Chapter 4

Husbandry of Spanish Ribbed Newts (*Pleurodeles waltl*)

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Abstract

Research on urodele amphibians, such as newts, is constantly contributing to our understanding of fundamental biological processes. In the present chapter, we present detailed husbandry protocols for the Spanish ribbed newt (*Pleurodeles waltl*). We describe the main phases of their life cycle, with emphasis on the progressive development of sensory, motor, and integration systems, which lead to the acquisition of specific stereotyped (and conditioned) behaviors. The methods are outlined to manage housing, feeding, handling, captive breeding, health monitoring, and euthanasia in this species under laboratory conditions. With minor changes, these protocols can also be applied to other species of urodele amphibians commonly used in laboratory research.

Key words Salamander, Housing, Environmental enrichment, Reproduction, Breeding, Larvae, Development

1 Introduction

Urodele amphibians became well known by scientists in the past, mostly due to their large embryos, the relatively easy manipulation, and their external and slow development that can be modulated by temperature [1–4]. All these facts together permitted to easily analyze the key stages during ontogenetic development, and as a result, research in urodeles provided significant knowledge regarding the ontogeny of vertebrates [2, 5–9]. In addition, the absence of rejection after transplant [2, 10], their extraordinary regenerative abilities [11–15], the large size of their neurons and neuroendocrine cells [2], and the secondary simplification that characterizes their nervous system [16, 17] have attracted the attention of generations of researchers. Since urodeles have become amenable to modern molecular technologies, such as transgenesis and genome editing, they provide reemerging animal models in particular for studying development and regeneration [2, 15, 18–31]. The Spanish ribbed newt is a relatively large urodele species natural of the Iberian Peninsula and Morocco that can reach 31 cm long,

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although most individuals' length ranges between 14 and 18 cm [32–35]. Wild populations develop two types of lifestyle, aquatic and terrestrial, although the latter mostly happens in order to survive either the drying of water masses in which they live or extremely cold environments [33, 35]. In the cases in which the place does not dry up and remain suitable, the Spanish ribbed newts carry out an entirely aquatic lifestyle [32, 36]. Accordingly, in captivity, they can be housed in entirely aquatic systems, provided with environmental enrichments, such as floating surfaces that some individuals eventually use [32]. Wild *Pleurodeles* can be found in shores, shallow and deepest parts of ponds, streams, and small lakes [33], although some authors suggest that they are more difficult to be seen during daytime, when they seem to remain in the deepest parts, hidden either among the aquatic vegetation or under stones [35, 37]. Therefore, for environmental enrichment, it is important for the adults to facilitate them the access to floating surfaces, aquatic plants, and hiding places. The diet during the adult aquatic stage is based on a wide spectrum of invertebrate and vertebrate species such as worms, crustaceans, snails, aquatic insects, small fish, and amphibians, even practicing cannibalism. Similarly, the larvae prey principally on different species of zooplankton, insects, and amphibian larval forms, including conspecifics [33, 35]. Two main conclusions can be obtained from this information: (1) it is recommended to provide more than just one source of food, and (2) the animals must be housed with tank mates of the same size in order to avoid loss of animals due to cannibalistic behaviors.

The breeding season varies in the different wild populations depending upon the environmental conditions, being restricted to the spring time in places where winter is harsh. However, in some temperate areas, the breeding season usually extends from September to April, coinciding the starting with the first rains of autumn [33, 35]. Mating occurs by spermatophore transfer after amplexus, in which the male holds the female with his forelimbs, placing her on his back. After several hours, the male twists around and deposits a spermatophore which is up taken by the female after the male manipulates her body [33, 35, 37–39]. 24–48 h later, the female start to lay a variable number of eggs, which is correlated with her age and size [33, 35, 39]. The embryonic and larval development takes 2–6 months in the wild, as is dependent on environmental factors, such as temperature, and the size of juvenile newts at metamorphosis is very variable [33, 35]. The specific developmental stages can be defined as outlined in Table 1 [40–47]. According to Gallien and Durocher [41], the different stages of the Spanish ribbed newt development are grouped in three principal periods: embryonic life (till hatching), initial larval period (ending at the beginning of feeding),

Table 1
Comparative depiction of developmental periods of commonly used salamander species based on external characters and physiological and behavioral key events

Life history	Am	Pw	Nv	Development of external characters	Development of key physiological and behavioral events
Embryonic stages	1–41	0–34	Tailbud stage, limb field stage bud stage	– Fertilization, first cleavages (*Pw* St. 0–4). – Blastulation (*Pw* St. 5–7). – Gastrulation (*Pw* St. 8–13). – Neurulation, somatogenesis (5 pairs of somites), eye primordium formation (*Pw* St. 14–21) – Somatogenesis (from 5 to 9 pairs of somites): brain flexure and growth, pronephros and tailbud formation, otic placode, and gill primordia appearance (*Pw* St. 22–24) – Tailbud (from 10 to 19 pairs of somites): brain growth and early segmentation, branchial furrowing, body axis straightening, tailbud enlargement, olfactory organ primordium, melanophores in trunk, and balancer appearance (*Pw* St. 25–29) – Limb field (from 20 somites on): brain regionalization, lateral line formation, appearance of melanophores in head region, unbranched gills enlargement, and circulation establishment in gills and tail (*Pw* St. 30–32) – Bud stage I: beginning of brain maturation, forelimb bud primordium formation, eye maturation, operculum and mouth formation, gill branching and elongation, second lateral line bypasses the forelimb on the ventral side (*Pw* St. 33) – Bud stage II: growth and maturation of hindbrain, mouth opening, forelimb bud formation, balancer elongation (*Pw* St. 34) *Peculiarities*: – In Notophthalmus viridescens, the embryo is encapsulated in a series of five concentric jelly layers. Due to the limited sac space and the strong layers covering it, the embryo is specially curved as growing proceeds – *Ambystoma mexicanum* don't develop balancers	Development of sensory systems – Mechanoreceptive endings in the skin – Skin excitability to noxious stimuli – A visual system firstly based on the pineal eye, which is excited by dimming Development of locomotion in urodeles – First movements (head flexure stage) – C-coil stage – S-wave stage – Swimming Miscellaneous – Primitive reflex mechanism: Muscular response to a mechanical stimulus – Heart beating appearance – Spontaneous muscular movements – Sporadic swimming – Hatching
Initial larval stages	42–44	35–38	Peg stage, e2D stage	– *Peg stage*: larva transparent, internal organs including heart, stomach, visceral arches, and auditory capsules are visible; balancer at the maximum point of development, hindbrain and midbrain show more growth and maturation than forebrain areas (*Pw* St. 35–36) – *e2D stage*: forelimb bud elongation (final shape: paddle), mouth opening, gill branching and elongation, yolk absorption, hindbrain, and midbrain show more growth and maturation than forebrain areas (*Pw* St. 37–38)	– Use of balancers to remain adhered in all surfaces – Steady behavior – Escape response triggered by visual/vibrational stimuli – Feeding: stereotyped suction marks the end of this period

(continued)

Table 1
(continued)

Life history	Am	Pw	Nv	Development of external characters	Development of key physiological and behavioral events
Early active larval stages	45–52	39–45	*Forelimb*: 2D, e3D, 3DI, 3DII, 4D *Hind limb*: Limb field, bud I, bud II, bud III	– Formation of three fingers and elbow articulation in forelimbs, hind limb bud primordia appearance, balancer thinning, gill branching and elongation, numerous melanophores distributed in the caudal fin, main subdivisions of the brain already present (*Pw* St. 39–42) – Formation of the forth finger in forelimbs, hind limb bud formation, balancer reduction, differential growth of the forebrain areas (*Pw* St. 43–45)	– Scouting: head movements from side to side – Feeding: acquisition of lunging – Learning: modulation of suction + lunging – Cannibalistic behavior, more common at high density of larvae – Coordinated walking in the bottom with the forelimbs
Late active larval stages	53–57	46–55c	*Hind limb*: eT, 2T, 3T, 4T, 5T	– Balancer disappearance, formation of two fingers in hind limbs (*Pw* St. 46–49) – Formation of the five fingers and articulation in hind limbs, gills at the maximum point of development, growth and maturation of the brain, specifically forebrain basal ganglia (*Pw* St. 50–55a) – Gills reduced to half of their size, fin regression, limbs thickening, skin transformation in trunk region (*Pw* St. 55b) – First molting, skin transformation in the whole animal, flattened head, gills extremely reduced (*Pw* St. 55c)	– Scouting: head movements combined with walking in the active seeking for food – Feeding: acquisition of the use of olfactory cues – Learning: care taker recognition – Cannibalistic behavior modulated by learning – Progressive use of the hind limbs – Pulmonary breathing – Floating
Juvenile, adult	57, 57+	56, 56+	Eft, adult	– Metamorphosis I: complete disappearance of fin and gills, skin change, eyes reorientation (*Pw* St. 56) – Body growing, maturation of the brain (specifically hypothalamus), sexual maturation (*Pw* St. 56+) – Metamorphosis II: skin change, fin development, brain completely developed (*Pw* St. 56+) – *Ambystoma mexicanum* don't metamorphose	*Notophthalmus viridescens* and *Pleurodeles waltl* – Terrestrial lifestyle of the eft – Aquatic lifestyle of breeding adults – Terrestrial and aquatic lifestyles of nonbreeding adults depending on environmental cues – Feeding: jaw prehension or biting – Feeding: tongue prehension *Ambystoma mexicanum* – Completely aquatic lifestyle

Am Ambystoma mexicanum, *Nv Notophthalmus viridescens*, *Pw Pleurodeles waltl*, St stage

and active larval life (in which limbs and complex behaviors develop) finishing at metamorphosis. In the following paragraph, more details of the different stages are given.

Throughout embryonic development (stages 0–34), in addition to the body plan formation, visual and olfactory primary organ primordia develop, together with the mechanoreceptory and chemoreceptory lateral line [41, 48]. The motor system also appears, and basic stereotyped movement acquisition ends up in swimming [49–51]. Before hatching, at each side of the head a balancer appears (a filament-like appendix used for remaining adhered to different surfaces), followed by the development of three branchial arches. During initial larval life (stages 35–38), behind the branching gills, the forelimb buds arise, and the visual and lateral line systems mature along with the escape response associated to visual and mechanical stimuli, although most of the time the larvae remain steady, absorbing the remaining yolk [26, 27, 52–54]. By the end of this period, larvae start to eat by stereotyped suction mechanism, driven also by visual and vibrational stimuli, but without active food searching. Consequently, in the laboratory, live foods such as *Artemia* nauplii should be provided. At this point, the active larval life begins and lasts about 3 months in *Pleurodeles waltl*, during which many morphological variations occur related to key physiological and behavioral changes [26, 27, 41, 45]. The active larval period can be further divided into two phases: early active larval period (stages 39–45, when forelimbs develop) and late active larval period (stages 46–55c, when hind limbs develop) [26, 27]. In the early active larval period, the most remarkable external changes include the loss of the balancers and forelimb development. In terms of hunting behavior, this period is driven by a refinement of the suction technique, related to scouting the surroundings by first forelimb-driven walking steps together with head movements from side to side, and the acquisition of lunging. During the late active larval period, the hind limbs are formed, a major growth in the body occurs, and the main structures present in the adult brain can be recognized [26, 27, 41, 45]. From this period onwards, the olfactory system start to be important for feeding, as active seeking followed by sniffing results in the capacity of predating on immobile food such as commercial fish pellets or frozen food. In addition, learning processes by classical conditioning can be assessed during this period, as larvae show different behaviors when raised in different conditions.

The present chapter describes husbandry protocols for successfully caring, breeding, and raising *Pleurodeles waltl* in the laboratory in a simplified manner with the aim of making the caretaker job as easy and efficient as possible.

2 Materials

2.1 Tanks, Containers, and Environmental Enrichment Complements

In general, most of the supplies available from Aquarium suppliers are sufficient.

1. Rack system: optional (Fig. 2a).
2. Plastic boxes in different sizes: pay attention to the maximum number of animals that are recommended to be housed together (Fig. 2b).
3. Aquaria: pay attention to the maximum number of animals that are recommended to be housed together (Fig. 2b).
4. Gravel: large size to avoid accidental ingestion.
5. Tiles: can be used either whole for big setups or broken in pieces for smaller tanks/containers.
6. Stones: select the irregular ones that can provide hiding places.
7. Natural or artificial plants.
8. Cork pieces/styrofoam: the borders ought to provide a shore-like structure.

2.2 Aquarium Setup, Maintenance, and Food

1. Filters (e.g., Fluval U2, U3, or U4 internal filters) or similar biofilters.
2. Air pumps with accessories.
3. Syphon (e.g., Marina Easy clean gravel cleaner).
4. Nets and filter-cleaning brushes.
5. *Artemia* hatchery.
6. Tap water conditioner (e.g., Nutrafin Aqua Plus Tap water conditioner).
7. Broad spectrum antibiotic/antimycotic (e.g., General tonic from Tetra Medica).
8. Marine salts (artificial aquarium sea salt mixture).
9. Live *Tubifex* worms.
10. Live Grindal worms.
11. Artemia cysts.
12. Frozen food and pellets.

2.3 Laboratory Supplies and Equipment

1. Surgical no. 5 forceps.
2. Plastic Pasteur pipettes.
3. Surgical scalpels.
4. 24-well plates.
5. Petri dishes (150 mm × 25 mm cell culture dish).
6. Stereomicroscope with fiber optic illumination.

2.4 Reagents and Solutions

1. Ultrapure water (distilled water).
2. Human chorionic gonadotropin (e.g., Sigma-Aldrich, cat no. C8554).
3. Cysteine (L-cysteine hydrochloride hydrate).
4. MS-222 (Tricaine methanesulfonate): Dissolve 1 g of MS-222 in 1 L of dechlorinated tap water.
5. 10× Modified Holtfreter's solution (MHS): Dissolve 34.6 g of NaCl, 0.5 g of KCl, 1 g of $CaCl_2$, 2 g of $NaHCO_3$, and 2 g of $MgSO_4$ in 1 L of ultrapure water. Store at 4 °C.
6. 20 % MHS (for embryos): Dilute 20.4 mL of 10× stock MHS in 1 L of distilled H_2O. Add 10 mL of penicillin-streptomycin. Adjust the pH to 7.6 with 1 N NaOH.
7. 40–50 % MHS (for larvae and adults): Dilute 41.6–52.6 mL of 10× stock MHS in 1 L of distilled H_2O. Adjust the pH to 7.6 with 1 N NaOH.
8. 1× MHS (for recovering adults, unfertilized eggs, and sperm solution): Dilute 111.1 mL of 10× stock MHS in 1 L of distilled H_2O. Add 10 mL of penicillin-streptomycin. Adjust pH to 7.6 with 1 N NaOH.
9. Marine salt solution: dissolve 30 g of marine salts in 1 L of distilled H_2O.

3 Methods

3.1 Housing Metamorphic/Adult Newts

Although the metamorphic ribbed newts are air breathers, in nature this species is mostly aquatic and remains all the year in water if the conditions are favorable (*see* **Note 1**). As the laboratory conditions are quite stable, there is no need for the animals to undergo hibernation or aestivation, and newts can be housed in an aquatic-based environment described herein, supplemented with just a floating surface (Fig. 1a).

1. Prepare the location in which the animals will be settled, having in mind ventilation, light/darkness (*see* **Note 2**) conditions, and possible temperature fluctuations (*see* **Note 3**).
2. Select the appropriate container according to the number of animals that are to be housed (Fig. 2).
3. Brush the tank (*see* **Note 4**) under hot tap water, and give a final rinse it in cold tap water.
4. Fill up the tank with chlorine-free water (*see* **Note 5**).
5. Add environmental enrichment objects (*see* **Note 6**) distributed evenly in the tank, providing at least a floating surface, artificial plants, and hiding places (Fig. 1a).
6. Add the filter (*see* **Note 7**), heater, and air pump if desired.

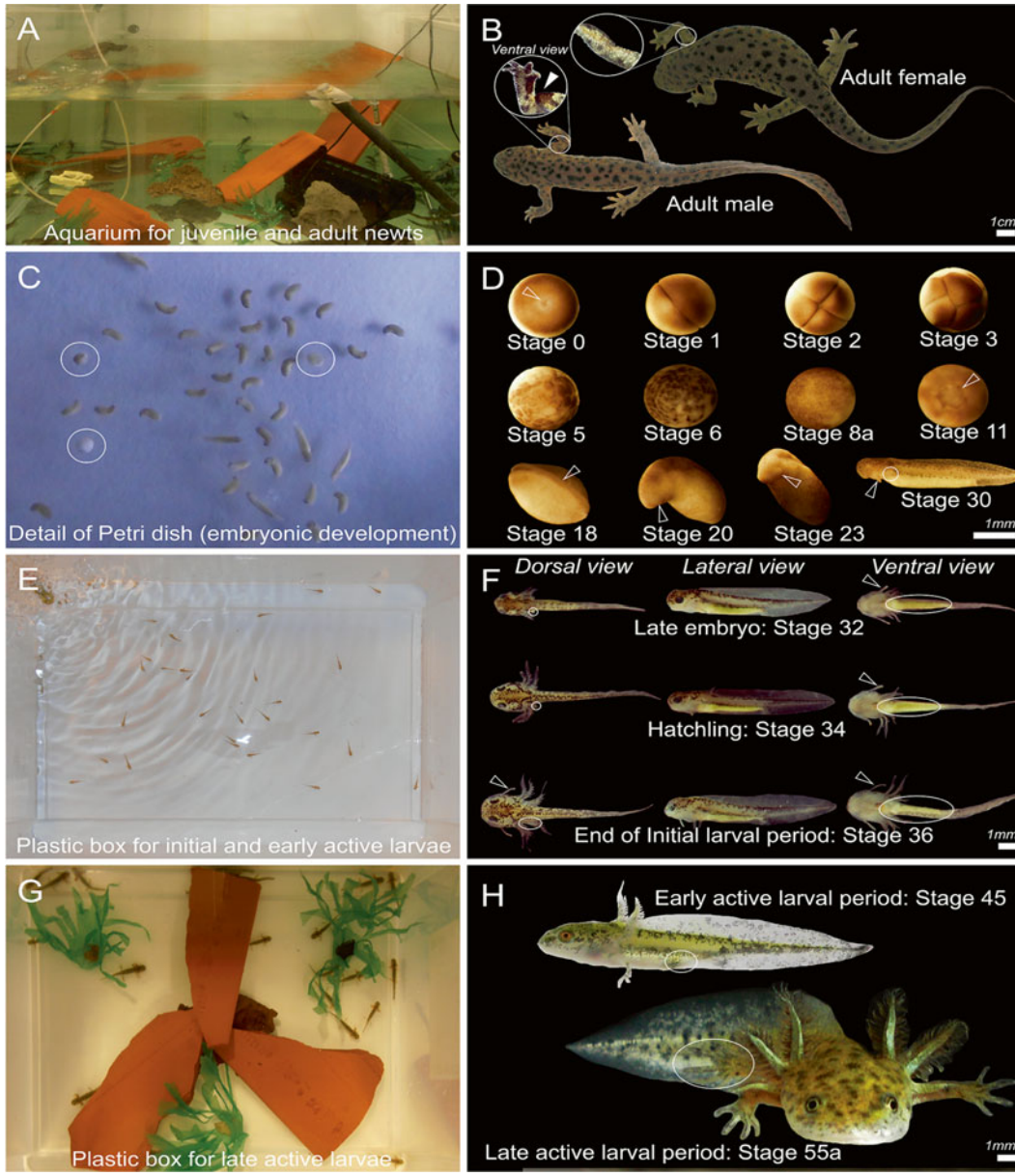
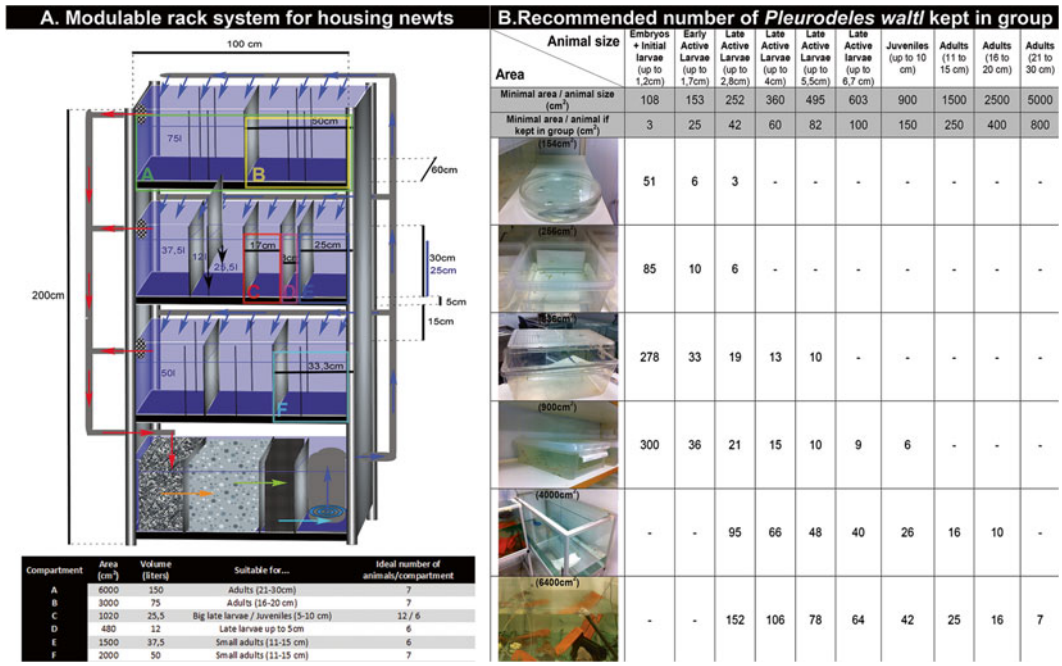


Fig. 1 Setups for Spanish ribbed newts and key features of the different developmental stages. (**a**) Aquarium for juveniles and adults. (**b**) Adult newts. Note the difference in the shape of the body and the specialization of the male forelimbs (*arrowhead*). (**c**) Detail of a Petri dish containing *Pleurodeles* embryos. Note the contaminated eggs that should be removed. (**d**) Representative stages of embryonic development, according to Gallien and Durocher [41]. The *arrowheads* point to the blastopore at stage 11, the closure of the neural tube at stage 18, the eye protrusion at stages 20 and 23, and the balancer at stage 30; the gill primordia are *encircled* at stage 30. (**e**) Box for housing initial and early active larvae. (**f**) Dorsal, lateral, and ventral views of late embryo, hatchling, and initial larva; note the progressive development of the forelimb buds (*small circles* in dorsal view), the development of the digestive system (*big circles* in ventral view), and the maintenance of the balancer (*arrowheads*). (**g**) Box for late active larvae. Observe the even distribution of environmental enrichment objects. (**h**) Early and late active larvae, showing the appearance and development of the hind limbs (*circles*)



Compartment	Area (cm^2)	Volume (liters)	Suitable for...	Ideal number of animals/compartment
A	6000	150	Adults (21-30cm)	7
B	3000	75	Adults (16-20 cm)	7
C	1020	25,5	Big late larvae / Juveniles (5-10 cm)	12 / 6
D	480	12	Late larvae up to 5cm	6
E	1500	37,5	Small adults (11-15 cm)	6
F	2000	50	Small adults (11-15 cm)	7

B.Recommended number of *Pleurodeles waltl* kept in group

Area \ Animal size	Embryos + Initial larvae (up to 1,2cm)	Early Active Larvae (up to 1,7cm)	Late Active Larvae (up to 2,8cm)	Late Active Larvae (up to 4cm)	Late Active Larvae (up to 5,5cm)	Late Active larvae (up to 6,7 cm)	Juveniles (up to 10 cm)	Adults (11 to 15 cm)	Adults (16 to 20 cm)	Adults (21 to 30 cm)
Minimal area / animal size (cm^2)	108	153	252	360	495	603	900	1500	2500	5000
Minimal area / animal if kept in group (cm^2)	3	25	42	60	82	100	150	250	400	800
(154cm²)	51	6	3	-	-	-	-	-	-	-
(256cm²)	85	10	6	-	-	-	-	-	-	-
(836cm²)	278	33	19	13	10	-	-	-	-	-
(900cm²)	300	36	21	15	10	9	6	-	-	-
(4000cm²)	-	-	95	66	48	40	26	16	10	-
(6400cm²)	-	-	152	106	78	64	42	25	16	7

Fig. 2 Examples of housing systems for Spanish ribbed newts and advisable maximum number of animals that should be housed together. (**a**) Hypothetical rack system. (**b**) Housing in Petri dishes, plastic boxes and aquaria of different sizes

7. Check that the temperature of the water ranges between 15 and 28 °C.
8. Transfer the newts to the tank (*see* Subheading 3.3 for appropriate handling).
9. Ensure that the lids of the tank allow air exchange with the room and prevent newts from escaping.

3.2 Feeding Metamorphic/Adult Newts

Feed the animals every 2–3 days (*see* **Note 8**).

1. Select the appropriate food types (Table 2), weigh the desired quantity, and mix altogether (*see* **Note 9**).
2. Distribute the food evenly in the tank.
3. If some newts are found in the floating land areas, offer them food (*see* **Note 10**) helped by forceps (*see* **Note 11**).

3.3 Handling and Sexing Post-metamorphic/Adult Pleurodeles waltl

1. Use a net to take a newt out of the tank (*see* **Note 12**).
2. Grip the newt by the base of the tail with one hand.
3. Observe the cloaca (*see* **Note 13**).
4. Place it calmly in the palm of your other hand.
5. Observe the forelimbs (*see* **Note 14** and Fig. 1b).

Table 2

Types of food, sources, sizes, adequateness, and suggested quantities

Type	Name (and reference where possible)	Where to find it?	Size (mm)	Adequate for…	Quantity for feeding 200 late active larvae of ~0.2 g/larvae	Quantity for feeding 50 metamorphic juveniles of ~2 g/newt
Free-living food	*Artemia* nauplii from commercial cysts	Pet shop/online	0.4	Early active larvae	Inadequate	Inadequate
	Tubifex worms	Pet shop/aquarist	0.5 × 2 to 1 × 4	Early and late active larvae	Can be used as a supplement: ~500 worms	Inadequate
	Grindal worms (*Enchytraeus* sp.)	Pet shop/aquarist	0.5 × 10 to 1 × 40	Early active larvae	Can be used as a supplement: ~500 worms	Inadequate
Frozen food	Cyclops	Pet shop/online (e.g., zooschatz@t-online.de)	0.5–2	Early and late active larvae	8–12 g	Inadequate
	Bloodworms	Pet shop/online (e.g., zooschatz@t-online.de)	3–10	Late active larvae	8–12 g	12–15 g
	Mysis	Pet shop/online (e.g., zooschatz@t-online.de)	2 × 8	Late active larvae	8–12 g	12–15 g
	Black mosquito larvae	Pet shop/online (e.g., zooschatz@t-online.de)	3–10	Late active larvae	8–12 g	12–15 g
	White mosquito larvae	Pet shop/online (e.g., zooschatz@t-online.de)	3–10	Late active larvae	8–12 g	12–15 g

Pellets	Powder Microvit BASIC (70342)	Pet shop/online (www.tropical.pl)	0.1	Early active larvae	Inadequate	Inadequate
	Tropical energy food S (TENF190)	Pet shop/online (www.aquatic-nature.com)	0.4–0.8	Early and small late active larvae	0.5–1 g	Inadequate
	Cichlid mini granules (T506387-CE)	Pet shop/online (www.tetra.net)	1–2	Late active larvae	0.5–1 g	Inadequate
	TetraMin granules (T505322CE)	Pet shop/online (www.tetra.net)	1–3	Late active larvae and small juveniles	0.5–1 g	1–2 g
	New Life SPECTRUM Community fish formula	Pet shop/online (www.nlsfishfood.com)	1–3	Late active larvae and small juveniles	0.5–1 g	1–2 g
	Tropical Spirulina granulat (60434)	Pet shop/online (www.tetra.net)	1 × 2	Late active larvae and small juveniles	0.5–1 g	1–2 g
	New Life SPECTRUM Amphibian formula	Pet shop/online (www.nlsfishfood.com)	2	Metamorphic juveniles and adults	Inadequate	1–2 g
	Hikari tropical sinking wafers (21521)	Pet shop/online (www.hikari.info)	6 × 12	Metamorphic juveniles (previously broken) and adults	Inadequate	1–2 g
	JBL Pond Sterlet (4020000)	Pet shop/online (www.jbl.de)	7–8	Big adults	Inadequate	Inadequate

3.4 Cleaning Post-metamorphic/ Adult Pleurodeles waltl

For small experimental setups lacking filter, partial cleaning should be done the day after feeding, and a thorough cleaning is recommended at least once every 7 days. In the case of large tanks equipped with filter and air pump, partial cleaning should be done once a week and thorough cleaning once a month.

Partial cleaning

1. Take the tube of the syphon and fill it up with water.
2. Holding both endings of the tube with your thumbs, place one of them inside the tank and direct the other one towards an empty bucket (*see* **Note 15**).
3. Release completely the ending of the tube that is inside the tank, and modulate the vacuum strength with your finger by opening and closing the opposite ending.
4. Use the vacuum to take out of the tank at least one third of the water from the bottom part, absorbing as much detritus as possible (*see* **Note 16**).
5. Refill the tank with chlorine-free tap water (*see* **Note 5**).

Deep cleaning

1. Set up a new tank using the procedure described in Subheading 3.1.
2. Transfer the animals to the new tank using a net.
3. Empty the water of the old tank. Rinse the filter, stones, cork mat, gravel, and plant over a bucket to collect the water.
4. Brush the stones and the cork mat, clean the filter (and all its components), and move the gravel under hot tap water and natural plants under cold tap water.
5. Brush the old tank under hot tap water.
6. Rinse everything in cold tap water.

3.5 Promoting Natural Breeding

Animals kept in good conditions will eventually produce natural spawns if both sexes are housed together, so regular ocular inspections shall be done to detect eggs (*see* **Note 17**). Remove the eggs from the breeding tank to avoid predation from the adults which subsequently provide the best conditions for the offspring to develop (*see* Subheading 3.7). Natural breeding can be promoted by mimicking the conditions in which the Spanish ribbed newts reproduce in their natural environment:

1. Decrease the water level gradually during 15–30 days, increase the temperature to 30 °C, and feed the newts only once a week during that period (*see* **Note 18**).
2. Increase the level of water in three consecutive days, decrease the temperature, and start feeding daily (*see* **Note 19**).

3. Observe the behavior of the newts (*see* **Note 20**) and look for eggs in the tank.
4. Collect all the eggs from the breeding tank using a wide-mouthed plastic pipette (*see* **Note 21**), and place them in a clean container (*see* **Note 22**).

3.6 Artificial Fertilization

For in vitro fertilization, males and females are kept separately, as the female newts are able to keep the sperm for several months after mating [55]. This ensures that fertilization occurs exactly when you want to.

1. Select adult newts that display breeding morphology (*see* **Note 23**).
2. Under anesthesia (*see* **Note 24**), give the female a dose of 50–200 IU of human chorionic gonadotropin (*see* **Note 25**) by injecting it subcutaneously in the lower jaw (*see* **Note 26**).
3. After 8–18 h, place the female in 100 % MHS (*see* **Note 27**); she will start to lay eggs that must be collected every 15 min to perform the in vitro fertilization (*see* **Note 28**).
4. Under anesthesia, squeeze the male abdomen by pressing gently to collect the sperm in a 2 mL tube containing 100 % MHS supplemented with antibiotics (*see* **Note 29**).
5. Place the collected eggs in a Petri dish and remove the excess of MHS. Add some sperm solution over the eggs, and then mix it by collecting the eggs with a widemouthed plastic Pasteur pipette (*see* **Note 30**).
6. Wait 15 min (*see* **Note 31**), and add 20 % MHS (supplemented with antibiotics) to the fertilized eggs to allow water absorption by the jelly (*see* **Note 32**).

3.7 Egg and Embryo Maintenance

A daily careful inspection of the eggs is essential to prevent microbial growth. Remove any unfertilized or contaminated eggs (*see* Fig. 1c), and change the medium (chlorine-free tap water or MHS) every 2–4 days.

1. Fill up the new housing place with chlorine-free tap water or 20 % MHS.
2. If necessary, release the egg from the jelly (*see* **Note 33**) either mechanically using no. 5 forceps or chemically by rinsing them in a solution of 2 % cysteine in 20 % MHS at pH 8.0 for 2–5 min. In the latter case, several washes in freshwater/20 % MHS must be done afterwards.
3. Follow the correct development of the embryos (Fig. 1d), and separate those that show abnormalities.
4. After hatching, when larvae start swimming, monitor daily the development of the embryos for free-living food to be supplemented at appropriate timing (*see* **Note 34** and Fig. 1f).

3.8 Housing Newt Larvae

1. Select an appropriate container (*see* **Note 35**) according to the number of animals that are to be housed and their developmental stage and size, in order to provide them with the required area (*see* **Note 36** and Figs. 1d–h and 2b).
2. Brush the tank under hot tap water, and give a final rinse it in cold tap water.
3. Fill up the tank (*see* **Note 37**) with chlorine-free water (*see* **Note 5**).
4. Fix the heater and air pump (*see* **Note 38**).
5. Add environmental enrichment objects in the case of late active larvae (*see* **Note 39** and Fig. 1g).
6. Check that the temperature of the water ranges between 15 and 28 °C.
7. Transfer the larvae to the tank (*see* Subheading 3.10 for appropriate handling of larvae).

3.9 Feeding Newt Larvae: General Procedures

1. Maintain the alternative sources of *Artemia* (*see* **Note 40**) regarding free-living food: *Tubifex* (*see* **Note 41**) and *Grindal* worms (*see* **Note 42**).
2. Select the appropriate food (Table 2) according to the developmental stage (*see* **Note 43**).
3. Chop the food in small pieces if necessary (*see* **Note 44**).
4. Mix the desired quantity in 500 mL of chlorine-free water.
5. Use a wash bottle (*see* **Note 45**) to evenly distribute the food among all the larvae (*see* **Note 46**).
6. Clean the wash bottle under tap water, and let it dry completely.

3.10 Handling Newt Embryos and Small Larvae

The big larvae can be easily collected using a net, but embryos and small larvae are more fragile and can be easily killed when trying to pick them up with a net. Use Pasteur pipette as described below.

1. Cut the tip of a plastic Pasteur pipette to have an opening larger than the size of the larvae to be transferred.
2. Calmly approach the larva with the widemouthed Pasteur pipette.
3. Capture the larva by a quick vacuum movement.
4. Use your finger tip to close the opening of the pipette (*see* **Note 47**).
5. Release the larva into its new location.

3.11 Cleaning Boxes and Tanks for Larvae

Clean the nonfeeding larvae setups (Petri dishes) at least once a week. The feeding larval containers require cleaning every second day (*see* **Note 48**).

3.11.1 Petri Dishes

1. Collect the eggs/embryos/initial larvae with a tip-cut pipette, and place them directly into a new one.
2. Clean the old Petri dish brushing it with hot tap water, give a final rinse in cold tap water, and let it dry completely. If contamination detected, discard the Petri dish.

3.11.2 Plastic Boxes

1. Place a large net over an empty plastic box or a bucket.
2. Prepare a small container with freshwater.
3. Take out the stones and artificial plants, and brush them under hot tap water.
4. Transfer the entire volume from the container with the newt larvae through the net.
5. Gently move the net to discard detritus and transfer the larvae into the small container with freshwater (*see* **Note 49**).
6. Remove any remaining debris using a pipette from the temporary tank containing the larvae.
7. Check in the empty container that no larvae escaped in the filtering process and discard the water.
8. Brush the plastic tank under hot tap water, and give a final rinse in cold tap water.
9. Fill the tank with chlorine-free water.
10. Replace the stones and artificial plants and gently place the larvae into the clean tank.

3.12 Health Inspection and Treatment of Diseases

Periodically observe the animals for signs of abnormalities in the skin (i.e., bleeding, ulceration, pigmentation abnormalities, fungus), behavior (i.e., slow moving, floating, or upside-down animals), and body constitution (either bloating or extremely skinny animals) (*see* **Note 50**).

1. If a sick animal is detected, isolate it from the main tank.
2. Place the animal in a temporary plastic container filled with marine salt solution for 1–5 min. Skip this step if the affected newt is a larva.
3. Transfer the newt into a tank with an easy access to an artificially created land area and containing 40–50 % MHS supplemented with antibiotic/antimycotic (follow manufacturer's instructions) (*see* **Note 51**). The depth should be around 10 cm.
4. Change the water of the tank every second day.
5. Daily follow the state of the sick newt and check the other newts in the tank in which the sick animal was found.
6. If more animals are affected in the same tank, empty it, brush it with marine salt solution, autoclave the stones/tiles, submerge the filter for 1 h in salt solution after cleaning it, replace

the plants with new ones, and treat all the tank mates as described in **steps 2** and **3**. Refill the tank with chlorine-free water and add antibiotic/antimycotic solution.

7. Every 2 or 3 days, change one half of the volume of the tank.
8. Stop the treatment when the animals look healthy, 2 days after the signs disappear.

3.13 Euthanasia

In those cases where no signs of improvement are observed after treatment, the animal should be euthanized.

1. Place the animal in 0.1 % Tricaine so the depth of the water is about 1–3 cm.
2. Wait a minimum of 30 min to ensure that the newt is deeply anesthetized.
3. Using sharp scissors, cut through the neck of the salamander to remove the head.
4. Quickly crush the skull with the flat side of the scissors or a blade.
5. Freeze the rests of the animal inside a paper towel and discard it according to the local regulations of the laboratory.

4 Notes

1. During the periods of extreme temperatures, *Pleurodeles waltl* may undergo hibernation and aestivation. During these processes, the skin becomes thicker and less permeable; Animals decrease food intake and slow down the metabolic rate. Nevertheless, Spanish ribbed newts have been found all along the year having an active lifestyle in the water [32–36].
2. Natural populations experience slight variations along the year in the light-dark (LD) cycle which approximately ranges from (LD 15:9) in summer to the opposite in winter (LD 9:15). They can be kept in a (LD 12:12) in laboratory conditions, although placing the newts under the influence of natural light (i.e., close to a window) or alternatively adapting the timer of the animal facility trying to mimic the natural LD cycle (±15 min of light/week) may benefit the adult newts and induce the natural breeding at the corresponding time of the year.
3. The Spanish ribbed newt has a high temperature tolerance. In nature, this species survives in regions in which absolute temperatures range from bellow −10 °C to over 40 °C. In the laboratory, the stable temperature prevents most of the newts undergoing hibernation and aestivation. Nevertheless, some animals may decide to spend several days/weeks in the floating surfaces, eating very little, moving slowly, and showing

skin changes typical of those two processes. Unless an animal experiences an alarming loss of weight (such as two thirds of their initial body mass), there is no reason to worry. They will sooner or later go back to the water and eventually breed.

4. You must not use any soap or detergent. Usually, brushing the different components under hot tap water is sufficient, but if deep cleaning is desired, a good option is to use marine salt solution described in Subheading 2.4, **item 9**. Disinfection by autoclaving is advisable for stones, rocks, and tiles. In some cases, 10 % bleach can be used for cleaning or disinfecting filters, but it is very important to rinse the tanks and filters several times with tap water, let it dry for 24 h, and rinse it again several times before use.
5. Keep tap water in a separate container for 24 h to remove chlorine, and to equilibrate with the room temperature. Alternatively, anti-chlorine product can be added to temperate tap water (15–25 °C) according to the manufacturer's instruction.
6. Newts should have the opportunity of choosing between climbing on floating devices (such as cork mat or Styrofoam) or remaining in the water. Under the water, they appreciate hiding places. Entire or broken tiles are probably the best option for this purpose, as they are easy to clean and offer a good hiding area. Live or artificial plants available from aquarium suppliers are a good option to enhance the atmosphere in an aquarium. For making an artificial plant, place some gravel in a light-colored plastic bag (better green color) and make a tight knot close to the base; then, cut the remaining plastic in stripes of 1–2 cm thickness. The use of gravel or stones is optional and it makes cleaning more laborious. If included, the size of the gravel should be large enough so that the newts do not swallow them by mistake.
7. The filter should direct the water flow towards the surface or one of the corners of the tank in order to avoid strong flow.
8. Newts do not require feeding every day; two to three feedings per week is sufficient. The frequency of feeding can be modulated according to the season to mimic natural conditions. Animals can be offered food once a week for mimicking winter time (LD 9:15) and every day for mimicking summer time (LD 15:9).
9. Alternatively, choose one type of food for each feeding day.
10. To avoid rejection of the food in land, the dry pellets may be placed in water for some min before offering them to the newts and the frozen food shall be defrost.
11. In land, newts will rely mostly on their visual system, and thus they recognize the food only if it moves. For that reason, one should offer different types of food with a forceps, moving it

back and forth as if it would be a living prey (or alternatively living preys such as earthworms can be offered with the forceps). After a first recognition, some newts will approach the prey with snout and catch them by jaw prehension, while others may grab them directly by tongue protrusion [38, 57].

12. Regarding body proportions, males present larger tails, while females tend to show a thicker body [32, 33, 35, 54].
13. Males' cloaca shows papillary appearance, whereas females' cloaca presents inward borders. Males' cloaca swells up during the breeding season [32, 33, 35, 54].
14. Males' forelimbs are proportionally longer and stronger than females' ones, and during breeding season the male forelimbs develop black rugosities (nuptial pads) that are used to grip the females during amplexus [32, 33, 35, 54].
15. For the vacuum to work, the level of the water in the tank must be higher up than the bucket.
16. Avoid vacuuming close to the newts. If an animal gets stuck into the tube, simply close the outflow ending of the tube and the animal will be able to swim out of the tube.
17. Eggs can be found attached by the jelly coat either in the substrate, stones, or artificial plants. The eggs have an upper dark animal pole and a lower opaque vegetal pole, so they are usually detectable after softly moving those objects in where they may be present.
18. For the best results, coinciding with the decrease of water level and feeding frequency, gradually lengthen the light cycle to mimic the summer period (LD 15:9). Return slowly towards the autumn-like light cycle (LD 12:12) starting 1 week before increasing water level and food intake.
19. Not only increasing the quantity but also the variety of food may have a positive effect on the induction of natural breeding.
20. The behavior of interest is the amplexus, as it precedes the spawn. It can be recognized easily (*see* Subheading 1).
21. The plastic pipette should be cut in order to get a 3–4 mm tip diameter. The jelly coat surrounding the egg is highly sticky; therefore, once the eggs are drawn into the pipette, close the opening with a finger.
22. Petri dishes can be used to facilitate oxygen exchange in water, or alternatively an air pump can be supplied if the eggs are placed in a large plastic container.
23. Breeding ribbed newts have swollen cloacae and flattened tails.
24. To anesthetize a newt, place it in a plastic box containing 2 cm depth Tricaine solution (*see* Subheading 2.4, **item 4**) and close the lid. Wait a minimum of 10 min before seeing if it is

anesthetized by placing the animal on its back. If it doesn't move within 5–10 s, it is anesthetized.

25. Eggs can be collected from the same animal every 2–4 weeks, although it is better to wait 2–3 months to avoid female's stress.
26. The hormone can alternatively be injected intramuscularly in the dorsal musculature, although the results are less satisfactory.
27. 1× MHS is used rather than water, as it prevents the jelly to absorb water, which in turn inhibits fertilization.
28. Eggs remain fertile for about 30 min. The female can be gently squeezed in order to collect the eggs ready to be laid in the oviduct every hour, although the best results are obtained by simply collecting the eggs every 15 min, avoiding the stress produced by squeezing.
29. Sperm can be collected from the same animal every 2–3 weeks.
30. This step improves the fertilization rate, as the sperm motility is activated by the jelly coat proteins, and the plastic pipette mimics the oviduct in which eggs are fertilized in the case of natural spawns.
31. Do not let the eggs completely dry at any time.
32. Eggs can be kept in chlorine-free tap water; however, the best survival rates are obtained when artificial freshwater is used, especially when supplemented with antibiotics/antimycotics (See Subheading 2.4, **item 6**).
33. Egg jelly is a natural protection of the amphibian eggs but sometimes sticks to undesirable material, such as detritus. In this case, eggs shall be released from the jelly coat.
34. A good knowledge regarding newts' life cycle is crucial for free-living food to be supplemented at appropriate timing, preventing the loss of animals due to cannibalistic behaviors (Table 1). A sign indicating that larvae are ready to start hunting is the disappearance of the whitish/yellowish color in the belly.
35. For eggs and embryos, Petri dishes are a good option, as the oxygen requirement of eggs and early embryos is low and it facilitates daily monitoring to separate unfertilized/dead eggs from the healthy ones. For larvae, plastic tanks with external air pumps are recommended, as they are more adaptable, and it will allow to separate the animals based on size.
36. The surface area of the tank is more important than the volume, as newt larvae mostly lay in the bottom. The abrupt increase in areal requirements from sedentary to early active larvae as shown in Fig. 2b is due to the beginning of feeding. This is to minimize cannibalistic behavior.
37. It is better to keep small larvae (initial and early active) in a container with 5–10 cm depth of water, so they can find the

free-living food easily, they do not accidentally desiccate, and the waste derived from non-eaten food and detritus will not strongly decrease oxygen content. The water depth can be increased for late larvae around 3–4 cm until 15 cm, as they start to use all levels of the water column, albeit 10 cm is enough. This will also help them to get air from the water surface, after they form the lungs and combine oxygen intake from water and air sources (stage 55a).

38. Both the heater and the air pump are recommendable in larval setups, although not essential. The former can be used to increase the growth rate of the larvae, and the latter improves the oxygen concentration of the water, preventing the growth of undesirable microbiota and the consequent death of larvae, especially when the water temperature is high.
39. The environmental enrichment of larvae of stages 45–55a is based on subaquatic objects (i.e., hiding objects and natural/artificial plants). When signs of metamorphosis are seen, such as absorption of the gills and a major change in pigmentation, a floating surface should be provided. In addition, a lid is necessary at this point to prevent young metamorphic newts escaping out of the tank.
40. *Artemia* nauplii provide adequate free-living food for the early active larvae [55, 56]. Commercial *Artemia* cysts will hatch in 1–3 days, and hatched nauplii can be used as food as long as they remain alive, which is only possible if oxygen and food is given. It's good to start a new culture every second day. Filter the *Artemia* to discard the salt water of the culture and distribute the nauplii evenly among the newt larvae using a wash bottle.
41. Keep the *Tubifex* sp. in a plastic tank filled up with water and provided with oxygenation through an air pump. Small doses of fish pellets can be provided as food, but it is not essential. Check the transparency of the water in the *Tubifex* culture; if it is cloudy, change it by chlorine-free tap water (it must be done every 1–3 days). Discard the dead worms. For feeding the larvae, collect some living worms and chop them in small pieces using the lid of a Petri dish and a pair of scalpels.
42. Keep the *Grindal* worms (*Enchytraeus* sp.) in several small plastic boxes filled up with moist soil, avoiding flood and in dark conditions. Pre-soaked dry dog/cat food or fish pellets can be used as food source. Worms can be easily collected with forceps no. 5, a couple of hours after providing a food pellet to the culture (they will surround the pellet). If the worm culture grows too fast (i.e., the food is consumed within 24 h), split the culture into two.

43. During the initial larval period, hatchlings live on their remaining stalk; thus, no feeding is necessary. Once the bellies start to lose their whitish-yellow appearance of the stalk, the initial larval period is about to start, and the first meal (free-living food) should be added. In the early active larval period, larvae start to prey over small live food, which is recognized usually by a combination of visual and vibrational cues. They do not seek for food at these stages, but prey only over the small creatures that pass by their heads. Cannibalistic behaviors become frequent; thus, it is recommendable to keep them at low densities and "ad libitum" conditions. At the beginning of the late active larval period, larvae start to use odor cues to discriminate also steady food, and learning processes modulate food intake. As a consequence, cannibalistic individuals should be isolated if detected. Once cannibalism is solved by housing larvae at appropriate densities, newts can learn that dry pellets are a source of food. The pellet size after hydration should be slightly smaller than the mouth of the animal.
44. For small and medium active larvae (stages 45–54), cut the frozen pellets as small as you can before the tablet gets defrost (also the *Tubifex*). Mix it in freshwater and filter it.
45. Cut the tip of the wash bottle to increase the flow and to avoid that food gets stuck inside the outflow tube.
46. The food tends to sink; thus, shake the wash bottle before adding the food into every tank.
47. Closing the opening of the pipette will prevent the larvae to escape from the pipette and end up on the floor or another dry surface. If they escape, try to save the larvae using a net or a wet piece of paper. Avoid catching them with the fingers or forceps.
48. If the tank is equipped with aeration, partial cleaning can be done every second day (*see* Subheading 3.4), followed by total cleaning after the next 2 days. Do not vacuum the larvae, and always filter the discarded water through a net before disposal to ensure that no larvae are being lost in the process.
49. Try to avoid touching the new clean water with the net; instead, let the larvae gently drop down at a short distance of the water surface. This procedure will prevent transfer of any debris from the net to the water.
50. The Caudata Culture website (www.caudata.org) provides information on the common symptoms and diseases in urodeles [58].
51. You may need to contact the university veterinarian for the treatment.

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