Vestnik zoologii, **46**(5): e-32–e-39, 2012
DOI 10.2478/v10058-012-0038-5

UDC 591.471.4:598.115.31

POLAR CARTILAGES AND FORMATION OF CRISTA SELLARIS IN GRASS SNAKE, *NATRIX NATRIX* (OPHIDIA, COLUBRIDAE), CHONDROCRANIUMAT THE EARLY STAGES OF EMBRYOGENESIS

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Received 6 March 2012
Accepted 14 September 2012

Polar Cartilages and Formation of Crista Sellaris in Grass Snake, *Natrix natrix* (Ophidia, Colubridae), Chondrocranium at the Early Stages of Embryogenesis. Sheverdyukova H. V. — The initial developmental stages of grass snake's, *Natrix natrix* Linnaeus, 1758 chondrocranium are described. Three paired structures form the floor of *N. natrix*'s neurocranium: cranial trabeculae, polar cartilages, and parachordals. The primordiums of polar cartilages and their independent centers of chondrification are identified at the stage 26 of development for the first time for *N. natrix* and snakes in general. The participation of these structures in the formation of crista sellaris and carotid foramina is proved.

Key words: polar cartilage, crista sellaris, chondrocranium, embryonic development, carotid foramina, *Natrix natrix*.

Полярные хрящи и формирование crista sellaris в хрящевом черепе ужа обыкновенного, *Natrix natrix* (Ophidia, Colubridae), на ранних стадиях эмбриогенеза. Шевердюкова А. В. — Описаны начальные этапы развития хрящевого черепа *Natrix natrix* Linnaeus, 1758. Три парные структуры: черепные трабекулы, полярные хрящи и парахордалии образуют дно нейрокраниума *N. natrix*. Зачатки полярных хрящей и их независимые центры охрящевения определены на 26-й стадии развития впервые для *N. natrix* и змей в целом. Доказано участие этих структур в формировании crista sellaris и сонных отверстий.

Ключевые слова: полярные хрящи, crista sellaris, сонные отверстия, эмбриональное развитие, хрящевой череп, *Natrix natrix*.

Introduction

The trabeculae cranii and basal plate (planum basale) are the first elements of the chondrocranium, which appear in the cranial embryogenesis of vertebrates, particularly snakes, and form the floor of neurocranium (Parker, 1878; Bäckström, 1931; Chekanovskaya, 1936; Bellairs, Kamal, 1981). Trabeculae appear in the form of separate paired bars. Later, their oral ends merge into the trabecula communis, and the aboral ones extend and merge with the oral-lateral edges of the basal plate. Triangular fenestra, limited laterally by the trabeculae, orally by the trabecula communis, and aborally by the oral edge of the basal plate, is called the pituitary fenestra (fenestra hypophysale).

The way of basal plate's primordium appearance in snake embryogenesis remains controversial. A common statement about initially independent primordiums of parachordals merging later into a basal plate in all vertebrates (de Beer, 1937), is questioned by researchers who studied the embryogenesis of a snake skull (Bäckström, 1931; Chekanovskaya, 1936; Bellairs, Kamal, 1981; Haluska, Alberch, 1983). The authors described a single primordium of the basal plate in the form of mesenchymal tissue, enveloping the notochord at the early stages of development.

According to most researchers, the fenestra basicranialis in snakes' chondrocranium is formed at the later stages of embryogenesis as a result of the cartilage resorption in the basal plate; it is limited by the oral portion of the basal plate, which is called crista sellaris. Thus, crista sellaris is a transverse bar that separates the pituitary and the basicranial fenestrae. *N. natrix* and *Lamprophis inornatus* (Colubridae) have foramina in the lateral parts of the crista sellaris for the internal carotid arteries — carotid foramina — to pass (Parker, 1878; Bäckström, 1931; Chekanovskaya, 1936; Pringle, 1954).

In most existing works structures of chondrocranium are described at fairly late stages, when the floor of neurocranium is already fully developed. There are only a few studies in which primordiums and formation of the basic structures of the snakes' chondrocranium (trabeculae and basal plate) at the initial stage of its development are described (Parker, 1878; Bäckström, 1931; Chekanovskaya, 1936).

The aim of our study was to determine the timing and the way of appearance and development of the chondrocranium basic elements at the early stages of normal *N. natrix* embryogenesis.

Material and methods

The material was collected in June—July 2010—2011. Pregnant *N. natrix* females were caught in their habitat and placed in terrariums, where they oviposited. The eggs were incubated in wetted vermiculite at a temperature of 27—30° C. Two eggs of each clutch were taken every day. At a point of egg laying *N. natrix* embryos are at stages 27+ of normal development. The stages of embryo development were determined by a table of normal development, worked out for *Thamnophis sirtalis* (Colubridae) (Zehr, 1962).

Embryos at 25—27th stages of development were extracted during caesarean section. The operative technique follows H. Clark (1937). Immobilization and anesthesia were performed according to the method introduced by D. B. Vasiliev, A. M. Timerina (2000) especially for reptiles. Medetomidin 50—70 mg/kg was used as an anesthetic. After oviposition, performed operations and rehabilitation all the females were released in their place of their capture.

The embryonic material was fixed in 4 % formalin solution. The dry-out material was placed into paraffin, thereafter serial histological 5—7 microns thick sections were prepared. The sections were stained with alcianblue — hematoxylin — eosin.

Twenty-five embryos at stages 25—32 of normal development were examined. A few embryos were investigated at one stage. The stage on which the embryo development has substantially advanced, but not yet reached the next one is marked with the sign "+".

Photographs of microsections were made with a microscope Zeiss Axio Imager M1 and software Zeiss Axio Vision v. 4.63 in the Centre of Common Access to Equipment at the I. I. Schmalhausen Institute of Zoology, NAS of Ukraine.

Cleared and stained embryos were prepared as described by E. Simons, D. R. Van Horn (1971). The whole mounts do not show structures, where the processes of chondrification is uncompleted, so some stages of development are illustrated by cleared and stained embryos at later stages.

Result

Stage 25

The first primordiums of the future elements of *Natrix natrix* chondrocranium appear at the stage 25 of normal development: ventral to the forebrain and ventro-medial to eyes, in the general mass of friable mesenchyme, paired clusters of mesenchymal cells in the form of parallel beams are seen (fig. 1, *a*). These are the mesenchymal primordiums of cranial trabeculae. Their aboral ends lay in front of carotid arteries.

At this stage, at the sides of the notochord head end the primordiums of parachordals appear in the form of elongated mesenchymal plates (fig. 1, *b*). As a result of the well-marked at this stage cervical flexure, the primordiums of trabeculae and parachordals are almost orthogonally situated.

Stage 26

The mesenchymal primordiums of another structures are visible dorsal to the parachordal primordiums, which tend to merge with oral ends of the latter. They are situated in one plane with the primordiums of parachordals. At the pituitary level their oral ends are bent towards each other; they are medial to the carotid arteries (fig. 2). Based on the topography of these mesenchymal structures, we believe them to be the primordiums of the polar cartilages (cartilago polaris), described in some cartilaginous fishes, bony fishes and birds (de Beer, 1937; Kovtun et al., 2008). The head end of the notochord, the primordiums of polar cartilages and of prachordals are situated ventrally to the hindbrain.

At this stage the cervical flexure remains, so the primordiums of polar cartilages and of parachordals are orthogonal to the primordiums of the cranial trabeculae.

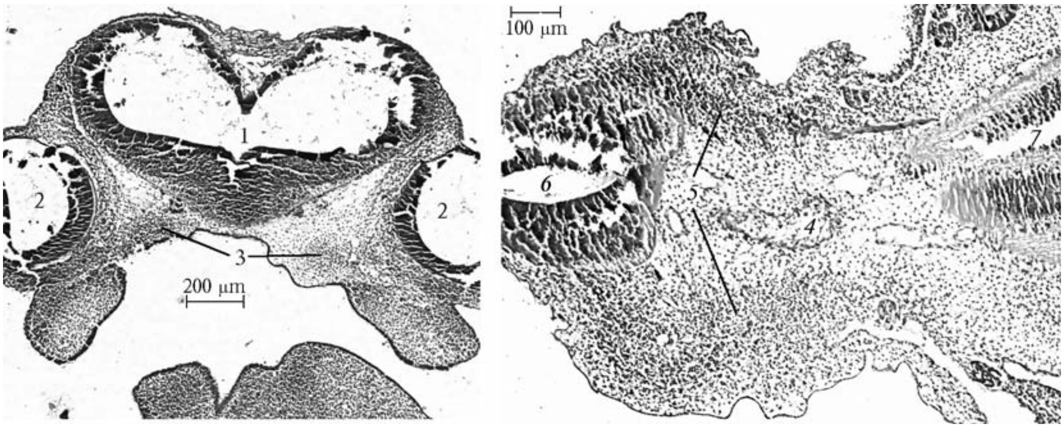

Fig. 1. Cross-section of *N. natrix* embryo's head at the stage 25 of the development: *1* — forebrain; *2* — eye; *3* — mesenchymal primordiums of the trabeculae cranii; *4* — notochord; *5* — mesenchymal primordiums of the parachordals; *6* — spinal cord; *7* — hindbrain.

Рис. 1. Поперечные срезы головы эмбриона *N. natrix* 25-й стадии развития: *1* — передний мозг; *2* — глаз; *3* — мезенхимные закладки черепных трабекул; *4* — хорда; *5* — мезенхимные закладки парахордалий; *6* — спинной мозг; *7* — задний мозг.

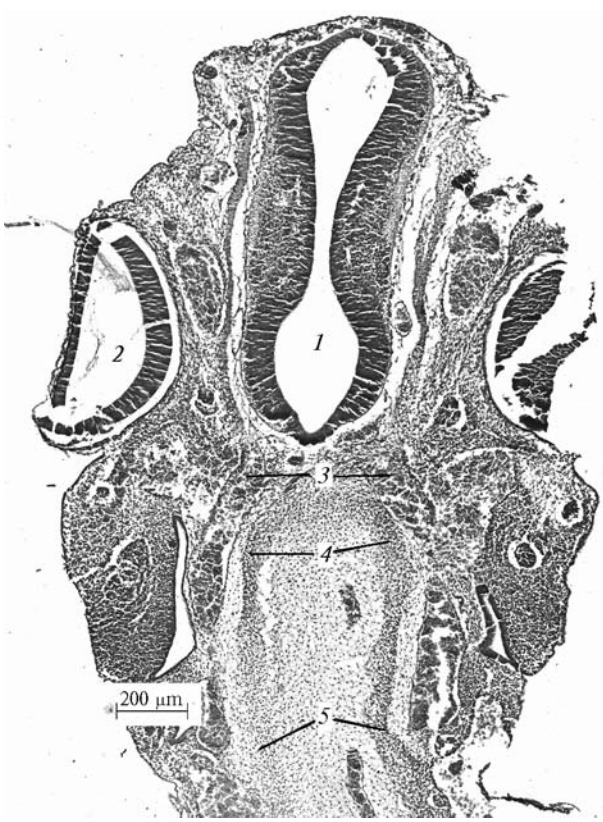

Fig. 2. Cross-section of *N. natrix* embryo`s head at the stage 26 of the development: *1* — midbrain; *2* — eye; *3* — carotid arteries; *4* — mesenchymal primordiums of the polar cartilages; *5* — mesenchymal primordiums of the parahordals.

Рис. 2. Поперечные срезы головы эмбриона *N. natrix* 26-й стадии развития: *1* — средний мозг; *2* — глаз; *3* — сонные артерии; *4* — мезенхимные закладки полярных хрящей; *5* — мезенхимные закладки парахордалий.

Stage 27

The cervical flexure is less marked. As a result, the structures of the future chondrocranium, described above, changed their relative positions: the angle between the primordiums of the trabeculae and the polar cartilages becomes blunt; the primordiums of the polar cartilages surround the internal carotid artery from the aboral side. The medial parts of the polar cartilages' primordiums are merged with the oral ends of the parachordals' primordiums at right angles in the transverse plane. At this stage the processes of chondrification are visible in all three structures: trabeculae, polar cartilages and parachodrals (fig. 3). Each of them has an independent center of chondrification. The processes of chondrification are seen in the medial zone of the trabeculae' and polar cartilages' primordiums. The aboral halves of the parachordals are merged, forming a basal plate (fig. 3, *c*). The processes of chondrification are more marked in the basal plate, that is chondrification of this structure proceeds in the aboral-oral direction.

Stage 27+

The aboral mesenchymal ends of the trabeculae merge with the lateral mesenchymal edges of the polar cartilages approximately orthogonally (in the transverse plane)

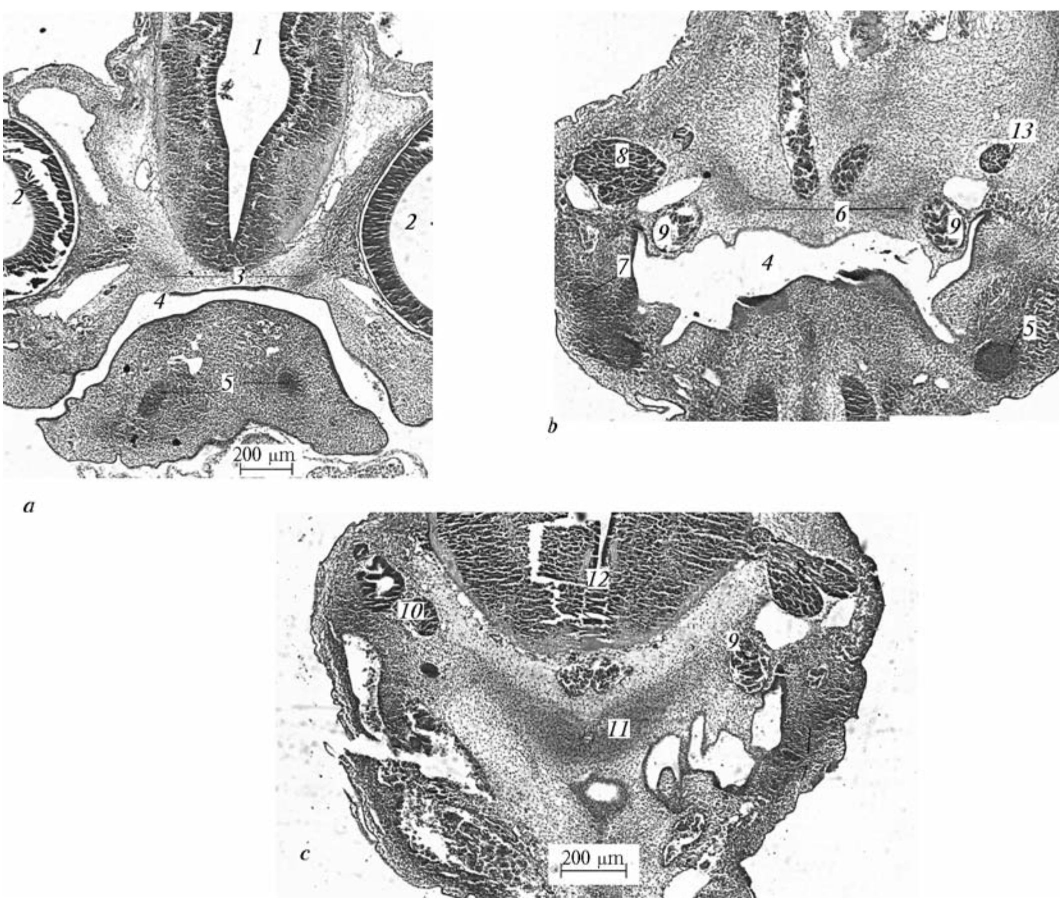

Fig. 3. Cross-section of *N. natrix* embryo`s head at the stage 27 of the development at different levels: *1* — forebrain; *2* — eye; *3* — primordiums of the trabeculae cranii; *4* — rostrum; *5* — Meckel's cartilage; *6* — primordiums of the polar cartilages; *7* — quadrate cartilage; *8* — ganglion of the trigeminal nerve; *9* — carotid arteries; *10* — semicircular canals; *11* — basal plate; *12* — hindbrain; *13* — oculomotor nerve.

Рис. 3. Поперечные срезы головы эмбриона *N. natrix* 27-й стадии развития: *1* — передний мозг; *2* — глаз; *3* — закладки трабекул; *4* — рострум; *5* — Меккелев хрящ; *6* — закладки полярных хрящей; *7* — квадратный хрящ; *8* — ганглий тройничного нерва; *9* — сонные артерии; *10* — полукружные каналы; *11* — базальная пластинка; *12* — задний мозг; *13* — глазодвигательный нерв.

(fig. 4, *a*). The medial edges of the polar cartilages are merged with the oral ends of parachordals also orthogonally so that the boundary between them is impossible to distinguish.

Stage 28

All the basic structures, described above are completely cartilaginous. The aboral ends of the trabeculae are merged with the lateral edges of the polar cartilages. The places of their junction are located lateral to the internal carotid arteries: at the point where the latter bend dorsally, going to the brain. At this stage the chondrifying processes from the medial edges of the polar cartilages depart in the oral direction. They surround the internal carotid arteries from the medial side, forming the so-called carotid incisures (fig. 4, *b*).

Stage 29

The processes of the polar cartilages are merged with the medial surface of the trabeculae, closing the passages for the internal carotid arteries and forming the carotid foramina (fig. 4, *c*). Transverse bar, the so called crista sellaris, connects the medial borders of the fully formed carotid foramina. The initial stage of its chondrification is marked. The crista sellaris divides the space between the beams formed by the trabecula, the polar cartilage and the parahordalia's oral end on each side into two fenestrae: pituitary and basicranial fenestrae. Thus, the pituitary fenestra is triangular in shape: it is limited by

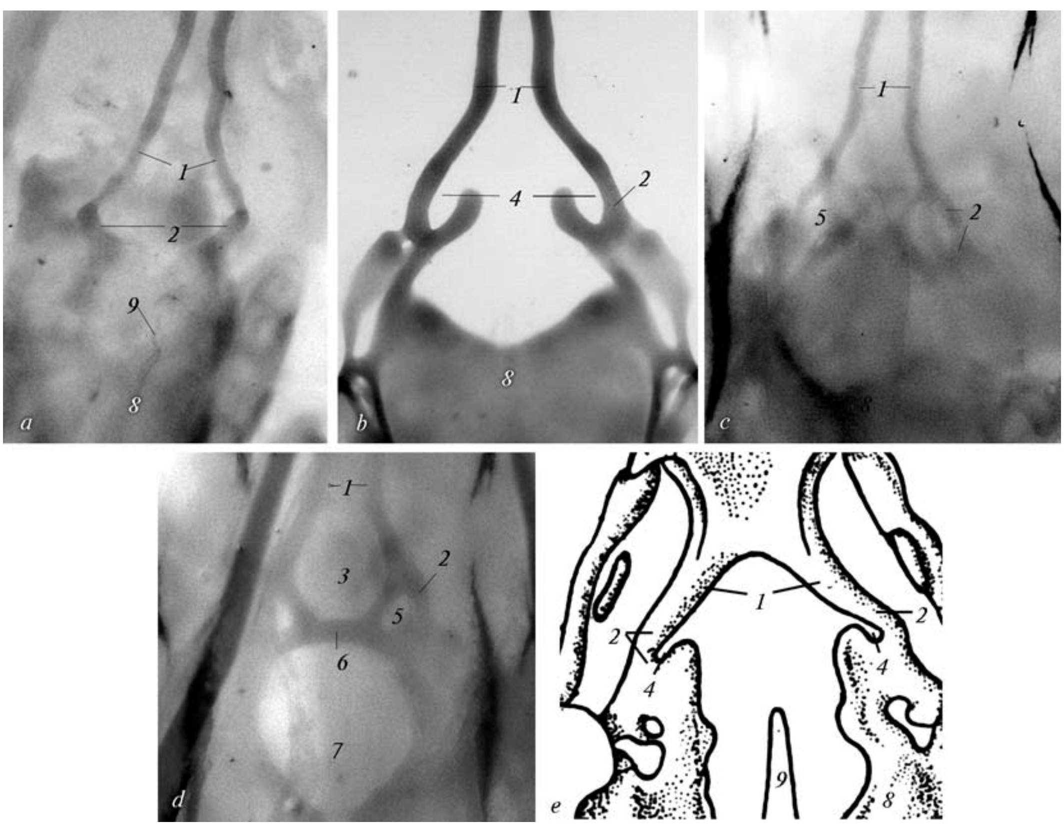

Fig. 4. Cleared and stained cranium of *N. natrix* embryo in dorsal view: *a* — stage of development 29; *b* — 30+; *c* — 31; *d* — 32; *e* — the future crista sellaris region in chondrocranium of embryo *Amia calva* (graphic reconstruction after Pehrson, 1922): *1* — trabecula; *2* — polar cartilage; *3* — pituitary fenestra; *4* — carotid incisure; *5* — carotid foramen; *6* — crista sellaris; *7* — basicranial fenestra; *8* — basal plate; *9* — notochord.

Рис. 4. Тотальный препарат черепа эмбриона *N. natrix* вид сверху: *a* — 29; *b* — 30+; *c* — 31; *d* — 32-я стадия развития; *e* — область формирования будущей crista sellaris в хрящевом черепе эмбриона *Amia calva* (графическая реконструкция из Персон, 1922): *1* — трабекула; *2* — полярный хрящ; *3* — гипофизарное окно; *4* — сонная вырезка; *5* — сонное отверстие; *6* — crista sellaris; *7* — базикраниальное окно; *8* — базальная пластинка; *9* — хорда.

the trabecula communis orally, by the trabeculae laterally and by the crista sellaris aborally. The basicranial fenestra is limited by the crista sellaris orally, by the free ends of the parachordals laterally and by the basal plate aborally (fig. 4, *d*).

At the later stages of development**,** the above mentioned structures grow and the processes of chondrification become more intensive in them. No other visible changes were observed. The chondrification of the crista sellaris ends at the stage of development 32.

Discussion

As already mentioned, there is no consensus on the way the basal plate's primordiums emerge in snakes' chondrocranium. We observed a pair of the parachordal primordiums at Stage 25, which confirms the E. Gaupp's (1906) and G. R. de Beer's (1937) findings. We were unable to determine whether each parachordal has an independent center of chondrification or the chondrification occurs after the mesenchymal aboral ends of parachordal primordiums merge, i.e., already in the basal plate. We believe that the parachordals merge at the mesenchymal stage around the head notochord, forming the so-called "enveloping mass" of mesenchymal cells, which most researchers described as an unpaired primordium of snakes' basal plate (Bäckström, 1931; Chekanovskaya, 1936; Bellairs, Kamal, 1981). According to our findings**,** the basal plate is formed by the confluence of the aboral ends of parachordals; the oral ends remain free, and the space between them is a future basicranial fenestra. As opposed to the authors mentioned above, we observed no resorption of the basal plate cartilage.

The polar cartilages we discovered in *N. natrix* between trabeculae and parahordals are described for the first time in snakes. The only mention of these structures in the snakes' chondrocranium is found in H. R. Srinivasahar (1955), who studied the embryo of *Vipera russelii* at one late stage, when the chondrocranium is fully formed. The author took note of the characteristic flexure of the aboral parts of trabeculae and its localization (lateral to the internal carotid arteries). At that stage it was impossible to determine, whether these zones were formed by separate cartilages, located between the trabeculae and parahordals, or they are actually trabeculae. However, the author suggested that if these zones appear as separate cartilages, they should be considered as polar cartilages, described in fishes and birds. We managed to find independent primordiums of these structures in *N. natrix* that confirms H. R. Srinivasahar's assumption. For the first time we discovered the primordiums of polar cartilages at the stage 26 of development, when they begin to merge with the oral ends of the parahordals' primordiums; an independent center of chondrification in these structures is observed at the stage 27 of development. Thus, the floor of the cartilage neuro**c**ranium of *N. natrix* is formed by three paired structures — trabeculae, polar cartilages and parahordals — probably homologous to those found in fishes and birds. The same as in fishes and birds, the polar cartilages in *N. natrix* merge with trabeculae lateral to the internal carotid arteries. In fish embryos at the late stages of development, the individual polar cartilage could not be discerned, but the researchers **(**Pehrson, 1922; de Beer, 1937**)** assumed that the place of their junction with the trabeculae is located lateral to the carotid arteries. In *N. natrix* at stage 27+ the polar cartilages do not look as separate structures**,** too.

The process of crista sellaris' formation in snakes was described in the works of those authors who managed to study the early stages of embryo development (Parker, 1878; Bäckström, 1931). The authors, who claimed that basicranial fenestra is the result of cartilage's resorption of the basal plate identified the oral margin of the latter as crista sellaris (Backstrom, 1931; Bellairs, Kamal, 1981). W. K. Parker (1878) described another way of crista sellaris formation. He believed that the transverse bar, called by him "post-pituitary bridge", is formed by two processes that deviate medially toward each other from the aboral ends of the trabeculae (in the place of their junction with parachordals) and merge.

We found that the crista sellaris in *N. natrix* is formed by the polar cartilages and their processes, not the processes of the trabeculae, as W. K. Parker considered.

The transverse bar, dividing the pituitary and basicranial fenestrae is described in all vertebrates as crista sellaris in amphibians and snakes, acrochordal cartilage in lizards, turtles and birds, dorsum sellae in mammals (de Beer, 1937). Different names of this structure in fishes are mentioned: crista sellaris (*Squalus acanthias*), postpituitary commissure (*Scyllium canicula*), dorsum sellae (*Torpedo ocellata*), acrochordal cartilage (*Callorhynchus antarcticus*), prootic bridge (*Lepisosterus osseus, Salmo fario, Exocoetus, Anguilla vulgaris, Gadus merlangus, Neoceratodus*). The different ways of its formation are described in the fishes: in *Lepisosterus osseus* and *Salmo fario* prootic bridge appears as an independent structure. In *Acipenser ruthenus* as in amphibians the crista sellaris is formed by the confluence of the oral parahordals' ends (de Beer, 1937).

The process of crista sellaris formation we observed in *N. natrix* is similar with those of some cartilaginous fishes whose polar cartilages' processus merged with each other forming transverse bar (de Beer, 1937).

There is no consensus on how the snakes' carotid foramina are formed. E. Gaupp (1906) believed that the carotid foramina in snakes are formed mainly by trabeculae and lateral processes, departing from their bottom and connecting to the basal plate. According to him, trabeculae surround carotid arteries medially. K. Beckstrom (1931) believed that the trabeculae surround carotid arteries laterally and the latter are medially surrounded by medial trabeculae' processes.

Among Ophidia the carotid foramina are described only in two species of the family Colubridae: *Natrix natrix* (Parker, 1879; Bäckström, 1931; Chekanovskaya, 1936) and *Lamprophis inornatus* (Pringle, 1954). J. A. Pringle believed its presence is a primitive feature of colubrid snakes, inherited from ancestral forms. It should be noted that in most other studied snakes the orally open notches, not enclosed foramina, are described for the passage of the internal carotid arteries. These notches are located at the oral edge of the crista sellaris, where they merge with trabeculae (Pringle, 1954; Srinivasachar, 1955; Bellairs, Kamal, 1981; Haluska, Alberch, 1983). We observed such carotid notches in *N. natrix* at the stage 28 of development. According to our findings, they are formed by the polar cartilages and by their processes. The similar carotid notches are described in *Amia calva* (Pehrson, 1922) (fig. 4, *e*).

In the literature, different ways of the crista sellaris' (acrochordal cartilage's) formation are described in lizards. G. R. de Beer (de Beer, 1937) discovered processes, going medially from the junction of trabeculae and parachordals, which merge with each other. Such a way of the crista sellaris' formation is similar to the way of formation of the «post-pituitary bridge», described by W. K. Parker (1878) in *N. natrix*. According to some authors (Bellairs, Kamal, 1981), crista sellaris is the oral edge of the basal plate: crista sellaris is recognized as a separate structure only after the formation of the basicranial fenestra as a result of cartilage resorption. According to A. N. Yarygin (2009), the acrochordal cartilage in *Lacerta agilis* appears originally as an independent structure. The similar way of the acrochordal cartilage's formation is described in turtles (de Beer, 1937; Bellairs, Kamal, 1981) and birds (de Beer, 1937; Kovtun et. al., 2008).

Some authors (Rieppel, Zaher, 2001) have called crista sellaris of snakes "the acrochordal cartilage", although, as follows from our investigation, the way of its formation differs from that of turtles, lizards and birds. According to our findings, an "ancient" way of the crista sellaris' formation in *N. natrix* is preserved. It should be homologous to that described in some fishes, so we consider crista sellaris to be an analogous structure to the acrochordal cartilage of turtles, lizards and birds.

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