

Motor neurons in the escape response circuit of white shrimp (*Litopenaeus setiferus*)

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Many decapod crustaceans perform escape tailflips involving giant interneurons, which includes a specialized fast flexor motor giant (MoG) neuron, and populations of larger, less specialized fast flexor motor neurons and fast extensor motor neurons. These escape-related neurons are well described in crayfish (Reptantia), but not in more basal decapod groups. To clarify the evolution of the escape circuit, I examined the fast flexor and extensor motor neurons of white shrimp (*Litopenaeus setiferus*; Dendrobranchiata) using backfilling. In crayfish, the MoGs in each abdominal ganglion are a bilateral pair of separate neurons. In *L. setiferus*, the MoGs have massive, possibly syncytial, cell bodies. The non-MoG fast flexor motor neurons and fast extensor motor neurons are generally found in similar locations to where they are found in crayfish, but the number of motor neurons in both the flexor and extensor pools is smaller than crayfish. The loss of fusion in the MoGs and increased number of fast motor neurons in reptantian decapods may be correlated with an increased reliance on non-giant mediated tailflipping.

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12 Introduction

13 Decapod crustaceans escape from predators and other sudden stimuli by tailflipping.
14 The neural basis of escape tailflips has been well-studied (Wine & Krasne 1972; Wine &
15 Krasne 1982; Edwards, Heitler & Krasne 1999; Krasne & Edwards 2002; Faulkes 2008),
16 particularly in Louisiana red swamp crayfish (*Procambarus clarkii*). The core of the escape
17 circuit consists of medial giant interneurons (MGs) and lateral giant interneurons (LGs) that
18 drive fast flexor motor neurons, including a specialized fast flexor motor giant (MoG) neuron.
19 Some of these neurons are found in non-decapod crustaceans (Silvey & Wilson 1979),
20 indicating that having this escape circuit is an ancestral condition for the decapods.

21 Crustacean escape behaviour is an excellent model for studying the evolution of neural
22 circuits: the behaviour has an obvious survival value (Herberholz, Sen & Edwards 2004);
23 many of the responsible neurons have no function other than escape, and; the circuit is found
24 in thousands of crustacean species (Faulkes 2008). Indeed, many curious features of the
25 neural circuit cannot be understood without thinking about the evolutionary history of the
26 circuit (Edwards, Heitler & Krasne 1999; Krasne & Edwards 2002), leading Krasne and
27 Edwards (2002) to write, “it may follow that reasonable understanding of the nervous system
28 may be impossible without evolutionary analysis, a most sobering possibility.”

29 Most escape circuit research has been done on crayfish, which belong to Reptantia, a
30 more derived taxon of decapod crustaceans. The more basal decapod taxa,
31 Dendrobranchiata, Caridea, and Stenopodidea (Dixon, Ahyong & Schram 2003; Porter,
32 Perez-Losada & Crandall 2005) are less well studied, but shrimps and prawns differ in
33 several ways from crayfish. First, crayfish neurons are unmyelinated, but giant interneurons
34 are myelinated in all three non-reptantian taxa (Holmes, Pumphrey & Young 1941; Heuser &

35 Doggenweiler 1966; Xu & Terakawa 1999). Because of the combination of giant interneurons
 36 and myelin, shrimp giant axons have the fastest conduction velocity known (Xu & Terakawa
 37 1999). Second, the left and right fast flexor motor giant neurons (MoGs) are separate in
 38 crayfish (Mittenthal & Wine 1978), but the MoG axons fuse in caridean shrimp and prawns
 39 (Johnson 1924; Holmes 1942; Friedlander & Levinthal 1982). Axonal fusion may promote
 40 greater synchrony in muscle activation, which should in turn lead to more powerful tailflips.
 41 This should reduce response latency, leading to greater chance of escape. It is surprising that
 42 this has been lost in crayfish. Third, at the behavioural level, crayfish giant mediated tailflips
 43 are stereotyped and propel the animal in a single plane (Reichert & Wine 1983), but some
 44 shrimp can perform a lateral roll during an escape tailflip (Arnott, Neil & Ansell 1998). It is not
 45 known how shrimp achieve this, particularly given the bilateral fusion of the MoG axons.

46 Dendrobranchiate shrimp are the most basal decapod crustacean taxa (Dixon, Ahyong
 47 & Schram 2003; Porter, Perez-Losada & Crandall 2005), and thus are in an interesting
 48 position for evolutionary studies of the escape response, but little is known about the motor
 49 neurons neurons involved in that group. Here, I examine the fast flexor and fast extensor
 50 motor systems of white shrimp, *Litopenaeus setiferus*. Some of this work has been presented
 51 in abstract (Faulkes 2007).

52 Methods

53 Live white shrimp, *Litopenaeus setiferus* (Linnaeus, 1767), fished from waters around
 54 South Padre Island, Texas, were purchased from commercial seafood stores in Port Isabel,
 55 Texas and housed in aquaria. Individuals were anaesthetized by chilling on ice and dissected
 56 in physiological saline. The abdominal nerve cord was removed.

Neurons were backfilled (Pitman, Tweedle & Cohen 1972; Quicke & Brace 1979; Altman & Tyrer 1980; Jones & Page 1983). The nerve containing the neurons of interest was placed in a well of petroleum jelly containing 0.3 M solution of either nickel chloride or cobalt chloride, while the remaining tissue was bathed in physiological saline (mM: 410 NaCl, 12.7 KCl, 10.3 CaCl₂, 10 MgCl₂, and 14 Na₂SO₄, 10 tris[hydroxymethyl]aminomethane (Trizma Base); pH adjusted to pH 7.4). The tissue was incubated in a refrigerator for 7-18 hours, precipitated with ammonium sulfide or dithiooxamide (a.k.a. rubeanic acid), fixed in 4% formalin in saline, dehydrated with a progressive ethanol series (70% for 10 minutes, 90% for 10 minutes, 100% for 10 minutes, and 100% again for 5 minutes), and cleared in methyl salicylate. When precipitated with dithiooxamide, neurons containing cobalt ions turn yellow, and those containing nickel ions turn blue (Quicke & Brace 1979; Jones & Page 1983). Neurons containing some mixture of the two ions turn an intermediate colour, ranging from dark orange to purplish-red (Quicke & Brace 1979; Jones & Page 1983).

The third nerve (N3) was filled 42 times in 30 abdominal ganglia of 14 individuals. The second nerve (N2) was filled 98 times in 61 ganglia of 23 individuals. Abdominal ganglia 1 through 5 were filled, although most fills were of the anterior four ganglia.

Cleared backfills were viewed on an Olympus CX41 microscope, and photographed using an attached Olympus C-5050Zoom digital camera. Images were assembled into final figures using Corel Photo-Paint 12. Some large images were stitched together from multiple photographs.

77 Results

78 **Fast flexor motor neurons**

79 The MoG cell bodies in *L. setiferus* have a variegated appearance, irregular shape,
80 and press closely together so closely that they look like one large mass covering much of the
81 ventral surface of the abdominal ganglion. They are not two separate, bilateral, spherical cell
82 bodies reported in caridean prawns (Holmes 1942). In *L. setiferus*, each MoG is ~300 μm
83 across the ventral surface of the ganglion, and about 100 μm when viewed in the sagittal
84 plane. Other fast flexor motor neurons in *L. setiferus* are ~50-100 μm in diameter.

85 Although the MoG cell bodies are pressed so close together to be almost
86 indistinguishable as two cell bodies, two axons emerge from the MoG cell bodies, project
87 dorsally a short distance within the ganglion before fusing as previously reported (Johnson
88 1924; Holmes 1942), continuing into the nerve cord as one axon until slightly anterior of the
89 point where N3 exits the body, when it bifurcates and sends a branch both left and right.

90 The non-MoG fast flexor cell bodies are found in three clusters, as in other decapods
91 (Mittenthal & Wine 1978). The flexor medial contralateral (FMC) cluster is contralateral and
92 anterior of the filled N3 (in the terminology of Mittenthal and Wine, “posterior” refers to the
93 position of the axon relative to the cell body). The flexor posterior ipsilateral (FPI) cluster is
94 ipsilateral and anterior of the filled N3. The flexor anterior contralateral (FAC) cluster of cell
95 bodies is contralateral and posterior to the filled N3. As in other species (Mittenthal & Wine
96 1978), there is some slight segmental variation in the number of cell bodies in each ganglion,
97 with the more posterior showing the greatest deviation (Table 1). White shrimp have one or
98 two fewer cell bodies in each cluster than most other decapods examined to date (Table 2).
99 The FMC cell bodies are more widely separated in *L. setiferus* than crayfish, with one anterior

of the MoG cell body and near the midline, and the other more posterior and lateral of the MoG cell body. Although this separation means these two cells would not normally be described as being in a “cluster,” the FMC in other species is rarely a tight grouping of cell bodies. Fast flexor cell bodies are often pairs or singletons, depending on the number, somewhat separated from other cells in the cluster (Mittenthal & Wine 1978; Espinoza et al. 2006) (e.g., Figure 3a, b in Espinoza).

The fast flexor motor axons appear slightly “haloed” compared to the smooth axons seen filled though N2 (below).

Fast extensor motor neurons and other N2 neurons

In crayfish, the second nerve (N2) of abdominal ganglia is a mixed nerve that splits some distance from the ganglion. The anterior branch (N2a) contains tactile afferents (Leise, Hall & Mulloney 1987); the posterior branch (N2p) contains fast extensor motor neurons (Treisman & Remler 1975; Drummond & Macmillan 1998), slow extensor motor neurons (Drummond & Macmillan 1998), and neurons associated with muscle receptor organs (MROs) (Leise, Hall & Mulloney 1987).

In *L. setiferus*, N2 splits into two branches very near the ganglion, with the anterior branch slightly thicker than the posterior. Fills from N2a revealed many fine processes projecting to the middle of the ganglion and no cell bodies. This is a probably a purely sensory branch containing only tactile afferents, as in *Pacifastacus leniusculus* (Leise, Hall & Mulloney 1987).

Many cell bodies fill through N2p. Reasonable hypotheses about the identity of cell bodies can be based on their sizes and homology with other species (Table 3). Fast extensor motor neurons are usually double or more the diameter of slow extensor motor neurons

(Otsuka, Kravitz & Potter 1967; Wine & Hagiwara 1977), although the largest slow extensor motor neurons approach the size of the smallest fast extensor neuron (Wine & Hagiwara 1977; Drummond & Macmillan 1998). Fills of N2p revealed four large cell bodies located along the posterior margin of the ganglion, one contralateral and three ipsilateral, which are putative fast extensor motor neurons. All abdominal ganglia showed this pattern and no segmental variation was evident. In other decapods, the contralateral cell body is an inhibitory motor neuron and the ipsilateral cell bodies are excitatory motor neurons (Otsuka, Kravitz & Potter 1967; Treisman & Remler 1975; Wine & Hagiwara 1977); the same is likely true in *L. setiferus*.

The other small cell bodies filling through N2p are located in several places. One is found contralateral and posterior, near the putative fast extensor inhibitor; about 3-5 small cell bodies sit along the posterior lateral margin; two are lateral, sitting in the notch between the exit paths of N1 and N2; one small cell body is located anterior of the exit point of N1 (seen in abdominal ganglia 1 and 2; presence in other ganglia unknown); one small cell body is near the exact center of the ganglion.

Two axons from N2p bifurcate near the midline, and send processes both anterior and posterior for unknown distances. These are almost certainly axons of the stretch receptors of MROs, which have been described in many species, and are almost always present as a pair of bifurcating axons (Sillar & Heitler 1985a; Leise, Hall & Mulloney 1987; Wallis et al. 1995).

At least three small axons turn posterior and run along the lateral margin of the nerve cord (ganglia 1-3); one exceptionally clear fill in ganglion 1 revealed five such axons. I was unable to fill any cell bodies associated with these axons; fills rarely extended past the exit point of N3. Despite this incomplete picture of their anatomy, these neurons are probably accessory neurons related to the MROs (Wine & Hagiwara 1977; Leise, Hall & Mulloney

147 1987). There are 4 accessory neurons in abdominal ganglion 2 of *Procambarus clarkii* (Wine
148 & Hagiwara 1977)

149 The backfilled axons of fast extensor motor neurons do not have the “haloed”
150 appearance of fast flexor motor neurons.

151 Discussion

152 The fast flexor motor giant neurons (MoGs) in *Litopenaeus setiferus* have a structure
153 unlike that reported for any other decapod crustacean. The structure suggests that the MoGs
154 may be syncytial cells formed by the fusion of many small neurons. The situation is
155 reminiscent of the third-order giant neurons in squid stellate ganglia, which are also syncytial
156 cells formed from many cell bodies, and also involved in an escape response (Young 1936;
157 Young 1939). Annelid worms also have syncytial giant neurons (Nicol 1948; Günther 1975)
158 that are involved in escape responses (Nicol 1948; Günther 1975; O'Gara, Vining & Drewes
159 1982).

160 Although the size and probable number of cells in the MoG cell body fusion is
161 unexpected, it is entirely consistent with the long-known fusion of the MoG axons in other
162 caridean shrimp species (Johnson 1924; Holmes 1942; Friedlander & Levinthal 1982). Given
163 that there are genetic mechanisms to fuse the MoG axons during development (Friedlander &
164 Levinthal 1982), the same mechanisms could be used to fuse cell bodies. Reduction of fusion
165 appears to be an evolutionary trend in the decapods, starting with extensive fusion of both
166 MoG cell bodies and axons in dendrobranchiates, to fusion of the MoG axons only in
167 carideans (Holmes 1942), to no MoG fusion in reptantians (Mittenthal & Wine 1978).

168 The remaining fast flexor and fast extensor motor neurons appear to be found in
169 homologous positions to the better studied reptantian species. In almost every case, there are

fewer cell bodies in *L. setiferus* than in the homologous groups of neurons in most reptantian decapods. If other non-reptantian species have similarly small numbers of motor neurons, it would suggest that duplication of fast abdominal motor neurons occurred during decapod evolution. The loss of the massive fused MoG cell bodies may be correlated with the increase number of fast abdominal motor neurons in reptantians: the amount of ganglionic “real estate” consumed by the MoGs may have constrained the addition of any new fast motor neurons.

The “haloed” appearance of the fast flexors, but not fast extensors, may be indicative of myelination. In *Palaemon serratus*, the MoG axons are myelinated in the periphery, and references to other axons of similar size being myelinated suggest the other fast flexor motor neurons are also myelinated. (Holmes 1942).

The smaller number of fast abdominal neurons might indicate that shrimp have less fine grained control over the fast flexor motor muscles than crayfish. In many reptantian crustaceans, the fast flexor muscles are used in two distinct forms of tailflipping. The MoGs and other fast flexor neurons are used in single stereotyped escape tailflips triggered by giant interneurons. The MoGs are not involved in repetitive variable tailflipping, which is controlled by an undescribed system of non-giant interneurons (Reichert, Wine & Hagiwara 1981; Reichert & Wine 1983; Sillar & Heitler 1985b; Wilson & Paul 1987; Faulkes 2004); non-giant tailflipping would be generated by the remaining pool of fast flexor motor neurons. A larger pool of motor neurons may allow for some of the fine control necessary for such variability. Previously, I suggested that non-giant tailflipping originated at the base of the decapod clade (Faulkes 2008), but I did not express this hypothesis as tentatively as it should have been. It is not known if non-reptantian decapods have variable, non-giant tailflipping behaviour like many reptantians do. Indeed, the myelination of the entire population of fast flexors and the axonal fusion of the MoGs point to a circuit specialized for explosive starts. It may be that

194 tailflipping cannot occur without giant neurons activity in shrimp, and that a small pool of
195 neurons is sufficient to generate shrimps' more consistently explosive tailflips. Alternately, the
196 variation in motor neuron number may be trivial and have little functional impact, because
197 crustacean muscles generally have sparse polyneuronal innervation. In *Munida quadrispina*,
198 the FAC cluster of fast flexor motor neurons was lost with no visible change in tailflipping
199 behaviour (Wilson & Paul 1987).

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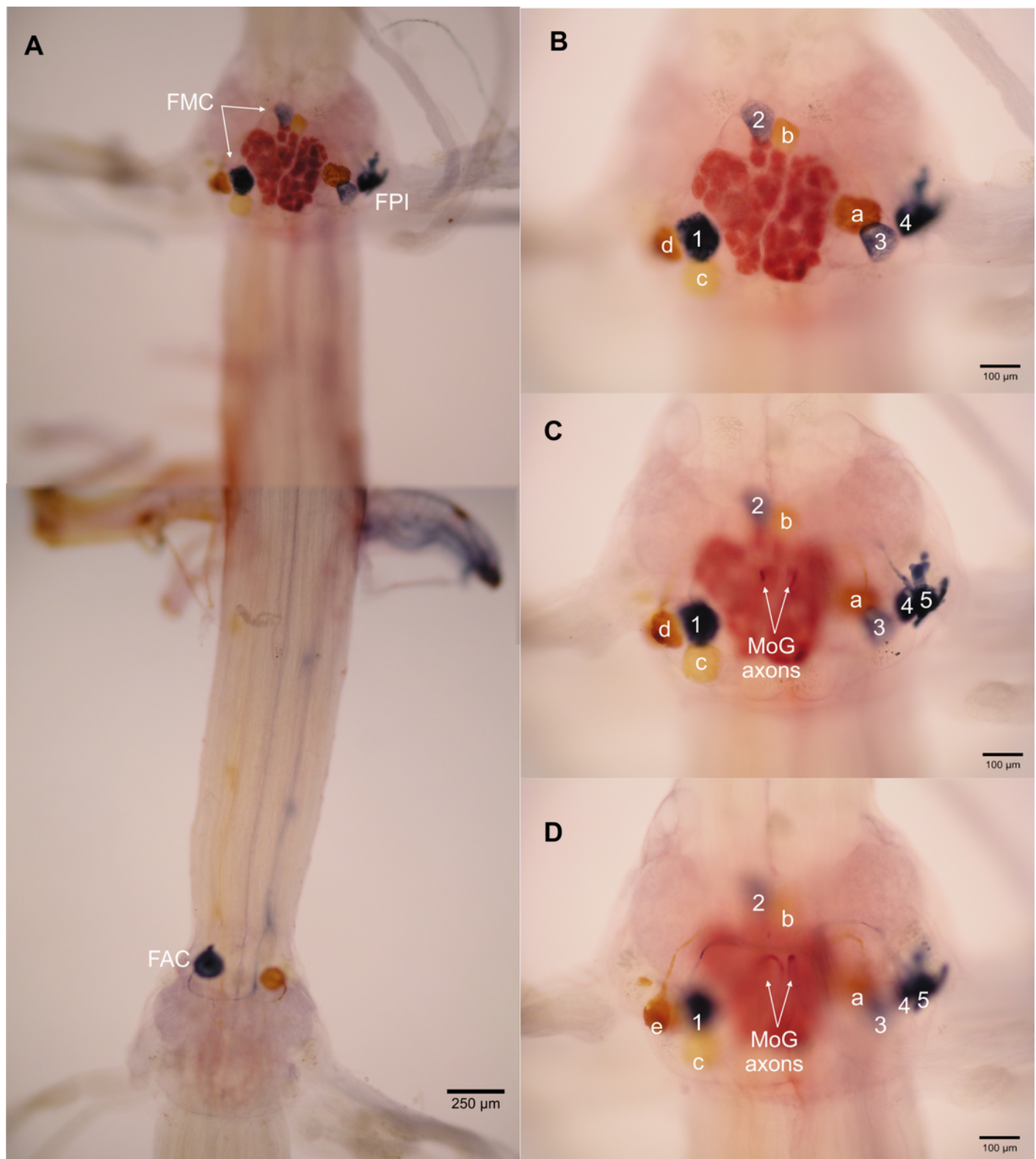
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Fast flexor motor neurons in *L. setiferus*.

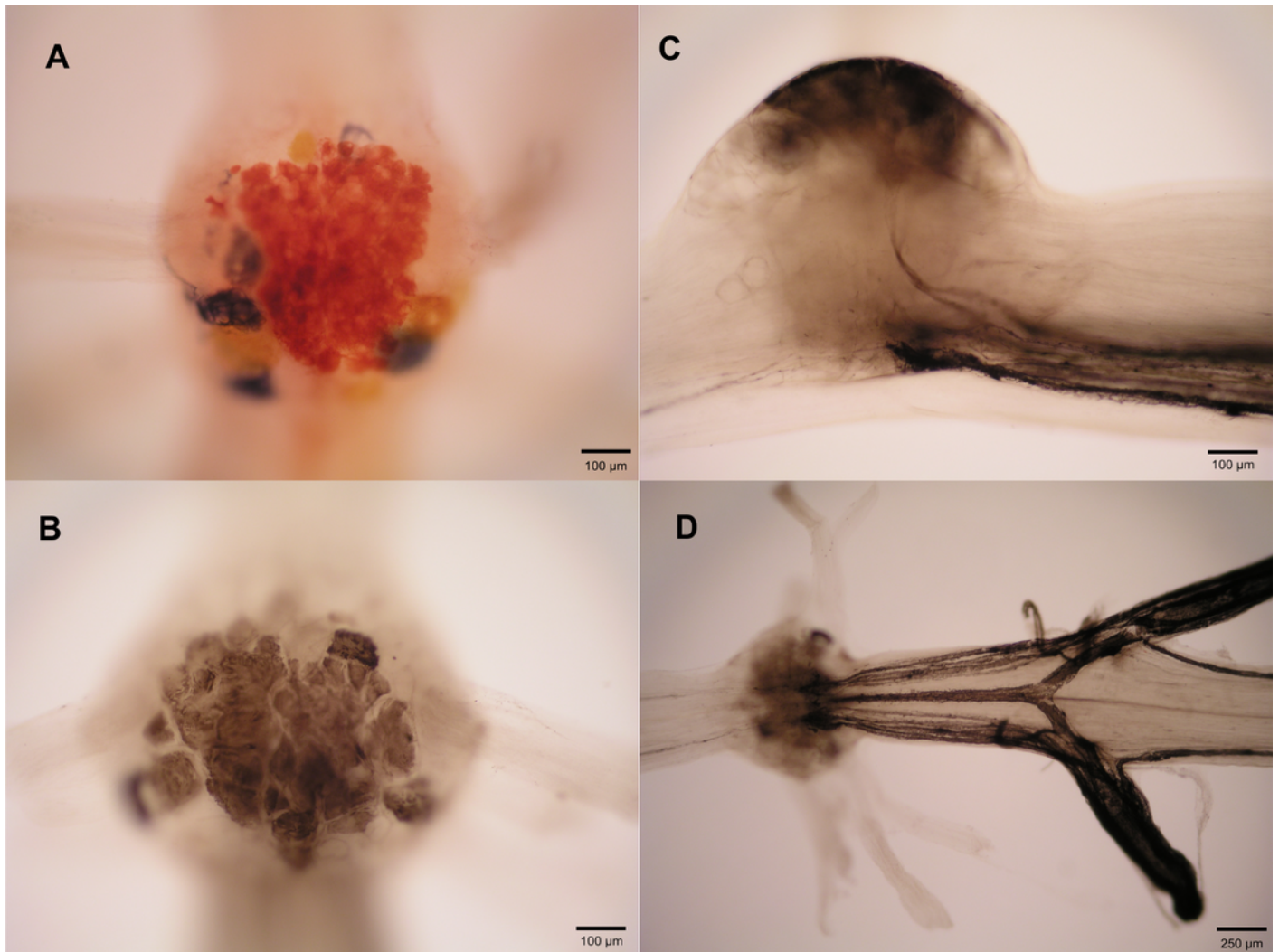
(A) Overview of complete fill of all fast flexor neurons, showing both all clusters of motor neuron cell bodies in abdominal ganglia 2 and 3, filled by N3 of abdominal ganglion 2. Cluster identifications for neurons in blue filled from right nerve. (B-D) Varying focal planes of the same individual in A, showing numbers of FMC and FPI cell bodies not visible in a single focal plane, and MoG axons. Fills using cobalt chloride and nickel chloride precipitated with rubeanic acid. Anterior towards top.



2

Motor giant neuron structure in *L. setiferus*

(A, B) MoG structure in abdominal ganglion 1 and 3, respectively. (C) Lateral view of MoG in abdominal ganglion 3. (D) Fused MoG axons in abdominal ganglion 3. Fills using cobalt chloride and nickel chloride precipitated with rubeanic acid in A; fills using cobalt chloride precipitated with ammonium sulphide in B-D. Anterior toward top of page A, B; anterior towards left in C, D.



3

Extensor-related neurons in *L. setiferus*.

(A, B) Bilateral fill of nerve 2 in abdominal ganglion 1. Putative sensory neurons filled by anterior branch of nerve 2 shown in A; motor neurons filled by posterior branch of nerve 2 shown in focus in B. (C) Muscle receptor organ (MRO) axons filled through posterior branch of nerve 2 in abdominal ganglion 1. Fills made using cobalt chloride and nickel chloride precipitated with rubeanic acid. Anterior towards top.

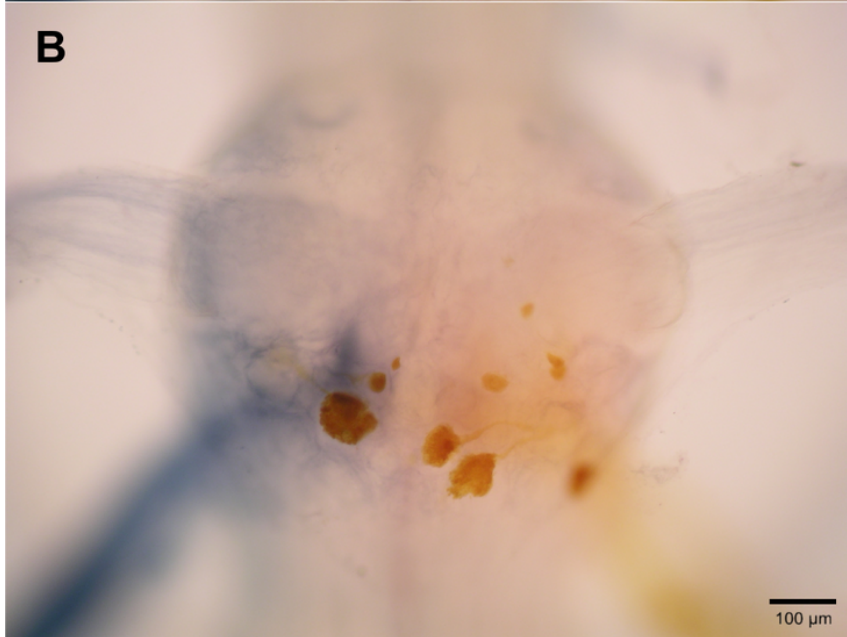
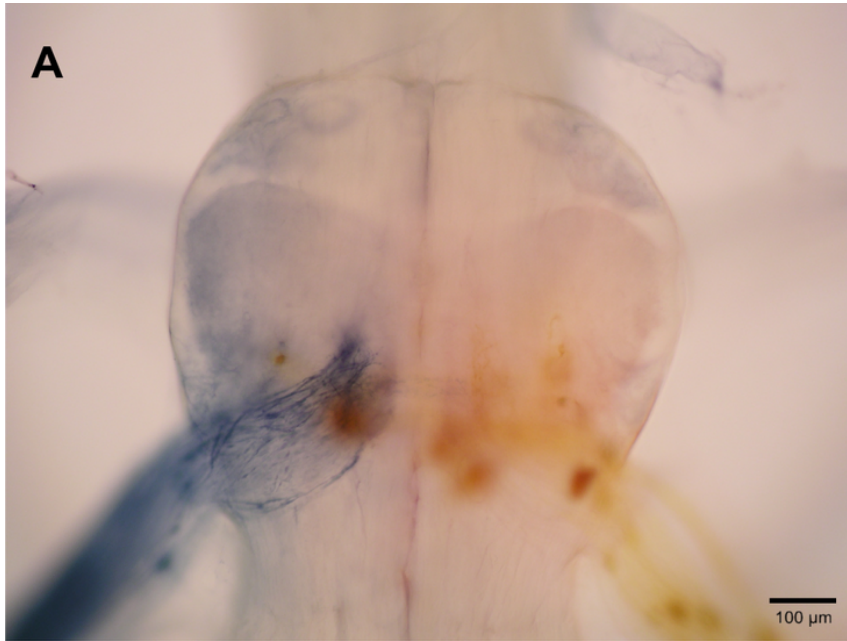


Table 1 (on next page)

Number of fast flexor motor neurons in each abdominal ganglion of *L. setiferus*

FMC = flexor medial contralateral; MoG = motor giant fast flexor motor neuron; FPI = flexor posterior ipsilateral; FAC = flexor anterior contralateral

Abdominal ganglia(on)	FMC (Non-MoG)	MoG	FPI	FAC
A1-4	3	1	3	1
A5	3	1	2	0
A6	?	?	?	0

2

Table 2 (on next page)

Extensor motor neurons and MRO related neurons

FEMNs = fast extensor motor neurons; SEMNs = slow extensor motor neurons; EE = extensor excitors; I = inhibitor. ¹ Treistman and Remler, 1975; Wine and Hagiwara, 1977 (but see Leise et al., 1987, which notes that Wine & Hagiwara misidentified some extensor neurons), ² Leise et al., 1987, ³ Drummond and Macmillan, 1998a; Drummond and Macmillan, 1998b, ⁴ FEMNs: Otsuka et al., 1967, SEMNs: Jones and Page, 1986, ⁵ Sillar and Heitler, 1985a; accessory neurons are shown in Figure 9, but the exact number is not mentioned in the text, ⁶ Wallis et al., 1995; assignment of fast and slow based on examination of Figure 5.

Species	Ganglia	Ipsilateral FEMNs	Contralateral FEMNs	Ipsilateral SEMNs	Contralateral SEMNs	Accessory neurons
White shrimp (<i>Litopenaeus setiferus</i>)	A1-4	3	1	5?	1	3
Louisiana red swamp crayfish (<i>Procambarus clarkii</i>) ¹	A1-4	5	1 (I)	5	1	4
Signal crayfish (<i>Pacifastacus leniusculus</i>) ²	A2-5	5	3	4	1	4
Australian yabby (<i>Cherax destructor</i>) ³	A3	5	1 (I)	5	1	4
American clawed lobster (<i>Homarus americanus</i>) ⁴	A1-4	3 (EE)	1 (I)	4	1	?
Squat lobster (<i>Galathea strigosa</i>) ⁵	A2	4-5 (EE)	1 (I)	4	1	3?
Squat lobster (<i>Munida quadrispina</i>) ⁶	A2-3	4	1	3	1	3

Table 3(on next page)

Number of fast flexor motor neurons in abdominal ganglion 2 of different species

FMC = flexor medial contralateral; MoG = motor giant fast flexor motor neuron; FPI = flexor posterior ipsilateral; FAC = flexor anterior contralateral. ¹ Espinoza et al., 2006, ² Mittenthal and Wine, 1978, ³ Sillar and Heitler, 1985a, ⁴ Wilson and Paul, 1987, ⁵ Otsuka et al., 1967, ⁶ See discussion in Mittenthal and Wine, 1978

Species	FMC (non MoG)	MoG	FPI	FAC
White shrimp (<i>Litopenaeus setiferus</i>)	2	1	3	1
Spiny lobster (<i>Panulirus argus</i>) ¹	3	0	4	3
Crayfish (<i>Procambarus clarkii</i>) ²	3	1	4	3
American clawed lobster (<i>Homarus americanus</i>) ⁵	3	1 ⁶	4	3
Squat lobster (<i>Galathea strigosa</i>) ³	4	0	4	2
Squat lobster (<i>Munida quadrispina</i>) ⁴	3	0	4	0

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