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THE DEVELOPMENT OF OLFACTORY ORGAN OF *LISSOTRITON VULGARIS* (AMPHIBIA, CAUDATA)

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The Development of Olfactory Organ of *Lissotriton vulgaris* (Amphibia, Caudata). Kovtun, M. F., Stepanyuk, Ya. V. — Using common histological methods, the morphogenesis of olfactory analyzer peripheral part of *Lissotriton vulgaris* (Amphibia, Caudata) was studied, during the developmental period starting with olfactory pit laying and finishing with definitive olfactory organ formation. Special attention is paid to vomeronasal organ and vomeronasal gland development. Reasoning from obtained data, we consider that vomeronasal organ emerged as the result of olfactory epithelium and nasal cavity differentiation.

Key words: amphibia, olfactory analyzer, vomeronasal organ, olfactory epithelium.

Развитие органа обоняния тритона обыкновенного, *Lissotriton vulgaris* (Amphibia, Caudata). Ковтун М. Ф., Степанюк Я. В. С помощью общепринятых гистологических методов исследован морфогенез периферического отдела обонятельного анализатора *Lissotriton vulgaris* (Amphibia, Caudata), от закладки обонятельных ямок до формирования definitiva обонятельного органа. Особое внимание уделяется развитию вомероназального органа и вомероназальной железы. На основании полученных данных считаем, что вомероназальный орган возник в результате дифференциации обонятельного эпителия и носовой полости.

Ключевые слова: амфибии, обонятельный анализатор, вомероназальный орган, обонятельный эпителий.

Introduction

Olfactory epithelium of nasal cavity and vomeronasal organ (or Jacobson's organ) are referred to peripheral part of olfactory analyzer. There are two hypothesis, which explain its origin: the first regards it as a new formation, related to terrestrial lifestyle adaptation (Bertmar, 1981); the second regards the emerge of vomeronasal organ as the result of aquatic tetrapods olfactory analyzer peripheral part differentiation (Eisthen, 2000). The variability of vomeronasal organ is in complete contradiction with its availability in all classes of terrestrial vertebrates (Medvedeva, 1975), as far as its availability implies its necessity, while structure variability — to the extent of its full reduction — implies the absence of common function.

There is a belief that the Jacobson's organ is synapomorphous for modern tetrapods (Trueb, Cloutier, 1991). Obviously it heightens the interest in the morphogenesis of vomeronasal organ. The definitive vomeronasal organ is covered with sensory epithelium which goes to the vomeronasal nerve with the termination in accessory olfactory bulb. A characteristic feature of the vomeronasal organ is the high variability of its topography, morphology, developmental level in different vertebrate taxa. For example, it is well-developed in lizards and snakes, and is absent in adult crocodiles, sea turtles, birds. Vomeronasal organ is well developed in most mammals, but it's absent in secondary aquatic mammals, in some of cheiroptera and primates. However, in some primates and cheiroptera, it exists only during the prenatal ontogenesis. As for amphibians, different species of this class have the vomeronasal organ with significant specific morphological features. The main differences relate to the general plan of its structure, peculiarities of the sensory epithelium and vomeronasal organ topography. Most studies of the olfactory organ morphogenesis are based on studying the different species of anuran amphibians (Tsui, 1946; Taniguchi et al., 1996; Jermakowicz, 2004; Wang et al., 2008; Jungblut et al., 2011; Benzekri, Reiss, 2012; Královec et al., 2013). As regards the tailed amphibians, the morphology of the olfactory organ is described mainly in mature forms (Dawley, Bass, 1988; Dawley, Crowder, 1995; Eisthen, 2000), and there is a small number of papers dedicated to the morphogenesis of this structure (Medvedeva,

1975; Eisthen et al., 1994; Stuelpnagel, Reiss, 2005). Moreover, there is no consensus among researchers about the structural rearrangements of the olfactory analyzer during the period of transition from aquatic to terrestrial stage and about functional meaning of the vomeronasal organ.

In this work we focus on the morphogenesis of the nasal cavity and vomeronasal organ in *Lissotriton vulgaris*.

Material and methods

The structures of the olfactory organ of the *Lissotriton vulgaris* (Pallas, 1771) were studied during different ontogenesis periods. There were studied 39 larvae of different developmental stages and mature individuals ($n = 5$). Eggs and larvae were kept in aquariums, with constant temperature and photoperiod ($22 \pm 20^\circ \text{C}$; 12 hours dark : 12 hours light). Ontogenesis stages (Gl 33–56) were determined according to the tables of *Lissotriton vulgaris* normal development (Glaesner, 1925). The material was fixed in 5 % neutral formalin or 2 % solution of glutaraldehyde with a 0.1 M cacodylate buffer. After thorough abluion, the material was set in homogenized paraffin mixture Histomix®. Blocks cutting was performed serially in frontal and sagittal planes, the thickness of slices was in range from 10 to 15 μm ; the sections were stained with Cresil Violet according to Nissl and with hematoxylin-eosin according to Boehmer. The photographing of the histological specimen was performed with the Zeiss Axio Imager M1 microscope and Zeiss Axio Vision software v. 4.63 in the center for collective use of unique equipment at the Schmalhausen Institute of Zoology. NAS of Ukraine.

Research results

The laying and development of the olfactory placode during embryonic developmental stages is similar for all amphibians (Medvedeva, 1975). Therefore, the study of the olfactory analyzer development in the smooth newt was started with the formation of the olfactory pits (stage Gl 33). We consider it to be expedient to divide all the morphogenesis of the olfactory analyzer in three periods, which simplify outlining, summarizing and comprehension of the material.

During the first period of development (Gl 33–40 stages) olfactory pits are formed, which are subsequently transformed into olfactory sacs.

During the second period of development (Gl 41–46 stages) the formation of the nasal cavity finalizes, the formation of inner nostrils finalizes and vomeronasal organ is laid.

During the third period of development (Gl 47–56 stages) the offset of the vomeronasal organ takes place, the laying of the vomeronasal gland and the Bowman's glands as well as the growth and differentiation of the olfactory analyzer structures take place.

Stages Gl 33–40. During the 33 stage of development of *T. vulgaris* the olfactory placodes are already well differentiated from the head ectoderm and lie ventral in the rostral part of the larva head. The placodes border dorsolaterally with the eye germs, and dorsomedially – with neural tube (fig. 1, *a*). In the central part the olfactory placodes create the invagination, resulting in formation of the olfactory pits. During the time period from Gl 34th to Gl 37th stages of development, the invagination increases, resulting in formation of the external nostrils germs which open at first ventrally, and then (Gl 38–40 stages) shift laterally. The invaginations of olfactory pits increase during Gl 38–39 stages and form closed sacciform patterns — olfactory sacs. During subsequent developmental stages (Gl 39–40) the volume of the olfactory sacs cavity increases due to the invagination of the dorsocaudal wall. The olfactory epithelium covers dorsomedially the surface of the olfactory sacs. In the ventrocaudal part of the olfactory sacs and dorsal surface of the anterior gut the new invaginations are formed, which are called choanal excrescences.

Stages Gl 41–46. The external nostrils increase in size, shift more laterally. On the 41 stage the internal nostrils (choanae) are formed as the result of the join of the choanal excrescences of olfactory sacs and anterior gut, and they open into the buccal cavity. Due to these morphological changes the olfactory sacs are transformed into the nasal cavity. In the place of choanae formation, the dorsal side of the nasal cavity is covered with well developed olfactory epithelium (fig. 1, *b*). During next stages the size of choanae increases significantly. The nasal cavity also increases and shifts to the ventrolateral surface of the olfactory bulbs germs (fig. 2, *a*). Olfactory epithelium becomes thicker, covers only

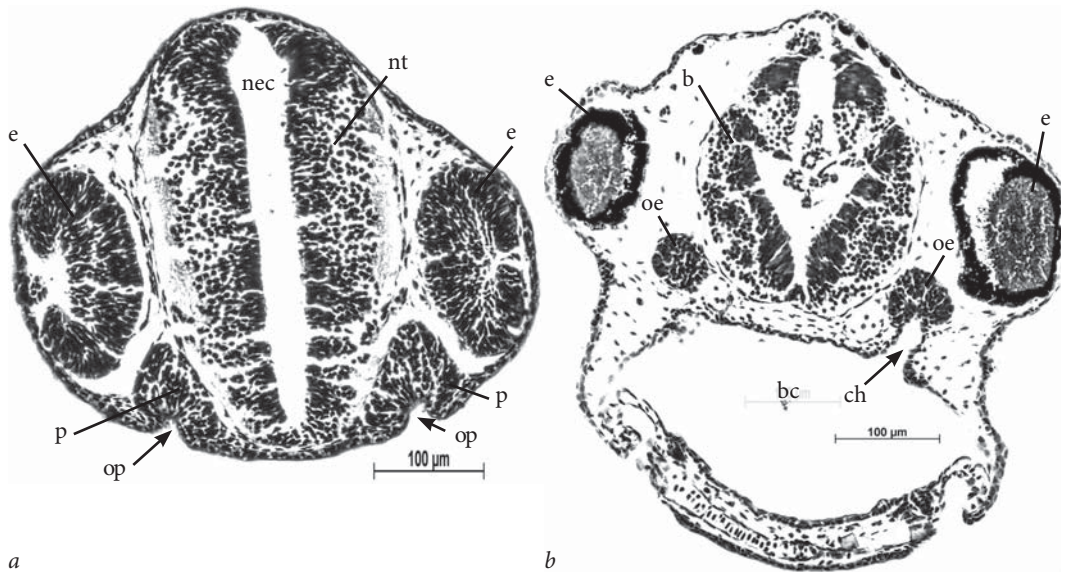


Fig. 1. Cross-section of the *L. vulgaris* larvae head at the level of the olfactory placode (a) and choanae (b): a — stage 33; b — stage 41. Legend: op — olfactory pits; e — eyes; nt — neural tube; nec — neurocoel; p — olfactory placode; ch — choanae; oe — olfactory epithelium; bc — buccal cavity; b — brain.

dorsomedial surface of the nasal cavity, it has the visible cilia on its surface.

During the Gl 42–43 stage nasal cavity acquires a tubular shape. The external nostrils acquire definitive condition and topography during the Gl 45–46 stage.

During the Gl 44 stage the germ of the vomeronasal organ appears in the ventral part of the olfactory epithelium, before the choanal duct, it has the form of small oval thickening (fig. 2, b). The rostral ends of the olfactory bulbs are differentiated, they have well-defined fibrous layer formed by fibers of the olfactory nerves.

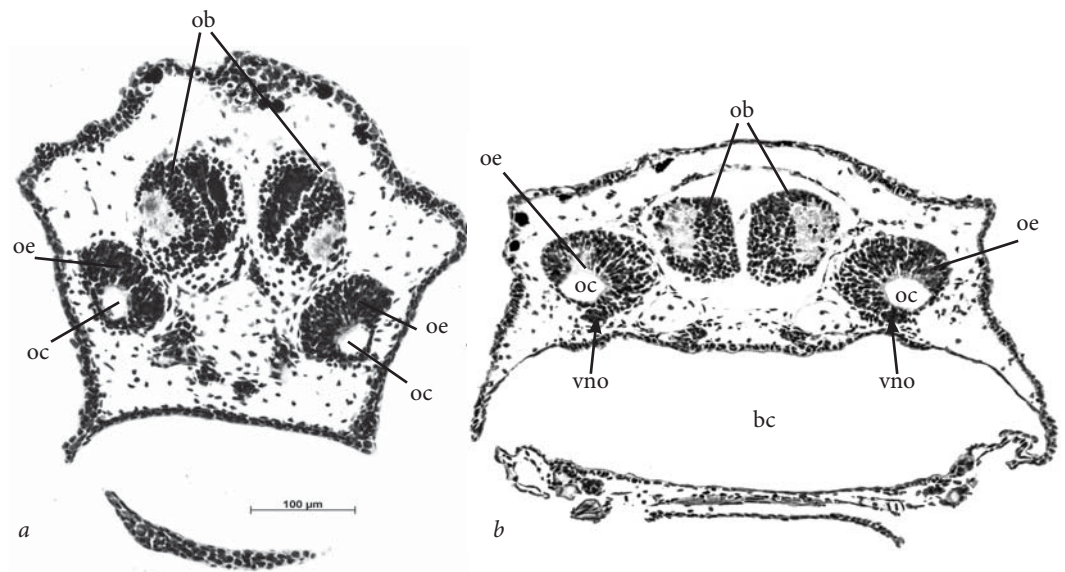


Fig. 2. Cross-section of the *L. vulgaris* larva head at the level of the nasal cavity (a) and vomeronasal organs laying (b): a — stage 41; b — stage 43. Legend: oc — nasal cavity; oe — olfactory epithelium; bc — buccal cavity; ob — olfactory bulb; vno — vomeronasal organ.

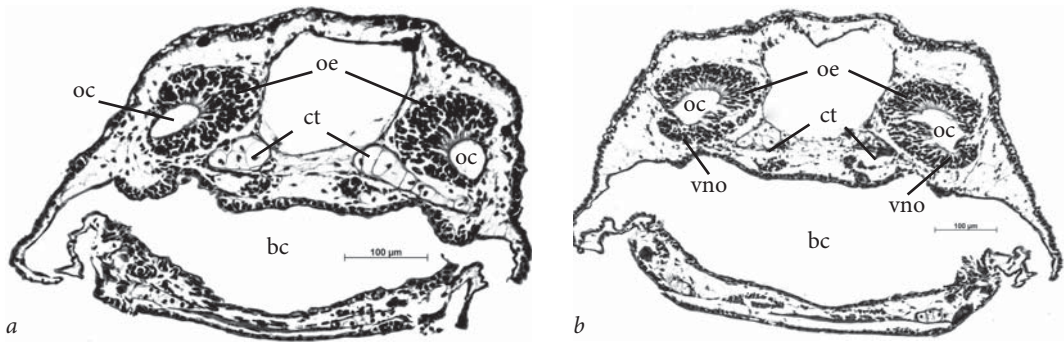


Fig. 3. Cross-section of the *L. vulgaris* larva head at the level of the nasal cavity (*a*) and vomeronasal organs (*b*): *a* — stage 47; *b* — stage 52. Legend s: oc — nasal cavity; oe — olfactory epithelium; bc — buccal cavity; vno — vomeronasal organ; ct — trabecular lamina.

Stages G1 47–56. Due to the uneven increase of the olfactory epithelium thickness and its expansion on the lateral surface of the nasal cavity the nasal cavity acquires the oval shape (stages G1 48–49) (fig. 3, *a*).

During the next stages, the volume of the nasal cavity increases, its internal surface, except the lateral area, is covered with olfactory epithelium (stage G1 51). In the ventral and dorsal wall of the nasal cavity the clusters of respiratory epithelium cells can be found among cells of the olfactory epithelium (stage G1 51). During the subsequent stages the number of respiratory cells increases significantly. The blood vessels grow under the basal layer of the respiratory epithelium. As a result, respiratory cells protrude above the surface of the olfactory epithelium forming a peculiar islets and trenches covered with sensory epithelium.

The germ of the vomeronasal organ increases in size, creates has chink-like invagination (fig. 3, *b*) and shifts laterally. During G1 54 stage it reaches its definitive topography. The laying of the vomeronasal gland takes place during G1 56 developmental stage. During the same period the single tubular-alveolar Bowman's glands are laid in the olfactory epithelium, in addition to the vomeronasal organ. Secretory sections of Bowman's glands are located on the basal membrane of the olfactory epithelium, excretory ducts are open on its surface. Olfactory bulbs acquire laminar structure. Fibrous and glomerular layers are differentiated (stage G1 49).

The structure of the olfactory analyzer after the metamorphosis

After the metamorphosis and until the mature state, the volume of the olfactory organ cavity in *L. vulgaris* continues growing. The external nostrils are opened ventrolaterally in

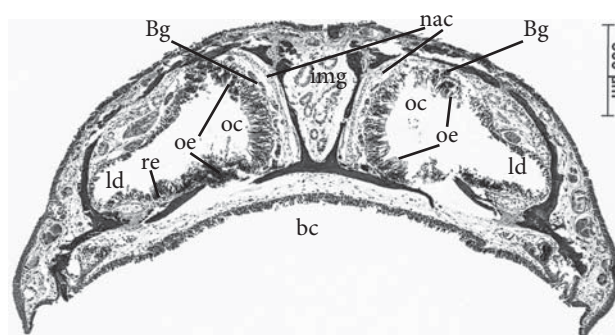


Fig. 4. Cross-section of mature *L. vulgaris* head at the level of the anterior part of the nasal cavity. Legend s: oc — nasal cavity; oe — olfactory epithelium; bc — buccal cavity; ld — lateral diverticulum; re — respiratory epithelium; nac — olfactory capsule; img — intermaxillary gland; Bg — Bowman's glands.

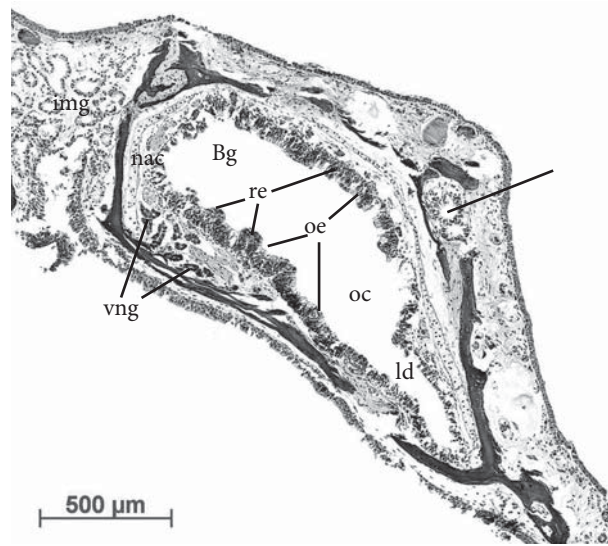


Fig. 5. Cross-section of mature *L. vulgaris* head at the level vomeronasal gland. Legends: oc — nasal cavity; oe — olfactory epithelium; ld — lateral diverticulum; re — respiratory epithelium; nac — olfactory capsule; nld — nasolacrimal duct, img — intermaxillary gland; Bg — Bowman's glands.

rostral part of the head. In the region of external nostrils the nasal cavity is small and has a round shape. Just behind the external nostrils the volume of the nasal cavity increases due to lateral diverticulum of its walls (fig. 4). The ventrocaudal wall of the nasal cavity is opened into the buccal cavity of a large choanae. The lateral diverticulum forms a groove, which connects the lateral margin of the external nostrils and lateral margin of choanae. The nasolacrimal duct flows dorsally into the middle part of the groove, and divides the groove into two parts — rostral and caudal. The walls of the rostral part of the groove are covered with respiratory epithelium, and the caudal part is covered with sensory epithelium and represents the vomeronasal organ. At the place of the confluence of nasolacrimal duct the lateral nasal gland is opened. The nasolacrimal duct bifurcates near the eye and is opened by two foramens on the inner surface of the lower eyelid. The olfactory epithelium covers the entire surface of the nasal cavity, except the region, which located aborally to external nostrils and the rostral part of the lateral diverticulum. The Bowman's glands are located in the major part of olfactory epithelium, except lateral diverticulum; most of them lay the area of the external nostrils (fig. 4). Secretory section of the vomeronasal gland lies under ventrocaudal part of the nasal cavity, its long secretory duct is opened ventrally at the beginning of the vomeronasal organ. The vomeronasal nerve passes between the dorsal wall of the nasal cavity and cartilaginated olfactory capsules.

Comparative analysis and discussion

The development and formation of the nasal cavity in vertebrates goes on according to the similar scheme: olfactory placode, olfactory pits, olfactory sacs, nasal cavity (fig. 6). During the development process the volume of the nasal cavity and the surface area of the sensory epithelium increase, the differentiation of the olfactory epithelium and its redistribution on the inner surface of the nasal cavity takes place. The increase in the volume and area of the nasal cavity (except the general growth) occurs by invagination of its walls.

The olfactory organ of *L. vulgaris* has a pipe-like form at the end of metamorphosis. This form is described for other members of the tailed amphibians: ambistoma (*Dicamptodon tenebrosus*) (Stuelpnagel, Reiss, 2005), mudpuppies (*Necturus maculosus*), amphiuma (*Amphiuma tridactylum*), sirens (*Siren intermedia*) (Eisthen, 2000).

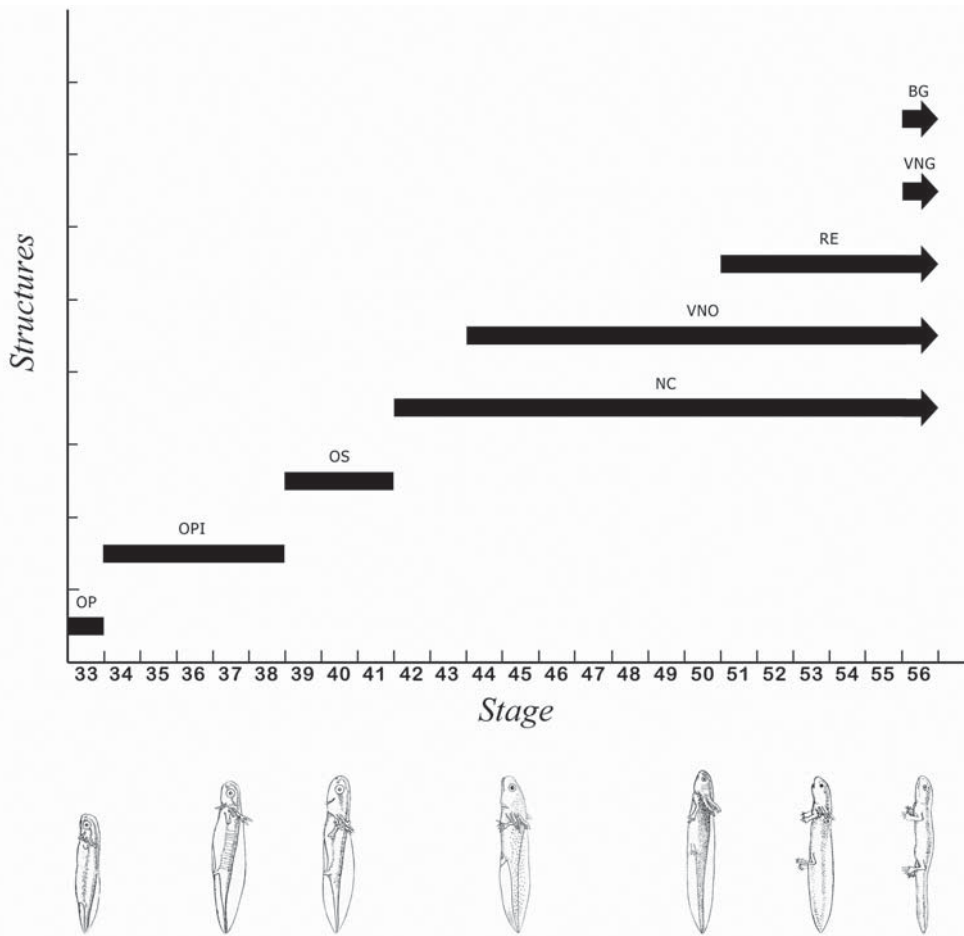


Fig. 6. Temporal morphogenesis of the *L. vulgaris* olfactory analyzer structures. Legend: OP — olfactory placode; OPI — olfactory pits; OS — olfactory sac; NC — nasal cavity; VNO — vomeronasal organ; RE — respiratory epithelium; VNG — vomeronasal gland; BG — Bowman's glands.

As for shape and topography, the definitive olfactory organ of *L. vulgaris*, like in other caudate amphibians, differs significantly from that of anura. It is more differentiated in anura (Tsui, 1946; Jungblut et al., 2011; Wang et al., 2008; Jermakowicz et al., 2004).

As mentioned above, the wall of the caudal part of the lateral diverticulum is covered with sensory epithelium which is innervated by vomeronasal nerve. This innervation indicates that this part of the lateral diverticulum is the vomeronasal organ. The vomeronasal epithelium of tailed amphibians and anura is deprived of Bowman's glands, which are typical for olfactory epithelium. In our opinion, the rostral section of the protrusion, which has no sensory epithelium, is homologous to lateral diverticulum of the lower cavity of anura. This location of the vomeronasal organ is described in *Plethodon cinereus* (Dawley, Bass, 1988), *Amphiuma tridactylum* and *Ambystoma mexicanum* (Eisthen et al., 1994; Eisthen, 2000). However, other variants are also described: the vomeronasal organ of sirens (*Siren intermedia*) is located in the nasal cavity more medially (Eisthen, 2000); in abystoma *Dicamptodon tenebrosus*, the vomeronasal organ is located ventrolaterally in the middle part of the nasal cavity (Stuelpnagel, Reiss, 2005). The mudpuppy *Necturus maculosus* has a lateral diverticulum, covered with non-sensory epithelium and has no accessory olfactory bulb (Eisthen, 2000), that indicates a complete loss of the vomeronasal system.

The vomeronasal organ forming is gemology in tailed amphibians and anura and develop in ventromedial part of the olfactory epithelium. During the further development,

the germ of *L. vulgaris* vomeronasal organ shifts laterally. The similar type of the vomeronasal organ laying in tailed amphibians and anura was noted by Medvedeva (Medvedeva, 1975).

The interaction of the vomeronasal organ glands raises great interest. We have already described that the vomeronasal epithelium of *L. vulgaris* originates at the confluence of nasolacrimal duct, lateral nasal gland and vomeronasal gland. In our opinion, the opening of two large glands ducts in front of the vomeronasal organ has a certain functional role. Thus, the lateral diverticulum is not in a horizontal plane, and is lowered in the direction from the outer nostrils to choanae, so we assume that the secret of both glands gets on the sensory epithelium of the vomeronasal organ. It is obvious that orbital glands secretion also gets through the nasolacrimal duct into the vomeronasal organ. It is believed that orbital glands of tailed amphibians are poorly differentiated in contrast to those of other four-legged vertebrates (Hillenius, Rehorek, 2005) and some of them perform the function of Harderian gland (Sakai, 1981), which is opened into the vomeronasal organ in reptiles. This interaction of vomeronasal organ with glands is apparently related to necessity of moisturizing the vomeronasal epithelium.

At the moment, the mechanism of odorants transport to the vomeronasal epithelium remains still not fully clarified. We support the hypothesis, which exists in the literature, that aforementioned glands can take part in providing this transportation mechanism. Based on studies of the lateral nasal gland in three species of anura it was shown, this gland is involved in transportation of chemical stimuli from the external nostrils to the vomeronasal organ (Nowack, 2011). As well, we support the idea of Nowack and Wöhrmann-Repenning (2009) that not only the vomeronasal gland but also the lacrimal gland is involved in the functioning of the vomeronasal organ — the lacrimal gland secretion through the nasolacrimal duct enters the olfactory organ.

The absence of Bowman's glands in the vomeronasal epithelium may indicate that it was formed somewhat later after the formation of nasal cavity sensory epithelium, and its moistening is realized due to vomeronasal gland, orbital gland, and the lateral nasal gland.

Concerning the nature of the vomeronasal organ, there is no data that would unambiguously imply the origin of the vomeronasal system. However, some indirect evidences incline us to think that the vomeronasal organ is a product of the olfactory organ differentiation.

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