

The evolutionary transition to sideways-walking gaits in brachyurans was accompanied by a reduction in the number of motor neurons innervating proximal leg musculature



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ABSTRACT

The forwards-walking portly crab, *Libinia emarginata* is an ancient brachyuran. Its phylogenetic position and behavioral repertoire make it an excellent candidate to reveal the adaptations, which were required for brachyuran crabs to complete their transition to sideways-walking from their forwards-walking ancestors. Previously we showed that in common with other forwards-walking (but distantly related) crustaceans, *L. emarginata* relies more heavily on its more numerous proximal musculature to propel itself forward than its sideways-walking closer relatives. We investigated if the proximal musculature of *L. emarginata* is innervated by a greater number of motor neurons than that of sideways-walking brachyurans. We found the distal musculature of spider crabs is innervated by a highly conserved number of motor neurons. However, innervation of its proximal musculature is more numerous than in closely-related (sideways-walking) species, resembling in number and morphology those described for forwards-walking crustaceans. We propose that transition from forward- to sideways-walking in crustaceans involved a decreased role for the proximal leg in favor of the more distal merus–carpus joint.

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1. Introduction

The behavioral repertoire and research amenability of decapod crustaceans have secured their choice as model systems for well over a century (for overview see Atwood, 1977; Hoyle, 1976; Schram, 1986; Wiens et al., 1988). Reptantian species in particular have been the subject of intense research on the production of legged locomotion (Macmillan, 1975; Ayers and Clarac, 1978; Clarac, 1977; Sillar and Skorupski, 1986; Jamon and Clarac, 1995; Martinez, 1996; Martinez et al., 1998; Cattaert and Le Ray, 2001). Much of what we now know about crustacean locomotion comes from studies on forwards-walking macrurans (Macmillan, 1975; Jamon and Clarac, 1995; Cattaert and Le Ray, 2001), or sideways-walking brachyurans (Clarac et al., 1987; Martinez et al., 1998). An integrative picture of crustacean locomotion is undoubtedly being consolidated at the moment. Researchers, however, often find themselves mixing and matching knowledge from disparate species with very

diverse ancestries or behavioral repertoires in order to arrive at a comprehensive approach to the study of the production of behavior. The need to find a system able to bridge the gap between the macruran and brachyuran literatures led us to select the ancient portly spider crab, *Libinia emarginata* for our studies. *L. emarginata* is a brachyuran that walks preferentially forwards 80% of the time (Schreiner, 2004). As a majoid, *L. emarginata* belongs to the group thought to have first evolved the crab form from lobster-like ancestors (Rice, 1983; Morrison et al., 2002). As an evolutionary and behavioral midpoint between typical macrurans and modern brachyurans, *L. emarginata* is an ideal candidate to study the adaptations that accompanied the transition from forwards-walking in ancient brachyuran ancestors to the modern, and highly successful, sideways-walking strategy unique to this group.

Previously, we showed that the skeletal anatomy of *L. emarginata* has adaptations that allow for the housing of more muscle bundles than those found in related sideways-walking species (Vidal-Gadea et al., 2008; Vidal-Gadea and Belanger, 2009). This permits the possibility of *L. emarginata* having some of these additional bundles independently innervated and activated (addressed in a future manuscript).

The typical brachyuran limb consists of six segments articulating with each other by bicondylar joints that restrict motion to

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single planes perpendicular to the adjacent joints (Fig. 1). During locomotion, crustaceans walking sideways rely heavily on the distal MC joint (merus–carpus, see Table 1 for abbreviations used), while they use primarily their proximal joints (TC, CB) while walking forwards (Ayers and Clarac, 1978). The motor neurons innervating the distal leg musculature of crustaceans (that of segments distal to the plane of autotomy) are highly conserved (in number, morphology and location) among decapods (Faulkes and Paul, 1997; Wiersma and Ripley, 1952). They consist of a pair of excitatory motor neurons innervating each muscle (with the exception of the MC-flexor which has four), a common inhibitory neuron (CI) that innervates every leg muscle, and an opener inhibitor that innervates the opener muscle of the dactyl. Greater variability has been reported in the (more numerous) motor neurons innervating the proximal musculature of the legs (Antonsen and Paul, 2001; Bévangut et al., 1983; El Manira et al., 1991a,b).

This work is the third in a series studying the neuroethology of forwards-locomotion in the spider crab, *L. emarginata*. We had two aims: to provide an anatomical framework for future neuroethological studies in *L. emarginata*; and to compare the neural anatomy of a forwards-walking brachyuran to that described for sideways-walking brachyurans, and forwards-walking macrurans. We employed standard physiological techniques to identify the nerves serving the leg musculature, and standard anatomical techniques to label and characterize the numbers, and location of all the motor neurons serving the leg musculature.

2. Materials and methods

2.1. Species used

L. emarginata crabs ($n > 100$) were obtained from the Marine Resources Center of the Marine Biological Laboratories in Woods Hole, Massachusetts. Crabs were kept at 20 °C in artificial seawater until used.

2.2. Dissections

Animals were cold anesthetized and euthanized before each dissection. We performed dorsal dissections by removing the carapace and inner organs until exposing the thoracic ganglia (TG) and endophragmal skeleton housing the proximal musculature. Alternatively, we dissected ventrally by removing the abdomen and ventral skeleton to expose TG. This was particularly useful since in *L. emarginata* the TG is not occluded ventrally by leg musculature. The preparations were immersed in 4 °C crab saline (Rathmayer

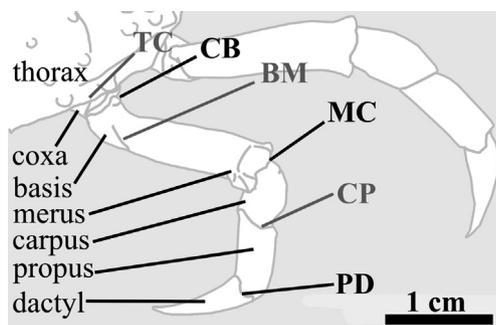


Fig. 1. The segments and joints composing the legs of *L. emarginata*. The six segments that make up the legs of *L. emarginata* articulate with each other via bicondylar joints that restrict movement to the sideways direction (black), of the forwards direction (gray). Anterior is up.

Table 1
Abbreviations used throughout this work.

| AcF | Accessory flexor excitor motor neuron |
|-----------------|--|
| BE α | Anterior bender excitor motor neuron |
| BE β | Posterior bender excitor motor neuron |
| BM | Basischiopodite-meropodite joint |
| CB | Coxopodite-basipodite joint |
| CE | Closer excitor motor neuron |
| CI | Common inhibitor motor neuron |
| CP | Carpopodite-propodite joint |
| EE | Extensor excitor motor neuron |
| EMG | Electromyography |
| Flex | Extensor excitor motor neuron |
| IGP | Inter-ganglionic processes |
| LTN1-8 | Left thoracic neuromeres one through eight |
| MC | Meropodite-carpopodite joint |
| OI | Opener inhibitor |
| Op \equiv Str | Opener and stretcher excitatory motor neuron |
| PD | Propodite-dactylopodite joint |
| RE α | Anterior remotor excitor motor neuron |
| RE β | Posterior remotor excitor motor neuron |
| RTN1-8 | Right thoracic neuromeres one through eight |
| SI | Stretcher inhibitor |
| TAG | Thoraco-abdominal ganglia |
| TC | Thorax-coxopodite joint |
| TG | Thoracic ganglia |

and Erxleben, 1983) with 1% methylene blue to stain the leg nerves. Throughout the dissection, methylene blue was added with a pipette to freshly dissected areas.

2.3. In vitro physiological recordings

Hook electrodes were mounted proximally on leg nerves for stimulation of individual roots. An *en passant* suction electrode was then placed distal to the stimulating electrode to record neural activity. Lastly, a pair of silver wire electrodes was inserted in the muscle of interest for EMG confirmation of the motor nerves activated during stimulation. The recorded signals were amplified by an amplifier (AM Systems) and fed into Datapak 2K2 software (Run Technologies, Mission Viejo, CA) for storage and analysis. Stimuli were administered using an AM Systems stimulator, and consisted of 1 ms pulses of increasing amplitude and/or frequency. We confirmed the identity of the nerves stimulated by observing the motion produced in the limb following stimulation, as well as by the presence of EMG activity in the muscle in question. If a nerve innervated multiple muscles, a glass electrode was used to defasciculate the different populations and to proceed with the stimulation and retrograde staining.

2.4. Retrograde staining

Once a nerve containing a motor neuron population of interest was identified the nerve was cut distally, removed with the TG still attached, and backfilled using standard techniques. We performed backfills using CoCl₂ (Pitman et al., 1972), or using CoCl₂ and NiCl₂ + CoCl₂ for dual color labeling (Quicke and Brace, 1979). After processing, the ganglia were dehydrated in alcohol series and cleared in methyl salicylate.

2.5. Image capture and manipulation

We photographed the resulting backfills using a camera-mounted Leica dissecting stereomicroscope. Images were adjusted for brightness and contrast using Corel Photo-Paint. Optic sections though backfills were traced on Corel Photo-Paint to combine different focal planes or partial backfills together.

3. Results

3.1. Thoracic nervous system and general ganglia morphology

Ventral dissection of the skeleton of *L. emarginata* revealed the thoracic ganglia (TG) to be located medially and anteriorly in the thorax of the animal. Because the ventral aspect of the thorax is not laterally compressed, the ganglia lie in close apposition with the skeleton (and removed from the muscular machinery that occludes it in many decapods). This allows direct ventral access to the TG without disturbing the leg musculature. Although in smaller specimens (2–3 cm wide) the ganglia are located medially within the thorax, in larger animals (5–7 cm wide) they are found more anteriorly. This causes most of the leg nerves (except those of the first pair) to travel posteriorly a considerable distance to the muscles they serve. This observation allowed us to stimulate and record from the exposed leg nerves of *L. emarginata* without disturbing the leg musculature.

The TG of *L. emarginata* consist of the fused thoracic neuromeres (one through eight, TN1–8) and the fused abdominal pleomeres partially overlying the last pair of thoracic neuromeres. The thoracic

sternal artery that bisects the TG of *Procambarus*, *Callinectes* and *Carcinus* does not pierce through *L. emarginata*'s. Instead this artery covers the dorsal surface of the ganglia and bifurcates into smaller vessels that follow the leg nerves into the pereopods. Mirroring the radial arrangements of the pereopods around the thorax, the ganglia also assume a radial orientation as one moves posteriorly. This difference in orientation becomes clear when observing the position of homologous motor neurons in different neuromeres.

3.2. Nerve roots

The exit routes of the leg motor neurons follow a similar plan to that described for *Procambarus* (see Cattaert and Le Ray, 2001) and *Carcinus* (Bévengut et al., 1983), but we observed variability between specimens. This led us to use physiological methods to confirm the identity of the motor neurons contained in any particular nerve. Although we did not quantify it here, stimulation of the different nerve roots exiting the ganglia revealed the location of motor nerves to vary between large and small specimens, as well as between anterior and posterior neuromeres (Fig. 2). The number

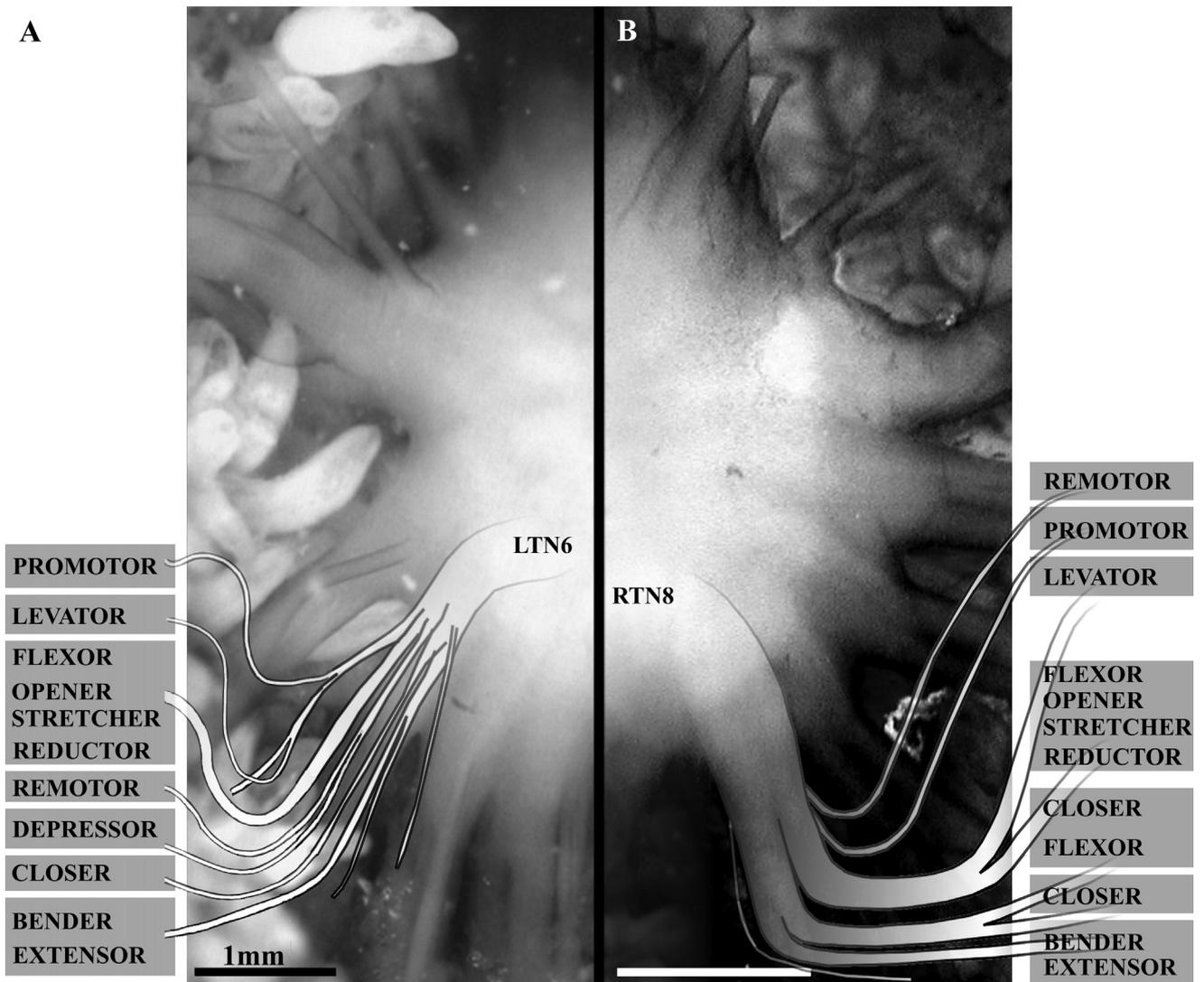


Fig. 2. Comparison between the thoracic ganglia (TG) of a medium (A), and small (B) sized *L. emarginata* showing examples of the exit routes of motor axons to the third (A), and the fifth (B) legs. The nerve roots, which exit the ganglia and innervate the leg musculature, show different degrees of defasciculation in animals of varying sizes as well as between anterior and posterior legs. The degree to which nerves defasciculate en route to their target muscles increases with the distance between the TG and the leg musculature. Nerve roots emerging from the neuromeres of larger animals show more defasciculation (A) than those from smaller ones (B). Ventral view, anterior is up.

of individual nerves arising from each neuromere increased for larger animals and for the posterior pereopods. This becomes obvious when looking at the anastomosis described in *Carcinus* and *Procambarus* (Moffett et al., 1987; Cattaert et al., 1992), in *L. emarginata* it is most pronounced for posterior limbs and all but disappears for anterior ones.

3.3. Distal innervation

A total of 19 somata were backfilled from nerves innervating the distal musculature of the legs of *L. emarginata*. We will describe the location of the motor neurons innervating the distal leg musculature and for practical reasons have chosen to group this description by the exit routes of the motor neurons rather than by their target muscles.

3.4. Closer excitators

The single closer muscle of *L. emarginata* is responsible for flexion of the dactyl segment at the propodite-dactyl joint. It is innervated by two motor excitatory neurons (Fig. 3A) and by the common inhibitor neuron that innervates every muscle in the leg of *L. emarginata* (not shown). Backfills of this nerve yielded two closely associated somata. These are located dorsally on the medial anterior surface of the ganglion and send their processes a short distance ventrally and then they turn posteriorly converging into a

common track that turns distally and exits along a private nerve. The number of somata was confirmed by physiological recordings from the same nerve (Fig. 3B).

3.5. Bender excitators

The bender muscle is responsible for flexion of the propodite in the anterior direction and (with the exception of the first pereopod), consists of a single muscle. It is innervated by two excitatory somata, and by the common inhibitor neuron (Fig. 4A, C and D). The two bender excitators lie dorsally on the ganglion but differ otherwise in location. The most anterior bender excitator ($BE\alpha$) lies anteriorly on the medial half of the ganglion. The second bender ($BE\beta$) lies posteriorly on the ganglia and tends to be found more distally than the $BE\alpha$.

3.6. Extensor excitators

The extensor muscle is responsible for extending the carpopodite (at the merus–carpus joint). There are two excitator cells for this muscle and they usually travel on the same nerve root as the bender, often requiring defasciculation from the latter prior to backfilling. These two cells lie dorsally on the ganglion (Fig. 4B, D) and are distal to the aforementioned closer excitators. The most medial of these cells is slightly larger and more anterior than its counterpart.

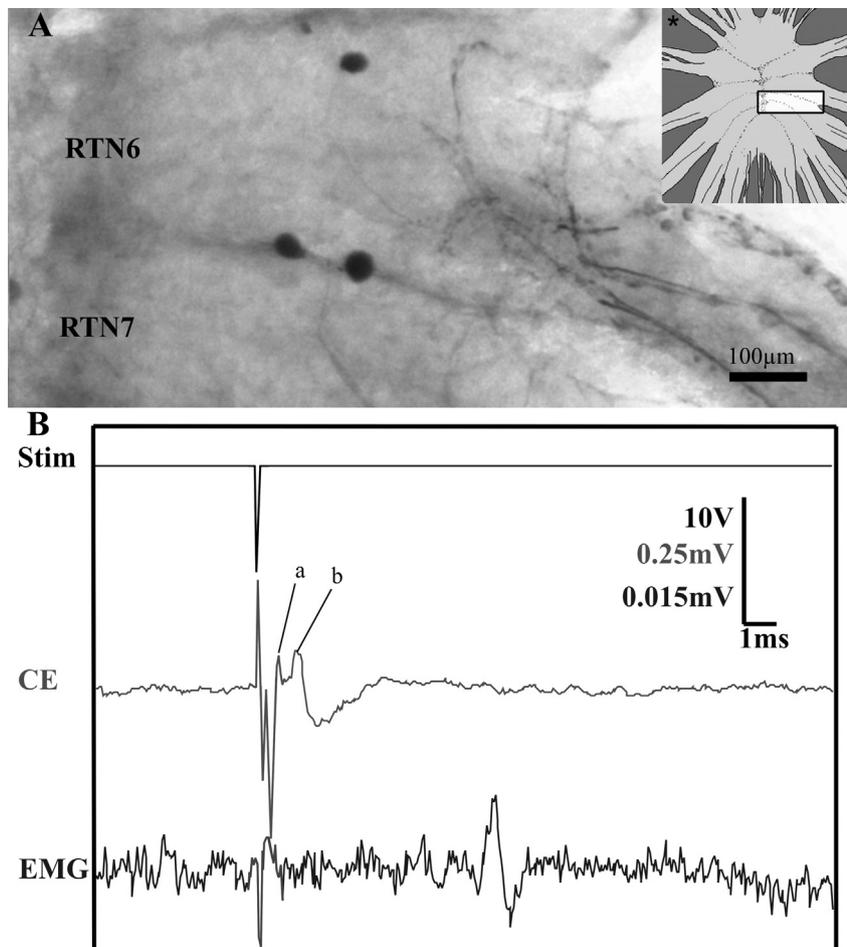


Fig. 3. A) Closer excitor somata of the right fifth and sixth thoracic neuromeres. B) Recording from the closer excitor neurons (CE) confirming the presence of two different (a and b) cells in the closer nerve. The ensuing activation of the single closer muscle is observed a few milliseconds later as a compound potential. Left is proximal and anterior is up.

3.7. Flexor excitators

The flexor musculature of *L. emarginata* is responsible for the flexion of the merus–carpus joint. The flexor motor neurons exit the ganglia and enter the leg in the same nerve as the reductor excitators, and the single opener \equiv stretcher excitators (Fig. 2). Backfills of this nerve revealed the presence of seven different cells (Fig. 5A and B). All the cells were found dorsally on the ganglion with seven clustering anteriorly on the middle of the ganglionic surface.

In addition to the somata backfilled, several inter-ganglionic processes (IGP) were also consistently filled in flexor preparations. Several of these axons originated from cells lying medially (both ipsilaterally and contralaterally) on the anterior neuromeres (Fig. 5D and F).

3.8. Reductor excitators

Two reductor muscles within the basi-ischiopodite of *L. emarginata* are responsible for pitching the leg axis forwards at the basis–merus joint. Two dorsal excitatory motor neurons were found to innervate this muscle. The most anterior lies medially on the ganglia, just distal to the flexor excitators. The second reductor excitator soma is located dorsally on the center of the ganglia (Fig. 5C).

3.9. Accessory stretcher and additional somata

In addition to the anteriorly located somata described above, we stained four additional somata posteriorly on the ganglion (Fig. 5D and F). Based on the similar location of these cells to the ones described in previous work on other decapod crustaceans (Faulkes and Paul, 1997) we believe these to be the somata of the common inhibitor (CI), located on the posterior medial corner of the ganglion, deeper than the excitatory somata. Closely associated with CI (but more dorsal and distal), a second cell was stained in the same location as the previously described Stretcher Inhibitor (SI, Wiens and Wolf, 1993). A third cell also lying posteriorly was more ventral than the others and behind the flexor cluster. This is likely the opener inhibitor (OI). The most distal of the four posterior somata lies adjacently to OI, although not as ventrally and is likely the accessory flexor soma previously described in related species (see Faulkes and Paul, 1997).

3.10. Opener \equiv stretcher excitators

The opener muscle is responsible for extension of the dactyl and shares a single excitatory innervation with the stretcher muscle (responsible for extension of the propodite). By defasciculating the opener and backfilling the remaining nerve, we were able to identify this neuron (Op \equiv Str) to be adjacent

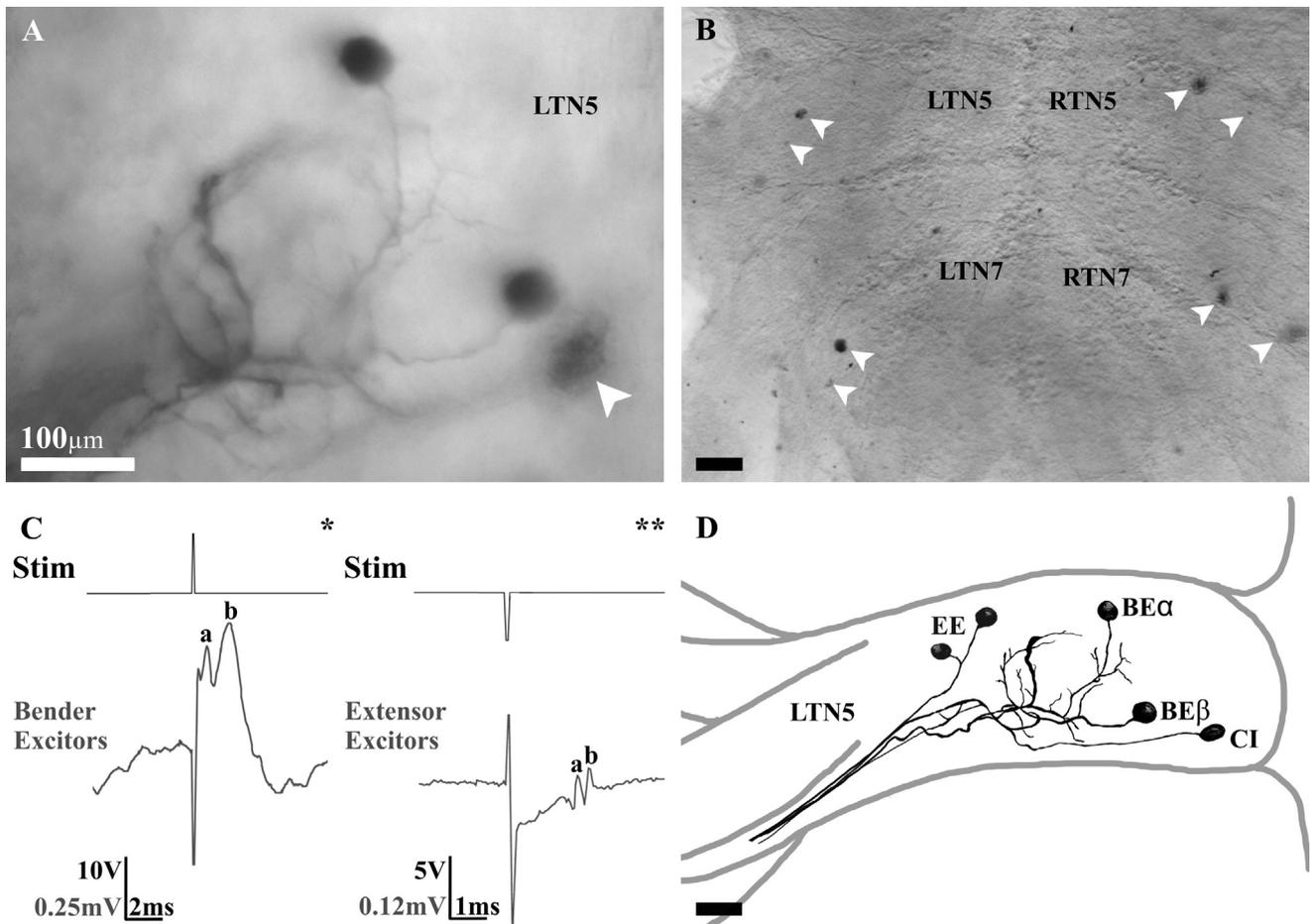


Fig. 4. Dorsal view of retrograde stainings showing the bender and extensor excitators. **A**) Two bender excitators ($BE\alpha$ and $BE\beta$), were stained with $NiCl_2 + CoCl_2$ for precipitation with Rubeanic acid. The white arrow head shows the position of the common inhibitor neuron. The two extensor excitators (EE) often leave the ganglia in the same nerve root as the bender excitators. Anterior is up and medial is left. **B**) Backfill with $CoCl_2$ showing the pair of extensor excitators (arrow heads) innervating the extensor muscle of the second and fourth pair of pereopods. Anterior is up. **C**) Physiological recordings confirming the number of cells innervating the bender (*), and extensor (**) musculature. **D**) Diagram showing the relative position of the benders, extensors, and the common inhibitory neurons within the ganglion. Dorsal view, anterior is up and medial is right.

to the flexor excitors and between the two reductor somata (Fig. 5E).

3.11. Proximal innervation

Retrograde staining of the nerves serving the proximal musculature revealed more than 64 somata lying on the surface of the ganglia.

3.12. Levators

The levator muscles of *L. emarginata* are located in the thorax and the coxa and elevate the leg off the substrate. A total of 16 somata were observed when backfilling the nerve serving the flexor musculature (Fig. 6). These were clustered in three groups, two lying dorsally on the anterior distal surface of the ganglion, and a smaller group lying ventrally on the posterior distal surface of the ganglion. Of the two anterior groups, the most medial consists of five cells and the distal one consists of eight cells. The axons of each anterior group travel posteriorly to a converging point before turning in the distal direction to form a common track on their way to the musculature they innervate. The posterior somata cluster also converges on this track.

As described for the flexors, levator backfills also stained inter-ganglionic processes that turned anteriorly after exiting the ganglia medially (Fig. 6C and D).

3.13. Depressors

The depressor musculature is responsible for lifting the animal off the substrate and so play an important role in locomotion as well as stance. We were able to backfill a maximum of 20 somata innervating this musculature (Fig. 7). A group of ten tightly clustered motor neurons were located medially, on the ventral-posterior surface of the ganglion. The remaining somata were all found dorsally with one single cell lying distally and anteriorly. A second cell located in the center of the dorsal ganglionic surface, and the last eight more somata were all anterior and dorsal, just proximal to the location of the ventral cluster.

3.14. Promotors

The promotor musculature is responsible for promotion of the leg in the anterior direction and consists of at most three muscle heads. We found that 17 cells were labeled by backfilling the promotor nerve (Fig. 8). Fifteen of the somata are dorsal and two are ventral. The dorsal somata are clustered in two groups; the largest

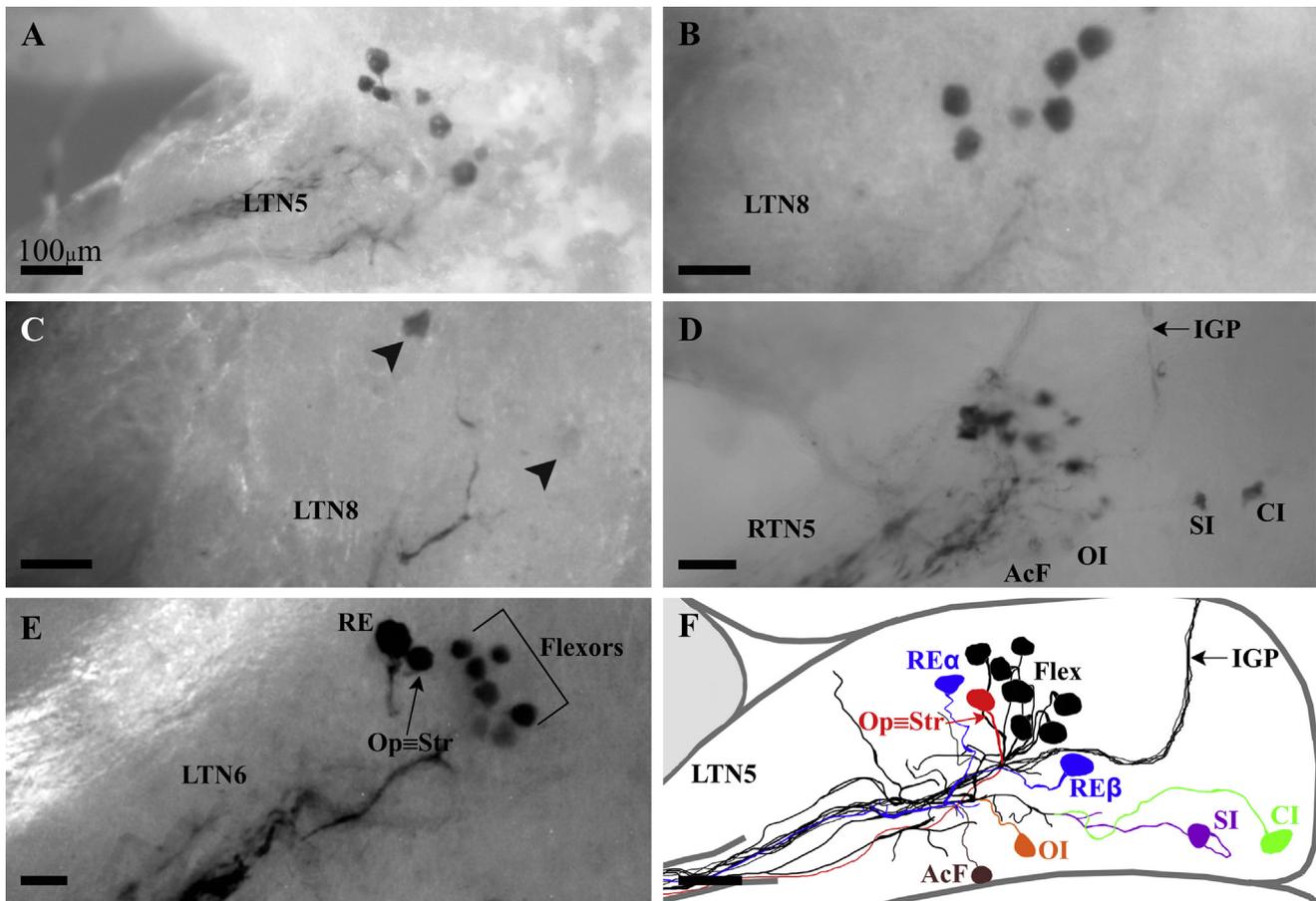


Fig. 5. Retrograde staining showing the number and location of flexor, reductor, and opener–stretcher motor neurons. Backfilling the flexor nerve revealed seven motor neurons clustered anterior dorsally on the thoracic neuromeres of *L. emarginata*, here shown for the left second (A) and fifth (B) walking legs. Note the rotation of the cluster due to the radial arrangement of the ganglia. C) Location of the two reductor excitator motor neurons (arrow heads). D) Ventral view of a flexor stain showing four somata tentatively labeled as the Common Inhibitor (CI), Stretcher inhibitor (SI), Opener inhibitor (OI), and Accessory flexor (AcF) based on previous work on related species (Faulkes and Paul, 1997). Most flexor backfills included a set of inter-ganglionic processes (IGP) that traveled to the midline and then turned anteriorly to their origin in anterior ganglia. E) Backfill showing the location of the opener and stretcher excitator (adjacent to the anterior reductor excitator). F) Diagram showing the relative positions of the motor neurons labeled. Anterior is up and proximal is left. All views are dorsal except in D.

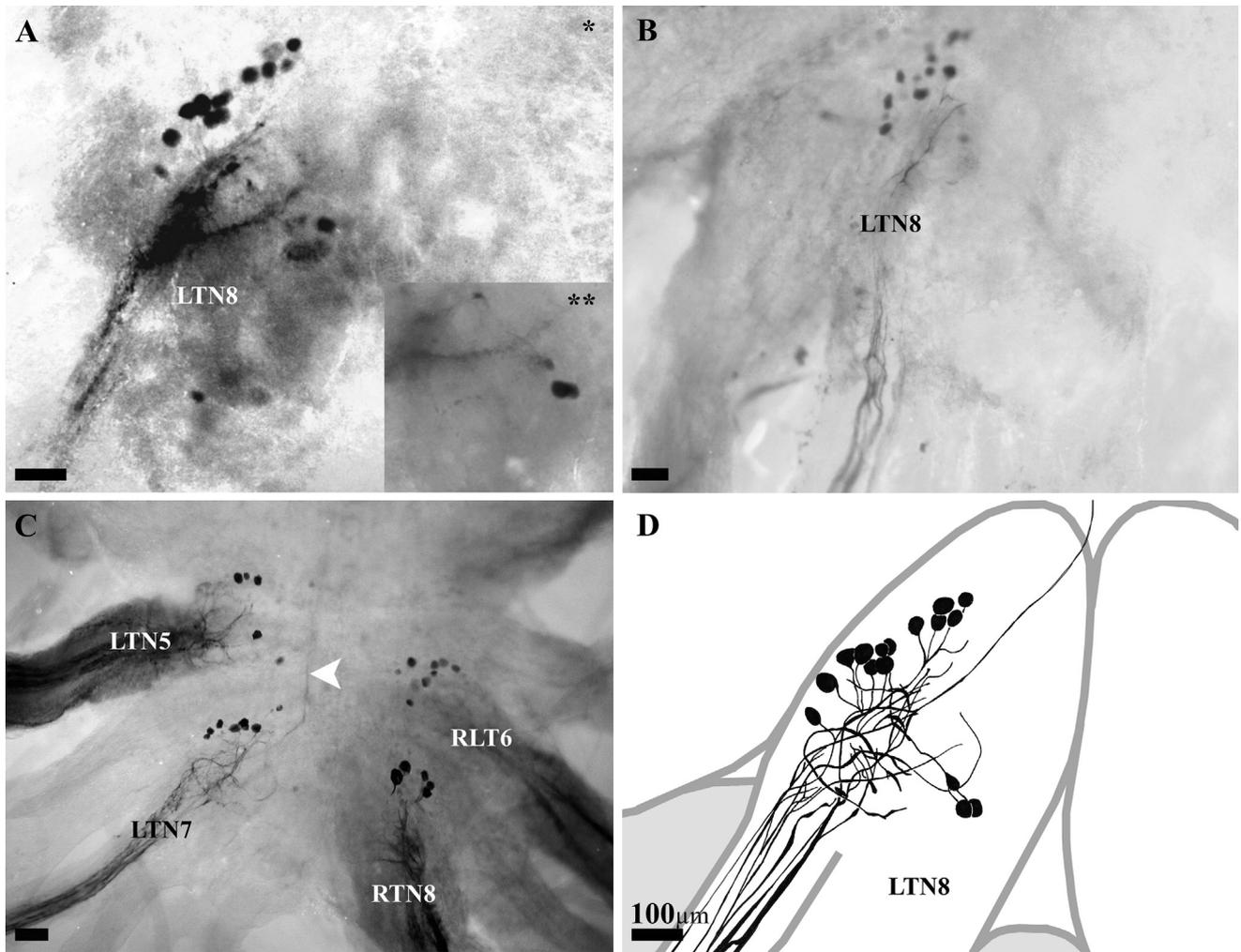


Fig. 6. Retrograde stainings showing the location and numbers of motor neurons innervating the levator musculature of *L. emarginata*. **A** and **B**) Examples of left eighth neuromere showing the anterior location of 13 levator somata. **C**) Partial backfills of the levators of the last four thoracic neuromeres showing the radial arrangement of the ganglia. The inter-ganglionic processes observed in the flexor backfills were also observed in levator backfills (arrow head). **D**) Diagram of the 16 motor neurons filled through the levator nerve. Anterior is up and proximal is right (except in **C**).

of which consists of thirteen located anteriorly and proximally on the ganglion. The smallest dorsal cluster is composed of two closely associated somata on the center of the dorsal ganglionic surface, and a third cell located more proximally. The remaining two cells lie just medial to the later group on the ventral surface of the ganglia. The axons of the promotor motor neurons form a common track that travels distally towards the leg and is located anterior to the levator track described above.

3.15. Remotors

The remotor musculature of *L. emarginata* is responsible for moving the leg in the posterior direction and consists of one to three (depending on the pereopod) muscle heads. Backfills of the remotor nerve revealed 11 somata (Fig. 9). A tightly clustered group of nine cells was located mid-posteriorly, on the ventral surface of the ganglion close to the midline. Additionally, one cell was located more distally and anteriorly on the ganglia, and a second cell was backfilled more posteriorly than those of the main cluster. Differential backfills with NiCl_2 and CoCl_2 revealed the main remotor cluster to be very closely associated with the ventral depressor cluster described above.

In addition to the somata described above, backfills of the remotors of the eighth neuromere revealed some somata that were adjacent to those of the contralateral remotor. A third bilaterally symmetrical soma close to the midline was backfilled sending extensive processes to both fifth neuromeres before joining the remotor tracks of each pereopods on their way to the musculature (Fig. 8C and D).

4. Discussion

4.1. Limitation of methods

The process of backfilling neurons is not without potential drawbacks. In any given preparation, usually only a random subset of the total number of neurons becomes dye-labeled. Therefore, determination of the total number of neurons requires the use of multiple preparations to establish the total number of somata in the population. Dye coupling between the targeted and unexpected nerves is another potential source of error. To minimize this risk we backfilled nerves that were cut close to the muscle they innervated. To minimize the errors involved in dye-filling neurons we only backfilled nerves whose electrical stimulation resulted in the

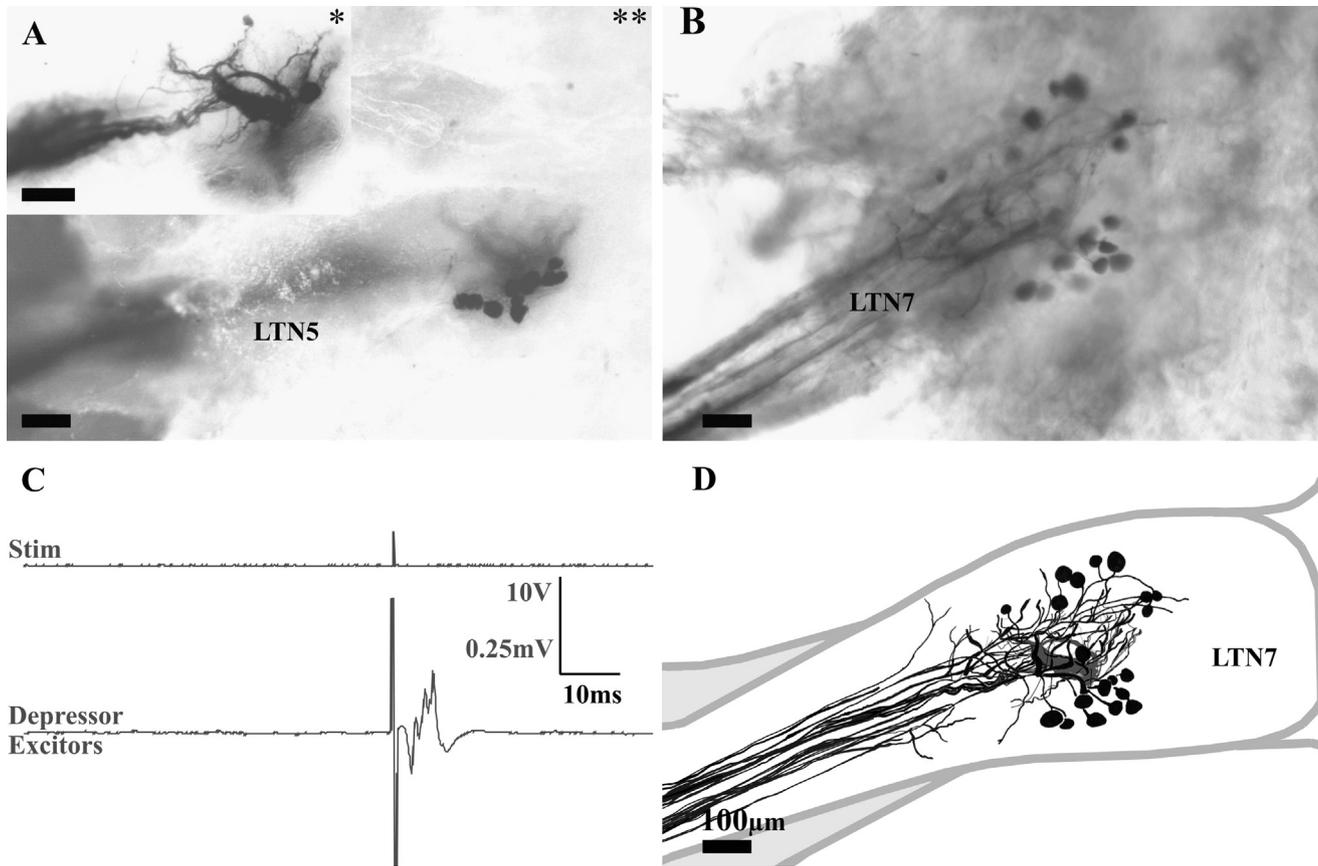


Fig. 7. Motor neurons innervating the depressor musculature of *L. emarginata*. **A)** Dorsal (*) and ventral (**) views of a partial backfill of the depressor motor neurons of the left fifth thoracic neuromere showing the extensive integrating zone in the center of the ganglion. **B)** Composite showing a backfill of the complete depressor motor neuron complement innervating the left fourth walking leg of *L. emarginata*. **C)** Elicited compound action potential able to produce leg depressions used in the identification of the population serving a particular muscle. **D)** Diagram showing the position of the depressor somata in the ganglion. Anterior is up and proximal is right.

production of discrete muscle contractions. We confirmed these both myographically, and visually by the resulting joint motion. Stimulation of neurons innervating the distal musculature produced myograms and neurograms where the numbers of units stimulated were discernable (Figs. 3 and 4). However, the large number of neurons involved, and the proximity between stimulating and recording electrodes, prevented us from physiologically confirming the number of neurons innervating the proximal musculature. For this reason, we restricted the use of the myographic and neural information to the identification of muscles innervated by a particular nerve.

4.2. Thoracic ganglia

We found the thoracic nervous system of *L. emarginata* to conform to the established crustacean plan (Wiersma and Ripley, 1952; Bévengut et al., 1983; Elson, 1996; Cattaert and Le Ray, 2001). The variability on axonal exit routes observed (Fig. 2) has been reported for other species (Faulkes and Paul, 1997) and in *L. emarginata* is likely the product of capricious defasciculation during development. As the animal increases in size, the thoracic ganglia become more removed from the musculature they innervate and motor axons previously traveling together in a nerve root could become pulled away from each other by the musculature they serve. This effect could be magnified in *L. emarginata* due to the lack of ventral skeletal compression present in this species (Vidal-Gadea et al., 2008).

Indeed, we observed occasional differences in the relative orientation of the ganglia with some neuromeres being slightly

rotated compared to their neighbors. This variability compared to other described decapods can be understood when considering that neurogenesis has been described well into the zoeal stages of other spider crabs, while it is over before metamorphosis in macrurans (Harzsch et al., 1998).

Differences in size between ganglia (the fourth and eighth thoracic neuromeres are larger than the rest) are possibly due to the larger neuropilar size correlated with an increased behavioral repertoire for these two legs (Mulloney et al., 2003). Recall that the fourth thoracic neuromere controls the claws, and the eighth neuromere is greatly responsible for the righting behavior of overturned crabs and poses an increased behavioral repertoire than anterior pereopods (in preparation).

4.3. Motor neuron pools

The layout within the ganglia of the motor pools resembles that of related species (Bévengut et al., 1983). Most of the somata are located on the dorsal surface of the ganglia and segregated into specific regions according to their function. As previously described for other crustaceans, the levator neurons congregate on the anterior lateral surface of the ganglia; the promoters are anterior and medial; the depressors posterior and lateral, and the remoters are posterior and medial. The central portion of the thoracic neuromeres is reserved for neuropils. Previous work has shown between 51 and 81 motor neurons innervating the leg musculature of decapod crustaceans (for review see Faulkes and Paul, 1997). We identified 84 potential motor neurons

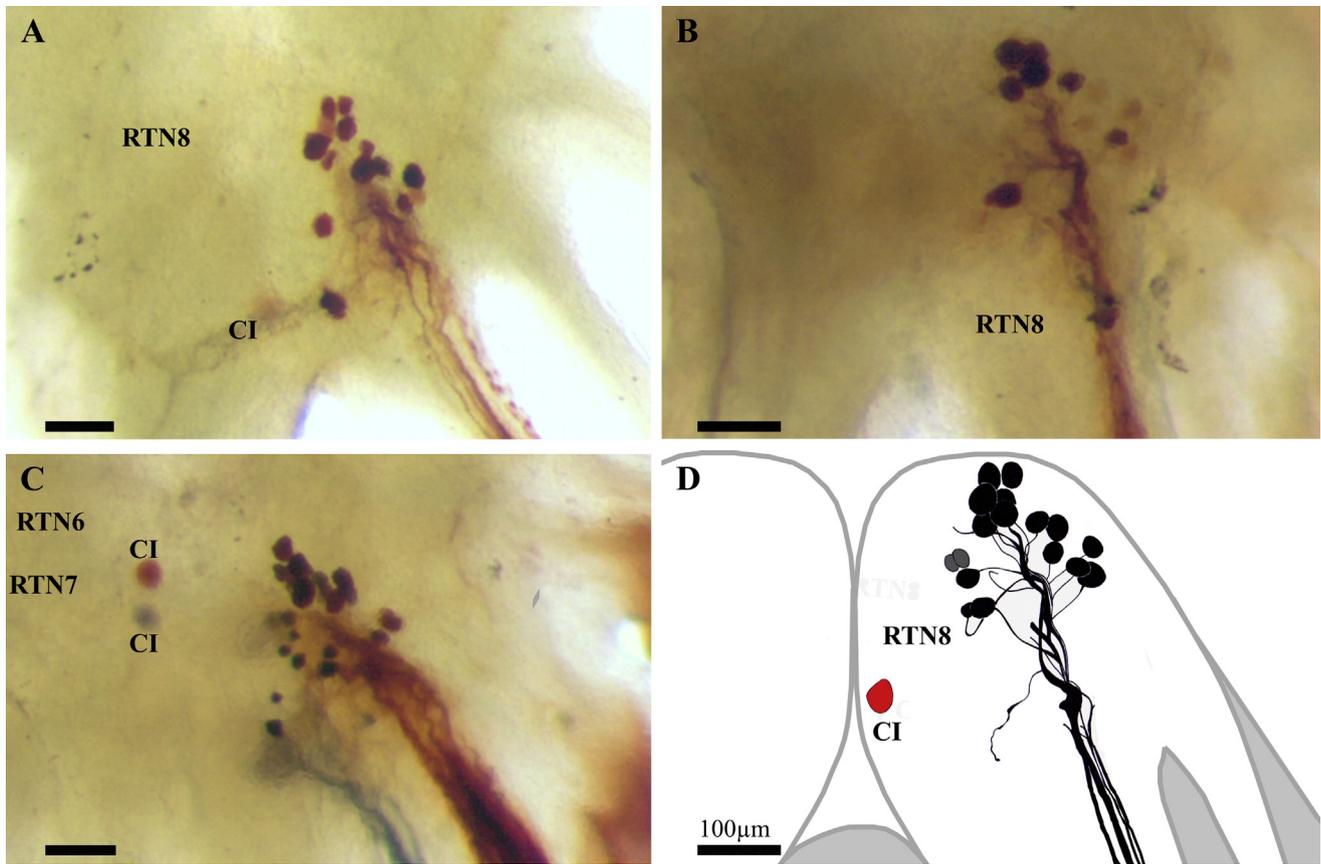


Fig. 8. Motor neurons innervating the promotor musculature of *L. emarginata*. **A)** Dorsal view of a $\text{NiCl}_2 + \text{CoCl}_2$ backfilled promotor nerve showing the 15 dorsal somata and the common inhibitor (CI) of the last right thoracic neuromere. **B)** Partial backfill of the same promotor population as in **A**. **C)** Differential backfill of the same promotor population of the right sixth neuromere (with $\text{NiCl}_2 + \text{CoCl}_2$: red) and the seventh neuromere (NiCl_2 : blue) showing the variability in somata size between the two neuromeres. The common inhibitor (CI) is visible in both backfills. **D)** Diagram of the last thoracic ganglion showing the location of the promotor somata, the two ventral somata not seen in dorsal view (for a total of 17 promotor neurons) are shaded gray, and the common inhibitor is colored red. Anterior is up and proximal is left. All views are dorsal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

innervating the legs of *L. emarginata*. The possibility remains that some of the cells backfilled in our studies were in fact sensory in nature. Alternatively, motor axons serving different muscles could have been inadvertently stained if they failed to elicit muscular activity upon stimulation. An example of this danger was evident in backfills of the remotor. Due to the radial arrangement of the legs in *L. emarginata*, the remotor nerves must travel posteriorly to reach their target. In some preparations, the abdominal flexor inhibitors (described for *Munida quadrispina* by Paul et al., 1985) were backfilled along with the remotor nerve of the eighth neuromere. Because of their contralateral projections and their unique morphology, these cells were easily recognized. However, the spatial segregation of functionally related somata within the ganglia minimized this risk. Additional somata were also labeled that were in all likelihood secretory in nature (see below) and could contribute to inflated somata numbers. Whenever any of these cells were identified, we left them out of our count for the motor pool under study. Notwithstanding the possibility of labeling non-motor cells, a considerably larger pool of motor neurons than that of *Carcinus* and other sideways-walking brachyurans likely innervates the proximal musculature of *L. emarginata*. Generally, the number of motor neurons was larger in populations innervating muscles with more muscle heads. The depressor musculature, for example, received a total of 20 motor cells (Fig. 7), and has also the largest number of muscle heads of any leg muscle in *L. emarginata* (Vidal-Gadea and Belanger, 2009).

4.4. Motor neurons innervating the distal musculature

The number of motor neurons innervating the distal musculature of crustaceans described in the literature is fairly conserved and ranges between 14 in the American lobster (*Homarus americanus*; Wilson and Sherman, 1975), 16 in the spiny lobster (*Jasus novaehollandia*; Silvey, 1981), and 17 in the squat lobster (*M. quadrispina*, Faulkes and Paul, 1997). We backfilled up to eighteen somata innervating the distal musculature of *L. emarginata*, the location of which were similar to those described for related species.

4.5. Closer excitors

The number and location of the neurons innervating the closer musculature closely resemble those described for several crustaceans (Faulkes and Paul, 1997; Govind and Lang, 1981; Wiersma and Ripley, 1952; Wilson and Mellon, 1982).

4.6. Bender and extensor excitors

The bender and extensor excitors exit the ganglia in a common nerve. Separating the two nerves revealed a pair of bender excitors lying on similar location to those described for other decapod species by Faulkes and Paul (1997). The two extensor excitors also

were located on the anterior and distal surface of the ganglia as described for the crayfish (Bradacs et al., 1996).

4.7. Opener \equiv stretcher excitors

Lesion experiments allowed us to selectively include or exclude the opener \equiv stretcher excitator from our backfills. This revealed a single soma in close association with the anterior reductor excitator, just distal to the flexor somata (Fig. 5E).

4.8. Flexor excitors

Previous work on *Carcinus maenas* (Parsons, 1982) demonstrated the departure of at least this one species from the accepted distal leg innervation plan established by Wiersma and Ripley (1952). Backfills of the nerve innervating the flexor muscle in *L. emarginata* legs revealed a group of seven closely associated somata (Fig. 5). The flexor muscle of the legs of *L. emarginata* differs from that of *C. maenas* in having additional muscle heads; one of which is bisegmental (Vidal-Gadea and Belanger, 2009). The additional somata seen for the flexor muscle could be serving (exciting or inhibiting) these additional muscle heads. The study of the physiological properties of the motor neurons identified was beyond the scope of this study.

4.9. Accessory flexor excitator

Our methodology did not permit us the discrimination between the flexor and accessory flexor muscles. Based on previous work (Faulkes and Paul, 1997) we inferred the identity of the accessory flexor somata to be the cell located posterior and distally on the ganglia (Fig. 5D and F). Additional innervation of the accessory flexor muscle has been revealed in several decapod species (Govind and Wiens, 1985). It is possible that one (or several) of the neurons backfilled in our experiment supply this muscle in lieu of the main flexor. This is however unlikely, based on previous work on the accessory flexor innervation of *L. emarginata* (Govind and Wiens, 1985).

4.10. Reductor excitors

There are two distinct reductor muscles on the basis of *L. emarginata*. Previous work on other decapods has identified two reductor excitator neurons to innervate them (Faulkes and Paul, 1997). Based on our defasciculation experiments of the nerve root

carrying the flexors, reductors, opener \equiv stretcher, and distal inhibitory neurons, we were able to identify two large somata as the reductor excitors (Fig. 5C). The reductor somata lie in close proximity to the flexor somata. This relationship echoes what goes on in the musculature where the reductor apodeme serve as attachment site for a bisegmental muscle head originating on the flexor apodeme (Vidal-Gadea and Belanger, 2009).

4.11. Inhibitors

4.11.1. Common inhibitor

The common inhibitor neuron (CI) previously shown to innervate the entire musculature of the leg in brachyurans (Rathmayer and Bévangut, 1986; Wiens et al., 1988), was repeatedly labeled and presented no difficulty in identification. In *L. emarginata* (as in other species) it lies posterior and medial in the ganglia. In the case of *L. emarginata*, CI is not contralateral (as described for *M. quadrispina*, Faulkes and Paul, 1997) but is instead ipsilateral (albeit very close to the midline, Fig. 5D and F).

4.11.2. Opener and stretcher inhibitors

Based on work carried on other species (Wiens and Atwood, 1978; Wiens and Wolf, 1993; Faulkes and Paul, 1997), we inferred the identities of the opener and stretcher inhibitors backfilled from nerves serving the distal musculature.

4.12. Motor neurons innervating the proximal musculature

Previous work on crustacean innervation has shown the motor neuron pools innervating the proximal musculature to vary considerably more than that to the distal muscles (Antonsen and Paul, 2000; Wilson and Sherman, 1975).

4.13. Levator excitors

The role of the levator musculature and its innervation has received particular attention in relation to the process of leg autotomy (McVean and Findlay, 1976). Moffett et al. (1987) described 12 levator motor neurons (plus the common inhibitor) innervating the different heads of the levator muscle in *C. maenas*. Although we backfilled more neurons in *L. emarginata* than those described by Moffett, the location of the somata within the ganglia were similar to those described for *C. maenas* (Fig. 6 here; Fig. 3 in Moffett et al., 1987). In *L. emarginata* the posterior levator muscle splits into two different muscles in the third to fifth pereopods

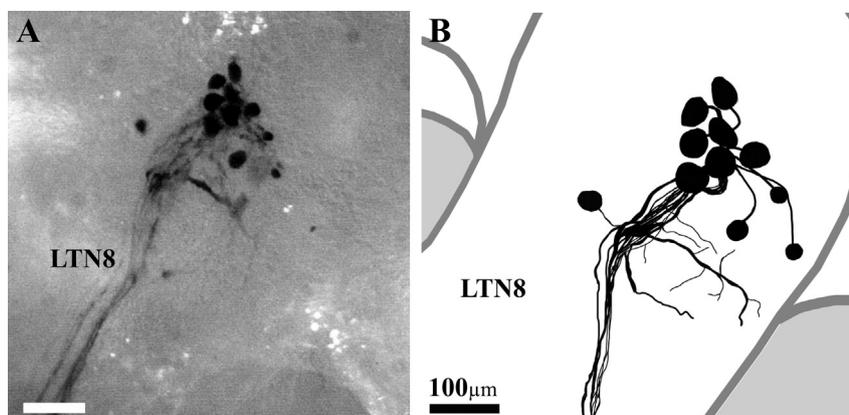


Fig. 9. Retrograde staining of the remotor nerve labeled a total of 11 somata on the ventral ganglionic surface. **A)** Backfill of the left eighth thoracic neuromere showing the population of remotor motor neurons. **B)** Diagram showing the position of the remotor motor neurons within the ganglion. All views are ventral. Anterior is up, and proximal is right.

(Vidal-Gadea and Belanger, 2009), the additional somata seen in *L. emarginata* could potentially be differentially associated with these heads, although this was not investigated in this study (Fig. 9).

4.14. Depressor excitors

We found the depressor motor neurons to closely resemble (in number and location) those described for the forwards-walking squat lobster (Fig. 7 here; Fig. 10 in Antonsen and Paul, 2000), over those described by Bévengut et al. (1983; Fig. 7C) for the shore crab, *C. maenas*. The large number of somata backfilled with the depressor nerve seems in agreement with the trend of increased number of somata accompanying increased number of muscle heads.

4.15. Promotor and remotor excitors

The promotor and remotor excitors were located in the same ganglionic region as those described for other crustaceans (El Manira et al., 1991b; Bévengut et al., 1983). As in the case of the levator and the depressor (above), the promotor and remotor pools had more motor neurons in *L. emarginata* than those described for *C. maenas* (Bévengut et al., 1983). As with all our backfills, the possibility exists that we inadvertently stained sensory cells along with motor neurons (as those described by Paul and Bruner, 1999; Paul and Wilson, 1994). Due to the radial arrangement of the legs, the last pair of pereopods lies directly caudal to TG. During our experiments, backfilling the remotor nerve also backfilled motor neurons innervating the abdomen of the crab. This was further confounded by the fact that the abdominal neuromeres of *L. emarginata* lie directly above the medial portion of the eighth thoracic neuromeres. The abdominal flexor inhibitor described by Paul et al. (1985) often exited the TG (really it should be renamed TAG for brachyurans) in a single posterior traveling nerve and split only after traveling most of the length of the thorax as one root. For this reason, when reporting the number of remotor somata we only reported the ones we observed in anterior neuromeres.

Table 2
Comparison of leg motor neurons between *L. emarginata* and other crustaceans.

| Species | <i>Carcinus maenas</i> ^a | <i>Libinia emarginata</i> | <i>Procambarus clarkii</i> ^b | <i>Munida quadrispina</i> ^c |
|--------------------|-------------------------------------|---------------------------|---|--|
| Walking preference | Sideway | Forward | Forward | Forward |
| Infraorder | Brachyura | Brachyura | Astacidea | Galatheidae |
| Muscle | Number of somata present | | | |
| Closer | 2 | 2 | 2 | 2 |
| Opener/stretcher | 1 | 1 | 1 | 1 |
| Bender | 2 | 2 | 2 | 2 |
| Extensor | 2 | 2 | 2 | 1 |
| Flexor | 6 | 7 | 4 | 2 |
| Acc. flexor | 1 | 1 | 1 | 1 |
| Reductor | 2 | 2 | 2 | 2 |
| Levator | 8 | 16 | 19 | |
| Depressor | 10 | 20 | 12 | 17 |
| Promotor | 7 | 17 | 19 | |
| Remotor | 7 | 11 | 13 | |
| Inhibitors | 3 | 3 | 3 | 3 |
| Total estimate | 51 | 84 | 80 | |

^a After Bévengut et al., 1983; Moffett et al., 1987; Parsons, 1982.

^b After El Manira et al., 1991a; El Manira et al., 1991b; Pearlstein et al., 1995.

^c After Faulkes and Paul, 1997; Antonsen and Paul 2000. Spaces signify numbers not reported.

4.16. Non-motor cells

Backfills of the nerve roots containing the flexor motor neurons, or the levator motor neurons also stained at least two inter-ganglionic processes (IGPs). These processes traveled to the proximal end of the ganglion and there joined a track containing other axons traveling in the rostral direction. On occasion, somata were seen on anterior ganglia close to the midline as far anterior as the third thoracic neuromere. Faulkes and Paul (1997) reported seeing similar processes in *M. quadrispina*, and suggested that they might be secretory in nature. Previous work on secretory neurons in crustaceans reveals somata with similar location to the ones we observed, however the inter-ganglionic processes derived from them do not match those in the cells we observed (Siwicki et al., 1985; Rossi-Durand, 1993; Antonsen and Paul, 2001).

5. Conclusions

Notwithstanding potential errors incurred by the intrinsic limitations of the technique, we have determined that the neural anatomy of *L. emarginata* is in keeping with that described for other decapod crustaceans. Proximally, it resembles that of distantly related (but forwards-walking) anomurans, rather than the closely-related (and sideways-walking) brachyurans (See Table 2, Supp. Table 1.). We have previously shown that the transition to sideways-walking in brachyurans (from forwards-walking lobster-like ancestors) involved changes in the skeletal structure where segments that propelled the animal sideways (MC joint) were favored in place of those that propelled the animals forward (Vidal-Gadea et al., 2008). This change is also reflected in the leg musculature where the number of proximal muscle heads is markedly larger in *L. emarginata* (and other forwards-walking crustaceans) compared to sideways-walking brachyurans (Vidal-Gadea and Belanger, 2009). This study provides further evidence of the evolutionary changes involved in this transition between gait strategies by showing that forward walking crustaceans have higher number of motor neurons innervating their proximal musculature than sideway walking brachyurans. In our next manuscript we will study whether the additional proximal musculature (and motor neuron) in *L. emarginata* result in functionally distinct muscles that are independently activated during locomotion. The study of legged locomotion in crustaceans benefits from the existence of distinct strategies which, when compared, can help us reveal underlying principles governing the performance of this behavior. As a forwards-walking brachyuran, *L. emarginata* is a convenient system for comparative studies in crustacean locomotion and will likely continue to help us bridge the gap between the work in the macruran (forwards-walking) and brachyuran (sideways-walking) literature.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.asd.2013.07.003>.

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