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Growth and function of modified gnathopods and walking legs in *Dikerogammarus villosus* (Sowinsky, 1894). A comparative study based on tomographic 3D images.

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Introduction

Crustaceans greatly vary in size (0,1mm to 3,5m) and the habitats they inhabit. They live terrestrially or aquatically, inhabiting both fresh- and saltwater. Their big diversity in body plans makes them very interesting for researching evolutionary transitions in morphology and development. Between different crustacean groups, biggest differences occur in patterns of tagmatization and modification of body appendages (Westheide and Rieger 2007).

Within crustaceans the Malacostraca represent the group with the highest extent of organization (Westheide and Rieger 2007). In Malacostraca, the first peraeon (thorax) appendages are modified to assist in feeding and other manipulating movements. These modified legs are called maxillipeds reflecting their dual nature of mouthpart and leg. A number of Malacostracan groups possess one pair of maxillipeds (e.g. Isopoda) while in Decapoda three of the eight pairs of peraeopods are maxillipeds. Posterior to the maxillipeds, other modifications occur that make these legs very different from normal walking legs, too. For example, Decapoda have modified the fourth peraeon appendages to huge claws. Currently toady, there are only few studies dealing in detail with functional aspects of Malacostracan leg morphology such as joints and musculature that compare normal walking legs with derived appendages. Recently, a description of the distal leg musculature of Decapoda (*Libinia emarginata*) was given based on dissections of the limbs by Vidal-Gadea and Belanger (2009). The most extensive work on Malacostracan functional leg morphology focuses on Isopoda, giving detailed descriptions of joints and muscles in isopod walking legs (Hessler 1982).

My thesis focuses on modified peraeon appendages in Amphipoda. Similar to isopods they have one pair of maxillipeds, but different to isopods, in amphipods the second and third peraeon appendages are modified grasping legs, so-called gnathopods. Detailed data on joints and muscles of these gnathopods concerning adults as well as developmental stages are still missing. The amphipod body-plan shows a multiplicity of modified appendages on repeating segmental units (Westheide and Rieger 2007). Amphipoda have laterally compressed bodies and sessile compound eyes. Like in other malacostracans, the head includes six segments plus the first segment of the peraeon, which is fused to the cephalon to a so-called cephalothorax. This first peraeon segment bears a pair of maxillipeds, which are extensively modified to assist in feeding (Browne, Price et al. 2005). The head segments bear five appendages (first antenna, second antenna, mandibles, first maxillae and second maxillae). Only the anteriormost preantennal segment carries no appendage. All the following seven peraeon-segments carry one pair of uniramous appendages. The second and third segment carries the gnathopods, which are subchelate (clawed) and function in grasping and mating. The five posterior peraeon segments (segments 4-8) carry normal walking legs used for locomotion. Amphipods show a typical orientation of the walking legs. The two anteriormost walking legs are oriented anteriorly and the others oriented posteriorly, thus the name for the group (amphipod). All walking legs possess a modified appendage branch (gill) that appears underneath the animal as a respiratory organ. In addition, females possess an additional appendage branch

(endit) on the peraeon segments 2-5 that form a ventral brood pouch (marsupium) (Westheide and Rieger 2007). Posterior to the peraeon there is the pleon consisting of six segments. The appendages of the pleon and the terminal structure called telson are basically used for locomotion. The anteriormost three segments constitute the pleosome and each bear a pair of biramous appendages termed pleopods. The other segments constitute the urosome and also carry one pair of biramous appendages on each segment termed uropods (Browne, Price et al. 2005).

My study object for this work is the gammarid amphipod *Dikerogammarus villosus* (Sowinski, 1894) (Crustacea: Malacostraca: Amphipoda: Gammaridae). This large (up to 3cm) predatory gammarid is native to the Ponto-Caspian region of Eastern Europe/Ukraine and has invaded Western Europe via the Main-Danube canal (Dick and Platvoet 2000) and poised to invade North America (Bollache, Dick et al. 2008). *D. villosus* is known to eliminate both native and other invasive species (Buřič, Kočí et al. 2009; Emde, Rueckert et al. 2012). It reaches sexual maturity at a very early stage (at a body length of 6mm) and reproduces all year long (Devin, Piscart et al. 2004).

Before mating, the larger male takes a reproductive female in a characteristic pre-copulatory amplexus (Plavoet, Song et al. 2006) to defend her against predators and competitive males. This pre-copulatory stage takes up to more than a week, depending mainly on water temperature and predation pressure. After this stage the female moults and they copulate in a specific belly-to-belly position. The eggs are released from the ovaries into the brood pouch and fertilized by the male. The male doesn't release the female until her cuticula is hardened. For a specific lock-on mechanism (during amplexus), large unusual pores in the female peraeonites one and five and the first pair of male gnathopods are involved (Plavoet, Song et al. 2006). When the development of all eggs in a single marsupium is completed the embryos were deposited within one hour (Browne, Price et al. 2005). Many amphipod species show a sexual dimorphism, and males are generally larger and possess larger second gnathopods (Crozier and Snyder 1923; Birkhead and Clarkson 1980; Adams and Greenwood 1983; Ward 1984; Elwood, Gibson et al. 1987; Ward 1993; Ward and Porter 1993; Dick and Elwood 1996; Hume, Elwood et al. 2002; Bollache and Cézilly 2004). The proportion of individual sizes between female and male in a pre-copula pair can vary a lot, but a field study showed that the pairing success is better for larger males (Wellborn and Bartholf 2004).

The focus of my work is based on showing the differences in the six distal segments (dactylus, propodus, carpus, merus, ischium and basis; the coxa is not investigated in the present study) between the modified first and second gnathopods (peraeopods 2 and 3) and a regular walking leg (peraeopod 4). My analysis focuses on three aspects: (1), I describe the eidonomy of leg segments, the leg musculature, and the plane of movement for each joint. This results in the first functional analysis of amphipod gnathopods. (2), I analyze intersexual differences in the growth of leg muscles, since gnathopods are sexually dimorphic in *Dikerogammarus villosus*. (3), I investigate appendages of pre-hatching embryos and freshly hatched specimens in order to see whether the walking leg-to-gnathopod transition occurs in early development or after hatching.

Materials and Methods

Study animals

For the research on leg musculature I used specimens of *Dikerogammarus villosus* (Sowinski, 1894). This predatory invasive species lives on the bottom of stony riverbanks including the river Danube and its tributaries in central-eastern Europe. Specimens for investigation were sampled in the main Danube-river near Vienna as well as in the Danube-channel close to Friedensbrücke. For analysis we choose sexually mature animals (that were in pre-copulatory amplexus from all size classes), ranging from the smallest mated specimens to the largest specimens found in Danube populations. In addition to sexually mature animals we collected freshly hatched specimens and embryos.

Captive breeding

In order to collect different pre-hatching developmental stages I kept females with embryos in their brood pouch in little plastic bowls while monitoring the development of the embryos (Figure 1). By harvesting a couple of embryos every two or three days I obtained the different embryonic stages required for analysis. Every bowl was filled with two individuals, a few stones and leafs taken out of their natural habitat, for feeding. In addition they were fed with living copepods bought in a pet shop. The bowls were positioned in between double windows, to create similar water-temperature, to the river Danube they were taken from.



Figure 1: Captive breeding in a plastic bowl equipped with two female specimen, stones and leaf litter.

Harvesting of embryos

Every two or three days, depending on water temperature, a couple of embryos were collected from the marsupium using a plastic pipette while the specimen was held in a fixed position using forceps (Figure 2). The received embryos were fixed in 70% ethanol. Under a microscope the stage of development was determined.



Figure 2: Harvesting of embryos from the marsupium of a female *Dikerogammarus villosus* using forceps and a pipette in a glass bowl filled with water.

Fixation, Staining and Mounting

To prepare the specimen for scanning with Xradia micro XCT they were fixed with 70% ethanol. After dehydration in a graded series of ethanol the animals were stained in 1% iodine in absolute ethanol for 24 hours (Metscher 2009). After staining, they were rinsed for several hours using 100% ethanol to remove unbound iodine from tissues to reduce background noise. For scanning samples were mounted in heat-sealed pipette tips.

The embryos which were scanned with a confocal microscope had a slightly different preparation. Embryos were also fixed in 70%, and the egg shell was removed with sharp forceps. Single legs were cut off the body and mounted on slides using the mounting medium fluoromount-G.

Image acquisition

Micro XCT scans were made with an Xradia micro XCT (Metscher 2009). Two scans were recorded for every individual. One of the whole animal and one high-resolution scan focusing on the appendages of interest. The embryos (specimens before hatching) were scanned with using upright Leica and an inverted Zeiss confocal microscope (Shotton 1989) to obtain cellular information of the very small structures of embryonic legs. For each leg a z-stack was recorded. This yielded very high resolution for the appendages, but based on the limited penetration depth of the laser we were not able to scan the whole specimen after the dissection of the appendages of interest. Since the tissue showed sufficient autofluorescence at excitation with a 405 nm laser diode, no staining was applied.

Reconstruction

Image data from both modalities was imported into the 3D-software-package Amira 5.3.3. With this program, every single muscle was reconstructed by hand with a drawing tablet. This means every slice of the picture-series has certain information (Figure 3), which needed to be interpreted, and each muscle of interest was marked it in every slice it appears.

Based on this segmentation procedure I was able to create 3D models of the legs and to calculate volume data for each. Based on the whole-body scans I measured the length of head and body for each individual.

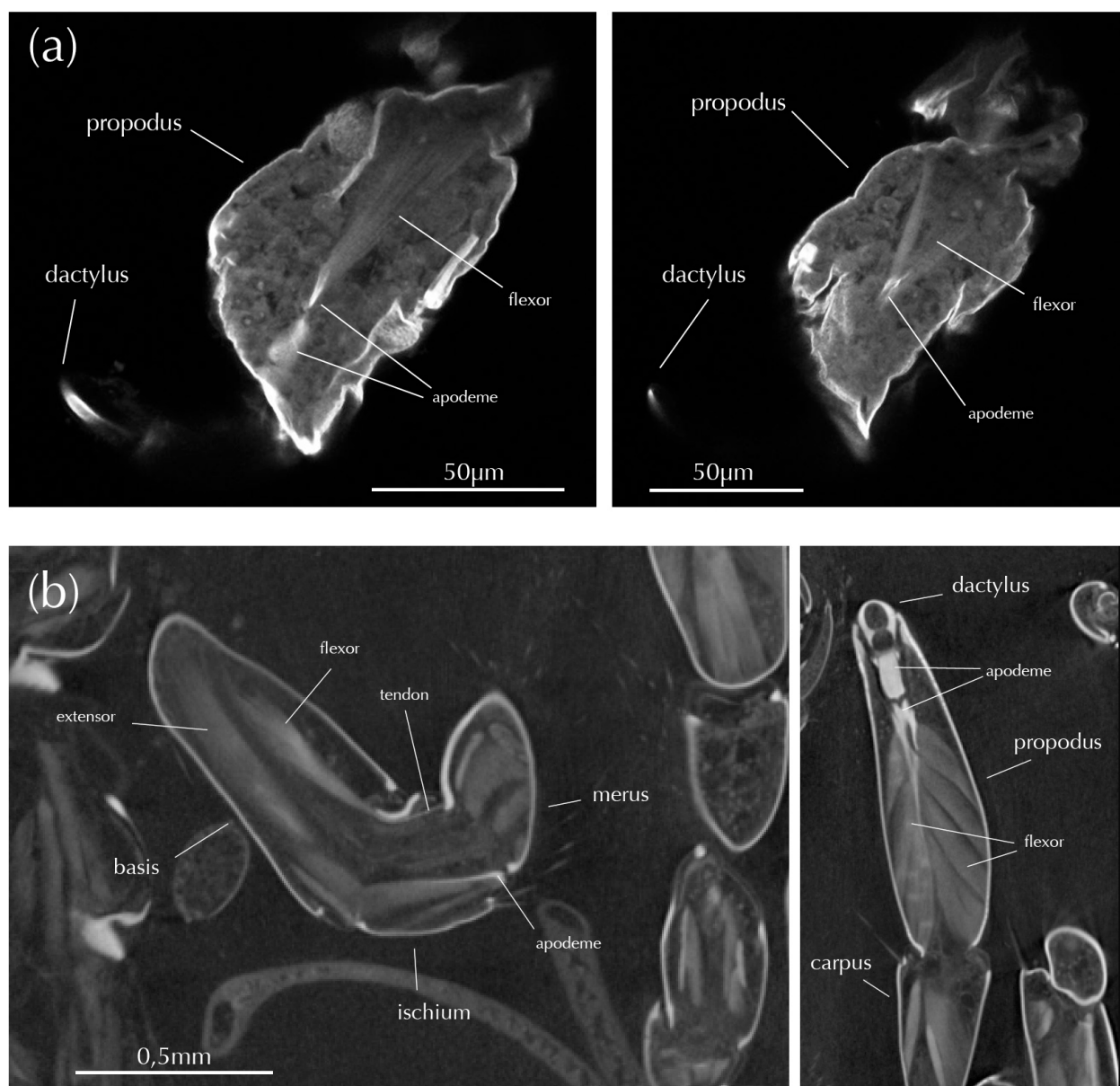


Figure 3: Images visualized with Amira 5.3.3. a.) Optical sections of a z-stack made with a confocal microscope. b) Image slices made with Xradia micro XCT.

Results

1. Eidonomy and exoskeleton

1.1 Eidonomy of the legs

Gnathopods differ from walking legs (peraeopods) significantly in size and shape of the six distal segments. In the following all descriptions will focus on these six segments, while the coxa was not subject of the present investigation. The propodus of gnathopods is much bigger than in a normal walking leg and represents the biggest segment within the gnathopods. Also the shape of the propodus is very different between the two legs. In walking legs it is elongated and slim, while in gnathopods it is stretched in the dorso-ventral axis and laterally depressed. In gnathopods, the dactylus can be moved against the propodus by a so-called subchelate joint, which is very effective to manipulate things like food. The segments carpus and merus are also elongated in walking legs and deformed in gnathopods. Ischium and basis show more or less the same size and shape in both legs (Figure 4).

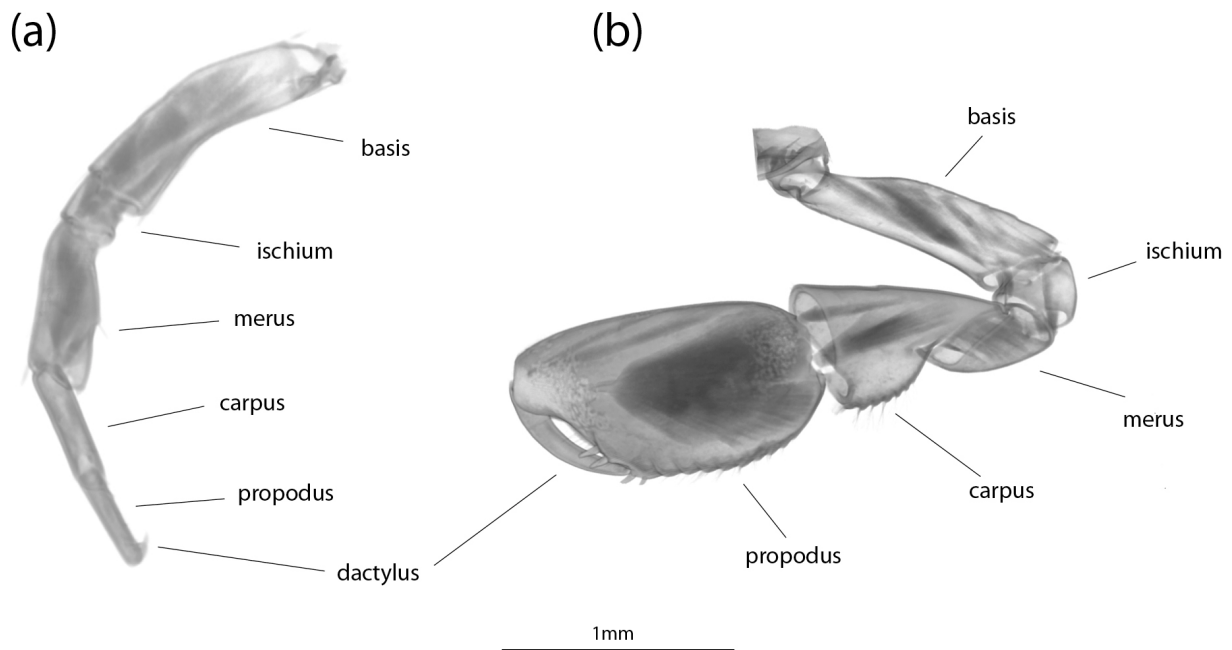


Figure 4: Eidonomy of two different leg-types of *Dikerogammarus villosus* (Sowinsky, 1894). a) The six distal segments of a normal walking leg (peraeopod 4); b) The six distal segments of a modified leg (gnathopod 2).

1.2 Articulations

In amphipod peraeopods, all joints distal to the coxa are dicondylic. Each joint is moved by a pair of antagonistic muscles, the flexor and the extensor. The flexor muscle decreases the angle between the two leg segments of the joint, while the extensor muscle increases it. The axis running towards the two condyles of the joint (hinge line) is perpendicular to the axis running through the insertions of the two muscles.

In the normal walking leg, the whole leg roughly lies in one plane, the limb plane, which passes through the long axis of all leg segments. Except the merus-carpus joint, the hinge line of joints is perpendicular to the limb plane, while the hinge line of the merus carpus joint is parallel to the limb plane (this roughly resembles the situation of isopods described by Hessler 1982). This means that all articulations except the merus-carpus joint move the leg in one plane (Figure 5b). The animal can move the leg up and down (with basis and ischium) outside and inside (with merus) and angular sort of up and down with the carpus and propodus musculature. The two distal-most articulations joints (propodus-dactylus, carpus-propodus) of the walking leg show that both articulations operate nearly (about 15° distorted) in the same direction (Figure 5a). The flexor moves the dactylus towards the propodus and the extensor moves the dactylus in the opposite way. In the carpus-propodus joint, the flexor bends the propodus in the direction to the carpus and the extensor the other way round. The third distal articulation is moved by the muscles of the merus-segment (Figure 5b). It is the only articulation that lies parallel to the limb plane. Here the muscles bend the distal segment inside and outside from the center of the animal. This axis of movement is nearly 90° distorted compared to the two distal-most articulations of the peraeopod.

The next two articulations (Figure 5c) moved by the ischium and basis have exactly the same planes of movement. Both axis of movement are 90° distorted to the merus. Therefore these articulations lay about in the same plane as the two distal-most segments do. Here the muscles move the distal segments up and down from the animal's body. The special thing about those articulation-muscles is the basis-ischium-flexor (Figure 5c) which finds its origin in the basis. This muscle flexes both following segments (ischium and merus). There are two main flexor muscles, one has its insertion at the ischium and the other runs through the ischium and finds its insertion (linked to a tendon) on the merus, while the ischium lacks an own flexor muscle. This is one reason why these two articulations need to operate exactly in the same plane (Figure 5c).

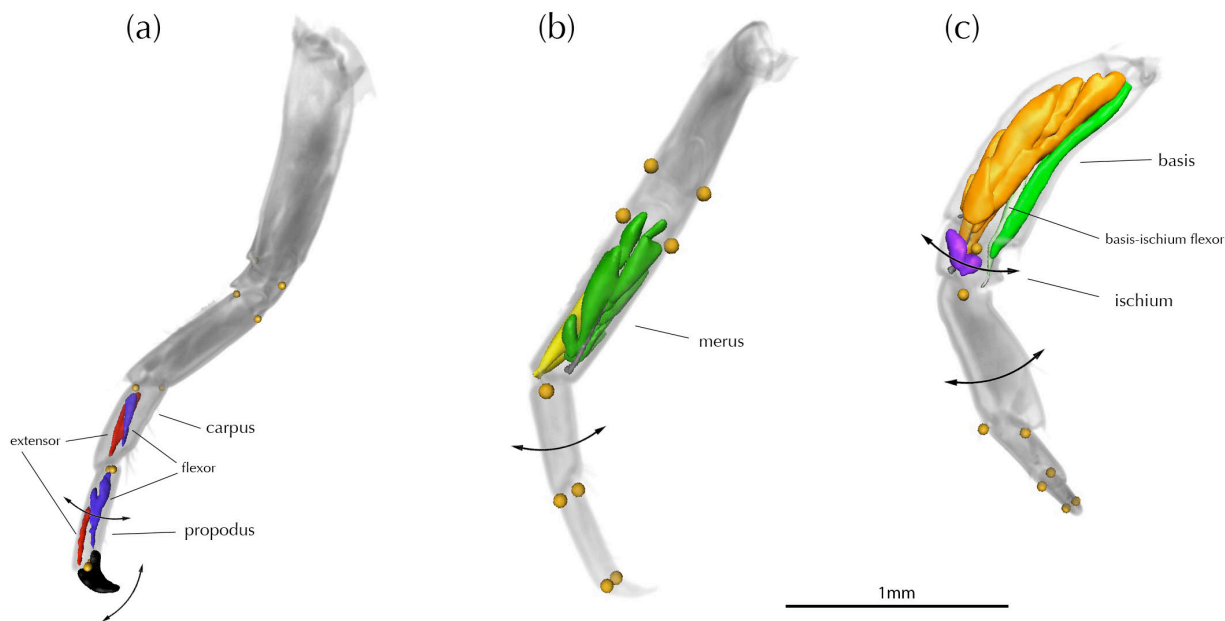


Figure 5: Planes of movement in a regular walking legs (peraeopod 4). a) one plane of movement within two articulations, moved by two antagonistic muscles in the propodus and the carpus. b) main distortion in the joint axis of the leg lays in the articulation of the merus. c) Articulation four and five are moved in the same plane, the basis-ischium-flexor moves both segments (ischium and merus).

The modified gnathopods show more different planes of movement (Figure 6) than the walking leg. As in walking legs, basis and ischium move the leg up and down from the animal. The special thing on this leg lies in the articulation of the merus. This one can tip the distal segment in a clockwise or anti-clockwise direction (Figure 6c). The distal segments (propodus) which closes and opens the dactylus is in the same plane as the next articulation, moved by the muscles of the carpus.

The distal-most articulation of the gnathopod (Figure 6a) moving the dactylus against the propodus includes the strongest and biggest muscle-bundle of all five distal segments. This muscle closes the subchelate dactylus and is used to manipulate or crush things. The so called flexor inserts to a complex of two apodemes that are connected to the dactylus. In this segment the closer-muscle (extensor) is very little compared to the flexor. The next articulation of the gnathopod (Figure 6b) is moved by the muscles of the carpus. This articulation-axis is distorted about 35° to the first one. The third distal articulation (Figure 6c) is very complex and differs significantly from walking legs. Those muscles are used for tipping the two distal segments around the anterior-posterior axis of the animal. So the articulation axis is about 90° distorted to the carpus articulation. The bigger sized muscle (Figure 6c, blue muscle) tips the distal segment in a clockwise direction. The last two gnathopod-segments have also, like the walking leg the same plane (Figure 6d) and also move the leg up and down. Here the articulations are about 80° distorted to the merus-articulation. As in the walking leg, both segments (ischium and merus) are moved by the basis-ischium-flexor which finds its origin in the basis and the ischium just shows an extensor muscles.

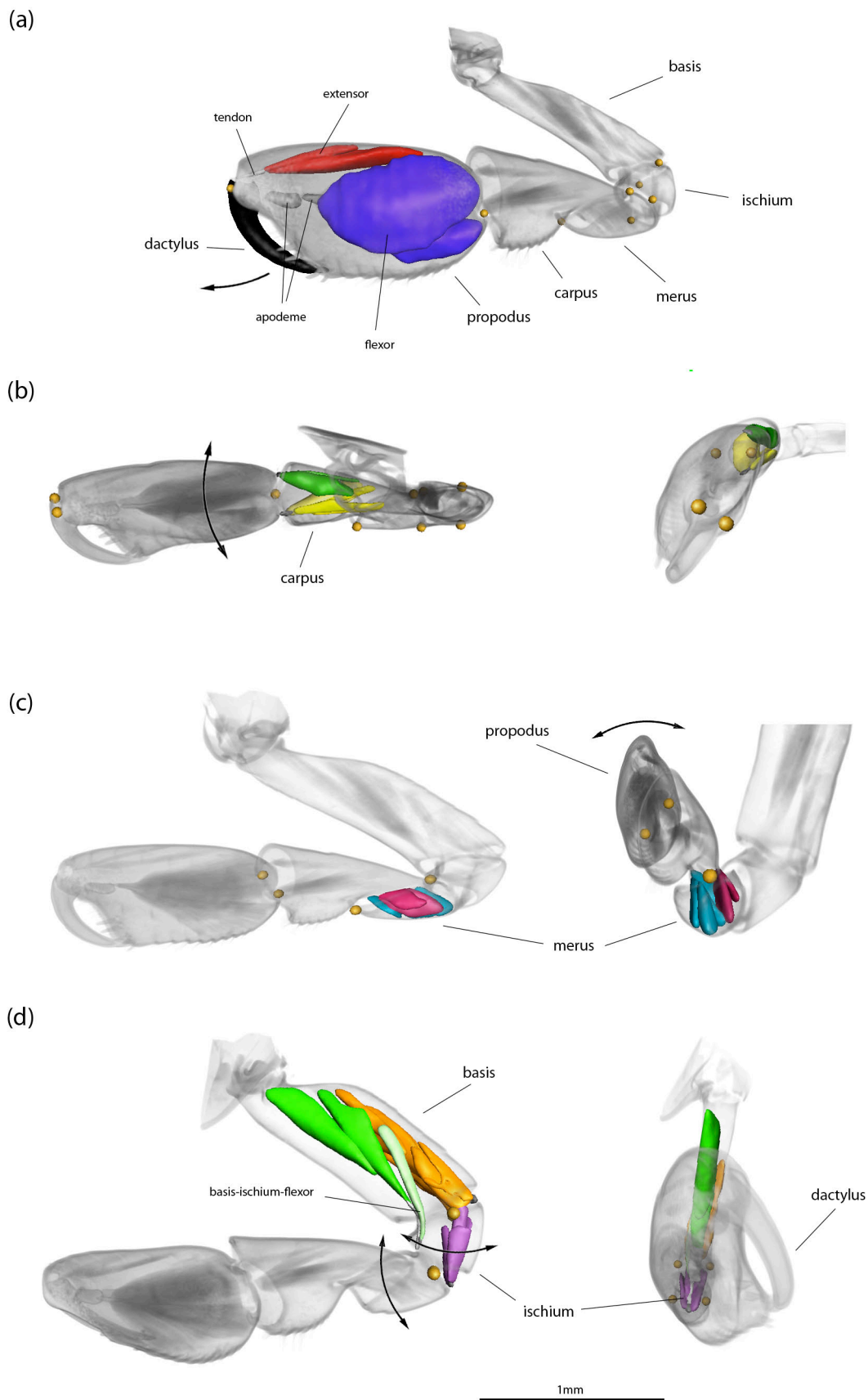


Figure 6: Planes of movement in gnathopods. a) Movement of the dactylus managed by flexor and extensor muscles. b) Articulation-axis of the carpus is about 35° distorted to the first one. c) Third articulation is used for tipping the two distal segments. d) Last two segments are moved with the same flexor muscle and have identical joint-planes

2. Leg musculature

2.1 Description of walking leg and gnathopod

As described in the previous section every joint is moved by two antagonistic muscles (Figure 5,6). Those two work in opposite directions. For example, in the gnathopod (Figure 6a) the red colored extensor of the propodus opens the dactylus and the much bigger flexor in blue close it to grab something. Based on the two muscles and the dicondylic articulations every segment can just be moved in one axis. Muscles are made of several bundles, number of bundles varies between 2 and 11, and some of them are pinnate inserting in broad apodemes. Every muscle has its origin in one segment and its insertion in the next one. There is one exception: the basis-ischium-flexor (light green) (Figure 5c,6d) bypasses a segment and they insert in the next one. It has its origin in the basis and its insertion in the merus. This special situation is shown in both gnathopods and walking leg. Muscles insert either via an apodeme or a tendon.

All together I reconstructed muscles on 21 legs (two walking legs, 19 gnathopods) out of ten specimens (Figure 7). Out of those ten individuals, nine were scanned with micro XCT and one (embryo, two to three days from hatching) with a confocal microscope because it was too small for the micro XCT. On eight specimens we also made scans from the complete animal so we can compare the sizes of the legs to body size. On two amphipods we could not do so because we made a dissection to get a better view on the appendages. After the dissection we could not save the body in one piece to scan it for better comparison.

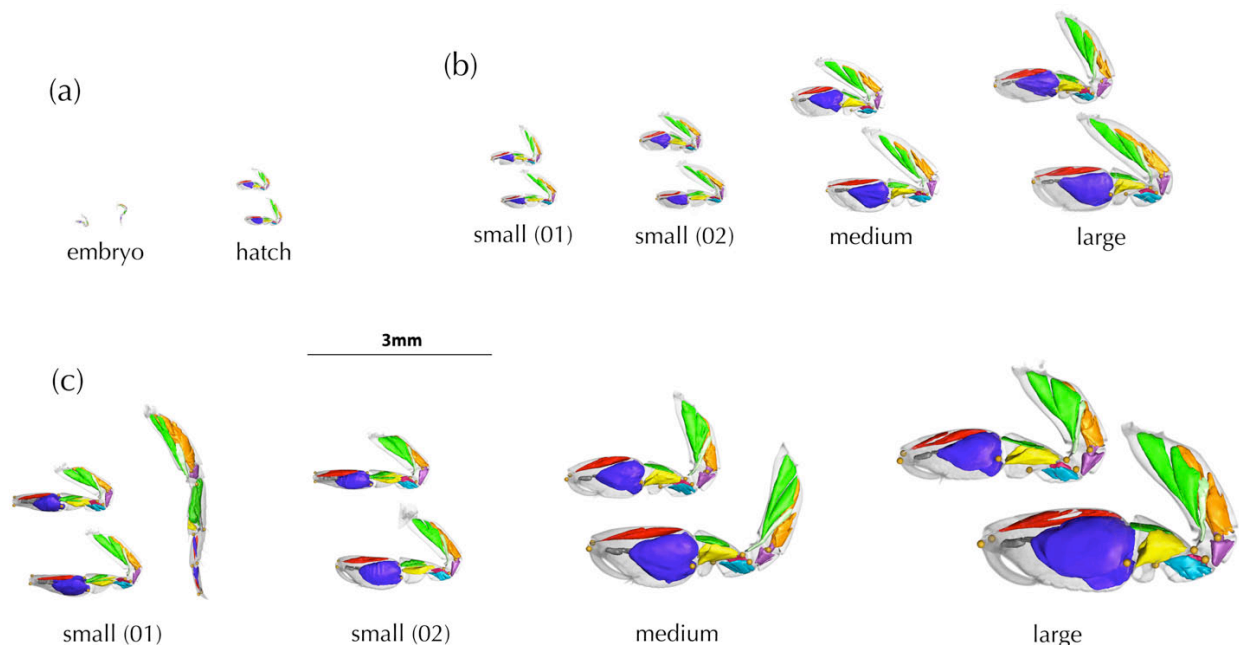


Figure 7: Reconstructed peraeon appendages of *Dikerogammarus villosus* specimens. a.) gnathopod and walking leg of an embryo (two to three days from hatching) and next to it, two gnathopods from a freshly hatched specimen. b.) gnathopods from four females of different size classes. c.) gnathopods and one walking leg out of four male individuals of different size classes. The same titled males and females were found as pre-copula pairs.

2.2 Comparison of females and males

Between males and females within the biggest differences are found in the sizes of the gnathopods. In both sexes the second gnathopods are bigger than the first ones but within the males this differences between those two modified legs is even bigger (Figure 8). Especially the propodus of the second gnathopods within large males is strongly sexually dimorphic.

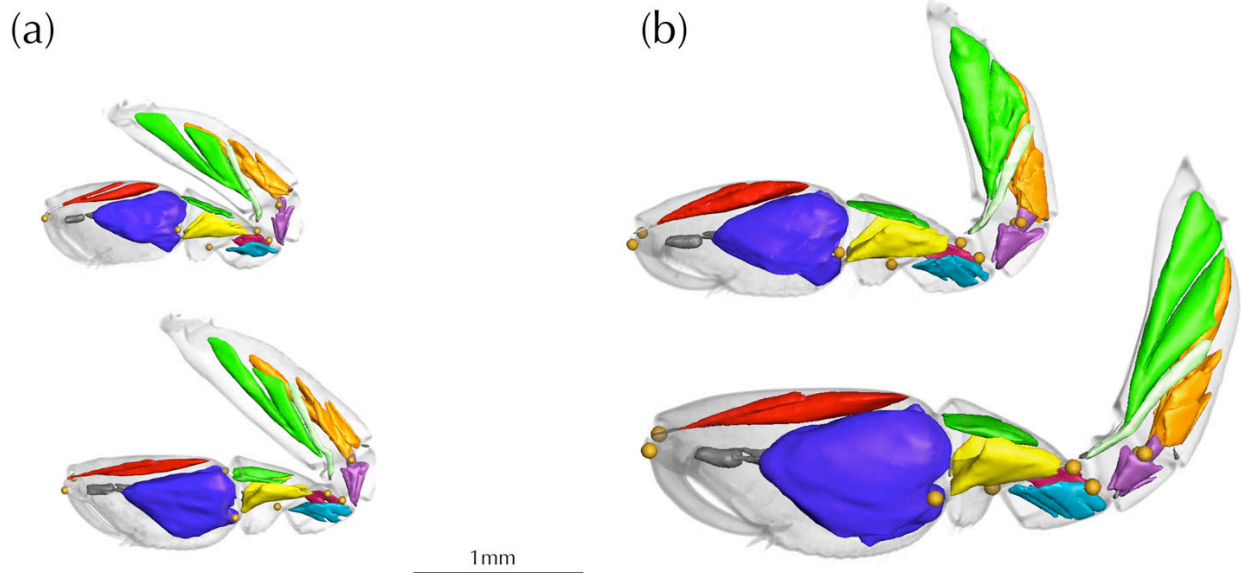


Figure 8: Modified gnathopods of medium sized amphipods which were found as a pre-copula pair
a) First (on top) and second (bottom) gnathopod of a female. b) Male gnathopods

3. Growth of leg musculature

3.1 Lifelong growth of the amphipod body

In order to relate muscle growth to lifetime growth of the amphipod body we measured the length of the head and the main body. The body length was measured from the first peraeonite (second peraeon segment, the first segment is fused with the cephalon) to the last segment of the pleon which includes 13 Segments (Figure 9) Head and body length was measured for all eight individuals (four females and four males) (Table 1). Those data were compared with the muscle volumes of first and second gnathopods of the same specimens.

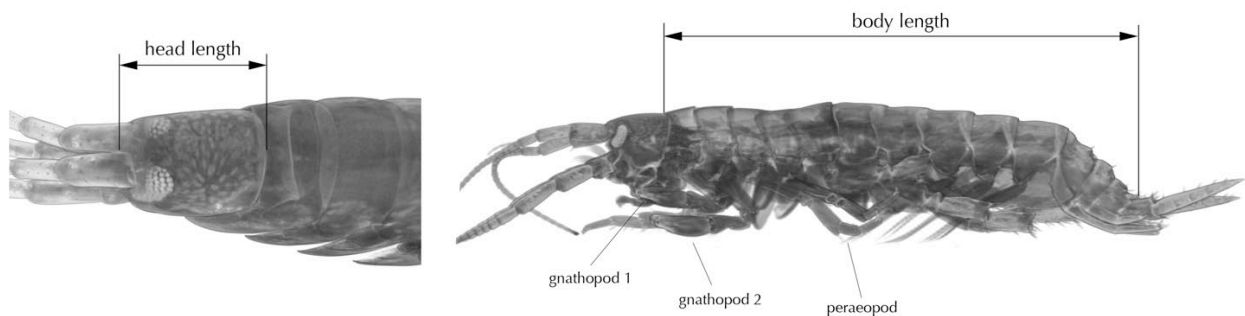


Figure 9: Measurement of head and body length.

Table 1: Summary of measured muscle volumes of first and second gnathopods and head and body lengths.

Gnathopod 1	Muscle volume (mm ³)	Body length (mm)	Head length (mm)	Proportions body length and head length
Small female (01)	0,0024	6,46	0,82	7,9 : 1
Small male (01)	0,037	9,09	1,13	8 : 1
Small female (02)	0,0065	8,24	0,95	8,7 : 1
Small male (02)	0,055	10,34	1,31	7,9 : 1
Medium female	0,033	11,54	1,37	8,4 : 1
Medium male	0,132	15,42	1,67	9,2 : 1
Large female	0,059	14,58	1,62	9 : 1
Large male	0,326	18,20	2,11	8,6 : 1
<u>Gnathopod 2</u>				
Small female (01)	0,0028	6,46	0,82	7,9 : 1
Small male (01)	0,043	9,09	1,13	8 : 1
Small female (02)	0,0072	8,24	0,95	8,7 : 1
Small male (02)	0,065	10,34	1,31	7,9 : 1
Medium female	0,053	11,54	1,37	8,4 : 1
Medium male	0,33	15,42	1,67	9,2 : 1
Large female	0,107	14,58	1,62	9 : 1
Large male	1,088	18,20	2,11	8,6 : 1

3.2 Allometry and sexual dimorphism in leg muscle growth

In both sexes, muscle growth is roughly exponential when plotted to body length (Figure 10-13). In small females, muscles of first and second gnathopods show similar volumes. In female gnathopods the highest rate of growth occurs between small (8mm body length) and medium (12mm body length) sized individuals (Figure 10) In contrast, males show high growth rates in the second gnathopod between small (10mm) and large (18mm) specimens (Figure 11), and in the first gnathopod muscles grow most between medium and large sized individuals. The lowest rate appears in females between medium and large specimens in both legs and in males this happens between the small (01) and the small (02). Comparing males and females of similar body sizes one can see that the developmental trajectories of gnathopod muscle growth are pretty similar regarding growth rates at specific body sizes (Figure 12).

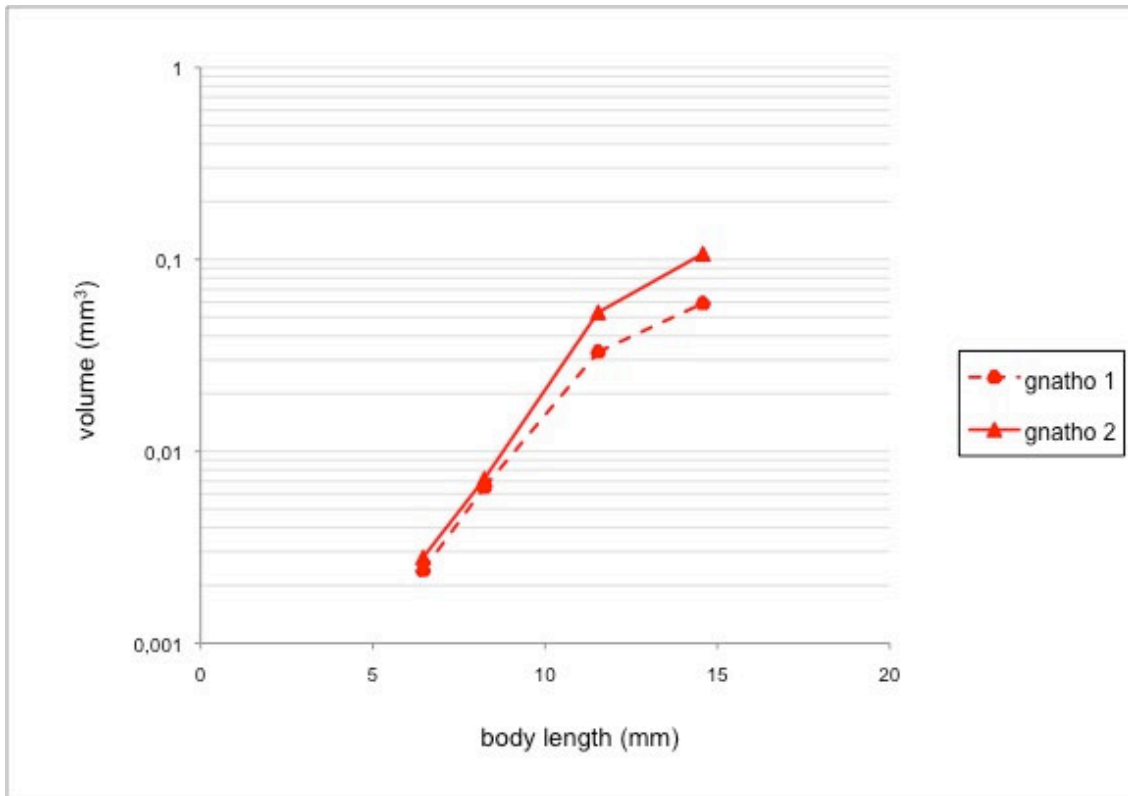


Figure 10: Comparison of female gnathopods. Dots with the dotted line represent the first gnathopods and triangles with the solid line the second ones. Shown are muscle volumes of five distal segments.



Figure 11: Comparison of male gnathopods. Dots with the dotted line represent the first gnathopods and triangles with the solid line the second ones. Shown are muscle volumes of five distal segments.

3.3 Increase in total leg muscle volume

The volumes of all muscles in five distal segments of gnathopods grow in a lifetime of a *Dikerogammarus villosus* enormously. The second gnathopod in an individual is 1,5 to nearly 3,5 times bigger than the first limb, when you just look at the total muscle-volume of the five segments. The differences between those modified peraeon appendages are bigger in older individuals. So this means that the second gnathopods have a faster growth. When in small individuals, male or female, the sizes of the modified legs are nearly the same; the differences after following moults are huge (Figure 12).

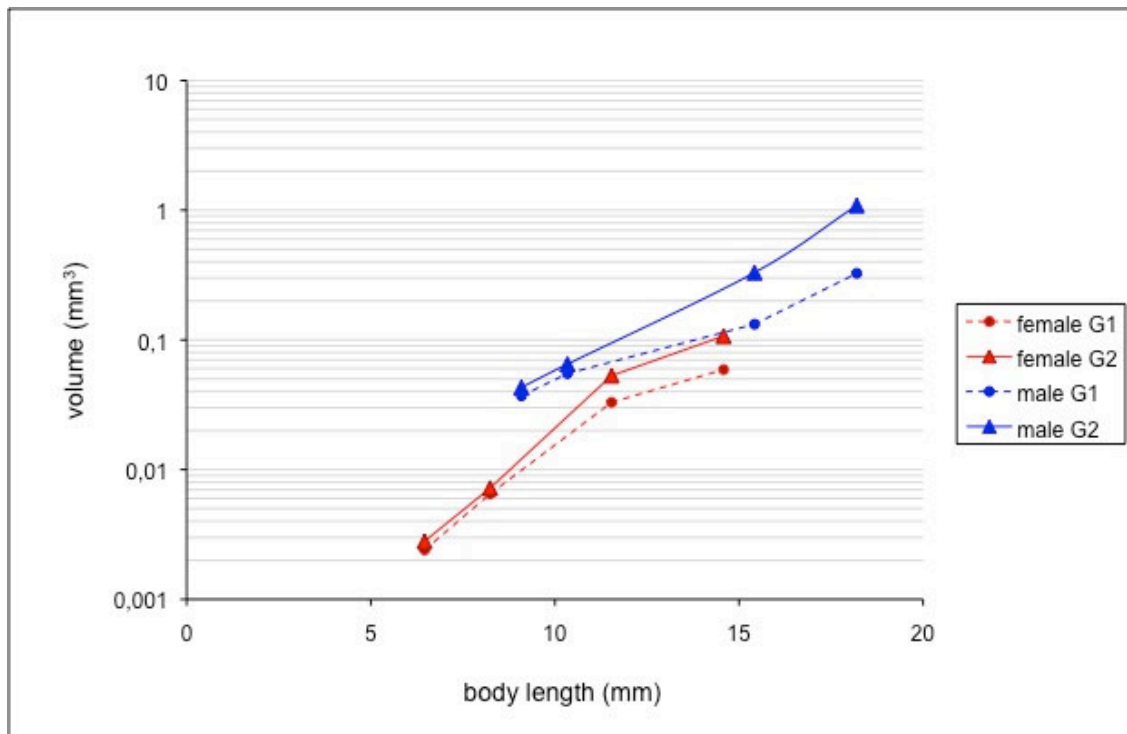


Figure 12: Comparison, gnathopod growth between males and females. Triangles and dots show males and female in four different body length and muscle volume. Measured were volumes of the muscles within five distal segments (propodus, carpus, merus, ischium and basis). Blue lines show male gnathopods and red lines show females. Dotted lines show the first gnathopod whereas the solid lines show the second gnathopod. On all the shown specimens the second gnathopod has more muscle-volume than the first one. Despite male gnathopods are consistently larger, the growth trajectories for gnathopod muscles at specific body sizes are pretty similar.

3.4 Increase in propodus muscle volume

The muscles within just the propodus of gnathopods show the greatest enlargement of all the leg segments. The relative increase of muscle volume in this segment is even bigger as we know it from the total gnathopod muscle mass. Here the small individual does not show a strong difference (especially the small females) between the two modified appendages. In the small (01) female it even seems that the first gnathopod includes more muscle volume than the second limb (Figure 13). But in larger individuals the differences between those legs are enormous.

Also here, the large male shows the biggest disparity in the second appendage (Figure 13) and reveals a nearly five times greater muscle-volume than in the first one. Compared to that, the second appendage of the large female is only 2.1 times bigger than the first gnathopod.

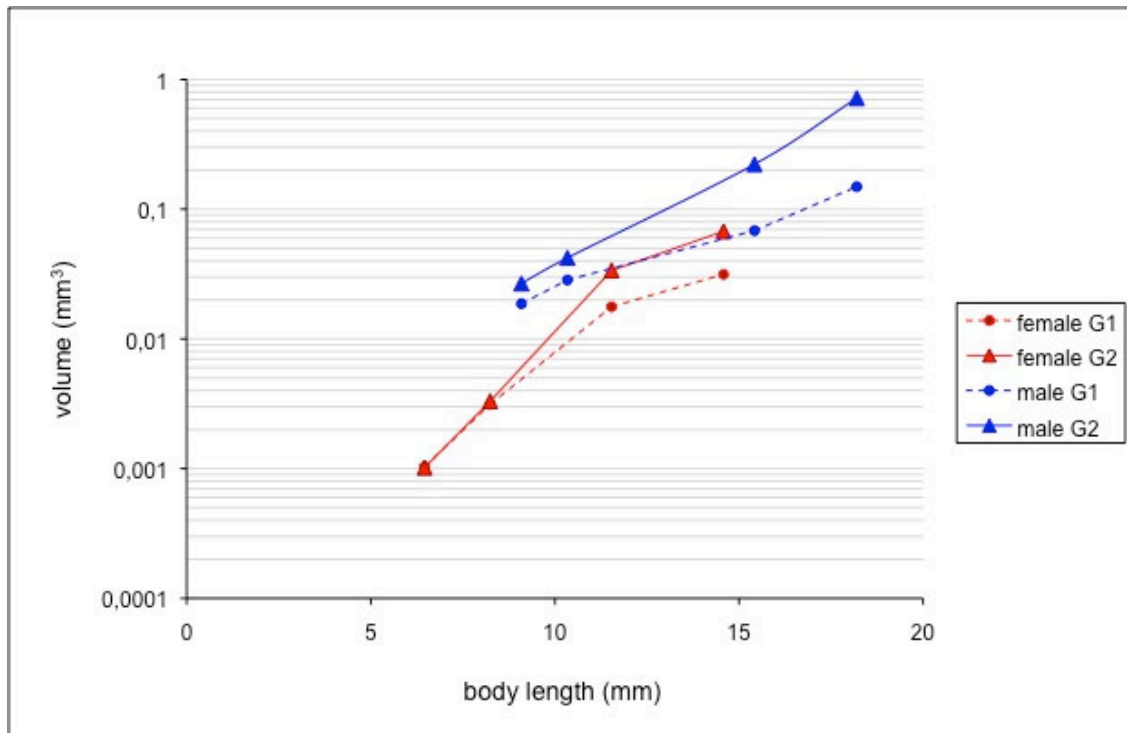


Figure 13: Growth of muscle volume in the propodus. Triangles and dots show males and female in four different body length compared to muscle volume of the propodus. Blue lines show male gnathopods and red lines show females. Dotted lines show the first gnathopod whereas the solid lines show the second gnathopod.

The muscles of the propodus have more muscle volume than any other segment of the legs (Table 2). In comparison the females show a bigger rate of growth (Figure 14) in the propodus but the proportion between propodus and the muscle mass including all segments is a little smaller. For example, gnathopods in small females have less than half the (37-48%) amount out of the whole muscle-volume just in the propodus. Medium to large females possess more than a half of the amount (53-64%). This rate is shown within both gnathopods. The first gnathopods in males have about half of the (49-52%) muscle volume in the propodus within all the individuals, in the second gnathopod they possess about two-third (63-67%) of the complete muscle mass just in the propodus. This rate is about the same in all the developmental stages. This means that within large males just one-third (33-37%) of all the muscle volume in those five segments together is separated in the other four segments (carpus, merus, ischium and basis). Exact data of the distribution in all five segments is shown in Table 2. So the biggest difference between females and males is located in the propodus of the second gnathopod in small individuals. Here the female's propodus includes 37-46% whereas the male's propodus includes 63-65%.

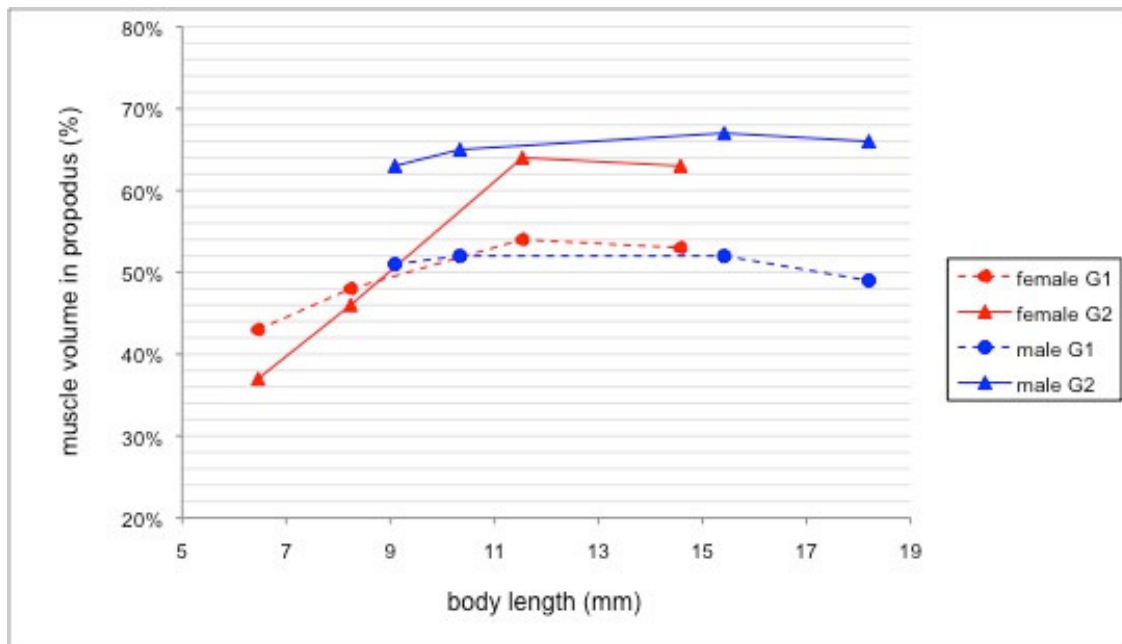


Figure 14: Distribution of the muscle mass in the propodus compared to the total muscle volume of all the five leg segments. The complete muscle mass in one leg of every individual represents 100%.

4. Embryonic development

4.1 Development of gnathopods

The last two to three days in the marsupium the growth of the muscles is enormous. The muscle volume is very small in all segments of pre-hatching embryos (Figure 15a). Some muscles of the carpus and merus are not even visible at this point. All other muscles are visible but very tiny, and muscle cells are not yet fully differentiated (Figure 3). The muscles of the hatchling (Figure 15b,c) which is about two to three days older than the embryo nearly fill out the inner space of the leg segments, whereas the embryo possesses still a lot more unfilled space in the haemocoel.

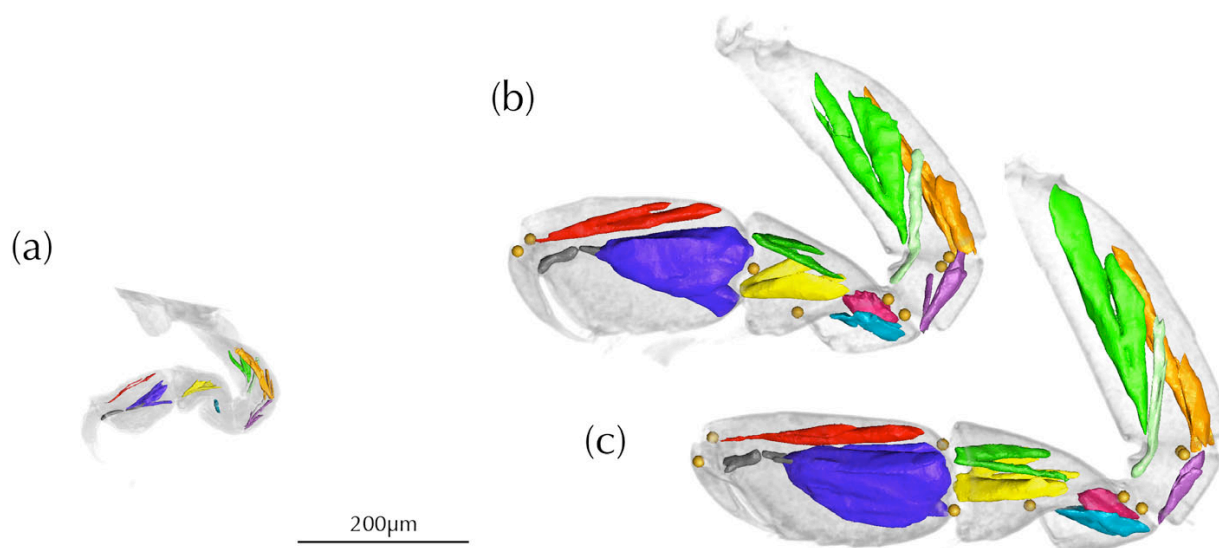


Figure 15: Differences in two different development stages of gnathopods from *Dikerogammarus villosus*. a) Gnathopod of an embryo, two to three days before hatching. b) First gnathopod and c) second gnathopod of a freshly hatched individual.

In a lifetime of *Dikerogammarus villosus*, the cuticula of leg segments grows each time the specimen moults. In the eidonomy of gnathopod, biggest differences from embryos until sexual maturity occur in the propodus-dactylus complex. An embryo, which is two to three days from hatching, has still a deformed propodus and the dactylus quite different in shape from adult gnathopods (Figure 16b) that does not show the typical subchelate form. It somehow resembles a mixture of a regular walking leg and a gnathopod (Figure 5,16). In embryos the cuticula is not yet hardened or mineralized, thus it shrinks during dehydration and mounting on slides. In contrast, the mineralized adult cuticula does not shrink during dehydration (Figure 16a).

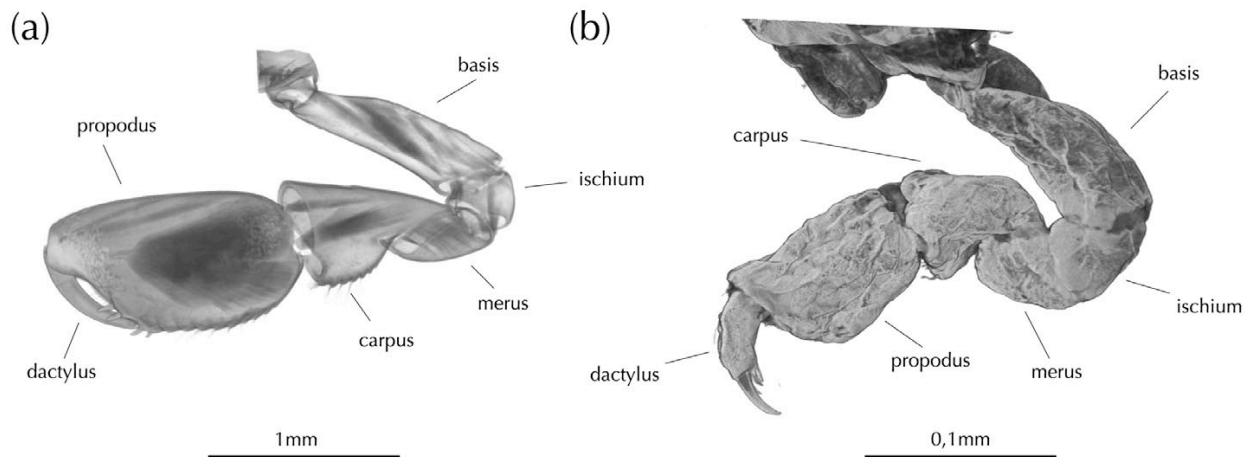


Figure 16: Comparison of leg eidonomy of a medium sized male and an embryo. a) Smooth surface in all segments on a medium sized male gnathopod. Image made with Xradia micro XCT and Amira 5.3.3. b) Shrinkage of the cuticula of a gnathopod in an embryo (two to three days from hatching). The main difference in leg morphology is shown in the propodus-dactylus-complex. The dactylus is not yet able to close fully to the propodus. This will change within two to three days of development. Image made with a confocal microscope and Amira 5.3.3.

At the time around hatching the biggest growth of the muscle volumes in the five distal segments happens in the propodus. Muscles of this segment grow in approximately three days up to 114-157 times (Figure 17). Second with an increase of 90-149 times is the merus. This is not a surprise knowing that within the embryo this segment there is just one tiny muscle yet developed. During the last days inside the marsupium the carpus grows 50-60 times. The least expansion happens in the basis-ischium flexor, this muscle shows also the least muscle volume in full-grown specimens. The complete muscle mass including five segments is growing in those two to three days, 67 times when the embryo's leg represents the first gnathopod and 88 times when it would be the second one.

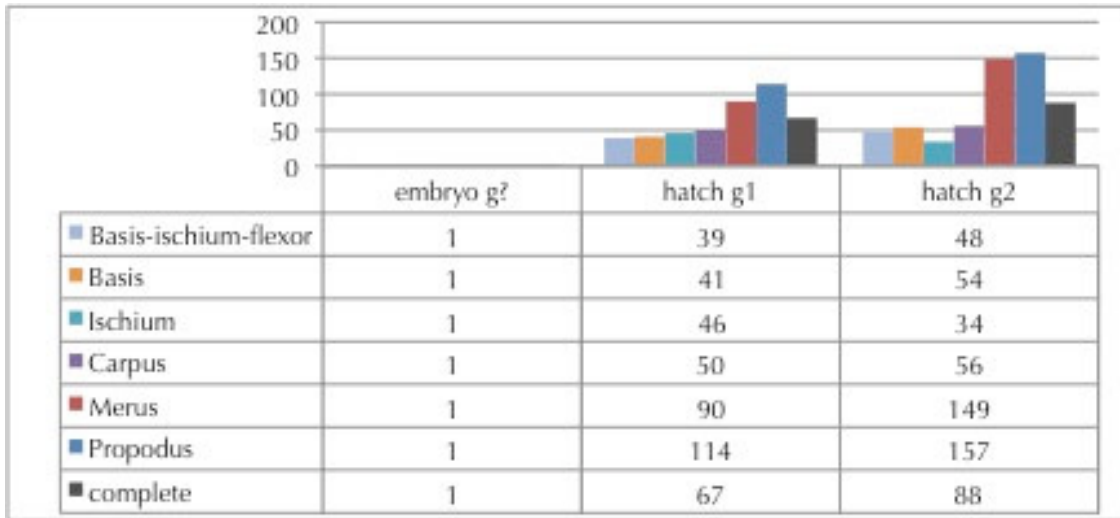


Figure 17: Rate of growth in gnathopods from an embryo (about two to three days from hatching) to a freshly hatched individual. The modified leg of the embryo is not dedicated to one specific gnathopod.

4.2 Development of walking legs

In regular walking legs the distribution of muscles in the single segments significantly differs from gnathopods. Here the basis represents the segment with the biggest muscle mass (Table 2). In gnathopods the merus is the segment with the second most musculature. In walking legs (Figure 18) the merus includes the second least muscle volume, followed by the propodus, but only in the reconstructed embryo not in the compared small male. Here the propodus is the segment with the least muscle mass (Figure 19).

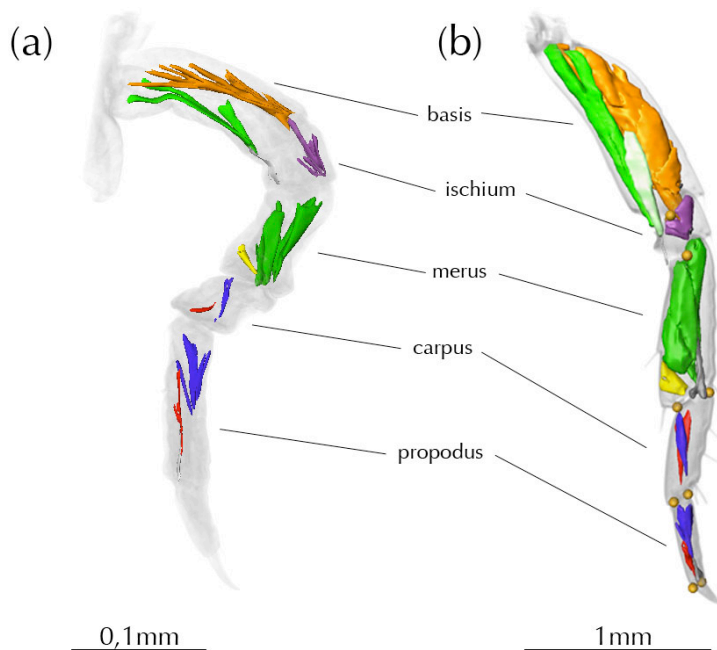


Figure 18: Regular walking legs (peraeopod) of *Dikerogammarus villosus*. a) Walking leg of the embryo (two to three days from hatching). b) Walking leg of a sexual mature small (01) male.

Table 2: volume table of all reconstructed pereaeon appendages. All data in mm³.

Gnathopod 1	Embryo (g1 or g2?)	hatchling	Small (01) female	Small (02) female	Medium female	Large female	Small (01) male	Small (02) male	Medium male	Large male
Propodus	0,00000394	0,000451	0,001035	0,003128	0,01768	0,03145	0,018701	0,028455	0,06831	0,14925
Carpus	0,00000167	0,000084	0,000308	0,000768	0,00304	0,00687	0,003355	0,004778	0,01416	0,03829
Merus	0,00000041	0,000037	0,000135	0,000334	0,00116	0,00246	0,001719	0,002529	0,00492	0,01058
Ischium	0,00000071	0,000033	0,000094	0,000242	0,00075	0,00167	0,001343	0,001985	0,00363	0,01115
Basis	0,00000553	0,000228	0,000797	0,001904	0,00945	0,01586	0,011012	0,016618	0,03877	0,10127
Basis-ischium-flexor	0,00000054	0,000021	0,000042	0,000106	0,00063	0,00088	0,000539	0,000615	0,00236	0,00599
Sum	0,00001281	0,000853	0,002411	0,006483	0,03271	0,05919	0,03667	0,05498	0,1321	0,3255
Gnathopod 2	Embryo (g1 or g2?)	hatchling	Small (01) female	Small (02) female	Medium female	Large female	Small (01) male	Small (02) male	Medium male	Large male
Propodus	_____	0,000618	0,001016	0,003304	0,03370	0,06751	0,026729	0,042165	0,22192	0,7181
Carpus	_____	0,000093	0,000350	0,000898	0,00442	0,00967	0,003395	0,005892	0,02601	0,09599
Merus	_____	0,000061	0,000258	0,000593	0,00219	0,00415	0,001546	0,002387	0,01129	0,03319
Ischium	_____	0,000024	0,000109	0,000217	0,00119	0,00199	0,0009	0,001305	0,00488	0,01771
Basis	_____	0,000301	0,000938	0,002083	0,01037	0,02213	0,009505	0,012243	0,06184	0,21443
Basis-ischium-flexor	_____	0,000026	0,000098	0,000125	0,00084	0,00144	0,000427	0,000759	0,00362	0,00887
Sum	_____	0,001124	0,002771	0,007221	0,05272	0,1069	0,042503	0,064752	0,3296	1,0883
Pereopod 4	Embryo (g1 or g2?)	hatchling	Small (01) female	Small (02) female	Medium female	Large female	Small (01) male	Small (02) male	Medium male	Large male
Propodus	0,0000022						0,000586			
Carpus	0,00000041						0,000624			
Merus	0,0000089						0,008233			
Ischium	0,0000013						0,001141			
Basis	0,0000104						0,019069			
Basis-ischium-flexor	_____						0,001177			
Sum	0,0000232						0,03083			

Also the rates of growth within regular walking legs vary a lot to gnathopods. The fastest growing segment with the highest increase of muscle volume is the basis (Figure 19). This segment grows from an embryo until it becomes a sexual mature small (01) male 1833 times in sum of volume size. Second is the carpus, which represents the segment with least muscles in the embryo's limb (Figure 19).

These muscles grow in the same development experiment 1560 times. The propodus once again bottoms of the table. In this segment the growth on muscle volume is comparatively small (266 times). Muscles in the ischium and merus grow approximately equivalent and represent the muscles with the second least increase.

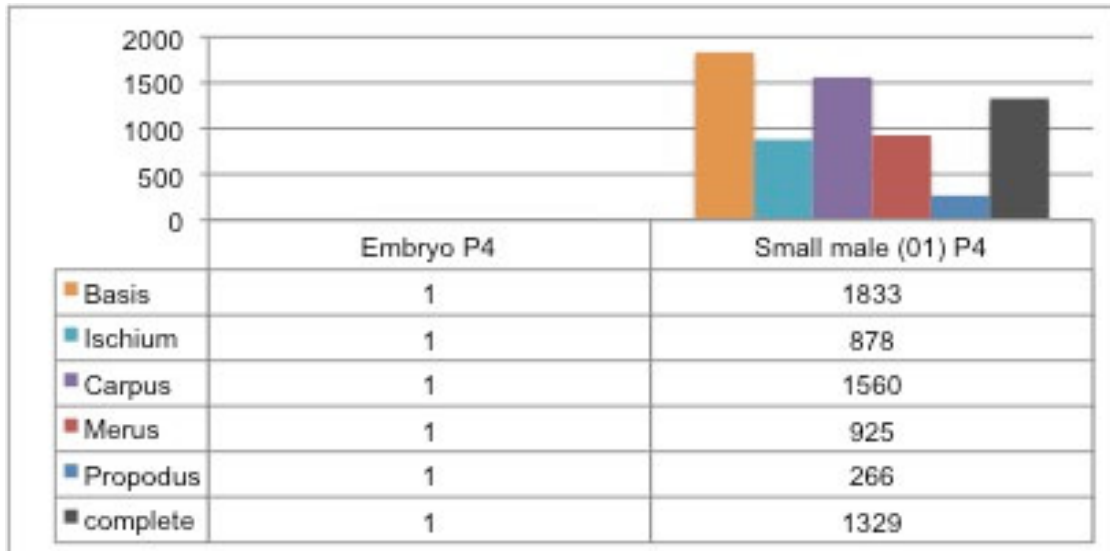


Figure 19: The rate of growth (walking leg) between an embryo (about two to three days from hatching) and a small (01) sexual mature male. The basis-ischium-flexor is not listed because it is not yet developed in the embryo.

5. Size-assortativity in pre-copula pairs

The comparison between males and females in body length show that the proportions between them stay about the same in every developmental stage (Figure 20). In the four investigated pre-copula pairs, mating was size-assortative.

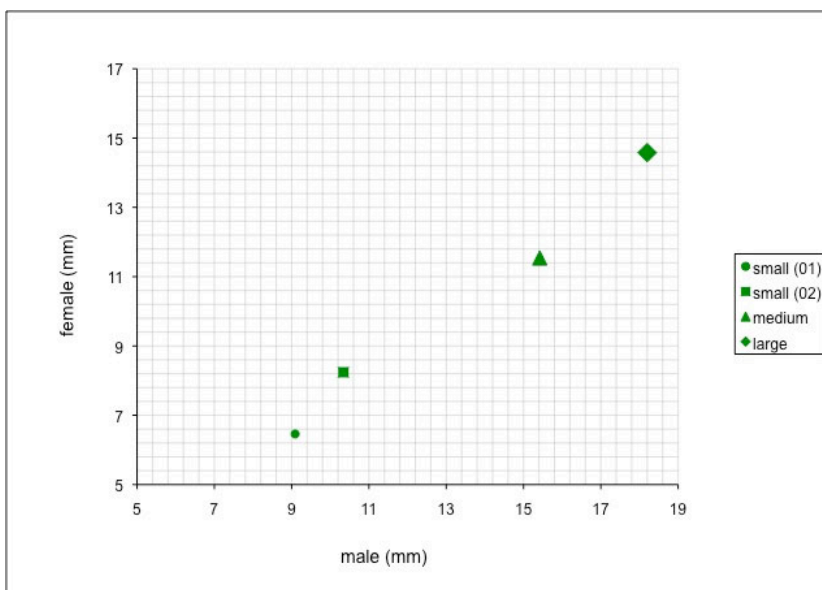


Figure 20: Size relation (body length) of paired specimens. The dot stays for small (01), the square for small (02), the triangle for medium sized and the rhomb for large pre-copula pairs.

Discussion

Differences in articulations between walking legs and gnathopods

The normal walking leg (peraeopod) of *Dikerogammarus villosus* (Sowinsky, 1894) has only two main planes of movement in the five distal articulations in contrast to the three main different planes of movement, which are found in the modified leg (gnathopod). More planes of movement are a prerequisite for more complicated and precise movements like manipulating things and for the typical amplexus where the first and second gnathopods of males are used to hold the female in a fixed position. The three different planes of movement in gnathopods were managed with shortened merus and carpus segments, distorted articulation-planes and for this, the distorted insertion of the muscles. The main reason for the additional degree of freedom within modified gnathopods is the distorted articulation-plane of the merus. This segment which is connected to the carpus is about 90° distorted compared to a regular walking leg. The novelty of this modification makes the animal able to tip the distal segments in a clockwise or anti-clockwise direction (Figure 6c). Due to those modifications it is possible to move the propodus-dactylus complex more precisely. Besides the possibility to carry out very fine and precise manipulations with gnathopods, the subchelate dactylus and the large volume of the closer muscle, which lies in the propodus, allows exerting of big bite forces on food items.

Leg musculature

Every segment is moved by two antagonistic muscles. For that reason every segment possesses one possible plane of movement. One muscle modification can be found in the basis and ischium segments, where the flexor muscles in the basis moves both following segments. Therefore these segments are in the same plane of movement. These muscles have its origin in the basis and its insertion in the ischium as well as the merus segment. The so-called basis-ischium-flexor, connected to a tendon, moves the merus up and the antagonistic muscles to that (extensor muscle in the ischium) moves the ischium down from the animal.

One reason for the combined flexor is the very short ischium segment, which does not include extensor muscles. Both articulations need to manage a broad distance of movement and so the muscles of the ischium would be too short to manage such a long distance.

In general, the modified gnathopod is far stronger which might be the reason for their higher selective pressure using them for amplexus.

The stronger musculature in the gnathopod is also important for the use of the dactylus to crush and manipulate food items whereas the walking legs dactylus is just used for clamping on substrate and walking.

Leg muscle growth

Comparing males and females of similar body sizes one can observe that the developmental trajectories of gnathopod muscle growth are pretty similar regarding growth rates at specific body sizes (Figure 12).

Still, male gnathopods are larger in every stage, meaning that they already grow faster before the animals

reach sexual maturity. Large males have up to 900 times bigger muscle volume in the five distal segments of the second gnathopod than the hatchling. The female muscle volume is just 90 times bigger in the large female than in the hatchling (Table 2). In this work we can only compare those rate of growth with just one hatchling on which we do not know the gender. The growth between small, already sexual mature individuals and large ones is 38 times in the second and nearly 25 times in the first gnathopod within females. Male modified legs grow 25 times in the second limb and nearly nine times in the first. Showing anew that males have larger gnathopods in every period of their development but the legs in females grow faster after they reach sexual maturity. This also shows that the proportions between first and second gnathopods rise the whole developmental period and the increase is even bigger in males. For example, the second gnathopod in the large male is 3,3 times bigger and in the large female it is 1,8 times bigger than in the first gnathopod. In small (01) individuals the proportions are 1,16 in male and also in the female. For propodus muscles the proportions between legs are even bigger but the differences between sexes are about the same as in all segments together. Another difference comes with the ratio of muscles in every segment. The second gnathopod in males has up to 67% of all the muscle volume within the five distal segments just in the propodus. In large females the propodus includes 63% of all the muscle mass within the second gnathopod. This shows the importance of this special segment in gnathopods to move the dactylus and crush things with it. The only disparity on those proportions is found in small female individuals where the first gnathopod includes more muscle volume in percent within the propodus as the second one. Another special thing on those proportions (Figure 14) is found in female's first gnathopods. When comparing same sized individuals of both sexes one can see that medium and large sized females include more muscle volume in the propodus than equal sized males do. Different to the second gnathopods, where the propodus includes more muscle volume in every developmental stage.

Walking legs are not as specific as modified gnathopods and do not show the typical subchelate propodus-dactylus joint. Here the propodus and the carpus are including the least muscle mass, whereas in modified gnathopods the ischium and merus have the smallest muscle mass. The muscle volume in walking legs is highest in the basis segments in both individuals I worked on and shows the biggest analogy to modified gnathopods where the basis includes the second most muscle volume and has about the same eidonomy as the regular walking leg does (Figure 4). This is followed by the merus, which owns just a little less muscle mass. Also the rate of growth between the embryo and the small (01) male (Figure 19) is highest in the basis and here the carpus represents the segment with the second highest rate of growth. The rate of the carpus is that high because of the fact that the muscle volume in this segment is the far smallest of all segments in the embryo. The rate of growth is smallest within the propodus and shows the biggest disparity between regular walking legs and modified gnathopods.

These legs are used just for moving the body and the remaining segments do not need as much power as modified legs do. Because of the very little sample size of only two individuals the result can differ a little when investigating a larger sample.

Embryonic development of gnathopods

The most important time in muscle development happens in the last two to four days within the brood pouch. Embryos, which are about four days away from hatching, do not show any differentiated muscles in their legs. The embryo I worked with was one to three days from hatching because here every muscle except two (in carpus and merus) were visible in the gnathopod (Figure 15) and had about the same shape as in older individuals. So the next two to three days the muscles grow up to 88 times in volume, when we suppose that the scanned gnathopod of the embryo represents the second modified leg, and after that the hatchling nearly fills out the cuticula with muscles. Not only the muscles grow, also the outer surface of the gnathopods, more than double their length. The rate of growth is about the same as in sexual mature individuals which means that the propodus is on top of this rate. This segment grows in those two to three days 114 to 157 times depending on which gnathopod the scanned embryo represent (Figure 17). Against the rate of growth in sexual mature individuals where the merus and ischium grow least, the merus in the embryo represents the segment with the second most increase of muscle volume until they hatch. One reason for that is the fact that in the embryo's gnathopod there is just one muscle yet developed (Figure 15). The other three segments (basis, ischium and carpus) show about equal rate of growth with an increase of muscle volume between 39 to 56 times.

In the embryo the dactylus propodus complex does not have the typical subchelate form (Figure 16b). They gradually obtain their gnathopod identity around the time of hatching.

Against the literature (Browne, Price et al. 2005) where all embryos are deposited from the marsupium within one hour the time, in the present study depositing of embryos took up to two days. The embryo I worked with was removed from the marsupium, three days before every embryo was released from the female as a hatchling. After I collected the embryo the female deposited hatchlings every following day. So the embryo in this was one to three days from hatching. Because of the muscle development we estimate that the embryo need to be two to three days from hatching.

Technical issues

Every single muscle was reconstructed by hand with the computer program Amira 5.3.3. This process can falsify the results in every muscle on every leg. Specially the small (01) female, the legs of the hatchling and the embryo were very difficult to reconstruct because of the low resolution of the scans. This complicated the work because sometimes it was hard to find the border of single muscles (Figure 3). The confocal scans of the embryo were even worse because of the fact that sometimes it was hard to say if certain structures represent muscles or yet undifferentiated cells.

This problem changes the result just a little bit because the amount of muscle volume in one leg is so little and there are up to 30 muscle bundles in every appendage. Only the proportions between the appendages can deviate a little bit from the reality especially in the rate of growth within the embryo's legs.

The shrinkage of the muscles, conditioned by the fixation in 70% ethanol, is one more reason why the results deviate from the living. Still, the proportions between the individuals should not be affected because the effect of shrinkage is equal to every individual, whereas the absolute volume data of the muscles is a little smaller then it would be without the fixation.

The measurement of head and body length can diversify a little bit. Especially in the body length, the measured data can vary from real length, since body segments can interlock with each other. When the animals are straightened the body length is smaller compared to crooked bodies. In this work the body lengths were measured in straightened bodies and so the measurement error for body length is expected to be very small. For the accuracy of the body length the length of the head was measured. The proportions between head and body length were very similar and varied between 1 : 7,9 and 1 : 9,2 (Table 1). In this work I only used the length of the body for better representative result.

Abstract

Using micro-CT and a confocal microscopy, tomographic 3D images of different peraeon appendages of the invasive gammarid *Dikerogammarus villosus* (Sowinsky, 1894) were acquired.

The emphasis of the study was placed on the six distal leg segments including the leg musculature, its origins and insertions in comparison of both sexes. We measured the volume for all muscles of these leg segments and analyzed the planes of movement for every dicondylic joint of two different leg types. To compare different sexes and different developmental stages of the gammarid we also measured the length of the head and body. The subchelate form of the dactylus and the shortening of the merus and carpus is one of biggest modifications of the gnathopods eidonomy in comparison to regular walking legs. In addition to the shortened leg segments, the articular facets are displaced in respect to the leg axis, as are the insertions of the musculature at the joints. Both leg-types have unusual basis-flexor-muscles, which move both the ischium and merus segments. This is the reason why the ischium has no extensor-muscles. Based on these modifications, the gnathopods have three main different planes of movement within the five distal articulations, whereas walking legs possess only two main planes of movement in these parts of the leg. This gain of an additional degree of freedom makes the modified gnathopod able to move very smooth and much more precise than a normal walking leg.

Gnathopods within pre-hatching embryos in a developmental stage where the muscles start to grow do not show the typical subchelate form of the dactylus. In this stage they look very similar to normal walking legs and differ to the shape of gnathopods within the next days.

Within male individuals both modified gnathopods are larger in every developmental stage compared to females. Also the proportions between first and second gnathopods are bigger in males. Only the rate of growth between small and large sexual mature amphipods is higher within females.

Zusammenfassung

Micro-CT und Konfokalmikroskop wurden verwendet um dreidimensionale Bilddaten von drei verschiedenen Extremitäten des Pereons eines invasiven Gammariden, *Dikerogammarus villosus* (Sowinsky, 1894), zu erstellen. Dabei lag das Hauptaugenmerk auf den sechs distalen Beinsegmenten inklusive Muskulatur, sowie den Muskelansatzstellen an der Kutikula. Diese wurden dann zwischen den beiden Geschlechtern verglichen. Für alle Beinsegmente wurde das Muskelvolumen gemessen und die Bewegungsebenen der einzelnen Gelenke von zwei unterschiedlichen Beintypen analysiert. Für den Vergleich der beiden Geschlechter und den verschiedenen Entwicklungsstadien der Gammariden wurde die Länge des Kopfes und des Körpers gemessen.

Die subchelate Form des Dactylus und die Verkürzung der Segmente Merus und Carpus sind eine der größten Modifikation der Gnathopoden im Vergleich zu einem herkömmlichen Laufbein. Zusätzlich sind die Gelenksflächen und die daraus resultierenden Muskelansatzstellen verschoben. Beide Beintypen haben ungewöhnliche Basis-Beuger-Muskeln welche die beiden folgenden Segmente, Ischium und Merus, bewegen. Deswegen besitzt das Ischium keine Streckermuskeln. Basierend auf diesen Modifikationen erlangt der Gnathopod drei Hauptbewegungsebenen in den fünf distalen Segmenten. Das herkömmliche Laufbein weist bei gleichen Bedingungen nur zwei Bewegungsebenen auf. Dieser zusätzliche Freiheitsgrad des modifizierten Gnathopoden ermöglicht eine exaktere und präzisere Bewegung als es dem herkömmlichen Laufbein möglich ist. Gnathopoden eines noch nicht geschlüpften Embryos, dessen Muskeln bereits begonnen haben zu wachsen, zeigen noch nicht die typische subchelate Form des Dactylus. In diesem Stadium sehen sie einem herkömmlichen Laufbein sehr ähnlich und differenzieren sich erst innerhalb der folgenden Tage.

In jedem Entwicklungsstadium sind beide Gnathopoden der männlichen Individuen größer als bei Weiblichen. Auch die Proportionen zwischen erstem und zweitem Gnathopoden sind bei Männchen immer größer. Nur die Wachstumsrate der geschlechtsreifen kleinen bis großen Amphipoden ist bei weiblichen Tieren größer als bei männlichen.

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