INTERSEGMENTAL COORDINATION OF CENTRAL NEURAL OSCILLATORS FOR RHYTHMIC MOVEMENTS OF THE WALKING LEGS IN CRAYFISH, *PACIFASTACUS LENIUSCULUS*

BY KEITH T. SILLAR*, FRANÇOIS CLARAC† AND BRIAN M. H. BUSH

Department of Physiology, University of Bristol, Bristol, BS1 5LS, UK

SUMMARY

The neuronal circuits underlying rhythmical movements of the walking legs in crayfish *Pacifastacus leniusculus* are organized into central oscillators within each thoracic hemiganglion. These segmental networks are coupled in different modes of coordination during activities such as walking and limb waving. This paper examines the nature of the coupling between hemisegmental oscillators in the absence of sensory feedback, and the effect of phasic input from a major limb proprioceptor.

1. In isolated preparations of the thoracic nerve cord, adjacent ipsilateral hemiganglia can generate a rhythmic motor output pattern in which homologous motor roots discharge approximately synchronously (the 'in-phase' rhythm). In contrast, contralateral hemiganglia show no evidence of any such phase-locking in their activity.

2. A single proprioceptor at the base of each limb, the thoracic-coxal muscle receptor organ (TCMRO), can influence the timing and intensity of the rhythmic output of two or more ipsilateral thoracic ganglia. Rhythmical stretch–release of the TCMRO of the fourth ganglion on one side can entrain the motor rhythms of both the third and fourth ipsilateral hemiganglia, in the in-phase pattern of coordination.

3. The TCMRO also elicits intra- and intersegmental reflexes during expression of the in-phase motor rhythm. These reflexes are centrally modulated in a phase-dependent manner. Thus mechanical stimulation of the TCMRO co-activates homologous sets of motoneurones in adjacent ipsilateral hemiganglia only during their active (spiking) phase in the motor rhythm.

We discuss the implications of these results for the generation and coordination of walking and other rhythmic behaviour of the thoracic limbs in decapod Crustacea. Our data shed further light on the properties of the motor rhythm generator for walking in crayfish, and suggest that the concept of a central pattern generator cannot be rigorously applied in this system.

* Present address: Department of Zoology, University of Bristol, Woodland Road, Bristol, BS8 1UG, UK.
† Present address: CNRS Laboratoire de Neurobiologie et Physiologie Comparées, Université de Bordeaux I, Place du Dr Peyneau, Arcachon, 33120, France.

INTRODUCTION

The coordination of the limbs during walking, in both vertebrates (Grillner, 1985) and invertebrates (Heitler, 1983; Pearson, 1972, 1976), is highly flexible and adaptable to environmental variation. It is now generally agreed that the basic patterns of reciprocal motor output underlying walking in animals are driven by central pattern generators (CPGs) located within the central nervous system (Delcomyn, 1980). Behavioural flexibility must therefore arise from the interactions between CPGs and the sensory feedback resulting from limb movements (Andersson, Grillner, Lindquist & Zomlefer, 1978; Clarac & Barnes, 1985; Zill, 1985). Stereotyped walking may only occur when the sensory feedback is constant on a cycle-by-cycle basis. Thus in animals as diverse as cats, cockroaches, stick insects and crayfish, the neural output coordinating the limbs can be entrained by a moving treadbelt (Grillner, 1985; Pearson, 1972; Bässler, 1985; Clarac & Chasserat, 1983). However, our knowledge of how such regulation is achieved is still rudimentary.

Most recent analyses of the walking behaviour of decapod Crustacea have been concerned with the organization of the motor output in intact animals, the principles of interlimb coordination and the parameters which regulate the stepping cycle (see, for example, Chasserat & Clarac, 1983; Cruse & Müller, 1986; for reviews see Clarac, 1982; Evoy & Ayers, 1982; Clarac & Barnes, 1985). Free-walking patterns are very complex and usually variable. Many species are able to walk in all directions by changing the synergism (or antagonism) of muscles operating at different intrinsic joints of the leg. However, most decapods show a preference for walking in a particular direction: crabs usually walk sideways while crayfish and lobsters walk forwards or backwards. During straight sequences of walking on a flat surface, a given leg is usually on the ground when adjacent and contralateral legs are raised (Bowerman, 1977), and interleg coordination is predominantly out of phase.

That peripheral inputs establish and maintain interleg coordination is supported by two observations in which reduced sensory feedback modifies the out-of-phase pattern. Rock lobsters, *Jasus lalandii*, like many other decapods, are able to autotomize each walking leg at the level of the ischiopodite. If one leg is removed the remaining stump continues to oscillate during walking but its movements are now in phase with the leg in front. A fully in-phase pattern develops progressively with sequential autotomy until, when all the legs are removed, the remaining stumps wave to and fro approximately synchronously (Clarac, 1982). The in-phase motor pattern has also been recorded in the intact animal during 'waving' behaviour (Pasztor & Clarac, 1983). Here the animal supports its weight with the abdomen and back legs while the front legs, together with the maxillipeds, wave backwards and forwards in large, slow oscillations. The pattern is metachronal, consisting of alternating promotion and remotion of each appendage. Waving behaviour can involve both sides of the animal, or alternatively each side can be activated separately.

These two observations indicate that substrate contact and feedback from power stroke movements are important factors in producing the out-of-phase pattern which underlies walking. In waving behaviour and after leg autotomy, decapod crustaceans

exhibit a different motor programme associated with the absence or diminution of sensory feedback. The resulting in-phase patterns suggest that the segmental oscillators for rhythmic limb movement may be centrally coupled in an in-phase mode of coordination (Heitler, 1983).

Little is known about the neural control of locomotory patterns of the thoracic limbs in Crustacea, and the existence of a central oscillator network for each walking limb was until recently only inferred. However, long stable sequences of rhythmical bursts of motor impulses have now been recorded *in vitro*, in the nerve roots of the isolated thoracic ganglia of the crayfish, with phase relationships between the basal limb muscles similar to those which underlie walking in the intact animal (Skorupski, Sillar & Bush, 1984; Sillar & Skorupski, 1985, 1986). The thoracic ganglia, like their abdominal homologues, are organized into a ladder-like network of segmental oscillators (Sillar, 1985), each able to generate rhythmic motor output. Coupling between the motor rhythms produced by different hemiganglia has not hitherto been analysed in detail.

The present study was undertaken to determine, first, the extent and nature of any intrinsic neuronal coupling between these segmental oscillators of the thoracic ganglia in the absence of sensory feedback and, second, the effect of afferent input from a major proprioceptor, the thoracic-coxal muscle receptor organ (TCMRO) from the basal joint of one leg (for reviews see Bush, 1976; Bush & Laverack, 1982). To this end we have developed a more extensive dissection of the crayfish's thoracic nervous system, allowing simultaneous recording from two or more hemiganglia during rhythmic activity. In such preparations the paired hemiganglia of one segment show no obvious phase coupling, whereas ipsilateral hemiganglia are coordinated loosely in phase. A single TCMRO can entrain this in-phase pattern, thereby stabilizing the rhythmic motor bursts. A preliminary account of these data has been published elsewhere (Clarac & Sillar, 1985).

MATERIALS AND METHODS

Experiments were performed on male or female crayfish, *Pacifastacus leniusculus*, measuring 9–12 cm from the tip of the rostrum to the caudal edge of the telson. These were obtained from local suppliers and maintained in aerated freshwater aquaria. The preparation used here was similar to that described by Sillar & Skorupski (1986). In brief, the thoracic nerve cord was dissected from the animal while the sternal artery was perfused at 3–5 ml min^{-1} with oxygenated saline (van Harreveld, 1936) at room temperature (18–22°C). Usually a bilateral chain of the posterior four of the five thoracic pereiopod ganglia (T2–T5) were excised. The thoracic-coxal muscle receptor organ (TCMRO) of the right fourth walking leg was left attached by its sensory nerve to the T4 ganglion. The other limb nerves of each ganglion were cut so as to allow recordings to be made of motor output in the promotor, remotor, depressor and levator muscle nerves (Fig. 1A). *In vitro*, the preparation was secured dorsal side up with insect pins to the Sylgard base of a Perspex chamber and superfused with oxygenated saline.

The TCMRO was pinned to the Sylgard at its proximal end, and clamped distally in a servo-controlled pulling device for mechanical stimulation. Up to four simultaneous extracellular recordings were made from motor nerve roots of thoracic ganglia, using fine-tipped polythene suction electrodes. Intracellular recordings were made from the neuropile of the right third and fourth thoracic ganglia after they had been mechanically desheathed. Microelectrodes filled with Lucifer yellow (Stewart, 1978), of resistances 30–80 MΩ, were used routinely. Neurones were identified by a combination of physiological and anatomical criteria, following Lucifer yellow injection (see Sillar & Skorupski, 1986). All physiological data were stored on an FM tape recorder for subsequent analysis and filming. Ganglia with Lucifer yellow stained cells were processed conventionally, viewed under ultraviolet light and drawn with the aid of a *camera lucida*. The experiments described below are based on over
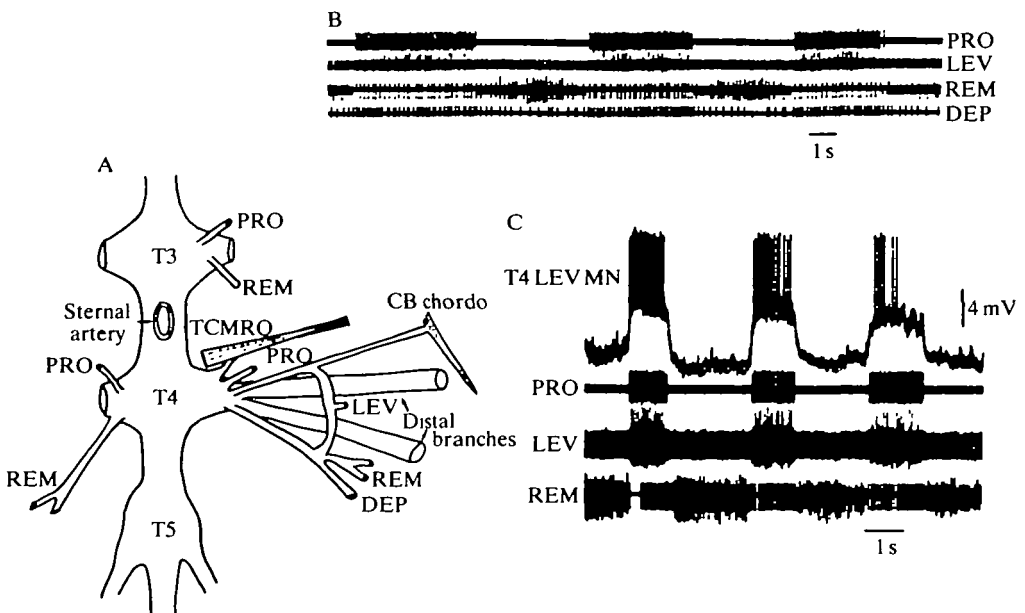

Fig. 1. (A) The isolated preparation of the crayfish thoracic nerve-chain, with the thoracic-coxal muscle receptor organ (TCMRO) of the right fourth hemiganglion left attached for mechanical stimulation (see Materials and Methods). T3, T4, T5: bilateral third, fourth and fifth thoracic ganglia, CB, coxo-basal chordotonal organ. (B) Each hemiganglion can produce sequences of rhythmic motor activity in which promotor (PRO) and levator (LEV) nerves discharge together and in antiphase with the remotor (REM) nerve. Depressor (DEP) motoneurones are usually inactive but here the tonic firing frequency of a single unit is modulated in time with the rhythm. Note that a single unit in the remotor nerve fires in the interburst intervals. This unit has not been identified, but a small branch of the levator nerve runs close to the main remotor root and therefore this unit may be either a levator excitor motoneurone (MN) or a remotor inhibitor MN (see also Figs 5–7). (C) Intracellular recording (top trace) from a T4 levator MN during the rhythm reveals oscillations in membrane potential phase-locked to the extracellular activity (depressor trace not shown). The levator MN depolarized and fired impulses during promotor bursts and was silent while remotor MNs were active.

30 successful experiments on the isolated preparation, with the TCMRO of the right fourth leg under strict experimental control.

<div align="center">RESULTS</div>

<div align="center">*Motor output from isolated thoracic ganglia*</div>

As noted above, the thoracic ganglia of crayfish are capable of producing rhythmic motor bursts when completely isolated from the rest of the animal (Fig. 1; see also Sillar & Skorupski, 1986, fig. 4). During this rhythm, remotor and promotor motoneurones (MNs) of each hemiganglion generally discharge in strict alternation, and levator MNs most commonly fire in phase with the promotors. These phase relationships broadly resemble those obtaining in the intact animal during forward walking (Skorupski, 1985). However, depressor MNs are usually not phasically active in these isolated preparations, although any tonic depressor units may be weakly modulated in time with the other roots (Fig. 1B).

In this and other respects, the motor pattern recorded in the absence of sensory feedback differs from the motor programme for walking. Are these differences reflected in the coupling between ganglia? We have made simultaneous recordings from nerve roots of adjacent ipsi- or contralateral hemiganglia during endogenous rhythmic activity.

<div align="center">*Coupling between hemiganglia in deafferented preparations*</div>

Contralateral coupling

Extracellular recordings from two homologous roots of paired hemiganglia of a single thoracic segment reveal at least three different types of activity. First, both roots may be tonically active; second, one hemiganglion can display rhythmic motor output while the other is tonically active; and third, both may be rhythmically active simultaneously. In none of these types of activity was there any obvious coordination between the two sides of one ganglion. In the preparation from which the two sequences shown in Fig. 2A were obtained, for example, the left and right promotor roots of T4 were both rhythmically active, but cycled more or less independently. There appeared to be a slight tendency for the rather variable impulse bursts on the two sides to occur roughly simultaneously but this was not common. A similar lack of phase-coupling between contralateral hemiganglia was recorded in four other preparations. This suggests that the neuronal pathways mediating cross-ganglion coordination in intact animals are activated either by sensory feedback, or by descending systems in more rostral ganglia (or both).

Ipsilateral coupling

In contrast, there is a very strong tendency for adjacent ipsilateral ganglia to burst in phase (Fig. 2B–D). Thus when two or even three ganglia are rhythmically active, the remotor roots of adjacent hemiganglia fire approximately synchronously (Fig. 2B,C). The levator root of each hemiganglion usually discharges in phase with
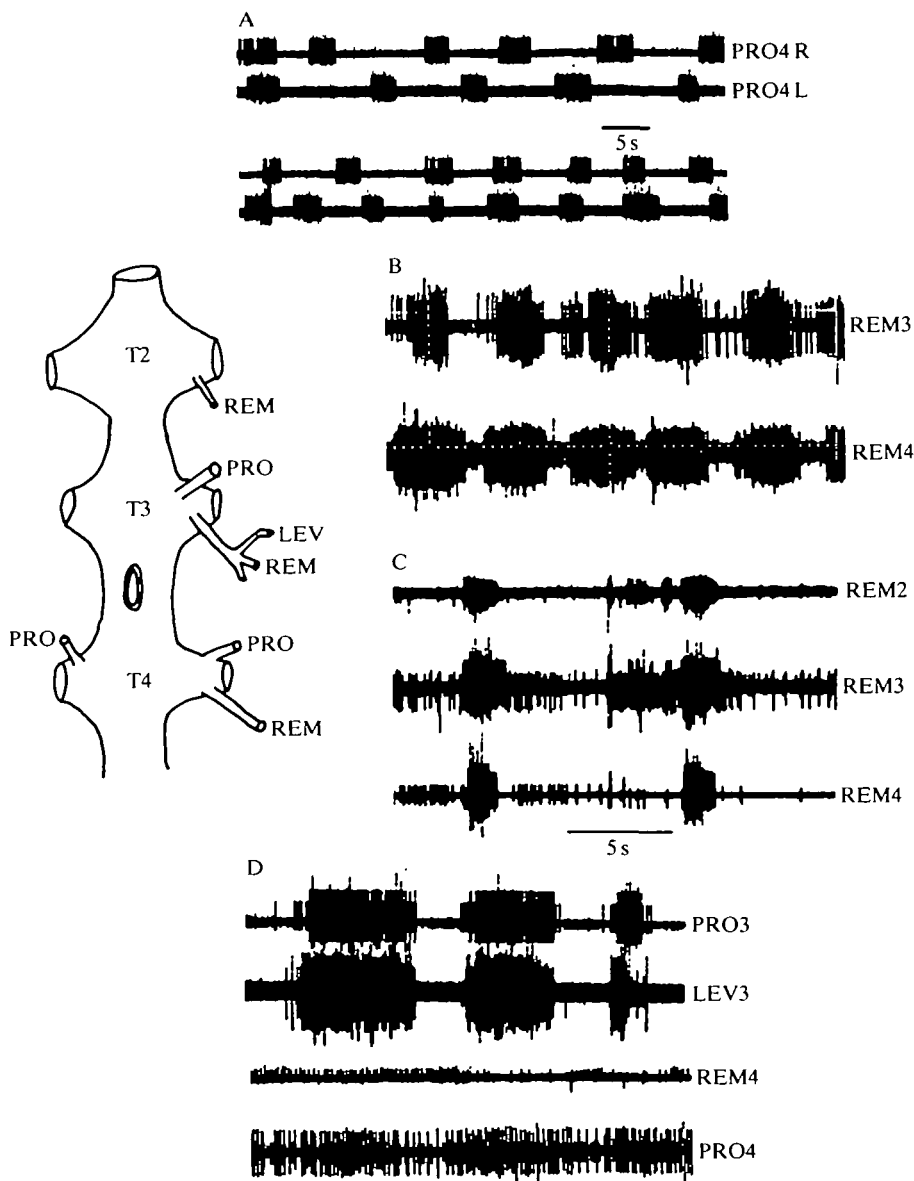
Fig. 2. Coupling between rhythmic motor patterns of adjacent hemiganglia in the isolated preparation. (A) *Contralateral hemiganglia* display little or no interganglionic coupling. Usually there is no consistent relationship between the rhythms on the two sides of the segmental ganglion (upper record). Occasionally, however, a weak drift-and-lock coordination may occur (lower record, same preparation). (B–D) Adjacent *ipsilateral hemiganglia* always burst in phase when rhythmic motor output is present. Extracellular recordings from homologous remotor roots of two (B) or three (C) ipsilateral ganglia reveal phase-locked rhythmic activity. (D) The rhythms of adjacent hemiganglia can vary widely in intensity. Here the third ganglion showed intense bursting in the promotor and levator roots, while a weaker in-phase rhythm was recorded simultaneously from the promotor and remotor roots of the fourth ganglion. Note the long and variable cycle periods recorded in these isolated preparations.

the promotor roots of the same and adjacent hemiganglia, and in antiphase with the remotor roots (Fig. 2D). In other words, the patterns of rhythmic activity in ipsilateral hemiganglia are basically similar and tend to occur in phase. The intensity of bursting in each hemiganglion can, however, vary enormously. The third ganglion may show strong, intense bursts, while the fourth ganglion may burst weakly, although a clear in-phase pattern is evident (Fig. 2D). The timing of bursts in homologous roots of adjacent ganglia does not appear to be strictly controlled, since the activity in one ganglion may either precede or follow that in adjacent ganglia by up to 500 ms or more.

Interganglionic coupling in the in-phase pattern may be influenced by the relative levels of excitability of each ganglion. In the experiment illustrated in Fig. 3A, the in-phase pattern was recorded in a preparation in which the right fourth leg was left attached to the CNS by the two main limb nerves, but all other ganglionic roots as well as the longitudinal connectives above the second and below the fifth ganglia were severed. The third and fourth right hemiganglia were bursting in phase, so that the two remotor roots (only the third is shown) discharged loosely in alternation with the two promotor roots (only that of T4 is shown here). Mechanical stimulation at the distal end of the intact fourth limb (at arrow) resulted in an increase in the intensity of T3 remotor discharge and a sharpening of the alternation between antagonist MNs: the two ganglia were now bursting strictly in phase. This suggests that ascending excitation between synergistic MN pools (or their antecedents) may be involved in production of the in-phase pattern.

Antidromic stimulation of the remotor nerve can initiate rhythmic activity in an otherwise tonic isolated preparation (Sillar & Skorupski, 1986) and can also modulate ongoing rhythmic activity. We therefore used such stimulation during the in-phase rhythm to help assess whether coupling between adjacent ipsilateral ganglia involved ascending or descending influences, or both (Fig. 3B–E). Stimulation of the remotor nerve of T4 had excitatory effects on the in-phase rhythm in the seven preparations on which it was tested, while stimulation of the third ganglion remotor nerve had similar effects in six preparations. In Fig. 3B, for example, a brief stimulus train to the T4 remotor root initiated an intense promotor burst earlier than would have been predicted from the previous cycle, followed by a (T3) remotor burst in which larger amplitude units were recruited. Later, in the same preparation, the fourth ganglion developed a tonic promotor discharge while the third ganglion continued bursting rhythmically. Now a similar T4 stimulus, this time applied shortly after the onset of a T3 remotor burst, immediately terminated this burst and restarted the in-phase rhythm, with clear remotor–promotor alternation in both ganglia (Fig. 3C). In preparations in which both T3 and T4 were tonically active, rhythmic in-phase activity could sometimes be induced by continuous stimulation of the T4 remotor root at 5–10 Hz (e.g. Fig. 3D). Another tonically active preparation responded to a brief high-frequency stimulus to the T3 remotor root with a single cycle of in-phase activity in both ganglia (Fig. 3E). In general, electrical stimulation of the remotor nerve of either T3 or T4 had both descending and ascending excitatory effects on the in-phase rhythm of ipsilateral ganglia.
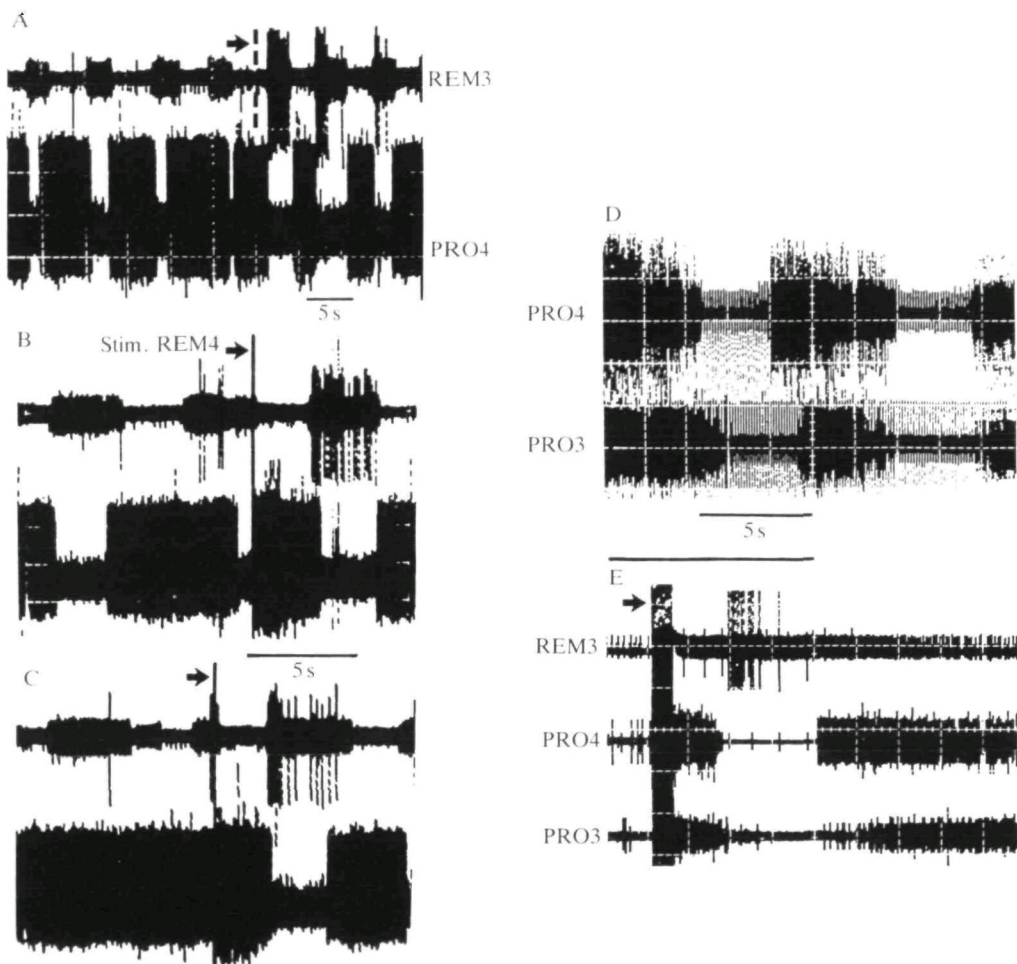
Fig. 3. Effects of mechanical and electrical stimulation on the in-phase rhythm of ipsilateral hemiganglia. In A, B and C (all from the same preparation), the in-phase pattern is recorded (represented here as alternating burst activity) in the remotor nerve of T3 and the promotor of T4. In A, the thoracic ganglia are semi-isolated (see text for explanation): pinching the intact fourth limb distally (at arrow) increases the intensity of the third ganglion remotor bursts and tightens the coupling between ganglia. (B) After isolation, a comparable effect was achieved through electrical stimulation of the fourth ganglion remotor root (50 Hz for 200 ms at arrow). (C) Later, in the same preparation, when the fourth ganglion had become tonically active, a similar electrical stimulus (arrowed) just after the onset of a remotor burst in the third ganglion, increased the intensity of its subsequent bursts, and also restarted the rhythm in the fourth ganglion. (D) In a previously non-rhythmic preparation, continuous electrical stimulation of the fourth ganglion remotor nerve at 10 Hz initiated intense in-phase bursts in the promotor roots of the third and fourth ganglia. (E) Brief, high-frequency stimulation of the third ganglion remotor root (arrowed) elicited a single cycle of in-phase bursting.

Intracellular recordings in T3 and T4

Motoneurones penetrated in these two hemiganglia showed rhythmical oscillations in membrane potential during the in-phase pattern. In one such recording the
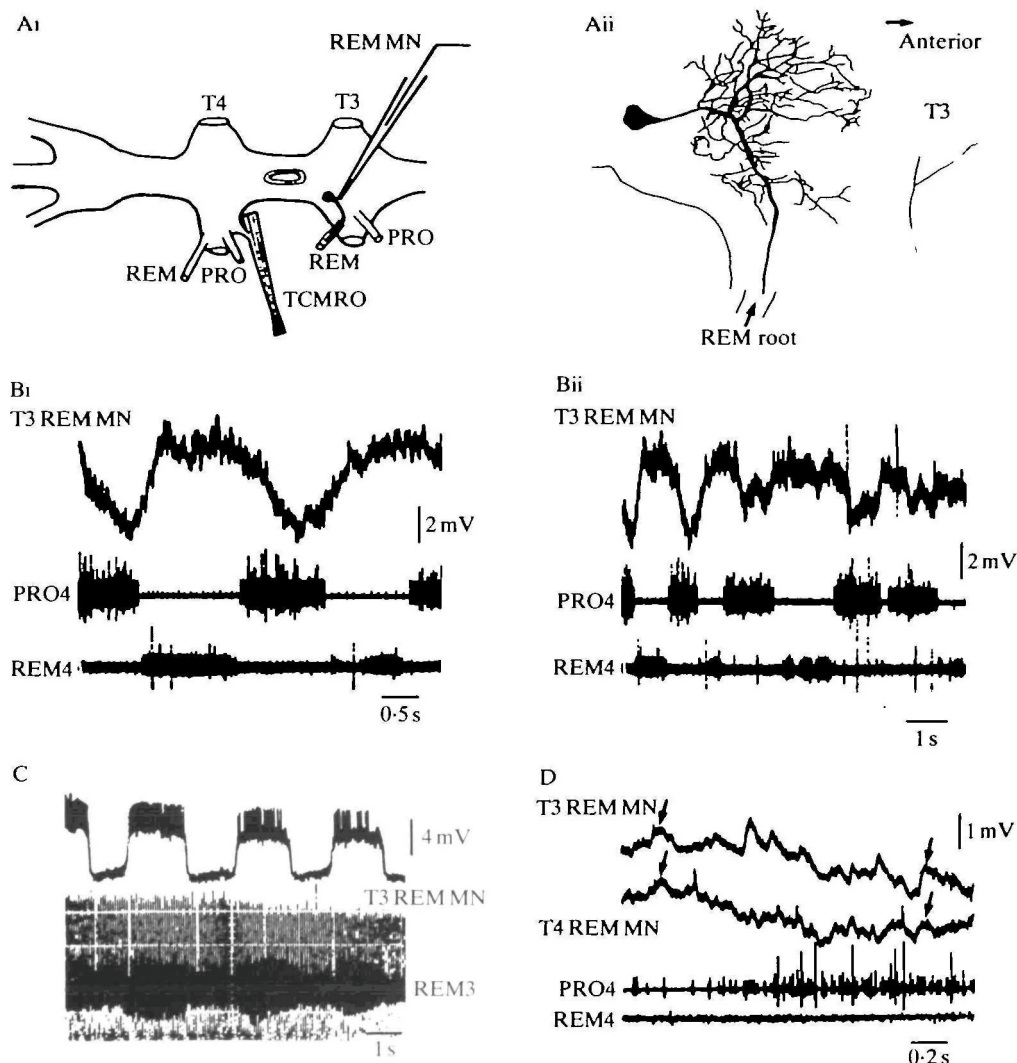
Fig. 4. Central synaptic drive to motoneurones (MNs) during the in-phase pattern. (Ai) Diagram of preparation and recording site. (Aii) *Camera lucida* drawing of the T3 remotor MN recorded in B–D and subsequently stained with Lucifer yellow. (Bi, Bii) This MN showed subthreshold oscillations in membrane potential in phase with the fourth ganglion spike discharge. (C) During tonic stimulation of the T4 remotor root a more intense and stable in-phase rhythm was elicited. The T3 MN was now depolarized above spike threshold and discharged in the T3 remotor burst and in antiphase to the T4 promotor root (not shown). (D) Simultaneous intracellular recordings from the same T3 remotor MN (top trace) and a T4 remotor MN (second trace) during a promotor burst elicited by prior electrical stimulation of the T4 remotor root. Note the general similarity in the fluctuations in the two membrane potentials. Arrows indicate synaptic inputs which may be common to the two cells.

membrane of a third ganglion remotor MN (anatomy shown in Fig. 4A) depolarized in phase with the fourth ganglion remotor output (Fig. 4B). This cell's oscillation was subthreshold for spike initiation during spontaneous bursting, although other

third ganglion remotor MNs (not shown) discharged impulses in phase with the fourth ganglion. The membrane potential of the MN depolarized and hyperpolarized about its apparent resting potential of approx. $-60\,\mathrm{mV}$, recorded in quiescent periods between bouts of the in-phase pattern. Tonic stimulation of the fourth ganglion levator nerve root initiated more intense bursting in this preparation (Fig. 4C). The stimulus drove an in-phase pattern in which the third ganglion remotor MN was now excited above threshold and fired in synchrony with the fourth (not shown) and third ganglion remotor bursts.

Later, in the same preparation, a remotor MN of the fourth ganglion was penetrated simultaneously with a second electrode. Rhythmic activity then ceased to occur spontaneously, but high-frequency stimulation of the third ganglion remotor nerve could still elicit a single cycle of in-phase activity. Following the stimulus, both remotor MNs depolarized together as the T4 promotor root fell silent. The promotor root then discharged again and the two remotor MNs hyperpolarized, revealing similar subthreshold synaptic inputs (Fig. 4D), some of which appeared to be common to the two cells (e.g. at the arrows).

Proprioceptor effects on intersegmental coupling

The preceding results show that adjacent ipsilateral segmental oscillators are coordinated loosely in-phase in the absence of sensory feedback, while contralateral hemiganglia are not coupled in any obvious way. The in-phase pattern of activity is clearly reminiscent of the motor patterns observed during waving behaviour and after autotomy, but the *in vitro* rhythm is less stable and its cycle period more variable. It seemed likely that proprioceptive feedback might stabilize the centrally generated rhythm in the intact animal. Since the movements of the limbs in waving and following autotomy mainly comprise alternate remotion and promotion at the thoracic–coxal (T–C) joints, the single muscle receptor organ (TCMRO) at this joint of each leg will contribute much of the proprioceptive feedback in these conditions. We therefore stimulated an individual TCMRO mechanically to investigate its possible influence on the coordination of the *in-vitro* rhythms of the different thoracic ganglia. One precedent for this analysis was the recent finding that the TCMRO has powerful timing effects on the central rhythm originating in its own ganglion (Sillar, Skorupski, Elson & Bush, 1986).

The TCMRO of one leg can have powerful interganglionic effects on the in-phase pattern of motor output from adjacent ipsilateral ganglia. In the experiment illustrated in Fig. 5 the preparation was spontaneously rhythmic. In the absence of sensory input, the remotor roots of T3 and T4 discharged approximately synchronously, albeit irregularly, in an in-phase pattern (Fig. 5A). Low-frequency sinusoidal stretch of the TCMRO of T4 entrained the rhythm of T3 as well as T4 (Fig. 5B–D, see legend). At 0·1 Hz the rhythm followed the stimulus 1:1, with the remotor bursts of both ganglia entrained to the stretch phase of the stimulus (Fig. 5B). When the stimulus frequency was almost doubled, however, the main motor bursts in both segments escaped from 1:1 entrainment, and were now driven by the briefer stretch phase of every second stimulus cycle (1:2 entrainment, Fig. 5C). Thus the

fundamental cycle period of the in-phase rhythm hardly altered, although the burst duration was considerably reduced. Further increasing the stimulus frequency to 0·33 Hz maintained 1:2 entrainment, so both cycle period and remotor burst duration now decreased (Fig. 5D). The weaker bursts on alternate cycles in the fourth ganglion traces in Fig. 5C,D probably represent activity in the small levator motor branch picked up by the T4 recording electrode, though it could be a remotor inhibitor (see legend to Fig. 5; also Fig. 1). It, too, is clearly modulated by TCMRO stimulation, particularly at the higher frequency.

Inspection of the time relationship between remotor bursts of T3 and T4 during entrainment indicates that the remotor bursts of T4 drive an ascending excitatory
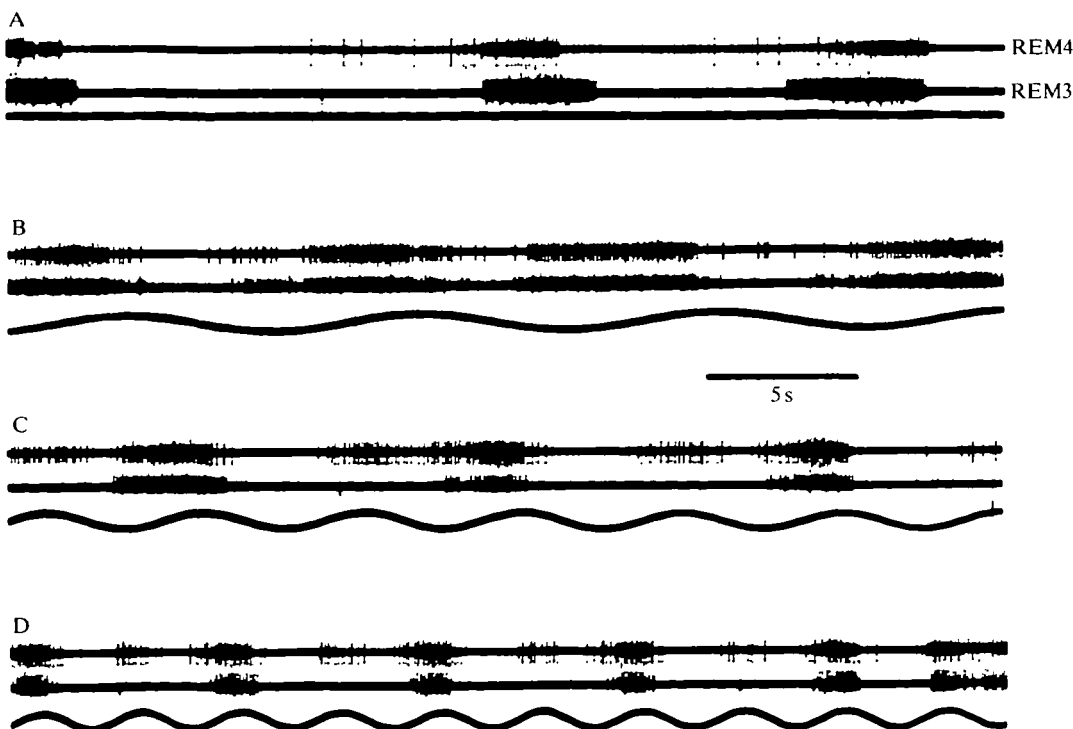
A

REM4

REM3

B

5 s

C

D

Fig. 5. Entrainment of the in-phase pattern by periodic mechanical stimulation of the fourth ganglion TCMRO. (A) In the absence of sensory input this preparation displayed an in-phase pattern which was variable in frequency. The impulse bursts in both nerves recorded from here (the right remotor roots of ganglia T3 and T4) were characterized by the intense discharge of several units. (B) Sinusoidal stimulation of the TCMRO at 0·1 Hz (lower trace, 0·5 mm amplitude, stretch upwards) entrained the in-phase rhythm such that the remotor bursts occurred predominantly during the stretch phase of each stimulus cycle. (C) Increasing the stimulus frequency to almost 0·2 Hz entrained the rhythm on every second cycle (1:2 entrainment). (D) At approx. 0·33 Hz, 1:2 entrainment was maintained, but the cycle period of the rhythm and the durations of the remotor bursts were reduced. Note that a small branch of the levator motor root was also picked up by the T4 electrode, so the large-amplitude unit firing in the REM4 interbursts in A was probably a single levator MN (see also Fig. 1B legend). In C and D the same large spike, presumed levator MN, now fires rhythmically on intermediate cycles. The effects of further increasing the stimulus frequency are shown in Fig. 7.
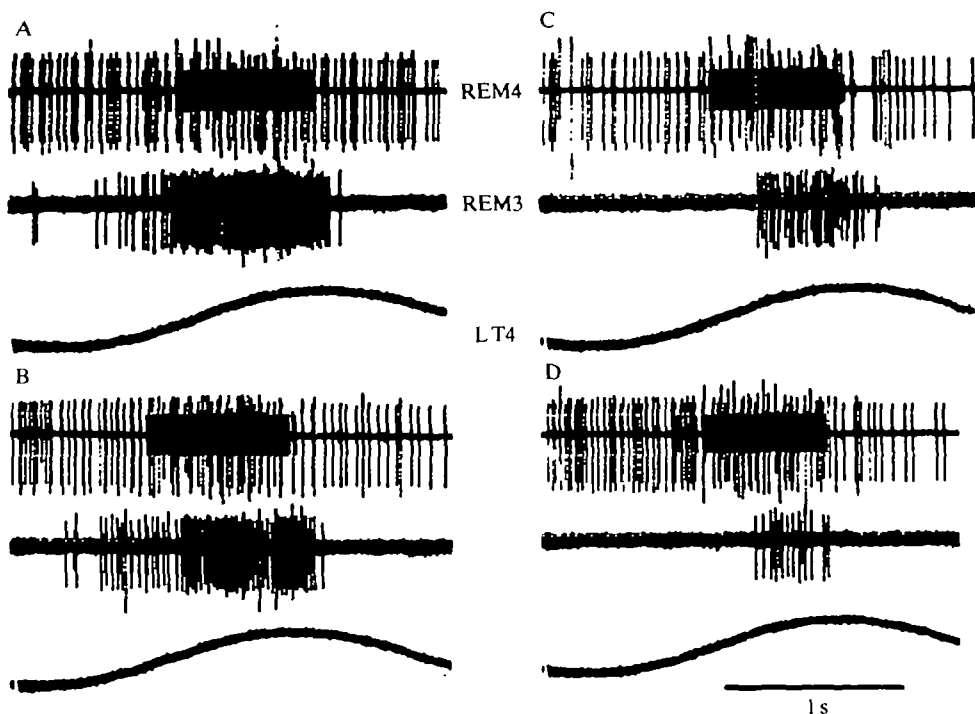
Fig. 6. Phase relationships of entrained remotor bursts in T3 and T4. (A–D) Four examples of in-phase remotor bursts in T4 (top traces) and T3 (middle traces) entrained by the stretch phase of sinusoidal TCMRO stimulation (bottom traces). The T4 remotor bursts are phase-locked to the stretch. The onset of T3 bursts is more variable (see text for further description).

pathway to the T3 remotor burst generator. In Fig. 6 four examples of remotor bursts entrained by a 0·33-Hz stimulus are shown. The delay between remotor bursts in the two ganglia was variable, and the onset of T3 bursts could precede (Fig. 6A,B) or follow (Fig. 6C,D) the onset of T4 bursts. When the T3 burst preceded that in T4, however, the frequency of firing of units within the burst was initially low; intense discharge of T3 remotor MNs then followed the onset of the T4 burst after a delay (Fig. 6B) comparable to that occurring when T3 bursts started after those of T4 (Fig. 6C). These data suggest that the anterior ganglion, T3, was cycling at a slightly different frequency from T4, and that an excitatory pathway, driven by the remotor burst of T4 and reinforced by periodic TCMRO stimulation, was resetting the T3 oscillator into an in-phase rhythm. This is consistent with analogous observations on the less regular, free-running in-phase rhythm where T4 remotor bursts more commonly, though not invariably, precede T3 bursts (Fig. 2B, cf. Fig. 5A).

When the frequency of TCMRO stimulation was increased beyond 1 Hz, outside the range at which clear entrainment occurred, the central rhythm became fragmented by the stimulus (Fig. 7A). Within each centrally originating remotor burst of T4, one or more remotor MNs (small spikes) were driven at high frequency

on each stretch. During the interburst intervals when the remotor MNs were inactive, a larger, tonically active, presumed levator MN (see Fig. 1 legend) was also driven on stretch, but this reflex was largely abolished during the central remotor bursts. Thus the TCMRO stimulus evoked phase-dependent reflex effects similar to those described previously (Skorupski & Sillar, 1986). Simultaneously the T3 remotor bursts, still occurring in the in-phase pattern (Fig. 7A), were also disrupted by the stimulus, so that each reflexly driven T4 discharge on stretch of the T4 TCMRO was generally followed by a discharge of spikes in T3 (Fig. 7B–E). The correlation between reflexes in the two ganglia is well illustrated in the central burst shown in Fig. 7E, where a progressive increase in strength of the T4 remotor reflexes
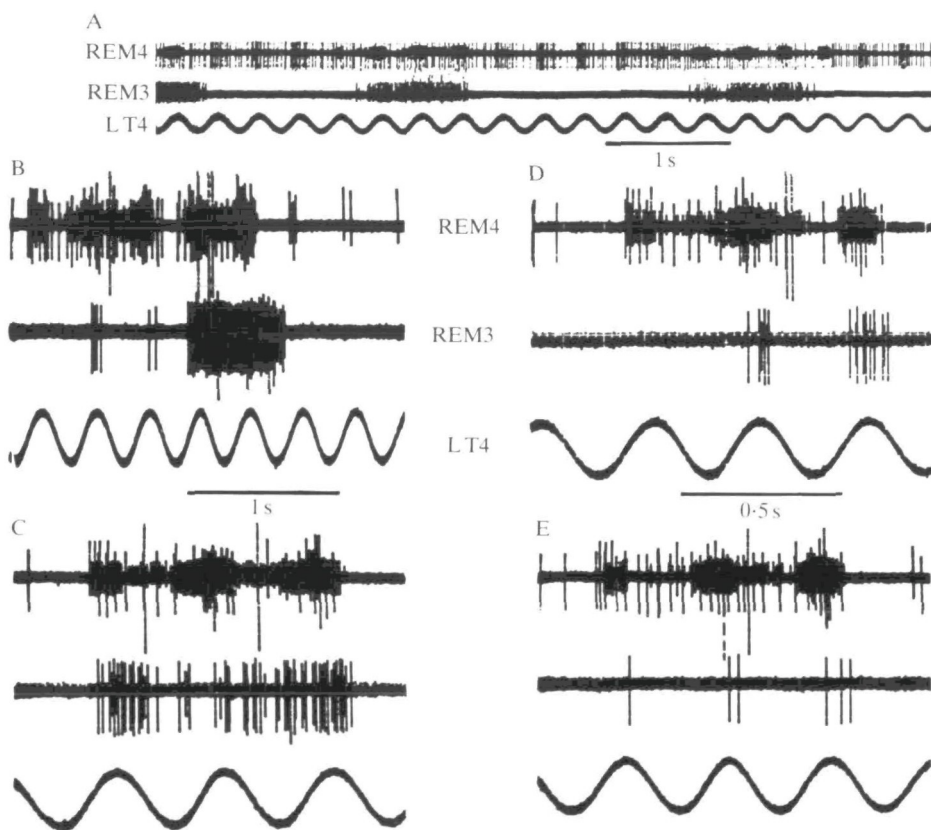

Fig. 7. Phase-dependence of TCMRO reflexes in the in-phase pattern. (A) At high frequencies of TCMRO stimulation the in-phase pattern is not obviously entrained, but motor bursts are fragmented by each stimulus cycle. Fourth-ganglion remotor motoneurones (MNs) are driven at high frequency on stretch, though only within each remotor burst; in the interburst intervals a single, presumed levator, MN (see legends to Figs 1B and 5) is excited by TCMRO stretch. The TCMRO of the fourth ganglion also drives a phase-dependent reflex in the third ganglion remotor root, whose central bursts occur in phase with T4. (B–E) Four different in-phase remotor bursts from a long sequence of activity similar to that in A. Reflexly driven third ganglion discharges *follow* those of the fourth ganglion, and may be correlated with the intensity of remotor discharge in T4. Records taken from the same experiment as shown in Fig. 6.

during the burst is accompanied by a parallel increase in T3 remotor spike discharge. These results show that the TCMRO has access to an ascending interganglionic pathway which links T3 and T4 in phase. As a consequence, reflexes from the TCMRO are preserved within this pathway and ascend to T3 where they are expressed in a phase-dependent way. The pathway is thus operated like a gate since it is cyclically opened and shut during rhythmic motor output.

Subthreshold reflex effects

Interganglionic reflexes can sometimes be recorded in the absence of overt rhythmic activity. For example in Fig. 8A, sinusoidal stretch of the TCMRO of T4 evoked reflex excitation of remotor MNs in T4, and correlated inhibition of T4 promotor MNs (a positive feedback reflex). The stimulus also elicited an interganglionic reflex in the remotor nerve of T3. An intracellularly recorded T3 remotor MN (top trace in A) showed slow, subthreshold fluctuations in membrane potential approximately in phase with the stimulus and extracellularly recorded T3 reflex. The background of synaptic inputs impinging on this MN consisted of numerous brief, apparently unitary depolarizing potentials on stretch (Fig. 8B). When hyperpolarizing potentials occurred, they did so predominantly on release (Fig. 8A). The occurrence of unitary synaptic potentials in this T3 MN in response to stretch of the fourth ganglion TCMRO suggests that the interganglionic effects are mediated *via* ascending excitation from one or more spiking interneurones.

Contralateral TCMRO effects

In view of the relative weakness or lack of coupling between the endogenous motor rhythms on the two sides of individual segmental ganglia, noted above, it was of interest to know whether TCMRO stimulation might influence cross-ganglionic coupling. This possibility was investigated in two preparations in which rhythmic motor activity was recorded from the left and right sides of T4 (not illustrated). In both cases sinusoidal TCMRO stimulation entrained the output of its own, ipsilateral hemiganglion, and also elicited phase-dependent reflex effects at higher stimulus frequencies. However, the contralateral hemiganglion continued to cycle independently, and neither entrainment nor any reflex modulation was observed.

DISCUSSION
Occurrence of the in-phase pattern

Interlimb coordination during walking in decapod Crustacea can vary on a cycle-by-cycle basis, and individual legs show a high degree of independence (Chasserat & Clarac, 1980, 1983). However, the basic pattern during forward walking is best described as an alternating tetrapod gait (Bowerman, 1977) in which adjacent ipsilateral and contralateral pairs move in antiphase. This mode of coordination may result from the requirement for mechanical support of the body, since the animal's weight must be evenly distributed over the legs. Experimental variations in load can
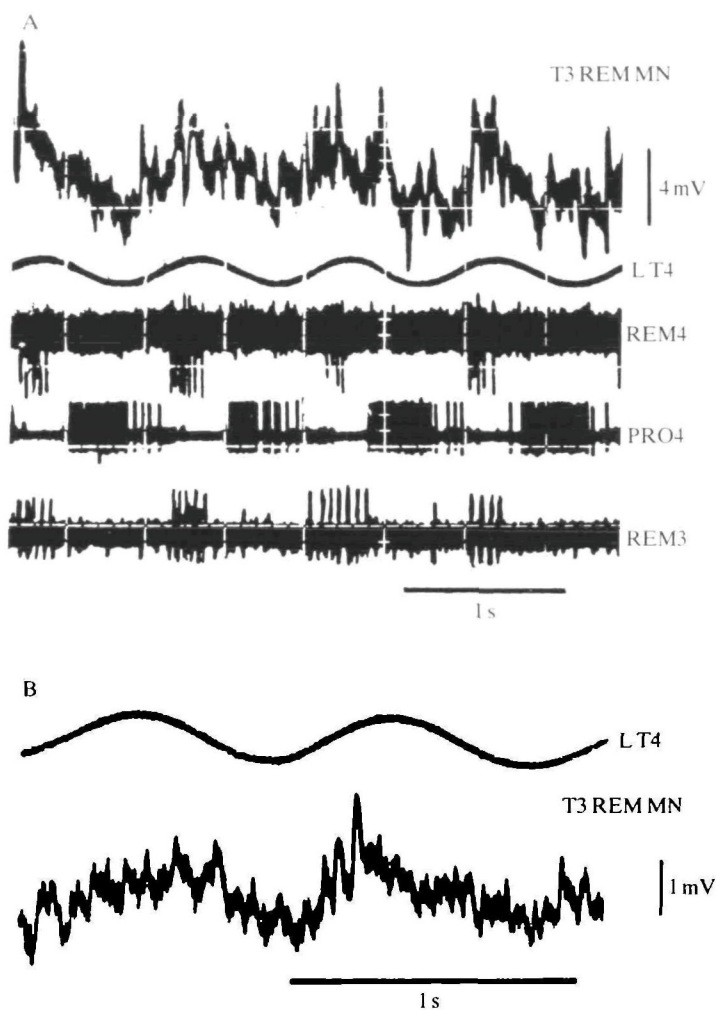
Fig. 8. Intersegmental reflex from the TCMRO of T4 to third ganglion motoneurones (MNs) in the absence of overt rhythmic activity. (A) Sinusoidal TCMRO stimulation at 1 Hz evoked a positive feedback (assistance) reflex in MNs of the fourth ganglion, with remotor MNs firing on TCMRO stretch and promotor MNs on release. Simultaneously the third ganglion remotor nerve discharged in phase with the fourth ganglion remotor activity. An intracellularly recorded third ganglion remotor MN showed small subthreshold oscillations in membrane potential, phase-locked to the stimulus. (B) Unitary EPSPs could sometimes be observed in the third ganglion MN upon stretch of the fourth ganglion TCMRO. Indications of brief hyperpolarizing potentials occasionally appeared on TCMRO release (e.g. last two cycles in A).

modify the strength of interlimb coordination. The phase relationships between the legs of crayfish walking under water are highly variable when the animal is unloaded, but a tight antiphasic pattern is observed when the animal is loaded (Clarac & Barnes, 1985; Cruse & Müller, 1986). When all load-related sensory feedback is removed, the alternating tetrapod gait disappears and adjacent legs move approximately synchronously. During waving behaviour (Pasztor & Clarac, 1983), for

example, or following autotomy (Clarac & Chasserat, 1979; Clarac, 1982), an in-phase pattern is expressed with the usual characteristics of a metachronal rhythm.

These alternative motor programmes suggest that the thoracic central nervous system of decapod crustaceans contains a coordination mechanism for near synchronous or in-phase motor output which is suitable for movement in an aquatic environment (Heitler, 1983). This coordination must be reorganized by the sensory feedback associated with substrate contact to produce an antiphasic pattern appropriate for load compensation during walking. The present data support this idea since we show, for the first time, that in the absence of all sensory feedback, the thoracic ganglia *in vitro* can produce in-phase coordination between rhythmically active ipsilateral ganglia.

The walking legs of crayfish are thought to have evolved from simpler biramous appendages resembling the abdominal swimmerets. Consequently the neural networks for rhythmic movements of the thoracic limbs are likely to have diverged from a system similar to that which coordinates the activity of the swimmerets. If this were true then it might be expected that the basic mechanisms for oscillatory motor output in the isolated thoracic ganglia would have general features in common with the swimmeret CPG (Heitler, 1981). The occurrence of in-phase coupling between ipsilateral segmental oscillators in the thoracic CNS may, therefore, represent a phylogenetic vestige of the neural networks from which the walking system evolved and upon which the wide behavioural repertoire of the legs has radiated.

Central coordination

The rhythmic motor output expressed by the thoracic ganglia *in vitro* has characteristics in common with the motor programmes used during both walking and waving behaviour. Each hemiganglion can produce rhythmical activity with the appropriate reciprocity between motoneurone pools of the different muscles for forward walking (Skorupski *et al.* 1984; Sillar & Skorupski, 1986). Thus the promotor and levator MNs discharge together and in strict alternation with remotor MNs of the same leg (Fig. 1B,C). During walking, however, depressor MNs are driven powerfully in phase with remotor MNs, whereas in the isolated preparation depressor bursts are rarely observed (Fig. 1B). The isolated rhythm is also highly variable in frequency, and the duration of motor bursts does not seem to be strictly controlled (Skorupski, 1985).

The clearest difference between the isolated and *in vivo* conditions, as we show here, is that adjacent ipsilateral hemiganglia discharge in phase in the isolated preparation, while during forward walking they are strictly antiphasic. In this respect the isolated preparation produces activity similar in its intersegmental coordination to that occurring after autotomy, or during waving behaviour; yet the levator muscle is not phasically active during waving of the intact legs or autotomized stumps. It is conceivable that the centrally driven levator bursts recorded in isolated preparations and after autotomy are inhibited during waving. Whether or not these different motor programmes seen in the intact animal represent modifications of the same central oscillator system is a matter for further study. However, the presence of

separate hemisegmental oscillators which produce a basic pattern with several characteristics common to both waving and walking suggests that these behaviours are not generated by wholly different mechanisms. It seems more likely that the central oscillator circuits in each hemiganglion generate a basic reciprocal output pattern which is sufficiently flexible to be modified under different conditions, depending on the nature of the sensory feedback they receive.

Peripheral feedback from the TCMRO

If the preceding hypothesis is true, that a ladder-like network of flexibly wired CNS oscillators underlies a variety of rhythmic behaviour of the walking legs, then the sensory feedback associated with different behaviours must be able to modify the structure and timing of oscillator output. In waving and after autotomy, the principal movement of each leg (or stump) consists of alternate remotion and promotion about the T–C joint. The most conspicuous proprioceptor in this region, the TCMRO, originates in the thorax, terminates on the anterior rim of the coxa, and is stretched by limb retraction (remotion). Its two primary afferent neurones, the S and T fibres, provide movement-related feedback concerning limb position and the velocity of limb displacement about the T–C joint (Bush, 1976; Sillar & Skorupski, 1986). Thus the TCMRO is likely to be a major source of sensory feedback during waving and after autotomy, and it will also be stimulated during walking.

Periodic stretching of the TCMRO will entrain the rhythmic activity of the hemiganglion from which it arises (Elson, Sillar & Skorupski, 1986), and a possible mechanism for this entrainment has recently been postulated (Sillar *et al.* 1986). In isolated preparations the TCMRO of one segment will also entrain the in-phase rhythm of adjacent ipsilateral, though not contralateral, hemiganglia (Figs 5, 6). Rhythmically stretching the TCMRO of the fourth ganglion can time the onset of motor bursts in both the fourth and third ganglia. Since the central arborizations of the S and T fibres and of walking-leg MNs are restricted to their own ganglia (Skorupski, 1985; Skorupski & Sillar, 1986), the TCMRO presumably excites one or more interganglionic interneurones in order to exert these effects. Furthermore, the occurrence of in-phase activity in the absence of TCMRO stimulation suggests that the same coordinating interneurones are driven by the rhythm-generating network. During entrainment, the TCMRO may excite these interneurones indirectly, by timing the phase of the oscillator, and thus introduce an ascending interganglionic delay (Fig. 6). The identity of these interneurones, however, has yet to be determined.

It should be emphasized that *in vitro* the TCMRO entrains an *in-phase* pattern, similar to that seen in waving. Clearly this pattern is not appropriate for walking, when the TCMRO of each leg will be naturally stimulated in the normal alternating sequence of adjacent legs. Presumably the interganglionic effects of the TCMRO, as described here, are overridden during walking. It is conceivable that the entraining influence of the TCMRO is targeted primarily on its ganglion of origin, and that concurrent load-related inputs suppress any ascending effects. To test this hypothesis a future aim of our research will be to manipulate the TCMRO in combination

with other leg proprioceptors, especially load-sensitive receptors such as the cuticular stress detectors.

Stretching the TCMRO also elicits reflexes among homoganglionic MNs which reverse in sign during rhythmic activity (Skorupski et al. 1984; Skorupski & Sillar, 1986). Resistance reflexes are elicited during the promotor phase of activity, that is, promotor MNs are excited by TCMRO stretch and remotor MNs are inhibited. In the remotor phase the opposite reflex effects are evoked: the resistance reflex is now absent, promotor MNs are inhibited and remotors are excited in a positive feedback, 'assistance' reflex. During the in-phase activity recorded in the present study, phase-dependent reflexes also occur in adjacent ipsilateral hemiganglia (Fig. 7). Thus assistance reflexes evoked in fourth ganglion remotor MNs by their own ipsilateral TCMRO during the remotor burst phase are followed by reflex excitation of homologous (remotor) MNs in the third ganglion. Such reflexes may serve an important function in interlimb coordination during waving and after autotomy, since they would ensure strict phase-locking of adjacent limb movements when environmental perturbations might otherwise disrupt the normal in-phase pattern of activity. During walking, however, the interganglionic pathways leading to in-phase activity must be centrally suppressed, and these reflexes then presumably do not occur.

Is there a central pattern generator for walking?

The data presented in this paper suggest that the central pattern generator concept cannot be rigorously applied to the walking system in crayfish. The thoracic ganglia may produce a rhythmic motor pattern in the absence of peripheral feedback, but the characteristics of the pattern indicate that it does not represent a specific rhythmic behaviour in the intact animal. As discussed earlier, the rhythm of each individual hemiganglion appears superficially to be appropriate for forward walking. When the chain of ganglia are studied together, however, the in-phase pattern we report here is clearly inappropriate for walking, but more closely resembles the motor programmes for waving behaviour and for the leg stump movements seen after autotomy.

Our deduction is that each hemiganglion contains a central oscillator network capable of producing a fundamental reciprocal motor output to antagonistic muscles. Taken out of context, in the absence of feedback, each oscillator essentially lacks a functional identity (see Pearson, 1985). Motor output is given identity by the prevailing sensory feedback the oscillators receive. One conclusion is that the rhythm generators for the walking legs are 'soft wired'; loosely organized to produce a basic reciprocity but reorganized by feedback to produce adaptive motor output.

Zill (1986) has recently highlighted the risk of erroneously ascribing functional significance to motor patterns recorded in reduced preparations. Decapitated cockroaches with cut thoracic connectives and restrained on their backs can show rhythmic bursting in leg motoneurones that persists after limb de-afferentation (Pearson & Iles, 1970) and has many characteristics in common with walking. Although this preparation has been used to model pattern generation in cockroach locomotion (Pearson, 1972, 1976), the activity most probably represents attempted

righting behaviour, since motor bursts are completely inhibited when the cockroach stands upright and the usual sensory inputs present in walking are operative. Zill emphasizes that while such preparations can still provide insight into the neural mechanisms for the production of walking patterns, they are of little value in assessing the role of proprioceptive feedback in the control of walking. A major advantage of isolated crustacean preparations is that sensory feedback from individual sense organs normally active during specific behaviours can be selectively manipulated by the experimenter.

REFERENCES

ANDERSSON, O., GRILLNER, S., LINDQUIST, M. & ZOMLEFER, M. (1978). Peripheral control of the spinal pattern generators for locomotion in cat. *Brain Res.* **150**, 625–630.

BÄSSLER, U. (1985). Proprioceptive control of stick insect walking. In *Coordination of Motor Behaviour* (ed. B. M. H. Bush & F. Clarac), Soc. exp. Biol. Seminar Series **24**, 271–281. Cambridge: Cambridge University Press.

BOWERMAN, R. F. (1977). The control of arthropod walking. *Comp. Biochem. Physiol.* **56**A, 231–247.

BUSH, B. M. H. (1976). Non-impulsive thoracic-coxal receptors in crustaceans. In *Structure and Function of Proprioceptors in the Invertebrates* (ed. P. J. Mill), pp. 115–151. London: Chapman & Hall.

BUSH, B. M. H. & LAVERACK, M. S. (1982). Mechanoreception. In *The Biology of Crustacea*, vol. 3, *Neurobiology: Structure and Function* (ed. H. L. Atwood & D. C. Sandeman), pp. 399–467. New York: Academic Press.

CHASSERAT, C. & CLARAC, F. (1980). Interlimb coordinating factors during driven walking in Crustacea: a comparative study of absolute and relative coordination. *J. comp. Physiol.* **139**, 293–306.

CHASSERAT, C. & CLARAC, F. (1983). Quantitative analysis of walking in a decapod crustacean, the rock lobster *Jasus lalandii*. II. Spatial and temporal regulation of stepping in driven walking. *J. exp. Biol.* **107**, 219–243.

CLARAC, F. (1982). Decapod crustacean leg coordination during walking. In *Locomotion and Energetics in Arthropods* (ed. C. F. Herreid & C. R. Fourtner), pp. 31–71. New York: Plenum Press.

CLARAC, F. & BARNES, W. J. P. (1985). Peripheral influences on the coordination of the legs during walking in decapod crustaceans. In *Coordination of Motor Behaviour* (ed. B. M. H. Bush & F. Clarac), Soc. exp. Biol. Seminar Series **24**, 249–269. Cambridge: Cambridge University Press.

CLARAC, F. & CHASSERAT, C. (1979). Experimental modification of interlimb coordination during locomotion of a crustacean. *Neurosci. Letters* **12**, 271–276.

CLARAC, F. & CHASSERAT, C. (1983). Quantitative analysis of walking in a decapod crustacean, the rock lobster *Jasus lalandii*. I. Comparative study of free and driven walking. *J. exp. Biol.* **107**, 189–217.

CLARAC, F. & SILLAR, K. T. (1985). Coordination between thoracic oscillators for rhythmic limb movement in the crayfish, *Pacifastacus leniusculus*. *Soc. Neurosci. Abstr.* **11**, 510.

CRUSE, H. & MÜLLER, U. (1986). Two coupling mechanisms which determine the coordination of ipsilateral legs in the walking crayfish. *J. exp. Biol.* **121**, 349–369.

DELCOMYN, F. (1980). Neural basis of rhythmic behavior in animals. *Science* **210**, 492–498.

ELSON, R. C., SILLAR, K. T. & SKORUPSKI, P. (1986). Stretch reflexes from the thoracic-coxal muscle receptor organ entrain a central locomotor rhythm of the crayfish. *J. Physiol., Lond.* **373**, 13P.

EVOY, W. H. & AYERS, J. L. (1982). Locomotion and control of limb movements. In *The Biology of Crustacea*, vol. 4, *Neural Integration and Behaviour* (ed. D. C. Sandeman & H. L. Atwood), pp. 62–105. New York: Academic Press.

GRILLNER, S. (1985). Neural control of vertebrate locomotion – central mechanisms and reflex interaction with special reference to the cat. In *Feedback and Motor Control in Invertebrates and Vertebrates* (ed. W. J. P. Barnes & M. H. Gladden), pp. 35–56. London: Croom Helm.

HEITLER, W. J. (1981). Neural mechanisms of central pattern generation in the crayfish swimmeret system. In *Neurobiology of Invertebrates* (ed. J. Salanki), Advances in Physiological Science 23, 369–383. Oxford: Pergamon Press.

HEITLER, W. J. (1983). The control of rhythmic limb movements in Crustacea. In *Neural Origin of Rhythmic Movements* (ed. A. Roberts & B. L. Roberts). *Symp. Soc. exp. Biol.* 37, 351–382.

PASZTOR, V. M. & CLARAC, F. (1983). An analysis of waving behaviour: an alternative motor programme for the thoracic appendages of decapod Crustacea. *J. exp. Biol.* 102, 59–77.

PEARSON, K. G. (1972). Central programming and reflex control of walking in the cockroach. *J. exp. Biol.* 56, 173–193.

PEARSON, K. G. (1976). The control of walking. *Scient. Am.* 235, 72–76.

PEARSON, K. G. (1985). Are there central pattern generators for walking and flight in insects? In *Feedback and Motor Control in Invertebrates and Vertebrates* (ed. W. J. P. Barnes & M. H. Gladden), pp. 307–315. London: Croom Helm.

PEARSON, K. G. & ILES, J. F. (1970). Discharge patterns of coxal levator and depressor motoneurones of the cockroach, *Periplaneta americana. J. exp. Biol.* 52, 139–165.

SILLAR, K. T. (1985). Comparative overview and perspectives. In *Coordination of Motor Behaviour* (ed. B. M. H. Bush & F. Clarac), Soc. exp. Biol. Seminar Series 24, 303–316. Cambridge: Cambridge University Press.

SILLAR, K. T. & SKORUPSKI, P. (1985). Phase-dependent central modulation of proprioceptive input to crayfish walking-leg motoneurones. *J. Physiol., Lond.* 364, 26P.

SILLAR, K. T. & SKORUPSKI, P. (1986). Central input to primary afferent neurons in crayfish, *Pacifastacus leniusculus*, is correlated with rhythmic motor output of thoracic ganglia. *J. Neurophysiol.* 55, 678–688.

SILLAR, K. T., SKORUPSKI, P., ELSON, R. C. & BUSH, B. M. H. (1986). Two identified afferent neurones entrain a central locomotor rhythm generator. *Nature, Lond.* 323, 440–443.

SKORUPSKI, P. (1985). Central nervous and proprioceptive control of crayfish walking leg motoneurones: an intracellular microelectrode study of the isolated 4th thoracic ganglion. Ph.D. thesis, University of Bristol, Bristol, UK.

SKORUPSKI, P. & SILLAR, K. T. (1986). Phase-dependent reversal of reflexes mediated by the thoracocoxal muscle receptor organ in the crayfish, *Pacifastacus leniusculus. J. Neurophysiol.* 55, 689–695.

SKORUPSKI, P., SILLAR, K. T. & BUSH, B. M. H. (1984). Central modulation of proprioceptive input to the isolated 4th thoracic ganglion of the crayfish. *Soc. Neurosci. Abstr.* 10, 627.

STEWART, W. W. (1978). Functional connections between cells as revealed by dye coupling with a highly fluorescent naphthalimide tracer. *Cell* 14, 741–751.

VAN HARREVELD, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. exp. Biol. Med.* 34, 428–432.

ZILL, S. N. (1985). Proprioceptive feedback and the control of cockroach walking. In *Feedback and Motor Control in Invertebrates and Vertebrates* (ed. W. J. P. Barnes & M. H. Gladden), pp. 187–208. London: Croom Helm.

ZILL, S. N. (1986). A model of pattern generation of cockroach walking reconsidered. *J. Neurobiol.* 17, 317–328.