

Appearance of muscle elements in the embryogenesis of Anomopoda and Ctenopoda (Crustacea: Branchiopoda: Cladocera)

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ABSTRACT: Despite employing modern genetic approaches, the phylogenetic relationships between recent orders of the Cladocera (Crustacea: Branchiopoda) remain unclear. Reconstructions of phylogenetic relationships within the class Branchiopoda (containing both cladocerans and large branchiopods) based only on morphological features are vulnerable for criticism. Therefore the incorporation of data on anatomical changes during embryonic development in the phylogenetic analysis looks extremely promising. In this article, we present data on muscle development in Cladocera obtained via confocal laser scanning microscopy. We developed an original technique for labeling the musculature and embryo nuclei in two model species, *Daphnia magna* Straus, 1820 (Anomopoda) and *Sida crystallina* (O.F. Müller, 1776) (Ctenopoda), using phalloidin and methyl green. Periodization of musculature development according to the key events of embryogenesis was carried out using *D. magna* as a model. The general order of muscle formation in *D. magna* and *S. crystallina* is found to be similar. However, musculature of thoracic limbs, dorsoventral and oblique lateral muscles in *S. crystallina* have more pronounced metameric structure as compared to *D. magna*. Presence of traces of segmentation posteriorly to the fifth pair of thoracic limbs and sixth pair of dorsoventral muscles in ventral muscle band in *D. magna* can be interpreted as a rudiment of the sixth segment and sixth pair of thoracic limbs, which, however, are not visible as any, even minute, rudiments during embryogenesis. As compared to *Cyclestheria hislopi* Baird, 1859 (order Cyclestherida, a sister group of the cladocerans), the muscles of swimming antennae and thoracic limbs in *D. magna* and *S. crystallina* are more oligomerized. Specific traits of the musculature development have a phylogenetic signal on order level and can be used to reconstruct phylogenetic relationships within the class Branchiopoda.

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Появление мышечных элементов в эмбриогенезе Anomopoda и Stenopoda (Crustacea: Branchiopoda: Cladocera)

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РЕЗЮМЕ: Несмотря на использование технологичных генетических подходов, филогенетические связи между современными отрядами Cladocera (Crustacea: Branchiopoda) остаются неясными. Реконструкции филогенетических связей внутри класса Branchiopoda (включающего как кладоцер, так и крупных жаброногих ракообразных), основанные только на морфологических признаках, уязвимы для критики. В связи с этим, включение в филогенетический анализ данных об анатомических изменениях в ходе эмбрионального развития выглядит чрезвычайно перспективным. В этой статье мы приводим данные по изучению развития мышц у Cladocera с помощью конфокальной лазерной сканирующей микроскопии. Для этой работы мы разработали оригинальную методику окрашивания мускулатуры и ядер эмбрионов у двух модельных видов, *Daphnia magna* Straus, 1820 (Anomopoda) и *Sida crystallina* (O.F. Müller, 1776) (Stenopoda), с помощью фаллоидина и метиленового зеленого. Периодизация развития мускулатуры в соответствии с ключевыми событиями эмбриогенеза была проведена с использованием *D. magna* в качестве эталона. Общий порядок формирования мышц у *D. magna* и *S. crystallina* оказался сходным. Однако мускулатура торакальных конечностей, дорсовентральные и косые латеральные мышцы у *S. crystallina* имеют более выраженное метамерное строение по сравнению с *D. magna*. Наличие следов сегментации позади от пятой пары торакоподов и шестой пары дорсовентральных мышц в вентральном мышечном тяже у *D. magna* можно интерпретировать как зачаток шестого сегмента и шестой пары торакальных конечностей, которые, однако, сами не видны в виде каких-либо, даже мелких, зачатков в ходе эмбриогенеза. По сравнению с *Cyclothemia hislopi* Baird, 1859 (отряд Cyclothemia, родственная кладоцерам группа), у *D. magna* и *S. crystallina* мышцы антенн II и торакальных конечностей более олигомеризованы. Особенности развития мускулатуры несут филогенетический сигнал на уровне отрядов и могут быть использованы для реконструкции филогенетических связей внутри класса Branchiopoda. Как цитировать эту статью: Antonovskaya K.A., Neretina A.N., Voronezhskaya E.E., Kotov A.A. 2026. Appearance of muscle elements in the embryogenesis of Anomopoda and Stenopoda (Crustacea: Branchiopoda: Cladocera) // Invert. Zool. Vol.23. No.3. P.423–445. doi: 10.15298/invertzool.23.3.05

КЛЮЧЕВЫЕ СЛОВА: Cladocera, Branchiopoda, анатомия, мышцы, онтогенез, филогенетический связи, эволюция.

Introduction

The cladocerans, or water fleas (Crustacea: Branchiopoda: Cladocera), are among the most successful microscopic invertebrates in recent inland water bodies (Kotov, 2013; Van Damme, Kotov, 2016; Korovchinsky *et al.*, 2021). To date, more than 850 species of Cladocera have been described (Kotov, 2013; Van Damme, Kotov, 2016). Over the last thirty years, significant progress has been made in the taxonomy of many species groups (Kotov, 2013), a number of genera and species new to science have been described (e.g., Dumont *et al.*, 2013; Jeong *et al.*, 2017; Sousa, Elmoor-Loureiro, 2018). Also, the taxonomy of Cladocera at the family level is relatively well developed, and has been accepted by most cladocerotologists to date (Kotov, 2013).

Subdivision of Cladocera into orders has not undergone significant changes since Sars (1865). Nowadays most cladocerotologists consider this group in a superorder rank, including four recent orders (Anomopoda Sars, 1865; Ctenopoda Sars, 1865; Haplopoda Sars, 1865 and Onychopoda Sars, 1865). Two more extinct orders (Cryptopoda Kotov, 2007 and Proanomopoda Kotov, 2013) are known only from Mesozoic (Kotov, 2013). Cladocerans are considered to be the most advanced monophyletic group of Branchiopoda. Monophyly of the latter, regarded in a separate class rank, has been confirmed by both morphological and molecular phylogenetic studies (Kotov, 2013; Van Damme, Kotov, 2016; Van Damme *et al.*, 2022). Monophyly of recent orders within Branchiopoda (as well as within Cladocera group, which unites Cladocera and Cyclotherida), was also confirmed recently by molecular phylogenetic studies (Van Damme *et al.*, 2022). At the same time, phylogenetic relationships between four orders within Cladocera are not fully resolved despite employing genetic and even full-genomic approaches (Kotov, 2013; Xu *et al.*, 2021; Van Damme *et al.*, 2022).

Anomopoda and Ctenopoda include the largest number of cladoceran species with phyllopodous non-segmented thoracic limbs while Haplopoda and Onychopoda (predators with segmented limbs) include considerably fewer species. Sars initially proposed the group Calyptomera for the former two orders, and Gymnomera for the latter two orders (Sars, 1865). During the 20th century, several authors

were dubious of cladoceran monophyly, as well as Gymnomera and Calyptomera monophyly (Fryer, 1987a–b). In some subsequent studies, Haplopoda and Onychopoda were again united into the monophyletic group Gymnomera based on the thoracic limb structure (Olesen, 1998). Van Damme *et al.* (2022) based on both morphological and full-genomic data proposed a division of Cladocera into two other superorders: Magipoda (Ctenopoda + Haplopoda) and Oligopoda (Onychopoda + Anomopoda), but this system is not yet widely accepted. Moreover, although support of the Onychopoda + Anomopoda clade is high, grouping of Ctenopoda + Haplopoda is not well-supported and could be an artifact, e.g. due to an insufficient number of taxa included in such analysis.

Phylogenetic reconstructions based on morphological characters of adult cladocerans (Olesen, 1998) are vulnerable to criticism (Korovchinsky, Boikova, 2023). The main defect of recent cladoceran “non-genetic” phylogenies is rare use of anatomical characters, which traditionally represent a basis for phylogenetic reconstructions in many other groups of animals, like nematodes, mollusks and chordates. Therefore, the incorporation of data on anatomical structures and their formation during embryonic development into the phylogenetic analysis of the cladocerans looks promising for order-level phylogeny. However, existing data on cladoceran anatomy are sporadic and still not generalized (Kotov, 2013; Smirnov, 2017). Structure of muscles has been described only via light microscopy and mainly in adults (e.g., Hérouard, 1905; Binder, 1931; Sebestyén, 1931; Morduhai-Boltowsky, Rivier, 1987; Fryer, 1991; Smirnov, 2017; Sigvardt *et al.*, 2020; Korovchinsky *et al.*, 2021). Detailed investigations of the muscle elements development during ontogeny in Cladocera are urgently needed.

In general, the muscle system of invertebrates shows a great diversity, and its study in different groups may be interesting in the context of the formation of different body plans and separation of different evolutionary lineages (Haszprunar, Wanninger, 2000; Bergter *et al.*, 2007). Studies of myogenesis enable us to draw even more general conclusions (Wanninger, Haszprunar, 2002). In some cases, although the data on muscle structure do not permit final conclusions about the phylogeny of a group, they can still be used to

trace developmental patterns common to groups adapted to different environmental conditions (Hunnekuhl *et al.*, 2009). Anatomical data, especially the data on most conservative muscular and nervous systems, often provide much more reliable information about the formation of a particular body plan or taxonomic group than external morphology (Yap *et al.*, 2023).

Some reliable data on musculature structure in adult females of cladocerans were obtained via different techniques by previous authors (e.g. Christensen *et al.*, 2018; Sigvardt *et al.*, 2020; Korovchinsky *et al.*, 2021), but musculature of their embryos has never been studied before. In the present paper we describe in detail the muscle system development in embryogenesis of two cladoceran species belonging to two different orders. The main attention was paid to the order of various muscle differentiation in two cladoceran species and finding the traces of segmentation. We also discuss possibility of using data on the muscular system development for the reconstructions of phylogenetic relationships between the cladoceran orders.

Material and methods

MATERIAL. We studied embryos of *Daphnia* (*Ctenodaphnia*) *magna* Straus, 1820 (Cladocera: Anomopoda) extracted from brood pouches of parthenogenetic females in laboratory culture and embryos of *Sida crystallina* (O.F. Müller, 1776) (Cladocera: Ctenopoda) from females caught in a natural water body as the material for our study. Two approaches to periodization of the cladoceran embryogenesis were used in the frame of our study. Developmental stages of *D. magna* were identified according to Mittmann *et al.* (2014). Development of *S. crystallina* was described according to Kotov and Boikova (1998).

The laboratory culture of *D. (C.) magna* (collection number Ru-MZ-ad 30.04.17) is a male-free clone obtained from a female caught in Pelicaniy Pond, located on the territory of the Moscow Zoo on April 30, 2017. The culture was maintained at Koltsov Institute of Developmental Biology (Russian Academy of Sciences) under constant light and temperature of 18 °C. The density of individuals was maintained at a level of 2 to 20 parthenogenetic females per 100 ml of medium. Artificial *Daphnia* culturing medium (ADaM) (Klüttgen *et al.*, 1994) was used for female cultivation. The total volume of the medium was 6 liters. During the cultivation of *D. magna*, the medium was changed every two months. Feeding of the culture was carried out three times a week by adding a single drop of the culture of algae *Tetrademus obliquus*

(Turpin) M.J. Wynne, 2015 (Goulden *et al.*, 1982; Sterner *et al.*, 1993; Tambovtseva *et al.*, 2020).

Parthenogenetic females of *S. crystallina* were sampled in the coastal zone of floating leaves, formed mainly by *Nuphar lutea* (L.) Smith, at the Hydrobiological station “Glubokoe Lake” of A.N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences (Ruza District, Moscow Area) via a plankton net with a diameter of 20 cm and a mesh size not exceeding 50 µm.

Parthenogenetic females of *D. magna* and *S. crystallina* with eggs and developing embryos were individually dropped by pipette into Petri dishes. Then, under a stereomicroscope Leica S9D (Leica Microsystems, Germany), eggs and embryos were extracted from the brood chambers of females using tungsten dissecting needles sharpened by the electro-galvanic method (Frey, 1986), trying, if possible, not to damage the adults. The extracted embryos were individually transferred by pipettes into Petri dishes with culture medium (in the case of *D. magna*) or lake water (in the case of *S. crystallina*) to keep them alive until fixation.

PREPARATION OF SPECIMENS FOR CONFOCAL LASER SCANNING MICROSCOPY. We used modified protocols for fixation and fluorescent dyes labeling of the bivalve larvae (Dyachuk, Odintsova, 2009), annelid embryos (Kerbl *et al.*, 2016) and adult *D. magna* and *S. crystallina* (Christensen *et al.*, 2018), but we used MethylGreen as a DNA marker (Prieto *et al.*, 2015) instead of DAPI or Hoechst dyes. Additional steps for adaptation of these techniques for cladocerans are described below in the special subsection.

Selected *D. magna* and *S. crystallina* embryos were transferred from Petri dishes into 1.5 ml Eppendorf tubes with culture medium and lake water, correspondingly. Then liquid was carefully withdrawn with a pipette and replaced with 4% paraformaldehyde (PFA) prepared in 0.01 M sodium phosphate buffer (PBS) at pH 7.4. In this fixative, embryos were placed in a refrigerator at 4 °C for 1–2 days. The fixed material was washed in 0.1% Triton X-100 solution in the same buffer (further TX-PBS) three times for 5 minutes, and three times for 15 minutes. Then washed samples were placed in a refrigerator at 4 °C overnight. Muscle (fibrillar actin) labeling was performed with labeled CF@543 Phalloidin (Biotium, USA). Samples were kept in the dye overnight. Methyl green dye MethylGreenHY-D0163 (MedChemExpress, USA) was used for labeling nuclei (DNA). All dyes were used at a dilution of 1:400 in 0.1% TX-PBS. The samples were then washed 3 times in 0.01M PBS and gradually transferred into 10%, 30% and 60% fructose solution in PBS. Then, under a stereomicroscope, the specimens were carefully transferred to slides and covered by coverslips for examination under a confocal laser scanning microscope.

INVESTIGATION OF SPECIMENS AND DATA PROCESSING. Material viewing and imaging were

performed using a laser confocal scanning microscope LSM Leica SP5 (Leica Microsystems, Germany) at the Core Centrum of IDB RAS. The series of optical sections were taken with 20× objective and digital zoom was used for selected region if necessary. Lasers corresponding to the excitation spectra of the dyes were used: 543 nm for phalloidin and 633 nm for methyl green. For each sample, laser power and detector gain were selected according to general guidelines (Hibbs, 2004) to ensure the maximal visibility of elements of interest. For each sample serial optical sections with 3 µm intervals between sections and 1024×1024 pixels/inch resolution were obtained to visualize the whole depth of sample with maximal resolution. Maximal intensity projection using several selected optical sections was made using FIJI free software, only brightness and contrast of images were corrected. The images were combined in plates using Adobe Photoshop 2022 (Adobe, USA).

GENERAL REMARKS TO ADAPTATION OF STAINING PROTOCOLS FOR LABELING OF FIBRILAR ACTIN AND DNA IN THE CLADOCERAN EMBRYOS OF DIFFERENT AGES. Since studies with staining of Cladocera embryos muscles via labeled phalloidin have not been performed before, we modified the fixation and staining protocols used for other invertebrates, such as bivalve larvae and annelids, for this work. As a starting point, we used the fixation protocol developed for *Bythotrephes longimanus* Leydig, 1860 (Onychopoda) embryos with short fixation in cold PFA (4 °C, 15–30 min) (Alwes, Scholtz, 2014).

In the early version of our protocol, labeling of muscles and nuclei was performed with both aforementioned fluorescent labels at a dilution of 1:800 in 0.1% TX-PBS. Confocal microscope examination of the samples revealed that the signal from both nuclei and muscle bundles was weak or almost absent. To understand the reason for such signal lack, we performed several experiments: we changed fixation protocols and varied the dilution of fluorescent labels. Some examples of the obtained images are shown in Fig. 1.

At the same fixation time (15–30 minutes) we used both fluorescent labels at a higher dilution (i.e. 1:400 and 1:200). In both cases, the embryos showed a signal from the nuclei on the confocal microscope, but cell and muscle boundaries were not visible (Fig. 1). Early embryos under this fixation regime had a cloudy white color, indicating underfixation (Fig. 1D).

Then we decided to increase the exposure time in the cold PFA (4 °C) to a full day (24 h). Fluorescent labels were taken at a dilution of 1:200. With this protocol, late embryos of *D. magna* showed good fixation quality, their muscles and nuclei were clearly visible, but the earlier embryos were still under-fixed, with no cell boundaries visible, only some of them had visible muscle bands. Nuclei were stained only in a portion of the early embryo bodies (Fig. 1A).

Presumably, the cold PFA penetrates through the egg membranes of early embryos slowly, resulting in their under-fixation. Embryos of Anomopoda and Ctenopoda sequentially shed off two egg membranes and two embryonic membranes during their development, with time somewhat different in these two orders (Kotov, Boikova, 1998; Kotov, 2013). All membranes may be considered as barriers for penetration of the fixative. Thus, a short fixation with heated PFA for 30 minutes was used. The embryos fixed in 45 °C PFA acquired yellowish color. On the confocal microscope, no signal from muscles was observed at any stage, probably due actin destruction. The signal from the nuclei was weak. After fixation in 30 °C PFA, nuclei and muscles were visible only in a fraction of embryos at late stages (Fig. 1C).

To minimize the temperature effect on embryos, PFA at room temperature (20–25 °C) was used, and fixation was carried out during two hours (Fig. 1B) or for a full day. The clearest signal from actin filaments within the muscle and cell borders was observed when fixation was carried out overnight. This fixation mode proved to be optimal and allowed us to obtain data on the beginning of muscle band formation in early embryos. Fluorescent labels were used at dilutions of 1:400 and 1:200. In the latter case, the signal from already formed muscles was too strong as compared to the forming muscles. A dilution of 1:400 was found to be an optimal solution.

In the first versions of the protocol, glycerol was used to clear samples already exposed in fluorescent labels. However, it turned out that most late embryos were dehydrated when transferred from 30% glycerol to 60% glycerol, and virtually all early embryos did so in 30% glycerol, and they could not be used for confocal microscope analysis thereafter. Glycerol was used in different versions of the fixation experiments, but even with a reasonably good degree of fixation, dehydration of most samples occurred. To avoid this, we used a solution of fructose in PBS instead of glycerol in the remaining experiments. Gradual treatment of samples via fructose solution ensures good tissue clarification necessary to obtain fluorescent confocal images.

For reasons that are not entirely understandable, the specimens that were kept in Petri dishes for longer than 10–15 minutes already showed a lower quality of fixation. Some of these specimens changed shape and became whitish in color; when the material was viewed with a confocal microscope, the signal from muscles and nuclei was very weak or almost absent (Fig. 1D).

Actin filaments turned out to be the most sensitive to the quality of fixation. If the fixation time is insufficient, they are destroyed and give a weak signal after reaction with phalloidin.

Thus, we have tested the following fixation protocols:

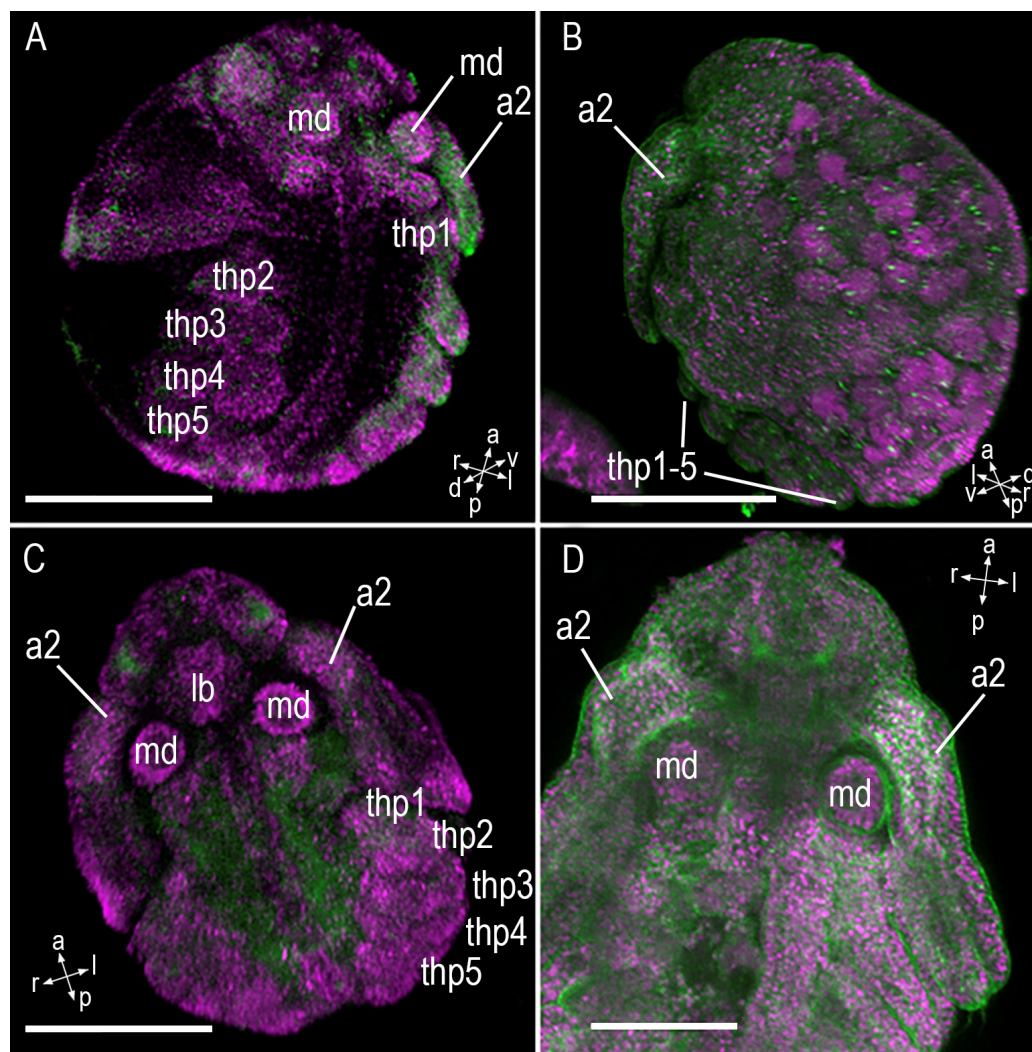


Fig. 1. Embryos of *Daphnia magna* at stage 7.5 according to Mittmann *et al.* (2014) obtained during various fixation and staining protocols: A — an underfixed specimen, PFA 4 °C, fixation for one day, fluorescent labels at dilution 1:200; B — underfixed specimen, PFA 20–25 °C, fixation for two hours, fluorescent labels at dilution 1:400; C — a specimen fixed in PFA 30 °C, fixation for 30 minutes, fluorescent labels at dilution 1:400; D — a specimen with long time interval between removal of an embryo from the brood pouch and fixation, PFA 20–25 °C, fixation for a day, fluorescent labels at dilution 1:400.

Fluorescent labeling of actin (green) and DNA (magenta). A–C — maximal intensity projection of whole embryo; D — maximal intensity projection of 5 selected optical sections.

Abbreviations: a2 — antenna II; lb — labrum; md — mandible; thp1–5 — thoracic limbs (or thoracopods) 1–5. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μ m.

1. Cold PFA (4 °C), fixation during 1 day;
2. Room temperature PFA (20–25 °C), fixation during 2 hours;
3. Room temperature PFA (20–25 °C), fixation during 1 day;
4. Hot PFA (30 °C), fixation during 30 minutes;
5. Hot PFA (45 °C), fixation during 30 minutes.

We also varied the dilution of fluorescent labels in 0.1% PBS: 1:800, 1:400 and 1:200.

For the specimens' clearing we tried two types of solutions: glycerol and fructose. Both solutions were graded at concentrations of 30, 60 and 90% diluted by 0.1% PBS.

In addition, the time between the extraction of embryos from the brood pouch of the adult female and their fixation varied. Initially, the time interval was 30–60 minutes, later it was decided to reduce it to 10–15 minutes.

The final version of specimen preparation for confocal microscopy is presented above in the corresponding subsection of “Material and Methods”.

The main methodological difficulty concerning the cladoceran embryos is the following: at early stages of muscle band formation it is difficult to distinguish absence of a local phalloidin binding to fibrillar actin, and absence of muscle fibers at such stage at all. Therefore, in our case, we evaluate the quality of labeling by the presence of signal from cell borders containing bands of actin filaments. They bind to phalloidin in the same way as actin in muscles. In the frame of our work, we changed fixation protocols and varied the dilution of fluorescent labels in order to obtain stable reproducible results. This approach was tested on *D. magna* embryos and further used for *S. crystallina* embryos presumably covered by thinner membranes as compared to *D. magna* (Kotov, 2013), which greatly facilitates the process of fixation and labeling. For *S. crystallina* embryos, fixation can be carried out for 1–2 hours and fluorescent labels can be used at lower dilution (1:400 or 1:600). For other groups of the Cladocera these parameters may also vary due to individual characteristics of their integuments.

Results

PERIODIZATION OF MUSCULATURE DEVELOPMENT ACCORDING TO THE KEY EVENTS IN EMBRYOGENESIS OF *DAPHNIA MAGNA* (Figs 2–10, 12B). In *D. magna*, the first muscle elements appear at the late phase of stage 7 according to Mittmann *et al.* (2014) (Fig. 2). At this stage, the buds of antennae I and antennae II with exo- and endopods are already formed; mandibles are represented by rounded rudiments; the maxillae I and II begin to form; and the labrum is already well-formed. At stage 7, thoracic (postnaupliar) segments develop and, at the same time, furrows marking thoracopod buds appear on their posterior sides. By the end of this stage, the valves cover approximately half of the dorsal side of the embryo.

At stage 7.3 (Fig. 2), thoracic segments 1–3 are formed and the segment 4 begins to form. Slightly thickened cell borders are visible in the center of the basipodite. The forming segments (Fig. 2D) and developing thoracopods (Fig. 2A–C) show a bright signal at the cell boundaries.

However, this signal is not associated with the beginning of muscle bands formation.

Later, at stages 7.4 and 7.5, a strong signal can be noted from the distal part of the exo- and endopod branches of antennae II and from the distal part of antennae I, which is associated with the formation of setal buds (Fig. 3). Also, elongated cell bands with large amounts of fibrillar actin at the base of antennae II become to be visible. The exopod and endopod of antenna II increase gradually in size. In the head region, the first signs of musculature near oesophagus and mouth can be seen as thin bands. An accumulation of actin also is noticeable in the mandibles, which at these stages are represented by rounded bodies. Maxillae II appear at stage 7.4 as an elongated hillock (Fig. 3A). At stage 7.5 they look like small tubercles located posteriorly to maxillae I (Fig. 3F).

At stage 7.4, the buds of maxillae II are formed, antennae II are elongated, and the carapace is formed (Fig. 3A). Furrows separating future limb exopodites and endopodites are formed on the thoracic segments. Rows of elongated cells with large amounts of fibrillar actin become visible in the basipodites of antennae II (Fig. 3B). Cells with a thickened actin layer also appear at the base of the exopodite and endopodite; at the distal end, actin marks the points of setae formation (Fig. 3C). In the head portion, disordered bands of actin and oesophageal extensor muscles form at the base of antenna II (Fig. 3B). Mandibles demonstrate an enlarged actin layer in the cells occupying their central positions (Fig. 3C). A muscular ring around the anus (Fig. 3D) is not so pronounced as that around the mouth and the oesophagus (Fig. 3B).

At stage 7.5, the segment 5 is formed (Fig. 3E), maxillae II is represented by a rounded formation located posteriorly to maxillae I (Fig. 3F). In the exo- and endopod of antenna II, cell bands with a thickened actin layer become more clearly visible (Fig. 3F).

At stage 8, antennae I shift medially and partially overlap the labrum (Fig. 4A, F). Antennae II elongate; their distal ends reach thoracic segment 1 by the end of stage 8 (Fig. 4A, I). Maxillae I are displaced laterally (Fig. 4F). Thoracopods are represented by plates of approximately rectangular shape (Fig. 4G–H). Thoracopods 1–4 have deepening furrows separating their basal segment, exopodite and endopodite (Fig. 4G). Cells with a thickened layer of fibrillar actin

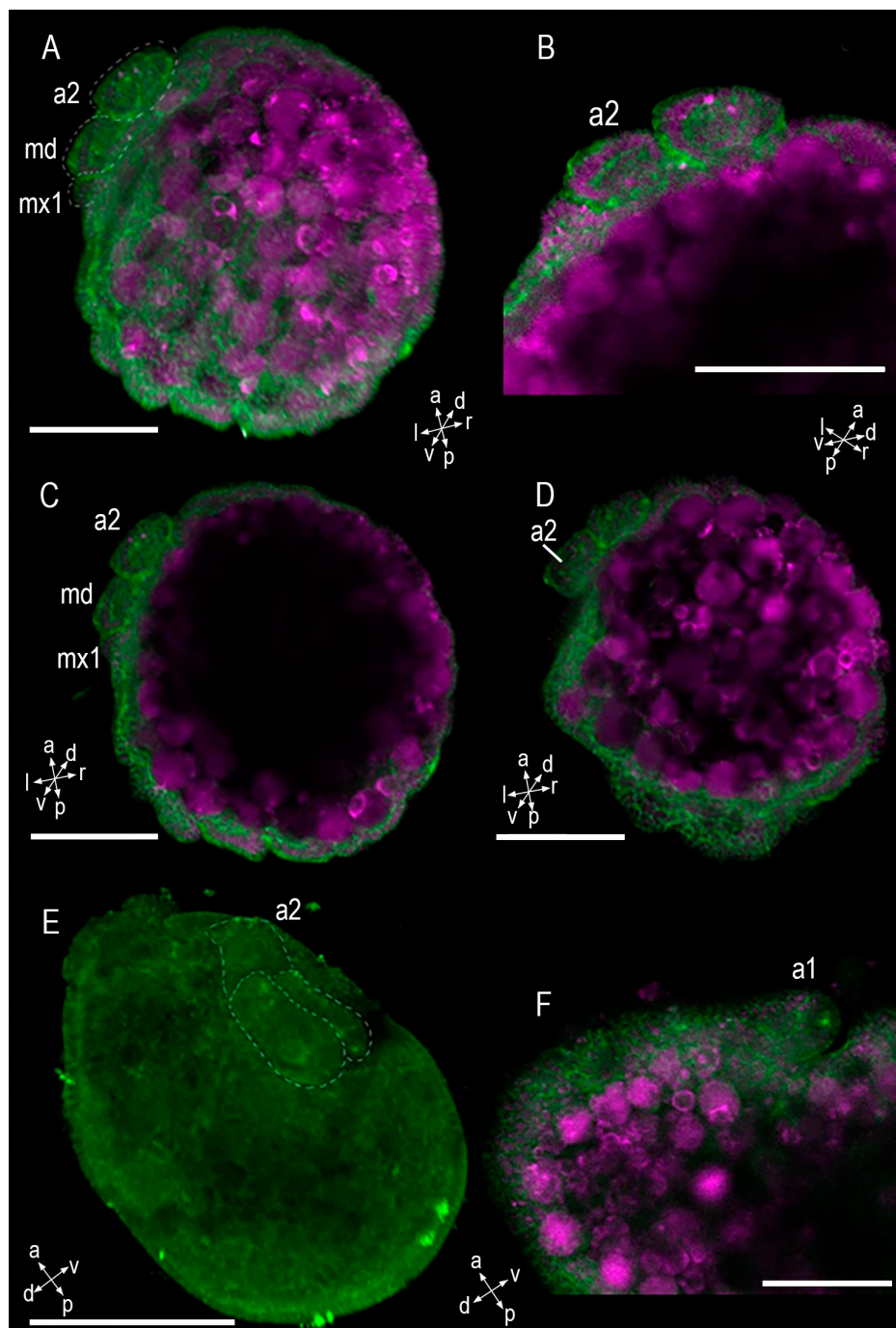


Fig. 2. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 7.3 according to Mittmann *et al.* (2014). A, E — maximal intensity projection of whole embryo; B, C, D, F — maximal intensity projection of 5 selected optical sections. Dotted lines show antenna II. Fluorescent labeling of actin (green) and DNA (magenta). Abbreviations: a2 — antenna II; md — mandible; mx1 — maxilla I. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μm.

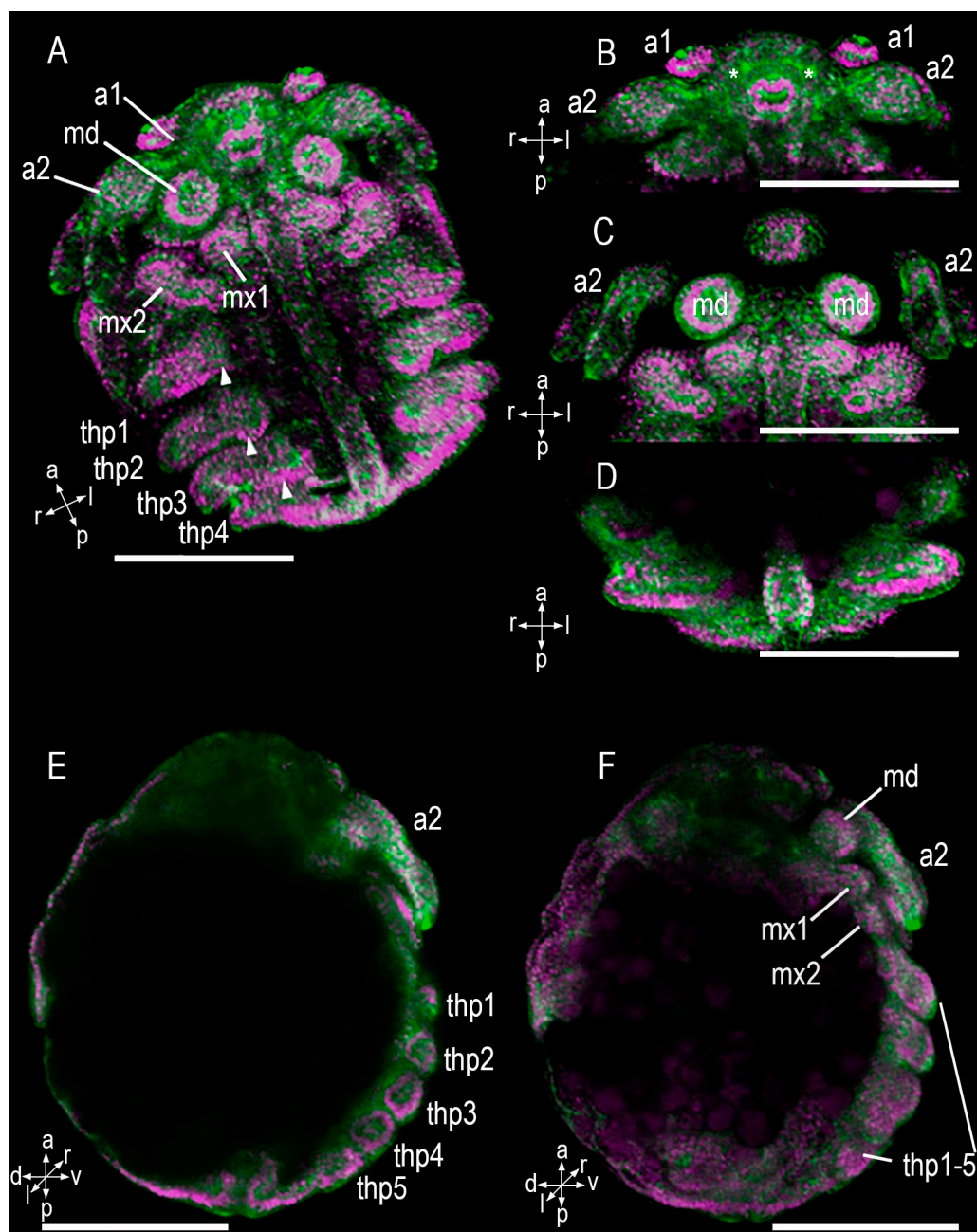


Fig. 3. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 7.4 (A–D) and stage 7.5 (E–F) according to Mittmann *et al.* (2014). A — maximal intensity projection of whole embryo; B–F — maximal intensity projection of selected optical sections in anterior, middle and caudal parts, respectively. White arrows mark the rudiments of the thoracopods. Asterisks (**) mark oesophageal extensor muscles. Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a1 — antenna I; a2 — antenna II; md — mandible; mx1 — maxilla I; mx2 — maxilla II; thp1–5 — thoracic limbs (or thoracopods) 1–5. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μm .

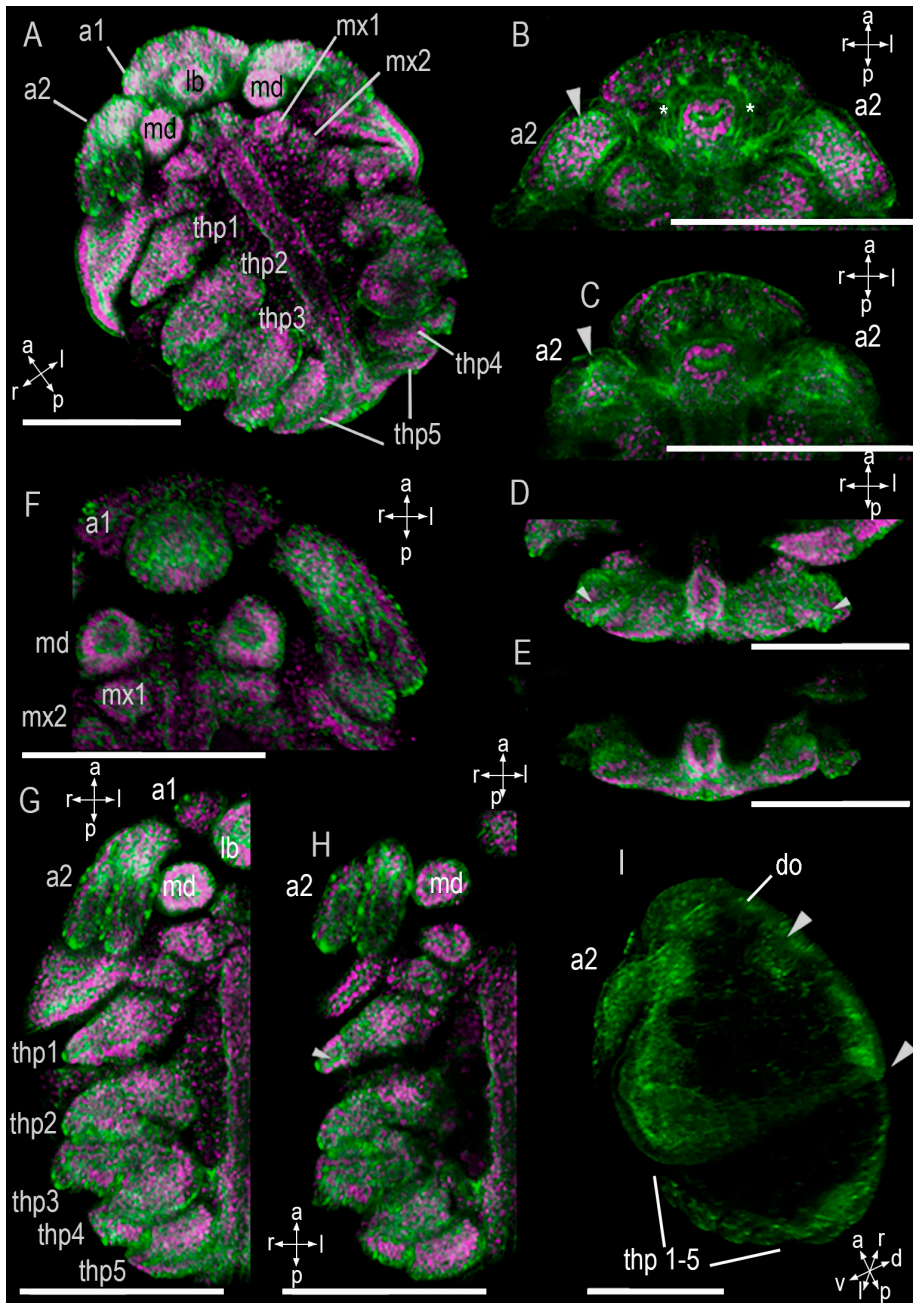


Fig. 4. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 8 according to Mittmann *et al.* (2014). A, I — maximal intensity projection of whole embryo; B–E — maximal intensity projection of selected optical sections, taken from anterior (B, C, F) and caudal (D, E) regions. G, H — maximal intensity projection of selected optical sections taken on various optical depth. White arrows mark absence of formed muscle bands at this stage. Asterisks (**) mark oesophageal extensor muscles.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a1 — antenna I; a2 — antenna II; do — dorsal organ; lb — labrum; md — mandible; mx1 — maxilla I; mx2 — maxilla II; thp1–5 — thoracic limbs (or thoracopods) 1–5. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μm.

appear in exopodites of thoracopods 1–4, which, however, at this stage do not have any traces of formed muscle bands (Fig. 4D, H, white arrows). The valves cover about half of the thorax (Fig. 4I). The dorsal organ also appears (Fig. 4I). A thin layer of muscles is formed around the intestine (Fig. 4B).

A cluster of cells with a large amount of actin is formed in the basal segment of antennae II (Fig. 4B, white arrow). At the base of antennae II, individual muscle fibers begin to form, penetrating deeper into the head, these are rudiments of future retractor and levator muscles (Fig. 4B–C: arrows). Muscle tracts in antennae II become more clearly visible, actin fibers become more ordered in the basal portion of antennae II. Protractor and retractor muscles begin to form within the branches of antennae II (Fig. 4G). At this stage, each of them is represented by a row of cells with a thickened actin layer. In addition, a cluster of cells with an additional volume of actin appears in the mandible. At this stage, the mandible has the rounded shape (Fig. 4G). The muscular lining of the oesophagus and the muscular ring around the mouth become clearly visible (Fig. 4B). At this stage, the muscular lining of the hindgut and the musculature around the anus are formed (Fig. 4D–E). As in the previous stage, small aggregations of actin are present at the sites of bristle formation on the antennae I and II.

The antennae II (Fig. 5) and mandibles (Fig. 5E) begin to elongate at the end of stage 8. The latter reaches the thoracic segment I (Fig. 5F) and shows more clear muscle bands (Fig. 5B–D); thin bands extend from the base of antenna II to the dorsal head part (Fig. 5B). Dorsal and ventral muscle bands probably begin to form (Fig. 5A, white arrows), but they are not well observable due to presence of numerous fat droplets.

At stage 9, the labrum is noticeably elongated (Fig. 6A–D). Within it, labral glands become visible (Fig. 6C, large cells marked with an asterisk), and transverse muscles are formed (Fig. 6C–D), which narrow and widen the groove on the inner side of the labrum and serve to squeeze secretion from the glands. Antennae I converge, their bases fuse to the end of this stage (Fig. 6A). On antenna II, segments of the exo- and endopodite become clearly visible for the first time (Fig. 6A). By the end of this stage, they elongate and their tips reach the thoracopod 2. The eye capsule begins to form (Fig. 6E).

Mandibles take a cylindrical shape (Fig. 7B, C), and musculature providing their movement is formed. Maxillae II become smaller and not clearly visible near mandibles. Segmentation of the thoracopods 1–4 continues, and at stage 9 their lobes enlarge and elongate. The postabdomen enlarges and begins to buckle on the ventral side.

Musculature of antennae II at this stage is fully formed, but individual muscle bands in basal segment and branches are rather thin (Fig. 7A, C). Bands attached to the dorsal surface of the head are thickened as compared to stage 8.

At stage 9, dorsal and ventral bands composed of thin muscle fibers become clearly visible (Fig. 7A–D). The ventral band shows segmentation in the form of thickenings opposite each thoracopod. On the dorsal side, the heart is formed in the region between the head and thoracic portions (Fig. 7C). The valve closing muscles are formed (Fig. 7A), at this stage they are represented only by thin fibers and a group of large cells with a thickened actin layer.

At stage 10, the postabdomen continues to bend ventrally and increase in length (Fig. 8A). Antennae II elongate, and their tips reach the thoracopod 3 by the end of the stage (Fig. 8). Thoracopod exopodites increase in size (Fig. 8C). Maxillae II decrease in size and resemble small nodules under maxillae I. The carapace expands not only posteriorly, but also laterally, and does not yet cover the thoracopods.

At stage 10, the muscle bands in antennae II thicken, and massive attachment of antennae II muscles to the dorsal surface of the head is formed (Fig. 8C–D). In addition to the transverse muscles, a labrum levator muscle appears within the former (Fig. 8B). The mandibular musculature is represented by the posterior and anterior rotating muscles of the mandible, the transverse mandibular musculature. The dorsal and ventral muscle bands thicken, the ventral band enters the postabdomen and attaches to its dorsal surface at three points (Fig. 8B). The musculature of the postabdomen is formed (Fig. 8A). By the end of stage 10, thin ventral bands of thoracopod muscles are formed simultaneously. Dorsoventral muscles also appear (Fig. 8C–D).

At stage 11, the carapace valves almost completely cover the thoracopods (Fig. 9). The labrum continues to elongate and becomes narrower. Aesthetascs appear at the tips of antennae I. Tips of antennae II at this stage reach

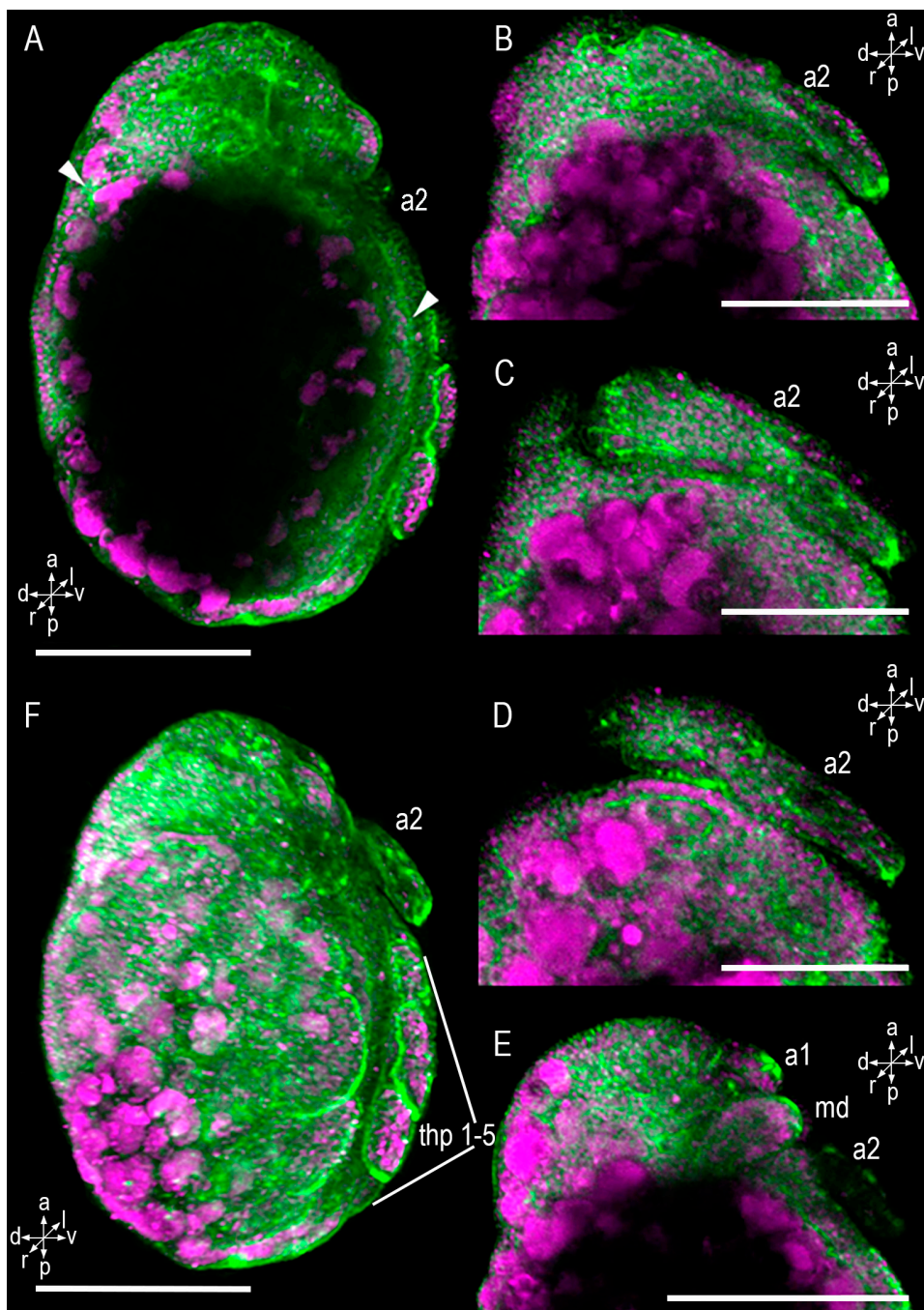


Fig. 5. Localization of actin and nuclei in embryos of *Daphnia magna* at the end of stage 8 according to Mittmann *et al.* (2014). A, F — maximal intensity projection of whole embryo and selected optical sections; B–E — anterior region maximal intensity projection of selected optical sections taken on various optical depth. White arrows mark forming dorsal and ventral muscle bands. Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a1 — antenna I; a2 — antenna II; md — mandible; thp1–5 — thoracic limbs (or thoracopods) 1–5. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μ m.

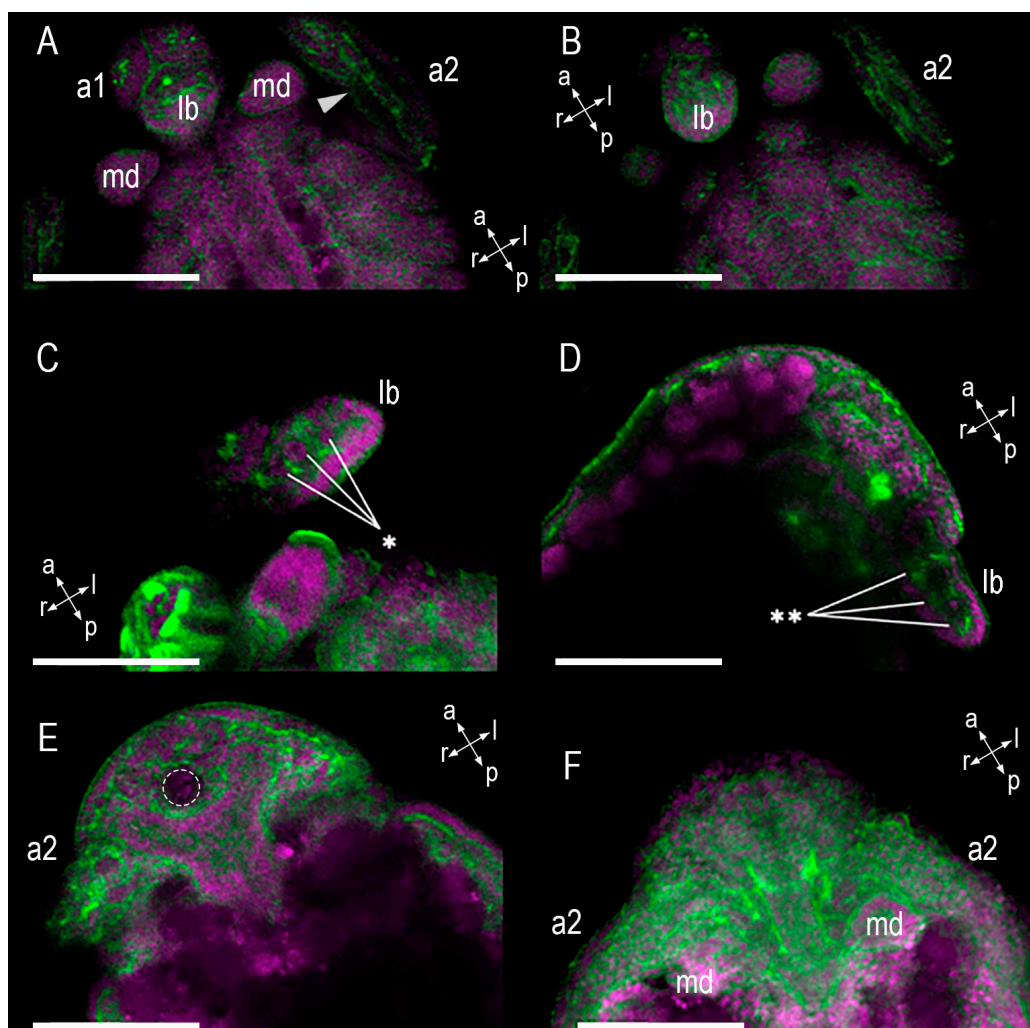


Fig. 6. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 9 according to Mittmann *et al.* (2014). A–F — anterior region, maximal intensity projection of selected optical sections taken on various optical depth. White arrow marks segmentation of antenna II. Dotted line shows eye capsule. An asterisk (*) marks labral gland cells. Two asterisks (**) mark transverse labral muscles.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a1 — antenna I; a2 — antenna II; lb — labrum; md — mandible. Axes directions: a — anterior; p — posterior; r — right; l — left. Scale bars: 100 μm .

the thoracopod 4. Maxillae I increase in size; maxillae II are no longer distinguishable (Fig. 9B). The shape of thoracopods does not change significantly, but muscle fibers in them become enlarged. The postabdomen continues to increase in size. According to Kotov & Boikova (2001), the first heartbeats are registered in cladoceran embryos at this stage. The heart is completely formed and located between the muscles of antennae II, mandibles, and bands of dorsal muscles

attached to the dorsal surface of the body. At this stage, the formation of the most groups of muscles is completed (Fig. 9A–D).

At stage 12, the labrum is still elongating and thinning. The valves completely cover the thoracopods. The musculature assumes definitive state (Fig. 10A–D).

BRIEF COMPARATIVE NOTES ON MUSCULATURE DEVELOPMENT IN *SIDA CRYSTALLINA* (Figs 11, 12A). Below we draw

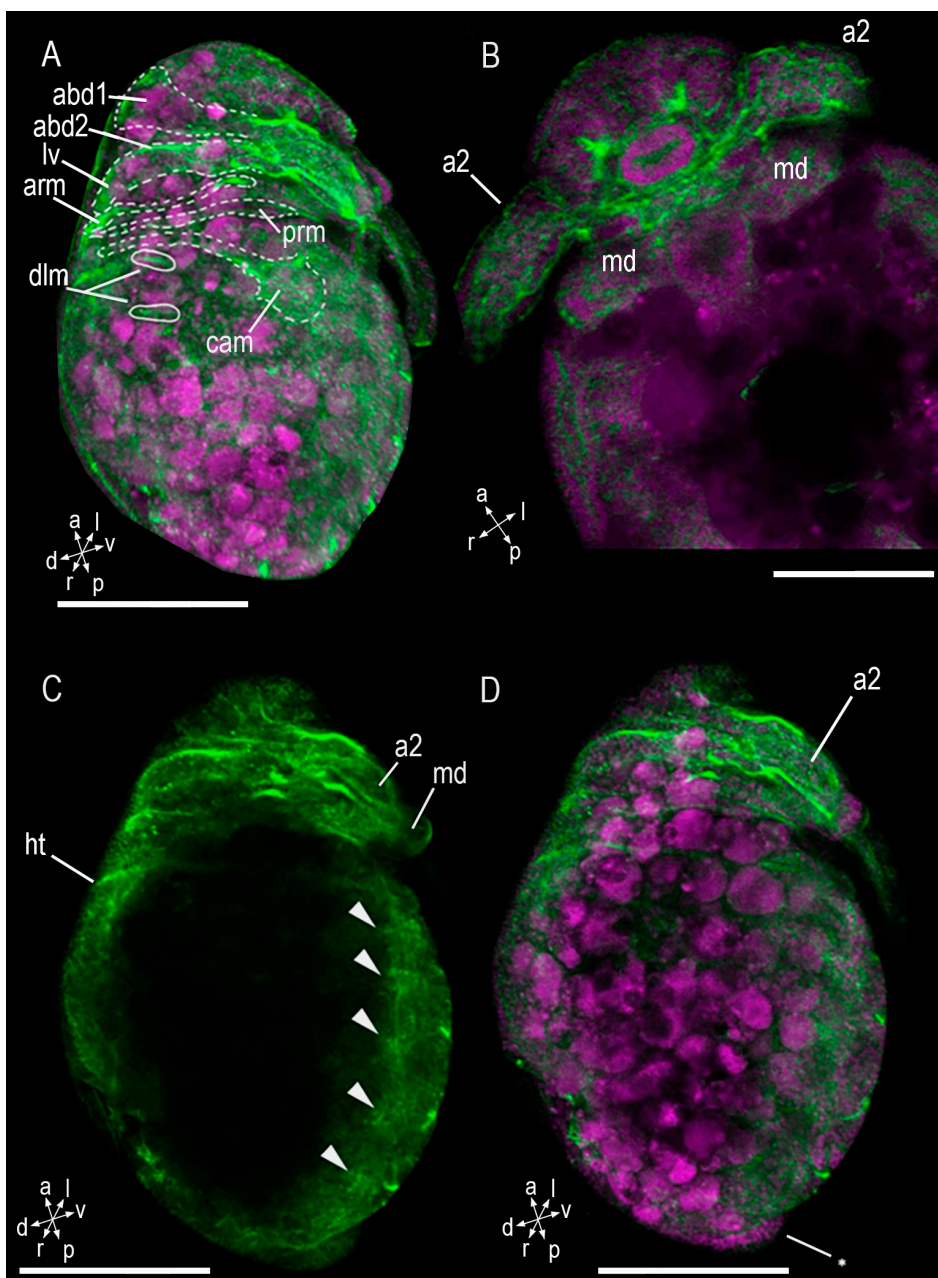


Fig. 7. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 9 according to Mittmann *et al.* (2014). A — maximal intensity projection of whole embryo; B — maximal intensity projection of selected optical sections from the anterior region; C, D — selected optical sections of the same embryo as in A. Dotted lines show boundaries between muscle groups. White arrows mark forming segments. An asterisk marks the forming postabdomen.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a2 — swimming antenna (or antenna II); abd1–abd2 — antennal musculature bands; arm — anterior carapace adductor muscle; cam — carapace muscles; dlm — dorsal longitudinal trunk muscles; ht — heart; lv — levator muscle of labrum; md — mandible; prm — posterior carapace adductor muscle. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μm.

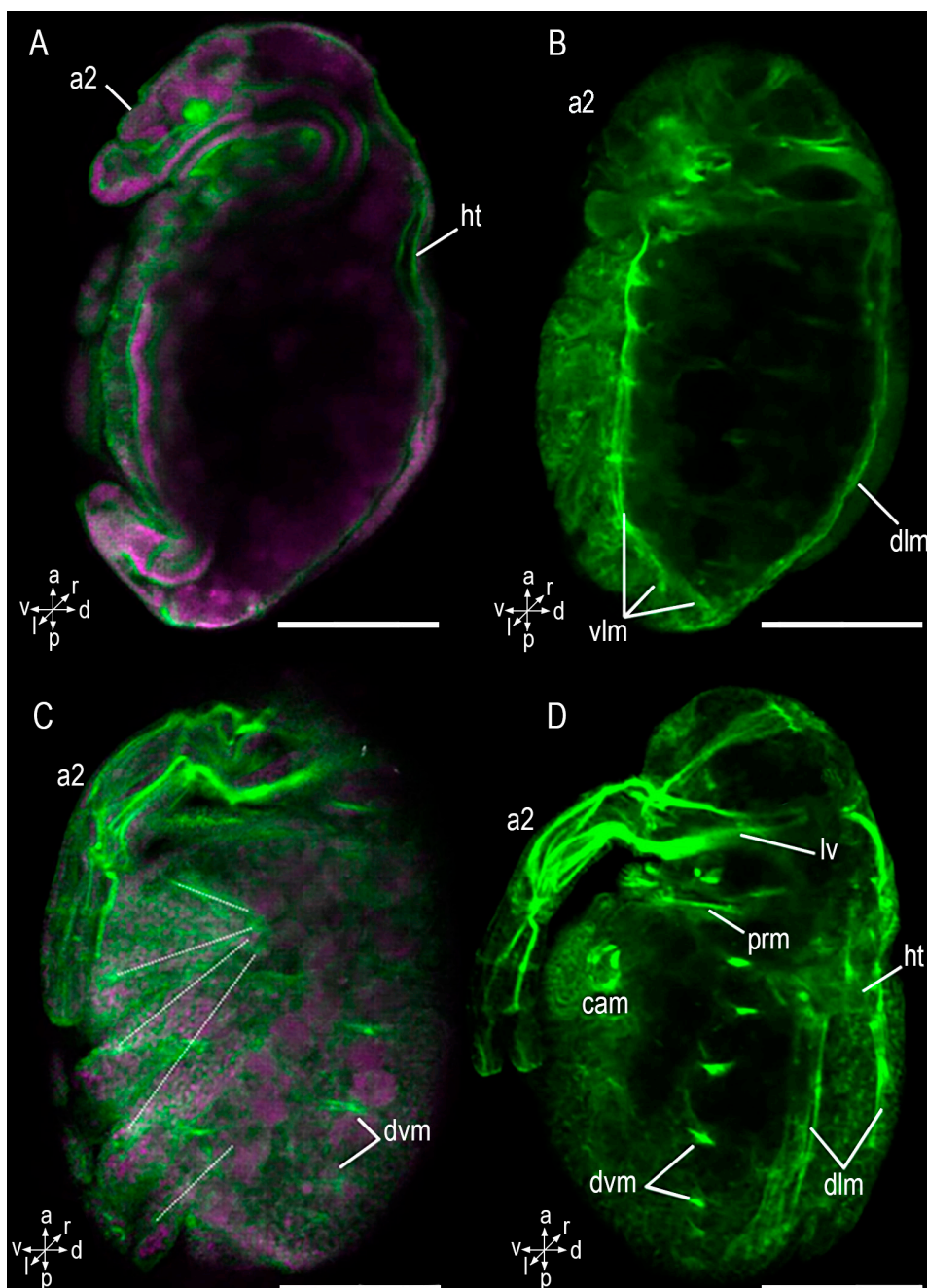


Fig. 8. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 10 according to Mittmann *et al.* (2014). A–D — maximal intensity projection of selected optical sections taken on various optical depth. Dotted lines show thoracic limbs musculature.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a2 — swimming antenna (or antenna II); cam — carapace muscles; dlm — dorsal longitudinal trunk muscles; dvm — dorsoventral muscles; ht — heart; lv — levator muscle of labrum; prm — posterior carapace adductor muscle; vlm — ventral longitudinal trunk muscles. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μ m.

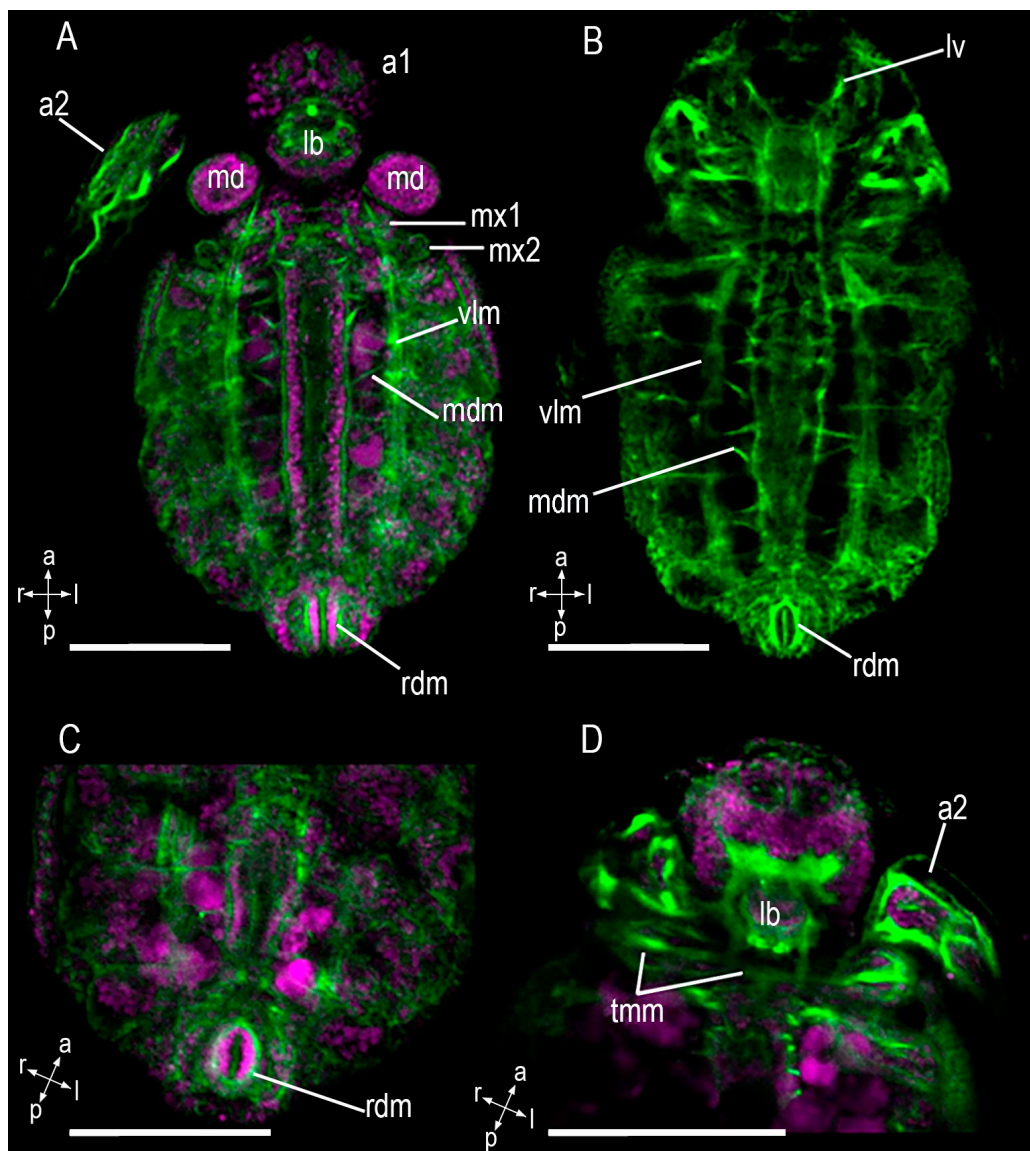


Fig. 9. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 11 according to Mittmann *et al.* (2014). A, B — maximal intensity projection of selected optical sections taken on various optical depth. C, D — maximal intensity projection of selected optical sections of caudal (C) and anterior (D) regions.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a1 — antennule (or antenna I); a2 — swimming antenna (or antenna II); lb — labrum; lv — levator muscle of labrum; md — mandible; mx1 — maxilla I; mx2 — maxilla II; mdm — midgut dilator muscles; rdm — rectal dilator muscle; tmm — transverse mandibular muscle; vlm — ventral longitudinal trunk muscles. Axes directions: a — anterior; p — posterior; r — right; l — left. Scale bars: 100 μ m.

a general pattern of the muscle system formation in *Sida* as compared to *Daphnia*. The first clearly visible muscle bundles in *Daphnia* are formed at late stage 7 in antennae II, around the mouth, oesophagus and anus (Fig. 3A–D). At

this stage, the muscles of antennae II are visible as separate rows of cells with a thickened actin layer, not organized into bands (Fig. 3C). Later on, the development of the muscles of antennae II and the lining of the digestive system portions

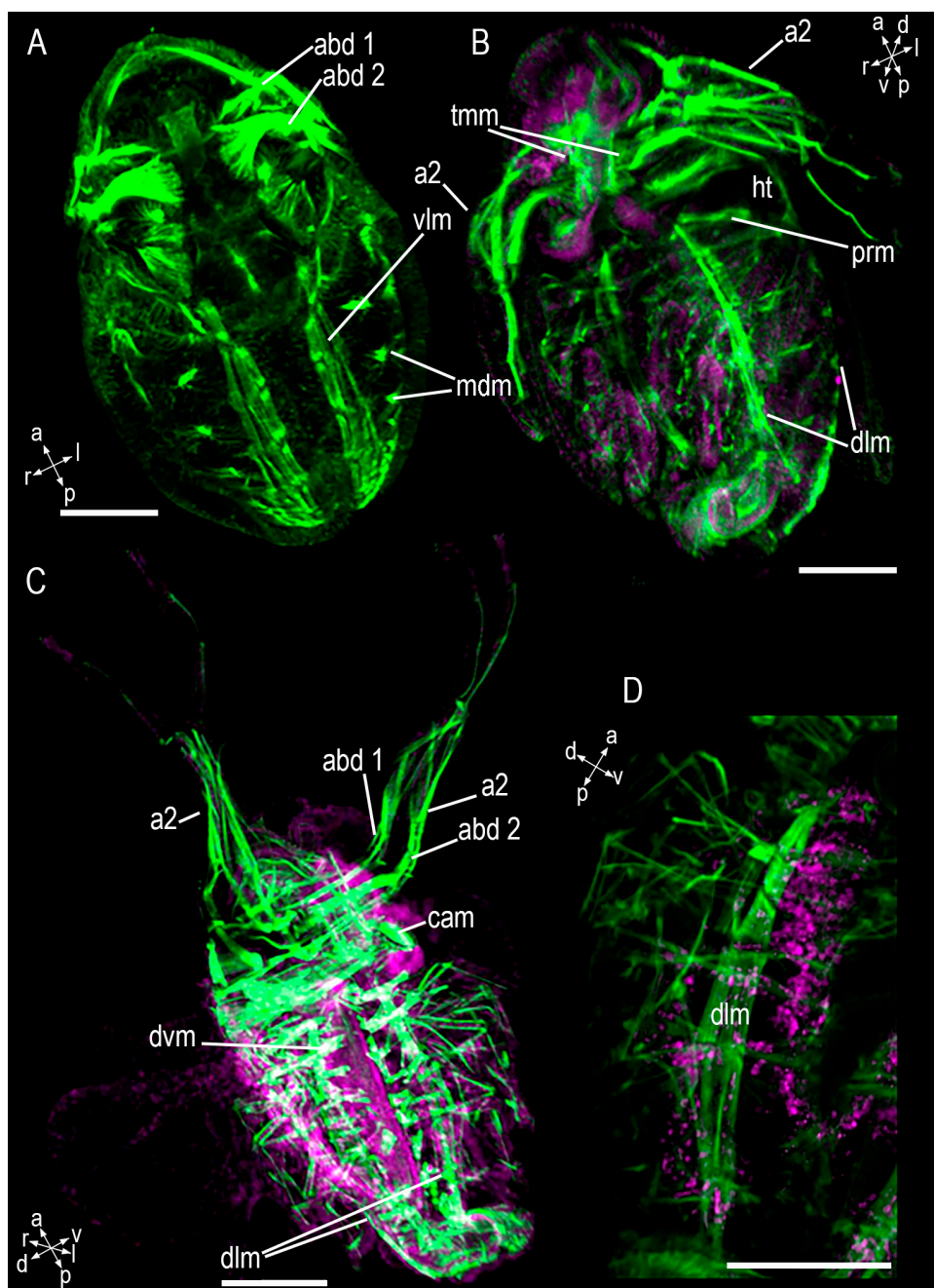


Fig. 10. Localization of actin and nuclei in embryos of *Daphnia magna* at stage 12 according to Mittmann *et al.* (2014). A, B — maximal intensity projection of selected optical sections taken on various optical depth. C — maximal intensity projection of whole embryo. D — close view of mid body region, single optical section. Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a2 — swimming antenna (or antenna II); abd1–abd2 — antennal musculature bands; cam — carapace muscles; dlm — dorsal longitudinal trunk muscles; dvm — dorsoventral muscles; ht — heart; mdm — midgut dilator muscles; prm — posterior carapace adductor muscle; tmm — transverse mandibular muscle; vlm — ventral longitudinal trunk muscles. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μm.

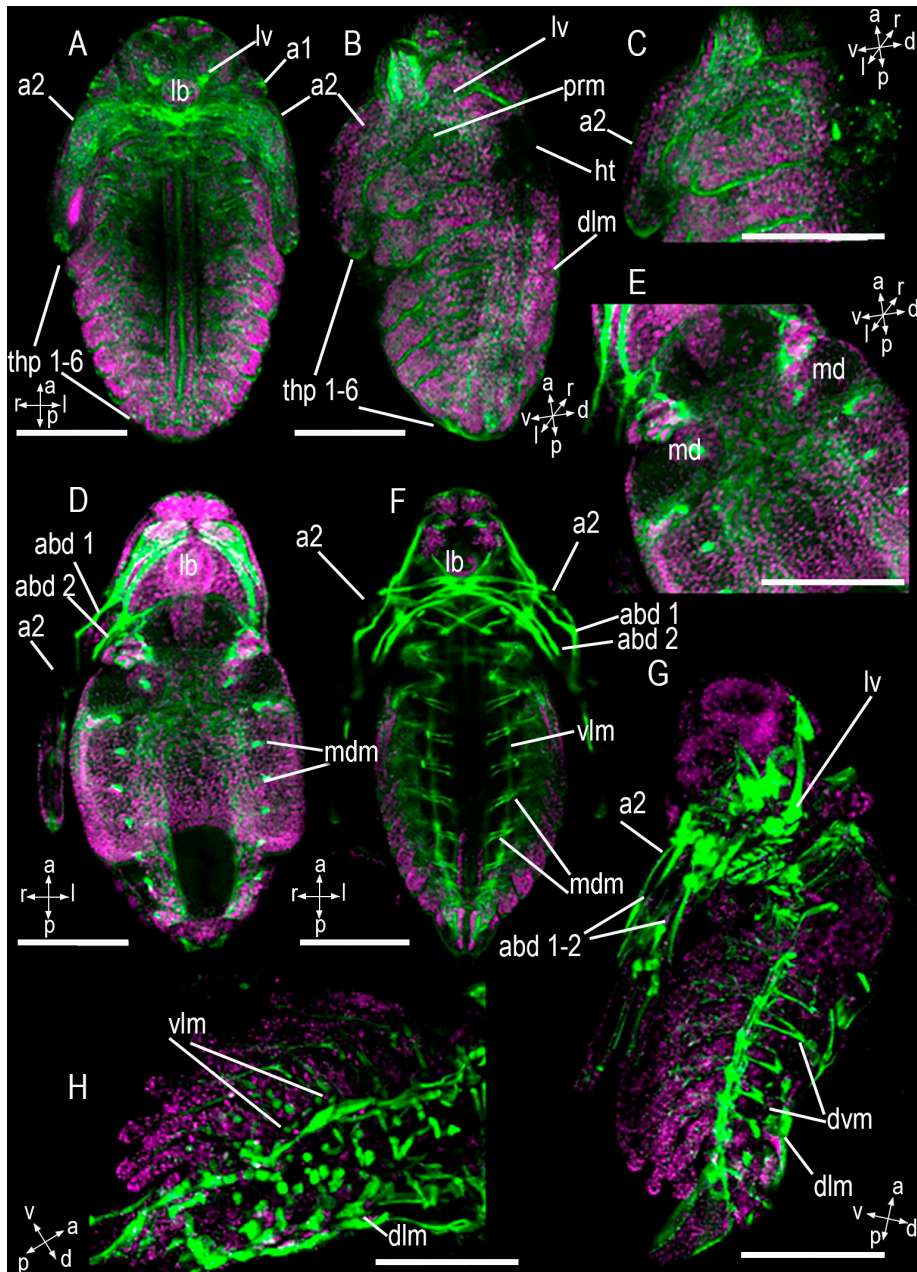


Fig. 11. Localization of actin and nuclei in embryos of *Sida crystallina* at different developmental stages. A–C — stage 1; D–F — stage 3; G, H — stage 4 according to Kotov and Boikova (2001). A, B, D, F, G — maximal intensity projection of selected optical sections. C, E, H — close view of anterior, mid and caudal body regions.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a1 — antennule (or antenna I); a2 — swimming antenna (or antenna II); abd1–abd2 — antennal musculature bands; lb — labrum; lv — levator muscle of labrum; md — mandible; dlm — dorsal longitudinal trunk muscles; dvm — dorsoventral muscles; ht — heart; mdm — midgut dilator muscles; prm — posterior carapace adductor muscle; thp1–6 — thoracic limbs (or thoracopods) 1–6; tmm — transverse mandibular muscle; vlm — ventral longitudinal trunk muscles. Axes directions: a — anterior; p — posterior; d — dorsal; v — ventral; r — right; l — left. Scale bars: 100 μm.

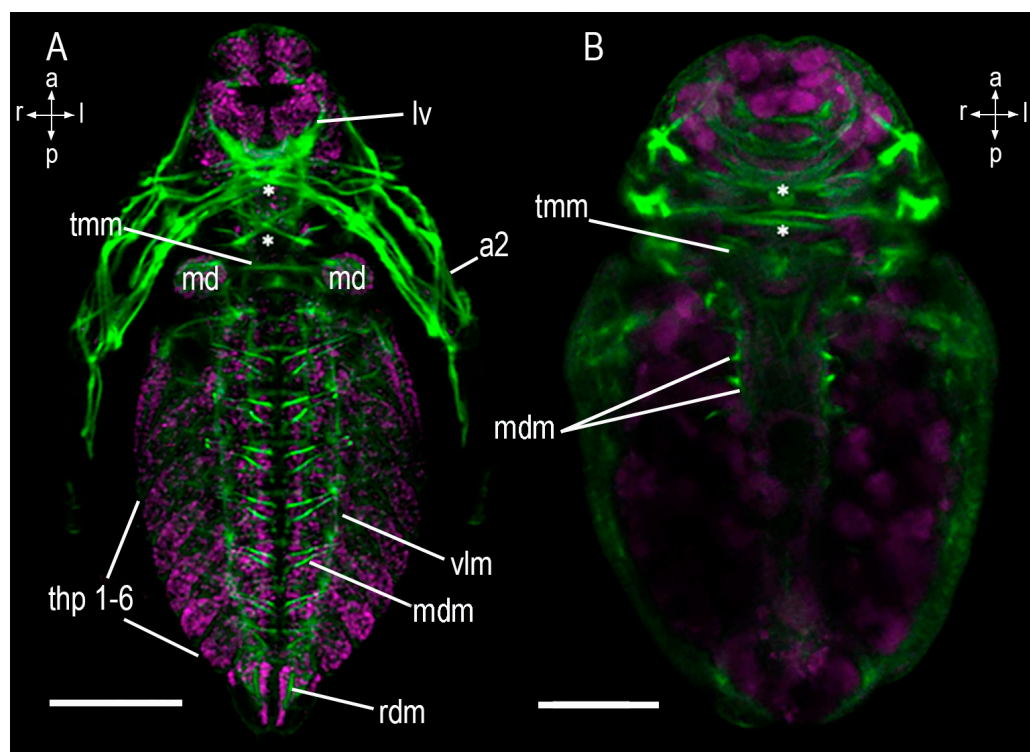


Fig. 12. Arrangement of muscles in swimming antennae. A — *Sida crystallina*, stage 4 according to Kotov and Boikova (2001); B — *Daphnia magna*, stage 12 according to Mittmann *et al.* (2014). A–B — maximal intensity projection of selected optical sections taken on approximately similar optical depth in two species. Asterisks mark the cross-overlaps of the muscles of antennae II.

Fluorescent labeling of actin (green) and DNA (magenta).

Abbreviations: a2 — swimming antenna (or antenna II); lv — levator muscle of labrum; md — mandible; mdm — midgut dilator muscles; thp1–6 — thoracic limbs (or thoracopods) 1–6; tmm — transverse mandibular muscle; vlm — ventral longitudinal trunk muscles. Axes directions: a — anterior; p — posterior; r — right; l — left. Scale bars: 100 μ m.

proceeds faster than in the rest of the embryo. Mandibular musculature is also formed rather quickly; at the initial stages, the forming muscle bands on transverse optical sections represent groups of 2–3 large cells with a thickened layer of fibrillar actin (Fig. 3C).

In *Daphnia*, at stages 8–9 (Figs 4–7) the muscles in the proximal segments and the bases of antennae II elongate and attach to the inner surface of the head capsule. Thin muscle bands responsible for rotation of mandibles are formed. At stage 8, the paired ventral and dorsal longitudinal muscles begin to form as separate thin bands, thickening in subsequent stages. At stage 9, longitudinal muscles compressing the labrum are formed, muscles of antennae II and mandible thicken, and valves closing muscles are formed.

The formation of musculature providing mobility of thoracopods and abdominal-postabdominal muscles in *Daphnia* occurs relatively late: at the end of stage 9 (Fig. 7). Ventral muscle groups are formed sequentially, beginning with the thoracopod 1. The ventral muscles are attached to the dorsoventral muscles, which provide connection with the longitudinal muscles and coordinate the movements of the thoracopods. At stage 10, the levator labrum muscle also forms (Fig. 8D). To the stages 11 and 12 (Figs. 9–10), the formation of the thoracopod muscles is completed, and the muscles of antennae II are elongated. The heart can be seen only from stage 10 (Fig. 8D).

In the course of our work, we failed to identify the muscles providing eye mobility, although they should be present in adults (Kotov, Boikova, 2001).

In general, in *Sida crystallina*, the muscles of antennae II, oesophagus and hindgut are formed first in development in the same way as in *D. magna* (Figs 11, 12A–B). The muscles of antennae II of *S. crystallina* are larger relatively to its body as compared to *Daphnia*. Already at the initial stages of muscle formation, it is possible to distinguish separate muscle bands in the rows of cells (Fig. 11A–C), whereas in *D. magna* they are indistinguishable at corresponding developmental stage. The muscles of antennae II in *S. crystallina* are more sparsely arranged relative to each other than in *D. magna* due to the differences in head shape (Fig. 12A–B). It can also be noted that the metameric nature of ventral and dorsal muscle bands (Fig. 11F–G) is more prominent in *S. crystallina* than in *D. magna* which has much more differentiated muscles (Fig. 10C–D). Ventral muscles of thoracopods and dorsoventral muscles in the development of *S. crystallina* are formed at late embryonic stages, similarly to that in *D. magna*. However, the muscle organization pattern is formed as similar in different thoracopods of *S. crystallina* (Fig. 11H), while *D. magna* has different patterns of muscle organization in different thoracopods (Fig. 10C).

Large cells with a thickened actin layer and large nuclei are visible in the heart of *S. crystallina* (Fig. 11D–E).

Discussion

In contrast to large branchiopods, in cladocerans the thoracic and abdominal portions occupy a larger volume of the body relatively to the head bearing antennae II and a system of oral appendages. It is probably related to the absence of a free-swimming nauplius in the cladoceran life cycle. Their naupliar appendages (antennae I and II and mandibles) remain unused until the “embryonised larva” (“metanauplius”) stage (Korovchinsky *et al.*, 2021). However, despite the smaller volume of this body portions, musculature begins to develop already during the “larval” stage. In contrast, thoracic limb musculature is formed at the late embryonic stages.

In Ctenopoda and Anomopoda, the segments are formed at rather early stages, before the time when the “embryonised larva” stage begins. Formation of buds of the thoracopods 1–2 occurs approximately at the same time as the

formation of the last thoracic segment (segment 6 in Ctenopoda and segment 5 in the daphniid Anomopoda). Segments on antennal branches are also formed before the time when the “embryonised larva” begins to grow. In Cladocera, the number of trunk segments and segments of antennal branches does not increase at stage of the “embryonised larva” in contrast to Cycletherida, the sister group of the cladocerans where the final number of segments is increasing in the larva and is finally established only at the first postlarval stage (Olesen, 1999).

Branches of antennae II in Cycletherida, a sister group to Cladocera, have a greater number of segments: 7–10 vs. 2–4 segments (Sigvardt *et al.*, 2020). In addition, antennae II is relatively shorter in *Cyclestheria* than in most cladocerans. In many anomopods, antennae II are rather short and comparable in its length to that in “Conchostraca”. In contrast, daphniid antennae II are strongly elongated due to their predominantly planktonic mode of life (Kotov, 2013; Korovchinsky *et al.*, 2021). No observations of the musculature development in *C. hislopi* are available, but based on data on muscle structure in adults (Sigvardt *et al.*, 2020), we can conclude that the muscle system of antennae II and thoracic limbs in *D. magna* and *S. crystallina* is more oligomerized (*sensu* Dogiel, 1929, 1954 and Smirnov, 1969) as compared to *C. hislopi*. Such oligomerization could be traced already in the cladoceran embryos: no additional segments are observable in antenna II rudiments even at the earliest stages of their differentiation.

Branches of antenna II of adult *C. hislopi* consist of more numerous segments; branch musculature is represented by flexor and extensor muscles, each of which reaches the distal segment (Sigvardt *et al.*, 2020). The musculature of antennal branches of *S. crystallina* is simplified as compared to *C. hislopi* — the two-segmented endopod of the former contains only two bands of muscles, and the three-segmented exopod contains only a sole band, all these muscles do not enter the distal exopod segments. In antenna II of *D. magna*, two muscles enter the exopod, and a sole muscle enters the endopod. Their distal segments are strongly thinned and are not well-observable in confocal microscope images.

In addition, adult Ctenopoda and Anomopoda are characterised by the cross-overlap of the muscles of antennae II (Fryer, 1991) in the

cephalic region (Fig. 12A–B), which is absent in adult *Cyclestheria* and other branchiopods (Sigvardt *et al.*, 2020), but present in the branchiopod nauplii (Kiernan, Hertzler, 2006). Therefore the cross-overlap of the antenna II muscles in both “larvae” and adults is a synapomorphy of Anomopoda + Ctenopoda. According to Fryer (1991), the crossing of muscles in Ctenopoda and Anomopoda causes their total length to increase, which gives a marked advantage in flexibility of the antennae II during fast swimming, since long muscles contract faster than short muscles. Antennal muscles are differentiated in the anomopod and ctenopod embryos in a non-crossed state (Figs 10A, 11D), but then cross-overlap (Figs 10C, 11F, 12). We have inspected separate stages of the embryogenesis, and the exact mechanism of such muscle re-arrangement is not clear from our study. Probably, it could be adequately described in course of *in vitro* observations. In the future, other cladoceran orders also need to be studied on this issue.

Thoracic limb musculature in *C. hislopi* is represented by a large number of muscle bands distributed relatively regularly throughout the limb, some muscles also penetrate into the endites. This arrangement of muscles provides *C. hislopi* limbs with a rather large freedom of movement (Sigvardt *et al.*, 2020). Thoracic limb musculature in *S. crystallina* is organized into larger muscle bands. Homologous bands could be found in *C. hislopi* based on their position and points of attachment. Sigvardt *et al.* (2020) found that homologs of other muscles of *C. hislopi* are absent in *S. crystallina*. She suggested that such oligomerization of muscles correlates with the increase in beating rate of *S. crystallina* thoracic limbs. Concerning the homology of *D. magna* musculature portions, it is difficult to formulate similar conclusions at this point: in our work, confocal images were taken at a lower resolution as compared to the work of Sigvardt *et al.* (2020). This did not allow us to visualise muscle bands and their attachment sites in detail.

We can confirm previous ideas (Sigvardt *et al.*, 2020) that musculature development may provide useful information for comparison of different cladoceran orders based on characters having a phylogenetic signal and can be used as an argument to reconstruct phylogenetic relationships within the class Branchiopoda along with data on morphology, anatomy and

molecular phylogeny. At different stages of their development, cladoceran embryos demonstrate the characters absent in adults, such as: other proportions of body parts and muscle band sizes, “additional” thoracic segments (lost in the adults), non-crossed muscles of the antennae II, non-oligomerized initial thoracic muscle system in the anomopod embryos. We also found that the timing of the muscle band formation is different in *Daphnia* and *Sida*, but to date the information is too scarce for any phylogenetic conclusions based on such differences.

We believe that just anatomical data could contribute to the final resolving of the cladoceran order phylogeny which is still a mystery, even after applying of full-genome methods (Xu *et al.*, 2021; Van Damme *et al.*, 2022). However, our study represents only a first step in such direction, and to date it is prematurely to reconstruct the cladoceran phylogeny based on muscle structure and development, these studies need to be continued.

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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