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Comparative morphological analysis during larval development of three syntopic newt species (Urodela: Salamandridae)

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Abstract

We provide comparative and updated staging tables for the larvae of three syntopic newt species – *Triturus carnifex*, *Lissotriton italicus* and *Ichthyosaura alpestris inexpectatus* –living in Calabria. A detailed description, stage by stage, of the full larval period until completion of metamorphosis is furnished, accompanied by photographic tables of the most important stages. The appearance of the most prominent and easily identifiable morphological features (i.e. development of gills, balancers and limbs; and body pigmentation), and length, have been used as staging criteria. Ontogenic development in the three considered species is compared with that of other Caudata species. For the three newt species, we also performed a comparative analysis of the developmental larval phases, emphasising the shared and species-specific features. The present descriptive study adds comparative information on distinctive morphological traits that may be useful for the recognition of newts during larval ontogeny and, in some cases, when sampling in the field.

Keywords: *Urodela, newt, larvae, normal development, staging tables*

Introduction

Biphasic development is one of the key features of amphibians and, after metamorphosis, many species remain aquatic or become partially terrestrial (Griffiths 1996; Wells 2007). Due to this complex life cycle, amphibians are a highly vulnerable taxon, and the increasing disturbance of water environments in particular is likely to have a great impact on aquatic larvae (Brunelli & Tripepi 2005; D'Amen et al. 2007; Quaranta et al. 2009; Egea-Serrano et al. 2012; Bernabò et al. 2016; Brunelli 2018). Amphibians are well acknowledged to exhibit an extreme plasticity that can be recognised in almost every trait, ranging from behaviour to morphology to life-history traits, as the result of a complex array of environmental influences (Relyea 2001). Such a level of plasticity also involves phenotypic characters of larvae (Fox 1984), which represent a good model to study amphibian ecology and populations.

In this context, the importance of distinguishing species on the basis of their larval stages is clear, and thus the implementation of staging tables along with descriptions of normal development could be useful. As outlined by Nye et al. (2003) and by Watson and Russell (2000), it is evident that the available data on the entire larval period are scarce or rather old, in particular for newts from different genera. Taking into account this paucity of information, here we provide an updated, comparative staging table with a detailed description of the whole larval period for three newt species, *Ichthyosaura alpestris inexpectatus*, *Triturus carnifex* and *Lissotriton italicus*.

These three species are syntopic in Calabria, where they frequently coexist in the same water bodies in sites on the Catena Costiera (Giacoma et al. 1988). When glaciations reached southern Italy, Calabria served as a refuge for the mesothermal species and as a long-term hotspot of intraspecific diversity (Canestrelli et al. 2012 and references therein). Moreover, Calabria is acknowledged as

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a fundamental area from a zoogeographic and a conservationist point of view, being an important centre of endemicity and representing the southern-most borders of many amphibian species distributions (Giacoma et al. 1988). The Alpine newt, *Ichthyosaura alpestris*, is present in the study area with the subspecies *inexpectatus* (formerly *Triturus alpestris inexpectatus* Dubois & Breuil 1983). This endemic subspecies, recently discovered, is a post-glacial relict and has a restricted distribution, populating only five sites on the Catena Costiera (Dubois & Breuil 1983; Tripepi et al. 1999; Sindaco et al. 2006). The Italian crested newt, *Triturus carnifex* (Laurenti, 1768), is part of the *T. cristatus* complex and it is distributed in both continental and peninsular Italy, up to the central part of Calabria. The Italian newt, *Lissotriton italicus* (formerly *Triturus italicus* Peracca, 1898), is limited to the central and southern portions of the Italian peninsula. *I. alpestris inexpectatus*, *T. carnifex* and *L. italicus* are listed on the Red List of Italian Vertebrates as "Endangered", "Near Threatened" and "Least Concern", respectively. The main potential threats are habitat alteration and/or loss, aquatic pollution, and introduction of alien species such as predatory fishes (Rondinini et al. 2013).

From an analysis of the literature it is evident that only partial descriptions are available. The development of *T. carnifex*, from deposition to hatching, was studied by D'Amen et al. (2006). Embryogenesis and passive larval stages of *L. italicus* have been described, with particular emphasis on the effects of temperature on developmental rate (Tripepi et al. 1998). The embryonic stages of *I. alpestris* have been described (Epperlein & Junginger 1982), although for the subspecies *inexpectatus*, only a brief description up to stage 44 (four-fingered larvae) is available (Bonacci et al. 2005).

To the best of our knowledge, a table of normal stages encompassing the entire course of larval development until metamorphosis has not yet been developed for the three considered species. For each species we updated normal developmental tables by re-examining previous data and restaging according to our observations; moreover, we extended the available tables to completion of metamorphosis, also including a detailed description of metamorphic stages.

As staging criteria, we used the appearance of the most prominent and easily identifiable morphological features (i.e. development of gills, balancers and limbs; and body pigmentation) as well as length. Moreover, through a stage-by-stage comparison, we aimed to identify distinctive features that may be useful for the recognition of newts, including in the field.

Material and methods

Study species

Ichthyosaura alpestris inexpectatus is a medium-sized semiaquatic newt that mainly inhabits deep ponds, with both aquatic and riparian vegetation, transparent waters and short periods of winter frost (Lanza et al. 2007). *Triturus carnifex* is the largest Italian newt and colonises various terrestrial habitats, while breeding in lentic aquatic habitats, both natural and artificial (Tripepi et al. 1999; Sindaco et al. 2006). *Lissotriton italicus* is the smallest newt in Europe; it breeds in lentic or slow-flowing water and often colonises man-made water bodies (Sindaco et al. 2006).

Study site and collection of newts

The newts were captured in a natural permanent lake (Lake Trifoglietti) located within a Site of Community Interest (Laghi di Fagnano – site code IT9310060) on the Catena Costiera in Calabria, southern Italy (39° 32′37″N, 16°01′20″E; see http://eunis.eea.europa.eu/sites/IT9310060 for more information) at an elevation of 1048 m above sea level. In this area, as stated above, the three considered species are syntopic.

Adult newts were captured during the breeding period (March and early April) using a landing net. For each species, four males and four females were transferred to our laboratory and then housed in separate glass aquaria for each species to allow breeding. Each aquarium (60 × 40 × 40 cm), filled with 40 L of dechlorinated tap water, was equipped with stones and artificial vegetation (plastic strips) necessary for oviposition. Breeding individuals were fed every other day with *Lumbriculus* spp. Water in aquaria was filtered and replaced weekly. At the end of oviposition, adults were released in the capture site.

Raising embryos and larvae

Aquaria were maintained at a temperature of 19 ± 1°C and under a natural photoperiod (Bonacci et al. 2005 and reference therein).

Newts began laying eggs within 3 days after capture. The newly laid eggs were gently collected from the plastic strips and placed in 15-L glass aquaria (35 × 19 × 22 cm) filled with aerated and dechlorinated tap water at a median pH of 7.3. Eggs that were not developing were removed daily. In order to minimise overcrowding, the number of embryos for each aquarium was kept to no more than 15

individuals. Two times a week the water volume was completely renewed, to prevent fungal infections. After yolk reabsorption, larvae were fed daily *ad libitum* with the nauplii of *Artemia salina* (Brine shrimp); at later stages, the diet was supplemented with *Tubifex* spp. 3 times per week. When the metamorphosis began, each tank was provided with two rocks as a refuge and the water depth was reduced, allowing metamorphs to emerge.

Staging methodology and measurement

For the purposes of the present work, we followed the developmental chronological table for *Lissotriton helveticus* of Gallien and Bidaud (1959), starting from stage 33 (emergence of fore limb bud), through both the passive and active larval phases, until the completion of metamorphosis. The developmental chronological table of Gallien and Bidaud (1959) provided us with a general staging scheme, with precise timing and morphological criteria for stage designation, applicable to all three considered species (both early life-history and larval developmental stages).

Stage definitions were based exclusively on external morphological features (eye pigmentation, development of gills and balancers, the appearance of the front and hind limbs, digit formation, and pigmentation pattern). We did not consider the timing between consecutive stages.

For evaluation of ontogenic development, randomly selected larvae of each species (n = 10) were examined daily under a stereomicroscope (Leica MZ APO, Leica Microsystems, Wetzlar, Germany), and then photographed with a digital camera (Canon Power Shot S40). For better embryo description, jelly coats and egg membranes were manually removed using fine forceps. Larval length (from the tip of the snout to the end of the tail) was measured using a Petri dish placed over 1-mm graph paper. Metamorphosing larvae were photographed in a glass chamber containing stones and vegetation, using a tripod-mounted camera (Sony DSC-F707).

Post-metamorphic juveniles were released at the capture site. All procedures for animal handling were carried out according to the recommendations of the Ethical Committee of our University and with the approval of the "Ministero dell'Ambiente e della Tutela del Territorio (Direzione per la Protezione della Natura)", permit number 2004/30911.

Statistical analysis

Values are presented as mean ± standard deviation of the mean (SD). Prior to analyses, data were checked for normality and homogeneity of variances (Kolmogorov–Smirnov test for normality and Bartlett's for homogeneity).

We used a two-way analysis of variance (ANOVA) to test for differences in the larval length, with "species" and "stages" as factors. The statistical significance of an interaction between species and stages, at some levels, indicates differences in species-specific growth. To explore interspecific differences in length, stage by stage, we performed Bonferroni's post hoc test. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using Graph Pad Prism 5.00 (GraphPad Software Inc., San Diego, CA, USA).

Results

We defined 26 distinct larval stages, from stage 33 to stage 53 which indicates the completion of metamorphosis, on the basis of the appearance and modification of the external morphological features (Table I; Figures 1–5). External morphological traits, stage by stage, are described in detail in Table I, which shows both the characters shared by the three species and the species-specific features.

The most significant steps of larval development are illustrated by a set of photographs from living specimens of *I. alpestris inexpectatus*, *T. carnifex* and *L. italicus* (Figures 1–5).

Two-way ANOVA clearly indicated a significant difference in length during development among the considered three species (df = 25,702; F = 5927.13; Figure 6). In more detail, the dimensional difference is greater between *T. carnifex* and *L. italicus* ($p < 0.001$ at all considered stages). When comparing *T. carnifex* and *I. alpestris inexpectatus*, lengths became significantly different starting from stage 41 ($p < 0.001$). Concerning *I. alpestris inexpectatus* and *L. italicus*, the difference in body size became highly significant from stage 44 ($p < 0.001$; except for stage 51b).

Discussion

Although a number of developmental tables have been produced for amphibians (Duellman & Trueb 1986), the ontogenic development of Urodela has received relatively little attention. Depending on the intentions of the various authors, the available studies have considered specific development phases (such as embryonic or larval phase), and only a few studies, some of which are rather old, have been conducted on the development as a whole (Glücksohn 1932; Gallien

Table I. Stages of development of *Ichthyosaura alpestris inexpectatus, Triturus carnifex* and *Lissotriton italicus* described considering the major common characters and highlighting species-specific features. Length is expressed as the mean ± standard deviation.

Stage	Common characters	Diagnostic features	Length
33	Fore limb buds are cone shaped. Gills and balancers are distinct with a cylindrical-elongated shape. Developing eyes appear as a slightly depressed area. The tail is well distinct and equipped with developing transparent fins. Melanophores form three longitudinal compact stripes: one dorsally and two ventrolaterally. The dorsal band splits at the level of the fore limbs and continues in two lines leaving the medium-cranial area without pigments. The ventrolateral bands extend up to the gills. Stains are visible on fore limbs, in the throat area and behind the eyes.	***I. a. inexpectatus*** Background colouration: straw yellow.	6.4 mm ± 0.4
		T. carnifex Background colouration: greenish yellow.	7.6 mm ± 0.2
		L. italicus Background colouration: straw yellow. The two lines that derive from the dorsal band join between the eyes.	6 mm ± 0.1
34	Fore limb buds become conical-elongated. The gills lengthen. Balancers are club shaped. A few melanophores are scattered on the base of the dorsal fin.	***I. a. inexpectatus*** Sub-branch buds appear on the first and second gill filaments. Beginning of pigmentation of the eye.	7.2 mm ± 0.2
		T. carnifex Sub-branch buds appear on the first and second gill filaments.	8.1 mm ± 0.1
		L. italicus Beginning of pigmentation of the eye.	6.2 mm ± 0.2
35	Fore limb buds have a cylindrical shape. Lengthening of both the club-shaped balancers and of gills. Pigmentation of the eye with melanophores, iridophores and xanthophores is evident.	***I. a. inexpectatus*** Sub-branches buds appear also on the third filament. Ocular pigmentation increases by about 50%. Xanthophores appear scattered on the ventral and dorsal fins. Fins are well formed: the dorsal fin extends nearly to the head and has a rectilinear profile.	7.8 mm ± 0.2
		T. carnifex Sub-branches buds appear also on the third filament. Beginning of ocular pigmentation. Several melanophores are visible on the gills. Fins are well formed: the dorsal fin extends nearly to the head and has a markedly convex profile.	8.8 mm ± 0.2
		L. italicus Sub-branch buds appear on the first gill filament. Ocular pigmentation increases by about 50%. Fins are well formed: the dorsal fin extends nearly to the head and has a convex profile.	7.1 mm ± 0.1
36	Fore limb buds still have a cylindrical elongated shape.	***I. a. inexpectatus*** First stage of hatching. Melanophores appear on the well-branched gills. Iris pigmentation is more than 80%. Balancers are elongated and slightly swollen at their distal ends.	8.3 mm ± 0.2
		T. carnifex Ocular pigmentation increases by about 50%.	9.1 mm ± 0.1
		L. italicus First stage of hatching. Sub-branch buds appear on the second gill filament. Melanophores are visible on the gills. Iris pigmentation is more than 80%.	7.3 mm ± 0.06
37	Fore limb bud presents a paddle form.	***I. a. inexpectatus*** Iris pigmentation is completed. Numerous chromatophores are scattered on dorsal fin.	8.8 mm ± 0.2
		T. carnifex First stage of hatching. Gills are thickened, more pigmented and branched. Iris pigmentation is more than 80%.	9.9 mm ± 0.4
		L. italicus Sub-branch buds appear on the third gill filament. Balancers are elongated and slightly swollen at their distal ends. Iris pigmentation is completed.	7.9 mm ± 0.3

(*Continued*)

Table I. (Continued).

Stage	Common characters	Diagnostic features	Length
38	Fore limb is paddle-shaped with two finger buds in formation.	***I. a. inexpectatus***	9.3 mm ± 0.4
		Late stage of hatching (the greater part of embryos has already hatched by this time). Eye is well formed. Beginning of the resorption of the yolk. Opening of the mouth. The bands appear less compacts.	
		T. carnifex	10.5 mm ± 0.1
		Iris pigmentation is completed. Balancers are elongated and slightly swollen at their distal ends.	
		L. italicus	8.5 mm ± 0.2
		Late stage of hatching (the greater part of embryos has already hatched by this time). Eye is well formed. Beginning of the resorption of the yolk. Opening of the mouth. The bands appear less compacts.	
39	Larvae show fore limbs longer and with two digits well distinct. Balancers are long and thin.	***I. a. inexpectatus***	10.2 mm ± 0.3
		The gills further lengthen. A grouping of melanophores is visible at the apex of the caudal fin.	
		T. carnifex	11.3 mm ± 0.6
		Late stage of hatching (the greater part of embryos has already hatched by this time). Eye is well formed. Beginning of the resorption of the yolk. Opening of the mouth.	
		L. italicus	9 mm ± 0.1
		The gills further lengthen.	
40	Fore limb displays a third finger bud.	***I. a. inexpectatus***	10.8 mm ± 0.8
		The still less-compact bands are formed by rounded and spaced melanophores.	
		T. carnifex	12 mm ± 0.5
		The stripes appear less compact. Several melanophores and xanthophores are scattered on fins, whereas iridophores are distributed on the margins of caudal region of the fins.	
		L. italicus	9.3 mm ± 0.2
		The still less-compact stripes are formed by rounded and spaced melanophores.	
41	Fore limb displays three digits well formed. The elbow joint is visible. Beginning of the regression of balancers appearing longer and thinner.	***I. a. inexpectatus***	11.4 mm ± 0.5
		T. carnifex	13.3 mm ± 0.6
		L. italicus	10.5 mm ± 0.3
		Melanophores are scattered on the dorsal fin.	
42	Further elongation of the three digits in fore limbs is evident. Hind limb bud can be seen.	***I. a. inexpectatus***	11.9 mm ± 0.1
		T. carnifex	14.3 mm ± 0.3
		The gills are long and branched. Both fins have a markedly convex lateral profile and end with a pointed apex, which extends into a filament.	
		L. italicus	11.8 mm ± 0.1
43	The active larval life starts at this stage. Complete yolk consumption; the larva begins to feed. Fore limb shows a fourth finger bud and hind limb buds are cone shaped. The gills are long and branched. Balancers reduced by half or almost completely reabsorbed. There is no longer a clear distinction in stripes: rounded melanophores evenly distributed over the whole body except for the ventral zone that remain free of pigmented cells. Dorsal and caudal fins are dotted by melanophores, xanthophores and iridophores.	***I. a. inexpectatus***	13.2 mm ± 0.7
		T. carnifex	18 mm ± 1.7
		Iridophores appear on the gills. Fore limbs appear slender and elongated.	
		L. italicus	12 mm ± 0.2
44	Fore limb shows the fourth finger well formed. Hind limb bud becomes conical-cylindrical.	***I. a. inexpectatus***	14.5 mm ± 0.5
		T. carnifex	21.7 mm ± 0.6
		L. italicus	12.5 mm ± 0.5

(*Continued*)

Table I. (Continued).

Stage	Common characters	Diagnostic features	Length
45	Balancers have completely disappeared. Lengthening of the fourth finger in fore limb is visible. Hind limb bud displays a cylindrical-elongated shape.	***I. a. inexpectatus***	15.8 mm ± 0.2
		T. carnifex	24 mm ± 0.1
		On the margin of both fins, black blotches appear.	
		L. italicus	13.2 mm ± 0.2
46	Hind limb presents a paddle form.	***I. a. inexpectatus***	16.4 mm ± 0.2
		T. carnifex	25.2 mm ± 0.1
		In the mid-cranial area, a darker V-shaped spot with the apex facing posteriorly appears.	
		L. italicus	13.5 mm ± 0.1
47	Paddle-shaped hind limb shows a bifurcation distally.	***I. a. inexpectatus***	17 mm ± 0.5
		Iridophores appear on gills, which are long and well branched.	
		T. carnifex	25.8 mm ± 0.3
		Further elongation of the four fingers in fore limb is evident.	
		L. italicus	13.8 mm ± 0.1
48	Hind limb presents two digits well formed.	***I. a. inexpectatus***	17.2 mm ± 0.3
		T. carnifex	27.8 mm ± 0.2
		L. italicus	14.4 mm ± 0.8
49a	Hind limb shows a third toe bud.	***I. a. inexpectatus***	17.8 mm ± 0.3
		T. carnifex	28.1 mm ± 0.4
		Iridophores appear on gills, belly and throat area.	
		L. italicus	15.5 mm ± 0.4
		Iridophores appear on gills, which are long and well branched.	
49b	Hind limb shows three digits well formed.	***I. a. inexpectatus***	20 mm ± 0.1
		T. carnifex	28.2 mm ± 0.2
		L. italicus	16.1 mm ± 0.7
50a	Hind limb displays a fourth toe bud; the knee joint begins to differentiate.	***I. a. inexpectatus***	20.1 mm ± 0.2
		T. carnifex	33.3 mm ± 2.8
		Iridophores appear on the flanks between the limbs.	
		L. italicus	16.8 mm ± 0.2
		Iridophores appear on the posterior limbs.	
50b	Hind limb has four toes well formed; an elongation of all digits is evident.	***I. a. inexpectatus***	20.2 mm ± 0.2
		T. carnifex	33.6 mm ± 2.3
		L. italicus	17.8 mm ± 0.3
51a	Hind limb presents a fifth toe bud.	***I. a. inexpectatus***	22.7 mm ± 2
		Iridophores appear on the flanks between limbs and in the throat area.	
		T. carnifex	35.3 mm ± 1.5
		The V-shaped stain becomes more evident.	
		L. italicus	20 mm ± 0.05
51b	Hind limb has five digits well formed. Larvae characterised by fully developed limbs and tail fin.	***I. a. inexpectatus***	23 mm ± 1.7
		T. carnifex	42.4 mm ± 0.6
		Iridophores appear on limbs.	
		L. italicus	23 mm ± 0.1

(*Continued*)

Table I. (Continued).

Stage	Common characters	Diagnostic features	Length
52a	The gills reach their maximum development. Further elongation of the five digits in the hind limb. Beginning of the thickening of the limbs.	***I. a. inexpectatus***	30.2 mm ± 1
		Dorsally, the body has a pigmentation ranging from greenish to light brown dotted with brownish-black veins. The belly is yellow-orange. The iridophores on the flanks are well visible, and others emerge, arranged in line and symmetrically with respect to the median plane, on the dorsal margin of the tail. The dorsal and ventral fins have a rectilinear side profile and join posteriorly forming a pointed and slightly curved apex. There are 12–13 costal grooves.	
		T. carnifex	47.7 mm ± 1.5
		Dorsal areas of the body of yellowish colour densely dotted with melanophores. The belly is yellow-orange. The dorsal and ventral fins have a convex side profile and join posteriorly forming a pointed apex that extends into a long filament. Rounded and large black spots are clearly visible on the fins. 15–16 costal grooves.	
		L. italicus	28 mm ± 1
		Dorsally, the body has a pigmentation ranging from dark yellow to light brown, dotted with brownish-black veins. The belly is yellow-orange. The iridophores on the flanks are well visible, and others emerge, arranged in line and symmetrically with respect to the median plane, in dorsolateral position and on the dorsal margin of the tail. In some individuals, the flanks may acquire a bluish pigmentation. The dorsal fin has a rectilinear side profile, whereas the ventral one has a convex profile. The fins join posteriorly forming a slightly pointed or rounded apex. There are 10–11 costal grooves.	
52b	Regression of the fins occurs along with a slight reduction of the gills. The limbs are more robust. Morphological changes occur in the skin.	***I. a. inexpectatus***	31.2 mm ± 1
		The gills acquire a brownish colour. Metamorphosing larvae are dorsally brown-green, with lighter spots given by groups of iridophores; numerous black spots are evident scattered dorsally, whereas larger roundish stains are distributed in line on the ventrolateral side from the head to the end of the tail. Dorsally, behind the gills, a median orange streak may be visible. Laterally, the grey-blue colour, typical of the species, is distinguishable, whereas the ventral part of the body and tail is yellow-orange. The eye is orange.	
		T. carnifex	50 mm ± 1.1
		The gills acquire a bright red colour. Metamorphosing larvae are dorsally dark with colouration from brown to blackish, dotted by lighter spots and veining given by groups of iridophores. A thin dorsal median orange streak is clearly visible from the gills along the entire body. The ventrolateral area of the body is whitish and dotted with large black spots, whereas the ventral part of the body and tail is bright orange. The ventral area of the head is also orange but thickly speckled with white. The eyes are bulging and orange in colour.	
		L. italicus	30.3 mm ± 1.1
		The gills acquire a red-brownish colour. Metamorphosing larvae are dorsally grey-green or brown-green, with lighter spots given by groups of iridophores; laterally the body is whitish in colour dotted with black spots (in some individuals the flanks may acquire a bluish pigmentation). Dorsally, behind the eye, appears a more or less evident cream-coloured spot (temporal mark). The ventral part of the body and tail is pink-orange dotted with small black spots. The eye is grey.	
52c	Premetamorphic stage. Dorsal and ventral fins have almost completely disappeared. Gills are reduced by half.	***I. a. inexpectatus***	34 mm ± 0.6
		T. carnifex	60 mm ± 1
		L. italicus	30.7 mm ± 0.6

(*Continued*)

Table I. (Continued).

Stage	Common characters	Diagnostic features	Length
53	Metamorphosis is completed. Gills and fins have completely disappeared.	***I. a. inexpectatus***	36 mm ± 1
		The larva has a darker brownish colour. The dorsal orange streak turns red. Belly bright orange in colour and free of blotches, throat with very few and small black spots.	
		T. carnifex	61 mm ± 1.2
		The larva has a darker colour with white dots spread along the midline and on the head; the tail is darker in colour. The median dorsal streak turns yellow.	
		L. italicus	31 mm ± 1
		The larva has a darker brownish-green colour with lighter spots. The temporal marks assume a pink colouring.	

Figure 1. Micrographs of *Ichthyosaura alpestris inexpectatus, Triturus carnifex* and *Lissotriton italicus* specimens at representative stages 33, 35, 37, 39 and 40. Dorsal and lateral views. Scale bar: 1 mm. A detailed description is given in Table I.

& Bidaud 1959; Iwasawa & Kera 1980; Iwasawa & Yamashita 1991; Shi & Boucaut 1995; Tarkhnishvili & Serbinova 1997; Lukanov & Tzankov 2016). Other studies are limited to the early developmental stages, from egg fertilisation to the emergence of fore limb buds and/or hatching (Harrison 1969; Schreckenberg & Jacobson 1975; Epperlein & Junginger 1982; Marks & Collazo 1998; Tripepi et al. 1998; D'Amen et al. 2006; Hurney et al. 2015). Lastly, post-hatching developmental staging tables are available for two species belonging to the genus *Ambystoma* (Watson & Russell 2000; Nye et al. 2003), until metamorphosis. Besides the difference in the choice of the development period to be studied and in the staging criteria, an explicit comparison with data in the literature is made difficult by the quite uneven amount of detail of examination and description. An attempted comparison of our developmental table with those proposed for other Urodela species is summarised in Table II.

In this study, for each species, we updated normal developmental tables by re-examining previous data (Epperlein & Junginger 1982; Tripepi et al. 1998; D'Amen et al. 2006). Our results are quite consistent with available data on embryo development (until stage 31–32, i.e. appearance of fore limb buds); for the post-hatching developmental stages, we propose a staging system that does not exactly correspond to those previously described. In summary, here we provide a complete description of the larval development of three European newts, thus contributing to the scarce amount of research on this topic, particularly evident for some genera of Salamandridae.

Throughout the detailed stage-by-stage description, and by the use of several distinctive features, we also performed a comparative analysis of the

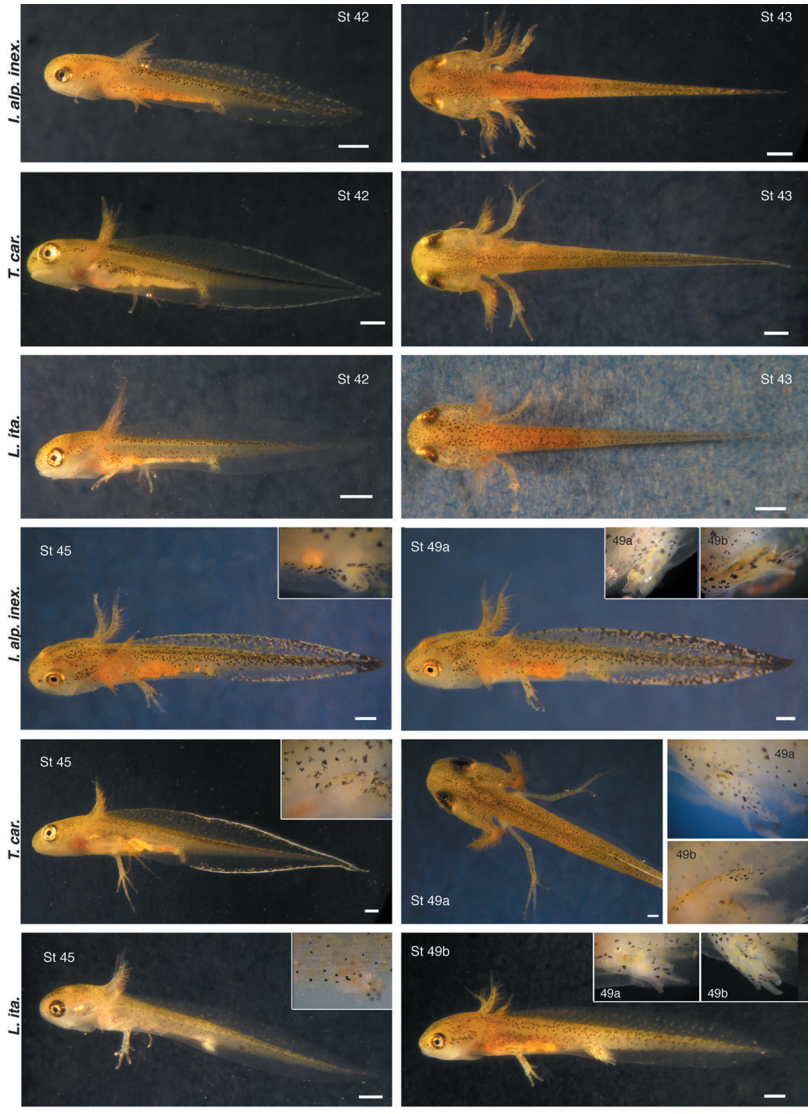

Figure 2. Micrographs of *Ichthyosaura alpestris inexpectatus, Triturus carnifex* and *Lissotriton italicus* specimens at representative stages 42, 43, 45, 49a and 49b. Dorsal and lateral views. Hind limb development is illustrated in insets. Scale bar: 1 mm. A detailed description is given in Table I.

developmental larval phases for the three considered species, with an emphasis on both shared and species-specific features. The most significant morphological traits arising from this study are detailed below.

Length

The larvae of the three species show highly significant differences in their length throughout development. Specifically, until stage 42, the growth trend is similar in the three species but as development proceeds, *T. carnifex* larvae considerably lengthen with respect to the other two (Figure 6), and the dimensional gap is particularly evident between *T. carnifex* and *L. italicus*. This dimensional pattern is easily noticeable in the metamorphosed larvae, reflecting the typical adult size.

Eye

Iris pigmentation begins at stage 34 in both *I. alpestris inexpectatus* and *L. italicus* and, by the end of stage 38, the eye development is completed. In *T. carnifex*, in contrast, ocular pigmentation starts at stage 35 and the eye is well formed during stage 39 (i.e. when embryos hatch).

Gill development

Gill development proceeds more slowly in the Italian newt, and the branching of the first gill filament begins

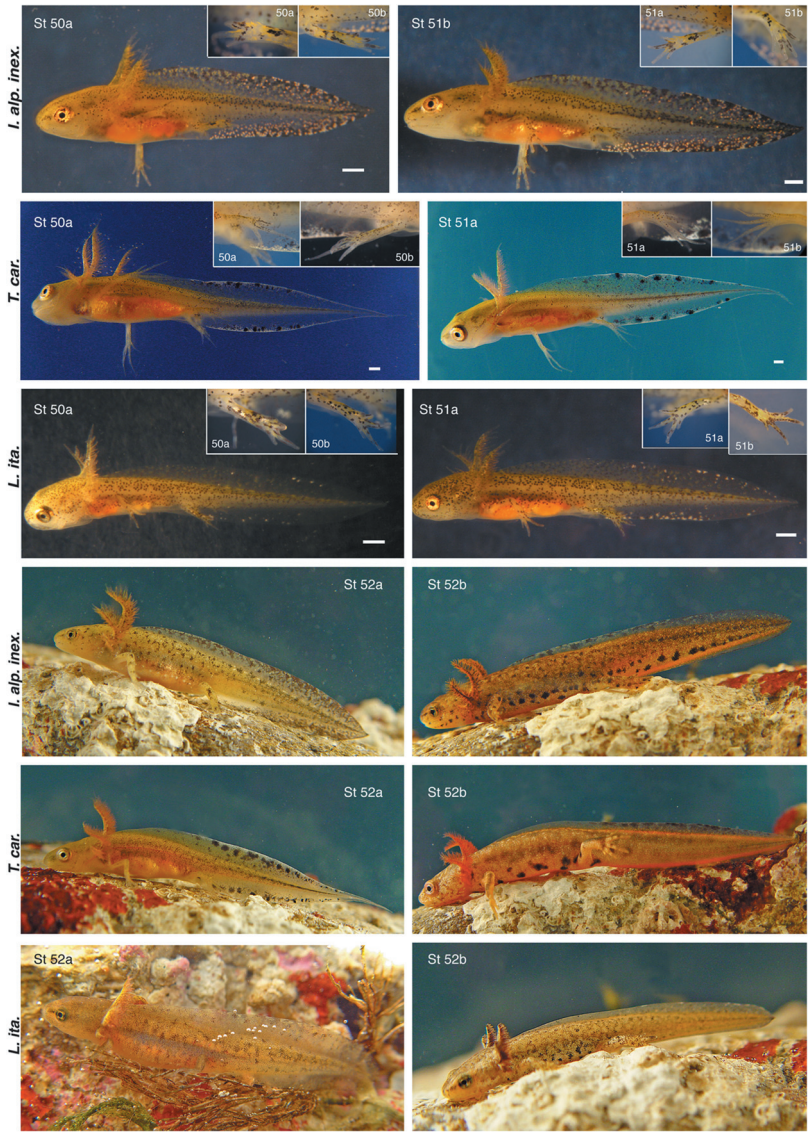

Figure 3. Pictures of *Ichthyosaura alpestris inexpectatus*, *Triturus carnifex* and *Lissotriton italicus* specimens at representative stages 50a–52b. Hind limb development is illustrated in insets. Scale bar: 1 mm. A detailed description is given in Table I.

at stage 35. In contrast, in *I. alpestris inexpectatus* and *T. carnifex*, the gill apparatus appears well formed and branched already at stages 36 and 37, respectively.

Differentiation of balancers

The balancers are rod-like organs, located behind and under the eyes, which allow the larvae to attach to the substrate (Brunelli et al. 2007). Balancer development proceeds similarly in the three species except for a slight difference in time. The balancers lengthen early in *I. alpestris inexpectatus* (stage 36) and *L. italicus* (stage 37), and slightly later in *T. carnifex* (stage 38). In all considered species, regression begins at stage 41 and the balancers have completely disappeared by stage 45.

Hatching

The stage of hatching is almost equal in the three species. In both *I. alpestris inexpectatus* and *L. italicus*, hatching begins at stage 36 (when fore limb buds have an elongated cylindrical shape), and the majority of embryos have already hatched by stage 38 (fore limbs with a bifurcation). In *T. carnifex*, hatching begins at stage 37 (fore limb buds have a paddle shape) and ends at stage 39 (fore limbs with two well-formed digits).

We observed that hatchlings of all three species start feeding (beginning of the active larval life) by the end of stage 42, concurrently with the completion of yolk reabsorption, as previously reported in both *L. italicus* and *T. helveticus* (Gallien & Bidaud

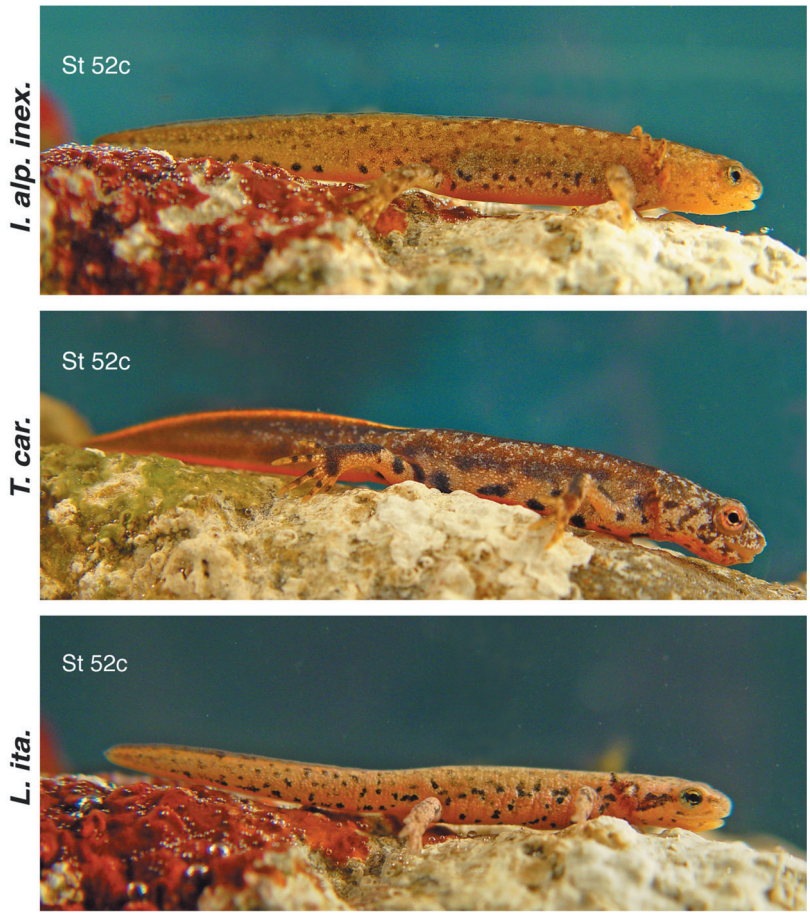

Figure 4. Premetamorphic stage (52c). A detailed description is given in Table I.

1959; Tripepi et al. 1998). Epperlein and Junginger (1982), however, reported that larvae of *T. alpestris* start to feed at stage 37.

Pigmentation pattern of the body

From the early stages of development (stage 33), a distinctive element is the background staining; the larvae of *T. carnifex* are distinguishable from those of the other two species by the greenish-yellow background colour. In all three considered species, the pigmentation in the early stages (33–37) is due to the presence of gathered melanophores that originate three compact stripes. Afterwards, the pigmentation pattern is modified and the melanophores are arranged uniformly throughout the body. More precisely, this occurs at stage 38 in both *I. alpestris inexpectatus* and *L. italicus*, and at stage 40 in *T. carnifex*.

Profile and pigmentation of the fins

The dorsal fin profile is different in the three species, with the fin appearing rectilinear in *I. alpestris inexpectatus*, convex in *L. italicus* and markedly convex in *T. carnifex*. This distinctive feature becomes more evident as development progresses (stages 42–52a). Also, fin pigmentation may represent a distinctive trait useful for larvae recognition. Around stage 40, the presence of numerous chromatophores scattered throughout the dorsal and ventral fins is a common trait for all the considered species. However, in *I. alpestris inexpectatus* a group of melanophores is placed at the apex of the caudal fin, remaining clearly visible until the beginning of metamorphosis. In *T. carnifex*, in contrast, iridophores are distributed on the fin margins in the caudal region, whereas from stage 45 several black blotches on the margins of both fins are evident.

Limb formation

According to other authors (Wong & Liversage 2005; Lukanov & Tzankov 2016), it seems the general pattern of limb development is similar across different groups. In the three considered species, the limb development shows no differences. The cone-shaped

Figure 5. Dorsal and ventral views of newly metamorphosed *Ichthyosaura alpestris inexpectatus*, *Triturus carnifex* and *Lissotriton italicus*. A detailed description is given in Table I.

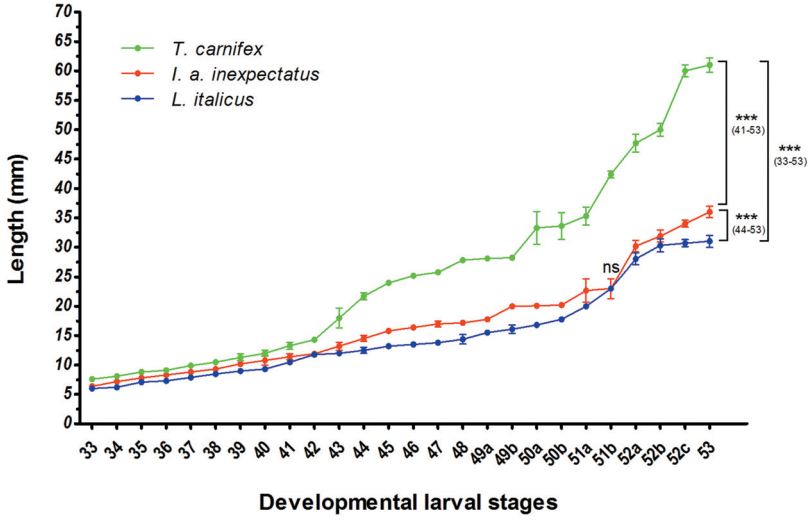

Figure 6. Total length trend during larval development in *Ichthyosaura alpestris inexpectatus*, *Triturus carnifex* and *Lissotriton italicus*. Data are presented as a mean ± standard deviation (n = 10 in each species). Species differences, stage by stage, were assessed using two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test; levels of significance are indicated as *** $p < 0.001$; ns = not significant.

limb buds lengthen, becoming first cylindrical, then paddle-shaped and finally polygonal, just prior to development of the digits. The hind limb buds typically develop after the appearance of the fourth finger of the fore limb (stage 43). Starting from stage 44, the digits of *T. carnifex* assume a very slender shape, and this distinctive feature is maintained until metamorphosis.

In summary, we have furnished staging criteria of *Triturus carnifex*, *Lissotriton italicus* and *Ichthyosaura alpestris inexpectatus*, which could represent a basis and a guideline for future studies on Urodela larval development. Our descriptive and comparative analysis of macroscopic interspecific differences also provides a guide that may be useful, in some cases, for recognition in the field.

Table II. Comparison among different staging systems. Larval developmental stages for *Ichthyosaura alpestris inexpectatus*, *Triturus carnifex* and *Lissotriton italicus*, presented here, compared with developmental stages previously described for other Urodela species. Stages numbers are approximately consistent with our stage description.

Present study	*I. alpestris* [a]	*L. vulgaris* [b]	*L. helveticus* [c]	*L. italicus* [d]	*T. carnifex* [e]	*T. cristatus* [b]	*T. ivanbureschi* [f]	*A. m. krausei* [g]	*P. waltl* [h]	*H. scutatum* [i]	*M. caucasica* [j]
33		36–37	32–33	33	32	36–37	13	1	33–35	23	34
34	31–32	38	34	34–35	33	38		2			35
35		39	35–36	36	34–35	39			35		
36	33	40		37	36	40		3		24	36
37	34	41	37–38	38	37	41					37
38	35	42–43		39	38–39	42	14	4	37		38
39	36	44	39		-	43–45	15	5	38	25	39
40	37	45	40	40	-	46	16	6			40
41	-	46	41	41	-	47		7		26	
42	-		42		-	48–49			39		41
43	-	47–48	43–44	42	-	50–52	17	8	40		42
44	-	49	45	-	-			9		27	43
45	-	50	46	-	-	53			41		
46	-	51–52	47	-	-						44
47	-	53	48	-	-	54		10	42	28	
48	-	54	49	-	-	56		11		-	
49a	-	55	50	-	-	57	18	12	43	-	
49b	-	55–56	51	-	-	58		13		-	45
50a	-	57	52a	-	-	59		14	44	-	
50b	-	58	52b	-	-	60		15		-	46
51a	-		53	-	-	61		16	45	-	48
51b	-	59–60	54	-	-	62	19	17	46	-	49
52a	-	61	55a	-	-	63		18	47	-	52
52b	-	-	55b	-	-	-		19	48	-	53
52c	-	-	55c	-	-	-		20/21	49	-	54
53	-	-	56	-	-	-		22	50	-	56

[a] Epperlein and Junginger (1982); [b] Glücksohn (1932); [c] Gallien and Bidaud (1959); [d] Tripepi et al. (1998); [e] D'Amen et al. (2006); [f] Lukanov and Tzankov (2016); [g] Watson and Russell (2000); [h] Shi and Boucaut (1995); [i] Hurney et al. (2015); [j] Tarkhnishvili and Serbinova (1997).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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