Environmental factors and the incidence of neoteny in *Ambystoma gracile* (Baird) (Amphibia: Caudata)

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Laboratory experiments were conducted to evaluate the effects of temperature, water evaporation rate, and food availability on the incidence of neoteny in *Ambystoma gracile* and to assess the nature of its genetic control. Larvae hatched from six clutches of eggs from ponds in British Columbia were reared until they were about to metamorphose. Six experiments were then initiated, each involving larvae from a single distinct clutch. Results indicated that at 12°C larvae metamorphosed later and the incidence of neoteny was higher than at 19°C; similarly, larvae fed once per week transformed more slowly and were more frequently neotenic than larvae fed three times per week. The rate at which water evaporated, and associated changes in salinity and depth appeared to have no effect. With one exception, under standard conditions there were no differences in the timing of metamorphosis of larvae from different clutches and different populations. It appears that populations of *A. gracile* are highly polymorphic, comprising some individuals which always metamorphose, some which never do, and others which may or may not transform, depending on the nature of the habitat.

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Des expériences en laboratoire ont permis d'estimer les effets de la température, du taux d'évaporation d'eau et de la disponibilité de la nourriture sur la fréquence de la néoténie chez *Ambystoma gracile*; on a cherché à déterminer également la nature du contrôle génétique de la néoténie. On a donc procédé à l'élevage de larves provenant de six masses d'oeufs recueillies dans des étangs de Colombie Britannique. Les larves, un peu avant la métamorphose, ont ensuite été soumises à six expériences différentes, chacune impliquant des larves d'une même masse d'oeufs. A 12°C, les larves se métamorphosent plus tard et la fréquence de la néoténie est plus grande qu'à 19°C; de même, les larves nourries une seule fois par semaine se développent plus lentement et sont plus souvent néoténiques que les larves nourries trois fois par semaine. La vitesse d'évaporation d'eau et, conséquemment, les changements de salinité et de profondeur ne semblent pas avoir d'effet. A une exception près, dans des conditions normales, il n'y a pas de différences dans le moment de déclenchement de la métamorphose chez les larves de différentes masses d'oeufs et de différentes populations. Les populations d'*A. gracile* semblent donc polymorphes, contenant des individus qui se métamorphosent et d'autres qui ne se transforment jamais, ainsi que des individus qui peuvent ou non se transformer, selon la nature de l'habitat.
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In a recent paper (Sprules 1974), I hypothesized that paedogenetic salamanders were adapted to habitats such as high altitudes and parts of the eastern coastal plain where terrestrial conditions for amphibians are relatively severe. The specific nature of this adaptation, however, is imperfectly understood. Whether for a given *Ambystoma* species some individuals in a population always metamorphose and others never do, whether metamorphosis depends on the physical conditions in the habitat, or whether some combination occurs, is not known. In this paper, the results of laboratory experiments on *Ambystoma gracile* are presented which indicate that not only are environmental factors of considerable importance in the determination of neoteny but other mechanisms are also involved.

With the exception of some work by Humphrey (e.g. Humphrey and Bagnara 1967), most of the evidence on the genetic control of neoteny in *Ambystoma* is indirect. The majority of individuals in populations of the Mexican axolotls appear never to transform (Deuchar 1957; Tihen 1969), suggesting that these species are obligate neotenes. On the other hand, in populations of *A. gracile* which consist of both transformed and neotenic adults, it would seem that some sort of polymorphism exists. My own observations on *A. tigrinum nebulosum* (Sprules 1972) indicate that the physical nature of the habitat alone may determine whether or not adults will be neotenic. Most of the available information on the effects of environmental factors on amphibian metamorphosis is for anurans (Moore 1964; Frieden 1968; Kaltenbach 1968); few experiments have been conducted with salaman-

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ders and even fewer have involved neotenic species. Shrode's (1972) experiments with *A. tigrinum* and Snyder's (1956) with *A. gracile* demonstrated that the rate of metamorphosis is a direct function of temperature; Snyder found that more neotenic individuals occurred at low than at high temperatures. Powers (1903) provided preliminary experimental evidence that reduced food intake accelerates metamorphosis in *A. tigrinum*, and Wilbur and Collins (1973) showed that food availability affects the growth rate and hence the timing of metamorphosis for *A. maculatum*. Indirect evidence from field studies suggests that for a variety of salamander species the drying of ponds and associated changes in salinity and oxygen may initiate transformation (Anderson 1967; Dempster 1930; Powers 1903; Kezer and Farner 1955; Hassinger *et al.* 1970; Gehlbach 1965; Graf 1949; Healy 1970; Reed 1949). It thus appears as though temperature, rate of evaporation, and food quantity may be important in the regulation of metamorphosis, and hence neoteny, in some species. In the experiments presented here, the effects of these environmental factors on the incidence of neoteny in *A. gracile* are investigated and some observations on its genetic control are presented.

Methods

I had originally hoped to obtain eggs for these experiments from known transformed or neotenic adults. However, the difficulty of breeding the animals in the laboratory made it necessary to collect fertilized *A. gracile* eggs from the field without knowing for certain the status of the adults. The eggs were collected from three localities in southwestern British Columbia during April and May, 1972, immediately after they appeared in the ponds. A continuous uniform mass of eggs was considered to have been laid by one female although it could have been fertilized by more than one male. Six separate clutches were collected. Clutches 1 and 2 came from two separate ponds in a river overflow in the Surrey area at a 60-m altitude. Clutches 3, 4, and 5 came from a single pond associated with the Little Campbell River about 2.5 km southwest of the first site and at an altitude of 75 m. Clutch 6 was collected in a small permanent pond (12 by 6 m, 1.2 m deep) above Horseshoe Bay at an altitude of 275 m and about 50 km northwest of the other ponds. The eggs were hatched and the larvae reared until just before metamorphosis, when the experiments were initiated.

During rearing, the larvae from different clutches were kept separate but treated identically. The eggs were shipped immediately to the laboratory where they were placed in small aquaria filled with well aerated, dechlorinated, tap water maintained at room temperature. From hatching until about 1 month of age, the larvae were kept in 300-ml glass preparation dishes, from 1 to 3 months of age in 450-ml plastic containers, and from 3 to 6 months of age in 800-ml plastic containers. To minimize mortality by cannibalism, salamander densities in these containers were gradually reduced from about five per bowl to one per bowl as the larvae grew larger. At 6–8 months of age they were transferred to 250-l fish tanks, where they were separately maintained in 3.4-l screened enclosures. Soon thereafter they were permitted to swim freely in the tanks.

Newly hatched larvae were fed day-old brine-shrimp larvae which had been carefully rinsed in fresh water. At approximately one month of age and 2 cm total length the larvae began to feed on *Daphnia magna* which were netted from a culture and dropped into the bowls. By the time they were 3 months old and approximately 1.5 cm long snout to vent (SV), and throughout the rest of the study, they were fed exclusively tubifex worms which were dropped in small clumps into the bowls. For one brief period just before the experiments were started, newly hatched *Bufo americanus* larvae were used as food. During the first 6–8 months of the rearing period, water temperatures were maintained at 19–20°C, except during the first 2 months, when half the larvae from each clutch were held at 16°C in an unsuccessful attempt to reduce high rates of mortality observed at higher temperatures. As no differences in growth were associated with this latter treatment, the larvae were recombined for the experiments. Water temperatures of 16°C were maintained in the fish tanks where the larvae spent the latter portion of the rearing period. The photoperiod was maintained constantly at 14 h light : 10 h dark.

On March 23, 1973, one larva from clutch 5 began metamorphosis, and the experiments were initiated on March 28. Six experiments were set up, each involving only larvae from a single clutch to minimize genetic differences among individuals. The numbers of reared larvae and their ages at the beginning of the experiment were:

Clutch	Number at Hatching	Number after *ca.* 1 year	Age (days from hatching)
1	55	34	343
2	66	16	343
3	49	27	332
4	134	50	332
5	78	23	332
6	128	32	317

Slight differences in age result from the various times at which eggs became available. With one exception, when too few individuals had survived rearing, the larvae in each experiment were divided into a "control" and one or more "experimental" groups (Table 1). Fiberglass fish tanks ranging from 150 to 640 l and, in one case (clutch 5), 190-l glass aquaria were used; within the limits of available tanks, identical sizes were used within each experiment to minimize density differences. Dechlorinated tap water was used in all tanks and

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conditions of temperature, evaporation, and food were maintained as indicated in Table 1. The photoperiod during the experiments was 14 h light : 10 h dark. In all tanks, regular determinations were made of salinity with a Hach model 2200 conductivity meter, of dissolved oxygen with a Yellow Springs Instruments model 51A oxygen meter, of temperature with a simple mercury thermometer, and of water depth. Transforming larvae showing dorsal and tail fin reduction, gill reduction, increased prominence of eyes, and the appearance of distinct costal grooves were removed and their lengths determined. All length measurements were taken from snout to vent. Individuals which died during the experiment were examined for signs of metamorphosis, measured, and removed. When tanks dried up during the evaporation experiments, the larvae were removed, the tanks cleaned, refilled to their original levels with dechlorinated tap water, and the larvae replaced.

Results

The growth and onset of metamorphosis for these laboratory-reared larvae compares favorably with that determined by Neish (1971) for natural populations of *A. gracile*. He found that larvae transformed in late August of their second year when they were about 15 months old and 4.5–5.0 cm long. A sample of 80 laboratory larvae metamorphosed primarily from April to June, 1973, when they were 12–14 months old with a mean (± S.E.) length of 43 ± 0.4 mm. Higher average winter temperatures in the laboratory than in the field probably account for the slightly younger age and smaller size at which laboratory larvae metamorphosed.

Figure 1 shows that for clutches 1 and 3, at 12°C a smaller proportion of larvae metamorphosed than at 19°C and in general they did so later. Curves for the low-temperature groups fall consistently below those for the high-temperature groups and, during the first 180 days, have lower slopes. By a Mann–Whitney U Test (Campbell 1967), the median times of metamorphosis shown in Table 2 are statistically different for groups 1A and 1B ($U = 188.5$, $P < 0.002$) and groups 3A and 3B ($U = 147$, $P < 0.02$). The effect of temperature is more pronounced on clutch 1 than on clutch 3, because in group 1B no individuals metamorphosed throughout the experiment, whereas in group 3B 71% of the larvae transformed. Since these two clutches came from different localities, the results may be indicative of differences in response between populations. For clutch 3, transformed larvae were larger on the average at 12°C than at 19°C, probably reflecting longer larval periods; but in both clutches 1 and 3, untransformed

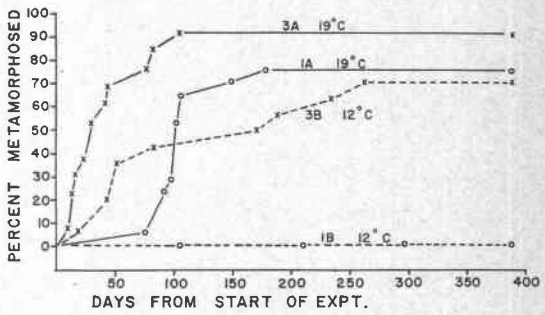

FIG. 1. Effect of temperature on metamorphosis. Clutches and groups are designated.

TABLE 1

Initial experimental conditions[a]

Clutch	Group	No. of larvae at start	Starting depth (cm)	Starting volume (l)	Evaporation	Food	Temperature (°C)
1	A	17	20	282	none	normal	19
	B	17	60	335	none	normal	12
2	A	16	11	73	high	normal	18
3	A	13	35	203	none	normal	19
	B	14	35	203	none	normal	12
4	A	18	28	160	none	normal	18
	B	18	13	75	medium	normal	20
	C	14	13	75	high	normal	18
5	A	12	32	130	none	normal	19
	B	11	32	130	none	low	19
6	A	16	27	154	none	normal	19
	B	16	11	73	high	normal	18

[a]Boxes indicate the factor varied in each experiment. High and medium rates of evaporation were achieved by placing the tanks at various distances from the major forced-air flow in the laboratory. To simulate no evaporation, regular additions of distilled water were made to maintain starting water levels. Normal food means feeding three times per week; low food means feeding once per week.

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TABLE 2

Median time of metamorphosis and size of experimental animals

Group	Experimental condition	Median time of metamorphosis (days from start of experiment)	All transformed individuals[a]		First 50% to transform (length, mm)	Second 50% to transform (length, mm)	Untransformed individuals[b]		
			Length (mm)	Number			Length (mm)	Number	% of original number
1A	19°C (control)	104	45±0.6[c]	14	43±0.2	46±1.0	69±2.4	3	18
1B	12°C	388	—	0	—	—	54±0.8	13	76[d]
2A	High evaporation	70	45±1.0	16	42±0.3	47±1.5	—	0	0
3A	19°C (control)	38	41±0.9	12	39±0.4	43±1.4	74	1	8
3B	12°C	170	46±2.2	10	41±1.1	52±2.5	56±0.2	4	29
4A	No evaporation (control)	96	47±0.7	17	45±1.0	49±0.5	73	1	6
4B	Medium evaporation	89	45±0.7	18	44±1.1	46±0.9	—	0	0
4C	High evaporation	84	46±0.7	14	45±0.8	46±1.1	—	0	0
5A	Normal food (control)	88	48±1.3	12	45±1.4	52±1.0	—	0	0
5B	Low food	140	44±0.8	9	43±0.9	46±0.9	50±2.0	2	18
6A	No evaporation (control)	110	50±1.4	15	45±1.0	54±1.3	71	1	6
6B	High evaporation	147	47±1.5	11	45±1.2	49±2.3	—	0	0[e]

[a]Measured on day transformation completed.
[b]Measured on day 388 when experiments terminated.
[c]Standard error.
[d]Four untransformed larvae died before the end of the experiment.
[e]One untransformed larva died during the experiment and four died on day 196 when tank dried up.

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larvae grew larger by day 388 at 19°C than at 12°C (Table 2).

On April 19, 1974 (day 388 of the experiment), 13 untransformed larvae 2 years old and averaging 54 mm long, and four averaging 56 mm remained in groups 1B and 3B, respectively (Table 2). Larvae of this age and size in nature are usually sexually mature (Neish 1971; Efford and Mathias 1969), and hence are neotenic. To check whether this was also the case for the experimental animals, one 53-mm larva from group 1B was killed and dissected. It had prominent well developed testes and hence was neotenic. On this basis, it is considered that all other untransformed larvae of this approximate size or greater appearing in these experiments are also sexually mature neotenes. Additional such larvae were not killed because of their small numbers and so that their progress could be followed beyond day 388. The primary effect of low temperature is thus to prolong the larval period and increase the incidence of neoteny. Frieden (1961) and Snyder (1956) both suggest that individuals with unusually long larval periods are progressively less likely to metamorphose. Although less numerous, some neotenic 2-year-old larvae also remained in the 19°C groups (Table 2).

Figure 2 shows that food deprivation also prolongs the larval period. In the group fed normally (5A), all larvae had metamorphosed by day 124, whereas the larvae in group 5B, fed only one-third as often, took longer to transform and two had not metamorphosed by day 388. The median time of metamorphosis for group 5A was statistically different from that for group 5B ($U = 93.5$, $P < 0.05$). The two individuals left in group 5B were almost 2 years old but their small size (52 and 48 mm), which probably reflects the reduced food intake, indicates that they may not have been sexually mature. Food deprivation, however, prolonged the larval period considerably and it is likely that they would have become neotenic. In both groups 5A and 5B, larvae which transformed early were smaller than those which transformed later (Table 2), simply reflecting continued growth throughout the larval period.

The effect of water-evaporation rate was tested on clutches 2, 4, and 6. Figure 3 and Table 2 indicate that medium- and high-evaporation rates appear to have little effect but may accelerate metamorphosis slightly for clutch 4. The median times of metamorphosis for groups 4A, 4B, and 4C are not statistically different by a Kruskal-Wallis test ($H = 1.02$, $P > 0.5$) (Campbell 1967). For clutch 6, a high rate of evaporation slightly prolongs the times taken for metamorphosis (Fig. 4), but the difference is not statistically significant ($U = 83.5$, $P > 0.2$). Although too few larvae were available to provide a control, the trend for the high-evaporation group in clutch 2 was similar to that for clutch 4, as was the median time of metamorphosis

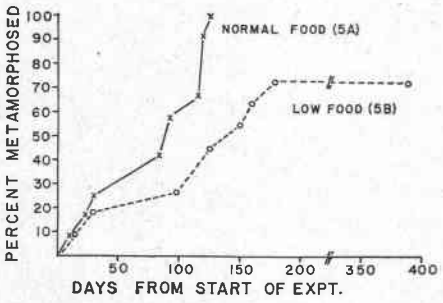

FIG. 2. Effect of food availability on metamorphosis in clutch 5.

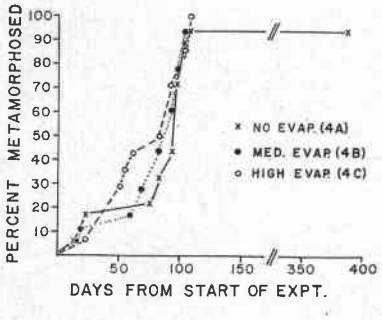

FIG. 3. Effect of evaporation rate on metamorphosis in clutch 4.

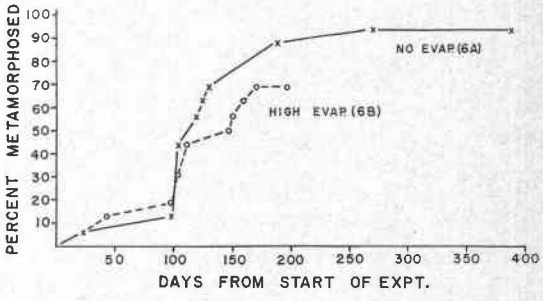

FIG. 4. Effect of evaporation rate on metamorphosis in clutch 6.

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(Table 2). Clearly, the effects of evaporation are not nearly as pronounced as those of temperature and food, and it is concluded that it does not have a significant effect on metamorphosis in this species. This is quite remarkable, considering that some of these tanks dried up almost completely leaving the larvae floundering in local areas where water was less than 1 cm deep and dissolved solids were eight times more concentrated than initially. There were no detectable changes in temperature or dissolved oxygen during these periods. Thus even drastic changes in water depth and dissolved solids appeared to have little effect on metamorphosis. Only two 2-year-old untransformed larvae, both of sufficient age and size to be neotenic, remained on day 388 from the evaporation experiments (Table 2).

The "controls" for all of these experiments were maintained under comparable conditions, 19°C, no evaporation, and normal feeding three times per week, although there were some differences in water depth and volume (Table 1). These can be considered standard conditions, and a comparison of times taken for metamorphosis in the different clutches will reflect differences between populations within a pond and between populations from different localities. Table 2 shows that median time of metamorphosis is very similar for clutches 1, 4, 5, and 6, ranging from 88 to 110 days, but is much shorter for clutch 3 (28 days). Clutches 3, 4, and 5 all came from the same pond near the Little Campbell River, so that the lower value for clutch 3 reflects differences in larval periods among progeny from different females within a population. The differences may simply be within the normal range of genetic variation or they may reflect the variation between neotenic and transformed adults. Clutches 1, 4 and 5, and 6 came from three different localities, that for clutch 6 being approximately 200 m higher above sea level, and yet the median times of metamorphosis are all comparable. This suggests that observed differences in timing of metamorphosis in natural populations from different locations may simply reflect varied responses to local conditions rather than inherited adaptations to these conditions.

It is also of interest to compare the number of neotenic individuals produced under these standard conditions from different clutches. Presumably this reflects differences in inherited tendencies towards neoteny among the various clutches. Although the numbers are small, indicating that few individuals are genetically fixed or obligate neotenes, Table 2 shows that major differences do not exist among control groups of the various clutches. In particular, neotenic individuals are not more abundant in clutch 6, which came from a considerably higher altitude. Of the controls for all clutches, 6 out of 76 larvae, or approximately 8% were neotenic under the standard conditions. To emphasize further the effects of the experimental conditions on the incidence of neoteny, out of a total of 81 larvae in experimental groups, 19 (*i.e.* 23%) were neotenic.

In summary, low temperature and reduced food intake prolong larval periods and increase the incidence of neoteny; rate of evaporation has little, if any, effect; and under standard conditions, there are only small differences in median times of metamorphosis (except for clutch 3) and the incidence of neoteny among the different clutches.

Discussion

The results of these experiments suggest that the determination of neoteny in *A. gracile* is more complex than might have originally been suspected. The sampled populations appear to be made up of at least three types of individuals: (a) those which always metamorphose regardless of environmental conditions; (b) those which are always neotenic; and (c) those which may or may not transform, depending on the habitat. Specifically, under experimental conditions for all groups except 1B, there were always some individuals which metamorphosed. They would probably have transformed under control conditions as well, and hence are the obligate "transformers" of (a) above. Similarly, except for group 5A, under control conditions some individuals had not metamorphosed by the time they were 2 years old and hence were neotenic; they would probably also have been neotenic under experimental conditions and hence are the obligate neotenes. As expected on the basis of these results, there are many examples of transformed and neotenic *A. gracile* coexisting under apparently uniform conditions (Watney 1941; Neish 1971; Farner and Kezer 1953). Finally,

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within single clutches, low temperature and reduced food availability delayed metamorphosis and produced neotenic forms, whereas high temperature and an abundance of food accelerated metamorphosis and produced transformed adults, thus suggesting that some individuals, corresponding to group (c), would be facultatively neotenic. On the basis of these latter results, one would predict that neoteny in *A. gracile* would be more common in permanent, cold, unproductive lakes and ponds, and this is generally the case (Sprules 1974). Similar temperature effects were also observed by Snyder (1956), who found that *A. gracile* sampled from a variety of altitudes transformed less readily at low temperatures, and he suggested that this could account for the higher incidence of neoteny at high altitudes. Once again, the primary significance of the experiments reported here is that all three types of individuals may coexist in a single polymorphic population.

It is impossible on the basis of the data presented here to suggest the genetic basis of this phenomenon except to speculate that heterosis may be involved, for it would seem that those individuals which are facultatively neotenic (*i.e.* the heterozygotes) would have a selective advantage over those which are certain to metamorphose or to become neotenic no matter what (the homozygotes). Breeding the progeny of known neotenic or transformed adults would be of interest in this regard. Factors determining the relative selective advantages of these phenotypes are also unknown. The probability of a pond drying up or the proportion of permanent ponds in an area could possibly be of importance.

The apparent unimportance of evaporation *per se* as a determinant of the incidence of neoteny is curious, especially given the observation, made by a number of investigators, that ponds in the last stages of drying up contain many transforming larvae. In view of the temperature effects noted here it is more likely that, in nature, temperature increases rather than evaporation and associated changes in salinity account for metamorphosis during pond drying. Oxygen availability could be of some additional importance, but at least for *A. tigrinum* larvae it has no effect on metamorphosis (Shrode 1972). Kirschner *et al.* (1971) found that *A. tigrinum* larvae can survive many weeks at 15°C and in 137 mM NaCl, and perhaps *A. gracile* larvae are also tolerant of a wide range of dissolved salt concentrations.

Recently, Wilbur and Collins (1973) proposed that the length of the larval period is quite variable within certain amphibian populations as a result of high variation in individual growth rates in response to environmental factors, notably availability of food. Under extreme conditions, they suggest, slower growing individuals are at a competitive disadvantage and would metamorphose to escape competition with faster growing larvae, which eventually become so large and old that metamorphosis is unlikely and they become neotenic. There is no suggestion in the results of the experiments reported here that slow-growing larvae transform and fast-growing ones do not. In contrast, underfed slow-growing larvae (group 5B) are more likely to become neotenic. This implies that neotenic individuals are more likely to develop in larger colder ponds, which are relatively unproductive, rather than in shallow warm ponds, which are likely to be less permanent and hence unsuitable for them. Only further experimentation on a variety of urodele species can resolve the effect of food availability. The suggestion by Wilbur and Collins that variable growth rates in larval populations serve as the basis for the evolution of different amphibian life histories, including neoteny, is certainly in accord with results presented here.

Neoteny in *Ambystoma* appears to be an adaptation to habitats where terrestrial conditions are less suitable than to aquatic conditions (Sprules 1974; Wilbur and Collins 1973). I suggest that this strategy has evolved in *A. gracile* through a selection for individuals showing decreased rates of metamorphosis in response to low temperatures and perhaps reduced food availability associated with the more favorable aquatic environment. This has led to complex polymorphic populations comprising facultative and obligate neotenes.

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