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Biogenic amines, behaviour, and the multifunctional depressor muscle in the squat lobster *Munida quadrispina* (Anomura, Galatheidae)

by

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Bachelor of Arts and Science (B.Sc.)
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In the Department of Biology

We accept this dissertation as conforming to the required standard

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ABSTRACT

The biogenic amines serotonin (5-HT) and octopamine (OA) have many roles in neurological systems in decapod crustaceans influencing processes as diverse as sensitivity of individual sensory neurons and agonistic behaviours. I examined aspects of the aminergic system in the squat lobster *Munida quadrispina* (Anomura, Galatheidae) and compared my results with data on aminergic systems and behaviours in more extensively studied species. *M. quadrispina* has a complex set of agonistic behaviours, and in comparisons with crayfish and lobster behaviour one major difference stands out: squat lobsters do not normally fight. Injecting carefully controlled doses of 5-HT induces *M. quadrispina* to perform stereotypical aggressive behaviours in the absence of any additional stimulation, and animals under the influence of injected 5-HT will fight. Animals under the influence of injected OA are much more likely to initiate escape responses to a standardized stimulus than are untreated animals, and assume, under certain circumstances, a submissive stance in the absence of additional external stimulation. The distributions of serotonergic and octopaminergic neurons in *M. quadrispina* are overall fairly similar to those of crayfish, lobsters, and crabs. However, several important differences, such as a lack of unpaired medial serotonergic neurons and far fewer octopaminergic "crotch" cells in *M. quadrispina* than in lobsters may relate to functional differences in the aminergic systems and other systems influenced by the amines. The pereiopod depressor muscles lift the body of the animal above the substrate and, therefore, are important in aggressive, and other behaviours. In *M. quadrispina*, as in all decapods, the depressor muscle and its antagonist, the levator muscle, are composed of multiple anatomically distinct heads. Most published studies have treated the

depressor muscle as a single functional unit, despite documented differences in the population of depressor excitatory motor neurons. In *M. quadrispina*, each head has individualistic patterns of excitatory innervation, and the heads are activated differentially during walking and maintained stance. These differences reveal a functional subdivision among the heads of the depressor muscle, with different combinations of heads responsible for movement of the leg, stance maintenance, and joint tension. Injecting 5-HT into freely moving animals increases the excitatory input to all of the heads of the depressor muscle, whereas injecting OA decreases excitatory input.

Examiners:

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LIST OF ABBREVIATIONS

5-HT	Serotonin (5-hydroxytryptamine)
A1 (A2...A6)	Abdominal ganglion 1 (2-6)
APH	Arthrophragm
BI	Basis (or basi-ischium) of leg
CB	Coxo-basal (usually in reference to the joint)
CDc	Caudal head of the caudal depressor muscle
CDcx	Coxal head of the caudal depressor muscle
CDd	Dorsal head of the caudal depressor muscle
CDm	Medial head of the caudal depressor muscle
CDMN	Caudal group of depressor motor neurons
CEG	Circumesophageal ganglia
CFB	Center fiber bundle
CLc	Caudal head of the caudal levator muscle
CLr	Rostral head of the caudal levator muscle
CI	Common inhibitor
CSD	Cuticular stress detector
C-Th-C	Caudal thoracico-coxal nerve root
Cx	Coxa of leg
Dc	Caudal head of the depressor muscle
Dcx	Coxal head of the depressor muscle
Dd	Dorsal head of the depressor muscle
DEP	Leg depressor muscle
DLN	Distal leg nerve
Dm	Medial head of the depressor muscle
Ds	Sternal head of the depressor muscle
Dv	Ventral head of the depressor muscle
Dvc	Caudo-ventral head of the depressor muscle
Dvr	Rostro-ventral head of the deressor muscle
LEV	Leg levator muscle
ENP	Endopleurite
ENS	Endosternite
EPM	Epimeron

FOE	First observable effect (of injected amines)
HP (HPR, HPC)	Horizontal process of endopleurite (rostral, caudal)
HRP	Horseradish peroxidase
LFB	Lateral fiber bundle
LN	Lateral neuron filled from the depressor muscle
MFB	Medial fiber bundle
MPH	Mesophragm
N1, N2, N3	Segmental nerves 1, 2, and 3
OA	Octopamine
PBS	Phosphate-buffered saline
PPH	Paraphragm
PRO	Leg promotor muscle
RD	Rostral depressor muscle
RDMN	Rostral group of depressor motor neurons
RLc	Caudal head of the rostral levator muscle
RLd	Dorsal head of the rostral levator muscle
RLs	Sternal head of the rostral levator muscle
REM	Leg remotor muscle
RLm	Medial head of the rostral levator muscle
RLr	Rostral head of the rostral levator muscle
R-Th-C	Rostral thoracico-coxal nerve root
SA	Sternal artery
SEG	Subesophageal ganglion
STE	Sternum
SuON	Supraesophageal nerve
T1 (T2...T8)	Thoracic ganglion 1 (2-8)
TC	Thoracico-coxal (usually in reference to the leg joint)

ACKNOWLEDGEMENTS

First I would like to thank Dorothy Paul for starting me on this work and teaching me about science, research, and the value of comparative studies. She has given me the kind of support which too few graduate students enjoy, namely constructive criticism, the opportunity to attend many conferences (11 in 6 years as I recall), and the freedom to explore the area around my research, within reason of course. Next I would like to thank my wife, Nadja, without whom this dissertation would not have been completed on time. She kept me sane during far too many very long days. My committee members past and present, Robert Burke, Nancy Sherwood, Geri Van Gyn, Mike Corcoran, and Ben Koop, have provided support and guidance when I needed it, but were never destructive. Zen Faulkes showed me how to do EMG recordings, and was an excellent lab mate and source of information on animal behaviour. In and around U. Vic., people too numerous to mention their individual contributions helped with various aspects of this work. In no particular order, these are Louise Page, Pat Kerfoot, Neil Mossey, Gerry Horne, Tom Gore, Heather Down, Sally Leys, Anne Pound, Manish om Prakesh, George Mackie, Chaman Singla, John Morrison, Patrick von Aderkas, and Eleanore Floyd. I would also like to thank Don Edwards, Barbara Musolf, and Ulrike Spörhase-Eichmann at Georgia State University for teaching me how to do immunocytochemistry the right way, and allowing me to use the confocal microscopes at that institution.

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Chapter 1: Introduction

Background and Overview

I undertook this study with the goal of using comparative methods to further understand how the amines serotonin (5-HT) and octopamine (OA) elicit behavioural changes in decapod crustaceans at a cellular level. When I began this work, the classic paper from Kravitz's laboratory (Livingstone et al. 1980) was widely cited as the example of how a single modulator could induce dramatic behavioural changes when injected into freely moving animals. Livingstone et al. (1980) showed that injecting lobsters with octopamine caused the animals to assume a stance that is typical of submissive animals, and injecting with serotonin caused them to assume an aggressive stance. A few other papers from the early 1980's showed similar results with other species, but not much had been done since that time. Additionally, a few studies tried to determine some of the cellular mechanisms responsible for these changes. These investigations showed some specific influences of the amines on particular neuron types, but nobody had yet found how the amines influence the pathways as a whole.

As it turns out, the initial studies had been blown up to something more than they really were. The initial paper in the journal *Science* from Kravitz's laboratory was written in the best possible light. They didn't falsify anything, of course, but neither did they substantiate the limitations of the study quite as clearly as they might have, namely that the animals were injected with massive doses of the amines and didn't move, often for hours, after receiving injections (E.A. Kravitz, 1995, personal communication). After this, authors citing this *Science* paper made the study out to be something it was not, namely that injection of the amines could influence behaviour (e.g. Kravitz, 1988), or that the amines facilitated motor output (e.g. Pearlstein et al., 1998A). This latter statement is likely true in some way, and subsequent studies have begun to reveal how this may be done, but without an understanding of what is actually happening to the motor circuits during these experiments, such statements were and are somewhat premature and misleading. By the time I found this out, however, I had already completed a controlled dose response curve in the squat lobster *Munida quadrispina* (Anomura, Galatheidae), and found that by injecting carefully controlled amine doses, behaviour can be changed in a way that mimics naturally occurring agonistic interactions (Chapter 2). During the course of these experiments I also did preliminary studies on the effects of injected 5-HT and OA on the sand crab *Emerita analoga* (Anomura, Hippidae) (Chapter 4).

Biologically meaningful behavioural changes have now been induced in several other species, including crayfish and lobsters, and more studies are being undertaken.

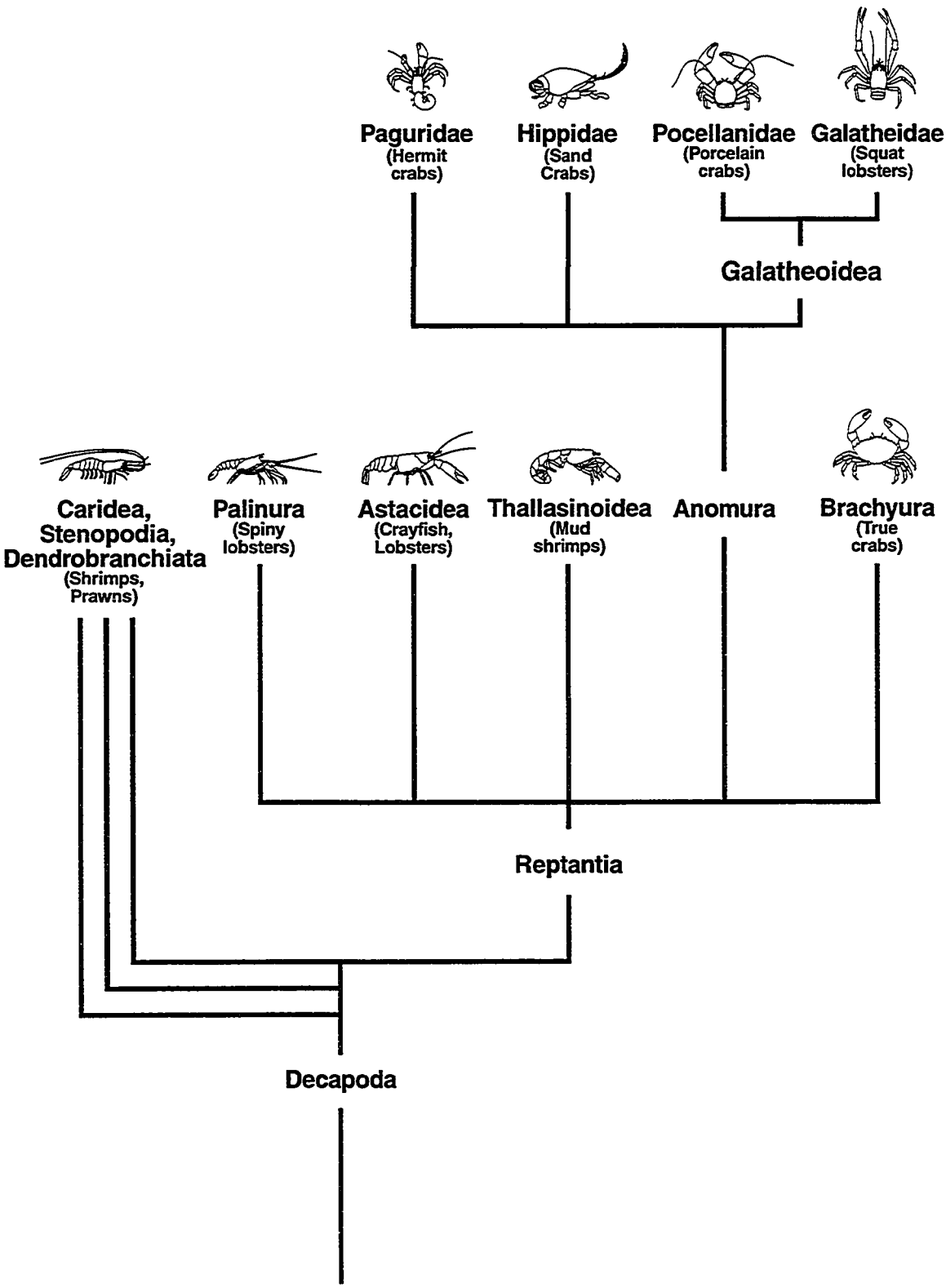
From this point I wanted to look at some of the changes induced by the amines at the neuronal and muscular levels. I chose to look at the depressor muscle because it is involved in producing aggressive stances and behaviours, more was known about the cellular effects of 5-HT than OA, and 5-HT is much easier to work with than OA. Furthermore, since *M. quadrispina* turns out to have fewer serotonergic (and octopaminergic) neurons than any other decapod studied so far (Chapter 3), I felt I stood a relatively better chance of finding the aminergic neurons involved in the behavioural changes. I started these studies with some success, but mostly the results were confusing and often contradictory (Chapter 4).

The pereiopod depressor muscle of decapods is usually treated as a single functional unit, even though it has been known for some time to be composed of multiple heads, as are the other proximal pereiopod muscles. Furthermore, there is a small body of evidence that the levator muscle has different activation patterns for different functions, and there is even one hint that the depressor muscle may not act as a single unit (Pearlstein et al., 1995). My initial results suggested that the depressor muscle definitely was not a single functional unit, and so I switched gears to find out just what the different parts of the depressor were doing. Four years later those studies are finished (Chapters 5 & 6), and my plan to look at cellular influences of the serotonergic cells has not come to pass, other than the initial preliminary data that sent me off on this other tack. As often seems to be the case with researchers in general, and graduate students in particular, I really had no idea what I was biting off when I started this - it looked like a simple problem.

Munida quadrispina Natural History

The decapods are the largest crustacean order, with approximately 1200 genera and over 10 000 species described (Bowman & Abele, 1982), and include most of the more familiar crustaceans, such as shrimp, crabs, and lobsters (Fig. 1.1). The general consensus is that there are eight infraorders within the Decapoda, although the relationships between the infraorders are in debate (e.g. see Burkenroad, 1981; Bowman & Abele, 1982; Abele, 1983; Schram, 1986; Kim & Abele, 1990; Katz and Tazaki, 1992). Most authors agree that Anomura and Thalassinoidea are closely related (Burkenroad, 1981; Abele, 1983; Martin and Abele, 1986), but the hypothesis that

Fig. 1.1. Phylogenetic relationships among decapods. This scheme is based on a summary of several proposed phylogenies (Burkenroad, 1981, 1983; Bowman and Abele, 1982; Abele, 1983; Felgenhauer & Abele, 1983; Abele and Felgenhauer, 1986; Schram, 1986; Kim and Abele, 1990). The three infraorders of shrimp are not a monophyletic group, but are commonly placed together in the "Natantia", or non-reptantian decapods. The relationships between the five reptantian infraorders are not clear. Most authors agree that Thalassinoidea and Anomura are closely related, and Brachyura are the most recently derived infraorder, but the relationships between these and the Astacidea and especially the Palinura (see Williamson, 1988) are not clear (Bowman and Abele, 1982; Abele, 1983; Schram, 1986). Only four of the 13 families of anomurans are shown; the others are the Pomatochelidae, Diogenidae, Coenobitidae, Lomisidae, Lithodidae, Parapaguridae, Aeglidae, Chirostylidae, and Albuneidae (Schram, 1986).


Paguridae
(Hermit crabs)
Hippidae
(Sand Crabs)
Pocellanidae
(Porcelain crabs)
Galatheidae
(Squat lobsters)
Galatheoidea
Caridea, Stenopodia, Dendrobranchiata
(Shrimps, Prawns)
Palinura
(Spiny lobsters)
Astacidea
(Crayfish, Lobsters)
Thallasinoidea
(Mud shrimps)
Anomura
Brachyura
(True crabs)
Reptantia
Decapoda

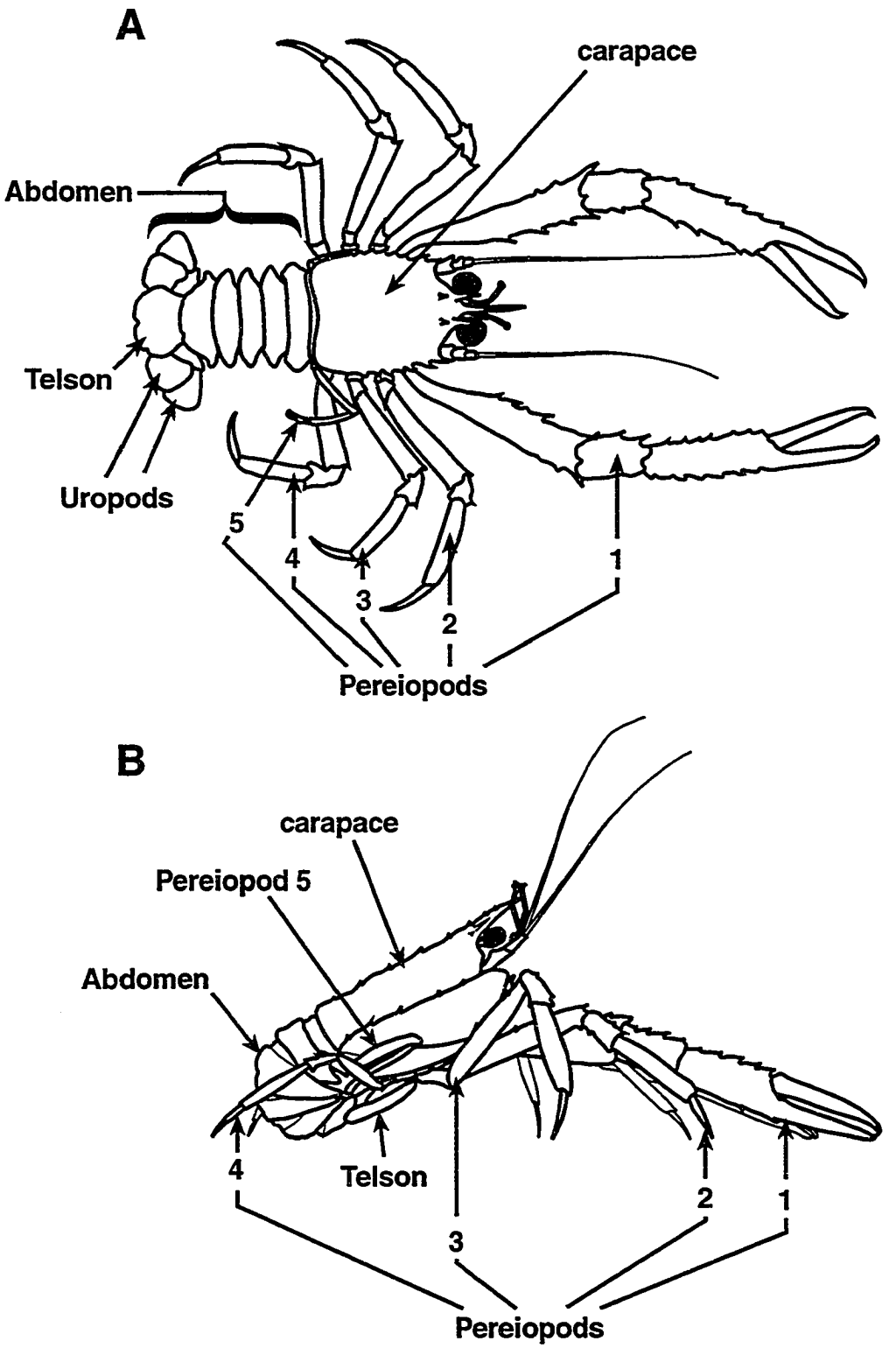
thalassinoideans are part of the Anomura has been discredited (Bowman & Abele, 1982; McLaughlin & Holthuis, 1985). Furthermore, Brachyura are believed to be a relatively recently evolved group (Glaessner, 1969; Schram, 1986) closely related to both the anomurans and thalassinoideans, although the exact nature of this relationship is not clear (Williamson, 1976, 1982; Rice, 1980, 1981A, 1981B, 1983).

M. quadrispina, commonly known as the squat lobster (Fig. 1.2), is one of the two species of galatheid anomurans found in waters near Victoria, B.C., the other being *Munidopsis quadrata*. There are 258 described species of galatheids, the majority of which live in deep water, even at abyssal hot vents (V. Tunnicliffe, 1993, personal communication). The exceptions to this general rule are in the South Pacific and Indian Ocean, where many species, often very colourful, are abundant on shallow water reefs, and a few species spend at least part of their adult lives pelagically (McWhinnie & Marceniak, 1964; Griffin & Yaldwyn, 1968; Blackburne, 1977). The closest local relatives to the galatheids are the porcelain crabs, whose external morphology superficially resembles true crabs (Brachyura) (Fig. 1.1). There are 11 other families in the infraorder Anomura, including the hermit crabs and sand crabs (Bowman & Abele, 1982). The anomurans can be thought of as the invertebrate analogue of marsupials in terms of their variety of form and ecological habits. Anomurans have forms that superficially resemble crabs, shrimp, and lobsters, as well as a number of forms that are unique to the infraorder, and they have evolved to exploit a tremendous array of ecological niches.

Very little is known about the biology and ecology of *M. quadrispina* in the wild. Their preferred habitat is deep, sometimes even extending into periodically anoxic zones in deep inlets such as Saanich Inlet near Victoria, B.C. (Levings, 1980; Tunnicliffe, 1981; Burd, 1983), although they do occasionally venture into shallower water and the intertidal zone (unpublished observations). They are generalist feeders, scavenging, filter feeding, or preying on small organisms as opportunities arise. They are quick to feed on any dead or unhealthy organism, leading to some anecdotal reports of their being active predators on conspecifics and other crustaceans, but this is not true.

In their preferred habitat *M. quadrispina* can reach incredible densities, often forming a solid mat of organisms on every exposed surface (Levings, 1980; V. Tunnicliffe, 1993, personal communication). In the laboratory squat lobsters are often seen literally piling on top of one another, even when space is available for them to spread out, and they are generally gregarious, with limited social conflict (see Chapter 2).

Fig. 1.2. The squat lobster *Munida quadrispina*. Note the reduced fifth pereiopod, the three pairs of walking pereiopods (pereiopods 2-4), and the very small first abdominal somite barely visible at the caudal edge of the carapace. (A) Dorsal view, with the abdomen straightened to show it clearly. (B) Side view of the animal in its normal resting (squatting) position, with the abdomen in its normal, folded position. Note the tilt to the thorax.


A
carapace
Abdomen
Telson
Uropods
5
4
3
2
1
Pereiopods
B
carapace
Pereiopod 5
Abdomen
4
Telson
3
2
1
Pereiopods

They are as likely to be found hanging upside down or off a sheer face as sitting upright. Little time is spent foraging, rather they are usually found sitting still and filter feeding, apparently waiting for food to come to them. Foraging would likely be wasted effort for the most part, because where these animals live in high densities, the substrate is picked clean of anything edible.

A Morphology Primer

M. quadrispina external morphology

Upon first observing *M. quadrispina*, many people mistake them for crabs, shrimps, or lobsters. This is no doubt due to inexperienced observers associating them with things familiar, as they do have features in common with all of these animals. Nevertheless, closer inspection reveals characteristics that distinguish *M. quadrispina* from each of these groups, and clearly identify them as members of the Anomura.

M. quadrispina is a typical decapod, with 5 pairs of pereiopods, a large carapace which completely covers the dorsal and lateral surfaces of the cephalothorax, and a large muscular abdomen which terminates in a broad tailfan, formed from the telson and uropods (Fig. 1.2). The body is dorso-ventrally flattened and the sternum on the ventral thorax is broad. The animals are red to brownish red in colour with off-white stripes, and have many small spines lining most edges of the exoskeleton, including the four distinct spines above the rostrum from which comes their scientific name.

The first pair of pereiopods is very long, thin, and chelate, with sharp hooks at the tips of the "pincer". These chelae are very effective at reaching into confined spaces and gripping soft items, but of little use for fighting or defense, and almost useless at crushing hard objects. Many other decapods have large chelate first pereiopods, and the crayfish and lobsters also have chelate second and third pereiopods, although these are no larger than typical walking pereiopods. *M. quadrispina* has three pairs of long, thin walking pereiopods that are held loosely flexed to the sides. Typical of anomurans, the last pair of pereiopods is much reduced in size, chelate, highly flexible, held folded alongside the thorax, and used as cleaning appendages. Modification of the fourth or fifth pair of pereiopods also occurs in some of the Dendrobranchiata and Brachyura (McLaughlin, 1982).

The pereiopods of decapods have seven articulated segments that are joined by flexible arthrodial membranes and allow freedom of movement of the limb tip in any

plane (Fig. 1.3A). The basis and ischium are tightly fused and often referred to together as the basi-ischium. Between these two segments is the plane of autotomy, which is the point at which a decapod may shed its appendages in response to trauma. The distal end of the pereiopod may be chelate, with the distal end of the propodus becoming elongated and forming a fixed finger against which the dactyl moves opposably. The propodus or carpus may be flexible, as is the case for the propodus of the fifth pereiopod of *M. quadrispina*.

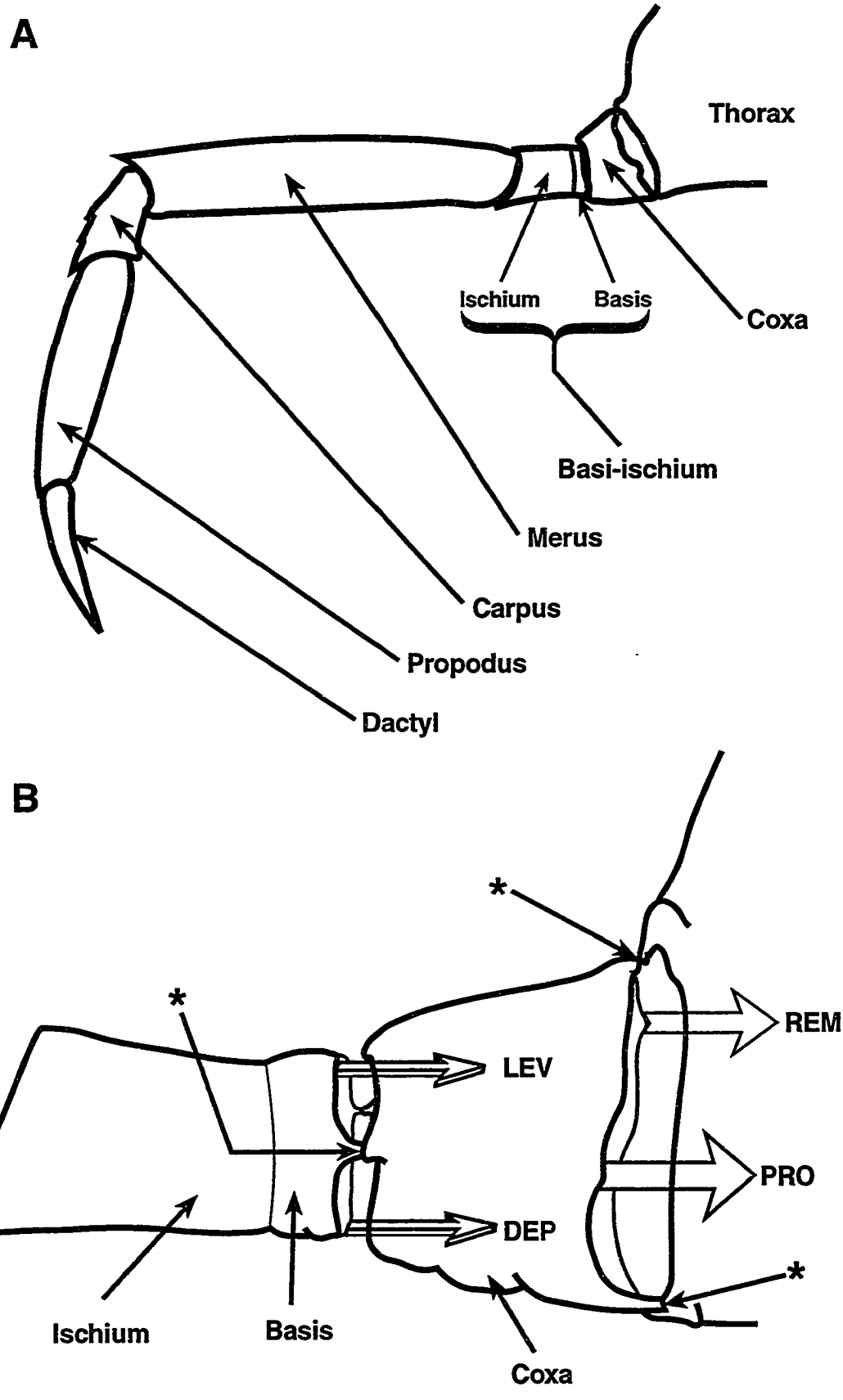
Of particular interest to this work is the way the proximal pereiopod segments, the coxa and basis, are articulated and controlled (Fig. 1.3B). The thoracico-coxal joint is controlled by the promotor and remotor muscles which move the pereiopod rostrally and caudally, respectively. The promotor muscle inserts on the rostral lip of the coxa, and the remotor muscle on the caudal lip, with the condyles (articulation points) on the dorso-ventral axis. The coxo-basal joint is articulated on the rostro-caudal axis at approximately a 90° angle to the thoracico-coxal joint. The depressor muscle inserts to the ventral lip and the levator muscle inserts to the dorsal lip of the basis, respectively lowering and raising the pereiopod.

The muscular abdomen is normally held loosely curled under the thorax, never straight as in crayfish or shrimp (Fig. 1.2B). The abdomen is used for tailflipping locomotion, as in shrimps and crayfish, although galatheids lack the giant escape circuits of these animals (Sillar & Heitler 1985; Wilson & Paul 1987). *M. quadrispina*'s normal resting posture (squatting) is with its head tilted slightly up, and supported by its folded abdomen, and tips of its walking pereiopods and chelae (Fig. 1.2B).

Endophragmal skeleton

The endophragmal skeleton of decapods is a complex set of columns and walls that provides rigidity for the thorax and attachment points for thoracic, proximal pereiopod, and other muscles. The parts of the endophragmal system are made up of infoldings of the cuticle, or exoskeleton, and as such are continuous with the exoskeleton and are shed with it during molting. The various parts of this system have been given many names by many different researchers; for a fairly complete list of the various systems of nomenclature see Rayner (1965). Without disputing the merits of the various nomenclatures, for clarity I will use only the terminology from Huxley's (1880) classic treatise, which seems to have fairly broad acceptance.

Fig. 1.3. External anatomy of a decapod walking pereiopod and the muscles that control its proximal joints. (A) The seven segments of a typical decapod walking pereiopod (*M. quadrispina*'s). Note the fusion of the basis and ischium. (B) The actions of the four proximal pereiopod muscles. The coxal muscles are the promotor (PRO), which moves the pereiopod rostrally, and the remotor (REM), which moves the pereiopod caudally. The basal muscles are the levator (LEV), which moves the pereiopod up, and the depressor (DEP), which moves the pereiopod down.

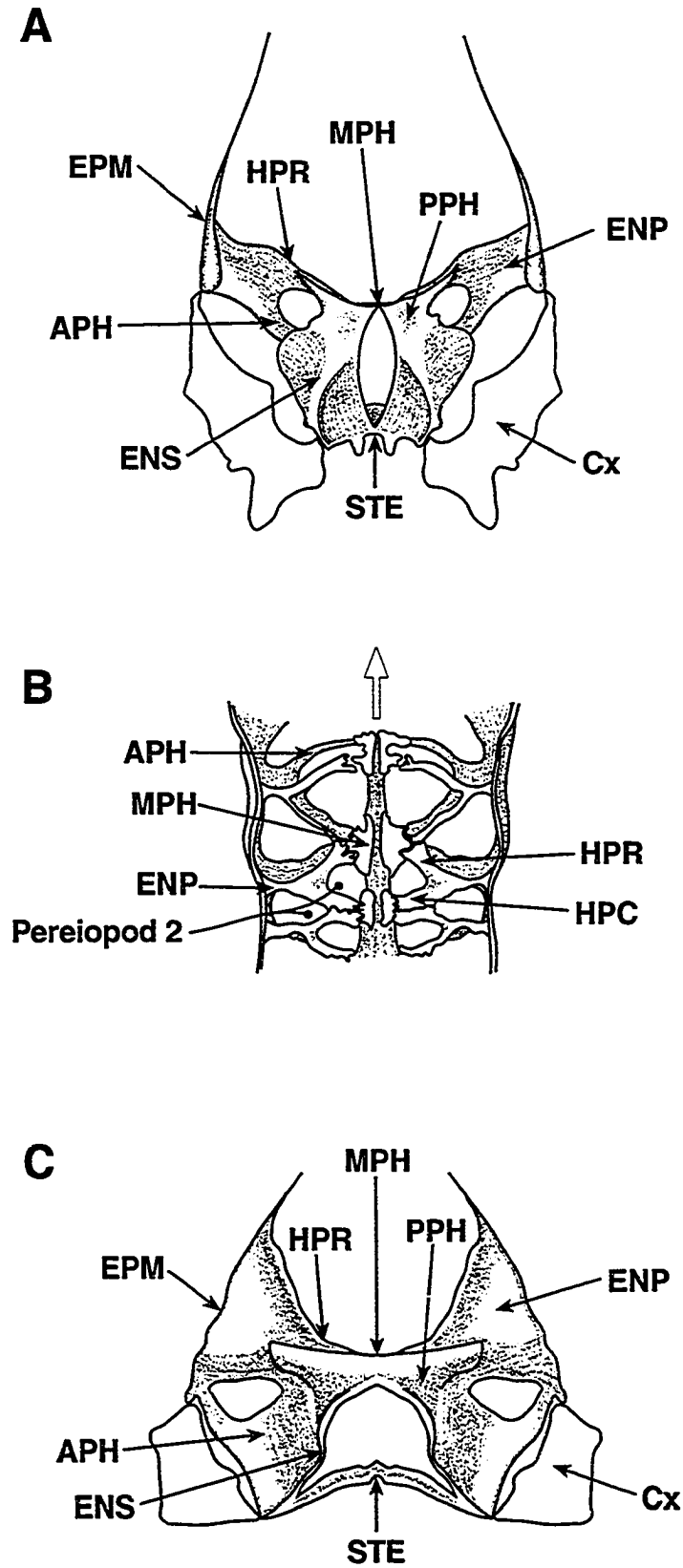

A
Thorax
Ischium
Basis
Coxa
Basi-ischium
Merus
Carpus
Propodus
Dactyl
B
*
*
REM
LEV
PRO
DEP
*
Ischium
Basis
Coxa

Detailed studies of endophragmal skeletal systems of decapods have been limited to crayfish (Huxley, 1880; Schmidt, 1915; Pilgrim and Wiersma, 1963; Rayner, 1965), two palinurans (Paterson, 1968; Schram, 1986), some crabs (Bourne, 1922; Cochran, 1935), a hermit crab (Pilgrim, 1973), and a few notes on an unidentified galatheid (Schram, 1986). None of these authors have suggested what might be the ancestral form of the endophragmal skeleton, or what form a typical skeleton may take. Nevertheless, it is clear from these few studies that thorax morphology can be quite different, even between closely related species, and among all decapods the diversity is extensive. Crayfishes, the white rats of the Crustacea, are used as a baseline for comparison in many of these studies, although there is no evidence that ancestral decapods had an endophragmal skeleton resembling that of modern crayfish (Schram, 1986).

The endophragmal skeleton consists of four infoldings of the cuticular wall between every two thoracic somites (segments). These infoldings surround the segmental chambers that contain the appendage neuromusculature, called the arthrodial cavities. The medial pair of infoldings (one per side) are called endosternites, and the lateral two are called endopleurites (Fig. 1.4A). Both of these structures are deeper invaginations of the primary intersegmental fold, which is essentially continuous around the circumference of the animal (Rayner, 1965). Between these structures are the arthrophragms, derived partly from the primary intersegmental fold of the sternum and epimera, and the endopleurites. The endosternites ascend dorsally, turn medially, and suture tightly together forming an elongate bar (Fig. 1.4A & B). The medial portion of this bar is the mespophragm and the lateral is the paraphragm. The endopleurites project medially and branch obliquely into an anterior and posterior horizontal process. The anterior horizontal process connects with the corresponding paraphragm of the same intersegment, the posterior with the next posterior paraphragm (Fig 1.4B) (Huxley, 1880; Pilgrim, 1973; Schram, 1986). Therefore, the arthrodial cavity has two parts: a lateral part in which most of the proximal pereiopod muscles arise, and a medial part in which the largest head of the promotors arises. The sternites make up the framework of the entrance to the arthrodial cavity and, dorsal to the arthrodial cavities, the sternites and epimera are tightly joined.

M. quadrispina shares the same four basic parts of the endophragmal skeleton with crayfish, but there are three main differences that concern this study. First, the sternum is much wider, resulting in the pereiopods extending more to the side rather than down as in crayfish (Fig. 1.4C). Second, the arthrophragms are much broader, forming

Fig. 1.4. The endophragmal skeletons of *M. quadrispina* and the crayfish *Cambarus bartonii*. The nomenclature is based on Huxley (1880). (A) A transverse section, viewed from rostral, of *C. bartonii*'s endophragmal skeleton at the level of pereiopod 2. (B) Dorsal view of *C. bartonii*'s endophragmal skeleton (modified from Huxley, 1880). (C) Transverse section, viewed from rostral, of *M. quadrispina*'s endophragmal skeleton at the level of pereiopod 2. APH, arthrophragm; Cx, coxa; ENP, endopleurite; ENS, endosternite; EPM, epimeron; HPC, caudal horizontal process of the endopleurite; HPR, rostral horizontal process of the endopleurite; MPH, mesophragm; PPH, paraphragm; STE, sternum.


A
EPM
HPR
MPH
PPH
ENP
APH
ENS
STE
Cx
B
APH
MPH
ENP
Pereiopod 2
HPR
HPC
C
MPH
HPR
PPH
EPM
ENP
APH
ENS
STE
Cx

an almost complete wall between the arthrodial cavities of the pereiopods. Finally, the endopleurites extend dorsally and rostrally, forming almost completely enclosed dorsal continuations of the lateral part of the arthrodial cavity.

This is by no means a complete description of the endophragmal skeleton in these two animals. In both species, there is considerable variation between somites from anterior to posterior, and there is a significant reduction of the posterior endopleurites and endosternites and fusion of the anterior endosternites in *M. quadrispina.* For clarity, I have limited this review to the parts that are directly relevant to this study.

Some Notes on Terminology

One of the greatest obstacles to the clear enunciation of scientific ideas is the prevalence of contradictory, duplicated, and misused terminology. A common example of this in recent literature, which has been the subject of several letters to leading journals, is the incorrect use of the term homology to describe the percentage of sequence similarity in genes or proteins. Several less grotesque examples occur in the research that preceded this dissertation, and I will take this opportunity to explain some of the terminology I use, while other instances are discussed later.

In many publications on crustacean (and other invertebrate) anatomy, the terms anterior and posterior have been used interchangeably with the terms rostral and caudal to describe the front and back, respectively, of the animal. In most cases this poses no problem, but when comparing these animals to animals who do not move with their head forward, such as humans, sessile animals, and many animals that live in the water column, this becomes confusing as rostral is no longer anterior, at least in common usage. Therefore, I use the terms rostral, meaning towards the head, and caudal, meaning towards the tail, exclusively.

Peripheral nerve branches leaving the ganglia are often called nerve roots in crustaceans, and nerves in every other invertebrate. The addition of "root" to the name is pointless, and implies that they may somehow be different than the nerves of, say, insects. They are not, and therefore should be called nerves, and the term "nerve roots" reserved for the bundles of axons contained within a ganglion and leading to the periphery (see Leise et al., 1986).

The Techniques

Amine Injection

When injecting substances into a living organism in order to determine their systemic effects, one must choose an injection site which will allow as rapid as possible dispersion through the body. The two most important reasons for this are: 1) to reduce possible confounding artifacts caused by systems being activated sequentially as they are reached by the injected substance and 2) to reduce any effects of higher concentrations near the injection site. Of course, if one is trying to study the effects on a single behaviour or process, using substances with far ranging effects such as 5-HT or OA (for reviews see Beltz & Kravitz, 1986; Bicker & Menzel, 1989; Beltz, 1999), it would be better to deliver the substances to a restricted area and avoid potentially confusing influences on other systems. However, the technology to do this in a freely moving crustacean has so far not been developed, so those of us doing this research have to keep potentially confounding effects in mind when doing our analysis.

The injection sites most often used in large crustaceans are the pericardial organs, paired sinuses located on the dorsal surface just under the carapace, which serve as major secretory sites for neurogenic bioactive molecules (Beltz & Kravitz, 1986). In *M. quadrispina*, however, the pericardial organs proved too small and easily damaged to be used reliably, so I injected along the midline into the ventral hemolymph sinus. In *M. quadrispina*, and other animals with a similar thorax plan, such as brachyuran crabs, the ventral hemolymph sinus is a large, open cavity in the ventral part of the thorax (and abdomen, although it is not as extensive there) filled with loose connective tissue. It can be easily penetrated with a rostrally-directed needle inserted at the thoracic-abdominal juncture. The ventral nerve cord lies immediately dorsal to the sinus, and the sternum with attached muscles (the sternal heads of the levators and depressors-see Chapter 5) is immediately below. Careful injection into this area can avoid the ventral nerve cord and musculature, and cause little noticeable permanent damage to the animal, even after many injections. Tests using dye indicated that the injected substance was completely dispersed throughout the animal less than 30 seconds after injection.

Some experiments using larger crustaceans (e.g. see Huber et al., 1997A & B) have used permanently mounted catheter tubes to deliver substances into the general circulation. I tried this with *M. quadrispina*, but found that the highly flexible fifth

pereiopods proved very adept at loosening the catheter tube in the animal, often leading to injected substances leaking out, or, at worst, infection at the implant site or death.

Immunolabelling

In whole mount, the ventral nerve cord of *M. quadrispina* proved very resistant to penetration of antibodies and other substances used in affinity reactions, such as avidin. Various combinations of Triton-X 100, acetone, Dent's solution (80% methanol, 20% dimethyl sulfoxide), propylene oxide, and the proteolytic enzymes collagenase, Proteinase K, and pronase were used to try to increase permeability. However, it was the rare preparation that had both good labeling and no destruction of fine neurites by the methods used to permeabilize the tissue. To exacerbate this problem, the ventral nerve cord of *M. quadrispina* is highly autofluorescent on its own, and aldehyde fixatives make this problem far worse. Standard methods of decreasing autofluorescence, such as incubation in multiple changes of sodium borohydride or pre-exposure to intense light, reduced the autofluorescence but not to a satisfactory degree. However, somewhat surprisingly, rhodamine labels were obscured much more than carboxyfluorescein, so this problem could be overcome to a large extent by using only fluorescein-based photofluors. Horseradish peroxidase-conjugated secondary antibodies were tried, but these proved to have more difficulty penetrating the ganglia than the fluorescent antibodies. Reliable and consistent immunolabels were difficult, but not impossible, to get. Crayfish nerve cords, in which the distribution of serotonergic and octopaminergic neurons is known and which do not have serious penetration or autofluorescence problems, were often run side by side with *M. quadrispina* nerve cords as positive controls to ensure that the technique was working.

Thick sections of paraffin-embedded thoracic and abdominal ganglia were cut and labeled with antibodies against 5-HT and OA; these helped to confirm the locations of somata, and got around the penetration problems of whole mounts. However, they were of limited usefulness in following neurites, as I found that it was easy to confuse neurites in areas where they were packed tightly together, whereas with careful microscopy in whole mount, neurites could usually be followed.

Electromyograms (EMGs)

Electromyograms (EMGs) are very useful for demonstrating the excitatory nervous stimulation reaching muscles in freely moving animals. However, as Bernstein

(1984) points out, there is no clearly definable relationship between an organism's movement and the activity of the nervous system. This is because external factors such as load and terrain can shape the final movement as much as the nervous input received by the muscles. Also, EMGs cannot directly record peripheral inhibitory input, which is known to exist in crustacean pereiopod muscles (Dudel & Kuffer, 1961; Atwood & Walcott, 1965; Clarac et. al., 1987; Clarac & Cattaert, 1996). The consequences of this were demonstrated very clearly by Faulkes and Paul (1998), who showed that although the patterns of EMG potentials in the stretcher and bender muscles in sand crab digging pereiopods are identical (they share excitatory innervation), the movement patterns of these two muscles are not, due to the actions of inhibitory motoneurons specific for each muscle (Faulkes and Paul, 1997A). Therefore, records of movement, such as video recordings, should accompany EMGs. While temporal resolution of video is not very good, with a sampling rate of 30 frames per second, it is sufficient for the relatively slow movements involved in postural changes and walking which I wanted to observe. I synchronized the video with the EMG records with a device which, when triggered, would simultaneously light a small LED bulb and send an electrical pulse to a dedicated channel on the tape recorder.

Ayers and Clarac (1977) and Clarac et al. (1987) reported that they were able to distinguish activity of single neurons on the basis of the size and shape of the EMG spikes. For the most part, I found this not to be possible. Factors such as muscle movement, time since electrodes were implanted (amount of healing/scarring), and position of the electrodes all influence EMG trace shape, making it impossible to follow activity patterns of individual neurons over long periods of time within an animal, or between animals, with any reliability. However, activity in individual neurons could be followed for short periods of time in a single recording, as long as I could continuously monitor traces and note any subtle changes in EMG spike shape. In order to minimize position shifts during contraction of the muscle, I implanted the electrodes as close to the origin of the muscle as possible.

Chapter 2: Serotonin and octopamine elicit stereotypical agonistic behaviours in Munida quadrispina[1]

Introduction

Serotonin (5-HT) and octopamine (OA) modulate many physiological processes involved in agonistic behaviours in crustaceans, as does 5-HT in vertebrates, although the effects of serotonin in vertebrates appear to be opposite those in crustaceans (Raleigh et al., 1991; Olivier et al., 1995). Although some examples of aminergic influences on neural circuits have been published, the neurological pathways by which these influences are effected are not well understood. Invertebrates make ideal subjects for study of the cellular effects of 5-HT and OA because there are far fewer neurons involved in producing their effects, and their processes can be, in general, more completely and exactly mapped out than in vertebrates (Paul, 1976; Getting, 1986 & 1989; Mulloney et al., 1993; Pearlstein et al., 1998A). Additionally, single cells are, with the exception of certain examples in fish (e.g., Eaton & Hackett, 1984; Grillner et al., 1991), more easily and reliably isolated for study than in vertebrates. This is demonstrated in Table 1 in Pearson (1993), that directly compares studies on fictive motor patterns in vertebrates and invertebrates. However, many of the basic motor, command, and modulatory elements have been conserved through evolution, and therefore lessons learned in invertebrates often have functional applications in vertebrate neurobiology as well (Arbas et al., 1991; Heiligenberg, 1991; Pearson, 1993).

Injecting American lobsters or crayfish with 5-HT or OA induces postures resembling typical aggressive or submissive stances, respectively (Livingstone et al., 1980). In crabs injected 5-HT elicits postural flexion and OA injection elicits extension (Bevengut & Clarac, 1982; Wood et al., 1995). Similar postural outputs are induced in both groups, but neither study reported induction of complex behaviours typical of normal agonistic encounters by either amine, or any influence of amine dose.

A clear behavioural change is elicited in submissive crayfish perfused with 5-HT: both incidence and length of fights with dominant animals increase (Huber, 1995; Huber et al., 1997A, B). Glanzman & Krasne (1988) implicated 5-HT and OA in gain-setting in the lateral giant interneuron escape circuit in crayfish. Current and prior social status has been found to influence serotonergic modulation of this circuit: 5-HT increases responsiveness to sensory input in dominant animals and decreases responsiveness in

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[1] This chapter has been published in a slightly different form (Antonsen & Paul, 1998)

submissive animals (Yeh et al., 1996, 1997; Edwards & Kravitz, 1997). Additionally, serotonergic cells act as gain-setters specific for the flexion control circuit (Ma et al., 1992).

Injected amines also affect posture of the amphipod *Gammarus lacustrus* (Helluy & Holmes, 1990) and the hippid sand crab *Emerita analoga* (Chapter 4). In both species injected 5-HT elicits postural flexion; in neither species, however, does injected OA alone affect posture or behaviour, but it inhibits the actions of injected 5-HT. The behavioural roles and perhaps also the mechanisms of action of 5-HT and OA clearly differ among crustaceans.

I have studied the serotonergic and octopaminergic influences on agonistic behaviours of the squat lobster *Munida quadrispina* (Anomura, Galatheidae), a decapod crustacean distantly related to the more thoroughly studied lobsters and crayfish. Galatheid crustaceans usually hold their muscular abdomen loosely curled under the thorax. Their long first pair of pereiopods is chelate, the second through fourth pairs are non-chelate walking pereiopods, and, typical of anomurans, the fifth pair is modified as cleaning appendages. *M. quadrispina* is native to deep marine waters off the west coast of North America. They live in rocky areas, often crowded together on accessible surfaces. Most wild populations are deep, making studies difficult, and no reports exist, even anecdotal, of agonistic interactions in the wild.

Several neuromuscular systems involved in agonistic encounters differ between galatheids and lobsters and crayfish. Among these are absence in galatheids of both giant escape tailflip circuits (Sillar & Heitler, 1985; Wilson & Paul, 1987), differences in abdominal proprioceptors (Wallis et. al., 1995), tailfan neuromusculature (Paul et al., 1985), and walking pereiopod musculature organization (Chapter 5). In this chapter I describe the normal behaviours of interacting *M. quadrispina* and examine the extent to which postures and behaviours typical of interacting animals can be induced by 5-HT and OA.

Methods

M. quadrispina were collected by trawl in Saanich Inlet near Victoria, British Columbia, Canada, and maintained in approximately 10° C recirculating natural sea water tables. Rocks and bits of netting were provided for the animals to climb. Animals were kept at densities approximating those in the wild and were fed regularly on mixed fish

and algal diet. One tank had a substrate and light regime which approximated their natural rocky habitat. They were observed for any evidence of developing dominance hierarchies or agonistic interactions. Animals of both sexes between 4 and 10 grams and in apparent good health were selected from this population for amine injection and isolation experiments. Some of the animals used for amine injection tests were selected directly from the main populations, while most were isolated in small groups prior to the experiments.

The small isolation aquaria had flat, textured bottoms; no attempt was made to reproduce a natural environment. I felt this was justified because previous, long term observations had shown that relative positions of animals on complex substrates had no effect on the frequency, course, or outcome of agonistic encounters. Detailed observations were made of these small groups to characterize behaviours typical of normally interacting animals for use as a baseline for the amine injection experiments. Normal stances and movements of non-interacting individuals and changes during agonistic encounters were recorded with videotape and still photographs. Pereiopod joint angles, measured to within 1° of accuracy, and drawings representative of each behaviour were taken from individual frames. I recorded relative positions of the antennae, claws, walking pereiopods, and abdomen, and any movements that occurred during interactions. Frequency and course of agonistic interactions between animals of various sizes and states of hunger, with and without food present, were noted. Signs of developing or prolonged dominance were sought in all groups. To mimic threatening situations, individual animals were challenged from outside the tank by sudden presentation of artificial visual stimuli: an artificial squat lobster and several dark shapes resembling fish. I chose to present these stimuli from outside the tank because there was no difference in responsiveness to these and stimuli approaching through the tank, and to avoid technical problems associated with pushing these sometimes quite large objects through small tanks. Additionally, squat lobsters in tanks were challenged by the introduction of an additional squat lobster to determine the reaction of animals to the appearance of nonfamiliar conspecifics. Results were pooled for all animals, with each group of animals serving as its own control in all comparisons of pre- and post-injection data presented in the Results. Food intake was controlled while animals were observed: some groups were kept satiated, while food was withheld from others for periods of up to 14 days.

Solutions of 5-HT and OA (Sigma) were prepared at concentrations between 0.01 and 1 mg/ml in physiological saline of the following composition: 460mM NaCl, 13.7mM $CaCl_2$, 12.7mM KCl, 10mM $MgCl_2$, 14mM Na_2SO_4, 5mM Maleic Acid, 10mM Tris Base, pH 7.4. All amine solutions were stored at 4°C and were less than 48 hours old when used.

Injections were made into the ventral hemolymph sinus at the thoracic-abdominal juncture, through a 27-gauge needle. I found that this injection point gave the fastest entry into the general circulation, the fastest distribution of the injected substance (tests using dye injection indicated less than 30 seconds to enter the vasculature in the ventral nerve cord along its entire axis), the least chance of damaging the ventral nerve cord or muscles vital to the experiments, and the most reproducible results. Injections of each amine started at 0.0001mg/g body weight, and were increased and decreased from this point until threshold and maximum non-toxic doses were found. Dose responses between these points were characterized. The concentration of the injected solution was such that the volume injected was never more than 5% of the animal's weight. Tests performed at the start of the experiments indicated that injecting a volume higher than 7-8% of the animal's weight resulted in erratic behaviour and sometimes death. However, injecting highly concentrated amines (above approximately 1 mg/ml) in low volumes had the same effect, so a balance was reached between volume and concentration that did not have any immediate detrimental effects on the animals. At least 6 hours were given between injections for recovery, with no more than two injections per day. I found that this injection rate did not result in any chronic effects on behaviour. Control injections of saline were done at 5% of the animal's weight. Food was offered every second day, after the conclusion of that day's experiments, and any left after 30 minutes was removed.

Forty-four animals received 5-HT dose series, 47 received OA dose series, and 21 received dose series of both amines. Starting immediately after each injection, changes in posture and behaviour of the animals, and responses to the same artificial stimuli used to challenge the untreated animals, were recorded continuously until behaviour appeared to have returned to normal. Postural and behavioural responses to injections were recorded as for the untreated, normally interacting animals. Mortality of injected animals during the injection experiments was not elevated above the low level normal in our larger sea water tables and was only slightly elevated following the experiments. Animals that recovered fully received repeat doses under a different set of conditions, such as a change in the stage of the molting or breeding cycle, or longer times in the

laboratory. Following characterization of the dose-response curves of animals which had been isolated in small groups, animals with similar responsiveness to the amines were placed together, and were observed for any behavioural changes. Injections were repeated after an additional period of one to three weeks in the new groups to determine if a change in social setting could induce a change in dose responsiveness.

Results

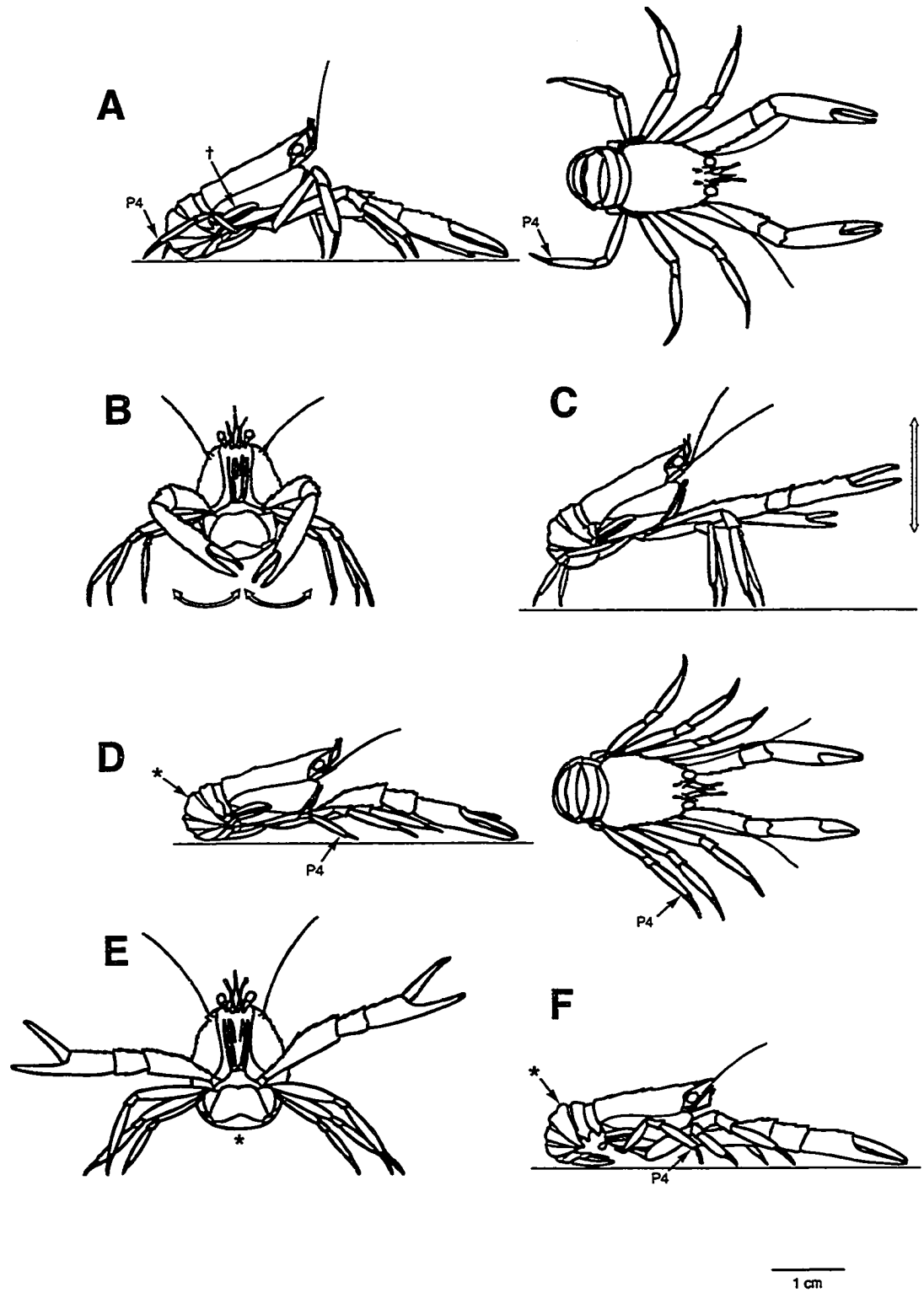
Posture and behaviour during normal interactions

All *M. quadrispina*'s postures and behaviours were consistent regardless of body orientation (i.e. sitting flat on a rock or hanging from an overhang), light levels within the limits of visual observation, or time of day or year. These animals normally rest in a squatting posture, with the head slightly elevated and the abdomen curled under the thorax, the dorsal surface of the tailfan resting on the substrate (Fig. 2.1A). The claws rest on the substrate to the front, and the three pairs of walking pereiopods are arrayed to the sides. *M. quadrispina* move by walking around the benthos and tailflipping short distances.

I observed no lasting dominance hierarchies among *M. quadrispina* in any of the sea tables or observation tanks. They did not have territories of any kind and were rarely aggressive towards each other. Transient agonistic interactions were always resolved without lasting effects. Dominance hierarchies did not develop and the frequency of agonistic encounters did not increase during mating periods. At least in captivity, the successful male in the competition for mates was the one who got to the receptive female first, regardless of size. Frequency of aggressive displays decreased in the presence of food. Feeding was a free-for all, success depending less on size than on speed in grabbing a piece of food and escaping to a safe spot. Larger individuals commonly used their closed claws to block competitors and shove them away, however, if many other animals were around some quickly found ways to reach in to steal the food.

No consistent movements or actions preceded the onset of either of *M. quadrispina*'s aggressive displays. Both aggressive behaviours involved depression at the coxo-basal (second) joints and flexion at the mero-carpal (fourth) joints of the walking pereiopods, which elevated the body relative to the substrate, while the abdomen remained loosely flexed. The first behaviour, which occurred while the animals were some distance apart, consisted of holding the claws in front, slightly elevated at the coxo-

Fig. 2.1. Postures and behaviours of normally interacting *M. quadrispina*. (A) side and top views of normal resting posture, with partial flexion of the walking pereiopods at the meral-carpal joint maintaining the head-up tilt of the cephalothorax. Note pereiopod four (P4) is remoted and the small fifth pereiopod (†) is held curled alongside the carapace. (B) shaking-claws aggressive behaviour. (C) raised-claws aggressive behaviour. (D) side and top views of the submissive, prostrate posture. Note that the abdomen is tightly curled (*) and all walking pereiopods, including the fourth pair (P4), are fully promoted alongside the thorax. (E) defensive stance, with raised claws held far apart. Note the abdomen (*) is tightly curled against the underside of the thorax, well off the substrate. (F) startle response. The rostral part of the abdomen is extended (*), the walking pereiopods, including the fourth pair (P4), are extended forward, although not to the same degree as in the submissive posture (compare with D). See Results for detailed descriptions of these behaviours.

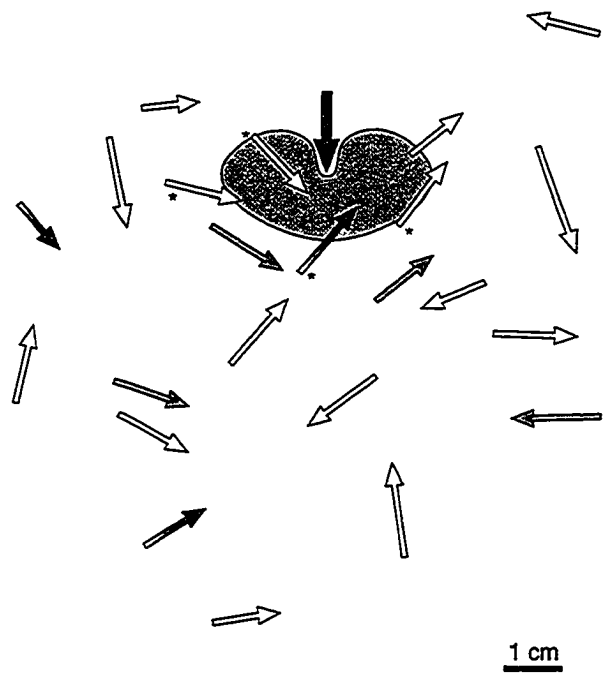

A
†
P4
P4
B
C
D
*
P4
P4
E
*
F
*
P4
1 cm

basal joint and slightly flexed, and intermittently shaking them horizontally and rapidly (approximately 2-5 Hz) at the thoracico-coxal joint in what appeared to be a warning display (Fig. 2.1B). During this "shaking-claws" display, the tips of the claws were kept at or below the level of the head, and the displaying individual would sometimes advance slowly towards its opponent. The second form of aggressive behaviour, the "raised-claws" behaviour, occurred when the animals were close or in contact. The advancing, aggressive animal extended both claws at the mero-carpal joint and elevated one or both claws high overhead to get its claws above the opponent's to push them down and its opponent back or to the side (Fig. 2.1C). I observed no consistent movements of the antennae at any time during agonistic interactions.

An aggressive individual usually turned to within approximately 45° of face on to its opponent but occasionally faced away (Fig. 2.2). The actions of the claws appeared to be the most important cues for the opponent, and the claws were visible regardless of the aggressive animal's orientation. Grasping rarely occurred, and the aggressive behaviours ended when one animal retreated or clearly showed itself to be submissive, or when both appeared to lose interest. Fighting was never seen in any encounter in the laboratory; the shed pereiopods and claws occasionally found in the densely populated sea tables were probably the result of cannibalism of recently molted or unhealthy animals, which was observed in a few instances. Size was not a factor in determining whether an aggressive display would be performed as small animals displayed to large ones as often as large animals displayed to small ones. Relative size of the animals also did not influence whether the non-aggressive animal would retreat or respond in kind to the display. The only exception to this was that very large animals very rarely responded at all to aggressive displays, unless they were by equally large animals. In this case, posturing by both animals sometimes continued for several hours. Only recently molted individuals were less likely than any other to perform aggressive displays and more likely to retreat from other squat lobsters; no animal at any other time was found consistently to be particularly aggressive or submissive.

Three types of reaction to aggressive displays occurred with different frequencies (Fig. 2.2), and their frequencies were the same regardless of which of the two aggressive displays initiated them. In only about 10% of agonistic encounters (22 of 225 observations) did individuals respond to aggression with aggressive displays of their own. In more than 70% (164 of 225 observations) of the encounters, the aggressors were ignored or pushed away without any reciprocal displays. Pushing was done with closed

Fig. 2.2. Composite diagram of 23 agonistic encounters between *M. quadrispina* individuals. The large black arrow is the animal that initiated each encounter with an aggressive display; each of the other arrows represents the second animal in different encounters. Arrows indicate the direction each animal was facing, and carapace length is represented by arrow length. Reactions of the second animals are as follows: (⟹) no reaction, (⟹) an aggressive reaction, (⟹) a submissive reaction. The shaded area is the claw reach of the first animal. An asterisk (*) at the base of the arrow indicates short-range encounters in which the first animal was performing the "raised-claws" behaviour. No asterisk indicates a "shaking-claws" display by the first animal.


1 cm

claws, and was performed with sweeping claw motions or extensions directly away from the body. The claws and body were not usually raised during pushing, and motions were not repeated, as in the aggressive "shaking claws" behaviour discussed above (Fig. 2.1C), unless the second animal continued to advance. Pushing never became violent, and transitions between pushing and any aggressive behaviours were rarely seen; an individual whose space was repeatably infringed upon usually moved away.

Behaviours which could be classified as submissive occurred in fewer than 20% (39 of 225 observations) of all agonistic encounters. Escape was the most common; animals retreated from an aggressor by walking or tailflipping away, and each behaviour occurred with approximately equal frequency. The bodies of retreating animals may or may not be lowered while walking backwards or sideways away from the aggressor. Tailflipping was not directional; the individual often came to rest temporarily as close or closer to the aggressive animal than when it started, in which case a second attempt to retreat often quickly followed. Occasionally, submissive animals prostrated themselves before an aggressor without retreating, by lowering the body to the substrate, extending the pereiopods, and promoting the fourth pair of pereiopods alongside the second and third pairs (Fig. 2.1D). This most often occurred when no route for retreat was open.

M. quadrispina occasionally performed a behaviour in response to a threat which could be analogous to a crayfish defense response (Wiersma, 1952; Glantz, 1977; Kelly & Chapple, 1990): the body was elevated, the animal turned toward the threat, and the open claws were held wide apart (Fig. 2.1E). This behaviour occurred only in response to handling or the sudden appearance of a potential predator (e.g. human hand, artificial fish shape), never in response to conspecifics, and was rare (approximately 15% of threatening situations not involving conspecifics). Defensive animals grasped the threatening object, sometimes violently, if it came within reach. More common were tailflipping away (50%) or startle responses with no retreat (22%). Startle responses involved a quick extension of the pereiopods and abdomen, resulting in an almost prostrate posture resembling the first stage of an escape tailflip when the rostral abdomen has extended (Fig. 2.1F). The startle position (Fig. 2.1F) was transient, unlike the prostrate posture that I observed to be held for periods up to 15 minutes. Most individuals were consistent in their defensive reactions, although no correlation existed between how an individual would react to a conspecific of any size and how it would react to potential predators.

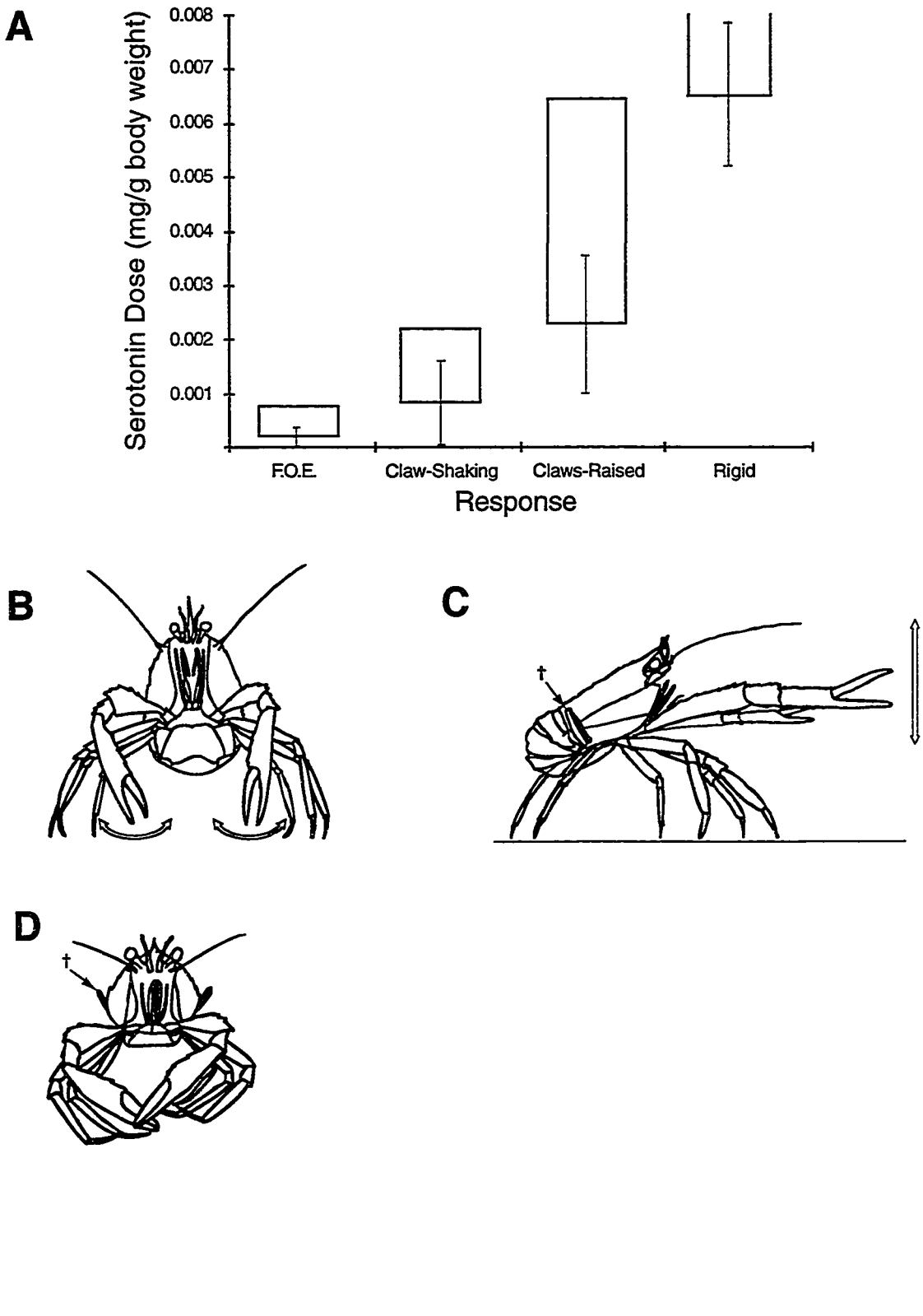
Effects of injected 5-HT

Almost every animal responded to amine injection with dose-dependent classes of behaviours that could be ranked in a dose-response curve. Although not all behaviours described could be elicited in every individual tested, and the dose required to produce a particular response in different animals varied considerably, the order in which the responses were elicited with increasing dose was consistent for all animals. Furthermore, individual animals' dose-response curves were consistent in order of responses and were not dependent on recent social experiences, changes in social environment, molting stage, reproductive state, or time in captivity. Control saline injections caused individuals to tailflip around the tank, assume defensive postures, and to explore the injection site with their fifth pair of pereiopods, but these behaviours never lasted for more than 30 seconds. In the first 30 seconds following amine injections, only behaviours that were very clearly different from these control responses were recorded as part of the aminergic effect. The characteristic amine-induced behaviours usually began 30 to 60 seconds following the injection.

Clear dose dependent responses to injected 5-HT occurred in every animal tested, with four distinct classes of response elicited by the range of doses used in these experiments (Fig. 2.3A). These classes were quite discrete, although intensity of a particular induced response often increased within the dose range capable of eliciting that response. Transitional stages between classes of behaviours occurred only rarely, and could not be repeatably induced. On two occasions, animals injected with large doses of 5-HT proceeded, within one minute, through all four of the response classes in order, and the transitions between classes were remarkably abrupt.

The first observable effect of injected 5-HT was an increased likelihood and sometimes intensity of aggressive reactions to real or artificial squat lobsters, but without a sustained change in posture. If undisturbed after the injection, their behaviour under this lowest effective dose of 5-HT was indistinguishable from that of untreated squat lobsters. However, these animals responded aggressively to untreated squat lobsters immediately after the latter had been placed in the observation tank in more than 90% (40 of 43 observations) of all tests, as compared to untreated animals responding aggressively in fewer than 10% of similar encounters (3 of 35 observations). Aggression by untreated animals towards treated animals was always responded to in kind. On two occasions (in 43 tests), animals injected with low doses of 5-HT actively pursued other squat lobsters, initiating combat when successful in catching their opponent, a behaviour never seen in

Fig. 2.3. *M. quadrispina*'s responses to injected 5-HT. (A) Dose-response graph for injected 5-HT. The responses correspond to the four classes of behaviour described in the Results. The boxes are the dose ranges that elicit each class of behaviour. Thresholds and maxima (respectively, bottom and top of boxes) for each behaviour class are means of all tests. Standard error bars are shown for the threshold dose for each behaviour. *F.O.E.*, first observable effect. (B) induced "shaking-claws" behaviour typical of early aggressive displays. (C) induced "raised-claws" display performed late in aggressive performances. Note that in the induced stances, the walking pereiopods are more depressed and flexed, and the body tilt is greater than in the natural behaviours (compare with Fig. 1B, C). Also note the fifth pereiopods, which are elevated and remoted, stick straight up above the animal, but remain flexed at the meral-carpal joint (†). (D) The rigid posture elicited by very high doses of injected 5-HT. The animal is illustrated upright, but often tips over to lie on its side or back. The walking pereiopods and chelae are very strongly depressed and flexed; the flexed fifth pereiopod sticks straight up (†).


A
Serotonin Dose (mg/g body weight)
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
F.O.E.
Claw-Shaking
Claws-Raised
Rigid
Response
B
C
†
D
†

untreated animals. Potentially threatening stimuli usually elicited aggressive responses in animals injected with their lowest effective dose, as opposed to the defensive or escape responses elicited in untreated animals. The effects of this dose abated 20-30 minutes after injections.

The next two classes of behaviour induced by larger doses of injected 5-HT (Fig. 2.3 A, B, C) closely resembled the two classes of aggressive display seen in normally interacting animals (see Fig. 2.1B, C). Several points distinguish the induced responses from natural behaviours, however. Most importantly, the aggressive displays induced by injected 5-HT were performed in the absence of any external stimulation, and were performed with greater frequency and for longer periods than the intermittent displays of untreated animals. Furthermore, as was the case under lowest effective doses, aggressive acts towards conspecifics increased in frequency and intensity, and threats were usually responded to aggressively. The degree of pereiopod flexion and depression was greater and the cephalothorax, therefore, more tilted than during aggressive displays by untreated animals (Table 2.1; compare Fig. 2.3B, C with Fig. 2.1B, C), and the differences between elicited and normal aggressive displays increased with increasing 5-HT dose. 5-HT doses which produced the "raised-claws" behaviour also caused the folded fifth pereiopod to rotate up from its usual horizontal position alongside the body until it stuck straight up above the thorax (Fig. 2.3C). The induced "shaking-claws" and "raised-claws" behaviours lasted for 30 to 90 minutes, depending on dose, and were followed by a period of increased aggressiveness similar to that induced by the lowest effective 5-HT doses.

Even higher doses of 5-HT caused extreme rigidity, with the pereiopods and claws depressed and tightly flexed under the thorax (Fig. 2.3A, D). Animals in this state would often tip over and remain on their side or back for up to several hours, depending on dose. If sufficiently disturbed by handling, however, these animals could overcome their rigidity and tailflip away, with apparently normal progression (movements), but their rigidity returned immediately after they settled down. Aggressive responses to any external stimulus could not be elicited from animals in this rigid state, or for several hours after they regained mobility.

Effects of injected OA

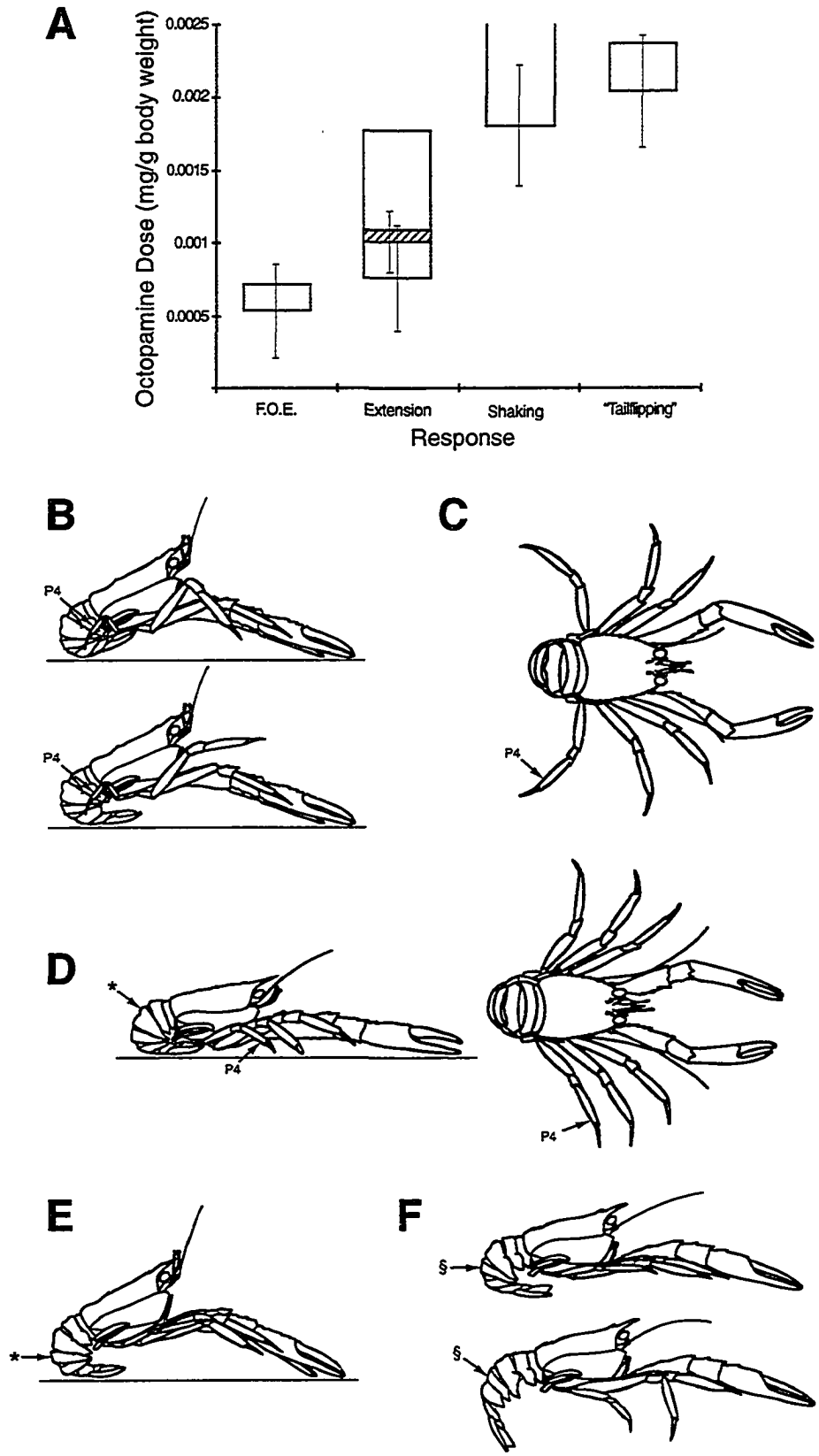
M. quadrispina's responses to injected OA also fell into four classes (Fig. 2.4A), although the divisions between them were not as distinct as those of the response classes

Table 2.1. Thoracic tilt and pereiopod 2 joint angles (means ± standard errors, N = 15) of *M. quadrispina* in normal resting posture, normal aggressive postures, and 5-HT-induced aggressive postures. Data for the two types of aggressive behavior, "shaking-claws" and "raised-claws", were pooled, as the angles were similar. Thoracic tilt is the angle of the dorsal surface of the carapace from the horizontal; the coxo-basal joint angle, relative to the dorso-ventral axis of the animal, corresponds to pereiopod depression (angles > 90° indicate the pereiopod is elevated relative to the thorax); the mero-carpal joint angle, measured from straight, corresponds to pereiopod flexion.

Table 2.1

Posture	Thoracic Angle	Coxo-Basal Angle	Mero-Carpal Angle
Normal Resting	22.0±3.4	99.0 ± 2.5	55.2 ± 7.5
Normal Aggressive	22.5±2.6	72.8 ± 3.1	40.2 ± 2.3
Induced Aggressive	26.1±4.0	66.5 ± 5.2	62.0 ± 4.3

Fig. 2.4. *M. quadrispina*'s responses to injected OA. (A) Dose-response graph for injected OA. The responses correspond to the classes of behaviour described in the text. The prostrate response occurs in the hatched area within the range of doses that elicit extension of the walking pereiopods and abdomen. The boxes are dose ranges that elicit each behaviour class. The threshold and maximum dose of each class (bottom and top of boxes, respectively) are means of all tests, and standard error bars are for each threshold dose. *F.O.E.*, first observable effect. (B,C) postures of one individual induced by OA doses within the range which causes extension of the pereiopods and abdomen, but which are not typical submissive postures: (B) (*top*) low dose within this range and (*bottom*) high dose. (C) top view of animal injected with a high dose to show clearly the position of the fourth pair of pereiopods (P4), which are only slightly promoted from their normal position. The claws are also kept close to their normal resting position, resulting in the walking pereiopods being elevated off the substrate (evident in B). (D) side and top view of prostrate stance induced by injected OA. The fourth pair of pereiopods (P4) are promoted to lie alongside the second and third pairs, but the walking pereiopods are not held as tightly to the thorax, and the abdomen is more extended (*), than in the natural submissive stance (Fig. 1D). (E) the most extended position during a typical example of the slow "tailflipping" behaviour induced by injected OA. The animal is balanced on the tips of its claws and dorsal surface of the tailfan. (F) positions of the pereiopods and abdomen at the end of the extension phase during normal slow (*top*) and fast (escape) tailflipping (*bottom*) (Wilson and Paul, 1987; Antonsen & Paul, unpublished observations). The abdominal extension (*) during the induced "tailflipping" is within the range of maximal abdominal extension (§) during normal tailflipping


A
Octopamine Dose (mg/g body weight)
0.0025
0.002
0.0015
0.001
0.0005
F.O.E.
Extension
Shaking
"Tailflipping"
Response
B
P4
P4
C
P4
D
*
P4
P4
E
*
F
§
§

in the 5-HT dose-response curve. The lowest doses of OA to have an observable effect caused no sustained change in posture or behaviour of undisturbed animals, but increased the likelihood of escape responses when another, untreated squat lobster was put in the tank from 30% (15 of 45 observations) to more than 90% (23 of 25 observations) as compared to untreated animals. Likewise, escape or startle responses when presented with an artificial threatening stimulus increased from 55% (110 of 200 observations) in untreated animals to almost 100% (123 of 125 observations) in OA treated animals. Escape responses consisted of two behaviours which occurred with approximately equal frequencies: tailflipping, which was indistinguishable from tailflipping in untreated animals, and rapid scuttling away from the perceived threat, a behaviour rarely seen in untreated animals.

Extension of the walking pereiopods and abdomen was elicited by a range of OA doses, beginning at a point slightly higher than that required to induce the increased escape tendencies (Fig. 2.4A, B). Within this range, increases in OA dose produced increasing extension of the walking pereiopods and abdomen. Extension and elevation of the walking pereiopods could become so extreme that they stuck straight out and up, leaving the animal balanced on the tips of its claws and dorsal surface of the tailfan (Fig. 2.4B). Pereiopod extension and elevation as extreme as in Figure 2.4B (bottom) could not be elicited in every individual, but all showed increasing extension with increasing dose. The fourth pair of pereiopods was not promoted alongside the second and third pairs in these elicited postures (Fig. 2.4C). With increasing OA dose within this range, frequency of escape reactions increased to 100%, and intensity and/or duration of escape or startle responses increased. At the highest doses within this range, the animals were very jumpy in the absence of any additional stimulation, scuttling and tailflipping around the tank seemingly without provocation.

A posture resembling normal submissive postures (see Fig. 2.1D) was elicited by a narrow dose range within the dose range which produced the increasing extensions of the pereiopods and abdomen just described, in 14 of the 47 animals tested. In this elicited prostrate stance, in contrast to the other OA-elicited extended stances, the claws were elevated, resulting in the body being lowered to the substrate, and the fourth pair of pereiopods were promoted alongside the second and third pairs (Fig. 2.4D). This induced prostrate posture differed from submissive postures in untreated animals in the greater extension of the rostral abdomen and flattening of the caudal abdomen against the substrate, as well as the more elevated and lateral position of the walking pereiopods

(Fig. 2.4D; compare with Fig. 2.1D). Additionally, the animals would scuttle about, usually backwards, and periodically assume the "pereiopods-extended" postures described above. The narrowness of the dose range over which prostrate stances could be induced (<0.0002 mg/g body weight) could explain why this behaviour was not induced in most of the test animals, but for those animals in which this stance could be induced, the result was reproducible.

The third class of response to injected OA was rapid shaking involving the walking pereiopods, claws, and abdomen This response was elicited by OA doses at the high end of the dose range which induced increasing postural extension. The degree of elevation of the walking pereiopods and extension of the walking pereiopods and abdomen decreased at the threshold dose for shaking, but always remained greater than in the normal resting posture. Further increases in OA dose resulted in shaking of increasing severity. Shaking could be quite violent, and could be either periodic or continuous for up to several minutes. Tailflipping and scuttling without any apparent provocation were frequent during these periods. Animals in this condition did not often respond to visual stimuli, but tailflipped immediately when touched.

At a dose slightly higher than the threshold required to induce shaking, the animals performed an unusual behaviour which resembled very low repetition tailflipping. This behaviour consisted of abnormally slow extensions punctuated by sudden, normal-looking, fast flexions. Starting with the pereiopods and abdomen somewhat extended and the body balanced on the tips of the claws and the dorsal surface of the tailfan (as in Fig. 2.4B top), the animal slowly extended, elevated, and promoted its walking pereiopods alongside the thorax and extended its abdomen (Fig. 2.4E). The extension phase took from two to 90 seconds, and was followed by a quick abdominal flexion, which brought the animal to a posture similar to the resting posture of untreated animals. Within 10 seconds, the pereiopods again became extended, stiff, and shaky, and the cycle began again, to be repeated for periods of up to several hours, depending on OA dose. The fully extended abdomen and the positions of the pereiopods alongside the thorax very closely resembled those assumed during the return stroke of a normal tailflip. In particular, the degree of extension of the abdomen during this OA-induced behaviour was well within the normal range for tailflipping in untreated animals (Fig. 2.4F; Wilson and Paul, 1988). Much higher OA doses resulted in very severe shaking and completely immobilized the animals.

Effects of 5-HT and OA injected together

In almost all instances where 5-HT and OA were injected together, the animals did not perform typical behaviours of any sort. At low doses, the animals would grab at themselves, tearing appendages off, and their whole bodies would shake, sometimes violently. At higher doses the two animals I tested became very rigid, dropped limbs and died. The exception to this was when very high doses of 5-HT and low doses of OA were injected together; in these cases the animals would perform aggressive behaviours, but whole-body shaking and sporadic tailflipping would interrupt the aggressive acts.

These results are in contrast to those in *G. lacustrus* (Helluy and Holmes, 1990) and *E. analoga* (Chapter 4), in which injected OA inhibited the effects of injected 5-HT. This indicates that in *M. quadrispina*, these two amines are not direct inhibitors of one another's actions along the entire pathway leading to agonistic behaviours; rather it seems from watching the animals under the influence of both amines together that crossed or muddled signals are reaching the motor systems from the command pathways which are being influenced.

Discussion

Posture and behavior during normal interactions

The social structure of *M. quadrispina* populations differs substantially from that of crayfish in that aggressive acts are rare and dominance hierarchies do not exist. Conversely, in populations of crayfish and American lobster, aggression and fights are common and used to establish dominance hierarchies (Scrivener, 1970; Bruski & Dunham, 1987; Huber & Kravitz, 1995). Aggression is used solely for transient space claims in *M. quadrispina* populations, and most aggressive acts appear to be bluff, for threats that are ignored are almost never followed by aggressive acts. *M. quadrispina*, like most benthic galatheids, are omnivorous scavengers and filter feeders (Nicol, 1932; Antonsen & Paul, unpublished observations). In high population densities, "scramble competition", similar to what we regularly observe in our laboratory populations, is likely the most efficient method of scavenging food items of unpredictable availability. The frequency of aggressive acts does not increase during the mating season, in keeping with the absence of territoriality.

Several crustacean species that normally live in low population densities become less aggressive towards conspecifics if kept at high densities (Hazlett, 1968; Courchesne & Barlow, 1971; Ulmer & Grant, 1971; Dunham, 1972; Vannini, 1981). However, *M.*

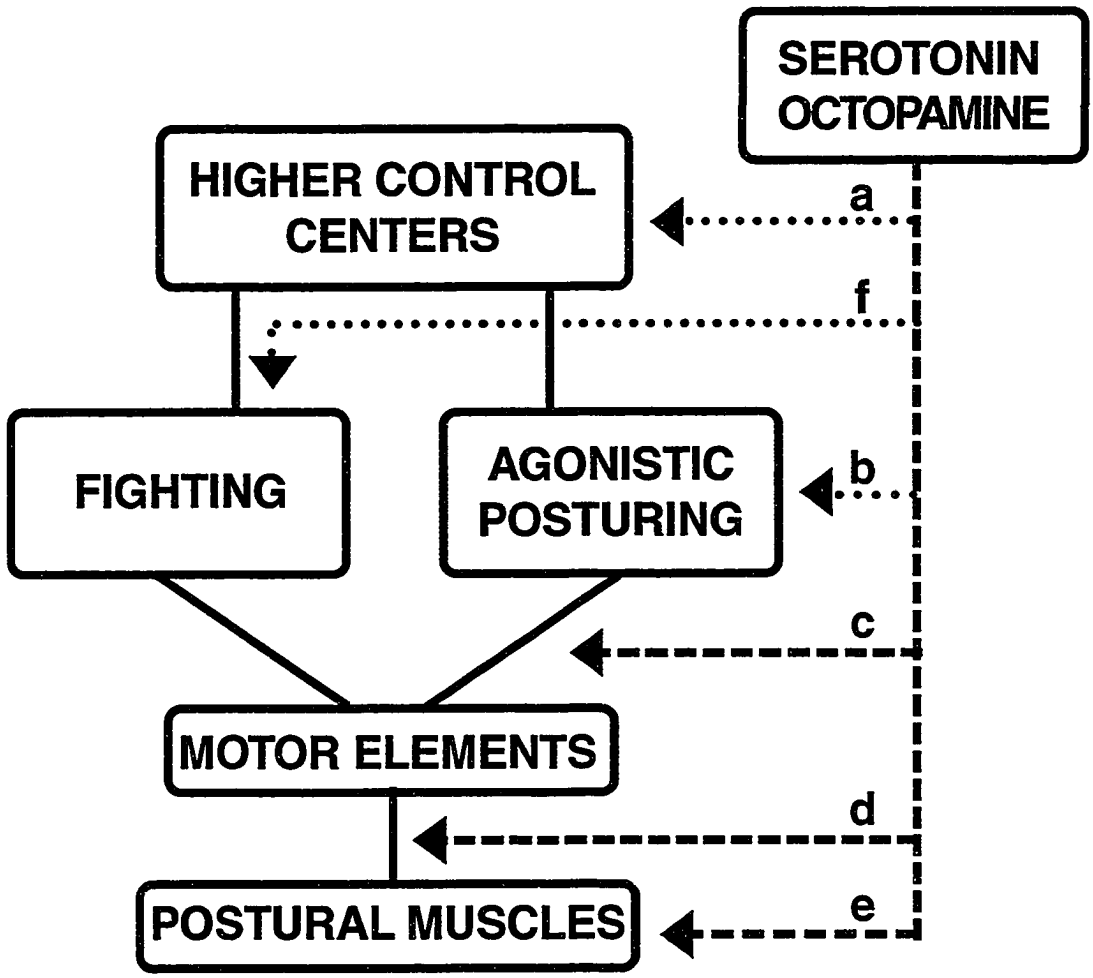
quadrispina (this study) and the hermit crab *Pagurus pubescens* (Ulmer & Grant, 1971), both of which live at high population densities, do not become more aggressive when kept at low density. This indicates that although aggressive species can be conditioned to become less aggressive, the lack of aggression in gregarious species is more permanent.

Effects of injected amines

These experiments implicate 5-HT and OA in the induction of aggressive and submissive behaviours in *M. quadrispina*. Firstly, complex behaviours exhibited by untreated, normally interacting animals can be mimicked in isolated individuals, in the absence of any additional stimulation, by injection of one of the amines. Secondly, injected amines prime the animals to react so that frequency and intensity of responses to external stimuli are increased. Thirdly, injection of an amine can, in at least one situation, reverse an untreated animal's typical response. That is, animals that are under the influence of injected 5-HT react aggressively to an artificial potential predator, as opposed to displaying their normal escape or defense reactions. These data indicate that the amines act on one or more higher centers responsible for the induction and orchestration of agonistic behaviours (Fig. 2.5a). Given the influence 5-HT and OA have on these behaviours in *M. quadrispina*, it seems reasonable to anticipate that future studies on crayfish will uncover additional roles beyond that in fighting readiness which has been reported (Huber, 1995).

The results of this and other studies suggest that 5-HT and OA also influence pathways for postural control directly, independently of behavioural control pathways. In *M. quadrispina* certain doses of OA cause extension of the walking pereiopods and abdomen without concurrent submissive postures or behaviours. Likewise, large doses of 5-HT, above those that produce aggressive behaviours, result in pereiopod flexion far more severe than during normal behaviours. Additionally, within the dose range for each class of behaviour induced by the amines, the degree of postural modulation by each of the amines increases with increasing dose. Large doses of 5-HT or OA induce, respectively, flexion or extension of the pereiopods and abdomen in lobsters and crayfish without inducing any distinct natural behaviours (Livingstone et al., 1983). These results suggest an influence in the areas controlling postural coordination (Fig. 2.5b) and postural extension and flexion (Fig. 2.5c). Additionally, some sites of action of the amines within postural control circuits have been identified in lobsters and crayfish: at the level of command neuron interaction with motor elements (Fig. 2.5c) (Harris-Warrick

Fig. 2.5. Possible sites of serotonergic modulation on agonistic control circuits. The boxes represent centers for the production or control of aggressive behaviours. The dashed lines indicate modulatory sites confirmed physiologically in lobsters and crayfish. Dotted lines indicate additional possible sites of influence suggested by comparisons of behavioural studies on lobsters and crayfish and this study on *M. quadrispina*. Arrows pointing to boxes indicate serotonergic effects directly on the hypothetical centers; arrows pointing to lines indicate modulatory influences on the circuits involved in communication between two levels of control.


SEROTONIN
OCTOPAMINE
HIGHER CONTROL CENTERS
a
f
FIGHTING
AGONISTIC POSTURING
b
c
MOTOR ELEMENTS
d
POSTURAL MUSCLES
e

& Kravitz, 1984; Harris-Warrick, 1985; Ma et al., 1992), at the neuromuscular junction (Fig. 2.5d) (Florey & Rathmayer, 1978; Glusman & Kravitz, 1982; Breen & Atwood, 1983; Fischer & Florey, 1983; Dixon & Atwood, 1985), and on the muscles themselves (Fig. 2.5e) (Kravitz et al., 1980).

Perfusion of 5-HT into crayfish promotes fighting readiness in an animal which would normally be submissive, but does not influence any other aspects of the aggressive encounter (Huber, 1995), suggesting direct or indirect serotonergic influences on putative fighting centers in crayfish (Fig. 2.5f). Injection of 5-HT can also induce fighting in *M. quadrispina*. Considering that fighting is not a part of *M. quadrispina*'s normal behavioural repertoire, its induction by injected 5-HT suggests that intact behavioural pathways for fighting exist but are never activated in untreated animals. This implies the loss of fighting behaviour in this species, and may explain how bluffs that are never followed up by action could have evolved. Alternatively, 5-HT's influence could be on a higher center which is separate from the aggression pathways per se. A candidate could be within the defense reaction pathway, as defensive reactions in *M. quadrispina* can involve violent grasping, which could appear as fighting among conspecifics.

This study implicates OA in control of tailflipping circuitry in *M. quadrispina*. High doses of OA induce motions that are very much like individual tailflips performed by untreated animals, except that the extension phases are very much slower and, therefore, the frequency is much lower. OA facilitates giant interneuron-mediated escape tailflip reactions in crayfish (Glanzman & Krasne, 1983), but high doses of injected OA have not been reported to induce escape tailflips in lobsters or crayfish, or to influence any aspect of non-giant interneuron-mediated tailflipping.

Although doses of either amine required to produce any single response varied between individuals, dose-response curves for individual animals remained consistent over time and regardless of physiological state or social experience. Interestingly, dose-response curves for individual animals remained consistent even under circumstances that alter behavioural repertoires of untreated animals. For example, animals are more likely to retreat from conspecifics when they had recently molted, but responses of individual animals to injected amines did not change in the period following molting. This leaves unanswered the question of the causes of the observed individual variation in responsiveness. Environmental conditions during the egg or larval stages may influence development of the modulatory circuits, or the variability in the circuits may be genetically determined.

Clearly, both amines act at two levels, postural and behavioural, in influencing agonistic encounters. While these two levels are interconnected, our results reveal that they are subject to (at least partially) independent, aminergic influences. The higher control centers that are responsible for behaviour are influenced by lower doses of the amines than are the postural circuits, as evidenced by behavioural facilitation at low doses in the absence of sustained postural change. This influence may be on central neurons, or on sensory afferents with inputs to these higher centers. Aminergic influences on several sensory pathways with potential influences on agonistic behaviours or posture have been described (El Manira et. al., 1991B; Pasztor & MacMillan, 1990; Pasztor & Golas, 1993; Rossi-Durand, 1993; Yeh et. al., 1996). Increasing doses of the amines have a punctuated effect on behaviour, with changes between classes occurring over a very narrow dose range. This suggests that changing concentrations of the amines could change recruitment in sets of higher level neurons responsible for effecting behavioural choice. At the level of postural control, the aminergic influences start at a higher dose, and are graded, increasing in intensity with increasing dose.

These results place aminergic neurons at the center of behavioural and postural induction. Activity of single aminergic neurons could influence behavioural and motor output by changing local amine concentrations in a way that was mimicked by systemic injections. Identification of individual neurons involved in modulating agonistic behaviours may prove easier in *M. quadrispina* than in lobsters and crayfish since there are relatively fewer aminergic neurons in *M. quadrispina* (Antonsen & Paul, 1994; Chapter 3).

Chapter 3: The distribution of serotonin- and octopamine-immunoreactive neurons in Munida quadrispina

Introduction

The biogenic amines serotonin (5-HT) and octopamine (OA) have important influences on many neural systems and behaviours in crustaceans. A few examples include agonistic posture and behaviour (Livingstone et al., 1980; Harris-Warrick & Kravitz, 1984; Huber et al., 1997A & B; Chapter 2), function of the neural systems controlling the heart and stomach (Florey & Rathmayer, 1978; Flamm & Harris-Warrick, 1986), and sensitivity of sensory systems (Pasztor & MacMillan, 1990; Rossi-Durand, 1993) and strength of synaptic outputs of sensory fibers to other neurons (Yeh et al., 1996) (for a review see Beltz, 1999). Most work on aminergic systems and the influences of 5-HT and OA has been done in American lobsters and several species of crayfish, although some studies have been done in crabs, spiny lobsters, and a few non-decapod crustaceans.

The distribution of serotonergic neurons has been described in only a few crustacean species and octopaminergic neurons in even fewer. Fairly complete maps of serotonergic neurons in the central nervous system are available for lobsters (Beltz & Kravitz, 1983 & 1987; Langworthy et al., 1997), crayfish (Elofsson, 1983; Sandeman & Sandeman, 1987; Sandeman et al., 1988; Real & Czternasty, 1990), a crab (Harzsch & Dawirs), several species of isopods (Thompson et al., 1994), and the primitive crustacean *Anaspides tasmaniae* (Harrison et al., 1995). Another study compared the distribution of serotonergic neurons in the olfactory lobes between several species, including two shrimp and a galatheid (Johansson, 1991). Biochemical analyses have demonstrated OA in discrete regions or structures in the nervous systems of lobsters (Evans et al., 1975 & 1976A, B; Livingstone et al., 1981), but antibodies to OA which produce highly specific and reproducible immunoreactivity have only been produced quite recently. To date, the American lobster is the only crustacean species in which the distribution of octopaminergic neurons has been mapped by immunocytochemical techniques (Schneider et al., 1993 & 1996). A recent review by Beltz (1999) discussed the functional anatomy of aminergic neurons, focusing mainly on the American lobster, but functional comparisons between diverse crustacean taxa have not been made.

The structure and physiology of the neuromuscular systems involved in producing many of the behaviours modulated by 5-HT and OA differ between decapod species. For example, 5-HT and OA influence the lateral giant escape circuit in crayfish (Glanzman & Krasne, 1983; Yeh et al., 1996 & 1997), and 5-HT-containing neurons are excited by both lateral and medial giant escape neurons (Hörner et. al., 1997). However, giant escape circuits do not exist in the anomurans, except for the medial giant in hermit crabs. Non-giant tailflipping circuitry, which is present in all macruran crustaceans but in crayfish is responsible only for repetitive swimming tailflips, also mediates initial escape responses in anomurans (Wine & Krasne, 1982; Sillar & Heitler, 1985; Wilson & Paul, 1987; Paul, 1989 & 1991). Furthermore, brachyuran crabs and some anomurans have lost the ability to tailflip at all. Studying the changes in aminergic systems associated with the modifications of loss of these circuits or loss of tailflipping behaviour could inform us about how the systems function and have evolved. Towards the goal of using comparative methods to study evolution and function of aminergic systems, in this chapter I describe the distribution of serotonergic and octopaminergic neurons in the central nervous system of the galatheid anomuran *Munida quadrispina*.

Methods

M. quadrispina were collected by trawl from Saanich Inlet near Victoria, B.C. and kept in recirculating 10°C seawater aquaria in the laboratory. Animals were used within four months of collection, although no evidence that long periods of confinement in the laboratory could change the patterns of immunoreactivity was ever found. Animals between 0.4 and 1.1 cm carapace length were used, and no differences based on sex or time of year were found. Most of the information was obtained from whole-mount material, but thick sections were also made to confirm positions of some structures. Animals were anesthetized in sea water ice, and ventral nerve cords were dissected under cold physiological saline (see Chapter 2 for constituents). Pre-treating nerve cords with 10^{-5} M 5-HT or OA in physiological saline, or injecting animals with 10^{-4} M 5-HT or OA just prior to dissection, improved the strength of the label, but it also resulted in labeling of somata and some processes which did not label otherwise, presumably due to uptake mechanisms in non-serotonergic cells. For this reason these nerve cords were used only for tracing processes which were identified in non-pre-treated animals and no illustrations from animals prepared using this technique are presented. Nerve cords were placed in

fixative as quickly as possible after dissection, or after a 30-minute rinse if they had been pre-treated with an amine solution. Nerve cords were carefully desheathed after fixation; some damage to the immunolabeled structures in some nerve cords was unavoidable, due to the brittleness of the fixed tissue, but desheathing the ganglia before fixation diminished the strength of the label considerably. I did not look at the optic lobes, or the cardiac or stomatogastric ganglia.

Serotonin immunolabeling

Sixty-seven whole mounts and four sectioned nerve cords were analyzed. Nerve cords were fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer, pH 6.5, overnight at room temperature, then washed in several changes of 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 1% Triton X-100 (PBS-Triton). After several additional washes in PBS, permeability of the tissues was improved by treatment with 0.1 mg/ml Pronase (Sigma) or collagenase-dispase (Boehringer Mannheim) for 5-10 minutes, followed by two to four, 10 minute washes in freshly made 0.5% (w/v) sodium borohydride in PBS. The tissues were dehydrated through a graded ethanol series, washed for 10 minutes in either propylene oxide or Dent's solution (80% methanol, 20% DMSO), and rehydrated. After several washes in PBS-Triton, tissues were treated overnight in a blocking solution of 5% normal goat serum in PBS-Triton then incubated in a 1:5000 dilution of a polyclonal antibody to 5-HT raised in rabbit (Incstar). The primary antibody incubation buffer was prepared as follows: to PBS was added 1% sodium metabisulfite, 0.25% Triton X-100, 0.25% BSA, 3% dry milk powder; the solution was homogenized, then centrifuged, and the supernatant was filtered through #1 filter paper (Whatman). After several more rinses in PBS-Triton, the tissues were incubated with a 1:500 dilution of a carboxyfluorescein-conjugated secondary antibody raised in goat against rabbit IgG (Vector). Both antibody solutions were incubated with the tissue for 3-4 days at 4°C. The tissue was dehydrated, cleared in methyl salicylate, and mounted in Cytoseal (VWR). For thick sections, the tissue was fixed as above, washed in PBS, embedded in 5% agarose, and 100μm sections were cut on a vibratome. The tissue was washed twice for 20 minutes in 0.5% sodium borohydride, then the blocking solution and the same series of antibody incubations were applied as above, except that each was left overnight. The sections were dehydrated, cleared with toluene, and mounted in Cytoseal. Specificity controls were done by omitting the primary antibody or by preadsorbing the primary antibody in an excess (1 mg/ml) of 5-HT

creatinine sulfate (Sigma). Both methods eliminated 5-HT immunoreactivity in the tissues. Positive controls using nerve cords from crayfish (*Cambarus bartonii*) were done, and showed similar patterns of 5-HT-like immunoreactivity as have been published for the crayfish *Procambarus clarkii* (Real & Czternasty, 1990).

Octopamine immunolabeling

Forty-one whole mounts and two sectioned nerve cords were analyzed. Because glutaraldehyde fixatives were used, I chose the smallest adult animals available, which were between 0.3 and 1.1 cm carapace length. There were two reasons for this: first, glutaraldehyde cross-reacts tissues very tightly, which tends to inhibit antibody penetration and second, glutaraldehyde causes much more autofluorescence in these animals than does paraformaldehyde, which can obscure weakly labeled structures in larger animals. Two fixation procedures were used: 1) 1.5% glutaraldehyde in 0.1M cacodylate buffer containing 1% sodium metabisulfite, pH 6.8 for 2 hours and pH 4.0 overnight at room temperature or 2) a mixture of 10 ml 25% glutaraldehyde, 30 ml saturated aqueous picric acid, 0.2 ml acetic acid, and 1% sodium metabisulfite (Schneider et al., 1993) overnight. Nerve cords fixed in the picric acid fixative had very weak immunolabeling. The procedure after fixation for both wholemounts and sections was the same as for the 5-HT immunolabeling, with the following antibodies. The blocking solution was 10% normal goat serum, the monoclonal primary antibody was raised in mouse against octopamine conjugated to thyroglobulin, used at 1:10 000 dilution (BIOMAR Diagnostic Systems Inc) (Agricola et al., 1999), and the secondary antibody was biotylated goat anti-mouse (Vector Laboratories) (1:250 dilution). CY3-conjugated streptavidin (1:250 dilution) was reacted with the biotylinated secondary antibody for visualization (Jackson ImmunoResearch). Specificity controls omitted the primary antibody or preadsorbed the primary antibody in 1 mg/ml DL-octopamine or 5-HT creatinine sulfate. The first two procedures eliminated and the latter did not effect immunolabeling.

Pictures and reconstruction

Epiflourescent photographs and camera lucida drawings were made using a Leitz Aristoplan microscope. Confocal images were obtained using Zeiss LSM 410 and Zeiss LSM 510 laser scanning confocal microscopes. Although the confocal microscopes could, in general, produce clearer images with a much greater depth of field than the

epiflourescent system, the thickness of the wholemount tissue prevented the confocal microscopes from being used to their full advantage. Therefore, the camera lucida and epifluorescent system was used to follow individual processes and to produce most of the drawings in the results. Reconstructions of confocal images were done using Adobe Photoshop 5.0 software on an Apple Power Macintosh 7500 computer.

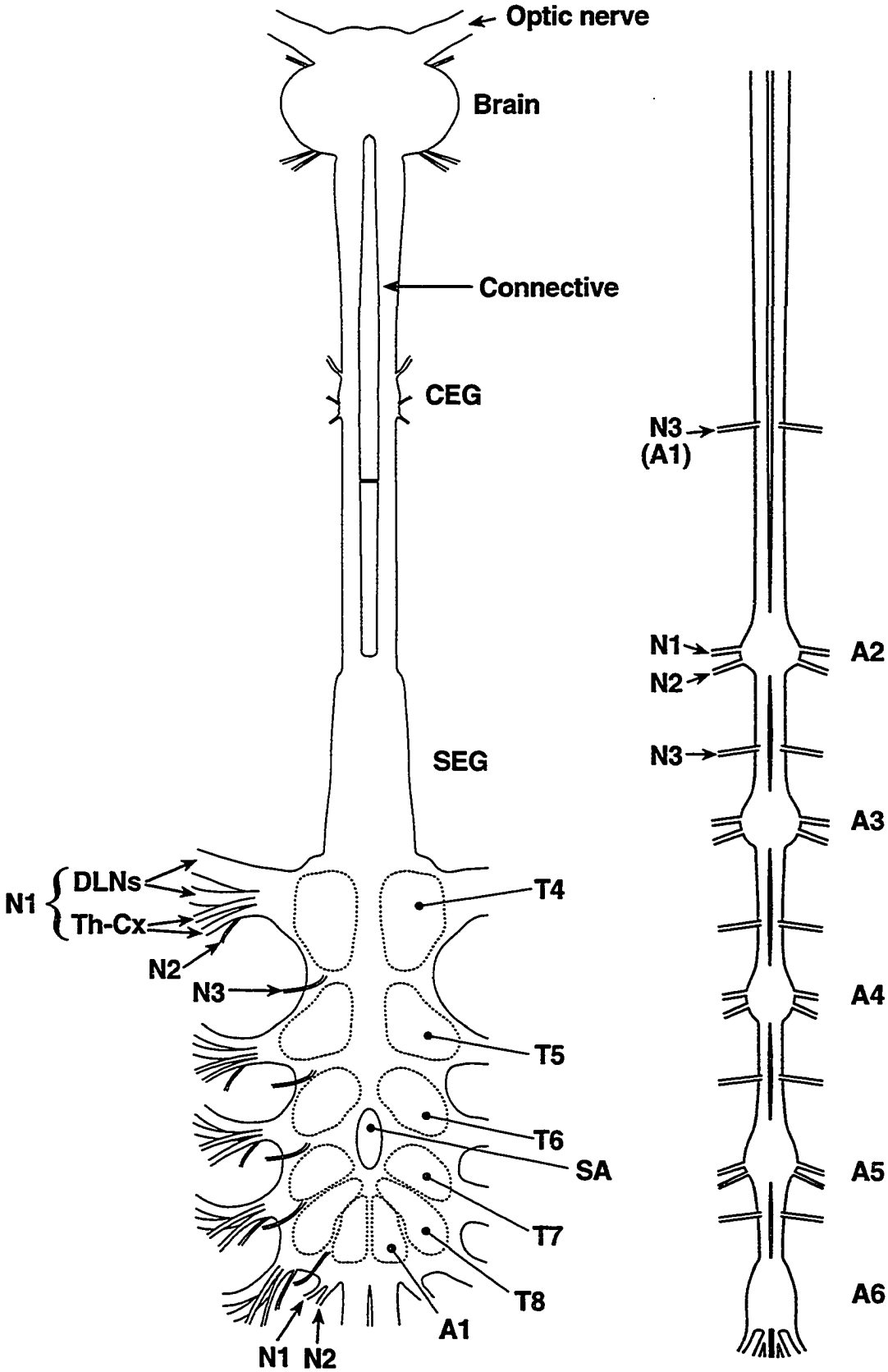
Results

Anatomy of M. quadrispina's central nervous system

The central nervous system of *M. quadrispina* is typical of decapods, with a rostral brain, a pair of circumesophageal ganglia, a subesophageal ganglion consisting of six fused ganglia, five ganglia innervating the pereiopods (thoracic ganglia 4-8, the first three thoracic ganglia being part of the subesophageal ganglion), and six abdominal ganglia (Fig. 3.1). The subesophageal, five pereiopod, and first abdominal ganglia are fused, and the connectives between them have been virtually eliminated. The first abdominal ganglion is medial and slightly caudal to the eighth thoracic ganglion. The sternal artery passes vertically through a conspicuous orifice on the midline of the ganglionic mass between the sixth and seventh thoracic ganglia. A similar fusion of ganglia occurs in other, but not all, anomurans, brachyurans, and some other decapods.

The organization of the segmental nerves leaving the abdominal ganglia is similar to those in crayfish and lobsters. The first and second segmental nerves leave laterally from ganglia 2 – 5 (or from the fused ganglionic mass in the case of abdominal ganglion 1), and the third leaves the connective dorsally, caudal to the ganglion (Fig. 3.1) (Sillar & Heitler, 1985; Wilson & Paul, 1987). Seven nerves leave from each side of the sixth abdominal ganglion, although in *M. quadrispina* these nerves are initially fused at their exit from the ganglion into two or three (depending on the branch points) combined nerves (Paul et al., 1985). The first segmental nerve of the pereiopod ganglia leaves the ganglion as several separate nerves, the largest of which are the two distal leg nerves and two thoracico-coxal nerves. In the 8th thoracic ganglion, the thoracico-coxal nerves leave the ganglion dorsal to the distal leg nerves, as they do in crayfish (Elson, 1996). However, in the more rostral pereiopod ganglia, the thoracico-coxal nerves are displaced increasingly caudally relative to the distal leg nerves. The positions of the thoracico-coxal nerves in the 4th and 5th thoracic ganglia (the claw and first walking pereiopod) are somewhat variable between animals, sometimes emerging from the ganglion dorsal to or

Fig. 3.1. Dorsal view of *M. quadrispina*'s central nervous system. Note the fused mass containing the subesophageal ganglion (SEG), thoracic ganglia 4-8 (the pereiopod ganglia) (T4-T8) and the first abdominal ganglion (A1). The dotted lines outline the approximate boundaries of the pereiopod and first abdominal ganglia. The segmental nerves of the pereiopod ganglia are shown on one side only (for clarity). A2-A6, abdominal ganglia 2 through 6; CEG, pair of circumesophageal ganglia; DLNs, distal leg nerves; N1-N3, segmental nerves 1 through 3; SA, sternal artery; Th-Cx, thoracico-coxal nerves.


Optic nerve
Brain
Connective
CEG
SEG
N1
DLNs
Th-Cx
N2
N3
T4
T5
T6
SA
T7
T8
A1
N1
N2
N3
(A1)
N1
N2
N3
A2
A3
A4
A5
A6

even caudal to the second thoracic nerve, which emerges just caudal to the distal leg nerves. The third segmental nerve emerges dorsally from the caudal region or just caudal to the ganglion.

Distribution of serotonin-like immunoreactivity

Approximately 120 neurons with 5-HT-like immunoreactivity were found in *M. quadrispina*'s central nervous system (Fig. 3.2), with soma diameters ranging from 10 to 85 μm. Of these, approximately 60 are located in the brain, two in the circumesophageal ganglia (one in each ganglion), approximately 24 in the subesophageal ganglion, two each in thoracic ganglia 4 – 7, four each in the 8th thoracic ganglion and abdominal ganglia 1 – 4, and six in the 6th abdominal ganglion. Perhaps the most striking feature of the overall distribution is the lack of unpaired medial cells in the abdominal ganglia, conspicuous in crayfish and lobsters, although there may be some unpaired medial cells in the rostral parts of the brain of *M. quadrispina* and possibly in the subesophageal ganglion. Except in the brain, all of the 5-HT-immunoreactive cell bodies are in the ventral half of the ganglion. In the brain, the cell bodies are distributed throughout the dorso-ventral axis.

The brain and circumesophageal ganglia

Many small (10-25 μm) labeled cell bodies are located in the rostral medial cell cluster of the brain, all but two of which send processes caudally which could not be followed far from the somata (Fig. 3.3 & 3.4A). The two that could be followed have large, lateral somata and send strongly labeled processes out the optic nerves. Each optic nerve contains at least 15 immunoreactive processes, but none except the two just mentioned could be traced to their cell bodies of origin. Eight additional immunoreactive cell bodies which are not in similar locations to any cells in crayfish (Sandeman et al., 1988), three commissures with immunoreactive axons, and several areas of neuropil are also in the rostral brain. However, the detailed cytoarchitecture of the brain of *M. quadrispina* has not been studied and, therefore, I cannot specify the anatomical locations of the immunoreactive structures of the rostral brain as well as I might like, or compare the fine structure to that of crayfish (Sandeman et al., 1988).

Only very weak immunolabeling was seen in the olfactory lobes (Fig. 3.4B), even in nerve cords that were pre-soaked in 5-HT. Two fairly large (45-50 μm) cells are just caudal to the optic nerves which could be homologues to the giant neurons associated

Fig. 3.2. Distribution of 5-HT-immunoreactive neurons. The size of each dot roughly corresponds to the size of the cell body. The connectives between the circumesophageal and subesophageal ganglia and between the first and second abdominal ganglia are not shown. Abbreviations as in Fig. 3.1.

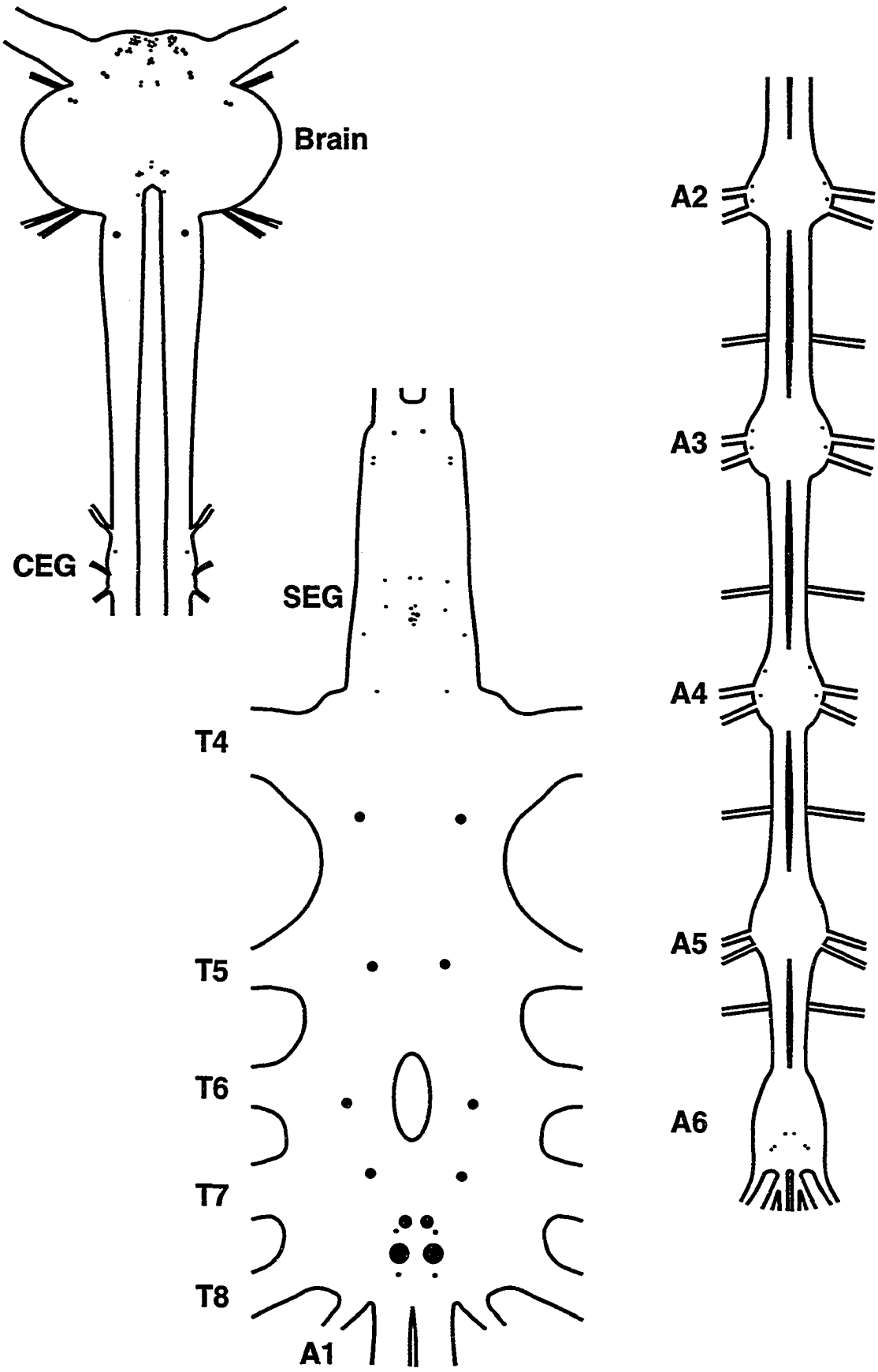

Brain
CEG
SEG
T4
T5
T6
T7
T8
A1
A2
A3
A4
A5
A6

Fig. 3.3 5-HT-immunoreactivity in the brain and circumesophageal ganglia. This is a composite drawing of a single nerve cord constructed from camera lucida drawings and traces of confocal micrographs. The solid arrows in the brain indicate the three tracts of fibers crossing the midline in the rostral brain; the open arrowhead points to one of the large immunoreactive cell bodies in the connectives. The stippled areas indicate immunoreactive neuropil; the density of the stippling roughly corresponds to the intensity of the label. LFB, lateral fiber bundle; MFB, medial fiber bundle; SuON, supraesophageal nerve.

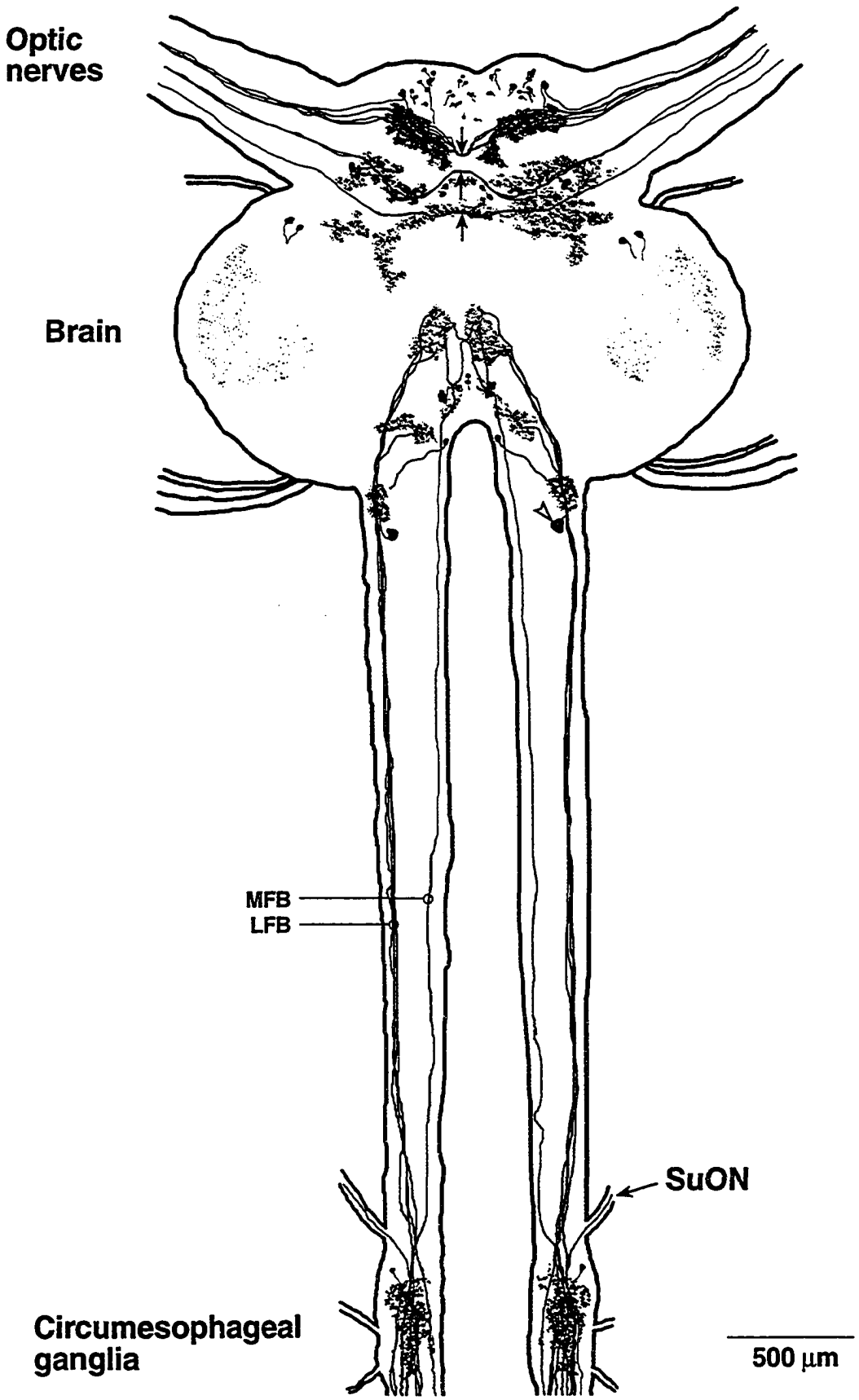
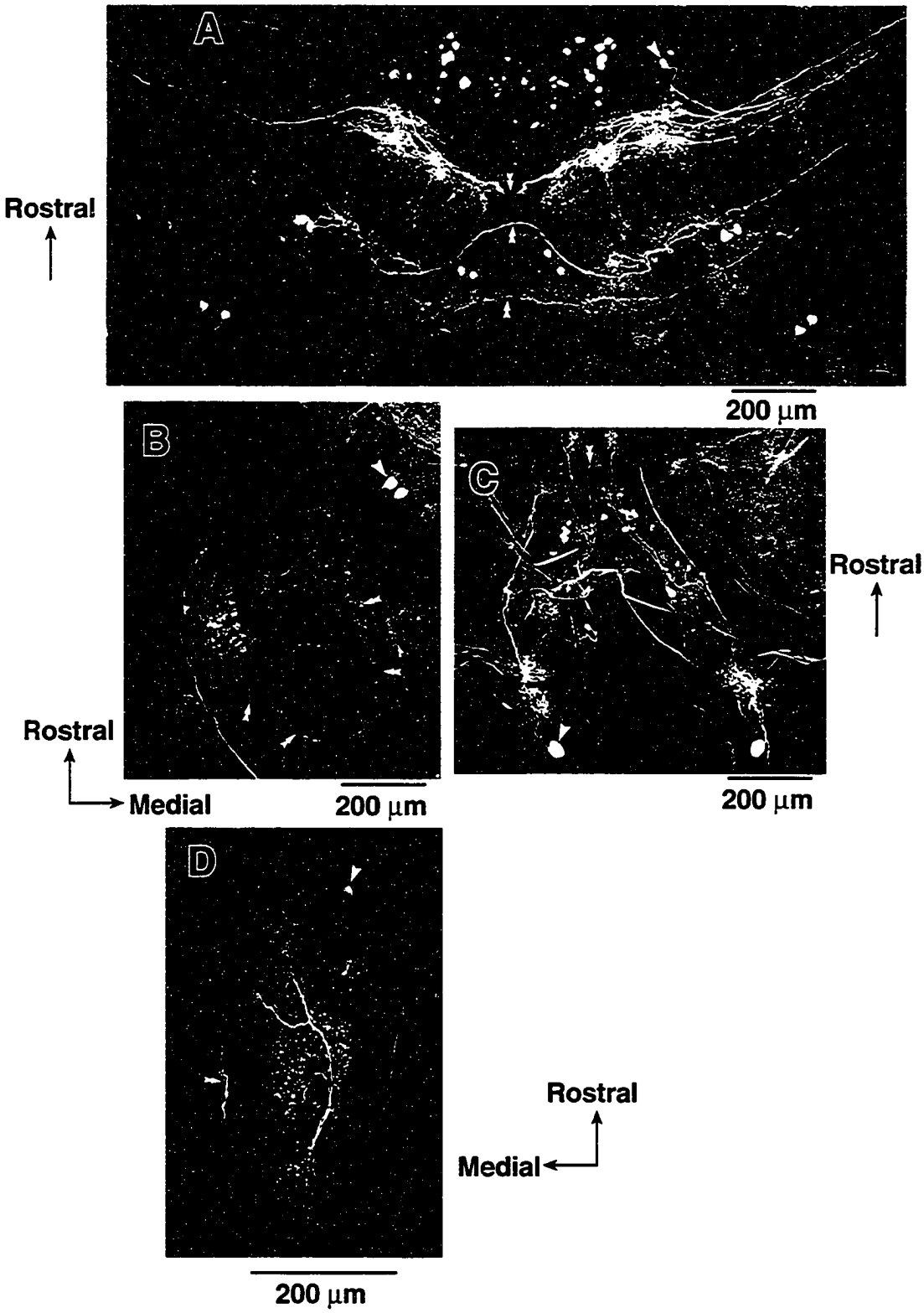

Optic nerves
Brain
MFB
LFB
SuON
Circumesophageal ganglia
500 μm

Fig. 3.4. Confocal micrographs of 5-HT-immunoreactivity in the brain and one circumesophageal ganglion. (A) The rostral brain. The arrowhead indicates one of the cell bodies whose axon could be followed into the optic nerves; the double arrowheads indicate the three tracts of fibers in the rostral brain. (B) The lateral deutocerebrum, showing the weak immunolabeling in the olfactory lobes (double arrowheads) and the two large cell bodies which send processes in that direction. (C) The caudal brain. The arrowhead points to one of the large immunoreactive cells in the rostral connective; the double arrowheads indicate the branches from fibers in the lateral fiber bundle which cross the midline. (D) A circumesophageal ganglion. The arrowhead indicates the weakly-immunoreactive cell body; the double arrowhead indicates the single fiber that runs medially through the ganglion.


A
Rostral
200 μm
B
Rostral
Medial
200 μm
C
Rostral
200 μm
D
Rostral
Medial
200 μm

with the olfactory and accessory lobes in crayfish (Sandeman et al., 1988); their processes initially head in that direction, but could not be traced into the lobes. The accessory lobes of anomurans are very small (Sandeman et. al., 1992), and I could not distinguish any immunoreactivity in these structures in *M. quadrispina*.

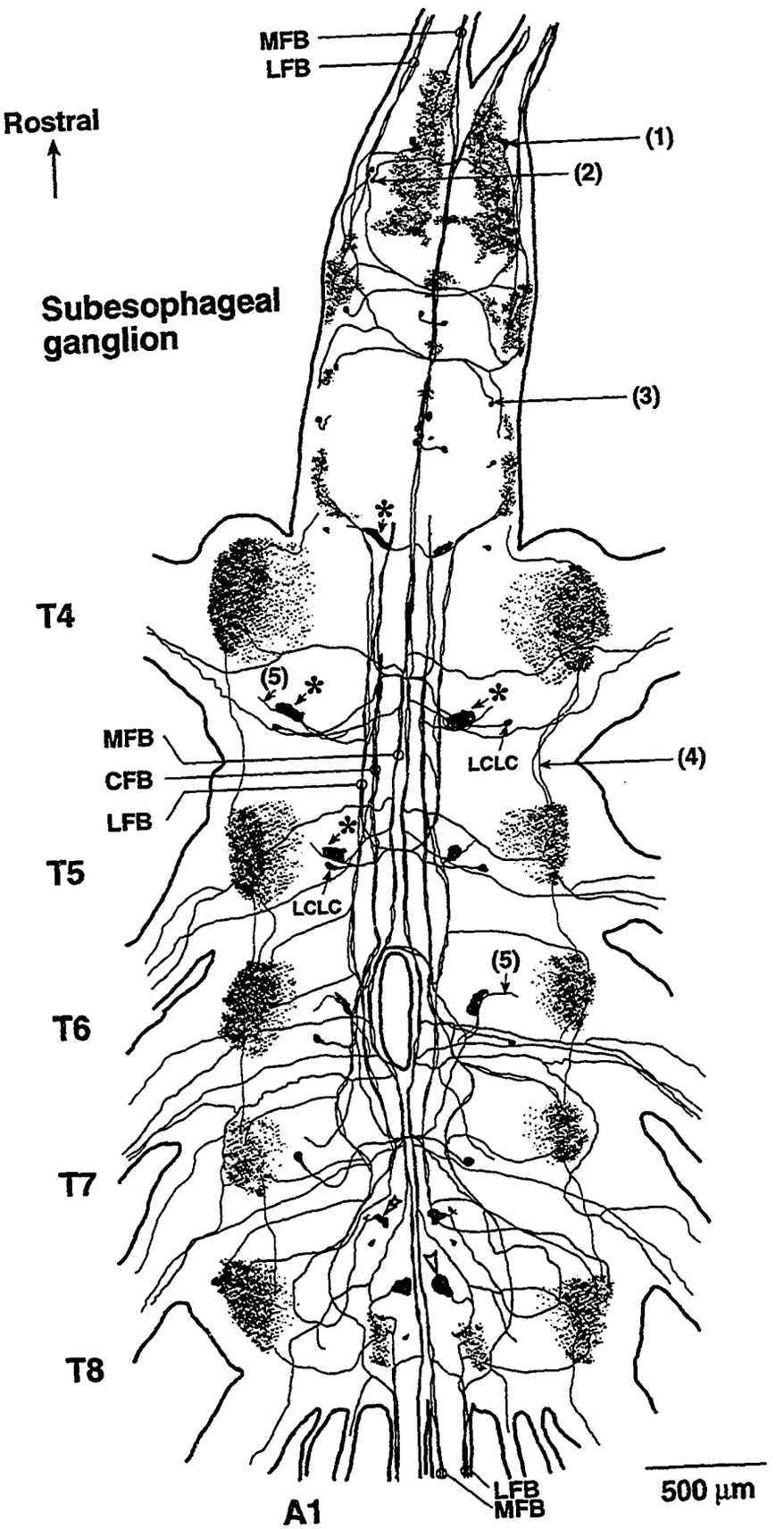
Sixteen cell bodies with 5-HT-like immunoreactivity are in the caudal brain. Four of these cells, including one pair of very large (70-80 μm) cells in the connective caudal to the connective neuropil, send processes into this neuropil. The processes of the rest of the cells could not be followed individually. Five axons are in each connective caudal to the brain, one medial and four in a lateral bundle. Each of these axons could be followed into one or more of the three distinct areas of immunoreactive neuropil in the ipsilateral caudal brain, and the medial axon also sends a process into the most rostral contralateral neuropil.

Each of the circumesophageal ganglia contains one weakly-immunoreactive cell body and a very dense immunoreactive neuropil (Figs. 3.3 & 3.4C). The soma (15μm) is located in the rostro-lateral part of the ganglion and sends a process caudally into the neuropil, where I was unable to follow it farther. The connective caudal to each circumesophageal ganglion contains nine immunoreactive axons in strongly-immunolabeled nerve cords, five of which continue through to the brain. Six fibers are in the lateral bundle and three are in the medial bundle in the caudal connective (Fig. 3.4C). Two axons in the lateral bundle and one in the medial bundle could be followed between the circumesophageal neuropil and cells in the subesophageal ganglion (see below), but the cell bodies of the remaining six axons have not been identified. Within each circumesophageal ganglion, eight of the axons are located centrally within the dense neuropil, and at least five contribute to the neuropil. The ninth axon passes through the ganglion medially, and has a short enlarged region with a dense plexus of beaded immunoreactive fibers surrounding it. This axon is part of the lateral fiber bundle in the connectives rostral and caudal to the ganglion. One strongly immunoreactive fiber, whose cell body of origin has not been identified, enters each of the nerves leading to the stomatogastric ganglion, the surpraesophageal nerves.

The subesophageal ganglion

Approximately 10 small (10-20 μm) immunoreactive cell bodies are located along or close to the midline in the caudal half of the subesophageal ganglion (Fig. 3.5). The fibers leaving these cells initially project rostrally in the medial fiber bundle, although

Fig. 3.5. Camera lucida drawing of 5-HT-immunoreactivity in the subesophageal, pereiopod, and first abdominal ganglia. Areas of immunoreactive neuropil are represented by stippling; intensity within the neuropils of the pereiopod ganglia was highest laterally. In the subesophageal ganglion, note the lateral cell bodies with caudal projections (1), with contralateral rostral projections in the medial fiber bundle (2), and with contralateral rostral and caudal projections in the lateral fiber bundle (3). Note also how the lateral fiber bundle turns laterally upon entering the subesophageal ganglion from thoracic ganglion 4 (T4). One large rostral medial cell in each of abdominal ganglion 1 and thoracic ganglion 8 is indicated by the open arrowhead and double arrowhead respectively. The lateral (LFB) and central (CFB) fiber bundles are dispersed and difficult to follow through the caudal pereiopod ganglia, although the medial fiber bundle is distinct (MFB). All three fiber bundles are distinct in the rostral ganglia. (4) indicates some of the lateral fibers that may be part of the lateral fiber bundle. Large caudal lateral cells (LCLC) in thoracic ganglia 4-7 form the unusual putative neurosecretory structures (*) in the contralateral caudal part of the next rostral ganglion (see fig. 3.6B). Close to these structures are branches of the large rostral medial cells of thoracic ganglion 8, which project into segmental nerve 3 (5).


MFB
LFB
Rostral
(1)
(2)
Subesophageal ganglion
(3)
T4
(5)
MFB
CFB
LFB
LCLC
(4)
T5
LCLC
(5)
T6
T7
T8
LFB
MFB
A1
500 µm

none of them could be followed for more than about 100 μm. Pairs of lateral cell bodies in the second, third, and fourth neuromeres (neuromere #1 being the most rostral) with fibers which cross the midline were consistently seen in well-immunolabeled nerve cords. Each of these cells projects a fiber rostrally that could be followed to the neuropils of the circumesophageal ganglia. The most rostral of these pairs of cell bodies (neuromere 2) project rostrally in the contralateral medial fiber bundles. The two more caudal pairs send contralateral branches both rostrally and caudally along the lateral edges of the subesophageal ganglion. The caudal branches of these cells project to lateral immunoreactive neuropils in the next caudal neuromere, while the rostral branches extend out of the ganglion as part of the lateral fiber bundles. Additional pairs of lateral cell bodies in neuromeres 2 and 3 have ipsilateral, caudally projecting fibers which could be followed through the next two caudal neuromeres. The processes leaving the final two pairs of lateral immunoreactive cell bodies, in neuromeres 5 and 6, could not be followed.

The medial fiber bundles are continuous through the central nervous system from the base of the brain to abdominal ganglion 6, although individual fibers within the bundle do not project over the entire length. The lateral fiber bundles could not be traced continuously through the subesophageal ganglion, although they are continuous rostrally and caudally. Fibers from the lateral fiber bundle of the more caudal ganglia could not be followed rostral to the lateral neuropils of the caudal two neuromeres of the subesophageal ganglion.

Thoracic ganglia four through seven

Three rostro-caudally oriented immunoreactive fiber bundles pass through the pereiopod ganglia (Fig. 3.5). Two, the medial and lateral fiber bundles, are continuous with fiber bundles in the abdominal nerve cord, and the medial fiber bundle is also continuous rostrally to the base of the brain. The third, the central fiber bundle, is only present in the pereiopod ganglia and appears to contain mostly, if not exclusively, fibers from the large caudal lateral cells of thoracic ganglia 4 to 7. The extent of the lateral fiber bundle in the pereiopod ganglia is unclear. One distinct bundle of fibers which is continuous with the lateral fiber bundle in the abdominal nerve cord is present quite medially on each side of the ganglia, but lateral to the medial and central fiber bundles. However, a second bundle that was never seen to contain more than three fibers is present laterally on each side of the ganglia. This bundle appears discontinuous; it passes between the lateral neuropils of the pereiopod ganglia, but individual fibers could not be

followed through the lateral neuropils. Fibers from this bundle join with the continuous lateral fiber bundle at the caudal edge of the subesophageal ganglion, forming the most caudal part of the lateral fiber bundle of this ganglion. Fibers lead from the continuous lateral fiber bundle to the lateral neuropils, but these were not seen to be continuous with any of the more lateral fibers. It is not clear whether these lateral fibers are part of the lateral fiber bundles, are branches of fibers in the lateral fiber bundles, or are something else. None of the cell bodies of origin of any of the fibers in the lateral bundle has been identified. With the exception of the possible division of the lateral fiber bundle in the pereiopod ganglia, the fibers constituting each bundle are, in general, very closely associated with one another in the dorsal part of the ganglia.

One pair of large immunoreactive cell bodies which send processes contralaterally occurs in each of the fourth through seventh thoracic ganglia (pereiopods 1-4) (Figs. 3.5 & 3.6A). Real and Czternasty (1990) called the homologues of these cells in crayfish "large caudal lateral cells", and I have adopted their terminology (they used posterior rather than caudal). These cells increase in size from the more rostral to the more caudal ganglia. Branches of each extend rostrally, initially in the contralateral lateral fiber bundle, then crossing to the central fiber bundle in the next rostral ganglion, and caudally in the contralateral central fiber bundle (Fig. 3.7A). The terminations of the rostral and caudal branches have not been found; although the rostral branches enter the caudal end of the subesophageal ganglion, the immunoreactivity fades before any branching can be seen.

Each of the large caudal lateral cells forms a putative neurosecretory structure unlike anything described in other species (Fig. 3.6B). These tubular structures are oriented dorso-ventrally in the caudal part of the contralateral hemiganglion rostral to each cell, where they are angled along a medio- caudal to latero-rostral axis. They are slightly rostral and ventral to the roots of the third segmental nerves, except in the fourth thoracic ganglion, where they are just ventral to the third nerve roots. Branches of the large rostral medial cells of thoracic ganglion 8 (see below) pass close to these structures before leaving the ganglia through the third nerves and may contribute to them (Fig. 3.6B). I have injected dye into the circulatory system and found small hemolymph vessels in the area of these structures, but I have not successfully immunolabeled a nerve cord with a filled circulatory system, so I cannot determine whether these structures surround vessels. Fibers from the large caudal lateral cells do not enter the third segmental nerves.

Fig. 3.6. Confocal micrographs of 5-HT-immunoreactivity in the pereiopod and first abdominal ganglia. (A) The caudal region of thoracic ganglion 5, showing the large caudal lateral cells of this ganglion (arrowhead) which send processes contralaterally (arrow) and the unusual, putative neurosecretory structures (double arrowhead) formed by the contralateral cells in thoracic ganglion 6. The lateral (LFB), central (CFB), and medial (MFB) fiber bundles are distinct at the level of this ganglion. (B) Higher magnification micrograph of one of the putative neurosecretory structures formed by the large caudal lateral cells. This example is from a thoracic hemiganglion 5; the cell that forms it is in thoracic ganglion 6. Note the branch of the thoracic ganglion 8 large rostral medial cell (arrowheads) which passes in close proximity to this structure and may contribute to it. The picture on the right is a single slice taken by the confocal microscope that clearly shows the circular form of this structure. (C) The large rostral medial cells of abdominal ganglion 1 (arrowhead) and thoracic ganglion 8 (double arrowhead. The medial fiber bundle (MFB) is distinct at this level, and one or two fibers can be seen in the lateral fiber bundle (LFB), but the central fiber bundle cannot be distinguished. The small pair of cell bodies in abdominal ganglia 1 is caudal to this image.

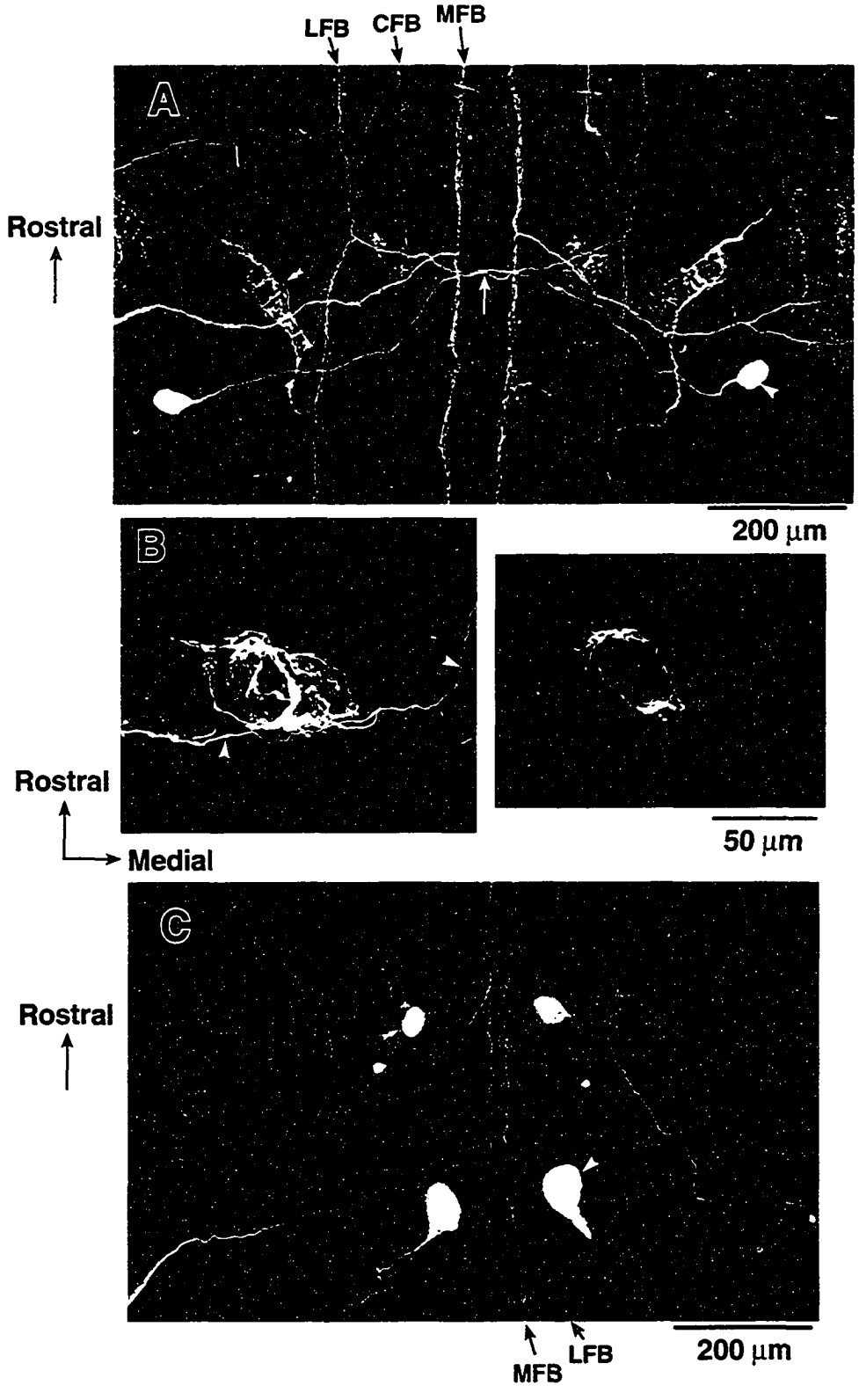
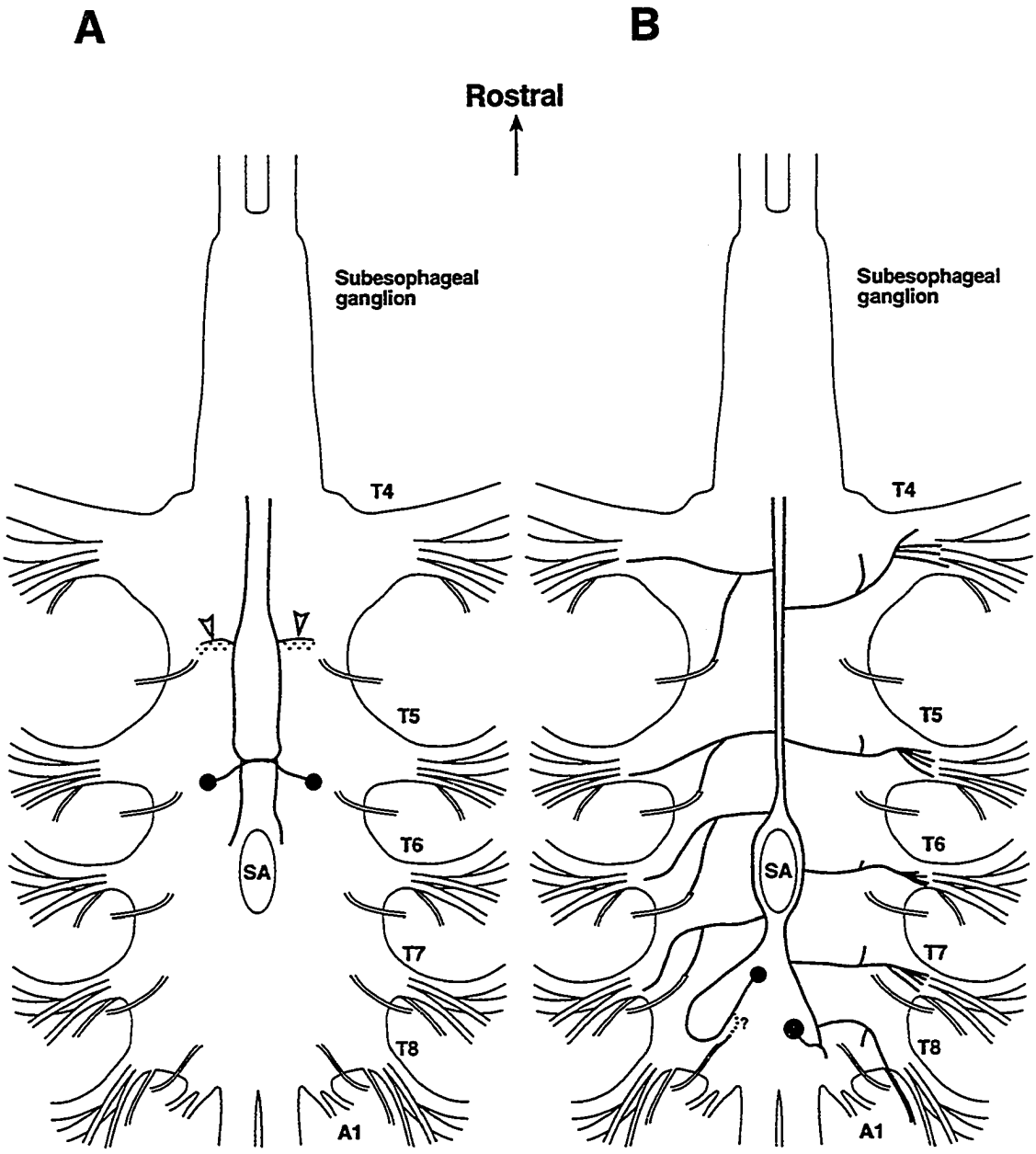

LFB
CFB
MFB
A
Rostral
200 µm
B
Rostral
Medial
50 µm
C
Rostral
MFB
LFB
200 µm

Fig. 3.7. Schematic drawings of the large 5-HT-immunoreactive cells of the pereiopod and first abdominal ganglia. (A) The large caudal lateral cells of thoracic ganglion 5. These cells project contralateral processes rostrally and caudally, and a branch of the rostral projection forms the putative neurosecretory structures (arrowheads) in the caudal part of thoracic ganglion 4. The morphology of the other three pairs of large caudal lateral cells (T4, T6, and T7) are similar. (B) The large rostral medial cells of abdominal ganglion 1 (A1LRMC) on the right and thoracic ganglion 8 (T8LRMC) on the left. Note that the rostrally projecting fibers of these cells join the medial fiber bundle in the next rostral ganglion. See Table 3.1 for a summary of the peripheral projections of these cells. SA, sternal artery.


A
B
Rostral
Subesophageal ganglion
Subesophageal ganglion
T4
T4
T5
T5
T6
T6
SA
SA
T7
T7
T8
T8
A1
A1
?
T8LRMC
A1LRMC

The eighth thoracic and first abdominal ganglia

Two pairs of 5-HT immunoreactive cell bodies are present medially in each of thoracic ganglion 8 and abdominal ganglion 1 (Figs. 3.5 & 3.6C). One pair of cells in each ganglion is small (10-15 μm) with processes which I have been unable to follow beyond a few micrometers. The other pairs of immunoreactive cells are homologues to the large rostral medial cells described in several other species (Beltz & Kravitz, 1983 & 1987; Real & Czternasty, 1990; Harzsch & Dawirs, 1995) (Fig. 3.7B). These two pairs of cells are responsible for all of the identified fibers that enter the segmental nerves of the pereiopod ganglia (Table 3.1). As in lobsters and crayfish, the fibers that enter the segmental nerves form dense plexuses of fine beaded fibers around the periphery of the nerves.

The large cells (50-60 μm) in thoracic ganglion 8 send large processes caudally and laterally into that ganglion's neuropil which then loop rostrally to project into the medial fiber bundle (Figs. 3.6C & 3.7B). Branches of these cells enter nerves 2 and 3 of thoracic ganglia 4-7, but the only branches of this cell seen in thoracic ganglion 8 form a small area of neuropil just rostral to the cell bodies. A fiber enters nerve 3 of this ganglion, but it has not been traced back to its cell body of origin. Each large (70-85 μm) rostral medial cell of abdominal ganglion 1 sends a process laterally, then rostrally to join the medial fiber bundle, with small branches leading from the rostral turn point to a small caudal area of neuropil in abdominal ganglion 1 (Figs. 3.6C & 3.7B). Large branches of this cell enter nerve 2 of all five pereiopod ganglia and nerve 1 of pereiopod ganglia 1-4. Additionally, branches of this cell arborize in the lateral neuropil of each pereiopod hemiganglion, and appear to be, together with the unidentified branches from the lateral fiber bundles, the main contributors to the lateral serotonergic neuropils. Other than the cell bodies and their projections, the 5-HT immunoreactive map for the first abdominal ganglion is similar to those for the second through fourth abdominal ganglia (see below). Unlike the large rostral medial cells in lobsters and crayfish (Beltz & Kravitz, 1983 & 1987; Real & Czternasty, 1990), in which the rostral projections initially enter the lateral fiber bundle and cross into the medial fiber bundle in the next rostral ganglion, the rostral projections of these cells in *M. quadrispina* do not join the lateral fiber bundles. However, the processes of these cells do not join with the medial fiber bundle until they have entered the next rostral ganglion, and perhaps these differences are a result cytoarchetectural rearrangements which occurred with the fusion of ganglia in *M.*

Table 3.1. Sources of 5-HT immunoreactive fibers in the segmental nerves of the pereiopod ganglia. Immunoreactive fibers entering nerve one project branches into both distal leg nerves and both thoracico-coxal nerves. A1LRMC and T8LRMC, abdominal ganglion 1 and thoracic ganglion 8 large rostral medial cells; T4-T8, thoracic ganglia 4 through 8. An immunoreactive fiber projects into nerve 3 of thoracic ganglion 8, but I have been unable to trace it to its cell body of origin.

Table 3.1

Ganglion	Nerve	Source of 5-HT-immunoreactive fibers
T4 (Claw)	1	A1LRMC
	2	A1LRMC, T8LRMC
	3	T8LRMC
T5	1	A1LRMC
	2	A1LRMC, T8LRMC
	3	T8LRMC
T6	1	A1LRMC
	2	A1LRMC, T8LRMC
	3	T8LRMC
T7	1	A1LRMC
	2	A1LRMC, T8LRMC
	3	T8LRMC
T8	1	None
	2	A1LRMC
	3	?

quadrispina. Their fibers are the largest (10-12 μm) and most strongly labeled in the medial bundles and are, therefore, easy to follow.

Abdominal ganglia 2-6

There are no immunoreactive cell bodies in abdominal ganglion 5. All of the 5-HT-immunoreactive cell bodies in abdominal ganglia 2-4 and 6 are small (10-15 μm) and have fibers which are initially directed rostrally when they leave the cell, but I was unable to follow any individual fibers to any areas of neuropil or out of the ganglia (Figs. 3.8 & 3.9). The medial and lateral fiber bundles are continuous through the abdominal nerve cord to ganglion 6.

The distribution of 5-HT-immunoreactive neurons is basically identical in abdominal ganglia 2 – 4 (Fig. 3.8 & 3.9A). The four cell bodies are located laterally, one pair rostrally and the other about mid-way back in the ganglia. This is similar to the positions of their homologues in lobster (Beltz & Kravitz, 1983) and crayfish (Real & Czternasty, 1990), although in lobsters the cell bodies are located more medially, and crayfish also have a large medial unpaired cell body in abdominal ganglion 4. The lateral fiber bundles on each side of these ganglia contain three immunoreactive fibers, and the medial bundles contain two fibers, for a total of 10 immunoreactive fibers in each pair of connectives. I was unable to trace any of these fibers to its cell body and therefore I do not use the terms *entering* or *leaving* to describe these fibers as they pass between the connectives and ganglia, since I do not know their source or whether they are ascending or descending. In each ganglion, one of the fibers from the lateral fiber bundle of the rostral connective terminates in the dense lateral neuropil, while two pass through to the caudal connective. Both of the fibers which pass through the ganglion also branch within the lateral neuropil, and one makes a lateral turn and sends a fine (1 μm), faintly-immunoreactive fiber into the second nerve. The immunoreactive processes in the second nerves were unbeaded and could not be followed more than about 20-40 μm out the nerve. The fibers in the lateral bundles are only very faintly immunoreactive in the middle of the connectives, and therefore I was not able to follow individual fibers for more than two ganglia and could not determine the extent of any single fiber's projections (i.e. I could not confirm that the fiber which sends the branch out nerve 2 is the same fiber in each ganglion).

The two fibers from the medial fiber bundle separate within each ganglion, one going slightly more lateral than the other, although the amount of separation is variable.

Fig. 3.8. 5-HT immunoreactivity in abdominal ganglia 2 through 6. These drawings are composites of camera lucida drawings and traces of confocal micrographs from a single nerve cord. Features are identified in abdominal ganglia 2 (similar in ganglia 3 and 4), 5, and 6, although ganglia 2 through 4 (A2-A4) have similar patterns of 5-HT immunoreactivity. Note the small lateral cell bodies (open arrowheads), the branches of the fiber from the medial fiber bundle which cross the midline (open double arrowhead), the area of fine processes which form the medial fiber bundles (MFB) in the ganglia (*), the fine branches of the most lateral fiber which project into nerve 2 (1), and the fibers which cross between the lateral fiber bundles (LFB) and medial fiber bundles in the caudal part of the ganglia (2) (identified in A3). Between abdominal ganglia 1 and 5, three fibers comprise the lateral fiber bundle and two comprise the medial fiber bundle. Between ganglia 5 and 6 (A5 and A6), there are two and one in the lateral and medial bundles, respectively. In abdominal ganglion 6, note the fiber from the lateral bundle that forms a dense plexus of beaded fibers in the connective (3) and a second in the caudal part of the ganglion and out nerve 7 (4).

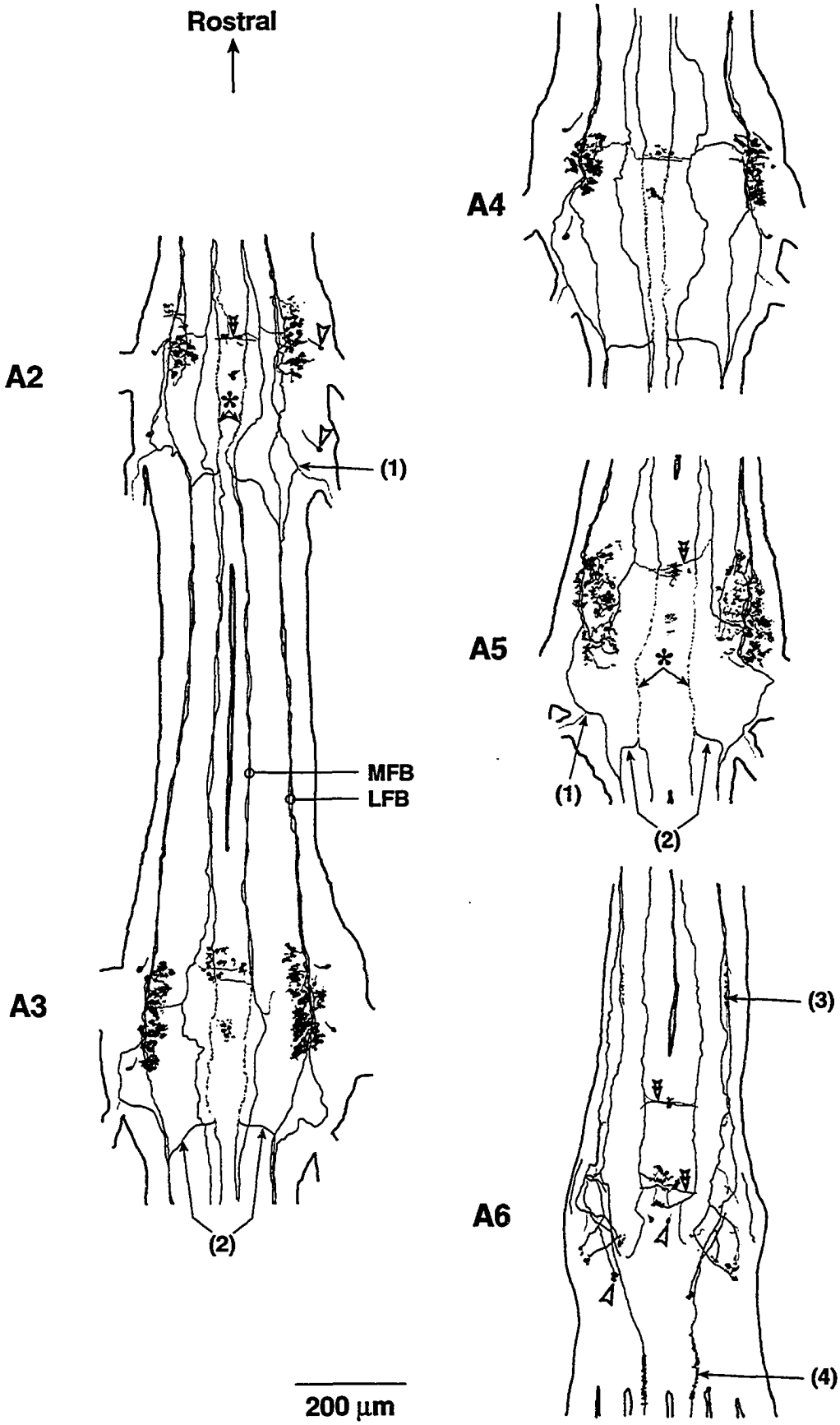
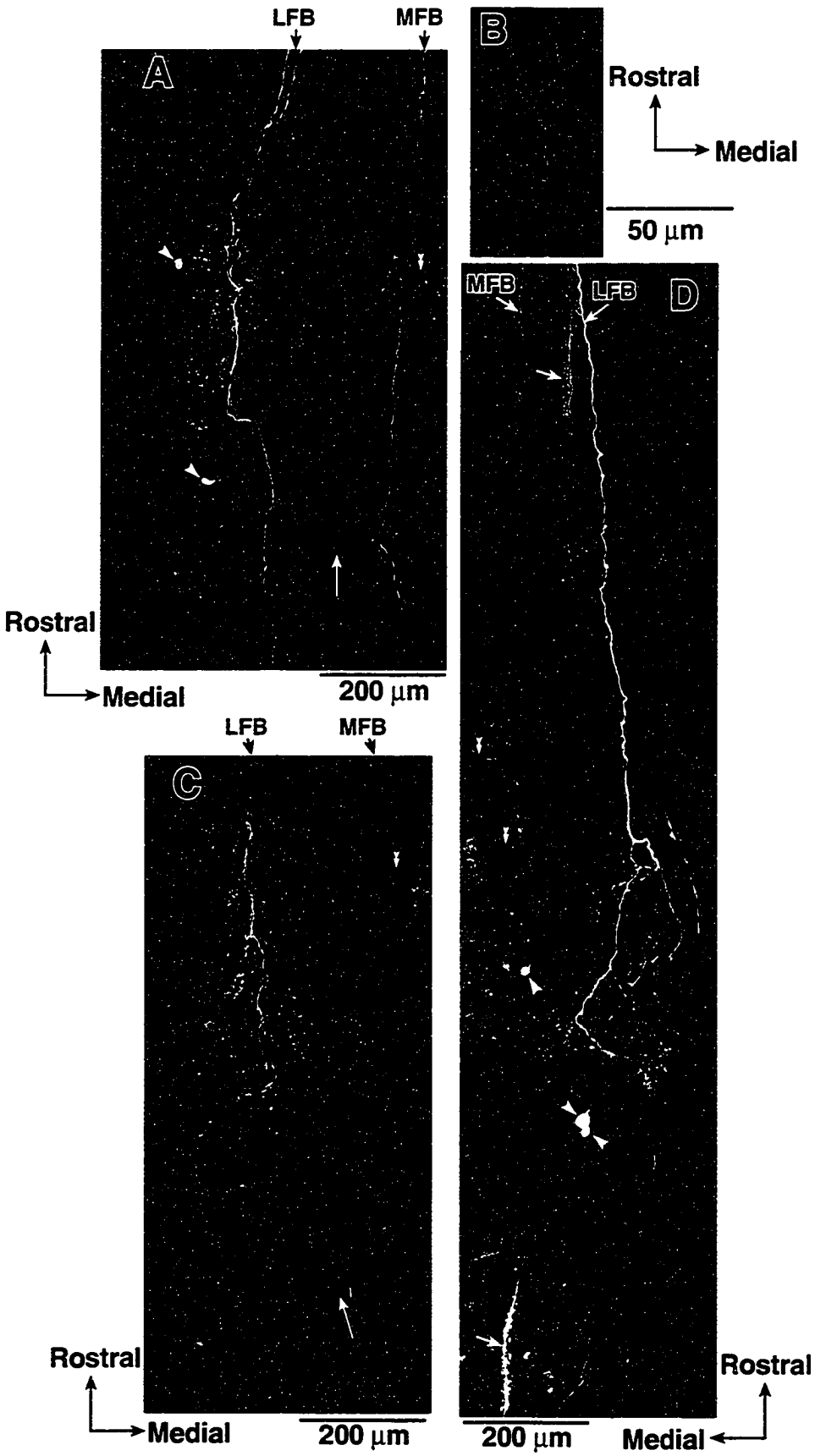

Rostral
A2
A3
A4
A5
A6
(1)
MFB
LFB
(2)
(1)
(2)
(3)
(4)
200 µm

Fig. 3.9. Confocal micrographs of 5-HT immunoreactivity in the abdominal ganglia. (A) One hemiganglion of abdominal ganglion 4. Abdominal ganglia 2 and 3 are essentially identical. There are three fibers in the lateral fiber bundle (LFB) and two in the medial fiber bundle (MFB) in the connectives rostral and caudal to the ganglion. The small lateral cell bodies are indicated by the arrowheads, the branches of the fiber from the medial fiber bundle that cross the midline are indicated by the double arrowhead, and the fiber that crosses between the medial and lateral bundles is indicated by the arrow. There is not much separation between the more lateral and medial fibers of the medial fiber bundle within the ganglion. (B) A high magnification view of the fine, dense fibers of the medial fiber bundle in the ganglion. (C) A hemiganglion of abdominal ganglion 5. Conventions as in (A). There are three fibers in the lateral fiber bundle and two in the medial fiber bundle rostral to the ganglion, but two in the lateral bundle and one in the medial bundle in the connective between ganglia 5 and 6. (D) A hemiganglion of abdominal ganglion 6. The small cell bodies are indicated by the arrowheads. The same fiber from the lateral fiber bundle forms dense plexuses of fine beaded fibers in the connective rostral to the ganglion and in the caudal part of the ganglion at the exit of nerve 7 (arrows).


LFB
MFB
A
Rostral
Medial
200 µm
B
Rostral
Medial
50 µm
MFB
LFB
D
C
LFB
MFB
Rostral
Medial
200 µm
200 µm
Rostral
Medial

One branch from the more lateral fiber enters the ipsilateral lateral neuropil and one branch passes contralaterally, where in well-labeled ganglia it could be followed to about half way between the midline and the contralateral lateral neuropil. This fiber could be followed from abdominal ganglion 1 through 5. Many fine fibers surround the more medial fiber in the middle part of the ganglion (Fig. 3.9B) , through which it was impossible to follow the course of any fiber. A similar feature has been described in lobsters (Beltz & Kravitz, 1983). The medial fibers from the rostral and caudal connectives contribute to this plexus of fine fibers, but it is not clear if they are the only contributors or if the more medial fiber is continuous through each ganglion. Near the caudal edge of each ganglion, but within the plexus of fine fibers, one fiber leaves the medial bundle and travels laterally to join the lateral bundle. This fiber appears to arise (or end) within the plexus of fine fibers, and therefore its source is not known. Along the midline, there are two small areas of immunoreactive neuropil, one of which is in the area of the branches from the medial fiber bundle which cross the midline, and one of which is slightly caudal to this point. The source of these immunoreactive neuropils could not be traced.

The immunoreactive map in the fifth abdominal ganglion is similar to the more rostral abdominal ganglia, except that there are no immunoreactive cell bodies and only three, rather than five, immunoreactive fibers are in the connective caudal to it (Fig. 3.8 & 3.9C). Two of the three fibers of the lateral bundle in the rostral connective terminate in the lateral neuropil of abdominal ganglion 5. The third, which passes through the ganglion, is similar to fibers seen in the more rostral abdominal ganglia in that it turns laterally and sends a fine branch out the second nerve. As in the more rostral ganglia, the two fibers in the medial bundle in the rostral connective separate in the ganglion, but the more lateral fiber terminates in the lateral neuropil.

Four of the immunoreactive cell bodies in the sixth abdominal ganglion are located caudally and the remaining two are close to the midline in the center of the ganglion (Fig. 3.8 & 3.9D). All three of the fibers in each rostral connective contribute to the fairly sparse lateral neuropil. The lone fiber of each medial fiber bundle sends branches contralaterally at two points, the more caudal of which contributes to caudal medial immunoreactive neuropil. I could not determine whether the more rostral branches contribute to the rostral medial neuropil. One of the fibers in the lateral fiber bundle produces two dense plexuses of fine beaded processes: one in the rostral connective and a second that begins in the caudal part of the ganglion and continues into

the seventh nerve and presumably innervates the caudal gut. This is different than the situation in crayfish, where no immunoreactive fibers are seen in the seventh nerve of abdominal ganglion 6 unless the nerve cords are pre-soaked in 5-HT, or adjacent sensory nerves are stimulated for one hour following dissection (Musolf et al., 1997). Immunoreactive fibers have been found to exit nerves of the sixth abdominal ganglion in lobsters (Beltz & Kravitz, 1983), but the innervation field of the nerves were not identified. Two large (7-8 μm) very strongly immunoreactive fibers (one per side) were consistently seen along the lateral surfaces of the ganglion, just below the sheath, but the source of these fibers could not be traced and they did not send off any visible branches.

Distribution of octopamine-like immunoreactivity

Forty-four OA-immunoreactive cells are in the central nervous system of *M. quadrispina* (Fig. 3.10). Ten of these are in the brain, 22 in the subesophageal ganglion, and 12 in the pereiopod ganglia. The largest cells are the rostral "crotch" (Schneider et. al., 1993) cells of thoracic ganglia 4 and 5, at 60-70 μm diameter, while the remainder of the cells are between 15 and 50 μm diameter. There are far fewer crotch cells in *M. quadrispina* than in American lobsters (Schneider et. al., 1993). Most suprisingly, there are no immunoreactive cell bodies at all in thoracic ganglia 6 and 7.

The brain and circumesophageal ganglia

Ten OA-immunoreactive cell bodies are located at the base of the brain; two pairs are medial and three pairs are slightly more lateral (Figs. 3.11 & 3.12A). The somata of one pair of medial and one pair of lateral cells have diameters between 15 and 20 μm, while the remaining three pairs are between 25 and 35 μm. The processes of the more lateral cells initially project rostrally into areas of immunoreactive neuropil, but the processes of the more medial cells were never visible. The distribution of OA-immunoreactive neuropil in the brain is very similar to 5-HT-immunoreactive neuropil. However, the intensity of the OA-immunoreactivity was much lower, both in whole mount and in thick sections. The differences between 5-HT and OA-immunoreactivity cannot be strictly due to differences in affinity or binding properties of the respective antibodies, because some OA-immunoreactive cell bodies consistently labeled very intensely, while others consistently labeled weakly. Also, because the intensity of the label was generally similar between whole mounts and thick sections, it is unlikely that penetration or endogenous autofluorescence problems caused by the glutaraldehyde

Fig. 3.10. Distribution of OA-immunoreactive neurons. The size of each dot roughly corresponds to the size of the cell body. The connectives between the circumesophageal and subesophageal ganglia and the first and second abdominal ganglia are not shown. A1-A6, abdominal ganglia 1-6; CEG, circumesophageal ganglia; SEG, subesophageal ganglion; T4-T8, thoracic ganglia 4-8 (the pereiopod ganglia).

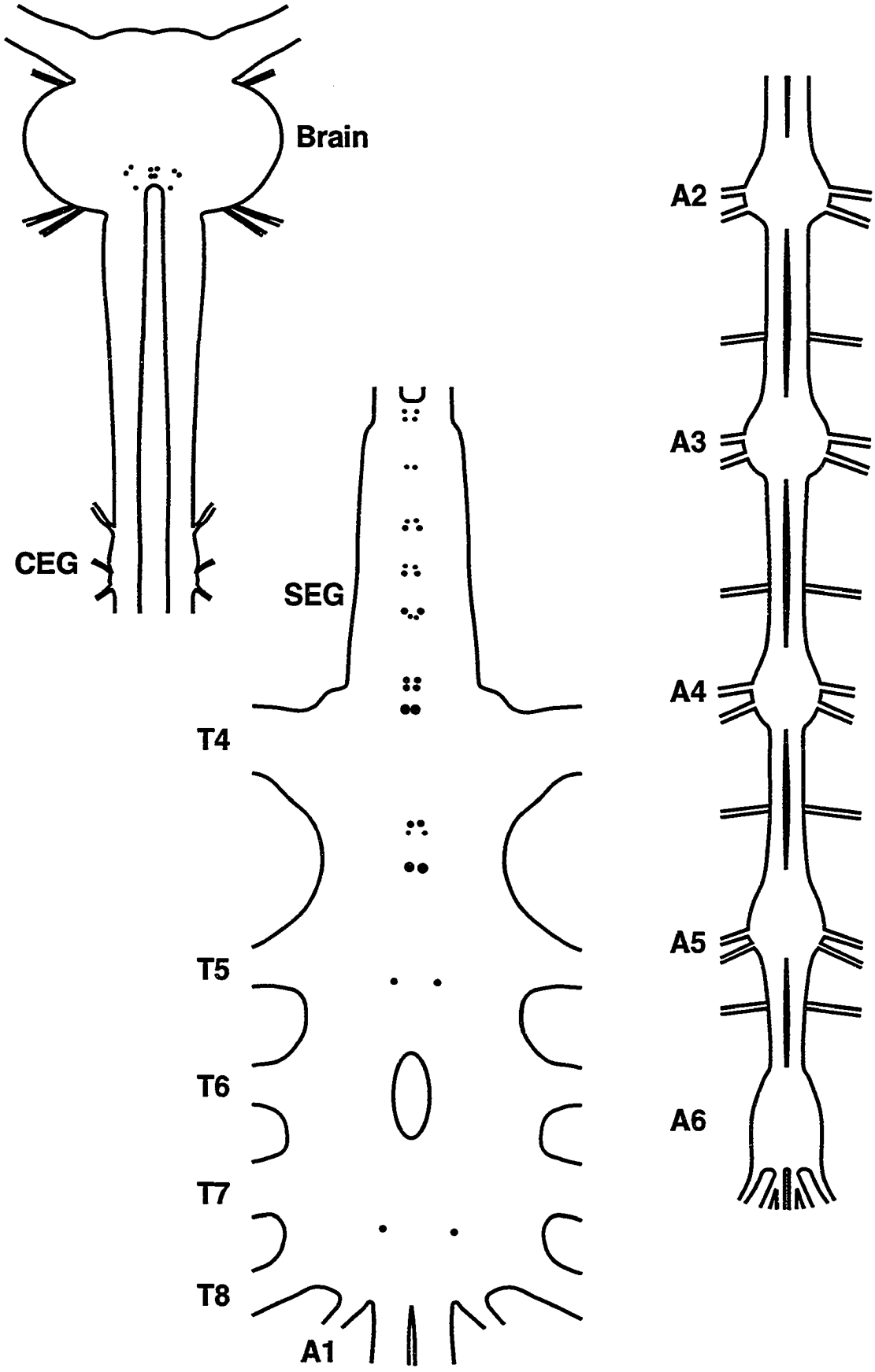

Brain
CEG
SEG
T4
T5
T6
T7
T8
A1
A2
A3
A4
A5
A6

Fig. 3.11. Camera lucida drawing of OA-immunoreactivity in the brain and circumesophageal ganglia. The ten immunoreactive somata in the brain are clustered at the caudal edge (arrowhead). Weakly immunolabeled neuropil areas are distributed through the brain, including at the edges of the olfactory lobes (double arrowheads). Note the two areas of dense immunoreactive fibers in each circumesophageal ganglion, one in the lateral neuropil (1), and one surrounding a group of fibers in the medial part of the ganglion (2).

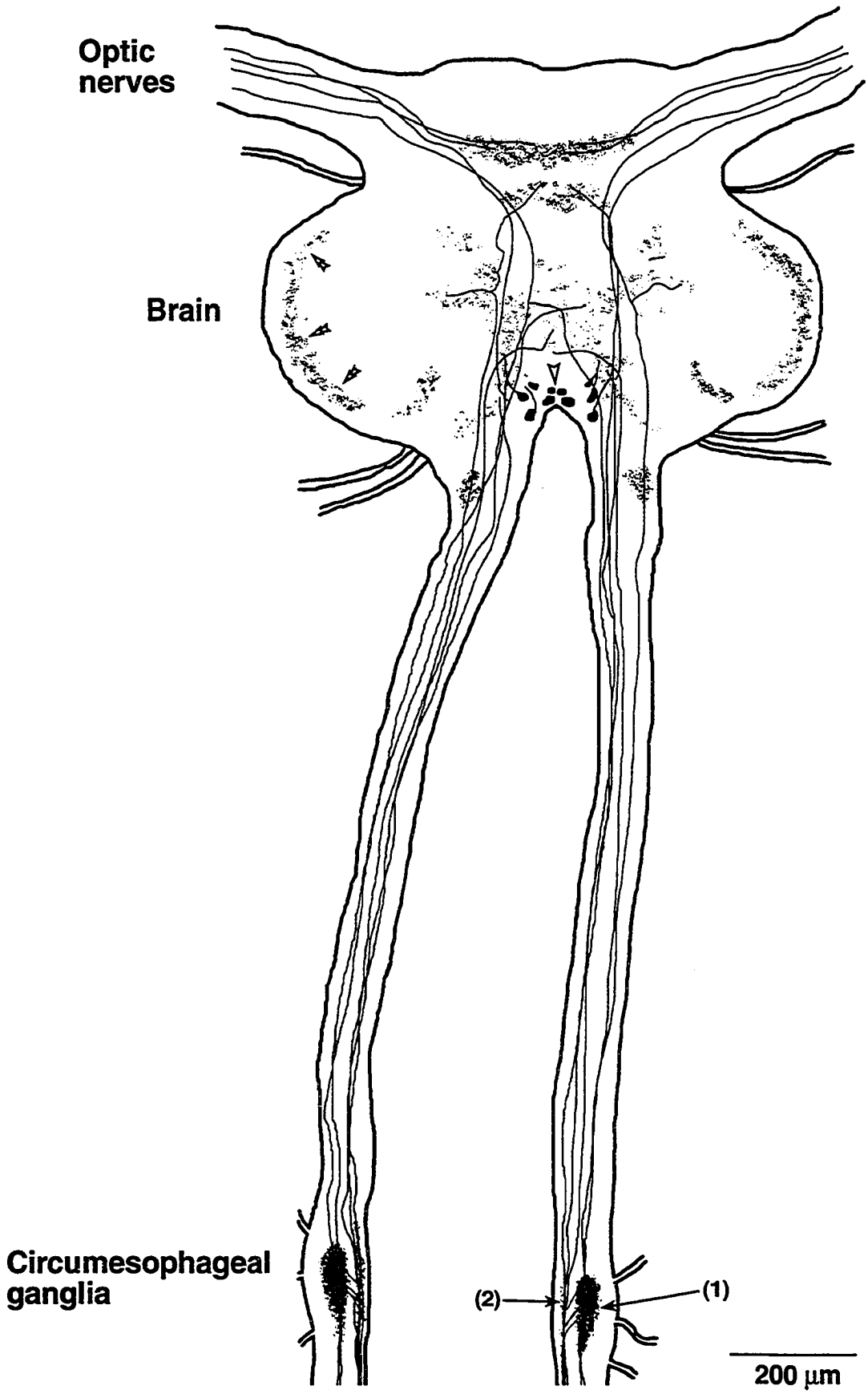
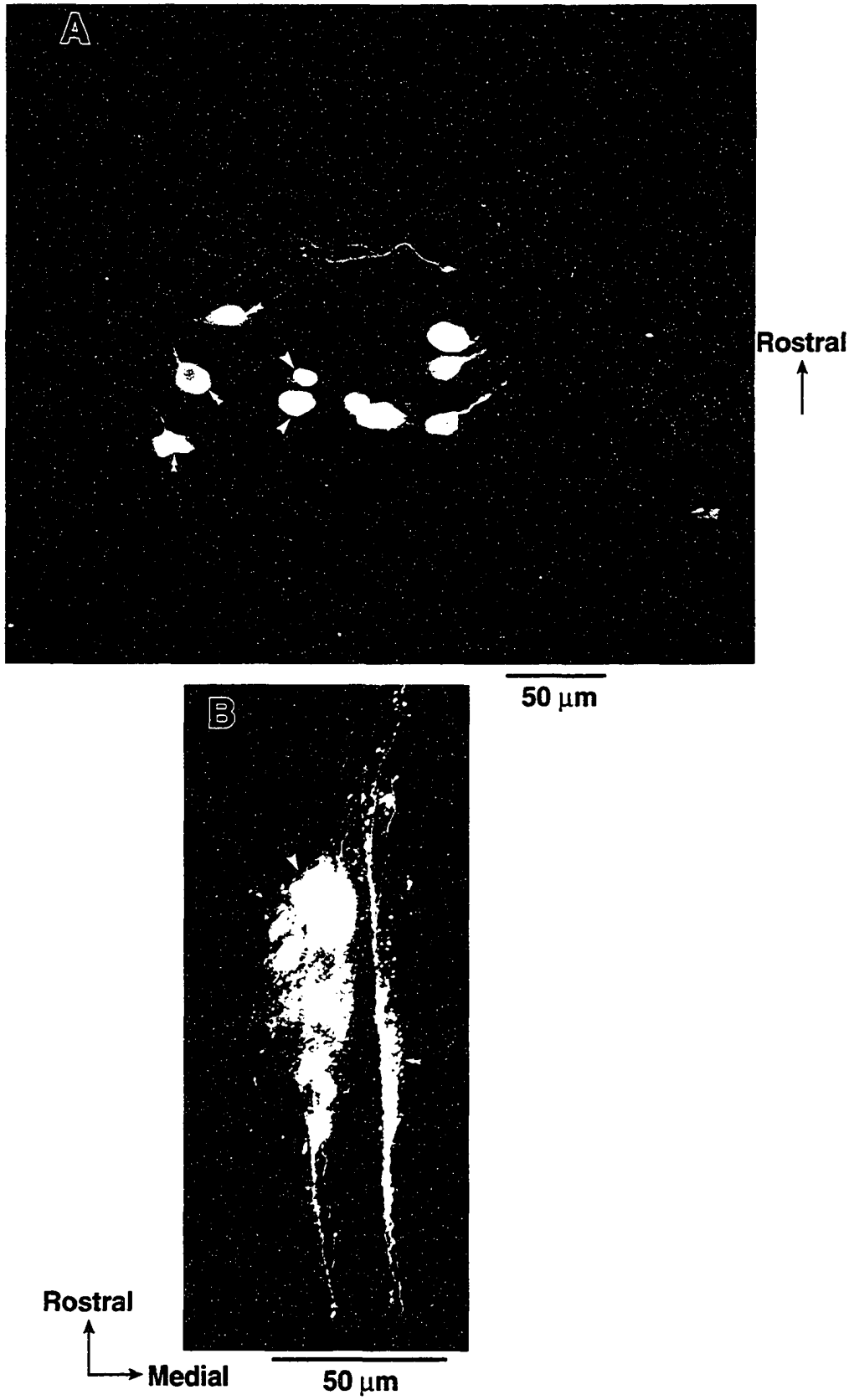

Optic nerves
Brain
Circumesophageal ganglia
(2)
(1)
200 µm

Fig. 3.12. Confocal micrographs of OA-immunoreactivity in the brain and a circumesophageal ganglion. (A) The cluster of immunoreactive somata at the base of the brain. Four of the cells are medial (arrowheads), and six are slightly more lateral (double arrowheads). The processes of the more lateral cells project rostrally into areas of immunoreactive neuropil. (B) One circumesophageal ganglion. Two areas of dense immunoreactive fibers are present; one in the lateral neuropil of the ganglion (arrowhead) and one surrounding a group of medial immunoreactive fibers (double arrowhead).


A
Rostral
50 μm
B
Rostral
Medial
50 μm

fixative could explain the weak label. Finally, larger immunoreactive fibers within areas of neuropil, and a few smaller fibers, were usually very strongly labeled, suggesting that the weak label may be due to low concentrations of OA in the neuropil. Fine labeled processes could not be followed easily in areas of neuropil; neuropil appeared as a pattern of unconnected beads of immunoreactivity. A similar appearance of OA-immunoreactive neuropil has been described in lobsters (Schneider et al., 1993).

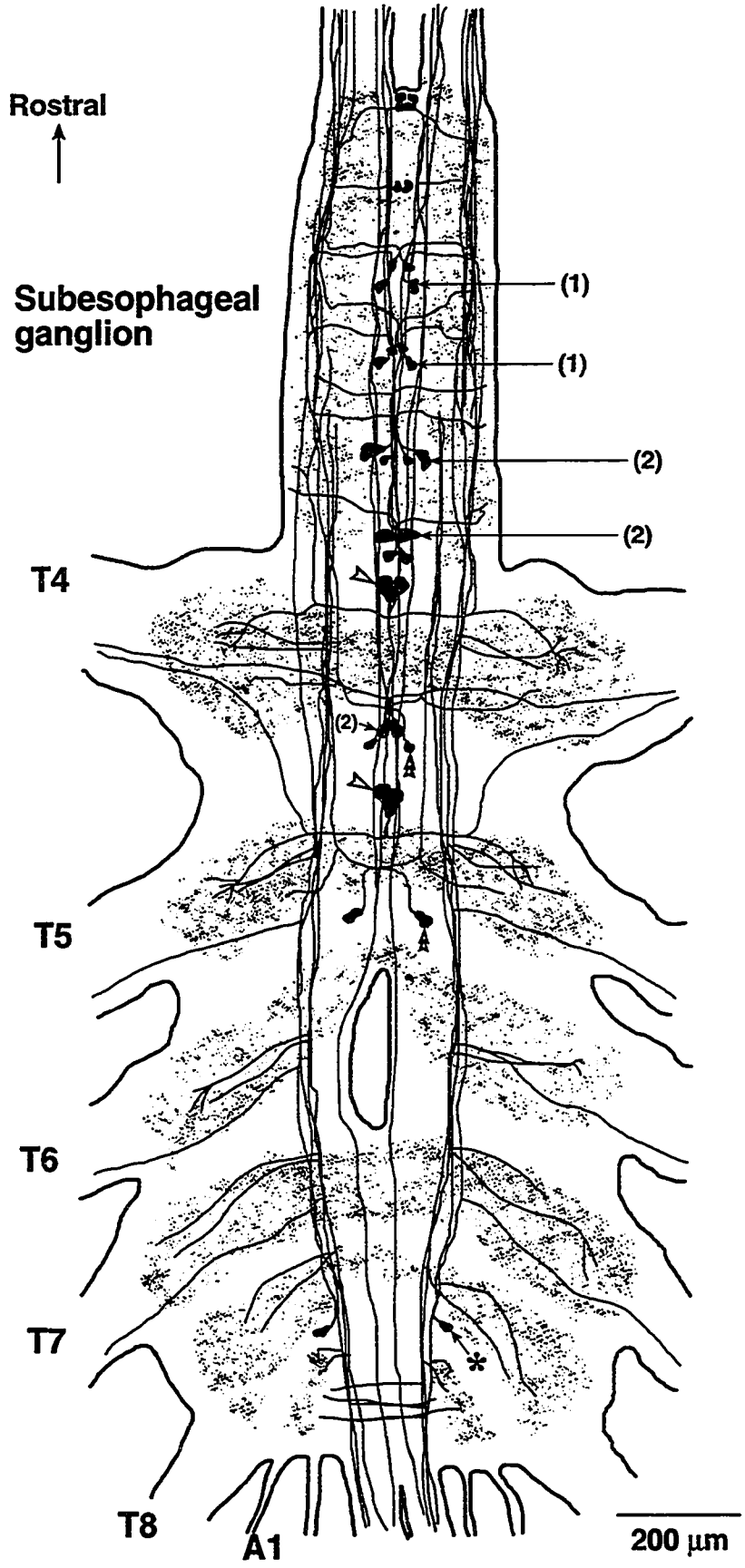
In well-labeled nerve cords, four or five OA-immunoreactive fibers were present in each optic nerve (Fig. 3.11). The cell bodies of origin of these fibers were not identified, but one of the fibers was consistently traced caudally to the subesophageal ganglion. Four additional fibers that arborize in the neuropils of the brain are present in the connectives caudal to the brain. All of these fibers were traced to the subesophageal ganglion, but none of the cell bodies of origin were identified. The only OA-immunoreactive fibers seen to enter the olfactory lobes were branches of one of the fibers in the connectives.

There are no OA-immunoreactive cell bodies in the circumesophageal ganglia, but these ganglia contain the most intensely labeled plexuses of fibers in the entire central nervous system (Figs. 3.11 & 3.12B). The relative intensity of labeling was consistent in whole mounts and thick sections, so it is likely not an artifact of differential antibody penetration. Eight immunoreactive fibers are arranged in a medial and lateral fiber bundle in the connective caudal to each ganglion, three of which are ascending fibers which terminate in the neuropils of these ganglia. The remaining five immunoreactive fibers also contribute to the circumesophageal neuropils, but pass through the ganglia, and have not been followed to their cell bodies of origin. Two distinct areas of dense immunoreactive fibers are present in each ganglion. The first, to which all of the immunoreactive fibers contribute, is in the lateral neuropil of the ganglion. The second is a plexus of beaded processes surrounding a group of fibers in the medial part of the ganglion. At least some of the medial fibers contribute to the medial plexus, but I have not seen any evidence to suggest that the more lateral fibers send any branches to it. No immunoreactive fibers were seen entering any of the segmental nerves of the subesophageal ganglia.

The subesophageal and pereiopod ganglia

Twenty-two OA-immunoreactive cell bodies are in the subesophageal ganglion (Fig. 3.13). Most of them are roughly the same size, between 20 and 30 μm. The four

Fig. 3.13. Camera lucida drawing of OA-immunoreactivity in the subesophageal, pereiopod, and first abdominal ganglia. (1) indicates the cells in the third and fourth subesophageal neuromeres that send processes caudally to the sixth abdominal ganglion. (2) indicates the cells in the fifth and sixth subesophageal neuromeres and fourth thoracic ganglion that send contralateral processes into the second segmental nerves. The open arrowheads indicate the rostral crotch cells; the open double arrowheads indicate the caudal crotch cells with contralateral rostral projections. The asterisk indicates the cells in thoracic ganglion 8 which send branches into the segmental second nerves of each of the fifth through eighth thoracic ganglion, and also may be the source of the fiber in the second nerve of the fourth thoracic ganglion which was not traceable to its cell body of origin (not visible on this drawing).


Rostral
Subesophageal ganglion
(1)
(1)
(2)
(2)
(2)
T4
T5
T6
T7
T8
A1
200 µm

immunoreactive cell bodies in neuromere 1 are located just at the rostral edge of the ganglion, about midway in the dorso-ventral axis. The pair of cell bodies in neuromere 2 is located dorsally. The processes leaving the six most rostral cells did not label and therefore could not be traced. All of the remaining immunoreactive cell bodies in the subesophageal ganglion, in neuromeres 3 through 6, are located ventrally and caudally in the neuromere.

Two pairs of immunoreactive cell bodies, one each in neuromeres 3 and 4, send ipsilateral processes caudally to abdominal ganglion 6 (Fig. 3.13 & 3.14A). They are similar to cells described in the American lobster (Schneider et al., 1993). These cells contribute to the ipsilateral lateral neuropil of their own ganglion and all ganglia caudal to them and, in the abdominal ganglia, they also contribute to the contralateral neuropil. A second pair of immunoreactive cell bodies is in each of the third and fourth neuromeres which project contralaterally, then rostrally. These fibers project to the next rostral neuromere, where they branch and appear to contribute to the lateral immunoreactive neuropil of these neuromeres (Fig. 3.14B). Neuromeres 5 and 6 each contain two pairs of immunoreactive cell bodies. One pair in each neuromere project ipsilateral fibers rostrally, close to the midline. These fibers did not send off any visible branches, although they could be followed for two rostral neuromeres, after which they faded. The second pair of cell bodies in the fifth and sixth neuromeres project fibers contralaterally out segmental nerves of their own neuromeres. These cells are slightly larger (30-50 μm) than the rest of the immunoreactive cells in the subesophageal ganglion, and are likely segmental homologues of the immunosecretory caudal crotch cells described in lobsters (Schneider et al., 1993). The positions of these cell bodies are variable; in some animals they are located on top of one another along the midline, while in others they are separated and located as much as one-third of the way laterally in the neuromere (e.g. the cells of neuromere 5 in Fig. 3.13).

Most of the crotch cells present in the pereiopod ganglia of lobsters (Schneider et al., 1993) are missing in *M. quadrispina* (Fig. 3.13). Dorsally-located rostral crotch cells are present in the fourth and fifth thoracic ganglia, and project laterally, then rostrally, with branches continuing to the lateral neuropil (Fig. 3.14C & 3.15A). The rostral branches of these cells turn laterally in the next rostral ganglion and enter the second segmental nerves, where they contribute to a dense plexus of peripheral immunoreactive fibers. As in lobsters, these cells are large (60-70 μm) and directly overlie one another along the midline and, therefore, determining whether their projections are ipsilateral or

Fig. 3.14. Schematic drawings of OA-immunoreactive neurons. (A) the descending interneurons in the third and fourth subesophageal neuromeres that send ipsilateral processes caudally to the sixth abdominal ganglion. (B) Cells with rostral projections. The rostral fibers of all of these cells faded without visible terminal arborizations. (C) the putative OA-neurosecretory system. Two pairs of cells in the caudal two neuromeres of the subesophageal ganglion and three pairs in the first and second pereiopod ganglia contribute to this system. All of these cells have branches that enter second segmental nerves and form dense peripheral plexuses. Note that three different fibers enter the second nerve of thoracic ganglion 4 (the claw), one of which was not traced to its cell body of origin, but may have come from the cells in thoracic ganglion 8.

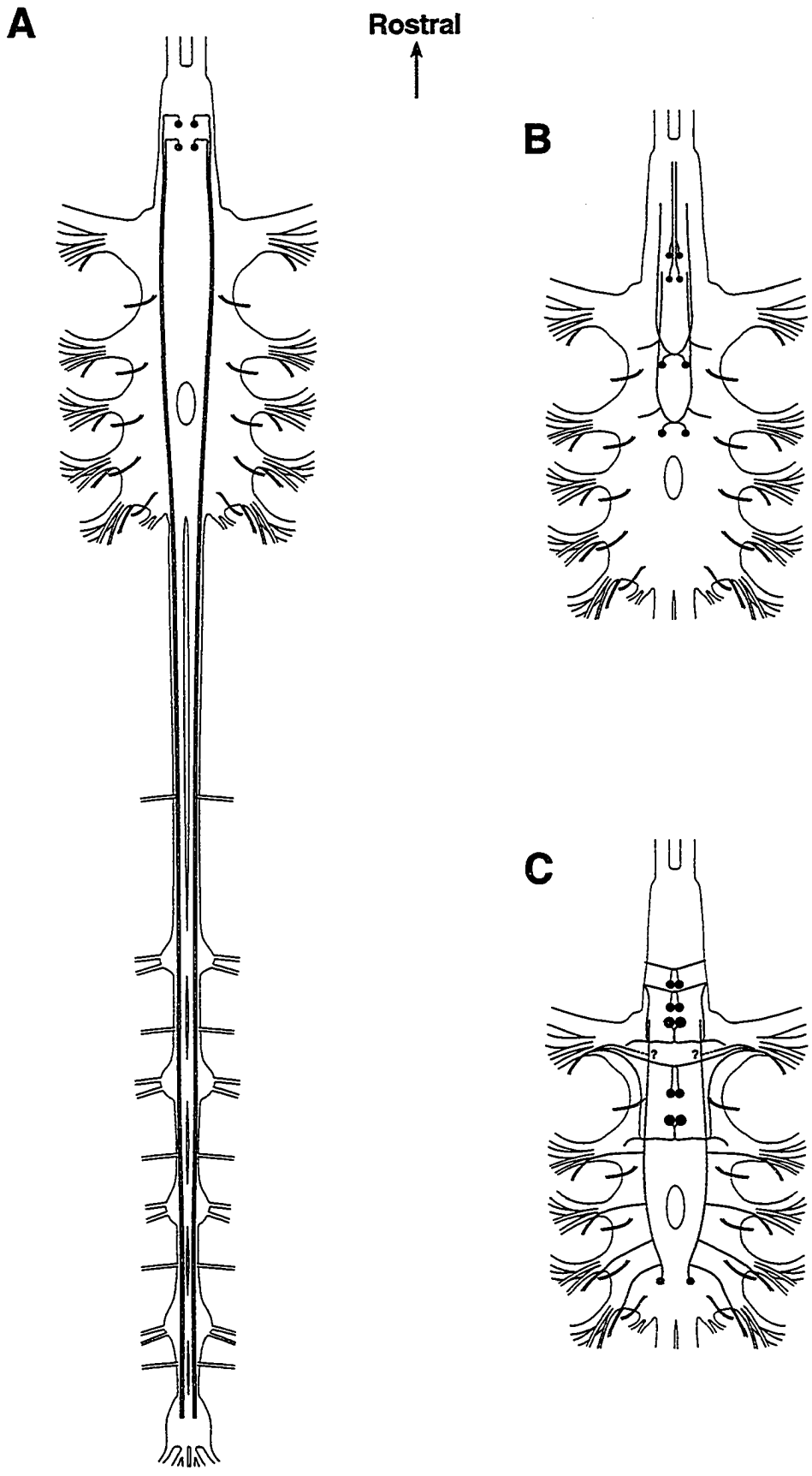
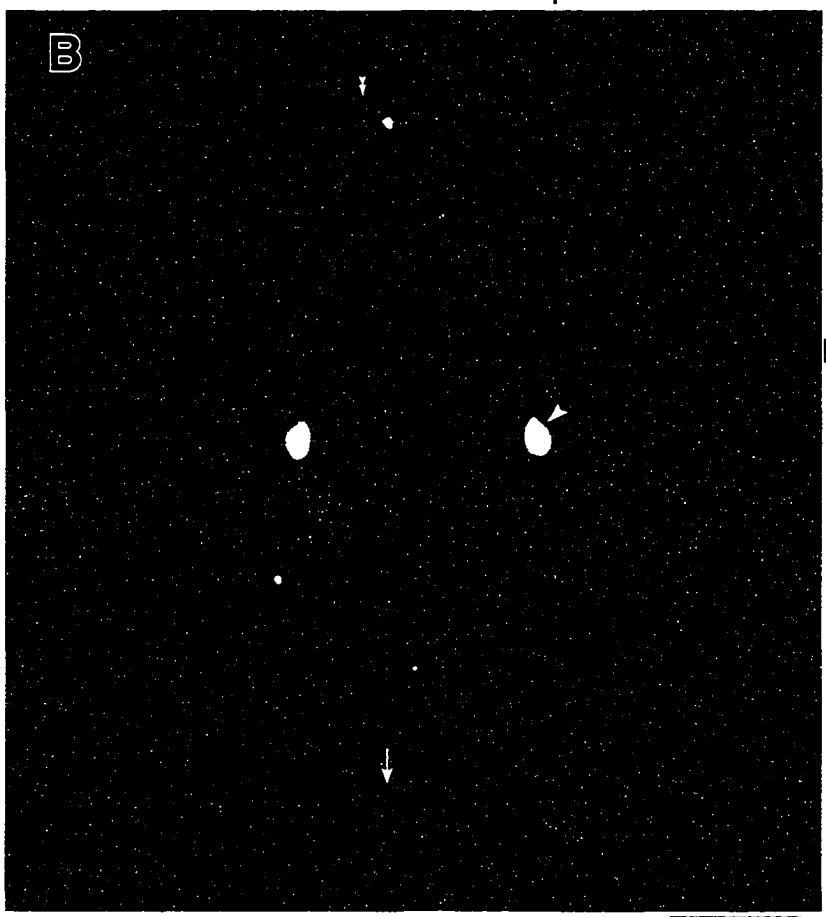

A
Rostral
B
C
?
?

Fig. 3.15. Confocal micrographs of OA-immunoreactive neurons in the pereiopod ganglia. (A) The middle part of the fourth thoracic ganglion. The solid arrowhead indicates the rostral crotch cells, and the solid arrows point to the processes of this cell on one side of the ganglion. The asterisk indicates one of the fine beaded processes that project from these cells near the midline. The double arrowhead indicates the caudal crotch cells that send processes out nerve 2 (double headed arrows). The more lateral caudal crotch cells (open arrowhead) send processes across the midline (open arrow), then rostral. (B) The immunoreactive cells in thoracic ganglion 8 (arrowhead). These cells project rostrally and send branches out nerve 2 of at least the next three rostral ganglia. Note the weakly immunoreactive processes along the midline of thoracic ganglion 8 (double arrowhead) and the contralaterally-projecting branches of the descending interneurons in abdominal ganglion 1 (arrow).


A
Rostral
100 μm


B
Rostral
100 μm

contralateral was not possible. Two types of OA- immunoreactive caudal crotch cells are present. One type, represented by a single pair in thoracic ganglion 4, has cell bodies located close to the midline and projects into nerve 2 of thoracic ganglion 4 (Figs. 3.13, 3.14C & 3.15A). In some animals, these cells are located almost on top of one another along the midline, but in others they were displaced slightly laterally and I could determine that their projections are contralateral. These two cells are likely segmental homologues of the two cells with similar projections in the caudal two neuromeres of the subesophageal ganglion. The second type of caudal crotch cell has cell bodies located slightly off the midline and sends projections contralaterally, then rostrally, with branches entering the lateral neuropil of the contralateral side (Figs. 3.13, 3.14B & 3.15A). Two pairs of these cells exist, and the cells in the fourth thoracic ganglion are slightly smaller than the ones in the fifth thoracic ganglion (15-25 μm vs. 30-40 μm). In general, these cells did not label as strongly as did the other crotch cells in the pereiopod ganglia. Their rostral projections were followed to the caudal part of the subesophageal ganglion. Despite their lateral displacement in comparison to the crotch cells described in lobsters (Schneider et al., 1993), the projections of these cells in *M. quadrispina* suggest that they are homologues of the crotch cells in lobsters.

The final pair of OA-immunoreactive cell bodies in *M. quadrispina* are located ventrally in the eighth thoracic ganglion (Figs. 3.13, 3.14C & 3.15B). The fibers of these cells project rostrally and send branches into segmental nerve 2 of thoracic ganglia 5 through 8, where they form peripheral plexuses of fine fibers. Unidentified immunoreactive fibers enter segmental nerve 2 of thoracic ganglion 4 which may be branches of these cells, but I was unable to confirm this. These two cells appear to be responsible for most of the putative peripheral neurosecretory fibers in the thoracic ganglia.

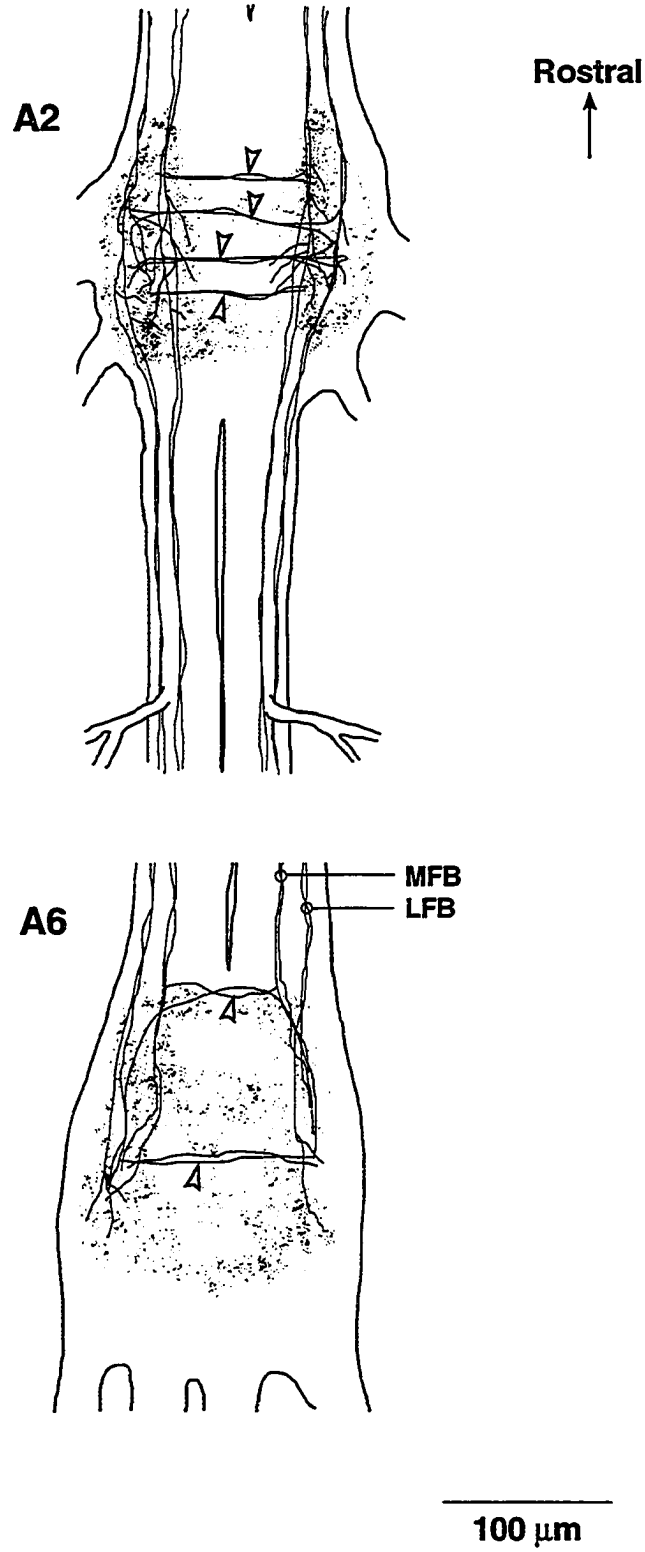
Rostro-caudally directed OA-immunoreactive fibers in the subesophageal and pereiopod ganglia are loosely organized into more lateral and more medial groups, but they are not as tightly associated as the bundles of serotonergic fibers. The octopaminergic fibers are much more dispersed both laterally and over the dorsal third or so of the ganglion. Furthermore, the degree of fiber dispersion is variable between animals, and in some animals the two groups are barely distinguishable. Therefore, the term *fiber bundle* is not really appropriate for OA-immunoreactive fibers in these ganglia, and simply describing a fiber's relative position as lateral or medial may be more accurate.

The abdominal ganglia

The are no OA-immunoreactive cell bodies in the abdomen of *M. quadrispina* (Fig. 3.16); this is similar to crayfish (Spörhase-Eichmann et al., 1998), but unlike American lobsters, in which abdominal ganglia 4 and 5 each contain a pair of immunoreactive cell bodies (Schneider et al., 1993). Eight descending interneurons, four on each side, contribute to the lateral neuropil of every abdominal ganglion in *M. quadrispina*. In the abdomen, these fibers are grouped into lateral and medial fiber bundles (2 fibers per bundle), but as in the rest of the central nervous system these fiber bundles are not in the same position as similarly named bundles of serotonergic fibers (see above) and are not associated with them. The pairs of fibers in the lateral fiber bundle were followed to cells in the third and fourth neuromere of the subesophageal ganglion (see above). The fibers in the medial fiber bundle were not traced to their cell bodies of origin, although in well-labeled nerve cords one of them could be traced to the brain and the other to the lateral region of the second neuromere of the subesophageal ganglion. However, since there are no immunoreactive cell bodies in the abdomen, these fibers, and the fibers in the lateral bundle, must be descending. Although I could follow some of the initial branches of these fibers in each abdominal ganglion, I was unable to determine if the individual fibers have different innervation fields.

As in the rest of the central nervous system, weakly OA-immunoreactive processes are distributed throughout each abdominal ganglion (Fig. 3.16). An obvious feature of labeled abdominal ganglia is the large number of contralateral branches of the descending fibers. In well-labeled examples of abdominal ganglia 1 through 5 contralateral branches of every descending fiber were seen, and in some ganglia two or three contralateral branches of a single fiber were identified. In all well-labeled ganglia, some additional decussating fibers passed between lateral neuropils but could not be traced to their fiber of origin. Although branches of each of the descending interneurons could be followed to the ipsilateral and contralateral lateral neuropil, the dense, weakly immunoreactive processes along the midline could not be associated with any particular descending fiber. In lobsters, the descending fibers from the third and fourth subesophageal neuromere contribute to the medial processes (Schneider et. al., 1993). In abdominal ganglion 6, usually only two or three pairs of contralaterally-projecting fibers were seen. The obvious swellings in abdominal ganglion 6 of the descending interneurons from the subesophageal ganglion described in lobsters (Schneider et. al.,

Fig. 3.16. Cameral lucida drawings of OA-immunoreactivity in the second and sixth abdominal ganglia. Immunoreactivity is basically identical in the second through fifth abdominal ganglia. The four descending fibers are arranged into medial (MFB) and lateral (LFB) fiber bundles. Note the many immunoreactive fibers crossing the midline (arrowheads).


A2
Rostral
A6
MFB
LFB
100 μm

1993) were not seen in *M. quadrispina*. No OA immunoreactive fibers were found entering any of the segmental nerves in the abdomen.

Discussion

Reproducible patterns of immunolabeling were obtained with antibodies directed against 5-HT and OA. The 5-HT antibody is commercially available and has been fully characterized. Specificity tests and controls indicate that it performed reliably under the conditions used in this study. Specificity of the OA antibody used in this study has been shown (Agricola et al., 1999), and tests using 5-HT or OA as blocking reagents indicated that it labeled structures with high specificity in *M. quadrispina* nerve cords. Additionally, in lobsters this antibody produced patterns of immunoreactivity similar to those produced by a polyclonal OA antibody whose specificity in lobsters has been characterized (Schneider et al., 1993 & 1996).

The very weak OA-like immunoreactivity of most of the fine dendritic processes and neuropil as compared to the larger fibers and somata is a matter of some concern. In American lobsters, the fine processes and neuropil are quite strongly immunoreactive (Schneider et al., 1993 & 1996). Consistently strong labeling of large fibers among the fine dendritic processes and of some fine fibers deep in the brain of *M. quadrispina*, and similar results in labeled thick sections of nerve cords indicate that neither insufficient penetration of reagents nor high autofluorescence obscuring the fine processes was responsible for the weak immunoreactivity. Pre-soaking the nerve cords in OA increased the strength of the label in the fine fibers, but not to the levels of the larger structures. Unfortunately, pre-soaking the nerve cords also resulted in labeling of somata and fibers that were never labeled in untreated nerve cords, and differentiating the fine processes was not possible. It is possible that concentrations of OA in most of the fine dendritic processes are normally low in *M. quadrispina*, but this seems unlikely. Another possibility is that the trauma associated with dissection causes massive release of OA. To investigate this possibility, I tried perfusing anaesthetized animals with the glutaraldehyde solution to fix the nerve cord in situ, and perfusing animals with low calcium, high magnesium saline. Perfusion with low calcium saline did not result in increased immunoreactivity, but the procedure used for perfusion of the animals is itself stressful, and therefore this result is not conclusive. Fixation in situ resulted in greatly increased background, which partially obscured the fine fibers. This observation could

be consistent with a massive dump of OA into tissues surrounding the fibers, if the fixation was quick enough to retain the released OA in the tissues, but not quick enough to prevent release. Another observation that is consistent with this possibility is that in nerve cords that were desheathed prior to fixation, immunoreactivity in the fine fibers was essentially eliminated. In order to test this hypothesis, a faster method of fixing the tissues must be developed. Why this should happen in *M. quadrispina* but not in American lobsters is not clear, but if OA is dumped as a result of the dissection procedure, this could be indicative of a natural physiological response to trauma.

Comparisons between the serotonergic and octopaminergic systems in M. quadrispina

Although the overall distributions of 5-HT- and OA-immunoreactivity in *M. quadrispina* are fairly similar, there are far fewer OA-immunoreactive cells. The large number of 5-HT-immunoreactive cell bodies in the rostral brain accounts for most of this difference. There are also far fewer segmentally repeated OA-immunoreactive cells than 5-HT-immunoreactive cells. The large caudal lateral 5-HT-immunoreactive cells are repeated in thoracic ganglia 4 through 7, and two pairs of small immunoreactive cells are present in abdominal ganglia 2 through 4, while a few of the OA-immunoreactive crotch cells have segmentally repeated branching patterns over no more than two ganglia. Segmentally repeated neurons provide a ready means to differentially control nervous output, but output of an aminergic neuron which projects over several ganglia could theoretically also be differentially controlled via presynaptic inputs at the terminals, or the actions of the amines could be differentiated by postsynaptic factors. For segmentally differentiated octopaminergic output in the abdomen of *M. quadrispina* to occur, such peripheral control systems would be required, as there are no OA-immunoreactive cell bodies located in the abdomen.

5-HT-immunoreactive fibers are present in every segmental nerve of the pereiopod ganglia, except for nerve 1 of the fifth pereiopod ganglion, and most of the nerves leaving the subesophageal ganglion. However, OA-immunoreactive fibers are present only in segmental nerve 2 of the pereiopod ganglia and the caudal three neuromeres of the subesophageal ganglion. This indicates that 5-HT could potentially be released directly onto most peripheral muscles, sensory neurons, and other tissues in the thorax and caudal head. OA has actions on peripheral systems in areas not innervated by the nerves which contain OA-immunoreactive fibers (Batelle & Kravitz, 1978; Kravitz et al., 1980; Pasztor & Bush, 1989; Pasztor & Macmillan, 1990), although OA could reach

these peripheral sites through systemic circulation. In American lobsters, concentrations of OA sufficient to activate most of the known peripheral targets are found in the systemic circulation (Livingstone et al., 1980). However, direct release onto peripheral targets allows for higher local concentrations while reducing the possibility of influences on non-target systems, and a greater level of control over the time course of peripheral actions. This suggests that serotonergic modulation could be targeted differentially to specific sites in the periphery, whereas OA acts as a systemic modulator on most peripheral OA-sensitive tissues.

Comparisons with other species

Organizational similarities exist in the aminergic systems of diverse crustacean taxa, but there are some important differences between *M. quadrispina* and other species that could have functional consequences. In general, *M. quadrispina* has fewer aminergic neurons than other decapods studied to date. Furthermore, the fusion of the pereiopod and first abdominal ganglia has resulted in changes in the organization of the ganglia which appear to be reflected in the morphology of some of the aminergic neurons, particularly in the more caudal ganglia, in which ganglionic cytoarchetecture has become rather distorted.

The serotonergic system

Comparisons between *M. quadrispina* and other decapod species reveal that many 5-HT-immunoreactive neurons are highly conserved among the decapods. The large immunoreactive neurons of the pereiopod ganglia are present in lobsters (Beltz & Kravitz, 1983), crayfish (Real & Czternasty, 1990), and crabs, although the caudal lateral cells of thoracic ganglion 6 may be missing in this group (Harzsch & Dawirs, 1995). These cells are also present in the primitive crustacean *Anaspides tasmaniae* (Harrison, MacMillan, & Young, 1995) and the large caudal lateral cells may be present in isopods, although in these animals the rostral projections of the cells are ipsilateral (Thompson et al., 1994). The large cells of the pereiopod and first abdominal ganglia in these diverse crustaceans are almost certainly homologous, and the remarkable conservation of morphology they exhibit implies functional conservation as well.

5-HT influences the lateral giant escape circuit in crayfish, and serotonergic nerve endings come into close apposition with the lateral giant neurons at several locations in the abdominal ganglia (Glanzman & Krasne, 1983; Yeh et al., 1996 & 1997). Although

the two serotonergic fibers (one per side) which give rise to these nerve endings have been traced into the thorax, the cell bodies of origin of these processes are unknown (Yeh et al., 1997). It is interesting that *M. quadrispina*, which has lost both sets of giant escape interneurons (Paul, 1989 & 1991; Wilson & Paul, 1987), is also missing the medial unpaired serotonergic neurons present in some abdominal ganglia in crayfish and lobsters (Beltz & Kravitz, 1983; Real & Czternasty, 1990). Of course, any attempt to associate these two elements is speculative at this point, but comparative observations such as these can point to potential places to look for physiological interactions.

An interesting segmental difference in the peripheral serotonergic system is the difference between the first segmental nerves of the first through fourth pereiopods and the fifth pereiopods. In crayfish, in which the fifth pereiopod is locomotory, 5-HT-immunoreactive fibers are in all of the first nerves, although the serotonergic fibers in the fifth pereiopod are branches of their thoracic ganglion 8 large rostral medial cells, whereas the fibers in the more rostral pereiopods are branches of the abdominal ganglion 1 large rostral medial cells (Real & Czternasty, 1990). The role of pereiopod 5 in crayfish walking is quite different from the roles of the other walking pereiopods (Jamon & Clarac, 1995). The different sources of the 5-HT-immunoreactive fibers indicate that the control of 5-HT release in the fifth pereiopods is different than in the first through fourth pereiopods, which in turn suggests the possibility of different roles for 5-HT in the fifth pereiopod. In *M. quadrispina*, branches of the first abdominal large rostral medial cell project into all of the first segmental nerves entering pereiopods 1 through 4, but no 5-HT immunoreactive fibers are present in nerve 1 of pereiopod 5. In all anomurans, the fifth pereiopods are drastically reduced in size and no longer contribute to locomotory behaviours (Schram, 1986). Loss of these fibers in the fifth pereiopods of *M. quadrispina* could be associated with the changed functional role of these limbs in anomurans.

The putative neurosecretory structures formed by the large caudal lateral cells in *M. quadrispina* have no anatomical equivalent in lobsters or crayfish. The only neurosecretory structure formed by any of these neurons described in any other species is in nerve 1 of thoracic ganglion 8 in crayfish, which is formed by the cells of thoracic ganglion 7 (Real & Czternasty, 1990). At this point, it is impossible to make even a reasonably informed guess as to the function of these unusual structures in *M. quadrispina*.

The octopaminergic system

Many of the OA-immunoreactive cells described in American lobsters (Schneider et al., 1993 & 1996) are not present in *M. quadrispina*. The loss of immunoreactive neurons at these locations could be explained by one of two phenomena: a change in pharmacological profile of the neurons, as has been shown to occur in crustaceans (e.g., see review in Katz & Tazaki, 1992), or a loss of the neurons themselves. Two possible reasons for the loss of segmentally repeated neurons are loss of function or consolidation of function into the remaining neurons. I suggest that both of these mechanisms may have occurred in the loss of OA-immunoreactive neurons in *M. quadrispina*.

In lobsters, a total of 28 segmentally repeated OA-immunoreactive crotch cells in the pereiopod ganglia and the caudal two neuromeres of the subesophageal ganglion contribute to the peripheral neurosecretory system in the second segmental nerves. At least two different neurons project into each nerve 2 except for the fifth pereiopod ganglion, which does not contain any immunoreactive fibers. In *M. quadrispina*, only 12 of these cells are present, although the morphologies of most of them are identical to their homologues in lobsters. In particular, there are no OA-immunoreactive crotch cells in the third and fourth pereiopod ganglia. However, a pair of caudal cells in the fifth pereiopod ganglion sends branches out the second segmental nerves of at least the next three rostral pereiopod ganglia. A similar pair of cells in the American lobster's fifth pereiopod ganglion sends processes to nerve 2 of pereiopod ganglion 4, but apparently to no further rostral ganglia (Schneider et al., 1993). The sources of peripheral OA neurosecretion in the second segmental nerves of pereiopod ganglia 3 and 4 have been changed from segmentally repeated neurons in the lobster to a single pair of cells in *M. quadrispina*, suggesting that consolidation of function has occurred. It is not known if the different neurons projecting into the second nerves of lobsters serve different functions; it is possible that some change in function has also occurred in association with the loss of segmentally repeated OA-immunoreactive neurons in *M. quadrispina*.

Of further note in the loss of OA-immunoreactive cells in the third and fourth pereiopod ganglia of *M. quadrispina* is that among these cells in American lobsters are the six claw octopamine cells (CLOCs) (Bräunig et al., 1994). Projections of these cells enter nerve 1 of the first pereiopod ganglia. American lobsters show subordinate behaviour if they lose one of their claws (Lang et al., 1977), and the potential roles of the CLOCs in this behaviour are being investigated by Kravitz and co-workers (Beltz, 1999). *M. quadrispina* do not become submissive after losing a claw (Antonsen & Paul,

unpublished observations); loss of these neurons could be associated with loss of this behaviour.

Summary

Despite overall similarity in the serotonergic and octopaminergic systems of decapods, several significant differences exist which are likely to have functional consequences. Very few functional roles have been assigned to any individual aminergic neurons. Comparative studies between species with different behaviours, anatomies, and neuromuscular circuits provide hints as to where to look for some of the interactions between aminergic neurons and other systems.

Chapter 4: Interesting sidelines

Injected Serotonin Inhibits Sand Crab Digging

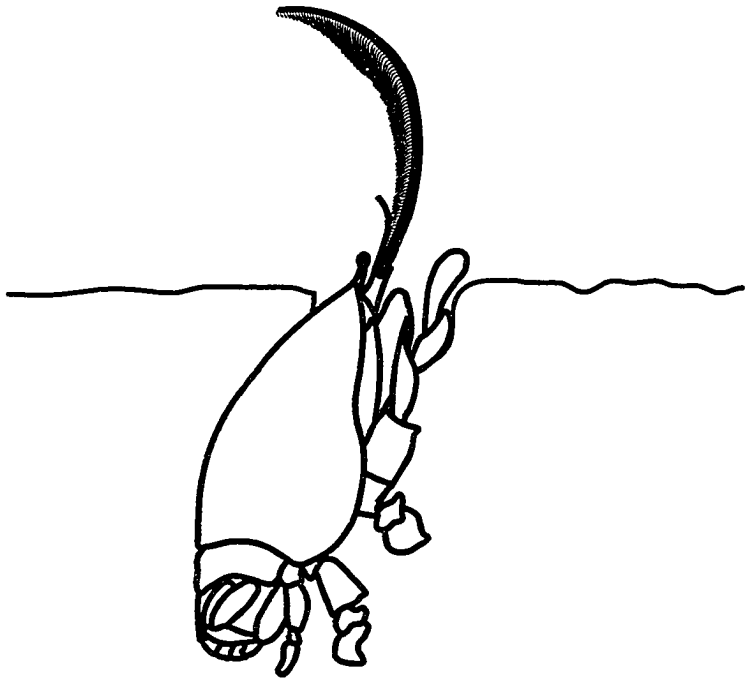
Serotonin (5-HT) and octopamine (OA) are involved in a wide range of behaviours in invertebrates. Some examples are feeding in leeches and nematodes (Horvitz et al., 1982; Lent & Dickenson, 1984), reproduction in bivalve molluscs (Fong et al., 1993; Fong et al., 1996), locomotion in nematodes, insects, leeches, crustaceans, and molluscs (Willard, 1981; Horvitz et al., 1982; Kinnamon et al., 1984; McPhee & Wilkens, 1989; Katz & Frost, 1995), mating behaviours in nematodes and crustaceans (Loer & Kenyon, 1993; Wood et al., 1995; Reinitz & Stretton, 1996), and agonistic behaviours in decapod crustaceans (see Chapter 2). 5-HT is also involved in locomotion, feeding, reproduction, and aggression in vertebrates (Schotland & Grillner, 1993; Sillar & Simmers, 1994; Olivier et al., 1995), although in some cases the effects on a particular behaviour are opposite to those in invertebrates. OA is present in vertebrates, but it is considered a trace amine and has not received much research attention (David & Coulon, 1985). Involvement of 5-HT and OA in similar behaviours in such distant taxa suggests that tracing behavioural (and cellular) effects of 5-HT and OA through evolution may provide crucial insights to their mechanisms of action.

The sand crab *Emerita analoga* digs into sandy beaches where it feeds, with its head and long antennae exposed in the surf (Fig. 4.1A), or submerges beneath the surface for protection from predators or from exposure in the intertidal zone. They can also swim by very rapid beating of the uropods (Paul, 1971), a behaviour which is homologous to the tailflipping of many decapods (Paul, 1981A). The first pair of pereiopods are paddle-shaped and used in swimming and digging behaviours, while the next three pairs are highly specialized digging pereiopods (pereiopods 2-4) (Faulkes & Paul, 1997B & C, 1998). *E. analoga* can occur in high densities, and have not been observed to be aggressive towards conspecifics in any way.

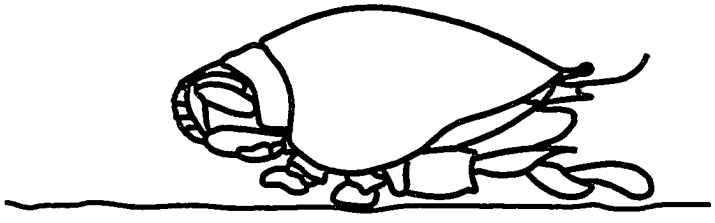
I injected doses of 5-HT and OA through the ventral surface of the caudal tip of the telson of freely moving, healthy *E. analoga*, taking care not to damage the musculature responsible for uropod beating. I chose the telson because the pericardial region proved too delicate and the ventral hemolymph sinus is much smaller than in *Munida quadrispina*, as well as being very difficult to penetrate due to the greatly reduced size of the abdomen relative to the thorax. However, hemolymph circulation through the telson is good, and tests with dye showed that substances injected at this site

Fig. 4.1. The mole sand crab *Emerita analoga* in its normal feeding and 5-HT-induced positions. (A) *E. analoga* feeds with its body buried in the sand and its head and long filtering antennae exposed to the water. They retract their antennae and dig deeper into the sand for protection from predators or from exposure during low tide. (B) Injected 5-HT inhibits digging and causes the animals to lie on the surface of the sand with their digging pereiopods tightly flexed.

A



B



are quickly distributed through the body. After injection, animals were released either above the sand in the water column, or were carefully buried in the sand in their natural position.

Injecting low doses of 5-HT into freely moving *E. analoga* inhibits digging, causing them to remain immobile at the surface of the sand with their digging pereiopods tightly flexed beneath them (Table 4.1, Fig. 4.1B). If the animals were buried into the sand after injection they would dig their way out of the sand and sit on the surface, most often almost immediately. Swimming is not fully inhibited by low doses of 5-HT, as the animals will swim when disturbed or occasionally spontaneously. However, it was difficult to determine whether the animals were more or less likely to swim as they normally sit at the surface of the sand only if they have been disturbed repeatedly, which does not make for an ideal control situation. The animals did not extend their antennae and feed while under the influence of injected 5-HT, but they normally only feed when partially buried in the sand, so this is not surprising and may not be a direct effect of the 5-HT injection. Increasing doses of 5-HT caused progressively tighter flexion of the digging pereiopods and reduced tendency to swim. Furthermore, the animals became increasingly rigid and immobile, and less likely to dig their way out of the sand if buried after injection (Table 4.1). No aggressive behaviours were elicited by any dose of 5-HT in *E. analoga*.

Injecting OA alone did not have any observable influence on the behaviour of *E. analoga*, except at very high doses where toxic effects were observed such as violent shaking, autotomising limbs, and death (Table 4.1). Injecting OA together with 5-HT, however, inhibited the effects of 5-HT, resulting in the animals burrowing normally. In order for the effects of 5-HT to be fully inhibited, the dose of OA had to be slightly higher than the dose of 5-HT (Table 4.1). Animals injected with both amines did not feed for some time after being returned to the tank, but neither did animals injected with control doses of saline or animals disturbed by handling, although they dig normally. It is therefore likely that this may be, at least in part, an effect of the disturbance caused the animal by the injection, and a less disruptive means of introducing the amines would have to be employed in order to study any possible effect of amines on feeding behaviour.

Similar behaviours have been induced by injected 5-HT and OA in the amphipod *Gammarus lacustris* (Helluy & Holmes, 1990), in which injected 5-HT causes the amphipods to swim to the surface of the water and cling to debris. Clinging is

Table 4.1. The effects of injected 5-HT and OA on *Emerita analoga*. The dose-response curve was not completely consistent between individuals; the data presented here are the most common responses to any given dose. Doses are given in mg amine per gram body weight; nc means no observable effect; -Digging, the animals' digging was reduced from normal, but it did make digging movements which sometimes resulted in partial burrowing; --Digging, the animals' digging behaviours were totally inhibited; stiff: the animals did not move at all for some time after the injection; shaky: the animals' limbs shook synchronously and rapidly.

Table 4.1

5-HT Dose	OA Dose (mg/g)				
(mg/g)	0	0.0005	0.001	0.002	0.005
0	nc	nc	nc	nc	shaky/death
0.0005	-digging	-digging	nc	nc	shaky/death
0.001	--digging	-digging	-digging	nc	shaky/death
0.002	--digging	--digging	--digging	-digging	shaky/stiff
0.005	stiff	stiff	stiff	stiff	shaky/stiff

accomplished by postural flexion. Injected OA by itself elicits no response, but when injected together with 5-HT it inhibits the swimming and clinging effects. Serotonin has been shown to decrease photonegative behaviour in a crab (McPhee & Wilkens, 1989), which may also be the cause of the upward movements of *E. analoga* and *G. lacustris*, but effects on statolithic systems cannot be ruled out.

An acanthocephalan parasite of the amphipod causes effects similar to injected 5-HT, making the amphipod easy prey for ducks, the parasite's definitive host. Helluy & Holmes (1990) hypothesized that the parasite may co-opt the serotonergic system of the amphipod to induce it to swim to the surface. *E analoga* also hosts a parasite which causes it to remain on the surface of the sand (K. Rafferty, 1994, personal communication), but the biology of this parasite is not known and there is no direct evidence in either of these crustaceans that the parasites are influencing the serotonergic systems in any way.

These data suggest that the influences of 5-HT and OA on crustacean behaviour have changed with species' habits. The simplest explanation for what occurs when 5-HT or OA is injected into *E. analoga* is that this species has lost aggressive behaviours as part of its normal repertoire, having no apparent need for them and little means to carry them out. Therefore, other behaviours on which 5-HT has an influence may be more noticeable than in species exhibiting agonistic interactions. Other than the few described here, no studies of the behavioural effects of injected 5-HT and OA other than on agonistic behaviours have been done in crustaceans, perhaps because changes in agonistic behaviours mask other changes in the behaviour of the aggressive crustacean species studied.

Initial Attempts to Record Depressor Muscle Electromyograms

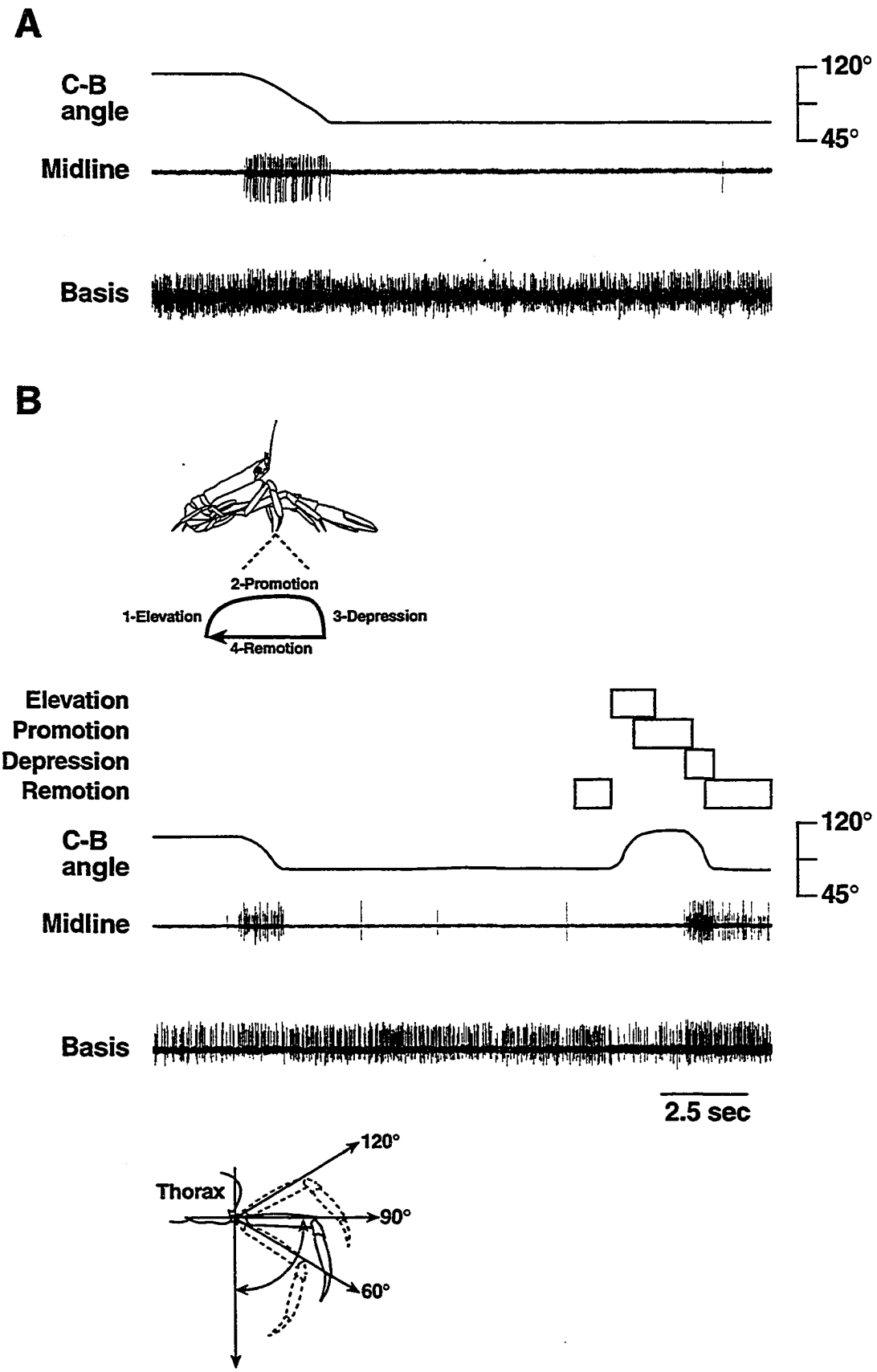
When I began this part of the study, I was operating under the assumption that all heads of the depressor muscle were activated more or less synchronously during all behaviours. The anatomy of the depressor muscle had been described in a few species, and it was known to contain multiple heads, but the vast majority of literature dealing with the physiology of the depressor muscle ignored this point. Furthermore, it was known that there were at least two, and possibly more, functional parts to the levator muscle of crabs (Moffett, 1975; McVean & Findlay, 1976), with roles in posture, locomotion, and pereiopod autotomy, and with largely separate innervation (Moffett et al., 1987). Just as I was beginning this study, one report was published which described

differential recruitment of depressor muscle motoneurons during postural changes and walking in crayfish (Pearlstein et al., 1995).

I implanted EMG electrodes in two positions initially (see Chapter 6 for details on methods); medial leads along the midline of the sternum in what I would later come to call the sternal head, and distal leads along the dorsal or ventral surfaces of the base of the depressor tendon, which laid alongside the depressor muscle. These distal leads recorded from what I now know could have been any combination of the six heads of the depressor muscle (see Chapter 5). EMGs recorded from these two positions were often not similar (Fig. 4.2). The leads along the midline of the sternum would give fairly consistent results: the depressor was active when the pereiopod was moving, and was not when the pereiopod was still. The leads implanted near the base of the depressor tendon, however, produced completely inconsistent records. Dissections confirmed that the leads were among the heads of the depressor muscle, but they often recorded continuous EMG activity in the depressor muscle (Fig. 4.2), and the traces were not consistently patterned in any way I could discern. Additionally, in some preparations leads in these positions recorded activity with clear patterns related to movements of the animal (e.g. Fig. 4.2B), but only rarely were these results similar to those recorded by the midline leads, and again these results could not be reliably reproduced between animals.

My initial explanation for these results was that the leads implanted at the base of the tendon had been picking up neuronal activity from the distal leg nerves, which are not far from where these electrodes were implanted. This was not an entirely satisfactory explanation, as when the depressor was active, as indicated by activity recorded by the medial leads, I did not see much larger depressor EMG activity drowning out the background distal leg nerve activity, as you might expect. This, together with analysis of the first EMG traces from animals injected with 5-HT or OA cast doubt on my initial hypothesis. Injected 5-HT slightly increased EMG activity recorded by the sternum leads when the joint was not moving as compared to activity in the same animals prior to injection. However, the leads near the base of the depressor tendon recorded a substantial increase in EMG activity, in a manner that appeared to be dose-dependent. Injected OA did little to depressor muscle EMG activity near the midline, but it decreased EMG activity recorded by the distal leads, again in what appeared to be a dose-dependent manner. I have since learned that although it acceptable in larger crustaceans to implant EMG electrodes alongside a muscle without penetrating it, this does not often work in *M. quadrispina*. The small size of these animals results in potentials from other muscles and

Fig. 4.2. Electromyograms from some initial attempts to study depressor muscle activity in freely moving *M. quadrispina*. Leads were implanted near the midline of the sternum in the origin of a large depressor muscle head and at the base of the depressor apodeme near the ventral lip of the basis. (A) Recordings from both sets of leads aligned with a plot of the coxal-basal joint angle (C-B angle). The midline leads recorded very little activity when the coxal-basal joint was still, but high activity when the pereiopod was being depressed. The leads at the base of the depressor apodeme recorded high activity continuously. Activity was slightly higher when the pereiopod was being depressed (95 Hz vs. 91 Hz when the pereiopod was still). (B) Traces from a different preparation showing a pereiopod depression (stance change) and, about 10 seconds later, one forward walking cycle, aligned with step component durations and coxal basal joint angles. As in A, the leads implanted medially recorded activity when the pereiopod was depressing but very little activity when the coxal-basal joint was still. The leads implanted at the base of the depressor apodeme recorded continuous activity, but the pattern was slightly different than in A. EMG spike frequency before the stance change (36 Hz) was significantly lower than during (51 Hz), and lower still than after (64 Hz) the stance change. In this animal, a pattern of EMG activity could be seen during walking: activity was lower while the pereiopod was being elevated and promoted, and stronger while the pereiopod was being depressed and remoted. This pattern was not consistent between animals with leads implanted in this way, however.


A
C-B angle
120°
45°
Midline
Basis
B
2-Promotion
1-Elevation
3-Depression
4-Remotion
Elevation
Promotion
Depression
Remotion
C-B angle
120°
45°
Midline
Basis
2.5 sec
120°
Thorax
90°
60°

nerves being picked up if the electrodes are not fully embedded in the target muscle, making for some rather confusing EMGs which no doubt added to my initial problems.

Up until this point I had not been particularly interested in describing the detailed anatomy of the depressor muscle. I was aware of its multiple muscle bundles from my initial dissections looking for places to implant electrodes, but my primary interest was in finding out how it was controlled, not describing its anatomy. I had not carefully mapped out the positions of the heads, assuming from the literature that the depressor muscle was all one functional unit. Dr. Paul and I began to believe that this may have been a mistake, so I went back and did the detailed descriptions of the anatomy of the depressor muscle, then I implanted leads in each of the heads and recorded from them independently. The results of these experiments are described in the next two chapters.

Chapter 5: The depressor muscle of Munida quadrispina: multiple heads with disparate innervation[1]

Introduction

The complex series of muscles controlling the six joints of the pereiopod in various species of decapods have been studied for more than a century (Atwood, 1977). The relatively small number of motoneurons controlling these muscles, and the ability, as in many invertebrates, to isolate single motor, command, or sensory neurons for detailed study (Weins and Atwood, 1978; Nagayama et al., 1983; Moore & Larimer, 1987; Chrachri & Clarac, 1989; Cattaert et al., 1995; Bévengut et al., 1996) have provided insights to the control of neuromuscular systems in general.

Most of the work on pereiopod neuromusculature in decapods has focused on the muscles distal to the autotomy plane (Wiersma & Ripley, 1952; Atwood & Walcott, 1965; Govind & Lang, 1981; Silvey, 1981; Parsons, 1982; Wiens, 1990; Faulkes and Paul, 1997A). The muscles controlling the two proximal leg joints, the levator, depressor, promotor, and remotor muscles, have not received nearly as much attention, although these joints are functionally very important in postural and locomotory behaviours (Evoy & Ayers, 1982; Kelly & Chapple, 1990; Müller & Cruse, 1991; Jamon & Clarac, 1995). Furthermore, although the anatomy of the proximal pereiopod muscles has been described in detail for only a few decapod species (Schmidt, 1915; White & Spirito, 1973; McVean & Findlay, 1976; Ayers & Clarac, 1978; Bévengut et al., 1983), it is clear that the proximal muscles are anatomically much more complex than the distal muscles, and are often comprised of multiple heads (or fiber bundles) with widely separated origins and, sometimes, separate insertions.

Descriptions of the proximal muscles' activity (Ayers & Davis, 1977; Ayers & Clarac, 1978; Chasserat & Clarac, 1983; Klärner & Barnes, 1986; Clarac et al., 1987; Bévengut & Clarac, 1990; Faulkes and Paul, 1997B & C, 1998), and the physiology of their motoneurons (Sillar et al., 1986; Heitler & Fraser, 1989; Head & Bush, 1992; Skorupski et al., 1992; Pearlstein et al, 1995, 1998A & B) have mostly not dealt with the possibility of functional subdivisions within the muscle. The levator muscle of crabs, in which the heads of the rostral and caudal levators have different roles in locomotion and limb autotomy, is the only proximal pereiopod muscle in which functional divisions

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[1] Parts of this chapter have been published in abstract form (Antonsen and Paul, 1995 & 1997)

between the heads of a proximal muscle have been studied in detail (McVean, 1973; Moffett, 1975; Moffett et al., 1987). Otherwise, data on functional adaptation of the proximal limb muscles and comparative analyses between species are sparse.

I undertook this study with two goals. First, to add to the few descriptions of decapod depressor neuromuscular anatomy by providing a description of the anatomy and innervation patterns of the different heads of *M. quadrispina*'s depressor muscle, with comparison to anatomy of *M. quadrispina*'s levator muscle and the depressor and levator muscles of the crayfish *Cambarus bartonii*. Second, to provide an anatomical base for studying potential functional differences revealed by electromyograms (EMGs) from the different depressor muscle heads, and differential effects of serotonin and octopamine on postural mechanisms (Chapter 6).

Methods

Munida quadrispina were collected and kept as described in Chapter 2. They were fed regularly on a diet of mixed seafood, with an emphasis on crustaceans, as I found that too much fish resulted in a decrease in pigmentation, which would in turn make the internal anatomy more difficult to resolve. *Cambarus bartonii* were obtained from a commercial supplier, kept in flow through fresh water aquaria, and fed a diet of mixed fish. All animals were debrained by cutting the ventral nerve cord just caudal to the brain, and the carapace, viscera, and abdomen were then removed.

Observations on muscular and peripheral nervous system anatomy were made on fresh specimens, and specimens preserved in formalin or 70% ethanol. Peripheral nervous tissue in fresh specimens was stained by adding reduced methylene blue (Baker, 1958) to physiological saline to a final concentration of about 0.5%. The tissue was kept at 5°C until well stained. For sectioning, depressor and levator muscle heads from *M. quadrispina* of 1.3-1.4 cm carapace length were fixed *in situ* with the coxal-basal joint at its normal resting angle in 2% glutaraldehyde for 2 hours at room temperature. Fixed tissues were rinsed, embedded in Epon and 0.25μm cross and longitudinal sections were cut and stained with Richardson's stain. Pictures were taken with a Leitz Aristoplan microscope and digitized using a Polaroid film scanner.

Nerves leading to the depressor muscle heads were backfilled with cobalt chloride (Pitman et al., 1972) and silver intensified (Bacon & Altman, 1977), or with Neurobiotin (Vector Labs) reacted with avidin-horseradish peroxidase (HRP). Nerve branches were

carefully isolated, leaving muscle heads and small pieces of supportive connective tissue attached. The target head was placed in a petroleum jelly well containing either 0.3 M cobalt chloride or 0.5% Neurobiotin in 1 M sodium acetate, and the fine nerves leading to the muscle head were then cleaned of connective tissue to ensure a good seal with the petroleum jelly and cut, leaving the freshly cut ends in the filling solution. The well was sealed and the preparation was left to backfill for 12-20 hours at 5°C.

Intracellular cobalt was precipitated by adding a few drops of ammonium sulfide to 4-5 ml of saline, and the preparations were then fixed for 2-20 hours in 10% formalin in physiological saline. I then cleaned the nerves and ganglia of any extra tissue, taking care to leave small bits of the depressor muscle heads attached to their nerves for reference, dehydrated the tissue through an ethanol series, and cleared them in methyl salicylate. Neurobiotin backfills were fixed in freshly made 4% paraformaldehyde in 0.1 M phosphate buffer, pH 6.5, then washed in several changes of phosphate-buffered saline (PBS) with 0.4% Triton-X 100 (Sigma). Two or three washes in PBS were done to remove the Triton-X 100 before incubating the ganglia in 3% hydrogen peroxide for 10 minutes to eliminate endogenous peroxidase activity, and rinsing them again in several changes of PBS. The nerves were then cleaned of excess bits of tissue, again taking care to leave small bits of the depressor muscle heads attached, and incubated for 12-40 hours at 4° C in 10 ng/ml avidin-HRP complex in PBS. Following several more rinses in PBS, the HRP was reacted with diaminobenzidine (DAB) and hydrogen peroxide to produce a brown precipitate, and the preparations were dehydrated, cleared and mounted in methyl salicylate. Photographs were taken and drawings were made using a camera lucida attached to a Leitz Aristoplan microscope.

Results

Pereiopod 2 proximal joint mechanics

The ability of decapod pereiopods to move freely through all planes has allowed for adaptation for roles in a variety of behaviours, such as feeding, swimming, walking, and burrowing. In vertebrates, this sort of freedom of movement can be accomplished across a single ball and socket type joint. With a few exceptions, such as the multidirectional coxal-basal joint in some isopods (Lochhead, 1961) and the unicondylar uropod joint of hippids which Paul (1981B) called the crustacean equivalent to a ball and socket joint, the rigid crustacean exoskeleton is not suited to this sort of joint. In

decapods, a typical pereiopod joint is an uni- or bicondylar structure between two segments joined by flexible arthrodial membrane. Full mobility of the pereiopod as a whole is provided by six joints, each restricted to movement in more or less a single plane but oriented at approximately right angles to the adjacent joints. Angles of joint articulation and variable degrees of flexibility of the joints themselves combine to provide the freedom of movement of the pereiopod as a whole (Young, 1959; Hessler, 1982; McLaughlin, 1986).

The two proximal pereiopod joints of *M. quadrispina*, the thoracico-coxal and coxal-basal joints, are bicondylar. The condyles of the coxal-basal joint are very rigid, strictly limiting movement to a single plane. The dorsal condyle of the thoracico-coxal joint is also very rigid, but the ventral condyle is quite loose, allowing a small degree of rotation about the dorsal condyle. The maximum rotation allowed by the joint is approximately 5°, but the maximum rotation I observed an animal make was approximately 0.5°. The potential advantages of a rotating joint in terms of freedom of movement are clear, assuming the animal possesses appropriate neuromuscular control mechanisms, but with this may come a decrease in limb rigidity which could be detrimental. It is not clear to what, if any, extent *M. quadrispina* makes use of this ability in locomotion or any behaviour.

In the simplest theoretical case, the articulation plane of the thoracico-coxal joint is parallel to the dorso-ventral axis of the animal, allowing promotion and remotion of the pereiopod along the rostro-caudal axis. The coxal-basal articulation plane is parallel to the rostro-caudal axis, allowing elevation and depression of the pereiopod along the dorso-ventral axis. However, in *M. quadrispina* the articulation planes of the proximal joints of all three walking pereiopods are rotated, so that the dorsal condyle of the thoracico-coxal joint is more rostral than the ventral condyle and the rostral condyle of the coxal-basal joint is more ventral than the caudal condyle (Fig. 5.1A). For pereiopod 2, which is the focus of this study, the resulting tilt to the thoracico-coxal articulation plane is 10° from the dorso-ventral axis of the animal, and the coxal-basal articulation plane is tilted 21° from the dorso-ventral axis of the animal (Table 5.1). During most behaviours, *M. quadrispina*'s thorax is tilted about 22° from the horizontal, with the head up (Fig. 5.1B). Therefore, the elevation-depression plane of pereiopod 2 is almost perpendicular to the substrate (Fig. 5.1B). Promotion of the pereiopod also causes a depression relative to the animal, but an elevation relative to the substrate, and vice versa for remotion of the pereiopod (Fig. 5.1B). The movements produced by pereiopod 3 are

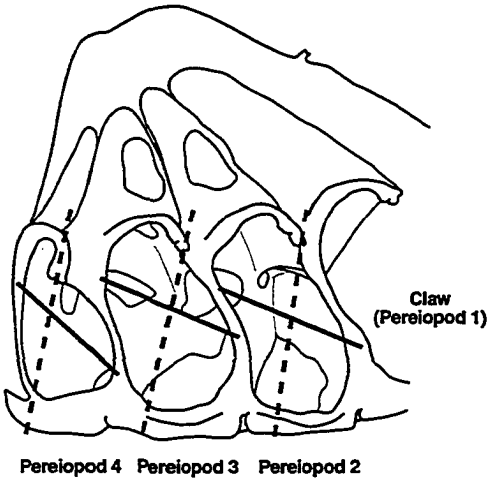
Table 5.1. Planes of articulation and movement ranges of the two proximal walking pereiopod joints. The angles of the thoracico-coxal (T-C) articulation are measured from the dorso-ventral axis of the thorax; the planes of the coxal-basal (C-B) articulation are measured from the rostro-caudal axis (see Fig. 5.1A). Joint ranges are given as minimum and maximum angles allowed by the joint, with angular excursion in brackets. The numbers in bold are the maximum joint angles recorded from freely moving animals. Thoracico-coxal joint angles are measured from the rostro-caudal axis; coxal-basal joint angles are measured from the dorso-ventral axis (see Fig. 5.1C).

Table 5.1

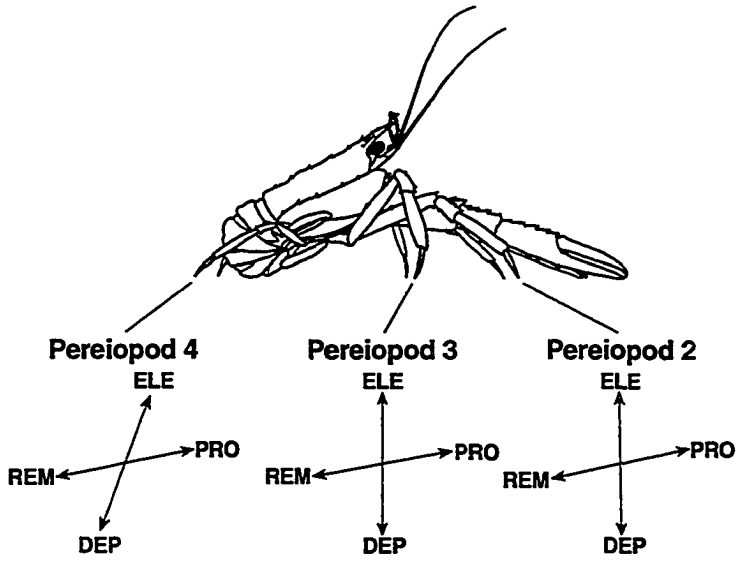
Pereiopod Number	T-C articulation angle	T-C joint range	C-B articulation angle	C-B joint range
2	10°	23°-120° (97°)	21°	30°-145° (115°)
		27°-115° (88°)		**44°-122° (88°)**
3	12°	24°-121° (97°)	22°	35°-135° (100°)
		30°-117° (87°)		**51°-120° (69°)**
4	11°	20°-145° (125°)	43°	25°-140° (115°)
		22°-139° (117°)		**33°-138° (105°)**

Fig. 5.1. The two proximal joints of the walking pereiopods of *M. quadrispina*. (A) Side view of a thoracic skeleton with soft tissues removed. The thoracico-coxal articulation planes are indicated by the heavy dashed lines; the coxal-basal articulation planes are indicated by the heavy solid lines. (B) *M. quadrispina* in its normal resting position, with directions of proximal joint movements relative to the substrate. (C) The measurement systems used to determine thoracico-coxal (T-C) and coxal-basal (C-B) joint angles. Numeric values for the articulation angles and joint movement range angles are given in Table 5.1. C-B, Coxal-basal; DEP, limb depression; ELE, limb elevation; PRO, limb promotion; REM, limb remotion; T-C, thoracico-coxal.

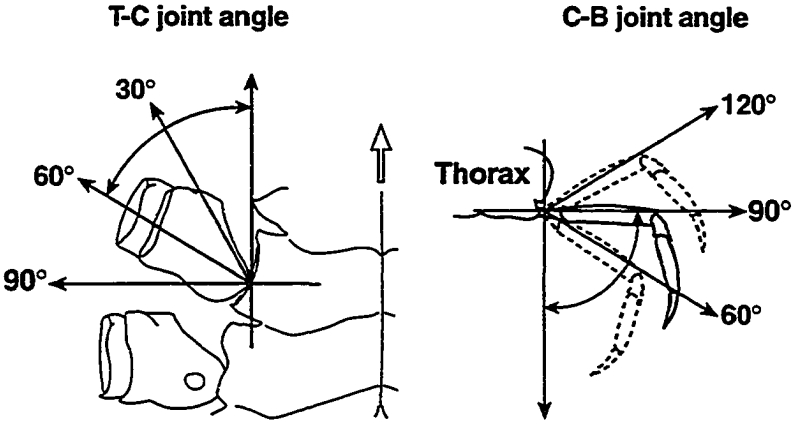
A


Claw
(Pereiopod 1)
Pereiopod 4
Pereiopod 3
Pereiopod 2

B


Pereiopod 4
ELE
REM
PRO
DEP
Pereiopod 3
ELE
REM
PRO
DEP
Pereiopod 2
ELE
REM
PRO
DEP

C


T-C joint angle
C-B joint angle
30°
60°
90°
Thorax
120°
90°
60°

almost identical to pereiopod 2, but the coxal-basal joint of pereiopod 4 is considerably more rotated, resulting in a substantial caudal motion during pereiopod 4 depressions. In *C. bartonii*, the thorax is normally held more or less parallel to the substrate and, not surprisingly, the condyles of the thoracico-coxal joint are oriented very close to the dorso-ventral axis of the animal, while the condyles of the coxal-basal joint are almost perfectly in the rostro-caudal plane.

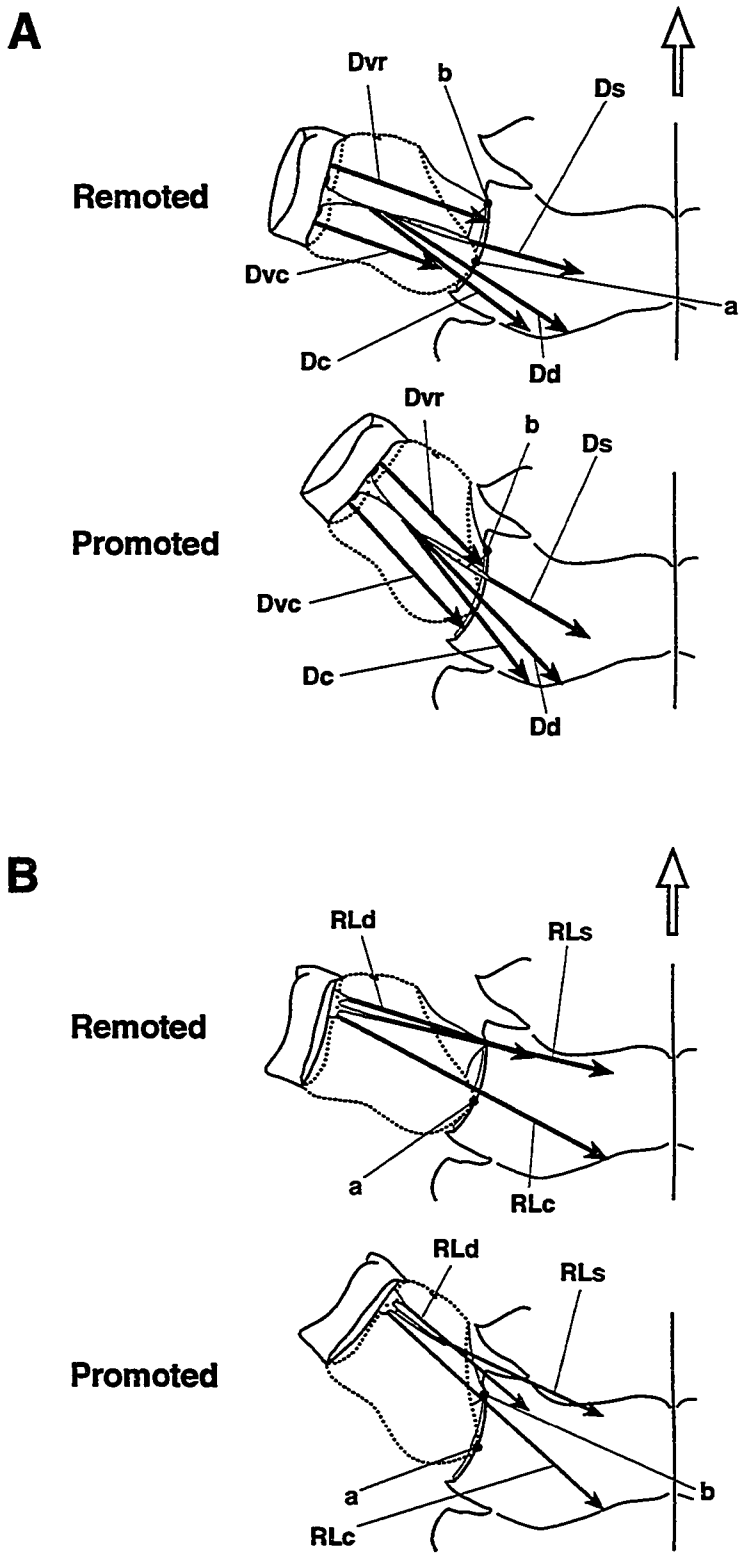
The observed proximal joint excursions in freely moving *M. quadrispina* were slightly lower than the maximum possible movements allowed by the joints for both of the proximal joints in all three walking pereiopods (Table 5.1). During normal walking and posturing on flat substrates, pereiopod 2's coxal-basal joint angles ranged between 44 and 122 degrees; coxal-basal joint angles greater or less than these were only observed on uneven substrates, or in animals under the influence of injected serotonin or octopamine (see Chapter 6).

Although the rigid coxal-basal joint restricts movement to one plane, some of the heads of the depressor and levator muscles are biarticular, crossing the thoracico-coxal joint as well. The origins of these heads are widely arrayed in the thorax and could potentially contribute to movements across the thoracico-coxal joint as well (Fig. 5.2) (see below for anatomical descriptions of these heads). From their positions relative to the thoracico-coxal articulation plane, the sternal and rostral-ventral heads of the depressor muscle and the sternal and dorsal heads of the rostral levator muscle could contribute to pereiopod promotion. The dorsal, caudal, and ventral-caudal heads of the depressor muscle and the caudal head of the rostral levator muscle could contribute to pereiopod remotion. However, in order to study any effects the basal muscles may have on thoracico-coxal joint movements, one would have to determine forces exerted by each of the heads across the thoracico-coxal joint either during natural behaviours or via artificial stimulation. This is a substantial undertaking and is beyond the scope of this study.

Anatomy of the pereiopod depressor muscle

I chose to study the pereiopod 2 depressor and levator muscles in detail mainly due to their larger size and accessibility, particularly in *M. quadrispina*. The proximal musculature of the other walking pereiopods is similar, although reduction or loss of parts of the endophragmal skeleton in the caudal somites necessitates some changes in the organization of the muscle heads within the thorax.

Fig. 5.2. Mechanical action of the heads of the depressor and levator muscles across the thoracico-coxal joint, viewed from ventral. (A) Depressor muscle, which inserts on the ventral lip of the basis. (B) Levator muscle, which inserts on the dorsal lip of the basis. The ventral and dorsal thoracico-coxal joint condyles are indicated by a and b respectively; compare with Fig. 5.1A for their relative positions in the dorso-ventral axis, and see below for the relative positions of the muscle heads in this axis. The solid arrows indicate force vectors of each of the muscle heads; the open arrow denotes rostral; Dc the caudal head of the depressor muscle; Dd, the dorsal head of the depressor muscle; Ds, the sternal head of the depressor muscle; Dvc the ventral caudal head of the depressor muscle; Dvr the ventral rostral head of the depressor muscle; RLc, the caudal head of the rostral levator muscle; RLd, the dorsal head of the rostral levator muscle; RLs, the sternal head of the rostral levator muscle.


A
Remoted
Dvr
b
Ds
Dvc
a
Dc
Dd
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B
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RLd
RLs
a
RLc
Promoted
RLd
RLs
a
b
RLc

The pereiopod 2 depressor muscle of *M. quadrispina* is composed of six distinct heads with widely distributed origins, but which insert on one apodeme attaching to the ventral lip of the basis (Fig. 5.3). With the exception of the dorsal and coxal heads, which fuse shortly before their insertion points, all of the heads are completely separate along their entire length. All the heads are closely apposed near their insertions along the complex depressor apodeme, but not even loose connective tissue binds them together.

The sternal head is by far the largest of the depressor heads, and is the largest head of all the proximal pereiopod muscles in *M. quadrispina*. It originates on small ridges along the medial and caudal edges of the pereiopod 2 sternite, extends laterally through the gap between the arthrophragms and sternite, and inserts on the lateral and ventral faces of the proximal end of the depressor apodeme (Figs 5.3B & C, 5.4). The origin of the sternal head does not extend to the medio-caudal corner of the sternite; it curves around leaving a small pocket in the corner (Fig. 5.3C). The caudal head originates on the ventero-medial part of the caudal arthrophragm, and passes laterally and slightly ventrally to insert on the dorsal ridge of the proximal end of the depressor apodeme, immediately dorsal to the insertion of the sternal head (Figs 5.3B, 5.4). The dorsal head originates on the caudal endopleurite, and the coxal head originates on the dorsal and caudal faces of the coxa (Fig. 5.3A, B). These heads pass ventrally and fuse shortly before their insertion on the dorsal ridge of the distal end and the dorsal projection of the depressor apodeme (Figs 5.3B, 5.4). By teasing the fibers of these two heads apart, I could determine that the insertion of the dorsal head is confined to the dorsal projection of the apodeme, while the coxal head inserted mostly on the dorsal ridge on the proximal part, but also has a few fibers inserting on the dorsal projection. The two very thin ventral heads (rostral and caudal) originate on the lip of the sternite, at the edge of the arthrodial cavity, and insert on the broad part of the depressor apodeme very close to the ventral lip of the basis (Figs 5.3C, 5.4).

The depressor apodeme of *M. quadrispina* is a complex structure which is able to translate forces from the flared depressor heads into an effective pull on the ventral lip of the basis, causing depression of the pereiopod (Fig. 5.4). Most of the heads pull more or less straight on the apodeme, but the coxal and dorsal heads pull dorsally, at almost a right angle to the longitudinal axis of the apodeme. The apodeme accommodates this with a thin bendable point just proximal to the dorsal projection, such that when the coxal and dorsal heads contract, the apodeme kinks dorsally. This relieves potentially damaging lateral force on the apodeme that would develop, were the apodeme rigid, and

Fig. 5.3. The six heads of *M. quadrispina*'s depressor muscle. Rostral is indicated by the open arrows; the fine dashed lines represent holes cut in the skeleton. (A) View from the right side, and slightly dorsal. (B) Transverse view of the right side looking caudally. The heavy dashed line represents the midline. (C) Ventral view; the insertion of the sternal head on the proximal part of the depressor muscle apodeme is just internal to the two ventral heads. Dc, caudal head; Dcx, coxal head; Dd, dorsal head; Ds, sternal head; Dvc, ventral-caudal head; Dvr, ventral-rostral head; *, the small pocket in the medio-caudal corner of the sternal head origin.

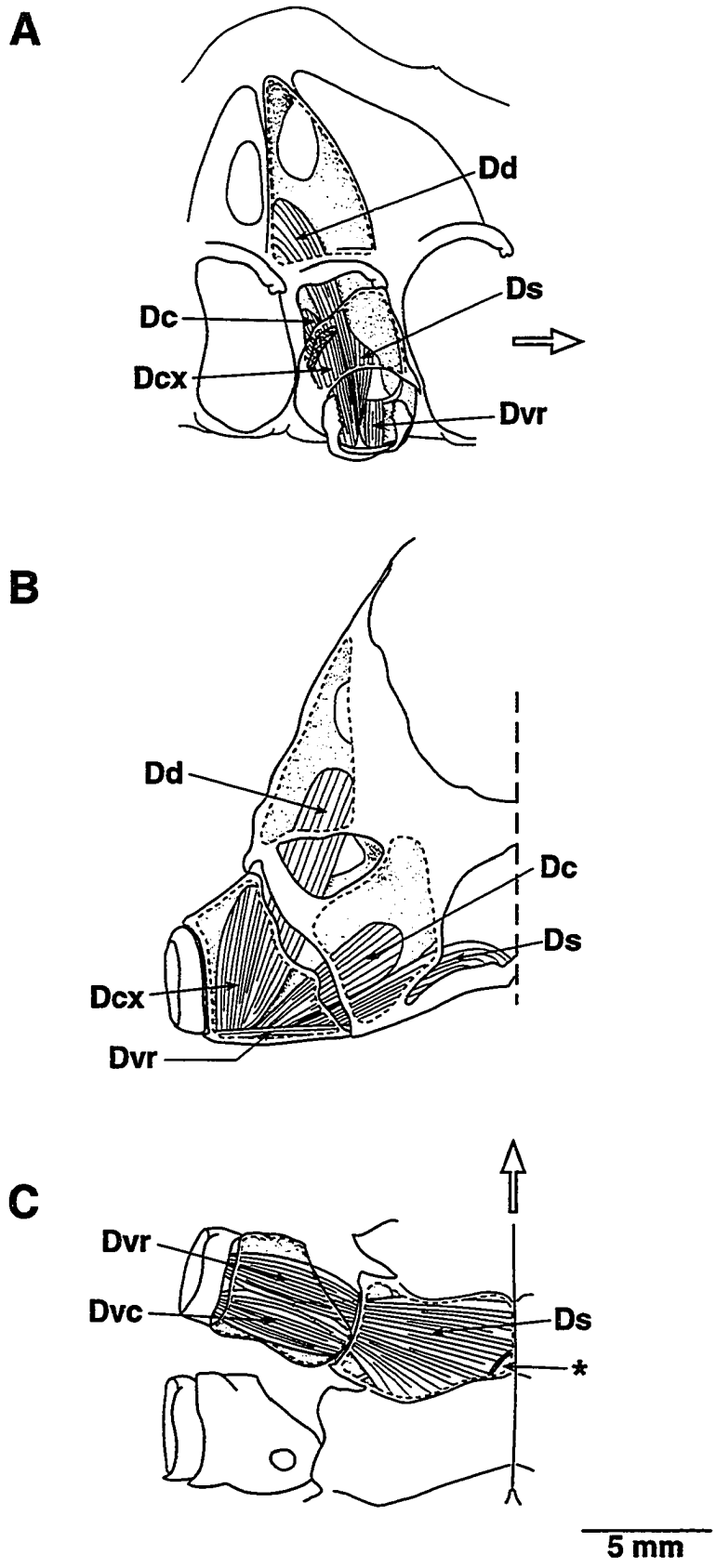
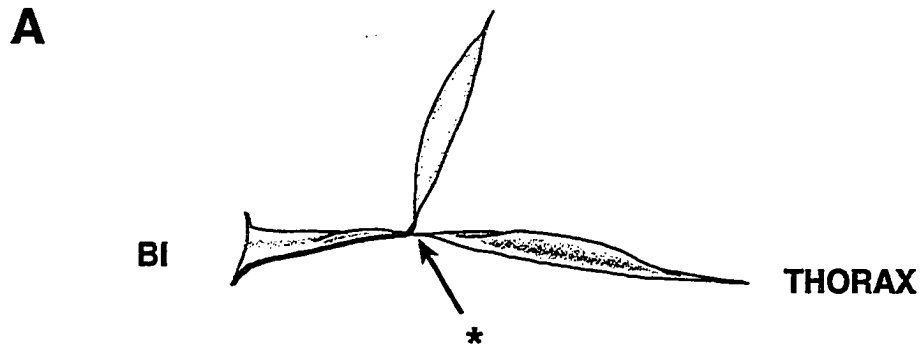
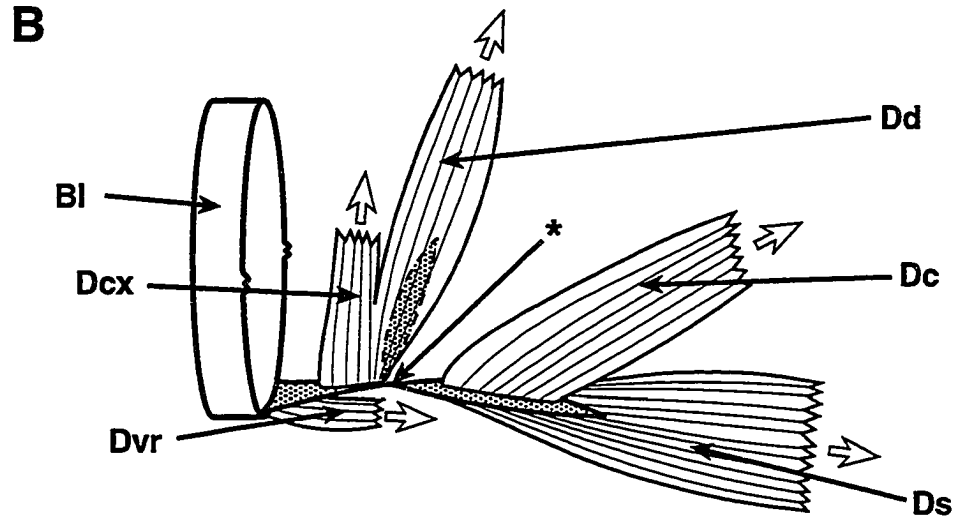

A
Dd
Ds
Dc
Dcx
Dvr
B
Dd
Dc
Ds
Dcx
Dvr
C
Dvr
Dvc
Ds
*
5 mm

Fig. 5.4. The depressor muscle apodeme in *M. quadrispina*. (A) A cleaned apodeme showing the thin bend point (*) on the thorax (proximal) side of the point where the dorsal projection diverges. Note the broad, thin distal end that attaches to the ventral lip of the basis (BI). (B) Diagrammatic rostral view showing directions muscles pull on the apodeme (arrows) (Compare with Fig. 5.3B). The dorsal head (Dd) and coxal head (Dcx) pull up, causing bending of the apodeme at the thinnest part. Dc, caudal head; Ds, sternal head; Dvr, ventral-rostral head.


A
Bl
THORAX
*


B
Dd
Bl
*
Dcx
Dc
Dvr
Ds

also frees these two heads from having to work to lift the large sternal and caudal heads. As the dorsal and coxal heads contract, tension is kept on the proximal part of the apodeme by the caudal head (see Chapter 6), preventing the proximal end from moving and resulting in force applied to the ventral lip of the basis. Although there are multiple heads pulling on the depressor apodeme from different directions, the rigid bicondylar articulation of the coxal-basal joint allows movement in one plane only. Therefore, the different heads cannot produce different directions of movement across this joint. However, all but the coxal head also cross the thoracico-coxal joint, which could potentially allow the heads of the depressor muscle (and the levator-see below) to have active roles in leg promotion and remotion (see Chapter 6).

Cochran (1935) described the depressor muscle for all five pereiopods of the crab *Callinectes sapidus*. She reported some variation between somites in the number and arrangement of depressor muscle heads, however, the arrangement of the origins of the heads in pereiopod 2 (Fig. 12, muscle 125 a-e in Cochran, 1935) is very similar to what is described here for *M. quadrispina*. In *C. sapidus*, there are depressor muscle heads with morphologies similar to *M. quadrispina*'s sternal head (125a), caudal head (125d), dorsal head (125e) and coxal head (125c) (Cochran, 1935). The final head of the second pereiopod (125b) originates on the rostral face of the coxa, not on the lip of the arthrodial cavity, as do the ventral heads in *M. quadrispina*. In *C. sapidus*, this head has similar morphologies in pereiopods 1-4, but in pereiopod 5, the origin is shifted to the endosternite just inside the ventral lip of the arthrodial cavity, a very similar position to the origin of the ventral-rostral head in *M. quadrispina*. In the fifth pereiopod of the crab *Carcinus maenas*, the depressor muscle has four heads, two of which originate in close apposition within the thorax, and two of which originate in the coxa, one ventrally and one caudo-dorsally (Bévengut et al., 1983).

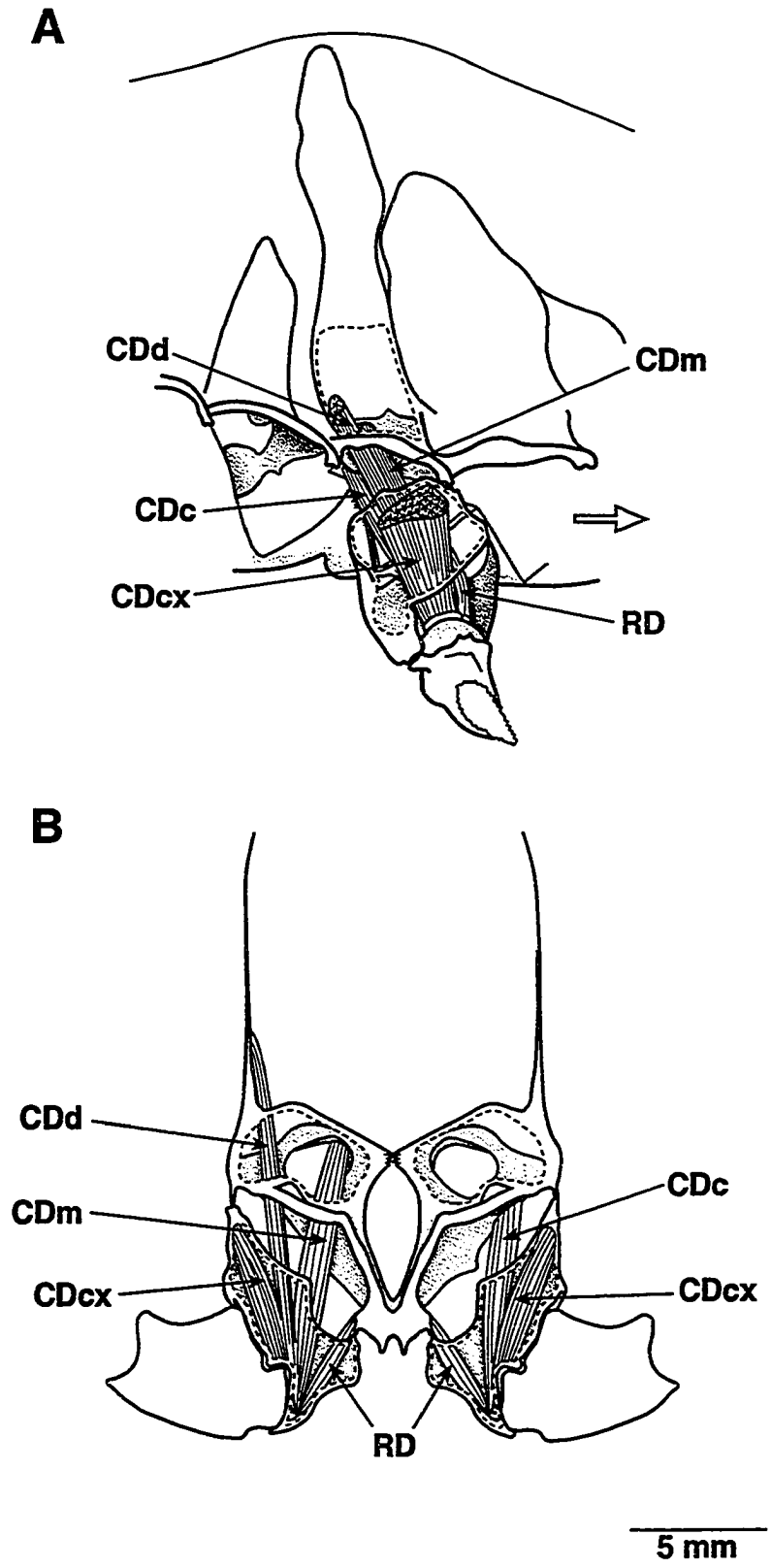
The presence of discrete heads responsible for controlling one direction of movement at a single joint raises the question of whether those heads can be considered a single muscle. Several authors have divided muscles based on their insertions, so that any group of heads sharing one apodeme is considered one muscle (McVean, 1973; White and Spirito, 1973; MacMillan, 1975; Ayers and Davis, 1977). Bévengut et al. (1983) disagree; they state that muscle heads should be shown to be functionally, as well as anatomically, distinct before they can be considered separate muscles. I support an anatomical distinction, with distinct origins and insertions, for separate muscles for two reasons. First, it seems logical that muscle heads which are completely separate from

beginning to end be considered separate muscles, regardless of whether they are co-activated under certain conditions or not, or whether they are derived through evolutionary duplication of a single muscle. Second, evidence suggesting or confirming division of function between heads which attach to a single apodeme is gathering (Moffett et al., 1987; Pearlstein et al., 1995; Chapter 6), and it seems unreasonably divisive to try to classify heads which share one end as separate muscles. For these reasons, I classify *M. quadrispina*'s depressor muscle as a single muscle with six heads.

The depressor muscle of *C. bartonii* has five heads organized in a similar manner to the depressor heads of *M. quadrispina* (Fig. 5.5), taking into consideration the considerable differences of the thorax, in particular the much narrower sternum, and coxa which lie almost directly under the thorax. There are two depressor apodemes in *C. bartonii*, which indicates, if one muscle is defined by insertion onto a single apodeme, that there are two depressor muscles. The rostral depressor muscle has a single head, and the caudal depressor muscle is comprised of four heads. The two apodemes are bound tightly by connective tissue and do not appear capable of being moved independently. The single, thin head of the rostral depressor muscle is morphologically similar to the ventral-rostral head of *M. quadrispina*'s depressor muscle. It originates on the lip of the sternite, at the edge of the arthrodial cavity, and inserts on the smaller and more rostral apodeme (Fig. 5.5A).

All four heads of *C. bartonii*'s caudal depressor muscle are bound together by loose connective tissue near their insertion point, and, as in *M. quadrispina*, the dorsal and coxal heads become fused near their insertion point on the dorsal surface of the large depressor apodeme. The dorsal head originates on the caudo-ventral part of the epimeron, while the coxal head originates on the dorsal surface of the coxa (Fig. 5.5). The caudal head originates on the lateral edge of the caudal arthrophragm, just inside the lip to the arthrodial cavity, extends ventrally and laterally and inserts on the ventral surface of the large depressor apodeme (Fig. 5.5). These three heads are quite similar anatomically to the dorsal, coxal, and caudal heads of *M. quadrispina*. The medial head of *C. bartonii*'s caudal depressor muscle originates on the lateral surface of the paraphragm, and inserts on the ventral surface of the large apodeme (Fig. 5.5). There is not a head with an origin quite like this in *M. quadrispina*, but the paraphragm is part of the endosternite, an intersegmental inward projection of the sternite, so it is possible that these heads are related. This paraphragm, however, originates from the endosternite from between the first and second pereiopods, while the sternal head in *M. quadrispina* (and *C.*

Fig. 5.5. The depressor muscles of *C. bartonii*. The fine dashed lines represent holes cut in the skeleton. (A) Lateral view of the right side, from slightly dorsal. Rostral is indicated by the open arrow. (B) Transverse view seen from rostral. On the animal's left, the dorsal and medial heads of the caudal depressor have been removed to give an unobstructed view of the caudal head. CDc, caudal head of the caudal depressor muscle; CDcx, coxal head of the caudal depressor muscle; CDd, dorsal head of the caudal depressor muscle; CDm, medial head of the caudal depressor muscle; RD, rostral depressor muscle.


A
CDd
CDm
CDc
CDcx
RD
B
CDd
CDm
CDcx
CDc
CDcx
RD
5 mm

sapidus) originates on the medial and caudal edges of the sternite, between pereiopods 2 and 3, so if these two heads are homologues, considerable rearrangement of their origins has taken place.

Schmidt (1915) described three heads for the depressor muscle of *Astacus fluviatilis*: depressor muscle *a*, which has a medial head originating in the area of the paraphragm and a lateral head originating on the epimeron (equivalent to the medial and dorsal heads of the caudal depressor described here), and depressor muscle *b*, which originates in the coxa. From Schmidt's figures, it appears that the lateral head may in fact have two origins, one of which may be ventral enough to be the equivalent of the caudal head of the caudal depressor described here, but it is difficult to say and Schmidt did not make it clear in his description. Schmidt noted nothing like the ventral head described here (Schmidt, 1915).

Two depressor muscles, rostral and caudal, have been described for lobsters (MacMillan, 1975; Ayers and Davis, 1977). The caudal depressor originates in the coxa and inserts on the caudal apodeme, while the rostral depressor has two heads that originate on the endophragmal skeleton and insert on the rostral apodeme. This is quite different from the arrangement in *C. bartonii*, where the rostral depressor muscle has only one head with its origin on the lip of the arthrodial cavity, and the caudal depressor muscle is comprised of the remaining four heads, including the coxal head. The depressor muscle in the shrimp *Palaemon squilla* is even more complex (Hessler, 1982). This species' depressor muscle has seven heads arising at various points in the coxa and thorax, with some inserting directly on the lip of the basis and some on a large depressor apodeme. The endophragmal skeleton of this species is extremely reduced, resulting in the origins of all the intrinsic proximal limb muscles being crowded very closely together. The depressor muscles of many non-decapod crustaceans also tend to be very complex, consisting of multiple heads with distributed origins and insertions (Hessler, 1982), as are the femoral depressor muscles of insects (Johnston & Levine, 1996; Brunn, 1998; Watson & Ritzmann, 1998; Lubischer et al., 1999), which are thought to be homologous to the crustacean basipodite depressor (Carpentier & Bartlett, 1959).

Anatomy of the pereiopod levator muscle

Studies in crabs have shown that the two levator muscles have different roles in two distinct behaviours, pereiopod elevation and autotomy (McVean & Findlay, 1976). Separate rostral and caudal levator muscles occur in all species studied so far, including

crabs (White & Spirito, 1973; MacMillan, 1975; Moffett, 1975; McVean & Findlay, 1976; Bévengut et al., 1983), lobsters (MacMillan, 1975; Ayers and Davis, 1977), hermit crabs (McVean & Findlay, 1976), and crayfish (below). In general, the heads of the caudal levator muscle originate in the coxa, and the heads of the rostral levator muscle originate in the thorax. In some species the caudal levator muscle has two heads, both originating in the coxa (e.g. *M. quadrispina* (see below) and *C. maenas*: McVean & Findlay, 1976; Bévengut et al., 1983), but in most species described to date, including *C. bartonii*, the caudal levator muscle has one broad head. Additionally, the rostral levator muscles of *C. maenas* (Bévengut et al., 1983) and the land crab *Cardisoma guanhumi* (Moffett, 1975) have heads originating in the coxa. The number of heads comprising the rostral levator muscle is more variable among species, ranging from one in *Pagurus bernhardus* and *Maia squinado* (McVean & Findlay, 1976) to four in *C. maenas* (Bévengut et al., 1983).

In *M. quadrispina*, the caudal levator muscle has two heads and the rostral levator muscle three heads (Fig. 5.6). Superficially, the caudal levator muscle appears to be composed of a single head. However, cross sections of the muscle (Fig. 5.7) and close examination of the insertions reveal two heads: a narrow caudal head and a more ventral, broader rostral head (Fig. 5.6A, C). Both heads originate together on the dorsal surface of the coxa and are indistinguishable in unstained muscles, but the insertions of the two heads are distinct. The insertion of the caudal head is on the edges and dorsal surface of the caudal levator apodeme, while the rostral head inserts on a small ventral projection near the rostral edge of the caudal apodeme (Fig. 5.8). The fibers of the caudal head of the caudal levator muscle are smaller than those of the rostral head and are surrounded by thick layers of connective tissue (Fig. 5.7).

The sternal head of the rostral levator muscle originates in the medio-caudal corner of the next rostral sternite, in the small pocket unoccupied by the origin of the sternal head of that somite's depressor (Fig. 5.6 B, C). This head extends laterally through the hole between the arthrophragms and sternite, and inserts along the ventral and lateral edges of the proximal part of the long caudal extension of the rostral levator apodeme (Fig. 5.8). The caudal head of the rostral levator originates on the caudal endophragmal wall, just medial and dorsal to the caudal head of the depressor, and inserts distal to the sternal head along the same projection of the rostral apodeme (Fig. 5.6). The dorsal head of the rostral levator originates on the rostral endopleurite (Fig. 5.6A, C), then passes ventrally and laterally to insert on the dorsal face and lateral edges of the

Fig. 5.6. The levator muscles of *M. quadrispina*. Rostral is indicated by the open arrows, the fine dashed lines represent holes cut in the skeleton. (A) View from the right side, and slightly dorsal. (B) Transverse view of the right side looking caudally. (C) Ventral view. CLc, caudal head of the caudal levator muscle; CLr, rostral head of the caudal levator muscle; RLc, caudal head of the rostral levator; RLd, dorsal head of the rostral levator; RLs, sternal head of the rostral levator.

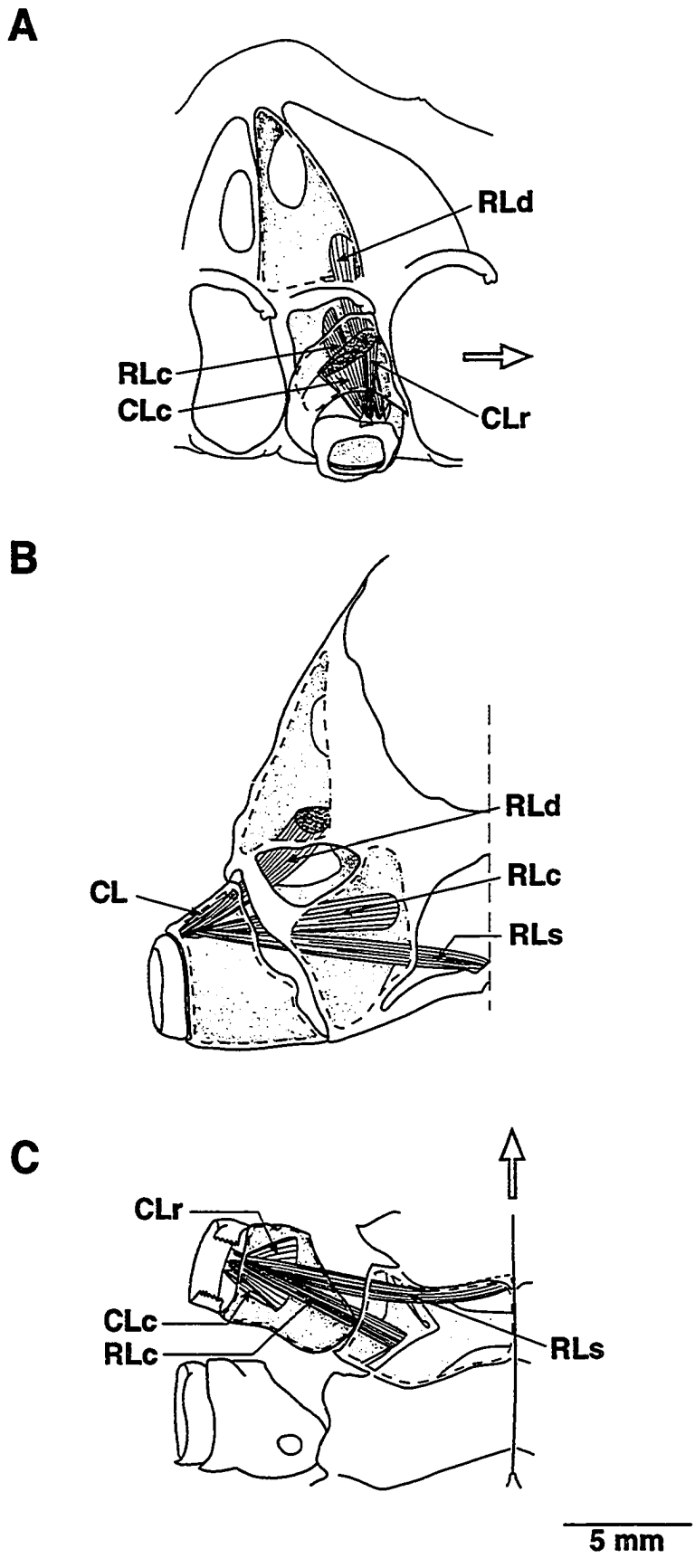
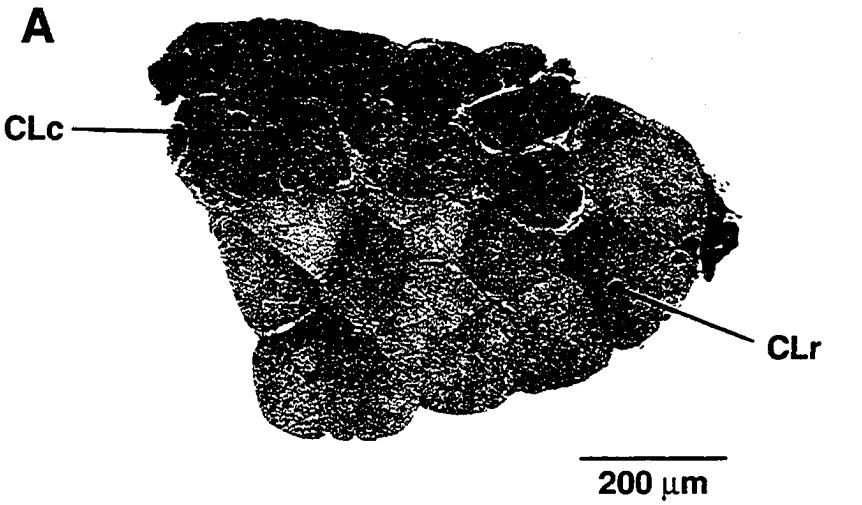
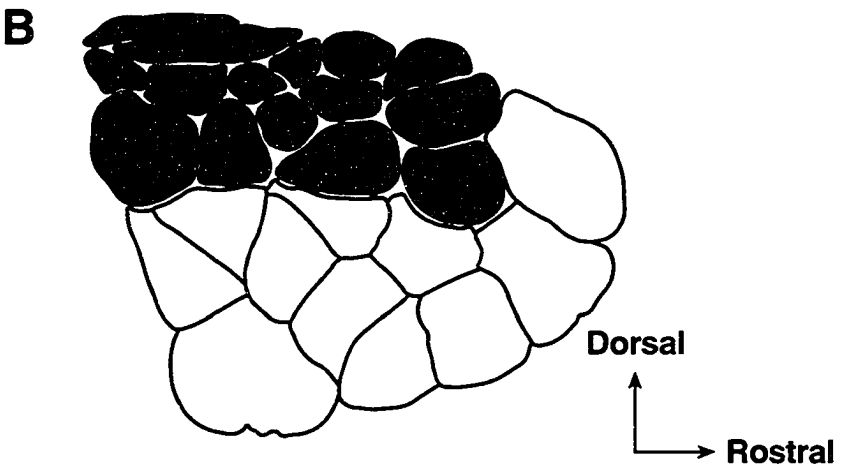

A
RLd
RLc
CLc
CLr
B
RLd
RLc
RLs
CL
C
CLr
CLc
RLc
RLs
5 mm

Fig. 5.7. Cross section of *M. quadrispina*'s caudal levator muscle. (A) The caudal (CLc) and rostral (CLr) heads are clearly differentiated in the Richardson's stained section. (B) The fibers comprising the two heads are drawn: the filled fibers are from the caudal head and the outlined fibers are from the rostral head. The identity of each head's fibers was confirmed by carefully separating the two heads, beginning at their insertions. (C) A closer view of part of the caudal and rostral heads. Note the extensive connective tissue between the fibers of the caudal levator. Both sections are taken from approximately the midpoint along the muscle's length. The arrows denote dorsal and rostral for all parts of the figure.


A
CLc
CLr
200 μm


B
Dorsal
Rostral

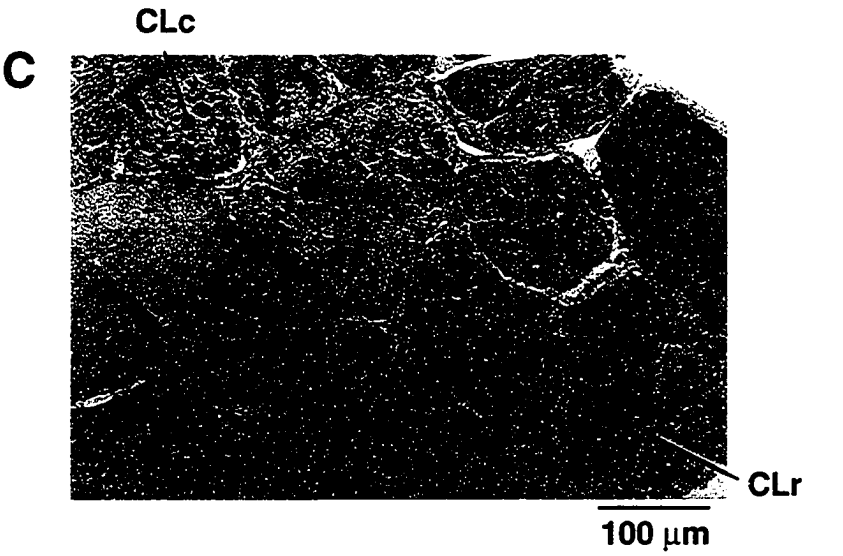
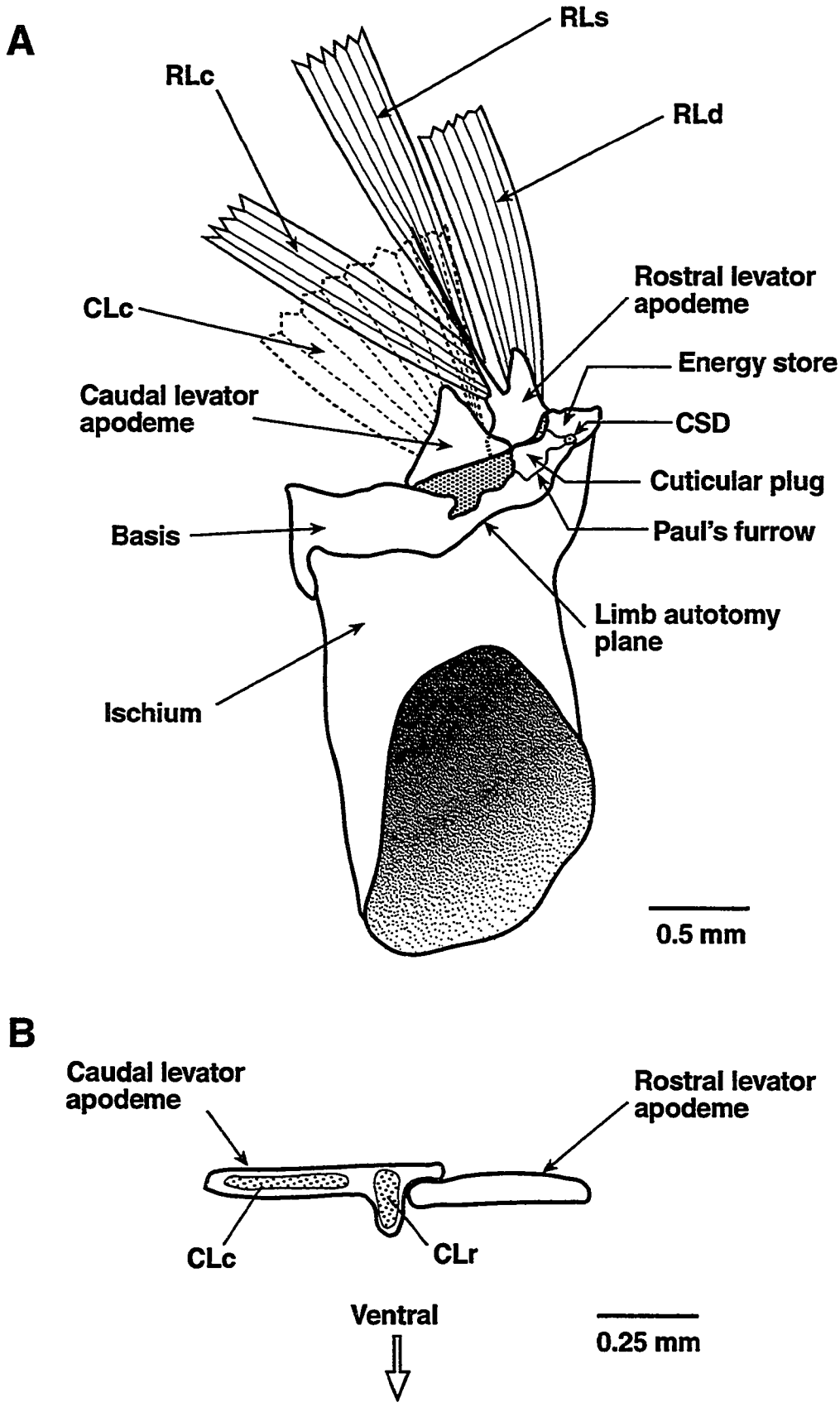

CLc
C
CLr
100 μm

Fig. 5.8. Dorsal view of the levator muscle apodemes in *M. quadrispina*, showing insertions of the levator muscle heads. (A) The caudal head of the caudal levator muscle (CLc) inserts on the lateral edges and dorsal surface of the caudal levator apodeme. The rostral head of the caudal levator (CLr), which is mostly hidden from view in (A), inserts on a small ventral projection of the caudal levator apodeme. Insertions of the two heads are indicated by the stippled areas in (B). The caudal levator muscle is dorsal to the rostral levator muscle, and is shown by dashed lines so as not to obscure the insertions of the rostral levator heads. The dorsal head of the rostral levator (RLd) inserts on a projection at the rostral edge of the rostral apodeme. The sternal (RLs) and caudal (RLc) heads insert on a long projection from the caudal part of the rostral apodeme; the sternal head on the proximal end of the projection and the caudal head on the caudal edge of the distal end of the projection. Each of the levator apodemes attaches to the dorsal lip of the basis at two discrete points, with arthrodial membrane (stippling) filling the gaps between the attachment points. The rostral levator apodeme attaches to the piece of cuticle called the energy store and to the cuticular plug (terminology from McVean & Findlay's (1976) study on the roles of the levator muscle in pereiopod autotomy). The cuticular plug is bounded by Paul's furrow and the membrane of the putative cuticular stress detector I (CSD) (Clarac et al., .1971; Wales et al., 1971;Marchand et al., 1995). The caudal levator apodeme attaches to the cuticular plug and to a more caudal point on the lip of the basis. (B) A cross-section of the two levator apodemes. The caudal levator apodeme is slightly dorsal to and overlaps the caudal part of the rostral apodeme, and the two apodemes are linked by connective tissue. The asterisk indicates the ventral projection of the caudal apodeme onto which inserts the rostral head of the caudal levator muscle.


A
RLs
RLc
RLd
Rostral levator apodeme
CLc
Energy store
Caudal levator apodeme
CSD
Cuticular plug
Basis
Paul's furrow
Limb autotomy plane
Ischium
0.5 mm
B
Caudal levator apodeme
Rostral levator apodeme
CLc
CLr
Ventral
0.25 mm

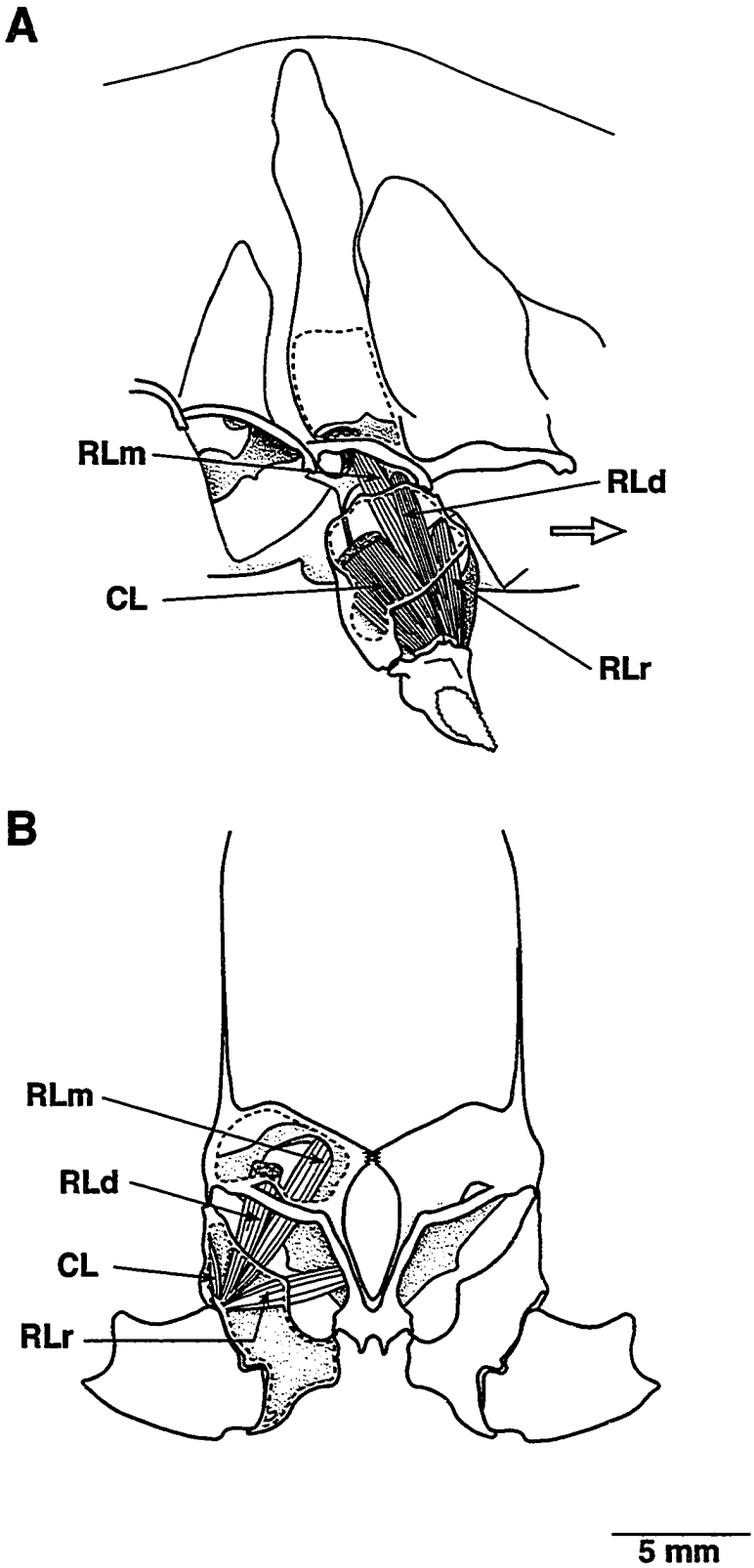
rostral projection of the rostral levator apodeme (Fig. 5.8). The long, caudal projection of the rostral levator muscle apodeme of *M. quadrispina* does not have bend points or dorsal projections, as does the depressor apodeme. However, the dorsal head of the levator does not pull at as sharp an angle as does the dorsal head of the depressor, and therefore this sort of modification may not be necessary.

The arrangement of the apodemes and cuticular structures of the basis associated with leg autotomy in *M. quadrispina* is similar to that described for the walking legs of crabs (McVean & Findlay, 1976). The two levator apodemes in *M. quadrispina* are held firmly together by connective tissue, although the attachment is not rigid and the two can be moved independently to a limited extent (Fig. 5.8). The caudal apodeme is dorsal and slightly overlapping the rostral apodeme, so that the caudal levator muscle lies dorsal to the heads of the rostral levator muscle (Fig. 5.8). Both apodemes attach to the dorsal lip of the basis at two discrete points (Fig. 5.8). The extent of the arthrodial membrane that fills the space between the attachment points, especially at the base of the caudal apodeme, is much greater in *M. quadrispina* than in other species that have been described (McVean & Findlay, 1976). For a detailed description of the structures involved and the process of leg autotomy, see McVean & Findlay (1976).

The origins of the pereiopod 2 levator muscle heads of *M. quadrispina* are not as similar to those in *C. sapidus* as are the origins of the depressor muscle heads (Cochran, 1935). There is a levator head in *C. sapidus* (124b in Fig. 12, Cochran, 1935) which arises from the dorsal endophragmal skeleton, but its position is far removed from the origin of the dorsal head of the rostral levator in *M. quadrispina*. There are two heads (124c and d) that arise in the area corresponding to that of the caudal head of the rostral levator of *M. quadrispina*, but no head which originates in the coxa, and no head arising near the midline on the sternum. The fourth levator head of *C. sapidus* arises on the face of the rostral endosternite; this could be related to the sternal head of the rostral levator of *M. quadrispina*, although its origin is considerably more lateral, and is within the pereiopod 2 somite, rather than the pereiopod 1 somite. Cochran (1935) also states that in all five pereiopods of *C. sapidus*, the heads of the levator muscle insert on a single tendon. However, since she did not describe a levator head with its origin in the coxa, and every other species looked at to date has separate rostral and caudal levators, it seems likely that the caudal levator was missed in this study.

The single head of the caudal levator of *C. bartonii* originates on the dorsal and caudal faces of the coxa and inserts on the caudal levator apodeme (Fig. 5.9). As in *M.*

Fig. 5.9. The levator muscles of *C. bartonii*. The fine dashed lines represent holes cut in the skeleton. (A) Lateral view of the right side, from slightly dorsal. Rostral is indicated by the open arrow. (B) Transverse view seen from rostral. CL, caudal levator; RLd, dorsal head of the rostral levator; RLm, medial head of the rostral levator; RLr, rostral head of the rostral levator.


A
RLm
RLd
CL
RLr
B
RLm
RLd
CL
RLr
5 mm

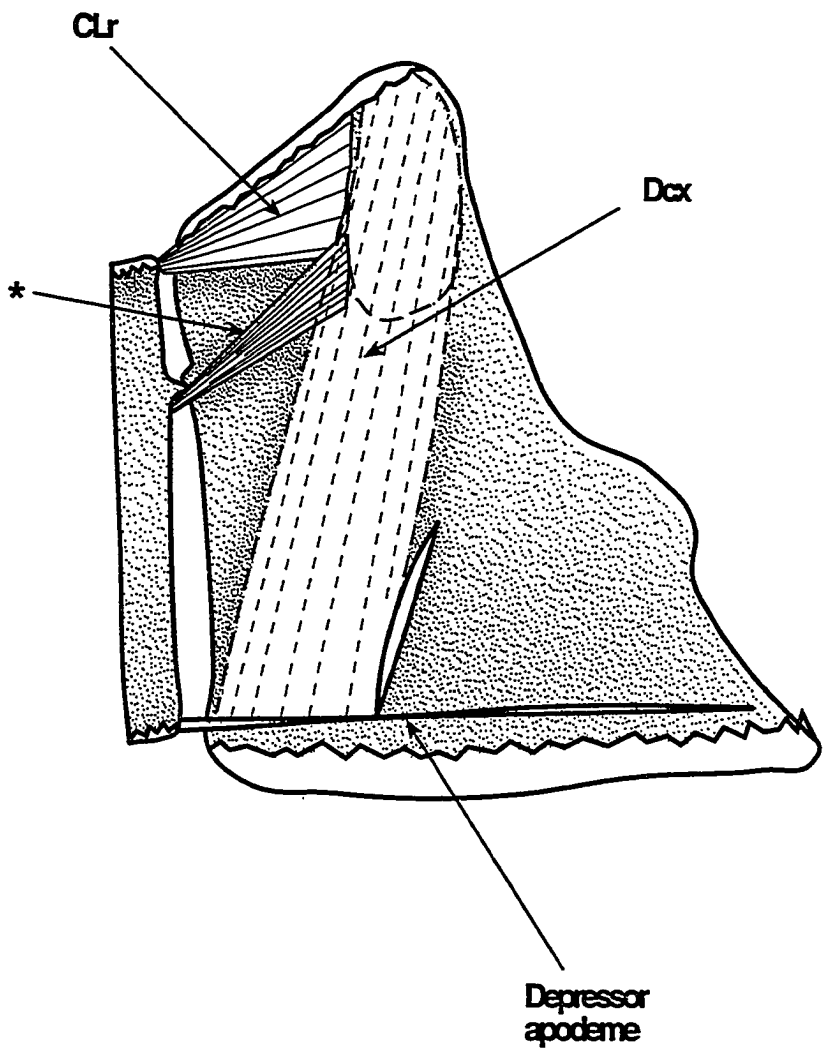
quadrispina, the rostral levator of *C. bartonii* is comprised of three heads, all of which originate in the thorax. The medial head of the rostral levator originates on the lateral face of the paraphragm, just rostral to the medial head of the depressor, extends laterally and slightly ventrally to insert on the dorsal face of the rostral apodeme (Fig. 5.9). This head could be related to the sternal head of the rostral levator of *M. quadrispina* for the same reasons as suggested for the sternal and medial heads of the depressor muscles of these two species. The dorsal head of the rostral levator originates on the ventral edge of the rostral endosternite, in a position corresponding to the origin of the dorsal head of the rostral levator muscle in *M. quadrispina*, passes laterally and ventrally and inserts on the dorsal face of the rostral apodeme. The rostral head of the rostral levator arises on the lip of the sternite leading to the arthrodial cavity, then passes laterally to insert on the ventral surface of the rostral apodeme (Fig. 5.9). Although the insertion of this head corresponds to the insertion of the caudal head of the rostral levator in *M. quadrispina*, the origins of these muscles are different. The three heads of the rostral levator in *C. bartonii* are loosely joined by connective tissue near their insertions.

A basal muscle of unknown function in M. quadrispina

A small muscle, that I call the caudal muscle, originates on the caudal face of the coxa and inserts on the rim of the basis just ventral to the caudal coxal-basal condyle (joint) (Fig. 5.10). As the insertion is ventral to the condyle, this muscle would act on the joint as a pereiopod depressor; this was confirmed by elevating the pereiopod in a partially dissected animal, which stretched the muscle. However, the insertion is so close to the condyle, and the muscle is so small, that it seems unlikely it could generate sufficient force to contribute to joint movement or support the animal's weight. Methylene blue staining revealed that this muscle is innervated by one motor neuron and no sensory neurons, indicating that it is not part of a stretch-receptor organ. I have not determined if the motor neuron is shared with another muscle or is unique to this muscle.

Another possible function of this muscle could be in maintaining joint rigidity. However, the coxal-basal joint of *M. quadrispina* is very strongly articulated, and does not appear to wobble or move at all other than in its designated plane. It is also possible that this muscle could be involved in pereiopod autotomy or molting, but without more data these possibilities are speculative.

Fig. 5.10. View from rostral of the caudal half of the coxa and basis to show the basal muscle in *M. quadrispina* that inserts just ventral to the caudal coxal-basal condyle. This muscle (indicated by the asterisk) originates near the coxal head of the depressor muscle (Dcx) and the most caudal part of the rostral head of the caudal levator muscle (CLr).

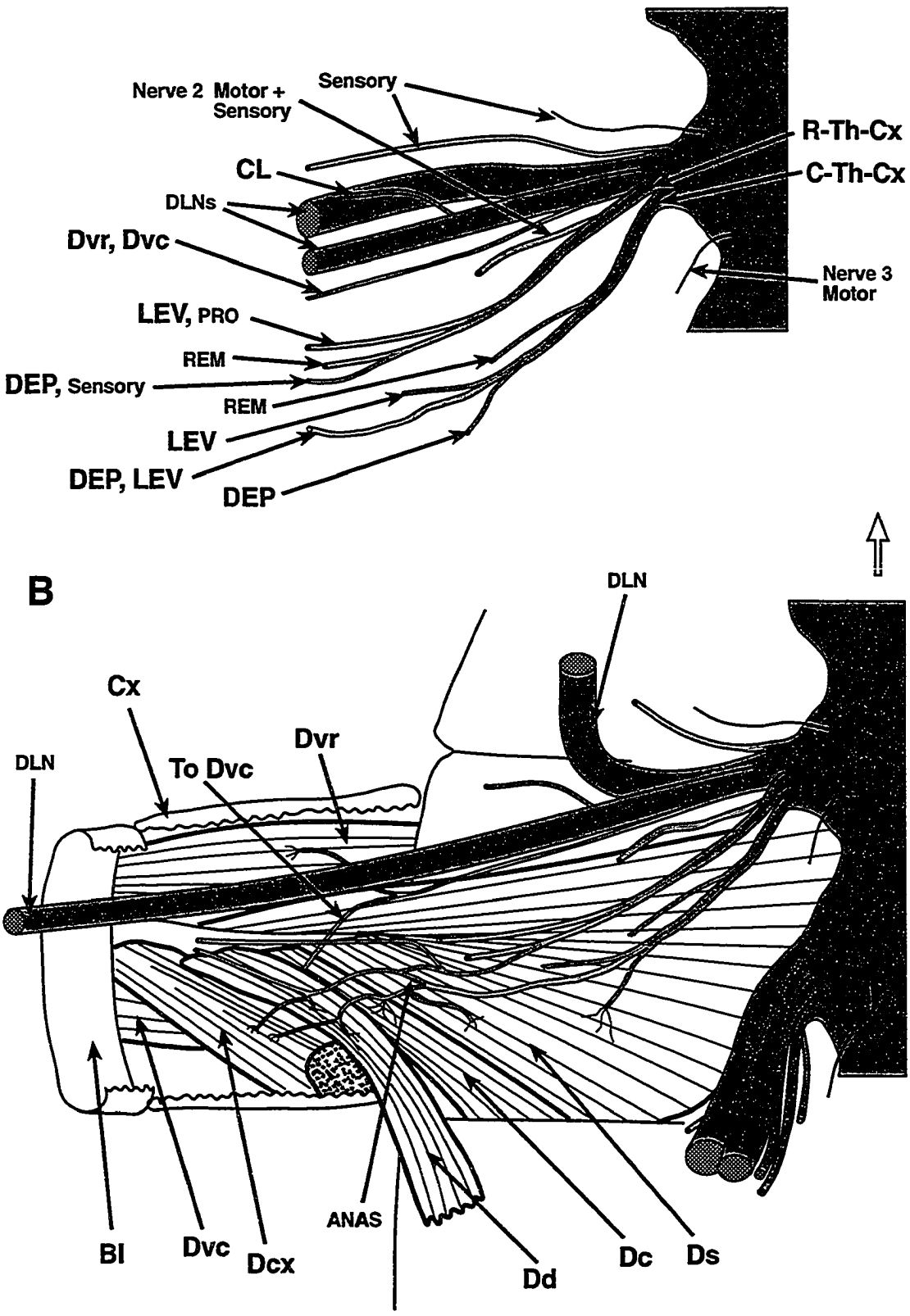

CLr
Dcx
*
Depressor
apodeme

Peripheral nerves innervating the proximal pereiopod muscles in M. quadrispina

Innervation of the proximal pereiopod muscles in *M. quadrispina* is almost entirely through the two thoracico-coxal nerves that leave the ganglion dorsal to the large distal leg nerves (Fig. 5.11). The position of the thoracico-coxal nerves in the rostro-caudal axis is variable between animals and between segments. In some animals the thoracico-coxal nerves leave the pereiopod 2 and 3 ganglia directly dorsal to the distal leg nerves, but more commonly they leave caudal to the distal leg nerves (as in the animal used for Fig. 5.11). However, the thoracico-coxal nerves innervating pereiopods 1, 4, and 5 always leave the ganglia directly dorsal to the distal leg nerves. The only proximal muscles not innervated through the thoracico-coxal nerves are the ventral heads of the depressor and parts of the caudal levator, which are innervated through branches from the caudal distal leg nerve. In *M. quadrispina*, the caudal distal leg nerve is smaller than and dorsal to the rostral distal leg nerve. The branch points of these depressor and levator nerves from the caudal distal leg nerve are quite variable; in some animals the nerves appeared to leave the ganglion directly, whereas in others they branch from the distal leg nerve only after it has entered the coxa. After leaving the caudal distal leg nerve, the ventral depressor nerve turns ventrally and splits into one branch for each ventral depressor head (Fig. 5.11B).

The innervation for the remaining heads of the depressor is split between the rostral and caudal thoracico-coxal nerves. The rostral thoracico-coxal nerve innervates the depressor (except the ventral heads), levator, remotor, and promotor muscles. There are also sensory axons in the rostral nerve with ganglionic processes resembling those described for coxo-basal chordotonal organ sensory afferents in crabs and crayfish, which in these species also leave the ganglion through the rostral thoracico-coxal nerve (Bévengut et al., 1983, El Manira et al., 1991A). The caudal thoracico-coxal nerve innervates the depressor, levator, and remotor muscles, and also contains some sensory axons of unknown function. A somewhat variably positioned anastimosis connects the two thoracico-coxal nerves. In some animals the anastimosis occurred before the nerves leading to the sternal and caudal heads branched, whereas in others it occurred distal to these branches. The dorsal and coxal heads always have axons in both the rostral and caudal nerves, whereas the axons to the sternal and caudal heads are usually confined to the caudal nerve (82 of 98 neurons from sternal and caudal head backfills). Rostral to the distal leg nerves are two small, purely sensory nerves that innervate cuticular sensory structures of the coxa and thorax.

Fig. 5.11. Dorsal view of nerves exiting the leg 2 hemi-ganglion of *M. quadrispina*, and innervation of the six heads of the depressor muscle. The open arrow indicates rostral for both illustrations. (A) Six large and two small nerves leave each hemiganglion. The thoracico-coxal nerves leave caudal, and slightly dorsal, to the distal leg nerves. The rostral thoracico-coxal nerve innervates the promotor (PRO), remotor (REM), levator (LEV), and the four heads of the depressor (DEP). It also contains a small number (a minimum of 10-13) of sensory axons. The caudal thoracico-coxal nerve contains only motor axons and innervates the remotor, levators, and the same four of the depressor. Innervation for the ventral heads of the depressor (Dvr, Dvc) and part of the innervation for the caudal levator (CL) are provided by branches of the caudal distal leg nerve. (B) Innervation of the depressor heads. ANAS, anastimosis between the rostral and caudal thoracico-coxal nerves; BI, Basis; Cx, Coxa; Dc, caudal head; Dcx, coxal head; Dd, dorsal head; Ds, sternal head.


A
Nerve 2 Motor + Sensory
Sensory
R-Th-Cx
C-Th-Cx
CL
DLNs
Dvr, Dvc
Nerve 3 Motor
LEV, PRO
REM
DEP, Sensory
REM
LEV
DEP, LEV
DEP
B
DLN
Cx
DLN
Dvr
To Dvc
ANAS
Bl
Dvc
Dcx
Dd
Dc
Ds

The superficial organization of the innervation to the proximal pereiopod muscles seen in *M. quadrispina* is quite similar to that in crabs (Bévengut et al., 1983) and crayfish (El Manira et al., 1991A; Elson, 1996). Two thoracico-coxal nerves leave each hemiganglion that contain in crabs the majority and in crayfish all, of the proximal muscle motor axons. In crabs, a nerve branch leading to the depressor muscle also leaves the smaller, but in this case more rostral, distal leg nerve. This small branch innervates the two thoracic heads of the depressor (El Manira et al., 1983). The rest of the innervation of the depressor muscle of crabs is from the caudal thoracico-coxal nerve. In crayfish, axons innervating the depressor muscle are contained wholly within the caudal branch (El Manira et al., 1991A; Elson, 1996). The strictly motor nerve, which lies between the distal leg and thoracico-coxal nerves and innervates muscles in the dorsal thorax, appears from its innervation field to be homologous to segmental nerve 2 of crayfish (Elson, 1996). Segmental nerve 3, which innervates segmental flexor muscles, leaves dorsally from the caudal edge of each hemiganglion.

Elson (1996) has proposed a new, anatomically based terminology for the distal leg nerves and thoracico-coxal nerves, which together are subdivisions of segmental nerve 1. This system names the nerves based on their dorso-ventral and rostro-caudal positions such that the more rostral of the distal leg nerves becomes the rostral ventral branch of nerve 1, the caudal thoracico-coxal nerve becomes the caudal dorsal branch of nerve 1, and so on. This system works well for crayfish and crabs, but if the thoracico-coxal nerves of these species are homologous to those of *M. quadrispina*, as appears likely, then this terminology would seem odd if applied to the latter, as these nerves are shifted caudally and no longer lie directly dorsally to the larger distal leg nerves. I recognize the need for a consistent means of nomenclature that recognizes the developmental and evolutionary origins of the various nerve branches, but, so far, no author has tried to assign the names distal leg nerve or thoracico-coxal nerve to anything other than homologues in different species. Paul (1981) and Paul et al. (1985) suggested a functional nomenclature, with names based on the innervation field of the nerve. I agree with this approach, and have chosen to remain with the functional nomenclature for the branches of segmental nerve 1, at least until the system proposed by Elson (1986) becomes widely accepted.

Central morphology of depressor motoneurons

Backfills of depressor muscle motor nerves revealed somata in the rostral part of the ganglion that innervated the ventral heads, and somata in the caudal region that innervated the remaining four heads. These cells have central morphologies typical of arthropod motor neurons (Evoy, 1977; Altman, 1981). Additionally, two other cell types were seen: one with its soma on the contralateral side of the ganglion and with a central morphology that closely resembles the common inhibitor, and one with its soma in the lateral part of the ganglion that does not resemble any neuron described to innervate a crustacean leg muscle. In the following descriptions, the counts of somata are of the maximum number seen in any fill; in incomplete fills the total number could usually be accounted for by partially filled axons out in the thoracico-coxal nerves.

A cell with a contralateral soma was filled from the nerve branches to all of the depressor heads (Fig. 5.12). This cell has a morphology essentially identical to the common inhibitor motoneuron previously described in a number of species (Bévengut et al. 1983; Moffett et al, 1987; Wiens and Wolf, 1993; Faulkes and Paul, 1997), which is known to innervate the depressor muscle (Moffett & Yox, 1986; Rathmayer & Bévengut, 1986). In crayfish (Wiens and Wolf, 1993; Bévengut et al., 1996) and crabs (Bévengut et al., 1983; Moffett et al., 1987), the soma of this neuron is located in the caudal region of the ganglion. In *M. quadrispina*, the soma was consistently seen near the midpoint along the rostro-caudal axis of the ganglion, although the projections of this cell are in similar positions to those described in other species. Interestingly, processes of this cell enter both the rostral and caudal thoracico-coxal nerves, in contrast to the situation in crabs, where a single axon exits the rostral nerve (Bévengut et al., 1983; Moffett et al., 1987).

Backfills of the nerve branches innervating the sternal head of the depressor revealed seven neurons with central somata (Fig. 5.13A). Five of the neurons have somata in a group in the caudal part of the ganglion, and a morphology and soma position similar to depressor motoneurons described in other species (Bévengut et al., 1983; Skorupski & Sillar, 1988; El Manira et al., 1991A; Pearlstein et al., 1998A, B). The somata of these cells are near the ventral surface of the ganglia at the caudo-medial periphery of the neuropil (Fig. 5.13A, B). For each, the neurite from the soma immediately curves dorsally and rostrally, leading to a large integrating segment located about two-thirds of the way dorsally in the ganglion (ca. 100-200 μm from the dorsal surface, depending on the size of the specimen). The integrating segments are oriented along a rostro-medial to caudo-lateral axis through the neuropil. From the medial end of

Fig. 5.12. Camera lucida drawing of the putative common inhibitor. This drawing was made from a backfill of the nerve branch to the caudal head. The heavy dashed line indicates the midline, the open arrow indicates rostral, the small arrow indicates the nerve through which the neuron was filled, and the fine dashed line outlines the neuropil area.

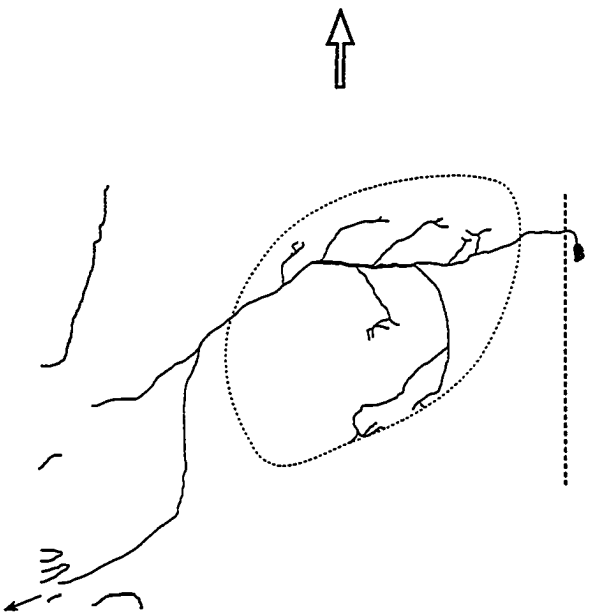
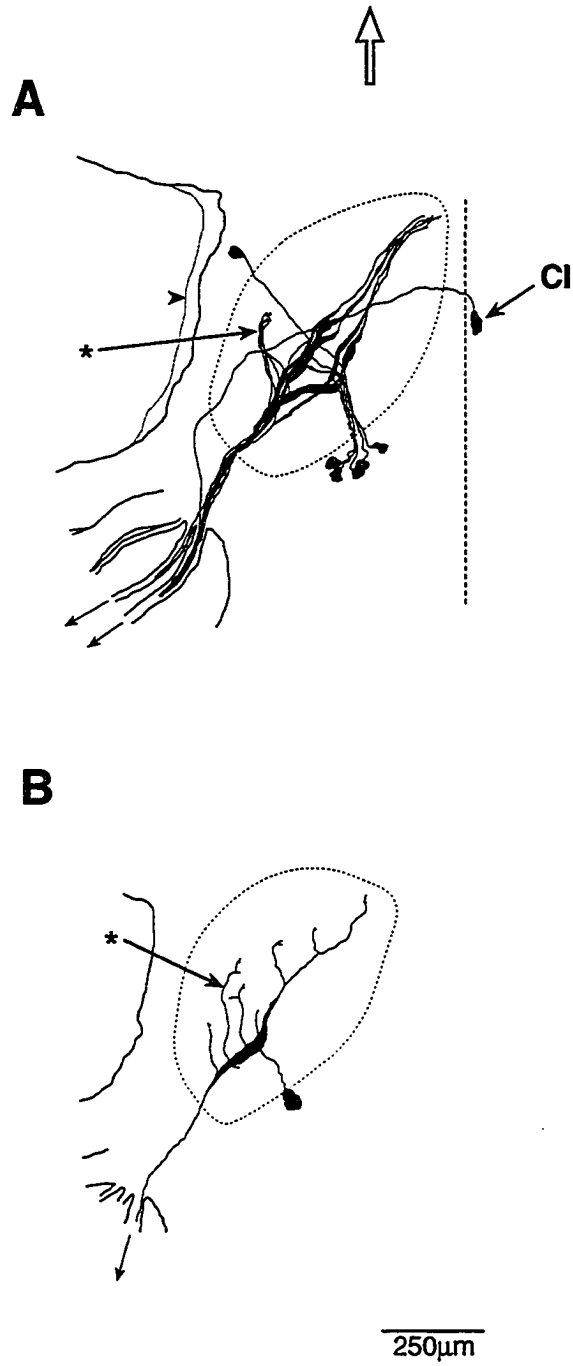

250µm

Fig. 5.13. Camera lucida drawings of neurons backfilled from the sternal head of the depressor of *M. quadrispina*, and their typical morphologies. The heavy dashed lines indicate the midline, the open arrow indicates rostral for all parts of this figure, the small arrows indicate the nerves through which the neurons were filled, and the fine dashed line outlines the neuropil area. (A) Composite drawing of all seven neurons filled: five neurons in a caudal group, one lateral neuron, and the putative common inhibitor (CI). The arrowhead points to the sheath of the ganglion. In A and B, the asterisk indicates the largest dendrites of these neurons. (B) A typical caudal group neuron, filled via the nerve to the sternal depressor head; caudal neurons filled from the dorsal, coxal and caudal heads are similar.


A
CI
*
B
*
250µm

each integrating segment a projection extends medially, ending in a small arborization near the medial edge of the neuropil area. Many rostrally and slightly ventrally projecting dendrites are given off from the integrating segment and medial projection. One of these dendrites was consistently larger than the others and projected to an area at the rostro-lateral edge of the neuropil, terminating with a distinctive rostral turn (Fig. 5.13A). The enlarged dendrite's position on the integrating segment and relative to the cell's other dendrites is variable. A small number of usually short caudal dendrites also project from the integrating segment. The efferent axon leaves the lateral end of the integrating segment and curves slightly ventrally to enter one of the thoracico-coxal nerves. The other two neurons filled were the putative common inhibitor, and an unusual cell with a lateral soma (see page 167).

Fills of the nerve branches innervating the caudal, coxal, and dorsal heads of the depressor also revealed neurons with a similar morphology to the one shown in Fig. 5.13B. Five of these neurons innervate the coxal and dorsal heads, which share innervation (Fig. 5.14A), and six innervate the caudal head (Fig. 5.14B). In general all of these neurons are roughly the same size and shape, but I found the size and location of the integrating segments varied somewhat between backfills (Table 5.2). In a small percentage of the cells, the integrating segment was either unusually large (e.g. the integrating segment of one neuron in Fig. 5.14A extends far towards the midline), or was bent rostrally or caudally on one or both ends (e.g. the neuron in Fig. 5.14A with the rostrally displaced medial end, and the neuron in Fig. 5.14B with the caudally directed lateral end). These oddities totaled fewer than 10% of all cells observed (18 of 201), and the displacements of their integrating segments was not associated with displacements of their somata or dendrites. It was not possible to diagram clearly the branching of the axons between the coxal and dorsal heads, due to the obscuring the axons by extracellular filling in these very short nerve branches. However, whether these heads were filled individually, or both heads were filled together, the maximum number of cells filled was identical.

Backfills of nerve branches to the sternal, caudal, and coxal-dorsal heads of the depressor muscle reveal that several axons are shared among them. Backfilling through the nerve branches to the sternal head showed four axons which bifurcate and enter the nerve innervating the caudal head, one of which also sends branches to the coxal-dorsal heads and to the levator and remotor muscles (Fig. 5.15A). The latter is presumably the common inhibitor, indicating that three other motoneurons are shared between the sternal

Table 5.2. Somata positions and cell dimensions of depressor muscle motoneurons in *M. quadrispina*, *Carcinus maenas* and *Procambarus clarkii*. Somata diameter and integrating segment size are given as range and mean (in brackets). Only the mean is given for axon diameter as the range was less than 10 percent. All measurements are in μm. Blank entries mean the data were not reported. References: (1) Bévengut et al., 1983; (2) Pearlstein et al., 1995; (3) El Manira et al., 1991.

Table 5.2

Species	Somata	Somata		Integrating segment size		Axon	
Muscle head	number	Position	Diameter	Length	Dia.	Dia.	n
M. quadrispina	16		31-73 (48.2)	225-515 (330)	14-64 (42)	7.3	146
Caudal	11	Caudo-medial	33-73 (49.6)	225-480 (322)	21-64 (43)	7.3	114
Rostral-straight	3	Rostro-lateral	31-71 (48.2)	245-295 (268)	14-49 (36)	8.2	18
Rostral-branched	2	Rostral	31-52 (37.1)	420-515 (485)	20-51 (39)	6.5	14
C. maenas[1]	10	Caudal (8) Rostral (2)	20-80				
P. clarkii[2, 3]	12-13	Caudo-medial					

Fig. 5.14. Camera lucida drawings of neurons backfilled from the caudal, coxal, and dorsal depressor heads in *M. quadrispina*. (A) The coxal and dorsal heads share innervation: five caudal neurons, one lateral neuron, and the putative common inhibitor (CI). (B) Backfills of the nerve to the caudal head reveal six caudal neurons, one lateral neuron, and the putative common inhibitor. The heavy dashed lines represent the midline; the fine dashed lines outline the neuropil areas; the open arrow indicates rostral for both illustrations; the small arrows indicate the nerves through which the neurons were filled; the arrowheads point to the ganglionic sheath.

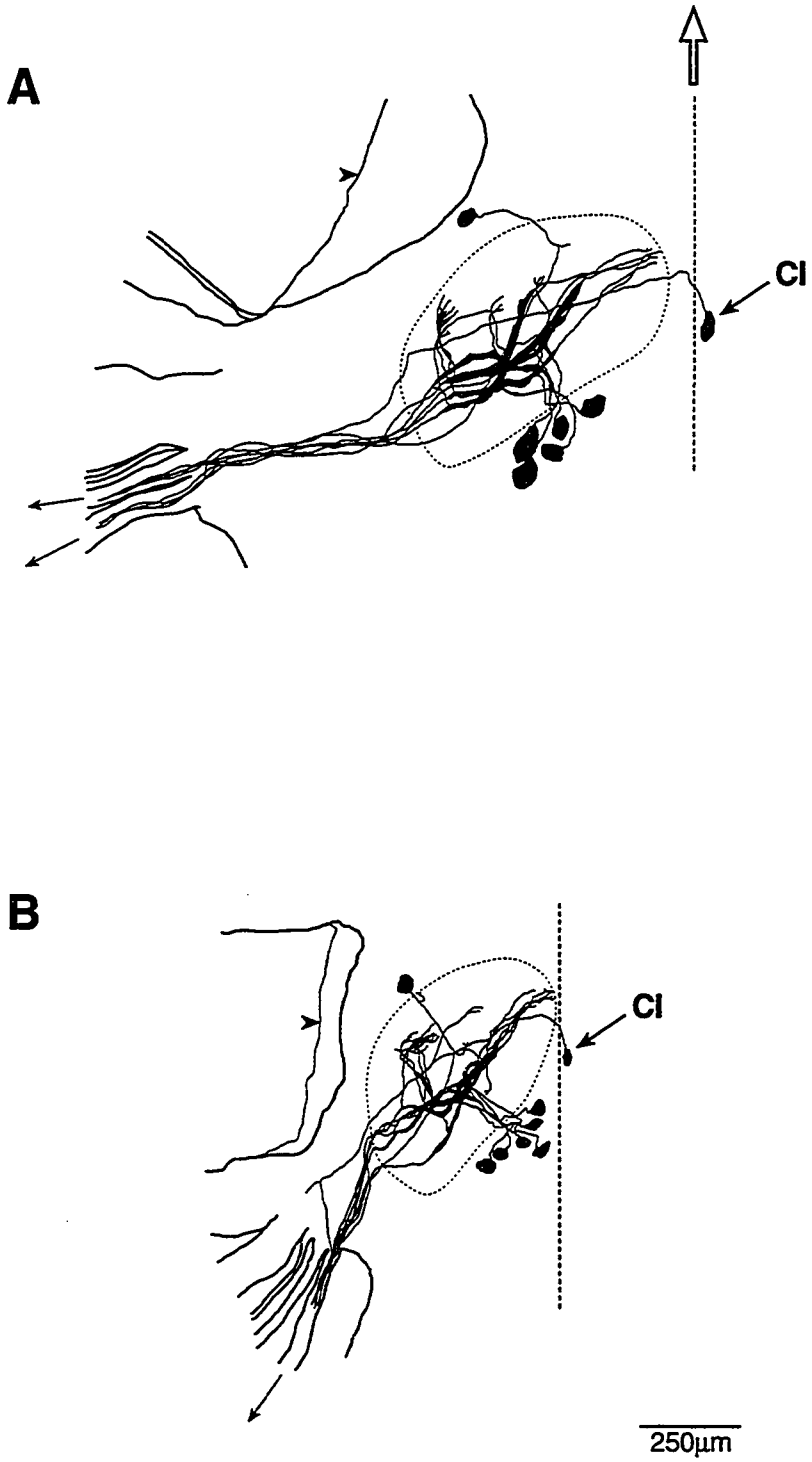
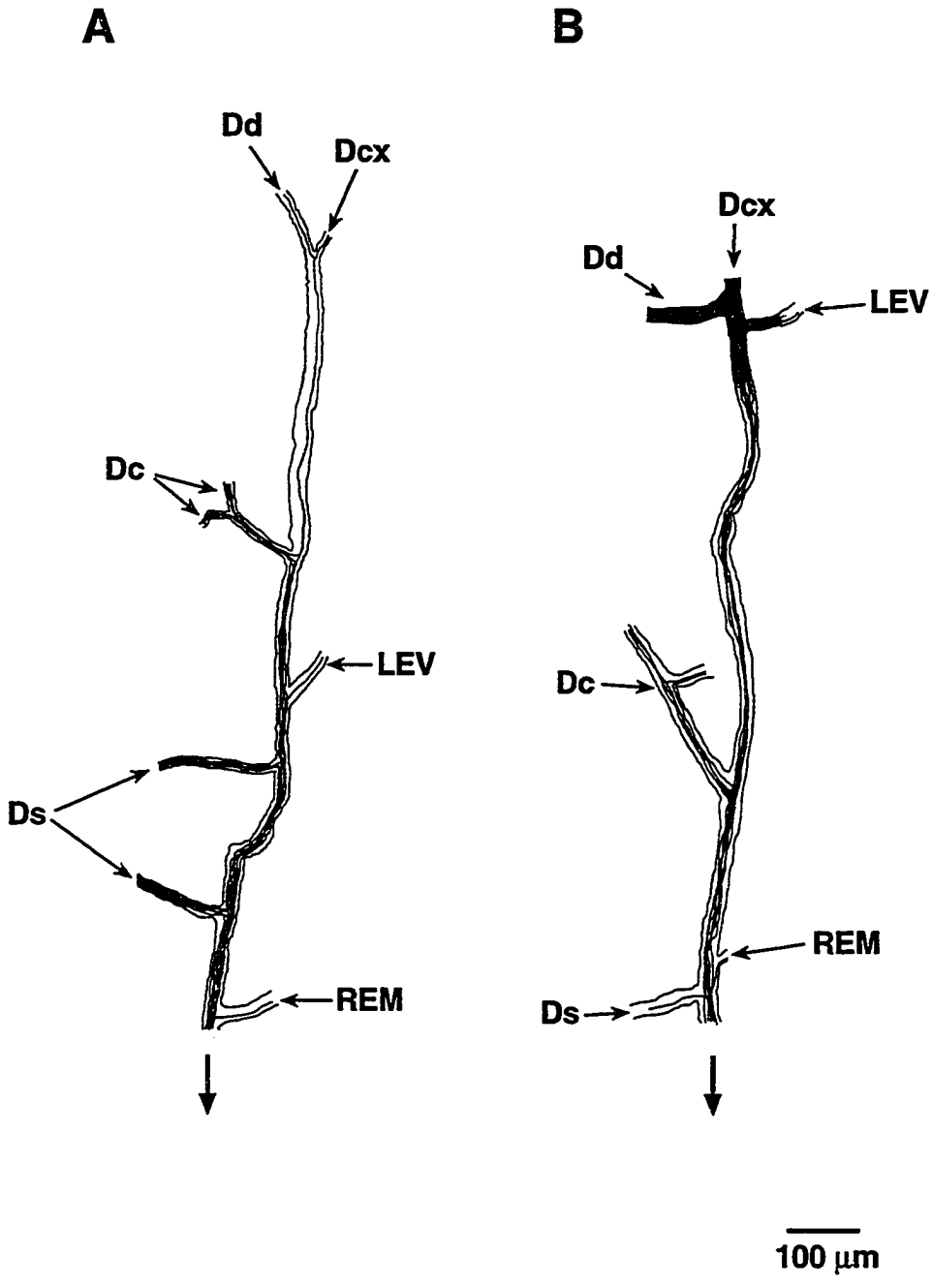

A
Cl
B
Cl
250µm

Fig. 5.15. Camera lucida drawings of peripheral branching patterns of axons innervating the depressor muscle heads in *M. quadrispina*. The dark peripheral ends are caused by extracellular filling, which obscures some detail in these very short nerve branches. The arrows point proximally, towards the ganglion. (A) A backfill from the sternal head (Ds), showing branches of four axons entering the nerve branch to the caudal head (Dc), one of which also sends branches into the nerves leading to each of the coxal (Dcx) and dorsal (Dd) heads of the depressor muscle, and the levator (LEV) and remotor (REM) muscles. (B) A backfill of the nerve branches to the dorsal and coxal heads of the depressor muscle, showing branches of three axons leading to the caudal head, one of which also branches to each of the sternal head of the depressor muscle, the levator and the remotor. One of the axons is greatly enlarged at its branch point to the caudal head.


A
Dd
Dcx
Dc
LEV
Ds
REM
B
Dcx
Dd
LEV
Dc
REM
Ds
100 µm

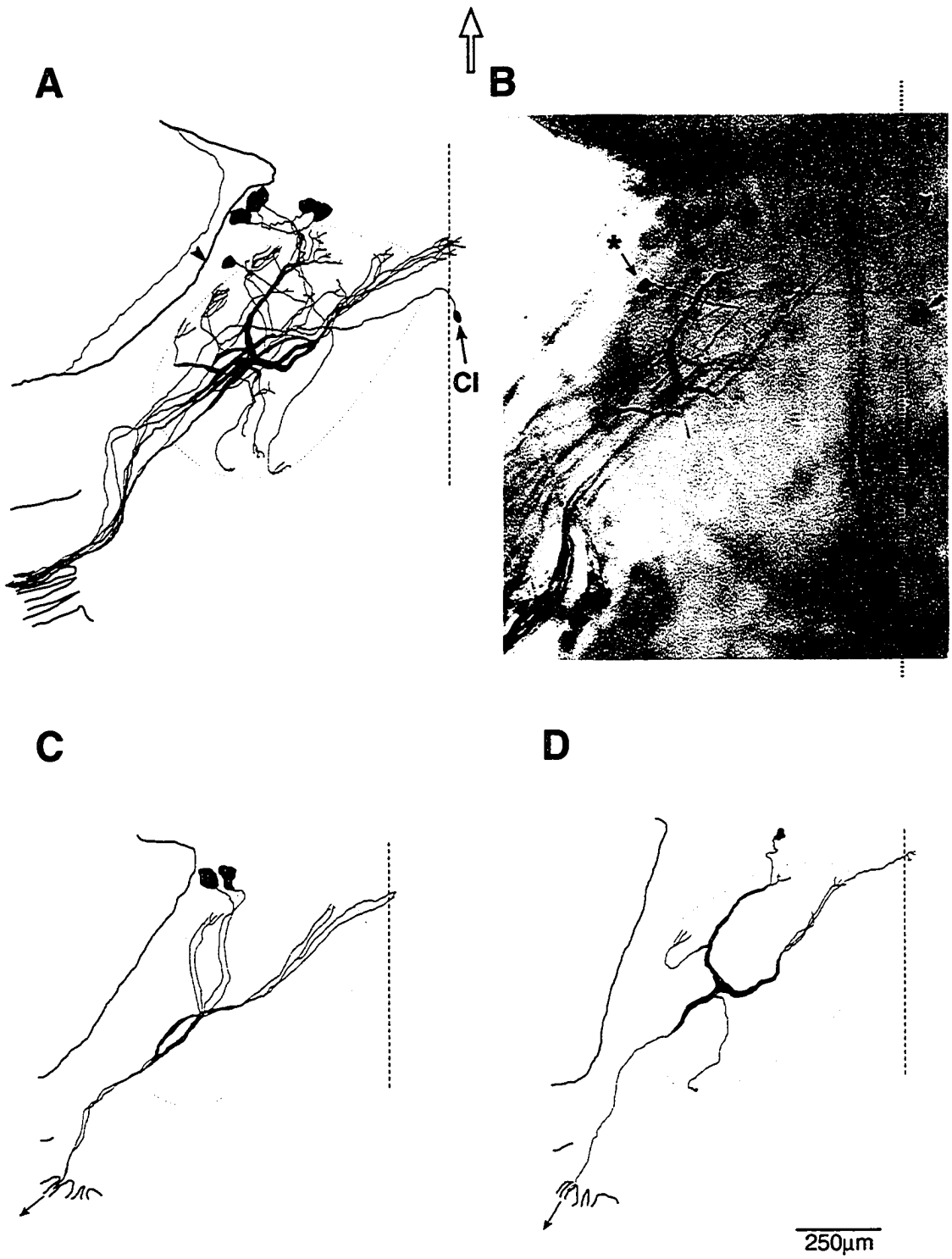
and caudal heads of the depressor muscle. Backfills of the nerve branch to the coxal and dorsal heads reveal three axons which branch and send processes to the caudal heads, one of which also sends branches to the sternal head, and to the levator and the remotor muscles (Fig. 5.15B). This suggests that the common inhibitor and two other motoneurons are shared between the caudal and coxal-dorsal heads, and only the common inhibitor is shared between the sternal and coxal-dorsal heads. Backfills from the caudal head reveal three branches leading to the coxal and dorsal heads, four leading to the sternal head, and one leading to the levator and remotor muscles. These data indicate that of the six caudal motoneurons backfilled via nerves leading to the caudal head of the depressor muscle, one is unique to this head, while three are shared with the sternal head, and two with the coxal-dorsal heads. Therefore, there are a total of 11 neurons in the caudal group: three unique to the coxal-dorsal heads, two unique to the sternal head, one unique to the caudal head, and five shared.

Backfills from the sternal, caudal, and coxal-dorsal heads never revealed processes entering the nerve leading to the ventral heads of the depressor muscle, although branches of the putative common inhibitor were seen entering the caudal distal leg nerve. Likewise, in backfills from the ventral heads of the depressor muscle, branches of the common inhibitor, but no other axons, were seen to enter the thoracico-coxal nerves, but did not project as far as the branches leading to individual heads of any muscle. The long distances involved may explain why common inhibitor branches to all the depressor heads were not filled.

Backfills of each of the two ventral heads of the depressor, the rostral and caudal, revealed that they share innervation. These heads are innervated by seven neurons, five with somata in a rostro-lateral group, one neuron with a lateral soma, and the common inhibitor (Fig. 5.16A & B). Clear branching in the periphery of all seven axons leading to the two ventral heads was seen, but is impossible to diagram clearly due to the overlapping axons in these small nerve branches. Depressor motoneurons with their somata in the rostral part of the ganglion have been described in crabs (Bévengut et al, 1983), but not for crayfish (Skorupski & Sillar, 1988; El Manira et al, 1991A). This is the first description of distinct separation of rostral and caudal motoneurons innervating different heads of the depressor muscle.

Two distinct morphological types of cells occur in the rostro-lateral group, one with a straight (Fig. 5.16C) and one with a branched (Fig. 5.16D) integrating segment. The somata, integrating segments, and dendrites of both types of rostro-lateral cells are at

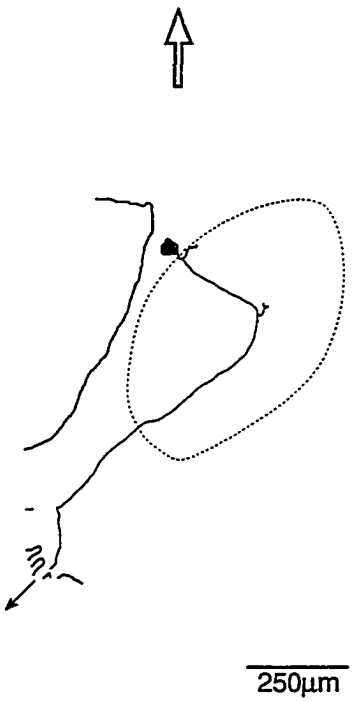
Fig. 5.16. Neurons backfilled from the ventral heads of the depressor muscle. The rostral and caudal ventral heads share innervation. (A) Five neurons in a rostral group, one lateral neuron, and the putative common inhibitor (CI) are filled. The arrowhead points to the ganglionic sheath. (B) A photomicrograph of the neurons innervating the ventral heads. The somata of the rostral motoneurons are out of the focal plane of this photomicrograph. The arrowhead indicates the processes which cross the midline; the double arrowhead indicates the putative common inhibitor; the asterisk indicates the unusual lateral cell (see Fig. 5.17). (C) Two typical neurons with unbranched integrating segment. (D) A typical example of a neuron with a branched integrating segment. The heavy dashed lines represent the midline (broken in (B) to avoid obscuring detail); the fine dashed lines outline the neuropil areas; the open arrow indicates rostral for all illustrations; the small arrows indicate the nerves through which the neurons were filled.


A
B
CI
C
D
250µm

similar levels in the dorso-ventral axis as for the caudal motoneurons described above, although the integrating segments of these cells are located more rostrally and laterally within the neuropil area. The straight type is morphologically very similar to the motoneurons described for the other heads, except that the somata are in a rostral location, the integrating segments are considerably smaller (Table 5.2), and there is an additional dendrite that terminates just across the midline, medial to the contralateral neuropil area (Fig. 5.16C). This is, I believe, the first description of a depressor motoneuron with contralateral projections. Otherwise, the locations of the remaining projections are similar between the rostral and caudal neurons. The integrating segments of the branched type neurons are the largest for all of the cells filled (Table 5.2); they have a distinctive "Y" shape, with the medial dendrites arising from the more caudal branch, and the neurite leading to the soma projecting off the rostral branch (Fig. 5.16D). The somata of these neurons are typically, but not always, smaller than those of the straight neurons, and the efferent axons of the branched cells are usually larger in the ganglion, but in the nerve the axons usually cannot be distinguished on the basis of size (Table 5.2). The medial dendritic projections of these neurons are similar to those of the straight neurons, including the branch that terminates across the midline. They also project one large dendritic process to the lateral edge of the neuropil area where it terminates with a distinctive rostral bend at its end, as for the other motoneurons, but in an area slightly caudal to the others. The branched-type neurons also have dendrites in two areas not seen for the other neurons. One is a short, medially projecting dendrite off the rostral branch of the integrating segment, the second is a caudal dendrite projecting from near the branch point of the integrating segment (distal to it in the example in Fig. 5.16D).

One unusual cell was also seen in backfills from every depressor head (Fig. 5.17). Its soma is at the rostro-lateral edge of the ganglion about midway along the dorso-ventral axis. The neurite leaving the soma curves slightly dorsally and projects caudo-medially to the center of the neuropil area, at about the same level as the integrating segments of the motoneurons, where it makes a sharp lateral turn and the axon leaves the ganglion through one of the thoracico-coxal nerves. These cells have no enlarged integrating segments and only two small dendrites, one projecting rostrally near the soma and one projecting medially near the sharp bend in the neuropil, could be discerned. Although I was not able to conclusively determine how many of these lateral neurons innervate the depressor muscle, two lines of evidence suggest that there are more than

Fig. 5.17. A typical example of the unusual lateral neuron backfilled from every depressor muscle head. This drawing was made from a backfill from the dorsal head of the depressor. The fine dashed line outlines the neuropil area; the open arrow indicates rostral; the small arrow indicates the nerve through which the neuron was filled.


250µm

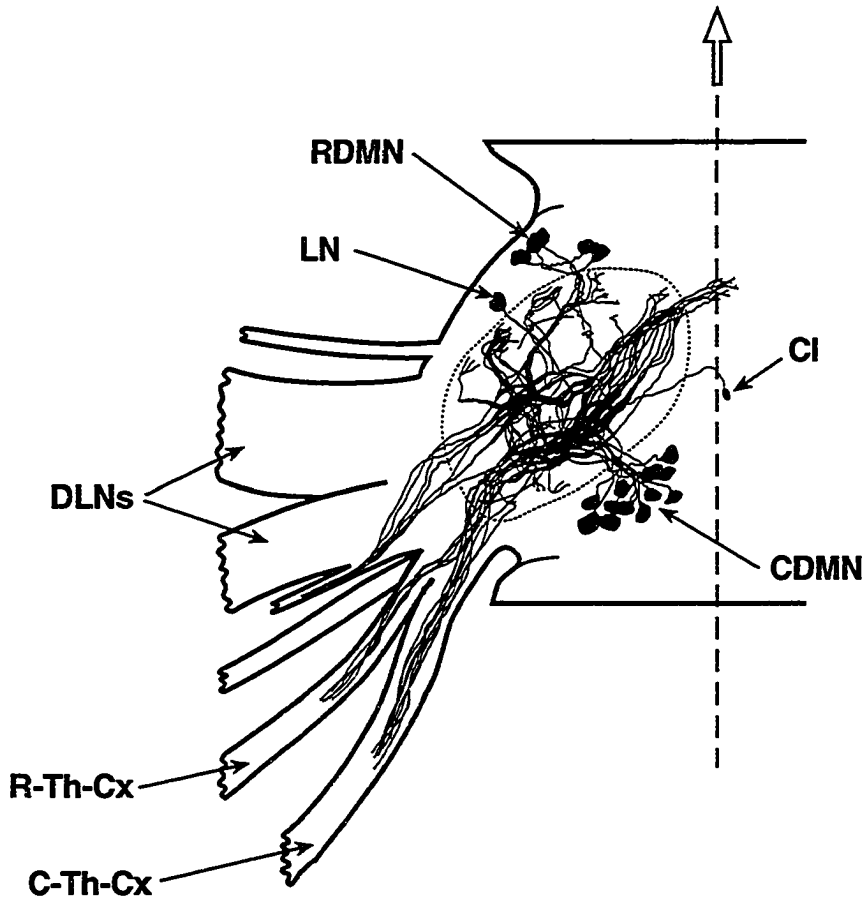
one. First, combined fills of more than one depressor head sometimes filled two of these cells. These fills were very difficult to do, because of the small size of the nerve branches involved, and nerve branches leading to other proximal muscles would sometimes be inadvertently filled; therefore, this observation alone is not conclusive evidence for more than one of these cells innervating the depressor. Second, in well-filled examples of this cell, branches leading to separately innervated heads of the depressor were never seen.

In total, backfills of the nerve branches leading to the six depressor heads revealed at least 18 neurons with central somata (Fig. 5.18). All of the somata are located around the edges of the neuropil areas, with those of the rostral and caudal groups and the common inhibitor located ventrally and the lateral neurons about mid level in the ganglion. The number of lateral neurons could not be confirmed, but evidence suggests it is likely more than one. Branching patterns suggest a total of 11 neurons in the caudal group and 5 in the rostro-lateral group, for a total of sixteen neurons with typical motoneuron-type morphology. The final motoneuron, the putative common inhibitor, has its soma contralateral to its axon.

Discussion

The most striking features of the skeletomusculature of the depressor and levator muscles in *M. quadrispina*, *C. bartonii*, and other species are the number of heads which comprise each of the muscles and the dispersion of the origins within the thorax and coxa. The most proximal pair of limb muscles, the promotor and remotor, are also comprised of multiple heads (White & Spirito, 1973; Bévengut et al., 1983; Antonsen & Paul, unpublished observations). However, in most species the promotor and remotor muscles have only two or three heads, and although the origins can be widely spaced in the thorax, the heads lie in parallel to each other and the directions of their force vectors are more or less similar. This is somewhat less true in *M. quadrispina* than in some other species, as only two of the three heads of each muscle in *M. quadrispina* are parallel while the other has a quite different orientation. The complex anatomy of the two basal muscles suggests that there may be underlying functional differences between these muscles.

Fig. 5.18. Composite drawing showing all neurons backfilled from depressor nerve branches in *M. quadrispina*. There are 11 neurons in the caudal group of depressor motoneurons (CDMN), five in the rostral group (RDMN), at least one of the lateral neurons (LN), and the putative common inhibitor (CI), for a total of at least 18. Neurons of the caudal group leave the ganglion by the rostral and caudal thoracico-coxal nerves (R-Th-Cx, C-Th-Cx), while those of the rostral group leave by a branch of the caudal distal leg nerve (DLNs). Lateral neurons have been filled through each of these nerves. The midline is represented by the heavy dashed line; the neuropil is outlined by the fine dashed line; the open arrow denotes rostral.


RDMN
LN
CI
DLNs
CDMN
R-Th-Cx
C-Th-Cx

Functional implications of depressor and levator muscle anatomy

The functional anatomy of the levator muscle has been described in crabs, where it has roles in postural and locomotory behaviours, as well as limb autotomy (Moffett, 1975; McVean & Findlay, 1976). The relative positions of the heads, or more specifically the directions of force vectors produced by their contractions, are critical for limb autotomy. Preliminary electromyographic data suggest that the heads of the levator muscle have similar roles in *M. quadrispina* (Antonsen and Paul, unpublished observations). The dorsal, coxal, caudal and sternal heads of the depressor muscle in *M. quadrispina* are active during limb autotomy (Antonsen and Paul, unpublished observations), but they do not appear to be activated in particular patterns as are the heads of the levator muscle. During autotomy one massive burst occurs simultaneously in all four of these depressor muscle heads and may act to stabilize the joint, facilitating the complex actions of the levator muscle.

All of the pereiopod muscles in walking decapods have roles in both forward and backward walking, but arguably it is the basal musculature that has the most important role in maintenance of stance. This is because the basal muscles are responsible for elevating and depressing the leg and, therefore, raising and lowering the animal, with the depressor bearing most of the responsibility for supporting the weight of the animal. This role could shift among muscles in animals, such as *M. quadrispina*, which live on irregular terrain and spend significant portions of their time in orientations other than upright. In such species, the promotor, remotor, and levator muscles could at certain times, depending on orientation, be directly responsible for supporting the animal's weight. This could explain the somewhat more complex anatomy of the promotor and remotor muscles in *M. quadrispina* as compared to species that maintain a more or less upright stance, but I did not look at this question in detail. In Chapter 6, I describe how the heads of the depressor muscle of *M. quadrispina* are activated differentially during different behaviours, and I hypothesize about why this division of function may have occurred. Briefly, the sternal head is the main depressor (mover) of the pereiopod, and the dorsal/coxal pair is responsible for maintaining the animal's stance against gravity, the caudal head is active synergistically with the sternal head and the dorsal/coxal pair. The function of the ventral heads is not immediately apparent, but may involve maintenance of joint tension or stance under different circumstances than those in which the dorsal/coxal pair perform this role.

The mechanics of the force applied by the heads of the depressor and levator muscles to the coxal-basal joint are extremely complicated and beyond the scope of this study. However, elementary physics dictates that force is applied more effectively in a direct line than at an angle. Therefore, the most effective direction to apply force for depression of the coxal basal joint should be perpendicular to the plane between the condyles of the joint and the ventral lip of the basis. In *M. quadrispina* all of the heads of the levator muscles are oriented close to this vector. In *C. bartonii*, the heads of the rostral levator are also oriented in this manner, but the heads of the caudal levator are not, and therefore are probably somewhat less effective than they could be at moving the joint. Only the rostral depressor is oriented perpendicular to the lip of the basis in *C. bartonii*, while the heads of the caudal depressor apply force in a more dorsal direction. There is a feature of the ventral lip of the basis, however, which may increase the effectiveness of this arrangement. The ventral lip of the basis projects quite far medially, and has a distinct dorsal bend near its tip (most easily seen in Fig. 5.7B), which effectively rotates the line between the condyles and the lip of the basis bringing it closer to perpendicular to the long axes of the muscles. The sternal, caudal, and ventral heads of *M. quadrispina*'s depressor muscle apply force along a relatively effective vector, but the dorsal and coxal heads do not. Furthermore, to allow for the angular pull of these two heads relative to the sternal and caudal heads, a rather complex apodeme has evolved. It is reasonable to assume that ancestrally the depressor muscle had a head or heads originating in the coxa. In extant decapods, the coxa is relatively short, while the angular excursion of the coxal-basal joint is quite large. The cross-section of the coxal head is quite large; if this muscle were oriented along the ventral surface of the coxa, the origins of some of the fibers would almost certainly be too far distal and, therefore, the fibers would be too short to accommodate the entire movement range of the joint. Dorsal displacement of the origin of this head may therefore be an adaptation to increase the length of the head, and thereby allow it to maintain a large cross section and retain strength. In the location of the origin of the dorsal head we may be seeing the effects of space considerations in the thorax. Essentially every exposed surface of the endophragmal skeleton in each arthrodial cavity is covered with muscle origins; there simply may have been no more room along a more mechanically advantageous vector. Alternatively, as the dorsal head evolved to function with the coxal head, its orientation may have also shifted to be parallel with it.

There are considerable differences between species in the origins and orientations of the heads of the basal muscles. I cannot determine at this point whether these differences are related to functional differences between the species or anatomical differences in the skeletal framework. Bévengut et al. (1983) describe some of the functional differences between muscles of the fifth pereiopods in two crab species with different behaviours, but their comparison does not extend to differences in the orientation of the muscle heads. Although there are substantial similarities in the organization of the pereiopod 2 basal musculature between *M. quadrispina* and crabs (Cochran, 1935), I cannot determine if this is due to retention of form from a common ancestor of the closely related anomurans and brachyurans (Williamson, 1976, 1982; Rice, 1980, 1981A, 1981B, 1983), or whether it is due to convergent evolution. Furthermore, if it is due to convergent evolution, was similarity of function or similarity of the underlying endophragmal framework the underlying influence?

Differences also exist between the organization of the basal muscles between serially homologous pereiopods in a single species. The organization of these muscles in the second and third pereiopods in *M. quadrispina* is quite similar, but there are substantial differences in the fourth pereiopod, such as loss of the dorsal, caudal, and ventral caudal heads of the depressor muscle. The orientations of the proximal pereiopod joints between the fourth and second/third pereiopods are quite different, as are the roles of these limbs in walking and stance maintenance. I did not look closely at differences between the innervation or activity patterns in the different pereiopods, but examining them within a single species could be a valuable source of information on the biomechanics of muscular systems.

Differences in innervation patterns among the heads of the depressor muscle

The only other study I am aware of which has described differences in innervation patterns between the heads of the depressor muscle is by White & Spirito (1973) on the crab *Callinectes sapidus*. However, in this study only five or six excitatory motoneurons are described, which is probably only a fraction of this muscle's innervation. The total number of motoneurons, not including the common inhibitor, backfilled from the depressor muscle of *M. quadrispina* is somewhat higher than the number found in crabs (10; Bévengut et al., 1983) or crayfish (12; Pearlstein et al., 1995). In crayfish, all of these motoneurons are located caudally in the ganglion, while in the crab, two are rostrally-located, one rostro-lateral and one rostro-medial, and the remaining eight are

caudal. The dendritic fields of all of the depressor motoneurons in *M. quadrispina* overlap extensively, facilitating connections between the motoneurons, common synaptic inputs, and inputs from populations of sensory neurons.

Contralateral projections of motoneurons are, in general, typical of bilaterally synchronous motor systems (Wine et al., 1974; Altman & Kien, 1985). Walking in decapod crustaceans is bilaterally asynchronous (Clarac & Barnes, 1985). Postural changes may be bilaterally synchronous on a flat substrate, but this is likely due to chance rather than design. *M. quadrispina* is the only species shown to have depressor muscle motoneurons with processes which cross the midline. Contralateral projections could allow for direct coupling of bilateral motoneurons, bilateral synaptic inputs, or even bilateral innervation of muscles (Altman & Kien, 1985). Even in the best backfilled cells, the contralateral processes terminated just across the midline, and did not project even as far as the contralateral neuropil, so the potential for contralateral interactions appears somewhat limited. However, their projections would allow coupling with contralateral ventral head motoneurons, and recently Pearlstein et al. (1994, 1998A & B) have shown that glutamatergic synaptic connections between proximal muscle motoneurons are inhibitory, raising the possibility that these motoneurons could be reciprocally inhibiting each other. These cells are different from other depressor motoneurons in *M. quadrispina* and other species in several additional ways: they project to the periphery through a different nerve, they have, except for one or two depressor motoneurons in crabs, unusual somata positions, and at least some of the cells (the Y-shaped ones) have an unusual central morphology. In addition to this, the ventral heads are activated generally out of phase with the rest of the depressor muscle heads during walking and posture maintenance (see Chapter 6). It is clear that these cells are very unusual, and more work is needed to determine their evolutionary and functional relationships to the rest of the depressor muscle's innervation.

Chapter 6: A division of labour among heads of the multifunctional depressor muscle of Munida quadrispina.[1]

Introduction

Individual limb muscles are often used to perform many different behaviours, such as walking, swimming, standing, and courtship behaviours. In many instances, single muscles are not activated as a homogenous unit; subdivisions among heads or regions exist which influence how a muscle is activated during behaviours. Examples include groupings of muscle fibers with similar physiological properties (e.g. fast and slow fibers) within an otherwise undivided muscle in arthropods (Müller et al., 1992; Günzel et al., 1993), differential activation of subdivisions of multi- or single-tendoned muscles in mammals (Chanaud & MacPherson, 1991; Fritz et al., 1992; Schieber, 1993; Schieber et al., 1997), restricted innervation fields of swim motor neurons of the pteropod mollusc *Clione limacina* (Satterlie, 1993), and differential activation of anatomically separate heads of the basipodite levator muscle in crabs during locomotory behaviours and pereiopod autotomy (Moffett, 1975; McVean & Findlay, 1976).

The proximal pereiopod muscles of decapods, the promotor, remotor, levator, and depressor muscles, are anatomically complex, each consisting of more than one head. Evolutionary duplication of individual muscles is common in animals, and although duplication is not always correlated with functional disparity, some authors believe that increased complexity of the muscular units mediating behavioural events may be indicative of increased functional diversity within a taxa, and may release evolutionary constraints on neuromuscular systems allowing functional diversity among the duplicated elements to evolve (Liem, 1980; Lauder, 1981, 1986 & 1991; Smith, 1994; Schaefer & Lauder, 1996; Friel & Wainwright, 1997 & 1998).

Despite the obvious complexity of the proximal pereiopod muscles in decapods, very few studies have been published concerning differential activation of their heads. In addition to the studies on crab levator muscles mentioned above, a study by Clarac (reported in Bévengut et al., 1983) indicated that the two heads of the remotor muscle of *Carcinus maenas* are activated together during walking, and Clarac et al. (1978) reported different activity patterns in the two levator muscles of crayfish in response to stimulation of the coxal-basal chordotonal organ, while the rostral and caudal depressor muscles are

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[1] Parts of this chapter have been published in abstract form (Antonsen and Paul, 1995 & 1997)

activated in a qualitatively similar, but not identical, manner. Furthermore, with a few exceptions (Hoyle, 1973; White & Spirito, 1973; Tse et al., 1983; Moffett et al., 1987), innervation patterns and distributions of muscle fiber types are largely unknown among the proximal muscles, although they have been studied extensively in the distal pereiopod muscles (e.g., Govind et al., 1981; O'Connor et al., 1982; Costello & Govind, 1983; Wiens et al., 1988 & 1991; Wiens & Govind, 1990).

Recent studies in crayfish have revealed differences in synaptic input to depressor motoneurons from sensory systems involved in locomotion and posture (El Manira et al., 1991A; Leibrock et al., 1996; Le Ray & Cattaert, 1997), and that different pools of depressor motoneurons are active during fictive posture and fictive locomotion (Pearlstein et al., 1995). I undertook this study when I found that electromyograms (EMGs) from different areas of the depressor muscle of the squat lobster *Munida quadrispina* reveal different activation patterns (Chapter 4). Here I report, for the first time in a decapod, differential activation of the heads of the depressor muscle that suggests a subdivision of labour among the heads under different behavioural circumstances.

Methods

Munida quadrispina were collected and kept as described in Chapter 2. One hundred and seventy six specimens were used, ranging in size from 2.9-3.7 cm carapace length. Very small animals were not used, as it proved too difficult to target the individual heads in these animals. No correlation was found between animal size or sex and depressor muscle activity. All recordings were done in a small aquarium with recirculating 10°C seawater and a flat, textured bottom that allowed the animals to move without slipping. Serotonin (5-HT) and octopamine (OA) were injected as described in Chapter 2. All observations on amine-injected animals were done between two and 15 minutes after injection, after which the influences of the injections decreased.

Movement recordings

Movements and proximal joint angles were recorded using a Panasonic Super-VHS PV-S770 video camera at 30 frames per second (period = 33 ms). The electronic shutter of this camera opens for less than 1 ms per frame, producing a very sharp image even during *M. quadrispina*'s fastest walking. A mirror was placed at a 45° angle in the

aquarium so the top and side of the animal could be seen by the camera simultaneously. The video was synchronized with the physiological recordings via a device which simultaneously triggers a small LED in the camera's field of view and sends a pulse to a dedicated channel on the tape recorder used to store EMG activity (see below).

Due to *M. quadrispina*'s sedate nature (they often spend hours without moving), for most animals I was forced to induce postural changes or walking. I did this by passing a small, round, red shape in front of the animals, but outside the tank. This stimulus was not meant to mimic anything in particular, and I have no idea what the animals might have recognized it as. However, it often resulted in the animals walking or raising their bodies above the substrate and holding this position for several minutes or more before lowering themselves. Sometimes the animals responded to the stimulus with an agonistic or defensive behaviour; these trials were analyzed separately.

Electromyograms

Electromyograms (EMGs) were recorded from each of the heads of the pereiopod 2 depressor muscle during postural behaviours and walking. In *M. quadrispina*, the second pair of pereiopods is the first walking legs; the first pair of pereiopods is chelate. Recordings were made through electrodes of 76.2 μm diameter silver wire insulated with Teflon except at the tips. A few recordings used Teflon-coated 50μm diameter tungsten wire, which is much stiffer than the silver wire and easier to implant reliably, but produced recordings with lower signal-to-noise ratio.

Implants were made in animals anesthetized by cooling in 1-2°C seawater, which improved survival and decreased the incidence of limb autotomy during the procedure. The animals were allowed to recover for 12-24 hours before any recordings were made. A maximum of four differential or single ended implants were done in any animal, as the time required for four implants was about the maximum an animal could spend out of water and still recover fully and rapidly, and additional wires began to impede the animals' movements. Differential recordings were made with two recording electrodes inserted into the muscle, and single-ended recordings with one recording electrode implanted in the muscle and a chloridated silver reference electrode immersed in the aquarium. In general, single ended recordings produced EMGs with more consistently shaped muscle potentials, but also with much poorer signal to noise ratio and larger movement artifacts, and therefore most experiments used differential electrodes.

In order to reduce movements of the muscle relative to the recording electrodes, leads were implanted within the muscle as close to the origins of the muscle heads as possible through holes drilled in the exoskeleton using a 100 μm carbide-tipped drill bit. Electromyographic cross-talk, where activity from an adjacent, non-target muscle is picked up by recording electrodes, can cause misleading traces in cats (English & Weeks, 1989), but as far as I am aware, no detailed studies on cross talk have been done in crustaceans. The small size of *M. quadrispina* and close proximity of nerves and muscle heads usually made recordings via electrodes implanted alongside the muscle useless because multiple extraneous field potentials were superimposed on the muscle's potentials. However, implanting recording electrodes fully in the body of the target head significantly decreases the occurrence and amplitude of cross-talk from other muscles or heads. Furthermore, because of the proximity of the heads near the base of the depressor apodeme, electrodes could not be reliably implanted into particular heads in this area, and activity was often recorded from multiple heads simultaneously (see Chapter 4). The electrodes were fixed to the cuticle by drying the area around the implant, sealing the electrode wire in the hole with a small bit of wax, then placing glue (Zap-A-Gap, a type of cyanoacrylate) over the wax and wire. Recordings could usually be made from such electrodes for five to seven days, before scar tissue around the electrode diminished the signal. Placement of all electrodes was confirmed by dissection following the experiments, and only recordings from electrodes that were fully inserted into the muscle head of interest were used. Due to the small size of the muscle heads and the difficulty in placing recording electrodes, usually only one or two of the pairs of electrodes were usable in any animal. In only four animals were all four of the electrode pairs useful.

Recording wires were fixed with wax at points along the exoskeleton in a way that did not impede joint movements, but protected them from damage due to the animals' movements and from being pulled out by the animal. At the dorsal midline, they were embedded in a wax saddle which was glued to the surface of the carapace, and then passed through a length of thin, highly flexible latex tubing to a float. This arrangement did not appear to hinder movements of the animal. Leading from the float were wires attached to a mini-connector that plugged into the recording preamplifier (Tektronix TM503). EMGs were simultaneously displayed on an oscilloscope (Tektronix 5113) and stored on an 8-channel Vetter D1 frequency modulated reel-to-reel tape recorder.

Data analysis

Videotape was analyzed by manually noting movements and measuring joint angles from individual frames displayed on a television. Tests using drawings of *M. quadrispina* and pre-positioned frozen animals which had been killed for use in other experiments but which had not had their thoraces damaged indicated that I could record joint angles in this way to within 1° of accuracy. Only recordings made from animals oriented within about 5° from facing the camera straight on were used, as accuracy of coxal-basal joint measurements fell if the animals were turned further away from the camera.

EMGs were digitized using a Labmaster TL-1 analogue to digital converter and Axotape 2 software (Axon Instruments). EMG traces were synchronized with the coxal-basal joint angles and pereiopod movements recorded from the video. Statistical analyses were done using SPSS (SPSS Inc.). To calculate EMG spike frequency as a function of coxal-basal joint angle velocity, EMG traces were synchronized with the video recordings, then spike frequencies and joint velocities were calculated for each inter-frame period ($1/30^{th}$ of a second). To ensure that I was not recording during changing joint velocities, I selected only records in which at least five sequential video frames showed a constant joint velocity, and the corresponding EMG traces showed constant spike frequencies. From these sequences, I eliminated the first and last frame, and calculated averages based on the remaining frames.

Results

Posture and locomotion in freely moving M. quadrispina

M. quadrispina are usually quite sedentary, squatting on the tips of their walking pereiopods and claws, and on their folded abdomen (see Chapter 2). When alerted, animals will often hold their claws above the substrate, or raise their bodies by depressing their walking pereiopods. Postural changes are fairly easy to elicit, as animals will often react to visual stimuli by raising their bodies and orienting towards the stimulus. The animals lower themselves back to the substrate after the disturbance has passed. After feeding they often hold a raised stance for extended periods of time.

Walking only occasionally occurred spontaneously, and could only rarely be induced by prodding, as even the gentlest touch usually causes the animals to tailflip away. Placing food in the tank seldom worked because, if the animals were interested in

the food, they usually ran towards it with such vigor as to make analysis of the EMGs impossible due to large movement artifacts. The visual stimuli mentioned above sometimes induced animals to walk, and when this or spontaneous walking did occur, it often lasted for a minute or more as the animals explored the aquarium. About 90% of the walking episodes observed during these experiments were either forwards or backwards. Lateral walking does occur, but it is more common on uneven substrates and the episodes recorded during the experiments lasted a maximum of two step cycles. Raising the body off the substrate precedes all forms of walking.

Differential activation of the heads of the depressor muscle

Analysis of EMGs from the six heads of the depressor muscle (see Chapter 5 for anatomical descriptions) during stance maintenance and walking revealed four different activation motifs. This activational disparity suggests a division of functional roles between the heads of the depressor muscle. This is the first demonstration of distribution of function among the heads of a depressor muscle, although division of labour among the heads of the levator muscle of crabs has been documented (Moffett, 1975; McVean & Findlay, 1976)

Co-activation of depressor muscle heads

EMG potentials in the dorsal and coxal heads of the depressor muscle match one to one (Fig. 6.1). This was expected since these two heads share innervation (see Chapter 5). Similarly, the two ventral depressor muscle heads (rostral and caudal) share innervation and are activated coincidentally (Fig. 6.2). Recordings made during many postural and locomotory behaviours confirmed that co-activation within these two pairs of heads is consistent. Several points suggest that these heads are truly co-activated and these results are not artifacts caused by electromyographic cross-talk. First, dissections confirmed that the recording electrodes were fully implanted in the target head in all animals used for analysis. Second, the delays seen in some animals were too long to be consistent with extracellular conduction. Third, in a small number of animals, one or two spikes were recorded from one head which were not simultaneously recorded from its synergist. In subsequent sections in this chapter I report results from one head of each pair only, usually the dorsal and ventral-caudal heads, because my experimental apparatus could record only four channels and I wanted to have each of these pairs of heads, plus the sternal and caudal heads, represented in most experiments. The dorsal

Fig. 6.1. Coincident activation of the dorsal and coxal heads of the depressor muscle. (A), (B), and (C) show the same trace on increasingly faster timebases. The arrow in (C) indicates the onsets of one pair of coincident muscle potentials, which in this animal are synchronous. Dcx, Coxal head of the depressor muscle; Dd, dorsal head of the depressor muscle.

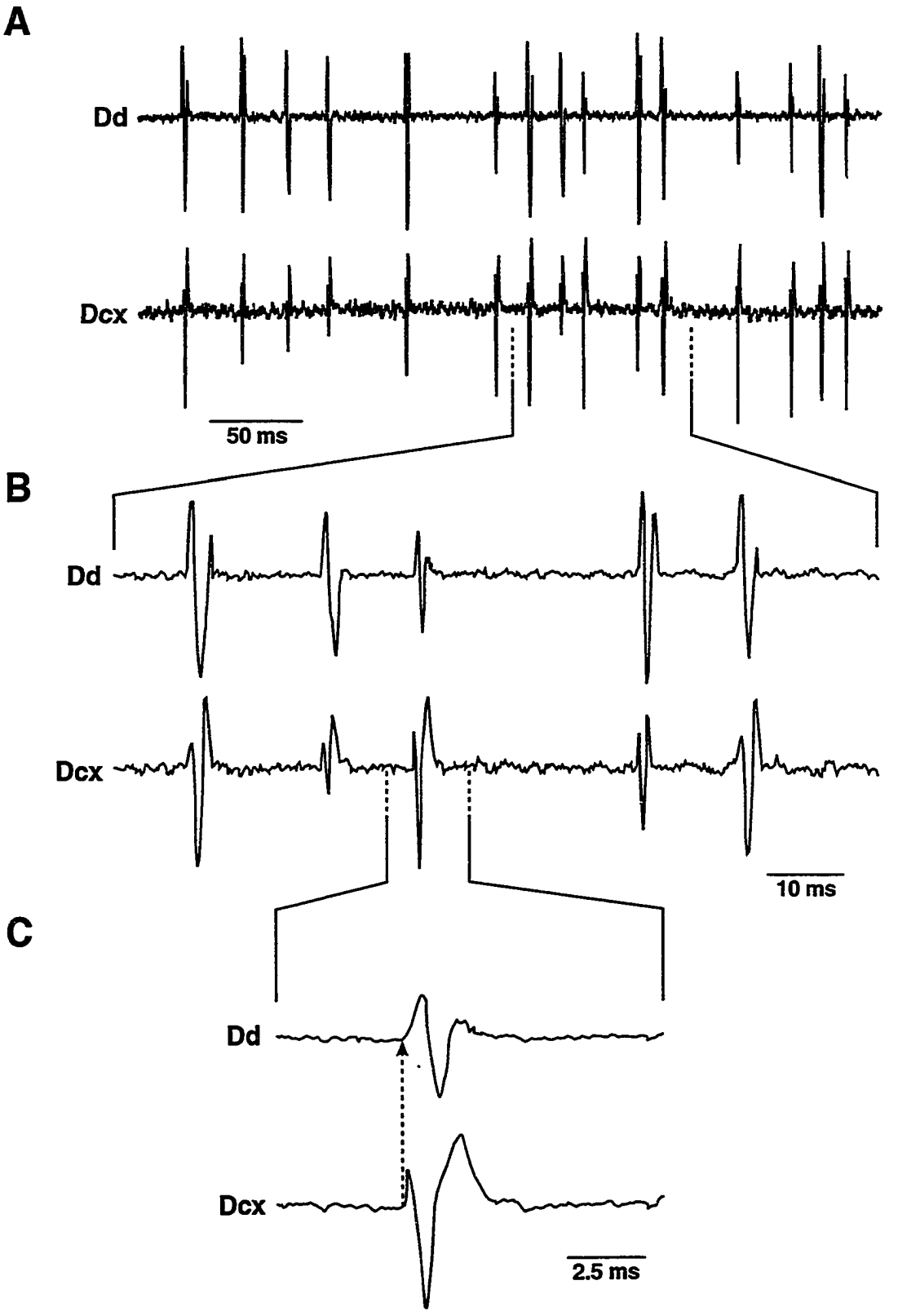
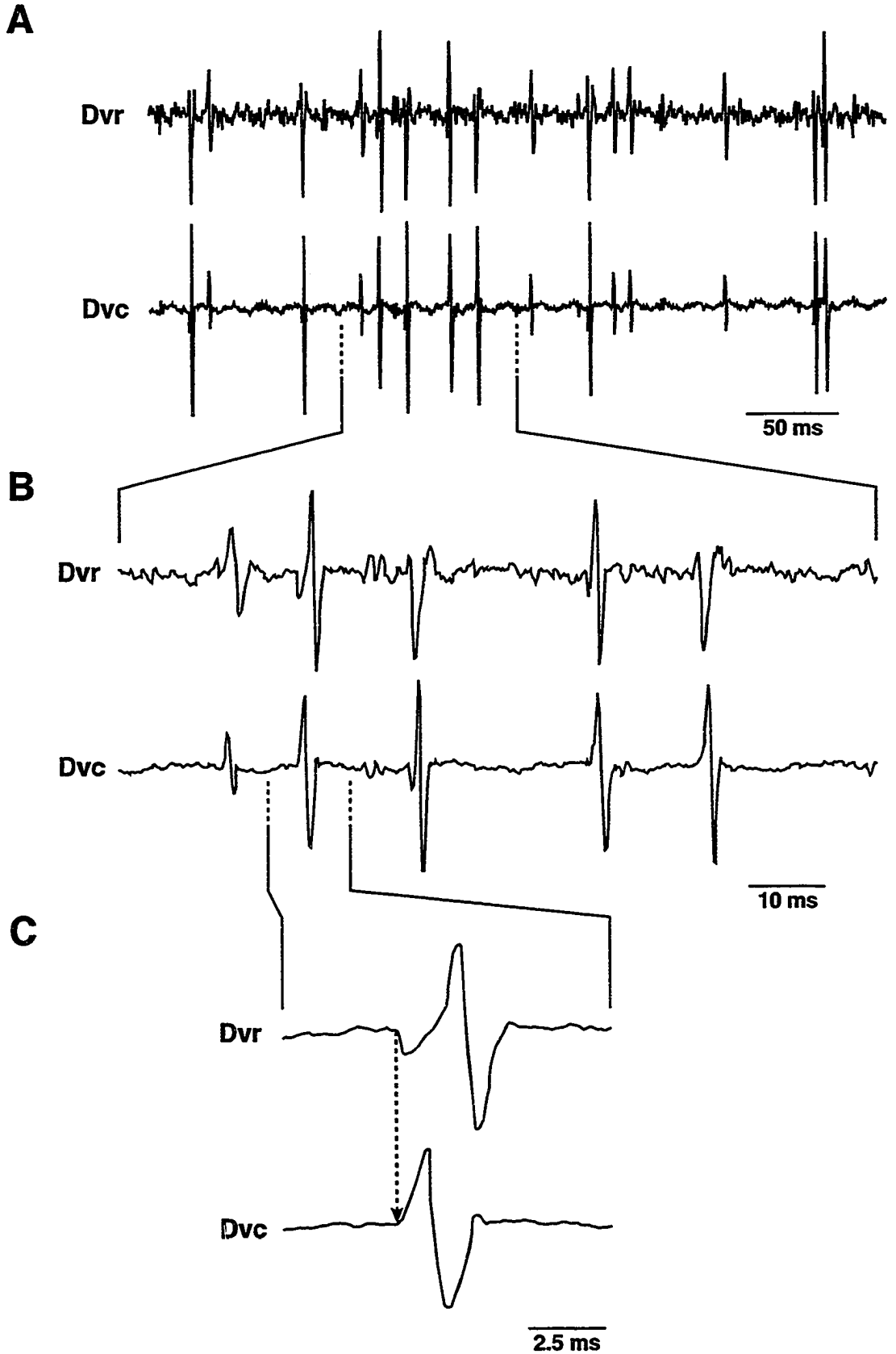

A
Dd
Dcx
50 ms
B
Dd
Dcx
10 ms
C
Dd
Dcx
2.5 ms

Fig. 6.2. Coincident activation of the two ventral heads of the depressor muscle. Dvc, ventral caudal head of the depressor muscle; Dvr, ventral rostral head of the depressor muscle; other conventions as in Fig. 6.1.


A
Dvr
Dvc
50 ms
B
Dvr
Dvc
10 ms
C
Dvr
Dvc
2.5 ms

and ventral-caudal heads were easier to implant reliably and the EMG leads were less prone to being damaged by the animal's movements when implanted in these two heads than in the coxal and ventral-rostral heads.

Spikes in the two ventral heads are never separated by more than 0.05 ms (Fig. 6.2C). In some animals, such as the one used for Fig. 6.1, potentials are completely synchronous between the dorsal and coxal heads. In other animals, potentials in the coxal head are delayed up to 0.2 ms from those in the dorsal head, but potentials in the coxal head never precede those in the dorsal head. The timing of the spikes in these two heads depends on the relative distances the impulses must travel from the central ganglia to the recording electrodes. The position of the recording electrodes was fairly consistent between animals (within about 1mm or so for these two muscle heads), but the lengths of the nerve branches innervating the two heads are quite variable between animals.

The dorsal and coxal heads of the depressor muscle are innervated by the common inhibitor and five other motoneurons, as are the two ventral heads (see Chapter 5). However, no more than four, occasionally three, and rarely, in the ventral heads, only two distinct potentials (different waveforms) could be resolved. The number of distinct potentials which could be resolved was not consistent within an animal over time, but these changes were not consistently correlated with any particular behaviours of the animal, with one exception in the sternal and caudal heads (see *Depressor muscle EMG activity during walking and postural changes,* below). There are several possible explanations for this discrepancy. First, there could be a second inhibitory motoneuron among the populations innervating each of the two pairs of heads. However, with the exception of the common inhibitor, no crustacean limb inhibitory motoneuron described to date has a morphology similar to the motoneurons found to be innervating these muscle heads (see Chapter 5), and the only demonstrated peripheral inhibitory input to the proximal leg muscles of decapods is the common inhibitor (Bévengut & Cournil, 1990). A second possibility is that subpopulations of the excitatory motoneurons innervating each pair of heads are sometimes activated synchronously. Coupling of excitatory motoneurons has been demonstrated for proximal muscles of crayfish (Skorupski & Sillar, 1988; Chrachiri & Clarac, 1989). Additionally, some of the potentials seen during these experiments had a form which suggested that they were comprised of two or more overlapping potentials (e.g. see the comparison between the sternal and coxal heads in *Depressor muscle EMG activity during walking and postural changes* below). A third possibility is that there may be graded activation or some other

form of disparate control on populations of motoneurons innervating each pair of heads. As traces during very fast movements or walking were usually not interpretable and I did not record during any situations where heavy load may have been placed on the muscle, such as when the animal was pushing at something, I cannot discount the possibility of graded recruitment of motor neurons. Another possibility is that potentials produced by two different motoneurons could theoretically have waveforms too similar to be distinguished. Although I believe that the second possibility is at least partly responsible for the observed "loss" of distinct potentials, contributions from the other three possibilities, and others, cannot be ruled out.

Under ideal conditions, activity of individual motoneurons could be followed in recordings from a single pair of implanted electrodes. Waveforms of EMG potentials induced by individual motoneurons remained relatively consistent provided the electrodes were stable within the muscle (i.e. did not move as the muscle contracted and relaxed) and so long as scar tissue did not form over the electrode tips. Atwood and Walcott (1965) and Ayers and Clarac (1978) were able to identify EMG potentials induced by particular motoneurons in different individuals, but except in rare cases I was unable to do this. The form of an EMG potential is dependent on the properties of the muscle cells producing it (Hoyle & Wiersma, 1958; Atwood & Walcott, 1965), activity of peripheral inhibitory neurons (Atwood & Walcott, 1965), the positions of the recording electrodes relative to the active muscle cells, and insulating properties of the surrounding tissues. Since it was impossible to implant electrodes in exactly the same place within a muscle every time, it is not surprising that I could not identify EMG potentials produced by individual motoneurons with any degree of certainty by their shape in different animals. However, one potential in the caudal head could be reliably identified by its activation pattern. Furthermore, high frequency muscle potentials in the sternal and caudal depressor muscle heads during fast pereiopod depressions were distorted due to summation and other factors, making individual potentials difficult to identify. Single-ended EMG electrodes recorded more consistent waveforms between animals than did differential electrodes, but problems with background noise and various artifacts made most of these recordings unusable, so I attempted very few of them.

In many less than ideal EMGs (i.e. low signal to noise ratio, unexplainable activity suggesting movement artifacts or cross-talk from adjacent muscle heads), and rarely in traces which otherwise appeared excellent, individual potentials were seen in one muscle head which did not have corresponding potentials in its synergist. This could

be due to transient conduction failure in a nerve branch to one of the two heads, but double recordings from a single muscle head also occasionally had this difference, suggesting that the recording electrodes could sometimes miss muscle potentials. In poor recordings, this result is easily explained by signals getting lost in the background noise or extra potentials being recorded from other sources. In otherwise good traces the nature of EMG recordings suggests some possible explanations. First, EMG electrodes record field potentials produced by electrical activity in their vicinity, the range being dependent on input impedance of the electrodes and conductive properties of the surrounding tissues, among other factors. Other studies on crustacean pereiopod muscles have shown heterogeneity in innervation of muscle fibers by individual motoneurons (Govind et al., 1981; Parsons, 1982; Rathmayer & Erxleben, 1983; Govind & Wiens, 1984; Mearow & Govind, 1986; Rathmayer & Maier, 1987; Wiens et al., 1990; Günzel et al., 1993); such variation could cause potentials from some motoneurons to be missed. A second, theoretical, possibility with differential recordings is that a single potential could reach both electrodes at exactly the same time with exactly the same waveform and be cancelled out. While this would be unlikely, a distortion of the waveform to the point that it is not recognizable above background is a distinct possibility.

Depressor muscle activity during walking and postural changes

The heads of the pereiopod 2 depressor muscle are activated in characteristically different patterns during postural changes and walking. In describing these behaviours, the terms postural depressions and postural elevations will refer to depression and elevation of the second pereiopod; raising and lowering will be used to refer to the movements of the animal's body relative to the substrate.

The sternal depressor muscle head has sporadic, low level activity when the animal is maintaining a stance (Figs. 6.3 & 6.4), but is strongly active during postural depressions (Fig. 6.3), and is activated for a short burst following postural elevations (Fig. 6.4). Similarly, the caudal depressor head is active during postural depressions (Fig. 6.3) and not during postural elevations (Fig. 6.4), but this head is also active while the animal is maintaining stance. Three different pairs of distinct potentials occurred coincidentally in the sternal and caudal heads during postural depressions (Fig. 6.5), with the potentials in the caudal head delayed by 0.2-0.6 ms from those in the sternal head (Fig. 6.5C). As delay between the two heads increases some variation occurs between the timing of different potential (0.02-0.05 ms), reflecting differences in conduction

Fig. 6.3. Activity in the depressor muscle heads during a large, followed by a small, postural pereiopod depression. The animal was supporting its weight on its walking pereiopods during the entire trace. Coxal-basal (C-B) joint angle is shown in the top trace; the diagram at the bottom shows how coxal-basal joint angles were quantified. Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Ds, sternal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle.

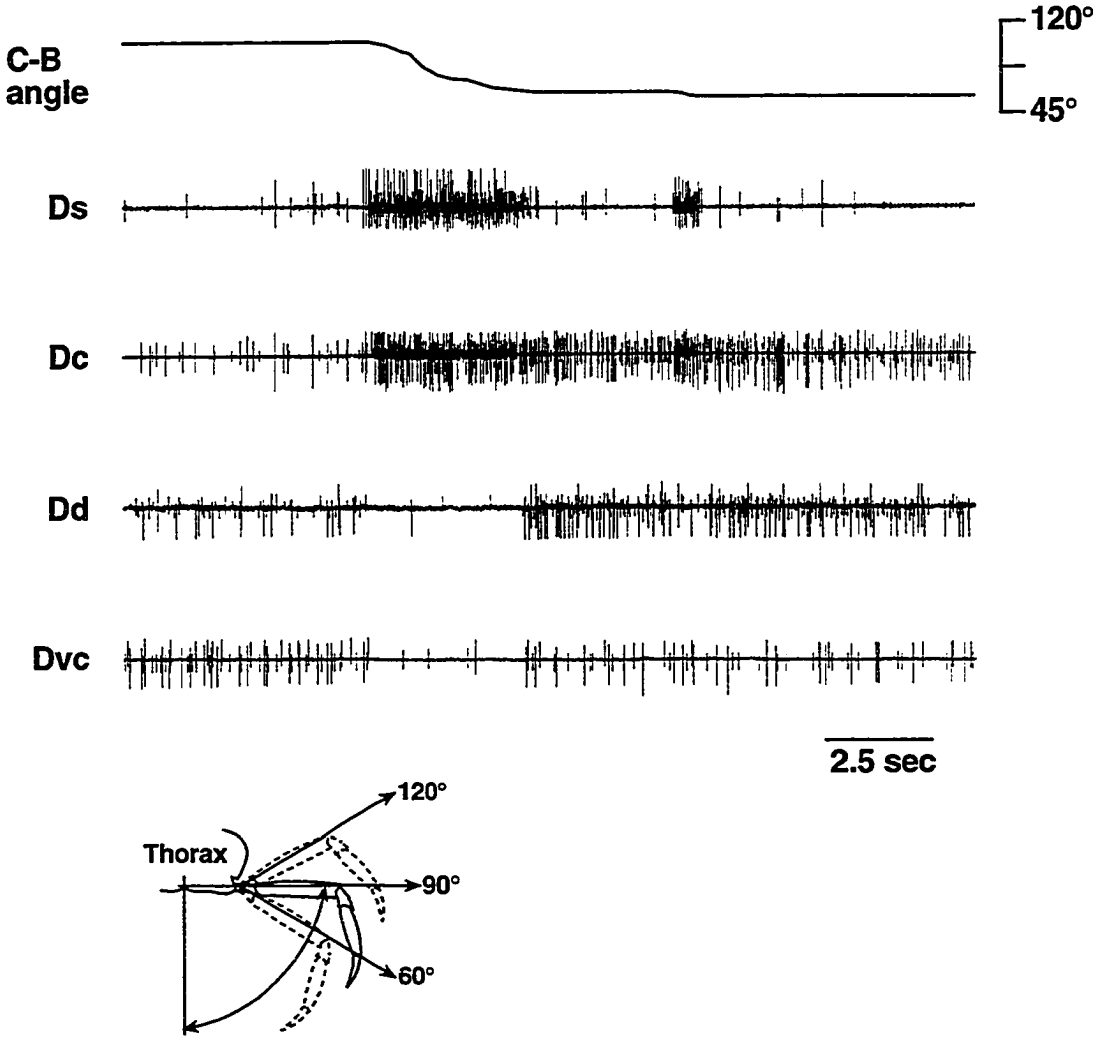

C-B angle
120°
45°
Ds
Dc
Dd
Dvc
2.5 sec
Thorax
120°
90°
60°

Fig. 6.4. Activity in the depressor muscle during a postural pereiopod elevation. The animal was supporting its weight on its walking pereiopods during the entire trace. Conventions and abbreviations as in Fig. 6.3.

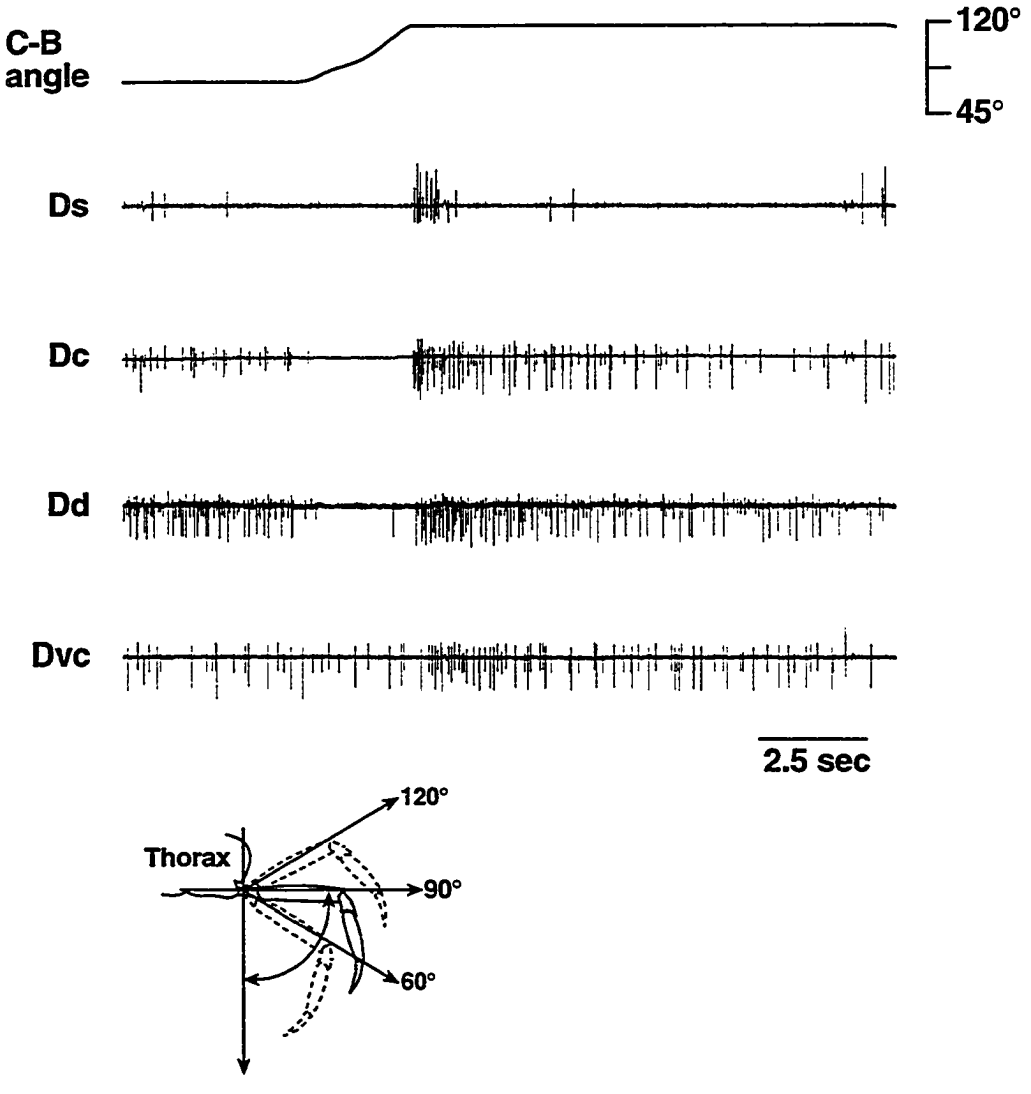
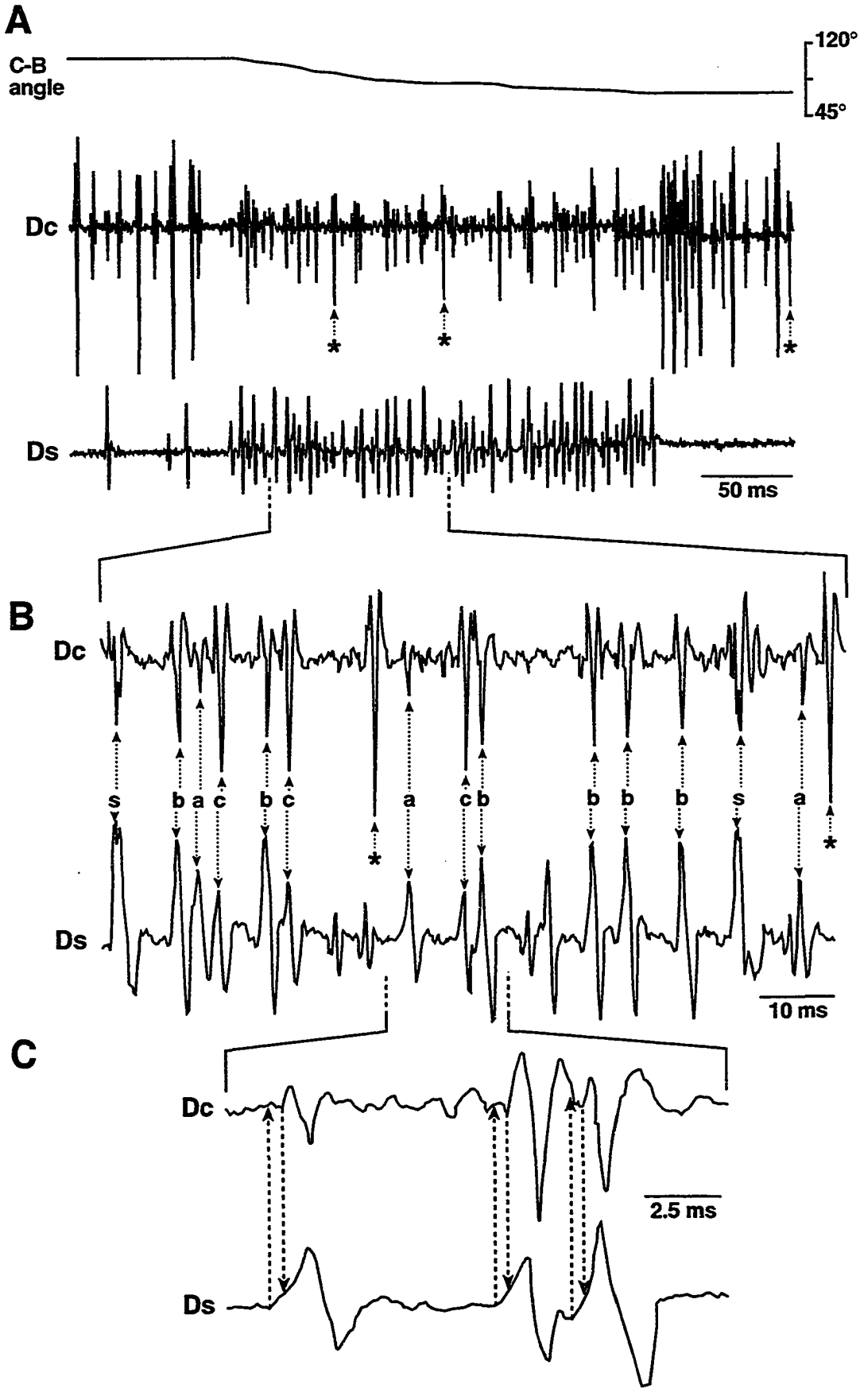

C-B
angle
120°
45°
Ds
Dc
Dd
Dvc
2.5 sec
Thorax
120°
90°
60°

Fig. 6.5. Coincident muscle potentials in the caudal (Dc) and sternal (Ds) heads of the depressor muscle during a postural pereiopod depression. Coxal-basal (C-B) joint angle is shown at the top. (A), (B), and (C) show the same trace at increasingly faster timebases. The asterisks (*) in (A) and (B) indicate the muscle potential which occurs at high frequency in the caudal head during pereiopod depressions and during stance maintenance, but is not coincident with any potential in the sternal (or dorsal) head. In (B), "a", "b", and "c" indicate three distinct forms of coincident potentials in the caudal and sternal heads. These potentials do not have a consistent waveform between preparations. "s" indicates instances of coincident pairs of partially superimposed potentials, which in this trace were not consistently superimposed. The dotted lines in (C) indicate the onset of the muscle potentials, in this animal the potentials in the caudal head were delayed 0.5 ms from those in the sternal head.


A
C-B angle
120°
45°
Dc
Ds
50 ms
B
Dc
s
b
a
c
b
c
a
c
b
b
b
b
s
a
Ds
10 ms
C
Dc
2.5 ms
Ds

velocity of the motoneurons. During walking, no more than two pairs of coincident potentials occur in the sternal and caudal depressor muscle heads. However, in many EMGs of walking animals, one of the pairs of coincident potentials (potential "d" in Fig. 6.6) appeared to be composed of two superimposed potentials. The traces in Fig. 6.6 were recorded approximately five minutes after the one in figure 6.5 from the same animal. One coincident pair of potentials ("a" in both Figures) is identical between the two traces, while potential "d" in Fig. 6.6 looks as though it could be composed of superimposed potentials "b" and "c" seen in Fig. 6.5. These data suggest that the command system for walking activates two of the motoneurons shared by the sternal and caudal depressor heads synergistically, while the postural command systems activate the same two neurons independently. Either two or, more often, one other distinct potential was seen in the sternal head for a total of up to five, which is the number of putative excitatory motoneurons backfilled from this head (Chapter 5). No potentials were seen which were clearly of a classic "phasic" type: I did not see any distinct potentials which discharged in short bursts, nor any rapid attenuation suggesting anti-facilitation of muscle potentials. However, I was not able to obtain usable EMGs from these muscles during very rapid movements, because large movement artifacts, cross talk from other muscle heads, and considerable overlap of potentials made individual potentials very difficult if not impossible to distinguish. Three other distinct potentials also occur in the caudal head which are not coincident with spikes in the sternal head. One occurs at high frequency during pereiopod depressions, and usually at slightly lower frequencies during maintenance of stance (Fig. 6.5A & B). The other two occur only very occasionally during coxal-basal joint movements, but at high frequency during stance maintenance, and are coincident with potentials in the dorsal head (Fig. 6.7). The potentials in the caudal head precede those in the dorsal head by 0.2-0.55 ms, with some variation between different potentials in animals with long delays (Fig. 6.7C). In total, up to six potentials with distinct waveforms were identified in the caudal head, which equals the number of putative excitatory motoneurons backfilled from this head.

During postural depressions, both the dorsal (and coxal) and ventral-caudal (and ventral-rostral) heads are relatively inactive (Fig. 6.3). The dorsal head is also quite inactive during postural elevations, but activity in the ventral heads remains at approximately the pre-movement level throughout the elevation (Fig. 6.4). The caudal, dorsal, and ventral heads are active whenever the coxal-basal joint is still and the animal is maintaining a stance (Figs. 6.3 & 6.4). Following postural changes, each of these

Fig. 6.6. Coincident muscle potentials in the caudal and sternal heads of the depressor muscle during a pereiopod depression during forward walking. This trace was recorded approximately 5 minutes after the one in Fig. 6.5, in the same animal. In (B), "a" indicates the same coincident muscle potential as in Fig. 6.5. "d" indicates a muscle potential which was not seen during postural pereiopod depressions, but its form suggests it may be a superposition of potentials "b" and "c" in Fig. 6.5. All other conventions and abbreviations are the same as in Fig. 6.5. Muscle potential delay in an animal's caudal head relative to its sternal head remains consistent regardless of the animal's behaviour.

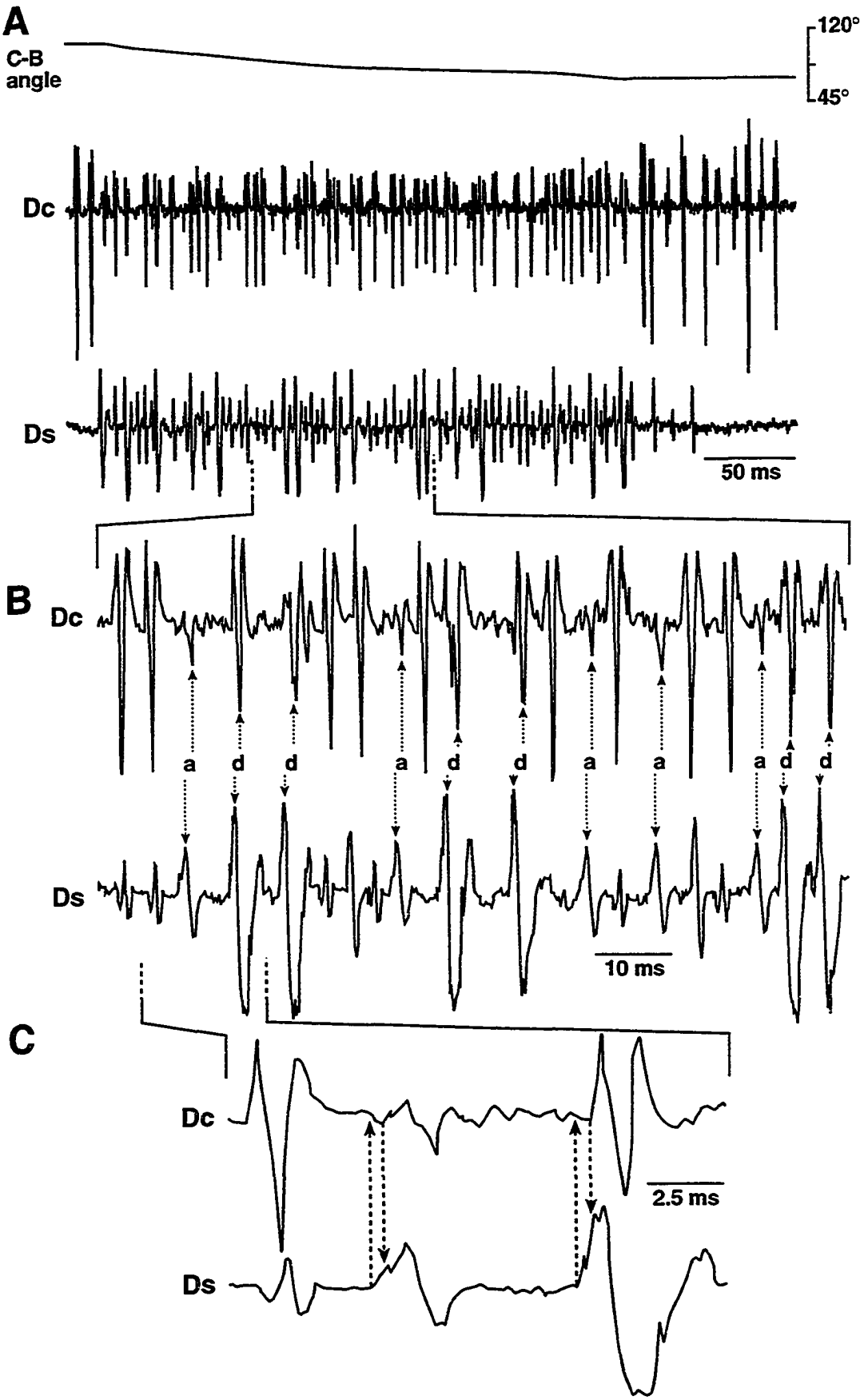
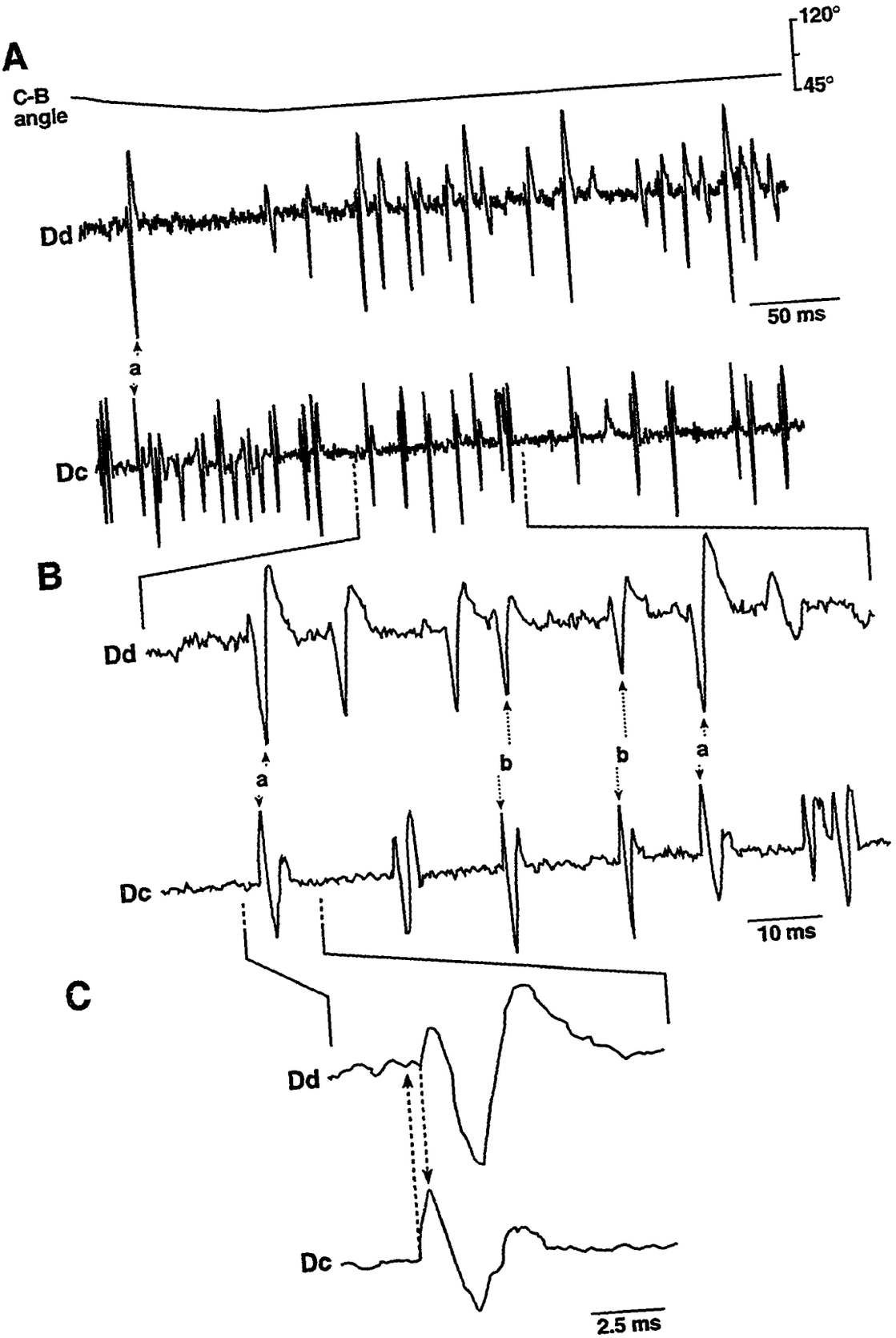

A
C-B angle
120°
45°
Dc
Ds
50 ms
B
Dc
a d d a d d a a a d d
Ds
10 ms
C
Dc
2.5 ms
Ds

Fig. 6.7. Coincident muscle potentials in the dorsal (Dd) and caudal (Dc) heads of the depressor muscle. (A) EMG during the last approximately 100 ms of a postural pereiopod depression, followed by a period of stationary posture maintenance. "a" and "b" indicate two distinct forms of coincident muscle potentials in the dorsal and caudal heads. In this animal, muscle potentials in the dorsal head are delayed 0.45 ms from coincident potentials in the caudal head. All other conventions as in Fig. 6.5.


A
120°
45°
C-B angle
Dd
50 ms
a
Dc
B
Dd
a
b
b
a
Dc
10 ms
C
Dd
Dc
2.5 ms

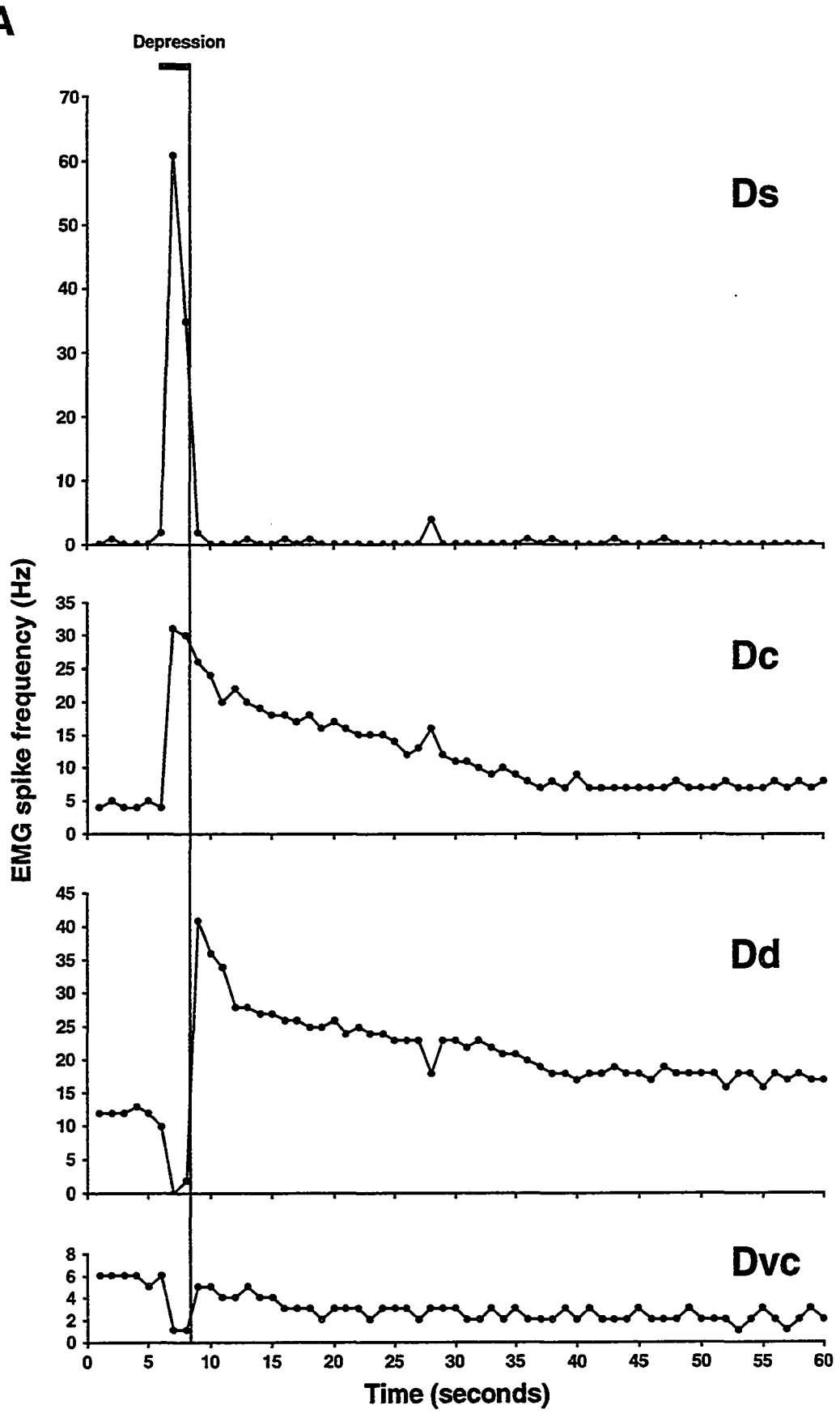
heads has an initial high frequency burst which quickly decays, usually within about 20 seconds after the movement, to a lower tonic rate (Fig. 6.8) which remains consistent for up to 16.5 minutes (the longest lasting stationary raised posture I recorded). The activity patterns of these five heads during stance maintenance are analyzed in more detail in the next section.

No observable attenuation of individual muscle potentials occurs at any time in any of the heads. Attenuation of muscle potential amplitude can be indicative of inhibitory influences postsynaptically on the muscle fibers or presynaptically on the motoneuron terminals, but a lack of attenuation does not necessarily indicate a lack of peripheral inhibition (Atwood & Walcott, 1965), and it is not possible to determine the existence or nature of inhibition from these recordings.

The sternal and caudal heads of the depressor muscle have patterns of activation during forward (Fig. 6.9A) and backward (Fig. 6.9B) walking which are similar to the patterns described for the depressor muscle in other species (MacMillan, 1975; Ayers & Davis, 1977; Ayers & Clarac, 1978; Clarac et al., 1987). These are the largest of the depressor heads and their activity dominates recordings from EMG electrodes implanted ventrally along the depressor muscle apodeme, near the lip of the basis. The sternal and caudal heads are very active during pereiopod depressions and continue activity during the power stroke, while the pereiopod is in contact with the substrate, although activity levels in the sternal head are very low compared to when the pereiopod is being depressed (Figs 6.9-6.10). Activity in both heads increases slightly over the course of pereiopod depressions, reaching a peak immediately before contact with the substrate (Fig. 6.10). This corresponds to movement at the coxal-basal joint: depressions during walking usually begin slowly, with velocity increasing near the end of the movement (see coxal-basal joint angle traces above the bar graphs, Figs. 6.10A & 6.10B), and activity in both muscles increases logarithmically with depression velocity during walking and postural changes (Fig. 6.11). However, activity levels in both the sternal and caudal heads are higher at a given angular velocity during walking than during postural depressions.

Activity in the remaining four heads of the depressor muscle is not typical of activity described for the depressor muscle in other species, and is not like activity in the sternal and caudal heads. The greatest activity levels in the dorsal (and coxal) heads are during the power stroke or stance phase, when the pereiopod is pushing against the substrate, with much lower levels of activity while the pereiopod is being actively

Fig. 6.8. Frequencies of depressor muscle potentials during and following postural changes. (A) A postural pereiopod depression. (B) A postural pereiopod elevation. Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Ds, sternal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle.


A
Depression
Ds
Dc
Dd
Dvc
EMG spike frequency (Hz)
Time (seconds)

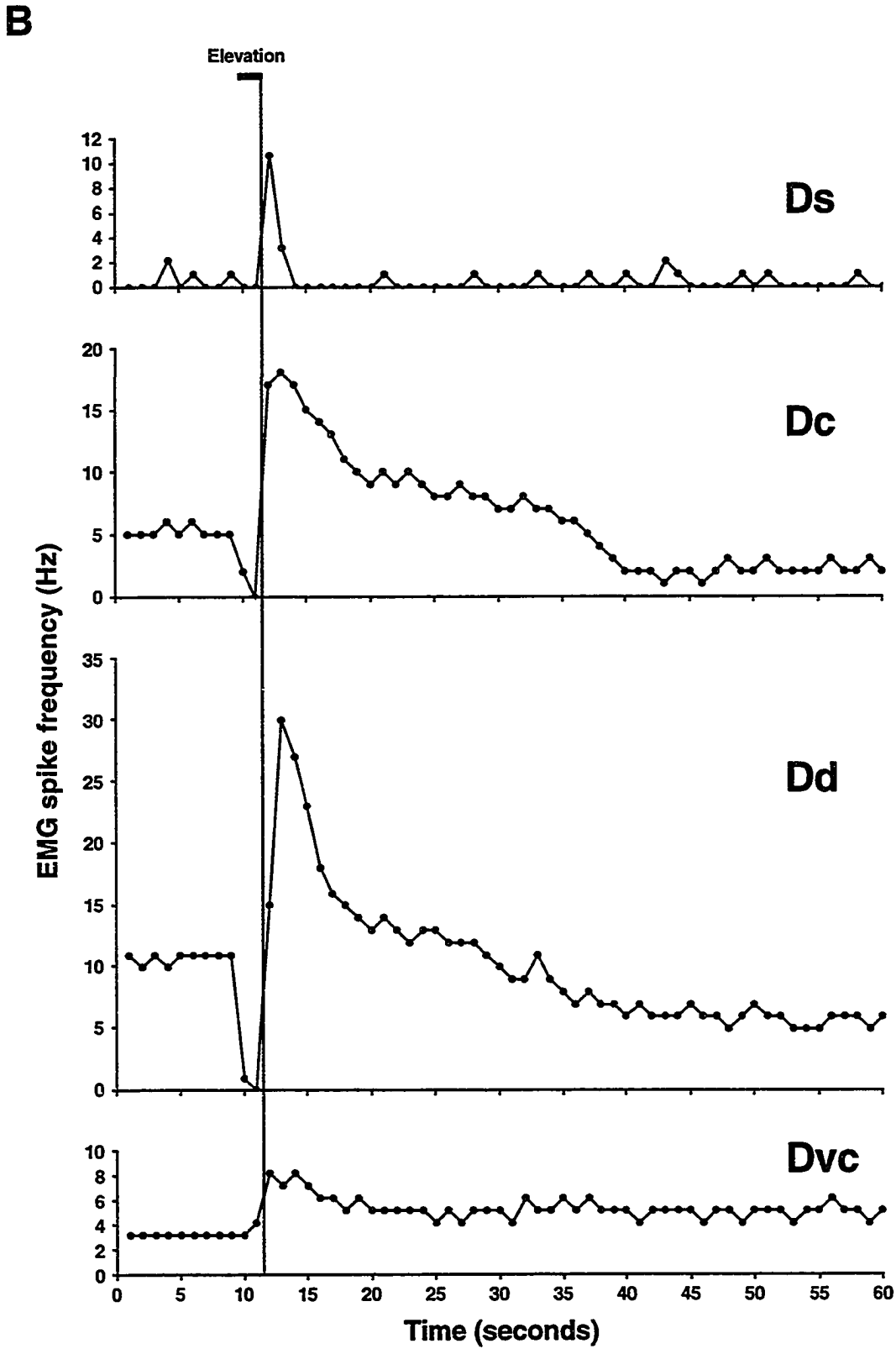
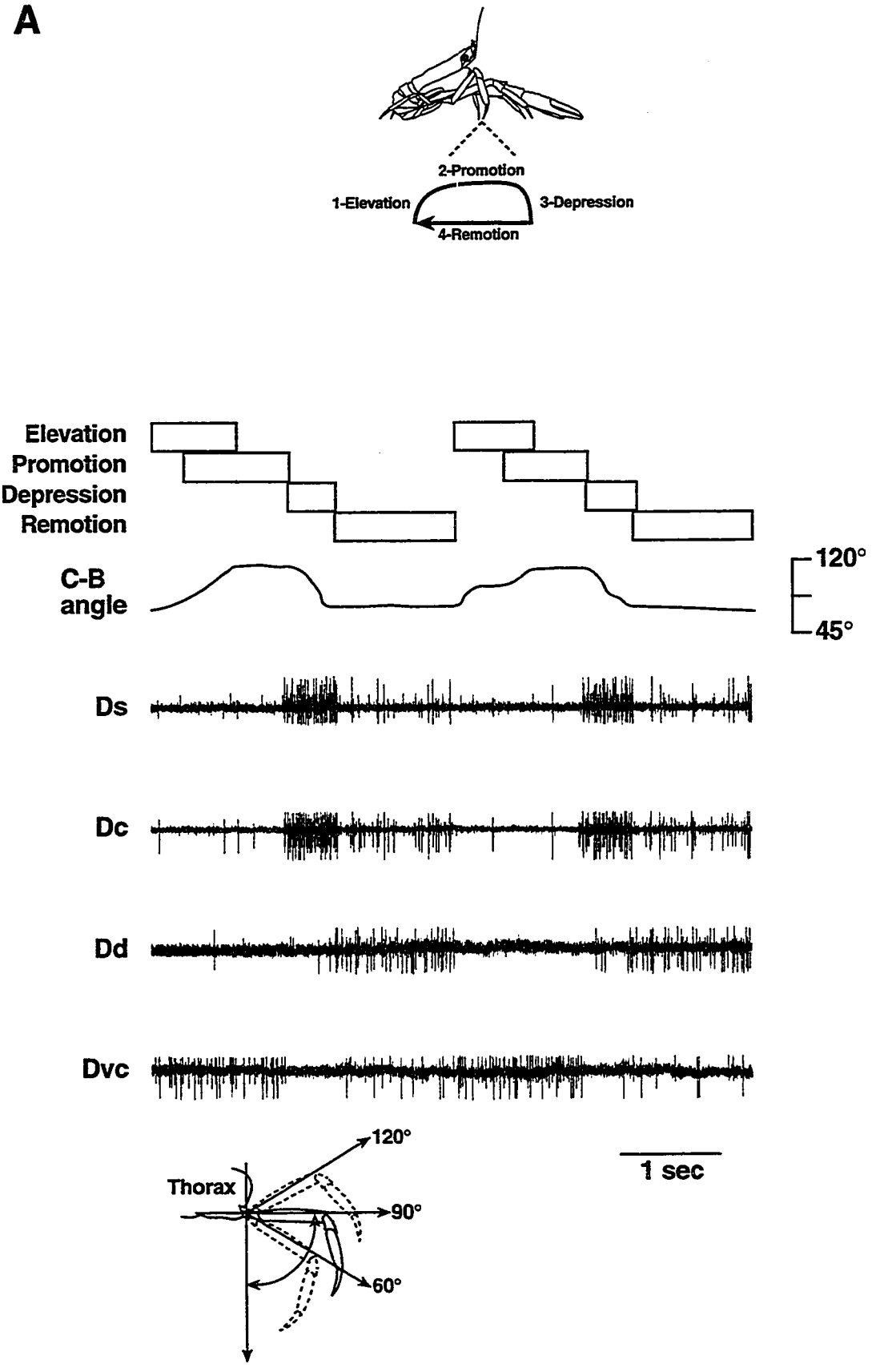

B
Elevation
Ds
Dc
Dd
Dvc
EMG spike frequency (Hz)
Time (seconds)

Fig. 6.9. Activity in the depressor muscle during forward and backward walking. The sequence of proximal joint movements is shown at the top of each figure. Two step cycles are shown, with the durations of the four proximal joint movements indicated by the boxes. (A) Forward walking. The power stroke occurs during pereiopod remotion, while the return stroke is comprised of elevation, promotion, and depression. (B) During backward walking, the power stroke occurs during promotion, while elevation, remotion, and depression occur during the return stroke. C-B angle, coxal-basal joint angle; Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Ds, sternal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle.


A
2-Promotion
1-Elevation
3-Depression
4-Remotion
Elevation
Promotion
Depression
Remotion
C-B
angle
120°
45°
Ds
Dc
Dd
Dvc
120°
Thorax
90°
60°
1 sec

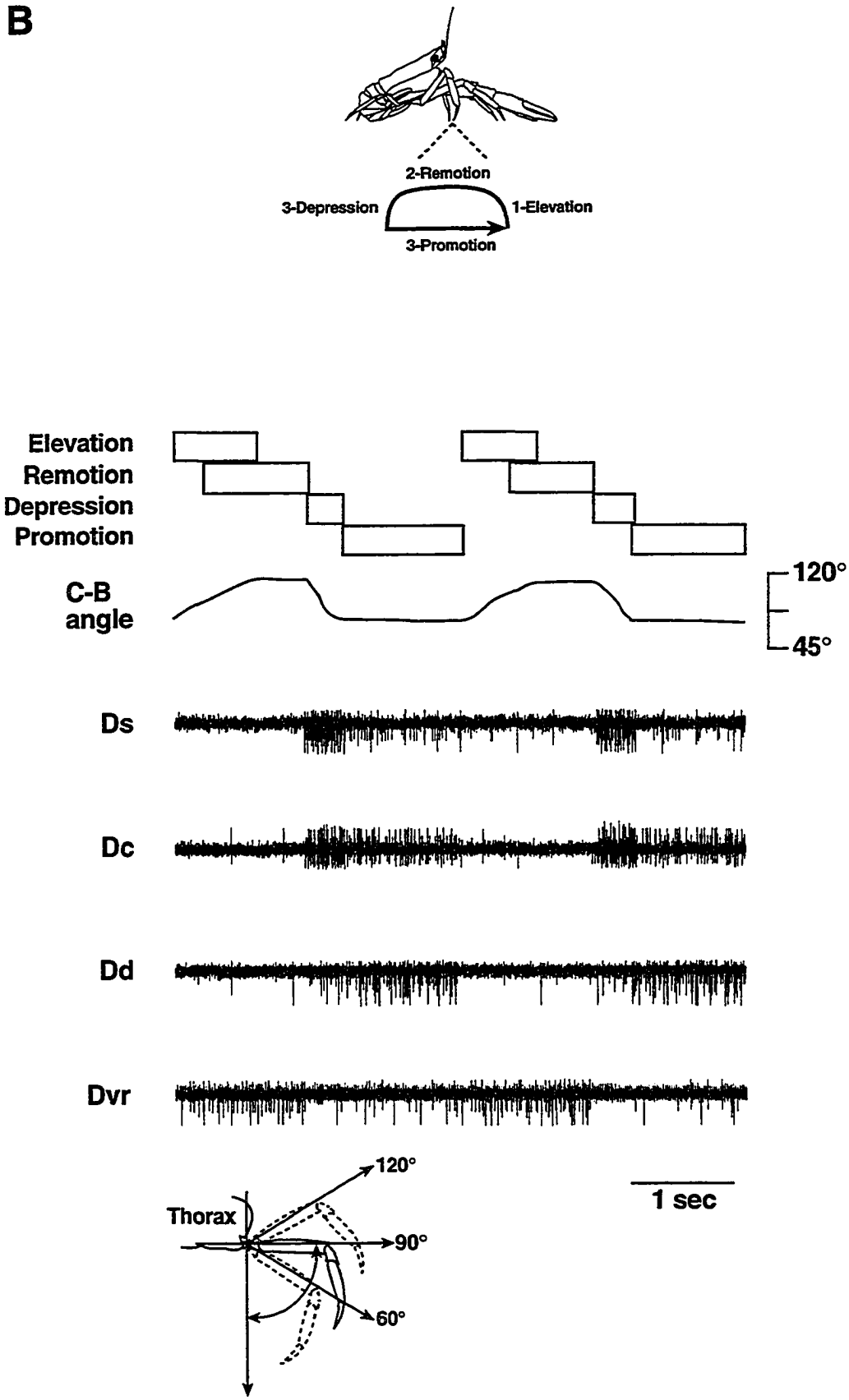
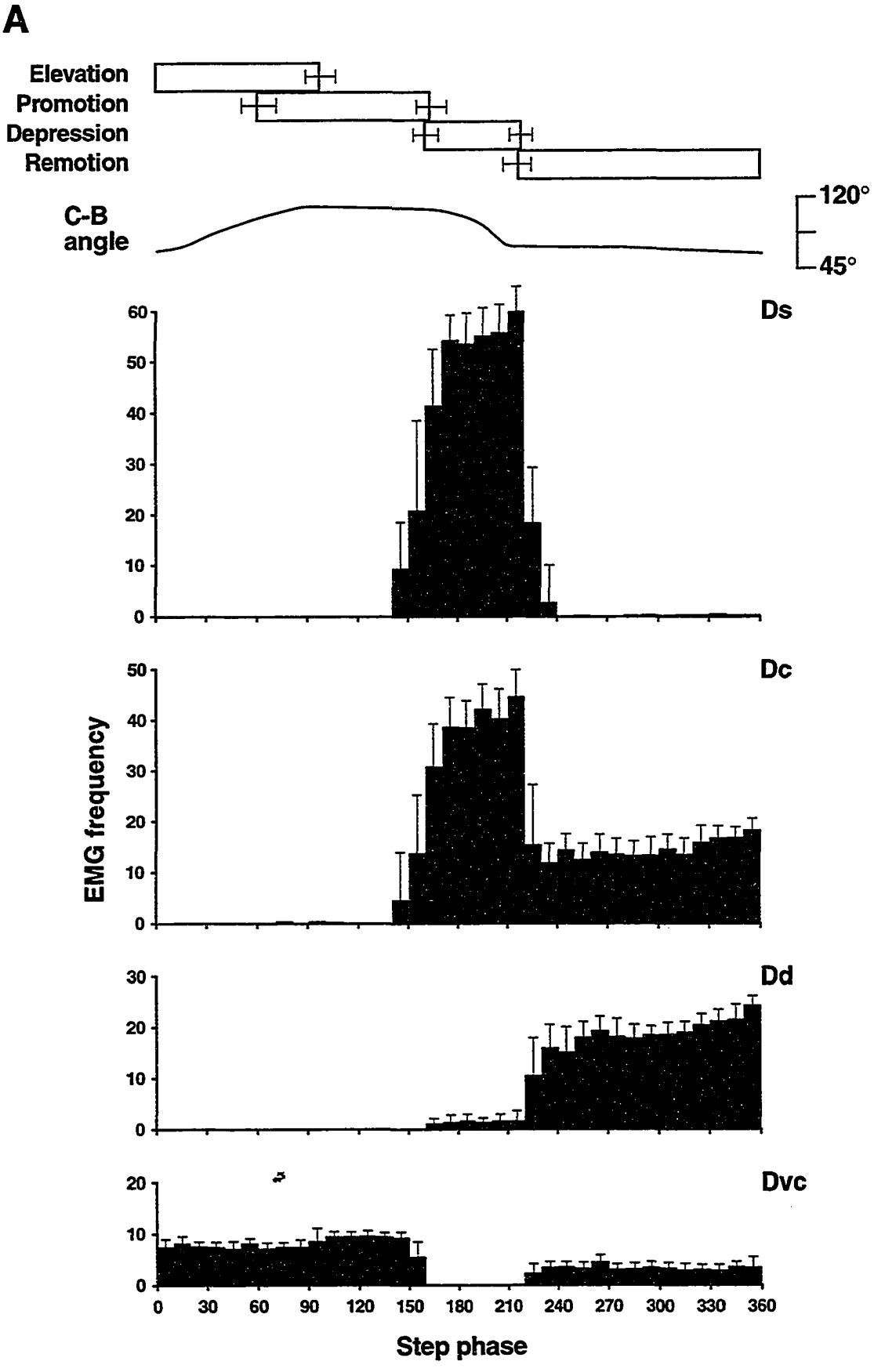

B
2-Remotion
3-Depression
1-Elevation
3-Promotion
Elevation
Remotion
Depression
Promotion
C-B angle
120°
45°
Ds
Dc
Dd
Dvr
120°
Thorax
90°
60°
1 sec

Fig. 6.10. Frequencies of depressor muscle potentials during forward and backward walking. EMG data were binned into 10° intervals (1/36th of the normalized step cycle). (A) Forward walking. Proximal joint movement durations, coxal-basal joint movements, and muscle potential frequencies are means of 38 normalized step cycles in 8 animals. (B) Backward walking. All data are the means of 24 normalized step cycles in 6 animals. The bars indicate standard errors of EMG spike frequencies and of the onset and termination of individual joint movements. Dvr, ventral rostral head of the depressor muscle. All other conventions and abbreviations are the same as in Fig. 6.9.


A
Elevation
Promotion
Depression
Remotion
C-B angle
120°
45°
Ds
Dc
Dd
Dvc
EMG frequency
Step phase
0
30
60
90
120
150
180
210
240
270
300
330
360

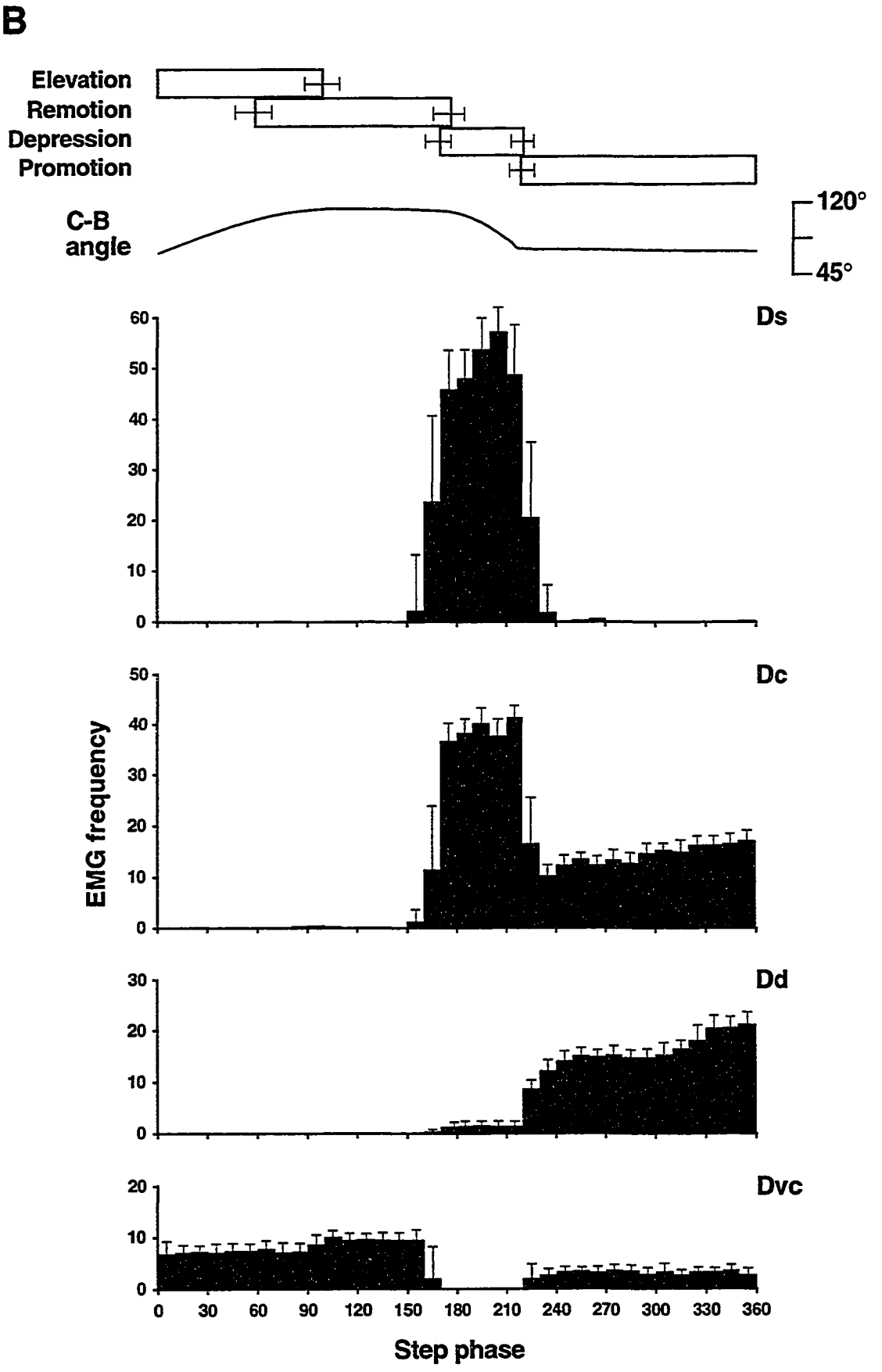
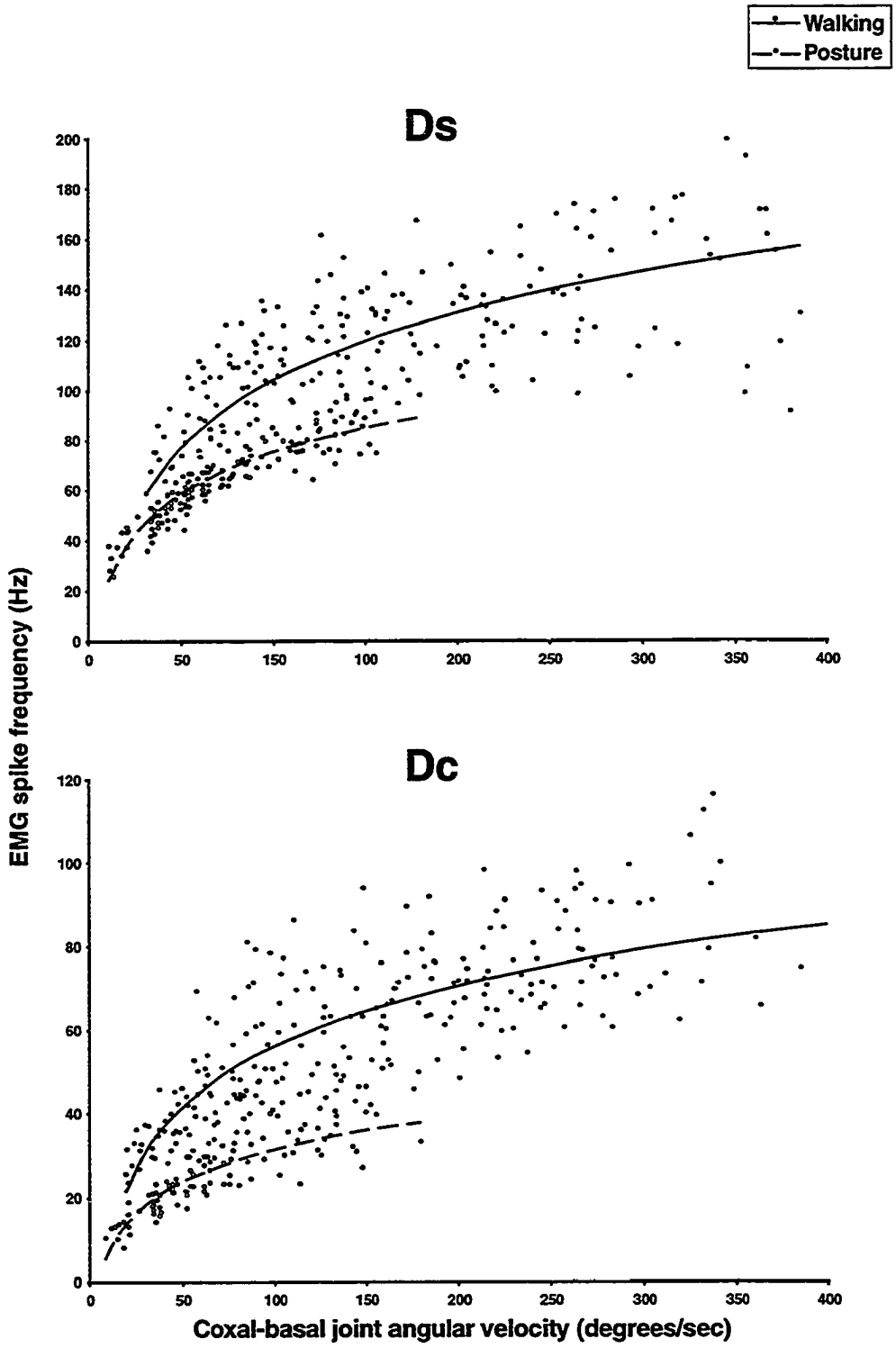

B
Elevation
Remotion
Depression
Promotion
C-B angle
120°
45°
Ds
Dc
Dd
Dvc
EMG frequency
Step phase
0
30
60
90
120
150
180
210
240
270
300
330
360

Fig. 6.11. EMG spike frequencies in the sternal and caudal depressor muscle heads as a function of depression velocity. Frequency of muscle potentials increases logarithmically with joint velocity, and frequency is higher during walking than during postural depressions. Ds sternal head of the depressor; Dc, caudal head of the depressor.


Walking
Posture
Ds
Dc
EMG spike frequency (Hz)
Coxal-basal joint angular velocity (degrees/sec)
0
20
40
60
80
100
120
140
160
180
200
0
50
100
150
200
250
300
350
400

depressed (Figs. 6.9-6.10). Activity increases during the stance phase, reaching the highest levels just before the pereiopod elevates for the beginning of the next cycle (Fig. 6.10). This pattern is similar in both forward and backward walking although, in general, potential frequencies are slightly higher during forward walking. During the stance phase, similar patterns of activity occur in the caudal head of the depressor muscle, although for this head there are no appreciable differences in spike frequencies between forward and backward walking, and activity levels during the stance phase are lower than during the depression phase (Fig. 6.10).

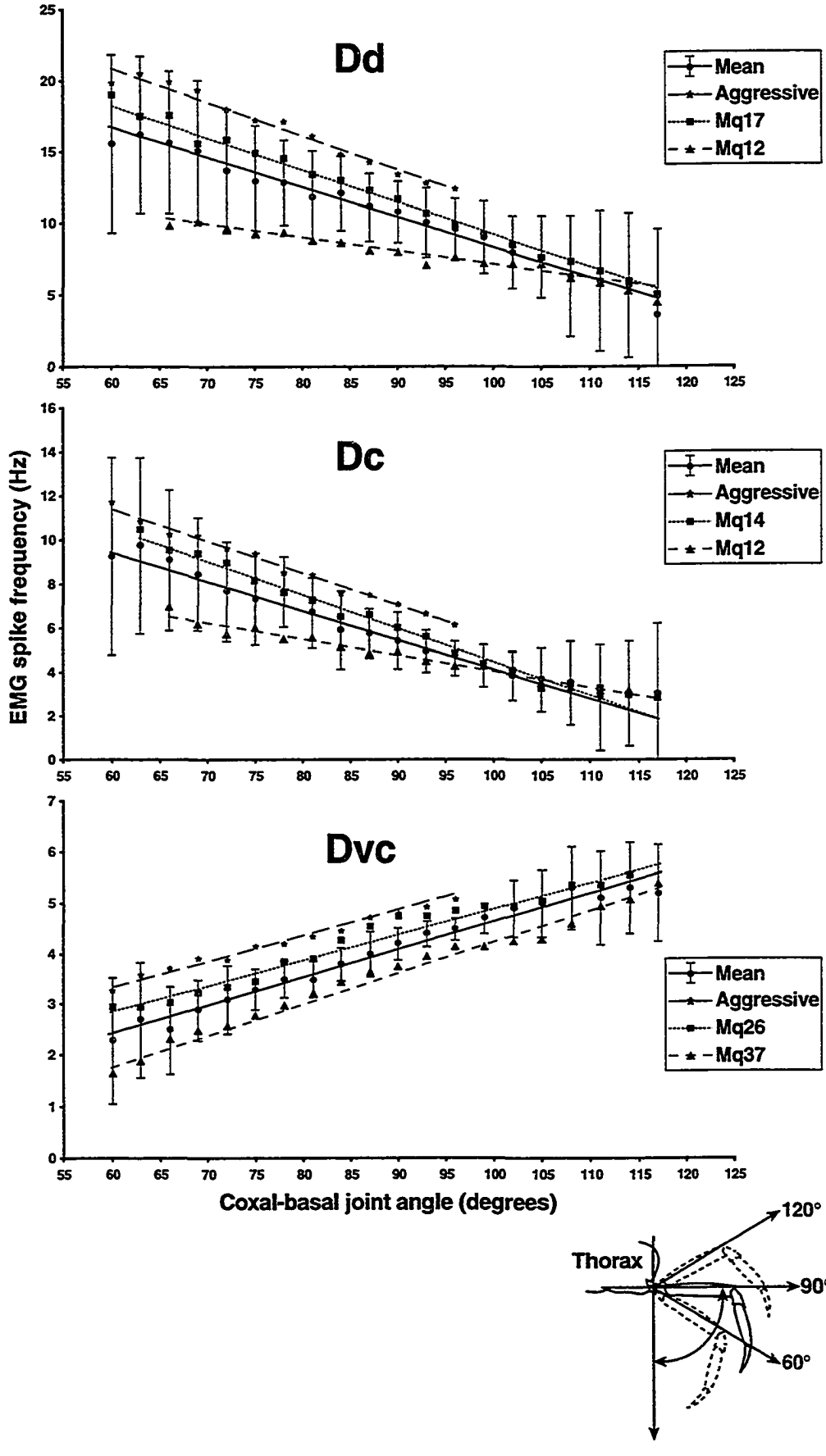
During walking, spikes occur only rarely in either of the ventral heads of the depressor muscle during the depression phase of the return stroke (Figs. 6.9-6.10). Spike frequency in these two heads is highest during the remaining two phases of the return stroke in both forward and backward walking, with activity slightly lower during elevations than during purely rostro-caudal movements. These heads are also active at low frequencies during the power stroke.

Depressor muscle activity during maintained stance

All of the depressor muscle heads except for the sternal head are active during postural maintenance. Recordings from animals which were actively supporting their bodies on the pereiopods (i.e. the claws or abdomen were not resting on the substrate) reveal higher activity in the dorsal (and coxal) and caudal heads with greater pereiopod depression. The reverse is true for the two ventral heads, which are more active when the pereiopod is more elevated (Fig. 6.12).

An important consideration when interpreting these data is the potential roles of agonistic behaviour, and therefore 5-HT and OA, in setting muscle potential frequencies during postural maintenance. Aggression (and 5-HT) cause animals to assume raised postures (Livingstone et al. 1980; Chapter 2), and increase excitatory output to the muscles involved in producing aggressive postures (Harris-Warrick, 1985; Ma et al., 1992; see *Effects of serotonin and octopamine on depressor muscle EMG activity,* below). Aggressively behaving *M. quadrispina* have greater activity levels in the dorsal, caudal, and ventral heads of their depressor muscles than do animals which are not displaying agonistic behaviours (Fig. 6.12). Activity in the sternal head is not increased in aggressive animals over the very low level normal for non-agonistic animals (data not shown). Very few records (a total of five data points) were obtained for animals in typical submissive postures, in part because *M. quadrispina*'s usual submissive response

Fig. 6.12. Frequencies of depressor muscle potentials during maintained stance. Means (± SE) were calculated from all interpretable traces from animals that were not behaving agonistically and were fully supporting themselves on the tips of their walking pereiopods. The high SE is reflective of inter-animal variability, intra-animal variability is very low, as can be seen for the individual animals plotted here: the error bars are not visible. Means without standard error (to avoid confusion) are given for all animals that were behaving aggressively. Animals Mq12 and Mq37 were undisturbed; Mq14, 17, and 26 were presented visual stimuli to induce them to change postures. Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle.


Dd
Mean
Aggressive
Mq17
Mq12
Dc
Mean
Aggressive
Mq14
Mq12
Dvc
Mean
Aggressive
Mq26
Mq37
EMG spike frequency (Hz)
Coxal-basal joint angle (degrees)
Thorax
120°
90°
60°

to a threat is to tailflip away, and submissive postures occur rarely under any circumstance (Chapter 2).

In only a very few instances (4 animals, 2 usable examples of the dorsal head, 3 of the caudal, 3 of the ventral) was I able to record muscle activity over a wide range of coxal-basal joint angles in completely isolated, undisturbed animals as the very long times required to complete such experiments in these very sedate animals often meant that the signals would degrade due to scar tissue forming over the implants before a sufficient number of records had been obtained. For most animals, I resorted to inducing the animal to change its posture by presenting visual stimuli, which occasionally elicited a clear defensive or aggressive posture as well as a raised posture. Although data from episodes in which animals displayed a recognizable agonistic reaction to visual stimuli were not used to calculate the means shown in Fig. 6.12 for non-agonistic animals, it is possible that physiological changes could have occurred which did not manifest themselves as observable behavioural changes. The data from the undisturbed animals (e.g. animals Mq12 and Mq37, Fig. 6.12) suggest that this is the case. Typically, animals not disturbed during the recordings had considerably lower activity in the dorsal, caudal and ventral heads of the depressor muscle when the leg was strongly depressed than the mean for the heads in all non-agonistically behaving animals, which includes episodes where the animals were disturbed with visual stimuli (Fig. 6.12). Furthermore, animals Mq14, 17, and 26, which did not assume any raised stances spontaneously, were typical of animals induced to change posture by visual stimuli, in having potential frequencies slightly above the mean, but below frequencies of aggressively behaving animals. Most of the EMGs used to calculate the mean values were from animals who made both spontaneous and induced postural changes. These results, together with those which will be presented in the next section, strongly implicate 5-HT, OA, and agonistic command circuitry in control of muscle potential frequencies in muscles involved in posture.

To determine the effect of load, in the form of the animal's weight, on activity in the depressor heads involved in stance maintenance, I compared EMGs recorded from animals which were resting their claws or their bodies (animals which were squatting) on the substrate with those which were fully supporting their weight on their walking pereiopods (Fig. 6.13 & 6.14). A short burst of activity occurs in the sternal, caudal, and dorsal depressor muscle heads immediately upon contact of the abdomen and claws with the substrate, followed by very low activity while the animals are resting on the substrate (Fig. 6.13). Muscle potentials occur at much lower frequencies for given coxal-basal

Fig. 6.13. Activity in the depressor muscle as the animal lowers itself to rest on the substrate. Dcx, coxal head of the depressor muscle. All other abbreviations as in Fig. 6.11.

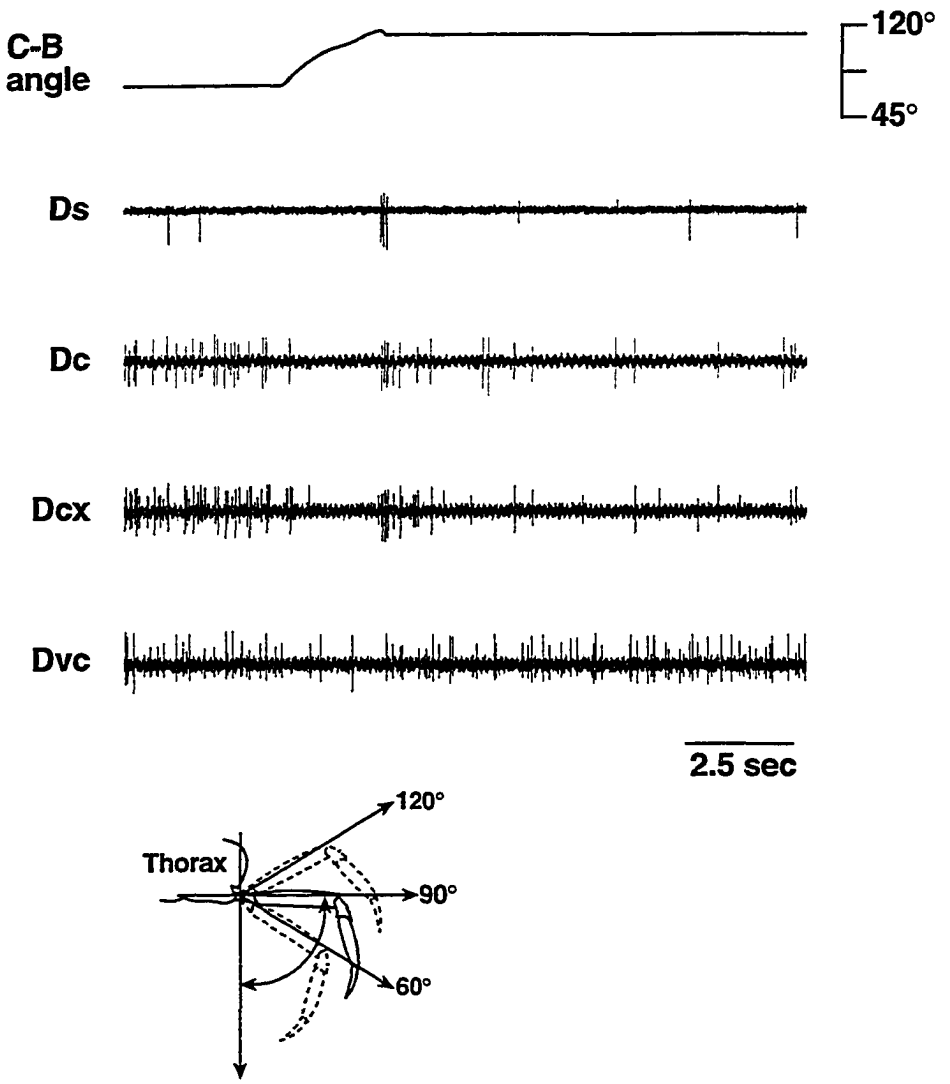
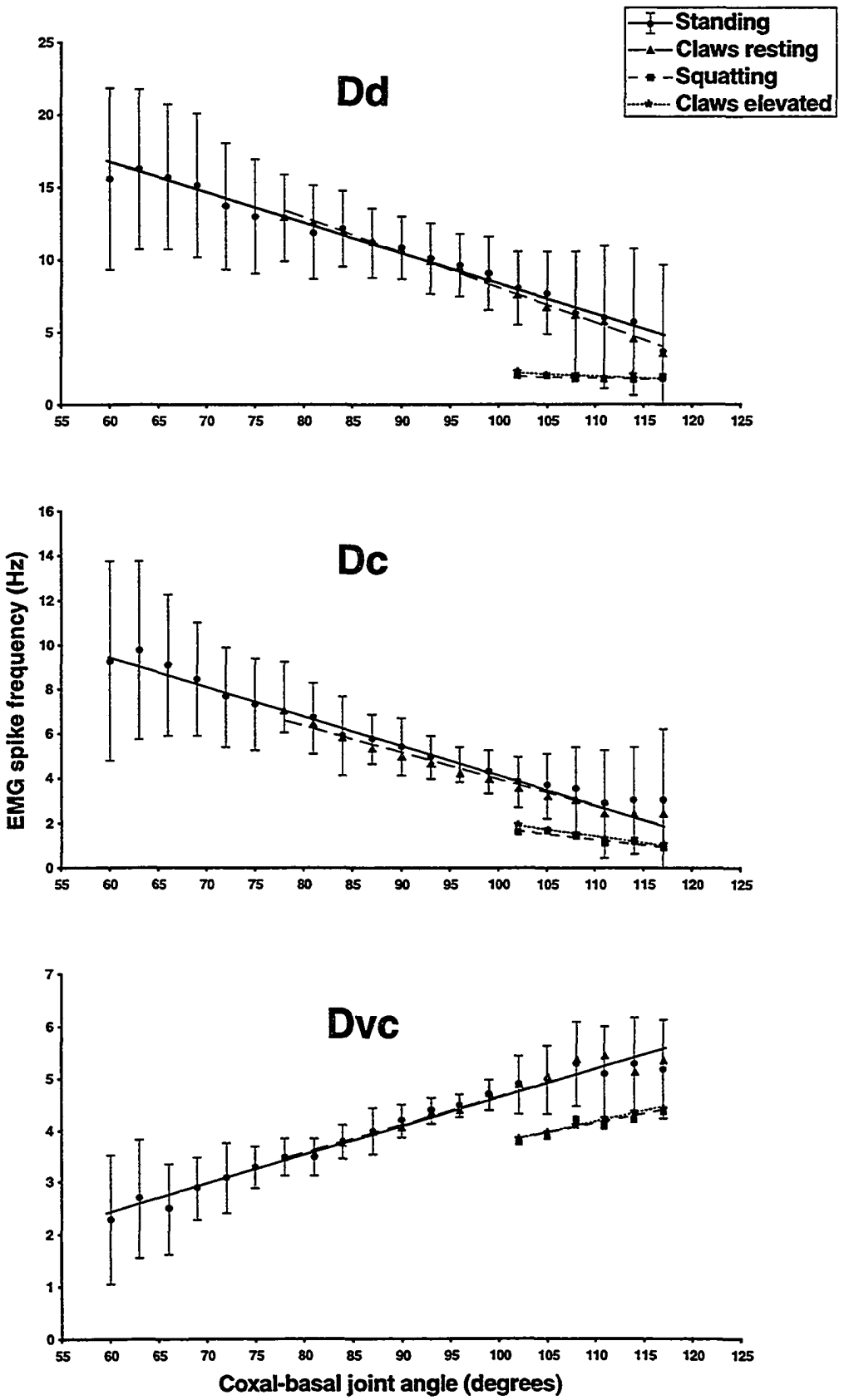

C-B
angle
120°
45°
Ds
Dc
Dcx
Dvc
2.5 sec
Thorax
120°
90°
60°

Fig. 6.14. Frequencies of depressor muscle potentials under different loads in maintained stances. Data are shown for animals that are squatting (abdomen and tips of claws in contact with the substrate) and animals which have their claws resting on the substrate but are otherwise supported by the walking pereiopods, animals which have their claws elevated but are resting their abdomen on the substrate, and animals which were fully supporting their weight with their walking pereiopods (same data as "mean" in Fig. 6.12). All other conventions and abbreviations as in Fig. 6.11.


Standing
Claws resting
Squatting
Claws elevated
Dd
Dc
Dvc
EMG spike frequency (Hz)
Coxal-basal joint angle (degrees)

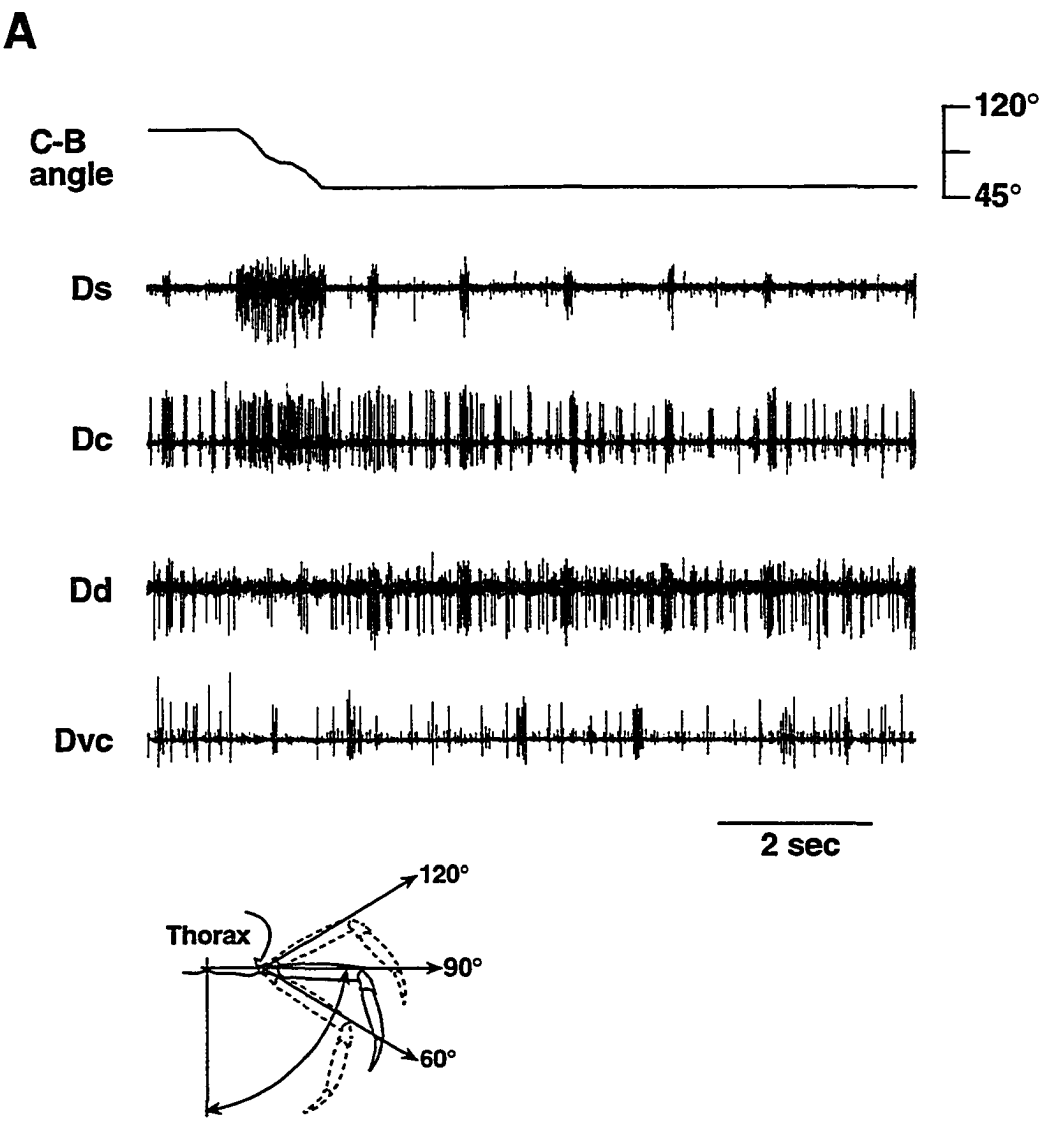
joint angles when the animal is resting on the substrate than while it is maintaining a stance with its body raised and supported by its pereiopods (Fig. 6.14). Activity in the ventral heads is also lower when an animal is resting its body on the substrate, but the decrease is proportionally less than in the caudal and dorsal heads (Fig. 6.14). These data strongly suggest a role for load-sensitive sensory systems in setting tonic motor output. Interestingly, elevating the claws while the abdomen is resting on the substrate has only a very small effect on muscle potential frequencies relative to when the claws are also resting on the substrate (Fig. 6.14). I would have expected the second pereiopods to support much of the weight of the claws when they are elevated and, therefore, claw elevation to have increased in activity in at least the dorsal and caudal heads of the depressor muscle. Additionally, there is no appreciable difference in activity levels when the claws but not the abdomen are in contact with the substrate, as compared to when the claws are also elevated (Fig. 6.13).

Effects of serotonin and octopamine on depressor muscle activity

5-HT increases and OA decreases muscle potential frequency in all of the depressor muscle heads in freely moving *M. quadrispina*. Additionally, amine injection causes bursting activity in all of the depressor muscle heads (Fig. 6.15). Frequency and strengths of the bursts are positively correlated with 5-HT dose but do not change with OA dose, except at very high doses when strong bursting in the depressor heads and violent shaking of the limbs (see Chapter 2) are induced. Bursts in the sternal, caudal, and dorsal (and coxal) heads are synchronous, and often coincide with small shakes or jerks of the walking pereiopods. Bursting in the ventral heads is normally out of phase with and of lower frequency than in the other heads.

During forward walking in animals injected with low doses of 5-HT or OA, the patterns of muscle discharge are essentially unchanged from those in untreated animals, however, the frequencies are higher in 5-HT-injected and lower in OA-injected animals (Fig. 6.16, compare with Fig. 6.10). Animals injected with high doses of the amines do not walk often, and when they do, the walking patterns are usually quite distorted and movements are shaky, producing EMGs that are difficult to interpret. Surprisingly, the average duration of the step cycle did not change in animals under the influence of low doses of injected amines, nor did the relative durations of the proximal joint movements, although under the influence of high doses of 5-HT a few very fast depressions occurred. Also, injected 5-HT increased, while OA decreased, spike frequencies associated with

Fig. 6.15. Depressor muscle activity in animals injected with (A) 0.002 mg/g serotonin or (B) 0.001mg/g octopamine. Note the short high frequency bursts in all of the heads. C-B angle, coxal-basal joint angle; Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Ds, sternal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle.


A
C-B angle
120°
45°
Ds
Dc
Dd
Dvc
2 sec
Thorax
120°
90°
60°

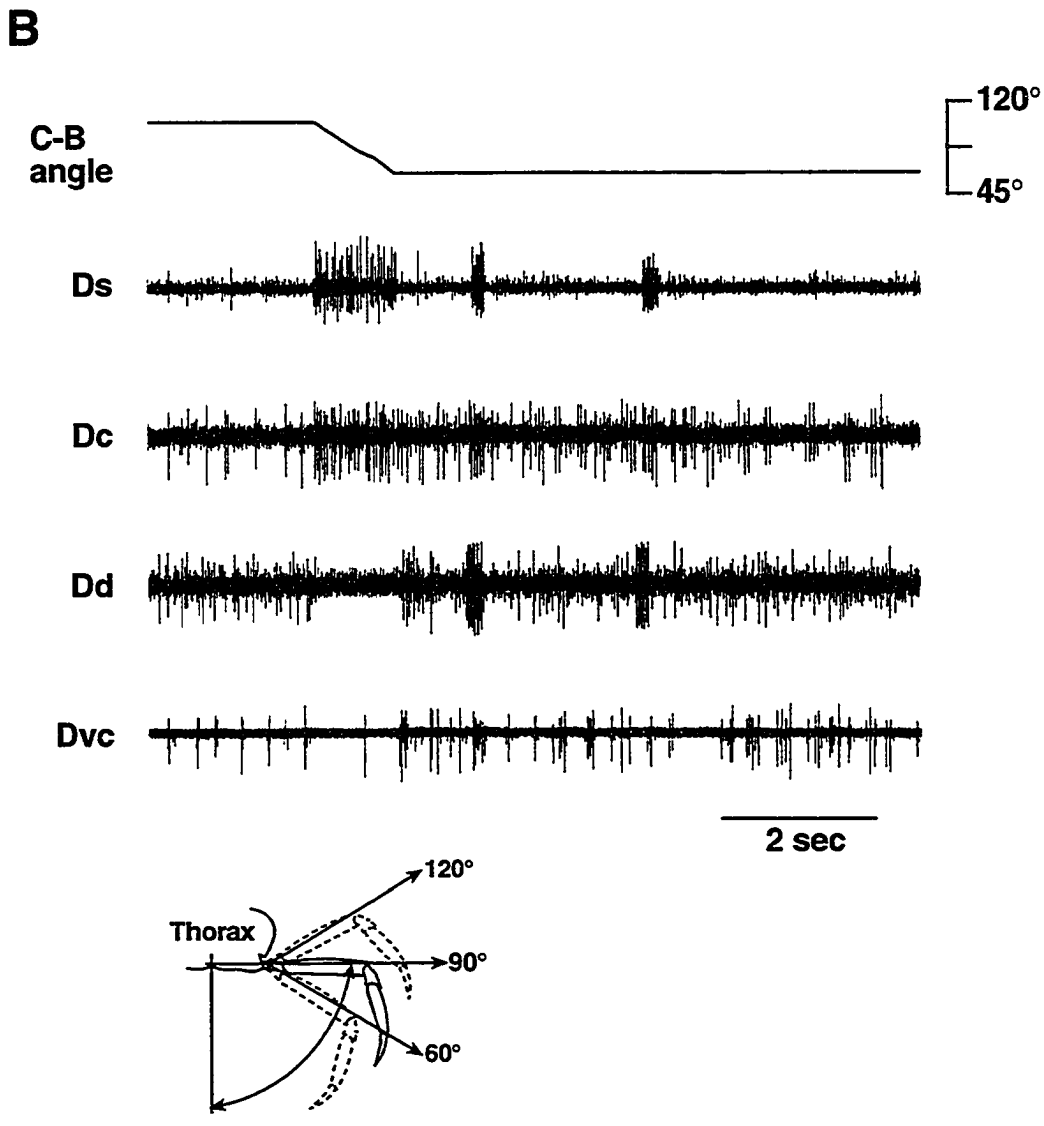
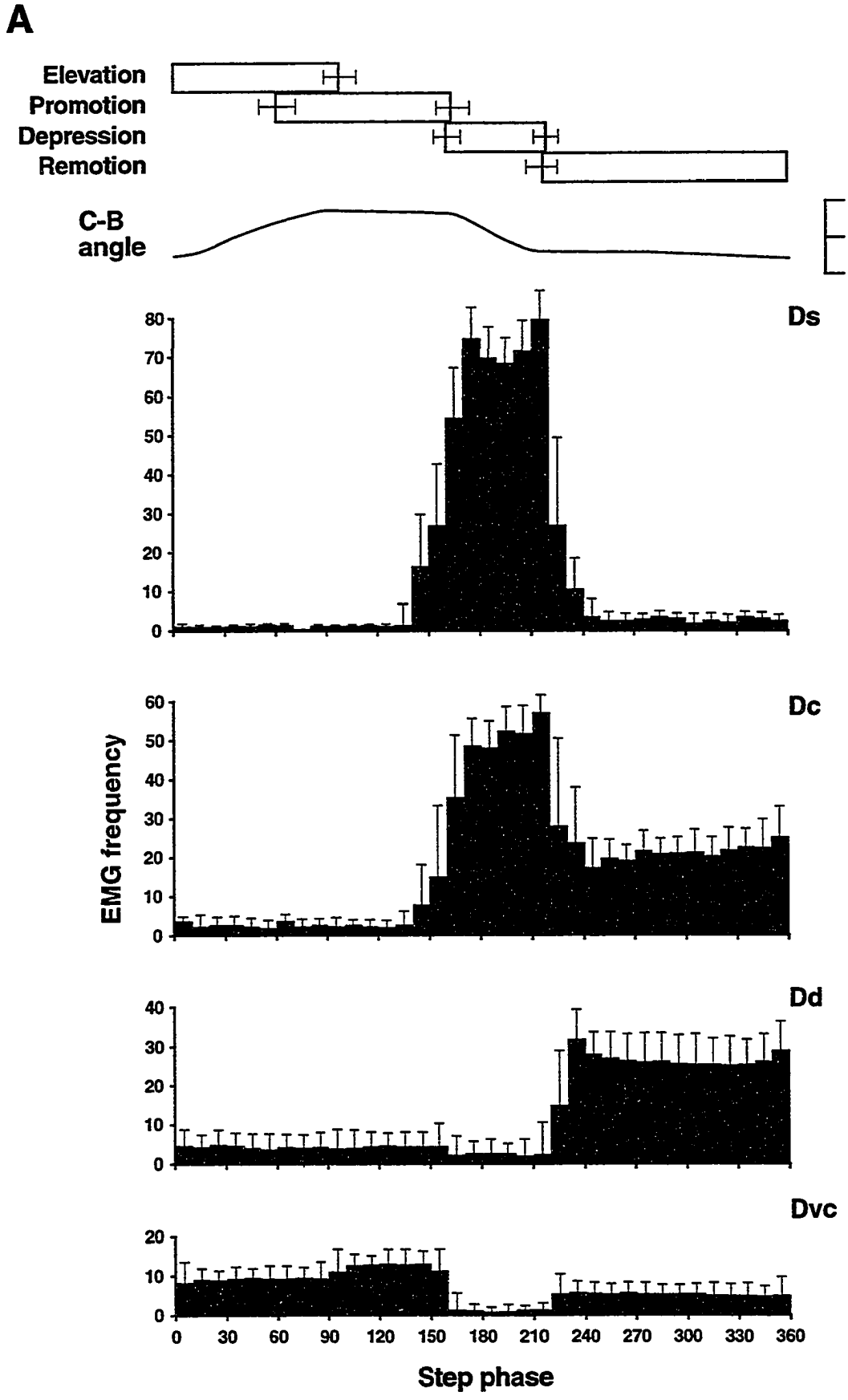
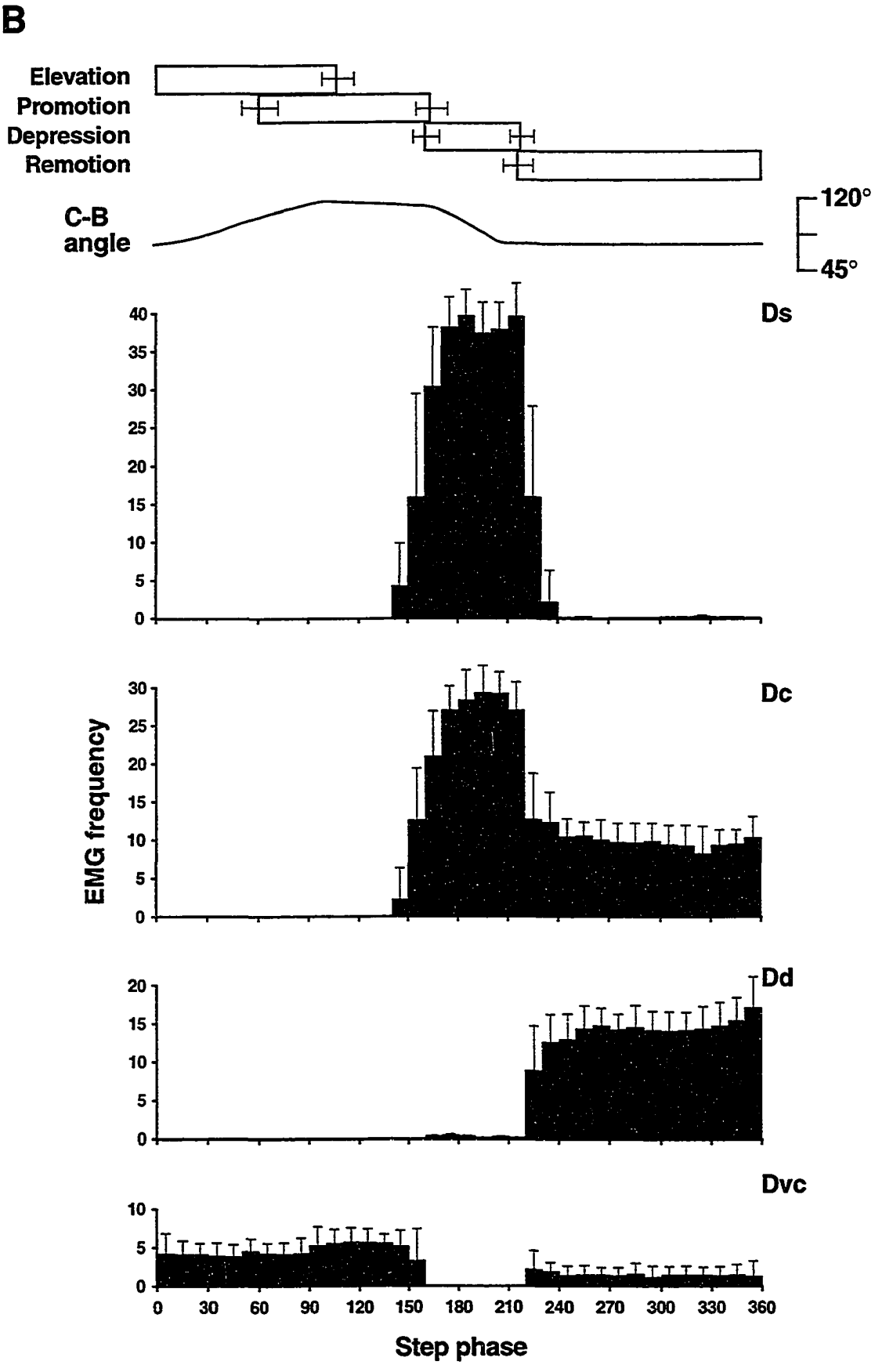

B
C-B angle
120°
45°
Ds
Dc
Dd
Dvc
2 sec
120°
Thorax
90°
60°

Fig. 6.16. Frequencies of depressor muscle potentials during forward walking in animals injected with serotonin or octopamine. (A) Animals injected with 0.002 mg/g 5-HT. Proximal joint movement durations, coxal-basal joint movements, and muscle potential frequencies are means of 38 walking cycles in 8 animals. (B) Animals injected with 0.001 mg/g OA. Data are the means of 36 walking cycles in 8 animals. The bars indicate standard errors of the EMG spike frequencies and onset and termination of joint movements; C-B angle, coxal-basal joint angle; Abbreviations for the muscle heads are the same as in Fig. 6.11.


A
Elevation
Promotion
Depression
Remotion
C-B angle
120°
45°
Ds
Dc
Dd
Dvc
EMG frequency
Step phase
0
30
60
90
120
150
180
210
240
270
300
330
360


B
Elevation
Promotion
Depression
Remotion
C-B angle
120°
45°
Ds
Dc
Dd
Dvc
EMG frequency
Step phase
0
30
60
90
120
150
180
210
240
270
300
330
360

given angular velocities during the depression movement (Fig. 6.17). I expected, due to the changes in muscle discharge, a decrease in the duration of depressions and possibly an increase in the duration of elevations in animals injected with 5-HT, and the opposite in animals injected with OA, but this did not occur. Apparently, discharge frequency of motoneurons to the depressor muscle is not the only factor controlling depression velocity and, therefore, duration during walking in animals injected with 5-HT or OA.

Injected 5-HT raises and OA lowers, in a dose-dependent manner, activity in the dorsal (coxal), caudal, and ventral depressor muscle heads during maintained stance (Fig. 6.18A). In individual animals, increasing the dose of either amine causes a progressively greater effect on muscle discharge (Figs. 6.18B & C). There is no evidence of punctuated changes in muscle activity correlated with the behaviour induced by the injected amines (see Chapter 2 for a description of the behaviours). This suggests an influence of the amines on the motor pathways independent of behavioural command circuits.

Although 5-HT also induces activity in the sternal head during maintained stance, the discharge frequency in this head is not dependent on joint angle, remaining consistent at about 3-5 Hz for 0.002mg/g injections, and 7 Hz for 0.005mg/g injections (averaged, but the actual discharge is bursty as described above), and therefore is not plotted. Muscle discharge frequencies in animals injected with 5-HT or OA are more variable both within and between animals for given coxal-basal joint angles than in untreated animals (see Appendix A, Table 1 for standard errors of the data plotted in Fig. 6.18A). Both amines also prolong the post-movement discharge frequency decay in the dorsal and coxal heads to between 30 and 100 seconds, indicating that both amines affect stability of the system (Fig. 6.19, compare with Fig. 6.8). Whether this instability is a natural effect of 5-HT and OA, or it is an artifact of widespread perturbations of many systems caused by the injections, is unknown.

Discussion

Functional implications of differential activation of depressor muscle heads

The multifunctional depressor muscle of *M. quadrispina* is responsible for pereiopod depression, supporting the weight of the animal against gravity (when the animal is standing upright), and maintaining tension at the coxal-basal joint. The activation patterns of the different heads suggest that each is specialized for a particular task (Table 6.1). The sternal head, which is the largest head by mass and is active mainly

Table 6.1. Activities of the heads of the depressor muscle and putative functions based on these data. (-) indicates the head is not active under the given conditions, (+++) indicates the greatest levels of activity for each head.

Table 6.1

Depressor head	Leg Action					Putative function
	Stationary Elevated	Stationary Depressed	Stance Depression	Walking Depression	Elevation	
Sternal	-	-	+++	+++	-	Mover
Coxal-Dorsal	+	+++	-	+	-	Stance
Caudal	+	++	++	+++	-	Stance and mover
Ventral (2)	+++	+	-	-	+	Joint tension/ stance?

Fig. 6.17. EMG spike frequency as a function of pereiopod depression velocity during walking in animals injected with 5-HT or OA. 5-HT increases and OA decreases EMG spike frequency without changing movement velocity. Data points were calculated as in Fig. 6.11; Ds, sternal head of the depressor, Dc, caudal head of the depressor.

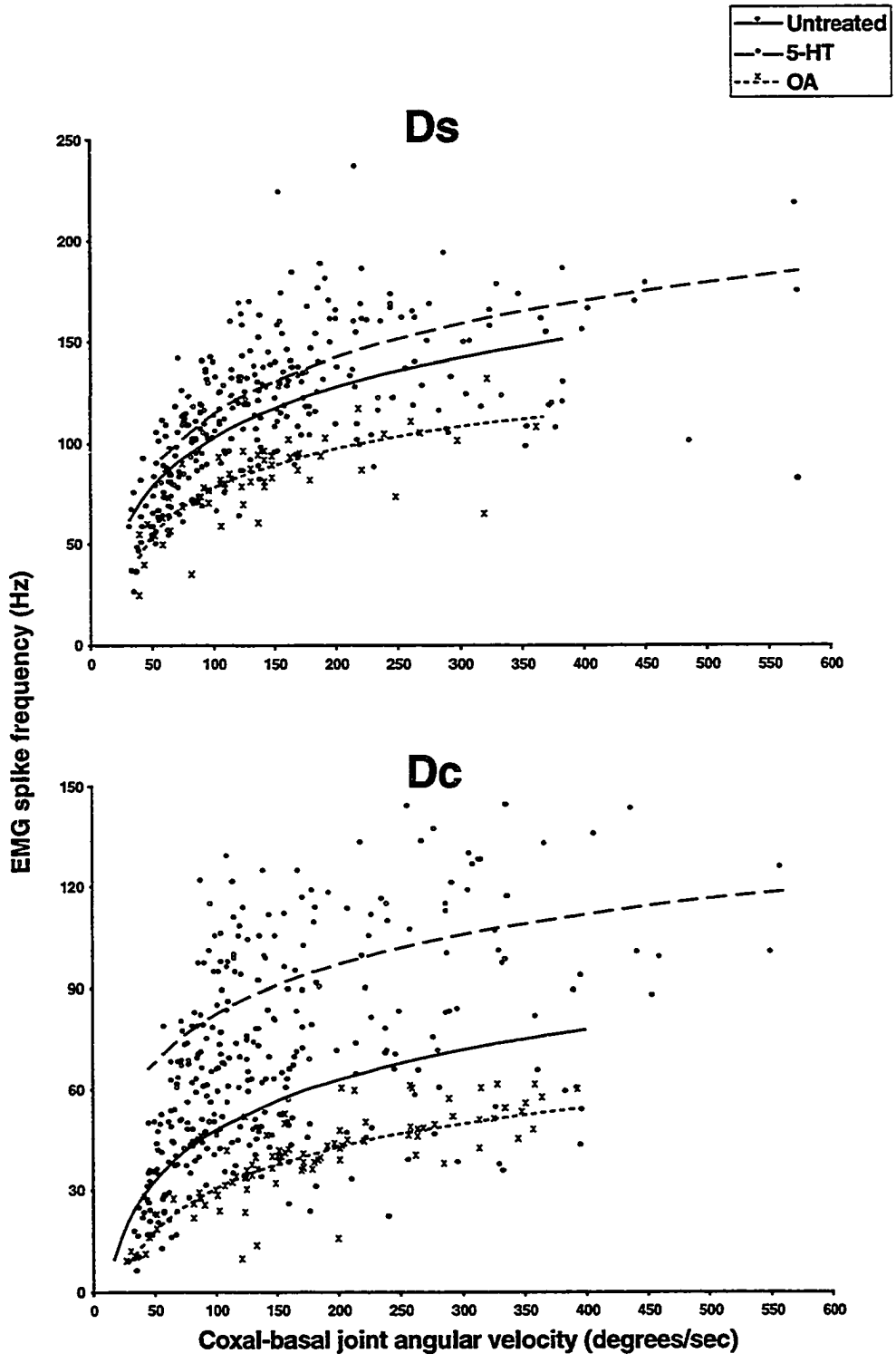
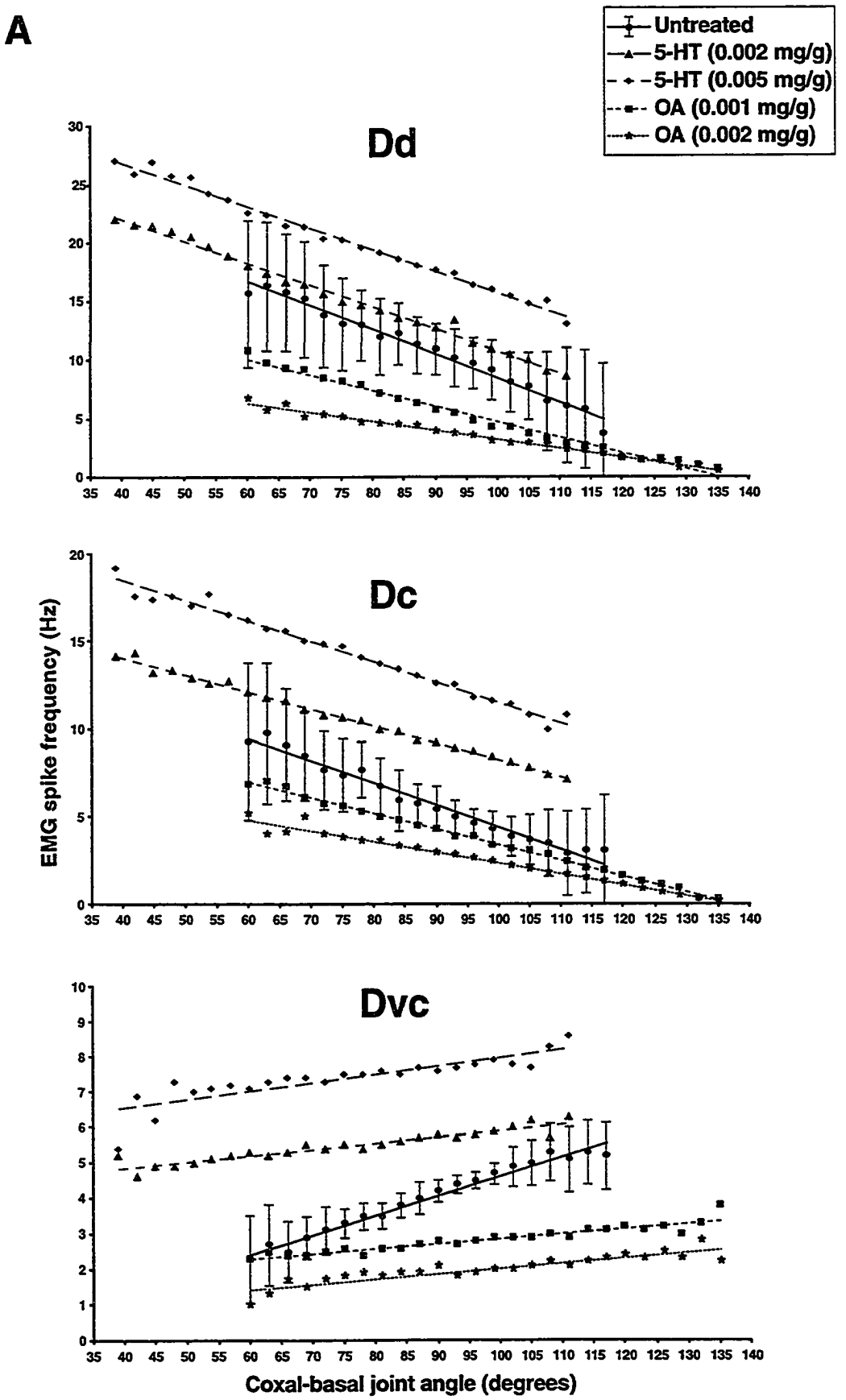
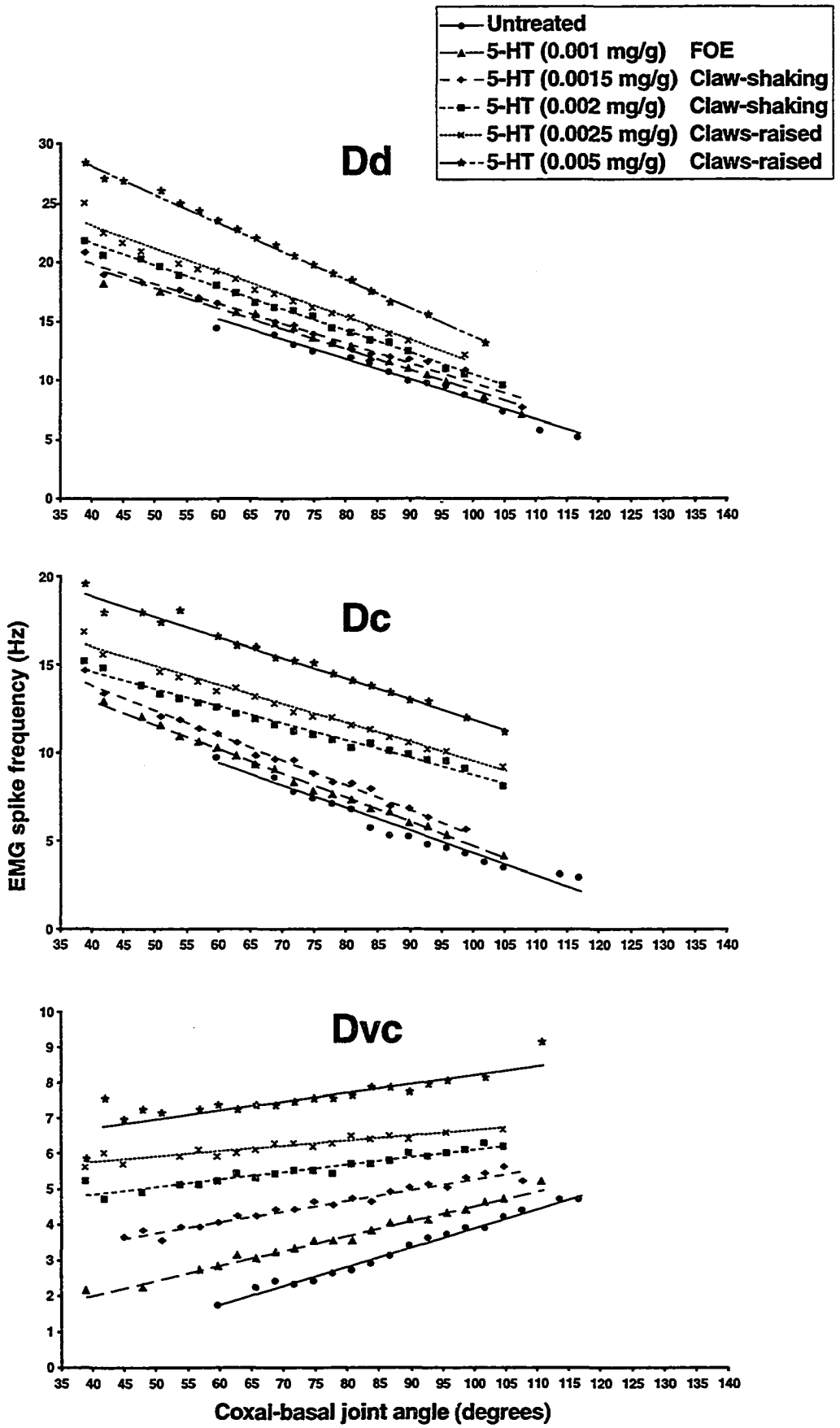

Untreated
5-HT
OA
Ds
Dc
EMG spike frequency (Hz)
Coxal-basal joint angular velocity (degrees/sec)

Fig. 6.18. Frequencies of depressor muscle potentials during maintained stance in animals injected with serotonin or octopamine. (A) Untreated is the mean (± SE) calculated from all interpretable traces from untreated animals that were not behaving agonistically. Means without standard error are given for animals that were injected with amines (see Appendix A for standard errors). (B) Data from an animal injected with a series of doses of 5-HT. (C) Data from an animal injected with a series of doses of OA. The legends in (B) and (C) give the amine doses and the behaviour induced (see Chapter 2 for a description of the behaviours). All data were taken from animals that were fully supporting themselves on the tips of their walking pereiopods. Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle; FOE, first observable effect.


A
Untreated
5-HT (0.002 mg/g)
5-HT (0.005 mg/g)
OA (0.001 mg/g)
OA (0.002 mg/g)
Dd
Dc
Dvc
EMG spike frequency (Hz)
Coxal-basal joint angle (degrees)

B


Untreated
5-HT (0.001 mg/g) FOE
5-HT (0.0015 mg/g) Claw-shaking
5-HT (0.002 mg/g) Claw-shaking
5-HT (0.0025 mg/g) Claws-raised
5-HT (0.005 mg/g) Claws-raised
Dd
Dc
Dvc
EMG spike frequency (Hz)
Coxal-basal joint angle (degrees)

C

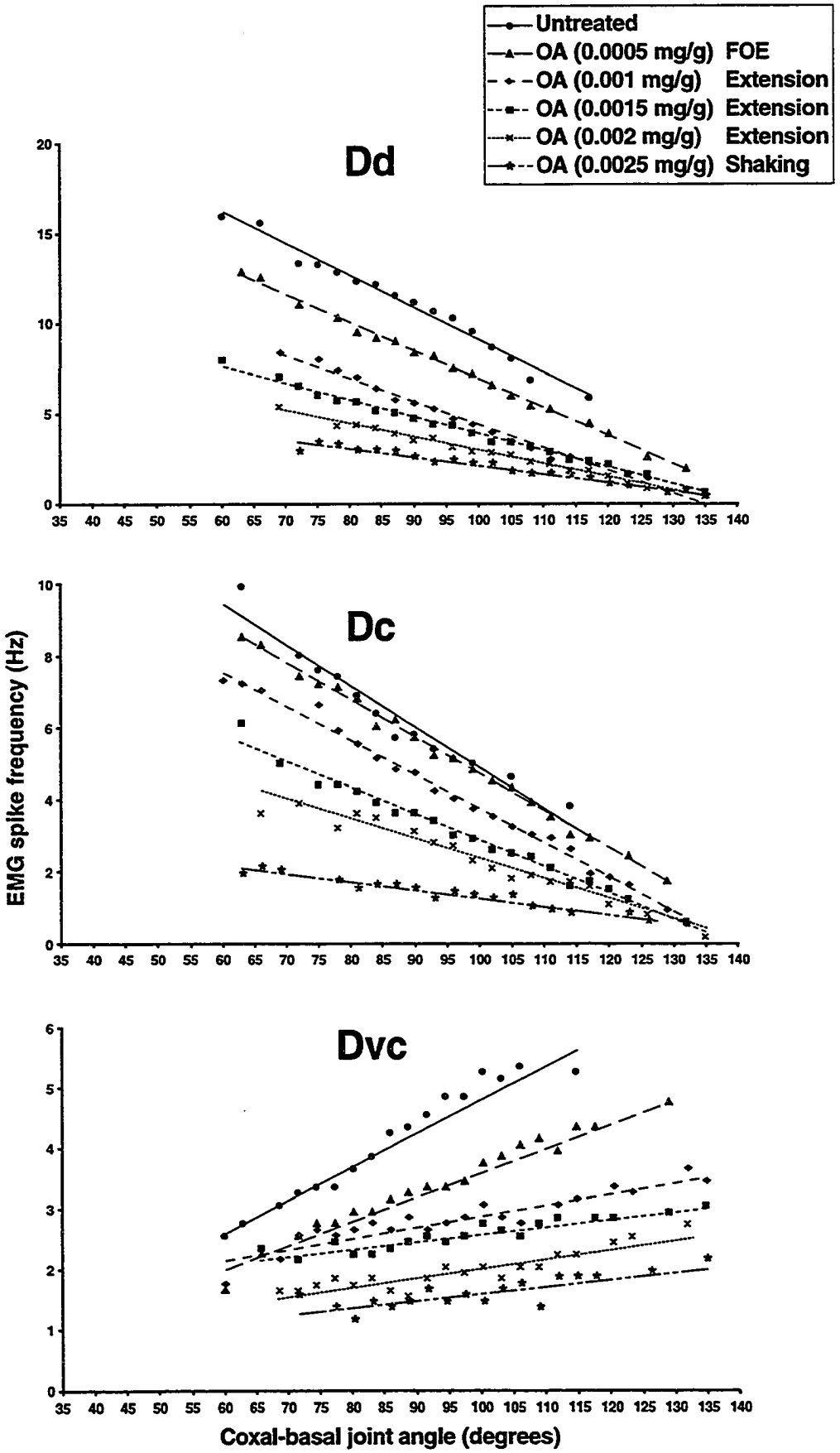
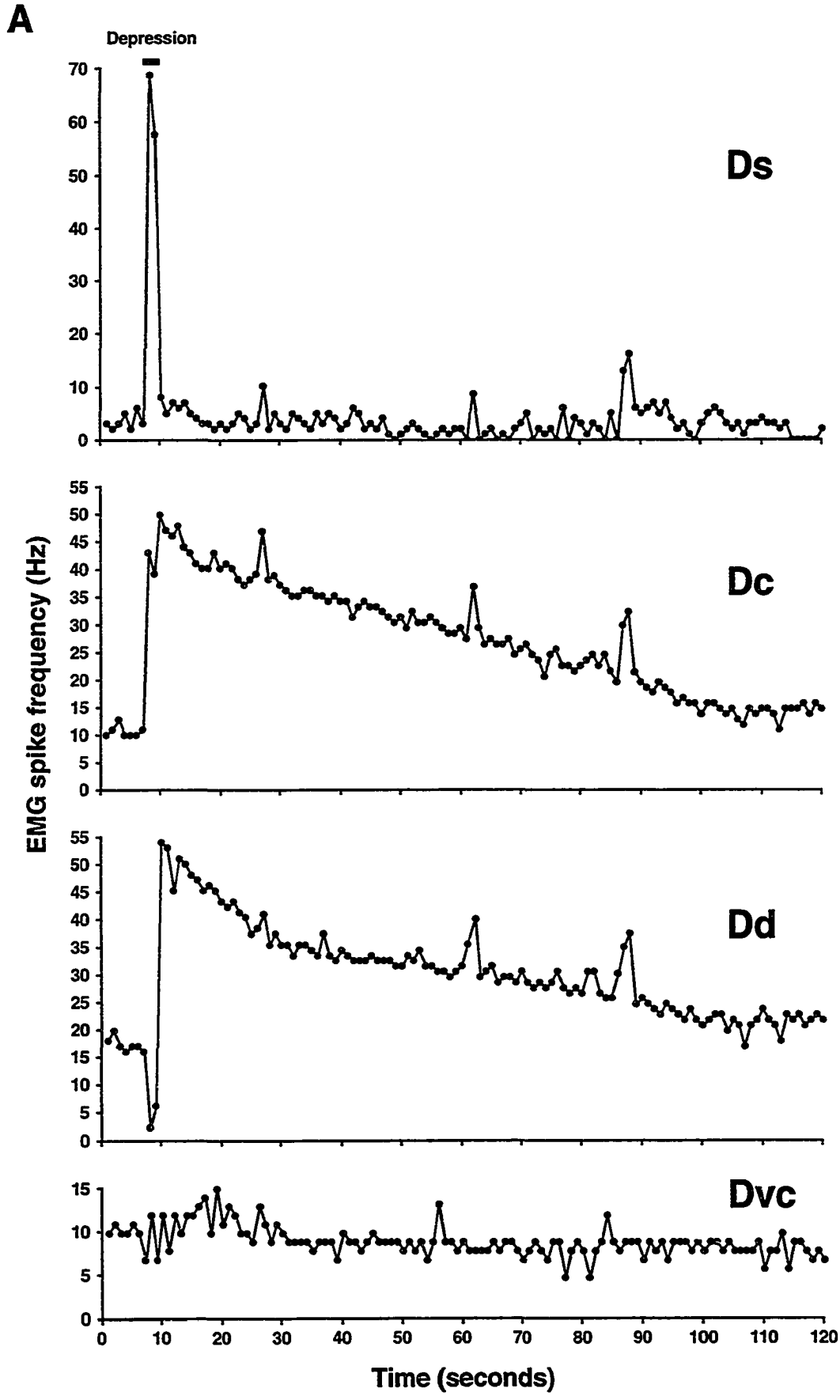
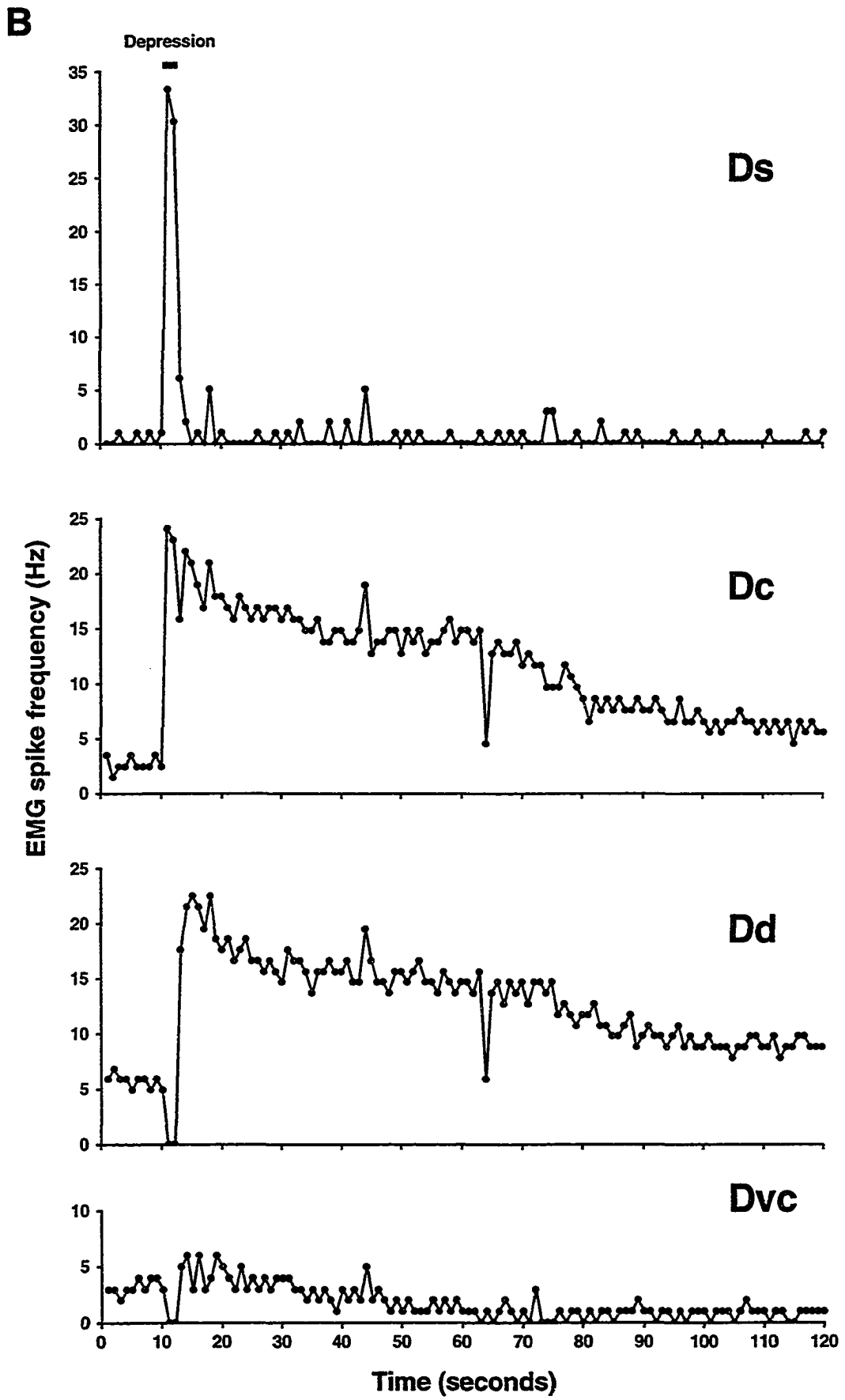

Untreated
OA (0.0005 mg/g) FOE
OA (0.001 mg/g) Extension
OA (0.0015 mg/g) Extension
OA (0.002 mg/g) Extension
OA (0.0025 mg/g) Shaking
Dd
Dc
Dvc
EMG spike frequency (Hz)
Coxal-basal joint angle (degrees)

Fig. 6.19. Frequencies of depressor muscle potentials during and after postural pereiopod depressions in animals injected with serotonin or octopamine. Results (A) from an animal injected with 0.002 mg/g 5-HT, (B) from an animal injected with 0.0005 mg/g OA. Dc, caudal head of the depressor muscle; Dd, dorsal head of the depressor muscle; Ds, sternal head of the depressor muscle; Dvc, ventral caudal head of the depressor muscle. Note differences in y-axis scales.


A
Depression
Ds
Dc
Dd
Dvc
EMG spike frequency (Hz)
Time (seconds)


B
Depression
Ds
Dc
Dd
Dvc
EMG spike frequency (Hz)
Time (seconds)

when the pereiopod is being depressed, is the main mover of the leg. The dorsal and coxal heads, which are active during maintenance of raised stances but not when the animal is resting on the substrate or the pereiopod is being depressed, are mainly responsible for supporting the weight of the animal against gravity. The caudal head is activated synergistically with the sternal head and with the dorsal/coxal heads, but it is much smaller than any of its synergists and, therefore, it may serve an accessory role to each of them. Until the physiological properties and contractile responses of the heads are determined, however, the roles of all the depressor muscle heads remain speculative.

The role of the two ventral heads is somewhat less clear. These heads' activation patterns are essentially opposite to the other heads, and their innervation is completely separate (see Chapter 5). Although these heads are active during maintenance of raised stances, activity is not diminished when the animal is resting its weight on the substrate. These heads could assist in maintaining the tilt of the thorax in the animal's resting position (i.e. holding the head up), but their activity under other circumstances suggests that this is probably not their only function. Another possibility is that they are responsible for maintaining joint tension when the leg is elevated, acting as an indirect inhibitory force on the levator muscles, but this is speculative.

The ability to attain and maintain tension in response to excitatory input, contraction speed, and contraction strength all vary between individual muscle fibers in crustaceans (e.g. Sherman & Atwood, 1972; Costello & Govind, 1983; Rathmayer & Hammelsbeck, 1985). Variation in physiological and biochemical properties of individual fibers has been found between and within heads of the levator muscles of swimming crabs (Hoyle, 1973; Tse et al., 1983). However, except for these studies, very little has been done on the physiological properties of any proximal muscle, possibly in part due to the anatomical complexity of these muscles. It is not known whether differences in physiological properties among the heads of the depressor muscle which correlate with differences in activation patterns and presumptive functions exist.

The evolutionary history of *M. quadrispina*'s depressor muscle is unknown, but it seems reasonable to hypothesize that its multiple heads are derived from a simpler precursor following muscle duplications. Examples of muscle duplication, where one ancestral muscle divided and resulted in several descendant muscles or heads, have been described in vertebrates and invertebrates. Duplication of muscles could release evolutionary constraints allowing for functional diversification to occur. In most examples, functional disparity between the heads has not evolved, and the new muscles

are activated as a single unit. This is true of the femoral depressor muscle of cockroaches, which is believed to be homologous to the depressor muscle of crustaceans (Carpentier & Barlet, 1959). The femoral depressor is divided into many heads with origins in the coxa and thorax, but all of the heads are innervated by only two motoneurons, one fast and one slow, and are activated synchronously (Watson & Ritzman, 1998). This may represent the simplest case of muscle duplication; multiple muscles or heads derived from a single ancestor have retained the plesiomorphic innervation and activation patterns.

Selective pressures for functional diversification of duplicated muscles could come from at least two sources. The first is the freedom to develop evolutionarily new functions. The ancestral function of the muscle could be retained by one or more of the new muscles while others are remodeled to perform a function which was not part of the ancestral repertoire, perform an ancestral function more effectively, or allow a refinement of the ancestral function, such as exploitation of a new prey species (e.g., the jaw muscles of tetraodontiform fishes; Friel & Wainwright, 1998). The second is an increase in efficiency. Efficiency is defined as the ratio of work performed to energetic input (Lauder, 1991). Efficiency of crustacean muscles has not, to my knowledge, received much attention, but plausible arguments can be made as to how selective forces acting to increase efficiency could shape crustacean muscles. It seems that intermixing muscle fibers specialized for different functions within a single anatomical muscle, as is the case in the distal pereiopod muscles (for a review see Rathmayer & Maier, 1987), should be inherently inefficient. This is because activated muscle fibers must not only perform the work they are assigned, but must also work to overcome the resistance of compressing or distorting attached, inactive fibers. Furthermore, a muscle must be large and powerful enough to perform its most strenuous function, and it is not energetically favourable to use a large muscle for a task which requires only a fraction of its strength. The evolution of abdominal musculature in macruran crustaceans may have been influenced by potentially greater considerations of efficiency achieved by separation of function. In the abdomen, the large fast flexor and extensor muscles are responsible for fast, powerful tailflips, while the much smaller slow muscles are responsible for postural changes. Similarly, efficiency could have influenced the functional evolution of the depressor muscle, resulting in functional separation among the different heads. It is difficult to hypothesize about the role of functional pressures on depressor evolution because, although the earliest arthropod thoracic appendages are believed to have been used for

locomotion, the details of the evolutionary history of this muscle are not known. However, limb autotomy is almost certainly a new behaviour that evolved in the crustaceans, since it is not present in the other arthropod taxa. Functional diversity has evolved among the heads of the levator muscle allowing performance of this new behaviour (Moffett, 1975; McVean & Findlay, 1976).

Differences in synaptic inputs among depressor motoneurons

Although differential activation of heads of the depressor muscle has not been reported in crayfish, a growing body of evidence reveals that depressor motoneurons in crayfish are not a homogenous population. Pearlstein et al. (1995) classified motoneurons into three groups based on their activation patterns during fictive posture and fictive walking in isolated nerve cords: motoneurons which are active only during posture, active only during walking, and active during both fictive behaviours. These authors also reported graded recruitment of depressor motor neurons during a burst. In contrast I saw no direct evidence of this in freely moving *M. quadrispina*. Depressor motoneurons in crayfish also differ from those of the other proximal leg muscles in their synaptic connections with other motoneurons. Most motoneurons are electrically coupled to one another within each of the motoneuron pools for the promotor, remotor, and levator muscles, but only some depressor motoneurons are electrically coupled while others appear to have only chemical synaptic connections (Chrachiri & Clarac, 1989). Graded inhibitory synaptic connections are made on depressor motoneurons by antagonistic levator motoneurons, but not all depressor motoneurons receive input from all levator motoneurons (Chrachiri & Clarac, 1989; Pearlstein et al., 1998B). None of these differences has been correlated with differential innervation of parts of the depressor muscle, but they do, along with the data reported here, suggest functional subdivision within the depressor muscle in decapods.

Several sensory systems have been found to directly or indirectly influence activity of depressor motor neurons. Cuticular stress detectors are organs located in the basis and ischium which respond to stress placed on the pereiopod, such as is produced by the load of an animal's weight (Klärner & Barnes, 1986; Marchand et al., 1995; Leibrock et al., 1996). Sensory afferents from the cuticular stress detectors make monosynaptic and polysynaptic connections with depressor motoneurons (Leibrock et al., 1996). Most of the inputs to depressor muscle motoneurons from afferents of cuticular stress detector 1 are excitatory, but afferents from cuticular stress detector 2 make both

excitatory and inhibitory inputs. Furthermore, individual sensory afferents of the cuticular stress detectors respond in very different manners to strains; some respond to the onset and some to the offset of a stimulus, their firing patterns can be phasic, phasotonic, or tonic, and some afferents of cuticular stress detector 1 only respond to very high amplitude vibrations (Marchand et al., 1995). Clarac et al. (1987) found that EMGs from pereiopod muscles of shore crabs walking on land and walking in water differ in ways that could be a response to the increased load on land. Furthermore, significant differences occur in leg movements and muscle activities between cockroaches walking upright and upside-down (Larsen et al., 1995). *M. quadrispina*'s natural habitat is on steep, rocky terrain, and they are found clinging to any available surface in any orientation. I did not examine the effects of different orientations on depressor muscle activity, but it is likely that cuticular stress detectors, as well as possibly statolithic organs (Hisada & Neil, 1985), could have roles in fine tuning depressor output to fit these circumstances.

Coxo-basal chordotonal organs and thoracico-coxal muscle receptor organs encode information concerning movement and position of their respective joints. The responses of their sensory afferents also differ according to phases and intensity of stimulation. Sensory afferents from both of these organs make synaptic connections with depressor motoneurons which vary in sign, with stimulus phase, or with stimulus intensity (El Manira et al, 1991A; Head & Bush, 1991; LeRay & Cattaert, 1997; LeRay et al, 1997). Several other sensory organs which have roles in posture and walking, such as contact-sensitive mechanoreceptive afferents of the dactyl (Libersat et al., 1987; Marchand et al., 1997) and tension receptors, which have only been described in crabs (MacMillan & Laverack, 1982), could also influence depressor muscle motoneurons.

It is clear from these experiments and my data that depressor motoneurons cannot be considered as a single functional pool. Differences in central control, feedback, and sensory input could allow each motoneuron to be controlled individually. Although I have not looked at the influences of any of these sensory systems on depressor motoneurons in *M. quadrispina*, and differences in sensory inputs among depressor motoneurons in crayfish have not been correlated with peripheral innervation patterns of the motoneurons, it is reasonable to expect that sensory inputs to the motoneurons controlling the individual heads of the depressor muscle are different. For example, inputs from cuticular stress detectors may influence the motoneurons innervating the dorsal, coxal, and caudal heads in a way which increases activity when the animal is

maintaining raised upright stance (supporting a load), and sensory afferents of the coxo-basal chordotonal organ could have opposite influences on the motoneurons of the dorsal and ventral heads. Taken together, these morphological and physiological data strongly suggest that functional subdivision has occurred in motor units of the depressor muscle and the sensory and feedback influences on them.

5-HT and OA have multiple influences on the systems which contribute to depressor muscle output. Contractility of muscle fibers, neurotransmitter release, postural circuits, and sensitivity and output of sensory organs are all influenced by one or both of the amines (for a review see Beltz, 1999). Although the end result, that 5-HT increases and OA decreases depressor muscle activity, is clear, the way this is accomplished is complex and not easily discerned. Studies in isolated nerve cords and partially-intact neuromuscular preparations have told us much about some of the individual sites of action of these amines, but only in intact animals can we see what the actions of all of the contributing systems combine to produce.

Chapter 7: Summary

Two important discoveries emerged from my research on the effects of injected serotonin (5-HT) and octopamine (OA) on agonistic behaviours in *M. quadrispina*. First is that complex aggressive behaviours can be induced in isolated animals by injection of carefully controlled doses of 5-HT, in the absence of any additional stimulation. Presumably the injections are mimicking endogenous release of serotonin during normal agonistic encounters, but it is not known where natural release may be taking place, or what specific neurological systems may be affected. I hypothesized that the effects of serotonin are at several levels, from the muscles involved in performing the behaviours to the higher command centers responsible for behavioural choice. The second important discovery was that a behaviour that appears to have been lost in *M. quadrispina*, aggressive fighting, can be induced by 5-HT injection. Defensive fighting does still occur in *M. quadrispina*, but this is a very different behaviour. In aggressive fighting, the aggressive animal grasps its opponent and systematically tries to pull it apart, much as occurs during crayfish fights. Defensive fighting, on the other hand, involves grasping and pushing in an attempt to shove the opponent away and escape. Furthermore, I observed defensive fighting only in response to threats from other species, never in conflicts with conspecifics, except by opponents of animals injected with 5-HT. This leads to the question of what exactly has been lost in *M. quadrispina*? The circuitry for fighting appears to be present, so what is it doing in normally interacting animals?

Injected 5-HT and OA change activity levels in the depressor muscle in a dose dependent manner. This is an important observation, as it suggests that the amines have effects on the postural control mechanisms that are independent of their effects on centers responsible for behavioural choice. Of course, gathering these data proved to be far more complex than anyone anticipated, as it was during the beginning of these experiments that I discovered that the depressor muscle is not activated as a single unit. It is rather curious that activity of all of the depressor heads are affected in more or less the same way by amine injections, I had expected some differences to occur. Not enough is known about the control of depressor muscle motoneurons to determine whether this similarity is due to common influences on all of the heads, or similar, but independent, influences on each of the heads. It is also possible, although the phenomenon is still interesting, that the similar effect is artificial and does not occur with natural 5-HT release.

The division of labour found among the heads of the depressor muscle in *M. quadrispina* raises several related questions, two of which I will consider now. First,

does it exist in other species, and if so, why hasn't anyone noticed? The answer to the first part of this question is very likely yes, although whether other species have the same pattern of division of labour as in *M. quadrispina*, or show different patterns adapted to their own particular habit, remains to be seen. The answer to the second part of the question is likely because no one has ever looked. In the vast majority of studies recording electromyograms from other species, leads were implanted near the depressor apodeme. I tried this in *M. quadrispina* as well, but because of the small size of the animals, this method did not work well. Leads implanted near the base of the depressor apodeme most often recorded from several heads simultaneously, although which heads depended on the precise location of the electrodes, which could not be precisely controlled. Therefore, my recordings from this location were inconsistent and forced me to find other places to implant. In larger species, the problems I had recording from the base of the depressor apodeme are less likely to occur, and the recordings would appear to be from one homogenous unit. The second question is, if a division of labour does not exist among the heads of a multi-headed muscle, why not? It has been shown that muscle duplication can occur without corresponding functional diversity (see Chapter 6), but why would this not be used to the best advantage? One possibility is that functional diversity may not be needed in a muscle if it performs a single function. This may be true of the remotor muscle of crabs (Bévengut et al., 1983), which appears to be involved mainly in locomotion. A second possible reason is that functional diversity may not have had time to evolve since muscle duplication. It is not known how long ago the subdivision of the proximal leg muscles into heads started, but since they occur in all decapods, and functional diversity has occurred in some of the muscles in at least some species, this possibility seems unlikely. Another possibility is that there may be constraints of which we are not aware that could be restricting functional diversification in some muscles.

Several differences in the serotonergic and octopaminergic immunoreactive maps between *M. quadrispina* and other decapods exist which may have important functional consequences. For example, loss of the unpaired medial serotonergic cells in *M. quadrispina* may be associated with loss of the giant tailflipping circuits, and loss of the so-called claw octopamine cells may be correlated with a difference in behaviour between *M. quadrispina* and American lobsters; the latter becomes submissive upon losing a claw, but the former does not. I was not able to confirm any of these potential functional changes, but, besides being interesting in its own right, this study did show

how comparative studies can be used to help determine places to look for functional interactions.

The Next Steps

As is the case with most research projects, this dissertation has raised far more questions than it has answered. It is clear from the amine-immunoreactive maps that significant differences exist between species, even between closely related species, such as lobsters and crayfish. Certainly independent work on the physiology and effects of the aminergic systems in all of these animals will not be done, but having such a range of immunocytochemical data will help perform this work in crayfish and lobsters, both by suggesting possible places to look for interesting interactions, and in providing a broader framework in which to interpret results.

The differential control of heads of the depressor muscle in *M. quadrispina* raises interesting questions with regard to the work which has been done on depressor motoneurons in crayfish. It has been shown that the depressor motoneurons are not a homogenous group with respect to sensory input and activation during fictive behaviours (see Chapter 6). It will be interesting to see if these differences in motoneuron properties correlate with differences in innervation patterns of the heads of the depressor muscle in crayfish. However, such studies will be very difficult to perform. In order to clearly separate the activity of the different heads in freely moving animals, electromyographic recording electrodes must be fully embedded within each muscle head of interest, near its origin. In crayfish, the origins of some of the heads are not as easily accessible as in *M. quadrispina*, as they are located deep in the thorax, with no unobstructed path from the cuticle through which recording electrodes may be placed. Furthermore, my feeling is that attempting to study this system in a partially intact animal, as is often done, may not give an accurate picture of what is happening in freely moving animals. In one experiment I did early in this study, I stimulated a depressor motoneuron in *M. quadrispina* via an intracellular electrode and recorded excitatory post synaptic potentials in the sternal, caudal, and dorsal heads of the depressor muscle, separated by only 1 ms. Evidence I gathered from backfills and electromyograms in freely moving animals indicates that the dorsal head does not share innervation with the sternal and caudal heads. Chrachiri & Clarac (1989) found that some depressor motoneurons are electrically coupled, while others apparently are not. This could indicate two things: first is that my data are misleading, and the sternal and dorsal heads do share innervation, or

second, that motoneurons innervating these heads are electrically coupled. I suspect that the latter is true, and at least some depressor motoneurons that innervate heads with different activation patterns are electrically coupled. If this is true, all of the heads could, therefore, be activated together, but because this does not occur during any of the natural behaviours examined in Chapter 6, the existence of some influence which inhibits electrical coupling under certain circumstances is implied. There are a number of questions raised by this theory which are relevant to understanding how pereiopods perform different behaviours.

The habitat of *M. quadrispina* is rocky and uneven, and as a result of this their normal stance is often not upright with respect to gravity. It would be interesting to determine if the other three proximal muscles, the levator, promotor, and remotor muscles, which are all responsible at different times for supporting the weight of the animal against gravity, have a division of labour similar to the depressor muscle. Furthermore, comparison with an animal that maintains a more or less upright stance, such as crayfish, could reveal if in fact the energetic and mechanical demands of body support vs. locomotion are driving functional diversification in the proximal muscles.

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Appendix A

Table A.1 EMG spike counts in the heads of the depressor muscle of *M. quadrispina* during maintained stance. Means and standard errors are given for untreated (normal) animals, and animals injected with doses of serotonin (5-HT) or octopamine (OA).

		Dorsal			
	Normal	5-HT		OA	
Joint Angles		0.002 mg/g	0.005 mg/g	0.001 mg/g	0.002 mg/g
39		22.08±10.24	27.12±12.35		
42		21.62±11.51	25.92±12.42		
45		21.48±10.34	27.03±13.04		
48		21.03±10.64	25.81±11.64		
51		20.52±9.88	25.70±10.95		
54		19.68±9.75	24.33±10.68		
57		18.84±9.95	23.74±10.21		
60	15.72±6.24	17.97±9.56	22.63±10.04	10.80±8.24	6.81±6.21
63	16.32±5.52	17.40±7.89	22.38±9.64	9.79±8.13	5.78±5.56
66	15.84±5.04	16.68±7.56	21.50±9.02	9.36±7.65	6.42±5.67
69	15.24±4.92	16.44±7.64	21.43±8.57	9.22±7.03	5.19±4.84
72	13.80±4.32	15.60±7.51	20.37±8.03	8.49±6.48	5.37±4.67
75	13.08±3.96	15.02±7.03	20.26±7.48	8.21±5.92	5.23±4.62
78	12.96±3.00	14.64±6.88	19.62±7.51	7.92±5.61	4.81±4.23
81	12.00±3.24	14.16±6.02	19.19±6.64	7.20±5.06	4.71±3.78
84	12.24±2.64	13.56±5.98	18.61±6.45	6.77±4.98	4.63±3.72
87	11.28±2.40	13.20±5.50	17.96±6.41	6.34±4.87	4.47±3.64
90	10.92±2.16	12.72±5.68	17.68±6.78	5.76±4.56	3.97±3.41
93	10.20±2.40	13.32±6.03	17.42±7.03	5.47±4.04	3.79±3.24
96	9.72±2.16	11.40±6.67	16.39±7.54	4.90±3.67	3.64±3.18
99	9.12±2.52	10.92±6.85	15.98±8.03	4.32±3.64	3.17±3.02
102	8.16±2.53	10.44±7.03	15.43±8.24	4.32±3.57	3.04±2.67
105	7.80±2.88	9.96±7.65	14.81±8.31	3.74±3.38	2.96±2.48
108	6.48±4.20	9.00±7.45	15.04±8.23	3.31±3.02	2.77±2.51
111	6.12±4.92	8.64±7.01	12.97±7.77	2.90±2.91	2.38±2.03
114	5.76±5.04			2.70±2.64	2.34±1.99
117	3.72±6.00			2.50±2.03	2.11±1.89
120				1.70±1.82	1.69±1.53
123				1.53±1.02	1.48±1.42
126				1.60±1.46	1.39±1.45
129				1.44±1.54	0.83±1.21
132				1.01±1.31	1.01±1.43
135				0.72±1.22	0.62±0.96

Table A.1 cont.

	Caudal				
	Normal	5-HT		OA	
Joint Angle		0.002 mg/g	0.005 mg/g	0.001 mg/g	0.002 mg/g
39		14.16±9.12	19.21±13.51		
42		14.32±9.23	17.58±10.24		
45		13.20±8.79	17.42±11.32		
48		13.36±8.74	17.57±10.24		
51		12.88±6.94	17.03±10.03		
54		12.56±6.75	17.76±9.65		
57		12.72±6.72	16.57±9.62		
60	9.28±4.48	12.08±6.58	16.20±9.04	6.88±4.75	5.18±4.41
63	9.76±4.00	11.56±6.52	15.68±8.24	7.04±4.46	4.02±3.98
66	9.12±3.12	11.60±6.03	15.63±7.65	6.72±4.23	4.13±3.87
69	8.48±2.56	11.12±5.92	14.96±7.23	6.08±4.31	4.98±3.81
72	7.68±2.24	10.80±5.64	14.76±6.99	5.76±4.27	4.04±3.67
75	7.36±2.08	10.64±5.45	14.72±6.84	5.60±4.18	3.81±3.54
78	7.68±1.60	10.48±5.23	14.08±6.60	5.28±4.06	3.60±3.02
81	6.72±1.60	10.00±4.87	13.66±6.00	4.96±3.91	3.58±3.10
84	5.92±1.76	9.84±4.65	13.36±5.54	4.80±3.85	3.31±2.98
87	5.76±1.12	9.36±4.46	13.03±5.23	4.48±3.81	3.17±2.84
90	5.44±1.28	9.20±4.43	12.60±5.03	4.32±3.67	2.86±2.73
93	4.96±0.96	8.88±4.37	12.51±5.02	3.84±3.21	2.79±2.30
96	4.64±0.80	8.72±4.86	11.83±5.21	3.84±3.05	2.64±2.03
99	4.32±0.96	8.40±4.90	11.56±5.23	3.36±3.11	2.43±1.98
102	3.84±1.12	8.08±5.13	11.35±5.36	3.20±2.81	2.20±1.93
105	3.68±1.44	7.76±5.24	10.82±5.68	3.04±2.64	2.01±1.72
108	3.52±1.92	7.44±5.50	10.03±6.91	2.88±2.43	1.82±1.64
111	2.88±2.40	7.12±5.89	10.77±7.26	2.40±2.01	1.66±1.05
114	3.04±2.43			2.08±1.86	1.51±1.13
117	3.04±3.20			1.92±1.67	1.27±1.10
120				1.60±1.30	1.11±0.98
123				1.28±1.22	0.93±0.86
126				1.12±1.13	0.74±0.64
129				0.96±1.03	0.45±0.66
132				0.32±0.51	0.31±0.54
135				0.32±0.87	0.22±0.69

Table A.1 cont.

	Ventral				
	Normal	5-HT		OA	
Joint Angles		0.002 mg/g	0.005 mg/g	0.001 mg/g	0.002 mg/g
39		5.19±3.96	5.41±4.02		
42		4.58±3.68	6.94±4.11		
45		4.88±3.56	6.22±3.92		
48		4.91±3.20	7.31±3.88		
51		5.03±3.41	6.98±3.80		
54		5.14±3.44	7.12±3.71		
57		5.18±3.42	7.20±3.62		
60	2.32±1.23	2.26±3.33	7.13±3.63	2.33±1.99	0.99±1.25
63	2.68±1.14	5.23±3.35	7.34±3.43	2.51±1.96	1.31±1.31
66	2.53±0.86	5.34±3.32	7.38±3.39	2.38±2.03	1.66±1.20
69	2.88±0.60	5.47±3.26	7.44±3.21	2.41±1.85	1.51±1.16
72	3.12±0.67	5.44±3.06	7.39±3.34	2.48±1.66	1.72±1.15
75	3.26±0.41	5.53±2.97	7.52±3.03	2.56±1.54	1.81±1.16
78	3.51±0.37	5.48±2.68	7.54±3.06	2.41±1.55	1.88±1.03
81	3.48±0.35	5.53±2.54	7.59±2.99	2.61±1.61	1.83±1.12
84	3.80±0.33	5.61±2.35	7.54±3.12	2.64±1.48	1.90±1.09
87	4.03±0.44	5.67±2.19	7.66±3.10	2.71±1.44	1.94±1.03
90	4.18±0.31	5.76±2.22	7.64±3.06	2.81±1.43	2.11±0.96
93	4.40±0.25	5.74±2.24	7.70±2.89	2.73±1.40	1.78±0.99
96	4.48±0.22	5.80±2.13	7.77±3.36	2.77±1.44	1.87±1.02
99	4.67±0.30	5.88±2.65	7.88±3.35	2.91±1.38	2.03±0.94
102	4.85±0.56	5.96±2.69	7.84±3.46	2.93±1.37	2.01±0.86
105	5.02±0.65	6.15±2.81	7.71±3.69	2.90±1.30	2.11±0.85
108	5.34±0.80	5.74±3.15	8.31±4.06	3.02±1.31	2.16±0.89
111	5.12±0.92	6.33±4.48	8.56±5.12	2.96±1.33	2.05±0.93
114	5.26±0.90			3.08±1.36	2.23±0.99
117	5.15±0.95			3.12±1.49	2.34±1.06
120				3.22±1.54	2.39±1.12
123				3.14±1.67	2.30±1.24
126				3.24±1.75	2.48±1.31
129				2.98±1.77	2.29±1.39
132				3.30±1.90	2.81±1.59
135				3.76±2.34	2.16±1.73