Amphibian Metamorphosis

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Amphibian metamorphosis is a dramatic developmental event during which major alterations in the animal's anatomy and physiology occur. The term "Amphibian" (from Greek *amphi* = two; *bios* = life) indicates the two completely different lives of the individual. The larva is aquatic: gill-breathing, and with a finned tail for swimming. The adult is (more or less) terrestrial: breathing with lungs, having four limbs, and a terrestrial-type, shedding skin. In *Urodeles*, the tail muscles are rearranged, and the fin disappears; in *Anurans*, the tail disappears completely during metamorphic development.

The Mechanisms of Metamorphosis

Anurans. The transition from the premature larval stage to metamorphosis is gradual, and the border is a matter of choice. The larval state is usually characterized as a period of growth, but differentiation and anatomical changes also take place throughout. Metamorphosis is initiated by secretion of hormones from the thyroid gland.

The first developmental features to be increased by thyroid hormones (TH) are the growth of limbs and differentiation of the central nervous system (CNS). While development of these adult characteristics is accelerated by TH, the tail and gastrointestinal (GI)-tract continue to grow in their larval form until mid-climax. (This is an interesting apparent contradiction, caused by the rigid dogma of a sharp boundary between developmental periods. The physiological interpretation is probably that the tail retains prolactin receptors and that achievement of TH receptors is delayed until mid-climax.) Tail resorption commences immediately after break-out of forelimbs from the branchial cavity, and thereafter development accelerates: the limbs grow; the tail shrinks; and the GI-tract is altered from vegetarian to carnivorous style, and its larval epithelium is resorbed and replaced by the permanent type. The larval skin is succeeded by the typical amphibian skin, which is shed as a coherent slough and differs in pigmentation from the larval skin. The CNS is further differentiated, and certain neurons, specifically associated with larval structures, disappear. The latter is the case for the Mauthner's cells, a pair of large neurons controlling the tail.

The thyroid. The characteristic follicle structure of the thyroid gland is apparent shortly after hatching. The follicles are small vesicles of monolayered epithelium, surrounding a colloidal mass. Follicle cells synthesize a protein, thyroglobulin, which is released and stored in the follicles. Iodide is taken up from blood/interstitial fluid by follicle cells, via a transport mechanism capable of concentrating I^-. From the internal side of the follicle cells, I^- is released into the colloid. During this process, I^- is oxidized into I, which immediately reacts with the tyrosine units of thyroglobulin. The tyrosine units, in juxtaposition with the tridimensional structure of the thyroglobulin chains, interreact to form ether-bridges. Thus, the thyroxine (T4) molecule is formed, still incorporated in the thyroglobulin structure. Secretion of T4 occurs by endocytosis of thyroglobulin fragments into follicle cells, followed by partial hydrolysis (S. Suzuki, pers.comm.) in secondary lysosomes. Eventually, this leads to the release of T4 into circulation. During larval life, T4 is not secreted in amounts detectable with existing methods.

Hormone secretion. Secretion of T4 is regulated by the secretion of *thyroid stimulating hormone* (TSH, thyrotropin) from the pituitary. The TSH-cells of the pituitary are, like the thyroid, developed and functional some time before metamorphosis. During the "mid-larval" stage, secretion granules may be seen in the cytoplasm in EM preparations. However, until metamorphosis TSH is not secreted in measurable amounts. The interconnected secretion of TSH and TH is detectable immediately before metamorphosis. TSH-secretion begins as a burst, initiated by a neurohormonal signal, arriving from the hypothalamus via the *eminentia mediana*, a local capillary network which facilitates immediate action. Pituitary activation is thus regulated by the brain, with some modifications (to be mentioned later).

Thyroidectomy (surgical/chemical). Larval thyroidectomy, or blocking of TH release,

leads to the absence not only of TH, but also of the signal from the hypothalamus, and of TSH secretion. The phenomenon is reversible: thyroidectomized (or TH-release-blocked) larvae may resume development of the TSH signal if T4 is supplied (i.e., submersion of larvae in a solution of T4). This observation proves both that hormone is being released from the thyroid during the larval period, although the amounts are too small to be detected with available methods, and that this secretion is crucial for CNS development leading to release of the neurohormonal signal. Various signs show that other neurons mature during this period besides those stimulating the TSH cells. The development of the signal is thus part of a general maturation of the CNS, induced by the previously mentioned, minor TH release. This maturation occurs as part of the development leading from the larval growth period into the metamorphic transition period.

Hormone receptors. A corresponding maturation occurs in the peripheral tissues, which are the targets of TH. The hormone's action on target cells is initiated by the reversible binding of hormone to receptor proteins in cell nuclei. However, thyroxine (= tetraiodothyronine, T4), as released from the thyroid, is not the most efficient form of the hormone. A specific enzymatic deiodination, removing the 5'I atom, increases the affinity for the receptor, and thus the effect of the hormone, by a factor of 10-20. The specific enzymatic system is activated by the low levels of TH, which also induce an increase in the number of TH receptors in target tissues (Galton 1985). The deiodinated hormone (triiodothyronine, T3) is rapidly absorbed through the gills and is routinely used for induction of metamorphosis.

Urodeles. The normal course of urodele metamorphosis is less studied and described than anuran metamorphosis. The alterations are less obvious, since the tail persists. Nevertheless, the metamorphosing urodele changes its appearance, since the tail fin is resorbed, and the skin, like anuran skin, is changed. The GI-tract is not altered as obviously as in anurans, probably because urodele larvae are carnivorous like the adults. The occurrence of a fasting period during metamorphosis suggests that some alterations of the intestinal epithelium take place. Urodele larvae have external gills, and the absence of a branchial cavity facilitates the study of gill resorption as well as of forelimb development.

Neoteny. Neoteny, also called larval reproduction or metamorphic failure, is the special developmental fate of some ambystomatid salamanders; instead of undergoing metamorphosis, the animals grow to adult size, and reproduce, still having the larval body form and aquatic behaviour. During this neotenic development, axolotls undergo some of the changes normally associated with metamorphosis. Limb formation (Rosenkilde et al. 1982), lung development, and the switch from larval- to adult-type erythrocytes (Turner 1988) occur during the second month posthatching. These features are neither prevented nor delayed in neotenic salamanders (although lung development may be incomplete), whereas gill resorption and alteration of oxygen affinity (adaptation to terrestrial behaviour) are directly related to thyroid-hormone-induced transformation.

In the axolotl, the property responsible for neoteny is transient, since later in life metamorphosis can be readily induced by treatment with thyroid hormone, both T4 (Prahlad and Delanney 1965, Taurog et al. 1974) and T3 (Prahlad and Delanney 1965) being efficient. In naturally metamorphosing amphibians, TH receptors are present or are formed as a response to hormone secretion (Galton 1989).

Regulation of Metamorphosis. The complex of developmental alterations constituting metamorphosis is triggered and regulated by hormones from the thyroid gland (Rosenkilde 1979, Galton 1988, Rosenkilde and Ussing 1990). In the axolotl, TSH cells and the thyroid gland are developed at the same time as the budding of the forelimbs (Pehlemann 1974). CNS maturation takes place during the stage when hind limbs are formed. Neoteny may be related to low activity of thyroid hormone (TH), or to lack of responsiveness of peripheral tissues. Low activity of thyroid hormone could be caused by impaired maturation of the hypothalamus, impaired release of thyrotropin releasing hormone (TRH), or lack of receptors for thyrotropin (= thyroid stimulating hormone, TSH). Either of these properties might be transient or permanent. In axolotl larvae 30-60 days posthatching, a transient surge in serum thyroxine from ≤ 20 nM to 40 nM has been measured (Rosenkilde et al. 1982). This stage, during which the limbs are formed, corresponds to metamorphosis in transforming species. After this period, serum thyroxine levels again become low, 2-6 nM.

Since this surge in serum thyroxine is not capable of inducing metamorphosis, the animals may be deficient either in their ability to deiodinate thyroxine (T4) into the biologically more active triiodothyronine (T3), or with respect to hormone receptors in peripheral tissues during the TH surge, or, possibly, with respect to some cellular post-receptor mechanisms (Rosenkilde et al. 1982). The increase in both the enzyme, 5'-deiodinase (5'D), that turns T4 into T3, and in the number of TH receptors requires a period of activation by T4. Time may have a key role in the ability of the axolotl to avoid metamorphosis and remain an aquatic, neotenic animal. Since the TH surge is brief, it may be over by the time the enzyme, 5'D, and the hormone receptors are established. In axolotl larvae, the observed surge (Rosenkilde et al. 1982) in serum TH may induce formation or maturation of TH receptors, though at a delayed rate, since the animals are able to respond to increased TH levels immediately after completion of limb formation (Rosenkilde 1985, and unpublished).

Experimentally induced metamorphosis. Axolotl metamorphosis is usually induced with TH, but TSH has also been used. Experiments with the hypothalamus hormone TRH have not been successful. The lack of effect is not merely a result of the short half-life of TRH in circulation. Infusion of TRH in cannulated axolotls has been shown to cause TH release in metamorphosed, but not in larval specimens (Jacobs et al. 1988, Kühn and Jacobs 1989). The results of hormone treatment of axolotls prove that the peripheral tissues of neotenic animals have the ability to respond to TH with the typical metamorphic changes, and that their own thyroid can release sufficient hormone to trigger metamorphic development when stimulated with TSH. The experiments also indicate that the hormone sensitivity of the thyroid and target tissues is not much different from that of metamorphosing species. Therefore, the idea has arisen that activation of the cerebral stimulating neurons might lead to metamorphosis. Injections of T4 into the cerebrospinal fluid (CSF) of the 3rd ventricle (the site of the hypothalamus) has induced metamorphosis in essentially smaller doses than those required by systemic treatment (Galton 1988), both in *Ambystoma tigrinum* from neotenic populations (Norris and Gern 1976), and in *Ambystoma mexicanum* (Rosenkilde et al. 1982). Immature larvae require a longer latency period and a higher dose of intrahypothalamic TSH than adult neotenic tiger salamanders (Norris and Gern 1976).

In our laboratory, metamorphosis is routinely induced by submerging axolotls in a 20 nM solution of T3 in tap water (the Danish tap water being non-chlorinated). Kept in this concentration of hormone, the animals will complete transformation within three weeks. In this way, we get axolotls of two different developmental forms (Figure 1), though of the same chronological age.

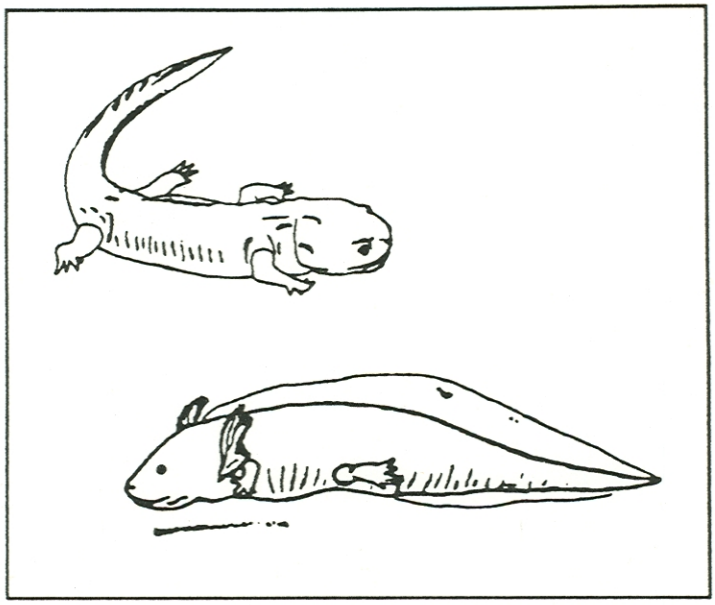

Figure 1. Mexican axolotls, *Ambystoma mexicanum*. Top: metamorphosed axolotl; bottom: neotenic (branchiate) axolotl.

Maintaining of Axolotls. Our axolotls are kept in (non-chlorinated) tap water. Adult neotenic animals are fed commercial trout pellets or beef liver. Metamorphosed animals are fed beef liver or live *Tubifex* worms. Newly hatched larvae from natural spawnings of eggs laid on plastic plants are raised on a diet of brine shrimp (*Artemia*). Older larvae are fed *Daphnia* or *Tubifex*. The axolotls are kept at 20°C (~68°F).

Induction of metamorphosis. Axolotls are kept in separate containers (5 *l* white plastic buckets with a loosely fitting lid), each containing 1.5 *l* 20 nM solution of T3 (3,3',5-triiodo-L-thyronine, sodium salt) in tap water (Figure 2). Non-metamorphosing control animals are kept in tap water. Hormone solution/water is changed twice a week. After 1 1/2 weeks in hormone solution, metamorphosing animals will cease to feed. During the following 1 1/2 weeks, neither of the animals are fed. To allow the nostrils to rest above the waterline, midmetamorphic axolotls are each given a piece of rock. After 3 weeks, metamorphosis is complete and the animals are transferred to tap water.

Overcoming rebound effect of TH treatment. In some of our experiments (Ussing 1991), newly metamorphosed axolotls failed to feed, and moulting was incomplete, leaving layers of dead cells on the skin surface. Incomplete moulting indicates insufficient or lack of production of thyroid hormone after the T3-treatment period. A "boost" of T3 will usually overcome the condition. The boost may be given as an *i.p.* injection of 2 -10 µg, or by immersion of the animal in an aqueous solution of hormone (2-20 nM has proven beneficial). In severe cases, postmetamorphic individuals may be kept in a 6 nM solution of T3. This is sufficient to ensure moulting of skin and alimentary canal function (feeding without regurgitation, and subsequent defecation).

Our metamorphosing axolotls usually appear healthy: fat bodies are large throughout, and the fasting period leads to no obvious

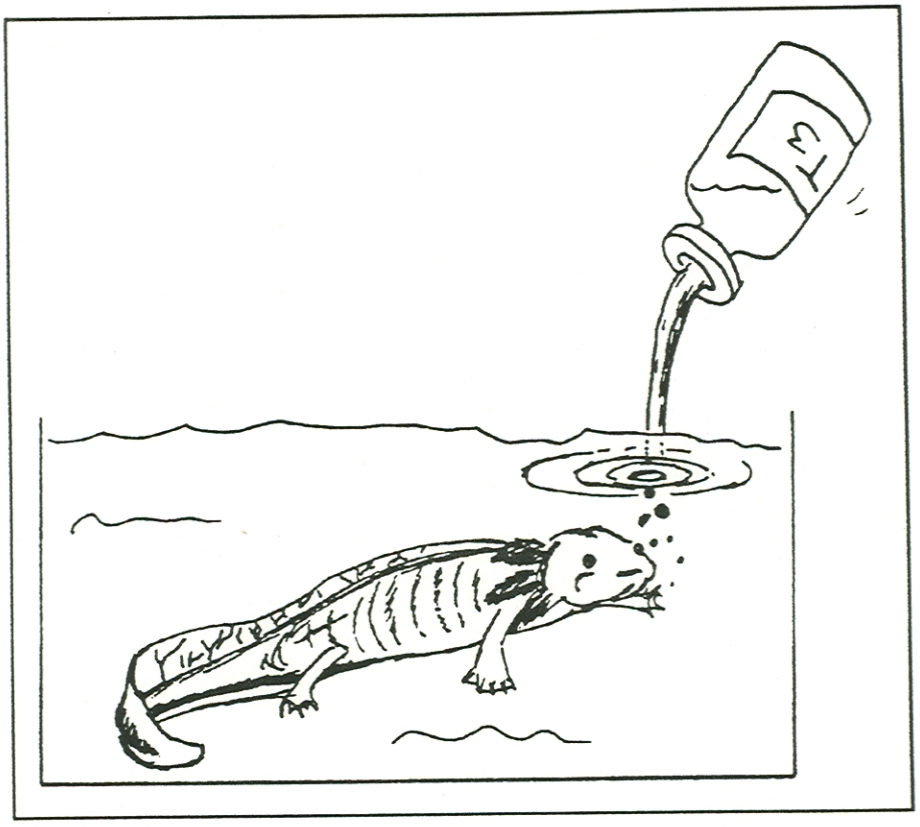

Figure 2. Experimental induction of metamorphosis in the axolotl: neotenic animals are kept in a 20 nM solution of T3. External signs of metamorphosis are evident after 5-6 days, and the animals are transformed by the end of 3 weeks.

fatigue or weakness. Axolotls one year old at the onset of experimental metamorphosis often become sexually mature (Ussing 1991) during the transformation. In our neotenic population, sexual maturity is usually reached at 2-3 years of age.*

After the "natural" fasting period during midmetamorphosis, newly metamorphosed axolotls may decline to feed. Live food is preferable. Although newly metamorphosed axolotls may be tempted to eat beef liver (held with tweezers), regurgitation may occur up to several days later.

During studies of induced metamorphosis, it became obvious that axolotls like to burrow! Metamorphosed axolotls want an "island" for resting with nostrils in the air. The container may be placed askew, with a support to secure a "shore of land" in one end. A piece of filter paper in the bottom keeps the animal from slipping. With this set-up (Figure 3), axolotls were often found burrowing under the paper, keeping the skin wet and the nose dry at the same time!

* Neotenic axolotls in the Indiana University Axolotl Colony routinely reach sexual maturity at 12-14 months of age. (Editor)

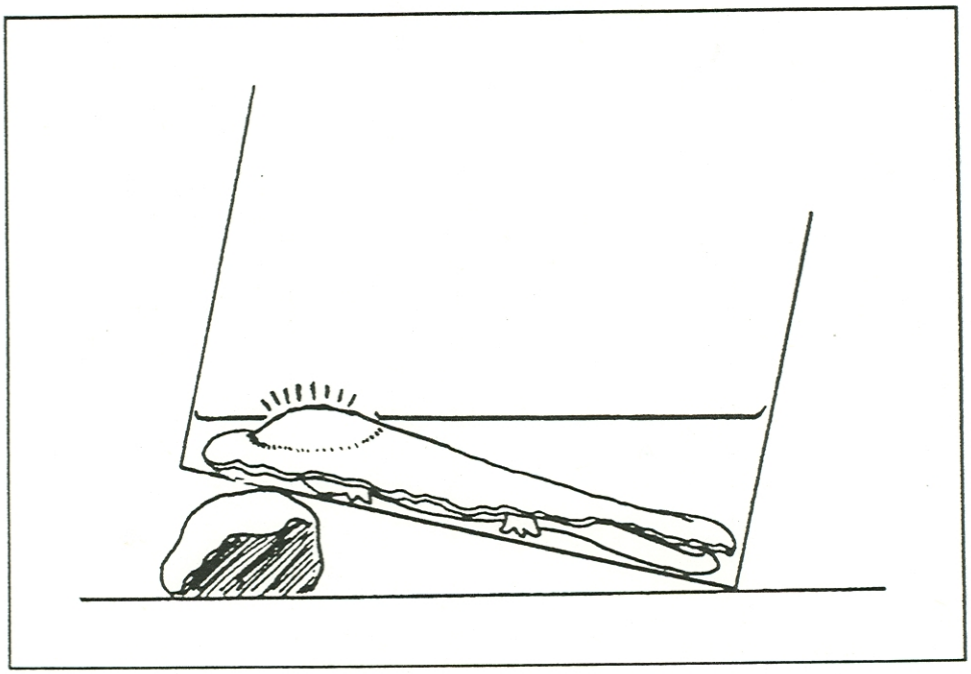

Figure 3. Metamorphosed axolotl burrowing under filter paper. NB: container not drawn to scale.

Drawings by Jonas Ussing.

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