THE POSSIBLE CAUSES OF "METAMORPHIC FAILURE" IN THE
URODELE AMPHIBIAN, *Ambystoma gracile* (Baird)

by

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of
Biological Sciences

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THE POSSIBLE CAUSES OF "METAMORPHIC FAILURE" IN THE URODELE AMPHIBIAN, AMBLYSTOMA GRACILE (BAIRD).

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ABSTRACT

Field and laboratory investigations were carried out on two populations of *Ambystoma gracile* from two different altitudes. Observations of animals reared under identical water, feeding and photoperiod conditions indicated that there was a greater tendency towards "metamorphic failure" (or neoteny) within the high-altitude population compared to the low-altitude animals, and that genetically determined physiological differences are involved. Larval high-altitude *Ambystoma gracile* exhibit greater growth rates and are significantly (*p* < 0.001) larger 7 months subsequent to hatching than low-altitude larvae. Physiological studies strongly indicated that a prolactin-like hormone is involved as the growth-promoting hormone for larval animals.

Investigations concerning thyroid function indicated that thyroid activity is generally lower during larval growth and development for high-altitude larvae compared to low-altitude larvae.

In *vivo* studies with larvae showed that thyroxine treatment (*10^-7 M*) activated the hypothalamo-hypophyseal-thyroid axis, whereas lower concentrations (*10^-8 M*) primarily reacted with peripheral tissues. At room temperature, high-altitude neotenous and larval animals exhibited greater peripheral tissue sensitivities, and hypothalamic activation of the pituitary-thyroid axis occurred at larger body sizes than for the low-altitude animals. Lower temperatures delayed hypothalamo-hypophyseal-thyroid activation with respect to body size for low-altitude animals, but the size for hypothalamic activation of pituitary-thyroid function for
high-altitude animals was not altered at the lower temperatures. These results indicated a greater proportion of animals with a phenomenal (temperature-dependent) response for the onset of metamorphosis within low-altitude populations of *Ambystoma gracile*, whereas a majority of high-altitude larvae have body size as a determining factor for the onset of metamorphosis.

Immunohistochemical and histochemical studies of pituitary thyrotrope and lactotrope function within developing *Ambystoma gracile* also strongly suggests a body-size dependency with relation to pituitary-thyroid activity. Generally, abundant thyrotropes are not observed within pituitaries of smaller (less than 6.0 cm snout to vent length) larvae from the high-altitude population, but large numbers of immunoreactive thyrotropes are seen within low-altitude larvae pituitaries from animals of 5.0 cm snout to vent length.

The results of these investigations strongly indicate that continuous environmental factors related to higher altitudes select towards faster growing larvae with lower thyroid activity, larvae that transform at larger body sizes, and the neotenic condition. Neoteny seems to be a result of selection for this accelerated growth accompanied by low thyroid activity and may be maintained by steroid hormone feedback altering (inhibiting) the hypothalamic activation center involved with pituitary-thyroid function. Aging of this center is discussed as another possible factor involved in the maintenance of the neotenic condition.
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I would like to dedicate this thesis to the memory of my father, Frank William Eagleson, who unfortunately passed away before the completion of this research. I would like to acknowledge the diligent help of Bruce F. McStay during the early phases of this work. I would also like to express my appreciation to Ron Long for his excellent photographic assistance, and I would also like to acknowledge Dave Morely for his assistance during the radioisotope portion of this thesis and for his help with statistical analysis of much of this work. I would also like to acknowledge the overall assistance of my major supervisor, Dr. Brian A. McKeown during the writing and later details of this thesis.
Amphibian metamorphosis is observed as the transformation of an aquatic gill-respiring larva to a lung-breathing terrestrial adult. It is accomplished by many morphological, physiological and biochemical changes which allow the animal to adapt to its new environment. These changes, which occur in an orderly sequence at precise stages during larval development, can be divided into two general processes: regressive changes and progressive transformations. Regressive processes in anurans include the resorption of the tail-fin (also in urodeles), the disappearance of the peribranchial cavities, the loss of the horny teeth, and the shortening of the intestine. The progressive processes in both anurans and urodeles include the reorganization of the eyes, formation of eyelids, changes in skin glands and pigmentation, and the development and differentiation of new brain centers. Regressive changes involve the loss (or resorption) of transitory aquatic structures, whereas progressive processes involve the development of structures adapted to a terrestrial mode of life.

The factors that determine the 'onset' of metamorphosis are incompletely understood, but they seem to be partially genetic and can be altered by the environment. Frogs and salamanders metamorphose after various periods of growth according to the species. It has been observed that the attainment of a critical species-specific size rather than the duration of growth is crucial (Wilbur and Collins, 1973). Bullfrog tadpoles within the southern, tropical
parts of the United States may transform before their first winter, whereas tadpoles within the northern areas may require three winters after hatching to attain the size for metamorphosis (Dent, 1968; Bently, 1975). A similar situation is observed for the Long-Toed Salamander, *Ambystoma macrodactylum*. Salamanders along the California Coast require only 90 - 120 days to attain the minimum size for metamorphosis, but montane populations from the Sierra Nevada of California require an additional year and occasionally two years to attain the same size for metamorphosis (Anderson, 1967). Other possible environmental factors that influence the onset of metamorphosis include food availability (Wilbur and Collins, 1973), temperature (Shrode, 1972), the salinity or acidity of the pond water (Plough, 1976), crowding (Wilbur and Collins, 1973), and recent rainfall (Healy, 1975). These environmental factors are thought to effect the timing of transformation through the activation of the endocrine glands or target tissues concerned with metamorphosis.

The endocrine control of metamorphosis involves the hypothalamus, pituitary, thyroid, and reactivity and developmental state of the target tissues (Kollros, 1961). A vast majority of studies dealing with the endocrine control of amphibian metamorphosis (for reviews see; Etkin, 1968; and Frieden and Just, 1970) have almost exclusively tested anurans, but recent studies with urodeles (Norris et al., 1973 and Norris and Gern, 1976) have revealed that control mechanisms are probably similar, although the transitions are more gradual and the tissue sensitivities are of a higher threshold.
Early studies implicated the thyroid hormones as the causal agents of metamorphosis (Guternatsch, 1912). If tadpoles were fed thyroid extracts, they soon metamorphosed precociously. Conversely, if tadpoles were fed antithyroid drugs, they attained gigantic sizes and only transformed when they were no longer fed the antithyroid drugs (Allen, 1938). Also, if the pituitary was removed from larval tadpoles, the animals did not metamorphose (Allen, 1938). Recently, investigations by Eddy and Lipner (1976) using rabbit antiserum to ovine-thyroid-stimulating hormone (TSH) indicated that a TSH-like factor is involved in anuran metamorphosis, and this factor from the pituitary is responsible for the activation of the thyroid during amphibian metamorphosis (Norris and Platt, 1973). Prolactin and thyroxine are antagonistic in their effects of amphibian metamorphosis in larval urodèles (Gona and Etkin, 1970) and anurans (Bern et al., 1967). Pituitary prolactin seems to be the larval growth-promoting factor (Derby, 1968; Etkin et al., 1969; and Frye et al., 1972) for both urodèles (Cohen et al., 1972) and anurans (Frye et al., 1972). The anti-metamorphic action of prolactin is still uncertain, though Gona (1967) suggests that it might act as a goitrogen and Derby (1970) suggests that it might also act at the peripheral level; but in many cases pharmacological doses were used. Its mode of action is still in doubt, thus requiring further investigations with lower doses.

The control of the pituitary TSH secretion is, in turn, mediated by the hypothalamus. Etkin (1966) found that the removal
of the pituitary from its intimate relation with the hypothalamus will hinder metamorphosis. Hanaoka (1967) discovered that hypothalectomized tadpoles underwent incomplete metamorphosis and attained nearly twice the size of sham-operated controls. Therefore, the hypothalamus is important for the 'onset' of spontaneous metamorphosis.

Other physiological factors concerned with the control of metamorphosis are the acquisition of reactivity (competence) to thyroxine and the maturational or developmental state of the target tissues. Kollros's (1961) and Moser's (1950) studies with anurans revealed that reactivity to exogenous thyroxine (T₄) is first observed when the gilled larva acquires an operculum, and that different tissues acquire T₄ reactivity in an orderly sequence during the next 21 days. An interesting feature of this sequence is that the tissues (i.e. hindlimbs) that are the last to become reactive to exogenous T₄ are the first to exhibit a (progressive) response during spontaneous metamorphosis. The first tissues to become reactive to T₄ are transitory larval structures that undergo regressive changes at a later sequence during spontaneous metamorphosis. Thus, the sequence in which tissues are reactive to thyroxine is drastically different from the sequence these tissues manifest their morphogenetic changes during natural metamorphosis.

Even though target tissues acquire the capacity to respond to T₄ early in ontogeny, it is evident by the studies of Kollros (1961) that different tissues exhibit different thresholds and that these
individual thresholds also vary according to the animal's developmental stage. Limb development, for example, exhibits a gradual increase in tissue sensitivity to low levels of $T_4$ during ontogeny. This is accompanied by subsequent increases in thyroid hormone thresholds in order to proceed to subsequent stages. The hindlimb of the tadpole will grow and differentiate under the influence of very low $T_4$ concentrations (0.02 $\mu$g/l) in the swimming media, but subsequent development of the digits of the foot and the formation of the webbing requires increases in $T_4$ concentration (Kollros, 1961). Thus, hypophysectomized tadpoles reared in these low concentrations will develop to a certain stage, and there will be no further development until the $T_4$ concentration is increased. These studies led Kollros to formulate a theory that states; "There is a progressive increase in reactivity to thyroid hormones..." with development and each metamorphic event requires its own 'individual threshold' of thyroxine to progress to a subsequent stage. Further studies by Kollros's group (Decker and Kollros, 1969) indicated that the environmental temperature greatly effects the thresholds of certain metamorphic events and the developmental stage when these metamorphic changes occur (i.e. at a lower temperature and the same $T_4$ concentration a particular event will occur at a larger body size than at the higher temperature).

These studies using exogenous $T_4$ are even more interesting in relation to spontaneous metamorphosis when one considers the fact that the $T_4$-dependent differentiation of the hypothalamic floor to
Figure 1. The role of hormones in the growth and development of the tadpole of a frog.
Upper. The development of the pituitary, especially in relation to the establishment of its connections to the hypothalamus.

Lower. Changes in the activity and hormone concentrations of the pituitary-prolactin axis and the pituitary-thyroid axis. Major events are the acquisition by the hypothalamus of a sensitivity to thyroxine, which hastens its development, and the onset of the definitive roles of TRH and P-R-IH, so that TSH and thyroxine secretion increases while prolactin decreases. (Based on Etkin, 1970.)
the median eminence is one of the last tissues to acquire reactivity (competence) to thyroxine. Since the median eminence forms an important link between the hypothalamus and the pituitary, its development and differentiation is necessary for full activation of the hypothalamo-pituitary-thyroid axis.

Taking most of these factors into account, Etkin (1970) (Figure 1) has developed a theory to explain the control of metamorphosis. The pattern of thyroid buildup that determines metamorphic changes is proposed to be moderated by a positive feedback mechanism of thyroxine on the hypothalamus. His theory also includes prolactin as the larval growth-promoting factor (Derby, 1968; Etkin et al., 1969 and Frye et al., 1972). According to this theory, the thyroxine levels within the animals are very low during the early phases of growth and development and remain low until the beginning of the emergence of the hindlimbs (prometamorphosis). Just prior to prometamorphosis, the hypothalamic thyrotropin-releasing factor (TRF)-cells become sensitive to a 'positive feedback' of thyroid hormones, and this initiates prometamorphosis (development of the hindlimbs). The increase in TRF promoted by this higher thyroxine level then stimulates pituitary TSH release which, in turn, promotes another increase in thyroid hormone concentration. This increase causes a spiraling effect leading to maximal activation of the thyroid (metamorphic climax). Prolactin concentrations are visualized as becoming progressively lower because hypothalamic T$_4$-dependent differentiation progressively
exerts an inhibitory control on pituitary prolactin secretion (Figure 1). This accounts for the retardation of growth observed during amphibian metamorphosis. After transformation, thyroxine is observed to exert a negative feedback effect upon the hypothalamus and pituitary. These negative feedback mechanisms are what are usually observed within adult amphibians. The negative feedback will then lower the overall thyroid activity.

Urodele amphibians exhibit a less dramatic transformation than anurans. Larval urodeles possess limbs, and the most obvious morphological changes include the resorption of the tailfin and gills. Urodele metamorphosis, like anuran metamorphosis, is under hypothalamo-pituitary-thyroid control (Norris and Gern, 1976). As in anurans, prolactin in high concentrations (pharmacological?) may delay and/or inhibit metamorphosis (Gona and Etkin, 1970). A number of urodeles (especially of the genus *Ambystoma*) are neotenic; they retain their larval characteristics and reproduce in this form. Populations of *Ambystoma gracile* in southwestern British Columbia and northwestern Washington exhibit these neotenic tendencies (Snyder, 1956). Previous investigations of neoteny within *Ambystoma gracile* populations (Snyder, 1956 and Sprules, 1974) have implicated cold temperatures as a factor determining neoteny. Wilbur and Collins (1973) have suggested that there might be a "progressive loss of sensitivity to thyroxine..." within such montane populations implying genetic selection as a factor. Dent (1968) has emphasized that further studies with such populations might prove interesting
and may divulge more testable hypotheses concerning the physiological control of amphibian metamorphosis. Since most studies concerning the effects of environmental factors on amphibian metamorphosis (Decker and Kollros, 1968; Frieden and Just, 1970; and Kaltenbach 1968) and nearly all investigations dealing with physiological changes during development (Chou and Kollros, 1974; Etkin, 1966; Kaye, 1961; and Kollros, 1961) have dealt with anurans; studies following physiological changes with development within different populations of *Ambystoma gracile* seemed warranted.

Before physiological studies could be initiated, it was proposed that one must first determine (a) the minimum size at sexual maturity and (b) the incidence of neoteny of *Ambystoma gracile* populations from different altitudes. Previous studies by Snyder (1956) implied that neotenous females attain sexual maturity at 70 mm snout to vent length (SVL) during their second or third year of growth. He also suggested that nearly all larvae from populations located in lakes at sea level transform prior to sexual maturation, and that montane populations were primarily neotenous. He concluded that the major factor involved with the determination of neoteny was the colder temperatures that occur at the higher altitudes. Since other investigators have assumed that sexual maturation occurs at 50 mm SVL (Effort and Mathias, 1969 and Sprules, 1975) and other scientists have implied that genetic selection may be a determining factor (Dent, 1968 and Wilbur and Collins, 1973); it was concluded that a reinvestigation of the
distribution of neotenes in habitats at different altitudes was in order. Field studies were, therefore, begun to investigate the proportion of neotenic individuals within lakes at different altitudes, the size at sexual maturation, and the possible physiological adaptations that might be of significance in relation to neoteny.

In order to investigate if neoteny might be genetic in nature, randomized hatchlings were reared under identical water, photoperiod, temperature and feeding conditions; but each randomized sample consisted of animals from a lake located at a different altitude. The growth rates, incidence of neoteny, the time at onset of metamorphosis, and the size at metamorphosis were recorded in order to ascertain if there were any 'genetic' differences between populations from different altitudes.

Concurrently with these field studies, physiological investigations were carried out in order to ascertain possible differences between populations with respect to thyroid activity, tissue sensitivities, pituitary changes and the influence of temperature upon these physiological parameters. These studies were carried out on animals of different phases of growth to compare the internal mechanisms involved in metamorphosis for these two populations. Since these tissues are critical links for amphibian metamorphosis in general; it seemed worthwhile to investigate which link might be 'defective' within the neotenous *Ambystoma gracile*. 
CHAPTER I

A comparison of the life histories and growth patterns of populations of the salamander Ambystoma gracile (Baird) from permanent low-altitude and montane lakes.
CHAPTER I

A comparison of the life histories and growth patterns of populations of the salamander *Ambystoma gracile* (Baird) from permanent low-altitude and montane lakes

**Introduction**

Neoteny is a condition observed in certain urodele amphibians where the 'adult' terrestrial form is suppressed, and the animals reproduce as aquatic larva. In certain species this larval form is the only mode of life, and these urodeles cannot be induced to metamorphose. Such species are referred to as obligate neotenes or paedogenetic species. Other species (especially of the genus *Ambystoma*) show a facultative neoteny. This is recognized as a condition whereby four forms exist: immature animals with larval characteristics; metamorphosed and terrestrial but sexually immature juveniles; sexually mature adults with larval characteristics; and sexually mature, transformed adults. Facultative neoteny may provide useful information concerning the normal physiology of metamorphosis as well as divulge evolutionary pathways by which developmental mechanisms might be altered (Etkin 1964).

Previously, physiological experiments involving neotenes dealt with either 'artificially selected' neotenes such as *Ambystoma mexicanum* (Frahled 1968) or neotenes from local populations. These modes of selection may have resulted in misinterpretations, for had the extent of the aberrant life pattern been better understood, a different interpretation of the data may have been evident. To alleviate this possible
difficulty, I attempted to assess the occurrence of spontaneous metamorphosis for *Ambystoma gracile* populations from lakes located at three different altitudes. Seeking clues towards the adaptive advantage of neoteny, I also examined possible life-history differences for these populations.

**Materials and Methods**

To eliminate possible density factors (Wilbur 1972) and other parameters not related to altitude, large permanent lakes of similar area were selected. Soon after spring and summer thaws, all three southwestern British Columbian lakes were in excess of 10 m in depth at their deepest portion. Lost Lake is at an altitude of 100 m, Petgill Lake is located at an altitude of 830 m, and Goldie Lake is situated at an altitude of about 1200 m.

During the study's 1st year (1974) animals were captured, measured (snout to vent and head to tail lengths) and weighed (towel-dried wet weight). Three techniques were employed for field captures; salmon nets and night lights were used to capture animals foraging along the shore at night, a 'slurp gun' was used for day and night captures, and funnel traps were used. Animals were measured by placing them into elevated, partially filled 20-l aquaria and measuring them to the nearest 0.05 cm with dissecting rulers placed beneath the aquarium. They were then towel dried and weighed (to the nearest 0.01 g) by placing them into preweighed beakers filled with dechlorinated water. Owing to Petgill Lake's inaccessibility, these animals could not be tagged, but animals from Lost Lake
and Goldie Lake were brought back to the laboratory, anaesthetized with MS-222 (1:2000), and branded with the number of the month captured, using copper-wire numbers cooled in liquid nitrogen. Two easily identifiable size cohorts were evident within Lost Lake; therefore salamanders of the smaller-sized cohort were tagged with horizontal numbers, and animals within the larger sized salamander cohort were tagged with vertical numbers. Tagged animals were returned to their respective lakes within a week and released at sites opposite from where they had been captured. To assess the period of egg laying and the time of hatching, skin-diving surveys and temperature readings were conducted throughout the field study.

To investigate the proportion of animals capable of spontaneous metamorphosis, 10 egg batches were brought back from Lost Lake and Goldie Lake (only two egg batches were discovered within Petgill Lake), and hatchlings were randomized within their respective lake populations. About 50 animals from each randomized sample were grown; and to follow growth rates, biweekly measurements of length (snout to vent and head to tail) and weight (wet weight) were taken. Animals were grown at room temperature (21 ± 2 °C), photoperiods of 12 h light:12 h dark, and were fed to satiation. They were initially fed (2 days after larvae hatched and had absorbed all their yolk) day-old shrimp (Artemia salina) or small mosquito larvae (Aedes aegypti). Groups of 10 hatchlings were kept in half-filled 20-1 aquaria. Aquaria were changed daily, cleaned and refilled with dechlorinated water. When larvae reached the hind-limb stage, they were reduced to five individuals per tank and fed tubifex worms (Tubifex tubifex). Once larvae were 4.0 cm snout to vent length (SVL), they were
reduced to two animals per tank and hand fed beef heart, liver, and earthworms.

To examine sexual and seasonal maturation during 1975, animals from Lost and Goldie Lakes were captured each month. Initially animals were captured before any oviposition while animals were observed to be breeding. During mating and before oviposition when metamorphosed animals were present in the lakes, neotenous and metamorphosed animals were equally catchable. Animals, captured before oviposition, were measured and weighed (as previously described) both before and after laboratory oviposition. Ova diameters and numbers were also recorded. Animals were then killed, preserved in 10% formalin, and used as standards for the minimal gonad state of sexually mature females. Subsequently, salamanders were captured each month, killed, and preserved in 10% formalin to follow seasonal changes in their reproductive state. The ovaries (or testes) and oviducts (or archinephric ducts) were dissected out, towel dried, and weighed. This weight was expressed as a proportion of body weight: gonadosomatic index (G.S.I.) = (gonad weight/body weight) x 100; and reproductive index (R.I.) = [(gonad weight + reproductive duct weight)/body weight] x 100. Only animals captured before egg deposition for March (1975) in Lost Lake and July (1975) in Goldie Lake were recorded as field captures, since after oviposition metamorphosed animals were selectively pursued to investigate the possibility of recapturing marked animals.

Statistical analysis were performed applying the Student's t-test, and data were analyzed using pooled (p.v.) (assumption equal variances;
Sokal and Rohlf 1969, p. 222) or separate (s.v.) variance (assumption of unequal variances; Sokal and Rohlf 1969, p. 374) estimates as prescribed by $F$ test values. Population estimates were calculated by the Lincoln index (Mosby 1963). The population size ($N$) was estimated by the equation: $N = Mn/m$, where $M$ is the number of marked salamanders, $n$ is the total number of animals caught during the period, and $m$ is the number of marked recaptures. The standard error (SE) could be calculated by $SE = \sqrt{[M^2n(n - m)]/m^3}$, and the population-size confidence limits were set by adding and subtracting $1.96 \times SE$ (95% confidence level) from the population size ($N$).

Results and Observations

Breeding and Egg Deposition

Figure 2 indicates that lower altitude lakes attained higher temperatures for longer periods than lakes at higher altitudes. During 1975 Lost Lake spring temperatures differed from the gradual temperature increases of 1974 (Fig. 2). Subsequent to melting during 1975, there was a 3-week period when Lost Lake exhibited negligible temperature changes and remained at 4-5 °C. During 1975 Goldie Lake melted 6 weeks earlier than the previous summer, but the steep temperature increase that followed melting during 1974 (Fig. 2) also occurred during 1975. Thus subsequent to melting, temperature changes within Lost Lake varied each year, but Lost Lake consistently melted during late February or early March (1974, 1975, and 1976). Soon after melting, Goldie Lake exhibited a consistent temperature increase each year, but the month it melted varied (probably
more dependent on the previous winter snowfall and the intensity of spring and summer heat).

The length of the period of oviposition (Fig. 2) is apparently dependent upon the rapidity of temperature change after lakes melt. The steeper the temperature rises, the shorter will be the period of oviposition. Gradual temperature rises seem to prolong oviposition. Within Lost Lake breeding and oviposition continued for a month during 1974, but lasted 7 weeks during 1975 when temperature changes were static for 3 weeks. Breeding and oviposition took less than a week within Goldie Lake during 1974 and 1975. The period of breeding and oviposition was determined by finding newly laid egg masses (recently deposited egg masses were about one-third the size of older masses, because they required about 48 h to imbibe water to saturation).

Mating within Goldie Lake occurred while the lake was more than half frozen. When the lake was still completely frozen, metamorphosed males were observed to enter under the frozen lake cap by melted creeks that flow into the lake. Metamorphosed males continued to immigrate up until the peak of egg deposition. Transformed females were rarely observed entering the lake, but a few were observed immigrating by crawling in from the partially melted shores or overhanging bushes. Metamorphosed females within Lost Lake and Goldie Lake were rarely captured at much distance from the shore and were usually found within partially submerged bushes. Metamorphosed males preceded transformed females within both Lost and Goldie Lake and remained throughout the period of oviposition. Transformed females spent less time within the lakes or were less abundant
Figure 2. Changes in temperature within three lakes of different altitudes (1974). Solid line represents Lost Lake (altitude 100 m). Broken line represents Petgill Lake (altitude 830 m). Broken-solid line represents Goldie Lake (altitude 1300 m). Temperatures were recorded at 0.5 m (usual depth of egg deposition). The length arrows represent the span of time when eggs were deposited.
than transformed males (i.e. unequal sex ratios).

Patterns of Larval Growth

Size-frequency distributions (Figs. 3, 4, and 5) indicated that growth patterns of high- and low-altitude larvae may differ. Within Lost Lake (Fig. 3), free-swimming larvae that hatched during mid-April of 1974 were first captured during late June. These were assumed to be 1st-year hatchlings, since they lacked fully developed hind limbs and were of a small size expected for recently hatched larvae. During June three size groups were observed within Lost Lake. They included these 1st-year hatchlings (sizes ranged from 1.5- to 2.0-cm SVL with weights ranging from 0.20 to 0.38 g), 2nd-year holdovers (sizes ranged from 3.0- to 5.5-cm SVL with weights ranging from 2.52 to 7.81 g), and suspected 3 year or older (neotenous?) animals (sizes ranged from 7.0- to 10.5 cm SVL with weights ranging from 17.8 to 32.3 g). Second year holdovers were animals that hatched during 1973 and were undergoing their 2nd year of growth. The subsequent growth of 1974 hatchlings substantiated this assumption, since animals that hatched in 1974 attained nearly the same average sizes the next April (1975) and May (1975) as the presumed 1974 2nd-year holdovers. These size groups (cohorts) were easily discernible within Lost Lake, therefore the growth of 1st- and 2nd-year larvae could be ascertained (Figs. 6 and 7). Animals grew most rapidly during the months of June, July, and August of their 1st year (Fig. 5) and June of their 2nd year (Fig. 7). During these months Lost Lake temperatures approximated the temperatures in the laboratory; and laboratory and lake larvae exhibited nearly identical growth rates (Figs. 6 and 7) for these
Figure 3. The size-frequency distributions of *Ambystoma gracile* captured from Lost Lake. The numbers in parentheses represent the number of animals captured during that month. Two cohorts were observed in April (not shown) and May of 1974. The first cohort consisted of animals that ranged from 2.5- to 6.0-cm SVL in May and 3.0- to 6.0-cm SVL during June. The second cohort consisted of animals that ranged in size from 7.0- to 11.0-cm SVL throughout the study.
The size-frequency distributions of *Ambystoma gracile* captured within Petgill Lake. The numbers in parentheses represent the number of animals captured during that month. Clear, distinct cohorts were less evident within this lake.
SNOUT TO VENT LENGTH (cm)

JULY 1974 (41)

AUGUST 1974 (51)

SEPTEMBER 1974 (29)

OCTOBER 1974 (23)

NUMBER OF ANIMALS
Figure 5. Size-frequency distributions of *Ambystoma gracile* captured from Goldie Lake. The numbers in parentheses represent the number of animals captured during the designated month. Animals captured during July (1975), which ranged from 2.5- to 2.8-cm SVL, were suspected holdovers of larvae that hatched during 1974 since they were captured before egg laying and were of a very small size.
Figure 6. Growth of 1st-year *Ambystoma gracile* hatchlings within Lost Lake (1974-1975). Dots represent mean SVL lengths (cm) for captured animals during the designated month. The vertical line represents the standard error of the mean. Numbers in parentheses represent number of captures during the designated month. Broken line represents laboratory and solid line represents lake growth curve.
Figure 7. Growth of 2nd-year holdover *Ambystoma gracile* within Lost Lake (1974). Dots represent mean SVL lengths (cm) for captured animals during the designated month. The vertical line denotes the standard error of the mean. Numbers in parentheses represent the number of captures during the designated month. Broken line represents laboratory and solid line represents lake growth curve.
months, suggesting that field larval growth was not limited by food supply.

Growth of Goldie Lake 1st-year hatchlings differed from Lost Lake hatchlings, since 1st-year Goldie Lake hatchlings were too small to be captured during their 1st year. Within Goldie Lake, eggs deposited during mid-August did not hatch until mid-September, and the lake froze over during July of 1975 (sizes were 2.5- to 2.8cm SVL), therefore little growth occurred during the 1st year. Most growth probably occurred during the 2 (or sometimes 3) warm months of the animals' 2nd year. Assuming maximal laboratory growth, larvae could conceivably attain sizes of 5.0- to 5.5-cm SVL during their 2nd year of growth, and a small proportion may metamorphose at this size; but most larvae remain within the lake a 3rd year. Goldie Lake salmanders captured while undergoing metamorphosis (or that underwent transformation during holding) were larger than the sizes that could be attained during the second season of growth ($n = 10$, SVL = 6.32 ± 0.132 (mean ± SEM)). Goldie Lake animals grew most rapidly during the warm summer months when feeding activity was greatest; but since Goldie Lake usually melted during July or August and refroze during October or early November (1973, 1974, and 1975), there were fewer months of rapid (exponential) growth. Slower growing older animals became indistinguishable from younger faster growing animals, and obvious body-size groups (cohorts) were not easily differentiated.

Recapture data (Table I) substantiated the continuance of growth patterns previously described for Lost Lake. First cohort (2nd-year holdovers) animals were abundant during May and June, became more difficult to capture, and none were captured during October (1974). These larvae either
Table I. Numbers of *Ambystoma gracile* captured from Lost Lake in 1974, 1975, and 1976*

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total unmarked</td>
<td>1st cohort</td>
<td>18</td>
<td>36</td>
<td>11</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2nd cohort</td>
<td>25</td>
<td>18</td>
<td>4</td>
<td>14</td>
<td>30</td>
<td>22</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Metamorphosed form</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total recaptured</td>
<td>1st cohort</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>(1)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2nd cohort</td>
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<td>0</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>0</td>
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<tr>
<td></td>
<td>Metamorphosed form</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total caught</td>
<td>1st cohort</td>
<td>18</td>
<td>38</td>
<td>13</td>
<td>11</td>
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<td>35</td>
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<td>2nd cohort</td>
<td>25</td>
<td>18</td>
<td>6</td>
<td>16</td>
<td>36</td>
<td>28</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Metamorphosed form</td>
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<td></td>
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<tr>
<td>Running total</td>
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<td>65</td>
<td>74</td>
<td>74</td>
<td>74</td>
<td>61</td>
</tr>
<tr>
<td></td>
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<td>47</td>
<td>61</td>
<td>91</td>
<td>113</td>
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<tr>
<td>Months of recaptures</td>
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<td>5,5</td>
<td>5,7</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd cohort</td>
<td>5,5</td>
<td>6,57</td>
<td>5,6,7,8,8</td>
<td>6,8,8,9,9</td>
<td></td>
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</tbody>
</table>

Only larval-form animals were tagged in 1974. No animals were tagged in 1975 or 1976.

Double recapture (triple capture).

First-cohort recapture within second-cohort size group.

Four recaptures were from the second cohort of 1974 (months were 57,9,0,0), 57 was a double recapture. One animal was from the first cohort of 1974 (month was 5).

All were recaptures from the second cohort of 1974 (months were 5,6,9,9).

All were recaptures from the first cohort of 1974 (months were 6,7,7,7).
Table II. Number of *Ambystoma gracile* recaptured from Goldie Lake in 1974 and 1975

<table>
<thead>
<tr>
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<th>1974</th>
<th></th>
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<td></td>
<td>(8)</td>
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</tr>
<tr>
<td>Total unmarked</td>
<td>74</td>
<td>59</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Total recaptured</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total caught</td>
<td>75</td>
<td>63</td>
<td>51</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Running total</td>
<td>74</td>
<td>133</td>
<td>183</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td>Months of recaptures</td>
<td>8</td>
<td>8,8,8,9</td>
<td>9</td>
<td>8,8</td>
<td></td>
</tr>
</tbody>
</table>

Note: Out of eight metamorphosed animals captured in July 1975, none were tagged. Only larval-form animals were tagged.
became second-coh ort (sized) animals or left the lake (metamorphosed). One first-cohort animal recaptured during March of 1975 was found to be the size of a second-coh ort salamander, and this recaptured female oviposited in the laboratory, suggesting that low-altitude neotenous females become sexually mature by their third spring. During 1975 none of the 61 transformed captures contained brands, and it was surmised that skin changes during metamorphosis might obliterate the cold brands. During 1976 this proved not to be true, because four of forty-one terrestrial captures had first-cohort (1974) brands (Table I). It was concluded that transformed salamanders required a year of terrestrial growth before they return as sexually mature terrestrial salamanders.

Since only the second-cohort animals remained within Lost Lake, only this cohort's population was estimated. The population estimate was 678 salamanders, with 95% confidence limits between 406 and 950 animals. The Goldie Lake population was estimated to be 4758 animals, with 95% confidence limits between 1524 and 7992 animals.

Laboratory studies performed under constant photoperiod, feeding, water, and temperature conditions indicated differences for larval growth patterns between randomized Lost Lake animals compared with Goldie Lake samples (Figs. 7 and 8). Three types of growth were evident for Lost Lake larvae. A small proportion (16%) of larvae grew faster than the rest of the larvae. After 6\(\frac{1}{2}\) months, eight animals were metamorphosing (\(n = 8\), SVL = 5.6 ± 0.20 cm, weight = 8.71 ± 2.262 g (mean ± SEM)) and these salamanders were larger (s.v.; \(P > 0.01\)) than the remaining larvae (\(n = 42\), SVL = 4.7 ± 0.06 cm, weight = 6.57 ± 0.385 g (mean ± SEM)). At this time seven of the
eight largest animals were undergoing metamorphosis, and the one larger animal not transforming later proved to be neotenous (when dissected it had mature, pigmented ova and convoluted oviducts). The faster growing animals were the first to metamorphose. Most remaining Lost Lake animals metamorphosed after 8 to 10 months of growth, but a small proportion metamorphosed (usually at larger sizes) after 10 months (Fig. 8).

Laboratory growth of Goldie Lake larvae differed from Lost Lake animals. Goldie Lake larvae were larger \( (p \cdot v.; P < 0.001) \) after 7 months of growth, but 14-month-old Lost Lake larval (neotenous) forms exceeded \( (s \cdot v.; P < 0.01) \) the body size of similarly aged Goldie Lake animals (Table III). Goldie Lake and Lost Lake larvae grew at nearly identical rates for their first 3 months (Figs. 8 and 9); but from the 3rd to 7th months Goldie Lake larvae grew faster (Table III). After 10 months of growth, the Goldie Lake animals' growth rate was dampened to a greater extent than Lost Lake animals (Table III; Figs. 8 and 9).

Metamorphic behavior was also different for these two populations. The Lost Lake sample exhibited 88\% (44 of 50) metamorphosis for the surviving (50 of 56) animals, whereas only 23.5\% (12 of 51) of the surviving (51 of 51) Goldie Lake larvae metamorphosed. Goldie Lake salamanders all transformed within a brief 2-week period after 9 months of growth (Fig. 9; Table IV; and they metamorphosed at larger sizes \( (s \cdot v.; P < 0.01) \) than did Lost Lake animals.
Figure 8. Growth of *Ambystoma gracile* reared from eggs from Lost Lake under constant temperatures (21 ± 2 °C). Circles represent the mean value of the size of larvae for that month (abscissa) since hatching. The solid dots represent the size and time (since hatching) when an animal was completely metamorphosed. Each dot represents a single metamorphosed animal. Note: all but one of the animals that metamorphosed from the 6th to 7th month had snout to vent lengths in excess of the mean values.
MONTHS SINCE HATCHING

MEAN SNOUT-VENT LENGTH (cm)
Figure 9. Growth of *Ambystoma gracile* reared from eggs from Goldie Lake under constant temperatures (21 ± 2 °C).
The circles represent the mean value for the size of larvae at the designated month since hatching (abscissa). Each solid dot represents the size and time in which a single animal was completely metamorphosed. Note: all animals metamorphosed between 9 and 9½ months after hatching. All animals that metamorphosed, metamorphosed in excess of 6.5 cm (SVL).
### Table III. A comparison of the sizes (mean ± SEM) of larval-form *Ambystoma gracile* from two separate populations during different phases of growth and maturation. SVL represents the snout to vent length

<table>
<thead>
<tr>
<th></th>
<th>Field females</th>
<th>Laboratory females</th>
<th>Both sexes (laboratory)</th>
<th>Laboratory females were 14 months old. Female animals reared in the laboratory are sexually mature, as judged by large extensive oviducts and large pigmented ova, after 12 months of growth at room temperature (21 ± 2°C).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>SVL, cm</td>
<td>Weight, g</td>
<td>n</td>
</tr>
<tr>
<td>Field females</td>
<td>10</td>
<td>9.9 ± 0.96</td>
<td>28.1 ± 2.46</td>
<td>10</td>
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<td>Laboratory females</td>
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<td>9.4 ± 0.24</td>
<td>35.3 ± 4.98</td>
<td>26</td>
</tr>
<tr>
<td>Both sexes (laboratory)</td>
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<td></td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>14 months old</td>
<td>6</td>
<td>9.3 ± 0.16</td>
<td>34.5 ± 3.44</td>
<td>38</td>
</tr>
<tr>
<td>7 months old</td>
<td>44</td>
<td>4.8 ± 0.09</td>
<td>7.0 ± 0.37</td>
<td>51</td>
</tr>
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</table>
Table IV. Timing and size range of metamorphosing *Ambystoma gracile* reared from eggs from different populations at room temperature (21 ± 2 °C). SVL represents the snout to vent length (cm)

<table>
<thead>
<tr>
<th>Population</th>
<th>Period of metamorphosis*</th>
<th>Mean days to metamorphosis (days + SEM)</th>
<th>Sizes at metamorphosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range (SVL)</td>
</tr>
<tr>
<td>Lost Lake</td>
<td>44</td>
<td>241 ± 7.5</td>
<td>4.7 - 7.4</td>
</tr>
<tr>
<td>Petgill Lake</td>
<td>5</td>
<td>236 ± 3.0</td>
<td>5.6 - 5.8</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>12</td>
<td>246 ± 2.0</td>
<td>6.4 - 7.2</td>
</tr>
</tbody>
</table>

* Period of metamorphosis is the period in which animals from each group underwent metamorphosis. Day 1 represents day of hatching. Animals were observed for up to 500 days. Lost Lake animals were significantly larger than Petgill Lake animals (p < 0.001) at metamorphosis.
Metamorphic Changes

Animals at different stages of development were observed and measured at different intervals. These measurements were expressed as snout to vent length (SVL) divided by tail width (TW) or SVL divided by hindlimb length (HLL).

Early larval animals (SVL/TW = 5.9 - 6.3) exhibited a consistent increase in general body growth without alterations in body proportional measurements (i.e. SVL/TW or SVL/HLL).

Late larval animals (SVL/TW = 6.3 - 6.9; Figure 10a.) exhibited a slight increase in hindlimb growth relative to body growth (SVL/HLL = 3.3 ± 0.20, n = 6 (X ± S.E.)) compared to early larval animals (SVL/HLL 3.6 ± 0.26, n = 6 (X ± S.E.)). Neotenous animals (SVL/HLL 3.1; SVL/TW = 6.0 - 6.35) had morphological characteristics that were similar to early larval forms.

Many larvae, especially larvae from the Lost Lake population, exhibited an increase in darkening (Figure 10b) just prior to any morphological metamorphic events (SVL/TW = 6.9 - 7.1). Animals undergoing the early phases (Figure 10c) and late phases of transformation (Figure 10d) exhibited a sharp increase in hindlimb growth relative to body growth (SVL/HLL = 2.5 ± 0.33, n = 6) and an increase in tail width compared to body length (SVL/TW = 7.0 - 9.0). Tail width and tail height measurements seemed to be the best indicators of the progression of metamorphosis. Other external changes included resorption of the gills, tail fin resorption (as measured by changes in tail height), resorption of the larval folds of the
lower jaw, fusion of the gill slits, development of the eyelids, protrusion of the eyes (Figure 10c.), development of the parotid glands at the upper surface of the head and tail (Figure 10e.), and changes in pigmentation.

Initially, these larvae were somewhat yellowish-brown with black pigment areas scattered over their bodies (Figure 10a.). Gills were long (8-12 mm) and highly branched. The gills began to regress, in length and number of branching filaments, concurrently with the resorption of the tailfin (Figure 10c.). During the final stages of tissue remodeling, the parotid glands become more prominent along the tail and head (Figure 10e.).

During the course of these experiments a number of individuals died, especially during the late phases of metamorphosis. Six larvae from Lost Lake perished during this phase, but all of the transforming Goldie Lake larvae survived through metamorphosis. Transformed individuals could not be maintained within the laboratory at room temperature as they underwent rapid dehydration. For this reason fully transformed salamanders were maintained within cooler temperatures (11 ± 2 °C) in moist terrariums. Transformed animals were fed mealworms and maintained for other experiments.
Morphological Changes during metamorphosis of *Ambystoma gracile*.

Changes in morphology of *Ambystoma gracile* is represented by:

(a.) Late larval form, SVL/HLL = 3.5 and SVL/TW = 6.8.

(b.) Prometamorphic form, SVL/HLL = 3.0 and SVL/TW = 6.9.

(c.) Metamorphic climax form, SVL/HLL = 2.4 and SVL/TW = 7.1.

(d.) Late Metamorphic form, SVL/HLL = 2.4 and SVL/TW = 7.5.

(e.) Fully Transformed form, SVL/HLL = 2.3 and SVL/TW = 8.1.

Note the protrusion of the eyes and the beginning of resorption of the gills and tailfin of 10 (c.). Also note the general changes in pigmentation.
Reproductive Biology

Sexual Maturation

Sexual maturation studies (Table V) show that sexually mature Goldie Lake field females were smaller (p<.01; P < 0.001; Table III) with greater G.S.I.'s than Lost Lake females. These size differences were observed despite comparable weight:length ratios for immature larvae suggesting that food may not be a factor. Neotenous females attained sexual maturity at a size of 7.1-cm SVL for both altitudes (see range of lengths, Table V), but low-altitude animals ultimately attained larger sizes. This is consistent with the observation of a greater dampening of growth rates for laboratory-reared Goldie Lake animals compared with Lost Lake animals.

A more variable picture was observed for males (Table V). Lost Lake neotenous males attained sexual maturity at 7.7-cm SVL and transformed males were mature at 7.4-cm SVL, whereas two neotenous Goldie Lake salamanders were mature at 6.4-cm SVL and 6.6-cm SVL, but one animal of 7.5-cm SVL was immature. Thus, Goldie Lake mature and immature males overlapped in body size, but animals in excess of 7.4-cm SVL were generally sexually mature since all smaller animals except two were immature. The sexes and morphological form for animals captured before egg deposition are illustrated in Table VI.

Seasonal Maturation

Seasonal ovarian and reproductive changes also differed for low- and high-altitude populations (Figs. 11 and 12; Table VII). Lost Lake
Table V. The snout-vent lengths, weights, and gonadosomatic indices (G.S.I.) (Mean ± SEM) of *Ambystoma gracile* collected from two different altitudes.
The numbers in parentheses represent the sample sizes.

<table>
<thead>
<tr>
<th></th>
<th>Sexually immature</th>
<th>Sexually mature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>larval forms</td>
<td>Metamorphosed forms</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Lost Lake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, cm</td>
<td>5.9 ± 0.42</td>
<td>5.5 ± 0.31</td>
</tr>
<tr>
<td>Length range</td>
<td>5.2 to 7.5</td>
<td>4.3 to 6.6</td>
</tr>
<tr>
<td>Weight, g</td>
<td>9.7 ± 1.32</td>
<td>9.4 ± 1.51</td>
</tr>
<tr>
<td>G.S.I.</td>
<td>0.09 ± 0.019</td>
<td>0.56 ± 0.080</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, cm</td>
<td>6.5 ± 0.10</td>
<td>6.1 ± 0.25</td>
</tr>
<tr>
<td>Length range</td>
<td>6.2 to 6.8</td>
<td>5.2 to 6.9</td>
</tr>
<tr>
<td>Weight, g</td>
<td>10.1 ± 0.43</td>
<td>9.4 ± 0.60</td>
</tr>
<tr>
<td>G.S.I.</td>
<td>0.34 ± 0.010</td>
<td>0.60 ± 0.070</td>
</tr>
</tbody>
</table>

Note: All sexually immature animals are significantly different, $p < 0.001$, from the sexually mature animals, except the Lost Lake females, which are significantly different by $p < 0.01$. See Figs. 12, 13, and 14 for photographic comparisons. All sexually mature females laid eggs within the laboratory (or had ovulated). Mature males were usually judged mature by swollen epididymis's. Goldie Lake females include both 1974 and 1975 captures.
Table VI. Composition of sexually mature *Ambystoma gracile* from two lakes of different altitudes captured before any egg deposition (1975)

<table>
<thead>
<tr>
<th></th>
<th>Neotenous</th>
<th>Metamorphosed</th>
<th>% mature animals in breeding condition</th>
<th>Mature animals, n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lost Lake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>85.7</td>
<td>14.3</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Males</td>
<td>17.7</td>
<td>82.3</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td><strong>Goldie Lake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>87.5</td>
<td>12.5</td>
<td>27.6</td>
<td>29</td>
</tr>
<tr>
<td>Males</td>
<td>88.9</td>
<td>11.1</td>
<td>100</td>
<td>27</td>
</tr>
</tbody>
</table>

**Note:** See Fig. 15 for photographs of females in breeding condition. Animals were judged to be mature by comparing their G.S.I.'s with those of known mature animals (Table 5). Females could also be visually assessed as mature, because they contained large, convoluted oviducts (see Fig. 14). Animals were known to be breeding since mating behavior was observed, and two Lost Lake females and two Goldie Lake females were captured while in the process of ovulation.
Table VII. Reproductive effort of neotenous female *Ambystoma gracile* from two lakes

<table>
<thead>
<tr>
<th>% weight loss after egg deposition</th>
<th>No. eggs per egg mass</th>
<th>Range of ovum diameters,* mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(8) Goldie Lake 38.80 ± 0.449</td>
<td>(8) 63.0 ± 2.41</td>
<td>2.6 - 3.4</td>
</tr>
<tr>
<td>(9) Lost Lake 30.96 ± 1.226</td>
<td>(9) 70.0 ± 5.00</td>
<td>3.2 - 4.5</td>
</tr>
</tbody>
</table>

* Ten eggs from each egg mass were measured. Larger animals usually had larger eggs (ovum diameter).

Included 1974 and 1975 females.

1975 animals only.
Figure 11. Seasonal changes in the reproductive states of neotenous female *Ambystoma gracile* from Lost Lake. Broken line represents the reproductive index (R.I.) and the solid line represents the gonadosomatic index (G.S.I.). The R.I. is staggered to the right for September. Numbers in parentheses represent number of animals. The dot represents mean values of the designated month, the rectangles denote the standard errors, and the vertical lines represent the standard deviations. Follicles within Lost Lake females began to become yolked during July and are first pigmented during August.
Figure 12. Seasonal changes in neotenous female *Ambystoma gracile* reproductive states from Goldie Lake. Data represented in same fashion as Fig. 11. Note: only 2 animals of the 10 captured in September had yolking eggs.
neotenous females exhibited little change in follicle size, gonad weight, or oviduct weight during the 2½ months (refractory period) after egg deposition. During late June and early July, the follicle sizes began to increase (vitellogenesis-yolking) as did the G.S.I. of Lost Lake females. During mid-August the yolked follicles first became pigmented, and their diameters nearly doubled between August and September. By September animals were prepared reproductively for the next spring, since follicles approximated the mature ova size and the proportion of weight 'allocated' towards reproduction (R.I.) corresponded with the weight lost during oviposition (Table VII).

Goldie Lake females did not exhibit synchronous ovarian changes nor did they reproduce annually. Most mature females, captured before any oviposition (Table VI) had undeveloped follicles (reminiscent of the previtellogenic seasonal state of May or June mature Lost Lake females). This indicated that a major proportion of mature Goldie Lake females were not ovipositing during 1975. During September (1975) most of the mature Goldie Lake females were also in the previtellogenic state. Only two animals contained yolking follicles or expanded (owing to oviducal jelly) oviducts. Since an even larger percentage of weight is allocated to reproduction (Table VII) for Goldie Lake females, only a small proportion of mature females are able to reproduce during any one season. Owing to the short summers at the high altitudes, females that have oviposited may require the full summer for recovery (refractory period), and ova and oviducts undergo seasonal maturation the next summer.

The number of eggs per egg mass was less than previously reported
Figure 13. Mature and immature males captured from Goldie Lake. Testes from the mature animals have a more dense, granular look. The vas deferens is much more extensive and convoluted within the mature males. Testes of immature animals contain spermatogonia, but no primary spermatocytes or later stages. Immature males captured soon after the lake melts usually have a much higher proportion of fatty deposits covering the testes.

Figure 14. Immature and mature *Ambystoma gracile* females captured from Goldie Lake. Note the lack of developed oviducts within the immature female. As in the immature males, the gonads of immature females have a much higher proportion of fatty deposits covering the gonads.
Figure 15. *Ambystoma gracile* females captured from Lost Lake before any egg deposition. Eggs are ovulated into egg sacs within the body cavity and are fertilized as they are deposited. Eggs removed from these sacs after dissection are infertile, whereas if animals are stripped the eggs are fertile (if the salamander has ovulated). Ovulation may be induced by tactile responses to males. Metamorphosed females and neotenous females captured before egg deposition have similar snout to vent lengths.
(Licht 1975; Watney 1941), and this may be a reflection of the more certain permanent lake environment compared with small stream or pond environment.

**Discussion**

Thermal profiles of lakes located at low and high altitudes reveal obvious differences for the annual time span when salamanders are capable of growth (Fig. 2). Lost Lake exhibited over 8 months of temperatures exceeding 4°C, whereas Goldie Lake rarely had ice-free seasons lasting longer than 4 months. Since amphibians do not grow at temperatures lower than 5°C, even if food is available (Wilbur and Collins 1973), their yearly growth is restricted to shorter time spans within the high-altitude lakes. This time and temperature restriction may also place constraints upon high-altitude mature salamanders with regard to energy allocation towards reproduction. Laboratory and field studies suggest larval growth, metamorphic, and reproductive adaptations to the short summers observed for the higher altitudes.

Possible genetic differences were implied by the laboratory growth experiments. Goldie Lake larvae grew faster between the sizes of 3.0- to 6.0-cm SVL than did the Lost Lake larvae (Figs. 8 and 9; Table III). Size-frequency data (Fig. 5, July 1975) for Goldie Lake may partially explain these differences. Eggs, laid soon after the lake melted, hatched during the late summer, and these hatchlings attained sizes of only 2.5- to 2.8-cm SVL because of their brief first summer. After the next summer melt, these larvae may grow very rapidly, taking full advantage of the rapid turnovers observed for subalpine eutrophic lakes. Thus, the faster larval growth at
this size may be an adaptation to the shorter growing seasons and rapid nutrient changes observed within high-altitude lakes.

The faster growing of the Lost Lake laboratory animals were the first to transform, and they metamorphosed at the smallest sizes. Shoop (1974), working with *Ambystoma maculatum*, observed that field animals transforming early in the summer were smaller than salamanders that metamorphosed later in the season. Discussing this phenomenon of early metamorphosis at smaller sizes Shoop (1974) stated, "the timing of metamorphosis began approximately the same time ... irrespective of conditions." This suggests a type of genetic heterosis related to fast growth and early metamorphosis, which was lacking within the high-altitude population. A few individuals from the Lost Lake population metamorphosed soon after 6 months of growth, whereas the first Goldie Lake animals to transform required 9 months of growth (Table IV). Animals within low-altitude lakes probably interbreed with salamanders that are adapted to ponds that often dry. This adaptation could be manifested by faster growth rates and early metamorphosis. Owing to the shorter seasons and the complete freezing of ponds during winters, such populations would be less likely to survive at the higher altitudes.

Goldie Lake animals metamorphosed at larger body sizes than did Lost Lake animals (Table IV). The greater chance of survival may select for larger body sizes (Wilbur and Collins 1973) since larger transformed individuals could more easily endure harsh winters and would have a greater range of prey sizes.
The laboratory experiments resulted in a very different incidence of metamorphosis for Lost Lake (88%) compared with Goldie Lake (23.5%) samples and suggested that the major factor determining neoteny may be genetic in nature. Though recapture data (Table I) were sparse, animals recaptured subsequent to the year of tagging (1974) indicated that a major proportion of Lost Lake field animals probably metamorphose. Four transformed animals captured during 1976 contained first-cohort 1974 tags and one (neotenous) animal captured in 1975 was tagged. Bell and Lawton (1975) studying the smooth newt Triturus vulgaris also found similarities in larval growth and metamorphosis (timing) for field and laboratory studies concerning the same pond population. These authors also discovered differences in developmental patterns when different pond populations were compared.

Goldie and Lost Lake laboratory animals exhibited unequal sex ratios with respect to neoteny. The neotenous female:male ratio for Lost Lake animals was 4:2 (the field ratio for neotenous animals, after August when they were easily sexed, was 45:13), and the laboratory Goldie Lake female:male ratio was 27:12. These unequal sex ratios seemed to be evident within the breeding population (Table VI) and unequal metamorphosed male:female ratios seemed to reestablish 1:1 sex ratios. These observations suggested that neoteny might be sex-linked for this species (perhaps sex-linked recessive since females are heterogametic within urodele species of the genus Ambystoma; Gallien, 1975). If this premise were true, it might account for the substantial number of neotenous Lost Lake females, and yet most offspring transform. Neoteny could be envisioned as a sex-linked recessive trait that might be selected for or
against in a clinal gene-frequency fashion (Endler, 1973) by various factors related to altitude. Selection parameters could be temperature regime, timing of oviposition, or the duration of early growth periods. A high gene frequency related to metamorphosis is maintained at the low altitudes even though there are large numbers of neotenic females.

The variability of the life cycle of Ambystoma gracile has been of interest for a long time (Carl, 1943). A recent study by Snyder (1956) discussed sexual dimorphic differences of Ambystoma gracile and suggested that temperature may play an important role in the attainment of neoteny. Snyder (1956) stated, "sexual maturity is attained during the second year when the animal reaches a snout-vent length of approximately 70 mm."

A reinvestigation confirmed this body size for sexually mature females (Table V), but the short seasons at the higher altitudes suggests that high-altitude animals require 3 or more years to attain this size. Generally, males larger than 7.3-cm SVL were sexually mature, but two exceptions of smaller, sexually mature males were captured within the high-altitude lake. An understanding of the body size for sexual maturity is essential if animals are to be designed as neotenic. Previous studies (Sprules, 1974) counting the number of captures per day as an indication of neoteny may be invalid since most animals may have been immature and capable of metamorphosis. The larger body size at metamorphosis for Goldie Lake animals seems to substantiate this point.

Many temperate amphibian species exhibit short breeding seasons when members of the locality aggregate at breeding places. The ripe, mature females empty their mature eggs during the few days (Goldie Lake)
or weeks (Lost Lake) that the breeding seasons last. After oviposition, temperate amphibians usually exhibit a resting (refractory) phase before the second growth phase (SGP: Jorgensen 1974) of follicles resumes. This resting phase lasted 2½ months for the annual breeders such as Lost Lake females. Owing to the short seasons within high-altitude lakes, the Goldie Lake females that had oviposited required the entire summer for this phase. Since oviposition within Goldie Lake occurred within a few days after capturing previtellogenic (resting) females, only a small percentage of mature females were destined to deposit eggs during 1975 (Table VI). This corroborates the supposition that high-altitude mature females alternate breeding and refractory seasons and it divulges a possible reproductive adaptation to the shorter seasons at the higher altitudes.

Initially this study attempted to eliminate all nonaltitudinal parameters that might affect Ambystoma gracile development, but it was later discovered that Petgill Lake contained fish, Salmo gairdneri. Comparing the sizes at metamorphosis of Petgill laboratory-reared animals with the other two lakes suggests that continued competition for food or habitat may have selected for a slower developmental rate and a smaller size (t v.; P < 0.01) at metamorphosis (Table IV; note time required before metamorphosing is quite similar to Goldie Lake animals, but animals are substantially smaller). This is consistent with the observations by Wilbur (1972) of low survivorship, smaller body sizes, and longer larval periods for Ambystoma maculatum when a competitive species was present. The addition of the fish competitor to Petgill Lake may
have influenced the developmental characteristics of *Ambystoma gracile* and therefore biased the altitudinal trends previously discussed. Previous studies (Efford and Mathias, 1969; Licht, 1975) neglected the possible competitive effects of fish on the growth of larval *Ambystoma gracile* populations.

Many environmental factors have been suggested to cause the initiation of metamorphosis. These include temperature (Shrode, 1972; Snyder, 1956), availability of food (Wilbur and Collins 1973), drying of ponds (Wilbur and Collins 1973), and recent rainfall (Healy, 1975). The present study suggests that the major factor involved with the occurrence of metamorphosis (or neoteny) is the genetic nature of the population. Animals destined to metamorphose are influenced by these environmental cues; and if these cues do not occur at the proper body size, the animal may overwinter to metamorphose the next summer. Neoteny seems to be maintained by genetic selection through constant environmental forces, and metamorphosis is retained within the population by gene flow against these forces.
CHAPTER II

Factors affecting the larval growth and development of laboratory-reared Ambystoma gracile (Baird) from permanent low-altitude and montane lakes.
CHAPTER II

Factors affecting the larval growth and development of laboratory-reared *Ambystoma gracile* (Baird) from permanent low-altitude and montane lakes

Introduction

Lower environmental temperatures are often correlated with slower growth rates and increased longevity for many poikilotherms (Bourliere 1957 and Beverton and Holt, 1959). Therefore, growth and physiological activity is temperature dependent within poikilotherms such as amphibians. Increases in physiological parameters might also effect pituitary and thyroid activity for poikilotherms such as larval amphibians. The possibility of a temperature dependence of the thyroid of larval *Ambystoma gracile* might be of particular interest since this species is a facultative neotene observed in lakes at high altitudes (Snyder, 1956) where there are great seasonal changes in temperature subsequent to the thawing of lakes. Studies investigating the effects of temperature-transfer upon larval growth and spontaneous metamorphosis for this species might reveal possible physiological adaptations to different habitats and might be more relevant to the animals' natural physiological regime. Considering these factors, an investigation was conducted to study the effect of cold to warm transfer upon larval growth and metamorphosis for larval *Ambystoma gracile* obtained from lake populations located at different altitudes.

Prolactin has been implicated as the larval growth factor within many anurans (Bern *et al*., 1967; Brown and Frye, 1968; and Etkin *et al*.,
1969) and is thought to inhibit metamorphosis in urodeles (Cohen et al., 1972; Gona and Etkin, 1970; and Platt, 1976) and anurans (Frye et al., 1972); but very few studies have investigated prolactin's possible physiological role in larval urodeles. Since only prolactin and not growth hormone has been detected in urodeles of the genus Ambystoma (Nicoll and Licht, 1971; Nicoll and Nichols, 1971; and Hayashida et al., 1973) and this prolactin-like hormone exhibits little immunochemical relatedness to somatotropin (Hayashida et al., 1973); I felt it warranted to investigate the possibility of a growth-promoting activity for prolactin in larval Ambystoma gracile.

Materials and Methods

Experiment 1:

In order to investigate the possible effects of temperature-transfer upon growth and metamorphosis of Ambystoma gracile, larvae were reared from eggs obtained from two separate populations. Animals were reared as previously described (Eagleson, 1976) except that larvae used in the temperature-transfer experiments were maintained at 11 °C ± 2 °C until they attained snout to vent lengths (SVL) comparable to second year larvae in the field (Chapter I). Once animals attained this size, 25 larvae from each randomized lake population were transferred from 11 °C to 21 °C. Growth, onset of metamorphosis, and size at metamorphosis were recorded as previously described (Eagleson, 1976). Growth was analyzed by length increase (SVL) and weight increase. Due to the subsequent dampening of growth during sexual maturation, growth was
followed until larvae attained a snout to vent length of 6.5 cm. The length increase was calculated by subtracting mean length (snout to vent length) from the observed initial snout to vent length.

These data were then plotted and fitted by the least squares linear regression analysis. Interpopulation differences were analyzed by the Student's t-test (two-tail analysis).

Experiment 2:

Due to limitations in the number of animals, hormone studies were performed on just the high-altitude (Goldie Lake) population. Larvae were maintained at 21 ± 2 °C in 1 litre preparation dishes and fed beef liver on alternate days. Animals were fed to satiation. In order to eliminate genetic differences in physiological response larvae utilized for test animals were from the same egg batch which was oviposited by a neotenous female within the laboratory during 1975. Larvae were reared as previously described (Eagleson, 1976) until they reached a snout to vent length of 4.5 cm when hormone treatments commenced. The two experimental groups consisted of 8 animals each. One group received a placebo injection of hormone diluant mixture without prolactin and the test group was injected with 10 μg of prolactin (NIH-P-S 11) dissolved with 2-3 drops 1% NaOH and diluted with amphibian saline. Animals were injected 6 times a week with a total of 60 μg/week of ovine prolactin. Larvae were injected approximately 2 to 3 hours following the onset of the photophase (12 L:12 D) and injected intraperitoneally. Animals were measured and weighed every 15 days and fed beef liver ad libitum every
other day.

Instantaneous growth rates (IGR) were estimated by:

\[
IGR = \frac{\log_{e} SVL_1 - \log_{e} SVL_0}{t_1 - t_0}
\]

where SVL represents snout to vent length and \( t \) represents time in days.

Absolute growth was also calculated for animals at different sizes.

Statistical analysis was performed by one-way analysis of variance and then by utilizing the Student's \( t \)-test (one-tailed).

Results

Absolute growth rates for Lost Lake larvae grown continuously at 21 °C (0.20 ± 0.014 mm/day) did not differ significantly from Lost Lake larvae maintained at 21 °C after transfer (0.20 ± 0.049 mm/day) from 11 °C. Goldie Lake larvae exhibited significantly greater growth rates (\( p < 0.05 \)) when continuously grown at 21 °C (0.23 ± 0.010 mm/day) than Lost Lake larvae, and temperature-transfer did not significantly alter Goldie Lake larval growth rates at 21 °C (0.23 ± 0.008 mm/day). Goldie Lake larvae grown at 11 °C also exhibited greater growth rates (\( p < 0.05 \); daily increment = 0.12 ± 0.007 mm/day) than Lost Lake larvae (0.09 ± 0.004 mm/day) reared at 11 °C. Temperature-transfer significantly (\( p < 0.001 \)) increased the growth rates for both populations. A 10 °C increase in temperature from 11 °C doubled the absolute growth rate (daily increment) for both populations (Figs. 16 and 17). Temperature-transfer did not significantly alter the size at metamorphosis or the
incidence of neoteny (Table VIII).

Prolactin treatment promoted faster growth rates (Fig. 18) and this was reflected by greater weights and lengths ($p < 0.025$) for treated larvae 60 days subsequent to treatment. Size-specific differences in absolute and instantaneous growth were observed for all but one size class (Table IX).
Figure 16

Effects of temperature-transfer upon growth of *Ambystoma gracile* larvae from Lost Lake.

Animals were grown either continuously at $21^\circ \pm 2^\circ$C (straight line with single slope) or grown at $11^\circ$C until they attained a mean size of 3.9 cm snout to vent length and then transferred to $21^\circ \pm 2^\circ$C (straight line with two slopes).

(a.) Length increase vs. time - Lost Lake larvae grown continuously at $21^\circ \pm 2^\circ$C.

Growth curve $= 0.627 + 0.609$

$R^2 = 97.7 \quad r = 0.988$

(b.) Length increase vs. time - Lost Lake larvae grown at $11^\circ \pm 2^\circ$C (prior to transfer).

Growth curve $= 0.677 + 0.296$

$R^2 = 99.0 \quad r = 0.995$

(c.) Length increase vs. time - Lost Lake grown at $21^\circ \pm 2^\circ$C (after transfer).

Growth curve $= -2.526 + 0.608$

$R^2 = 97.6 \quad r = 0.987$
Figure 17

Effects of temperature-transfer upon growth of *Ambystoma gracile* larvae from Goldie Lake.

Animals were grown either continuously at 21° + 2°C (straight line with single slope) or grown at 11° + 2°C until they attained a mean length of 3.4 cm snout to vent length and were then transferred to 21° + 2°C (straight line with two slopes).

(a.) Length increase vs. time - Goldie Lake larvae grown continuously at 21° + 2°C.

Growth curve = 0.523 + 0.693

R^2 = 97.9    r = 0.989

(b.) Length increase vs. time - Goldie Lake larvae grown at 11° + 2°C (prior to transfer).

Growth curve = 0.989 + 0.336

R^2 = 98.3    r = 0.992

(c.) Length increase vs. time - Goldie Lake larvae grown at 21° + 2°C (after transfer).

Growth curve = -1.514 + 0.699
MONTHS SINCE HATCHING

SNOUT-VENT LENGTH (cm)
Figure 18. Effects of ovine prolactin upon growth for larval *Ambystoma gracile*.

Triangles represent larvae injected with 10 μg/animal of ovine prolactin six times a week. The circle represents saline-injected controls. The vertical lines denote the 95% confidence limits. Days represents the days of treatment. Mean length (top graph) and mean weight (lower graph) are represented for the treatment period.
### Table VIII

**Effects of temperature-transfer on metamorphosis.**

<table>
<thead>
<tr>
<th>Lake</th>
<th>(n)</th>
<th>% metamorphosed</th>
<th>size (M ± SEM)*</th>
<th>Range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lost Lake</td>
<td>50</td>
<td>88.0</td>
<td>6.19 ± 0.173</td>
<td>4.7 to 7.4</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>51</td>
<td>23.5</td>
<td>6.67 ± 0.056</td>
<td>6.4 to 7.2</td>
</tr>
<tr>
<td>Lost Lake #</td>
<td>25</td>
<td>96.0</td>
<td>5.91 ± 0.100</td>
<td>4.8 to 6.7</td>
</tr>
<tr>
<td>Goldie Lake #</td>
<td>25</td>
<td>28.0</td>
<td>6.48 ± 0.130</td>
<td>6.0 to 7.0</td>
</tr>
</tbody>
</table>

* Metamorphosed size and range are in cm snout to vent length

# Temperature-transfer animals

Goldie Lake larvae transformed at a significantly greater size \((p < 0.01)\) than did Lost Lake larvae. Temperature-transfer did not significantly alter the mean size at metamorphosis.
<table>
<thead>
<tr>
<th>Size-class, CM.</th>
<th>Saline Controls</th>
<th></th>
<th>Prolactin Treated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>daily increment</td>
<td>IGR</td>
<td>daily increment</td>
<td>IGR</td>
</tr>
<tr>
<td>5.0</td>
<td>0.113 mm/day (0.0452)</td>
<td>0.220</td>
<td>0.260 mm/day (0.078)</td>
<td>0.547</td>
</tr>
<tr>
<td>5.5</td>
<td>0.167 mm/day (0.0429)</td>
<td>0.327</td>
<td>0.187 mm/day (0.101)</td>
<td>0.351</td>
</tr>
<tr>
<td>6.0</td>
<td>0.167 mm/day (0.0835)</td>
<td>0.300</td>
<td>0.360 mm/day (0.162)</td>
<td>0.641</td>
</tr>
<tr>
<td>6.5</td>
<td>0.227 mm/day (0.0480)</td>
<td>0.357</td>
<td>0.271 mm/day (0.071)</td>
<td>0.400</td>
</tr>
</tbody>
</table>

IGR represents the Instantaneous Growth Rate

Numbers in parenthesis denote the Standard Error of the Mean of the daily increment (mm/day).
Discussion

Temperature-transfer studies indicated that growth subsequent to transfer from 11 °C to 21 °C was not significantly altered from that of animals grown continuously at the higher temperature. This was observed despite the generally faster growth rates for larvae from the montane population. This suggests that natural selection has maintained higher growth rates for the montane population, perhaps by selecting for increased sensitivity to growth-promoting hormones (prolactin-like?) or an increased synthesis and/or release of these hormones. Since populations are exposed to rapid temperature-shifts in their natural environment, this type of regime might be more relevant to neoteny. The size at metamorphosis and the incidence of metamorphosis within the two lake populations (Lost Lake and Goldie Lake) was not altered by temperature-transfer, although the upper asymptote of body size at metamorphosis was lowered (Table VIII). These studies strongly implicate a "genetically selected physiological condition" with respect to neoteny as suggested by Sprules (1974a). Continual low temperatures may alter the incidence of neoteny (Sprules, 1974b; Snyder 1956); but such occurrences are probably rare in nature, because even montane populations are exposed to two or three months of warmer temperatures each year; and the low temperatures observed at the high altitudes are too low for growth and maturation, since montane lakes are either frozen or at warmer summer temperatures with rapid temperature shifts subsequent to melting.

This study also indicates that ovine prolactin exerts a somatotropic action upon larval Ambystoma gracile and that a prolactin-like hormone
may be responsible for increased growth, such as is observed following
temperature increases. This observation is in agreement with other
studies involving larval anurans (Bern et al., 1967; Brown and Frye, 1969;
and Frye et al., 1972) and implies that the function of the prolactin-
like hormone found in *Ambystoma* larvae (Nicoll and Licht, 1971) might
be somatotropic in nature.

Absolute and instantaneous growth rates indicated that prolactin-
injected larvae grew faster in length for three of the four size groups
compared to saline-injected controls. The 5.0-5.5 cm SVL size groups
had only a slightly higher growth rate when prolactin-injected larvae
are compared to saline injected controls. Subsequent investigations with
this lake population indicated that there was greater thyroid activity
and higher serum thyroxine levels for this size group, and the lowered
sensitivity to ovine prolactin at this size may be a reflection of thyroid/
prolactin antagonism. Gona et al. (1973) have observed prolactin/
thyroxine antagonism of water drive response for *Diemictylus* at relatively
high thyroxine concentrations, but these authors also observed a prolactin/
thyroxine synergism at low thyroxine concentrations. The lowered response
to prolactin-injections observed in this study (Table IX) suggests a
similar existence of both antagonism and synergism between prolactin and
thyroxine. Subsequent size groups exhibited a lowering of thyroid
activity (with the obvious exception of spontaneous metamorphosis!), and
these size groups responded to prolactin injection with increased growth
(Table II).
This study strongly suggests the involvement of a prolactin-like hormone with larval growth of *Ambystoma gracile*. Further studies should be pursued to determine if a growth hormone-like substance may also promote growth and if thyroxine and prolactin antagonism is in deed observed in a similar manner for larval urodele growth as is observed during amphibian metamorphosis (Etkin, 1964 and Platt, 1976).
CHAPTER III

Changes in thyroidal activity of *Ambystoma gracile* (Baird) during different phases of postembryonic growth: A comparison of two lake populations.
CHAPTER III

Changes in thyroidal activity of Ambystoma gracile (Baird) during different phases of postembryonic growth: A comparison of two lake populations

Introduction

Several studies have followed the development of thyroid activity in anuran larvae (Hanaoka et al., 1973; Kaye, 1961; and Saxen et al., 1957), but there have been no investigations following the thyroid activity of larval urodele amphibians. Since the thyroid plays such an important link in promoting the onset of metamorphosis, it seemed logical to investigate changes in thyroid activity of postembryonic Ambystoma gracile larvae. Investigations assessing changes during development might provide clues towards a possible developmental "flaw" that might promote neoteny in this species; therefore studies investigating possible variations in thyroid function with development were begun on two populations of Ambystoma gracile which exhibit differences in neotenic tendencies.

Since a thyrotropin-like hormone has been implicated as the factor responsible for mediating the amphibian thyroid and metamorphosis (Eddy and Lipner, 1976) and pituitary thyrotope malfunction has been suggested as the defective link in Ambystoma mexicanum (Turner and Bagnara, 1976), additional studies assessing the sensitivity of Ambystoma gracile thyroids to mammalian TSH (NIH-TSH-B7) during different phases of development were also performed. Other studies investigating the possible effects of steroid pretreatment upon thyroid function were also performed since other investigators have suggested that steroids might act synergistically (Frieden and Just, 1971) or antagonistically (Norris and Gern, 1976) with amphibian metamorphosis.
Materials and Methods

Animals:

The animals used in this study were reared during 1974 from eggs obtained from two populations of *Ambystoma gracile*. Hatchlings from these lakes were randomized within each population. The Lost Lake population exhibited approximately a 10% incidence of neoteny, whereas the Goldie Lake population was approximately 75% neotenic when animals were reared at room temperature (21 °C ± 2 °C). Animals were fed to satiation on alternate days, with beef liver, beef heart, and occasionally earthworms. The animals used for the estrogen studies were all from the same egg batch oviposited in the laboratory by a neotenous Lost Lake female during 1975. All experiments were performed at room temperature (21 °C ± 2 °C) on a 12 L: 12D photoperiod.

Experiment 1:

In order to investigate the time course of thyroid accumulation, radioiodide (1.25 μCi 125I, carrier free) was injected intraperitoneally in 10 μl of amphibian saline to untreated Lost Lake larvae (approximately 5.0 cm snout to vent length). Animals were killed by decapitation 12, 24, 48 and 96 hr after 125I injection. Animals were weighed and measured prior to injection; and after treatment, the thyroid area of the lower jaw was excised, weighed and placed in a plastic well-counter tube. A portion of the tailfin of similar area was also placed in a tube and radioactivity determined. The mean counts for each thyroid area were corrected for background by subtracting the mean tailfin counts. They were then compared as a percentage of the radioactivity of the injected dose.
Experiment 2:

To investigate the possible effects of thiourea (a thyroid inhibitor) and thyroxine upon thyroid activity, radioiodide uptake experiments were conducted on randomized Lost Lake larvae of 6.0 cm SVL. Larval animals of this length were utilized because this was the mean size for spontaneous transformation. All larvae were pretreated for five days prior to radioiodide injection. The thiourea group \((n = 4)\) was pretreated by immersing into 0.25% thiourea (Dent, 1961). One thyroxine treatment group \((n = 4)\) was pretreated with \(1 \times 10^{-7}\) M \(1\)-thyroxine (Sigma). A second thyroxine group \((n = 4)\) was pretreated with immersion into \(1 \times 10^{-8}\) M \(1\)-thyroxine. The control group \((n = 4)\) was maintained in dechlorinated tap water. Animals were fed to satiation on alternate days. Five days after pretreatment animals were injected intraperitoneally with radioiodide \((1.25 \mu\text{Ci} ~ ^{125}\text{I}, \text{carrier free})\) in 10 \(\mu\text{l}\) of amphibian saline. Animals were killed by decapitation 48 hours post-radioiodide injections. All animals were maintained in pretreatment solutions during the 48 hour period. Animals were weighed and measured as in Experiment 1. The percentage radioiodide accumulation was also calculated in the same fashion as Experiment 1.

Experiment 3:

To investigate possible changes in thyroid activity with development, randomized samples of salamanders reared from Lost Lake and Goldie Lake populations were injected in the same manner as Experiment 1, but during different phases of postembryonic growth. Animals were weighed and
measured as in Experiment 1, percentage radioiodide uptake was calculated in the same manner as Experiment 1, and dissections were carried out 48 hr post-radioiodide injections.

Experiment 4:

In a concomitant experiment to Experiment 3, investigations into possible changes in serum thyroxine levels with development of *Ambystoma gracile* were also carried out. During different stages of postembryonic growth, randomized *Ambystoma gracile* reared from eggs from Lost or Goldie Lake populations were anaesthetized by being placed in 1:2000 tricaine methanesulfonate (MS 222) until they no longer exhibited a muscular response. Two microliters of blood per grain body weight were taken by heart puncture with a 25 μl Hamilton syringe. The blood was immediately centrifuged in microhaematocrit tubes, plasma removed and then stored frozen (-20 °C) in heparinized microhaematocrit tubes until assayed.

The serum thyroxine concentrations were analyzed by using a commercial radioimmunoassay kit (Mallinckrodt, Inc.). To assure that the concentrations fell on the linear/log curve, 4 μl% of thyroxine was added to each "unknown" vial and this amount was then subtracted from the final calculated value. Serum calculations were done in duplicate for animals of 6.0 cm SVL and smaller and were done in triplicate for larger salamanders. Duplicate and triplicate values all varied by less then 10%.

Experiment 5:

In order to test the possible effects of estrogen and sexual maturity upon thyroid function, genetically similar larvae were utilized for hormone treatment studies. Sixteen immature larvae reared from the same egg batch
deposited by a Lost Lake female were divided into four groups of four larvae each. Larvae were grown to 6.5 cm SVL (a size just prior to sexual maturation) before hormone treatments started. One group was injected with 15 μg/animal/day of estradiol (Sigma) for 21 days. A second group received injections of NIH-TSH-B7 (500 ng/g body weight per/day for seven days). A third group was first injected with estradiol (15 μg/animal/day for 21 days) and then TSH (500 ng/g body weight/day for 7 days). A fourth group was injected with the saline vehicle for 28 days. On the last day of injections all animals from each group were given 2 single intraperitoneal injection of radioiodide (1.25 μCi 125I, carrier free) in 10 μl of saline subsequent to weighing and measuring. After 48 hours the thyroid area of the lower jaw was excised, weighed and placed in a plastic well-counter tube. The mean corrected counts were calculated as described in Experiment 1.

Experiment 6:

In order to investigate possible changes in sensitivities to mammalian-TSH, 4 older sexually mature laboratory reared animals from each of the two lakes were injected with 500 ng/g body weight of bovine TSH (NIH-TSH-B7) daily for 7 days. On the last day of injection all animals were given a single injection of radioiodide (1.25 μCi 125I, carrier free) subsequent to weighing and measuring. After 48 hours, the thyroid area was excised, counted, and corrected as described in Experiment 1. This treatment was duplicated for Lost Lake immature animals (4 experimentals; 4 controls) of approximately 6.0 to 6.5 cm snout to vent length.
Statistics

Intrapopulational data were subjected to one factor analysis of variance, followed by the Neuman-Keuls multiple comparison procedure. Interpopulational comparisons utilized one-way analysis of variance and was followed by the student's t-test (two-tailed).

Results

Experiment 1:

The results of this experiment are summarized in Table X. Untreated larvae of 5.0 cm SVL and of approximately 6.0 g wet weight exhibited their maximum radioiodide uptake 48 hrs after radioiodide injection with no significant increases in uptake at 72 or 96 hrs post-radioiodide injection.

Experiment 2:

The results of this experiment (Table XI) demonstrate an activation of the thyroid by $1 \times 10^{-7}$ M thyroxine and a reduction in thyroid radioiodide uptake by thiourea treatment. Treatment by immersion into $1 \times 10^{-8}$ M thyroxine did not significantly alter thyroid activity when compared to controls (Table XI).

Experiments 3 and 4:

Developmental studies demonstrated that of the Lost Lake larvae, spontaneously metamorphosing larvae exhibited significantly greater thyroidal uptake values than all untreated nonmetamorphosing larvae.
Table X

Experiment I: Thyroidal uptake of radioiodide by larval *Ambystoma gracile*.

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>mean body weight (± S.E.)</th>
<th>time (hrs.)</th>
<th>% Uptake (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>5.8 ± 0.22 g</td>
<td>12</td>
<td>1.66 ± 1.06</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>6.1 ± 0.43 g</td>
<td>24</td>
<td>8.32 ± 2.89</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>6.3 ± 0.13 g</td>
<td>48</td>
<td>14.31 ± 2.00</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>6.0 ± 0.29 g</td>
<td>72</td>
<td>14.19 ± 1.09</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>6.3 ± 0.52 g</td>
<td>96</td>
<td>14.22 ± 2.04</td>
</tr>
</tbody>
</table>

Thyroid accumulation of radioiodide was greatest at 48 hrs. postinjection ($p < 0.01$), and was not significantly greater 72 or 96 hrs. postinjection from this value.
Table XI

Experiment 2: Effects of thyroxine and thiourea upon thyroidal radioiodide uptake of *Ambystoma gracile* larvae

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Mean Body Weight (± S.E.)</th>
<th>% Uptake (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>4</td>
<td>12.3 ± 0.32 g</td>
<td>9.6 ± 2.33</td>
</tr>
<tr>
<td>1 x 10(^{-8}) M (T_4)</td>
<td>4</td>
<td>12.2 ± 0.30 g</td>
<td>7.2 ± 1.14</td>
</tr>
<tr>
<td>1 x 10(^{-7}) M (T_4)</td>
<td>4</td>
<td>13.0 ± 0.47 g</td>
<td>27.5 ± 3.84</td>
</tr>
<tr>
<td>Thiourea</td>
<td>4</td>
<td>11.7 ± 0.34 g</td>
<td>2.0 ± 1.89</td>
</tr>
</tbody>
</table>

Animals treated with 1 x 10\(^{-7}\) M \(T_4\) exhibited greater thyroidal uptake values \((p < 0.01)\) than control animals. Control animals had significantly greater thyroidal uptake values \((p < 0.01)\) than animals treated with thiourea.
and transformed forms; and they also were observed to have higher serum thyroxine levels \((p < 0.01)\) than all nonmetamorphosing and transformed animals (Figure 19). A significant increase in thyroidal radiiodide uptake was observed for larvae at sizes of 5.0 cm SVL \((p < 0.01)\) compared to the smaller larvae of 4.5 cm SVL. Larvae of sizes greater than 7.4 cm SVL (neotenic) had lower \((p < 0.01)\) thyroidal uptake values than smaller (5.0 cm or 6.0 cm SVL) larvae.

Goldie Lake larvae exhibited a similar rise in thyroid activity. Larvae of 5.0 cm SVL had significantly higher thyroidal radiiodide uptake values \((p < 0.01)\) than smaller larvae of 4.5 cm SVL. Metamorphosing larvae had higher thyroidal radiiodide uptake values \((p < 0.01)\) and greater serum thyroxine levels \((p < 0.01)\) than all nonmetamorphosing forms. Interpopulational differences were observed for only the 5.5–6.0 cm SVL size group. The Lost Lake larvae of this size (6.0 cm SVL) had greater thyroidal radiiodide uptake values \((p < 0.05)\) and serum thyroxine levels \((p < 0.05)\) than Goldie Lake larvae of the same size. Radiiodide uptake values for larvae of 4.5 cm SVL or neotenes (9.0 cm SVL) from either Lost Lake of Goldie Lake populations did not differ significantly from thiourea treated larvae (Experiment 2).

Experiment 5:

Pretreatment of larvae with TSH (500 ng/g body weight) or estrogen (15 μg/animal) and then 500 ng/g body weight of TSH promotes a significantly higher thyroidal radiiodide uptake \((p < 0.01)\) than pretreatment with just estrogen (15 μg/animal) or untreated controls (Table XII). Control larvae
Figure 19.

Postembryonic changes in thyroid activity of *Ambystoma gracile* reared from eggs from Lost Lake.

Clear bars indicate the mean percent uptake of injected $^{125}$I by the thyroid area of larvae at different sizes and developmental states. Hatched bars denote the mean thyroxine levels. The letter M signifies transformed animals (4 months post-transformation) and the small letter m represents animals undergoing metamorphosis. The standard errors are indicated by the vertical lines.

Spontaneously metamorphosing larvae (m) exhibited greater thyroidal radiiodide uptakes ($p < 0.05$) and serum thyroxine levels ($p < 0.01$) than all other groups. Thyroidal radiiodide uptake values were significantly greater ($p < 0.01$) for larvae of 5.0 cm and 6.0 cm than smaller larvae (less than 4.5 cm SVL). Animals greater than 7.4 cm SVL exhibited significantly less ($p < 0.01$) radiiodide accumulation values than did larvae of 5.0 or 6.0 cm SVL.
IODINE UPTAKE (% INJECTED DOSE)

SNOUT TO VENT LENGTH (cm)

SERUM THYROXINE CONCENTRATIONS (ug/100ml)
Postembryonic changes in thyroid activity of *Ambystoma gracile* reared from Goldie Lake eggs.

Clear bars denote the mean percent uptake of injected $^{125}$I by the thyroid area of larvae at different sizes and developmental states. Hatched bars indicate the mean serum thyroxine levels. The letter m signifies animals that were undergoing metamorphosis when injected. The standard error is indicated by the vertical lines.

Larvae of 5.0 cm SVL had significantly greater ($p < 0.01$) thyroidal uptake values than larvae of 4.5 cm SVL and greater values ($p < 0.05$) than larvae of 6.0 cm SVL. Metamorphosing larvae had significantly greater radiiodide accumulation values ($p < 0.01$) and serum thyroxine levels ($p < 0.01$) than all other developmental states. Larvae in excess of 7.4 cm SVL had significantly less active thyroids ($p < 0.01$) than larvae of 5.0 cm SVL.
IODINE UPTAKE (% INJECTED DOSE)

SNOUT TO VENT LENGTH (cm)

SERUM THYROXINE CONCENTRATIONS (ug/100 ml)
exhibited significantly higher thyroidal radioiodide uptake values ($p < 0.05$) compared to larvae pretreated with estrogens (Table XII).

Experiment 6:

Adult neotenous forms and immature larvae exhibited similar responses to TSH with no significant difference with respect to whole thyroid accumulation (Table XIII). In both cases, TSH treatment significantly stimulated thyroidal uptake ($p < 0.01$) to a higher level than untreated controls.
Table XII  
Effects of Different Hormone Treatments upon Thyroidal Activity of Ambystoma gracile

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>(n)</th>
<th>SVL (cm) ± S.E.</th>
<th>WEIGHT (g) ± S.E.</th>
<th>% UPTAKE ± S.E.</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen</td>
<td>4</td>
<td>6.6 ± 0.21</td>
<td>13.0 ± 1.69</td>
<td>4.6 ± 1.72</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>6.8 ± 0.07</td>
<td>14.0 ± 0.19</td>
<td>8.9 ± 3.30</td>
<td>—</td>
</tr>
<tr>
<td>Estrogen than TSH</td>
<td>4</td>
<td>6.6 ± 0.09</td>
<td>12.4 ± 0.37</td>
<td>16.0 ± 2.31 *</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>TSH</td>
<td>4</td>
<td>6.4 ± 0.09</td>
<td>10.6 ± 1.15</td>
<td>16.4 ± 2.36 **</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

Significance is value compared to saline injected controls.

Treatment: TSH: 500 ng/g body weight/day for 7 days
  : estrogen; 15 µg/animal/day for 21 days
  : all animals were from the same egg batch

* one animal was 2/3 transformed and had an uptake of 9.10%

** same as *, except uptake was 9.96%
Table XIII. Effects of bovine TSH (500 ng/g body weight) upon Thyroidal Uptake of \( ^{125}I \) by Ambystoma gracile during Different Phases of Growth

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Mean SVL (cm) ± S.E.</th>
<th>Mean Weight (g) ± S.E.</th>
<th>% Uptake ± S.E.</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLDIE LAKE: controls</td>
<td>8.97 ± 0.19</td>
<td>37.2 ± 2.09</td>
<td>6.1 ± 0.43</td>
<td>24</td>
</tr>
<tr>
<td>GOLDIE LAKE: treated</td>
<td>9.15 ± 0.13</td>
<td>38.7 ± 1.95</td>
<td>20.0 ± 1.44</td>
<td>24</td>
</tr>
<tr>
<td>LOST LAKE: treated</td>
<td>10.35 ± 0.09</td>
<td>49.1 ± 1.05</td>
<td>18.4 ± 1.64</td>
<td>26</td>
</tr>
<tr>
<td>LOST LAKE: controls</td>
<td>6.10 ± 0.07</td>
<td>10.5 ± 0.75</td>
<td>8.8 ± 1.29</td>
<td>9.5</td>
</tr>
<tr>
<td>LOST LAKE: treated</td>
<td>6.40 ± 0.09</td>
<td>10.6 ± 1.15</td>
<td>16.4 ± 2.36</td>
<td>9.5</td>
</tr>
</tbody>
</table>

All treated groups had greater \((p < 0.01)\) thyroidal uptake values than untreated controls.

Age is in months reared at room temperature.
Discussion

Several studies with facultative neotenes (Iturriza, 1971; Norris and Platt, 1973; and Prahlad, 1968) have demonstrated very low thyroid activities for mature neotenes, but these studies did not investigate possible changes in thyroid activity throughout early development. Larvae of 4.5 cm SVL exhibit a low thyroid activity which is similar to radioiodide values observed for larvae treated with a thyroid inhibitor. Radioiodide and serum T₄ assay experiments indicated that there is increased thyroid activity prior to the body size when spontaneous metamorphosis occurs and a decline in thyroid activity subsequent to this size. Thus, thyroid function definitely begins before the onset of metamorphosis, but serum levels of thyroxine are substantially less than animals undergoing transformation. This suggests that this early thyroid activity may be primarily concerned with iodine storage and accumulation rather than metabolism and secretion of thyroid hormones.

A decline in thyroid activity after larvae attained 5.0 cm SVL was evident for both neotenic and transformed salamanders. The obvious exception of this trend was the case of spontaneous transformation when thyroid activity and serum thyroxine levels were greatest. These early patterns of thyroid activity were observed for both populations of *Ambystoma gracile* (Goldie Lake and Lost Lake) even though Goldie Lake animals exhibited greater (76.5% vs 12%) neotenic tendencies (despite the fact that larvae from both populations were reared under identical lighting, temperature and feeding conditions). Therefore, it was concluded that neotenic "salamanders" exhibit an early thyroid activity analogous to
anuran "prometamorphosis", but do not undergo the climactic phase. Failure to metamorphose seems to reside in the larvae's inability to promote the thyroid to a higher level of activation, since neotenic adults possess functional thyroids capable of activation by mammalian TSH or exogenous thyroxine. Exogenous thyroxine may act by promoting morphogenetic (Goos, 1969) or maturational (Norris and Gern, 1976) differentiation of the thyrotropic center of the hypothalamus. These developmental changes may then later activate the pituitary-thyroid axis.

As has been observed in anurans (Dodd and Dodd, 1976; Kaye, 1961; and Saxen et al., 1957) Ambystoma gracile thyroid activity commences some time prior to the onset of metamorphosis. All Ambystoma gracile larvae probably enter a phase similar to anuran prometamorphosis when thyroid activity is increased with a similar small increase in thyroid hormone levels. During this phase, biochemical and physiological "prometamorphic events" probably occur. Studies by Ducibella (1974a, b) have indicated that hemoglobin and serum protein metamorphic events occur and are induced by lower levels of thyroxine than those required for anatomical metamorphosis ("climax"). Therefore, like anurans, this early increased thyroid activity could also be considered as a preparatory phase for climax when slow reacting low threshold events occur. A certain proportion of Ambystoma gracile larvae "fail" to engage into the next (climactic) phase. This failure may be due to pituitary hypofunction, and and developmental studies related to cell type changes might prove fruitful. Perhaps the generally lower thyroid activity preceding the size at transformation for the Goldie Lake population might delay or inhibit full
differentiation of the thyrotropic center of the hypothalamus, which may
in turn promote pituitary hypofunction.

Experiment 4 suggests that an early peripheral sensitivity to
estrogens may also alter pituitary function. Since estrogen treatment
did not alter the thyroid's ability to respond to TSH (at a minimal dose
that promotes significant differences in thyroidal radioiodide uptake
compared to saline-injected controls), the lowered activity of the
thyroid for estrogen pretreated larvae compared to controls may be due
to a feedback mechanism to the brain (hypothalamus?) or pituitary. Such
a feedback might alter pituitary TSH (or prolactin) secretion and promote
neoteny. Similarly, Norris and Gern (1976) have found an enhancement
of the metamorphosing inhibiting action of prolactin with the use of
corticosterone. Perhaps steroids might enhance the action of prolactin,
the secretion of prolactin, or effect thyrotrope cell secretion,
differentiation, or synthesis. Obviously, further studies in this area
require critical examination.

Interestingly, two immature animals treated with TSH underwent
an accelerated metamorphosis (Table XII), and they were 2/3 metamorphosed
when radioiodide uptakes were measured. Their uptakes were substantially
lower than the other animals within their groups even though they had
been injected with the same quantities of TSH. Norris and Platt (1973)
oberved a similar lowering in thyroid activity in Ambystoma tigrinum
salamanders when they were near complete metamorphosis. This phenomenon
could be due to an induction of thyroid refractoriness by TSH which has
recently been observed in higher vertebrates (Rapoport, 1976; and
Rapoport and Adams, 1976). Such a mechanism to explain the lowering of thyroid activity immediately following metamorphosis (and seemingly during late metamorphic climax) might seem more feasible than the positive feedback switch to negative feedback mechanism proposed by Etkin (1968). Again investigations into a possible thyroid refractoriness induced by TSH during transformation requires further study. Taking this into consideration, caution should be taken when interpreting the time of maximal thyroid stimulation, since high levels of thyroid activity may in turn promote a reduction in thyroidal uptake values. Thyroidal uptake values must be compared for transforming animals when they are at exactly the same stage of metamorphosis.
CHAPTER IV

Factors affecting thyroid hormone tissue sensitivity of *Ambystoma gracile*: A developmental study of two lake populations.
CHAPTER IV

Factors affecting thyroid hormone tissue sensitivity of Ambystoma gracile: A developmental study of two lake populations.

Introduction

Snyder (1956) investigated age-related changes in thyroid hormone sensitivity by performing studies with field-captured Ambystoma gracile larvae placed in solutions of thyroxine at different temperatures. He concluded that younger larvae responded more readily to the metamorphic effects of thyroxine (T₄) than did older animals. Due to differences in the lengths of seasonal periods of growth for high-altitude animals compared to low-altitude populations, it is difficult to determine the "true age" of field animals. It was considered therefore, that a reinvestigation of thyroxine sensitivity changes with age using laboratory-reared larvae of known ages grown under controlled conditions was warranted. In this manner, the effects of age and temperature upon tissue sensitivity could be more easily delineated. It was considered that in vitro studies with tailfin tissue might delineate the stimulatory effects of exogenous T₄ upon the in vivo hypothalamo-hypophyseal-thyroid axis (Norris and Gern, 1976; Iturizza, 1971; and Prahlad, 1968) from possible changes in other peripheral tissues. Eddy and Lipner (1976) have found that there is increased release of a TSH-like factor when premetamorphic tadpoles are immersed in thyroxine solutions. Norris and Gern (1976) have found that large intraperitoneal injections of thyroxine or intrahypothalamic injections of lower concentrations cause an activation of the hypothalamo-
hypophyseal-thyroid axis. Prahlad (1968) and Iturizza (1971) also found a similar stimulatory effect upon the hypothalamo-hypophyseal-thyroid axis of *Ambystoma mexicanum* with high thyroxine concentrations. Seemingly, low concentrations of thyroxine may act in a slow manner peripherally (Norris and Platt, 1974), whereas high concentrations may rapidly stimulate the hypothalamo-hypophyseal-thyroid axis (Prahlad, 1968). In this study *in vivo* and *in vitro* studies with a facultative neotene were conducted in an attempt to differentiate between tissue sensitivity (*in vitro*; tailfin) and stimulation of the hypothalamo-hypophyseal-thyroid axis (*in vivo*; whole animal). Relative changes in the differences between *in vivo* and *in vitro* tissue responses to $T_4$ with changes in age or temperature might divulge underlying mechanisms involved in the coordination of metamorphosis in response to environmental changes. Using laboratory-reared larvae obtained from two separate populations (low-altitude and montane) and comparing the responses of each population may also point to possible genetic differences related to the physiological coordination of metamorphosis.
Materials and Methods

Animals:

Unless otherwise noted, the animals used in this study were reared at room temperature (21 ± 2 °C) from eggs obtained from two populations of *Ambystoma gracile*. Hatchlings were randomized within each population. The Lost Lake population exhibited approximately a 10% incidence of neoteny and the Goldie Lake population was approximately 75% neotenic. During the three years of observation, only two animals (both males) spontaneously metamorphosed subsequent to attaining a snout to vent length (SVL) of 7.5 cm, therefore spontaneous metamorphosis of "designated" neotenic individuals was very rare. Animals were fed and cared for as previously described (Chapter 1). Changes in tail height and gill length (second right gill rachis) were measured daily during the treatment periods. Measurements were made by placing the animal in shallow water and measuring the tail and gill rachis to the nearest 0.5 mm with a dissecting ruler or a caliper.

Experiment 1:

In order to investigate *in vivo* sensitivities and metamorphic capability to respond to different concentrations of \( T_4 \), immature larvae of approximately 5.0 cm SVL were separated into 8 groups \((n = 8)\). One group acted as a control and was not subjected to hormone or thiourea treatment. Each larva was placed into a one liter preparation dish containing dechlorinated water at room temperature (20 ± 1 °C). Within the second group, each larva was placed into a 1 liter preparation dish containing 0.25% thiourea (Dent, 1961) (Sigma) diluted with dechlorinated water. Subsequent groups were subjected to different thyroxine concentrations with or without 0.25% thiourea and diluted with dechlorinated water. One group was placed into \( 10^{-6} \) M thyroxine \( (T_4) \)
(Sigma) and a second group was placed into $10^{-6}$ M $T_4$ and 0.25% thiourea. Another group was placed into $10^{-7}$ M $T_4$ and another group was subjected to $10^{-7}$ M $T_4$ with 0.25% thiourea. The last 2 test groups were subjected to $10^{-8}$ M $T_4$ and $10^{-8}$ M thyroxine with 0.25% thiourea. All groups were changed on alternate days and fresh solution or water was replaced for the appropriate group. All experiments were performed with larvae reared at $21 \pm 2 ^\circ C$, and these experiments were performed at $20^\circ \pm 1 ^\circ C$. Animals were fed to satiation every other day, and the photoperiods were 12 L: 12 D.

Experiment 2:

To investigate possible age-related changes in sensitivities to the metamorphic action of thyroxine, animals from each population and of different lengths (and age) were subjected to the minimum concentration of thyroxine ($10^{-7}$ M that induced all larvae to transform. These experiments were performed at $20 \pm 1 ^\circ C$ and a 12 L:12 D photoperiod. Animals were individually placed in 1 liter preparation dishes with $10^{-7}$ M $T_4$ diluted with dechlorinated water. Solutions were changed on alternate days. Animals were considered completely metamorphosed when their gills were completely resorbed and individuals consistently floated at the surface of the water.

Experiment 3:

In order to investigate the effects of temperature upon tissue sensitivity, animals were reared at $11 ^\circ \pm 2 ^\circ C$ from randomized hatchlings obtained from the same two populations. With the exception of the lower temperature, hatchlings were reared as described for the room temperature animals. Six groups ($n = 6$) of larvae were placed individually into a 1-liter preparation dish with $10^{-7}$ M $T_4$ diluted with dechlorinated water. Animals of
the same lengths as Experiment 2 animals were utilized from each population, and the criterion for complete metamorphosis was the same as used in Experiment 2. Experiments were conducted at 11 °C ± 1 °C with a 12L:12D photoperiod.

Experiment 4:

Immunization:

NIH prolactin (NIH-P-S11) and NIH thyrotropin (NIH-TSH-B7) were used as antigens. Three New Zealand white rabbits were injected with prolactin (2.5 mg/ml), two rabbits were injected with TSH (2.5 mg/ml), and one animal was used as an uninjected control. Immunization procedures were carried out as previously described (McKeown and van Overbeeke, 1971).

Specificity of the antiserum for mammalian anterior pituitary hormones was determined by the Ouchterlony double diffusion precipitin method. Only the antiseras that exhibited a single-specific line for prolactin and only the antiseras that gave a single-specific line for TSH were used for the T-4 induced metamorphosis experiments. These antiseras also exhibited heterologous cross-reactivity with Ambystoma gracile hormones as deduced by pituitary immunoflorescence (see Chapter V).

Two separate experiments were carried out in order to investigate the possible role of thyrotropin-like and prolactin-like hormones with relation to metamorphosis and neoteny. All animals used in these studies were from the same egg batch obtained from a Goldie Lake neotenous female which had oviposited in the laboratory. These larvae were approximately 6.5 SVL [6.5 ± 0.10 cm (Mean ± SEM)]. The first series of animals was divided into three groups (n = 6) and all were individually placed into
1 liter preparation dishes containing $10^{-7}$M thyroxine diluted with de-
chlorinated water. The control animals were injected with 7 µl of normal
rabbit antiserum; the second group was injected with 7 µl of prolactin
antiserum; and the third group was injected with 10 µg of prolactin. All
injections were intraperitoneal, and sera injections were done on alter-
nate days, whereas hormone injections were done 6 times a week. Gill
length and tail height were measured as previously described. The second
series of larvae was divided into two groups ($n = 6$). The control group
received injections of 7 µl of normal rabbit serum and the experimental
animals were injected with 7 µl of normal rabbit serum and the experimental
animals were injected with 7 µl of anti-TSH serum. Animals were exposed to
$10^{-7}$M thyroxine at day 0, and serum was injected intraperitoneally on alternate
days. All procedures were carried out at room temperature 20 ± 1 °C with
12L:12D photoperiods.

Experiment 5:

In order to investigate possible genetic differences in tissue sensi-
tivity for different populations, *in vitro* experiments were performed on
randomized samples of *Ambystoma gracile* using Derby's (1968) method of
culturing tailfin tissue. Animals were anaesthetized with MS-222 (1:2000)
until cessation of muscular response. Small pieces, approximately
4 mm X 6 mm, cut from the dorsal fin were kept in sterile Petri dishes in
Medium 199 diluted to amphibian concentrations and containing penicillin
(500 U/ml) and streptomycin (200 µg/ml). The Petri dishes were placed in
an incubator at a temperature of 20 ° ± 1 °C. After two days, fin tissues
that underwent damage-induced involution (Davis *et al.*, 1975) or that were
contaminated were removed. Only the discs that were completely healed
were used for experiments. Discs were assigned to experimental categories at random and were transferred singly to individual sterile Petri dishes (60 X 15 mm) containing 10 ml of control or experimental medium buffered to a pH of 7.4 with sodium bicarbonate buffer. Dishes and media were changed on alternate days.

Tailfin resorption was measured quantitatively by projecting the image of the tailfin onto the wall and tracing this magnified image onto a sheet of paper. In order to assure accurate comparisons, a grid of 1 cm$^2$ was also projected and traced with each tissue magnification. The magnified area of the tissue and the 1 cm$^2$ standard were then measured with a planimeter and the data were expressed in terms of per cent of original tailfin area.

(1.) The first series of experiments was carried out with randomized samplings of *Ambystoma gracile* at different stages of postembryonic development. Tailfin discs were cut from animals at 5.0 cm, 6.0 cm and 8.0 cm SVL. After two days within control media, experimental tissues were subjected to $10^{-8}$ M, $10^{-7}$ M, and $10^{-6}$ M 1-thyroxine (Sigma), and one group remained within the control media. Each group contained 12 tailfin discs obtained from 6 animals reared at 21 °C ± 2 °C and 12L:12D photoperiods. Petri dishes were marked with the concentrations, the lake of the animal, and the length of the animal. Tailfins were cultured at 20 °C ± 1 °C for 20 days.

(2.) The second series of experiments was carried out with adult, neotenic *Ambystoma gracile*. All animals were in excess of 8.0 cm SVL and were approximately 2 years old. Tailfin discs dissected from animals from each lake population were subjected to media containing $10^{-6}$ M thyroxine and media devoid of this hormone, and experiments were carried out at 15 °C ± 1 °C, 20 °C ± 1 °C, and 25 °C ± 1 °C.
Statistics

Intrapopulational treatment data were subjected to one-factor analysis of variance, followed by the Newman-Keuls multiple comparison procedure (Sokal and Rohlf, 1969).

Interpopulational comparisons were subjected to one-way analysis of variance and followed by the Student's \( t \)-test (two-tailed).
Results

Experiment 1:

The results of experiment 1 are illustrated in Table XIII. The rate of resorption of the gills (Figure 21) and of the tailfin (Figure 22) followed similar patterns, except that the initiation of resorption of the gills occurred later, and once resorption started it exhibited a somewhat faster rate of resorption. Due to its more gradual rate, decrease in tail height seemed to be a better indication of the rate of metamorphosis. The minimal thyroxine concentration capable of inducing metamorphosis for all the larvae was $10^{-7} \text{ M} \ T_4$. Of the larvae that transformed (6/8), at $10^{-8} \text{ M} \ thyroxine$ the time required for complete metamorphosis was significantly less ($p < 0.05$) for larvae treated with thiourea compared to immature larvae treated with just $10^{-8} \text{ M} \ thyroxine$. Larvae immersed into $10^{-7} \text{ M} \ thyroxine$ with 0.25% thiourea took significantly longer ($p < 0.05$) to transform than larvae immersed only in $10^{-7} \text{ M} \ T_4$. The rate of transformation was proportional to logarithm of the concentration of thyroxine (Table XIII and Figures 21 & 22).

Experiment 2:

Within the group of animals tested and reared at room temperature $(21 ^\circ \pm 2 ^\circ \text{C})$, adult neotenous forms required more time to transform ($p < 0.01$) when placed into $10^{-7} \text{ M} \ thyroxine$ than did immature larval forms. Adult neotenics from both populations (Goldie and Lost Lake) exhibited these age-related in vivo sensitivity differences to $10^{-7} \text{ M} \ thyroxine$ (Table XIV). Goldie Lake immature larvae of 5.0 cm SVL exhibited a slower ($p < 0.01$) rate of metamorphosis to $10^{-7} \text{ M} \ thyroxine$ compared to Lost Lake immature larvae of the same length. The per cent weight loss during metamorphosis
was generally greater at the higher temperature (20 °C ± 1 °C; Table XIV) than the lower temperature (11 °C; Table XV).

Experiment 3:

Animals reared and tested at the lower temperature (11 °C; Table XV) exhibited an age-related *in vivo* sensitivity to thyroxine (10⁻⁷ M) which was the reverse to the room temperature experiments. The larger, sexually mature Lost Lake larvae required less time (*p* < 0.01) to transform than did the smaller immature larvae, but the time required was greater (*p* < 0.001) than at the higher (20 °C ± 1 °C) temperature. The rate of transformation at 11 °C for immature larvae from Goldie Lake of 5.0 cm SVL was significantly greater (*p* < 0.01) than Goldie Lake larvae of 6.0 cm SVL or adult neotenous forms. The rate of T₄-induced transformation at 11 °C was not significantly different for Goldie Lake immature larvae of 6.0 cm SVL compared to adult neotenous forms.

Experiment 4:

Injections of prolactin antiserum did not significantly increase the rate of T₄-induced metamorphosis when compared to larvae injected with normal rabbit serum (Figure 23). Injections of NIH ovine prolactin significantly (*p* < 0.01) depressed the T₄-induced rate of tailfin resorption when compared to controls (Figure 23). Injections of antiserum to bovine NIH-TSH also significantly depressed (*p* < 0.01) the rate of T₄-induced resorption (Figure 24).

Experiment 5:

Tailfin discs could be maintained in good conditions for up to 21 days in control medium. The discs maintained their size, shape and general
thickness for up to 3 weeks, and most discs exhibited slight increases in size during this culture period. Control discs can survive up to 4 weeks, before fin tissue undergoes a gradual resorption. Tailfin discs obtained from immature larvae exhibit more variability in growth and/or resorption when cultured in control media as compared to tailfin discs from neotenous forms (Figures 25 and 26).

Tailfin discs from immature larvae treated with hormone initiate resorption at Day 4, whereas hormone treated tailfin discs from adult, neotenous forms do not begin shrinkage until Day 6.

Immature larvae tailfin discs treated with different concentrations of thyroxine demonstrate a quantitative relationship between hormone concentration and percent resorption (Figure 25A). Adult tailfin tissues also exhibit a quantitative relationship between hormone concentration and percent resorption; and if the percent remaining area after 8 days is plotted against the negative log of the molarity of thyroxine concentration in the incubation media, reasonably linear relations are observed (Figure 27).

Goldie Lake adult tissues exhibited a slightly greater sensitivity to thyroxine hormones, but these differences are not significant at 20 °C. Goldie Lake adult neotenous tailfin tissues do exhibit a significantly greater sensitivity to thyroxine ($10^{-6}$ M at 15 °C ($p < 0.01$) and at 25 °C ($p < 0.001$)) than tailfin discs from similarly-aged Lost Lake neotenous animals (Figure 28).
Figure 21. Decrease in gill length of larval *Ambystoma gracile* immersed in different concentrations of l-thyroxine.

The solid circle represent animals immersed in $10^{-6}$ M thyroxine. The triangles designate larvae subjected to $10^{-7}$ M thyroxine. The squares represent larvae immersed into $10^{-8}$ M thyroxine. Each percent original gill length is based on the mean percent for eight larvae, except the $10^{-8}$ M thyroxine group which included six larvae.
Figure 22. Decrease in tail height of larval *Ambystoma gracile* immersed in different concentrations of 1-thyroxine.

The solid circle represent animals immersed into $10^{-6}$ M thyroxine. The triangles designate larvae subjected to $10^{-7}$ M thyroxine. The squares represent larvae immersed into $10^{-8}$ M thyroxine. Each percent decrease in tail height is based on the mean percent for eight larvae, except the $10^{-8}$ M thyroxine group which included six larvae.
Table XIV. Effects of different hormone concentrations and a thyroid inhibitor upon thyroxine-induced and spontaneous metamorphosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days to metamorphose (± S.E.)</th>
<th>Proportion transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Controls</td>
<td>57.0 ± 1.83</td>
<td>7/8</td>
</tr>
<tr>
<td>2. Thiourea</td>
<td></td>
<td>0/8</td>
</tr>
<tr>
<td>3. $1 \times 10^{-8}$ M thyroxine</td>
<td>30.7 ± 3.47</td>
<td>6/8</td>
</tr>
<tr>
<td>4. Thiourea and $1 \times 10^{-8}$ M thyroxine</td>
<td>22.8 ± 7.17 *</td>
<td>6/8</td>
</tr>
<tr>
<td>5. $1 \times 10^{-7}$ M thyroxine</td>
<td>10.7 ± 0.33</td>
<td>8/8</td>
</tr>
<tr>
<td>6. Thiourea and $1 \times 10^{-7}$ M thyroxine</td>
<td>15.3 ± 1.93 *</td>
<td>8/8</td>
</tr>
<tr>
<td>7. $1 \times 10^{-6}$ M thyroxine</td>
<td>8.5 ± 0.31</td>
<td>8/8</td>
</tr>
<tr>
<td>8. Thiourea and $1 \times 10^{-6}$ M thyroxine</td>
<td>10.1 ± 0.77</td>
<td>8/8</td>
</tr>
</tbody>
</table>

* Thiourea treatment was significantly different from hormone treatment without thiourea ($p < 0.05$)

Thyroxine and thiourea were diluted by 1 liter of dechlorinated water and larvae were immersed within this swimming media.
Table XV. Effects of age upon in vivo thyroid hormone sensitivity.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>(n)</th>
<th>AGE (months)</th>
<th>SVL (cm) ± S.E.</th>
<th>% WEIGHT LOSS ± S.E.</th>
<th>DAYS to TRANSFORM ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lost Lake</td>
<td>6</td>
<td>7.5</td>
<td>5.18 ± 0.09</td>
<td>17.9 ± 3.00</td>
<td>11.0 ± 0.36^a</td>
</tr>
<tr>
<td>Lost Lake</td>
<td>6</td>
<td>8.5</td>
<td>6.00 ± 0.03</td>
<td>19.1 ± 1.13</td>
<td>11.3 ± 0.21^b</td>
</tr>
<tr>
<td>Lost Lake</td>
<td>6</td>
<td>19.0</td>
<td>8.68 ± 0.09</td>
<td>23.2 ± 0.42</td>
<td>15.2 ± 0.60^c</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>6</td>
<td>6.5</td>
<td>5.08 ± 0.06</td>
<td>14.2 ± 0.44</td>
<td>13.0 ± 0.25^d</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>6</td>
<td>8.0</td>
<td>6.00 ± 0.03</td>
<td>15.0 ± 1.21</td>
<td>10.5 ± 0.22^e</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>6</td>
<td>22.0</td>
<td>8.87 ± 0.14</td>
<td>27.6 ± 1.66</td>
<td>15.7 ± 0.56^f</td>
</tr>
</tbody>
</table>

^a vs. b (N.S.); a vs. c (p < 0.01); b vs. c (p < 0.01).

d vs. e (p < 0.01); d vs. f (p < 0.01); e vs. f (p < 0.01).

only a vs. d (p < 0.01) was significant for interpopulational comparisons of animals of similar sizes and ages.

All animals were reared at room temperature (21 ± 2 °C) and experiments were performed at 20 ± 1 °C.
Table XVI. Effects of lower temperatures on \textit{in vivo} thyroid hormone sensitivity

<table>
<thead>
<tr>
<th>GROUP</th>
<th>(n)</th>
<th>AGE (months)</th>
<th>SVL (cm) ± S.E.</th>
<th>% WEIGHT LOSS ± S.E.</th>
<th>DAYS to TRANSFORM ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lost Lake</td>
<td>6</td>
<td>14</td>
<td>5.07 ± 0.080</td>
<td>12.4 ± 2.63</td>
<td>31.7 ± 2.26 \textsuperscript{a}</td>
</tr>
<tr>
<td>Lost Lake</td>
<td>6</td>
<td>18</td>
<td>5.94 ± 0.080</td>
<td>13.5 ± 0.97</td>
<td>25.2 ± 1.86 \textsuperscript{b}</td>
</tr>
<tr>
<td>Lost Lake</td>
<td>4</td>
<td>30</td>
<td>7.30 ± 0.147</td>
<td>16.4 ± 1.04</td>
<td>20.3 ± 1.58 \textsuperscript{c}</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>6</td>
<td>11</td>
<td>5.30 ± 0.020</td>
<td>13.5 ± 2.08</td>
<td>29.5 ± 1.06 \textsuperscript{d}</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>6</td>
<td>16</td>
<td>5.97 ± 0.030</td>
<td>14.7 ± 2.64</td>
<td>22.8 ± 1.22 \textsuperscript{e}</td>
</tr>
<tr>
<td>Goldie Lake</td>
<td>6</td>
<td>22</td>
<td>7.45 ± 0.089</td>
<td>16.7 ± 1.02</td>
<td>20.5 ± 1.05 \textsuperscript{f}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} vs. \textsuperscript{b} (p < 0.01); \textsuperscript{a} vs. \textsuperscript{c} (p < 0.001); \textsuperscript{b} vs. \textsuperscript{c} (p < 0.01)

\textsuperscript{d} vs. \textsuperscript{e} (p < 0.01); \textsuperscript{d} vs. \textsuperscript{f} (p < 0.01); \textsuperscript{e} vs. \textsuperscript{f} (N.S.)

All animals were reared at 11 ± 2 °C and experiments were performed at 11 ± 1 °C, and larvae were immersed in $1 \times 10^{-7}$ M thyroxine.
Figure 23. Effects of prolactin antiserum and prolactin injections upon the $T_4$-induced rate of tail resorption.

Normal rabbit serum (NRS) and prolactin antiserum (AS) were injected (7 μl) on alternate days, and prolactin was injected 6 times a week. Prolactin injections significantly depressed the rate of tailfin resorption ($p < 0.01$). Antiserum to prolactin did not significantly alter the rate of tailfin resorption compared to NRS control injections. Vertical lines represent standard errors, and bars denote the main mean values.
Figure 24. The effects of TSH antiserum injections on T$_4$-induced tail resorption.

Normal rabbit serum (NRS) and antiserum to TSH (7 µl) were injected on alternate days. Antiserum to TSH significantly ($p < 0.01$) altered the rate of T$_4$-induced metamorphosis. The vertical lines represent the standard error about the mean (the bar).
Figure 25. *In vitro* analysis of the response of *Ambystoma gracile* tailfin tissue to thyroxine.

Graphs represent regression of tailfin tissue for discs dissected from *Ambystoma gracile* reared from eggs obtained from Goldie Lake. Section (A) represents fins from animals of 5.0 cm snout to vent length (SVL); section (B) denotes tailfin discs from larvae of 6.0 cm SVL; and section (C) denotes fins from neotenous larvae in excess of 8.5 cm SVL. The percent initial area is plotted against the time exposed to the different concentrations of 1-thyroxine. The small dot represents control tissue (no hormone exposure), the solid diamond denotes fins exposed to $10^{-8}$ M 1-thyroxine, the solid circle denotes fin discs exposed to $10^{-6}$ M thyroxine. Vertical lines represent standard deviations of the designated mean value. Each group contains 12 discs obtained from 6 animals. Experiments were performed at $20 \pm 1^\circ C$. 
Figure 26. *In vitro* analysis of the response of *Ambystoma gracile* tailfin tissue to thyroxine.

Graphs represent regression of tailfin tissue from *Ambystoma gracile* reared from eggs obtained from Lost Lake. Discs are represented as in Figure 25.
Figure 27. The percent of original area of tailfin discs remaining after 8 days of exposure to different concentrations of thyroxine.

The solid dot represents the mean value for 12 discs obtained from 6 neotenous Goldie Lake larvae. The solid square denotes the mean value for 12 discs obtained from 6 neotenous Lost Lake larvae. Experiments were performed at 20 ± 1°C. The vertical lines represent the standard deviation of the mean for each group.
Figure 28. The percent of original area of tailfin discs remaining after 8 days of exposure to $10^{-6}$ M thyroxine at different temperatures.

The solid square represents the mean value for 12 discs obtained from 6 neotenous Goldie Lake larvae. The solid dot denotes the mean value for 12 discs obtained from 6 neotenous Lost Lake larvae. The vertical lines represent the standard deviation of the mean for each group.
% INITIAL AREA AFTER 8 DAYS

TEMPERATURE

15°C 20°C 25°C
Discussion

Previous studies with *Ambystoma gracile* (Snyder, 1956) indicated that there was a decrease in sensitivity to thyroid hormones with age; and Snyder (1956) attributed neoteny to the colder conditions within the higher altitudes promoting progressive age-related decreases in tissue sensitivity. The present study with Lost Lake larvae indicates continued growth at lower temperatures (11 ± 2 °C) promotes an increased *in vivo* sensitivity to exogenous thyroxine with aging and increased body size; but a decreased *in vivo* sensitivity to exogenous thyroxine with age and body size was observed for those animals maintained and treated at the higher temperature. It might be argued that these differences could be accounted for by alterations in thyroxine turnover or metabolism, but *in vitro* experiments with tailfin tissues from donors of various sizes do not exhibit similar trends. In fact, *in vitro* studies indicated that there was no difference in tissue response to thyroxine, despite the age-differences for donating individuals. Tailfin tissue from large, neotenous and older (2 1/2 years) animals responded in a similar fashion as immature, younger larval (6 months) tailfin discs (Figs. 25 and 26). Therefore, the metabolic capabilities within the target tissues, such as deiodinating activity (Robinson and Galton, 1976), are not responsible for these conflicting *in vivo* developmental trends at the two temperatures. Robinson and Galton, (1976) also found that the specific activity of deiodination "remains constant throughout development .... " for the liver of amphibians suggesting that peripheral metabolism differences may not be responsible for these divergent trends at the two temperatures. There is an increase in general metabolism at the higher temperature compared to the lower temperature, and this is reflected in the greater weight loss at
higher temperatures for transforming animals; but this does not explain
the differences in response to exogenous thyroxine with development at
each temperature. The interpretation of these discrepant developmental
trends is more easily understood when one separates *in vivo* from *in vitro*
responses.

Prahlad (1968) and Iturizza (1971) demonstrated an activation of
thyroid activity with injections of large amounts of thyroxine (T$_4$) or
triiodothyronine (T$_3$). Norris and Gern (1976) have also shown that small
amounts of T$_4$ injected into the hypothalamus activate the hypothalamo-
hypophyseal axis in the neotenic *Ambystoma tigrinum,* and larger intraperitoneal
doses of T$_4$ are required for hypothalamo-hypophyseal-thyroid activation in
a similar fashion. Exogenous T$_4$ initiates both peripheral tissue changes
as well as hypothalamo-hypophyseal activation of the thyroid. That this
activation occurs when animals are exposed to exogenous thyroxine, was
demonstrated indirectly by the effects of thyrotropin antiserum upon T$_4$-
induced metamorphosis (Figure 24). Immature larvae exposed to 10$^{-7}$ M T$_4$
and injected with thyrotropin antiserum exhibited a depressed rate of T$_4$-
induced transformation when compared to immature larvae exposed only to
10$^{-7}$ M T$_4$ and injected with normal rabbit serum (Figure 24). Also thiourea
treatment of larvae immersed in 10$^{-7}$ M T$_4$ significantly depressed the rate
of T$_4$-induced metamorphosis (Table XIV). That the hypothalamo-hypophyseal-
thyroid axis is activated in *Ambystoma gracile* is further supported by the
fact that 10$^{-7}$ M T$_4$ increases thyroidal uptake of radioiodide compared to
control larvae, larvae immersed in 0.25% thiourea, and larvae immersed in
10$^{-8}$ M T$_4$ (Chapter III; Table XI).
Smaller doses of thyroxine may act primarily at the peripheral tissue level (Norris and Platt, 1974) and not induce complete activation of the hypothalamo-hypophyseal-thyroid axis. This also seems to be true for *Ambystoma gracile*, because lower T₄ concentrations (10⁻⁸ M) induced transformation in only 6 of 8 individuals (Table XIV) at 21 °C and 4 of 6 larvae transformed when immersed in 10⁻⁸ M T₄ for up to 180 days at 11 °C. This suggests the minimal concentration for the immersion solution which causes anatomical transformation is between 10⁻⁸ M and 10⁻⁷ M T₄. Though not all the T₄ enters the animal during *in vivo* immersion, it is assumed that the same proportion (or relative concentration) enters the animal (per unit surface area), and changes in permeability with development is not a factor.

*In vitro* sensitivities are not altered with age or body size of the donating Lost Lake (Figure 26) or Goldie Lake larva (Figure 25). These *in vitro* studies are consistent with the studies of Hsü et al., (1971). Hsü et al., (1971) using thyroidectomized tadpoles (therefore separating *in vivo* activation of the thyroid from peripheral tissue response) and low levels of exogenous T₄ also observed that there was "no discrepancy of tissue response to thyroxine ... in spite of age differences." It is proposed that the differences for trends of *in vivo* sensitivities to exogenous T₄ with development may be accounted for by changes in sensitivity of the portion of the hypothalamus responsible for pituitary-thyroid activation. This interpretation is consistent with the findings of Norris and Gern (1976) with *Ambystoma tigrinum*. Norris and Gern (1976) found that small, immature larvae were less sensitive to intrahypothalamic injections of
than large, neotenic larvae. They attributed this difference to the maturational effects of $T_4$ upon the hypothalamus of immature larvae requiring more time for its action than hypothalamo-hypophyseal-thyroid activation within mature neotenes.

The present study reports the effects of temperature upon the maturation of the "thyrotropic center" of the hypothalamus and possible genetic differences with respect to the effects of hypothermia. Comparisons of studies of in vivo $T_4$ reactivity of Lost Lake animals reared at the colder temperatures with Lost Lake larvae reared at the warmer temperatures indicate that older neotenous forms larger than 7.3 cm SVL are more sensitive to exogenous $T_4$ at 11 °C than are immature forms (5.0 cm SVL) reared at this temperature (Table XV), whereas the reverse trend is observed at 21 °C (Table XVI). Within anurans, colder temperatures have been observed to inhibit or delay the development of the hypothalamic "thyrotropic area" (Neuenschwander and Weber, 1970) in relation to general body growth (Voitkevitch, 1963; Decker and Kollros, 1969). Metamorphosis at cooler temperatures will occur at a larger body size (Wilbur and Collins, 1973) than at higher temperatures, due to a "delay" in the maturation of the hypothalamus. Since the differentiation of the hypothalamus and median eminence are also under the control of thyroid hormones (Goos, 1968) lower temperatures may interfere with thyroid hormone metabolism or target sensitivity (Kollros, 1961). Neuenschwander and Weber (1970) demonstrated that delay or "blockage of spontaneous metamorphosis at low temperatures must result from the inhibition of the hypothalamic center." and that the "responding capacity" of other larval tissues were less affected by lower temperatures than was the
hypothalamus. The Lost Lake larval neotenes continuously maintained at 11 °C are more prone to T₄-induced hypothalamo-hypophyseal-thyroid activation than the smaller immature larvae, and this is due to the delay in hypothalamic maturation at the lower temperature (Tables XV and XVI) compared to the higher temperature (21 °C).

Generally, the high-altitude (Goldie Lake) larvae were more resistant to low-temperature delay of hypothalamic development compared to the low-altitude (Lost Lake) population (Tables XV and XVI). Goldie Lake larvae attain their greatest in vivo sensitivity at 6.0 to 7.0 cm SVL irrespective of the temperature (11 °C or 21 °C) they are reared in and exposed to thyroxine.

Previous investigators (Gorbman, 1964; Wilbur and Collins, 1973) have suggested that neoteny may be due to a progressive loss of sensitivity to T₄ within certain populations. Thyroxine tissue (tailfin) sensitivity differences between populations were observed during these studies (Figure 28), but these sensitivity differences were contrary to what would be expected for predominantly neotenic populations (Goldie Lake). Tailfin discs from individuals reared from randomized Goldie Lake hatchlings exhibited greater sensitivity to T₄ at 15 °C and 25 °C compared to tailfin tissue discs from similarly raised neotenous Lost Lake animals (Figure 28). Neotenous high-altitude larvae show an increased rapidity of response to T₄ at each of the higher temperatures, whereas tailfin discs from the low-altitude larvae exhibit similar rates of resorption at 25 °C and 20 °C. It is possible that the metabolic enzymes (Davis et al., 1975) involved with T₄-induced resorption reach a temperature optimum at 20 °C for the low-altitude larvae,
whereas this optimum is higher for larvae from high-altitude populations. Since the high-altitude population rarely experiences these higher temperatures (Eagleson, 1976) this population has maintained a greater peripheral tissue response at certain temperatures. In this fashion, high-altitude larvae which do metamorphose will undergo a more rapid, coordinated transformation within an environment which often exhibits rapid changes in thermal profiles and shorter seasons.

The present study suggests that $T_4$-induced hypothalamic activation of the pituitary-thyroid function within *Ambystoma gracile* from montane populations occurs at approximately the same body size irrespective of the larvae's thermal history, but low-altitude animals exhibit a delay in hypothalamic development with respect to body size when they are subjected to lower temperatures. Previous studies (Eagleson, 1976) have shown that montane larvae transform at larger, more restricted body sizes; whereas the onset of metamorphosis can occur at much smaller sizes within low-altitude populations, and transformation occurs over a wide range of sizes. These factors indicate that a greater proportion of low-altitude larvae exhibit a phenomenal (temperature-dependent) response towards hypothalamic maturation and activation of the onset of metamorphosis, but montane populations have body size as the determining factor for the onset of metamorphosis. Once the hypothalamic activation center for pituitary-thyroid function is "activated" within montane larvae, peripheral tissues due to their greater sensitivity assure rapid transformation. The hostile terrestrial montane environment seems to have selected towards the neotenic condition (Wilbur and Collins, 1973; Sprules, 1974), and transformation at
larger body sizes. Late development of the hypothalamus (with respect to body size) irrespective of thermal history promotes this larger body size at transformation and may promote neoteny due to an inability to attain high enough $T_4$ levels to "activate" the hypothalamo-pituitary-thyroid axis. Prolactin may also be a factor in the attainment of neoteny (Figure 23) by either antagonizing the peripheral tissue response to $T_4$ (Derby, 1975) or elevating the $T_4$ threshold for hypothalamic activation (Platt, 1976).
CHAPTER V

Localization of the pituitary lactotropes and thyrotropes within
*Ambystoma gracile* (Baird) by histochemical and immunochemical methods;
A developmental study of two populations.
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Localization of the pituitary lactotropes and thyrotropes within *Ambystoma gracile* (Baird) by histochemical and immunochernical methods: A developmental study of two populations.

Introduction

Investigations dealing with amphibian metamorphosis have implicated both thyrotropin (Allen, 1938; Eddy and Lipner, 1976) and prolactin (Brown and Brown, 1972; Platt, 1976) as pituitary hormones that mediate the onset of metamorphosis. Very few studies have investigated the appearance and development of these cell types (Kerr, 1966; van Oordt, 1966) in larval and transformed amphibians, and these studies have been restricted solely to histochemical studies. Recently, immunohistochemical techniques have been applied to identify the prolactin-producing cells (Vellano *et al.*, 1973; Thompson and Trimble, 1975; Hansen and Hansen, 1976) and thyrotropin-producing cells (Hauser-Gunsbourg *et al.*, 1973) of urodele amphibians. Since many studies have implicated pituitary thyrotrope hypofunction as a factor involved with neoteny (Blount, 1950; Prahlad, 1968; Dent, 1968) and other studies have suggested that pituitary prolactin hyperfunction might promote neoteny (Bern *et al.*, 1967; Platt, 1976), the present study was undertaken in order to follow developmental changes in pituitary function of the facultative neotene, *Ambystoma gracile*. Immunohistochemical identification along with histochemical corroboration was utilized to follow pituitary distributions of thyrotropes and lactotropes throughout postembryonic development of larval, transformed and neotenous, *Ambystoma gracile* from two populations which exhibit differences in neotenic tendencies.
Materials and Methods

Animals:

The animals used in this study were reared during 1974 and 1975 from eggs obtained from two populations of *Ambystoma gracile*. Hatchlings from these lakes were randomized within each population. The Lost Lake population exhibited approximately a 10% incidence of neoteny, whereas the Goldie Lake animals were approximately 75% neotenic when larvae were reared at room temperature \((21 \pm 2 ^\circ C)\). All Goldie Lake larvae which transformed, metamorphosed subsequent to attaining a size of 6.0 cm snout to vent length (SVL), and nearly half of the metamorphosing Lost Lake larvae transformed prior to this size (Eagleson, 1976). On alternate days, larvae were fed beef heart, liver or earthworms until satiated. At different stages of postembryonic growth (5.0 cm SVL, 6.0 cm SVL, animals in the process of metamorphosis, animals 4 months subsequent to transformation, and neotenous adults) 4 animals from each lake population were killed by decapitation; brains and thyroids were dissected out, fixed in Bouin's mixture and later embedded in paraplast.

Hormone Preparations:

Hormones were generously supplied by the Endocrinology Study Section, the National Institutes of Health, Bethesda, Maryland. The hormones used as antigens were NIH-TSH-B7 (3.58 USP units/mg) bovine thyrotropin and NIH-P-S11 (26.4 IU/mg) ovine prolactin. Antigens were dissolved and injected as previously described (McKeown and van Overbeeke, 1971).

Immunoochemical Analysis:

Blood collected from injected rabbits was allowed to clot, refrigerated overnight, and the serum was removed by a Pasteur pipette. Merthiolate
(1:10,000) was added as a preservative and antiserum was stored frozen (-20 °C). Antiserum specificity was tested by Ouchterlony gel diffusion, using TSH, LH and FSH to test glycoprotein hormone specificity of antisera to TSH. Prolactin, growth hormone and TSH was utilized to test the prolactin antiserum specificity.

Immunohistochemical Studies:

Five-micrometer midsagittal sections of the brain containing the pituitary gland for each stage of Ambystoma gracile development were used. The sections were dewaxed in toluene, hydrated gradually in decreasing concentrations of ethanol, and washed in phosphate-buffered saline (PBS) buffered to pH 7.4. For fluorescence staining, the indirect method was employed. Prolactin antiserum was absorbed with growth hormone (NIH-GH-S10) for 24 hrs. at 4 °C. TSH antiserum was similarly absorbed with LH (luteotropin, Sigma). After application of the appropriate antisera, sections were incubated in a moisture chamber for 30 minutes at 37 °C. Sections were then rinsed in three changes of PBS (pH 7.4) for 1½ hrs. After rinsing in PBS, sections were incubated with FITC (flourescein isothiocyanate)-conjugated goat-anti-rabbit γ-globulin (Nutr. Biochem.; Lot No. 2622) for 20 minutes in a moisture chamber (room temperature). Sections were rinsed in three changes of PBS for 1 hr. These sections were then mounted in 10% glycerol diluted with PBS and viewed and photographed with a fluorescent microscope (Carl Zeiss, Universal). Control procedures included following the same procedure but using Normal Rabbit Serum (NRS-uninjected control rabbits) instead of the specific antiserum to a hormone.
Histochemical Studies:

Thyroid glands were subjected to the usual procedures of dehydration, embedding in Paraplast, and serial sections of 5-7 μ were cut. Thyroid sections were stained by eosin-hematoxylin.

After photographing the fluorescence, cover slips were floated off the sections, and sections were heated (40 to 44 °C) in 10% copper sulfate to precipitate or disengage attached antibodies. Sections were then stained according to one of the following methods:

(1) El Etreby and Tushous's (1973) modification of Brooke's trichrome method (1968) shortening times and utilizing azocarmine instead of Carmoisine -L

(2) Herlant's (1960) trichrome with shortened times as suggested by van Oordt (1974).

(3) Alcian blue-PAS-Orange G method for further differentiation of mucoid granules (Herlant as described by van Oordt, 1974).

Only sections in which the pituitary remained connected to the hypothalamus by the infundibular stalk were used for this study. Microscopic images of the pituitary sections were projected on to drawing paper. With the use of a planimeter, the total area of the pituitary and area occupied by thyrotropes, lactotropes, and gonadotropes were also determined. The area occupied by each cell type was then expressed as a percentage. An analysis of variance of the percentage of each cell type indicated that there were no significant differences for sections from different portions of the
same pituitary (within the infundibular stalk region). Immunofluorescent prints were also measured with a planimeter and were utilized to corroborate histochemical determinations for the percentage area of lactotropes and thyrotropes. G.S.I.'s (gonadosomatic indices) were determined as previously described (Eagleson, 1976).

Results

Immunochemical Analysis:

Individual rabbits receiving the identical hormone injections exhibited different specificities; and therefore, only antisera that reacted with a single precipitin line (Figure 29) were utilized for immunofluorescent studies. By the Ouchterlony gel diffusion tests (Figure 29), the antisera to prolactin reacted strictly with prolactin; and the antisera to TSH exhibited a single precipitation line for TSH.

Immunohistochemical Studies:

The distribution of the antiprolactin positive cells and anti-TSH positive cells for a large (9.0 cm SVL) neotenic male from Lost Lake is illustrated in Figure 30. The distribution of antiprolactin positive and anti-TSH positive cells for larval (6.0 cm SVL) Ambystoma gracile from Goldie Lake is illustrated in Figure 31.

Small larvae (5.0 cm SVL or less) from Goldie Lake (approximately 75% neotenic) exhibited a few clumps of anti-TSH reactive cells situated in the peripheral ventral and caudal regions of the pars distalis. Small Lost Lake larvae of similar length also contained many anti-TSH reactive cells
throughout the central regions of the pars distalis in 3 of 4 cases. A similar situation was observed for animals of 6.0 cm SVL (Figure 31); except that 1 of 4 Goldie Lake larva was observed to have a large number of anti-TSH reactive cells throughout the pituitary, whereas 3 of 4 of these larvae contained just sparse clumps of anti-TSH reactive cells (Figure 31). All 4 Lost Lake larvae of 6.0 cm SVL exhibited numerous anti-TSH reactive cells.

Animals undergoing metamorphosis exhibited very few anti-TSH reactive cells (usually a few cells located adjacent to the median eminence), unless the animal was dissected very early during the process of metamorphosis. One larva, dissected prior to any morphological changes except for early eye changes, contained numerous anti-TSH reactive cells throughout the pars distalis. Transformed animals (4 months post-transformation) contained very few TSH-reactive cells, and these cells were extremely small in size compared to larval anti-TSH reactive cells.

Prolactin reactive cells were observed to be localized throughout the pars distalis (Figure 31), but become situated more caudally after animals increase in size (Figure 30) or subsequent to transformation.

Histochemical Studies:

Smaller animals (less than 5.0 cm SVL) exhibited fewer thyroid follicles, and follicles were smaller in size compared to animals 6.0 cm SVL. Thyroid follicles progressively increased in number and size between the sizes of 4.0 to 6.0 cm SVL.
Figure 29.

Immunological characterization of hormone antisera.

(A.) Ouchterlony plate demonstrating the reaction of rabbit anti-ovine prolactin antiserum to ovine prolactin (20 ul of 0.5 mg/ml.), ovine growth hormone (20 ul of 0.5 mg/ml. of NIH-GH-S10) and bovine TSH (20 ul of 0.5 mg/ml.)

(B.) Ouchterlony plate demonstrating the reaction of rabbit anti- bovine TSH antiserum to ovine FSH (NIH-FSH-S5), ovine LH (Sigma), and bovine TSH (NIH-TSH-B7). All hormone preparations were 20 ul of 0.5 mg/ml of each hormone.
Immunohistochemical localization of TSH and prolactin in the pituitary gland of an adult neotenous *Ambystoma gracile*.

A.) represents NRS-controls

B.) represents sections treated with antiserum to ovine prolactin.

C.) represents sections treated with antiserum to bovine TSH.

Note (arrow) the nonstaining (B.) and Staining (C.) areas of adjacent sections. All three pictures are of identical magnification X125.
Figure 31.

Immunohistochemical localization of the TSH- and prolactin-reactive cells in the pituitary gland of larval, Ambystoma gracile.

A.) and B.) represent the TSH-reactive cells of two different larval (6.0 cm SVL), Ambystoma gracile reared from eggs obtained from Goldie Lake.

C.) and D.) represent the prolactin-reactive cells of two different larval (6.0 cm SVL), Ambystoma gracile reared from eggs obtained from Goldie Lake.

Note the differences in the number of TSH-reactive cells within B.) compared to A.). The fainter prolactin antiserum reactivity of the periphery cells of pituitary D.) may denote a greater activity for these cells compared to the brighter more central cells of the same pituitary. Sections A.) and C.) are from the same animal and are X 180. Sections B.) and D.) are from the same animal and are X 125.
Metamorphosing larvae exhibited a dramatic increase in thyroid activity as judged by morphological indices (Fig. 32), and thyroid epithelia become progressively flattened (squamous type) after salamanders transform (Fig. 32). Neotenous *Ambystoma gracile* thyroids appear inactive as judged by distended colloid and flattened epithelia (Fig. 32).

Further identification of the thyrotropes and lactotropes was substantiated by El Etreby's (1973) modification of Brooke's trichrome (1968) stain. This staining method was utilized in order to differentiate the acidophils and orangophils, but no orangophils could be detected. The carminophils were directly correlated with the cells which reacted with antisera to prolactin (Fig. 34). Subsequent studies with antisera to growth hormone yielded negative results (personal observation). Another acidophilic cell type (Table XVI) was detected. This cell type did not react to antisera to prolactin and was of a larger size than the other carminophilic cells with less affinity for azocarmine.

Three types of basophils (Table XVI) were detected within *Ambystoma gracile* pituitaries. The antisera to TSH reacted with a small to medium-sized cuboidal basophil (Fig. 34) which stained purple with El Etreby's trichrome technique (1973). This basophil was the first to appear during development of *Ambystoma gracile* (Table XVII), and this basophil type contained numerous, large, azocarminophilic inclusions. These inclusions became less prominent in older, larger animals. Transformed animals (4 months post-transformation) contained very few of this basophil type. One animal undergoing the early phases of transformation was observed to
Changes in thyroid morphology with development of *Ambystoma gracile*.

Changes in thyroid activity with development. A.) is from an animal of SVL = 6.0 cm. Note the large colloid-filled follicles. Each follicle is surrounded by epithelial cells of varying heights. B.) is a thyroid from a larva during early metamorphosis (protrusion of the eyes and the beginning of tailfin resorption). Note the intense activity represented by the greater epithelial size and small amount of colloid. C.) denotes the thyroid of a neotenous (SVL = 8.0 cm) larva. Note the squamous epithelia and large amount of colloid. D.) represents the thyroid activity of a transformed, *Ambystoma gracile* (four months after metamorphosis). Note the squamous epithelial cells and the immense amount of colloid. All sections were X and stained with eosin-hematoxylin. X 900.
Immunohistochemical localization of TSH- and prolactin-reactive cells in the pituitary of *Ambystoma gracile*.

Mid-sagittal sections of the pituitary of *Ambystoma gracile* (SVL = 7.0). A.) is a mid-ventral region of the pituitary and shows the prolactin-reactive cells of this region. B.) is more caudal to A.) and is from the same section. C.) denotes the TSH-reactive cells located along the caudal periphery of the pituitary of this animal. X900.
Figure 34.

Histochemical correlation of the immunoreactive cells in the pituitary of *Ambystoma gracile*.

Mid-sagittal sections (identical sections as Figure 33) of the pituitary of *Ambystoma gracile* stained by the technique of El Etreby (1973). A.) correlates with Figure 33 A. Note the azocarminophilic prolactin cells interdispersed with blue-staining gonadotropes surrounding a central vessel. B.) correlates with Figure 33 B. Note the prolactin-reactive cells interdispersed with the purple-staining TSH cells. C.) correlates with Figure 33 C. Note the deep purple-staining thyrotropes. These cells along the caudal periphery seem to have an excessive amount of cytoplasm denoting low secretory activity. X900.
contain numerous chromophobes in the ventral regions of the pituitary. These chromophobes were reminiscent of throidectomy cells (Dent, 1961). Basophils reappear during late metamorphosis (50% tailfin resorption). Large, cuboidal basophils begin to appear when animals are 7.0 to 7.5 cm SVL (neotenic). These basophils stain medium blue (El Etreby's trichrome) and first appear in a ventral position to the pars intermedia and are arranged near the median eminence. Numerous mitotic figures are observed at this stage in this area. These basophils increase in number until within large neotenic females, they may comprise nearly half the area of the pars distalis. A summary of these five different cell types is illustrated in Table XVII.

Discussion

The cells that reacted to anti- TSH were identifiable as the basophils Type I of van Oordt (1974). These cells first appear early in larval life (Table I) and increase in abundance as Ambystoma gracile larvae proceed towards their mean size for metamorphosis. These cells are first observed along the caudal periphery of the pars distalis and adjacent to the median eminence; and as they increase in number they can be found situated within the central regions of the adenohypophysis. Development of this cell type seems to be delayed with respect to body size for Goldie Lake larvae compared to Lost Lake larvae (Table XVIII) and this delay in pituitary thyrotrope formation may substantiate the theory that thyrotrope hypofunction promotes the neotenic condition (Blount, 1950; Prahlad, 1968; Dent, 1968). Insufficient development of increased numbers of thyrotropes at the proper
Table XVII

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Description</th>
<th>El Etreby stain</th>
<th>Alcian Blue Inclusions</th>
<th>First Appear*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophil I</td>
<td>Cuboidal; orient towards vessels</td>
<td>Purple</td>
<td>+++</td>
<td>4.0 cm SVL</td>
</tr>
<tr>
<td>Basophil II</td>
<td>Small; form follicles with blue staining or extensive cytoplasm</td>
<td>Pale Blue</td>
<td>+</td>
<td>5.0 to 5.5 cm SVL</td>
</tr>
<tr>
<td>Basophil III</td>
<td>Large cuboidal cells; orient towards blood vessels</td>
<td>Medium Blue</td>
<td>++</td>
<td>7.0 cm SVL</td>
</tr>
<tr>
<td>Acidophil I</td>
<td>Columnar cells that orient towards blood vessels</td>
<td>Red-Orange</td>
<td>-</td>
<td>3.5 cm SVL</td>
</tr>
<tr>
<td>Acidophil II</td>
<td>Large columnar cells located adjacent to the pars intermedia</td>
<td>Rose-Pink</td>
<td>-</td>
<td>4.5 cm SVL</td>
</tr>
</tbody>
</table>

* Size of larval *Ambystoma gracile* when this cell type first appears within their pituitary. Inclusions denotes carminophilic inclusions after staining according to El Etreby (1973).
Table XVIII

Developmental changes in pituitary cell types.

<table>
<thead>
<tr>
<th>Lake</th>
<th>(n)</th>
<th>% of pituitary consisting of;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>thyrrotropes</td>
<td>lactotropes</td>
<td>gonadotropes</td>
<td></td>
</tr>
<tr>
<td>Goldie Lake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 cm</td>
<td>4</td>
<td>5.57 ± 1.31</td>
<td>39.4 ± 1.37</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6.0 cm</td>
<td>4</td>
<td>15.51 ± 7.04</td>
<td>38.1 ± 1.72</td>
<td>10.1 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>*adults (neotenes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G.S.I. (X̄) = 0.94</td>
<td>3</td>
<td>11.82 ± 3.98</td>
<td>40.3 ± 1.80</td>
<td>19.1 ± 7.50</td>
<td></td>
</tr>
<tr>
<td>G.S.I. (X̄) = 2.52</td>
<td>3</td>
<td>8.63 ± 3.66</td>
<td>38.8 ± 3.14</td>
<td>43.3 ± 6.34</td>
<td></td>
</tr>
<tr>
<td>Lost Lake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 cm</td>
<td>4</td>
<td>19.31 ± 4.06</td>
<td>41.4 ± 1.84</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6.0 cm</td>
<td>4</td>
<td>26.82 ± 3.70</td>
<td>37.5 ± 1.59</td>
<td>4.6 ± 3.15</td>
<td></td>
</tr>
<tr>
<td>*adults (neotenes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G.S.I. (X̄) = 0.88</td>
<td>3</td>
<td>9.11 ± 3.44</td>
<td>44.6 ± 3.11</td>
<td>14.3 ± 5.08</td>
<td></td>
</tr>
<tr>
<td>G.S.I. (X̄) = 2.61</td>
<td>3</td>
<td>8.10 ± 1.10</td>
<td>39.0 ± 1.21</td>
<td>39.3 ± 6.60</td>
<td></td>
</tr>
</tbody>
</table>

*All adult neotenes were females and G.S.I. (X̄) represents the mean ovarian G.S.I.

Larvae with G.S.I. values in excess of 2.0 have mature, pigmented eggs whereas G.S.I. values of less than 1.0 indicate previtellogenic ovaries.
body size may delay or inhibit metamorphosis. Maintenance of the neotenic condition may involve other mechanisms such as blockage of releasing factors from the hypothalamus, because numerous thyrotropes are observed along the caudal periphery of neotenic individuals, yet these individuals exhibit inactive thyroids (Fig. 34). This pituitary cell type is drastically reduced in number within animals undergoing metamorphosis and salamanders dissected four months subsequent to transformation. The low number of thyrotropes within transformed individuals is consistent with the observation of a very inactive thyroid for such animals (Fig. 32). This low number of sparsely clumped clusters of these "thyrotrope" cells is in agreement with both the location and sparseness of the presumed thyrotrope of the Red-spotted newt after transformation (Dunn and Dent, 1976). This morphological similarity is observed despite the differences in staining characteristics for the Red-spotted newt presumptive thyrotropes and the present study.

The prolactin cells appear as heavily granulated, pleomorphic cells inserted among other cells within the pituitary. These cells are strongly carminophilic and erythrosinophilic and appear as columnar cells often orienting their cytoplasm towards blood vessels. That prolactin exists as a single hormone entity within *Ambystoma* has been demonstrated by Nicoll and Licht (1971). Nicoll and Licht (1971) could not detect a growth hormone-like factor within *Ambystoma* pituitaries which might explain the difficulty in detecting orangeophils within *Ambystoma gracile* pituitaries. Anti-prolactin positive cells are located throughout the pars distalis of larval *Ambystoma gracile*, but become situated mainly within the caudal
pars distalis of adult animals (Fig. 30 B). Their early appearance and extreme abundance in larval forms is consistent with the rapid growth phases of this animal (Eagleson, 1976) suggesting a growth-promoting function. Prolactin's possible function within adult animals requires further study.

A second acidophil was observed within this study (Acidophil II; Table XVII). This acidophil was presumed to be involved in the synthesis and secretion of adrenocorticotrophic hormone (ACTH). This cell type exhibited its greatest activity during spontaneous transformation.

Due to the build-up of the medium blue staining basophils (Table XVIII), it was concluded that these basophils represented goudotropes (LH-like?). Their late appearance with reference to body size (Table XVII) and sexual maturation corroborates this supposition.
While discussing the ecological and physiological factors influencing the body size at metamorphosis, Wilbur and Collins (1973) formulated a predictive synthetic theory for metamorphosis. They suggested that the magnitude of the body size range at metamorphosis and the degree to which the optimum size at transformation is accomplished is dependent upon the larval habitat. Thus, larval amphibians from a stable, permanent, aquatic habitat will remain until they attain the upper limit of body size for their species, and such a species will exhibit a narrower range in size at metamorphosis. One of the basic assumptions of their model is that each species of amphibian has a species-specific size range (upper and lower limits) in which metamorphosis can occur. If near such a stable, permanent, aquatic situation; the terrestrial habitat is hostile then selection will delay and ultimately eliminate metamorphosis (neotenic condition).

The present studies substantiate the main points of this hypothesis. Larvae, from the montane lake (Goldie Lake) which exhibits harsher terrestrial conditions than the low-altitude habitat (Lost Lake), transformed at larger body sizes and within a narrower range of body sizes when compared to larvae from a low-altitude habitat (Table IV). Also, neoteny was much more prevalent for larvae reared from the montane population compared to larvae reared from the low-altitude population. Within different Ambystoma gracile populations, there seems to be a continuum between populations in which a vast majority of individuals undergo metamorphosis (low-altitude populations) to populations in which almost all individuals are permanently aquatic in morphology (montane populations). The extent of either life history
pattern seems to be dependent upon the steepness of the environmental gradient (selection gradient). The selection forces related to altitude tend to establish a cline in gene frequency concerned with neoteny and other physiological parameters affecting metamorphosis. The purpose of this discussion is to attempt to describe these physiologically-related adaptive strategies and suggest how they might be advantageous to survival for this species within these different habitats.

Before proceeding to these "physiologically-related" adaptations, one must first present a generalized scheme for metamorphosis of *Ambystoma gracile* (Figure 35) and then note possible physiological differences between montane and low-altitude populations.

For *Ambystoma gracile* larvae destined to transform, the thyroids increase in size during the early stages of larval life, due both to proliferation of follicles and increase in follicular volume. The thyroid follicular epithelium becomes progressively more columnar as larvae proceed through metamorphosis (Figure 32). The pituitaries of larvae destined to transform change concomitantly with these thyroid transitions. Larvae first exhibit immunoreactive thyrotropes when they attain 4.0 cm SVL (Table XVII). This cell type becomes more numerous as larvae proceed towards the mean size for the onset of metamorphosis (Table XVIII). The complete extrusion of thyrotrope cell contents is observed for larvae during the early stages of transformation. Pituitaries of neotenic larvae contain numerous thyrotropes; but this basophil is less prominent, because of the increased number of gonadotropes (Table XVIII). Thyrotropes decrease in number and prominence within transformed, terrestrial salamanders. This decrease in the proportion
The Physiology of Metamorphosis of *Ambystoma gracile*.

Environmental cues (such as temperature), are received by sense organs and integrated within the hypothalamus. The thyrotropic center within the hypothalamus (preoptic nucleus, Goos, 1969) modulates pituitary thyrotrope development and activity. The development and maturation of the thyrotropic center of the hypothalamus is under control of circulating thyroid hormones. Once neural and vascular links between the thyrotropic center and the pituitary are fully developed releasing factors from this center act directly upon the pituitary. These neural and vascular connections also develop under the influence of thyroid hormones (Goos, 1969). Originally releasing factors from the hypothalamus enter the peripheral circulation (route #1) maintaining the low activity of the hypothalamo-pituitary-thyroid axis. Increased peripheral tissue avidity (tissue receptiveness to thyroid hormones) promotes a decrease in circulating thyroxine levels. The "thyrotropic center" overadjusts to this change in plasma thyroxine levels by secreting increased amounts of releasing factors which promote increased TSH levels (due to an increased number of thyrotropes). The result is an increased thyroid activity (first surge) which may promote the neural-vascular linkages between the hypothalamic thyrotropic center and the pituitary. Once this linkage is mature, releasing factors act directly upon the pituitary (route #2) promoting the second surge of thyroid activity (climax). Thyroid activity is depressed by TSH induced refractoriness. See text for more detailed discussion of this model.
ENVIRONMENTAL CUES

EXTERIORECEPTIVE AREAS OF THE BRAIN

HYPOTHALAMUS

PITUITARY

T_{4}, T_{3}  TSH

THYROID

PERIPHERAL TISSUES (AVIDITY)
of thyrotropes (Table XVIII) within the pituitary is consistent with decreased thyroid activity subsequent to transformation (Figure 19) and subsequent to attaining the upper limit of body size of metamorphosis (neotenic condition) within nontransforming larvae (Figure 19).

In vivo studies suggested that the greatest sensitivity to exogenous thyroxine (T₄) is attained just prior to the mean size for spontaneous transformation (Table XV). This was observed despite the lack of changes in peripheral tissue sensitivity with development (Figures 25 and 26). Further studies (Table XII and Figure 24) suggested in vivo changes in T₄ sensitivity might be due to alterations in the sensitivity of the hypothalamus with development. These observations are consistent with the studies of Etkin (1970), Goos (1968, 1969) and Norris and Gern (1976).

Etkin (1970) observed that the median eminence exhibited a gradual increase in sensitivity to T₄, and Goos (1969) concluded that T₄ promoted the maturation of the thyrotropic center in the dorsal preoptic nucleus of Xenopus laevis tadpoles. Norris and Gern (1976) found that T₄ injected directly into the hypothalamus of neotenic Ambystoma tigrinum promoted an activation of the pituitary-thyroid axis, whereas the same amount of T₄ injected intraperitoneally did not promote pituitary-thyroid activation. Norris and Gern (1976) also found that a similar intrahypothalamic injection of T₄ into small, immature larvae did not promote pituitary-thyroid activation. Seemingly, the thyrotropic center in the hypothalamus as well as the median eminence (Etkin, 1970) require low levels of T₄ for development and maturation and a small increase of T₄ is needed for the next level in order to promote the activation required for metamorphic climax.
Investigations following thyroid activity and plasma T$_4$ levels with development (Fig. 19) substantiate this pattern of thyroid activity prior to the onset of metamorphosis. Low levels of T$_4$ were detected prior to the size for transformation. Thyroid activity and T$_4$ levels increase prior to the mean size for metamorphosis, and thyroid activity decreases subsequent to this size (except for animals that enter metamorphic climax). Presumably, this initial (prometamorphic) surge is required for the maturation of the thyrotropic center and the median eminence (interestingly the median eminence is the last tissue to acquire its competence to T$_4$; Dodd and Dodd, 1976). Once these neural and vascular links have matured and direct connections between the thyrotropic center and the pituitary occur; a second surge of releasing factors will direct the pituitary thyrotopes to secrete their contents which will, in turn, cause the full activation of the thyroid required for metamorphosis.

The aforementioned studies and the experiments performed within this thesis have suggested the following theory to account for these observed phenomena related to metamorphosis (Fig. 35).

During the early periods of larval growth, circulating levels of T$_4$ and TSH are low. Due to the lack of developed pituitary thyrotopes, the thyroid may autonomously secrete low amounts of T$_4$ and undergo proliferation and development. As thyrotopes develop, low levels of TSH are secreted promoting further thyroid development and a gradual increase in T$_4$ secretion. These low T$_4$ levels bring about the gradual development of the thyrotropic center of the hypothalamus and effect other progressive changes in preparation for climax. As the thyrotropic center matures, it releases factors (TRF) which, in turn, promote the development of thyrotopes within the pituitary and
stimulate TSH secretion. Since vascular links between the hypothalamus
and the pituitary are not functional, TRF may be released into the general
circulation and its action is, therefore, diluted causing only gradual
maturational effects upon the pituitary thyrotropes. As the thyrotropic
center matures, a negative feedback by T4 acts upon the hypothalamus (Goos, 1968) with respect to the release of TRF. But TRF must be released in
much larger amounts to the peripheral circulation to compensate for its
indirect route to the pituitary. This establishes a deceptive maintenance
of hormone levels for the hypothalamo-hypophyseal-thyroid axis with high
peripheral TRF levels. At a definite stage of larval development certain
tissues of the larva begin to exhibit competence (or reactivity) to T4.
These tissues might include the hindlimbs (which grow relative to body
size; Fig. 10) or the median eminence. The target tissues' increased
avidity would then lower the circulating T4 levels which would cause an
increase in TSH levels by promoting secretion of TRF. This might be the
initial thyroid activity surge observed within Ambystoma gracile some
time prior to the mean size for metamorphosis (Chapter III). Subsequently,
the vascular links between the thyrotropic center of the hypothalamus and the
pituitary become established due to the morphogenetic action of T4 upon the
"competent" median eminence. Once this direct route between the hypothalamus
and pituitary is established a much greater concentration of TRF is released
directly upon the pituitary, and this second surge is the observed "hypo-
thalamo-hypophyseal-thyroid activation" which occurs during metamorphosis.
The resultant high increase in TSH due to this activation induces thyroid
refractoriness (Chapter III) which, in turn, reduces thyroid activity.
In view of this model for spontaneous metamorphosis, how do montane and low-altitude populations differ with respect to their apparent physiological adaptive strategies? Before considering these apparent differences, it must first be realized that a complete genetic divergence between these two populations does not exist, since terrestrial salamanders exist within montane field populations and a certain proportion of montane larvae do transform. Therefore, distinct physiological differences may be difficult to detect, because the small proportion of montane larvae destined to transform may have "masked" these possible differences. For this reason I shall discuss general trends toward physiological divergence, and relate them to neoteny.

The previous studies have suggested this limited genetic divergence with relation to the physiology of metamorphosis. Montane populations (laboratory reared under identical conditions as low-altitude animals) differ in developmental physiology from low-altitude populations in the following aspects:

(1) Montane larvae's pituitary thyrotropes develop later at a larger body size compared to low-altitude larvae (Table XVIII).

(2) General patterns of thyroid activity preceding the mean size for transformation are lower for montane populations compared to low-altitude animals (Figs. 19 and 20).

(3) Larval growth rates are greater for montane populations (Chapter II).
Peripheral tissue sensitivity to $T_4$ is greater (at most temperatures) for montane populations compared to low-altitude populations (Figure 28).

These aforestated trends may be responsible for neoteny through the following proposed mechanisms. Two "genetic differences" selected for by factors related to altitude may explain the neotenic condition. The first condition may be a genetic disability that promotes a generally lower thyroid activity during the early phases of development. Though this was detected at significant levels for only one size-group (Figs. 19 and 20), this condition may delay hypothalamic maturation and development and, in turn, pituitary thyrotrope development (Table XVIII), and the onset of metamorphosis could only occur at a larger body-size (near the upper limits for transformation). The lowered $T_4$ levels may also be insufficient enough to promote the neuro-vascular links during "prometamorphosis" preventing the second surge of $T_4$ needed for anatomical transformation.

A second genetic factor that might promote neoteny could be a delayed (with respect to body size) attainment of "competence" for $T_4$ by the tissues responsible for these neural-vascular links between the thyrotropic center and the pituitary. Once competence is attained, it may require greater levels of $T_4$ to promote complete maturation, and larvae that do not attain these $T_4$ levels may become neotenic. Montane populations would exhibit this competence at the optimum body size irregardless of temperature, whereas low-altitude larvae would exhibit competence for these tissues at a smaller body size. Lower temperatures within low-altitude environs would delay hypothalamic maturation (with respect to body size) by elevating the threshold
for $T_4$ required for morphogenetic changes (Kollros, 1961). Therefore, higher temperatures would hasten maturation, and the onset of metamorphosis would occur when terrestrial conditions (food availability) might be optimal. These explanations would explain the in vivo responses to temperature observed for the montane and low-altitude populations (Tables XV and XVI). Other factors that might elevate hypothalamic tissue thresholds could be prolactin (Platt, 1976), steroids (Table XVII), or aging. Metamorphosis for many older, neotenes is physiologically traumatic and the older the larvae the more stressful is metamorphosis (Snyder, 1956). Possibly the thyropic center and median eminence loses its ability to respond in a harmonious fashion with age (Dent, 1968).

The greater peripheral tissue sensitivity probably ensures for montane larvae that once metamorphic climax is initiated, it will proceed in a quicker, coordinated manner despite the possibility of rapid environmental changes. In this manner a low proportion of transformed individuals are maintained. If the aquatic environment remains stable (i.e. no droughts) than selection against the harsher terrestrial environment may ultimately eliminate metamorphosis.
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Educational Background

<table>
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<tr>
<th>Degrees</th>
<th>College, University or Inst.</th>
<th>Field</th>
<th>Year</th>
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<tr>
<td>B.Sc.</td>
<td>University of Calif., Riverside</td>
<td>Zoology</td>
<td>1968</td>
</tr>
<tr>
<td>Ph.D.</td>
<td>Simon Fraser University</td>
<td>Biology</td>
<td>1977</td>
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Membership in Learned Societies-

Canadian Society of Zoologists

Society for the Study of Amphibians and Reptiles

Herpetologist's League

External Professional Activities-

Conferences Attended-

1.) California Conference on Neoplasia; Fullerton, California (1972)
2.) Northwest Conference on Comparative Endocrinology; Vancouver (1974)
3.) Plains University Biological Seminars; Regina (1976)
4.) Conference for the Canadian Society of Zoologists; Victoria (1977)

Papers Given-


Publications-

### Teaching Activities

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<tr>
<th>Institution</th>
<th>Course</th>
<th>Year(s)</th>
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<tbody>
<tr>
<td>1.) Calif. State Univ.</td>
<td>T.A.- Molecular Biology</td>
<td>1969, 1972</td>
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<tr>
<td>2.) Toloa College, Kingdom of Tonga</td>
<td>Secondary Science</td>
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<td>T.A.- Endocrinology</td>
<td>1974, 1976</td>
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