may also be a difference in breeding peaks, although the actual breeding seasons overlap completely. A possible subtle difference in response to rainfall may help support isolation between two of the forms (*microcephala* and *phlebodes*).

If all these mechanisms fail, gametic isolation appears to be effective in interspecific crosses with female *ebraccata*, and crosses to male *ebraccata* result in early death of the few larvae that hatch. However, there appears to be no reduced fertility in crosses between the other two species, *microcephala* × *phlebodes*, nor any reduced hybrid viability, in fact there is a suggestion of heterosis. It is possible that these hybrids might die a genetic death before reaching maturity, but this is doubtful. There also remains the possibility of hybrid sterility and further hybrid breakdown, which were not investigated. However, in the absence of any records of natural hybridization, or even cross-clasping, it appears that reproductive isolation is effected by anti-mating and courtship mechanisms. Inasmuch as both species were commonly found calling and breeding within inches of each other, and because of the great overlap of most other factors which might support

isolation, it is concluded that the only factor exhibiting a consistent significant difference, namely the breeding call, must be the primary isolating mechanism operating here. This is equally true of isolation between *ebraccata* and these two species. There is no reason to assume that this courtship mechanism will be any less effective merely because a potential post-mating mechanism exists. In fact, as pointed out by Fisher (1930), Sturtevant (1938), Blair (1955), and others, selection against the wastage of gametes will ultimately cause reinforcement of the potential anti-mating and courtship mechanisms in the area where these species come into contact.

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