

Chapter 2

Maintaining Eastern Newts (*Notophthalmus viridescens*) for Regeneration Research

Hans-Georg Simon and Shannon Odelberg

Abstract

The adult Eastern newt, *Notophthalmus viridescens*, has long served as a model for appendage as well as heart muscle regeneration studies. Newt tissues include all major cell types known in other vertebrates and mammals, including bone, cartilage, tendon, muscle, nerves, dermis, and epidermis. Therefore, these aquatic salamanders make an excellent model for studying the regeneration of complex tissues. Regeneration of adult tissues requires the integration of new tissues with preexisting tissues to form a functioning unit through a process that is not yet well understood. Scale is also an issue, because the regenerating tissues or structures are magnitudes larger than their embryonic counterparts during development, and therefore, it is likely that different physics and mechanics apply. Regardless, regeneration recapitulates to some degree developmental processes. In this chapter, we will describe basic methods for maintaining adult Eastern newts in the laboratory for the study of regeneration. To determine similarities and differences between development and regeneration at the cellular and molecular level, there is also a need for embryonic newt tissue. We therefore also outline a relatively simple way to produce and raise newt embryos in the laboratory.

Key words Eastern newt, Red-spotted newt, *Notophthalmus viridescens*, Embryo, Larva, Red eft, Breeding, Spawning, Regeneration

1 Introduction

1.1 Eastern Newts

Eastern newts are urodele amphibians, commonly called salamanders [1]. A native of eastern North America, Eastern newts belong to the genus *Notophthalmus* of which there are three known species: *N. viridescens* (Eastern newt), *N. meridionalis* (black-spotted newt), and *N. perstriatus* (striped newt). *Notophthalmus* is one of only two genera of newts native to the United States, the other genus being *Taricha*, which inhabits primarily the coastal regions of western North America. Taxonomists currently classify Eastern newts into four subspecies—*N. v. viridescens* (red-spotted newt), *N. v. dorsalis* (broken-striped newt), *N. v. louisianensis* (central newt), and *N. v. piaropicola* (peninsula newt). Red-spotted newts are endemic to the northeastern region of the United States but

range as far north as the Canadian provinces Ontario and Quebec; as far south as Alabama, Georgia, and South Carolina; and as far west as Michigan, Indiana, Kentucky, and Tennessee. Central newts range as far west as Texas and Oklahoma and south to the Gulf of Mexico. Broken-striped newts are mostly restricted to the coastal regions of North and South Carolina, and peninsula newts are found in the Florida peninsula.

1.2 Life Cycle

Eastern newts have two distinct features that set them apart from most other vertebrate species—their remarkable regenerative abilities and their unusual and complex life cycle, which can be divided into four major stages, including the embryo, larval, red eft, and adult stages (Fig. 1). Newts have two fascinating courtship and breeding behaviors. Courtship may involve a stereotypical behavior known as “hula” during which the male undulates his body and tail in an effort to entice the female to nudge his tail. After receiving this stimulus, the male deposits a spermatophore. The female then uses her cloaca to pick up the sperm and stores them in a special cavity known as the spermatheca to be used later for fertilizing her eggs. Alternatively, a more common courtship behavior involves amplexus, in which the male grasps the female’s trunk with his large hind limbs and nuzzles her with his snout. Amplexus can go on for several hours before the male dismounts and deposits a spermatophore in front of the female. A female newt fertilizes

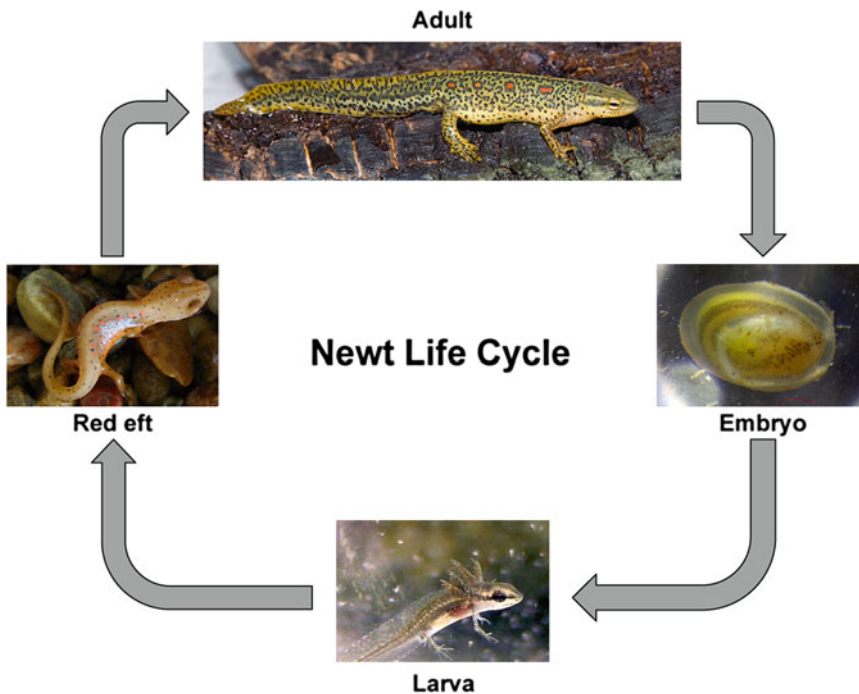


Fig. 1 Newt life cycle

each egg with stored sperm just before depositing the egg on an underwater leaf. She then gently wraps the egg with the leaf to form a protective shield. The fertilized egg develops into an embryo that hatches in about 20–35 days. Over a period of 2–5 months, the gilled larvae develop first forelimbs followed by the hind limbs. When the larvae reach a length of about 35–38 mm, they metamorphose into land-dwelling red eft. These red efts typically grow over a 3-year period before developing into mature adults that are mostly aquatic (Fig. 1). In the wild, newts can live for 12–15 years, which is remarkable longevity for a vertebrate that only weighs 2–3 g. More details concerning the life cycle of the newt have been described elsewhere [2].

1.3 Regenerative Abilities

Although the ancient Greeks knew about regeneration in vertebrate species as evidenced both by Aristotle's notation in *Historia Animalium* that lizards can regenerate their tails and the Greek myth of liver regeneration in Prometheus, the first known scientific study of regeneration in a vertebrate was published by the Italian scientist Lazzaro Spallanzani in 1768 [3]. Spallanzani was able to show that the aquatic salamander (most likely a newt) is able to regenerate its forelimbs and hind limbs, tail, upper and lower jaws, and caudal spinal cord. He also showed that aquatic salamanders could repeatedly regrow a limb even after multiple amputations. Over the past decades, major discoveries have been made using the newt as a model organism for regeneration. In fact, the adult Eastern newt (most often the red-spotted newt) has long served as a primary model for the study of epimorphic regeneration of amputated limbs and tails [4, 5]. Similar to the limb, in a study spanning 16 years, Goro Eguchi and coworkers demonstrated that the regenerative capacity of the newt lens is not altered by repeated regeneration and aging [6]. It has also been shown that during lens regeneration, pigment epithelial cells of the iris can transdifferentiate to lens cells [7]. The plasticity of differentiated cells has been a great focus in regeneration studies. Adult newt cardiomyocytes were shown to reenter the cell cycle [8, 9], and high resolution 3D imaging as well as modern lineage tracing methods have revealed that during limb regeneration, newt multinucleate myofibers dedifferentiate and fragment to form proliferating mononuclear cells that give rise to new skeletal muscle in the regenerated limb [10, 11, 12, 13]. Moreover, the newt not only regenerates the caudal spinal cord following tail amputation but can also regenerate the trunk spinal cord following a complete transection and regain function of initially paralyzed appendages caudal to the injury site [14, 15]. Modern cellular and molecular studies have also shown that parts of the brain can regenerate by activation of quiescent regions of the adult newt brain [16, 17].

As illustrated above, the advent of new methodologies has opened an era in which the newt has become an attractive model to study the cellular and molecular basis of regeneration in a vertebrate species.

This chapter focuses on the maintenance of Eastern newts in a laboratory setting for the purpose of studying their remarkable regenerative abilities.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. The housing and maintenance of animals must follow the guidelines of and be approved by the Institutional Animal Care and Use Committee.

2.1 Adult Newts

Notophthalmus viridescens can be purchased from Connecticut Valley Biological Supply Company (Southampton, MA) or Charles D. Sullivan Co (Nashville, TN) (*see* **Notes 1–3**).

2.2 Newt Water

Deionized and dechlorinated water supplemented with 0.0375 % Instant Ocean (Aquarium supply store). We use Instant Ocean salt at a very low concentration, which is not comparable to the concentrations typically used in salt-water aquariums (*see* **Note 4**).

2.3 Aquarium Setup and Maintenance

1. In a dedicated room with controlled temperature of 20–22 °C and day-night light cycle, set up 80 L glass tanks (61 cm×32 cm×42 cm) (Aquarium supply store) with lid (Fig. 2a).
2. Cover bottom of aquarium with a 3–4 cm layer of small pea gravel (Aquarium supply store) (Fig. 2a, b).
3. Fill aquarium halfway with approximately 38 L deionized water.
4. In a 4 L plastic beaker, dissolve 15 g Instant Ocean in 2 L of deionized water and add to tank.
5. The conductivity of the water in the aquarium should be approximately 600 µSI. Read the conductivity with a Nester Micro MHO Pen or similar device every month! When conductivity has doubled, dilute water with deionized water.
6. The water pH should be in a range of 7–8. Determine the pH with a calibration-free pH meter every 2 weeks! If pH is getting low, adjust with 1 M NaOH.
7. For water aeration, circulate water using a pump with integrated carbon filter. To maintain water quality, use a filtering system like the H.O.T. Magnum BIO-PRO System (Marineland), which contains in a canister a container with activated carbon (removes toxic chemicals and dissolved organic pollutants), micron cartridge (traps microscopic dirt particles), and Rite-Size sleeve prefilter (screens out free-floating

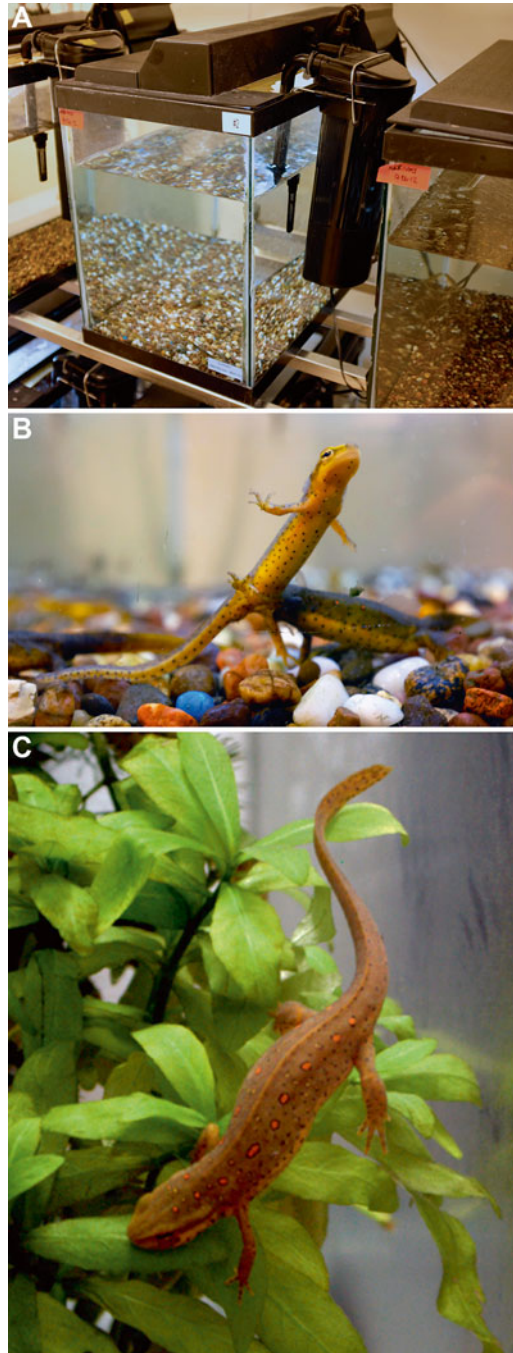


Fig. 2 Aquarium setup and swimming adult newts. (a) 80 L aquariums are supported by metal shelves in temperature- and light cycle-controlled room. (b) A layer of pea gravel covers the aquarium bottom with (c) aquatic plants rooted into the gravel

dirt and debris) plus a magnetic impeller module that pumps water through the filter. Adjust water level every week.

2.4 Food

1. Beef heart (local butcher). Mince in food processor and keep small portions packaged in plastic bags frozen at -20°C .
2. *Daphnia* sp., *Tubifex* sp., and California blackworms (Aquatic Foods; see **Note 5**). Note that newts prefer live worms over frozen food.

2.5 Breeding, Spawning, and Rearing of Newt Larvae

1. Environmental enrichment for breeding: In addition to the described aquarium setup, root into the gravel a mixed variety of aquatic plants, e.g., *Sagittaria* sp. and *Elodea* sp. (Fig. 2c). Newts also like to spend time out of the water, so you may want to add several rocks that generate a ramp out of the water.
2. Newt eggs and larvae: Keep deposited eggs and rear hatched newt larvae in small clear plastic containers ($30\text{ cm} \times 18\text{ cm} \times 13\text{ cm}$). Change water daily.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. The use of animals must follow the guidelines of and be approved by the Institutional Animal Care and Use Committee.

3.1 Animal Housing and Care

1. Keep groups of approximately 30 adult newts in a 20°C temperature-controlled room in 80 L aquariums with circulating water (Fig. 2a–c).
2. Feed newts live *Tubifex* sp., California blackworms, or frozen beef heart every 3 days.

3.2 Breeding, Spawning, and Rearing of Newt Larvae

We have experienced that females caught in the wild during spring will spontaneously deposit eggs if housed in an aquarium that contains a variety of aquatic plants (Fig. 2c).

1. Mix several sexually mature males and females in a tank and feed well. Noticeable gravid females (females with swollen abdomens) and males with blackened distal hind limb digit pads are the most suitable for mating (see **Note 6**). A supply of 100–200 eggs per aquarium with a group of 20 mixed female and male animals can be achieved over a period of 2–4 weeks.
2. Inspect your spawning chamber daily for deposited eggs. Typically, females will attach the eggs to the underside of the plant's leaves, at times rolling a leaf around a single egg or cluster of deposited eggs. Freshly laid eggs are about 1.5 mm in diameter.
3. Remove the egg-laden plant sprigs and transfer them to a clear plastic container. The eggs develop well in room temperature newt water and hatch in 20–35 days to produce free-swimming larvae.

4. Carefully transfer the larvae to a separate clear plastic container filled with newt water and supplemented with *Daphnia* sp., which the larvae will consume.
5. The larvae develop rapidly and once they mature, you can detect prominent gills. These larger larvae require live, moving food, and we found *Tubifex* sp. worms ideally suited.
6. Change the water daily in these small plastic containers. To enhance the animals' environment, add aquatic plants to float in the water.
7. The newt larvae will eventually metamorphose into red efts. This is a physiologically and morphologically significant event, as the animals will resorb their external gills and switch to lung breathing. When you notice signs of this transformation, transfer these more developed animals into a larger chamber that includes an aquatic area with floating *Elodea* sp. plants and a terrestrial component so that the animals can leave the water.
8. Continue feeding the red efts with *Daphnia* sp. and *Tubifex* sp. The mix of land and water with abundant food supply provides an environment the efts will thrive well in. We note that investigators have developed protocols for induced spawning, and we refer to this published work for a more detailed setup description [18, 19].

4 Notes

1. Newts freshly shipped from the vendor should be kept separate for 2 weeks before using for experimentation. Occasionally, a sick newt is among the shipments, and keeping animals "in quarantine" helps to prevent spreading of a potential disease.
2. Newts can be handled by gently picking them up by the tail with gloved hands. Unlike some lizards, which readily discard their tails as a defense mechanism, the tail of the newt cannot be discarded and therefore picking up the newt by the tail is an appropriate method for quickly transferring animals from one location to another. If the newt is to be handled for any length of time beyond a simple transfer, its body should be supported by the palm of the hand.
3. The skin of both Eastern and Western newts often contains a potent neurotoxin known as tetrodotoxin, which binds to and blocks voltage-gated sodium ion channels. The concentration of tetrodotoxin has been shown to vary widely between individuals depending on location, diet, and possibly other unknown factors. Although controversy still remains as to the origin of the tetrodotoxin, several studies suggest that in Eastern newts, the toxin might be of dietary origin and if newts

are fed a toxin-free diet, they can lose their toxicity over a period of several years [20]. Regardless, when handling newts at any stage of their lives, it is best to wear gloves. If a newt is handled without gloves, the hands should be washed immediately afterwards. Needless to say, unlike axolotls, which were regularly eaten in Mexico before they became critically endangered (or possibly extinct in the wild), newts should never be consumed.

4. In the wild, newts live in and by water streams, and therefore, in principle, they can be kept in tap water in the laboratory. However, in most cities, the drinking water is chlorinated to an extent that it could be harmful to the animals. Therefore, if chlorinated tap water is used, it should be sufficiently dechlorinated by allowing it to stand exposed to the air for a sufficient time period for the chlorine to evaporate (usually several days). However, many municipalities are treating their water supply with monochloramine, rather than chlorine. Monochloramine cannot be removed by evaporation. Therefore, we prefer to carefully prepare our own newt water by supplementing the deionized water supply in the laboratory with low amounts of Instant Ocean salt to achieve a conductivity of approximately 600 μSI (equivalent to Evian Water). We have found that the method described in this chapter produces reliable results that allow for the maintenance and care of a healthy population of newts.
5. California blackworms can also be purchased from Eastern Aquatics. Website: currently easternaquatics.com.
6. There is considerable competition among males for the right to mate with females. Often males will try to dislodge a male that is in amplexus with a female. Occasionally, a different male not in courtship deposits his spermatophore between the spermatophore of a male in courtship and the female in an attempt to get the female to collect sperm from his spermatophore rather than the spermatophore from the male in courtship.

Acknowledgments

We would like to acknowledge both current and former researchers in our respective laboratories who helped develop the protocols described in this chapter. These individuals include Paul Khan, Barbara Linkhart, Claudia Guzman, Sarah Calve, Sarah Mercer, Donald Atkinson, Vladimir Vinarsky, Tamara Stevenson, David Kent, and Katherine Zukor. We would also like to acknowledge several of our colleagues who provided us with invaluable advice when we were just beginning our studies on regeneration using this remarkable animal, including Cliff Tabin, Mark Keating, Jeremy Brockes, David Stocum, Roy Tassava, Panagiotis Tsonis, and Anthony Mescher.

References

1. Duellman WE, Trueb L (1986) *Biology of amphibians*. McGraw-Hill Book Company, New York, NY
2. Petranka JW (1996) *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, DC
3. Spallanzani L (1769) *An essay on animal reproductions*. Translated from Italian by M. Maty. Printed for T. Becket, and P. A. de Hondt, in the Strand, London. Available through UMI Books on Demand
4. Wallace H (1981) *Vertebrate Limb Regeneration*. J Wiley and Sons Ltd., Toronto, ON
5. Nye HL, Cameron JA, Chernoff EA, Stocum DL (2003) Regeneration of the urodele limb: a review. *Dev Dyn* 226:280–294
6. Eguchi G, Eguchi Y, Nakamura K, Yadav MC, Lillan JL, Tsonis PA (2011) Regenerative capacity in newts is not altered by repeated regeneration and ageing. *Nat Commun* 2:384. doi:[10.1038/ncomms1389](https://doi.org/10.1038/ncomms1389)
7. Eguchi G, Itoh Y (1982) Regeneration of the lens as a phenomenon of cellular transdifferentiation: regulability of the differentiated state of the vertebrate pigment epithelial cell. *Trans Ophthalmol Soc U K* 3:380–384
8. Oberpriller J, Oberpriller JC (1971) Mitosis in adult newt ventricle. *J Cell Biol* 49:560–563
9. Bettencourt-Dias M, Mitnacht S, Brockes JP (2003) Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. *J Cell Sci* 116:4001–4009
10. Calve S, Odelberg SJ, Simon H-G (2010) A transitional extracellular matrix instructs cell behavior during muscle regeneration. *Dev Biol* 344: 259–271
11. Calve S and Simon H-G (2011) High resolution 3D imaging: Evidence for cell cycle reentry in regenerating skeletal muscle. *Developmental Dynamics* 240:1233–1239
12. Lo DC, Allen F, Brockes JP (1993) Reversal of muscle differentiation during urodele limb regeneration. *Proc Natl Acad Sci U S A* 90: 7230–7234
13. Sandoval-Guzmán T, Wang H, Khattak S, Schuez M, Roensch K, Nacu E, Tazaki A, Joven A, Tanaka EM, Simon A (2014) Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell* 14:174–187
14. Piatt J (1955) Regeneration of the spinal cord in the salamander. *J Exp Zool* 129:177–207
15. Davis BM, Ayers JL, Koran L, Carlson J, Anderson MC, Simpson SB Jr (1990) Time course of salamander spinal cord regeneration and recovery of swimming: HRP retrograde pathway tracing and kinematic analysis. *Exp Neurol* 108:198–213
16. Okamoto M, Ohsawa H, Hayashi T, Owaribe K, Tsonis PA (2007) Regeneration of retino-tectal projections after optic tectum removal in adult newts. *Mol Vis* 13:2112–2118
17. Berg DA, Kirkham M, Beljajeva A, Knapp D, Habermann B, Ryge J, Tanaka EM, Simon A (2010) Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain. *Development* 137:4127–4134
18. Khan PA, Liversage RA (1995) Development of *Notophthalmus viridescens* embryos. *Dev Growth Differ* 37:529–537
19. Khan PA, Liversage RA (1995) Spawning of *Notophthalmus viridescens* embryos. *Herpetol Rev* 26:95–96
20. Yatsu-Yamashita M, Gilhen J, Russell RW, Krysko KL, Melaun C, Kurz A, Kaufenstein S, Kordis D, Mebs D (2012) Variability of tetrodotoxin and of its analogues in the red-spotted newt, *Notophthalmus viridescens* (Amphibia: Urodela: Salamandridae). *Toxicon* 59:257–264

Salamanders in Regeneration Research

Methods and Protocols

Kumar, A.; Simon, A. (Eds.)

2015, X, 357 p. 89 illus., 81 illus. in color., Hardcover

ISBN: 978-1-4939-2494-3

A product of Humana Press