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Constant morphological patterns in the hemolymph vascular system of crayfish (Crustacea, Decapoda)

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ABSTRACT

We present a study of the hemolymph vascular system of the marbled crayfish, *Procambarus fallax* f. *virginalis*, the only crayfish species known to be parthenogenetic. To identify potential evolutionary patterns, we compared data from a total of 48 specimens of *P. fallax* with 22 specimens of *Orconectes limosus*. Visualizations (2D and 3D) were carried out using a combination of classical and modern morphological techniques. Our data were compared to the existing literature.

Like all Decapoda, both *P. fallax* and *O. limosus* have a hemolymph vascular system, consisting of a globular heart with seven off-branching arteries. We were able to visualize in detail the heart of crayfish for the first time, i.e., the myocard with its clusters of muscles running through the lumen of the heart, the valves and flaps of ostia and arteries. Furthermore, the branching patterns of the seven artery systems were analyzed. Anatomical structures identified to be consistent in all specimens of both species were combined as ground pattern of hemolymph vascular system features for Astacida.

1. Introduction

Crayfish are frequently used as model organisms in research and education. Probably most students of biology have been confronted with crayfish biology at some time during their studies. Nonetheless, comprehensive morphological studies comparing different crayfish species are scarce. The majority of the literature dates from the beginning of the last century, thus, the investigations were carried out before modern advances in morphological methods became available (e.g., the 3D revolution *sensu* Murienne et al., 2008). This also applies to the comprehensive morphological investigation of entire organ systems such as the circulatory system.

Previous studies of the hemolymph vascular system of crayfish have established that, similar to vertebrates, intraspecific variation occurs with respect to the branching patterns of arteries (for *Astacus astacus*: Baumann 1917, 1921, Chatanay, 1907, for *Procambarus clarkii*: Imafuku, 1993). This was also shown in other arthropod taxa: Complex arterial systems are known to exist in horseshoe crabs (Göpel and Wirkner, 2015) scorpions (Klußmann-Fricke et al., 2012), spiders (Huckstorf et al., 2013, 2015, Runge and Wirkner, 2016) and anomalan decapods (Keiler et al., 2013, 2015a, b, 2016).

However, the cause for intraspecific variability, i.e., polymorphism versus epigenetic factors, remains largely unknown.

The study of the marbled crayfish, *Procambarus fallax* (Hagen. 1870) f. *virginalis* (Martin et al., 2010), because of its parthenogenetic, and thus clonal mode of reproduction (Scholtz et al., 2003, Martin et al., 2007), might reveal that morphological variations of the circulatory system are very likely generated by epigenetic factors. Preliminary data on the characteristics of the descending artery connecting the heart with the ventral vessel were published by Vogt et al. (2009). In our current study, we are presenting those morphological patterns of the hemolymph vascular system which we consider not to be subject to variability and which may therefore be regarded as inheritable patterns. A forthcoming study will describe those patterns which show variability, and may therefore be related to epigenetic changes, by comparison of both crayfish species.

As all decapods, crayfish possess a hemolymph vascular system made up of a globular heart (Balss et al., 1940-1961, Wirkner et al., 2013) from which two paired and three unpaired artery systems emanate (Balss et al., 1940-1961, Gruner 1993). Intraspecific variations are mainly observed in the secondary arteries of their arterial systems. In order to describe the morphological variability and to distinguish between constant and variable patterns, it is therefore necessary to study a larger number of individual animals. We used

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P. fallax f. *virginalis* as our model organism, as it can be easily kept under laboratory conditions.

Data gathered from *P. fallax* f. *virginalis* and *Orconectes limosus* using up to date morphological methods in combination with classical methods are compared with data from the literature. We report morphological patterns which were consistently identified as invariable in all animals of each species studied so far, and claim that these can be regarded as the ground pattern of the crayfish hemolymph vascular system.

2. Materials and methods

2.1. Species studied

Adult specimens of *P. fallax* f. *virginalis* were bought at a pet shop, and reared individually in 6-Liter-aquaria (20 cm × 20 cm × 15 cm) filled with tap water at 22 ± 3 °C; 48 specimens were studied. For histology we dissected one 0.3 cm long (carapace length) juvenile as well as 12 recently hatched animals. For injection preparations we used specimens with a carapace length of at least 1 cm. All animals and life stages were fed ad libitum with "Novo Crabs" food chips (JBL).

Adult specimens of *O. limosus* with a carapace length of about 4.5 cm were caught from a lake near Rostock (Hohensprenzer See) in November 2009 and kept together in a 300-Liter-aquarium (85 cm × 120 cm × 30 cm) filled with tap water at 5 °C and equipped with shelters; 22 specimens were studied. All animals and life stages were fed ad libitum with "Novo Crabs" food chips (JBL).

Additionally, three specimens of *Astacus leptodactylus* (Eschscholtz, 1823), of unknown origin were taken from the Zoologische Sammlung der Universität Rostock (ZSRO) to examine the pericardial septum.

2.2. Histology

The specimens were fixed in Bouin's fixative and dehydrated in ethanol. After an intermediate step in acetone the dissected parts were embedded in araldite epoxy resin. Series of semi-thin sections (1 μm thickness) were made with a rotary microtome (Leica, RM 2165) using either glass or diamond knifes. Sections were stained with a mixture of 1% toluidin blue, 1% sodium-tetraborat and 1% pyronin G in an aqueous solution for about 35 s at 60 °C.

2.3. Micro-CT

X-ray imaging was performed with a Nanotom® high resolution micro computed tomography system (phoenix|x-ray, GE Sensing & Inspection Technologies). Scans were obtained by using the following instrument adjustments: Molybdenum-target; voltage: 50 kV; amperage: 250 μA; Mode: 0; 1440 projections; detector-timing: 1000 ms; resulting voxel-size ca. 2–10 μm. Volume-files were generated using the software datos|x-reconstruction and a stack of virtual sections was exported with the software VG-Studio max (Volume Graphics GmbH). Some scans were performed using an X-ray Microscope, Xradia 410 Versa (Zeiss).

2.4. 3D reconstruction

Every fifth semi-thin section of the series was photographed with a Zeiss AXIOCAM ICc3 camera mounted with an adapter (TV 2/3"C 0,36x) on a Zeiss AXIO Imager.M1 microscope (used objective ZEISS A Plan 5×/0.12). Digitized sections were aligned automatically and corrected manually with the software Autoaligner 6.0.1. (Bitplane AG). Visualizations of both the digitized section and the micro-CT data were performed with the software Imaris (version 6.4. and 7.0) by Bitplane AG.

2.5. Corrosion casting

For in-situ vessel depiction and following preparation, casting resins were injected into the heart of specimens of *P. fallax* f. *virginalis* and *O. limosus*. Three different casting resins were applied; one based on polyurethane (PU4ii, vasQtec) and the others based on *methyl* methacrylate (Mercox II, Ladd Research and Mercox CL-2R-5, SPI-Suplies). *O. limosus* specimens were injected with PU4ii while all three resins were used for *P. fallax* f. *virginalis* specimens.

2.6. Scanning electron microscopy

Parts of corrosion casts or whole mount casts were sputter coated at the Electron Microscopy Center of the Universität Rostock with gold using a Bal-Tec SCD 004 (duration: 100 s) and at the Institut für Spezielle Zoologie und Phyletisches Museum of the Friedrich-Schiller-Universität Jena. Coating was performed with platinum using an Emitech, K500 (duration: 100 s). Scanning electron micrographs were taken with a scanning electron microscope in Jena (FEI Company, XL30 ESEM TMP) and in Rostock (Zeiss, DSM 960A).

2.7. Image management and processing

Irfan-View 4.0 software was chosen to convert the entire batches of the digitized pictures into grayscale, to invert and rename them. CorelDraw Graphics Suite X3 was used to create schematic depictions and the picture plates.

2.8. Terminology

The terminology used bases on the Ontology of the Arthropod Circulatory System (Wirkner et al., 2017). All parts of the circulatory systems are considered as morphemes (Richter and Wirkner, 2014). No implications on homology and function are intended.

3. Results

In this first step towards a definition of constant morphological features within the anatomy of the vascular system of crayfish, we investigated specimens of different age and body size, yet the descriptions relate to the adult conditions if not otherwise remarked.

The body of crayfish consists of two tagmata, i.e., cephalothorax and pleon. The cephalothorax is formed by the cephalic segments, i.e., the head, and the eight thoracic segments, which are covered by the carapace and its lateral branchiostegites. On each body side the latter forms a respiration chamber, which houses the gills (Fig. 1E). These gills are epipodial appendages with two different insertion sites (Fig. 3C). In the two species investigated in the current study, the gills are distributed as follows:

The podobranchs in *O. limosus* and *P. fallax* f. *virginalis* insert at the coxopodite of the 2nd up to the 7th thoracopod. The arthrobranchs occur at the same thoracomers. However, in *P. fallax* f. *virginalis* two arthrobranchs instead of one are present at the 3rd up to the 6th thoracomere. In *O. limosus* also two arthrobranchs occur in the 7th thoracomere. Each of the six pleomers bears one pair of pleopods the last one of which forms the uropods.

Following the tagmatization of the crayfish body, below we first describe non-vascular, i.e., lacunar parts, followed by the vascular parts and structures of the circulatory system in the cephalothorax