

UDC 591.3:591.486]:597.6/9

MORPHOGENESIS OF VOMERONASAL ORGAN OF *PELOPHYLAX RIDIBUNDUS* (AMPHIBIA, ANURA)

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Morphogenesis of Vomeronasal Organ of *Pelophylax ridibundus* (Amphibia, Anura). Stepanyuk Ya. V., Kovtun M. F. — The morphogenesis of the lake frog (*Pelophylax ridibundus*) vomeronasal organ was studied during different ontogenesis stages. The vomeronasal organ is laid after the formation of olfactory sacs, which are lined by olfactory epithelium, and after choan formation. Vomeronasal organ anlage takes place during G24 stage of larval development, which is the result of inflection and cell redistribution of olfactory epithelium rostroventral part. Formation of the vomeronasal organ finished at the beginning of metamorphosis. Apparently, vomeronasal organ appeared in aquatic Amphibia ancestors and after their transition from aquatic to terrestrial environment it developed new adaptive functions.

Key words: Anura, olfactory system, olfactory epithelium, vomeronasal organ, vomeronasal gland.

Морфогенез вомероназального органа *Pelophylax ridibundus* (Amphibia, Anura) Степанюк Я. В., Ковтун М. Ф. — Исследовано развитие вомероназального органа лягушки озерной (*Pelophylax ridibundus*) в разные периоды онтогенеза. Вомероназальный орган закладывается после образования обонятельных мешков, которые выстланы обонятельным эпителием, и хоан. Его закладка происходит на G24 стадии личиночного развития вследствие выпячивания и перераспределения клеток ростровентральной части обонятельного эпителия. Формирование вомероназального органа заканчивается в начале периода метаморфоза. Очевидно, что образование вомероназального органа состоялось в водных предках земноводных, и в результате выхода животных на сушу он приобрел новые адаптивные функции.

Ключевые слова: Анура, обонятельная система, обонятельный эпителий, вомероназальный орган.

Introduction

The olfactory system of vertebrates, including Amphibia, consists of central and peripheral parts. Amphibian olfactory system is the first one among vertebrates to be divided into main and accessory (vomeronasal) olfactory systems. Peripheral part of vomeronasal system is represented by vomeronasal or Jacobson's organ (VNO). VNO is rather different in various tetrapods. For instance, VNO is absent in teleost fishes, crocodiles, birds, major part of bats, marine mammals and Old World primates (Bertmar, 1981; Bhatnagar, Meisami, 1998; Halpern, Martinez-Marcos, 2003). Its functions in adult humans are still an unresolved problem (Halpern, 2003).

The VNO topography varies in different amphibian orders. In the majority of Anura, VNO is situated medially considering principal chamber (PC) (Tsui, 1946; Taniguchi et al., 1996; Jermakowicz et al., 2004; Wang et al., 2008; Jungblut et al., 2011). In Caudata VNO is laid laterally or ventrolaterally in relation to the nasal cavity (Dawley, Bass, 1988; Eisthen et al., 1994; Dawley, Crowder, 1995; Eisthen, 2000; Stepanyuk, Motuzyuk, 2010). The data on the morphogenesis of VNO of caecilians is lacking (Schmidt, Wake, 1990). Despite a great number of the studies dealing with VNO development, there is no common point of view on its phylogenetic origin, functional significance and development patterns. It was supposed, that VNO appeared as supplementary structure for smell perception in atmospheric air (Bertmar, 1981). Therein, Amphibia are rather interesting as an animal group, which mastered the water and terrestrial border niche during their evolution. The purpose of this research is to study VNO development in Anura starting from the beginning of its formation until the defined state. The lake frog, which is a typical anuran representative in the Ukrainian fauna, was chosen as a research object of the study.

Material and methods

The structures of peripheral part of the olfactory analyzer in *Pelophylax ridibundus* (Pallas, 1771) were studied during different ontogeny periods. The taxonomy of species was defined based on external morphological features, particularly, during G18–20 embryonic development stages, during G21–41 larval development stages and during metamorphosis period (stages G42–46). Juvenile specimens in the age of 14 days were also studied. Totally 49 embryos were studied. Eggs and tadpoles were kept in aquaria under invariable temperature and photoperiod ($22 \pm 2^\circ\text{C}$; 12 hr dark : 12 hr light). Ontogenesis stages were determined according to tables of normal Anura development (Gosner, 1960). Material was fixed in 5 % solution of neutral formalin or in 2 % solution of glutaraldehyde in 0.1M Cacodylate buffer. After thorough washing the material was placed into homogenized paraffin media HistoMix[®]. Block slicing was performed serially in frontal and sagittal planes, slice thickness was 10–15 μm ; slices were stained with cresyl violet according to Nissl and with hematoxylin-eosin according to Beemer. Histological preparation photography was conducted using Zeiss Axio Imager M1 microscope with Zeiss AxioVision v.4.63 software in a unique equipment collective usage center, located in Ukrainian NAS Schmalhausen Institute of Zoology. Morphometry was conducted from the very beginning of VNO cavity formation using “Morphologia 5.0” software. The nomenclature proposed by Jermakowicz et al. (2004) was chosen for description and identification of olfactory structures. Cranium structures definition was conducted according to Pugener, Maglia (2007).

The terminology of Jermakowicz et al. (2004) and Pugener, Maglia (2007) was used for description and identification of olfactory structures and cranium structures.

Result

According to our data, the olfactory placodes are the first structures of a peripheral part of a lake frog olfactory system appearing during embryogenesis (G18 embryogenesis stage). At the next stage they invaginate. This leads to formation of olfactory pits (G19) and olfactory sacs (G23–24). After the break of olfactory sacs ventrocaudal wall and secondary nostrils (choanea) formation, PC is formed, which dorsomedial wall is covered with olfactory epithelium (OE).

VNO primordium appears during 24 larval development stage of *P. ridibundus* ontogenesis as the result of OE cell inflation and redistribution in external nostrils area. The structure looks like a small oval bulge, which is located between ventral OE part and trabecular lamina horns. Earlier (G23 stage), as the result of dorsolateral OE part inflation, the lateral appendix (fig. 1, *a*) is laid. Internal nostrils are being laid on the same stage. From G24 till G26 stage, VNO doesn't change its topography and does not increase in size (fig. 1, *a*).

At the next developmental stage, VNO increases in size, expands caudally under ventral wall of PC (fig. 1, *b*). During this period the fissure-like cavity forms in VNO, this cavity increases in size and at G30 stage penetrates with caudal ending the ventral wall of PC. Thus, VNO cavity connects with the PC and becomes the predecessor of inferior chamber medial corner. At stage G27 the first secretory unit of vomeronasal gland (VNG), or Jacobson gland appears at the VNO dorsomedial wall.

Intensive formation of new secretory units and gland excretory duct formation goes on till G30 stage. Gland secretory units consist of single-layer gland epithelium; granulocytes nuclei are round, large and basally located (fig. 1, *b*). The vomeronasal epithelium (VNE) becomes more differentiated at G30 stage, its thickness is significantly less than OE thickness from the stage G27 till the stage G30 and varies in scope from $41.08 \pm 1.85 \mu\text{m}$ to $57.18 \pm 2.14 \mu\text{m}$, but volume increases in size from 0.00048 mm^3 (G27 stage) to 0.00247 mm^3 (G30 stage).

During the following developmental stages (G31–38) VNO stretches rostrally and considerably increases in size due to the increase of its own cavity that is shaped into duct form (fig. 2, *a*). The maximum width of VNE is in the medial part of medial corner. VNG increases in size and shifts caudomedially in the relation to VNO. VNE thickness, during the period of research, decreases from $53.52 \pm 7.2 \mu\text{m}$ (G31) to $40.35 \pm 5.86 \mu\text{m}$ (G38), while its volume, on the contrary, continues to increase significantly from 0.00106 mm^3 to 0.00645 mm^3 .

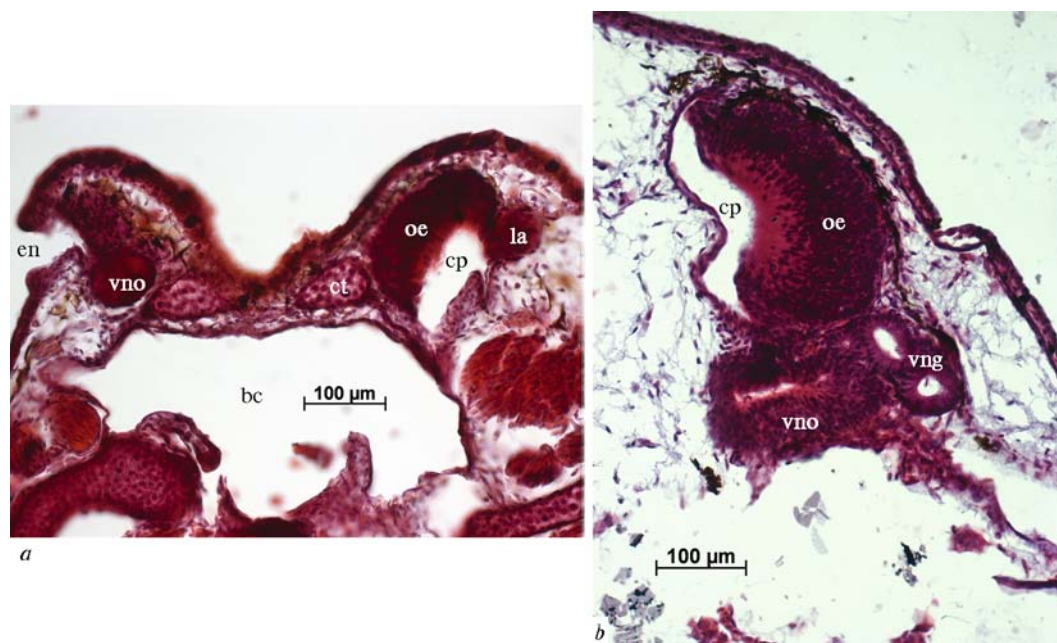


Fig. 1. Transverse section of *P. ridibundus* principal nasal cavity and vomeronasal organ: *a* — G26 larval development stages; *b* — G30 larval development stages; en — external nares; oe — olfactory epithelium; cp — principal chamber; bc — buccal cavity; ct — trabecular horn; la — lateral appendix; vno — vomeronasal organ; vng — vomeronasal gland.

Рис. 1. Поперечный срез через основную носовую полость и вомероназальный орган. *P. ridibundus*: *a* — G26 стадия личиночного развития; *b* — G30 стадия личиночного развития; en — внешние ноздри; oe — обонятельный эпителий; cp — основная полость; bc — ротовая полость; ct — рог трабекулы; la — латеральный аппендикс; vno — вомероназальный орган; vng — вомероназальная железа.

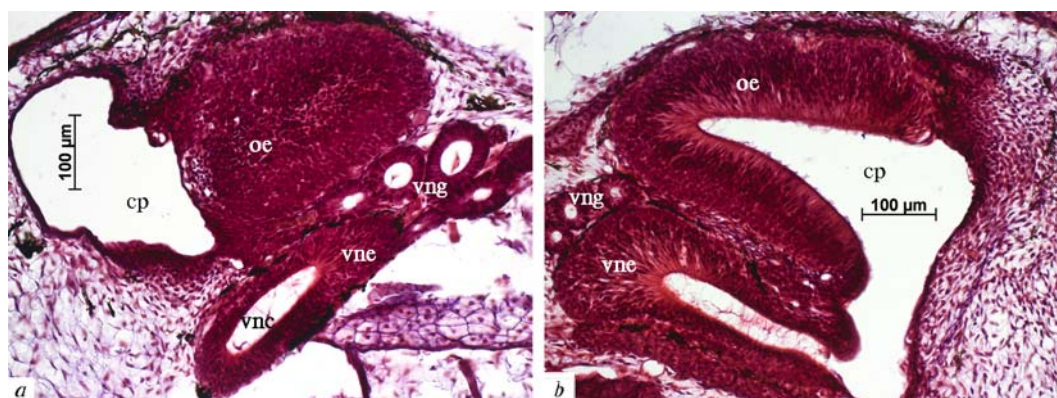


Fig. 2. Transverse section of *P. ridibundus* principal nasal cavity and vomeronasal organ: *a* — G38 larval development stages; *b* — G40 larval development stages. oe — olfactory epithelium; cp — principal chamber; vnc — vomeronasal epithelium; vnc — vomeronasal cavity; vng — vomeronasal gland.

Рис. 2. Поперечный срез через основную носовую полость и вомероназальный орган. *P. ridibundus*: *a* — G38 стадия личиночного развития; *b* — G40 стадия личиночного развития; oe — обонятельный эпителий; cp — основная полость; vnc — вомероназальный эпителий; vnc — вомероназальная полость; vng — вомероназальная железа.

During the G39–42 ontogenesis stages VNO moves medially by its rostral end and takes its definitive position under the PC (fig. 2, *b*). Sensor epithelium, especially in a medial VNO part, is differentiated into layers. VNG, which is located between VNO and nasal septum, significantly increases in size and also takes its definitive position. VNG duct pierces through mediocaudal VNO wall and opens in VNO cavity (fig. 3, *a*).

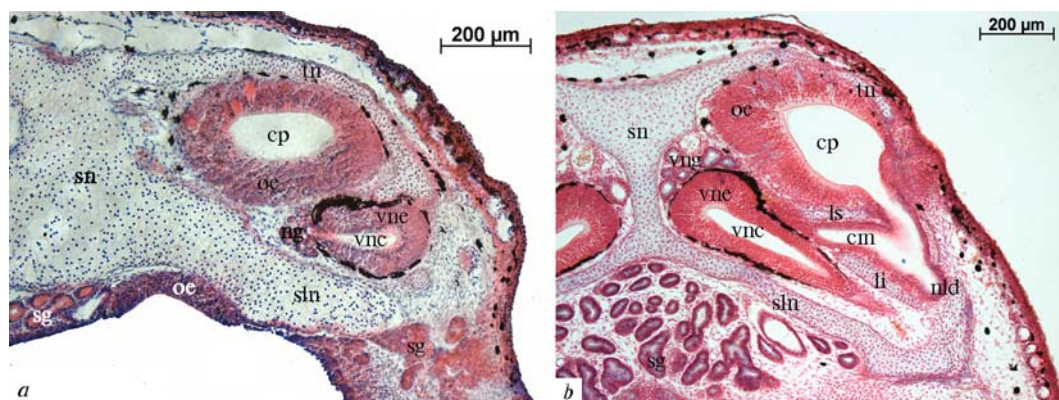


Fig. 3. Sagittal sections of *P. ridibundus* at G42 stage in medial part of principal nasal cavity (a); transverse sections at stage G44 (b); oe — olfactory epithelium; cp — principal chamber; cm — middle chamber; vne — vomeronasal epithelium; vnc — vomeronasal cavity; vng — vomeronasal gland; bc — buccal cavity; sg — salivary gland; sn — solum nasi; sln — solum nasi; tn — tectum nasi; ls — lamina superior; li — lamina inferior; nld — nasolacrimal duct.

Рис. 3. Сагитальный срез головы *P. ridibundus* в медиальной части основной носовой полости на G42 стадии (a) и поперечный срез головы на G44 стадии метаморфоза (b); oe — обонятельный эпителий; cp — основная полость; cm — средняя полость; vne — вомероназальный эпителий; vnc — вомероназальная полость; vng — вомероназальная железа; bc — ротовая полость; sg — слюнная железа; sn — септум nasi; sln — септум nasi; tn — тентум nasi; ls — lamina superior; li — lamina inferior; nld — носослезный проток.

During this developmental stage simple tubularalveolar Bowman's glands, that are absent in VNE, appear in OE depth. Three large (probably, salivary) glands, which ducts open in mouth cavity, are located caudally, rostrally and laterally from choanea. Additional olfactory nerve (vomeronasal) that lies between medial VNO part and nasal septum becomes visible. During this period, the middle chamber, a rostral inflection of PC over the VNO, is formed. In comparison with the previous development stage, VNE thick-

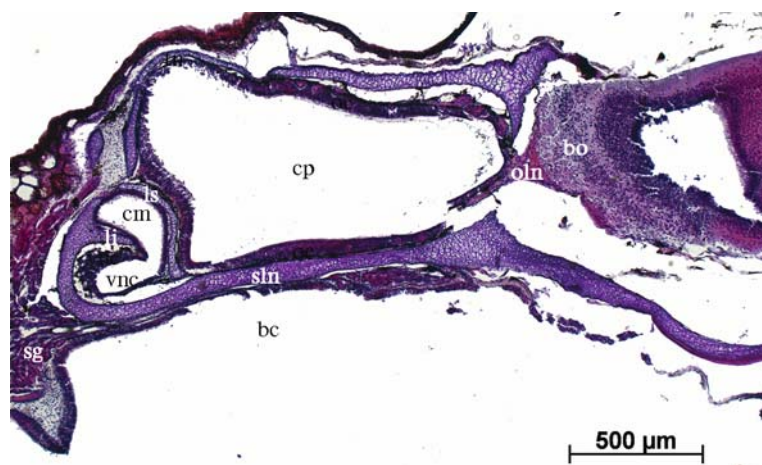


Fig. 4. Sagittal section of *P. ridibundus* at G42 stage at lateral part of principal nasal cavity after 14 days of metamorphosis finish: oe — olfactory epithelium; cp — principal chamber; cm — middle chamber; vne — vomeronasal epithelium; vnc — vomeronasal cavity; vng — vomeronasal gland; bc — buccal cavity; sg — salivary gland; oln — olfactory nerve; bo — olfactory bulb; sln — solum nasi; tn — tectum nasi; ls — lamina superior; li — lamina inferior.

Рис. 4. С агитальный срез головы *P. ridibundus* в латеральной части основной носовой полости на 14-й день после окончания метаморфоза: oe — обонятельный эпителий; cp — основная полость; cm — средняя полость; vne — вомероназальный эпителий; vnc — вомероназальная полость; vng — вомероназальная железа; bc — ротовая полость; sg — слюнная железа; oln — обонятельный нерв; bo — обонятельная луковица; sln — септум nasi; tn — тентум nasi; ls — lamina superior; li — lamina inferior.

ness increases from $59.85 \pm 2.56 \mu\text{m}$ to $60.89 \pm 4.45 \mu\text{m}$ and VNE volume increases from 0.009 mm^3 to 0.0094 mm^3 .

During the metamorphosis period from G43 till 46 stages VNO morphogenesis is associated with a further nasal cavity. During this period PC increases in size, but its OE doesn't become more differentiated. Rostral part of the PC in the region of external nostrils is connected with the fissure-like middle chamber, that increases in size (fig. 3, b). Nasolacrimal duct falls laterally into it and VNO is located ventromedially. VNO cavity through the isthmus is medially connected with lateral inferior chamber recess, which has no sensory epithelium. Thereby, at the end of metamorphosis period, the entire nasal cavity is interconnected and have definitive morphologies. VNG doesn't change significantly. VNE becomes more differentiated, in particular receptor cells in its medial part have significantly noticeable cilia and basal membrane is covered externally with the layer of numerous pigmental cells. VNE thickness decreases in comparison with the previous developmental stage and vary in narrow range from $45.19 \pm 8.14 \mu\text{m}$ to $49.09 \pm 2.83 \mu\text{m}$, but its volume dramatically increases, and on the G46 stage equals to $0,403 \text{ mm}^3$.

After metamorphosis (14 days) VNE thickness is unchangeable, while VNO volume considerably increases (0.651 mm^3) (fig. 4).

Discussion

In general, our data coincide with a slight difference with the other authors' data related to VNO anlage in other Ranidae representatives. However, these processes differ in the terms of structure formation, and, in a smaller extent, in their topography. The VNO primordium in *P. ridibundus* can be found at G24 stage (beginning of larval development period) in rostroventral OE part. Basically, our data on VNO development in *P. ridibundus* agree with the data of other authors concerning the other Ranidae. VNO of *Rana chensiensis* appears later than VNO of *P. ridibundus* at G26 stage of larval development between OE and trabecule horns (Wang et al., 2008); VNO of *Rhinella (Bufo) arenarum* occurs rostroventrally as a dorsal OE diverticulum during the G24–25 stages (Jungblut et al., 2011). Similar VNO development pattern was found in *Eleutherodactylus coqui*, which has no larval development period (Jermakowicz et al., 2004). VNO development of *Rana japonica* as OE inflection is observed on the 4th day after the tadpole escape from egg membranes (from the free floating larvae stage till the end of metamorphosis) (Taniguchi et al., 1996). Tsui (1946) names VNO an anterior inferior sac, and its anlage in *Rana nigromaculata* occurs as the result of superior sac ventrorostral part inflection.

VNO development in Bufonidae occurs considerably later. For instance in *Bufo americanus*, VNE appears in medial wall of PC only during the last stages of larval development (G34) (Jermakowicz et al., 2004). This probably connects with OE differentiation into dorsal and ventral parts in the rostral part of PC.

VNO of *Xenopus laevis* — the representative of one of the most primitive Anura families — Pipidae, is also laid during later stages of larval development (N/F37–38) (according to normal development tables, Nieuwkoop, Faber, 1956). In *Ascaphus truei* (Leiopelmatidae), that have some organization features bringing them closer to Caudata, VNO is layed at the larval development stage not medially, but in ventrolateral part of PC invagination and then goes on medially into OE (Benzekri, Reiss, 2012). Thereby, in comparison with other Anura, VNO anlage occurs laterally but not in rostromedial OE part. VNO of *A. truei* mature specimens looks like horizontal fissure covered by VNE, which shifts medially and is located under PC. Though VNO comes closer to “rana type” topography (Medvedeva, 1975), but its shape differs from *P. ridibundus* VNO shape.

VNO of mature caudates has rather different topography. VNO of *Plethodon cinereus* is located in ventrolateral diverticulum of PC (Dawley, Bass, 1988), VNO of *Amphiuma tridactylum* and *Ambystoma mexicanum* is located in lateral inflection of PC (Eisthen et

al., 1994; Eisthen, 2000). Therefore, there are some exceptions, e. g. VNO of *Siren intermedia* (Sirenia) is located more medially in relation to PC (Eisthen, 2000). Mentioned cases allow considering VNO of Anura to be more variable structure.

VNO of some amphibians has no sensor epithelium. Eisthen (2000) found that *Necturus maculosus proteus* has lateral evagination covered with non-sensory epithelium instead of VNE. These data need further studying and discussion. In this case, what is the function of this evagination in *Proteus*? There is no answer to the moment.

In *P. ridibundus*, VNG anlage takes place during the G27 stage of larval development period near dorsomedial wall of formed VNO. VNG is called medial nasal gland by Tsui (1946), obviously, due to its location. Gland begins developing after the beginning of VNO development can be found also in *R. (B.) arenarum* during G28–29 stages (Jungblut et al., 2011), *R. chensinensis* during G34 stage (Wang et al., 2008), *R. japonica* — on the 6th day after VNO anlage (Taniguchi et al., 1996), *X. laevis* during the N/F42 stages (Nieuwkoop, Faber, 1956) and *E. coqui* at the end of embryonic development during TS12 stage (Jermakowicz et al., 2004). In the last species, the gland is laid laterally in relation to VNO, in contrast to the majority of Anura. In certain species, e. g. *B. americanus*, VNG begins developing simultaneously with the beginning of VNO development (G34) (Jermakowicz et al., 2004). This can probably take place due to the late VNO formation. VNE of *P. ridibundus*, in contrast to OE, has no Bowman's glands, that appear during metamorphosis (G41 — 42), and this coincides with the data of other authors (Jermakowicz et al., 2004; Wang et al., 2008; Jungblut et al., 2011). Obviously, that VNG secretion begins earlier than the secretion of Bowman's glands. This indicates that VNO starts functioning in water environment (larval period), while OE starts functioning during transition to terrestrial environment (metamorphosis period). The early function of vomeronasal system is testified by its central part structure. It was shown that synaptic contacts between VNO and supplementary olfactory bulb are already formed at the beginning of larval development period (Jungblut et al., 2011), thus vomeronasal system is functionally ripe. Our results support Eisthen (2000) hypothesis that states that VNO first appeared in water tetrapods and it is not an adaptation of olfactory system to terrestrial life conditions. Interestingly enough, that mammal VNG develops at the end of embryonic period (Garrosa et al., 1998).

According to Nowack and Wöhrmann-Repenning (2009), not only VNG, but also lacrimal gland (harderian gland) takes part in VNO functioning. Gland secretion is exuded through nasolacrimal duct, opened near external nostrils, where it fixes chemical irritants that later get to VNO.

In this case, what is the function of VNG? It is important that snakes have lacrimal gland connected with VNO through nasolacrimal duct, and have no VNG at all (Holtzman, Halpern, 1990). Since VNE has no Bowman's glands, it is obvious that VNG function is executed by lacrimal gland.

In spite of VNO being relatively well developed in Amphibia, one hardly can suppose that VNO had emerged as the result of transition to terrestrial or amphibian way of life (Medvedeva, 1975). It is not correct to suppose that VNO is a typical feature of the animals with well-developed scent while vertebrates who belong to the microsmatic group (i. e., some Primates) have also VNO (Bhatnagar, Meisami, 1998). The specific feature of VNO is that some vertebrates have this organ only during early ontogenesis stages (Aves). Thereby, vertebrate VNO is characterized by wide variation: between different vertebrate taxons, interspecific, specific, both qualitative and quantitative. This obviously is the evidence of loss of VNO functional meaning during regressive evolution.

Concerning VNO origin, it is evident that it appeared first in water Amphibia ancestors as preadaptation and got new adaptive functions as the result of animals transition to terrestrial conditions.

We agree with Eisthen's idea (2000) as to the origin of VNO in aquatic tetrapods and consider it to be a preadaptation that got new functions in the result of transition to terrestrial conditions. According to Jarvik (1942) rhipidistia had VNO, but the conclusions were not final, because they were made based on presence of invagination in olfactory capsules.

VNO diversity in tetrapods can probably be the result of functional specialization in some lineages and loss of its function in some other lineages.

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Received 8 January 2013

Accepted 20 May 2013