

Chapter 3

Housing and Maintenance of *Ambystoma mexicanum*, the Mexican Axolotl

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Abstract

The aim of this paper is to assemble a significant amount of information on *Ambystoma mexicanum*, the axolotl salamander, to assist in the basic knowledge needed to raise, breed, and study most aspects of axolotl biology. It is important to understand the basic biology of the axolotl in order to make informed decisions on their proper care and use in experiments. Therefore, we will provide necessary information to the non-herpetologist that will assist in their study of this unique and fascinating animal. We also aim to provide a resource on the general anatomy, behavior, and experimental tips specific to the Mexican axolotl that will be of use to most axolotl laboratories. Axolotls have been actively researched since the 1860s, giving testament to their relatively straightforward maintenance and their versatility as an animal model for development and regeneration. Interest in using the axolotl in laboratory research has grown tremendously over the past decade, so dedicated resources to support the study of this species are needed and encouraged.

Key words Salamander, Limb regeneration, Animal model, Axolotl anatomy

1 Introduction

1.1 Taxonomy, Habitat, and the Laboratory Strain

A. mexicanum, commonly named the axolotl (Fig. 1), are members of the order Urodela (*oura* tail + *delos* evident, also called Caudata) or tailed amphibians, which constitute ten extant families of salamanders found mainly across the temperate regions of the northern hemisphere. Axolotls belong to the family Ambystomatidae, genus *Ambystoma*, which are commonly called mole salamanders and are comprised of approximately 30 species found across North America from southern Mexico to southern Alaska. There are 17 Mexican ambystomatid salamander species that inhabit the mountains of Central Mexico. Five of these species are primarily or obligatorily neotenic, meaning they do not undergo metamorphosis and can breed in the adult larval form [1]. Axolotls are endemic to the Lake Xochimilco area in the Valley of Mexico, which has been reduced over the centuries to a



Fig. 1 Image of female leucistic (*left*), female albino (*middle*), and male wild-type (*right*) axolotls. Notice the large bellies of the two females indicating they are filled with eggs and the large cloaca of the mature male wild-type

~40 km² area of artificial canals just outside the city limits of Mexico City [2]. This high-altitude lake system (~2,200 m above sea level) has been inhabited for centuries, most notably by the Aztecs. This is where the name axolotl originates, as the animal was named after the Aztec god Xolotl [3]. The water conditions of Lake Xochimilco between 1978 and 1988 were estimated to be between 16 and 20 °C, pH 7.4–8.0, with a conductivity between 975 and 1,650 microsiemens (μS)/cm [1]. Today, axolotls are critically endangered and are on the brink of extinction due to habitat loss, the introduction of invasive species, and shifts in water quality [4]. It has been estimated that densities were at 6,000 ind./km² in 1998, 1,000 ind./km² in 2000, and 100 ind./km² in 2008 [2], and only a few axolotls were cited in the lake system after months of surveying in 2013 [5]. Unfortunately, the majority of axolotls today are found in aquaria and laboratories around the world.

The modern axolotl strain used in most laboratories is a highly inbred population that most likely arose from a donation of seven wild axolotls (six wild-type and one white mutant) between 1863 and 1866 to the Paris Natural History Museum [6]. In fact, most modern-day laboratory axolotls likely have a direct lineage to these founders, and all white mutants are descendants from this single white animal [7]. A few wild-caught axolotls were introduced into the colony strain in the 1960s including an albino tiger salamander (*Ambystoma tigrinum*) [8], but overall the present-day laboratory strain is likely one of the most long-running inbred strains of any laboratory species. The 150-year history of laboratory breeding seems to have selected against spontaneous metamorphosis (currently <1 % frequency), as it is more prevalent in wild strains than the lab strain [7, 9]. The most extensive colony of laboratory axolotls is maintained at the Ambystoma Genetic Stock Center (AGSC) at the University of Kentucky (www.ambystoma.org), which is a continuation of the Indiana University Axolotl Colony initiated by

Humphrey R.R. in 1957 [10]. Over the past 50 years, the Axolotl Colony has provided the majority of axolotls and housing information to labs worldwide. Valuable information on housing, breeding, and diseases of axolotls can be found at www.ambystoma.org as well as in the archives of the Axolotl Newsletter.

1.2 Laboratory Research Using the Axolotl

Axolotls have classically been used in developmental biological research for practically every organ system due to their large egg size, external development, reliable breeding, acceptance of embryonic and adult tissue grafts, and large clutch sizes [11]. Historically, both newts and axolotls have been the primary types of salamanders used in lab research, but the unique advantage of axolotls is that they can be easily bred in a lab environment in the long term. What truly sets both newts and axolotls apart from other animal models is that they are our closest relatives that display a wide range of striking regenerative capabilities. In fact, axolotls can regenerate and recover from virtually any injury that does not kill them. Regeneration has been observed in parts of the heart, the tail, the jaw, the spinal column, the gills, the brain, and entire limbs (Fig. 2). This regrowth occurs without scarring and with full restoration of function. It can also presumably reoccur an indefinite number of times without any loss of fidelity, although a careful analysis of this point is needed in axolotls. Regeneration can be induced throughout all stages of life, although the process is faster in larvae and less reliable in animals that have been forced to undergo metamorphosis [12, 13].

Other amphibians besides axolotls are studied for their regenerative abilities. Tadpoles of the African clawed frog *Xenopus laevis* can regenerate their tails and spinal columns, while certain species of newts (*Pleurodeles waltl*, *Notophthalmus viridescens*, and *Cynops pyrrhogaster* are the most commonly studied species) can regenerate appendages and organs. Despite these similarities, the axolotl is not closely related to these species. As a member of the order Anura, *X. laevis* is only distantly related to urodele salamanders with a common ancestor approximately 260 million years ago [14], a divergence emphasized by the fact that *X. laevis* loses its regenerative abilities in late larval stages. Furthermore, although newts and axolotls are both urodeles with superficially similar anatomy, they diverged from a common ancestor at least 145 million years ago [14], and recent studies have shown that newt regeneration and axolotl regeneration differ in both mechanism [15–17] and recovery after denervation [18]. Consequently, caution is advised when attempting to compare axolotl protocols and findings with those of other amphibian models.

1.3 Gross Anatomy

Unlike the majority of urodeles, axolotls are obligate paedomorphs which do not undergo metamorphosis unless it is artificially induced via the addition of thyroid hormone to their environment [19]. Consequently, they grow to be large fully aquatic adults and

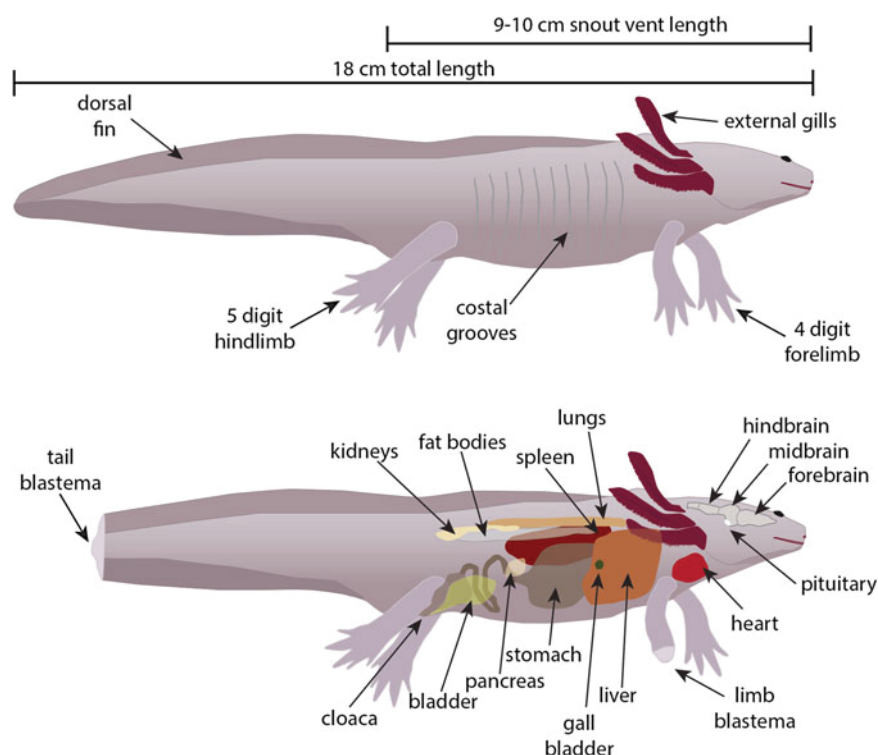


Fig. 2 Cartoons of the exterior (*top*) and interior (*bottom*) of an adult leucistic axolotl. Each organ is drawn to approximate scale and represents the approximate position in the animal. Colors also represent the approximate color of each organ. The regenerating limb and tail represent mid-bud blastema stages. Internal histology of a mid-bud blastema is represented in Fig. 3c

maintain the feathery external gills characteristic of ambystomatid larvae throughout their lives (Figs. 1 and 2). Males reach sexual maturity, indicated by a blackening of the nails, at around 10 months of age, while females tend to mature slightly later from 12 to 18 months. Sexual dimorphism is moderate but visible, as males are slimmer and longer than females and exhibit a cloacal bulge (Fig. 2). Sexually mature females are also more rotund due to their egg supply. While the lifespan of wild axolotls remains unknown, they generally live between 10 and 15 years. Animals can be induced to undergo metamorphosis, which become terrestrial and strongly resemble adult tiger salamanders (*Ambystoma tigrinum*). Axolotls are in fact closely related to tiger salamanders and are capable of crossbreeding with this species in addition to various other ambystomatid salamander species [20]. Studies of *A. tigrinum* have thus provided extensive insight into the anatomy and development of *A. mexicanum* [21].

Although the overall body plan of the axolotl is considered to be among the most primitive of the tetrapods, these salamanders nevertheless share a number of basal characteristics with other vertebrates and have proved useful for the study of many different systems.

Aquatic and lacking a middle-ear structure, axolotls rely on olfaction far more than audition when they are searching for food. The urodele olfactory system is quite complex and allows the animal to discriminate between very similar odorants and chemicals. Because their olfactory epithelium and olfactory bulb are large and easily accessible, tiger salamanders were a favored model for the study of olfaction and neuronal signaling during the 1970s and 1980s [22–24]. Axolotls possess true teeth and a calcified skeleton with cartilaginous joints that are anatomically similar to mammalian joints [25, 26].

Although their eyesight is poor and their vision is largely limited to the detection of movement, the axolotl retina displays the layered structure typically seen in vertebrates. In fact, many early studies of retinal intracellular signaling were conducted in salamanders because of their large and easy-to-access retinal cells [27–29]. Tiger salamanders can detect ultraviolet light, and their UV-sensitive photoreceptors express three different opsins [30, 31]. Furthermore, like fish and other aquatic amphibians, axolotls possess a lateral line system that is used to detect both electrical currents (via ampullary organs located on the head) and water movement (via mechanoreceptive neuromasts that run along the side of the animal). The lateral line develops from neurogenic placodes, and this developmental process may provide insights into the evolution of vertebrate sensory systems [32]. However, the study of the axolotl's development and evolutionary history has been complicated by its massive genome. Consisting of around 32×10^9 base pairs located across 14 haploid chromosomes [33], the axolotl diploid genome is among the largest of all tetrapods. Our understanding of this animal is sure to increase rapidly as genetic technology advances and more techniques are adapted for use with salamanders.

1.4 Structure of the Circulatory and Respiratory System

Axolotls possess a three-chambered heart consisting of two atria and one ventricle. As blood leaves the ventricle, it can pass to either the pulmonary arteries or through an aorta that leads to the rest of the body. Oxygenated blood leaves the lungs and is pumped back into the lone ventricle of the heart, where it mixes with deoxygenated blood that has already circulated through the body. This three-chambered system is consequently less efficient than the four-chambered system seen in mammals and birds, as tissues are nourished by blood that is not saturated in oxygen. The number of aortic arches can vary greatly between and within different urodele species, but axolotls always have four aortic arches [34]. Like all amphibians, axolotls are poikilothermic and their heart rate is strongly influenced by the temperature of their environment. Hematopoiesis arises from the adult liver and spleen [35] and their erythrocytes are very large, nucleated, and strongly autofluorescent. Injury induces rapid vasoconstriction to prevent excessive

blood loss, and clotting occurs very quickly even after injury to major arteries. As a result, axolotls are at only minimal risk of bleeding to death during and after surgery. They are a fairly popular model for the study of cardiac development because they can exhibit a recessive mutation, dubbed *c*, that is cardiac lethal within 2 weeks after hatching. The hearts of *c/c* mutants do not beat, and studies have found that these mutant hearts are largely differentiated but lack tropomyosin [36] and organized sarcomeres [37].

Aquatic throughout their lives, axolotls utilize multiple strategies for obtaining oxygen from their environment. They exchange oxygen with their three-paired external gills and can respire through their skin via cutaneous gas exchange. However, axolotls also possess rudimentary lungs and may obtain at least 40–60 % of their oxygen through surface breathing [38]. These lungs are elongated and translucent in appearance and run parallel to the spinal column for virtually the entire length of the body cavity (Fig. 2). Very little research on axolotl respiration has been conducted, but previous studies of tiger salamander larvae have provided likely insights into the mechanism of this process. These larvae inhale using a two-stroke buccal pump system that mixes expired and fresh air in the buccal cavity before pumping it into the lungs. Exhalation is active and very rapid, minimizing the amount of fresh air that is expelled through the mouth and gills. The ventilation efficiency of this system is comparable to that of mammalian ventilation [39]. Axolotls housed in hypoxic tanks make frequent trips to the surface to breathe [40], which can increase the probability that they will swallow air and cause the formation of an air bubble within the body that can disrupt the animal's locomotion and feeding. Because of their numerous options for respiration, axolotls can survive for hours outside of their tanks so long as they are not allowed to desiccate. This ability proves useful for the purpose of surgical procedures, as axolotls will heal very quickly if kept moist and left sedated after surgery.

1.5 The Immune System

Axolotls have a very primitive acquired immune system and are generally described as immunodeficient. They do not induce a humoral response to soluble antigens [41], they produce just two classes of immunoglobulins, and they generate antibodies to antigens extremely slowly if at all [42]. This immunodeficiency is common in urodeles and has worked to the advantage of researchers, as axolotls do not reject tissue from other salamanders and will even readily accept tissues [43] and tissue primordia [44] from other amphibian species such as *X. laevis*. Creative grafting experiments using embryos have done much to elucidate the development of axolotls, while grafting of larval or adult tissue has uncovered some of the mechanisms of regeneration. The recent production of GFP-expressing axolotls has opened up a new avenue of grafting possi-

bilities, and researchers have already begun grafting GFP tissues to white animals in order to reveal the basis of cellular plasticity and positional memory during regeneration [45].

Despite these deficiencies in the acquired immune system, the axolotl is still resistant to bacterial infections. This is likely due to their mucus coat and fairly robust innate immune system, which represent the main line of immune defense for urodeles. Abundant neutrophils and macrophages rapidly engulf and destroy foreign invaders, while antimicrobial peptides provide an additional layer of defense [46]. Past the age of 2 weeks, axolotls are at moderate to minimal risk of bacterial infection even after surgery. However, they are still susceptible to fungal and viral infections and can acquire bacterial infections after chronic stress. Exposure to *Ambystoma tigrinum* virus (ATV) can devastate both wild and laboratory populations, with mortality rates potentially exceeding 90 % [47]. This extreme susceptibility is likely due to a lack of lymphocyte proliferation upon exposure to the virus [48]. Another curiosity of the axolotl immune system is the fact that macrophages seem to be essential for regeneration, as early ablation of macrophages completely inhibits blastema formation and results in excessive collagen deposition after limb amputation [49].

Intriguingly, although they may undergo high levels of cellular proliferation in multiple tissues throughout their lives, axolotls are remarkably resistant to cancer. Repeated studies of various urodeles have failed to induce cancerous growth in regenerating tissues even upon administration of carcinogens [50, 51], although spontaneously occurring tumors have been described in veterinary literature [52, 53]. This curious resistance to malignant growth even during extreme cellular proliferation remains largely unexplored.

1.6 Pigmentation and Structure of the Epidermis

Wild-type axolotls are a mottled gray/brown/olive-green color, and this coloration arises from a combination of three different neural crest-derived pigment cells. Black melanophores are the predominant chromatophores in adult wild-type animals, while yellow xanthophores are more abundant early in development (Fig. 1) [54]. *Tip: shiny iridophores reflect light and are strongly reflective under fluorescent imaging.* White leucistic (white mutant, *d/d*) axolotls are the result of a recessive mutation in an unknown gene. They are oftentimes preferred over wild-type animals for research purposes, as pigment can negatively affect histological staining or fluorescent imaging. Leucistic animals are descendants of a single founder white axolotl that was donated to Auguste Duméril in 1866, hence the genetic designation *d* [55]. Leucistic larvae are lightly pigmented dorsally but lose almost all pigment shortly after hatching due to a lack of pigment cell migration to the flank of the animals [56]. In contrast, albino animals completely lack melanin and have a yellow appearance due to an overabundance of

xanthophores (Fig. 1). Although albino eggs are difficult to inject due to their lack of pigment, they are very useful for whole-mount embryonic staining methods. Adult leucistic salamanders can be easily distinguished from albinos by their black eyes.

The adult axolotl epidermis is similar to other larval amphibians and does not contain the distinct cornified layer (stratum corneum) that mammals or metamorphosed amphibians possess [57]. It is highly vascularized in order to facilitate efficient cutaneous gas exchange and is coated with mucus secreted by epidermal Leydig cells. Dermal mucous glands help retain moisture and withstand microbial threats. Leydig cells are large, club-shaped, and packed with secretory granules (Fig. 3a, b). As they fill with these granules, they rupture and release mucus into the intracellular space where it can then escape via pores onto the epidermal surface [58]. This mucus coat is very sticky and makes for an effective natural adhesive during surgical manipulation of the animals. Epidermal healing and regeneration is completely scar-free and occurs without extensive long-term fibrosis [57].

1.7 Regeneration

Limb regeneration is the classic paradigm of complex tissue regeneration and is currently the primary focus of laboratory research on the axolotl (Fig. 2). Axolotls regenerate limbs by generating a mass of highly proliferative cells called a blastema at the distal tip of a limb stump (Fig. 3c). After amputation, locally derived lineage-restricted progenitor cells or dedifferentiated cells proliferate and recapitulate developmental processes in order to regrow the missing portion of the limb [59]. As long as animals are well cared for, limb regeneration is a robust, dependable assay. A juvenile animal (8.5–10 cm SVL) will reach the differentiation stage of regeneration around approximately 32 days post amputation but will not replace 100 % of the missing limb for at least 100 days post amputation. Three-month-old axolotls (~5 cm SVL) will reach the differentiation stage of regeneration at approximately 22 days post amputation and will replace 100 % of their limbs by approximately 66 days post amputation [12].

Perhaps because axolotls are a neotenic member of an evolutionarily primitive order, their brains are very simple and share several similarities with the mammalian embryonic brain (Fig. 3d). The axolotl brain is relatively flat and elongated with clear delineations between the telencephalon, mesencephalon, and rhombencephalon (Fig. 2). The olfactory bulbs are large, but the optic lobes are small and poorly separated, and the cerebellum is relatively undersized and weakly developed as well [21]. The cerebellum contains the only neurons in the axolotl central nervous system that are not paraventricular—all other neurons remain stationary and do not migrate from the germinal site during development [60] (Fig. 3d). Though less is documented about the terrestrial axolotl brain, it is known that the brain undergoes

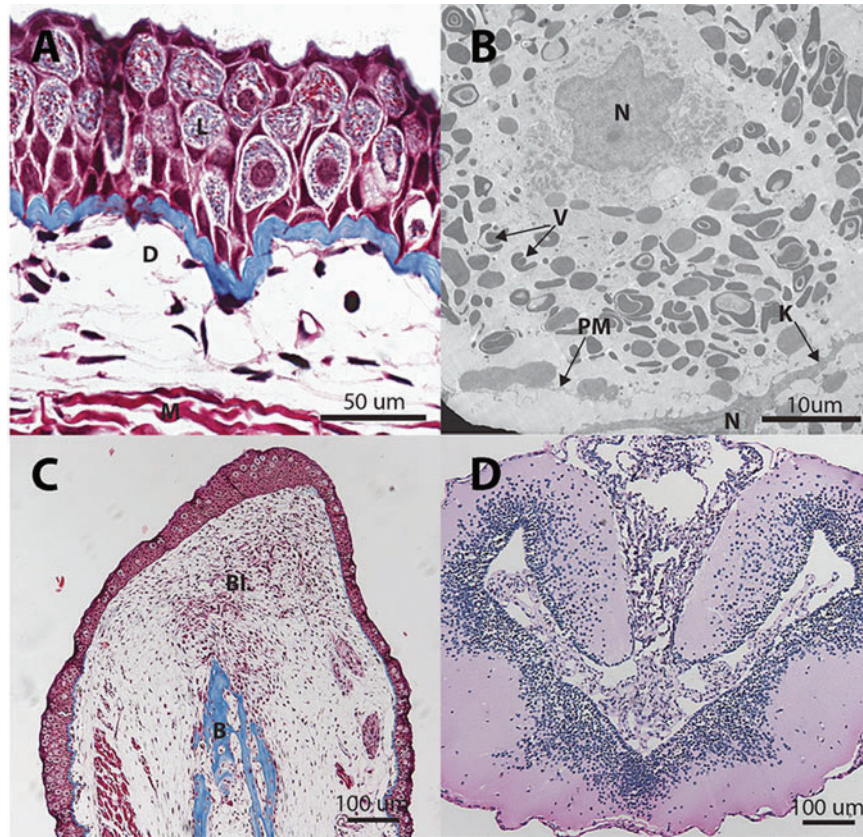


Fig. 3 Histological images of axolotl tissues. (a) Masson's trichrome histological staining of a juvenile axolotl epidermis. Notice the large Leydig cells (L) throughout the epidermis, dermal tissue underneath the epidermis (D), and muscle lying underneath the dermis (M). (b) A transmission electron micrograph of a Leydig cell in a juvenile axolotl epidermis. Notice the nucleus in the middle (N), which is surrounded by rough endoplasmic reticulum and a large number of dense vesicles (V). PM represents the Leydig cell plasma membrane. K represents a keratinocyte with N representing the nucleus of the keratinocyte. (c) Masson's trichrome histological staining of a juvenile mid-bud limb blastema (BL). The bone is indicated with a B. Notice the thickened epidermis on the distal tip of the blastema and mesenchymal cell that makes up the blastema. (d) Hematoxylin and eosin histological staining of a juvenile axolotl brain. The cross section is taken through the posterior telencephalon (forebrain; see Fig. 2). Notice the ventricular zone that contains highly proliferative neural progenitor cells [66]

structural changes during the metamorphic process, particularly in the optic lobes as they grow to accommodate the animal's new mode of binocular vision [61]. This suggests that the axolotl brain remains plastic and capable of drastic change throughout the animal's lifespan, possibly indicating that axolotls would make a favorable model for the study of neural plasticity. Although seemingly possessed of limited intelligence, laboratory axolotls will learn to associate humans with food and will move to the front of their tank in anticipation of feeding. Few studies on axolotl learning have been conducted, but classical conditioning studies

have been performed on tiger salamanders and seem to be most effective when the conditioned stimulus is olfactory in nature [62]. Tiger salamanders have also been trained to complete a T-maze test, a feat which occurs more rapidly and reliably in adult animals than in larvae [63].

Unlike mammals, which possess only limited regenerative potential in the central nervous system, the axolotl CNS can recover from extensive injury. Large portions of the axolotl brain can regenerate fully after injury, and studies have found that they can even recover from complete lobectomy [64, 65]. Neuronal proliferation has been observed all along the ventricular zone of adult brains [66]. Axolotls are also capable of fully recovering from spinal crush and regenerating their tail, spinal column included, after amputation. Both of these regenerative processes occur without the formation of a glial scar [67, 68], which in mammals is thought to inhibit axonal growth after CNS injury. Precisely why the CNS of these animals can heal without glial scarring remains under investigation.

The peripheral nervous system of the axolotl is simple but organized in a manner similar to other tetrapods. The axolotl PNS is of particular interest to researchers because of the phenomenon of nerve-dependent regeneration. If a limb is amputated and then denervated within approximately 7 days—easily accomplished by severing two of the three major nerves at the brachial plexus—the blastema does not form and regeneration does not occur. The cause of this nerve dependency is still under investigation, although it may be due to a loss of nerve-secreted mitogenic factors that are essential for supporting the regenerative process. Axolotl peripheral nerves are myelinated and generally easy to find and sever, particularly in young animals. However, they begin to regrow within 10 days and must be periodically re-severed in studies that last longer than this span. Denervation becomes successively more challenging over time, and consequently it is difficult to maintain a fully denervated state for longer than approximately 20 days.

1.8 Reproductive System Structure

Axolotls reproduce sexually and fertilize internally. Males lay spermatophores which are then taken up and dissolved by the female in the spermatheca, allowing spermatozoa to be stored in the cloaca until it is time for spawning to occur. As eggs leave the oviducts and enter the cloacal chamber, spermatozoa come into contact with the egg and more than one sperm enter the egg cytoplasm [69]. Eggs are usually laid in strings and are protected by a thick coating of sticky jelly, which must be removed prior to embryonic injections or grafting experiments. Though spawnings vary considerably in size, most females will lay between 200 and 1,000 eggs per spawn. Breeding ease and efficiency in the laboratory peaks in the spring and decreases throughout the summer, suggesting that axolotl breeding is seasonal even in animals which have been removed from

their natural environment. This seasonality is probably linked to spermatogenesis, which typically begins in early summer and results in the deposition of sperm into the vas deferens during the winter [70]. Some labs have attempted to overcome this seasonal impediment by hormonally inducing ovulation with injections of human chorionic gonadotropin (hCG) [71]. However, this technique does not seem to affect the rate of spermatophore deposition, which seems to be the limiting step in this process.

Despite the fact that axolotls are commonly and reliably bred in the laboratory setting, much remains unknown about the structure and development of their reproductive system. It is known that axolotls share a conserved inductive mechanism of germ cell development with mammals, rather than the oocyte-derived germplasm observed in *Xenopus laevis* and zebrafish [72]. Structural anatomy of the axolotl ovary has yet to be fully described or characterized, though it has been found that adult females constantly undergo oogenesis and thus their ovaries contain oocytes at all stages of maturation and development [73]. The axolotl ovary can take up a considerable portion of the peritoneum and resides next to the fat bodies (Fig. 2). Although breeding fidelity tends to decrease over time, oogenesis never halts completely. Therefore, although few studies of axolotl ovarian development and anatomy have been conducted, the animal remains an intriguing model of ovarian regeneration and long-term adult oogenesis.

Slightly more is known of the male reproductive system. The axolotl testis is comprised of a variable number of lobes, and older animals tend to have increased numbers of these lobes. Lobes are made of lobules, which resemble sacs and are themselves composed of small spermatogonial cysts, each of which arose from a single stem cell [70]. As noted previously, spermatogenesis begins in the early summer after old cysts from the previous reproductive cycle have been broken down and replaced. By August the testis is swollen and heavy, and sperm are released into the vas deferens in the following months as cysts rupture and then degenerate. Not all individuals adhere strictly to this seasonal pattern, and there is even some variation within a single testis as cysts may mature at different rates. Consequently, an axolotl testis is likely to exhibit cysts at many different stages of development.

2 Materials

2.1 Housing

1. A windowless (*see Note 1*) or blacked-out room held at a constant temperature of 16–20 °C (*see Note 2*).
2. An automatic light timer which controls lighting conditions for a 12L:12D light cycle (*see Note 3*).

3. Deionized water or dechlorinated tap water supplemented with a balanced salt solution at a pH between 7.0 and 7.5. Commercial artificial seawater is sufficient for housing all animals. Classically, 40–50 % Holtfreter's solution has been used for adult housing, which has a conductivity of approximately 2,300 $\mu\text{S}/\text{cm}$ ($\text{NaCl}=30\text{ mM}$, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}=0.4\text{ mM}$, $\text{CaCl}_2=0.45\text{ mM}$, $\text{KCl}=0.33\text{ mM}$) (*see Note 4*).
4. Water can be stored in a large recirculating storage tank or in a food service quality 45-gallon wheeled trashcan (*see Note 5*).
5. For animal storage, 6QT/5.7 L Sterilite plastic boxes or comparable plastic/glass containers.
6. Animals can also be housed in 5-gallon (18.9 L) aquaria with minimal flow through (*see Note 6*).
7. For high animal volume labs, automated flow-through systems can be used to house large quantities of both juvenile (2–3 L tanks) and adult (10 L tanks) animals (*see Note 7*). Water temperature should be set to 18 °C, pH 7.0, conductivity to 800–1,500 $\mu\text{S}/\text{cm}$, with 5 % daily water changes. The water flow in each tank should be kept low.

2.2 Raising Axolotls from Embryos to Adulthood

1. Soda-lime glass bowls, 8 in. diameter.
2. Soda-lime glass bowls, 3.5 in. diameter.
3. 20 % Holtfreter's solution.
4. Aeration bubblers.
5. Soft mesh nets.
6. Artemia brine shrimp and brine shrimp hatchery (*see Note 8*).
7. California Blackworms (*Lumbriculus variegatus*) (*see Note 9*).
8. Soft moist salmon pellets.

2.3 Breeding

1. 28-quart plastic container (58.4 cm L×41.3 cm W×15.2 cm H) lined on the outside with foil.
2. Reusable ice packs.
3. Broken terracotta pots or large flat natural rocks.
4. Soft mesh nets.

2.4 Disease Treatment

1. 35 g/L salt solution.
2. 5 mg/mL amikacin solution.

2.5 Shipping

1. At least two water-sealed containers.
2. Packing materials.
3. Thick, sealable plastic bags.
4. Disposable ice packs.

3 Methods

3.1 Housing

1. Three times a week, change water in animal containers manually using a plastic colander or soft mesh net.
2. Scrub containers thoroughly with a brush at each water change (*see Note 10*).
3. Feed animals on same schedule as water changes.
4. For aquaria, perform 10 % water changes weekly.
5. Check pH, conductivity, and water level of flow-through systems daily. Check ammonia levels every 2–4 weeks once systems are established.
6. Monitor animal health daily.
7. For further sources on axolotl housing, *see Note 11*.

3.2 Raising Axolotls from Embryos to Adults

1. Place embryos in groups of 100 animals in aerated 20 % Holtfreter solution (*see Note 12*).
2. The expected death rate is 10 %. Check for and remove dead embryos daily (*see Note 13*).
3. Change water three times a week.
4. Embryos will hatch out of their eggs on their own and should be separated from unhatched animals at the next water change.
5. Larvae (*see Note 14*) are housed in small groups of 20/container in 6QT plastic containers or 8 in. soda-lime glass bowls.
6. Feed larvae newly hatched artemia brine shrimp daily.
7. Change water three times a week using a soft mesh net.
8. Split groups in half at the first sign of cannibalism and house animals of a similar size together.
9. Transition animals from brine shrimp to aquatic California Blackworms, *Lumbriculus variegatus*, as soon as they are large enough to eat the worms (approx. 3 cm total length) (*see Note 15*).
10. At approximately 3 cm SVL, juvenile (*see Note 16*) house at a density of 5 per 6QT container or 8 in. soda-lime glass bowl.
11. Animals can be transitioned to sinking soft moist salmon pellets at approximately 7 cm SVL.
12. Housing water is changed and animals are fed every Monday, Wednesday, and Friday.
13. House animals that are being used for limb regeneration experiments alone in a 3.5 in. soda-lime glass bowl (*see Note 17*).

14. At approximately 5 cm SVL, animals are transitioned to individual 6QT housing containers or into the automated flow-through systems in 3 L housing containers.
15. Adult (*see* **Note 18**) axolotls are either housed in individual 6QT containers or automated flow-through systems.

3.3 Breeding

1. Place one male and one female together in a 28-quart plastic container covered with aluminum foil. Include two reusable ice packs and a substrate of broken terracotta pots or large flat rocks (*see* **Note 19**).
2. If spermatophores are observed in the tank after the first night, remove the male and add soft mesh nets to the container.
3. The female will lay eggs continuously over the next 12–24 h, usually laying between 200 and 1,000 eggs on the provided substrate.
4. The males can mate once a month and females at maximum every 3 months (*see* **Note 20**).

3.4 Disease Treatment

1. In order to prevent disease, keep animals well fed and in fresh water from the moment eggs are laid to the death of the animal. Chronic stress is the most common cause of illness.
2. Catch signs of poor health early before disease spreads. This usually is apparent by a lack of appetite, loss of coloration in the gills, floating continuously, or loss of the “feathery” appearance of the gills.
3. A *columnaris* bacterial infection manifests as a white fungus-looking growth on the animal’s gills or flank.
4. Treat *columnaris*-infected animals with a high salt bath of 35 g/L for 10 min for three consecutive days [74].
5. *Aeromonas* is a common parasite that manifests as red blotchy skin, oftentimes on the animal’s leg.
6. Treat *Aeromonas* with three intraperitoneal injections of 5 mg/mL amikacin solution at 5 mg/kg body weight separated 48 h apart.
7. For more information on axolotl diseases, please *see* [75].

3.5 Shipping

1. Always ship axolotls with proper institutional animal care approval (*see* **Note 21**).
2. Shipping should be performed in a water-sealed container such as a cooler or Styrofoam box inside a second cardboard box.
3. Place packing material within the interior container.
4. Place animals and water in a thick plastic bag, seal it, and place the bag within a second bag.
5. Seal bags with heat or securely tape them shut (*see* **Note 22**).

6. Disposable ice packs should also be added to the shipping container.
7. Ship overnight only and remove animals from shipping containers to fresh water as soon as they arrive at their destination.

4 Notes

1. Restricting sources of natural light will increase the chances of successful mating taking place during the typically nonbreeding months.
2. Water temperatures higher than 22 °C are stressful on the animals and can have long-term negative effects on axolotl health.
3. A dimly lit room is preferable to a bright one, as axolotls can be stressed by bright lights.
4. Success is also possible at a lower salinity of 750 $\mu\text{S}/\text{cm}$ [76]. Thus, axolotls thrive in a range of salinity conditions.
5. Free-standing water, especially tap water, should be treated with the water conditioners Amquel Plus and Novaqua Plus (Kordon LLC).
6. Aquarium filters should be used, but make sure that the water flow is kept to a minimum, as high flow is irritating to axolotls. Long-term housing in these tanks is reliable without any substrate, but small gravel can be used to act as a host for nitrifying bacteria and supplemented with 20 % crushed coral to facilitate pH balance (Jessica Whited, pers. comm.).
7. The advantage of these systems is that the footprint is relatively small and many of the daily tasks are automated and alarmed over network including water changes, water conductivity, pH, water flow, and temperature. Water-cooling is highly recommended with axolotls considering the heat that is generated by the automated systems. Most modern buildings can leverage process-cooling water, but if this is not available, air-cooled units can be used for chilling as long as an exhaust vent for hot air is built into the animal room. The downside to an automated system is that the water is shared between tanks so disease could spread between animals. Therefore, diligent observation of animal health is critical when using an automated system.
8. Commercial brine shrimp hatcheries work very well for high yields. If the axolotl colony is small, a simple brine shrimp hatchery can be generated using a 500 mL glass bottle with a bubbler set at the bottom placed next to a window. Hatching solution should include 35 g sea salt/L and 1.5 g brine shrimp cysts per 500 mL. Cysts will hatch after approximately 24 h depending upon salinity, heat, light, and batch of artemia so optimization will be needed for each lab.

9. A two-pound shipment of California Blackworms can be kept in the refrigerator in a large plastic container with just enough axolotl housing solution to cover the worms. Worms will survive for weeks if they are washed daily.
10. A small amount of sodium bicarbonate can assist in scrubbing.
11. For advice on establishing a colony, consult the AGSC, read over their guide to axolotl care (www.ambystoma.org), and read the several reviews on raising and caring for axolotls in captivity [11, 55, 76, 77].
12. Embryos left in their jelly coat will hatch in 2–3 weeks. The speed of development can be slowed if embryos are housed in colder temperatures.
13. There is substantial variability in clutch-to-clutch embryo survival so close examination is needed to ensure maximal viability. It is important to remove dead eggs as they will foul the water and negatively affect the development of other embryos. Aerating the water with bubblers will increase animal survival and leads to faster embryo development.
14. Hatched axolotls are considered larvae until their hind limbs have developed to the digit stage.
15. We have found that feeding larval axolotls live blackworms promotes rapid growth.
16. Juveniles are categorized as animals that have completed development, but are not yet sexually mature. Animals range from approximately 3 cm snout tip to vent (SVL) to 10–12 cm SVL. Axolotls do not undergo metamorphosis so it is not possible to determine a true transition from the larval to juvenile stage.
17. This ensures that limbs will be kept intact, as animals housed together may attempt to eat and injure each other.
18. Adults are categorized as animals that are sexually mature. Sexual maturity is highly variable in axolotls ranging from 8 to 15 months. For this reason, we categorize axolotls that are larger than 20 cm snout tip to cloaca length as adults and greater than 10 months of age.
19. Two reusable ice packs are included to lower the temperature of the water to induce male mating behavior. On the first night of mating, males lay spermatophores onto the bottom of the tank, and the female takes up one or more spermatophores into her cloaca. A substrate of broken terracotta pots or large flat natural rocks is added to provide substrate for the males to lay spermatophores.
20. We have found an approximate 30 % success rate in the mating of healthy animals. Reproductive success is variable depending on the season and age of the animals. Matings are generally

more successful during the winter and spring months, with lower success in the summer months.

21. Shipping axolotls is relatively straightforward and safe if the proper measures are taken to safeguard the welfare of the animal.
22. There is enough oxygen in the water for the animals to survive the trip.

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