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Glycoconjugate expression in the olfactory bulb of the premetamorphic larva of the Japanese sword-tailed newt (*Cynops ensicauda*)

Toshiyasu MATSUI[1] and Yasushi KOBAYASHI[1]*

[1]Department of Anatomy and Neurobiology, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

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ABSTRACT. We examined the organization of the olfactory organ and assessed the lectin histochemistry to investigate the glycoconjugate distribution of the olfactory bulb in premetamorphic larvae of *Cynops ensicauda*. The nasal cavity was an oval chamber that contained olfactory epithelium and a primitive vomeronasal organ. Secretory products were found in the supporting cells of the two sensory epithelia and in the respiratory cells. Ten lectins bound to the olfactory and vomeronasal nerve fibers as well as to the glomeruli in the olfactory bulb. The binding intensity in larvae was weaker than that reported previously in mature animals. This difference suggests a functional correlation between the expression change of glycoconjugates and the developmental refinement of the olfactory system during metamorphosis.

KEY WORDS: lectin, metamorphosis, olfactory system, salamander, vomeronasal organ

Salamanders inhabit a wide variety of habitats, from aquatic to terrestrial environments. Most salamander species undergo a transition from an aquatic larva to a more terrestrial juvenile form, and often cross the threshold between aquatic and terrestrial habitats in the adult form. Metamorphosis during ontogenesis provides amphibians, including salamanders, a competitive advantage by reducing the competition for food sources between the larvae and adults that feed in two distinct habitats. Olfaction is a chemosensory system to detect chemical molecules emanating from a distant source, such as food items and individuals of the same or different species [2, 13]. During metamorphosis, the olfactory system in salamanders must adapt to a perception of water-soluble and/or volatile odorant molecules.

The olfactory system of amphibians is divided into two subsystems: the main and accessory (vomeronasal) olfactory systems [2, 13, 19]. In the main olfactory system, the olfactory epithelium (OE) in the nasal cavity contains three types of cells: receptor cells, supporting cells, and basal cells as a progenitor of the above two cells. Axons of the receptor cells form the olfactory nerve that projects to the main olfactory bulb (MOB) in the brain. The accessory olfactory system comprises the sensory epithelium in the vomeronasal (Jacobson's) organ (VNE), the vomeronasal nerve, and the accessory olfactory bulb (AOB). The vomeronasal organ in amphibians is a diverticulum that branches off the main nasal cavity and its epithelium generally resembles the OE. Receptor cells in the VNE extend axons through the vomeronasal nerve to the AOB. The main olfactory system detects general odorants, while the accessory olfactory system detects unique stimuli, including some pheromones [6, 7, 13]. It is interpreted that the vomeronasal organ appeared in a common amphibian ancestor by the partitioning of a neuronal subpopulation of the OE that expressed vomeronasal-specific genes as a distinct organ [1, 13, 19]. In salamanders, the developmental variance of the accessory olfactory system seems to be complicated by both habitat diversity and metamorphosis. Schmidt *et al.* (1988) examined the primary olfactory and vomeronasal projections in ten salamander species with and without a habit transition (the biphasic and direct-developing species, respectively) [15]. Vomeronasal projections of the biphasic species exhibit higher complexity, with termination fields in the AOB, than those of the direct-developing species, whereas the olfactory projections are similar in both types of species. In addition, an aquatic salamander with paedomorphosis, the mudpuppy (*Necturus maculosus*), lacks the vomeronasal organ, but another neotenic salamander, the axolotl (*Ambystoma mexicanum*), has an anatomically independent vomeronasal organ [1, 3, 13]. Previous studies have, therefore, focused on morphological comparisons of the olfactory systems among salamanders, between larval and adult stages, and between habitat types [1, 13, 17, 18].

The Japanese sword-tailed newt, *Cynops ensicauda*, is a semi-aquatic salamander that occurs in woodland habitats and is endemic to the Ryukyu Islands of Japan. *Cynops ensicauda* is strictly aquatic in the larval stage, but progressively transitions to a more terrestrial habit in the juvenile and adult life stages through the metamorphosis. This change from aquatic to terrestrial

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*Correspondence to: Kobayashi, Y.: yasushi@ndmc.ac.jp

Table 1. Concentrations, sugar specificities, and binding intensities of the lectins used in the glycoconjugate expression study involving the olfactory bulb of larval *C. ensicauda*

Lectins (Abbreviation)	Conc. (μg/m*l*)	Primary sugar specificity	MOB		AOB	
			NL	GL	NL	GL
Wheat germ agglutinin (WGA)	1.0	GlcNAc	+	+	+	+
Succinylated wheat germ agglutinin (s-WGA)	10.0	GlcNAc	+	+	+	+
Lycopersicon esculentum lectin (LEL)	0.5	GlcNAc	+++	+++	++	++
Soybean agglutinin (SBA)	10.0	GalNAc	+	+	+	±
Bandeiraea simplicifolia lectin-I (BSL-I)	2.0	Gal	++	+	++	+
Peanut agglutinin (PNA)	10.0	Gal	-	±	-	±
Pisum sativum agglutinin (PSA)	5.0	Man	+	+	+	+
Lens culinaris agglutinin (LCA)	5.0	Man	±	±	±	±
Phaseolus vulgaris agglutinin-E (PHA-E)	5.0	Complex structures	+	+	+	+
Phaseolus vulgaris agglutinin-L (PHA-L)	5.0	Complex structures	±	±	±	±

The lectin binding intensity in the nerve and glomerular layers (NL and GL) of the main and accessory olfactory bulb (MOB and AOB) was evaluated on a five-grade scale: -, negative; ±, faint; +, weak; ++, moderate; +++, strong. Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Man, mannose.

habitats affords the opportunity to investigate the relationship between the development of the vertebrate olfactory system and the transition of living environments [1, 13, 18]. We previously determined the glycoconjugate expression in the primary olfactory center of mature *C. ensicauda* using the lectin histochemistry [9]. Lectins have a capacity to bind to specific sugar residues, and the lectin histochemistry is useful to visualize the glycoconjugate distribution in the olfactory system [6, 12]. Diverse glycoconjugates are expressed in the primary olfactory projection of vertebrates, and play an important role in cell-cell recognition and in guiding axons to their appropriate target during ontogenic development of the olfactory system [6, 12]. We hypothesized that if glycoconjugates act on the development and refinement of the primary olfactory projection during metamorphosis in salamanders, lectin bindings to the olfactory bulb should differ between larval and metamorphosed adult life stages of salamanders. We, therefore, determined the glycoconjugate distribution of the olfactory bulb in premetamorphic larvae of *C. ensicauda*, using the lectin histochemistry.

All procedures were carried out as approved by the Animal Care and Use Committee of the National Defense Medical College. Tadpoles of *C. ensicauda* from our laboratory stock were housed at approximately 20°C and fed three times a week with live brine shrimp. Sixteen- to eighteen-week-old larvae (n=6; body length: 32–38 mm; body weight: 0.28–0.35 g) that were grouped into developmental stage 58 based on the body appearance [20], were used in this study. Animals were anesthetized with 0.1% solution of Ethyl 3-aminobenzoate methanesulfonate (MS-222; MP Biomedicals, Santa Ana, CA, U.S.A.) and sacrificed by decapitation. Skulls and brains were placed in Bouin's solution without acetic acid overnight for fixation, and routinely embedded in paraffin. Sections of the olfactory organ and brain were cut transversely and horizontally, respectively, at 5 μm and subjected to the hematoxylin-eosin staining, periodic acid-Schiff (PAS) staining, and lectin histochemistry analyses.

Ten biotinylated lectins, which exhibited a binding reactivity in the primary olfactory center of adult *C. ensicauda* [9], were used in this study (Lectin screening kits I–III, Vector Laboratories, Burlingame, CA, U.S.A.). The applied concentration and sugar specificity of lectins is shown in Table 1. Lectin histochemistry was performed as described previously [8]. In summary, deparaffinized sections were blocked with 1% bovine serum albumin in phosphate-buffered saline and incubated overnight at 4°C with biotinylated lectins. The reactions were detected with the Vectastain ABC-Elite kit (Vector Laboratories) and diaminobenzidine solution.

A previous study noted that the vomeronasal organ in the coastal giant salamander is present in all life stages from small larvae to metamorphosed adults and does not arise from metamorphosis [17]. Therefore, we first examined general histology of the olfactory organ of premetamorphic *C. ensicauda* at stage 58 prior to the lectin-binding analysis in the primary olfactory and vomeronasal projections. No obvious individual differences were observed in the organization of the olfactory organs of larvae at this developmental stage. The nasal cavity was a single oval chamber, and the OE was present in the dorsal, medial, and ventral regions in each nasal cavity (Fig. 1a). The nonsensory ciliated epithelium formed a series of ridges and separated the sensory epithelium into several shallow grooves. The vomeronasal organ (also referred to as the lateral nasal sinus) was positioned ventrolaterally in the nasal cavity, but appeared immature compared to that in adult specimens. Nerve fibers from the sensory epithelium were observed in the lamina propria and extended caudally to the olfactory bulb. The branching of the vomeronasal organ was at a primitive phase of development in the larvae of this stage; however, the surface was fully covered with the VNE. The OE and VNE consisted of the pseudostratified epithelium and contained three types of cells: supporting, receptor, and basal cells (Fig. 1b), which presented elongated nuclei in the upper region of the epithelium, oval nuclei in the middle region of the epithelium, and nuclei just above the basal laminae of the epithelia, respectively. The nasal sections were subjected to PAS staining to evaluate mucosal secretion in the olfactory organ of the larvae. In larval specimens of stage 58, no PAS-positive glands (Bowman's glands) were found in the lamina propria of the sensory epithelium (Fig. 1c). In contrast, the OE and VNE showed a PAS reaction in the free border and in the supranuclear cytoplasm of the supporting cells (Fig. 1d). Weak reaction of PAS was also observed in cell bodies of the receptor neurons in both the OE and VNE.

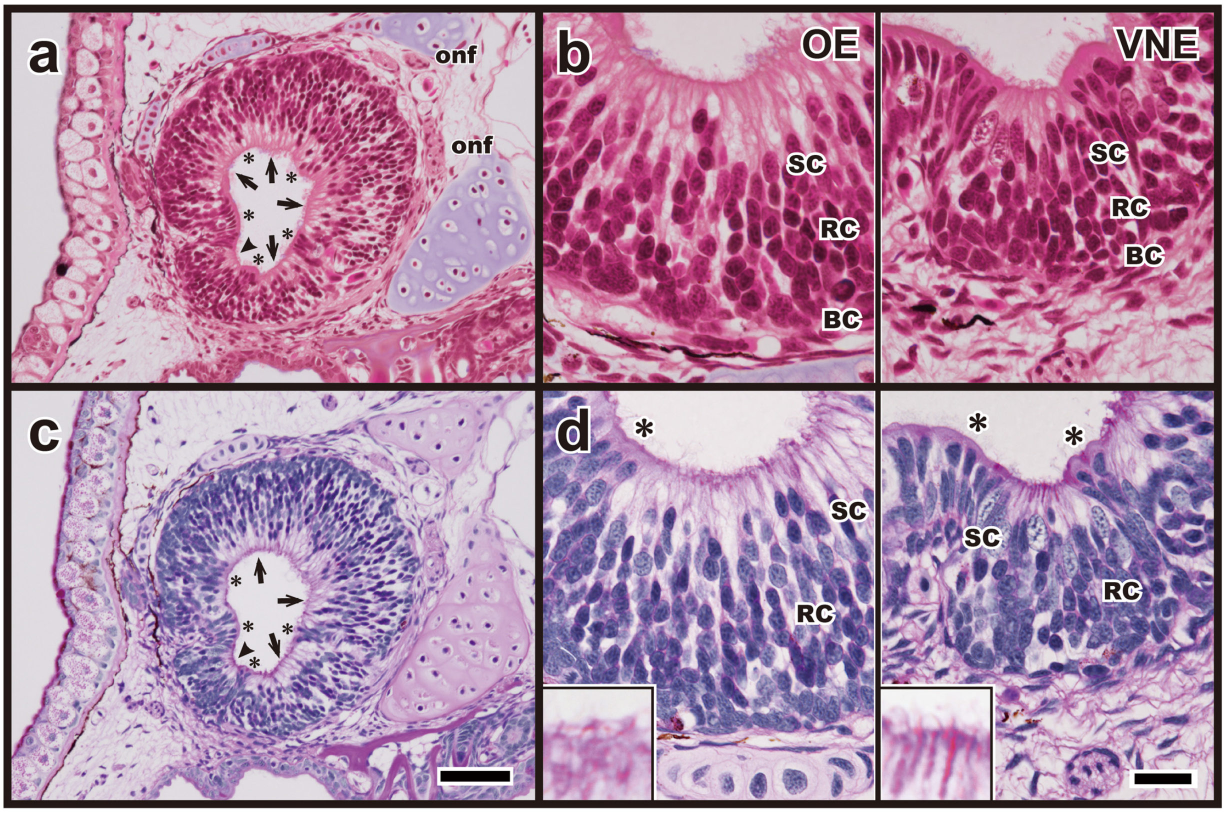

Fig. 1. Transverse sections of the olfactory organ of larval *Cynops ensicauda*. The left and top sides of panels (a, c) are lateral and dorsal portions, respectively. (a, b) Hematoxylin-eosin staining. In stage 58 larvae, the olfactory and vomeronasal epithelium (OE; arrows, and VNE; arrowhead) were partitioned by ridges of nonsensory epithelium (asterisks). Nuclei of supporting, receptor, and basal cells (SC, RC and BC) were located in the upper, middle, and basal regions of the OE and VNE, respectively. onf, olfactory nerve fibers. (c, d) Periodic acid-Schiff (PAS) staining. In both the OE and VNE, PAS reaction was found in the free border (insets in panel (d)) and the supranuclear region of the supporting cells. Respiratory cells in ridges were also positive with PAS (asterisks). Scale bars: 100 μm (a, c), 25 μm (b, d).

The olfactory bulb in larval *C. ensicauda* was found to be similar to that in adult animals [9]. The MOB occupied the rostrolateral region of the telencephalon, and olfactory nerve fibers covered the surface of the olfactory bulb (Fig. 2a). The AOB was positioned caudally to the MOB, and the vomeronasal nerve coursed laterally to the olfactory nerve. The olfactory glomeruli of the MOB and AOB were distributed broadly and directly beneath the nerve layer (NL) that comprised of the olfactory and vomeronasal nerve fibers. All ten lectins showed a binding reactivity to the NL and glomerular layer (GL) in the olfactory bulb (Table 1). We scored the binding intensity of lectins in the olfactory bulb on a five-grade scale (-: negative, ± : faint, +: weak, ++: moderate, +++: strong). *Lycopersicon esculentum* lectin (LEL) and soybean agglutinin (SBA) showed a different binding in the NL and the GL, when compared between the MOB and AOB. LEL showed strong labeling in the NL and GL of the MOB, but bound moderately to the both of the AOB (Fig. 2b). SBA bound weakly to the NL of the MOB and AOB, and also distinguished the GL between the MOB and AOB by a different binding intensity: weak in the MOB, and faint in the AOB (Fig. 2c). *Bandeiraea simplicifolia* lectin-I (BSL-I) and peanut agglutinin (PNA) showed a preferential reactivity to the NL and the GL, respectively, despite these having a similar reactivity between the MOB and AOB. The olfactory and vomeronasal NL were moderately labeled by BSL-I and the GL of the MOB and AOB were weakly labeled (Fig. 2d). PNA faintly labeled the GL of the MOB and AOB, but showed no labeling in the NL (Fig. 2e). The remaining six lectins [wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (s-WGA), *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* agglutinin-E (PHA-E), and *Phaseolus vulgaris* agglutinin-L (PHA-L)] exhibited a uniform binding reactivity in the NL and GL of the olfactory bulb. The two layers were labeled with weak intensity by WGA (Fig. 2f), s-WGA (Fig. 2g), PSA (Fig. 2h) and PHA-E, and with faint intensity by LCA and PHA-L.

Adult *Cynops* possess the olfactory organ in a wide dorsoventrally flattened nasal cavity, and a vomeronasal organ that arises laterally from the main cavity as a diverticulum [10, 14]. In contrast, the nasal cavity of *C. ensicauda* larvae was oval with a shallow groove lined with the VNE. Previous studies have shown that neotenes and larvae of several salamanders exhibit an immature lateral nasal groove that contains the vomeronasal organ, in contrast to metamorphosed adults [16, 17]. These findings suggest that endocrine and metabolic modifications during metamorphosis would induce the development and maturation of the vomeronasal organ of larvae in some salamander species including *C. ensicauda*. Larval *C. ensicauda* showed a differentiated

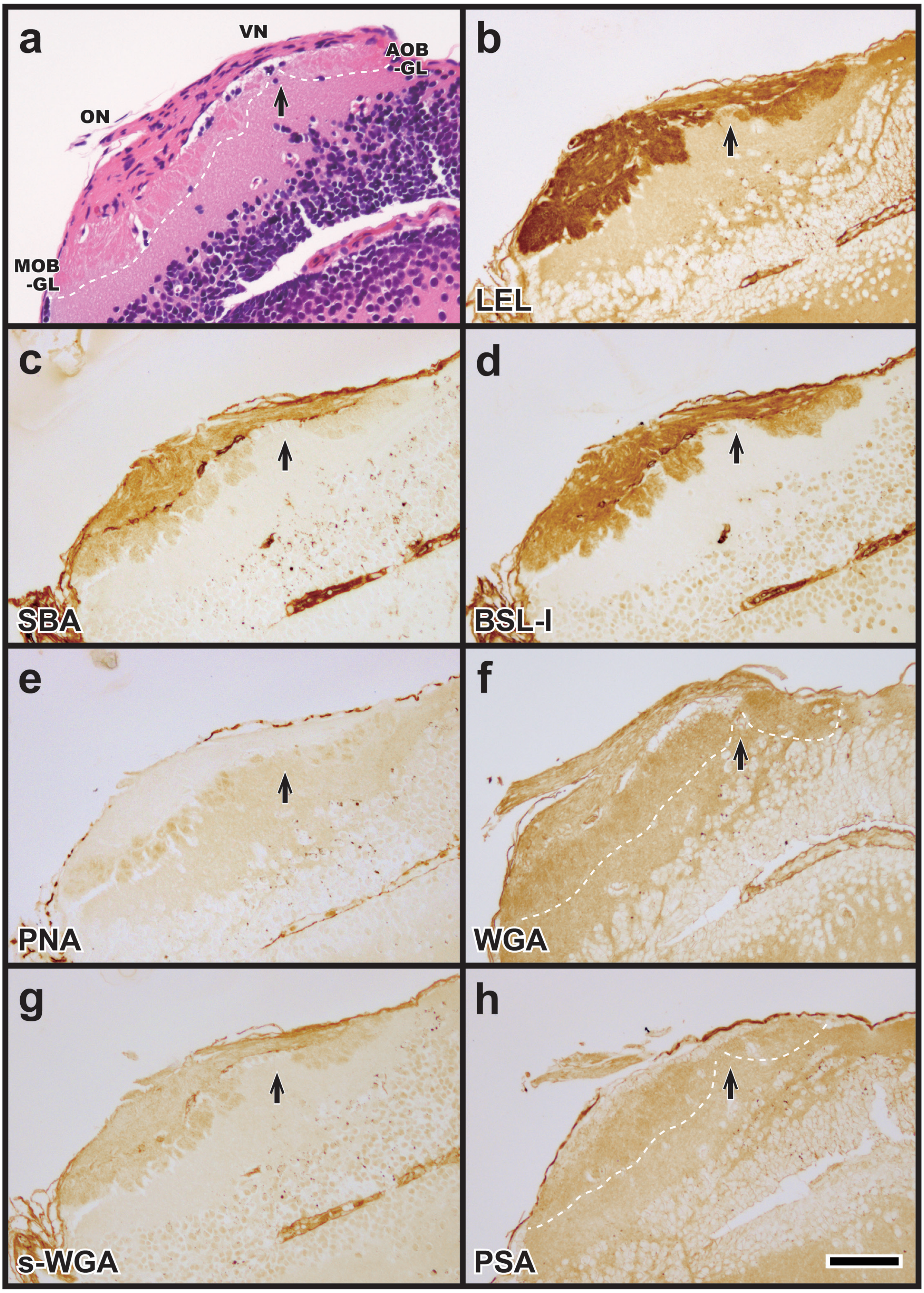

Fig. 2. Hematoxylin-eosin staining and lectin bindings in the main and accessory olfactory bulb (MOB and AOB) of larval *Cynops ensicauda*. The left and top sides of each panel indicate the rostral and lateral portions in hemispheres of the telencephalon, respectively. (a) Horizontal section of the olfactory bulb, stained with hematoxylin-eosin. The olfactory and vomeronasal nerves (ON and VN) terminated in the glomeruli of the MOB (MOB-GL) and in those of the AOB (AOB-GL), respectively. (b–h) LEL (b) and SBA (c) exhibited a different binding intensity between the MOB and AOB. BSL-I (d) and PNA (e) showed a different preference for binding between the nerve and glomerular layers. WGA (f), s-WGA (g), and PSA (h) bound with a uniform labeling to the ON and VN and to the MOB-GL and AOB-GL. Arrows in the panels represent the border between the MOB and AOB. Scale bar: 100 μm.

arrangement of cells in the OE and VNE, but lacked PAS-positive Bowman's gland in the lamina propria. In most tetrapods, respiratory and supporting cells and Bowman's glands secrete specialized products onto the surface of the olfactory sensory epithelium [2, 5]. These secretory products contain various compounds, including odorant binding proteins, and play a role in sensing odorant molecules. In general, Bowman's glands are absent in fish and some aquatic amphibians [5, 13]. We found PAS reactions in supporting cells of the OE and VNE and in respiratory cells of non-sensory ridges in *C. ensicauda* larvae. It appeared that PAS-positive products may have been secreted from the two cell types onto the free border of the sensory epithelium, where the PAS reaction was also observed. Our preliminary investigations showed that receptor cells in the OE of larval *C. ensicauda* expressed the subunit $G\alpha_{olf}$ of G-protein (unpublished data), which is an essential factor in the olfactory signal transduction [6, 10], suggesting a functional maturity of receptor cells. The olfactory reception in aquatic premetamorphic salamanders may, therefore, be sustained by secretory products of the respiratory and supporting cells.

Our results demonstrated that the glycoconjugate expression characterized the nerve fibers and olfactory glomeruli in both the MOB and AOB of premetamorphic *C. ensicauda*. Of the ten lectins used in the present study, LEL and BSL-I showed more preferential binding to the primary projections in the olfactory bulb than the others. LEL and BSL-I have an affinity for N-acetylglucosamine and galactose, respectively, and these glycoconujugate expressions in larval *C. ensicauda* may play a role in the axonal growth and fasciculation of the primary olfactory projection, as previously seen in rodents [12]. Regarding the comparison of lectin binding to the olfactory bulb among mature salamanders, a species-specific glycoconjugate expression was demonstrated by some lectins [3, 4, 8, 9]. In the present study, the ten lectins (see Table 1) showed a biding reactivity to the olfactory bulb of larval *C. ensicauda*. Labeling of these lectins also showed positive results with the olfactory bulb of the adults [9], and it suggests that the glycoconjugate types expressed in the primary olfactory projection are well conserved from the premetamorphic larval stage to the mature stage. Interestingly, in larvae of *C. ensicauda*, the lectin reactivity tended to exhibit a weaker binding reaction in both the MOB and AOB than that in the adult [9]. The developmental change of lectin bindings in the olfactory system has also been described in rodents [6, 11, 12]. In mouse (*Mus musculus*), *Dolichos biflorus* agglutinin (DBA) first labels axon bundles of the olfactory nerve at embryonic stage day 16, and subsequently labels the olfactory glomeruli of the MOB at the prenatal stages [11]. At the postnatal developmental stage, DBA binding is initiated in the vomeronasal nerve and the glomeruli of the AOB. This indicates a developmental change of the glycoconjugate expression in the primary olfactory center, and suggests that glycoconjugates in the primary projection may be involved in the maturation of axonal fasciculations and neuronal connections [12]. Our studies have revealed that there is a difference in the density of glycoconjugates in the olfactory bulb between premetamorphic and adult *C. ensicauda*, and suggest that there is a common correlation between the developmental change of lectin bindings and the maturation of the olfactory system among vertebrates. Interestingly, larval *C. ensicauda* did not represent a rostral subset in olfactory glomeruli of the MOB that was defined in adults by a dense expression of glycoconjugates [9]. Larval salamanders are fully aquatic and sense only water-soluble odorant molecules, so the primary projection to the rostral MOB may be immature in this stage of development. This seems to result in the homogenous glycoconjugate expression among glomeruli in the MOB of larval *C. ensicauda*. Further analysis of the anatomical comparison throughout various ontogenic stages of salamanders may provide greater insights into the development of the olfactory system in relation to habitat transition in metamorphic animals.

CONFLICT OF INTERESTS. None of the authors have any conflicts of interest associated with this study.

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