EXPERIMENTAL CROSSES BETWEEN
RANA AURORA AURORA BAIRD AND GIRARD AND RANA AURORA CASCADAE SLATER

by

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Two of the ranid frogs found in the Pacific Northwest, the
Red-legged frog and the Cascade frog, were originally treated as
separate species and more recently as conspecific subspecies. The
purpose of this study is to help clarify the taxonomic status of
these frogs by modern experimental methods.

For many years taxonomists followed the principles of Linnaeus
in defining the various taxonomic categories. To Linnaeus, each
species was a static entity, created as such and easily identified
from other species on the basis of morphological characteristics alone.
With the discovery of numerous variations within, and in many in-
stances intergrades between, these established morphological species,
this criterion became inadequate and a new basis for delimiting the
species level was needed. Although difficult to apply, the soundest
and most generally accepted criterion for a species is now con-
sidered to be reproductive isolation. Therefore, Mayr (17, p. 25)
defined biological species as follows: "Species are groups of
actually (or potentially) interbreeding natural populations which are
reproductively isolated from other such groups." Morphological dif-
ferences merely indicate the natural groupings and should only be
given primary consideration in those cases where the degree of repro-
ductive isolation has not been determined. Because fertility and

crossability may often vary independently of morphological characters the usefulness of morphology lessens in border line cases.

In contrast to species, adjacent subspecies interbreed, or are potentially capable of interbreeding if separated by extrinsic barriers. Because they will interbreed, only one subspecies of a polytypic species can breed in any one area. As a result of interbreeding, intermediate populations may often be found in the border zones between two distinct subspecies.

The species criterion of reproductive isolation has become strengthened in recent years as a result of experimental research which has borne out the merits of this theory. In the field of herpetology, speciation studies have centered around the research of John A. Moore, E. Peter Volpe, and W. Frank Blair on frogs and toads. For example, Moore (21, pp. 408-416) crossed individuals of Rana burnsi with those of Rana pipiens and found no obvious differences in development rate between crosses and controls. He concluded that Rana burnsi should not have the status of species or subspecies but should be reduced to synonomy with Rana pipiens and be referred to as the "burnsi mutant".

Sympatric populations can remain distinct, and be considered as species, only if gene exchange between them is limited or prevented by one or more reproductive isolating mechanisms. Allopatric populations may or may not exhibit reproductive isolation if they expand their distributional ranges and overlap one another. Volpe's studies (32, pp. 303-318) on the reproductive isolation between sympatric

and allopatric populations of Bufo americanus and Bufo fowleri
indicated that there is no positive correlation between the territo-
rial adjacency of the two species and the degree of reproductive
isolation between them.

In his study of interspecific hybridization in Bufo woodhousei
and Bufo valliceps, two distinct sympatric species, W. A. Thornton
(29, pp. 455-468) cross-mated female woodhousei with male valliceps
and found all zygotes died in the neurula stage. Reciprocal crosses
produced zygotes which developed through metamorphosis. However,
there was a high degree of inviability of these hybrids as the
majority of the back-crosses developed no further than the blastula
stage. Thus, there is an effective genetic block to the gene exchange
between these two species.

The Red-legged frog, Rana aurora aurora, and the Cascade frog,
Rana aurora cascadae, are adjacent allopatric forms which occur in
Oregon and whose precise taxonomic status is questionable. As a re-
sult of the questionable status of these two frogs this study was
suggested by my major professor, Dr. Robert M. Storm.

Rana aurora aurora was first described from the Puget Sound area
in 1852 by Baird and Girard (3, p. 174) and has been recognized as a
species from that time to the present. The Red-legged frog is proba-
bly distributed over most of Oregon west of the Cascade Mountains and
has been collected from the locations indicated on the map in Figure 1.
Non-breeding Red-legged frogs are found in down timber, dense patches
of vegetation and to some degree adjacent to ponds and small streams.
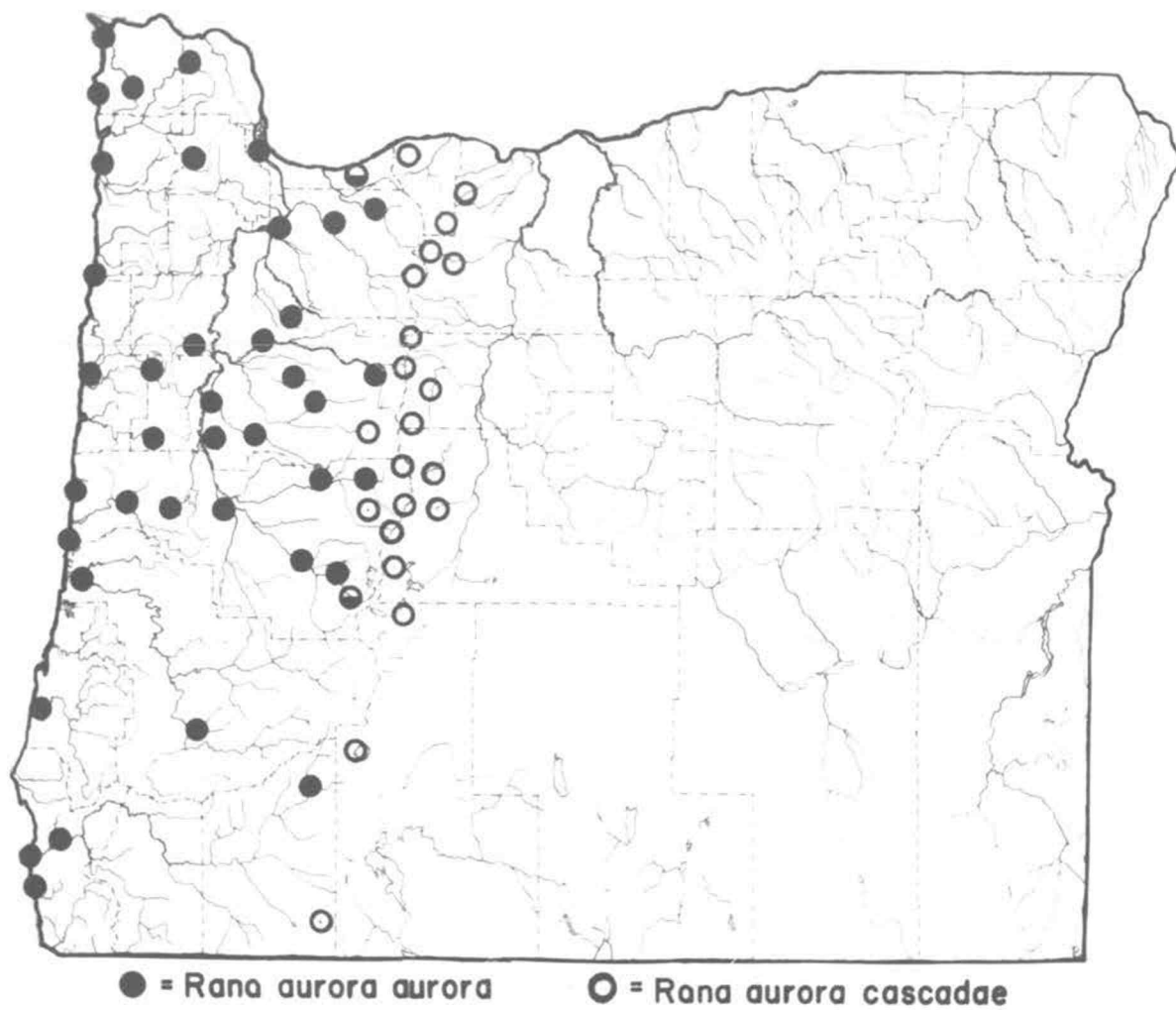
● = Rana aurora aurora   O = Rana aurora cascadae

Figure 1. Locality records of the Red-legged and Cascade frogs in Oregon.

The Cascade frog was originally described as a new species, Rana cascadae, by James R. Slater in 1938 (27, pp. 145-149). In his book, The Amphibians and Reptiles of Western North America, Robert C. Stebbins (28, p. 128) treated the Cascade frog as a subspecies, Rana aurora cascadae. In A Checklist of North American Amphibians and Reptiles by Karl P. Schmidt (26, p. 85) this frog is also designated as Rana aurora cascadae. The type specimen is an adult female collected by Slater June 19, 1938 from the Elysian Fields, Rainier National Park, Washington, at an elevation of 5,700 feet. The Cascade frog is found chiefly in the higher montane regions of the Cascades and has been collected in Oregon as far south as Crater Lake, as indicated on the map in Figure 1.

While the Cascade frog is generally found above the Transition Zone and the Red-legged frog is limited to lower elevations, being especially abundant in the valleys, their ranges do overlap to some degree in the Canadian Zone. Thus, they have essentially allopatric distributions characteristic of subspecies. However, Dunlap (11, p. 329) observed no overlapping in ventral coloration and concluded no genetic interchange was taking place in these zones of contact. The result of Dunlap's morphological studies, plus this lack of intergradation, indicates that the two frogs should be separate species.

Richard G. Zweifel (38, pp. 261-262) made a single cross between Red-legged and Cascade frogs and obtained abnormal embryos, although six out of forty-five hatched. He suggested that this single experiment indicated a specific level of difference had been reached by

these frogs but that confirmatory evidence in the form of more experimental crosses was desired.

A series of reciprocal crosses has been made in this study which was begun in the fall of 1957. The results of these first experiments were inconclusive, and the crossings were repeated on a larger scale in the fall of 1958, when conclusive results were obtained.

METHODS AND MATERIALS

Adult Cascade frogs were collected during August and the first
week of September from Wildcat Creek, Cedar Swamp, and Upper Horse
Creek, all within the Three Sisters Wilderness Area, Lane County,
Oregon; and from the meadows around Sparks Lake, Deschutes County,
Oregon. Mature female and immature Cascade frogs could be found
quite abundantly in these areas along the water's edge and in damp
situations within the streamside vegetation. Mature males were quite
difficult to find, however, except around Sparks Lake where five were
obtained in one collecting trip.

Numerous collecting trips for adult Red-legged frogs were made
during the fall, around the Corvallis area. The most productive
trips were those conducted in October and early November at night
when the temperatures were in the fifty-degree Fahrenheit range and
a slight rain was falling. Under these conditions the frogs could
be obtained quite readily by driving infrequently traveled roads and
spotting the migrating frogs in the headlight beams. Collecting
trips conducted early in the evening just before dark were usually
more profitable than those attempted later at night. Oddly enough,
it was found that these night "drives" very rarely produced mature
male frogs, whereas both mature female and immature frogs were found
quite regularly. Mature male Red-legged frogs were extremely scarce,
the few which were obtained being collected around ponds and streams
primarily at night by means of a flashlight and dip net. Adult

Red-legged frogs were collected from the following locations: Coffin Butte ponds, 10 miles north of Corvallis, Benton County, Oregon; a tributary of Champoeg Creek, approximately 2 miles southwest of Hubbard, Marion County, Oregon; in the vicinity of the Calapooya River west of Tangent, Linn County, Oregon; and from marshes near Florence, Lane County, Oregon.

A retention problem was created because the Cascade frogs had to be obtained prior to hibernation in September, and the Red-legged frogs were difficult to find until October and November when breeding migrations occurred. Therefore, after collection, the frogs were retained in large aquaria and kept in a temperature-controlled room at a temperature of 35° F. Approximately three inches of water were placed in the aquaria and then several inches of leaf litter were added which allowed the frogs to bury themselves in the material. This proved to be a very satisfactory method of holding the frogs with very little care being required at such a low temperature. Frogs were kept in this manner for as long as four months before induced ovulation was initiated. One female Red-legged frog was held for a full year under these conditions and appeared to be perfectly healthy upon being released, although no eggs could be obtained from her after repeated pituitary injections.

The pituitary method was used to induce ovulation. All instruments were sterilized in boiling water or 70% ethyl alcohol. Glassware was sterilized in a steam autoclave. Initially, eight pituitary glands were dissected from female Leopard frogs, or twice that number

from male frogs, for each female being injected. These pituitary glands were placed in Syracuse watch glasses containing distilled water. A separate glass was used for each female which was to be injected in order to facilitate injecting the same number of glands in each frog. A previously sterilized syringe was used to suck up the glands, a number 20 needle attached, and all but 1 cc. of the solution was forced out together with any air bubbles present. The glands were then injected abdominally. The needle was inserted obliquely to prevent internal injuries and the glands were injected into the body cavity, being mashed as they passed through the needle. As several mortalities resulted from this procedure, the dosage was changed to a subcutaneous injection of two female pituitary glands per female per day until ovulation was initiated. No mortalities occurred from this reduced dosage and ovulation was usually induced in from one to three days.

After being injected, the frogs were kept at room temperature in glass jars containing moist paper towels. The frogs were tested for ripeness after twenty-four hours and at that time, if they were not ripe, another injection of two pituitaries was made. It was found that females which had become ripe could be held quite success-fully for a few days in a refrigerator at a temperature of 40° F. without any apparent effect upon the fertility of the eggs, if the females were warmed to room temperature prior to ovulation. Thus, the eggs from several females could be fertilized at one time, re-sulting in a greater number of crosses per male than otherwise would

have been possible. A parallel control from eggs and sperm of the same type was made for each of the crosses.

The eggs were fertilized artificially for all cultures. The mature male frogs were killed and their testes dissected out. Each pair of testes was placed in a labeled Syracuse dish containing 10 cc. of tenth normal Holtfreter's solution prepared from the formula given by Rugh (25, p. 10). The testes were then mashed with a scalpel and forceps and the resulting suspension was allowed to stand for thirty minutes at room temperature to allow the sperm to become active before attempting to fertilize the eggs.

The eggs were stripped onto small pieces of glass made from halves of standard microscope slides. Approximately fifty eggs were placed on a glass. Each glass was then placed into a Syracuse dish which was labeled with a code number. The sperm suspension was applied to the eggs with an eyedropper, after which the eggs were allowed to stand for fifteen minutes. Separate eyedroppers were used for each sperm suspension to prevent contamination. An additional precaution consisted of using eyedroppers with black bulbs for the Cascade sperm and eyedroppers with red bulbs for the Red-legged sperm.

Fifteen minutes after application of the sperm the eggs were flooded with tenth normal Holtfreter's solution and allowed to stand for an additional thirty minutes. They were then rinsed off, transferred into numbered finger bowls, and approximately 200 cc. of tenth normal Holtfreter's solution was added for the culture medium.

The first twenty-four cultures were kept in a temperature

controlled room at a temperature of 68° F. while the last thirty-four
cultures were kept at a temperature of 60° F. The numbered finger
bowls were used for the culture containers, the numbers being re-
corded on the corresponding data sheet which indicated the actual
cross of that particular group of eggs. Tenth normal Holtfreter's
solution was used for the culture medium throughout the study.

An attempt was made to keep the concentration of eggs approxi-
mately the same within each bowl. Therefore, approximately fifty
egss were used for each cross and parallel control. Difficulty in
counting the eggs while they were being stripped from the females
resulted in a variation from this number, however, but there appeared
to be no crowding effect even in duplicate crosses made at a later
date when over one hundred eggs were cultured in each finger bowl.

As there was no other light source in the temperature controlled
room, the only radiation which the developing embryos received was
that from a 100 watt electric light bulb which was allowed to burn
for twelve hours during each twenty-four hour period.

Prior to hatching of the embryos, the culture media were changed
each 196 hours. This was accomplished by pouring off as much as
possible of the old medium, rinsing with fresh tenth normal Holt-
freter's solution, pouring this off, and adding about 200 cc. of
fresh medium. Although exact measurements were not made, an attempt
was made to keep approximately the same volume of medium in each
culture bowl.

None of the hybrid embryos hatched, and so each experiment was

terminated after all of the developing control embryos had hatched.
Consequently, the only feeding which was required was that necessary
to maintain the control tadpoles for a few days until it was ascer-
tained that all of the crosses had died. Pulverized commercial rat
food proved to be quite satisfactory for feeding purposes. The food
was left in the culture bowls for a maximum of twenty-four hours,
after which the culture medium was changed.

Records were kept on prepared forms during the development of
the embryos. Separate forms were maintained for each cross and each
control indicating the approximate number of eggs in the particular
cross, the time and date of fertilization, code number of male and
female parents, the number of eggs successfully fertilized, and the
stage of development at each twelve hour period. The stage of de-
velopment was based upon the maximum development attained by at least
fifty percent of the individuals of the culture, using the stages as
outlined by Rugh (25, pp. 61-72). Additional remarks were recorded
indicating mortalities, deformities, variation in growth, and unusual
abnormalities.

Examination of the developing embryos was made in the temperature
controlled room by means of a binocular microscope so that handling
of the embryos would have little effect upon their development.
Embryos which died at various stages of development were removed and
preserved in five percent formaldehyde so that photographs could be
made at a later date. These dead embryos were cut out of the egg

clusters with forceps and sharply pointed scissors so as not to damage adjacent embryos.

As soon as it became apparent that all development of the crosses had ceased, the glass slide was removed, along with excess jelly, and a final count was made of the embryos. This was accomplished by cutting the mass into several small clusters, after which the embryos were removed one at a time, the stage of development being recorded for each one. This was the only accurate count made of the egg masses, as an accurate total number was found to be impossible to derive from examination of the entire mass.

RESULTS

A total of 1,653 eggs were treated with foreign sperm in the cross cultures and 1,674 eggs were treated with natural sperm in the parallel control cultures. The percentages of the eggs which were successfully fertilized and the percentages of the original numbers which developed to various embryonic stages are indicated for each culture in Figure 2 and Figure 3. The parent frogs are indicated by a letter and a number, the letter designating the type of frog (C equals cascadae, A equals aurora) while the number indicates the individual frog involved.

Cultures of cascadae Eggs

There was no significant difference in fertilization between the 790 cascadae eggs treated with aurora sperm and the 661 cascadae eggs which were treated with cascadae sperm, the percentages being as high as 100% for both crosses and controls. Culture #56 was the only culture in which none of the cascadae eggs were successfully fertilized. This particular culture was exposed to A-9 sperm, which successfully fertilized only 3 out of the 318 eggs to which it was applied. Although all eggs were used in computing data, possibly the cultures involving this type of sperm should have been disregarded.

All cascadae eggs which were successfully fertilized developed through the late blastula stage with no apparent differences in structure or developmental rates between hybrids and controls.

Culture No.	No. of Eggs and Source	Sperm Source	Fertilized No. %	Gastrulae No. %	Neural Fold No. %	Tail Bud No. %	Response No. %	Hatched No. %
1	51 from C-1	A-1	47 – 92.2	47 – 92.2	47 – 92.2	18 – 35.3	4 – 07.8	0 – 00.0
2	34 from C-1	A-2	33 – 97.1	33 – 97.1	33 – 97.1	6 – 17.6	2 – 05.9	0 – 00.0
3	48 from C-1	A-3	38 – 79.2	38 – 79.2	35 – 72.9	1 – 02.1	1 – 02.1	0 – 00.0
4	27 from C-1	C-1	26 – 96.3	26 – 96.3	26 – 96.3	11 – 40.7	7 – 25.9	7 – 25.9
5	46 from C-1	C-2	33 – 71.7	33 – 71.7	33 – 71.7	1 – 02.2	1 – 02.2	1 – 02.2
6	43 from C-1	C-3	41 – 95.3	41 – 95.3	41 – 95.3	27 – 62.8	17 – 39.5	8 – 18.6
7	50 from C-2	A-1	46 – 92.0	46 – 92.0	46 – 92.0	6 – 12.0	4 – 08.0	0 – 00.0
8	41 from C-2	A-2	39 – 95.1	39 – 95.1	39 – 95.1	6 – 14.6	6 – 14.6	0 – 00.0
9	41 from C-2	A-3	41 –100.0	41 –100.0	41 –100.0	11 – 26.8	11 – 26.8	0 – 00.0
10	47 from C-2	C-1	45 – 95.7	45 – 95.7	45 – 95.7	45 – 95.7	36 – 76.6	19 – 40.4
11	43 from C-2	C-2	29 – 67.4	29 – 67.4	29 – 67.4	29 – 67.4	7 – 16.3	7 – 16.3
12	48 from C-2	C-3	41 – 85.4	41 – 85.4	41 – 85.4	41 – 85.4	27 – 56.3	27 – 56.3
13	94 from C-3	A-4	94 –100.0	94 –100.0	65 – 69.1	24 – 25.5	11 – 11.7	0 – 00.0
14	107 from C-3	A-5	107 –100.0	107 –100.0	96 – 89.7	13 – 12.1	0 – 00.0	
15	88 from C-3	C-4	81 – 92.0	81 – 92.0	81 – 92.0	81 – 92.0	62 – 70.5	0 – 00.0
16	70 from C-3	C-5	33 – 47.1	33 – 47.1	33 – 47.1	33 – 47.1	22 – 31.4	0 – 00.0
17	31 from A-1	A-4	22 – 71.0	17 – 54.8	0 – 00.0			
18	20 from A-1	A-5	9 – 45.0	1 – 05.0	0 – 00.0			
19	24 from A-1	C-4	8 – 33.3	0 – 00.0				
20	47 from A-1	C-5	15 – 31.9	0 – 00.0				
21	44 from A-2	A-4	44 –100.0	44 –100.0	0 – 00.0			
22	62 from A-2	A-5	62 –100.0	62 –100.0	0 – 00.0			
23	47 from A-2	C-4	14 – 29.8	9 – 19.1	0 – 00.0			
24	43 from A-2	C-5	8 – 18.6	0 – 00.0				
25	88 from A-3	A-6	81 – 92.0	81 – 92.0	64 – 72.7	41 – 46.6	41 – 46.6	31 – 35.2
26	66 from A-3	A-7	62 – 93.9	62 – 93.9	43 – 65.2	43 – 65.2	43 – 65.2	27 – 40.9
27	81 from A-3	A-8	4 – 04.9	4 – 04.9	4 – 04.9	4 – 04.9	4 – 04.9	4 – 04.9
28	76 from A-3	A-9	1 – 01.3	1 – 01.3	0 – 00.0			
29	67 from A-3	C-6	3 – 04.5	3 – 04.5	1 – 01.5	0 – 00.0		
30	84 from A-3	C-7	0 – 00.0					

Figure 2. Fertilization rates and embryonic development of cultures 1 - 30.

Culture No.	No. of Eggs and Source	Sperm Source	Fertilized No. %	Gastrulae No. %	Neural Fold No. %	Tail Bud No. %	Response No. %	Hatched No. %
31	72 from A-3	C-8	0 - 00.0					
32	39 from A-4	A-6	34 - 87.2	34 - 87.2	34 - 87.2	25 - 64.1	25 - 64.1	12 - 30.8
33	42 from A-4	A-7	39 - 92.9	39 - 92.9	39 - 92.9	14 - 33.3	9 - 21.4	5 - 11.9
34	35 from A-4	A-8	1 - 02.9	0 - 00.0				
35	47 from A-4	A-9	2 - 04.3	1 - 02.1	1 - 02.1	0 - 00.0		
36	46 from A-4	C-6	1 - 02.2	1 - 02.2	0 - 00.0			
37	53 from A-4	C-7	0 - 00.0					
38	59 from A-4	C-8	3 - 05.1	1 - 01.7	1 - 01.7	0 - 00.0		
39	47 from A-5	A-6	46 - 97.9	46 - 97.9	46 - 97.9	23 - 48.9	23 - 48.9	11 - 23.4
40	31 from A-5	A-7	28 - 90.3	28 - 90.3	23 - 74.2	23 - 74.2	15 - 48.4	8 - 25.8
41	51 from A-5	A-8	8 - 15.7	8 - 15.7	5 - 09.8	5 - 09.8	5 - 09.8	3 - 05.9
42	66 from A-5	A-9	0 - 00.0					
43	47 from A-5	C-6	4 - 08.5	4 - 08.5	4 - 08.5	0 - 00.0		
44	47 from A-5	C-7	1 - 02.1	1 - 02.1	1 - 02.1	0 - 00.0		
45	74 from A-5	C-8	2 - 02.7	2 - 02.7	1 - 01.4	0 - 00.0		
46	49 from A-6	A-6	49 -100.0	49 -100.0	49 -100.0	29 - 59.2	29 - 59.2	7 - 14.3
47	44 from A-6	A-7	41 - 93.2	41 - 93.2	41 - 93.2	17 - 38.6	16 - 36.4	7 - 15.9
48	53 from A-6	A-8	6 - 11.3	6 - 11.3	6 - 11.3	4 - 07.5	4 - 07.5	3 - 05.7
49	41 from A-6	A-9	0 - 00.0					
50	36 from A-6	C-6	6 - 16.7	6 - 16.7	4 - 11.1	1 - 02.8	0 - 00.0	
51	53 from A-6	C-7	1 - 01.9	0 - 00.0				
52	64 from A-6	C-8	2 - 03.1	1 - 01.6	0 - 00.0			
53	75 from C-4	A-6	75 -100.0	75 -100.0	5 - 06.7	5 - 06.7	5 - 06.7	0 - 00.0
54	92 from C-4	A-7	92 -100.0	92 -100.0	45 - 48.9	34 - 37.0	7 - 07.6	0 - 00.0
55	69 from C-4	A-8	69 -100.0	69 -100.0	22 - 31.9	22 - 31.9	13 - 18.8	0 - 00.0
56	88 from C-4	A-9	0 - 00.0					
57	102 from C-4	C-6	102 -100.0	102 -100.0	87 - 85.3	71 - 69.6	43 - 42.2	29 - 28.4
58	58 from C-4	C-7	56 - 96.6	56 - 96.6	56 - 96.6	56 - 96.6	52 - 89.7	47 - 81.0
59	89 from C-4	C-8	86 - 96.6	86 - 96.6	86 - 96.6	86 - 96.6	86 - 96.6	63 - 70.8

Figure 3. Fertilization rates and embryonic development of cultures 31 - 59.

The first discernible abnormalities involving these crosses occurred during the process of gastrulation when many of the hybrids exhibited various degrees of exogastrulation, as exemplified by the embryo from Culture #13 in Figure 4. Although a considerable number of the hybrid gastrulae were deformed in this manner, the majority of them developed through the neural fold stage. It is interesting to note, however, that in Cultures #53, 54, and 55 there was a greater mortality between gastrulation and the formation of the neural folds than in the other eight cultures of hybrids from cascadae eggs. These three cross cultures were kept at a temperature which was eight degrees colder than the earlier cross cultures. The control cultures, which were kept at the same temperature, did not show as appreciable an increase in mortality at this stage.

The cultures of hybrids derived from cascadae eggs which were kept at a temperature of 68° F. exhibited a greater increase in mortality between the neural fold stage and the tail bud stage than did the cultures which were kept at a temperature of 60° F. Those individuals which survived to the tail bud stage appeared to be normal in development of the anterior regions, whereas the posterior regions were abnormal in many individuals due to incomplete incorporation of the yolk material. The posterior disorders resulted in the tail developing at approximately a forty-five degree angle to the longitudinal axis of the body. The presence of internal disorders is indicated by the fact that none of the developing hybrids hatched,
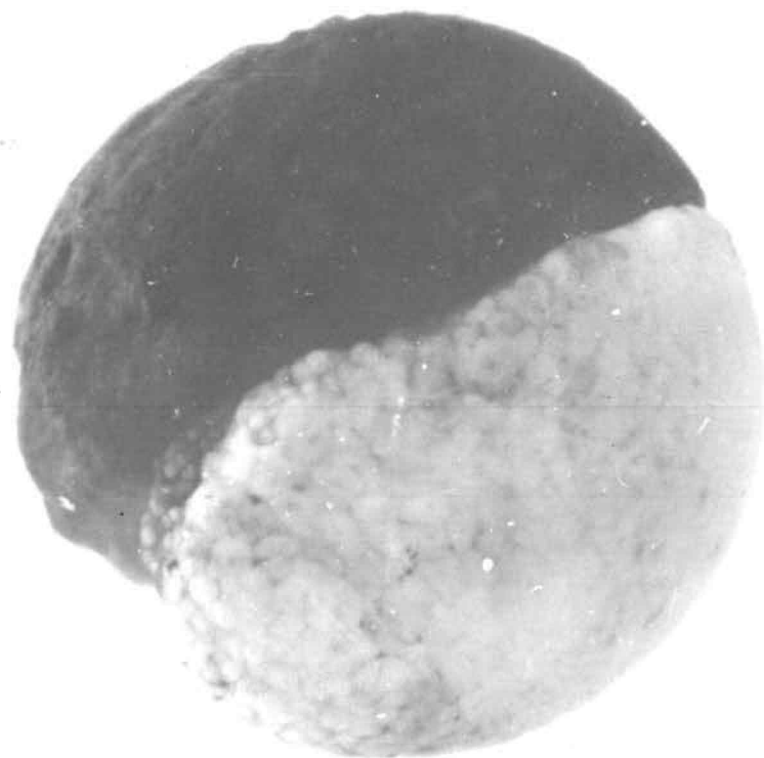
Figure 4. Exogastrulation of hybrid embryo from Culture 13.

although from external appearance several appeared to be quite normal
at the muscular response stage.

Cultures of aurora Eggs

In all of the reciprocal crosses, in which aurora eggs were used,
the percentage of eggs which were successfully fertilized was much
lower than that for the cascadae eggs. There was a considerable dif-
ference between the fertilization rates of the control cultures and
that of the cross cultures. Approximately 53% of the aurora eggs
which were treated with aurora sperm were fertilized, whereas only an
average of about 7% of those treated with cascadae sperm were
fertilized.

Abnormalities were evident in the cross cultures from the first
cleavage stages on through later development. Many of the eggs which
had appeared to have been fertilized failed to cleave and those which
did cleave were quite variable in their cleavage rates. Less than one
half of the aurora eggs which were fertilized by cascadae sperm devel-
oped to the gastrula stage. Many mortalities occurred in the late
blastula stages when the control embryos were beginning to gastrulate.

Because none of the cultures of aurora eggs which were maintained
at 68° F. developed beyond the gastrula stage this series of crosses
was repeated at a temperature of 60° F. The same abnormalities
occurred in the cultures maintained at 60° F. as had occurred previ-
ously at 68° F. However, twelve of the developing hybrids survived

to the neural fold stage and one of these continued to develop to the
tail bud stage after which it died. As embryos were successfully
reared through hatching in each of the cultures of controls at this
temperature no further crosses were made.

Summary of Results

The average fertilization rates and the extent of development of
the eggs from each source are indicated in Figure 5. The hybrid
embryos derived from cascadae eggs incurred the highest mortalities
after the neural fold stage, whereas the hybrid embryos derived from
aurora eggs showed the greatest increase in mortality prior to the
gastrulation process. These differences are summarized in Figure 6
which indicates the over-all averages for the two types of eggs.

Figures 7 and 8 indicate the relative fertilization rates and
the extent of development as related to the sperm source. From these
data it is obvious that some of the sperm suspensions, especially
that of A-8 and A-9, were quite dilute or inactive. It is also
apparent that the cascadae eggs were more readily fertilized than the
aurora eggs as evidenced by the results of fertilization attempts
with types A-4, A-5, A-6, A-7, and A-8 sperm with which 100% of the
cascadae eggs were fertilized as opposed to smaller percentages of
the aurora eggs which were fertilized in the corresponding control
cultures.

No. of Eggs and Source	Sperm Source	Fertilized No. %	Gastrulae No. %	Neural Fold No. %	Tail Bud No. %	Response No. %	Hatched No. %
133 from C-1	aurora	118 - 88.7	118 - 88.7	115 - 86.5	25 - 18.8	7 - 05.3	0 - 00.0
116 from C-1	cascadae	100 - 86.2	100 - 86.2	100 - 86.2	39 - 33.6	25 - 21.6	16 - 13.8
132 from C-2	aurora	126 - 95.5	126 - 95.5	126 - 95.5	23 - 17.4	21 - 15.9	0 - 00.0
138 from C-2	cascadae	115 - 83.3	115 - 83.3	115 - 83.3	115 - 83.3	70 - 50.7	53 - 38.4
201 from C-3	aurora	201 -100.0	201 -100.0	161 - 80.1	37 - 18.4	11 - 05.5	0 - 00.0
158 from C-3	cascadae	114 - 72.2	114 - 72.2	114 - 72.2	114 - 72.2	84 - 53.2	0 - 00.0
51 from A-1	aurora	31 - 60.8	18 - 35.3	0 - 00.0			
71 from A-1	cascadae	23 - 32.4	0 - 00.0				
106 from A-2	aurora	106 -100.0	106 -100.0	0 - 00.0			
90 from A-2	cascadae	22 - 24.4	9 - 10.0	0 - 00.0			
311 from A-3	aurora	148 - 47.6	148 - 47.6	111 - 35.7	88 - 28.3	88 - 28.3	62 - 19.9
223 from A-3	cascadae	3 - 01.3	3 - 01.3	1 - 00.8	0 - 00.0		
163 from A-4	aurora	76 - 46.6	74 - 45.4	74 - 45.4	39 - 23.9	34 - 20.9	17 - 10.4
158 from A-4	cascadae	4 - 02.5	2 - 01.3	1 - 00.6	0 - 00.0		
195 from A-5	aurora	82 - 42.1	82 - 42.1	74 - 37.9	51 - 26.2	43 - 22.1	22 - 11.3
168 from A-5	cascadae	7 - 04.2	7 - 04.2	6 - 03.6	0 - 00.0		
187 from A-6	aurora	96 - 51.3	96 - 51.3	96 - 51.3	50 - 26.7	49 - 26.2	17 - 09.1
153 from A-6	cascadae	9 - 05.9	7 - 04.6	4 - 02.6	1 - 00.7	0 - 00	
324 from C-4	aurora	236 - 72.8	236 - 72.8	72 - 22.2	61 - 18.8	25 - 07.7	0 - 00.0
249 from C-4	cascadae	244 - 98.0	244 - 98.0	229 - 92.0	213 - 85.5	181 - 72.7	139 - 55.8

Figure 5. The average fertilization rates and embryonic development as related to egg source.

No. of Eggs and Source	Sperm Source	Fertilized No. %	Gastrulae No. %	Neural Fold No. %	Tail Bud No. %	Response No. %	Hatched No. %
790 from 4 cascadae	aurora	681 - 86.2	681 - 86.2	474 - 60.0	146 - 18.5	64 - 08.1	0 - 00.0
661 from 4 cascadae	cascadae	573 - 86.7	573 - 86.7	558 - 84.4	481 - 72.8	360 - 54.5	208 - 31.5
1013 from 6 aurora	aurora	539 - 53.2	524 - 51.7	355 - 35.0	228 - 22.5	214 - 21.1	118 - 11.6
863 from 6 aurora	cascadae	68 - 07.9	28 - 03.2	12 - 01.4	1 - 00.1	0 - 00.0	

Figure 6. The over-all averages of the fertilization rates and extent of embryonic development as related to the two types of eggs and sperm.

Sperm Source	No. of Eggs and Source	Fertilized No. %	Gastrulae No. %	Neural Fold No. %	Tail Bud No. %	Response No. %	Hatched No. %
A-1	101 from cascadae	93 - 92.1	93 - 92.1	93 - 92.1	24 - 23.8	8 - 07.9	0 - 00.0
A-2	75 from cascadae	72 - 96.0	72 - 96.0	72 - 96.0	12 - 16.0	8 - 10.7	0 - 00.0
A-3	89 from cascadae	79 - 88.8	79 - 88.8	76 - 85.4	12 - 13.5	12 - 13.5	0 - 00.0
A-4	94 from cascadae	94 -100.0	94 -100.0	65 - 69.1	24 - 25.5	11 - 11.7	0 - 00.0
A-4	75 from aurora	66 - 88.0	61 - 81.3	0 - 00.0			
A-5	107 from cascadae	107 -100.0	107 -100.0	96 - 89.7	13 - 12.1	0 - 00.0	
A-5	82 from aurora	71 - 86.6	63 - 76.8	0 - 00.0			
A-6	75 from cascadae	75 -100.0	75 -100.0	5 - 06.7	5 - 06.7	5 - 06.7	0 - 00.0
A-6	223 from aurora	210 - 94.2	210 - 94.2	193 - 86.5	118 - 52.9	118 - 52.9	61 - 27.4
A-7	92 from cascadae	92 -100.0	92 -100.0	45 - 48.9	34 - 37.0	7 - 07.6	0 - 00.0
A-7	183 from aurora	170 - 92.9	170 - 92.9	146 - 79.8	97 - 53.0	83 - 45.4	47 - 25.1
A-8	69 from cascadae	69 -100.0	69 -100.0	22 - 31.9	22 - 31.9	13 - 18.8	0 - 00.0
A-8	220 from aurora	19 - 08.6	18 - 08.2	15 - 06.8	13 - 05.9	13 - 05.9	10 - 04.5
A-9	88 from cascadae	0 - 00.0					
A-9	230 from aurora	3 - 01.3	2 - 00.9	1 - 00.4	0 - 00.0		

Figure 7. The average fertilization rates and embryonic development as related to the aurora sperm.

Sperm Source	No. of Eggs and Source	Fertilized No. %	Gastrulae No. %	Neural Fold No. %	Tail Bud No. %	Response No. %	Hatched No. %
C-1	74 from cascadae	71 - 95.9	71 - 95.9	71 - 95.9	56 - 75.7	43 - 56.8	26 - 35.1
C-2	89 from cascadae	62 - 69.7	62 - 69.7	62 - 69.7	30 - 33.7	8 - 09.0	8 - 09.0
C-3	91 from cascadae	82 - 91.0	82 - 91.0	82 - 91.0	68 - 74.7	44 - 48.4	35 - 38.6
C-4	88 from cascadae	81 - 92.0	81 - 92.0	81 - 92.0	81 - 92.0	62 - 70.5	0 - 00.0
C-4	71 from aurora	22 - 31.0	9 - 12.7	0 - 00.0			
C-5	70 from cascadae	33 - 47.1	33 - 47.1	33 - 47.1	33 - 47.1	22 - 31.4	0 - 00.0
C-5	90 from aurora	23 - 25.6	0 - 00.0				
C-6	102 from cascadae	102 -100.0	102 -100.0	87 - 85.3	71 - 69.6	43 - 42.2	29 - 28.4
C-6	196 from aurora	14 - 07.1	14 - 07.1	9 - 04.6	1 - 00.5	0 - 00.0	
C-7	58 from cascadae	56 - 96.6	56 - 96.6	56 - 96.6	56 - 96.6	52 - 89.7	47 - 81.0
C-7	237 from aurora	2 - 00.8	1 - 00.4	1 - 00.4	0 - 00.0		
C-8	89 from cascadae	86 - 96.6	86 - 96.6	86 - 96.6	86 - 96.6	86 - 96.6	63 - 70.8
C-8	269 from aurora	7 - 02.6	4 - 01.5	2 - 00.7	0 - 00.0		

Figure 8. The average fertilization rates and embryonic development as related to the cascadae sperm.

DISCUSSION

The modern concept of a species stipulates that each species must be a population that is reproductively isolated from other populations and, in addition, is usually morphologically, physiologically, and ecologically distinct. As stated in the introductory remarks, the probability of reproductive isolation is the primary criterion for determining if a population has evolved to the species level. "No degree of morphological distinctness is in itself proof of specific distinctness. The probability of reproductive isolation is the primary criterion. To emphasize this point is important, because the degree of reproductive isolation of a geographically isolated form is not necessarily correlated with the degree of morphological distinctness, nor is the degree of morphological distinctness necessarily a good index of genetic distinctness. A single gene difference may produce a phenotypic difference which might cause some taxonomists to call the population carrying it a different species" (8, p. 135). Because there are generally several isolating mechanisms involved in keeping species separate, greater stress should be laid on intergradation under natural conditions than on potential interbreeding under artificial conditions. However, intergradation of any kind is evidence which would indicate that the populations involved are on the subspecies level of differentiation.

Based on this philosophy of reproductive isolation, the interspecific and intraspecific relationships of various herpetological

forms have been determined in recent years. One of four patterns of development generally occurs in zygotes derived from interspecific crosses. Some eggs will undergo no cleavage at all upon being fertilized by foreign sperm. This was found by Moore (20, p. 420) to be typical of eggs from Rana clamitans when fertilized by Rana sylvatica, Rana pipiens, Rana palustris, Rana catesbeiana, Rana septentrionalis, or Rana heckscheri sperm.

In the second pattern of development, hybrid zygotes will cleave in an apparent normal manner prior to gastrulation but fail to complete this process. These zygotes, which die in the process of gastrulation, generally develop an exogastrula condition which results from failure to incorporate the entire yolk mass. In these forms, the cleavage rate is generally reported to be maternal (20, p. 410) and apparently the sperm does not exert its influence until the late blastula and early gastrula stages. At this time incompatibility of the egg and the foreign sperm causes the death of the individual. Exogastrulation is found to be typical of hybrids derived from crosses between female Rana sylvatica and male Rana pipiens, Rana palustris, Rana catesbeiana, or Rana sphenocephala; female Rana pipiens and male Rana sylvatica or Rana catesbeiana; female Rana palustris and male Rana sylvatica; female Rana catesbeiana and male Rana pipiens (20, pp. 405-422). Zweifel (38, pp. 260-261) reports a high frequency of exogastrulation in crosses between female Rana muscosa and male Rana boylei and between female Rana (aurora) cascadae and male Rana (aurora) aurora. Volpe (34, pp. 61-74) also obtained exogastrulae

in crosses between female Bufo valliceps and male Bufo fowleri.

Hybrid larvae which survive the gastrulation process tend to continue to develop, in a manner which appears to be normal from external appearance, until they have reached the neural fold or tail bud stages. By the tail bud stage the majority of these hybrids will have developed a dorsal bending of the body, upturned tail, and an enlarged abdomen, and subsequently die. This type of abnormal development occurs in hybrids derived from crosses between female Bufo fowleri and male Bufo valliceps (34, pp. 61-74) and from crosses between female Scaphiopus couchi and male Scaphiopus hurteri (37, p. 324).

Closely related species are sometimes interfertile and hybrids derived from crosses between two such species may develop normally through embryonic and larval stages, even transforming in some instances. Thus, normal development of hybrids has been obtained in crosses between female Rana palustris and male Rana pipiens; female Rana pipiens and male Rana palustris or male Rana sphenocephala (20, p. 420); between female Scaphiopus hurteri and male Scaphiopus couchi (37, p. 329); and between female Bufo woodhousei and male Bufo valliceps (29, pp. 461-462). Even though these hybrids are able to develop normally, their rate of development is not consistent with that of the parent types, being either retarded or accelerated over the maternal rate.

The F-1 hybrid generations resulting from crosses between these closely related species are usually inviable, as indicated by the

back-crosses of the zygotes from cross-mating female Bufo woodhousei and male Bufo valliceps which developed no further than the blastula stage (29, p. 468). Hybrids of Scaphiopus hurteri - couchi were highly inviable resulting in some anomalous cleavage but no further development of the F-2 generation (37, p. 326). Thus, there is an effective block to the exchange of genetic materials between even these closely related species.

The results of the crosses made in the present study are very consistent with the first three patterns of interspecific hybrid development discussed above. Hybrid larvae derived from fertilizing cascadae eggs with aurora sperm generally followed the third pattern of development when the cultures were maintained at a temperature of 68° F. The maximum development of these hybrids was to the muscular response stage, while the majority developed no further than the neural fold stage. Those individuals which did develop to the tail bud stage exhibited the identical abnormalities described above; namely, a dorsal bending of the body, upturned tail, and an edematous abdomen.

Hybrids of this same type which were cultured at a temperature of 60° F. showed these same abnormalities, but the majority followed the second pattern of development terminating in an exogastrula condition. A comparison of the abnormal development of these hybrids, as illustrated in Figure 9, can be made with the normal development of cascadae larvae, as indicated in Figure 10.

12 hours 24 hours 36 hours 48 hours 60 hours 72 hours

84 hours 96 hours 108 hours 120 hours

132 hours 144 hours 156 hours 168 hours

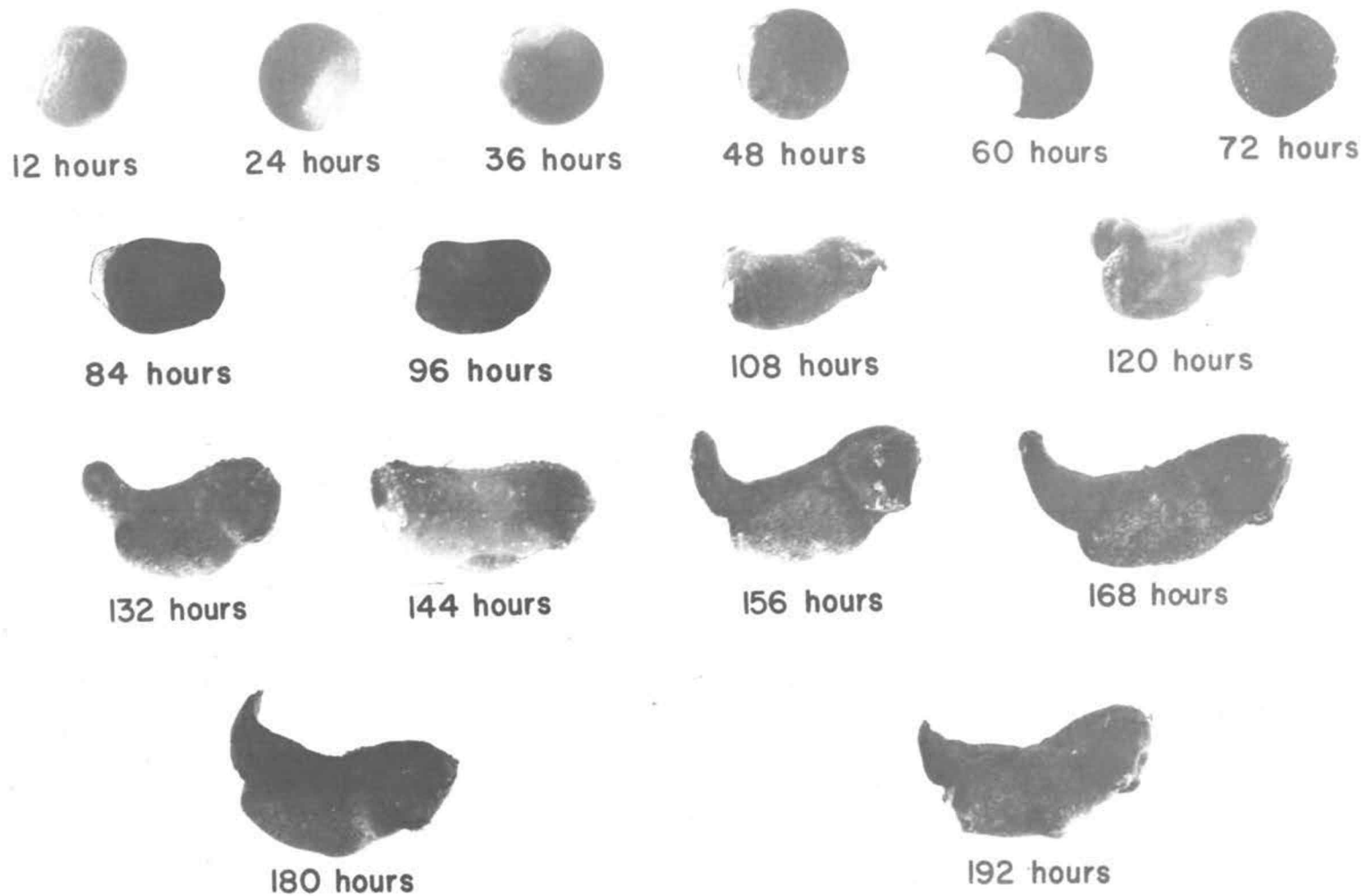180 hours 192 hours

Figure 9. Abnormal development of hybrids derived from cascadae eggs and aurora sperm.

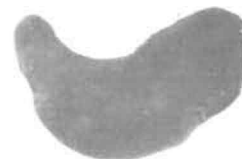12 hours   24 hours   36 hours   48 hours   60 hours   72 hours

84 hours   96 hours   108 hours   120 hours

132 hours   144 hours   156 hours   168 hours

180 hours   192 hours

Figure 10. Normal development of _cascadae_ larvae.

The hybrids derived from _aurora_ eggs and _cascadae_ sperm also exhibited these same characteristics of interspecific hybrids. A higher degree of incompatability of eggs and sperm in these crosses is indicated by the very reduced fertilization rates plus the smaller number of individuals which was able to survive the gastrulation process.

In summary, the results of these experimental crosses definitely indicate that an intrinsic reproductive isolating mechanism, characteristic of species, prevents the Red-legged and Cascade frogs from intergrading. The fact that none of the developing hybrids even hatched would indicate that not only are these two forms separate species but that they may not even be closely related species.

The Red-legged and Cascade frogs are ecologically quite distinct forms. The Cascade frog occurs principally in the upper elevations of the Cascade Mountains of Washington, Oregon, and California and in the Olympic Mountains of Washington. It is found at higher elevations than the Red-legged frog which occurs at lower elevations west of the crest of the Cascade Mountains of southern British Columbia, Washington, Oregon, and northern California.

Slater (27, p. 146) describes the Cascade frog as being typically a Hudsonian frog which overlaps the distribution of the Red-legged frog in the Canadian Zone. The Cascade frog is the only _Rana_ which Slater (27, p. 149) finds in the Hudsonian Zone of Washington. The author and others have found the Oregon distribution of the Cascade frog to be primarily in the Hudsonian, Canadian, and Upper Transition

Zones of the Cascades while the Red-legged frog is confined to the
Transition Zone, being most abundant in the lower elevations.
Stebbins (28, p. 148) states that the Cascade frog is principally in
the Boreal Zone (commonly the Hudsonian Zone) but that it is found as
low as the Upper Transition Zone. He also notes (28, p. 146) that it
is found from higher elevations than aurora which is found down to the
upper portions of the Lower Sonoran Life-Zone in California. Thus,
these two forms have a consistent altitudinal relationship to one
another, with a limited overlap of their ranges within the Transition
Zone.

The habitat preferences of the Red-legged and Cascade frogs are
also quite different. Except during the breeding season when they mi-
grate to ponds, adult Red-legged frogs are found most commonly among
down logs, ferns, blackberry thickets, and dense understory types of
vegetation. They may often be found in damp situations quite some
distance from the nearest body of water. The Red-legged frog popula-
tion at the Coffin Butte ponds, north of Corvallis, was the only ex-
ception to this habitat preference noted by the author. These frogs
are found only in the immediate vicinity of the ponds and apparently
spend a considerable amount of time actually in the water. There is
very little vegetative cover around these ponds and the surrounding
area is quite arid, being a rather barren southern exposure. There-
fore, the close relationship of these frogs to the ponds is almost a
necessity for the survival of this population. Dunlap (11, p. 327)
also found that these Coffin Butte frogs departed to the greatest ex-
tent from the general habitat type characteristic of Red-legged frogs.

In contrast to the Red-legged frogs, the Cascade frog is found most commonly in close proximity to small streams and sub-alpine meadow potholes, being rarely more than a few yards from permanent bodies of water. These frogs appear to prefer to inhabit stream banks which are covered with grass, ferns, or other types of low herbaceous vegetation.

The breeding season of the Cascade frog is from the latter part of May to July, while that of the Red-legged frog is from the middle of January until March. This difference undoubtedly arose as a result of physiological adaptations to the two different climatic conditions which prevail at the respective altitudes where these forms are found. Snow depth is probably a very important factor affecting the initiation of the breeding activity of the Cascade frog, as pointed out by Slater (27, p. 149). Night temperature and precipitation seem to be the primary factors which stimulate sexual activity in Red-legged frog populations.

The eggs of aurora are deposited in relatively deep and cool ponds while those of cascadae are deposited in the shallows of deep pools or in shallow ponds which are, consequently, warmer during the day. Therefore, although the Cascade frog is found at higher altitudes, its embryonic development is undoubtedly adapted to warmer daytime temperatures than that of the Red-legged frog.

The masses of aurora eggs are quite compact and ovoid in contrast to the more flattened cascadae egg masses. In addition, the cascadae egg masses are deposited at or near the surface of the water while

those of *aurora* are attached to vegetation at greater depths. Both of these characteristics of *cascadae* egg masses could be interpreted as adaptations to warmer surroundings, as discussed by Moore (19, pp. 89-90).

On the basis of morphology, *cascadae* frogs can always be distinguished from *aurora*. Adult morphological characteristics which distinguish *cascadae* from *aurora* have been described by Dunlap (11, pp. 314-331). It is very significant that Dunlap (11, p. 323) could find no instance where individuals showed an overlap or intergradation of morphological characteristics. It was noted by the author that the *cascadae* tadpoles generally were a darker shade of brown dorsally than the *aurora* tadpoles. The *cascadae* tadpoles also tend to have a more blunt tail fin than that of the *aurora* tadpole which is quite pointed. *Cascadae* and *aurora* eggs may be quite readily distinguished on the basis of color. In the *cascadae* eggs the animal pole is very darkly pigmented and greatly contrasts the vegetal pole which is bright creamish yellow in color. *Aurora* eggs, on the other hand, have a dark animal pole but a less contrasting gray-brown vegetal pole.

On the basis of the experimental crosses made in this study plus the single cross made by Zweifel (38, p. 261) it is apparent that *Rana aurora cascadae* and *Rana aurora aurora* are reproductively isolated and incapable of intergrading with one another. The morphological studies of Dunlap (11, pp. 314-331) and observations by the author and others leave no doubt as to the morphological distinctness

of these two frogs. Ecological and physiological differences between these forms have been established. In view of this overwhelming evidence it is concluded that the Red-legged and Cascade frogs meet all of the specifications required of biological species and should, therefore, be separate species, designated as Rana aurora Baird and Girard and Rana cascadae Slater.

The embryonic development of each of these species is greatly affected by the ambient temperature. Therefore, a further step in experimental crosses, such as have been conducted in this study, would be to determine the optimum temperatures for the development of Rana aurora and Rana cascadae respectively and then to determine the effects of various ambient temperatures upon the development of hybrids.

SUMMARY

1. The precise taxonomic status of the Red-legged and Cascade frogs has been questionable up to the present study.

2. The modern concept of a species stipulates that each species must be a population that is reproductively isolated from other populations and is usually morphologically, physiologically, and ecologically distinct.

3. The purpose of this study was, through experimental crosses, to determine if a reproductive isolating mechanism exists which would prevent Rana aurora aurora and Rana aurora cascadae from intergrading. The presence of such a reproductive isolation mechanism would indicate that a specific level of differentiation had been reached by these frogs.

4. A series of reciprocal crosses has been made under laboratory conditions, using the pituitary technique to induce ovulation and employing artificial fertilization. Parallel controls were established for each cross.

5. The results of the crosses are consistent with those of other interspecific hybridization studies. None of the hybrids hatched.

6. Hybrids derived from fertilizing cascadae eggs with aurora sperm developed no further than the muscular response stage. Individuals which developed this far exhibited a dorsal bending of the body, upturned tail, and an edematous abdomen. Hybrids of this type maintained at 68° F. tended to develop further than those maintained at 60° F.

7. Very few hybrids derived from _aurora_ eggs and _cascadae_ sperm survived the gastrulation process. A higher degree of incompatability of eggs and sperm in these crosses is indicated by a very reduced fertilization rate.

8. The results of these experimental crosses definitely indicate the presence of an intrinsic reproductive isolating mechanism between the Red-legged and Cascade frogs.

9. The Red-legged and Cascade frogs are ecologically and morphologically distinct and they exhibit different breeding habits.

10. On the basis of this evidence, it is concluded that the Red-legged and Cascade frogs meet the specifications for species and should, therefore, be separate species, designated as _Rana aurora_ Baird and Girard and _Rana cascadae_ Slater.

11. A further step would be to determine the effects of temperature upon the embryonic development of these two frogs and upon the development of hybrids.

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