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Abstract	The axolotl (*Ambystoma mexicanum*) has remained an important model for regeneration and developmental biology for over a century. Although axolotls in captive-bred colonies usually exist in an aquatic form, they retain the ability to undergo metamorphosis following exposure to thyroid hormone. Here we present a robust method for inducing metamorphosis in adult axolotls that results in high survivability and produces terrestrial animals that can be maintained in long-term captivity.	

Keywords (separated by " - ")	Metamorphosis - Thyroxine - T_4 - Thyroid hormone - Axolotl - *Ambystoma* - Salamander - Regeneration

Chapter 11

Thyroxine-Induced Metamorphosis in the Axolotl (*Ambystoma mexicanum*)

Peggy S. Coots and Ashley W. Seifert

Abstract

The axolotl (*Ambystoma mexicanum*) has remained an important model for regeneration and developmental biology for over a century. Although axolotls in captive-bred colonies usually exist in an aquatic form, they retain the ability to undergo metamorphosis following exposure to thyroid hormone. Here we present a robust method for inducing metamorphosis in adult axolotls that results in high survivability and produces terrestrial animals that can be maintained in long-term captivity.

Key words Metamorphosis, Thyroxine, T_4, Thyroid hormone, Axolotl, *Ambystoma*, Salamander, Regeneration

1 Introduction

Amphibian metamorphosis produces a suite of behavioral, morphological, physiological, biochemical, and genetic changes associated with the transition from a larval to adult form [1–3]. These changes are mediated by alterations in circulating thyroid hormones (T_3—triiodothyronine and T_4—thyroxine) and their interaction with thyroid hormone receptors in target cells [3, 4]. Among urodeles, however, paedomorphosis has evolved in several lineages to produce species that fail to undergo an obvious metamorphosis in natural populations [5]. Instead, these species become sexually mature while retaining many larval traits. Although the biochemical changes that mediate the failure to undergo metamorphosis in these paedomorphic lineages have not been identified, some urodeles, including *Ambystoma mexicanum* (axolotl) and *Ambystoma tigrinum* (tiger salamander), retain the ability to undergo metamorphosis following exposure to thyroid-stimulating hormone (TSH), T_3 and T_4 [2, 3, 6–9].

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In order to induce metamorphosis in axolotls, two basic methods using hormones are usually employed: peritoneal injection of the hormone [7] or addition of the hormone to the rearing water [2]. While the majority of studies using induced metamorphosis of the axolotl have focused on aspects of metamorphosis itself, some recent studies have sought to use captive terrestrial axolotls to explore mechanisms regulating regeneration [10, 11]. For these studies, animals must be healthy, actively eating, and able to survive in captivity for more than a year. Although more time intensive than single peritoneal injections, using the method presented here, >50 % of completely metamorphosed animals survive in long-term captivity.

2 Materials

1. Axolotls (*see* **Note 1**).
2. Round plastic containers (diameter = 21.5 cm, height = 15 cm).
3. Plastic shoeboxes with lids (width = 33.5 cm, depth = 19.5 cm, height = 9 cm).
4. L-Thyroxine (100 μM) stock T_4 solution: Add 100 mg L-thyroxine (T_4) (Sigma; Cat. no: T2376) in 10 mL 0.4 M NaOH in a 15-mL conical tube. Vortex the tube for 30 s and incubate the tubes in a water bath maintained at 37 °C for 5 min. Repeat vortex and heat cycle until L-thyroxine is completely dissolved. This solution is now 13 mM T_4. Take 5 mL of T_4 solution and add to 645 mL of sterile water to make 100 μM stock solution. The remaining 5 mL of 13 mM T_4 can be wrapped in foil and stored at 4 °C.
5. L-Thyroxine (T_4) working solution (50 nM): Make 50 nM T_4 as needed by mixing 100 μM T_4 stock solution with 40 % modified Holtfreter's solution at 1:2,000 dilution (*see* **Note 3**).
6. 40 % modified Holtfreter's solution: For a 1,136-L (300-gallon) container, add 1,589.7 g NaCl, 90.8 g $NaHCO_3$, 22.7 g KCl, 181.7 g $MgSO_4$–$7H_2O$, and 90.8 g $CaCl_4 \cdot 2H_2O$. Add all the dry components into the container and fill to volume with sterile water. Mix with circulation until all components have gone into solution. Add 75.7 mL Amquel and 75.7 mL Novaqua to this solution. Solution is now ready to use (*see* **Note 2**).
7. California blackworms (*Lumbriculus species*).
8. Night crawlers (earthworms).

3 Methods

1. To facilitate successful metamorphic transition, feed animals California blackworms every day for 1 week prior to the first addition of T_4 working solution (normal feeding schedule is every 3 days).
2. After 1 week, transfer axolotls into plastic containers with working T_4 solution (one animal per container). The amount of T_4 solution should fill the container (*see* **Note 4**).
3. Remove and replace working T_4 solution every 3 days.
4. Feed animals after each changing with a generous portion of California blackworms (approximately 20–25 worms). Since they will stop eating during metamorphosis, the goal is to get them to eat as much as possible.
5. Obvious morphological changes are rarely visible until approximately 2 weeks after T_4 exposure. The first visible changes occur in the third week and include noticeable weight loss, dorsal ridge recession, tail fin recession, and eye protrusion (Fig. 1) (*see* **Note 5**).
6. Concomitant with the above morphological changes, the gills begin to disappear. It is critical to begin lowering the water level as this happens. The water level should be reduced an inch per water changing. When the gills have nearly disappeared,

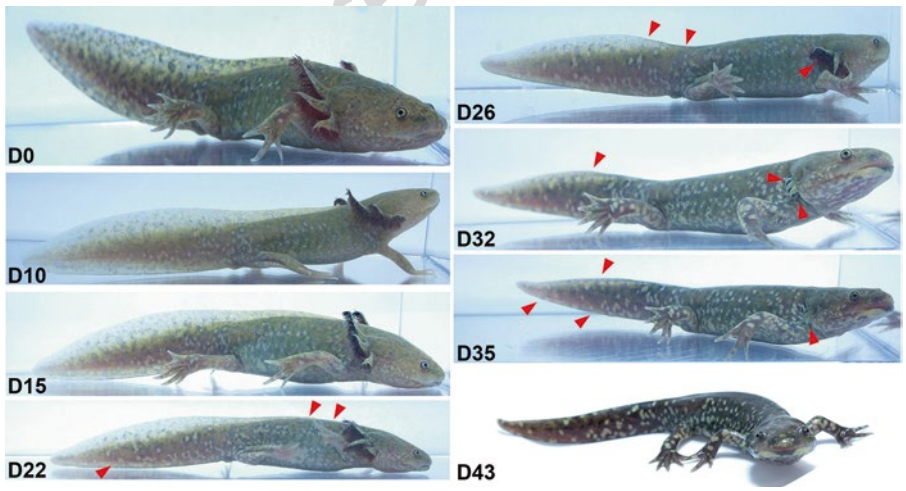

Fig. 1 Pictorial representation of morphological changes occurring during thyroxine-induced metamorphosis in adult axolotls. The first obvious changes are visible 2–3 weeks following thyroxine exposure. The dorsal fin begins to regress in a cranial to caudal direction, as does the ventral portion of the tail fin (*red arrows*). Four weeks after exposure, the gills have begun regressing and the dorsal and anal fins continue to disappear. The eyes begin to protrude from the head. The gills, dorsal, and anal fins have almost entirely disappeared in the fifth week (*red arrows*). By the sixth week, animals have metamorphosed to a completely terrestrial form

place two pieces of bunched-up paper towel at the bottom of the containers (*see* **Note 6**).

7. After 4–5 weeks, the gills have completely disappeared and T_4 administration is no longer required. Transfer each axolotl to a plastic shoebox container with lid, paper towel, and 1 in. of 40 % modified Holtfreter's solution. Feed each animal ½ or ¼ of an earthworm (*see* **Note 7**).
8. From this point on, animals can be maintained on paper towels in 1 in. of 40 % Holtfreter's solution. Animals should be offered food every 3 days (*see* **Note 7**).

4 Notes

1. In our experience, wild type and albino color morphs exhibit higher survivability than white mutants. We have used this method successfully on animals ranging in size from 7 cm TL to >20 cm TL adults. [AU1]
2. Although we maintain our colony of axolotls at a relatively constant water temperature of 18–20 °C, for metamorphosis we use Holtfreter's solution that is at room temp (~23–25 °C).
3. We typically add 50 mL of T_4 stock solution to a 44-gallon container (e.g., large Rubbermaid trash bin) to make 100 L working solution (with 40 % modified Holtfreter's solution). The working T_4 solution can be used for several days when morphing a large number of animals.
4. It is imperative that plastic containers used for induced metamorphosis are kept separately from normal housing containers because T_4 can induce metamorphosis at extremely low concentrations. Once containers are exposed to L-thyroxine, they should be clearly labeled.
5. As the skin matures to an adult phenotype, it is shed frequently and this will cause the water to appear dirty. In our experience, this does not require an increase in water changing frequency.
6. Paper towels provide a surface to help the axolotls reach the surface and acquire oxygen along with helping to keep the containers moist. Axolotls will eat significantly less at this point during metamorphosis and may refuse to eat at all. To help encourage consumption, place 5–10 blackworms directly in front of their mouth.
7. Resumption of feeding is critical for metamorphs to survive in captivity. A behavioral change is associated with transition to a terrestrial mode of feeding. Every animal must be offered food, although every axolotl may not eat at every feeding. This is normal. Patience is required while feeding metamorphs.

Earthworms should be offered with a tweezer and placed to the left or right of the axolotl's nostril to encourage eating. A light tap in front of the eyes can sometimes encourage the animal to bite for food. When animals are reluctant to eat earthworms, we offer blackworms.

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