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Vital fluorescent staining for non–destructive studies of neuromast topography in urodele amphibians

Uporaba vitalnega fluorescentnega barvila kot nedestruktiven pristop za raziskave razporeditve nevromastov pri repatih dvoživkah

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Abstract: Neuromasts are mechanosensory organs found in primarily aquatic vertebrates, including many species of amphibians, and are arranged as specific patterns to form the lateral-line system on the head and along the body. We used a hair-cell-specific fluorescent dye, DiASP, to analyze the distributional pattern of neuromasts in the lateral line system of live captive-born larvae of the Italian crested newt, *Triturus carnifex* (Laurenti, 1768). We confirmed that DiASP presents a safe and accurate alternative method for non-destructive studies of neuromast ontogeny and distribution in live amphibians. All newt larvae subjected to analyses survived and no teratogenic effects of DiASP on their further development were observed. We were able to use these data to completely characterize the distribution of neuromasts in this species and to infer the functional significance of this distribution. Cross-species comparison of general topography points to neuromast arrangement as a conserved trait in urodelans.

Keywords: fluorescent staining, DiASP, neuromasts topography, salamanders

Izvleček: Nevromasti so mehanosenzorični organi primarno vodnih vretenčarjev, vključno z mnogimi vrstami dvoživk. Nameščeni so v specifičnih vzorcih in oblikujejo sistem bočne linije na glavi in vzdolž telesa. Z nedestruktivno metodo barvanja z vitalnim fluorescentnim barvilom DiASP smo analizirali vzorec razporeditve nevromastov v bočni liniji v ujetništvu rojenih ličink velikih pupkov *Triturus carnifex* (Laurenti, 1768). Potrdili smo, da DiASP predstavlja varno in natančno alternativo za nedestruktivne študije ontogenije nevromastov in njihove razporeditve pri živih dvoživkah. Vse tretirane ličinke so preživele, teratogenih učinkov DiASP na nadaljni razvoj nismo zasledili. S pridobljenimi podatki smo v celoti karakterizirali razporeditev nevromastov pri tej vrsti in sklepali na funkcionalen pomen razporeditve. Medvrstna primerjava kaže na razporeditev nevromastov kot konzervativno lastnost repatih dvoživk.

Ključne besede: flourescentno barvanje, DiASP, topografija nevromastov, repate dvoživke

Introduction

Neuromasts are mechanosensory organs constituting the functional units of the lateral-line system of aquatic vertebrates including hagfish, lampreys, fishes and amphibians (Russell 1976, Duellman and Trueb 1986, Lannoo 1988, Coombs et al. 1988, Webb 2014). In amphibians they are present in aquatic larvae and adult salamanders that retain an aquatic lifestyle (e.g. *Amphiuma, Andrias, Cryptobranchus, Necturus, Pleurodeles, Proteus, Siren*, and some species of *Ambystoma*), as well as in permanently aquatic pipid frogs (e.g. *Pipa, Xenopus*). A few species of urodeles (e.g. *Notophtalmus viridescens*) retain their lateral line system throughout life, although it partially regresses during the terrestrial subadult ("red eft") phase (Dawson 1936); the neuromasts become fully functional again when the adults return to water.

Neuromasts in aquatic amphibians enable the animals to detect water disturbances caused by currents, the movements and sounds of nearby animals, and a variety of other sources (Dijkgraaf 1962, Shelton 1970, Russell 1976, Duellman and Trueb 1986, Lannoo 1987, Hong et al. 2000). They function as a "distant touch" sensory system important to locate objects in their environment and also play an active role in localization and orientation. Unlike other vertebrate sensory systems such as eyes and nostrils, which are located in a pair of complex organs on the head, the neuromasts are distributed widely over the head and body in stereotyped patterns established during embryogenesis, and function together with electroreceptive ampullary organs to form the lateral-line component of the functionally integrated vestibular system (Dijkgraaf 1962, Lanoo 1988, Coombs and Bleckmann 2014).

The neuromasts of amphibians lie in the epidermis of the skin and consist of three types of cells. The mantle cells lie peripherally and surround the centrally positioned sensory hair cells and supporting cells that in turn surround and separate the sensory cells (Shelton 1970, Sato 1976, Russell 1976, Lannoo 1985, Coombs, et al. 1988, Webb 2014). At the apical part of every sensory hair cell are a kinocilium and many stereocilia that decrease in length with increasing distance from the kinocilium. The sensory hair cells are covered with a gelatinous cupula secreted by the supporting cells of neuromast (Dijkgraaf 1962, Webb 2014). Each hair cell is polarized with the kinocilium always located on the periphery (Dijkgraaf 1962, Flock and Wersäll 1962, Flock and Duvall 1965). The neuromasts are also arranged in lines and in different orientations that maximize the overall sensitivity of the sensory system (Lannoo 1987). Individual neuromasts are maximally sensitive to water currents in one plane only along their long axis, and different neuromasts are oriented so that their planes of maximal sensitivity are in different directions (Dijkgraaf 1962, Flock and Wersäll 1962).

Embryonically, neuromasts develop from both the neural crest cells and epidermal placodes, specifically from pre- and post-auditory placodes, ectodermal thickenings of the temporal region of head, which are situated near the inner-ear primordia (Sato 1976, Northcutt et al. 1994, Colazzo et al. 1994). The lateral line system is completely formed right before hatching of larvae (Sato 1976, Smith et al., 1988, Northcutt et al. 1994). Each neuromast is innervated by one efferent and two afferent nerve fibers of the lateral-line nerves (Dijkgraaf 1962, Flock and Jørgensen 1974, Russell 1976, Webb 2014). Most neuromasts on the head are innervated by fibers of the anterior lateral-line nerve (lateralis anterior VII), and all remaining neuromasts by the posterior lateral line nerve (lateralis posterior X) (Russell 1976, Fritzsch 1981, Duellman and Trueb 1986).

The distribution of the neuromasts of the lateral-line system of amphibians, and especially that of urodeles, has been the subject of many studies (review of Fritzsch 1981, Lannoo 1985, 1987). However, previous studies of neuromast topography in urodelans have been done in a destructive way, requiring euthanizing the animal in order to obtain specimens suitable for conventional light microscopy and/or scanning electron microscopy. An alternative, non-destructive method using fluorescent staining for neuromast topography has been widely used for the study of neuromast topography in live fish specimens (Colazzo et al. 1994, Schuster and Ghysen 2011), but has never before been used on live urodeles. One of the most commonly used hair-cell-specific fluorescent dyes for the study of the topography of neuromasts in live zebrafish is the cationic styryl pyridinium dye DiASP (Colazzo et al. 1994, Schuster and Ghysen

2011). Here we test the applicability of DiASP fluorescent dye in live urodele larvae in order to optimize the procedure and to use it to analyze the ontogeny of neuromast distribution in larvae of the Italian crested newt (*Triturus carnifex*) and to compare with available data for other urodele species described in the literature.

Material and methods

We used a sample (n = 24) of larvae of the Italian crested newt (*Triturus carnifex* Laurenti, 1768) representing a range of ages from pre-hatching to approximately 10 weeks post-hatching. The larvae were obtained from eggs laid in captivity in the laboratory of the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana. Adult male and female newts (total body length 14-16 cm) were collected during the mating season from a pond in the University Botanical Garden in Ljubljana, Slovenia with the approval of the Slovenian Ministry of the environment and spatial planning (permit No. 35601-23/2016-4). The adults were kept in 20 liter tanks with aerated dechlorinated tap water at 20˚C with plastic strips for the attachment of eggs during laying. After spawning the adults were immediately released back into their natural habitat at the same location.

After hatching, the young larvae were kept in plastic containers (5 x 23 x 30 cm) at low density and later kept individually in smaller plastic containers (5x 10 x 18 cm) to avoid cannibalism. The larve were fed three times per week with artemia larvae and later with enchytraeid worms cultured in the laboratory. The water in the containers was replaced with decholorinated tap water three times per week after feeding.

For neuromast topography analyses we used the hair-cell-specific cationic styryl pyridinium fluorescent dye 2-Di-4-ASP (Sigma-Aldrich D3418), which has been used for studies of neuromasts in zebrafish (Colazzo et al. 1994). Live newt larvae were incubated in 5 μM Di-4-ASP in dechlorinated tap water for 10 min followed by anesthesia in 0.3 % tricaine methane-sulphonate solution (MS222, Sigma Chemical Co., St. Louis, Mo.) buffered with 0.2 % sodium bicarbonate (pH = 7) for 2 min. The lightly anaesthetized larvae were mounted in 0.5 % agar in a Petri dish for quick observation under a stereomicroscope (Leica MZ FLIII) using a GFP1 filter and photographed with a Leica DFC290 HD digital camera and Leica LAS 4 software. After observation, which usually took 10-15 minutes, the larvae were released by dissolving the agar in dechlorinated water. Larvae were then transferred back to their plastic containers in order to monitor their further development.

Results

The fluorescent dye has at a low concentration specifically labelled all neuromasts, following a short incubation period. The neuromasts could be well discerned from other, auto-fluorescing body parts (Figs. 1-4). The staining persistent sufficiently to allow the imaging of the living larvae, but faded away after 15 - 20 minutes under illumination. All larvae of *T. carnifex* used in this study survived and developed normally.

The neuromasts lie in the epidermis of the skin and are grouped together in specific clusters on the head and in lines along the rest of the body (Figs. 1A-C, 2A-B, 3). On the head they appear more closely clustered on the snout, while the rest of the other groups form rows around the eyes, in the posterior part of head, along the lower jaw and skeletal elements of the hyoid apparatus of the ventral side of the head (Figs. 1A-C, 2A-B, 3). The typical arrangement of neuromasts on the head and trunk was completely formed before the hatching of the larvae (Fig. 1A-C).

The rows of the neuromasts on the head were divided on the basis of their position and colocalization with the skull elements into eight major groups: nasal, maxillar, circumorbital (supraorbital and infraorbital), postorbital, parietal, postotic, mandibular and submandibular groups (Figs. 1A-C, 2A-B, 3). The supraorbital neuromasts are located dorsal and medial to the eyes, while infraorbital neuromasts are located posterior and ventral to the eyes (Figs. 2A-B). The orientation of neuromasts in each circumorbital row (supra and infraorbital) is tangentially to the eye (Fig. 4A). Nasal neuromasts are arranged in two rows anterior to the supraorbital and the rows are perpendicular to each other (Figs. 2A, 4A). The maxillary neuromasts form three to four rows

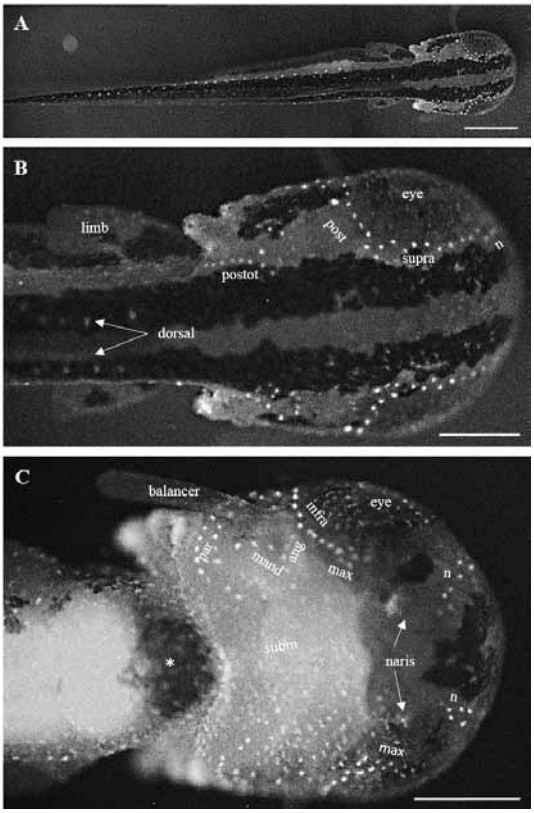

Figure 1: The neuromasts of a larva of the Italian crested newt *T. carnifex* before hatching. Fluorescent DiASP staining. **A**–Dorsal view of the whole body. **B**–Dorsal view of the head. **C**–Ventral view of the head. ang – angular group, asterisk – heart, infra – infraorbital group, max – maxillary group, mand – mandibular group, n – nasal group, par – parietal group, post – postorbital group, postot – postotic group, subm – submandibular group, supra – supraorbital group. Scale bar: 1 mm (A), 0,5 mm (B and C).

Slika 1: Nevromasti ličinke velikega pupka *T. carnifex* pred izleganjem. Fluorescentno barvanje z DiASP. **A**–Dorzalna stran telesa. **B**–Glava dorzalno. **C**–Glava ventralno. ang – angularna skupina, infra – infraorbitalna skupina, man – mandibularna skupina, max – maksilarna skupina, n – nasalna skupina, par – parietalna skupina, post – postorbitalna skupina, postot – postotična skupina, subm – submandibularna skupina, supra – supraorbitalna skupina, zvezdica – srce. Merilo: 1 mm (A), 0,5 mm (B in C).

anterior to the infraorbital group (Fig. 2B), mostly one medial and two lateral rows of neuromasts which are oriented with their long axis perpendicular to adjacent neuromasts (Fig. 2B). Both maxillary and nasal neuromasts continue in one line to the pre-maxilla part of the upper jaw. The postorbital group has fewer neuromasts that are located in one row behind the circumorbital row (supra and infraorbital) and perpendicular to the circumorbital row (Figs. 2B, 4A). The neuromasts in the postotic group form a loose pattern on the caudal portion of the dorsal part of the head and appear to be continuous with the medial line of the body neuromasts (Figs. 2A, 4A). The neuromasts in the postotic group are oriented parallel as well transverse to body axis (Fig. 4A). The

parietal group consists of two adjacent curved rows of neuromasts which transition into the submandibular group (Figs. 2B, 3). The rows of neuromasts in the parietal group are perpendicular to each other. The angular row lies posterior to the jaw angle, vertically to the infraorbital and continues to the mandibular row of neuromasts (Figs. 2B). On the ventral side of the head the submandibular group of neuromasts is arranged along the hyoid skeletal elements in two rows at the rostral part which converge to one row that extends to the caudal part of the head and joins the parietal group (Fig. 3). The rows of neuromasts at the rostral part of the submandibular group are oriented perpendicular to each other.

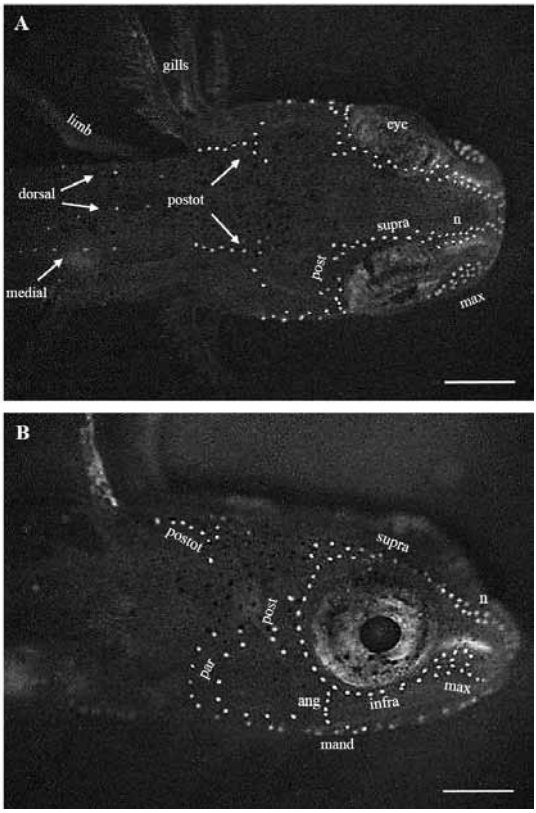

Figure 2: The head of post-hatched larva of the Italian crested newt *T. carnifex* with labeled neuromasts. Fluorescent DiASP staining. **A**–Lateral view. **B**–Dorsal view. The anterior part of trunk dorsal and medial line of neuromasts is also visible in A. ang – angular group, dorsal – dorsal line of neuromasts, infra – infraorbital group, max – maxillary group, mand – mandibular group, medial – medial line of neuromasts, n – nasal group, par – parietal group, post – postorbital group, postot – postotic group, subm – submandibular group, supra – supraorbital group. Scale bar: 1 mm.

Slika 2: Glava izležene ličinke velikega pupka *T. carnifex* z označenimi nevromasti. Fluorescentno barvanje z DiASP. **A**–Dorzalni pogled. **B**–Lateralni pogled. Na sliki A je viden tudi sprednji del dorzalne in mediane linije nevromastov trupa, ang – angularna skupina, dorsal - dorzalna linija nevromastov, infra – infraorbitalna skupina, man – mandibularna skupina, max – maksilarna skupina, medial – osrednja linija nevromastov linija nevromastov, n – nasalna skupina, par – parietalna skupina, post – postorbitalna skupina, postot – postotična skupina, subm – submandibularna skupina, supra – supraorbitalna skupina. Merilo: 1 mm.

The neuromasts on the trunk occur along three distinct lines: the dorsal, medial, and ventral line (Figs. 2A, 3). All three lines begin slightly rostral to the front limb and the medial line continues up to the tip of the tail, while the other two lines (dorsal and ventral) are shorter, and extend only to the hind limb. The dorsal line neuromasts are also very sparse and at the middle part of the trunk approach the medial line (not shown). The neuromasts in the medial and ventral lines lie parallel with the major body axis, although the rostral part of the ventral line also follows the curve of the pectoral girdle. (Figs 3, 4B). The dorsal line neuromasts are oriented dorso-ventrally, inclined at 45˚, and they are less numerous than those in the other two lines.

In young post-hatching larvae the neuromasts are situated individually in the rows on the head and along the body, but later approximately after one month after hatching the neuromasts occur in pairs (Fig. 4A-B). The total number of neuromasts on one side of the head in pre-hatched larvae is lower (75.5 ± 2.1) than in post-hatched larvae (137 ± 6.8) (Table 1) mostly due to lower number of neuromasts in the maxillary, nasal and submandibular group. The total number of neuromasts doubles in older larvae (277 ± 10.3) due to the formation of neuromast pairs. The largest numbers of neuromasts are found in the circumorbital, maxillar, nasal and submandibular group (Table 1). Along one side of the trunk, newly hatched larvae begin with approximately 56 single neuromasts but this number later doubles due to the formation of neuromast pairs. A rough estimate of the total number of neuromasts on the whole surface of the body in older larvae is approximately 750 to 800 neuromasts.

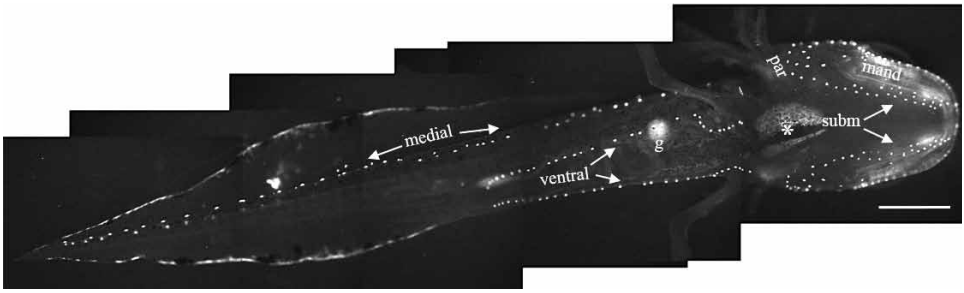

Figure 3: Fluorescent imaging of labeled neuromasts of the ventral side of the body of the Italian crested newt *T. carnifex* larva. asterisk – heart, g – gall blader, mand – mandibular neuromasts, medial – medial line of neuromasts, par – parietal neuromasts, subm – submandibular neuromasts, ventral – ventral lines of neuromasts. Scale bar: 1 mm

Slika 3: S fluorescenčnim barvilom označeni nevromasti ventralne stani telesa ličinke velikega pupka *T. carnifex.* g – žolčnik, medial – osrednja linija nevromastov, mand – mandibularni nevromasti, medial – osrednja linija nevromastov, par - parietalni nevromasti, subm – submandibularni nevromasti, ventral – ventralni liniji nevromastov, zvezdica – srce. Merilo: 1 mm.

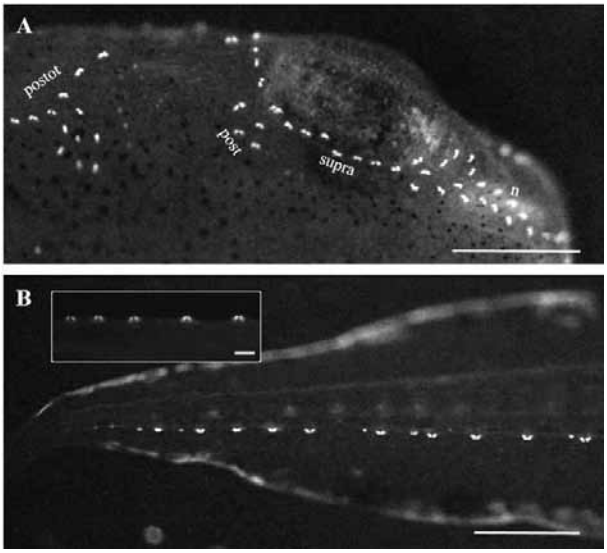

Figure 4: Older larva of the Italian crested newt *T. carnifex* with double neuromasts. Fluorescent DiASP staining. **A**–Head region with postotic (post), postorbital (post), supraorbital (supra), and nasal (n) group of neuromasts. **B**–Tail region with the medial line neuromasts. **Insert in B**–Double neuromasts of tail under higher magnification. Scale bar: 1 mm (A and B), 100 µm (insert in B).

Slika 4: Starejša ličinka velikega pupka *T. carnifex* z dvojnimi nevromasti. Fluorescentno barvanje z DiASP. **A**–Glavina regija s postotično (post), postorbitalno (post), supraorbitalno (supra) in nasalno (n) skupino nevromastov. **B**–Repna regija z mediano linijo nevromastov. **Manjša slika na B**–Dvojni nevromasti repa pod večjo povečavo. Merilo: 1 mm (A in B), 100 µm (manjša slika na B).

Table 1: The number of neuromasts on the head of larvae of Italian crested newt *T. carnifex* at different ages. The given number of neuromasts is for one side of the head.

Tabela 1: Število nevromastov na glavi pri različno starih ličinkah velikega pupka *T. carnifex*. Podano število nevromastov je za eno stran glave.

	Number of neuromasts			
Neuromast group	Before hatching (N=2)	Hatched larvae (N=2)	Post-hatched larvae (N=7)	Older larvae with double neuromasts in stitches (N=6)
angular	2.5 ± 0.7	2.5 ± 0.7	3.6 ± 0.8	7.3 ± 1.6
circumorbital	19.0 ± 1.4	20.5 ± 0.7	22.1 ± 2.7	45.7 ± 4.3
mandibular	7.0 ± 0.0	8.5 ± 0.7	9.9 ± 1.1	19.7 ± 2.3
maxillar	6.0 ± 1.4	7.0 ± 1.4	20.0 ± 1.0	40.0 ± 2.2
nasal	7.0 ± 1.4	11.0 ± 1.4	20.1 ± 2.0	42.7 ± 4.3
parietal	12.5 ± 0.7	12.5 ± 0.7	16.4 ± 1.9	34.0 ± 2.5
postorbital	5.0 ± 1.4	8.5 ± 0.7	8.4 ± 0.8	17.0 ± 1.7
postotic	9.0 ± 1.4	11.5 ± 0.7	13.6 ± 2.4	27.0 ± 5.3
submandibular	7.0 ± 0.0	21.0 ± 1.4	21.9 ± 1.9	44.3 ± 3.7
Total	75.5 ± 2.1	103.0 ± 7.1	137.0 ± 6.8	277.7 ±10.3

Discussion

The goals of this preliminary study of neuromasts of Italian crested newt *Triturus carnifex* larvae were two-fold, first to optimize a non-destructive method and to confirm that the DiASP fluorescent dye has no harmful effects on live larvae and, secondly, to perform a detailed description of neuromast distribution on the head and on the rest of the body and to compare with the available data for other urodele species.

Our work is the first non-destructive study of neuromast topography in urodele amphibians using the hair cell-specific fluorescent dye DiASP. Previous studies of neuromast topography in urodeles utilized destructive approaches that necessitated killing the animals for conventional light and electron scanning microscopy on whole specimens (review in Fritzsch 1981, Lannoo 1985, 1987). All newt larvae used in our study survived and no teratogenic effects of DiASP on their further development were observed. DiASP therefore presents an alternative, non-destructive method for studies of the ontogeny and evolution of distribution patterns of this functionally important sensory system in live amphibians.

Among aquatic amphibians, the arrangement of neuromasts in the lateral line system varies among and within the different orders (Lannoo 19987, 1988): caecilians have single rows of mechanoreceptive neuromast organs; generalized anurans have single rows of neuromasts that divide to form secondary neuromasts or "stitches"; generalized urodeles have transverse stitches, and double or triple rows of neuromasts. The general arrangement of neuromasts in *T. carnifex* larvae is similar to that described for other aquatic urodeles with three rows (dorsal, medial and ventral) on the trunk and many distinctive rows on the head divided into nasal, maxillary, supraorbital, infraorbital, postorbital, parietal, postotic, mandibular and submandibular groups (Lannoo 1987, 1988, Smith et al. 1988, Mali 1990). The neuromasts may be single, or clustered in parallel to form stitches which in turn, are organized into groups that are arranged in different orientations in a pattern that maximizes directionality (Lannoo 1985, 1987). Topographically similar neuromasts are oriented in the same direction (Lannoo 1987, 1988), which is consistent with what we found in *T. carnifex* larvae. The arrangement of circumorbital neuromasts is tangent to the eyes. The rest of the other groups of neuromasts on the head and body are oriented either parallel or perpendicular with the body axis making them sensitive to water displacements in all directions along a plane across the body surface (Lannoo 1987). Three groups of head neuromasts near the snout (nasal, maxillary and submandibular) have a more complex arrangement than the rest of the other groups: the rows are perpendicular to each other, thus allowing high resolution in prey detection (Dijkgraaf 1962, Russell 1976, Lannoo 1987).

The lateral-line system in *T. carnifex* is completely formed just before hatching, as is common for larvae of other urodelan species (Sato 1976, Smith et al. 1988, Northcutt et al. 1994), but the number of neuromasts in individual groups is lower than in post-hatching larvae of *T. carnifex.* This is especially prominent in the maxillary, nasal, and submandibular groups of neuromasts due to different head morphology which is rounder and smaller in younger larvae but then gradually elongates as it grows larger with age. Likewise, as in other post-hatching larvae of urodeles (Lannoo 1987), the neuromasts are single in younger larvae of *T. carnifex*, but later (in one month old larvae) are doubled in short stitches. The early division of individual (primary) neuromasts to form secondary neuromasts and stitches is a common characteristic for the older larvae of the salamander family Salamandridae to which *T. carnifex* belongs, and is also seen in the families Ambystomatidae, Cryptobranchidae, and Proteidae (Lannoo 1987). In most other salamander families (Hynobiidae, Dicamptodontidae, Plethodontidae, Amphiumidae and Sirenidae) the neuromasts remain single in older larvae as well in permanently aquatic adults (Russell 1976, Lanoo 1987). In all urodeles, the final number of neuromasts per stitch is variable and increases with age while the total number of stitches remains constant (Lannoo 1985). Some urodeles retain only two neuromasts per stich while others develop three or more and the number of neuromasts per stitch can even differ among the species of the same genus. For example, stitches with three neuromasts are predominant in *Ambystoma mexicanum,* while *A. triginum* has seven (Lannoo 1985). Because we did not analyze the larvae of *T. carnifex* older than three months, we

do not know how many neuromasts are found in fully formed stitches.

The total number of neuromasts not only varies among taxa, but even between the left and rights sides of the same individual (Lannoo 1987). Lannoo (1987) compared the total numbers of neuromasts from one side of the head of larvae in different species of seven urodeles families (Ambystomatidae, Amphiumidae, Dicamptodontidae, Hynobiidae, Plethodontidae, Proteidae, Salamandridae) that ranged from a mean of 94 in the smallest larvae in *Hynobius nebulosus* (with snout-vent length 14.5 mm) to a mean of 150 in larger sized larvae in *Necturus maculosus* (with snout-vent length 210 mm). The total number of single neuromasts on one side of the head in post-hatched larvae of *T. carnifex* (with snout-vent length 16.5 mm) was approximately 137 (range 126 to 148) and this is similar to that described by Lannoo (1987) for larve of *Notophthalmus viridescens* (with snout-vent length 17.5 mm). This number of neuromasts doubles in older larve of *T. carnifex* when stitches contain two neuromasts. Consistent with the literature (Lannoo 1985, 1987), the number of neuromasts in different groups on the head of *T. carnifex* varies and the groups with the largest number of neuromasts are the circumorbital, maxillar, nasal and submandibular groups. These observed patterns are important since neuromast density affects the mechanosensory ability of the neuromast system as a whole and the more neuromasts an animal has, the greater will be its ability to perceive water displacements (Lannoo 1985).

The increase in the number of neuromasts per stitch with age is accompanied by an increase in the number of hair cells in individual neuromasts (Lannoo 1985, Mali 1990). Mali (1990) described the progressive changes in the number of hair cells per neuromast in larvae of the alpine newt *Ichthyosaura alpestris*, with 2-5 hair cells in neuromasts at hatching, 3-10 hair cells after 25 days post-hatching and 11 -16 hair cells in 60-day old post-hatching larvae. The number of sensory cells per neuromast in older larvae of different species of newts (e. g. *Ichthyosaura alpestris, Lissotriton vulgaris, Triturus cristatus*) range from 12 to 20 (Fritzsch and Wahnschaffe 1983, Mali 1990). A similar number of sensory cells per neuromast was described for larvae of the fire salamander *Salamandra salamandra* (Fritzsch and Wahnschaffe 1983), adult axolotls *Ambystoma mexicanum* (Jørgensen and Flock 1973), as well as for adult European blind cave salamanders *Proteus anguinus* (Bulog 1988, Mali 1990). We did not quantify the number of hair cells in individual neuromasts because the specimens were examined under stereo microscope where individual hair cells could not be discerned. Nevertheless, it seems likely that the number of neuromast hair cells in *T. carnifex* is similar to that seen in other newts. However, further study should focus on the ontogeny of the stitches and numbers of hair cells per neuromast in *T. carnifex* larvae.

In conclusion, the DiASP fluorescent staining method is a safe and completely non-destructive method for studies of neuromast ontogeny and detailed arrangement in live urodele amphibians. Comparison of the general topography of neuromasts in *T. carnifex* larvae with other urodelan larvae confirms neuromast distribution to be a conservative trait in urodele amphibians.

Povzetek

Nevromasti so mehanosenzorični organi sistema bočne linije primarno vodnih vretenčarjev, vključno z dvoživkami. Pri slednjih so zastopani pri ličinkah in pri odraslih permanentno vodnih repatcih in brezjezičnicah. Nameščeni so v vrhnjici kože na glavi in vzdolž telesa in imajo pomembno vlogo v lokalizaciji plena in prostorski orientaciji. Razporeditev nevromastov v bočni liniji dvoživk je bila predmet mnogih raziskav, ki pa so bile izvedene na destruktiven način, saj je bilo potrebno živali evtanazirati. Alternativna nedestruktivna metoda, ki je v uporabi pri ribah in pri dvoživkah še ni bila preizkušena, je uporaba vitalnih fluorescentnih barvil, npr. kationskega barvila DiASP, ki specifično barva čutnice znotraj nevromastov. Namen naše raziskave je bil: i) preizkusiti uporabnost barvila DiASP na dvoživkah in ii) opisati ontogenijo razporeditve nevromastov pri ličinkah velikega pupka *Triturus carnifex* (Laurenti, 1768) in primerjati s podatki iz literature za druge vrste repatcev.

Za raziskavo smo uporabili v ujetništvu zležene ličinke velikega pupka v razponu starosti pred izleganjem do 10 tednov po izleganju. S pomočjo

fluorescentnega vitalnega barvila DiASP smo lahko natančno lokalizirali razporeditev nevromastov, saj je barvilo selektivno barvalo nevromaste, ki so se jasno razlikovali od morebitnih avtofluorescentih delov telesa. Vse z DiASP tretirane ličinke so preživele, prav tako nismo zasledili nikakršnih teratogenih učinkov na njihov nadaljni razvoj. Osnovna razporeditev nevromastov pri ličinkah velikega pupka je podobna razporeditvi pri ostalih predstavnikih iz družine pupkov in močeradov (Salamandridae). Nevromasti so urejeni v specifičnem vzorcu na glavi in treh paralelnih linijah na trupu. Glede na pozicijo in kolokalizacijo s skeletnimi elementi lobanje lahko nevromaste na glavi razdelimo v 8 glavnih skupin: nazalna, maksilarna, cirkumorbitalna (supra- in infraorbitalna), postorbitalna, parietalna, postotična, mandibularna in submandibularna. Najbolj kompleksna ureditev nevromastov je v predelu gobca (v nazalni, maksilarni in submandibularni skupini), saj so le-ti razporejeni v več linijah, ki potekajo pravokotno ena na drugo. Takšna ureditev omogoča zaznavanje premikov vode v različnih smereh, kar naj bi pripomoglo k učinkovitejšemu zaznavanju plena. Na trupu so nevromasti razporejeni v tri paralelne linije (dorzalna, mediana in ventralna linija), ki se pričnejo nekoliko pred sprednjimi okončinami. Medtem ko mediana linija poteka vse do konice repa, sta ostali dve liniji krajši in segata le do zadnjih okončin. Tipičen vzorec razporeditve nevromastov je pri velikem pupku izoblikovan tik pred izleganjem ličink, kar je značilno tudi za ostale repatce. Nevromasti so nameščeni posamično, kasneje (en mesec po izleganju) pa zasledimo dvojne nevromaste oz. nevromaste v parih, ki tvorijo kratke "šive" ali "stitches". Število nevromastov znotraj posameznih "šivov" je vrstno specifično, žal pa ne moremo trditi, da so dvojni nevromasti tudi končna oblika "šiva", saj ličink starejših od treh mesecev nismo vključili v študijo. Celokupno število nevromastov pred izleganjem je tudi bistveno nižje v primerjavi s starejšimi ličinkami. Slednje je še posebej izrazito na glavinem delu, predvsem na račun manjšega števila nevromastov v maksilarni, nazalni in submandibularni skupini, kar je v korelaciji s samo obliko glave, ki je pri mlajših ličinkah majhna in okrogla in se s starostjo ličink postopoma podaljšuje.

Naša raziskava je ena prvih nedestruktivnih pristopov za analizo topografije nevromastov pri dvoživkah. Potrdili smo, da je uporaba fluorescenčnega barvila DiASP varna, in da omogoča natančno analizo ontogenije nevromastov in njihove razporeditve na živih dvoživkah. Medvrstna primerjava kaže na splošno topografijo nevromastov kot konzervativno lastnost repatih dvoživk.

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