Intersegmental influences contributing to coordination in a walking insect

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Abstract

Locomotion depends on correct interaction of the nervous system, muscles and environment. A key element in this process is the coordinated interplay of multiple body parts to achieve a stable and adapted behavior. Different aspects of intersegmental coordination in the stick insect have been investigated in this thesis: the activation of the walking system, intersegmental information transfer in the connectives and the influence of load signals. I used a reduced preparation with only single intact front, middle or hind legs. The intact leg(s) performed stepping movements on a passive treadmill, hence providing, both sensory feedback and central input from its active pattern generating networks to the other hemiganglia. The activity of protractor and retractor motoneurons (MNs) was simultaneously recorded extracellularly in the other segments. The preparation allows investigating intersegmental influence of stepping single leg(s) on motoneural activity in the other deafferented hemisegments.

The experiments revealed that the stick insect walking system is constructed in a modular fashion. Stepping of a single leg does not imply that the animal is in a locomotor state. In the two leg preparation with two intact legs that stepped on two separate treadmills, stepping of one leg did not imply stepping of the second leg. The legs stepped independent of each other concerning coordination and frequency. In the single leg preparation stepping of a single leg did not activate pattern generating networks in all other hemiganglia. The different hemiganglia were obviously activated independently. Only forward stepping of the front leg and, to a lesser extend, backward stepping of the hind leg, elicited alternating

activity in mesothoracic protractor and retractor MNs. Motoneural activity in the other hemisegments increased and was slightly modulated during stepping sequences. Activation of the metathoracic ganglion required both ipsilateral front and middle legs stepping.

Furthermore, the stick insect walking system is constructed asymmetrically on the neural level concerning the contribution and importance of the different legs for intersegmental coordination. The influence of middle leg stepping was qualitatively different to the influence of front leg stepping. In the single leg preparation front leg stepping induced alternating activity in ipsilateral mesothoracic protractor and retractor MNs that was most probably shaped by pattern generating networks. Middle leg stepping did not induce alternating activity in MNs of its ipsilateral neighboring segments. In a two leg preparation with front and ipsilateral middle leg stepping the middle leg appears to have no influence on the timing of metathoracic motoneural activity whereas front leg stepping was able to entrain metathoracic MN activity.

The processing of intersegmental signals from other stepping legs appears to depend on the state of the receiving ganglion. Signals from the stepping front leg most probably reach the metathoracic ganglion as connective recordings show. If the metathoracic ganglion is active in the sense that the central pattern generating networks are active the signals from a stepping leg are treated differently. If the metathoracic ganglion was not active a general increase in motoneural activity was observed during front leg stepping. In case of an active metathoracic ganglion protractor and retractor MN activity alternated and was influenced by front leg stepping.

Sensory signals are particularly important for coordination of the legs in the stick insect. In experiments in which middle leg campaniform sensilla were stimulated during single front leg stepping sequences, mesothoracic levator and depressor motoneuron activity was coupled to the campaniform sensilla stimulation. Stimulation of middle campaniform sensilla pretends increased load on the leg and induced an increase in depressor and a decrease in levator motoneuron activity. In mesothoracic protractor and retractor motoneurons front leg stepping induced alternating activity. Depending on the phase of front leg step cycle middle leg campaniform sensilla stimulation increased retractor and decreased protractor motoneuron activity or the influence was reverse (around 180° of step cycle).

Zusammenfassung

Fortbewegung ist abhängig von der Interaktion von Nervensystem, Muskeln und Umwelt. Eine Schlüsselrolle nimmt dabei das koordinierte Zusammenspiel aller an der Bewegung beteiligten Körperteile ein. In der vorliegenden Arbeit wurden verschiedene Aspekte intersegmentaler Koordination an der Stabheuschrecke untersucht: die Aktivierung des Laufsystems, der Informationstransfer in den Konnektiven, und der Einfluss von Belastungssignalen. Es wurde ein reduziertes Präparat mit einem oder zwei intakten Vorder-, Mittel- oder Hinterbein(en) verwendet. Das intakte Bein bzw. die intakten Beine vollführten Schreitbewegungen auf einem passiven Laufband. Von diesen Beinen gehen somit sensorische Signale und der Einfluss aktiver Rhythmus generierender Netzwerke aus. Gleichzeitig wurde die Aktivität von Protraktor und Retraktor Motneurone (MNe) der anderen Hemiganglien extrazellulär abgeleitet. Diese Präparation erlaubt es einzelne intersegmentale Einflüsse wie die Laufbewegung eines Beines auf die motoneuronale Aktivität anderer deafferentierter Hemiganglien zu untersuchen.

Das Laufsystem der Stabheuschrecke ist auf neuronaler Ebene modular aufgebaut. Die Schreitbewegung eines einzelnen Beines ist nicht hinreichend um das gesamte Laufsystem zu aktivieren. In der Zweibein Präparation, bei der zwei Beine auf separaten Laufbändern laufen und daher mechanisch entkoppelt sind, machten beide Beine unabhängig voneinander Schreitbewegungen. Im Einbein Präparat aktivierte die Laufbewegung eines Beins nicht die Rhythmus generierenden Netzwerke in de anderen Hemiganglien. Die einzelnen Hemiganglien werden offensichtlich unabhängig voneinander aktiviert. Nur die vorwärts

gerichtete Laufbewegung eines Vorderbeins und in geringerem Maße die rückwärts gerichtete Laufbewegung eines Hinterbeins lösten alternierende Aktivität in mesothorakalen Protraktor und Retraktor MNen aus, wohingegen in den anderen Hemiganglien ein Anstieg motoneuronaler Aktivität zu beobachten war. Um das Metathorakalganglion zu aktivieren müssen sowohl das ipsilaterale Mittel- als auch Vorderbein intakt sein.

Desweiteren zeigte sich, dass das Laufsystem im Hinblick auf den Einfluss der einzelnen Beine auf neuronaler Ebene asymmetrisch strukturiert ist. Die Schreitbewegungen eines einzelnen Vorderbeins lösten in mesothorakalen Protraktor und Retraktor MNen alternierende Aktivität aus, die sehr wahrscheinlich auf die Aktivierung mesothorakaler Rhythmus generierender Netzwerke zurückzuführen ist. Die Schreitbewegung des Mittelbeins löste in keinem benachbarten Hemiganglion alternierende Aktivität in MNen aus. In der Zweibein Präparation mit laufendem Vorder- und ipsilateralem Mittelbein war das laufende Vorderbein in der Lage die Aktivität metathorakaler Protraktor und Retraktor MNe anzukoppeln, unabhängig davon, ob das Mittelbein Schreitbewegungen vollführte oder nicht.

Wie intersegmentale Signale von anderen Beinen verarbeitet werden, scheint vom Status der Netzwerke des "empfangenden" Ganglions abzuhängen. Signale des laufenden Vorderbeins erreichen sehr wahrscheinlich das Metathorakalganglion, wie Konnektivableitungen zeigen. Abhängig vom Status des Ganglions werden die Signale des laufenden Vorderbeins unterschiedlich verarbeitet. Waren die Rhythmus generierenden Netzwerke des Metathorakalganglions inaktiv, so führte die Laufbewegung des Vorderbeins zu einem gemeinsamen Anstieg der Aktivität der Protraktor und Retraktor MNe. Waren sie aktiv, so alternierte die motoneuronale Aktivität und wurden von den Schritten des Vorderbeins beeinflusst.

Sensorische Signale sind wichtig für die Koordination der Beine. Stimulation der Campaniformen Sensillen des Mittelbeins während Schrittsequenzen des Vorderbeins führte zu einem Anstieg der Aktivität in mesothorakalen Depressor MNen und einem Abfall der Aktivität in mesothorakalen Levator MNen. In mesothorakalen Protraktor und Retraktor MNen führte die Stimulation der Campaniformen Sensillen abhängig von der Phase des Schrittzyklus zu einem Anstieg der Aktivität in den Retraktor MNen und einem Abfall der Aktivität in den Protraktor MNen oder umgekehrt (ca. 180° des Schrittzyklus).

Contents

CHAPTER 1

Introduction

The ability to move is a characteristic trait of animals and humans. Locomotion emerges from a complex interplay of nervous system, muscles, sense organs and environment (Orlovsky et al. 1999). There are various ways of locomotion throughout the animal kingdom. Nevertheless, all ways of locomotion regardless if flying or swimming, walking or crawling meet the same principles. Locomotion is based on cyclic recurrent movements of specified body parts. This is achieved by coordinated, rhythmical contractions of antagonistic muscles that move the body, multijointed limbs or other appendages. Therefore, a complex rhythmic motor pattern has to be generated, coordinated intersegmentally and adapted to the environment. Rhythmic motor patterns are generated by neural networks within the central nervous system, called central pattern generators (CPGs) (Grillner 1985, 2003; Pearson et al. 1993; Büschges 2005). Besides the basic rhythmic motor output sensory signals from various sense organs are crucial for a functional motor program as they establish the only connection between animal and environment. They monitor parameters of behaviors and assure that movements are successfully and efficiently performed, they

aid in adjusting posture and locomotion to variations in the environment and they detect perturbations and contribute to the development of compensatory adjustment (Pratt 1995; Clarac et al. 2000; Grillner and Wallen 2002; Pearson 2000, 2004; Zill et al. 2004; Büschges 2005). The degree of central and peripheral control for the coordination of motor output varies with the way of locomotion and the surrounding environment. Central interactions are particularly well understood in swimming (*crayfish (swimmerets)*: Paul and Mulloney 1986; Tschuluun et al. 2001; *leech*: Weeks 1981; *lamprey*: Cohen and Wallén 1980; Grillner et al. 1981a, b; *tadpole*: Tunstall and Roberts 1990, 1994) whereas peripheral feedback has been thoroughly investigated in terrestrial locomotion (*stick insect*: Büschges 2005 (review); *crayfish*: Clarac 1985; Cattaert and Ray 2001 (review); *cat*: Shik et al. 1966; Grillner 1975; Wetzel et al. 1976; O. Andersson and Zomlefer 1978; Rossignol et al. 1981; Andersson and Grillner 1983; Conway et al. 1987; Lam and Pearson 2002 (review)). In general peripheral feedback appears to be more important for terrestrial locomotion. Here the surrounding environment is more irregular and requires constant adjustment. Furthermore, locomotor organs for terrestrial locomotion are often multi-segmented legs. Hence, in legged animals besides intersegmental coordination intrasegmental coordination of the leg segments is required.

"Model system" stick insect

A well established model system for slow walking behavior is the stick insect. Stick insects have a simply organized nervous system that is easily accessible and show only a simple behavioral repertoire. They naturally walk and climb on bushes they feed on. They have six multisegmented legs that have to be coordinated properly to achieve a stable locomotion pattern. Adult stick insects walk in tripod or tetrapod or an intermediate gait (Graham 1985b). During walking the cyclic pattern of a leg consists of the two phases: stance (power stroke) and swing (return stroke). During stance phase the leg is on the ground and yields propulsion to the body. During swing phase the starting position of the leg is re-established to generate the next stance. Each phase requires the orderly recruitment of specific muscles that move the different leg joints (Graham 1985b; Büschges and ElManira 1998; Büschges 2005). The leg is equipped with different sense organs that

provide information about the relative position of the leg segments (hair plates and hair rows) (Wendler 1964c; Tatar 1976; Bässler 1977), about load (femoral and trochanteral campaniform sensilla) (Tatar 1976; Bässler 1977; Hofmann and Bässler 1982; Akay et al. 2004) and the angle and movement of the femur tibia joint (femoral chordotonal organ) (Borchardt 1927; Bässler 1965, 1967; Füller and Ernst 1973).

Extensive behavioral studies have been performed on the stick insect. Observations and perturbations of the stepping pattern allowed proposing six rules that describe intersegmental coordination (Bässler 1979; Cruse 1979a, 1985; Graham 1985b; Cruse and Müller 1986; Cruse and Schwarze 1988; Dean and Wendler 1983; Cruse 1990).

1. Rostrad influence: Return stroke inhibits start of return stroke.
2. Rostrad influence: Start of power stroke excites start of return stroke.
3. Caudad influence: Caudal positions excite start of return stroke.
4. Position influences position at end of return stroke(targeting).
5. a) Increased resistance increases force (co-activation).
   b) Increased load prolongs power stroke.
6. Treading-on-tarsus reflex.

(adapted from Cruse et al. 1995b

These rules mainly serve the purpose to re-establish the walking pattern after perturbations (Cruse 1990) and are sufficient to generate a walking pattern that resembles closely to that of straight forward walking in the stick insect. This could be shown in simulation studies (Cruse et al. 1995b, a, 1996, 1998). Nevertheless, little knowledge exists about the neural basis of intersegmental coordination. From behavioral studies and amputation experiment it is known that sensory signals are important for intersegmental coordination. Amputation of one middle leg leads to an immediate adaptation to a slightly changed walking pattern (Hughes 1957; Bässler 1972; Graham 1977). When the amputated

middle leg is substituted by a stick that is glued to the coxa the original coordination is re-established (Wendler 1964c). In contrast, if the middle leg is immobilized instead coordination is destroyed (Graham 1977). Hence, sensory signals are necessary to assure coordination. Foth and Bässler (1985a, b) used two treadmills with five legs walking on one treadmill with a given velocity and one leg walking on another treadmill with a different velocity. All six legs were still coordinated and adapted to respective tread wheel velocity. This indicates that intersegmental coordination in the stick insect is not achieved primarily by central coupling between the legs. Mechanical coupling through the ground alone can also be excluded as coordination mechanisms. When the legs are decoupled by walking on a slippery surface still a coordinated walking pattern was observed (Graham and Cruse 1981; Epstein and Graham 1983).

The generation of a step in a single leg is well investigated on the neural level (Bässler and Büschges 1998; Ekeberg et al. 2004; Büschges 2005). The coordination between different legs is not sufficiently described on the neural level so far. Büschges et al. (1995) could show that each leg joint posses its own CPG. But no coupling of active CPGs in different segments could be observed (Ludwar et al. 2005a). Therefore the interaction between the CPGs appears to be comparably weak and is not responsible for intersegmental coordination. Ludwar et al. (2005a) show that stepping of a single front leg induces alternating activity in antagonistic MN pools of the ipsilateral middle leg. Sensory signals of the front leg contribute to this influence. Stimulation of the front leg chordotonal organ increases the probability for mesothoracic protractor and retractor MNs to switch from protractor to retractor MN activity (Ludwar et al. 2005a).

Issues

This thesis deals with different aspects concerning the neural basis of intersegmental coordination in walking in the stick insect *Carausius morosus*. The experiments were conducted on reduced preparations that allowed investigating isolated intersegmental influences. The following issues were investigated:

1. The activation and maintenance of rhythmic motor activity in the walking system.

2. The information transfer contributing to intersegmental coordination.

3. The processing of intersegmental signals and local sensory signals.

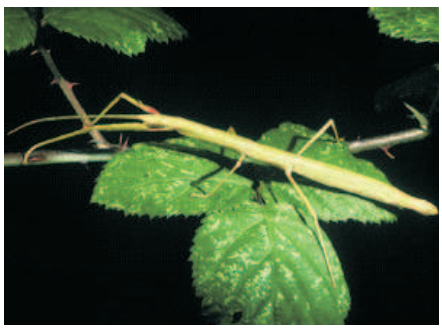
The results are structured in several subchapters. Each subchapter contains a short introduction that is focused on the specific topic of respective chapter. At the end of each result chapter is a short summary of the conclusions.

CHAPTER 2

Materials and methods

2.1 Experimental animal

All experiments were conducted on female stick insects of the genus *Carausius morosus* (*C. morosus*). The stick insect *C. morosus* belongs to the family Phasmatidae. It was originally domiciled in India and is also known under the trivial name Indian stick insect.



Order:	Phasmatoptera
Family:	Phasmatidae
Subfamily:	Lonchodinae
Genus:	Carausius
Species:	*C. morosus*

Figure 2.1: *Stick insect Carausius morosus on a blackberry leaf.*

Its wingless body is nearly cylindrical and varying in color from green to grey (Bückmann 1979). The adult females reach a size of approximately 7,5 cm. Male stick insects are smaller and rangier reaching a size of approximately 5,5 cm. In adult animals the inner femur of the front legs is colored in bright red.

Male individuals occur rarely as stick insects mainly proliferate by pathogenesis. The oogenesis takes four to eight months depending on the temperature. The larvae need another three months to develop to an imago.

C. morosus is crepuscular and nocturnal. Stick insects live on bushes and feed upon the leafs. There most important protective mechanism is twig mimesis. The twig-like form of their body masks them in the bushes and together with some behavioral components, such as catalepsy and rocking, this appears to reduce the probability of the insects being eaten (Bässler and Wegner 1983; Rupprecht 1971).

The animals that were used for the experiments came from the breed of the Institute of Zoology at the University of Cologne exclusively. The animals were kept under constant conditions at temperatures between 20° and 22° and artificial light in a twelve hour rhythm of light and dark. They were fed with blackberry leafs of the sort *Rubus fructiosus*.

2.2 Preparation and experimental setup

The experiments were solely performed on adult female stick insects of the genus *C. morosus* at room temperature and obscured light conditions. Aligned to the different experimental requirements different preparations were used.

2.2.1 Preparation

Single leg preparation

This semi-intact preparation allowed to study the influence of a single intact stepping leg on the motoneural activity of the other hemiganglia. All legs except one intact front, middle or hind leg were cut in the middle of the coxa (Fig. 2.2(a)). The amputation of all legs

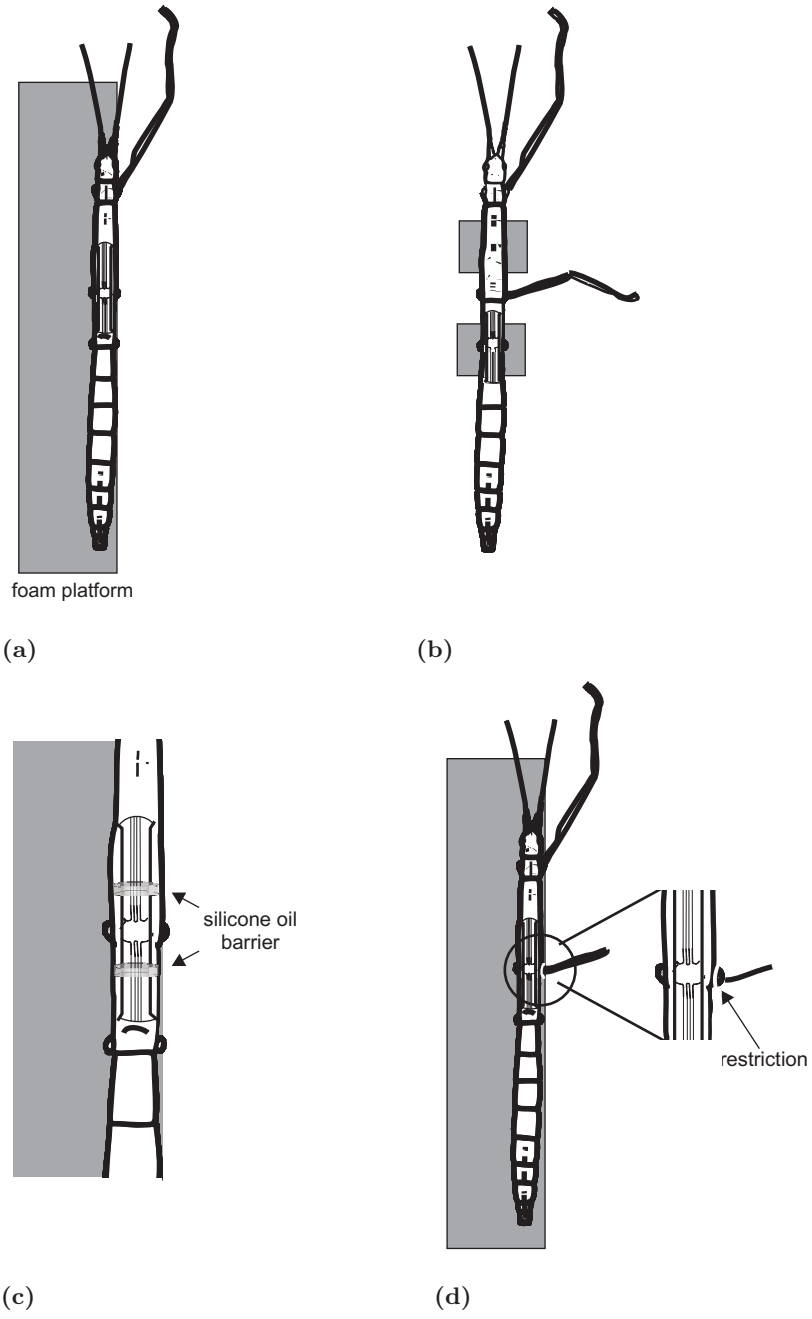


Figure 2.2: *(a) Single leg preparation. (b) Two leg preparation. (c) Split bath preparation. (d) Preparation for middle leg campaniform sensilla stimulation during front leg stepping. In the enlarged part the restriction around the coxa is visible that restricts movements of the thorax coxa joint.*

except one made sure that sensory signals of these legs were eliminated. The animal was fixed dorsal side up on the edge of a squared foam platform with dental cement (Protemp II, ESPE). The intact leg could move barely restricted. The thorax was opened with a sagittal cut along the midline and the cuticle was opened with four 0,1 mm or 0,2 mm insect pins, two on each side of the animal. The gut was moved aside and connective tissue was carefully removed to expose the various hemiganglia and their leg nerves for extracellular recording. The ganglion that was recorded from was completely deafferented by either cutting or crushing the nerves. The body cavity was filled with saline (composition according to Weidler and Diecke 1969). (Preparation modified after Karg et al. 1991; Fischer et al. 2001).

Two leg preparation

In the two leg preparation two legs were left intact while all others were cut in the middle of the coxa. I used three different combinations of intact legs. Either front and ipsilateral middle legs or front and contralateral middle legs or front and ipsilateral hind legs were left intact. In this preparation the platform the animal was fixed on was reduced to two small platforms (Fig. 2.2(b)). One platform supported the body of the animal beneath the ganglion that was recorded from. The other platform was placed between front and middle legs or at the abdomen respectively. The animal was fixed dorsal side up and dissected as described for the single leg preparation. The use of two small platforms had the advantage that both legs moved freely in each joint and two treadmills were easily positioned around the animal, one under each leg.

Split bath preparation

In different sets of experiments one ganglion was superfused with saline containing pilocarpine. Pilocarpine is a muscarinic Acetyl choline agonist. It is known to activate central pattern generating networks which elicit rhythmic activity in stick insect MNs. MNs to antagonistic muscles start alternating with a slow frequency (Büschges et al. 1995). For the preparation the animal was fixed on a foam platform and dissected as described for the

single leg preparation. To be able to superfuse only one ganglion with saline containing pilocarpine, half a centimeter rostral and caudal of respective ganglion a silicone oil barrier was built (Fig. 2.2(c)). Therefore two transversal sections were cut out of the cuticle, one rostral of the ganglion and one caudal. The connectives were left intact. The gap was kept dry and was completely filled with silicone oil (hochviskos, BAYER, Leverkusen). Three separate compartments were formed, one rostral and caudal of the ganglion in focus and one around the ganglion. Drops of stock solution with a pilocarpine concentration of $10^{-2}\ mol \cdot l^{-1}$ were delivered into the compartment with the ganglion in focus, to reach a final concentration in the range of $10^{-4}\ mol \cdot l^{-1}$ to $5 \cdot 10^{-3}\ mol \cdot l^{-1}$. The denoted concentration is known to reliably elicit a stable rhythm in respective MNs (Büschges et al. 1995).

Preparation for campaniform sensilla stimulation

In one set of experiments middle leg campaniform sensilla were stimulated. The leg was cut at two thirds of the femur. Coxa and trochanter and the proximal part of the femur were shaved with a scalpel to cut of sensory hairs. The stimulation was achieved by bending the femur (Schmitz 1993). This lead to an unspecific stimulation of femoral and trochanteral campaniform sensilla. For the stimulation it was necessary to exclude movements in the thorax coxa joint. The thorax coxa joint was fixed in a position where the femur and the body of the animal formed an angle of approx. 85°. The fixation was achieved with a small ring of dental cement around the coxa that prohibited pro- and retraction movements of the coxa (Fig. 2.2(d)). The front leg was left intact and the ipsilateral middle leg was prepared for campaniform sensilla stimulation (Fig. 2.2(d)). All other legs were cut. The further preparation was done analogous to the single leg preparation. The mesothoracic ganglion was deafferented (including nerves C1 and C2) except for the *nervus cruris* (ncr) that contains the campaniform sensilla afferents.

2.2.2 Extracellular recordings and electromyograms

Lateral nerves and connectives were recorded with extracellular hook electrodes (modified after Schmitz et al. 1991) filled with silicone oil (hochviskos, BAYER, Leverkusen). The

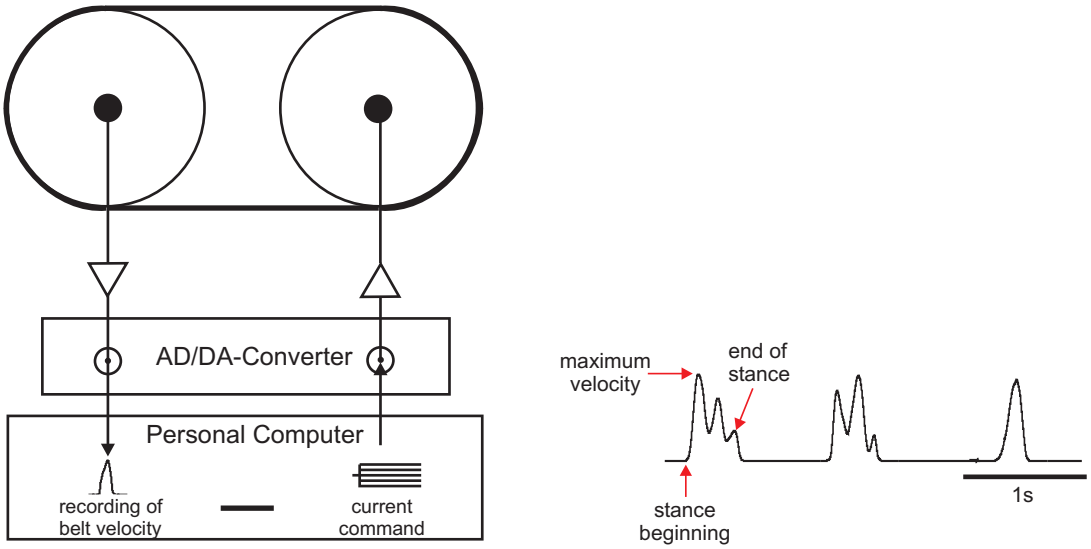
ganglion that was recorded from was completely deafferented by either cutting or crushing the nerves. The connectives were left intact. It was recorded from the lateral nerves nl2, nl5, C1 and C2 (nomenclature according to Marquardt 1940 and Graham 1985b) and from different connectives.

In some experiments electromyograms (EMGs) of the femoral muscles were done to monitor the movement of the leg. Therefore, two thin copper wires (diameter 50 μm, insulated except for the tips) were inserted closely together through the cuticle of the proximal femur. Depending on the insertion site, muscle potentials from the flexor tibiae muscle were recorded individually or together with potentials from the extensor tibiae muscle.

Both the extracellular recordings and the EMG recordings were amplified (500x - 5000x), filtered (250 Hz - 5 kHz, 50 Hz - 1 kHz) and digitized with a MICRO1401 A/D converter (sampling rate: 12,5 kHz) and recorded with SPIKE2 software (both CAMBRIDGE ELECTRONIC DESIGN, Cambridge, UK) on a personal computer.

2.2.3 The treadmill

The treadmill (Fig. 2.3(a)) consisted of two styrofoam drums (diameter 40 mm; width 28 mm) each mounted on a micro DC-motor (*DC1516*, FAULHABER, Schönaich, Germany) that had a center distance of 50 mm. Around them a belt made of light crepe paper (35 g/m^2) was placed. The tangential force that had to be applied to overcome belt friction was 4,0 $\pm$ 0,3 mN. The moment of inertia of the system, which is determined by the effective mass of the treadmill, was 1,1 g and thus equal to the mass of an adult animal (Gabriel 2005). One of the DC-motors served as a tachometer. The output voltage, which was proportional to the belt velocity, was smoothed (first order low-pass filter, time constant 20 ms) in order to eliminate voltage spikes. The tachometer was digitized with a MICRO1401 A/D converter (sampling rate: 6,5 kHz) and recorded with SPIKE2 software (both CAMBRIDGE ELECTRONIC DESIGN, Cambridge, UK) on a personal computer. With the other motor belt friction could be varied. By a voltage current converter (Peter Heinecke, self construction) a current could be applied that generated a torque and thereby changed the force required to move the belt without moving the



(a) (b)

Figure 2.3: *(a) Treadmill. The signal from the tachometer was filtered and digitized prior to recording. A current could be applied to the other motor to decrease or increase belt friction. (Picture modified after Gabriel 2005). (b) Tachometer trace.*

belt itself. By this, belt friction could be altered in a range from 1,5 mN to 6,5 mN. The treadmill was positioned below the leg. The height was adjusted so that the angle of the joint between femur and tibia was approximately 90° in mid-stance. The tachometer trace allowed determining the stance phase of a step (Fig. 2.3(b)). The beginning of stance was defined as the beginning of the raising edge. The end of stance was defined as the last maximum of the trace before it decreases back to zero. The falling edge is just determined by the inertia of the treadmill and does not contain any information about the status of the step. From the tachometer trace the maximum belt velocity was determined by the maximum of the voltage trace of respective step as the voltage output is proportional to the belt velocity. In all experiments not single steps were used for analysis but stepping sequences. A stepping sequence was defined as a minimum of three consecutive steps that had a maximum temporal distance of 3,5 s.

2.2.4 Piezoelectrical stimulation

For the campaniform sensilla stimulation a piezoelectric element that was driven by a ramp generator (electronic workshop, University of Cologne) was used. In these preparations, the left middle leg femur extended over the platform. The femur was attached to the piezoelectrical element. To adequately stimulate the campaniform sensilla, the femur was slightly bent horizontally (Schmitz 1993). The stimulation was carried out only in posterior direction. The ramp that was used had a fast 0,03 s rising edge followed by a 0,3 s plateau and a slow movement (1,5 s) back to zero. The amplitude was 530 μm. The stimulation was done with frequencies between 0,16 Hz and 0,7 Hz or single irregular stimulations.

2.2.5 Experimental setup

The experimental setup stood on damped table (MICRO-g, TMC, Peabody, MA, USA) surrounded by a Faraday cage. Next to the foam platform where the animal was mounted two to four micromanipulators were positioned for electrodes and the piezo element. A lamp with optical fibers illuminated the setup. Depending on the experiment one or two treadmills were positioned next to the platform under respective intact leg. The front leg treadmill was usually positioned in a approx. 40° angle to the animal as this corresponds to the position of the front leg in a standing animal (Cruse 1976). The middle leg treadmill was either positioned in parallel to the animal or perpendicular. For the single middle leg preparation, experiments were performed with the treadmill perpendicular to the animal as well as in parallel to the animal. With the treadmill perpendicular to the animal the middle leg stepped sideways. This corresponds to the traditional fashion of the single middle leg preparation (Fischer et al. 2001; J.P Gabriel and Büschges 2003). This walking situation is most comparable to an inner middle leg during curved walking or to front leg stepping. With the treadmill parallel to the animal the middle leg stepping resembles more closely the kinematics during forward straight walking (Cruse and Bartling 1995). For the two leg preparation the middle leg treadmill was positioned parallel to the animal. The animals performed spontaneous stepping sequences very seldom. Therefore

stepping sequences were elicited by gently touching the abdomen with a paintbrush. The paintbrush was removed as soon as the animal started a sequence of stepping movements.

2.3 Data analysis

The extracellular recordings and the tachometer trace were preprocessed in SPIKE2 for further data evaluation. Neuronal activity of the extracellular recordings and beginning and end of a stance phase in the tachometer trace were displayed as event channels. In order to estimate the gross activity of an extracellular recording the recording was rectified and smoothed (first order low pass filter, time constant $T_s = 70\ ms$). Custom SPIKE2 script programs were written to analyze the recordings and prepare it for further advanced analysis with MATLAB 7.0 (The MathWorks, Inc., Natick, USA).

Circular statistics

Stepping is a cyclic recurrent behavior. Therefore I used circular statistics to describe and evaluate motoneural activity in the step cycle and the relationship between two stepping legs. Polar plots show the mean phase of MN activity or mean stepping activity in the stepping legs step cycle. The vector length of the mean vectors was tested for significance with the Rayleigh-Test (Batschelet 1981). The following symbols show the level of statistical significance: ( ) not significant; (*) significant with $P < 0,05$. N gives the number of experiments or animals while n gives the number of steps. Phase histograms show the distribution of MN activity in the step cycle. On top of the histogram the mean end of stance phase is shown with the mean angular deviation (Batschelet 1981). This is equivalent to the standard deviation in linear statistics. Furthermore, the phase of occurrence of MN spikes in the step cycle was plotted against the step cycle duration to detect a possible phase or time dependence. In this plot the frequency of steps over the step cycle duration was plotted additionally. For a clearer illustration the curve was smoothed by a moving average.

Cross correlation and auto correlation analysis

Auto correlation analysis has been applied on rectified and smoothed extracellular traces to detect oscillations in the gross activity of the recording (see chapter 5).
Cross correlation analysis has been applied for different purposes. Cross correlation between the event channels of extracellular recordings was done for the whole time of recording including the time between stepping sequences. The event channel was "re-sampled" in this case as the mean number of events was determined in consecutive non-overlapping windows of 50 ms. In this case no bounds of significance were quoted for the cross correlation function. Because of the irregular episodes of neuronal activity due to the irregular occurrence of stepping sequences the cross correlation function often showed a drift. Hence, the interesting range in the cross correlation function around 0 s time lag was not necessarily symmetrical around R = 0. Therefore, the bounds of significance have no meaning. The cross correlation function between neuronal activities was calculated on the basis of the rectified and smoothed extracellular recordings for single stepping sequences only. Because of the high number of data points due to the high sampling rate of 12,5 kHz this was reasonable only for small time windows as for large time windows it takes far too long to calculate the cross correlation function.

Averaged overdraws

The course of the rectified and smoothed or the unprocessed extracellular recording was averaged with reference to a certain trigger for example the beginning of stance phase. The overdraws were done with respect to time as well as with respect to phase. The variance was indicated by the standard deviation.

Phase response curve (PRC) A phase response curve (PRC) allows determining and characterizing the phase dependent influence of a stimulus on the timing of an oscillator (Fig. **??**). The PRC was calculated by

$$PRC(\frac{d}{mean(Tp)}) = \frac{mean(Tp) - T}{mean(Tp)} \ .$$

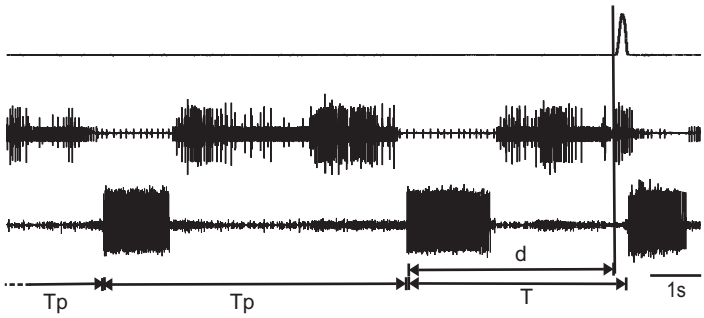


Figure 2.4: *Calculation of a phase response curve for front leg steps occurring during pilocarpine induced rhythm in mesothoracic MNs.*

Statistics

Regression analysis was used to analyze linear correlation between two variables. The correlation coefficient was determined and tested for significance with the Fisher test (Sachs 1972).

Mean values were compared to zero or among each other using a t-test. Means, samples and correlation coefficients were regarded as significantly different from zero or from each other at $P < 0,05$. The following symbols show the level of statistical significance: (n.s.) not significant $P \geq 0,05$; $(*) 0,01 \leq P < 0,05$; $(**) 0,001 \leq P < 0,01$; $(***) P < 0,001$. N gives the number of experiments or animals while n gives the sample size.

CHAPTER 3

Activation of the walking system

The stick insect is a well established model system for slow walking behavior. Each of the six legs is driven by its own walking pattern generator for walking (e.g. Foth and Bässler (1985a)), which contains at least three CPGs - one for each of the major leg joints (Bässler and Wegner 1983; Büschges et al. 1995). Intrasegmental coordination has been fairly well studied and is achieved by sensory feedback that couples the activities of each leg's three joints (reviewed in Büschges (2005)). In contrast, little is known about the neural mechanisms underlying intersegmental coordination among the individual legs. Behavioral experiments have led to the proposal of six rules that phenomenologically predict the interactions among the different legs (Bässler 1979; Cruse 1979b, 1985; Cruse et al. 1995b; Cruse and Schwarze 1988; Dean and Wendler 1983; Graham 1979; **?**). These rules mainly serve to re-establish the stepping pattern in case of disturbances and present evidence suggests that these rules act similar for all legs (Dürr et al. 2004). It remains unclear, however, whether these behavioral rules emerge from all legs interchanging similar information with each other or whether leg specific differences in information transfer

exist. Resolving this issue requires investigating how single leg stepping affects the other legs. Ludwar et al. (2005a) first addressed this issue by investigating the influence of a single stepping front leg on the ispilateral middle leg. Front leg stepping induced alternating activity in antagonistic motoneuron (MN) pools of the ipsilateral mesothoracic hemiganglion with a clear coupling to front leg activity. This may indicate that there are phasic influences from each walking leg on its neighbors, as previously suggested by the coordination rules. I investigated what influence single front, middle and hind leg forward and backward stepping has on motoneural activity in the other hemiganglia.

The animal walked with a single intact front, middle or hind leg on a passive treadmill while in the other hemisegments the activity of protractor and retractor coxae MNs was recorded extracellularly. In intact animals during forward walking protractor coxae MNs induce forward movement of the leg and are active mainly during swing, while retractor coxae MNs induce backward movement and are active mainly during stance (Graham and Wendler 1981; Graham 1985b). The recordings show that in the resting animal in nerves nl2 and nl5 of all hemisegments small units were tonically active (**?**Graham and Wendler 1981). With the beginning of a walking sequence activity in both nerves increased (data not specifically shown but see Fig. 3.2(a)). Shortly after the end of stepping the neural activity decreases back to tonic activity of few small units as before the stepping sequence.

3.1 Influences of single front leg stepping

3.1.1 Ipsilateral influences

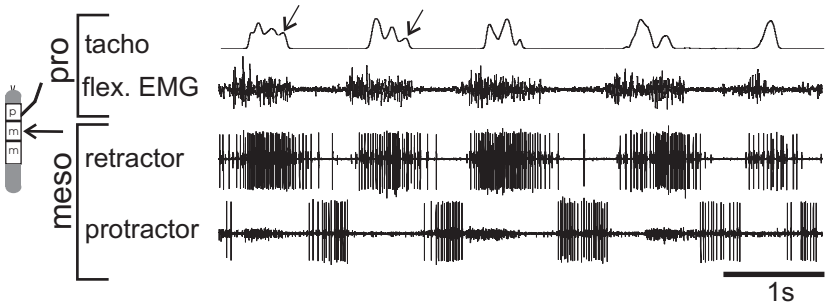
Single front leg stepping induces a general increase of activity in all mesothoracic leg MN pools (Ludwar et al. 2005a, b), with protractor MN activity decreasing and retractor MN activity increasing during stance (Ludwar et al. 2005a). I extended this work by determining whether the occurrence of protractor and retractor MN activity in the front leg step cycle is phase or time dependent and whether a systematic coupling between protractor and retractor MN activity exists. Data are shown for one representative experiment (Fig. 3.1).

Extracellular recordings from mesothoracic lateral nerves nl2 and nl5, containing protractor and retractor MNs, showed alternating activity during front leg stepping (Fig. 3.1(a)). Plots of protractor and retractor MN spike phase against step cycle period (Fig. 3.1(b), 3.1(c)) showed that, regardless of step cycle period, the protractor and retractor MNs were active in preferred phases of the front leg step cycle, as indicated by the dense horizontal bands in the plots. Protractor MN spikes primarily occurred between 180° and 360° of front leg step cycle and retractor MN spikes between 0° and 180°, independent of the actual step cycle duration. The red curve in figure 3.1(b) shows the frequency of steps over the step cycle durations. A clear peak is visible that shows that most of the steps had a step cycle duration around 1,5 seconds.

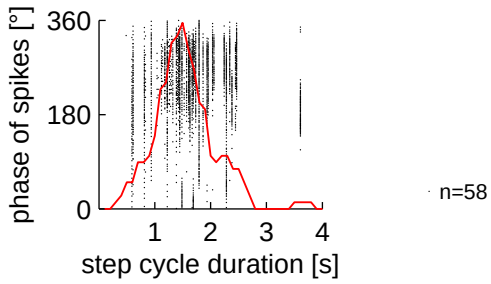
The histogram gives the distribution of protractor and retractor MN spikes in front leg step cycle for 58 steps (Fig. 3.1(d)). Retractor MN activity was maximal between 30° and 60° of front leg step cycle and sharply decreased at phase angles greater than 180°. Protractor MN activity increased at 180° of step cycle and was maximal between 270° and 300°. The mean end of stance was at 146° of the step cycle, as indicated by the black square (error bar is mean angular deviation after Batschelet (1981)) at the top of the histogram. Retractor MNs were thus active primarily during stance and protractor MNs during swing.

To investigate whether a systematic relationship between protractor and retractor MN activity existed, protractor and retractor MN activity were cross correlated (Fig. 3.1(e)). In the cross correlation function a clear oscillation is visible with a maximal negative correlation at 0 seconds time shift, indicating an alternating coupling between protractor and retractor MN activity. Although this analysis is independent of step cycle phasing, this alternating coupling is not surprising considering the phase dependency of protractor and retractor MN activity on front leg steps and the fact that the distribution of step cycle periods had a relatively sharp peak at 1,5 s (Fig. 3.1(b)). Nevertheless, it is important to show for comparison to the other data of the following experiments. Similar coupling was observed in nine of eleven experiments.

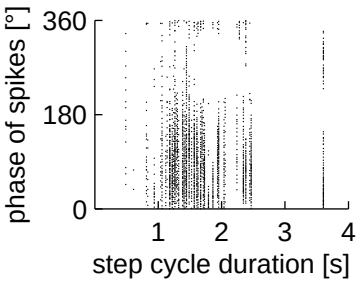
Figures 3.1(f) and 3.1(g) show polar plots of the mean vectors of protractor and retractor


pro
tacho
flex. EMG
meso
retractor
protractor
1s

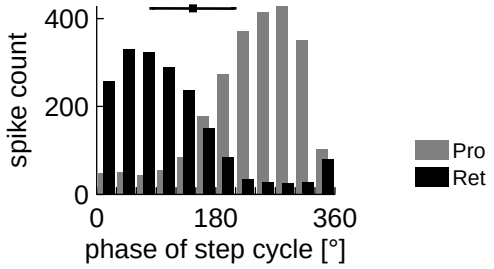
(a)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]
n=58

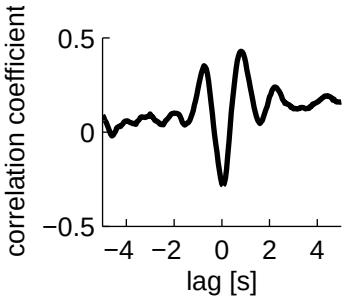
(b)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

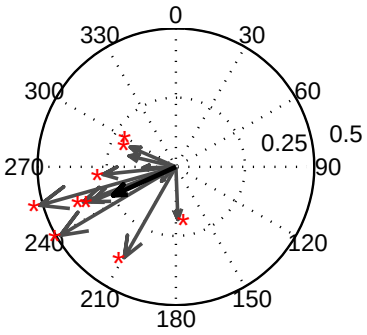
(c)


spike count
400
200
0
0
180
360
phase of step cycle [°]
Pro
Ret

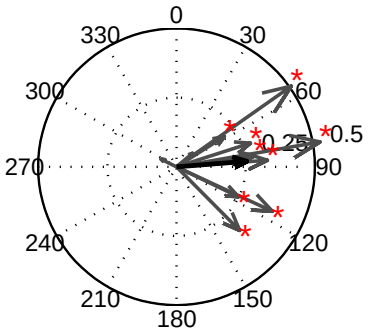
(d)


correlation coefficient
0.5
0
−0.5
−4
−2
0
2
4
lag [s]

(e)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5

(f)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5

(g)

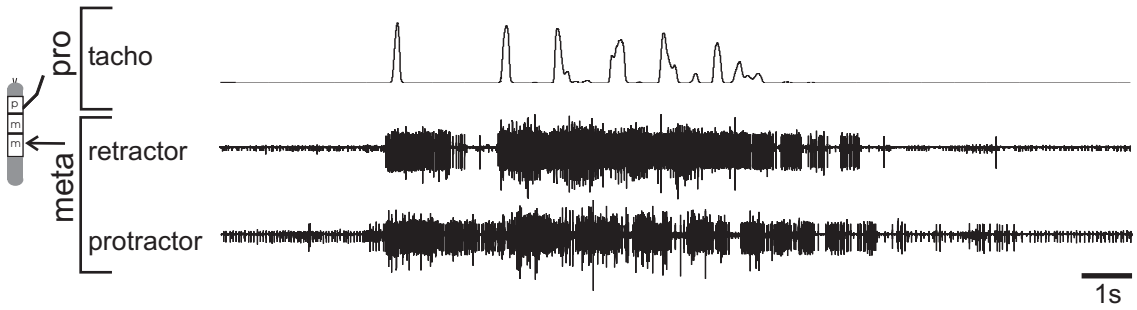
Figure 3.1: *(a) Mesothoracic retractor and protractor coxae MN activity recorded from nerves nl5 (retr.) and nl2 (protr.) while the ipsilateral front leg performed walking movements on a passive treadmill. Front leg flexor EMG and treadmill velocity were monitored. The end of stance phase is marked for two steps by black arrows. Phase of (b) protractor and (c) retractor MN spikes in front leg step cycle (beginning of stance to beginning of next stance) plotted against step cycle duration. Protractor MN spikes occurred between 180° and 360° of front leg step cycle and retractor MN spikes between 0° and 180°, independent of step cycle duration. The red curve (smoothed) shows the frequency of steps over the step cycle duration in arbitrary units. (d) Distribution of protractor (grey) and retractor (black) MN activity in front leg step cycle for 58 steps. Black square at top marks average end of stance phase, error bars are mean angular deviation. Protractor MN activity had a maximum between 270° and 300°. Retractor MN activity had a maximum during front leg stance phase between 30° and 60°. (e) Cross correlation function showing alternating coupling between protractor and retractor MN activity. Similar coupling observed in 9 of 11 experiments. Polar plots of (f) protractor MN activity and (g) retractor MN activity in front leg step cycle for eleven experiments (grey arrows) and mean vector of all experiments (black arrows) (radius of cycle = 0,5). Each vector points in direction of the mean phase of spike activity in front leg step cycle. Consistently for all experiments protractor MN activity had an overall mean phase of about 246° (182° - 315°) and retractor MN activity of about 86° (58° - 135°). Red stars mark significant vectors.*

MN activity in front leg step cycle for all eleven experiments. The plot summarizes the data from the experiments not shown in detail and shows how consistent the observed results were across the experiments. Each grey vector corresponds to one experiment and the black vector is the mean vector of all experiments. Each vector points in direction of the mean phase of activity and its length is between 0 and 1. The radius of the circle is 0,5. A vector length of one indicates that all the data points coincide. The mean phase of protractor and retractor MN activity was consistent throughout the experiments. Protractor MN activity covered an angle from 182° to 315° with a mean phase for all experiments of 246°. Retractor MN activity covered a range from 58° to 135° with an overall mean phase of 86° of front leg step cycle.

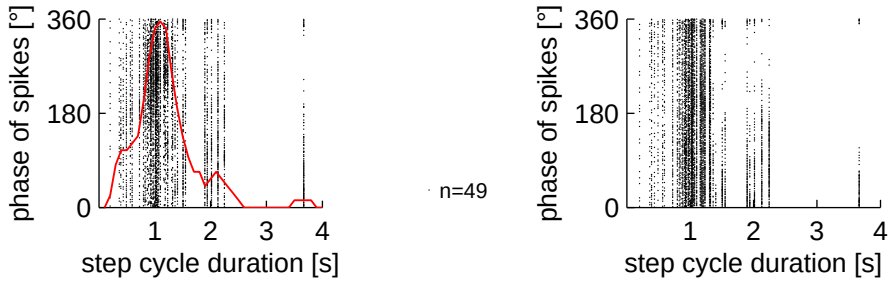
In summary, front leg stepping elicited alternating activity in ipsilateral mesothoracic protractor and retractor MNs. This pattern was phase coupled to the front leg step cycle. Middle leg retractor MNs were active during front leg stance and protractor MNs during front leg swing phase. Thus, front leg stepping had a patterning influence on mesothoracic protractor and retractor MNs.

Next, it was investigated whether and how single front leg stepping affected ipsilateral hind leg MN activity. During front leg stepping metathoracic protractor and retractor MN activity increased (Fig. 3.2(a)) (N=7), but this activity was less clearly structured than in the mesothoracic protractor and retractor MNs (Fig. 3.1(a)). The phase plots show that protractor and retractor MN spikes were widely distributed over front leg step cycle at all of step cycle lengths (Fig. 3.2(b), 3.2(c)) (that is, obvious no horizontal "bands" are present, compare to Fig. 3.1(b), 3.1(c)). The frequency of steps over the step cycle duration (red curve in figure 3.2(b)) shows a clear peak around one second step cycle period. The histogram (Fig. 3.2(d)) for 49 front leg steps reveals that protractor MN activity was maximal between 240° and 270° of the front leg step cycle and retractor MN activity was maximal at the beginning of stance phase between 0° and 30°.

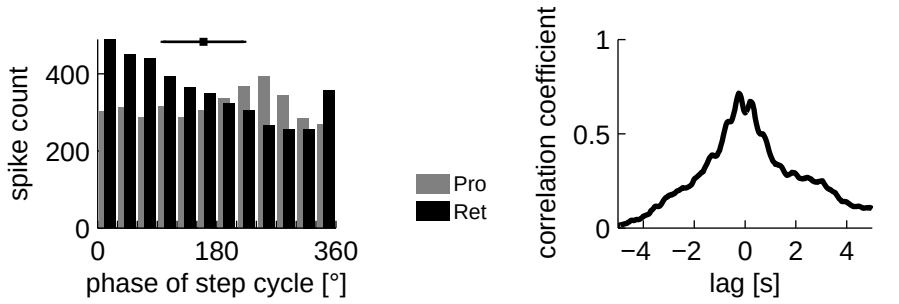
The cross correlation function (Fig. 3.2(e)) is dominated by a broad peak around zero. This is due to the fact that activity in both MN pools increased together during front leg stepping sequences. No clear oscillation can be seen in the cross correlation function although front leg stepping was regular and had a dominant step cycle period (Fig. 3.2(b)). This indicates that protractor and retractor MN activity was not permanently and sys-


pro
meta
tacho
retractor
protractor
1s

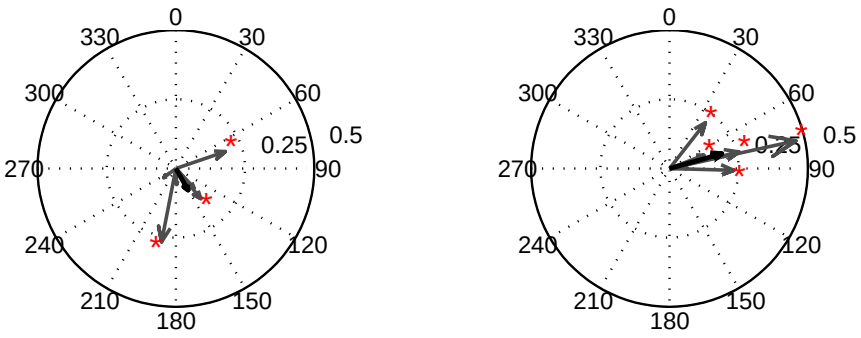
(a)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]
n=49
phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(b) (c)


spike count
400
200
0
0
180
360
phase of step cycle [°]
Pro
Ret
correlation coefficient
1
0.5
0
−4
−2
0
2
4
lag [s]

(d) (e)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5
0
30
60
90
120
150
180
210
240
270
300
330
0.5

(f) (g)

Figure 3.2: *(a) Metathoracic protractor and retractor MN activity during front leg stepping. No clear bursts are visible but a modulation of the motoneural activities. Phase of (b) protractor and (c) retractor MN spikes in front leg step cycle plotted against step cycle duration. No obvious phase or time dependence of MN activity on front leg step cycle is present. The red curve (smoothed) shows the frequency of steps over the step cycle duration in arbitrary units. (d) Distribution of metathoracic protractor and retractor MN activity in front leg step cycle for 49 steps. Retractor MN activity had a maximum between 0° and 30°. Protractor MN activity had a maximum between 240° and 270°. (e) The cross correlation function is dominated by a broad peak around zero, indicating that activity in both MN pools increased together during front leg stepping sequences. No clear oscillation can be seen in the cross correlation function indicating that protractor and retractor MN activity was not permanently and systematically phase coupled with a constant phase shift. Polar plots of mean phases of (f) protractor and (g) retractor MN activity in front leg step cycle for six experiments (grey arrows) and mean vector of all experiments (black arrows). Mean phases of protractor MN activity were variable between the experiments with an overall mean phase of 151° (71° - 240°). Retractor MN activity had an overall mean phase of 74° (38° - 92°) of front leg step cycle.*

tematically phase coupled with a constant phase shift.

The polar plots for the seven experiments show mean phases of activity for protractor and retractor MNs. The spike distribution is not normal (Fig. 3.2(d)) but unimodal, and as a consequence the mean vector does not point exactly in the direction of the maximum of the distribution. Nevertheless, this analysis shows the preferred phase of activity and the similarity between experiments. The polar plot reveals an overall mean phase for protractor MN activity at 151° (Fig. 3.2(f)), but the mean phases of the different experiments covered an angle from 71° to 240°. For retractor MNs the polar plot (Fig. 3.2(g)) reveals an overall mean phase at 74° of front leg step cycle with mean phases between 38° and 92° for the different experiments. Compared to the polar plots of mesothoracic protractor and retractor MN activity (Fig. 3.1(f), 3.1(g))), vector direction is much more variable, and vector length is generally shorter, indicating that each experiment's data points were less concentrated in one direction.

In summary, in metathoracic protractor and retractor MNs there was an increase in activity during front leg stepping. However, no rhythmic activity pattern was present, although

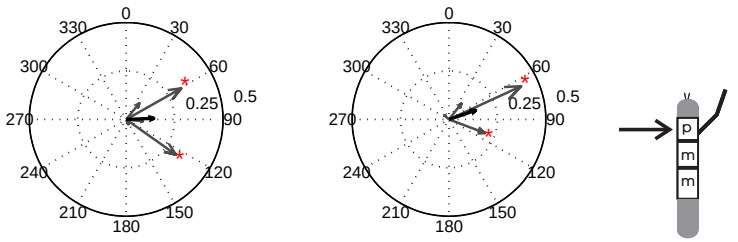
protractor and retractor MN activity increased with the beginning of a stepping sequence and was slightly modulated by the steps. No systematic coupling between protractor and retractor MN activity was observed. Thus, on the ipsilateral side the influence of front leg stepping appeared to decrease from rostral to caudal.

3.1.2 Contralateral influences

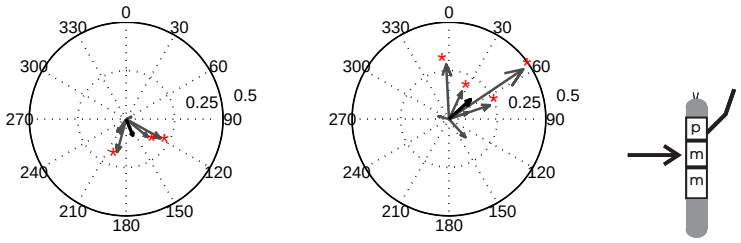
Front leg stepping likewise induced in the coxal MNs of all three contralateral hemiganglia a simultaneous activity increase that was slightly modulated with the steps. No systematic coupling between protractor and retractor MNs was observed for any of the contralateral hemiganglia. Only the polar plots for protractor and retractor MN activity in the front leg step cycle are shown (Fig. 3.3).

Front leg: Protractor and retractor MN activity showed a consistent and nearly identical phase preference in front leg step cycle throughout the four experiments (Fig. 3.3(a), 3.3(b)). The overall mean phase for protractor MN activity was 88° with the individual experiment mean phases covering angles between 39° and 125° (Fig. 3.3(a)). Retractor MN activity had an overall mean phase at 71° with individual mean phases between 38° and 111° for the different experiments (Fig. 3.3(b)). **Middle leg**: Contralateral mesothoracic protractor MN activity had an overall mean phase of 158° covering a range from 120° to 210° (N=7) (Fig. 3.3(c)). The overall mean phase for retractor MN activity was 48° of front leg step cycle, with mean phases for the individual experiments between 357° and 138° (Fig. 3.3(d)). **Hind leg**: Metathoracic protractor MN activity had a mean phase of four experiments of 78° (73° to 187°) (Fig. 3.3(e)). Retractor MN activity had an overall mean phase of 119° (21° to 114°) (Fig. 3.3(f)). Comparing the range of mean phases of protractor and retractor MN activity for the different contralateral ganglia shows that they overlap around 120° for protractor MN activity (Fig. 3.3(g)) and around 60° for retractor MN activity (Fig. 3.3(h)).

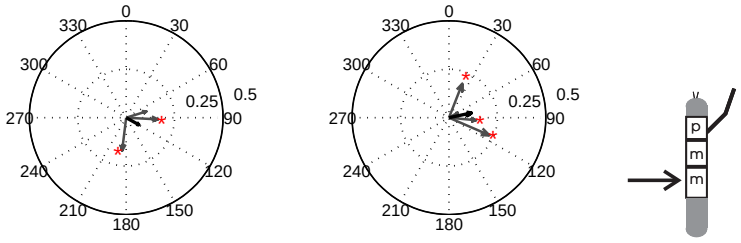
Taken together, these data show that front leg forward stepping had a strongly modulating influence eliciting alternating activity only on ipsilateral mesothoracic protractor and retractor MNs. In all other hemisegments front leg stepping lead to a general activity


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5
p
m
m

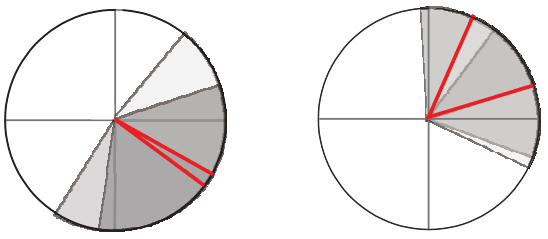
(a) (b)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5
p
m
m

(c) (d)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5
p
m
m

(e) (f)



(g) (h)

Figure 3.3: *Polar Plots summarizing influence of front leg stepping on protractor and retractor MNs of the hemiganglia contralateral the walking front. The radius of each circle is 0.5. (a), (b): Polar plots of mean phases of prothoracic (a) protractor and (b) retractor MN activity in front leg step cycle (N=5). The overall mean phase of protractor MN activity was 88° (39° - 125°). The overall mean phase of retractor MN activity was 71° (38° - 111°). (c), (d): Polar plots of mean phase of mesothoracic (c) protractor and (d) retractor MN activity in front leg step cycle (N=7). The overall mean phase of protractor MN activity was 158° (120° - 210°).The overall mean phase of retractor MN activity was 48° (357° - 138°). (e), (f): Polar plots of mean phases of metathoracic (e) protractor and (f) retractor MN activity in front leg step cycle (N=4). The overall mean phase of protractor MN activity was 78° (73° - 178°).The overall mean phase of retractor MN activity was 119° (21° - 114°). (g), (h): The ranges of mean phases for (g) protractor and (h) retractor MN activity in each of the contralateral hemiganglia (light grey: prothoracic contralateral hemiganglion; grey: mesothoracic; dark grey: metathoracic) and the sector where they overlap (red).*

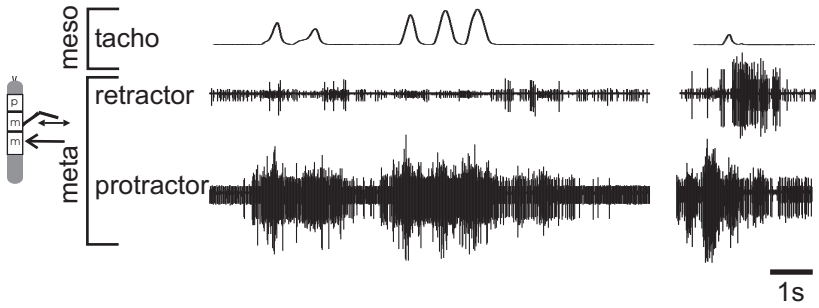
increase that was slightly modulated with the steps.

3.2 Influences of single middle leg stepping

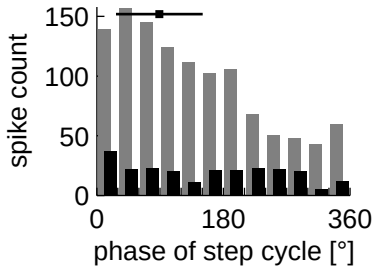
3.2.1 Ipsilateral influences

We next investigated the effect of middle leg stepping on the various hemiganglia. The single stepping middle leg experiments were performed with two different single leg preparations. In the first the middle leg stepped sideways on a treadmill perpendicular to the body axis, the traditional fashion for the single middle leg preparation (Fischer et al. 2001; J.P Gabriel and Büschges 2003) (N=5). In the second the treadmill was positioned parallel to the animal's body axis of resulting in walking more resembling natural forward walking (N=6).

In the first preparation, activity in ipsilateral metathoracic protractor and retractor MNs increased but no clear activity pattern was present (Fig. 3.4). Depending on the experiment, the increase in activity could be stronger in one of the two MN pools than the other. For instance in the 26 steps analyzed in figure 3.4(b), protractor MN activity increased during stance phase with a maximum between 30° and 60° of middle leg step cycle

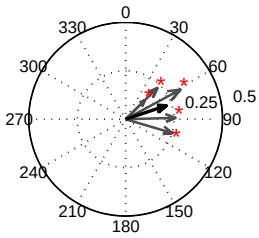

meso
tacho
retractor
meta
protractor
1s

(a)

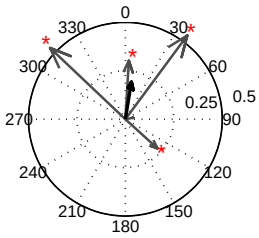

spike count
150
100
50
0
0
180
360
phase of step cycle [°]

Pro
Ret

(b)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5

(c)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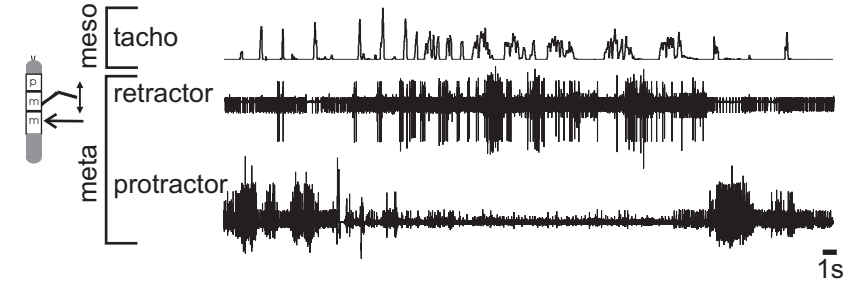
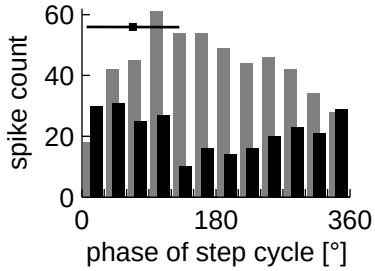
(d)

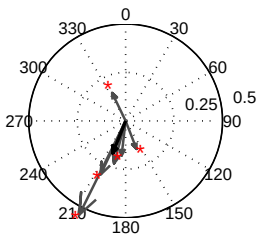

meso
tacho
retractor
meta
protractor
1s

(e)


spike count
60
40
20
0
0
180
360
phase of step cycle [°]

Pro
Ret

(f)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0.25
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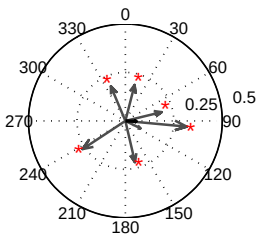
(g)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270
300
330
0.25
0.5

(h)

Figure 3.4: *(a) Metathoracic protractor and retractor MN activity during sideways middle leg stepping. (b) Distribution of protractor (grey) and retractor (black) MN activity in the middle leg step cycle for 26 sideways steps. Protractor MN activity was maximal during middle leg stance phase between 30° and 60°. Retractor MN activity was in this experiment averaged 20% of protractor MN activity and was maximal between 0° and 30°. Polar plots of mean phases of (c) protractor MN activity and (d) retractor MN activity for five experiments (grey arrows) and mean vector of all experiments (black arrow) for the sideways stepping preparation. The overall mean phase of protractor MN activity was 72° (45° - 107°). Retractor mean phase was highly variable with an overall mean phase of 9°. (e) Recording of metathoracic protractor and retractor MN activity during parallel middle leg stepping. It was often observed that either protractor or retractor MN activity was much higher during a walking sequence. (f) Distribution of protractor (grey) and retractor (black) MN activity in middle leg step cycle for 24 parallel steps. Metathoracic protractor MN activity was maximal between 90° and 120°. Retractor MN activity increased at the beginning and the end of the stepping cycle with a maximum between 30° and 60°. Polar plots of mean phases of (g) protractor MN activity and (h) retractor MN activity in the middle leg step cycle for five experiments (grey arrows) and mean vector of all experiments (black arrow) for the parallel stepping preparation. The overall mean phase of protractor MN activity was 202° (154° - 206°). Mean phases of retractor MN activity were again highly variable between experiments.*

(Fig. 3.4(b)), whereas retractor MN activity was approximately 75% lower than protractor MN activity and was widely distributed over the whole step cycle with a maximum between 0° and 30°. The polar plot of mean vectors for all five experiments shows a consistent phase preference for protractor MNs around 72° covering an angle of 45° to 107° (Fig. 3.4(c)). For retractor MN activity the mean phases were highly variable between experiments with an overall mean phase of 9° (Fig. 3.4(d)). Parallel stepping of the middle leg had comparable influences on metathoracic coxal MN activity. Although this walking pattern resembles more closely to normal forward walking no alternating activity was observed in metathoracic protractor and retractor MNs (Fig. 3.4(e)). Activity in both MN pools increased during walking sequences of the middle leg. The histogram for 24 steps shows an increase in protractor MN activity at the beginning of stance phase and a maximum between 120° and 150° of middle leg step cycle. Retractor MN activity was maximal between 30° and 60° (Fig. 3.4(f)). The mean phase of protractor MN activity in the middle leg step cycle was similar for five of the six experiments with mean phases between 154° and 206° and an overall mean at 202° (Fig. 3.4(g)). The mean phase for retractor MN activity was again highly variable between experiments (Fig. 3.4(h)).

In summary experiments for both preparations provided similar results. No alternating activity was observed in ipsilateral metathoracic protractor and retractor MNs in either preparation. The only difference between the two was a 130° shift of the overall mean phase of protractor MN activity.

Experiments with both, the sideways and parallel preparation, were performed to examine the influence of single middle leg stepping on prothoracic protractor and retractor MN activity (parallel stepping: N=2; sideways stepping: N=5). As the results again were similar in both preparations, data from only one typical experiment in the sideways stepping preparation is presented. Prothoracic protractor and retractor MN activity increased during middle leg stepping but no alternating activity was observed (Fig. 3.5(a)). In this experiment protractor MN activity increased at the beginning of stance and was maximal between 90° and 120°. Retractor MN activity increased in the first quarter of the step cycle with a maximum between 60° and 90° (Fig. 3.5(b)). The cross correlation function (Fig. 3.5(c)) is dominated by a broad peak around zero showing that although activity in both MN pools increased together during middle leg stepping, protractor and retractor

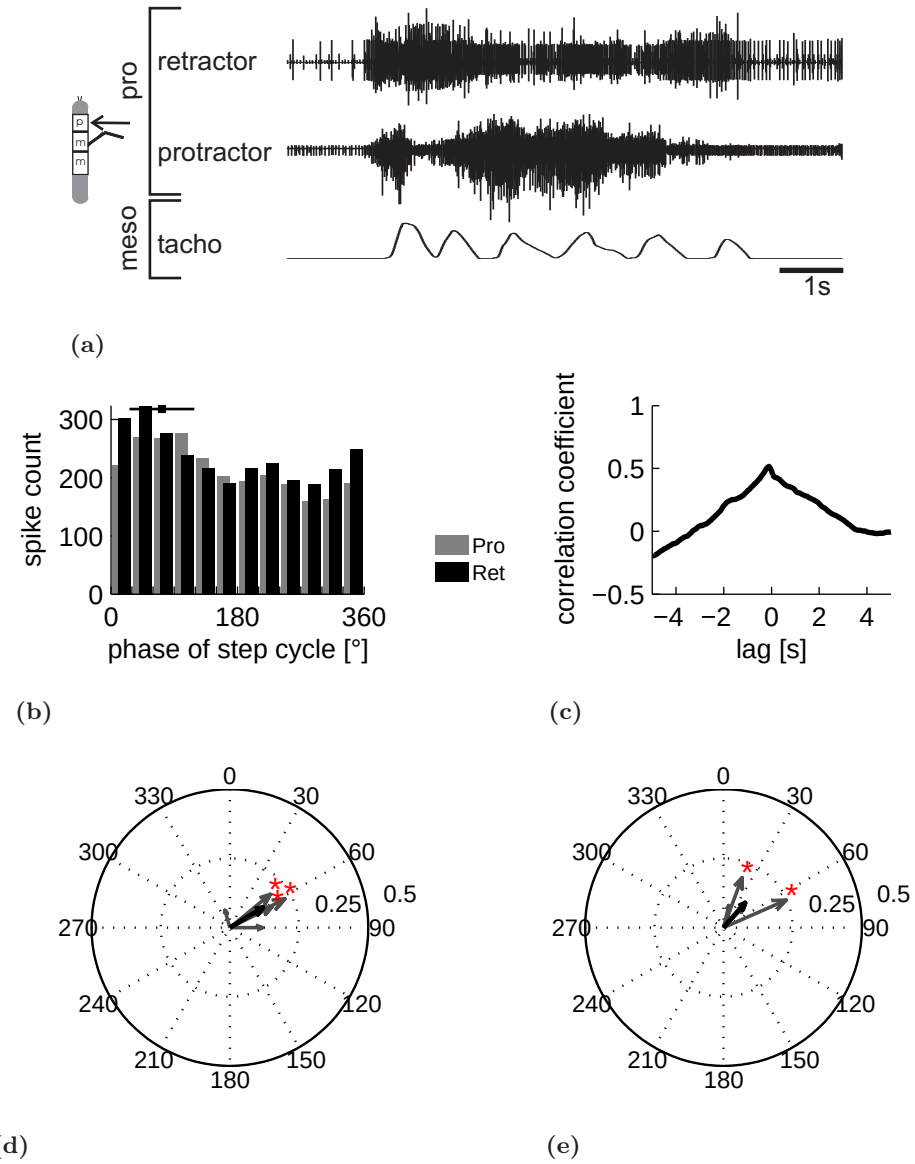


Figure 3.5: *(a) Prothoracic protractor and retractor MN activity during middle leg sideways stepping. (b) Distribution of protractor (grey) and retractor (black) MN activity in the middle leg step cycle for 37 steps. Protractor MN activity was maximum between 90° and 120°. Retractor MN activity was maximum between 30° and 60°. (c) Cross correlation function: The cross correlation function reveals no phase coupling between protractor and retractor MN activity. The broad beak is due to the fact that protractor and retractor MN activity increased together with each stepping sequence. Polar plots of mean phases of (d) protractor and (e) retractor MN activity in middle leg step cycle for five experiments (grey arrows) and mean vector of all experiments (black arrows). The overall mean phase of protractor MN activity was 59° (344° - 90°).The overall mean phase of retractor MN activity was 41° (14° - 66°).*

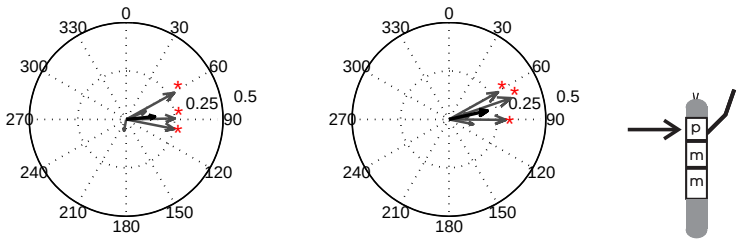
MN activity were not permanent and systematically phase coupled. The polar plots with mean vectors for five experiments show that mean phases for protractor and retractor MN activity were consistent across the experiments and similar for protractor and retractor MNs (Fig. 3.5(d), 3.5(e)). Protractor MN activity had an overall mean phase of 59° ranging from 344° to 90° (Fig. 3.5(d)) and retractor MN activity had an overall mean phase of 41° ranging from 14° to 66° (Fig. 3.5(e)).

3.2.2 Contralateral influences

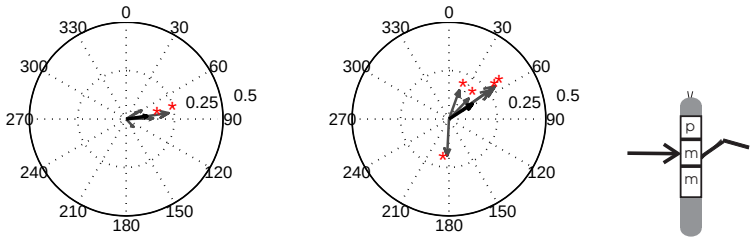
In all contralateral hemiganglia middle leg stepping induced a general increase in protractor and retractor MN activity that was slightly modulated with the steps. Experiments were performed only in the sideways stepping middle leg preparation. The influence of sideways middle leg stepping on protractor and retractor MN activity of the contralateral hemiganglia is summarized in figure 3.6. Polar plots for protractor and retractor MN activity in the middle leg step cycle are shown (Fig. 3.6).

Front leg: For prothoracic protractor and retractor MN activity preferred phases in the middle leg step cycle were consistent across the experiments (Fig. 3.6(a), 3.6(b)). The overall mean phase of six experiments was 85° (63° to 102°) (Fig. 3.6(a)) for protractor MN activity and 77° for retractor MN activity (60° to 98°) (Fig. 3.6(b)). **Middle leg**: Mesothoracic protractor and retractor MN activity had an overall mean phase of 82° (61° to 135°)(Fig. 3.6(c)) and 57° (19° to 182°) (Fig. 3.6(d)) of middle leg step cycle (N=5). **Hind leg**: For metathoracic protractor and retractor MNs the mean phases of activity in middle leg step cycle were similar between the four experiments (Fig. 3.6(e), 3.6(f)). Protractor MN activity had an overall mean phase of 28° (22° to 40°) (Fig. 3.6(e)) and retractor MN activity of 58° (60° to 98°) (Fig. 3.6(f)). Comparing the range of preferred phases of protractor and retractor MN activity one can see that they overlap around 90° of the middle leg step cycle (Fig. 3.6(g), 3.6(h)).

In summary, the middle leg induced a general activity increase in protractor and retractor MNs of all other hemiganglia, ipsilateral and contralateral, that was slightly modulated by the steps. Thus, the influence of middle leg stepping qualitatively differed from that of front leg stepping, in that front leg stepping induced alternating activity in mesothoracic


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5
p
m
m

(a) (b)


0
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60
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120
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180
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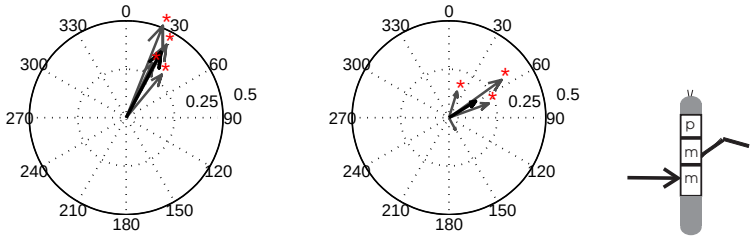
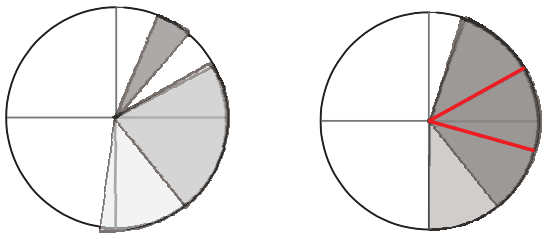
240
270
300
330
0.25
0.5
p
m
m

(c) (d)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5
p
m
m

(e) (f)



(g) (h)

Figure 3.6: *Influence of middle leg sideways stepping on protractor and retractor MNs of the hemiganglia contralateral to the walking middle leg summarized in polar plots. The radius of each circle is 0,5. (a), (b): Polar plots of mean phases of prothoracic (a) protractor and (b) retractor MN activity in middle leg step cycle (N=5). Overall mean phase of protractor MN activity was 85° (63° - 102°). Overall mean phase of retractor MN activity was 77° (60° - 98°). (c), (d): Polar plots of mean phases of mesothoracic (c) protractor and (d) retractor MN activity in middle leg step cycle (N=7). Overall mean phase of protractor MN activity was 82° (61° - 135°). Overall mean phase of retractor MN activity was 57° (19° - 182°). (e), (f): Polar plots of mean phases of metathoracic (e) protractor and (f) retractor MN activity in middle leg step cycle (N=4). Overall mean phase of protractor MN activity was 28° (22° - 40°). Overall mean phase of retractor MN activity was 58° (60° - 98°). (g), (h): Plot shows the range of mean phases for (g) protractor and (h) retractor MN activity in each of the contralateral hemiganglia (light grey: prothoracic contralateral hemiganglion; grey: mesothoracic; dark grey: metathoracic) and the sector where they overlap (red).*

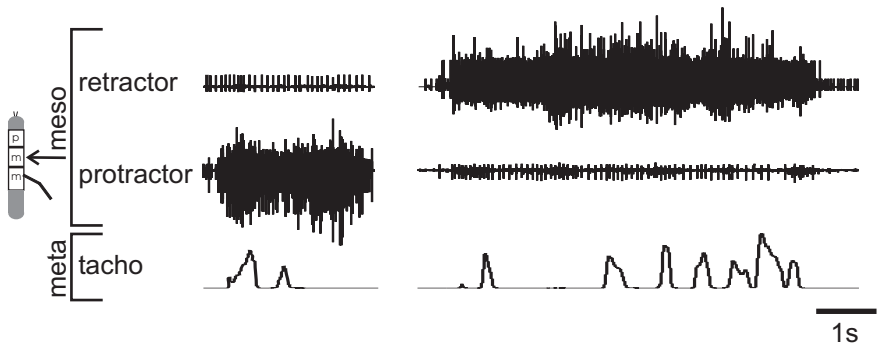


Figure 3.7: *Mesothoracic protractor and retractor MN activity during hind leg forward stepping. Protractor or retractor MN activity increased during stepping sequences.*

protractor and retractor MNs, but middle leg stepping induced only slightly modulated activity increase in all other hemiganglia.

3.3 Influences of single hind leg stepping

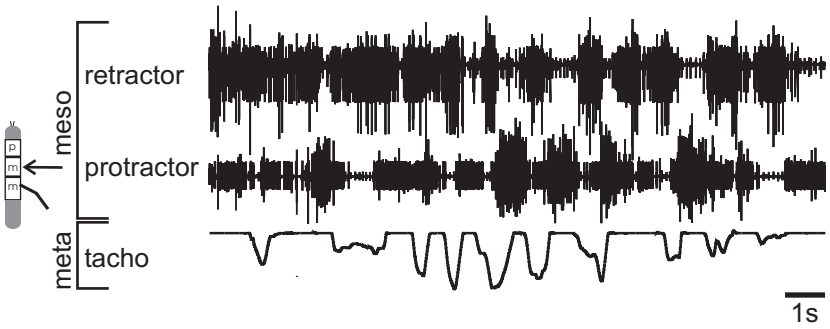
These experiments were very difficult to perform for the single hind leg preparation because the intrinsic walking direction of the hind legs is backward (U. Bässler and Breutel 1985), and consequently forward walking only very rarely (two experiments ($n_1 = 52, n_2 = 20$) out of eleven) occurred in the single hind leg preparation. In these experiments forward

hind leg stepping induced a general activity increase in mesothoracic protractor and retractor MNs (Fig. 3.7). Due to the small number of these experiments and the forward stepping sequences within them, no further evaluation was done for this experiments.

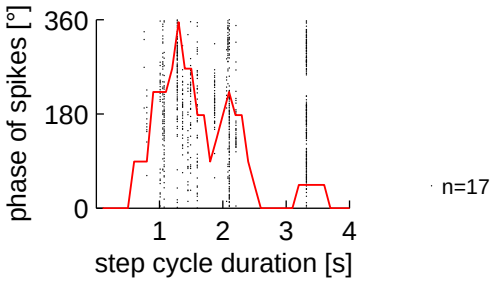
3.4 Influence of the walking direction

Next, I investigated whether walking direction altered the influence of single stepping legs using single backward stepping hind and middle legs. In three of nine experiments backward hind leg stepping elicited alternating activity in protractor and retractor MNs of the ipsilateral mesothoracic hemiganglion (Fig. 3.8(a)). The phase plots show no systematic phase dependency of protractor and retractor MN spikes on hind leg step cycle (Fig. 3.8(b), 3.8(c)). The histogram for 24 hind leg steps shows that retractor MN activity increased at the beginning and end of the step cycle with a maximum between 30° and 60° (Fig. 3.8(d)). Protractor MN activity had a maximum around 180° of hind leg step cycle (Fig. 3.8(d)). The cross correlation function shows a coupling of protractor and retractor MN activity with a phase shift of 180° indicated by a maximal negative correlation at 0 seconds time lag (Fig. 3.8(e)). In six of nine experiments hind leg backward stepping increased mesothoracic protractor and retractor MN activity. No alternating activity was observed. The polar plots (Fig. 3.8(f), 3.8(g)) summarize the mean phases of activity for all nine experiments. The mean phase of protractor MN activity was very variable between the experiments (Fig. 3.8(f)). The mean vectors of retractor MN activity revealed a consistent phase preference across the experiments with an overall mean phase of 90° and a range from 77° to 135° (Fig. 3.8(g)). In summary, in one third of the experiments the backward stepping hind leg stepping had a patterning influence on mesothoracic protractor and retractor MNs. This shows that the backward stepping hind leg stepping can elicit alternating activity in mesothoracic coxal MNs.

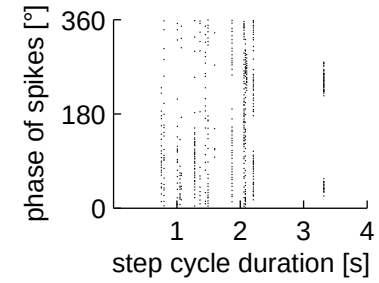
In contrast, middle leg backward stepping induced a general activity increase in ipsilateral metathoracic (Fig. 3.9(a)) (N=4) and prothoracic (Fig. 3.9(b)) (N=4) protractor and retractor MNs in four of four experiments. In summary, the influence of single middle stepping did not depend on the walking direction. Both forward and backward middle


retractor
protractor
meso
meta
tacho
1s

(a)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(b)


phase of spikes [°]
360
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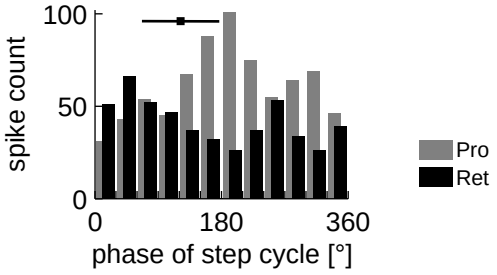
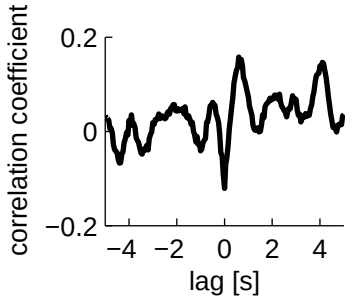
step cycle duration [s]

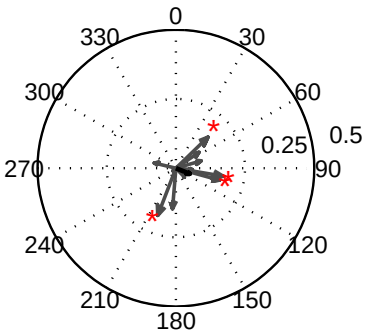
(c)


spike count
100
50
0
0
180
360
phase of step cycle [°]
Pro
Ret

(d)


correlation coefficient
0.2
0
−0.2
−4
−2
0
2
4
lag [s]

(e)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300
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0.25
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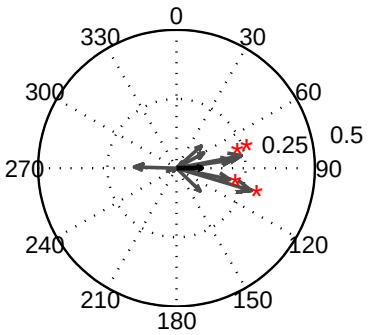
(f)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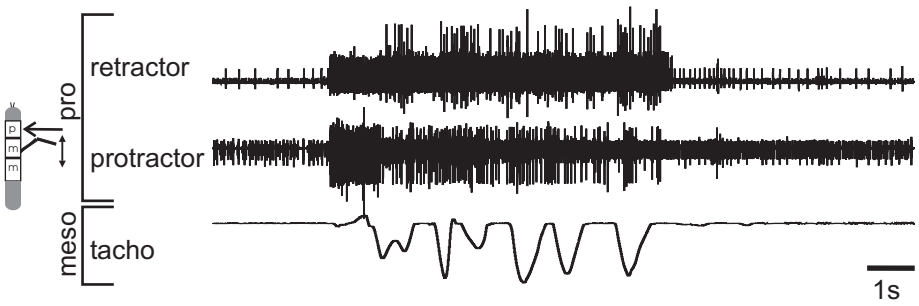
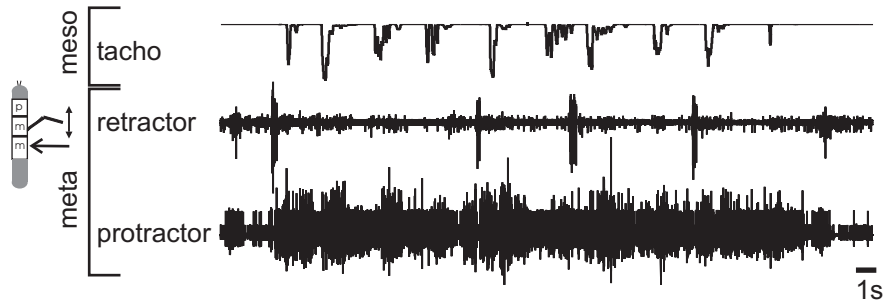
330
0.25
0.5

(g)

Figure 3.8: *(a) Mesothoracic protractor and retractor MN activity during hind leg backward stepping. In 3 of 9 experiments alternating activity occurred during hind leg stepping sequences. Phase of (b) protractor and (c) retractor MN spikes in hind leg step cycle plotted against step cycle duration. No obvious phase dependency of protractor or retractor MN activity on hind leg step cycle was present. (d) Distribution of protractor (grey) and retractor (black) MN activity in the hind leg step cycle for 24 steps. Protractor MN activity was maximum between 180° and 210°. Retractor MN activity had two maxima, one between 30° and 60° and the other between 240° and 270°. (e) The cross correlation function reveals an alternating coupling between protractor and retractor MN activity. Polar plots of mean phases of (f) protractor and (g) retractor MN activity in hind leg step cycle for nine experiments (grey arrows) and mean vector of all experiments (black arrows). Mean phases of protractor MN activity were variable across the experiments. Overall mean phase of retractor MN activity was 90° (77° - 135°).*



(a)



(b)

Figure 3.9: *(a) Prothoracic protractor and retractor MN activity during backward middle leg stepping. Protractor and retractor MN activity increased during stepping sequences. (b) Recording of metathoracic retractor and protractor coxae MN activity during backward middle leg stepping. Protractor and retractor MN activity increased during stepping sequences. Walking direction did not alter the influence of middle leg stepping.*

leg stepping induced a general activity increase in coxal MNs of ipsilateral neighboring hemiganglia. Backward hind leg stepping induced alternating activity in ipsilateral mesothoracic protractor and retractor MNs in one third of the experiments.

3.5 Conclusions

In this chapter I have investigated what influence signals from a single stepping leg has on motoneural activity in adjacent segmental ganglia.

Single front leg forward walking induced alternating activity in ipsilateral mesothoracic protractor and retractor MNs in almost all experiments. Protractor and retractor MN activity was phase coupled to front leg step cycle with retractor MNs being active in the first half of the step cycle and protractor MNs being active in the second. Hind leg backward stepping elicited alternating activity in ipsilateral mesothoracic protractor and retractor MNs in one third of the experiments. Protractor and retractor MN activity showed no clear phase dependence on hind leg step cycle in these cases. In two thirds of the experiments hind leg backward stepping induced a general activity increase in ipsilateral mesothoracic protractor and retractor MNs. In contrast, single middle leg stepping either sideways or parallel, did not induce alternating activity in protractor and retractor MNs of its neighboring segments. The influence of middle leg stepping thus is qualitatively different to that of the forward walking front leg and backward walking hind leg. Therefore, front, middle and hind legs appear to be unsymmetrical with respect to their influence on motoneural activity in other segments.

The influence of single front, middle and hind leg stepping on coxal MNs of the contralateral hemiganglia was similar. Stepping induced a general activity increase in protractor and retractor MNs of the contralateral hemiganglia.

One walking leg is apparently insufficient to generate an alternating motor output for antagonistic muscles in all other segments as one could infer as being typical for the locomotor state.

CHAPTER 4

Involvement of central pattern generators

Rhythmical recurring patterns are the basis for many different motor tasks. Basic underlying structures in the nervous system are so called central pattern generators (CPGs) (Grillner and Zangger 1975). A central pattern generator is characterized by its ability to generate rhythmic motor patterns even in the absence of timing cues from sensory neurons or other intrinsic inputs (Marder et al. 2005). It turned out that CPGs can be found throughout all classes of animals and in a variety of behavioral contexts as locomotion, breathing and feeding. Central pattern generators have successfully been studied in many different model systems in invertebrates as well as in vertebrates.

Locomotion, regardless if swimming, flying or walking, is generated by cyclic recurring activity patterns in respective muscles. These activity patterns underlie pattern generating networks that, in interplay with sensory organs, produce an adequate motor output. In different invertebrates and vertebrates the existence of CPGs and their contribution to

locomotor output has been demonstrated. The most direct demonstration of a CPG is the generation of fictive motor patterns in an in vitro preparation (Marder et al. 2005). It turned out that the pattern generating networks can be activated by pharmacological (locust: Ryckebusch and Laurent 1993; crayfish: Chrachri and Clarac 1987 neonatal rat: Cazalets et al. 1992; lamprey: Cohen and Wallén 1980; Grillner 1985; zebra fish: McDearmid and Drapeau 2006), electrical (goldfish: Fetcho and Svoboda 1993; turtle: Juranek and Currie 2000) or sensory stimulation (crayfish: Cattaert et al. 1992). The stimulation induces so called fictive locomotion, a coordinated motor output, in timing and shaping comparable to the observed motor pattern during locomotion, and reproducible without sensory input. The rhythmic motoneural activity resembles closely to that observed in vivo but may show some significant differences such as a longer cycle period (lamprey: Grillner 1981 crayfish: Chrachri and Clarac 1990 *Manduca sexta*: Johnston and Levine 1996 stick insect: Büschges et al. 1995). This has been explained with the lack of excitatory inputs from sensory organs (Grillner 1981; Pearson and Wolf 1987; Chrachri and Clarac 1990; Cruse 2002).

In the stick insect each leg appears to have three independent CPGs - one for each joint (Büschges et al. 1995; Bässler and Büschges 1998). The CPGs can be activated pharmacologically by the muscarinic Acetyl choline agonist pilocarpine (Chrachri and Clarac 1987; Büschges et al. 1995). Apart from this evidence of the existence of CPGs in the nervous system of the stick insect little is known about anatomy and behavioral context. nonspiking interneurons are most probably part of these networks (Büschges 1994a, b; Büschges and Wolf 1995; Cruse et al. 1995b). But it is still unknown how they contribute to different motor tasks like walking, rocking or climbing. In the stick insect the CPGs appear to be very variable and underlie sensory modulation. The coupling of the different joint CPGs for instance is due to sensory signals (Akay et al. 2004; Ekeberg et al. 2004; Büschges 2005).

In chapter 3 it was shown that front leg stepping elicited alternating activity in ipsilateral mesothoracic coxal MNs whereas stepping of front or middle leg induced a general activity increase in metathoracic coxal MNs and coxal MNs of all contralateral hemiganglia. These observations bring up two questions: 1) Do mesothoracic central pattern generating networks contribute to the observed alternating activity in protractor and retractor MNs?

2) Under which conditions is the metathoracic thorax coxa (TC-) joint CPG activated?

4.1 Activation of the mesothoracic thorax coxa joint CPG

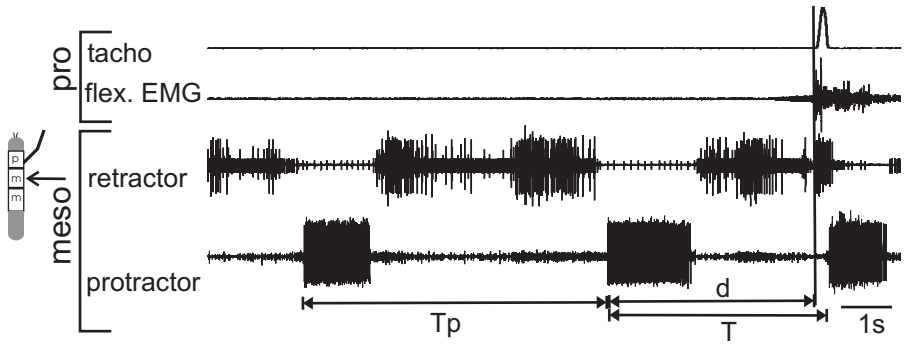
4.1.1 Activation of the mesothoracic ganglion by pilocarpine

In a first set of experiments I tested whether front leg stepping was able to influence the motoneural activity in the active mesothoracic ganglion. Therefore, the pattern generating networks of the ganglion were pharmacologically activated by superfusing the mesothoracic ganglion with saline containing pilocarpine (Chrachri and Clarac 1987; Büschges et al. 1995). Under the assumption that pilocarpine activates the same pattern generating networks as naturally activated during walking, these experiments show whether front leg stepping was able to influence the pilocarpine induced rhythmicity in mesothoracic protractor and retractor MNs.

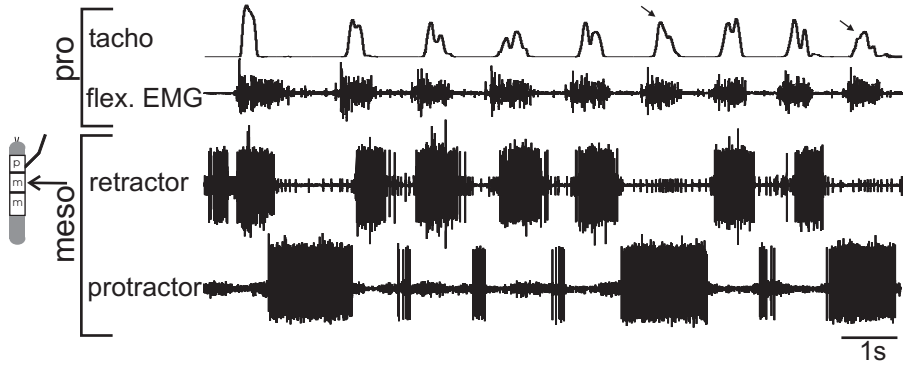
The experiments were performed after a stable pilocarpine induced rhythm had established in mesothoracic coxal MNs (Fig. 4.1(a)) (N=6). With the start of a front leg stepping sequence (Fig. 4.1(b)) the mesothoracic protractor and retractor MN rhythm changed. Still clear bursts occurred but they were shorter and appeared to be coupled to the step cycle in most cases (Fig. 4.1(b)).

The motoneural activity was analyzed with reference to front leg step cycle. Plots of protractor and retractor MN spike phase against step cycle period (Fig. 4.1(c), 4.1(d)) show that, regardless of step cycle period, retractor MNs were active particularly in the first half of the front leg step cycle, as indicated by the dense horizontal band (Fig. 4.1(d)). For protractor MN activity it was less clear in this experiment (Fig. 4.1(c)).

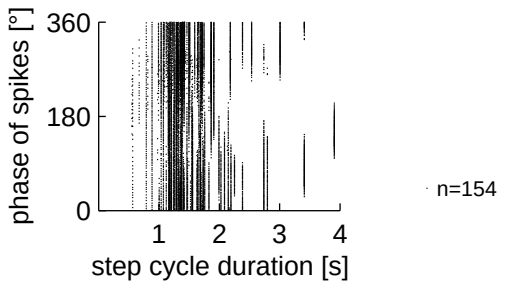
The histogram (Fig. 4.1(e)) shows the distribution of protractor and retractor MN spikes in front leg step cycle for 154 front leg steps. Retractor MN activity increased with the beginning of stance phase, had a maximum between 60° and 90° of the front leg step cycle and then decreased again. Protractor MN activity increased from 180° and had a maximum between 270° and 300°. The mean end of stance was 147°, as indicated by the black square (error bar is mean angular deviation) at the top of the histogram. Retractor MNs


pro
tacho
flex. EMG
meso
retractor
protractor
d
Tp
T
1s

(a)

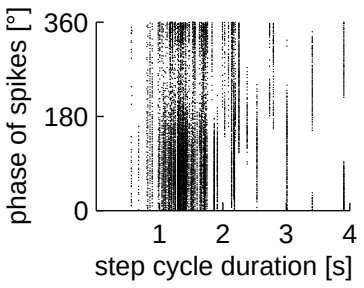

pro
tacho
flex. EMG
meso
retractor
protractor
1s

(b)

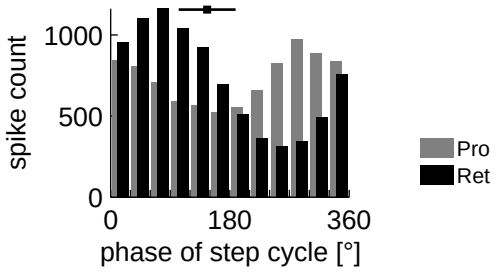

phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(c)

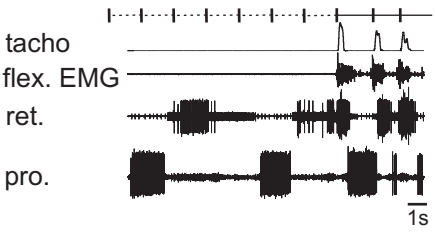
n=154


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(d)


spike count
1000
500
0
0
180
360
phase of step cycle [°]
Pro
Ret

(e)


tacho
flex. EMG
ret.
pro.
1s

(f)

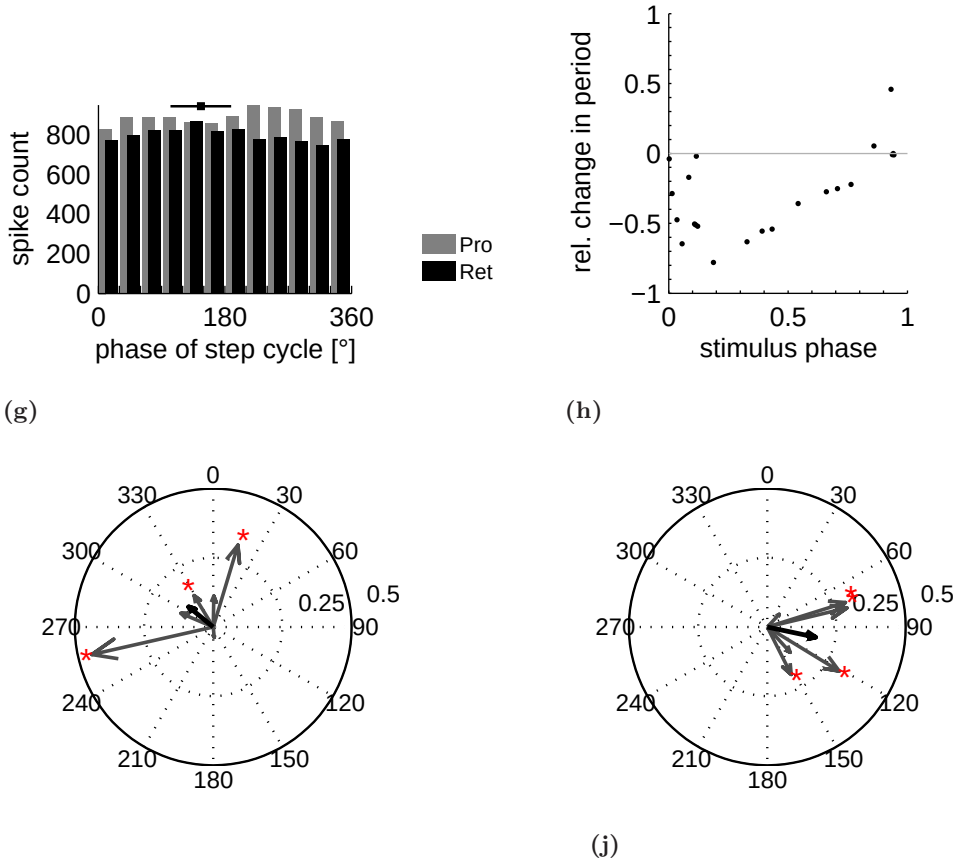


Figure 4.1: *Recording of mesothoracic protractor and retractor MN activity: (a) Protractor and retractor MNs show slow alternating pilocarpine induced rhythm. (b) During a front leg stepping sequence. Front leg stepping entrains the pilocarpine induced rhythm in most cases. Black arrows mark steps that did not influence the pilocarpine induced rhythm. Phase of (c) protractor and (d) retractor MN spikes in front leg step cycle (beginning of stance to beginning of next stance) plotted against step cycle duration. (e) Distribution of protractor and retractor MN activity in front leg step cycle for 154 steps. Black square at top marks average end of stance phase. Error bar is mean angular deviation. Protractor MN activity had a maximum between 270° and 300° and retractor MN activity between 60° and 90°. (f) Phase response curve: phase of first step in the protractor burst cycle is plotted against the relative change in cycle period for all six experiments. (g) Constructed step cycles in the pilocarpine induced rhythm between stepping sequences. The mean step cycle period of the following stepping sequence was calculated and assumed that the animal performed steps with respective step cycle period. (h) Distribution of protractor and retractor MN activity during the pilocarpine rhythm in constructed front leg steps. Both were uniformly distributed over the whole step cycle. Polar plots of (i) protractor MN activity and (j) retractor MN activity in front leg step cycle for six experiments (grey arrows) and mean vector of all experiments (black arrows). Each vector points in direction of the mean phase of spike activity in front leg step cycle. Protractor MN activity had an overall mean phase of about 309° (257° - 16°) and retractor MN activity of about 103° (44° - 153°). Red stars mark significant vectors.*

were thus active primarily during stance and protractor MNs during swing. It had to be excluded that there was an influence of the mesothoracic CPG activity on the stepping frequency of the front leg. To test this, for each stepping sequence the mean step cycle period was determined. Then the protractor and retractor MN activity of the pilocarpine induced motor rhythm between this stepping sequence and the one before was analyzed as if the animal performed steps with respective mean step duration (Fig. 4.1(f)). The distribution of protractor and retractor MN spikes in these artificially constructed step cycle is shown in figure 4.1(g). Protractor and retractor MN spikes were uniformly distributed in the constructed front leg step cycle. Hence, front leg stepping was not influenced in frequency by the mesothoracic pilocarpine activated ganglion.

A phase response curve was used to characterize the influence of front leg stepping on the pilocarpine induced rhythm in the coxal MNs. Therefore, the cycle period of the pilocarpine rhythm was averaged over at least four cycles often more. The beginning of a cycle was determined as the start of the protractor burst (Fig. 4.1(a)). The animal started stepping sequences in different phases of the pilocarpine induced rhythm (Fig. 4.1(a)). The phase of the first step of a stepping sequence was plotted against the relative change in cycle period (Fig. 4.1(h)) for all six experiments. The phase response curve is negative for all phases, indicating that the front leg step always advanced the next protractor burst. In the first 72° of the cycle a step advanced the next protractor burst by zero to 60%. Between 72° and 360° the protractor burst cycle was terminated by a step and a new cycle started as the curve is nearly linear in this range. The first 72° or 20% of protractor burst cycle correspond to approximately two thirds of the protractor burst (Tab. 4.1). Hence, during the protractor burst the front leg step did not break up the cycle immediately but shortened it. It cannot be excluded that a second step followed during respective cycle and contributed to the observed effect. Nevertheless the influence of front legs steps on the pilocarpine induced rhythm in mesothoracic coxal MNs appeared to be stronger at the end of the protractor burst and during the retractor burst. This corresponds to the observation that in a stepping sequence during protractor bursts a front leg step had less effect (Fig. 4.1(b)).

Figure 4.1(i) and 4.1(j) show polar plots of the mean vectors of protractor and retractor MN activity in front leg step cycle for all six experiments. For protractor MN activity

experiment	duty cycle of protractor burst
1	0,32
2	0,29
3	0,49
4	0,36
5	0,43
6	0,35

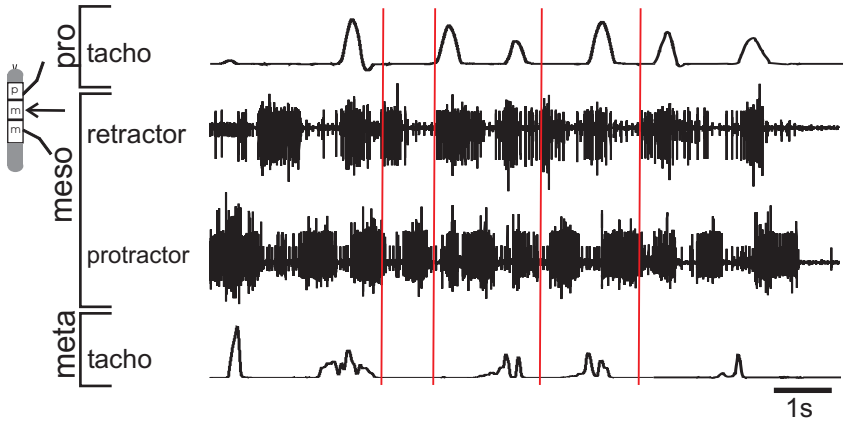
Table 4.1: *Average duty cycle of the protractor burst in the pilocarpine induced rhythm in mesothoracic protractor and retractor MNs for six experiments.*

mean phases were variable between the experiments with an overall mean phase of 309° (257° to 16°). The mean phases of retractor MN activity were consistent across the experiments. The overall mean phase was 103° (44° to 153°) of front leg step cycle.
In summary, these results show that front leg stepping was able to influence the pilocarpine induced rhythm in mesothoracic protractor and retractor MNs. Protractor and retractor MNs were active in distinct phases of front leg step cycle. The coupling of protractor and retractor MN activity to front leg steps corresponded to that observed in the non-pilocarpine activated mesothoracic ganglion (see also chapter 3).

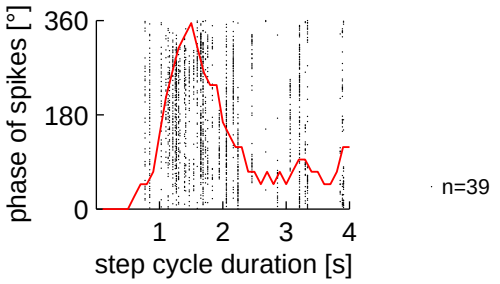
4.1.2 Influence of front and ipsilateral hind legs stepping

In a second set of experiments mesothoracic protractor and retractor MNs were recorded extracellularly during ipsilateral front and hind leg stepping. Both legs stepped on separate passive treadmills and therefore were mechanically uncoupled.

Both front and hind legs stepping elicited alternating activity in ipsilateral mesothoracic protractor and retractor MNs (Fig. 4.2(a)) (N=8). However, the coupling of mesothoracic protractor and retractor MN activity to front leg stepping was not as reliable as it was for the single leg preparation. As indicated by the vertical lines, onset of retractor MN activity in the step cycle was variable and switching from protractor MN activity to retractor MN activity occurred also between two front leg steps. Plots of protractor and

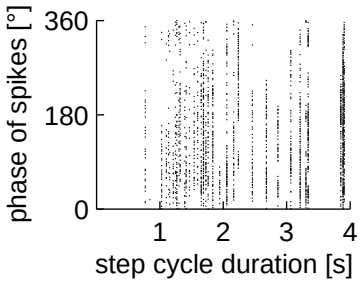

pro
tacho
meso
retractor
protractor
meta
tacho
1s

(a)

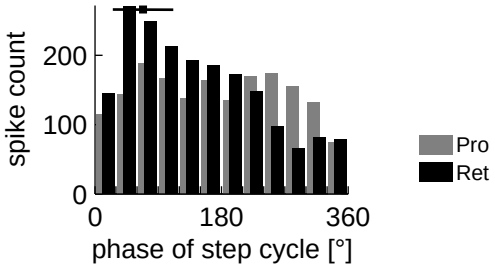

phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(b)

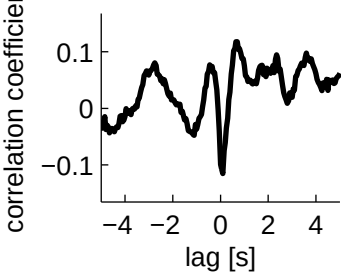
n=39


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

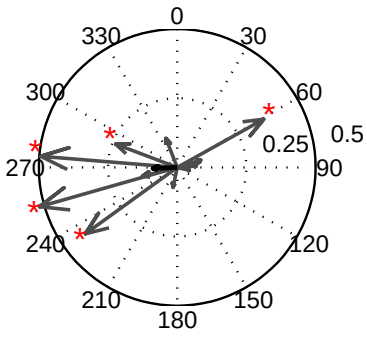
(c)


spike count
200
100
0
0
180
360
phase of step cycle [°]
Pro
Ret

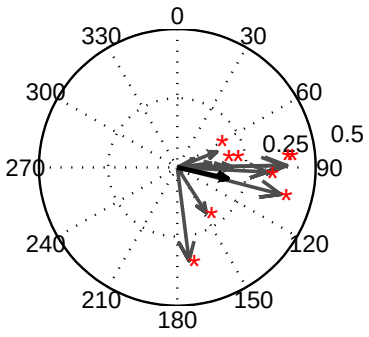
(d)


correlation coefficient
0.1
0
−0.1
−4
−2
0
2
4
lag [s]

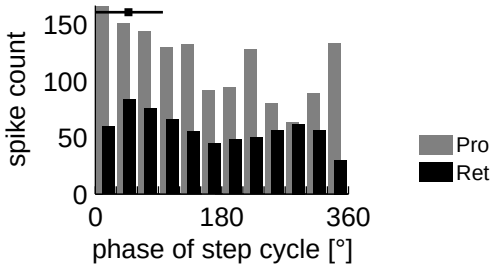
(e)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5

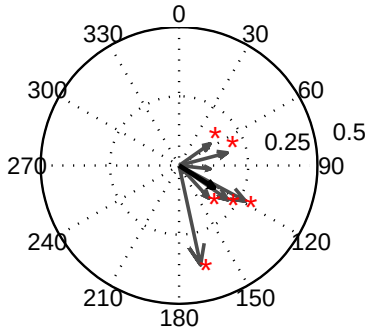
(f)


0
30
60
90
120
150
180
210
240
270
300
330
0.25
0.5

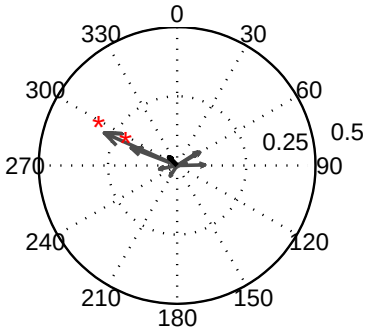
(g)



(h)



(i)



(j)

Figure 4.2: *(a) Mesothoracic protractor and retractor MN activity alternated during front and ipsilateral hind leg stepping. Coupling of protractor and retractor MN activity to front leg steps was comparably weak. Phase of (b) protractor and (c) retractor MN spikes in front leg step cycle plotted against step cycle duration. No obvious phase or time dependence of MN activity on front leg step cycle was present. The red curve (smoothed) shows the frequency of steps over the step cycle duration in arbitrary units. (d) Distribution of mesothoracic protractor and retractor MN activity in front leg step cycle for 48 steps. Retractor MN activity had a maximum between 30° and 60°. Protractor MN activity had no clear maximum. (e) Cross correlation function showing alternating coupling between protractor and retractor MN activity. Polar plots of mean phases of (f) protractor and (g) retractor MN activity in front leg step cycle for eight experiments (grey arrows) and mean vectors of all experiments (black arrows). Protractor MN activity had an overall mean phase of 226° (162°to 287°). Retractor MN activity had an overall mean phase of 76° (355° - 125°). (h) Distribution of mesothoracic protractor and retractor MN activity in hind leg step cycle for 24 steps. Retractor MN activity had two maxima at 30° and at 270°. Protractor MN activity had a maximum between 0° and 30°. Polar plots of mean phases of (i) protractor and (j) retractor MN activity in hind leg step cycle: mean phases of protractor and retractor MN activity were highly variable between the experiments.*

retractor MN spike phase against front leg step cycle period show that there was no phase dependence to the front leg step cycle (Fig. 4.2(b), 4.2(c), compare to Fig. 3.1). Neither for protractor MN spikes (Fig. 4.2(b)) nor for retractor MN spikes (Fig. 4.2(c)) distinct phases of occurrence are recognizable, as indicated by the uniformly distributed dots in the plot. The red curve in figure 4.2(b) shows the frequency of steps over the step cycle duration. In this experiment the front leg step cycle duration was around 1,5 s for most of the steps. The distribution of protractor and retractor MN activity in front leg step cycle for 48 steps (Fig. 4.2(d)) shows that retractor MN activity increased during first half of front leg step cycle with a maximum between 30° and 60°. Protractor MN activity increased during the whole step cycle with no clear maximum. The cross correlation function between protractor and retractor MN activity reveals a phase coupling with 180° phase shift (Fig. 4.5(g)). Protractor and retractor MN activity was clearly alternating. The period of the oscillation is around 1 s, indicating that protractor and retractor MN activity alternated on average with 1Hz. This is faster than front leg stepping. As shown in figure 4.2(b) front leg steps had a step cycle duration around 1,5 s and therefore a stepping frequency of 0,67 Hz.

The polar plots summarize the mean phases of protractor and retractor MN activity in front leg step cycle for eight experiments (Fig. 4.2(f), 4.2(g)). The overall mean phase of protractor activity was 226° (162° to 287°). Retractor MN activity had an overall mean phase of 76° covering a range from 355° to 125°. The whole analysis has also been done with reference to the hind leg step cycle. The distribution for 24 hind leg steps (Fig. 4.2(h)) shows that protractor MN activity increased during first half of the step cycle with a maximum between 0° and 30°. Retractor MN activity had two maxima, one at 30° and the other at 270° of hind leg step cycle. The polar plots of mean phases of protractor and retractor MN activity in hind leg step cycle show that there existed no overall mean phase for protractor (Fig. 4.2(i)) as well as for retractor (Fig. 4.2(j)) MN activity. In both cases the mean phases of the different experiments point all along the circle.

In summary, stepping of both front and ipsilateral hind legs lead to alternating activity in mesothoracic protractor and retractor MNs. Protractor and retractor MN activity was not coupled to front leg stepping rigorously. No coupling to hind leg stepping was observed either.

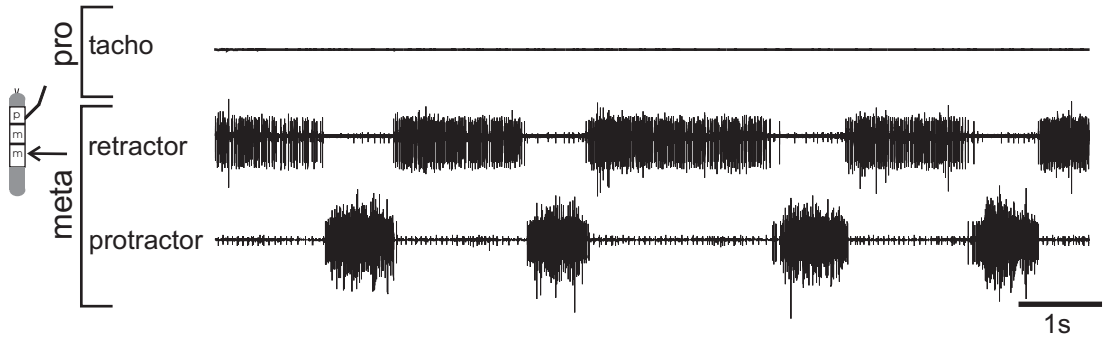
4.2 Activation of the metathoracic thorax coxa joint CPG

While the mesothoracic TC-joint CPG appeared to be activated by stepping of a single front leg the metathoracic TC-joint CPG was not (see chapter 3). Neither stepping of a single front leg nor stepping of a single middle leg elicited alternating activity in metathoracic protractor and retractor MNs. In both metathoracic coxal MN pools a general increase in activity was observed during front or middle leg stepping (Fig. 3.2, 3.4). If we take alternating activity in antagonistic MN pools as an indication for active pattern generating networks stepping of a single leg appears to be insufficient to activate those in the metathoracic ganglion.

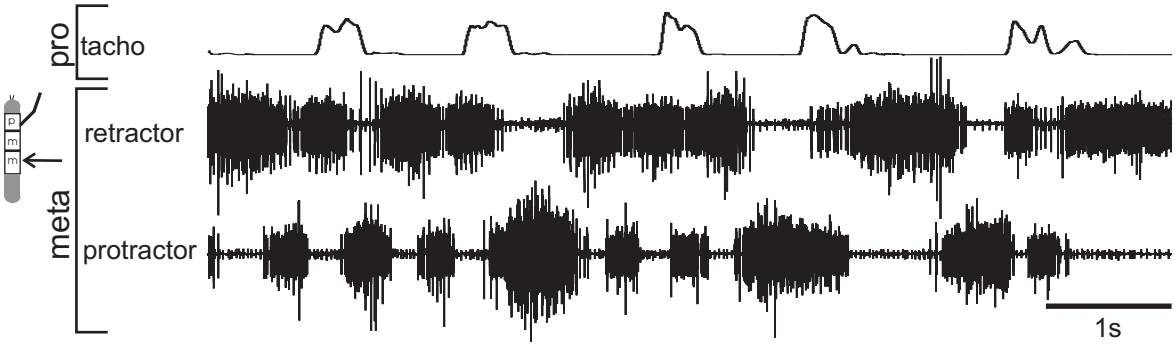
4.2.1 Activation of the metathoracic ganglion by pilocarpine

In this set of experiments the metathoracic ganglion was pharmacologically activated with pilocarpine (N=5). Metathoracic protractor and retractor MNs were recorded extracellularly. The intact front leg performed stepping movements on a passive treadmill. In four of five experiments an influence of front leg stepping on metathoracic coxal MNs was observed. Two experiments are presented here in more detail.

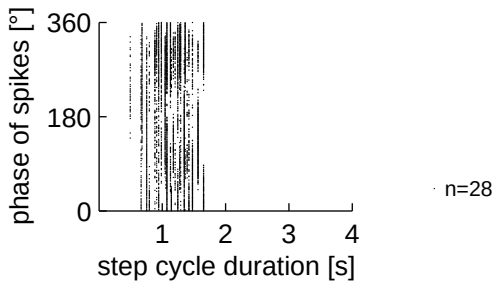
The extracellular recording of metathoracic protractor and retractor MNs (Fig. 4.3(a)) shows the slow pilocarpine induced rhythm. Front leg stepping obviously influenced this rhythm (Fig. 4.3(b)). The bursts in both coxal MN pools became shorter, the alternation became faster although it appears as if the pilocarpine induced rhythm was still superimposed. The alternation in protractor and retractor MNs did not show a coupling to front leg stepping. In the phase plots protractor (Fig. 4.3(c)) and retractor (Fig., 4.3(d)) MN spike phase is plotted against front leg step cycle duration. Protractor and retractor MN spikes were distributed over the whole step cycle, regardless of step cycle duration. The histogram (Fig. 4.4(e)) shows the distribution of protractor and retractor MN activity in front leg step cycle for 34 steps. The distribution was bimodal in this experiment. Protractor MN activity had two maxima, one between 90° and 120° and the other between


pro
tacho
retractor
meta
protractor
1s

(a)

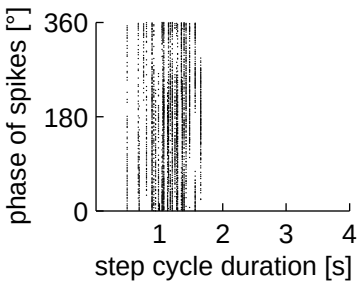

pro
tacho
retractor
meta
protractor
1s

(b)

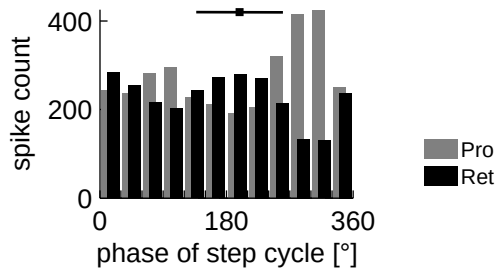

phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(c)

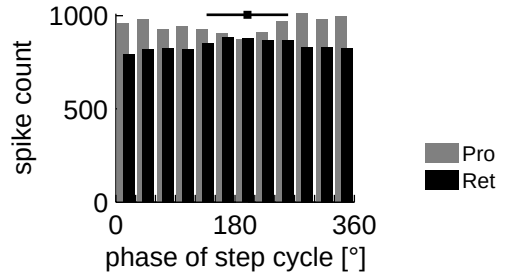
n=28


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(d)


spike count
400
200
0
0
180
360
phase of step cycle [°]
Pro
Ret

(e)


spike count
1000
500
0
0
180
360
phase of step cycle [°]
Pro
Ret

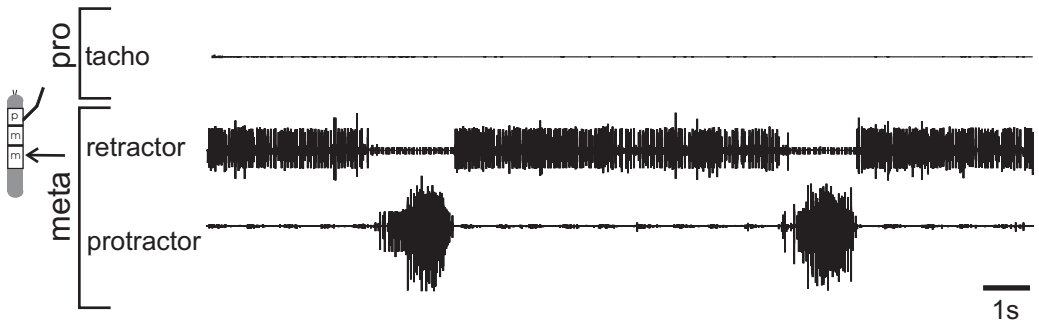
(f)

Figure 4.3: *(a) Metathoracic protractor and retractor MNs show slow alternating pilocarpine induced rhythm. (b) Metathoracic protractor and retractor MN activity during a front leg stepping sequence. Stepping changed the frequency of protractor and retractor MN alternation. Phase of (c) protractor and (d) retractor MN spikes in front leg step cycle plotted against step cycle duration. No obvious phase or time dependence of MN activity on front leg step cycle was observed. (e) Distribution of protractor and retractor MN activity in front leg step cycle for 34 steps. Black square on top marks average end of stance phase, error bar is mean angular deviation. Protractor MN activity had maxima at 90° and at 300°. Retractor MN activity had maxima at 0° and 180°. (f) Distribution of protractor and retractor MN activity during the pilocarpine rhythm in constructed front leg step cycles. Protractor and retractor MN activity were uniformly distributed over the whole step cycle indicating that there existed no influence of the pilocarpine induced rhythm on front leg stepping.*

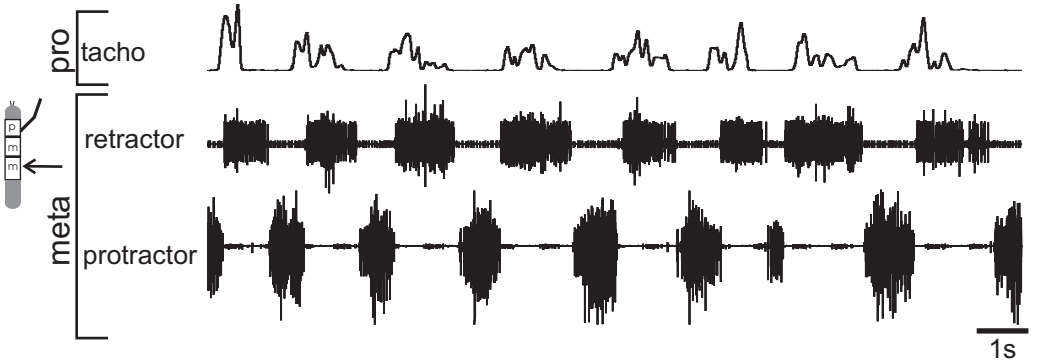
300° and 330°. The second maximum was more pronounced than the first one. Retractor MN activity was maximal between 0° and 30° and between 180° and 210°. To exclude an influence of the pilocarpine induced rhythm on front leg stepping the distribution of protractor and retractor MN activity in between stepping sequences for constructed step cycles is shown in figure 4.4(e) (see also 4.1(f)). Protractor and retractor MN activity are evenly distributed in this fictive front leg step cycle. The pilocarpine induced rhythm does not influence front leg stepping.

In summary, this experiment shows that there existed an influence of front leg stepping on the frequency of the pilocarpine induced rhythm in metathoracic protractor and retractor MNs. There appeared to be no clear coupling of the motoneural activity to front leg stepping.

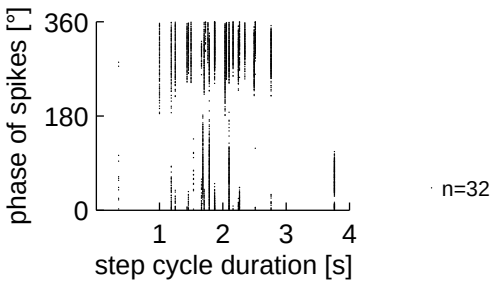
In three of five experiments the pilocarpine induced rhythm in metathoracic protractor and retractor MNs was entrained to front leg stepping. Superfusion with saline containing pilocarpine induced the characteristic slow rhythm in metathoracic protractor and retractor MNs (Fig. 4.4(a)). Stepping of the front leg completely entrained protractor and retractor MN activity (Fig. 4.4(b)). Phase plots of protractor and retractor MN spike phase against front leg step cycle duration show that protractor MN spikes (Fig. 4.4(c)) occur in the second half of front leg step cycle between 180° and 360°, retractor MN spikes in the first half (Fig. 4.4(d)), independent of step cycle duration. This is indicated by the


pro
tacho
retractor
meta
protractor
1s

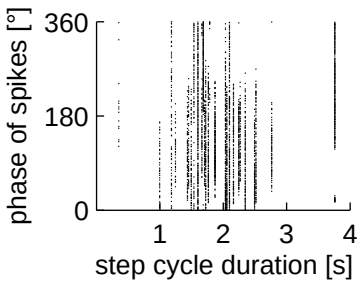
(a)


pro
tacho
retractor
meta
protractor
1s

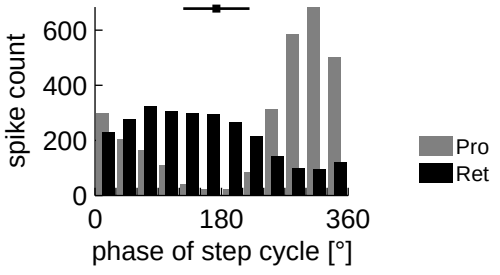
(b)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

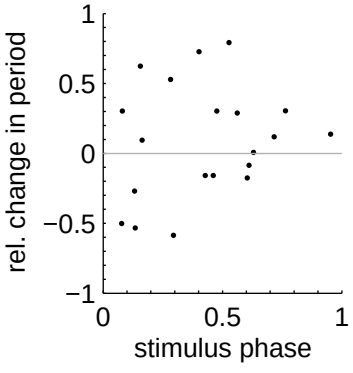
(c)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

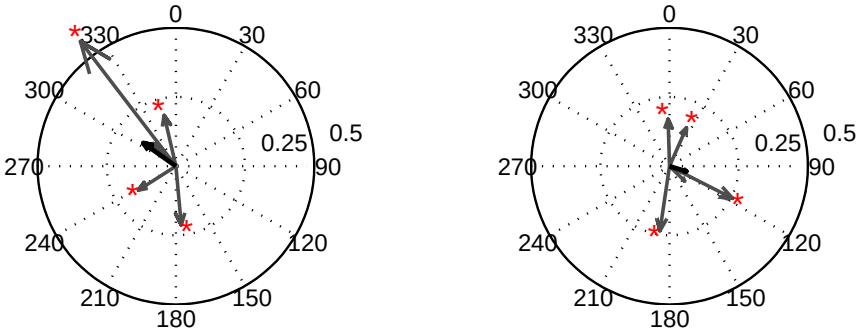
(d)


spike count
600
400
200
0
0
180
360
phase of step cycle [°]
Pro
Ret

(e)


rel. change in period
1
0.5
0
−0.5
−1
0
0.5
1
stimulus phase

(f)



(g) **(h)**

Figure 4.4: *(a) Protractor and retractor MNs show slow alternating pilocarpine induced rhythm. (b) Metathoracic protractor and retractor MN activity during a front leg stepping sequence. Front leg stepping entrains protractor and retractor MN activity. Phase of (c) protractor and (d) retractor MN spikes in front leg step cycle plotted against step cycle duration. Protractor and retractor MN spikes occurred in distinct phases of the front leg step cycle independent of step cycle duration. Protractor MN spikes occurred between 180° and 360° of front leg step cycle and retractor MN spikes between 0° and 180°. (e) Distribution of protractor and retractor MN activity in front leg step cycle for 32 steps. Black square on top marks average end of stance phase, error bars are mean angular deviation. Protractor MN activity had a maximum between at 300° and 330°. Retractor MN activity had a maximum between 60° and 90°. (f) Phase response curve: phase of first step in the protractor burst cycle is plotted against the relative change in cycle period for all five experiments. Polar plots of mean phases of (g) protractor and (h) retractor MN activity in front leg step cycle for five experiments (grey arrows) and mean vectors of all experiments (black arrows). Protractor MN activity had an overall mean phase of 289° (175° to 347°). Retractor MN activity had an overall mean phase of 103° (358° to 202°).*

dense horizontal bands in the phase plots. The distribution of protractor and retractor MN activity in front leg step cycle for 32 steps (Fig. 4.4(e)) shows that protractor MN activity had a maximum between 300° and 330°. Retractor MN activity had a maximum between 60° and 90°. Retractor MNs were mainly active during stance and protractor MNa during swing. The phase response curve (Fig. 4.4(f)) was determined for the first step of a stepping sequence for all five experiments. One cycle was defined from the beginning of a protractor burst to the beginning of the next one. The phase of the step in the pilocarpine induced rhythm was plotted against the relative change of cycle period.

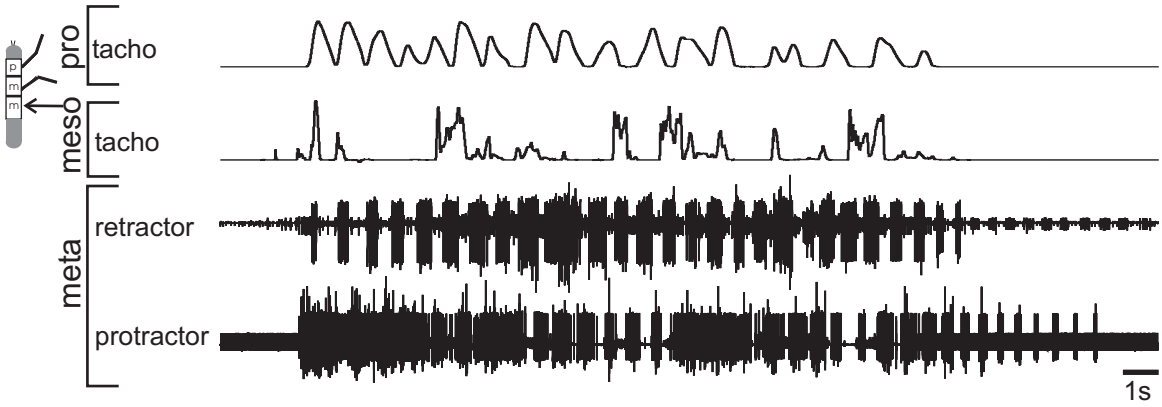
The phase response curve is not as clear as it was for the influence of front leg stepping on the mesothoracic protractor and retractor MNs (compare to Fig. 4.1(h)). The data points are spread wider. No systematic influence of front leg steps can be derived. In all phases of the metathoracic protractor burst cycle a front leg step could advance or delay the next burst. There appeared to be a tendency that a front leg step in the second half of the protractor burst cycle delayed the next protractor burst. Polar plots summarize the mean phases of activity in front leg step cycle for protractor and retractor MNs for all five experiments (Fig. 4.4(g), 4.4(g)). The overall mean phase of protractor MN activity was 289° (175° to 347°) (Fig. 4.4(g)). For retractor MN activity the overall mean phase was 103° (358° to 202°).

In summary, these data show that front leg stepping influenced the pilocarpine activated metathoracic protractor and retractor MNs. In three experiments motoneural activity was coupled to front leg steps. Retractor MNs were active in the first half of the step cycle and protractor MN in the second half. This coupling corresponds to that observed in the single front leg preparation for mesothoracic protractor and retractor MNs (see chapter 3). In one experiment front leg stepping influenced the frequency of alternation in protractor and retractor MNs. Hence, the processing of intersegmental information appears to depend on the status of the receiving ganglion.

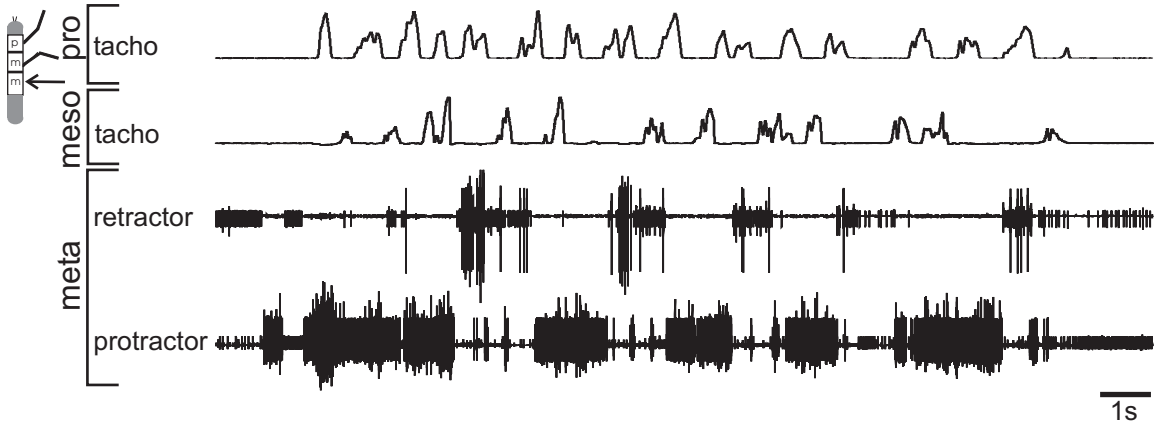
4.2.2 Influence of front and ipsilateral middle legs stepping

Stepping of both front and ipsilateral middle leg elicited alternating activity in ipsilateral metathoracic protractor and retractor MNs in eleven of twelve experiments. Following the characteristics of this activity pattern we could distinguish between three different classes of influences. The largest group showed alternating activity independent of front and middle leg stepping cycle (N=6). The second class showed coupling to front leg stepping (N=2) independent of middle leg stepping. In three experiments both characteristics occurred. One representative example will be shown for each class.

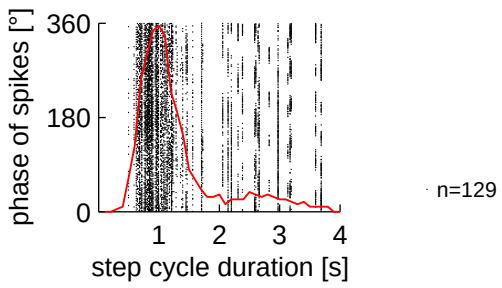
In six of the twelve experiments stepping of front and ipsilateral middle leg elicited alternating activity in metathoracic protractor and retractor MNs that was not coupled to front or middle leg steps. These oscillations were usually faster than the actual stepping frequency (Fig. 4.5(a)). In two experiments it occurred that they were much slower than the actual stepping frequency (Fig. 4.5(b)). A detailed analysis is presented here for one experiment that showed fast oscillations in protractor and retractor MN activity during front and ipsilateral middle leg stepping (Fig. 4.5(a)). Plots of protractor and retractor MN spike phase against front leg step cycle duration reveal no phase dependence on front leg step cycle (Fig. 4.5(c), 4.5(d)). Protractor (Fig. 4.5(c)) and retractor (Fig. 4.5(d)) MN spikes were uniformly distributed over the stepping cycle regardless of step cycle duration. The red line in figure 4.6(b) shows the frequency of steps over the step cycle durations. Most of the steps had a step cycle duration around 1 s. Figure 4.5(e) shows the distribution of protractor and retractor MN activity in front leg step cycle for 129 steps. Although the extracellular recording reveals no dependence of motoneural activity on front leg steps (Fig. 4.5(a)) the distribution shows small maxima and therefore preferred phases in front leg step cycle for protractor and retractor MN activity. Protractor MN activity had two maxima, one at 180° and the other one at 330°. Retractor MN activity was slightly increased at the beginning of front leg step cycle with a maximum between 0° and 30°. This analysis has also been done with reference to the middle leg step cycle. The distribution of protractor and retractor MN activity for 44 middle leg steps (Fig. 4.5(f)) shows a slight modulation. Protractor MN activity had a maximum between 120° and 180°. Retractor MN activity had two maxima, one at 90° and the other at 300° of middle leg step cycle.


pro
tacho
meso
tacho
meta
retractor
protractor
1s

(a)

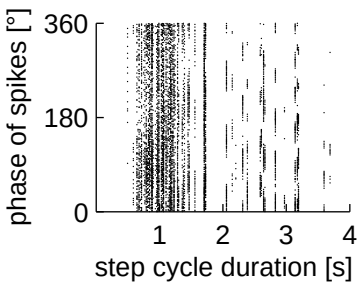

pro
tacho
meso
tacho
meta
retractor
protractor
1s

(b)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(c)

n=129


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

(d)

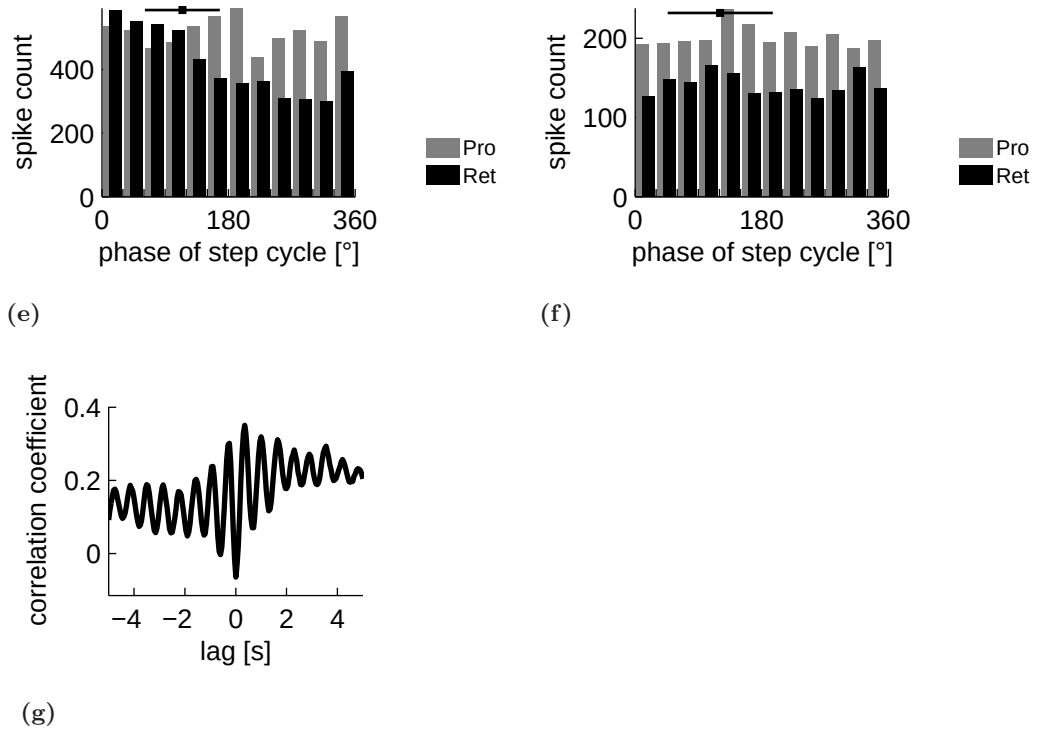
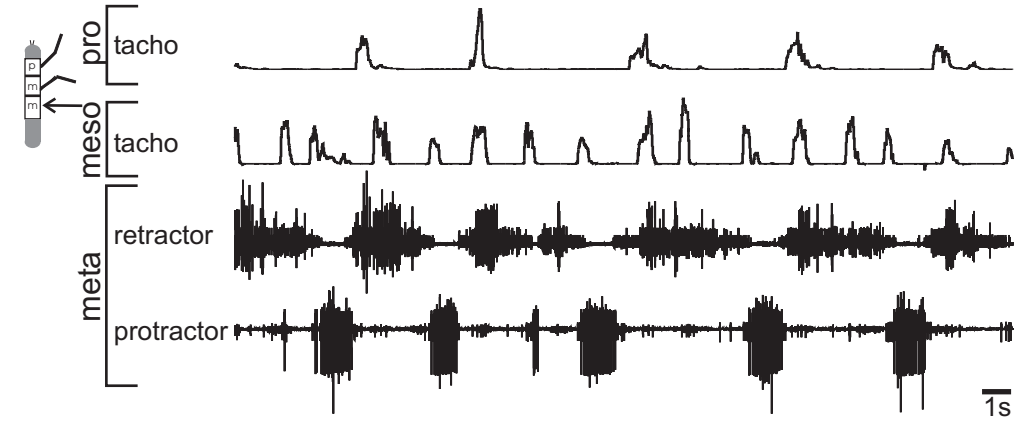


Figure 4.5: *(a) Metathoracic protractor and retractor MN activity during front and ipsilateral middle leg stepping. Protractor and retractor MN activity alternated faster than the actual stepping frequency. (b) Metathoracic protractor and retractor MN activity during front and ipsilateral middle leg stepping. Slow alternation in protractor and retractor MN activity occurred independent of front and middle leg stepping. Phase of (c) protractor and (d) retractor MN spikes in front leg step cycle plotted against step cycle duration. No obvious phase or time dependence of MN activity on front leg step cycle was present. The red curve (smoothed) shows the frequency of steps over the step cycle duration in arbitrary units. (e) Distribution of metathoracic protractor and retractor MN activity in front leg step cycle for 129 steps. Retractor MN activity had a maximum between 0° and 30°. Protractor MN activity had two small maxima at 180° and 330°. (f) Distribution of metathoracic protractor and retractor MN activity in the middle leg step cycle for 44 steps. Retractor MN activity had two maxima at 90° and at 300°. Protractor MN activity had a maximum between 120° and 180°. (g) The cross correlation function mirrors the fast alternation in protractor and retractor MN activity.*

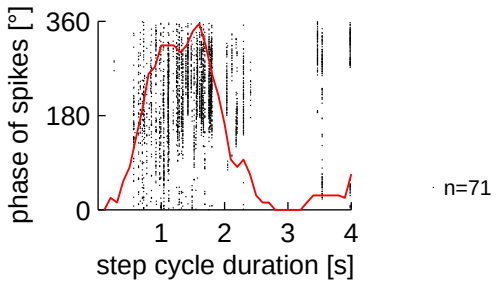
The cross correlation function between protractor and retractor MN activity (Fig. 4.5(g)) mirrors the fast alternation and reveals a phase shift of 180°.

In two of the twelve experiments coupling of metathoracic protractor and retractor MN activity to front leg step cycle was observed independent of middle leg stepping (Fig. 4.6(a)). Plots of protractor and retractor MN spike phase against step cycle duration of front leg steps (Fig. 4.6(b), 4.6(c)) reveal a clear phase dependence. Regardless of step cycle duration protractor MN spikes primarily occurred between 180° and 360° of front leg step cycle (Fig. 4.6(b)) and retractor MN spikes between 0° and 180° (Fig. 4.6(c)). The red curve in the left plot (Fig. 4.6(b)) shows the frequency of steps over the step cycle durations. Most of the steps had a step cycle duration around 1,6 s. The histogram (Fig. 4.6(d)) shows the distribution of protractor and retractor MN activity in front leg step cycle for 71 steps. Retractor MN activity had a maximum between 60° and 90° of front leg step cycle and sharply decreased at phase angles greater than 180°. Protractor MN activity increased at 180° of front leg step cycle and had a maximum between 180° and 210°. The mean end of stance was at 102° of the front leg step cycle. Retractor MNs were thus active primarily during stance and protractor MNs during swing. This corresponds to the coupling that was observed between front leg stepping and mesothoracic protractor and retractor MN activity in the single leg preparation (Fig. 3.1). Figure 4.6(e) shows the distribution of protractor and retractor MN activity in the middle leg step cycle for 54 steps. A slight modulation is visible here as well. Protractor MN activity increased with the beginning of the step cycle and had a maximum between 180° and 210°. Retractor MN activity had a maximum between 60° and 90°. The cross correlation function between protractor and retractor MN activity reveals a phase shift of 180° (Fig. 4.6(f)). The period of the oscillation in the cross correlation function corresponds with 1,7 s to the most frequent step cycle duration of 1,6 s.

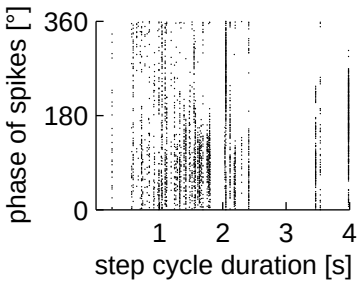
Three experiments showed both, coupling to front leg stepping as well as uncoupled oscillations even in one stepping sequence (Fig. 4.7(a)). One example is presented here without detailed analysis. The extracellular recording shows alternating activity in protractor and retractor MNs during the stepping sequence. The frequency of the oscillations changed within the stepping sequence from slow alternation coupled to front leg stepping to fast frequency oscillation not coupled to the steps.


pro
tacho
meso
tacho
meta
retractor
protractor
1s

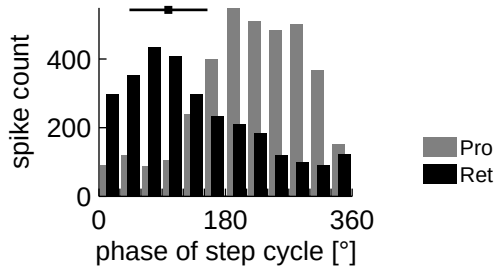
(a)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]
n=71

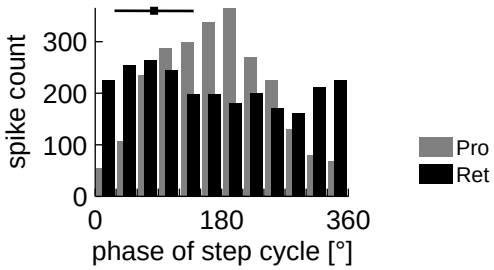
(b)


phase of spikes [°]
360
180
0
1
2
3
4
step cycle duration [s]

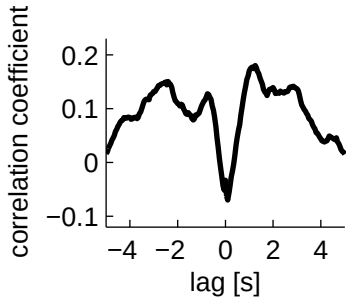
(c)


spike count
400
200
0
0
180
360
phase of step cycle [°]
Pro
Ret

(d)


spike count
300
200
100
0
0
180
360
phase of step cycle [°]
Pro
Ret

(e)


correlation coefficient
0.2
0.1
0
−0.1
−4
−2
0
2
4
lag [s]

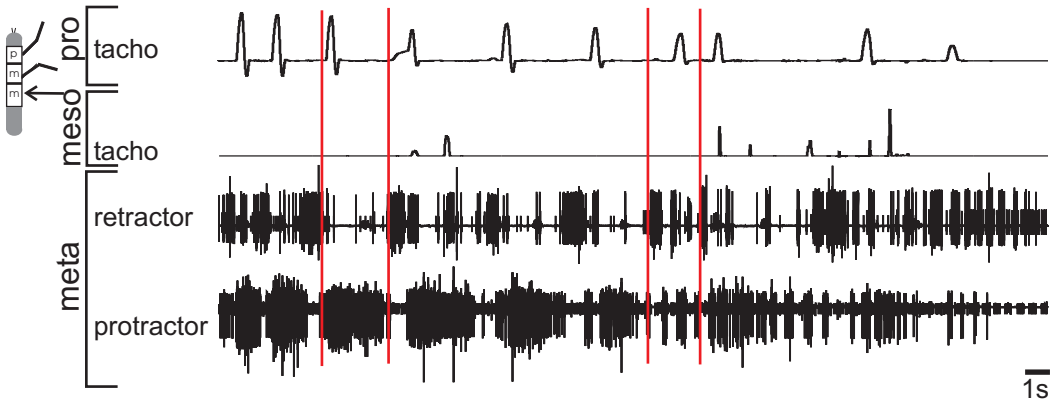
(f)

Figure 4.6: *(a) Metathoracic protractor and retractor MN activity during front and ipsilateral middle leg stepping. Protractor and retractor MNs were coupled to front leg steps regardless of middle leg stepping. Phase of (b) protractor and (c) retractor MN spikes in front leg step cycle plotted against step cycle duration. Protractor MN spikes occurred between 180° and 360° of front leg step cycle and retractor MN spikes between 0° and 180°, independent of step cycle duration. The red curve (smoothed) shows the frequency of steps over the step cycle duration in arbitrary units. (d) Distribution of mesothoracic protractor and retractor MN activity in front leg step cycle for 71 steps. Retractor MN activity had a maximum between 60° and 90°. Protractor MN activity had a maximum between at 180° and 210°. (e) The cross correlation function shows alternating coupling between protractor and retractor MN activity. (f) Distribution of metathoracic protractor and retractor MN activity in middle leg step cycle for 54 steps. Retractor MN activity had a maximum between 60° and at 90°. Protractor MN activity had a maximum between 120° and 210°.*

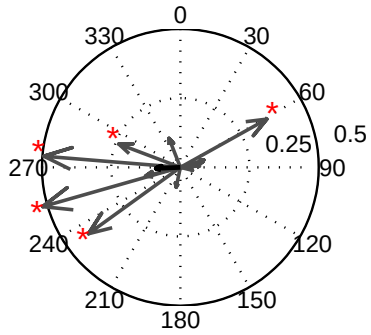
Polar plots summarize the mean phases of protractor and retractor MN activity for all twelve experiments in front and middle leg step cycle (Fig. 4.7). In front leg step cycle mean phases of protractor MN activity (Fig. 4.7(b)) were variable between the experiments with an overall mean phase of 268°. For retractor MN activity (Fig. 4.7(c)) the overall mean phase was 101° (62° to 173°) of front leg step cycle. In middle leg step cycle the overall mean phase of protractor MN activity (Fig. 4.7(d)) was 118° with mean phases of the different experiments pointing around the whole circle. The mean phases of retractor MN activity in middle leg step (Fig. 4.7(e)) cycle were very variable between the experiments. No overall mean phase was determined.

The presence of both front and ipsilateral middle legs and at least one of the two legs stepping was sufficient to elicit alternating activity in metathoracic protractor and retractor MNs (Fig. 4.8,Tab. 4.2). Figure 4.8 shows a stepping sequence of front and middle legs stepping (Fig. 4.8(a)) and one stepping sequence of the same experiments of only the front leg stepping while the middle leg rested on the treadmill (Fig. 4.8(a)). In both stepping sequences metathoracic protractor and retractor MN activity alternated.

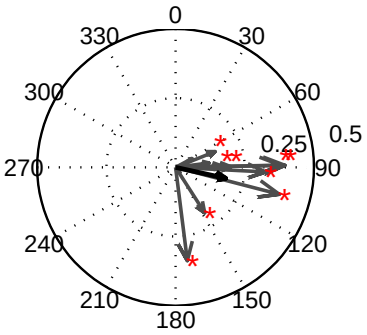
For a better overview the number of steps, the mean stepping frequency for front and middle legs and the frequency of alternation between protractor and retractor MN activity was summarized in table 4.2 for three experiments. The four experiments correspond to the four experiments that are shown in this chapter 4.2.2 in more detail in the order they



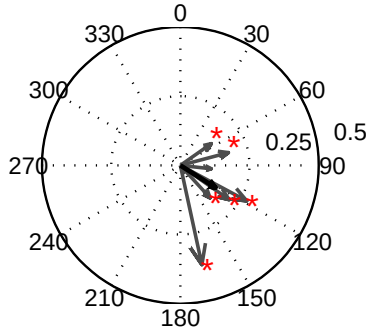
(a)



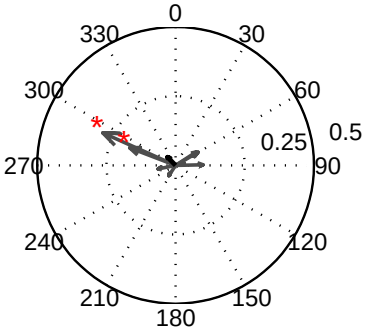
(b)



(c)



(d)



(e)

Figure 4.7: *(a) Metathoracic protractor and retractor MN activity during front and ipsilateral middle leg stepping. Protractor and retractor MN activity appears to be coupled to front leg stepping only for some steps. Polar plots of mean phases of (b) protractor and (c) retractor MN activity in front leg step cycle for twelve experiments (grey arrows) and mean vectors of all experiments (black arrows). Mean phases of protractor MN activity were variable with an overall mean phase of 268°. Retractor MN activity had an overall mean phase of 101° (62° to 173°). Polar plots of mean phases of (d) protractor and (e) retractor MN activity in middle leg step cycle. Mean phases of protractor MN activity were variable with an overall mean phase of 118°. For retractor MN activity no overall mean phase was determined.*

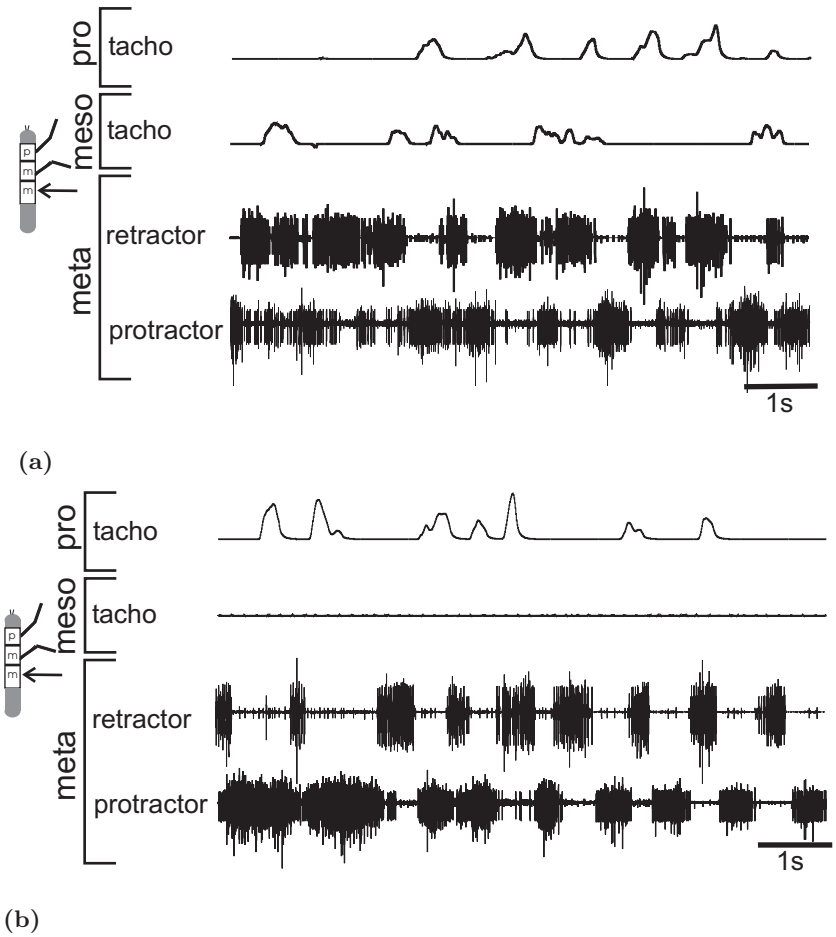
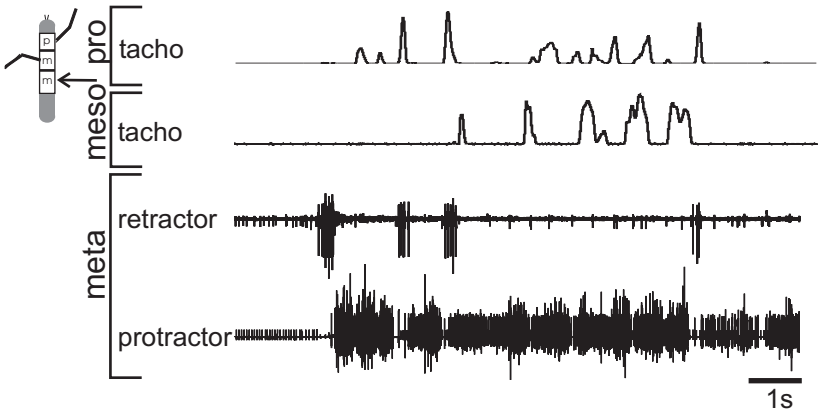


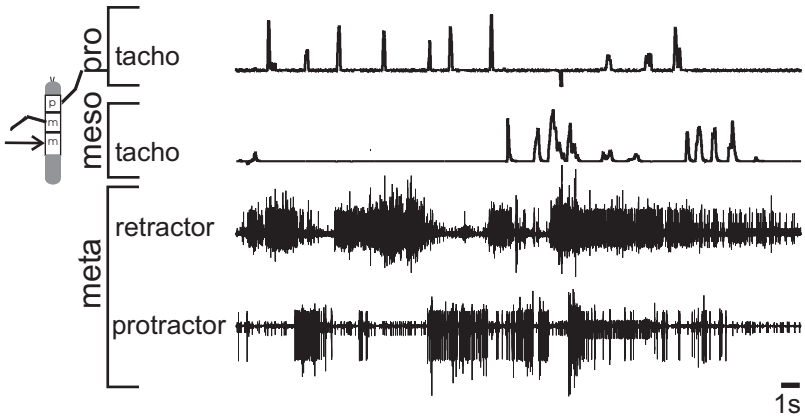
Figure 4.8: *(a) Metathoracic protractor and retractor MN activity during front and ipsilateral middle legs stepping. (b) Metathoracic protractor and retractor MN activity during front leg stepping. The middle leg did not perform stepping movements. This did not change the alternating activity in metathoracic protractor and retractor MNs.*

		front leg		**middle leg**		**metathorax**
experiment number	stepping sequence number	number of steps	mean frequency [Hz]	number of steps	mean frequency [Hz]	frequency alternation [Hz]
1	1	8	0,76	3	0,40	0,50
	2	19	1	6	0,30	0,59
	3	18	1	6	0,27	0,67
	4	10	0,75	3	0,40	0,69
	5	17	0,95	3	0,18	
	6	3	0,75	0		
	7	21	0,59	6	0,14	0,63
	8	33	0,52	12	0,15	0,64
	9	13	0,83	5	0,30	0,68
2	1	7	0,70	0		1,85
	2	7	0,64	0		0,91
	3	6	0,59	0		
	4	7	0,45	2		0,77
	5	12	1,50	0		0,56
	6	17	1,00	13	0,85	0,91
	7	16	1,10	9	0,84	2,38
	8	14	1,30	8	1,30	2,00
3	1	26	0,83	0		1,10
	2	18	0,70	0		0,63
	3	10	0,90	6	0,59	0,57
	4	5	0,20	17	0,56	0,32
	5	14	0,64	4	0,56	0,74
	6	5	0,20	18	0,71	0,21
	7	4	0,20	12	0,55	0,20
	8	8	0,55	0		0,56

Table 4.2: *Parameters of front leg and middle leg steps for different stepping sequences for three experiments and the frequency of alternation between metathoracic protractor and retractor MN activity.*



(a)



(b)

Figure 4.9: *Metathoracic protractor and retractor MN activity during front and contralateral middle leg stepping (a) ipsilateral to the stepping front leg (b) ipsilateral to the stepping middle leg. A general increase in activity was observed but no alternating activity.*

occur (experiment 1: see Fig. 4.5, experiment 2: see Fig. **??**, experiment 3: see Fig. **??**). The frequency of alternation between protractor and retractor MN activity was calculated from the period of the oscillation of respective cross correlation function. The table shows that there were several stepping sequences where the middle leg did not perform stepping movements. During these stepping sequences protractor and retractor MN activity were alternating as well. Hence, stepping of one leg and the presence of a second leg appears to be sufficient to elicit alternating activity in metathoracic protractor and retractor MNs.

In summary, stepping of both front and ipsilateral middle legs elicited alternating activity in ipsilateral metathoracic protractor and retractor MNs in eleven of twelve experiments.

Between the experiments a varying coupling to front leg steps was observed. For the majority of experiments no coupling was observed neither to front nor to middle leg stepping. The same experiments were conducted with intact front and contralateral middle legs (N=9) (Fig. 4.9). In protractor and retractor MNs ipsilateral to the stepping front leg (Fig. 4.9(a)) as well as ipsilateral to the stepping middle leg (Fig. 4.9(b)) no alternating activity was observed in eight of nine experiments during front and middle legs stepping sequences. Front and middle legs stepping induced a general activity increase in metathoracic coxal MNs. Hence, exclusively ipsilateral front and middle legs stepping was able to elicit alternating activity in metathoracic protractor and retractor MNs. This indicates that activating the pattern generating network in the metathoracic ganglion requires the presence of both ipsilateral legs when sensory input of the own leg is missing.

4.3 Conclusions

This chapter deals with the question to what extend single and two leg stepping activated central pattern generating networks in the mesothoracic and the metathoracic ganglion. Front leg stepping elicited alternating activity in mesothoracic coxal MNs (see chapter 3, Ludwar et al. (2005a)). This activity pattern was phase coupled to the front leg step cycle (chapter 3). Retraction of the front leg coincided with retractor MN activity in the mesothoracic ganglion. From these observations it was not clear whether pattern generating networks in the mesothoracic ganglion were activated and contributed to this effect. Two possible hypotheses would have been reasonable. 1) Front leg stepping could exhibit a direct influence on mesothoracic coxal MNs. 2) Front leg stepping could somehow activate the pattern generating networks in the mesothoracic ganglion that shaped the motoneural output in mesothoracic coxal MNs. The results presented in this chapter provide strong indications that front leg stepping activated pattern generating networks in the ipsilateral mesothoracic hemiganglion that form the observed motor output in protractor and retractor MNs. The data showed that front leg stepping was able to influence and to a great extend entrain the pilocarpine induced motor rhythm in mesothoracic protractor and retractor MNs. The phase relation between front leg stepping and protractor

and retractor MN activity was the same as in the single leg preparation without pilocarpine superfusion. Retractor MNs were active in the first half of front leg step cycle protractor MNs in the second half. The second set of experiments was performed in the two leg preparation. Stepping of ipsilateral front and hind legs elicited alternating activity in mesothoracic coxal MNs. The frequency of the alternation was similar to the stepping frequency of the legs. The rigorous coupling to front leg stepping that was observed in the single leg preparation (see chapter 3) was weakened in the two-leg preparation. Alternating activity in protractor and retractor MNs persisted but got to some extend independent of front leg stepping.

Stepping of a single front or middle leg induced a general activity increase in metathoracic coxal MNs (see chapter 3). No alternating activity was observed. Stepping of both front and ipsilateral middle leg reliably (in eleven of twelve experiments) elicited alternating activity in metathoracic protractor and retractor MNs.

In 50% of the experiments stepping of front and middle leg elicited alternating activity independent of front and middle leg stepping. The frequency of the alternation between protractor and retractor MN activity varied between 0,4 Hz and 1,8 Hz. In 16% of the experiments coupling of protractor and retractor MN activity to front leg stepping was observed. This coupling was equivalent to that between front leg stepping and mesothoracic protractor and retractor MNs (see chapter 3). Retractor MNs were active in the first half of front leg step cycle. Protractor MNs were active in the second half of front leg step cycle. The stepping movements of the middle leg appeared to be irrelevant for the coupling to front leg stepping in this experimental situation. This is a further indication that the front leg is dominant concerning the intersegmental influence compared to the middle leg. In 25% of the experiments both cases, alternating activity uncoupled and coupled to front leg steps, were observed even in one stepping sequence.

The influence of front leg stepping on the pilocarpine activated metathoracic ganglion was qualitatively the same as the influences observed in the two leg preparation. Alternating activity uncoupled of front leg stepping and faster than the stepping cycle (one of five experiments) occurred as well as complete entrainment (three of five experiments) to front leg stepping. Hence, the status of the "receiving ganglion" appears to be crucial for the processing of intersegmental signals.

The experiments show that it was not necessary that both front and ipsilateral middle legs performed stepping movements. It was sufficient to have one leg stepping and the other one just standing on the treadmill. Stepping of both front and middle legs occurred in 55,3% of all stepping sequences. In 41,2% only the front leg performed stepping movements and in 3,6% the middle leg performed stepping movements while the front leg did not move. This did not change the observed alternating activity in metathoracic protractor and retractor MNs.

CHAPTER 5

Intersegmental signals in the connectives

Intersegmental information transfer is a crucial part in establishing intersegmental coordination and assuring directed locomotion. In insects all ganglia, the head ganglia, thoracic ganglia and abdominal ganglia, are interconnected by pairs of connectives. The connectives act solely as cables and contain around 2000 axons (Leslie 1973). Dean (1989) and von Buddenbrock (1921) could show that intact connectives are crucial for intersegmental coordination and mechanical coupling alone is not able to establish a stable walking pattern. If both connectives between the pro- and meso- or the meso- and the metathoracic ganglia are cut, the legs posterior to the cut no longer participate in walking (von Buddenbrock 1921). At least a unilateral connection to the subesophageal ganglion seems to be a prerequisite for walking (Bässler 1983b).

The relevance of the connectives for coordination has also been investigated in context of other motor programs. The influence of cutting individual connectives on the flight activ-

ity of locusts (Ronacher et al. 1988) and the stridulatory movements of male grasshoppers (Ronacher 1988) was investigated by Ronacher et al.. The locust flight experiments (Ronacher et al. 1988) show that transection of one of the connectives between the mesothoracic and the metathoracic ganglion did not abolish free flight capability and the motor coordination was close to normal. This indicates that the neuronal information that is exchanged between the ganglia might be redundant in the two connectives in this case. In the stridulation experiments (Ronacher 1988) the metathoracic ganglion was hemisected and one of the neck connectives was transected. The animal stridulated only with the hind leg ipsilateral to the intact neck connective. This indicates that the initiation for this behavior is generated in the head ganglia. The neurons that are responsible for the initiation of the stridulation in the metathorax appear to remain in the ipsilateral connectives. In few studies the origin and destination of specific intersegmental interneurons was identified in the locust (Watson and Burrows 1983; Siegler and Burrows 1984; Burrows and Watkins 1986; Burrows and Pflüger 1992). For the stick insect no detailed knowledge exists about origin and destination of the axons in the connectives.
In this chapter neuronal activity in the connectives was investigated in more detail. Therefore the connectives ipsilateral to an intact stepping leg were recorded extracellularly and analyzed for different experimental situations. All connectives were intact. The ganglion that was recorded from was completely deafferented.

5.1 Connective recordings during single front leg stepping

The connectives serve the information transfer between the different ganglia. Figure 5.1 shows extracellular recordings of the ipsilateral connectives between the prothoracic and the mesothoracic (pro-meso connective) and the mesothoracic and the metathoracic (meso-meta connective) ganglion. When the animal is at rest the extracellular recordings showed a certain level of tonic activity (Fig. 5.1(a)). With the start of front leg stepping the neuronal activity in the pro-meso connective and the meso-meta connective increased (Fig. 5.1). The neuronal activity not only increased but appeared to be phasically modulated.

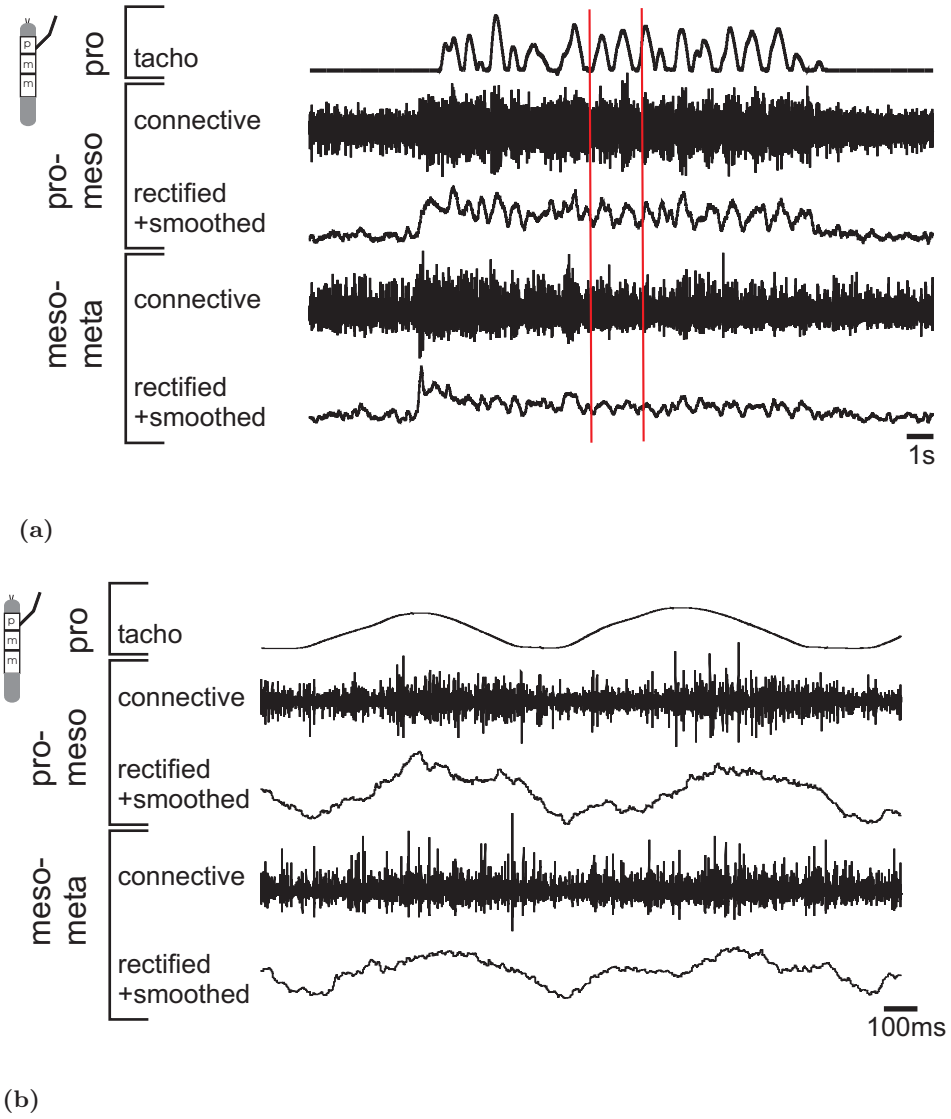


Figure 5.1: *(a) Extracellular recordings of the pro-meso and meso-meta connective during a front leg stepping sequence. Red vertical lines mark the range that is scaled up in (b).*

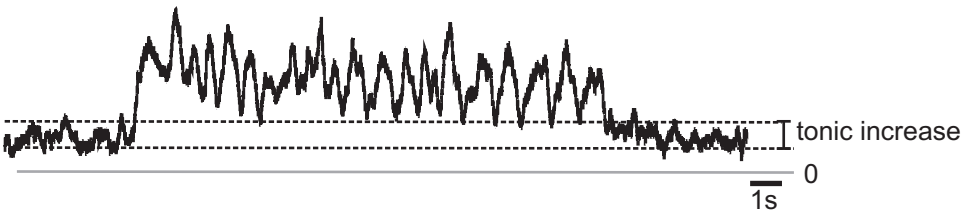


Figure 5.2: *Distinction of phasic and tonic component in the rectified and smoothed extracellular connective recording.*

The neuronal activity in the connectives was analyzed under different aspects. First the activity increase was characterized and compared between the two connectives. Second the phasic modulation in the connectives was analyzed in more detail and with reference to front leg steps. As for most of the analysis not single action potentials were of interest but the course of the mean neuronal activity, the extracellular recordings were rectified and smoothed ($T_s = 0,07s$). For the analysis two components were distinguished in the rectified and smoothed extracellular recording - a tonic component and a phasic component. The tonic component was defined as difference in mean activity before the stepping sequence to the smallest minimum of the phasic modulation during the stepping sequence (Fig. 5.2). The difference is given in percentage of the tonic component. The phasic component was defined as the oscillations above the tonic component (Fig. 5.2). This definition is in accordance to the definition used by J.P Gabriel and Büschges (2003) for intracellular recordings although the use of the same definition should not imply a functional correlation.

Tonic component

During a front leg stepping sequence the neuronal activity in the pro-meso and the meso-meta connective increased. The tonic component of the activity increase was determined for each stepping sequence of each experiment in the pro-meso and the meso-meta connective. Figure 5.3 shows the mean tonic increase in neuronal activity for five experiments. The mean increase in the pro-meso connective was between $36,2\%$ and $49,6\%$ (Fig. 5.3(a)). This was significantly different from zero for all five experiments (t-test). In the exemplary experiment presented here in more detail (Fig. 5.1) the mean activity increase was $36,2\%$. The tonic activity increase in the meso-meta connective was between $25,3\%$ and $49,3\%$ for the different experiments. This was significantly different from zero for all five

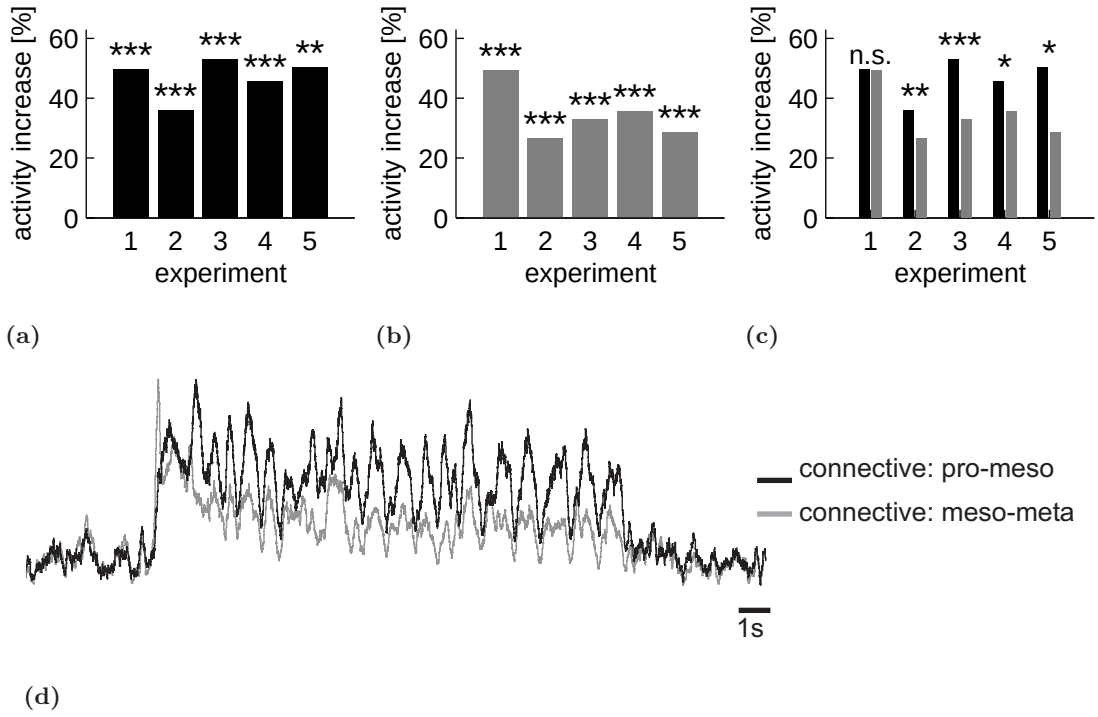


Figure 5.3: *Relative tonic activity increase in (a) the pro-meso connective, (b) the meso-meta connective and (c) the comparison of both during front leg stepping sequences. The stars on top mark the level of significance: (n.s.) not significant;* $(*)\ 0,01 \leq P < 0,05; (**)\ 0,001 \leq P < 0,01; (***)\ P < 0,001$. *(d) Overlay of the rectified and smoothed extracellular recordings of the two connectives during one front leg stepping sequence.*

experiments as well (t-test). In the experiment presented here (Fig. 5.1) it was $25,3\%$. For each experiment the tonic increase in neuronal activity in the pro-meso connective and the meso-meta connective was compared (Fig. 5.3(c)). In four of five experiments the tonic activity increase was significantly higher ($9,7\%$ to $21,3\%$) (t-test) in the pro-meso connective (Fig. 5.3(c)). In the experiment presented here in more detail the tonic activity increase in the pro-meso connective was $10,9\%$ higher than in the meso-meta connective. This becomes visible in the overlay of the rectified and smoothed recordings of both connectives for one stepping sequence as well (Fig. 5.3(d)).
These results show that during a front leg stepping sequence the neuronal activity was significantly increased in the ipsilateral pro-meso and the ipsilateral meso-meta connective. The increase was significantly higher in the pro-meso connective.
Phasic component

During front leg stepping sequences the neuronal activity in the pro-meso and the meso-meta connective appeared to be phasically modulated (Fig. 5.1). Therefore the phasic component was analyzed in more detail.
First auto and cross correlation analysis of the rectified and smoothed extracellular recording of the pro-meso and the meso-meta connective have been performed (Fig. 5.4) for each stepping sequence. The auto correlation functions of the pro-meso connective (Fig. 5.4(a)) show an oscillation. This indicates that the neuronal activity in the connective was modulated with a frequency around 1 Hz. The auto correlation functions of the meso-meta connective (Fig. 5.4(b)) show oscillations as well. In the cross correlation functions between the rectified and smoothed extracellular recordings of the pro-meso and the meso-meta connective oscillations are visible (Fig. 5.4(c)). This shows that both connectives oscillated with a similar frequency. The maxima of the cross correlation functions were slightly shifted against zero between $-0,071$ s and $0,007$ s for this experiment (Fig. 5.4(d)). As the maxima of the cross correlation functions was mainly left of zero this corresponds to an earlier maximum of neuronal activity in the meso-meta connective. These results were similar in four of five experiments. In one experiment the maxima of the cross correlation functions were shifted slightly to the right indicating that the maximum of the neuronal activity in the meso-meta connective was shortly before the maximum in the pro-meso

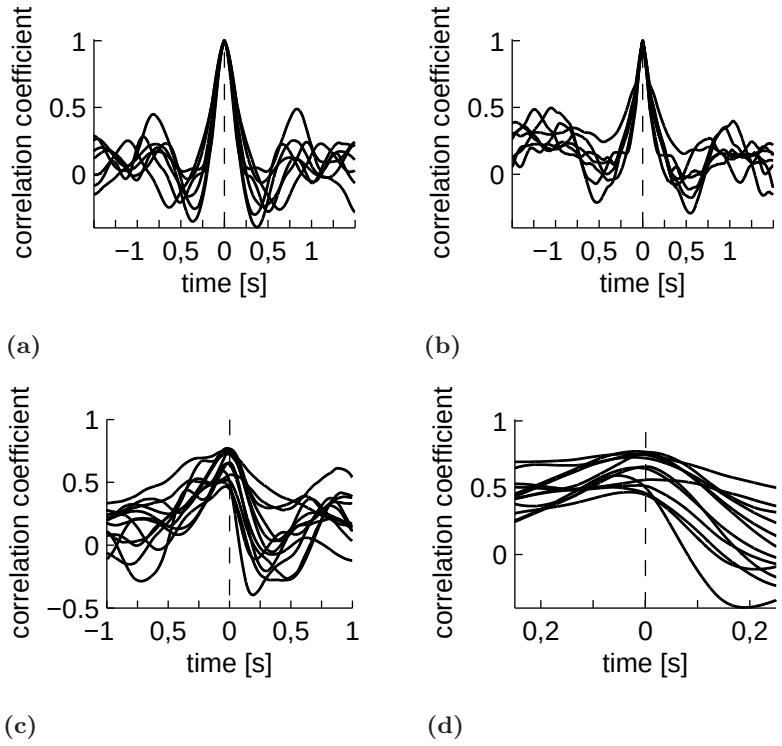


Figure 5.4: *(a) Auto correlation functions of the rectified and smoothed ($T_s = 0,07s$) extracellular recordings of (a) the pro-meso connective and (b) the meso-meta connective for seven front leg stepping sequences of one experiment for seven front leg stepping sequences. (c) Cross correlation function between the rectified and smoothed extracellular recordings of the pro-meso and the meso-meta connective for seven front leg stepping sequences.*

connective.

The analysis so far did not have any reference to the front leg step cycle. The cross correlation analysis showed that there existed a relationship between the phasic modulation in the pro-meso and the meso-meta connective. Therefore, overdraws of the rectified and smoothed extracellular recordings of the pro-meso and the meso-meta connective with reference to front leg stepping were performed and averaged. This was done referring to time as well as to phase. The overdraw referring to time was triggered by the beginning of front leg stance. The overdraws were done from 0,3 s before to 0,7 s after the beginning of front leg stance. The overdraws referring to phase were done for the whole front leg step cycle from beginning of stance to the beginning of next stance.

The time dependent averages show that in the pro-meso connective (Fig. 5.5(a)) as well as

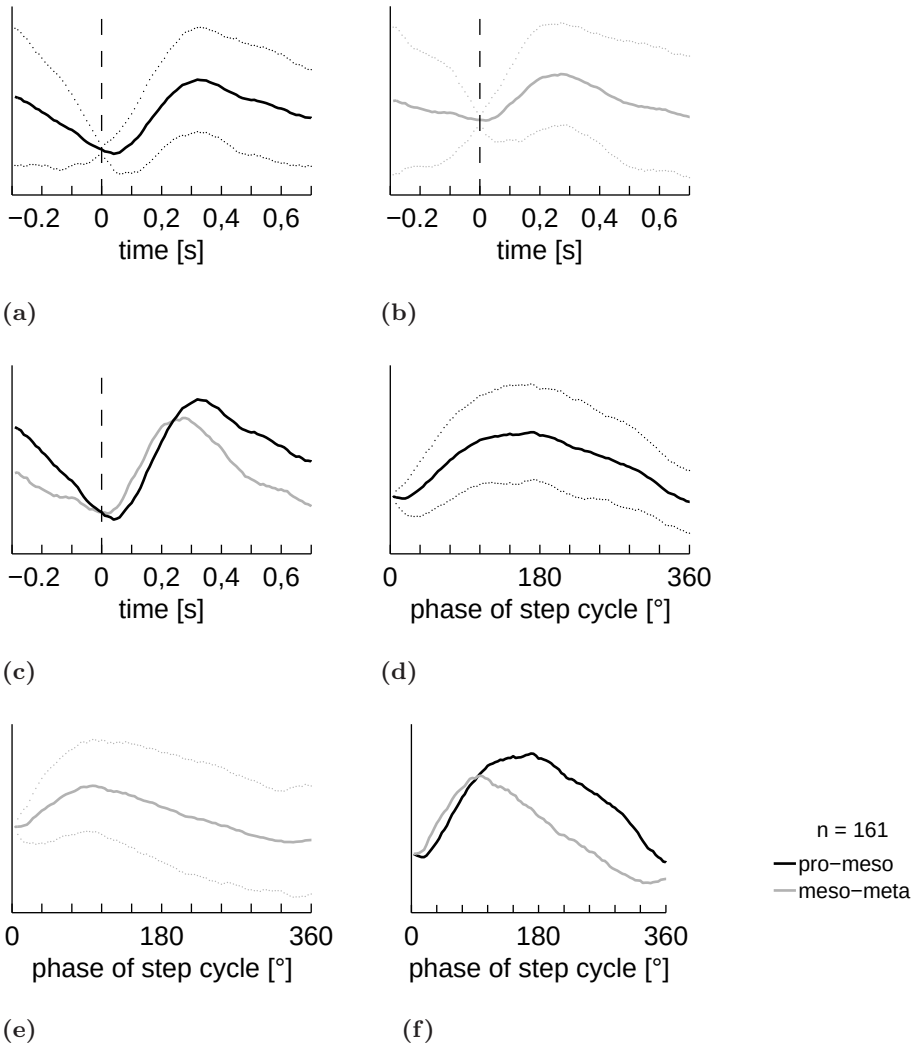


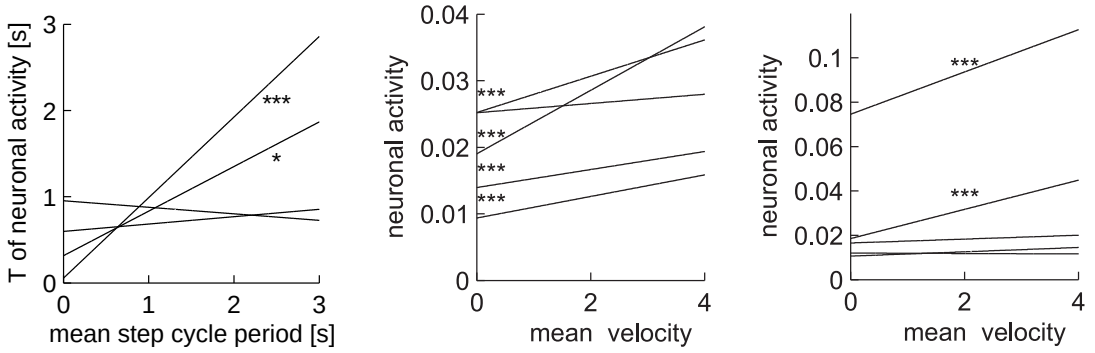
Figure 5.5: *(a)-(c) Average of overdraws of the rectified and smoothed ($T_s = 0,07s$) extracellular recording of (a) the pro-meso connective, (b) the meso-meta connective and (c) both connectives from 0,3 s before to 0,7 s after the beginning of front leg stance. (d)-(f) Average of overdraws with reference to front leg step cycle of the rectified and smoothed extracellular recording of (d) the pro-meso connective, (e) the meso-meta connective and (f) both connectives. The light lines mark the standard deviation. Y-axis in arbitrary units.*

in the meso-meta connective (Fig. 5.5(b)) the neuronal activity increased on average with the beginning of front leg stance. The neuronal activity in the pro-meso connective had a maximum 0,32 s after the beginning of stance phase and decreased afterwards (Fig. 5.5(a)). In the meso-meta connective the neuronal activity on average had a maximum 0,27 s after the beginning of stance (Fig. 5.5(b)). In figure 5.5(c) it becomes visible that the neuronal activity in the meso-meta connective on average raised earlier and reached its maximum before the neuronal activity in the pro-meso connective. This corresponds to the results from the cross correlation analysis that also showed a tendency for the meso-meta connective to be maximal shortly before the pro-meso connective.

The phase dependent averages show similar results. The neuronal activity in the pro-meso connective had a maximum at 169° of front leg step cycle (Fig. 5.5(d)). The neuronal activity in the meso-meta connective had a maximum at 97° of front leg step cycle (Fig. 5.5(e)). The neuronal activity in the meso-meta connective reached its maximum faster and decreased earlier than the neuronal activity in the pro-meso connective (Fig. 5.5(f)). Similar results were obtained in four of five experiments. From these results it cannot be concluded whether the observed phasic modulation was time or phase dependent. In both analysis, the time and the phase dependent overdraws, the results were similar.

In summary, this analysis shows that there existed a phasic modulation that occurred in both connectives and that can be related to the front leg step cycle.

The parameters of the neuronal activity were compared with parameters of the front leg steps (Fig. 5.6). First, it was tested whether the front leg step cycle duration can be related to the period of the phasic modulation in the two connectives. In the cross correlation functions for the different stepping sequences in most of the cases an oscillation was visible. The period of this oscillation corresponds to the mean period of the phasic modulation for respective stepping sequence. The question was whether there existed a linear relationship between the mean step cycle duration and the period of the oscillation of the cross correlation function. In two of five experiments a positive correlation existed (Fig. 5.6(a)). For one experiment the analysis was not possible as too few values could be determined. In two experiment the two parameters were uncorrelated. Second, it was analyzed whether the stepping velocity could be related to the neuronal activity in the connectives. Therefore, as a correlate of the mean neuronal activity in the connective,



(a) **(b)** **(c)**

Figure 5.6: *(a) Linear fit of the mean period in the cross correlation function vs. the mean step cycle period. (b) Linear fit of the mean activity (integral/step duration) per step of the pro-meso connective vs. the mean velocity of the treadmill (both in arbitrary units). (c) Linear fit of the mean activity (integral/step duration) per step of the meso-meta connective vs. the mean velocity of the treadmill (both in arbitrary units).The stars mark the level of significance: () not significant;* $(*)\ 0,01 \leq P < 0,05; (**)\ 0,001 \leq P < 0,01; (***)\ P < 0,001.$

the integral under the rectified and smoothed extracellular recording was determined for each step cycle and normalized by respective step cycle period. The normalized integral was plotted against the mean treadmill velocity (Fig. 5.6(b), 5.6(c)). The mean treadmill velocity was determined by the integral under the tachometer trace during the stance phase normalized by respective stance duration. For the pro-meso connective in four of five experiments a linear dependence existed (Fig. 5.6(b)). For the meso-meta connective only in three of five experiment a significant correlation existed (Fig. 5.6(c)).

In summary, these correlations show that the observed pattern of neuronal activity in the pro-meso and meso-meta connectives changed with parameters of front leg stepping. Therefore they strongly indicate the dependence of the neuronal activity in the connectives on front leg stepping.

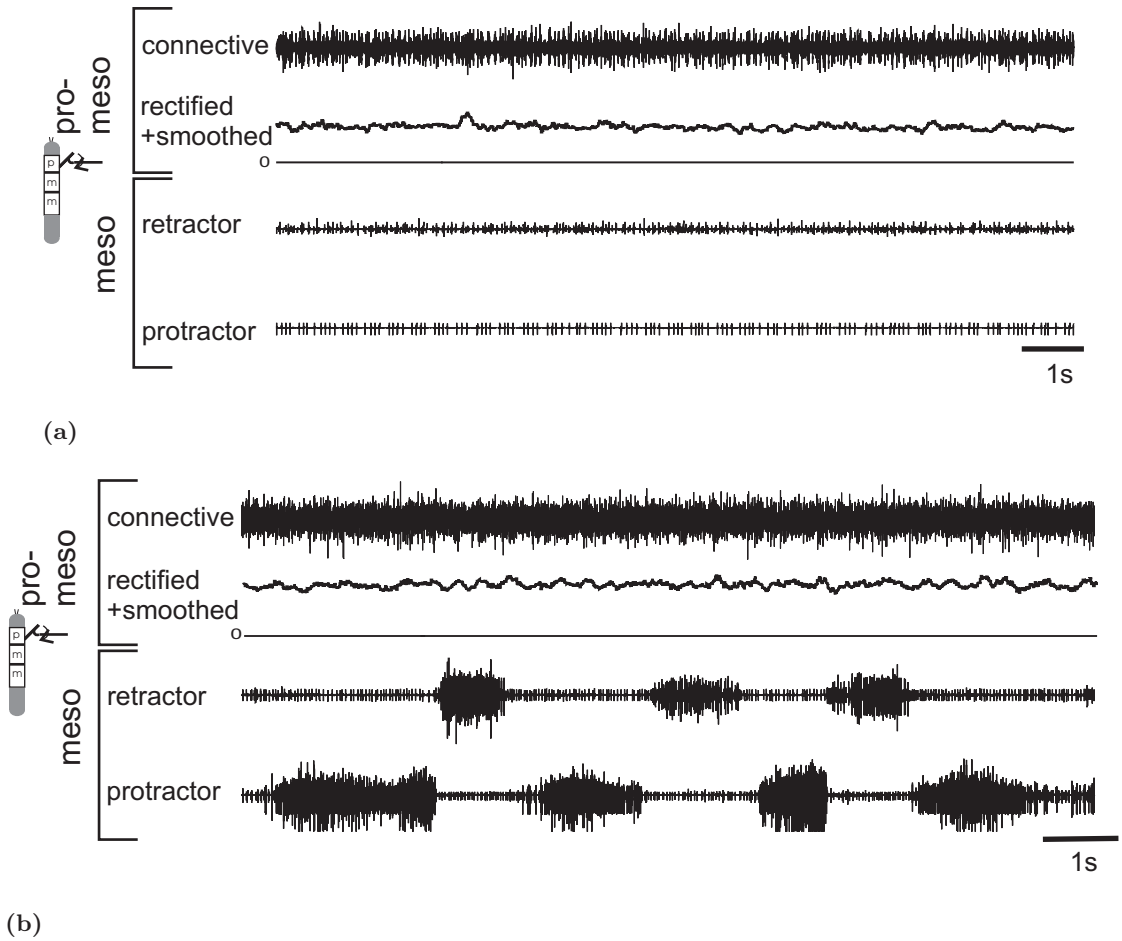


Figure 5.7: *Extracellular recording of the pro-meso connective and mesothoracic protractor (nl2) and retractor (nl5) MNs (a) without and (b) with pilocarpine superfusion of the mesothoracic ganglion.*

5.2 Origin of the tonic component

As shown in the previous section stepping of a single intact front leg induced an increase in neuronal activity in the ipsilateral pro-meso and meso-meta connective that was phasically modulated with front leg steps. This raised the question what contributes to the tonic increase and the phasic modulation of the neural activity in the connective.

In this set of experiments the mesothoracic ganglion was superfused in the split bath configuration with saline containing pilocarpine. The right pro-meso connective as well as ipsilateral mesothoracic protractor and retractor MNs were recorded extracellularly (Fig. 5.7). First, data was analyzed under the aspect of a measurable increase in activity.

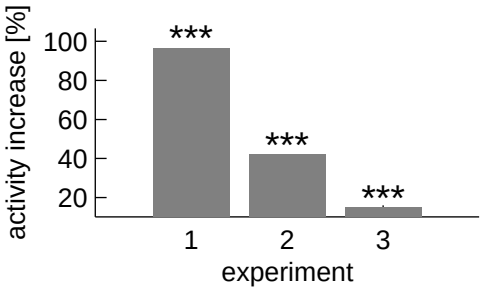
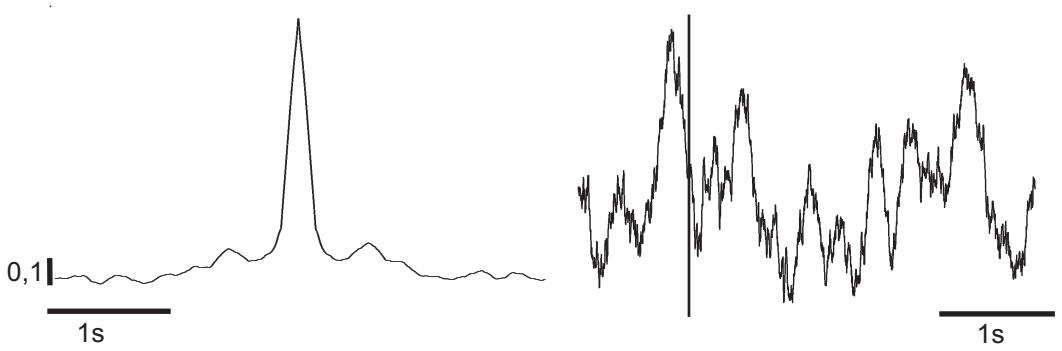


Figure 5.8: *Relative neuronal activity increase in the pro-meso connective during pilocarpine induced rhythmicity in the mesothoracic MNs.*

Did the activation of the mesothoracic ganglion with pilocarpine lead to a significant increase in neuronal activity in the connective? The extracellular recording (Fig. 5.7) of the pro-meso connective before (Fig. 5.7(a)) and after (Fig. 5.7(b)) the pilocarpine rhythm in mesothoracic MNs established, suggested that the neuronal activity increased. The mean value of the rectified and smoothed connective recording at rest and during pilocarpine rhythm in the mesothoracic ganglion was determined several times, averaged and compared. It turned out that in all three experiments neuronal activity was significantly increased (Fig. 5.8). Hence, activating the mesothoracic pattern generating networks with pilocarpine lead to a significant increase in neuronal activity in the pro-meso connective.

Second, the connective recording was analyzed for phasic modulations. The auto correlation function of the rectified and smoothed extracellular recording did not show any oscillating behavior (Fig. 5.9(a), compare to Fig. 5.4(a)). There are only small fluctuations visible in the autocorrelation function. Furthermore, overdraws with respect to the beginning of a pilocarpine cycle were done. The beginning of a pilocarpine cycle was defined as the beginning of the protractor MN burst. The average did not show any systematic modulation (Fig. 5.9(b)).

In summary, the presented results indicate that the activity of pattern generating networks in the mesothoracic ganglion contributed to a tonic increase in neuronal activity in the pro-meso connective.



(a) (b)

Figure 5.9: *(a) Autocorrelation function and (b) average of overdraws of the rectified and smoothed ($T_s = 0,07s$) extracellular recording of the pro-meso connective during pilocarpine induced rhythmicity in the mesothoracic MNs. The overdraw was triggered by the beginning of the protractor burst.*

5.3 Origin of the phasic modulation

The neuronal activity in the connectives was shaped by the activity emanating from the sensory organs of the stepping leg as the following results indicate. Stimulation of front leg sensory organs led to bursts of activity in the ipsilateral pro-meso connective. This was investigated in two different experimental situations.

First, the campaniform sensilla of the front leg were stimulated while the ipsilateral pro-meso connective was recorded extracellularly (for detailed information about the stimulation see chapter 2 "Materials and methods"). As figure 5.10 shows, a stimulation of front leg campaniform sensilla was followed by a short burst of neuronal activity in the pro-meso connective. The average of overdraws (Fig. 5.11) of the extracellular connective recording shows that the burst of neuronal activity was clearly related to the campaniform sensilla stimulation. The overdraw was triggered by start of the ramp for the campaniform sensilla stimulation. The average of the overdraws shows a clear burst. This indicates that the occurrence of the burst was very consistent for the different stimulations and therefore is visible as burst in the average of the overdraws as well. The mean latency between the stimulation and the burst of activity was determined by using the averaged overdraws of the extracellular connective recording (Fig. 5.11). The first maximum of the averaged

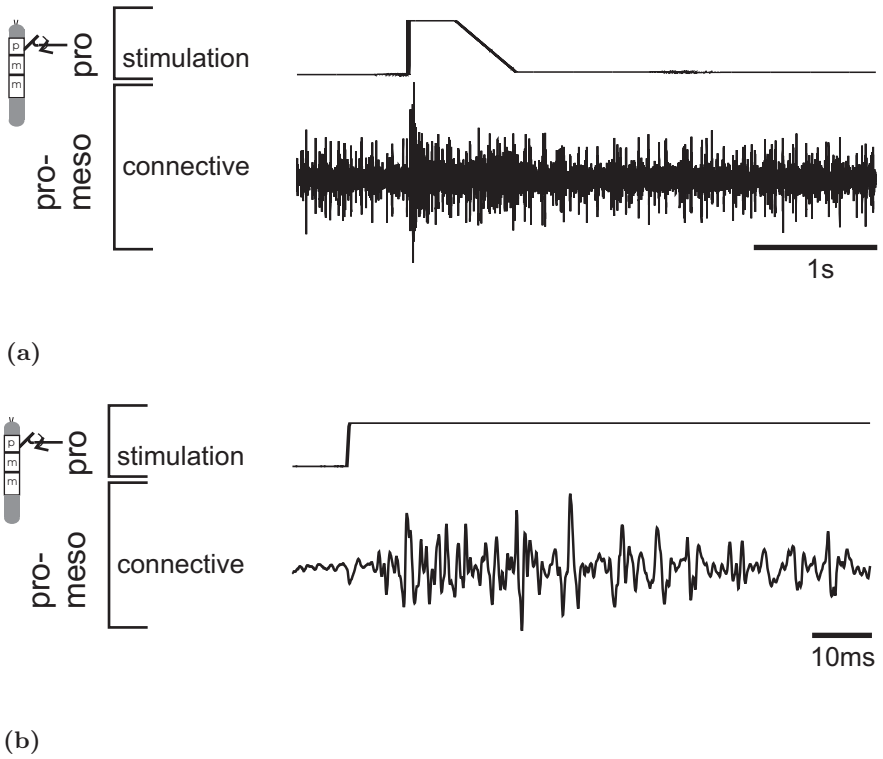
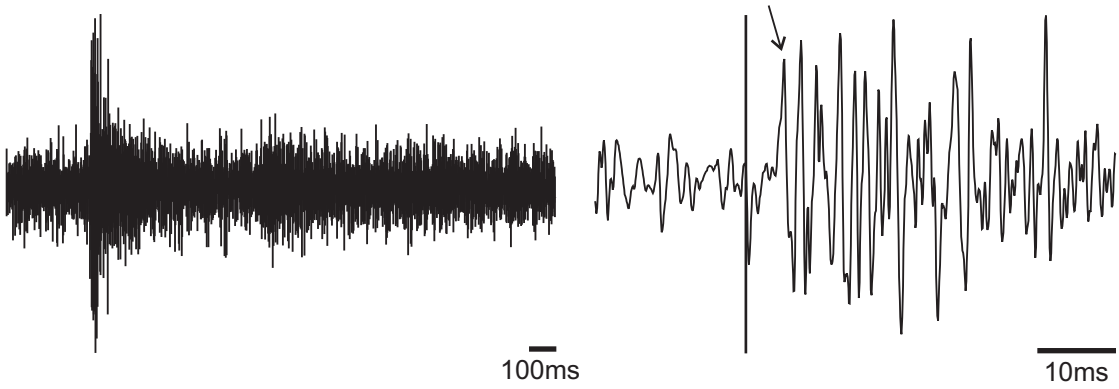


Figure 5.10: *Extracellular recording of the connective between the prothoracic and the mesothoracic ganglion during campaniform sensilla stimulation of the ipsilateral front leg, (a) for one stimulation and (b) zoomed in.*

burst was determined as the mean latency between the stimulation and the burst beginning (Fig. 5.11(b)). The latencies for the three experiments were between 4,9 ms and 9,1 ms (Tab. 5.1). The electrode was always positioned in the anterior third of the connective.

In a second experimental situation the neuronal activity in the pro-meso connective was compared for passive leg movements and active stepping movements of the intact front leg. Both cases are shown here for one experiment. The stepping movements of the front leg (Fig. 5.12(a)) lead to the above described general increase in neuronal activity in the pro-meso connective and the phasic modulation with the steps. For the passive movement of the leg, the leg was positioned on the treadmill when the animal was at rest. The treadmill and consequently the leg on the treadmill were moved back and forth without the animal actively contributing to this movement. Still bursts of activity were visible in the pro-meso connective recording (Fig. 5.12(b)). The general increase in activity vanished whereas the phasic modulations persisted (Fig. 5.12(b)). Only one experiment had been



(a) **(b)**

Figure 5.11: *Average of overdraws (n=25) of the connective recording triggered by the beginning of the campaniform sensilla stimulation, (a) for one stimulation and (b) zoomed in. Black arrow marks the maximum that was taken to determine the mean latency.*

experiment	mean Δt [ms]
1	4,9
2	9,1
3	7,6

Table 5.1: *Mean latency between front leg campaniform sensilla stimulation and occurrence of a burst in the pro-meso connective.*

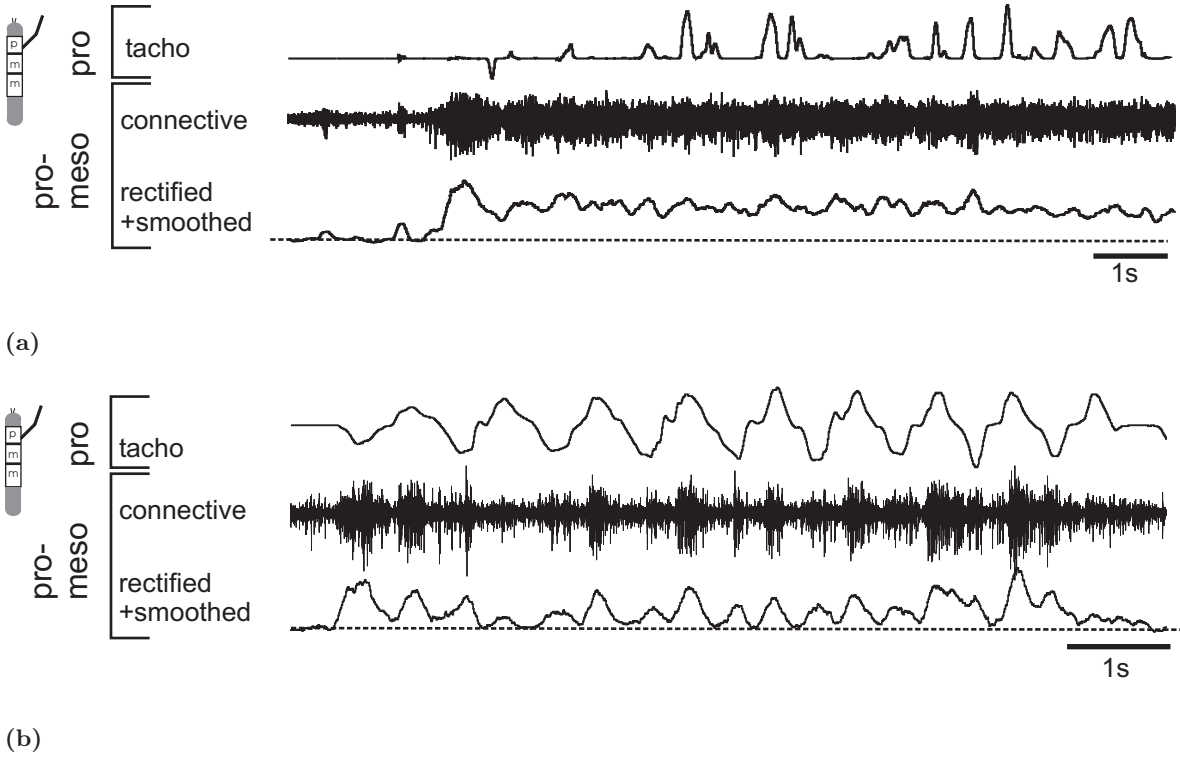


Figure 5.12: *Extracellular recording of the pro-meso connective during (a) front leg stepping and (b) passive movements of the front leg.*

done in this experimental setup but further experiments by a bachelor student support these findings (personal communication Dr. J. Schmidt).

Taken together, these results indicate that sensory signals of the stepping front leg shaped the phasic modulation whereas the activity of the pattern generating networks contributed to a general increase in neuronal activity.

5.4 Conclusions

In this chapter the neuronal activity in the pro-meso and the meso-meta connective ipsilateral to the stepping front leg were investigated. Both connectives were recorded extracellularly with hook electrodes (Schmitz et al. 1988).

Front leg stepping induced an increase in neural activity in the ipsilateral pro-meso and meso-meta connective. The activity was modulated with front leg steps. This phasic mod-

ulation was clearly assigned to the steps. The results indicate that the sensory influences of the stepping front leg might be the origin of the phasic modulation in the connectives. Front leg campaniform sensilla stimulation reliably elicited a burst in the ipsilateral pro-meso connective in all three experiments. When the front leg was moved passively the phasic modulations in the connectives persisted. Prothoracic pattern generating networks were most probably not active and therefore did not contribute actively to the movement. Active central pattern generating networks appeared to contribute to a general increase in neuronal activity in the connectives. The activation of the mesothoracic ganglion by pilocarpine induced an increase in neuronal activity in the pro-meso connective. No phasic modulations were observed in these experiments. When the front leg was moved passively the general increase in neuronal activity was not observed.

CHAPTER 6

Coordination of two legs

The stick insect is a "model system" for slow walking behavior since the 1920ies (von Buddenbrock 1921). Understanding walking patterns and the coordination of the legs was a major goal of behavioral studies. Therefore, walking patterns have been investigated, analyzed and described under various conditions (reviewed e.g. in Graham (1985b)).
Freely walking adult stick insects tend to walk on a horizontal surface in a tripod or tetrapod or an intermediate gate (Graham 1972). The use of self-propelled, moving or artificial surfaces such as the Kramer-Heinecke sphere (Kramer 1975, 1976), a self-propelled tread wheel (Wendler 1964a, 1966) or two light wheels (Graham 1981) and the slippery surface (Graham and Cruse 1981; Cruse and Epstein 1982; Epstein and Graham 1983; Gruhn et al. 2006) allowed to investigate insect walking under precisely defined walking conditions and with reduced preparations. On the split tread wheel the legs walked in the tripod gait over a large range of stepping frequencies (Graham 1981). The animal can spontaneously decouple the two body sides and walk with different frequencies on each side. Walking on a slippery surface mechanically decouples the legs that are otherwise

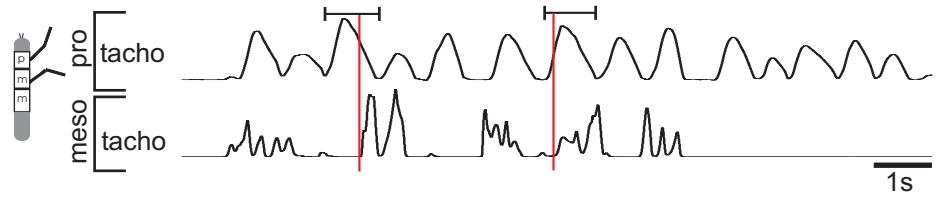
coupled via the substrate they walk on. The leg movements are only 70% coordinated compared to 90% for walks on rigid surface. The well coordinated walks closely resemble to the tripod gait of the first instar nymphs (Graham and Cruse 1981).

Amputation experiments were carried out to investigate the influences between the six legs. Amputation of any leg leads to subtle but quantifiable changes in the temporal and spatial patterning of movements of the remaining legs (Hughes 1957; Bässler 1972; Graham 1977). The amputee does not require any learning period to adopt to the new coordination. Middle leg amputation shows the clearest effect. The front leg takes up the same phase relation to the hind leg as before to the middle leg. This coordination is never observed in the intact animal (Bässler 1983b). The amputation of one or two legs in general leads to a gait very similar to the tetrapod gait (Graham 1977).

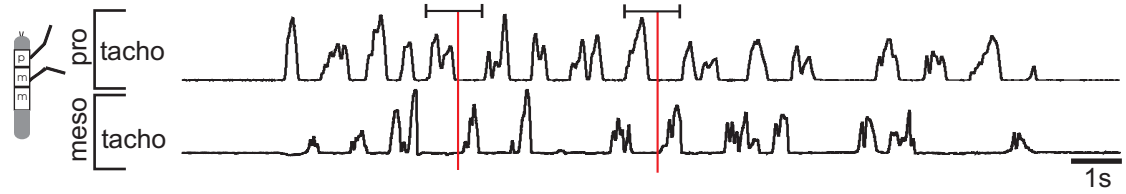
In the experiments presented in this chapter the coordination of two intact legs stepping on two separate passive treadmills was investigated. The animal was mounted dorsal side up on two small foam platforms and all legs except two were amputated. As the intact legs performed stepping movements on two separate passive treadmills they were mechanically uncoupled. The stepping pattern of the two intact legs was analyzed concerning stepping frequency, phase relations between the legs and relative coordination (Wendler 1964b). As walking patterns and leg coordination have been excessively studied under different walking conditions and in different reduced preparations it is important to regard these two leg experiments in context of the former experimental situations and results.

6.1 Coordination of ipsilateral front and middle legs

In this set of experiments the coordination between ipsilateral front and middle legs in the two leg preparation (see chapter 2 "Materials and methods") was investigated. The animal was fixed dorsal side up on two small foam platforms. Front and ipsilateral middle legs remained intact while all other legs were cut. The animal performed stepping movements with each leg on its own passive treadmill (J.P Gabriel and Büschges 2003). Both treadmills were arranged in parallel to the body axis. Therefore stepping of the front and middle legs resembles forward walking as close as possible. Because of the two small foam



(a)



(b)

Figure 6.1: *Stepping sequence of front and ipsilateral middle leg for (a) experiment one and (b) experiment two. Red lines mark the beginning of middle leg stance. Black bars on top mark front leg step cycles.*

platforms that supported the body beneath the meso- and the metathorax both legs were able to move freely in each joint. Nine experiments have been performed in this experimental configuration. Two representative experiments are presented here in more detail.

Figure 6.1 shows stepping sequences of two experiments with both front and middle legs stepping. It was not necessarily the case that both legs performed stepping movements in each stepping sequence. In the nine experiments that were conducted, stepping of both front and middle legs occurred in 55,3% of all stepping sequences. In 41,2% only the front leg performed stepping movements and in 3,6% the middle leg performed stepping movements while the front leg did not move. This shows that stepping of one of the two legs did not imply stepping of the other one. In the stepping sequence of the first exemplary experiment (Fig. 6.1(a)) the middle leg did fewer steps and stepped slower than the front leg. The different shapes of the tachometer traces of front and middle leg treadmill are due to different characteristics of the treadmills. The front leg treadmill had a higher moment of inertia and therefore the tachometer trace was smoother than the one of the middle leg treadmill. In the stepping sequence of the second exemplary experiment (Fig. 6.1(b)) the middle leg stepped with a similar frequency as the front leg did and appeared to start with the stance phase in around 180° of the front leg step cycle.

To characterize the stepping of front and middle legs, different parameters were collected for each experiment (Tab. 6.1 and appendix A). The table shows the number of steps per stepping sequence and the mean stepping frequencies for front and middle legs for the two experiments presented here. For the whole table of all nine experiments see appendix A. In the first exemplary experiment the animal performed eleven stepping sequences. The front leg performed between ten and 35 steps with mean frequencies between 0,73 Hz and 1,3 Hz. The middle leg performed stepping movements whenever the front leg did so, except for one very short stepping sequence. Nevertheless, the middle leg performed less steps (between three and twelve) in each stepping sequence and stepped with lower mean frequencies between 0,17 Hz and 1,14 Hz. In the second exemplary experiment the middle leg did not perform stepping movements during the first five stepping sequences of the front leg. The front leg did between six and seventeen steps per stepping sequence with mean frequencies between 0,45 Hz and 1,3 Hz. The middle leg performed less steps per stepping sequence (eight to thirteen). Mean stepping frequencies were in a range similar to that of the front leg (0,84 Hz to 1,3 Hz).

First, it was analyzed whether there existed a correlation between front and middle leg stepping frequencies. Figure 6.2(a) shows the mean middle leg stepping frequency of a stepping sequence plotted against the mean front leg stepping frequency of the same stepping sequence for all experiments. The correlation coefficient is 0,04 and not significantly different from zero. There existed no correlation between front leg stepping frequency and middle leg stepping frequency.

The parameters collected in table 6.1 do not give any information about the coordination between front and middle legs. Although stepping of the middle leg occurred more rarely and the middle leg stepping frequency could differ from the front leg stepping frequency this does not exclude that middle leg steps might occur at preferred phases of front leg step cycle. Circular statistics were used to analyze the coordination between front and middle stepping. The histogram (Fig. 6.2(b)) shows the distribution of middle leg steps in front leg step cycle for the first exemplary experiment (Fig. 6.1(a)). The beginning of middle leg stance was summed in each class. Front leg step cycle was defined from the beginning of stance to the beginning of the next stance. Middle leg steps were evenly distributed in front leg step cycle. This was observed in seven of nine experiments. For the

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
1	1	11	0,75	0	
	2	14	1,1	8	0,89
	3	30	0,83	7	1,14
	4	29	1	14	0,4
	5	24	0,73	21	0,51
	6	19	1,2	15	0,94
	7	10	1,3	4	
	8	35	0,88	11	0,29
	9	22	0,98	14	0,24
	10	15	0,78	7	0,17
	11	0		14	0,47
2	1	0	7	0,7	
	2	0	7	0,64	
	3	0	6	0,59	
	4	2	7	0,45	
	5	0	12	1,5	
	6	13	17	1	0,85
	7	9	16	1,1	0,84
	8	8	14	1,3	1,3

Table 6.1: *Parameters of front and ipsilateral middle leg steps for different stepping sequences of two experiments (see figure 6.1). For the whole table with all experiment see appendix A.*

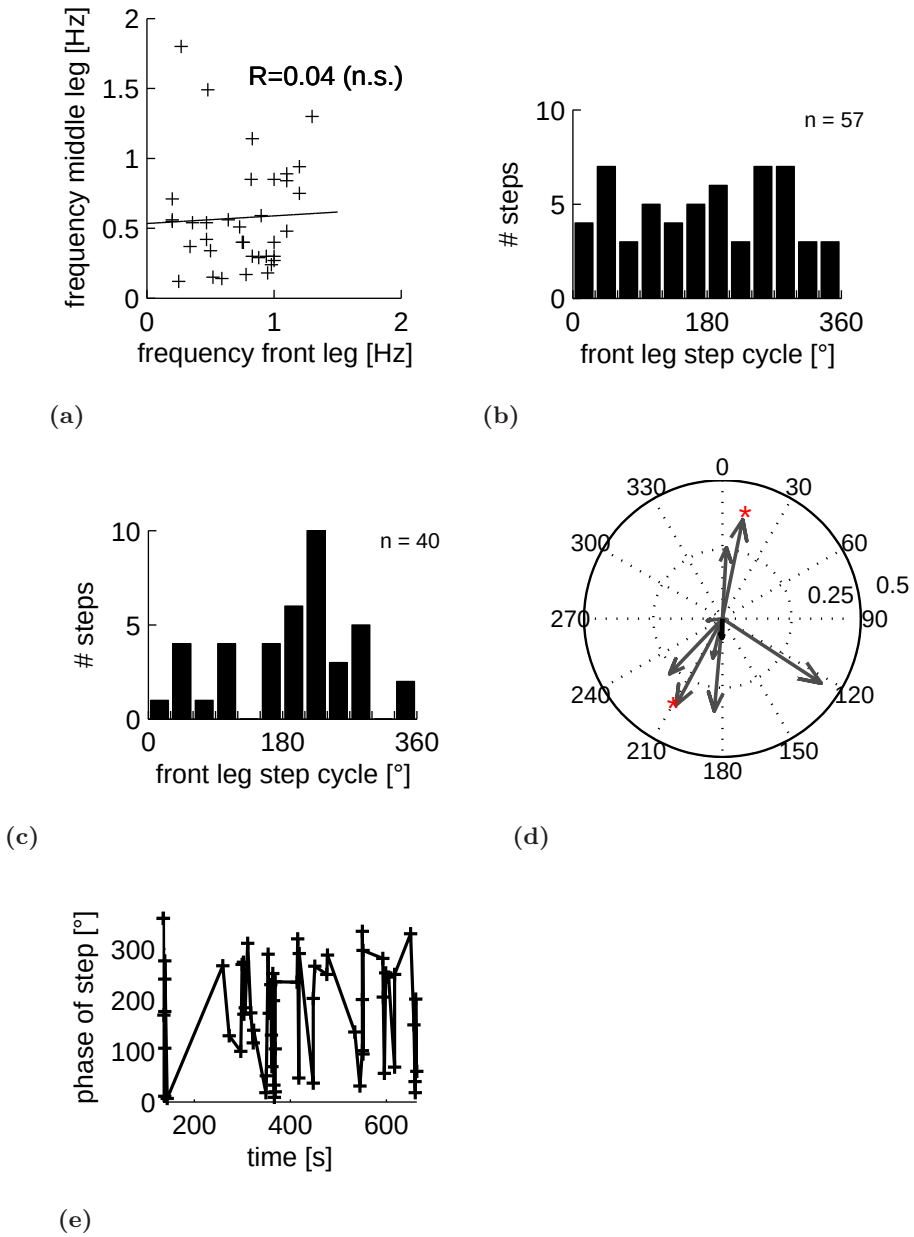


Figure 6.2: *(a) Middle leg stepping frequency plotted against front leg stepping frequency of respective stepping sequence for all stepping sequences of all experiments. (b)-(d) Circular statistics of middle steps in front leg step cycle. Distribution of middle leg stance beginnings in front leg step cycle for (b) experiment one and (c) experiment two. (d) Polar plot: vectors point in direction of mean phase of the middle leg stance beginnings in front leg step cycle for nine experiments. Red star marks the vectors with significant length. (e) Phase of middle leg stance beginnings in front leg step cycle is plotted against time for experiment one.*

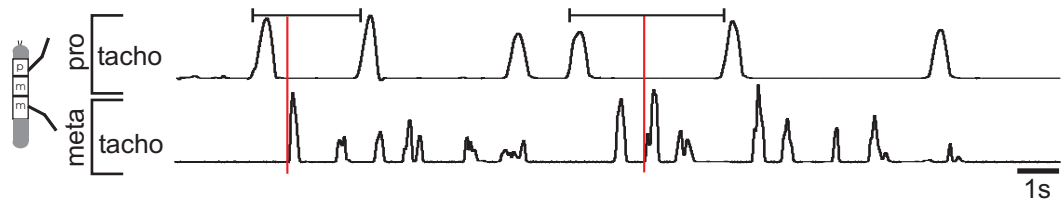
second exemplary experiment the histogram (Fig. 6.2(c)) reveals that the distribution of middle leg steps in front leg step cycle had a maximum between 210° and 240°. Middle leg steps started most frequently in the middle of front leg step cycle in this experiment. This would correspond to the phase relation between front and middle leg in a tetrapod gait. In the tetrapod gait front and ipsilateral middle legs have a phase relation of 240° (Collins and Stewart 1993).

The polar plot (Fig. 6.2(d)) summarizes the mean vectors of middle leg steps in front leg step cycle for all nine experiments. The mean phases appear to be clustered between 180° and 240° and around 0°. Only for two experiments the mean vectors differ significantly from zero (Rayleigh-Test). One significant vector points in direction of approximately 210° and belongs to the second experiment described above. The other significant vector belongs to an experiment not shown in detail. The mean phase of middle leg steps in front leg step cycle was 0° in this experiment. This indicates that front and middle leg tented to start a stance phase at the same time. This cannot be related to any known gait. In summary, in the majority (seven of nine) of the experiments no significant phase relation was observed. In the experiments where no phase preference existed this could be due to a relative coordination pattern between front and middle leg (Wendler 1964b). Therefore the phase of middle leg steps in front leg step cycle was plotted against time. This is shown in figure 6.2(e) for the first exemplary experiment. No systematic drifting of middle leg steps in phase over time is recognizable. Hence, front and middle leg stepping did not show relative coordination. This was true for all nine experiments.

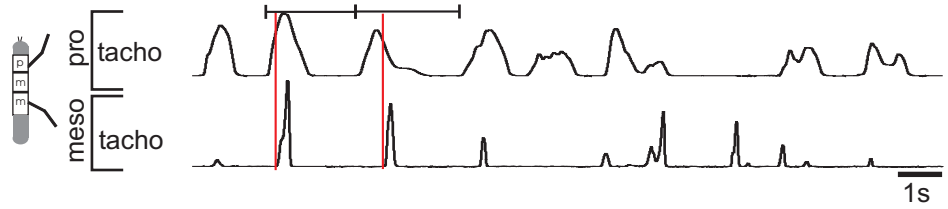
In summary, for front and middle leg stepping in this experimental situation no clear phase relation between the two legs was observed. Both legs appeared to step independent of each other concerning coordination, the number of steps and the stepping frequencies.

6.2 Coordination of ipsilateral front and hind legs

In this set of experiments the coordination between ipsilateral front and hind legs in the two leg preparation was investigated. The animal was fixed dorsal side up on two small foam platforms, one beneath the prothorax and one beneath the mesothorax. Front and hind leg remained intact while all other legs were cut. The animal performed stepping



(a)



(b)

Figure 6.3: *Stepping sequence of front and ipsilateral hind leg for (a) experiment one and (b) experiment two. Red lines mark the beginning of middle leg stance. Black bars on top mark front leg step cycle.*

movements with each leg on its own passive treadmill (J.P Gabriel and Büschges 2003). Both treadmills were arranged in parallel to the body axis. Seven experiments have been performed in this experimental configuration. Two representative experiments are presented here in more detail.

Figure 6.3 shows stepping sequences of these two experiments with both front and hind legs stepping. It was not necessarily the case that both legs performed stepping movements in each stepping sequence. In the seven experiments that were conducted, stepping of both front and hind legs occurred in 29,4% of all stepping sequences. In 66,3% only the front leg performed stepping movements and in 4,4% the hind leg performed stepping movements while the front leg did not move. Stepping of the front leg did not imply stepping of the hind leg. In approximately one third of all stepping sequences both legs performed steps. In the stepping sequence of the first exemplary experiment (Fig. 6.3) the hind leg appeared to step faster than the front leg and steps occurred in varying phases of front leg step cycle. In the stepping sequence of the second exemplary experiment (Fig. 6.3(b)) the stepping frequency appeared to be similar for both legs and hind leg steps started in the first third of front leg step cycle.

The parameters (number of steps per stepping sequence, mean stepping frequency) of front

and hind leg steps are summarized for the two presented experiments in table 6.2 and for the other five experiments see appendix A. In the first exemplary experiment the animal performed nine stepping sequences. The front leg performed between six and 19 steps. The hind leg performed between four and 33 steps in seven of the nine stepping sequences. The stepping frequencies of both legs were similar and lay between 0,31 Hz and 0,64 Hz for the front leg and between 0,45 Hz and 0,8 Hz for the hind leg. In the second exemplary experiment the front leg performed much more steps than the hind leg did, between six and 99 in five stepping sequences. The number of hind leg steps lay between one and 18. Front leg stepping frequencies ranged from 0,59 Hz to 0,73 Hz and were a little higher than the hind leg stepping frequency around 0,41 Hz. It was analyzed whether there existed a correlation between front leg stepping frequency and hind leg stepping frequency. Figure 6.4(a) shows the mean hind leg stepping frequency of a stepping sequence plotted against the mean front leg stepping frequency of the same stepping sequence for all experiments. The correlation coefficient is 0,18 and not significantly different from zero. There existed no correlation between front leg stepping frequency and hind leg stepping frequency.

Circular statistics were used to analyze the coordination between front and hind leg stepping. For the first experiment the histogram shows a wide distribution of hind leg steps in front leg step cycle (Fig. 6.4(b)). There was a tendency for hind leg steps to begin in the first half of front leg step cycle but no clear maximum was observed. In the second experiment the beginning of hind leg steps appeared to be more clustered around the beginning of front leg step cycle (Fig. 6.4(c)). The distribution of hind leg steps in front leg step cycle had a maximum between 0° and 30°. Hence, front and hind leg tended to start a stance phase simultaneously in this experiment. This corresponds to a tripod gait.

The polar plot (Fig. 6.4(d)) shows no consistent phase preference for hind leg steps in front leg step cycle across the experiments. Only two of the five mean vectors have significant length. One points at 26° of front leg step cycle and the other one at 294°.

In the experiments where no clear phase preference was determined this could be due to a relative coordination pattern between front and hind legs. Therefore the phase of hind leg

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
1	1	7	0,59	0	
	2	8	0,49	5	0,45
	3	6	0,59	0	
	4	11	0,53	11	0,45
	5	9	0,42	4	0,52
	6	19	0,54	16	0,76
	7	6	0,31	12	0,8
	8	14	0,33	33	0,55
	9	9	0,64	9	0,49
2	1	6	0,73	1	
	2	19	0,61	1	
	3	10	0,59	3	0,42
	4	99	0,65	18	0,41
	5	12	0,59	5	0,41

Table 6.2: *Parameters of front and ipsilateral hind leg steps for different stepping sequences of two experiments (see figure 6.3). For the whole table of all experiments see appendix A.*

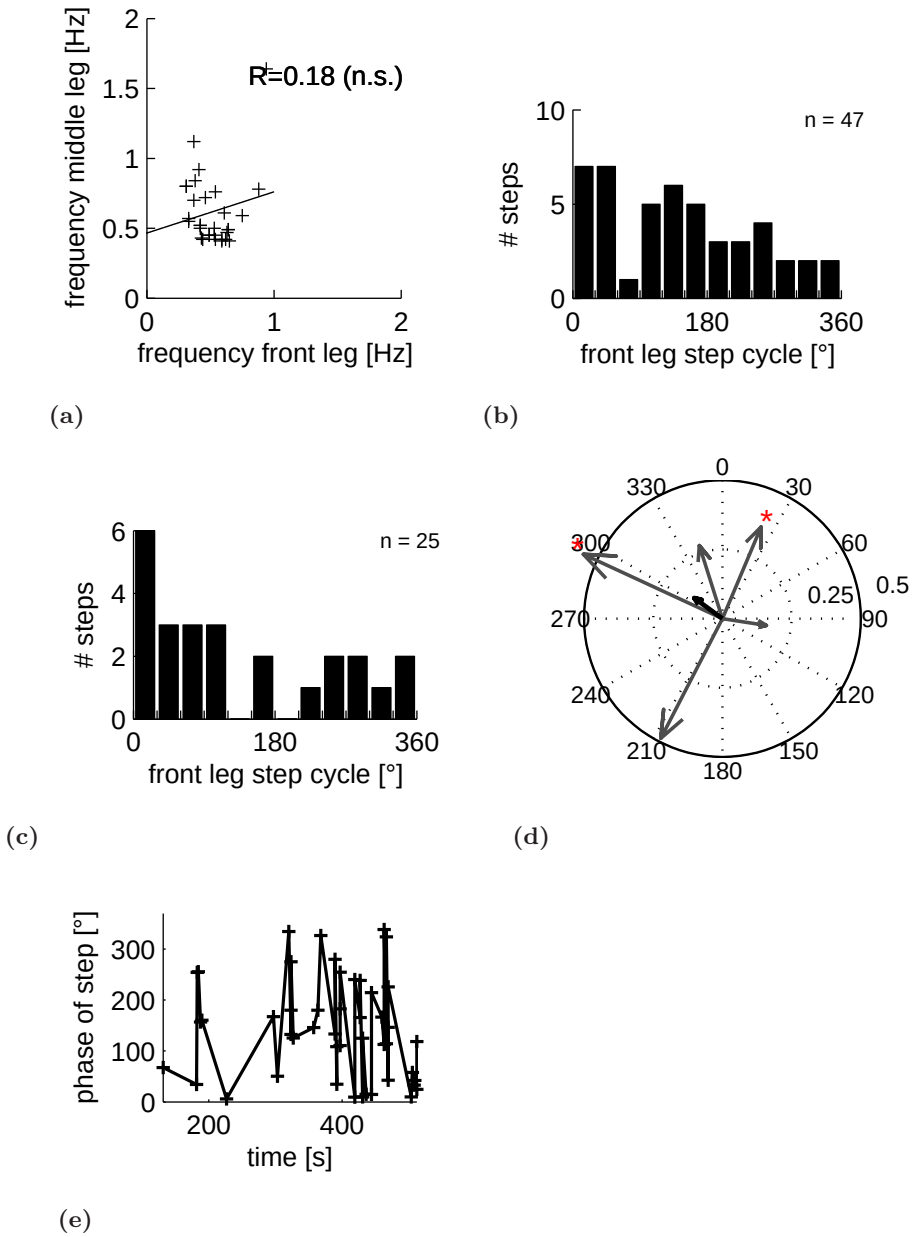


Figure 6.4: *(a) Hind leg stepping frequency plotted against front leg stepping frequency of respective stepping sequence for all stepping sequences of all experiments. (b)-(d) Circular statistics of hind leg steps in front leg step cycle. Distribution of hind leg stance beginnings in front leg step cycle for (b) experiment one and (c) experiment two. (d) Polar plot of mean phase of hind leg stance beginnings in front leg step cycle for five experiments. Red star marks the vectors with significant length. (e) Phase of hind leg stance beginnings in front leg step cycle is plotted against time for experiment one.*

steps in front leg step cycle was plotted against time. This is shown in figure 6.4(e) for the first exemplary experiment. No systematic drifting of hind leg steps in phase over time is recognizable. Hence, front and hind leg stepping did not show relative coordination. This was true for all five experiments.

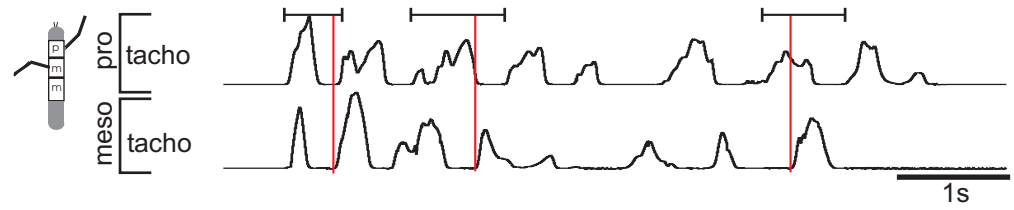
In summary, stepping of front and ipsilateral hind leg did not show a stable coordination pattern in this experimental situation.

6.3 Coordination of contralateral front and middle legs

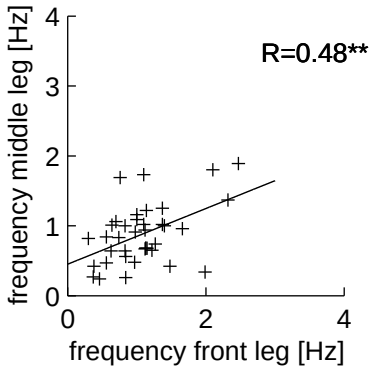
In this set of experiments the coordination between front and contralateral middle legs in the two leg preparation was investigated. The animal was fixed dorsal side up on two small foam platforms. Front and contralateral middle legs remained intact while all other legs were cut. The animal performed stepping movements with each leg on its own passive treadmill (J.P Gabriel and Büschges 2003). Both treadmills were arranged in parallel to the body axis. Nine experiments have been performed in this experimental configuration. One representative experiment is presented here in more detail.

Figure 6.5(a) shows a stepping sequence where the animal stepped with both front and middle legs. It was not necessarily the case that both legs performed stepping movements in each stepping sequence. In nine experiments, stepping of both front and middle legs occurred in 45,3% of all stepping sequences. In 36,1% only the front leg performed stepping movements and in 18,6% the middle leg performed stepping movements while the front leg did not move. Again stepping of one leg did not imply stepping of the other one. Table 6.3 shows the parameters of the stepping sequences for the presented experiment (for the other eight experiments see appendix A). The animal performed 19 stepping sequences. In 16 of these stepping sequences the front leg performed between three and 27 steps with stepping frequencies between 0,59 Hz and 1,37 Hz. The middle leg performed steps in 13 of the 16 stepping sequences. The number of steps varied between three and 22 and the stepping frequencies lay between 0,42 Hz and 1,09 Hz in a similar range as the front leg stepping frequencies.

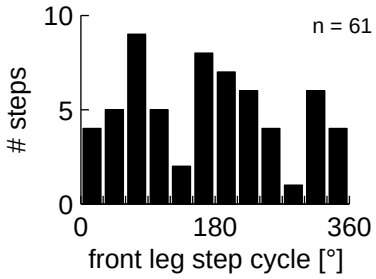
It was analyzed whether there existed a correlation between front and middle leg stepping frequencies. Figure 6.5(b) shows the mean middle leg stepping frequency of a stepping



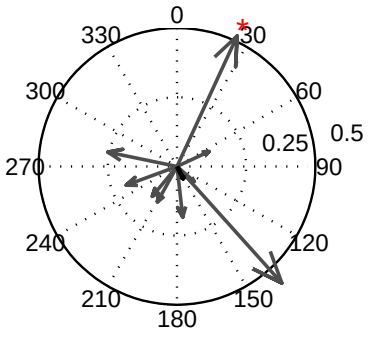
(a)



(b)



(c)



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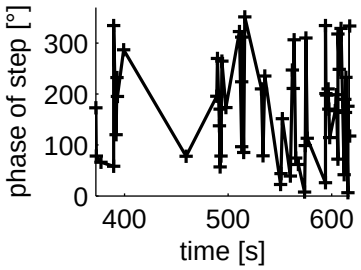


(e)

Figure 6.5: *(a) Stepping sequence of front and contralateral middle leg. (b) Middle leg stepping frequency plotted against front leg stepping frequency of respective stepping sequence for all stepping sequences of all nine experiments. (b)-(c) Circular statistics of middle leg steps in front leg step cycle. (b) Distribution of middle leg stance beginnings in front leg step cycle. (c) Polar plot of mean phase of middle leg stance beginnings in front leg step cycle for nine experiments. Red star marks the vectors with significant length. (d) Phase of middle leg stance beginnings in front leg step cycle is plotted against time.*

sequence plotted against the mean front leg stepping frequency of the same stepping sequence for all experiments. The correlation coefficient is 0,48 and significantly different from zero. It existed a correlation between front leg and middle leg stepping frequencies. In stepping sequences where the the front leg stepped faster the middle leg stepped at a higher frequency as well and vice versa.

The coordination between front and middle leg stepping was analyzed with circular statistics. The histogram (Fig. 6.5(c)) shows the distribution of middle leg steps in front leg step cycle. Three peaks are visible (Fig. 6.5(c)), one around 90° of front leg step cycle, one around 180° and one at 330°. No clear maximum was observed. The polar plot (Fig. 6.5(d)) summarizes the mean vectors of middle leg steps in front leg step cycle for all nine experiments. The mean vectors are distributed over the whole circle. For one of the nine experiments the vector length differs significantly from zero and a preferred phase of middle leg steps in front leg step cycle existed. This vector points in direction of 30°. For the majority of experiments no preferred phase of middle leg steps in front leg step cycle was observed. The stepping pattern of front and middle legs was tested for relative coordination. The phase of middle leg steps in front leg step cycle was plotted against time. This is shown in figure 6.5(e) for the presented experiment. No systematic drifting of middle leg steps in phase over time is recognizable. Hence, front and contralateral middle leg stepping did not show relative coordination. This was true for all nine experiments.
In summary, stepping of front and contralateral middle legs did not show a coordinated stepping pattern in this experimental situation but the front leg stepping frequency and the middle leg stepping frequency were correlated.

6.4 Conclusions

The different legs appear to be fairly independent in these experimental situations. Stepping of one of the two intact legs did not imply stepping of the second leg. This shows again how modular the locomotor system of the stick insect is constructed. There was no coordinated stepping pattern observed in any of the three preparations. The front leg performed steps most frequently. Hardly no stepping sequences were observed where only the

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
1	1	3	0,66	0	
	2	16	0,71	0	
	3	5	0,71	0	
	4	4	0,51	0	
	5	3	0,59	0	
	6	0		4	0,99
	7	14	1,48	3	0,42
	8	19	1,1	5	1,02
	9	0		3	1,43
	10	0		5	0,63
	11	11	0,85	0	
	12	2	0,83	3	0,64
	13	15	1,27	16	0,74
	14	6	0,64	8	1,01
	15	8	0,74	4	0,83
	16	7	1,37	3	1,02
	17	11	1,22	6	0,65
	18	7	1,12	4	0,94
	19	27	1	22	1,09

Table 6.3: *Parameters of front and contralateral middle leg steps for different stepping sequences of one experiments (see figure 6.5(a)). For the whole table of all experiments see appendix A.*

ipsilateral middle or the ipsilateral hind leg performed steps. The stepping frequencies of the two ipsilateral legs were not correlated. Front leg stepping frequency was uncorrelated to the ipsilateral middle leg stepping frequency and to the ipsilateral hind leg stepping frequency. Hence, the ipsilateral neighboring legs were uncoordinated and independent in their stepping frequencies. Stepping frequencies of the front leg and the contralateral middle leg were correlated.

The range of stepping frequencies of front, middle and hind legs was between 0,2 Hz and 2,4 Hz. This frequency range is within the range observed in freely walking animals. The stepping frequency in freely walking stick insects on a horizontal surface is between 0,5 Hz to 4 Hz (Graham 1985b).

CHAPTER 7

Intra- vs. intersegmental influences

In the experiments described in this chapter intra- and intersegmental influences were combined to investigate the effect on mesothoracic MN activity. The stick insect stepped with an intact front leg on a passive treadmill. In addition middle leg campaniform sensilla (CS) were stimulated. Meanwhile the activity of mesothoracic protractor and retractor and levator and depressor MNs was recorded extracellularly.

Campaniform sensilla are strain sensors and provide information about the load on the leg. Three fields of CS are located on the trochanter and one field is located on the femur (Bässler 1983b). The campaniform sensilla are known to play an important role in intrasegmental coordination. Akay et al. (2004) could show that load signals switch on retractor MN activity and switch off protractor activity. Furthermore, they assist the generation of movement-dependent reflex reversal in the femur-tibia joint (Akay and Büschges 2006). Ekeberg et al. (2004) could show in a biomechanical simulation that input from

sense organs is sufficient to coordinate the MN pools of the different joints in order to achieve a proper stepping pattern of the middle leg. Therefore, it is assumed that sensory signals of the middle leg sense organs play a major role in coordinating the motoneural activity in mesothoracic MNs. Sensory information appears to play an important role in intersegmental coordination as well (Graham 1977, 1985a; Ludwar et al. 2005a). As it is crucial for intersegmental coordination that not all ipsilateral legs lift off at a time, load signal should be particularly important.

As the results of chapter 3 and Ludwar et al. (2005a) show, front leg stepping elicits alternating activity in mesothoracic antagonistic MNs. Motoneural activity of the different mesothoracic MN pools is not properly coordinated (Ludwar et al. 2005a). Therefore two questions guided the experiments. 1. Does the middle leg campaniform sensilla stimulation influence mesothoracic MN activity during front leg stepping? 2. Is the effect phase dependent on the front leg step cycle?

7.1 Influences of middle leg campaniform sensilla stimulation on front leg stepping

First, the influence of middle leg campaniform sensilla stimulation on the front leg stepping pattern was investigated. Middle leg campaniform sensilla have been stimulated during front leg stepping. All other legs were cut off (see Materials and Methods 2.2.1, 2.2.4). 18 experiments have been performed in this set of experiments. For each of the 18 experiments multiple series of middle leg campaniform sensilla stimulations were conducted. The stimulations have been performed with different constant frequencies between 0,16 Hz and 0,7 Hz (Fig. 7.1).

In a first analysis it was tested whether the stimulation frequency of middle leg campaniform sensilla had any influence on the front leg stepping frequency. Therefore the stimulation frequency was plotted against the mean stepping frequency during respective stimulation (Fig. 7.2(a)) for all experiments. The correlation coefficient is 0,04 and not significantly different from zero. Hence, no correlation existed between the stimulation

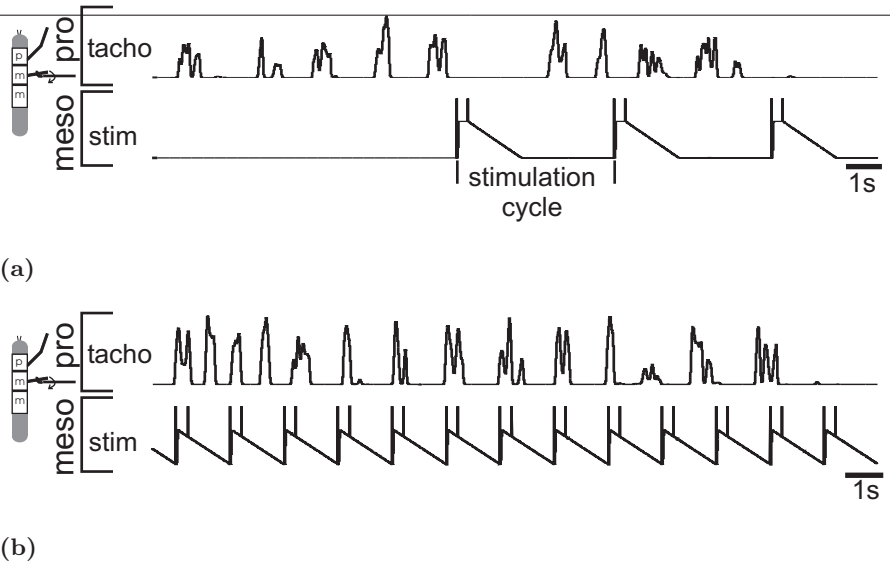


Figure 7.1: *Front leg stepping sequence during middle leg campaniform sensilla stimulation with (a) 0,25 Hz (b) 0,7 Hz.*

frequency of middle leg campaniform sensilla and the front leg stepping frequency.

Next, circular statistics were used to analyze the occurrence of front leg steps in the campaniform sensilla stimulation cycle. One stimulation cycle was defined from the beginning of the ramp to the beginning of the next one (Fig. 7.1(a)) The histogram (Fig. 7.2(b)) shows the distribution of front leg steps in the campaniform sensilla stimulation cycle for one experiment and 47 stimulations. The beginning of front leg stance was summed for each class. There are three maxima at 180°, 270° and 330° but no clear phase preference. Similar results were obtained for other stimulation frequencies and in the other experiments. The polar plot summarizes the mean vectors of front leg steps in the stimulation cycle for 18 different experiments and the different stimulation frequencies (Fig. 7.2(c)). The vectors are distributed over the whole circle. Only for one stimulation frequency of one experiment the vector length differs significantly from zero. Circular statistics did not reveal a phase preference of front leg steps in campaniform sensilla stimulation cycle. This could be due to relative coordination (Wendler 1964b). Therefore the phase of the step is plotted against time (Fig. 7.2(d)). No drifting and resetting of the phase is visible. Hence, relative coordination can be excluded.

In summary, no matter what stimulation frequency was used, the stimulation of middle

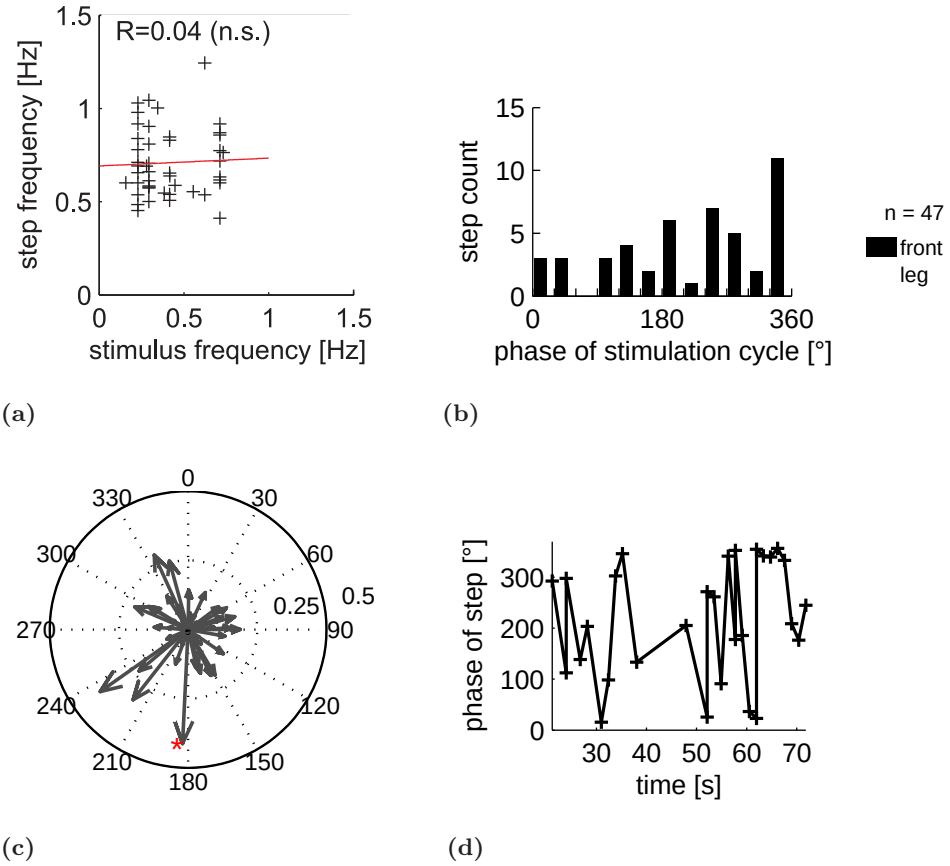


Figure 7.2: *(a) Mean stepping frequency of front leg steps plotted against respective stimulation frequency of middle leg campaniform sensilla. (b) Distribution of front leg steps in middle leg campaniform sensilla stimulation cycle (stimulation frequency 0,7 Hz) ($n = 47$). (c) Polar plot: mean vector of front leg stance in middle leg campaniform sensilla stimulation cycle for the different stimulation frequencies of 18 experiments ($N_{ges} = 46$). Red star marks the vectors with significant length. (d) Phase of the beginning of front leg steps plotted against time. No relative coordination was observed.*

leg campaniform sensilla did not have any influence on the beginning of front leg steps. This allowed to disregard the influence of middle leg campaniform sensilla stimulation on front leg stepping and to assume that the intersegmental influence of front leg stepping and the intrasegmental of the campaniform sensilla act unidirectional on the mesothoracic motoneuronal activity without affecting each other.

7.2 Influences on mesothoracic protractor and retractor MNs

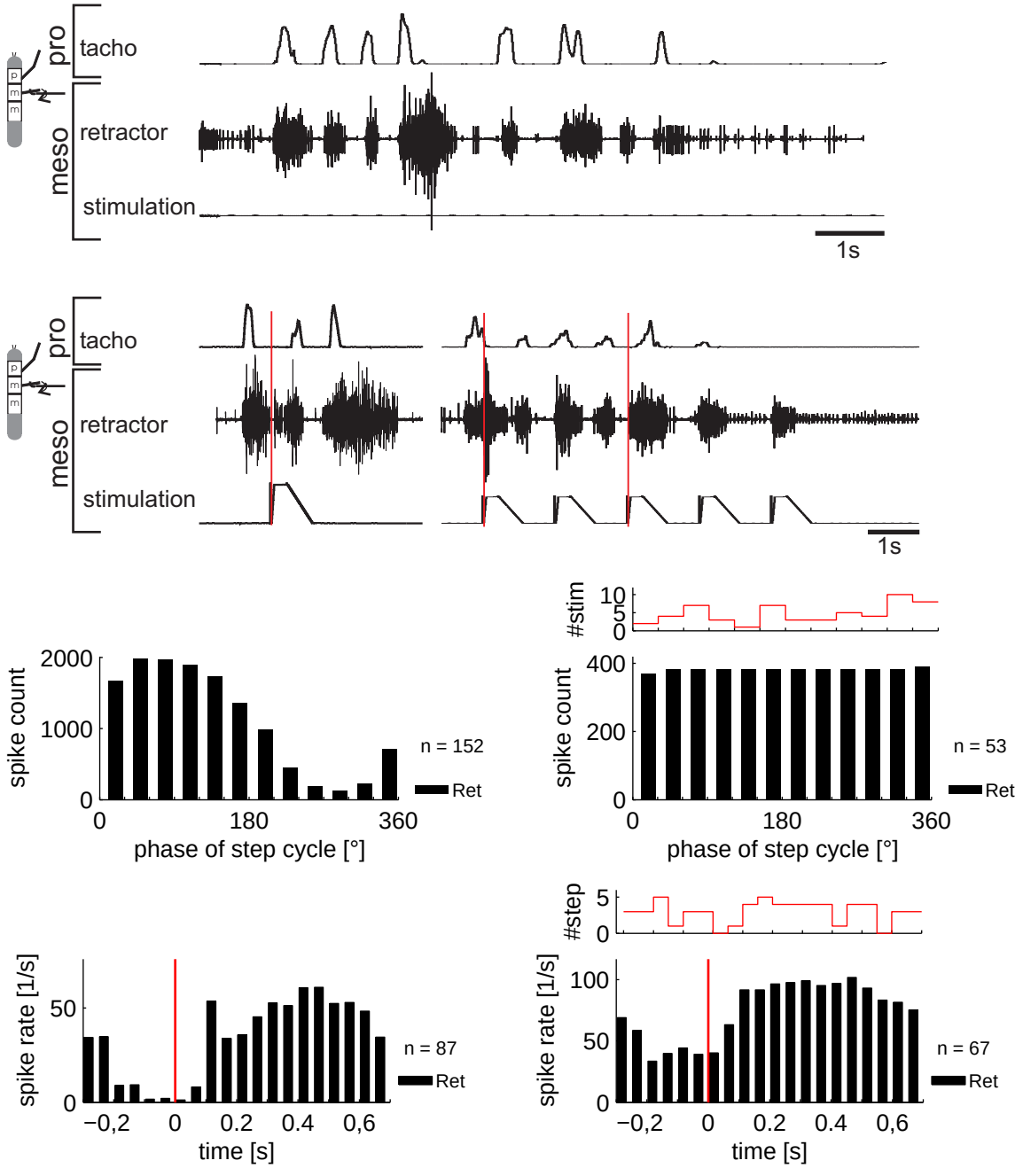
In this set of experiments the combined influence of front leg stepping and middle leg campaniform sensilla stimulation on mesothoracic protractor and retractor MNs was investigated. The intact front leg stepped on a passive treadmill while ipsilateral mesothoracic retractor MNs were recorded extracellularly. The mesothoracic ganglion was deafferented except for the *nervus cruris* that contains the afferent axons of the campaniform sensilla (Bässler 1983b). Middle leg campaniform sensilla were stimulated with frequencies between 0,16 Hz and 0,7 Hz. At low frequencies campaniform sensilla stimulation occurred in arbitrary phases of the front leg step cycle every few steps.

Figure 7.3(a) shows an extracellular recording of retractor MNs during a front leg stepping sequence. Mesothoracic retractor MNs were active in the first half of front leg step cycle. The additional stimulation of the middle leg campaniform sensilla caused an increase in retractor MN activity (Fig. 7.3(b)). Either retractor MNs became active or retractor MN activity further increased when retractor MNs had been active. Data was first analyzed with respect to front leg stepping and to campaniform sensilla stimulation separately. Phase histograms of retractor MN activity in front leg step cycle and peri stimulus time histograms with respect to CS stimulation were calculated. Figure 7.3(c) shows the distribution of retractor MN activity in front leg step cycle for 152 steps without campaniform sensilla stimulation. Retractor MN activity increased in the first half of the step cycle with a maximum around 90°. Figure 7.3(d) shows the distribution of retractor MN activity in front leg step cycle for 53 steps with campaniform sensilla stimulation. The red stair

function on top of the histogram shows the number of middle leg campaniform sensilla stimulations in each class. Retractor MN activity was evenly distributed in front leg step cycle. The campaniform sensilla stimulation switches on retractor MN activity also in the second half of front leg step cycle.

The influence of campaniform sensilla stimulation on retractor MN activity was analyzed with peri stimulus time histograms. Figure 7.3(e)) shows the averaged retractor MN activity from 0,3 s before to 0,7 s after the stimulus for 87 campaniform sensilla stimulations without front leg stepping when the animal was at rest. Before the stimulus retractor MN activity was low around 13 spikes/s. In the second bin 0,05 s after the stimulus mean spike rate increased until it reached a plateau of approx. 64,5 spikes/s 0,2 s after the stimulus. This equals a mean increase in retractor MN spikes rate of 79,9%. The second histogram (Fig. 7.3(e)) shows the mean spike rate before and after the stimulus for 67 stimulations during front leg stepping. The stair function on top of the histogram shows the number of steps in each class. Right before the stimulus mean retractor MN spike rate was around 39 spikes/s, increased in the second bin 0,05s after the stimulus and reached a plateau around 96 spikes/s 0,1 s after the stimulus. This corresponds to a relative increase in mean spike rate of 59,4%. For stimulation during a stepping sequence the retractor MN activity before the stimulus was on average higher and the relative activity increase after the stimulus was 20% lower although a higher absolute spike rate was reached. The increase in retractor MN activity in response to the stimulus appears to add up on the activity elicited by the steps.

The following analysis takes both influences, front leg stepping and middle leg campaniform sensilla stimulation, into account. Retractor MN activity before and after the stimulus was compared with respect to front leg step cycle. The front leg step cycle was divided into ten classes. The retractor MN activity 0.5 s before and 0.5 s after the stimulus was determined and averaged for the different classes depending on, in which phase of the step cycle the stimulus was given (Fig. 7.4(a)). The histogram shows the mean spike count of retractor MNs before the campaniform sensilla stimulation and afterwards for each class. The stair function on top of the histogram shows the number of stimulations in each class. The histogram shows that a campaniform sensilla stimulation given in the first 108° of a front leg step cycle increased retractor MN activity significantly. Between


pro
tacho
meso
retractor
stimulation
1s
pro
tacho
meso
retractor
stimulation
1s
#stim
10
5
0
spike count
2000
1000
0
n = 152
Ret
0
180
360
phase of step cycle [°]
#stim
10
5
0
spike count
400
200
0
n = 53
Ret
0
180
360
phase of step cycle [°]
#step
5
0
spike rate [1/s]
50
0
n = 87
Ret
−0,2
0
0.2
0.4
0,6
time [s]
#step
5
0
spike rate [1/s]
100
50
0
n = 67
Ret
−0,2
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0.2
0.4
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time [s]

Figure 7.3: *(a) Mesothoracic retractor MN activity during front leg stepping. (b) Mesothoracic retractor MN activity during front leg stepping and middle leg campaniform sensilla stimulation. Retractor MN activity increased with the stimulation. (c) Distribution of mesothoracic retractor MN activity in front leg step cycle for 152 steps without middle leg campaniform sensilla stimulation. Retractor MN activity had a maximum around 90° of front leg step cycle. (d) Distribution of mesothoracic retractor MN activity in front leg step cycle for 53 steps during middle leg campaniform sensilla stimulation. The red stair function on top of the histogram shows the number of stimulations in each class. Retractor MN activity was evenly distributed in front leg step cycle. (e) Stimulus time histogram: Distribution of retractor MN activity from 0,3 s before to 0,7 s after middle leg campaniform sensilla stimulation for 87 stimulations without front leg stepping. Stimulation increased retractor MN spike rate on average by 79,9%. (f) Stimulus time histogram: Distribution of retractor MN activity from 0,3 s before to 0,7 s after middle leg campaniform sensilla stimulation for 67 stimulations during front leg stepping. The red stair function on top of the histogram shows the number of steps in each class. Stimulation increased retractor MN spike rate on average by 59,4%.*

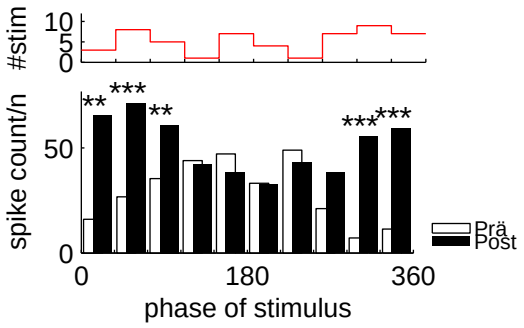
108° and 252° retractor MN activity did not change when a stimulus was given. From 252° to 360° campaniform sensilla stimulation increased retractor MN activity. The increase was significant in the last two classes. The second histogram (Fig. 7.4(b)) shows the relative difference between the activity before and after the stimulus for each class. In the first three classes the retractor MN activity increased after the stimulus by 50% and more. As well as in the last three classes. In between, in classes four to seven, retractor MN activity did not change or even decreased up to 20%. From these two histograms it appears as though the stimulus had less effect around 180° of front leg step cycle. This had to be tested. The mean values in the different classes were tested for significant differences (t-test). This is shown in the color plots (Fig. 7.4). On the x- and the y-axis of the square the ten classes of front leg step cycle (Fig. 7.4(a)) are assigned. In the first color plot (Fig. 7.4(d)) the mean retractor MN activity before the stimulus was compared for all classes (Fig. 7.4(c)). The value of the first class was tested for significant difference to the value of the second, the third, the fourth class and so on. The darker a color is the higher the level of significance between the two values. Blue colors signal a decrease, red colors an increase in activity from one class to the other. Grey fields mark fields where

not enough data existed to do a test for significance between values of respective classes, a white field means no significant difference. The plot shows that the mean spike count before the stimulus was significantly higher in the fifth class compared to that in class one and two and significantly lower in the classes nine and ten compared to classes two, three and four. Hence, activity before the stimulus tended to be higher around 180° of front leg step cycle than at the beginning and the end.

In figure 7.4(e) the mean retractor MN spike count after the stimulus (Fig. 7.4(e)) was compared between the different classes. The mean retractor MN spike count was significantly higher in the first three classes compared to the sixth class and in the second class compared to the values in the classes five, six, eight and nine. Hence, although the value before the stimulus was even higher in the classes around 180° of front leg step cycle the value after the stimulation was significantly lower in theses classes compared to the values of the classes at the beginning and the end of the step cycle. The same results were obtained by comparing the relative differences between the mean spike count before and after campaniform sensilla stimulation in the different classes (Fig. 7.4(f)). The relative change in mean retractor MN spike count before and after the stimulus was significantly lower in the classes five and six compared to that in the classes one and/or two. The relative increase was significantly higher in the classes nine and ten compared to that in the classes five and six and the classes two to eight.

This was true for six of eleven experiments where only the retractor MN activity was recorded. In three experiments the retractor MN activity strongly increased with every middle leg campaniform sensilla stimulation no matter in which phase the stimulus was given. In two experiments there were too few stimulations to analyze. This indicates that there existed a phase in front leg step cycle where retractor MNs did not increase or even decreased with middle leg campaniform sensilla stimulation.

In four experiments protractor and retractor MN activity had been recorded. In two of four experiments retractor MNs decreased and protractor MN activity increased for middle leg campaniform sensilla stimulations around 180° of front leg step cycle. This is shown here for one experiment in more detail. The extracellular recording of protractor and retractor MN activity during front leg stepping (Fig. 7.5(a)) shows the characteristic alternating activity with retractor MNs being active in the first half of front leg stepping


#stim
10
5
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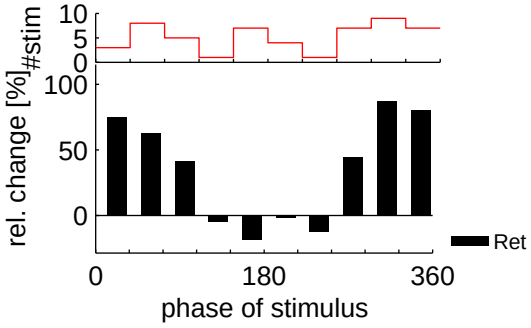
(a)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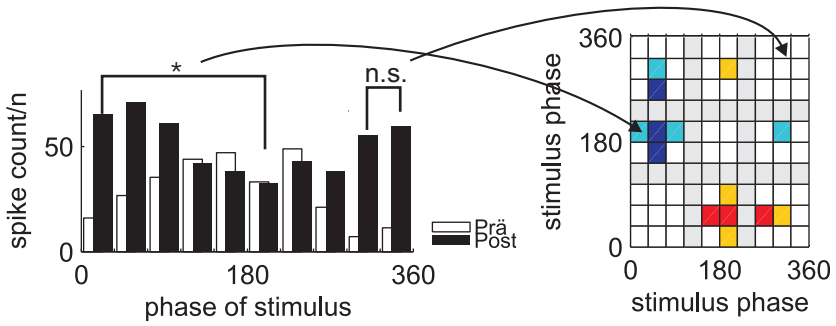
phase of stimulus

(b)


*
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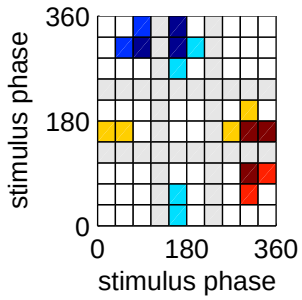
(c)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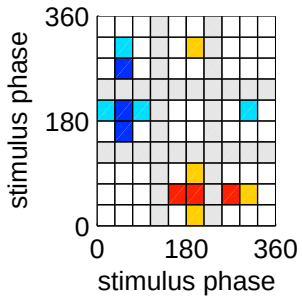
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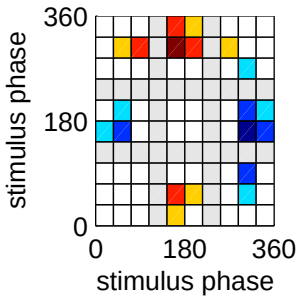
(e)


stimulus phase
360
180
0
0
180
360
stimulus phase

(f)

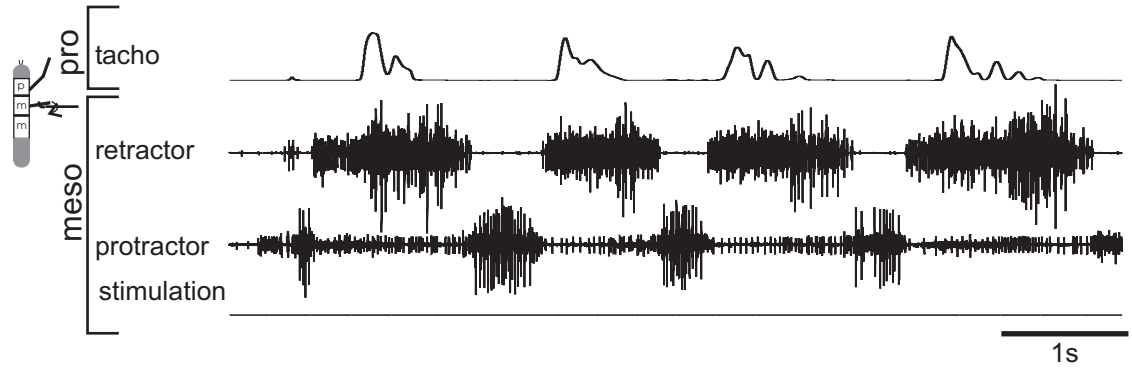
Figure 7.4: *(a) Mean spike count of retractor MNs 0,5s before (white bars) and 0,5s after (black bars) middle leg campaniform sensilla stimulation for different phases of front leg step cycle. Stars on top of the bars mark the level of significance for the difference in mean spike count before and after the stimulus (( ) not significant;* $(*)$: $0,01 \leq P < 0,05$; $(**)$: $0,001 \leq P < 0,01$; $(***)$ $P < 0,001$*). The red stair function on top of the histogram shows the number of stimulations in each class. Retractor MN activity was significantly increased by middle leg campaniform sensilla stimulation at the beginning and the end of front leg step cycle. (b) Relative change in mean spike count before and after the stimulus. Retractor MN activity increased by 50% and more when a stimulus was given between 0° and 108° and 252° and 360° and stayed the same or decreased for a stimulus in between. (c) Explanation to subfigure (d). The value of the first class was tested for significant differences to the value of the second, the third, the fourth class and so on. The darker a color the higher the level of significance (from* $P < 0,05$ *to* $P < 0,001$*) between the two values. Blue colors signal a decrease, red colors an increase in retractor MN activity from one class to the other. Grey fields mark fields where not enough data existed, a white field means no significant difference. (d) Significance plot between mean spike counts of retractor MNs before the stimulus for the ten classes (see histogram (a)). Retractor MN activity was significantly higher in classes between 108° and 252° before the stimulus. (e) Significance plot between mean spike counts of retractor MNs after the stimulus for the ten classes (see histogram (a)). Retractor MN activity was significantly higher in classes between 0°and 108° and 252° and 360° after the stimulus. (f) Significance plot between relative changes in retractor MN activity after the stimulus for the ten classes (see histogram (b)). The increase in retractor MN activity was significantly higher in classes between 0°and 108° and 252° and 360°.*

cycle and protractor MNs in the second half. Middle leg campaniform sensilla stimulation during front leg stepping increased retractor MN activity (Fig. 7.5(b)) even when retractor MNs were already active. The histogram (Fig. 7.4(a)) with the relative differences between the mean spike count before and after the stimulus shows that retractor MN activity increased between 42% and 96% after the stimulus and protractor MN activity decreased by 75% and more in all classes except for the two classes between 180° and 252°. In this classes the effect was reversed, protractor MN activity increased by 77,8% and 100% and retractor MN activity changed by -31% and 26%. It appears that around 180° of front leg step cycle the effect of middle leg campaniform sensilla stimulation on protractor and retractor MNs was reversed. In the single middle leg preparation this effect was not obtained (Akay et al. 2004). This has to be a phenomenon of intersegmental influences.

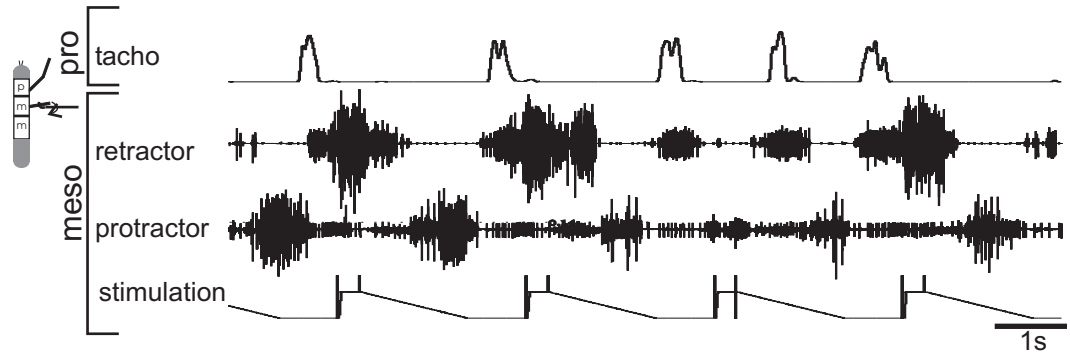
In two experiments the influence of front leg stepping was still present but clearly dominated by the influence of the campaniform sensilla stimulation. No matter in which phase of the step cycle the stimulus was given the retractor MN activity increased. The extracellular recording in figure 7.6(a) shows the alternating activity in mesothoracic protractor and retractor MNs. Stimulation of middle leg campaniform sensilla induced an immediate switch to retractor MN activity (Fig. 7.6(b)). The histogram (Fig. 7.6(c)) shows the relative change in mean spike count after the stimulus. In every phase of the step cycle campaniform sensilla stimulation increased retractor MN activity between 41,9% and 90,7% and decreased protractor MN activity (48,8% to 80,2%).

In all experiments a co-existence of the two influences, the intersegmental influence of front leg stepping and the intrasegmental one of middle leg campaniform sensilla stimulation, was observed. This becomes clearly visible in the series of stimulus histograms (Fig. 7.7). The vertical red line marks the phase of stimulation. The stimulation moves along in the step cycle. The influence of front leg steps is still visible. Retractor MNs were active in first half of the step cycle protractor MNs in the second half. Depending on the stimulus phase retractor MN activity was additionally increased or activity switched from protractor to retractor MN activity except for a small phase window around 180° of front leg step cycle. Here retractor MN activity was decreased and protractor MN activity was increased (Fig. 7.7).

In summary, mesothoracic protractor and retractor MNs were influenced by front leg



(a)



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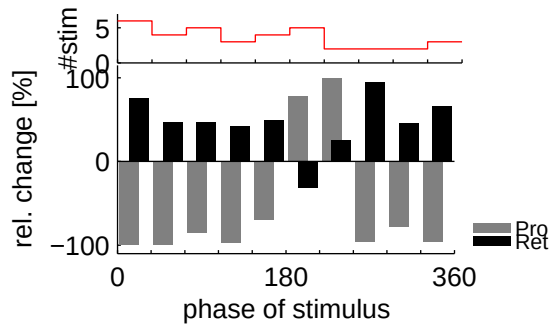
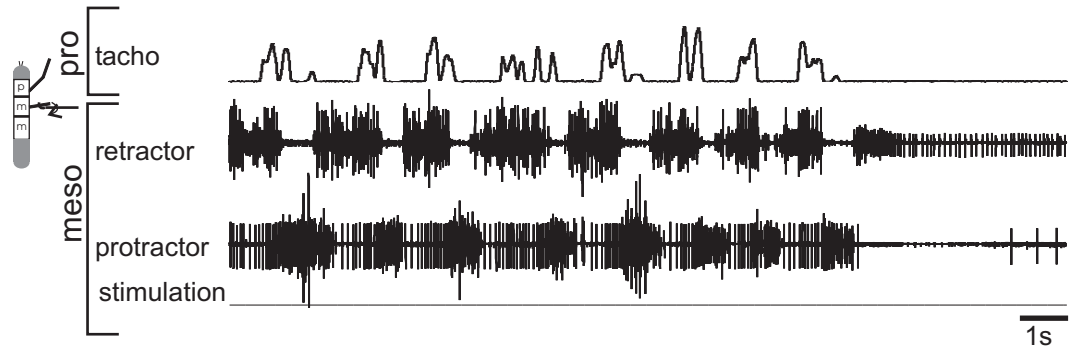
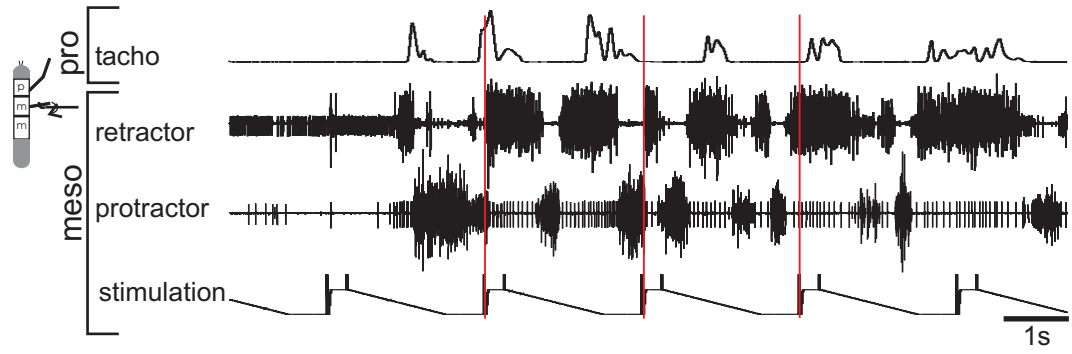


(c)

Figure 7.5: *(a) Mesothoracic protractor and retractor MN activity during front leg stepping. (b) Mesothoracic protractor and retractor MN activity during front leg stepping and simultaneous middle leg campaniform sensilla stimulation. Retractor MN activity increased with the stimulation. (c) Relative change in mean spike count before and after the stimulus for protractor (grey bars) and retractor (black bars) MNs. The red stair function on top of the histogram shows the number of stimulations in each class. Retractor MN activity increased between 42% and 96% after the stimulus and protractor MN activity decreased by 75% and more in all classes except for the two classes between 180° and 252°. Here protractor MN activity increased by 77,8% and 100% and retractor MN activity changed by -31% and 26%.*



(a)



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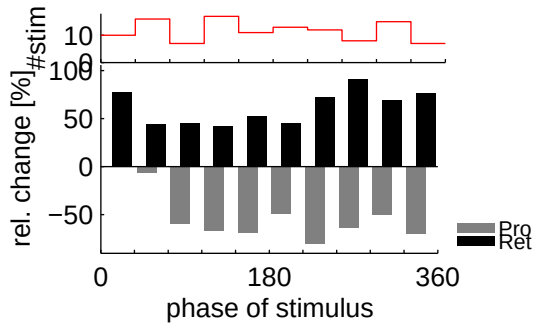

(c)

Figure 7.6: *(a) Mesothoracic protractor and retractor MN activity during front leg stepping. (b) Mesothoracic protractor and retractor MN activity during front leg stepping and simultaneous middle leg campaniform sensilla stimulation. Retractor MN activity increased with the stimulation. (c) Relative change in mean spike count before and after the stimulus for protractor (grey bars) and retractor (black bars) MNs. The red stair function on top of the histogram shows the number of stimulations in each class. Retractor MN activity increased between 41,9% and 0,7% after the stimulus and protractor MN activity decreased between 48,8% to 80,2% in all classes. No phase dependent insensitivity to middle leg campaniform sensilla stimulation was observed.*

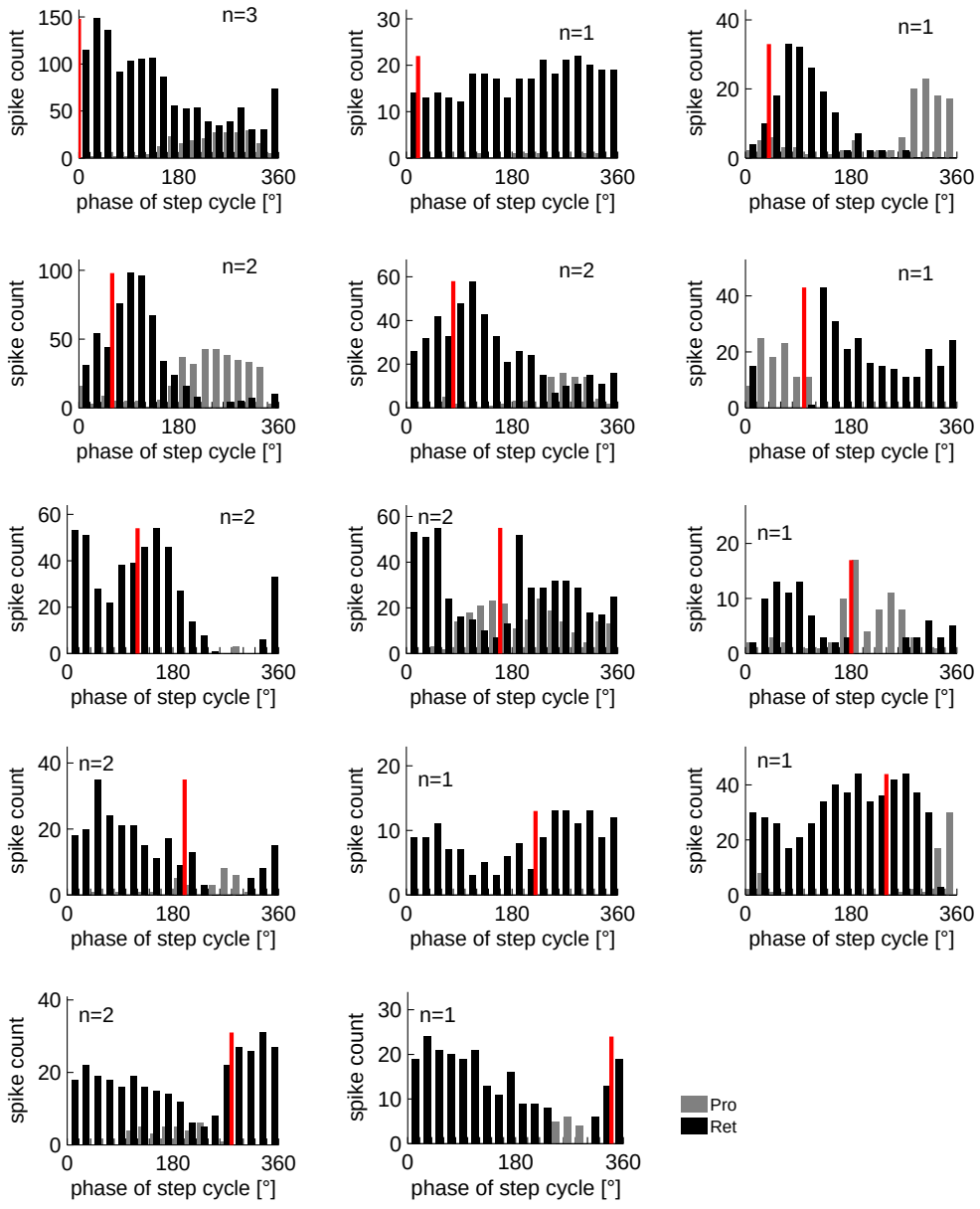

Figure 7.7: *Distribution of mesothoracic protractor and retractor MN activity in front leg step cycle for different phases of middle leg campaniform sensilla stimulation. The red vertical line marks the phase of stimulation. n accounts for the number of steps averaged in respective histogram. The series shows the co-existence of the intersegmental influence of front leg stepping and the intrasegmental influence of campaniform sensilla stimulation. The retractor MN activity increased in the first half of front leg step cycle induced by front leg stepping. The stimulus in addition increased retractor MN activity at the beginning and the end of front leg step cycle. Only around 180° the stimulation decreased retractor MN activity and increased protractor MN activity.*

stepping as well as by middle leg campaniform sensilla stimulation. In eight of 15 experiments around 180° of front leg step cycle middle leg campaniform sensilla stimulation decreased retractor MN activity.

7.3 Influences on mesothoracic levator and depressor MNs

In this set of experiments the influence of front leg stepping and middle leg campaniform sensilla stimulation on mesothoracic levator and depressor MNs was investigated.
Levator and depressor MN activity was dominated by the influenced of middle leg campaniform sensilla stimulation. The extracellular recording of levator and depressor MN activity during front leg stepping (Fig. 7.8(a)) shows increased levator MN activity whereas depressor MNs fell completely silent. Stimulation of middle leg campaniform sensilla switched on depressor MN activity whereas levator MNs fell silent (Fig. 7.8(b)). Sometimes depressor MN activity increased not only in response to the increase of strain but also to the release (Fig. 7.8(b)). Data was first analyzed with respect to front leg stepping and to campaniform sensilla stimulation separately. Phase histograms show the distribution of levator and depressor MN activity in front leg step cycle (Fig. 7.8(c), 7.8(d)). The first histogram (Fig. 7.8(c)) shows the distribution of levator and depressor MN activity for 82 front leg steps where no campaniform sensilla stimulation was present (Fig. 7.8(c)). Depressor MN activity was slightly increased at the beginning of front leg step cycle with a maximum of 117 spikes between 0° and 30° of front leg step cycle and then decreased. Levator MN activity was increased over the whole step cycle with a maximum of 848 spikes between 150° and 180°. The second phase histogram (Fig. 7.8(d)) shows the distribution of levator and depressor MN activity for 33 front leg steps with campaniform sensilla stimulation being present. The stair function on top of the histogram shows the number of stimulations in each class. Levator MN activity still showed a phase preference in front leg step cycle although the maximum of activity was slightly shifted to 120° of front leg step cycle. This may be in part due to the fact that in the range between 30° and 120° of front leg step cycle less stimulations occurred. Nevertheless, there was still a structure in the distribution of levator MN activity whereas depressor MN activity was evenly distributed

over the whole step cycle. Campaniform sensilla stimulation overwhelms the small effect of front legs steps.

The influence of middle leg campaniform sensilla stimulation on levator and depressor MNs was analyzed with peri stimulus time histograms (Fig. 7.8(e), 7.8(f)). The first of the peri stimulus histograms (Fig. 7.8(e)) shows the effect of campaniform sensilla stimulation on levator and depressor MN activity for 18 stimulations without front leg steps being present when the animal was at rest. Mean spike rate of levator MNs was around 63 spikes/s before the stimulus. In the second class 0,1 s after the stimulus the spike rate decreased to zero and started to increase again very slowly 0,5 s after the stimulus. Mean spike rate of depressor MNs was 7,6 spikes/s before the stimulus. In the second class 0,1 s after the stimulus the spike rate increased with a maximum of 67,8 spikes/s in the fourth class 0,15 s to 0,2 s after the stimulus. Then depressor MN spike rate slowly decreased again. Middle leg campaniform sensilla stimulation induced switching from levator MN activity to depressor MN activity. The second stimulus time histogram (Fig. 7.8(f)) shows the distribution of mean spike rates of levator and depressor MNs before and after campaniform sensilla stimulation (n=42) during front leg steps. The stair function on top of the histogram shows the number of steps in each class. The steps were not evenly distributed in this case. There were only two steps in the classes before the stimulation. Levator MN activity was on average increased before the simulation with approximately 77,5 spikes/s and decreased in the second class 0,2 s after the stimulation to zero. Spike rate started increasing again 0,3 s after the stimulus. Depressor MN spike rate was low before the stimulus (8,3 spikes/s) and increased in the second class after the stimulus with a maximum spike rate of 65,7 spikes/s in the fourth class 0,15 s to 0,2 s after the stimulus. Then spike rate slowly decreased again. There was hardly no difference in spike rate and distribution of levator and depressor MN activity no matter if the stimulation was given during front leg steps or not. The following analysis takes both influences, front leg stepping and middle leg campaniform sensilla stimulation, into account. Levator and depressor MN activity before and after the stimulus was compared with respect to front leg step cycle. The front leg step cycle was divided into ten classes. The depressor MN activity 0.5 s before and 0.5 s after the stimulus was determined and averaged for the different classes depending on, in which phase of the step cycle the stimulus was given

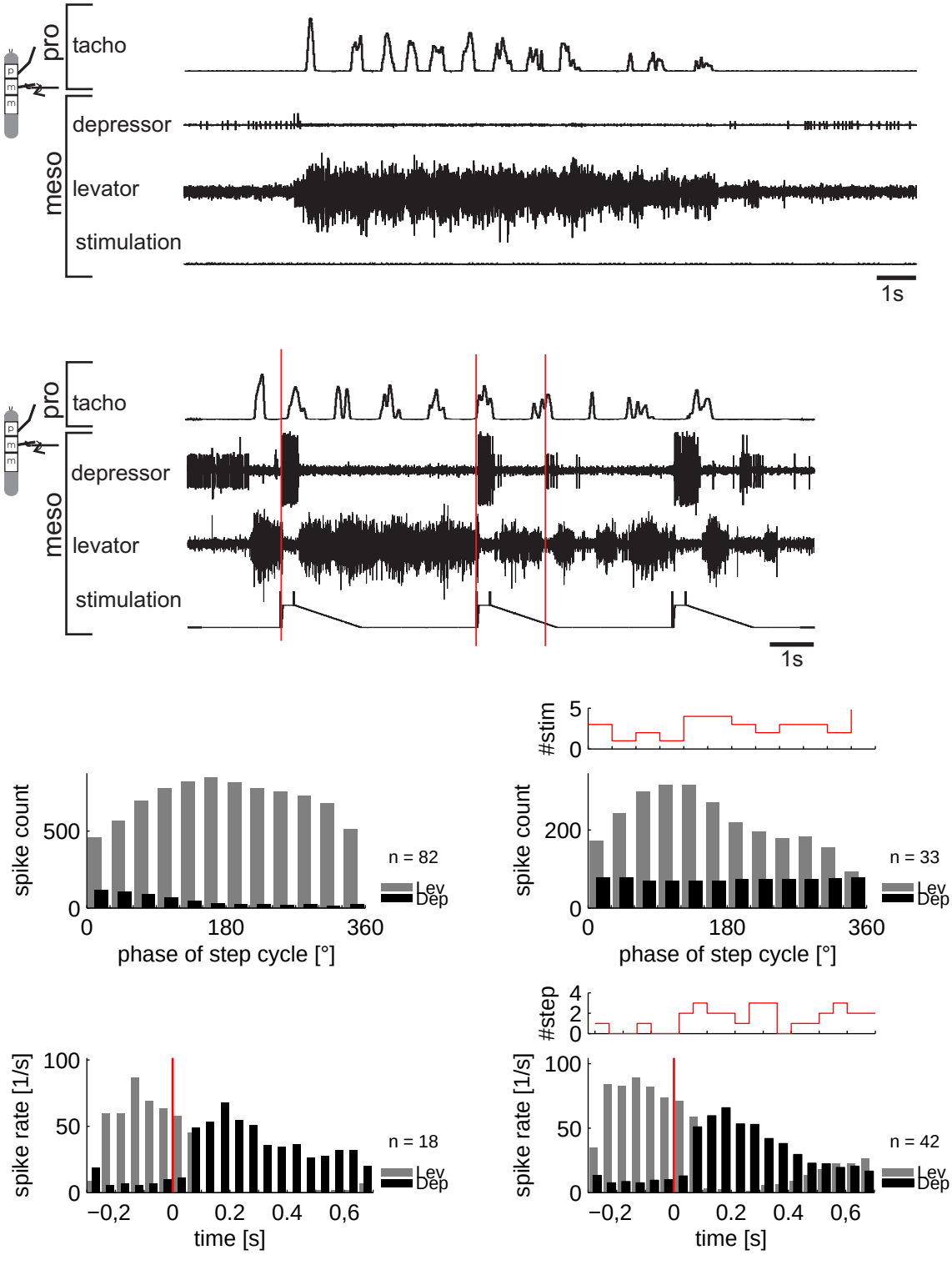
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Figure 7.8: *(a) Mesothoracic levator and depressor MN activity during front leg stepping. (b) Mesothoracic levator and depressor MN activity during front leg stepping and simultaneous middle leg campaniform sensilla stimulation. Stimulation switched on depressor activity and switched of levator activity. (c) Distribution of mesothoracic levator and depressor MN activity in front leg step cycle for 82 steps without middle leg campaniform sensilla stimulation. Levator MN activity was increased over the whole step cycle with a maximum between 150˚ and 180˚. Depressor MN activity had a maximum between 0˚ and 30˚ of front leg step cycle. (d) Distribution of mesothoracic levator and depressor MN activity in front leg step cycle for 33 steps with middle leg campaniform sensilla stimulation. The red stair function on top of the histogram shows the number of stimulations in each class. Levator MN activity had a maximum at 120˚. Depressor MN activity was evenly distributed in front leg step cycle. (e) Stimulus time histogram: Distribution of levator and depressor MN activity from 0,3 s before to 0,7 s after middle leg campaniform sensilla stimulation for 42 stimulations without front leg steps. Stimulation increased mean retractor MN spike rate by 79,9%. (f) Stimulus time histogram: Distribution of retractor MN activity from 0,3 s before to 0,7 s after middle leg campaniform sensilla stimulation for 67 stimulations during front leg steps. The red stair function on top of the histogram shows the number of steps in each class. Stimulation increased mean retractor MN spike rate by 59,4%.*

(Fig. 7.9(a)). The histogram shows the mean spike count of depressor MNs before the campaniform sensilla stimulation and afterwards for each class. The stair function on top of the histogram shows the number of stimulations in each class. Middle leg campaniform sensilla stimulation induced in all classes an increase in depressor MN activity that was in seven classes significantly different from zero. Figure 7.9(b) shows the levator MN activity 0.5 s before and 0.5 s after the stimulus. Levator MN activity decreased in nine of ten classes. The relative difference between motoneural activity before and after the stimulus is shown in figure 7.9(c). Depressor MN activity increased by 60% to 100% after CS Stimulation. No matter in which phase of front leg step cycle. Levator MN activity decreased in nine of ten classes by 80% to 100%.

The coupling to campaniform sensilla stimulation regardless of front leg stepping becomes visible in the series of phase histograms in figure 7.10. Distribution of levator and depressor MN activity in front leg step cycle is shown for different phases of stimulation. One can see how the stimulus moves along the phase of the step cycle and with it the

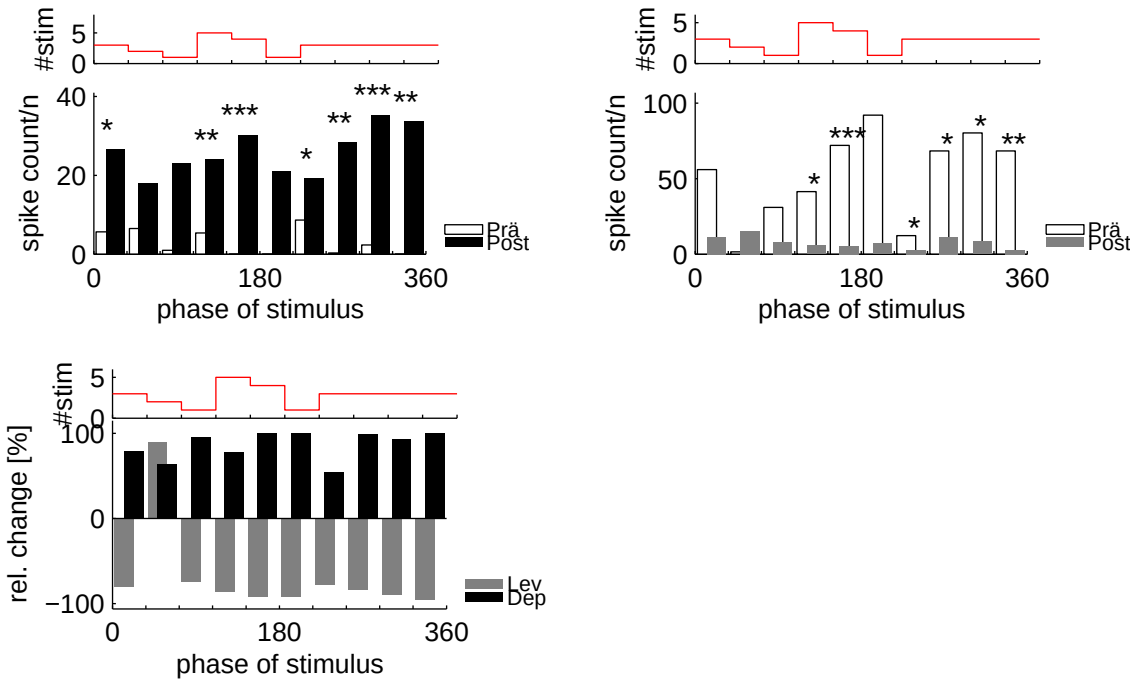

Figure 7.9: *(a) Mean spike count of depressor MNs 0,5s before (white bars) and 0,5s after (black bars) middle leg campaniform sensilla stimulation for different phases of front leg step cycle. Stars on top of the bars mark the level of significance for the difference in mean spike count before and after the stimulus (( ) not significant;* $(*)\ 0,01 \leq P < 0,05; (**)\ 0,001 \leq P < 0,01; (* \ * \ *)\ P < 0,001$*). The red stair function on top of the histogram shows the number of stimulations in each class. Depressor MN activity was significantly increased by middle leg campaniform sensilla stimulation for a stimulus in every phase of front leg step cycle. (b) Mean spike count of levator MNs 0,5 s before (white bars) and 0,5 s after (grey bars) middle leg campaniform sensilla stimulation for different phases of front leg step cycle. Levator MN activity was significantly decreased by middle leg campaniform sensilla stimulation for a stimulus in every phase front leg step cycle. (c) Relative change in mean spike count before and after the stimulus. Depressor MN activity increased by 80% and more when a stimulus was given and levator MN activity decreased by 80% and more.*

activation of depressor MNs.

Under these experimental conditions hardly any intersegmental influence of front leg stepping was observed. Front leg stepping led to an increase in levator MN activity and depressor MNs fell silent. This was the case in all five experiments. Levator and depressor MN activity was completely coupled to the campaniform sensilla stimulation and showed no alternating activity during front leg stepping.

7.4 Conclusions

The stimulation of middle leg campaniform sensilla did not have any measurable influence on front leg stepping. This does not necessarily mean that there is none but that it is very weak and not detectable in this experimental situation especially as front leg stepping as such is so irregular that a weak influence might be impossible to resolve. In the presented sets of experiments it was an advantage that the influence of middle leg campaniform sensilla stimulation was very weak. Therefore this influence could be disregarded.
The experimental setup allowed adding one sensory influence to the well known single front leg preparation (Ludwar et al. 2005a, b). Mesothoracic protractor and retractor MN activity and levator and depressor MN activity were investigated under the influence of front leg stepping and middle leg campaniform sensilla stimulation. Mesothoracic protractor and retractor MN activity was influenced by front leg stepping and middle leg campaniform sensilla stimulation. Front leg stepping induced alternating activity in protractor and retractor MNs. Retractor MN activity increased during front leg stance protractor MN activity during front leg swing. This corresponds to the results in the single leg preparation (see chapter 3 and Ludwar et al. (2005a)). The stimulation of middle leg campaniform sensilla induced an increase of retractor MN activity and a decrease in protractor MN activity for stimulations at the beginning and the end of front leg step cycle. This corresponds to the observations of campaniform sensilla stimulation in the single middle leg preparation (Akay et al. 2004). For middle leg campaniform sensilla stimulation around 180° of front leg step cycle the effect was reversed in 50% of the experiments. For mesothoracic protractor and retractor MN activity the phase of front leg

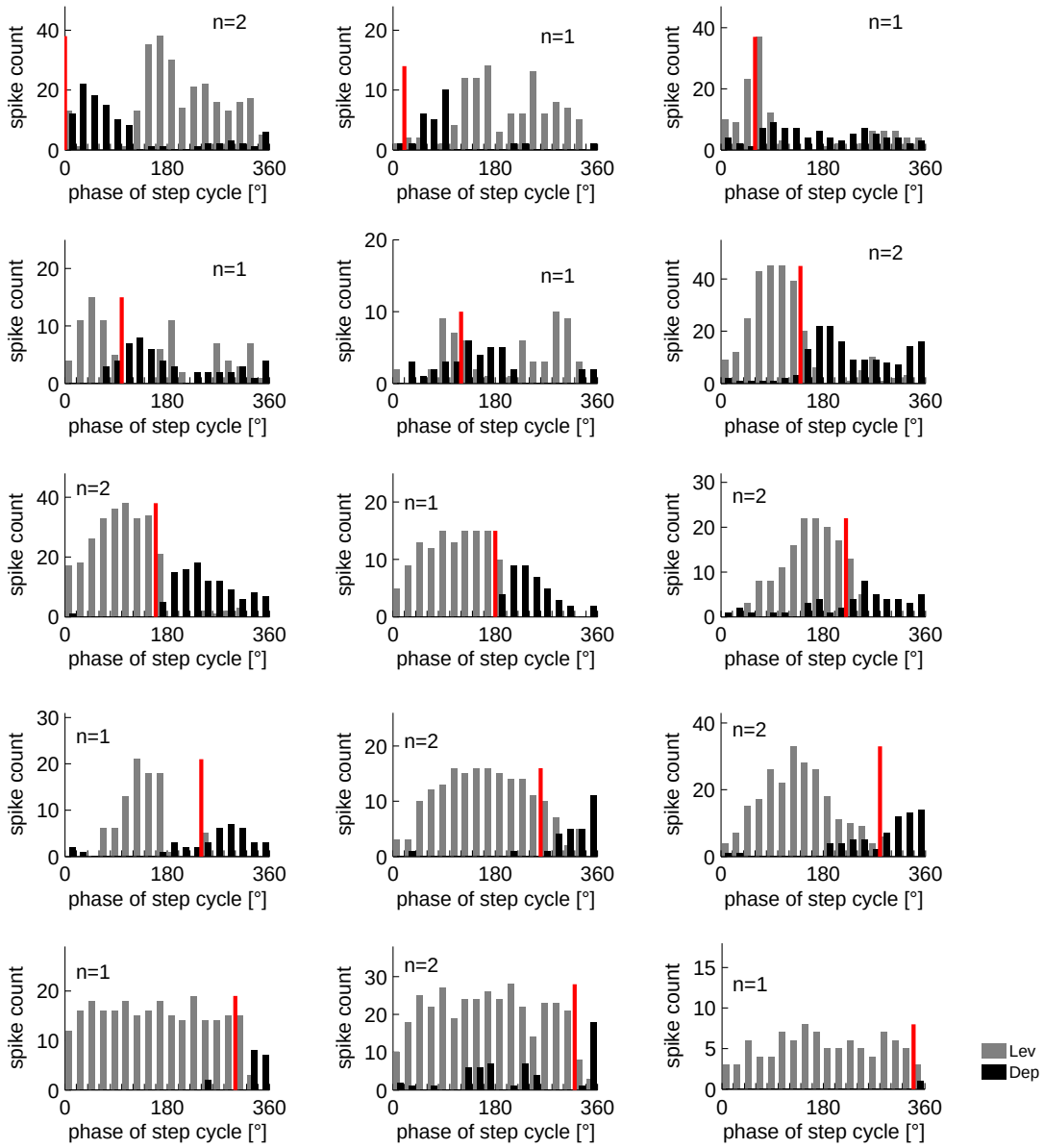

Figure 7.10: *Distribution of mesothoracic levator and depressor MN activity in front leg step cycle for different phases of middle leg campaniform sensilla stimulation. The red vertical line marks the phase of stimulation. n accounts for the number of steps in respective histogram. The series shows the coupling of depressor MN activity to campaniform sensilla stimulation regardless of front leg stepping. As the stimulus moves along the step cycle activation of depressor MN activity moves with it.*

step cycle appeared to have an influence on the processing of load signals.

In contrast, mesothoracic levator and depressor MN activity was coupled to middle leg campaniform sensilla stimulation. Front leg stepping induced a general activity increase in levator MNs and depressor MNs fell silent. No alternating activity was observed. For levator and depressor MNs the intrasegmental load signals appear to have a greater influence than intersegmental signals of the stepping front leg. Ludwar et al. (2005a) show that in the single leg preparation front leg stepping induces an increase in depressor MN activity with front leg stance. Levator MN activity increases with stance or swing. Both cases occur. In this experimental situation with the front leg stepping and the middle leg campaniform sensilla being present, levator and depressor MN activity were dominated by the intrasegmental influence of the middle leg campaniform sensilla. The intersegmental influence was not observable in mesothoracic levator and depressor MNs anymore.

CHAPTER 8

Discussion

Different aspects of intersegmental coordination in the stick insect have been investigated in this thesis. The activation of the walking system, intersegmental information transfer in the connectives and the influence of load signals. The results of the different chapters will be discussed in detail and with regard to a general overview and a connection between the subchapters.

8.1 Activation of the walking system

I investigated the influence a single stepping leg has on motoneural activity in adjacent segmental ganglia.

Front, middle and hind legs do not have the same influences on motoneural activity in adjacent segments

Single leg stepping was always accompanied in all hemisegments by a general increase in MN activity. Single front leg forward walking induced alternating activity in ipsilateral mesothoracic protractor and retractor MNs. Thereby, protractor and retractor MN activity was phase coupled to the front leg step cycle with retractor MNs active in the first half of the step cycle and protractor MNs active in the second. Hind leg backward stepping elicited alternating activity in ipsilateral mesothoracic protractor and retractor MNs in about 33% of the experiments. In the remaining 66% of the experiments protractor and retractor activities were associated with a general activity increase in MN activity. From behavioral experiments it is known that backward walking is not due to levator and depressor phase shifts, but instead arises from a general biasing of activity toward the rear of the animal (Graham and Epstein 1985). The front legs seem to give up their "leading" role as indicated by their stepping more slowly than the middle or hind legs and showing a larger range of movement (Graham and Epstein 1985). The hind legs become functional front legs with kinematics more closely resembling those of the front legs during forward walking. This behavioral data showing that during backward walking hind legs can assume a functional role equivalent to that which the front legs play during forward walking may have relevance to the observation that hind leg stepping can induce alternating activity in the mesothoracic motoneurons. Taken these previous results and my data together it appears quite conceivable that in the stick insect walking system stepping of the functional front leg is associated with activity in intersegmental neuronal pathways that leads, firstly, to alternating activity in coxal MNs of the functional next caudal segment and, secondly, to a phase coupling of the alternating activity in the ipsilateral mesothoracic hemiganglion with leg stepping of the functional front leg.

In contrast, single middle leg stepping did not induce alternating activity in protractor and retractor MNs of its neighboring segments. These experiments were done in two different preparations. The first resembles the traditional single leg preparation (Fischer et al. 2001) in which the middle leg performs sideways steps on a treadmill positioned perpendicular to the body's length. In this preparation the thorax coxa (TC-) joint of the middle leg does not move. The movement in one plane results in a different activation

of sense organs on the walking leg and thus might lead to a different influence of middle leg stepping on the neighboring ganglia compared to in vivo straight forward walking. Therefore, a second single leg walking position was used in which the middle leg performed stepping movements parallel to the body axis. This walking situation resembles more closely the kinematics during *in vivo* forward straight walking (Cruse and Bartling 1995). The results were the same for both preparations. Single middle leg stepping caused a general activation of protractor and retractor MNs and only a slight modulation with the steps in all other hemiganglia. Middle leg stepping did not induce alternating activity.

The influence of middle leg stepping thus is qualitatively different than that of the forward walking front leg and backward walking hind leg. Therefore, the neural networks controlling front, middle and hind legs might not be regarded as fairly symmetrical with respect to their influence on other segments (Cruse et al. 1998). This finding on the neural level parallels a previous behavioral study (U. Bässler and Breutel 1985) that also reported differences between the three pairs of legs and their neural control in the stick insect walking system. U. Bässler and Breutel (1985) have shown in experiments with decerebrated animals, that front and hind legs have different intrinsic walking directions when being the sole pair of legs present. When all other legs are amputated the front legs tend to walk forward, the hind legs tend to walk backward. However, middle legs have no preferred intrinsic walking direction. They follow either the front or the hind legs when a second pair of legs is present. These observations support the conclusion that the middle leg influence is different from that of the front and hind legs, as they show that in this experiment middle legs are dominated by the front and hind legs.

The influence of single front, middle and hind leg stepping on protractor and retractor MNs of the contralateral hemiganglia was similar. Stepping induced a general activation of coxal MNs of the contralateral hemiganglia. This corresponds to prior recordings from mesothoracic flexor MNs contralateral to the walking leg. Only small amplitude phasic modulations with variable coupling to the front leg step cycle was detectable in the intracellular recordings (Ludwar et al. 2005b).

Activation of the locomotor system

Single front leg stepping was always accompanied by a general activation of protractor and retractor MNs of the two caudal ipsilateral hemiganglia and all contralateral hemiganglia. Similar results were observed for single middle leg stepping. A phase coupled coordinated activity of coxal MNs was in addition generated in the ipsilateral mesothoracic segment upon front leg stepping.

To start a locomotor program descending signals from higher brain structures are generally needed. Two mechanisms have been described. Locomotion is either initiated by command neurons (*Clione limacine*: Panchin et al. 1995; Satterlie and Norekian 1995; Norekian and Satterlie 1996; leech: Brodfuehrer and Friesen 1986a, b, d, c; crayfish: Bowerman and Larimer 1974b, a; Wiersma and Ikeda 1964; Davis and Kennedy 1972a, b, c) or more complex descending signals from higher brain structures (lamprey: McClellan and Grillner 1984; Manira et al. 1997; Prisco et al. 1997; cat: e.g. reviewed by Mori et al. 1991. In insects the command system for walking initiation has not yet been identified, although Kien et al. (1990) showed in the locust that at least some of the 200 pairs of identified neurons that project to the thoracic ganglia are involved in walking initiation. In the stick insect there are indications from behavioral experiments that the start and end of a stepping sequence, as well as the walking direction, are determined in the subesophageal ganglion (U. Bässler and Breutel 1985). In these preparations the single leg performed stepping movements. This implies that the pattern generating networks for walking are activated in at least this hemiganglion. In many other well-investigated locomotor systems activation of one part of the system results in a coordinated motor output of the whole. This has been shown in reduced preparations in vitro in the leech (Debski and Friesen 1987) and in vivo and in vitro in crayfish (Larimer 1976; Cattaert et al. 1992; Wiersma and Ikeda 1964; Davis and Kennedy 1972a, b, c) and lamprey (Brodin et al. 1988). In the crayfish swimmeret system, for example, stimulating one swimmeret's CBCO induces rhythmical activity in the whole of the otherwise deafferented swimmeret system (Cattaert et al. 1992).

The stepping leg in this preparation was intact and only slightly restricted in its movement and therefore was providing sensory information and information about its own status to the nervous system. Ludwar et al. (2005a) showed that front leg stepping induced alternating activity in all antagonistic motoneuron pools of the ipsilateral mesothoracic ganglion. It was possible that this activation of alternating activity would have been present throughout the active walking system. My data show this is not the case. One walking leg is apparently insufficient to generate an alternating motor output for antagonistic muscles in all other segments as one could infer as being typical for the locomotor state (Bässler 1993b; Bässler and Büschges 1998). Taken together, these data emphasize how decentralized and modular the stick insect walking system is, a conclusion supported by behavioral studies showing that there is no fixed coupling between the legs and each leg possesses its own pattern generator (Foth and Bässler 1985a, b). My results strongly suggest that even the different hemiganglia are independently activated and highlights the importance of identifying the activating inputs to the walking system. They raise the question of whether these inputs actively participate in generating alternating activity in the individual hemiganglia.

Intersegmental influences of single stepping legs in the light of the coordinating rules governing stepping in the stick insect

What do the influences described above mean for intersegmental coordination, particularly in view of prior behavioral studies and the known behavioral rules for intersegmental coordination (e.g. Cruse 1990 and Dürr et al. 2004)? In general, these single leg experiments did not reveal neural mechanisms underlying coordination. Single leg stepping was associated with an increase in activity in all other hemisegments of the thoracic nerve cord, except for the hemisegment posterior to a functional walking front leg, which exhibited rhythmic activity in coxal MNs. In these cases, however, the coxal MNs of the functional caudal neighboring segment exhibited in-phase activity with the leading front leg, a pattern of coordination not found in vivo. Nevertheless, in several instances they do replicate on the motoneural level what has been before observed in behavioral experiments. In amputation

experiments in which the middle leg is cut off (Wendler 1964a; Cruse 1983) the stump of the amputated leg produces in forward walking retraction and protraction movement in phase with the walking front leg. This corresponds to the activation of mesothoracic protractor and retractor MNs observed here during front leg stepping. Retractor MNs were active during the first half of the step cycle, especially during stance, and protractor MNs were active in the second half of the step cycle. As retraction is performed in forward walking during stance, my data correspond to in phase retraction and protraction of the front and "middle" legs. Furthermore, in behavioral experiments a weaker coupling between contralateral legs has been observed in stick insects and crayfish (Cruse 1990). This corresponds to the data repeated here in that front leg walking had no phasic influence on motoneural activity of contralateral hemiganglia.

In the intact walking stick insect there are seven behavioral rules known that describe the coordination of ipsilateral neighboring legs activity (Cruse 1990; Dürr et al. 2004), three of which play the most important general role for coordination. Two of these rules describe a rostral effect, the first hindering an anterior leg from starting a return stroke while the posterior leg is performing its return stroke. The second assists the start of a return stroke in a rostral leg when the posterior leg starts a power stroke. The third influence is caudally directed and acts to advance induction of a return stroke with the ongoing power stroke of the rostral leg. These three rules are thought to function equally between ipsilateral neighboring legs (Dürr et al. 2004). From this one may expect that all ipsilateral neighboring legs have an equal influence on their ipsilateral neighbors on the neuronal level. It would thus be reasonable to expect middle leg stepping to be associated with alternating activity in coxal MNs of at least one of the ipsilateral neighboring hemiganglia. This was not observed in my experiments, and thus, despite this behavioral performance (Cruse 1990), on the neural level the different segments do not exert qualitatively equal influences on their neighbors. Only one of the coordination rules formulated for the stick insect walking system is partially fulfilled by the changes in motoneuron activity in adjacent segments upon single leg stepping, i.e. the co-activation rule number 5 (reviewed in Dürr et al. 2004). This rule describes that upon encountering an increased

resistance during stepping, like when walking uphill or when loading the animal, the force output of all legs is enhanced. From my results it is quite conceivable that pathways underlying the observed activation of motor activity in adjacent segments related to single leg stepping can contribute to this co-activation influence among walking legs. However, this interpretation is hampered by the fact that both pro- and retractor coxae activity were enhanced upon single leg stepping indicating that this influence might also have another function, i.e. the general and unspecific activation the locomotor networks in the adjacent hemisegments.

8.2 Involvement of central pattern generators

I investigated the influence of a single stepping leg on pharmacologically activated pattern generating networks and the influence of two stepping legs on motoneural activity in adjacent segments. This was done to clarify the following issues: 1) what is the neural substrate of the rhythmic activity observed in the mesothoracic ganglion upon ipsilateral front leg forward stepping? 2) Does the neural activity of intersegmental coordinating pathways rely on the stepping of multiple legs?

Activation of the mesothoracic CPG

As discussed above and described in chapter 3 front leg stepping induces alternating activity in mesothoracic protractor and retractor MNs (see also Ludwar et al. 2005a). Retraction of the front leg coincided with mesothoracic retractor MN activity and protraction with mesothoracic protractor MN activity. This leads to the question what is the neural substrate of this rhythmic activity. Two possible hypotheses appeared to be reasonable. 1) Front leg stepping could exhibit a direct influence on mesothoracic coxal MNs. This would probably be due to sensory signals of the front leg. Ludwar et al. (2005a, b) show that the active prothoracic CPGs do not exhibit this influence whereas signals from the front leg chordotonal organ contribute to the intersegmental influence observed. For the stick insect some sensory pathways are known intrasegmentally that make monosynaptic connections on MNs (Hess and Büschges 1997). Therefore, this hypothesis might

be reasonable for intersegmental pathways as well. 2) Front leg stepping could activate mesothoracic pattern generating networks that shape the motoneural output in protractor and retractor MNs. For the locust intersegmental pathways via interneurons on MNs are known as well as intersegmental pathways that converge on nonspiking interneurons (Laurent 1987; Laurent and Burrows 1989).

My results indicate that front leg stepping activates mesothoracic pattern generating networks that shape the alternating activity in protractor and retractor MNs, based on the following evidence. I could show that front leg stepping to a great extend entrained the pilocarpine induced motor rhythm in mesothoracic protractor and retractor MNs. Pilocarpine is known to elicit alternating activity in antagonistic MN pools (Büschges et al. 1995). This alternating activity is due to the activation of central pattern generating networks. Under the assumption that pilocarpine activates the central pattern generating networks usually activated during walking, the experiments allowed to test, whether a stepping front leg was able to influence this centrally generated rhythmicity in mesothoracic protractor and retractor MNs. Retractor MNs were active during front leg stance and protractor MNs during swing. This indicates that front leg stepping is able to influence active mesothoracic central pattern generating networks. The phase relation between front leg stepping and protractor and retractor MN activity was the same as in the single leg preparation without pilocarpine superfusion. Retractor MNs were active in the first half of front leg step cycle protractor MNs in the second half. Hence, front leg stepping was able to influence the pharmacologically activated pattern generating network in the mesothoracic ganglion.

Furthermore, I could show that during front and ipsilateral hind leg stepping the alternating activity in protractor and retractor MNs persisted and was only loosely coupled to front leg stepping. This supports the hypotheses that central pattern generating networks were activated in the mesothoracic ganglion. During single front leg stepping protractor and retractor MN activity was rigorously coupled to front leg step cycle. Most probably sensory signals contribute to this influence as Ludwar et al. (2005a) show. If the mesotho-

racic TC-joint CPG was not activated in this experimental situation another result would have been reasonable. The alternating activity in mesothoracic protractor and retractor MNs should be unchanged compared to the single front leg preparation. In case the hind leg also influences the protractor and retractor MNs the alternating activity might be destroyed. Hence, the persisting alternating activity in mesothoracic coxal MNs that is loosely coupled to front leg stepping indicates that the middle leg pattern generating networks were active.

Another set of experiments indicates that it might be crucial that the pattern generating networks of a ganglion are active to process signals from other stepping legs. Stepping of a single front or middle leg induced a general activity increase in metathoracic protractor and retractor MNs (see chapter 3). No alternating activity was observed. When the metathoracic ganglion was activated by pilocarpine front leg stepping was able to influence the slow pilocarpine induced motor rhythm. In three of four experiments pilocarpine rhythm was entrained to front leg stepping. Metathoracic retractor MNs were active during first half of front leg step cycle protractor MNs during second half. This corresponds to the coupling observed between front leg stepping and mesothoracic protractor and retractor MNs of the non-activated mesothoracic ganglion. In one of the four experiments front leg stepping did not entrain the pilocarpine induced motor rhythm, but had an influence on the frequency. With the start of a stepping sequence the frequency of alternation increased and was faster than the actual stepping frequency. These experiments show that a ganglion has to be active in the sense that its central pattern generating networks have to be active to process intersegmental signals from other stepping legs. When the metathoracic ganglion was activated by pilocarpine front leg stepping exhibited a comparable influence on metathoracic protractor and retractor MNs as it did on mesothoracic protractor and retractor MNs.

In summary, from my results it is quite conceivable that front leg stepping activates central pattern generating networks in the mesothoracic ganglion that generated the observed alternating activity in protractor and retractor MNs. 1) Front leg stepping is able to en-

train mesothoracic pilocarpine induced motor rhythm and therefore the influence active pattern generating networks. 2) The activation of pattern generating networks appears to be a pre-condition for the processing of signals from the stepping front leg. This could be shown for the metathoracic ganglion. 3) In the two leg preparation with front and ipsilateral hind leg stepping the pattern generating networks appeared to be active. Alternating activity in mesothoracic protractor and retractor MNs was present during front and ipsilateral hind leg stepping. The motoneural activity was only loosely coupled to front leg stepping.

Activation of the metathoracic TC joint CPG

This raises the question what activates the central pattern generating networks in the metathoracic ganglion? Stepping of both front and ipsilateral middle leg reliably (in eleven of twelve experiments) elicited alternating activity in metathoracic protractor and retractor MNs.

In 50% of the experiments protractor and retractor MN activity was clearly uncoupled to the front and middle leg stepping cycle. Protractor and retractor MNs started alternating with the beginning of a stepping sequence indicating that the metathoracic CPG was activated and shaped the observed motor output. The frequency of the alternation between protractor and retractor MN activity varied between 0,4 Hz and 1,8 Hz whereas fast rhythms occurred more often than slow ones. The wide range of frequencies that was observed resembles to the range of frequencies that is characteristic for different motor programs such as rocking (Pflüger 1977) or walking and pilocarpine induced motor rhythm (Büschges et al. 1995). Rocking describes a behavior stick insects use to mimic leafs (Bässler 1983a). The rocking frequency lies between 1,3Hz and 5,6Hz with a peak between 2Hz and 3Hz. The fast frequency alternations in metathoracic protractor and retractor MNs are at the lower end of the rocking frequencies. Frequency of stepping in freely walking stick insects on a horizontal surface is between 0,5Hz to 4Hz (Graham 1985b). The pilocarpine induced rhythm in mesothoracic protractor and retractor MNs alternates with a frequency of approximately 0,2Hz (Büschges et al. 1995). This is a little lower

than the lowest observed frequency. Nevertheless, the animal did not perform a specific motor task. Rocking behavior is known to be centrally generated and occurs in all legs at the same time with ipsilateral neighboring legs being strictly in phase and contralateral legs being 180° out of phase. It has been described that rocking can be superimposed on walking movements but usually occurs when the animal is at rest (summarized in Bässler (1983c)). In the two leg preparation the animal performed stepping movements with both front and middle legs. As rocking is centrally generated it should occur in all legs at the same time, whereas in these experiments two legs were walking. The observed alternating activity cannot be related to walking behavior either. The frequency of the alternation did not match the stepping frequency and no stable phase relation existed between the legs and protractor and retractor MN activity.

In 16% of the experiments with front and ipsilateral middle leg stepping coupling of metathoracic protractor and retractor MN activity to front leg stepping was observed. Retractor MNs were active in the first half of front leg step cycle. Protractor MNs were active in the second half of front leg step cycle. This coupling was equivalent to that between single front leg stepping and mesothoracic protractor and retractor MNs (see chapter 3 and Ludwar et al. 2005a). The stepping movements of the middle leg appeared to be irrelevant for the coupling to front leg stepping in this experimental situation. No matter if the middle leg was stepping or not metathoracic protractor and retractor MN activity was coupled to front leg steps. The influence of front leg stepping clearly dominated the influence of middle leg stepping. Hence, the middle leg has to be present to activate the metathoracic TC-joint CPG but it has no influence on the metathoracic protractor and retractor motor rhythm.

In another 25% of the experiments both cases, coupling of protractor and retractor MN activity to front leg stepping and uncoupled oscillations, were observed even in one stepping sequence. This might be an indication that I observed two different extremes of a continuum. On the one end metathoracic pattern generating networks were just activated and no coupling to stepping of any of the two legs was observed and on the other end

strong coupling to front leg stepping regardless of middle leg stepping occurred.

The metathoracic TC-joint CPG was activated and produced a great variety of different oscillating behaviors. One extreme might be the very fast oscillations similar to the ones observed in rocking behavior and on the other end the very slow oscillations similar to that induced by pilocarpine. This goes hand in hand with different grades of coupling to the front leg. This might allow the animal to adapt the activity of respective network to master different behavioral tasks such as rocking or walking.

How do the intact legs contribute to the activation of the metathoracic CPG?

The question arises what contributes to the activation of the metathoracic TC-joint CPG. Starting with the simple question: do both front and ipsilateral middle legs have to walk simultaneously to induce alternating activity in metathoracic protractor and retractor MNs? The experiments show that it was not necessary that both front and ipsilateral middle legs performed stepping movements. It was sufficient to have one leg stepping and the other one just standing on the treadmill. Stepping of both front and middle legs occurred in 55,3% of all stepping sequences. In 41,2% only the front leg performed stepping movements and in 3,6% the middle leg performed stepping movements while the front leg did not move. This did not change the observed activity pattern. It was apparently not the simultaneous stepping of both legs, and the network activity and sensory information associated with it, that elicited this alternating activity pattern in metathoracic protractor and retractor MNs. What could be a plausible explanation? Several explanations might be reasonable. 1) There might be a general higher excitation in the nervous system with more legs being present and sending their sensory input to the central nervous system. The higher excitation might result in crossing an activation threshold in the metathoracic ganglion. This could cause the TC-joint CPG to oscillate. A problem with this explanation is that in case of one of the two legs stepping there might not be very much sensory input from the resting leg. 2) The middle leg central pattern generating networks have to be active to activate the metathoracic CPG. This explanation is also very hard to hold as I could show that mesothoracic pattern generating networks are probably active during

front leg stepping. Furthermore, the additional activation of the mesothoracic ganglion with pilocarpine did not change the influence of single front leg stepping on metathoracic protractor and retractor MNs (no data shown). Furthermore, it is questionable to what extend the pattern generating networks are active if the leg is intact, getting feedback from its own sensory organs and standing on the treadmill. 3) A third explanation might be an unknown sensory influence of the middle leg that either gates intersegmental influences or contributes in some other way to the observed behavior.

The metathoracic TC-joint CPG was activated only by stepping of front and ipsilateral middle legs. Stepping of front and contralateral middle legs was accompanied by a general activity increase in metathoracic protractor and retractor MNs. Thus, the influence of front and middle leg stepping on metathoracic protractor and retractor MNs appeared to be restricted to ipsilateral hemisegments. This approves behavioral studies that show a weaker coupling between contralateral legs in stick insects (Cruse 1990).

Relation to other results of intersegmental coordination

In chapter 3 it turned out that front and middle leg stepping were different in their influence on motoneural activity of ipsilateral neighboring ganglia. Front leg stepping induced alternating activity in mesothoracic protractor and retractor MNs. Middle leg stepping induced a general increase in activity in pro- and metathoracic protractor and retractor MNs. The influence of middle leg stepping thus appeared to be "weaker" compared to that of the front leg. The results of chapter 4 confirm the minor importance of the stepping middle leg compared to the front leg. In the two leg preparation with intact front and ipsilateral middle legs stepping a strong coupling of metathoracic protractor and retractor MN activity to front leg stepping was observed at least temporarily in four of twelve experiments. Stepping of the middle leg was irrelevant. Middle leg stepping did not influence metathoracic protractor and retractor MNs.

The difference in the influence of front, middle and hind leg stepping appears to continue in different responses of respective ganglia to influences of other stepping legs. In metatho-

racic protractor and retractor MNs a great variety of different oscillating behaviors during front and ipsilateral middle leg stepping was observed. Mesothoracic protractor and retractor MN activity alternated as well during front and ipsilateral hind leg stepping. The frequency was similar to front leg stepping frequency and there was still a tendency to be in phase with front leg stepping. So the mesothoracic ganglion appears to be easier to activate and in general more accessible to intersegmental influences.

Pilocarpine is known to activate the pattern generating networks (Büschges et al. 1995). The induced motor rhythms tend to be much slower than usual stepping frequencies. This is around 0,2 Hz for the TC-joint CPG. In the two leg preparation stepping of front and middle leg elicited slow (0,4 Hz) alternating activity in metathoracic protractor and retractor MNs in some stepping sequences. The frequency did not match the one of the pilocarpine induced motor rhythm exactly but was much slower than the actual stepping frequency. This might be an indication that a pilocarpine-like motor rhythm could be elicited in this experimental situation.

8.3 Intersegmental signals in the connectives

I analyzed the neuronal activity in the pro-meso and the meso-meta connective ipsilateral to a single stepping front leg.

The neuronal activity in the connectives was recorded extracellularly. A general problem in doing extracellular connective recordings consists in the fact that there are around 2000 axons (Leslie 1973) in each connective. In the stick insect it is not known where in the connective the axons, relevant for the transmission of intersegmental signals concerning walking, are situated. It is not possible to say which action potential units are prominent in the recording and whether the observed results are determined by the location of the electrode that records such axons better that are closer to the electrode and further peripheral in the connective. Action potential units that are active at the same time sum up in the recording and the relevant signals for intersegmental coordination might be

masked. Nevertheless, the results were very consistent between the different experiments and showed a similar recurring behavior to comparable stimuli. They were obviously related to front leg stepping.

Front leg stepping sequences induced an increase in neuronal activity in the connectives. The neuronal activity was phasically modulated with front leg steps. This was observed in the pro-meso connective as well as in the meso-meta connective. In the meso-meta connective the effect of front leg stepping appeared to be weaker than in the pro-meso connective. The general increase in neuronal activity was lower in the meso-meta connective. Furthermore, the neuronal activity in the pro-meso connective was correlated to front leg stepping velocity. This correlation was not observed for the neuronal activity of the meso-meta connective. Comparing the phasic modulation in the pro-meso and the meso-meta connective shows that the increase in neuronal activity and the maximum appeared to be earlier in the meso-meta connective. The cross correlation functions between pro-meso and the meso-meta connective showed this as well. This is surprising as the phasic modulation clearly originated from the front leg. In case of the cross correlation function the time shift between the pro-meso and the meso-meta connective was so small and in range of the smoothing ($T_s = 0,07\ s$) factor that it is questionable how reliable these results are. Nevertheless, the same results were obtained in the averaged overdraws as well. One explanation might be that the neuronal activity increase in the pro-meso connective lasted longer and was more pronounced in the pro-meso connective. As a consequence the averaged maximum is situated later in time and phase. On the other hand it might very well be that at the same time signals are traveling from caudad to rostrad and therefore the maximum in the meso-meta connective is earlier than in the pro-meso.

My experiments allow conclusions about the source of the phasic modulation and the general increase in neuronal activity in the connectives. The phasic modulations appeared to be due to sensory signals from the stepping front leg whereas the activity of pattern generating networks appeared to contribute to the general increase in neuronal activity in the connectives.

Sensory signals are important for intersegmental coordination in legged animals. This is well known for the stick insect (Wendler 1964c; Graham 1977; Ludwar et al. 2005a). From my experiments there are several indications that the observed phasic modulations in the neuronal activity of the pro-meso and the meso-meta connective are due to sensory signals of the front leg. First, my experiments show that the phasic modulations in the pro-meso and the meso-meta connective were clearly related to front leg stepping. Averaged overdraws of the rectified and smoothed recordings showed that neuronal activity increased with the beginning of front leg stance. The front leg stepping velocity was correlated with the neuronal activity in the pro-meso connective. The higher the mean velocity of a step the stronger the neuronal activity in the connective. When the front leg was passively moved the phasic modulations in the connective recordings persisted but no general increase in activity was observed. Furthermore, front leg campaniform sensilla stimulation reliably elicited a burst in the ipsilateral pro-meso connective in all experiments. This burst was consistent in its occurrence. Taken together, these results strongly indicate that sensory signals from the front leg shape these modulations in neuronal activity of intersegmental pathways. Especially the campaniform sensilla contribute to it. As the action potential units carrying the phasic modulation had large amplitude this might indicate that their axons are located particularly peripheral in the connective.

The tonic increase in neuronal activity in the connectives appears to be at least partially due to the activity of pattern generating networks. During passive movements of the front leg, where pattern generating networks are not active, no general increase in neuronal activity was observed. Experiments in which the mesothoracic ganglion was superfused by saline containing pilocarpine show that the neuronal activity in the pro-meso connective increased when the mesothoracic ganglion becomes active. No phasic modulation was observed. One could argue that this might be due to the fact that the action potential units that might generate a phasic modulation in this case are situated unfavorable in the connective and therefore were not detected properly. On the other hand the autocorrelation function and the averaged overdraw of the connective recording did not show

any oscillations. It cannot be completely excluded that there are interneurons mediating phasic information in this experimental situation as well but with the different analysis used here no phasic modulation was detected.

In the stick insect a tonic increase in activity with the beginning of stepping appears to be a crucial part in activating the walking system. On the intracellular level a tonic increase is observed in all MNs (Ludwar et al. 2005b; Gabriel 2005). In extracellular recordings this was observed in protractor and retractor MNs (see chapter 3). Although this does not necessarily indicate a causal correlation it shows that this appears to be a global phenomenon in the stick insect walking system.

The phasic modulations during front leg stepping were observed in the pro-meso connective as well as in the meso-meta connective. Although the increase in neuronal activity and the modulation appeared to be weaker in the meso-meta connective signals from the stepping front leg appeared to reach the metathoracic ganglion as well. Even so metathoracic protractor and retractor MNs do not show alternating active during front leg stepping (see chapter 3) as it could be observed in mesothoracic protractor and retractor MNs. This appears not to be due to the transfer of the signals from the front leg but might lay in the metathoracic ganglion itself and the processing of intersegmental signals.

In general little is known about the information transfer in the connectives concerning the walking system of insects. In the cockroach it is known that especially the neck and circumoesophageal connective are important for posture and locomotion. This suggests that thoracic circuits are sufficient to produce leg movements but coordinated walking with normal motor patterns requires descending input from head ganglia (Ritzmann et al. 2005; Ridgel and Ritzmann 2005). Harris and Eisenstein (1999) could show that the connectives are important for the transfer of learned information between the ganglia. The right prothoracic leg was trained to lift in order to avoid a shock. It was found that this information transferred via the two interganglionic connectives from prothoracic ganglion to the mesothoracic ganglion so that now the right mesothoracic leg lifted to avoid shock

even though it was not directly trained.

8.4 Coordination of two legs

Stepping of two legs on separate treadmills was analyzed concerning their stepping patterns and coordination. The experiments were performed for front and ipsilateral middle legs, front and ipsilateral hind legs and front and contralateral middle legs. All other legs were amputated. The legs were mechanically uncoupled as they stepped on separate treadmills.

The legs walked fairly independent of each other for all three combinations of legs. Stepping of one of the two intact legs did not imply stepping of the second leg. Stepping of both front and ipsilateral middle legs occurred in 55,3% of all stepping sequences. Stepping of both front and contralateral middle legs occurred in 45,3% of all stepping sequences and stepping of both front and ipsilateral hind legs occurred only in 29,4% of all stepping sequences. The amount of steps of the second leg decreased from rostral to caudal. This might indicate that the hind leg is more autarkic from the front leg than the middle legs are. The decreasing amount of stepping sequences and steps from front to hind leg could also be an indication for an activation of the walking system from rostral to caudal in a forward walking situation. This resembles to the results obtained in chapter 3. Here the influence of single front leg stepping appeared to decrease from rostral to caudal as well.

The stepping frequencies of all three legs were between 0,2 Hz and 2,4 Hz. This frequency range is in the range observed in freely walking animals. Frequency of stepping in freely walking stick insects on a horizontal surface is between 0,5 Hz to 4 Hz (Graham 1985b). The stepping frequencies of the two stepping legs were not necessarily correlated. The front leg stepping frequency was uncorrelated to the ipsilateral middle leg stepping frequency and to the ipsilateral hind leg stepping frequency. The stepping frequencies of the front leg and the contralateral middle leg were correlated. Hence, the ipsilateral neighboring legs were independent in their stepping frequencies. This might be due to the fact that the legs were mechanically uncoupled. Epstein and Graham (1983) showed for intact animals on

a slippery surface when individual legs are not coupled mechanically through the surface they are capable of operating with significantly different step periods, as evidenced by the 'go slow' behavior of some legs. Reference to the step profiles of well-coordinated walks shows that even at these times individual legs may move at significantly different velocities over the surface and there is no apparent control mechanism to keep all legs retracting at the same rate.

The Foth and Bässler (1985a, b) show that the legs can step coordinated with different stepping frequencies if they are forced by different tread wheel velocities. In the experimental situation here where the legs stepped on passive treadmills no coordinated stepping pattern was observed in any of the three preparations. In the preparation with intact front and ipsilateral middle legs the polar plot showed a cluster of mean vectors around 180° of front leg step cycle. This phase relation corresponds to a tripod gate. Only one mean vector was significantly different from zero. Therefore the other mean vectors do not give a reliable result as they were not significant. For the front and ipsilateral hind leg and front and contralateral middle leg vectors of mean phase were widely distributed and mainly not significant. Hence, no preferred phase of hind and middle leg stepping in front leg step cycle was observed. This shows again how modular the stick insect walking system is constructed.

The front leg appears to take over a leading role in forward walking. It performed steps most frequently. In the preparations with two intact ipsilateral neighboring legs, front leg stepping occurred in over 95% of all stepping sequences. Hardly any stepping sequences were observed where only the ipsilateral middle or the ipsilateral hind leg performed steps. In general it appears that for the initiation of forward walking especially the front legs are important.

8.5 Intra- vs. intersegmental influences

I analyzed the influence of middle leg campaniform sensilla stimulation and front leg stepping on mesothoracic protractor and retractor MNs and levator and depressor MNs.

The stimulation of middle leg campaniform sensilla did not have any measurable influence on front leg stepping. This does not necessarily mean that there existed none but that it was very weak and not detectable in this experimental situation. Front leg stepping as such is so irregular that a weak influence might be impossible to resolve. In experiments with intact animals it is shown that increasing load on the middle leg induces a swing phase in the ipsilateral front leg (Cruse 1990). As no influence of middle leg campaniform sensilla stimulation on front leg stepping was detected it could be disregarded for the analysis.

Mesothoracic protractor and retractor MNs and levator and depressor MNs process intersegmental influence of front leg stepping and the intrasegmental influence of middle leg campaniform sensilla stimulation differently. Mesothoracic protractor and retractor MNs were influenced by front leg stepping and middle leg campaniform sensilla stimulation. Levator and depressor MNs were influenced only by middle leg campaniform sensilla stimulation.

For mesothoracic protractor and retractor MNs the influence of front leg stepping is well known. The same is true for the influence of middle leg campaniform sensilla stimulation. Front leg stepping while all other legs were amputated induced alternating activity in mesothoracic protractor and retractor MNs (Ludwar et al. 2005a). Retractor MNs were mainly active during stance and protractor MNs during swing. Middle leg campaniform sensilla stimulation increased retractor MN activity and decreased protractor MN activity (Akay et al. 2004). When both influences, front leg stepping and middle leg campaniform sensilla stimulation, were present at the same time their influences persisted. Front leg stepping induced alternating activity in protractor and retractor MNs. Simultaneous middle leg campaniform sensilla stimulation further increased or switched on retractor MN activity in most cases. The influence of front leg stepping and middle

leg campaniform sensilla stimulation on mesothoracic protractor and retractor MNs co-existed.

For mesothoracic levator and depressor MN activity the influence of front leg stepping was investigated by Ludwar et al. (2005a). Depressor MN activity increases with front leg stance. Levator MN activity increases with front leg stance or swing. Both cases occur. No experimental data about the influence of middle leg campaniform sensilla stimulation on levator and depressor MNs exists. But as levator and depressor MNs control lift off of the leg it is assumed that load signals are particularly important. When both influences, front leg stepping and middle leg campaniform sensilla stimulation, were present front leg stepping induced a general increase in levator MNs and depressor MNs fell silent. No alternating activity was observed. Middle leg campaniform sensilla stimulation decreased levator MN activity and increased depressor MN activity. Therefore, the intrasegmental load signals clearly dominated the influence of front leg stepping. In this experimental situation with the front leg stepping and the middle leg campaniform sensilla being present, levator and depressor MN activity did not increase with stance or swing respectively as described by Ludwar et al. (2005a). This appeared to be due to the presence of middle leg campaniform sensilla. No further indications were obtained what exactly contributed to this change in the influence of front leg stepping.

In 50% of the experiments the effect of middle leg campaniform sensilla stimulation on mesothoracic protractor and retractor MNs depended on the phase of the front leg step cycle. Mesothoracic protractor and retractor MN activity was influenced by front leg stepping and middle leg campaniform sensilla stimulation. Front leg stepping induced alternating activity in protractor and retractor MNs. Retractor MN activity increased during front leg stance protractor MN activity during front leg swing. This corresponds to the results in the single leg preparation (see chapter 3 and Ludwar et al. (2005a)). The stimulation of middle leg campaniform sensilla induced an increase of retractor MN activity and a decrease in protractor MN activity for stimulations at the beginning and the end of front leg step cycle. This corresponds to the observations of campaniform

sensilla stimulation in the single middle leg preparation (Akay et al. 2004). For middle leg campaniform sensilla stimulation around 180° of front leg step cycle the effect was reversed in 50% of the experiments. The phase of front leg step cycle appeared to have an influence on the processing of load signals for mesothoracic protractor and retractor MN activity. 180° of front leg step cycle corresponds to the phase where the transition from stance to swing takes place and the animal lifts off its leg. For a stable walking pattern the middle leg should start with a stance phase at this point. Especially as the stimulation of middle leg campaniform sensilla pretends increasing load on the middle leg. This could be due to the artificial stimulation of middle leg campaniform sensilla. The stimulation was conducted by bending the femur in a horizontal plane to the animal (the coxa trochanter joint was outstretched) from anterior to posterior. The activation of the campaniform sensilla does most probably not correspond to that during walking. Perhaps more sensory signals from the middle leg are needed to get in vivo like activation of protractor and retractor MNs. As it is known that many of the effects of load require integration of information from load and angle sensors. For example the transition from stance to swing (Cruse 1985) (summaries in Duysens et al. (2000); Zill et al. (2004)).

8.6 Conclusions on the organization of the stick insect walking system

Modularity of the stick insect walking system

My results have confirmed and extended previous observations indicating that the stick inset walking system is constructed in a modular fashion (Bässler 1993a; Cruse 1990; Büschges 2005). This becomes apparent in different experimental situations. First, stepping of a single leg does not imply that the animal is in a locomotor state. The different hemiganglia are obviously independently activated. In the two leg preparation with two intact legs that walked on two separate treadmills and therefore were mechanically uncoupled stepping of one leg did not imply stepping of the second leg. The legs stepped mostly independent of each other concerning coordination and frequency.

Processing of intersegmental signals

My results indicate that the state of a hemiganglion determines the processing of signals from the other legs. The connective recordings of the pro-meso and the meso-meta connective during front leg stepping show that the signals from the stepping front leg most probably reach the metathoracic ganglion. If the metathoracic ganglion is active in the sense that the pattern generating networks are active the signals from a stepping leg are treated differently as if it is not active. If the metathoracic ganglion was not active a general increase in motoneural activity was observed during front leg stepping. In case of an active metathoracic ganglion protractor and retractor MN activity alternated and were influenced by front leg stepping.

Asymmetry in the walking system

Different results show that the stick insect walking system is constructed asymmetrically concerning the contribution and importance for intersegmental coordination. The forward walking front leg has a stronger influence on motoneural activity of the other hemiganglia than the middle leg has. This is supported by different experiments. First, in the single leg preparation the front leg stepping induced alternating activity in ipsilateral mesothoracic protractor and retractor MNs (Ludwar et al. 2005a)(chapter 3) that is probably shaped by pattern generating networks in the mesothoracic ganglion (chapter 4). Middle leg stepping did not induce alternating activity in protractor and retractor MNs of its ipsilateral neighboring segments (chapter 3). Therefore, the influence of middle leg stepping was qualitatively different to the influence of front leg stepping.

The "weaker" influence of middle leg stepping and the dominance of front leg stepping is supported by the two leg experiments (chapter 4). Intact front and ipsilateral middle leg induced alternating activity in metathoracic protractor and retractor MNs. In four of twelve experiments protractor and retractor MN activity were coupled to front leg step cycle. Although the middle leg performed stepping movements simultaneously no coupling to middle leg steps was observed in any experiment. The middle leg appears to have no

influence on the timing of metathoracic motoneural activity whereas the front leg is able to entrain metathoracic protractor and retractor MN activity in one third of the experiments.

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APPENDIX A

Tables chapter 6

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
3	1	10	0,68	5	
	2	11	0,82	12	0,85
	3	3		3	1,7
4	1	19	0,25	6	0,12
	2	12	0,45	2	
5	1	8	0,34	8	0,37
	2	9	0,36	10	0,54
	3	5	0,52	0	
	4	7	0,64	0	
	5	4	0,52	0	
	6	6	0,53	0	
	7	6	0,78	0	
	8	4	0,68	0	
	9	8	1,2	4	0,75
	10	2		3	1,05
	11	1		7	0,64
6	1	3	0,48	14	1,49
	2	0		6	1,47
	3	3	0,27	3	1,8
	4	0		4	2

Table A.1: *Parameters of front and ipsilateral middle leg steps for stepping sequences of experiment three to six of twelve experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
7	1	8	0,76	3	0,4
	2	19	1	6	0,3
	3	18	1	6	0,27
	4	10	0,75	3	0,4
	5	17	0,95	3	0,18
	6	3	0,75	0	
	7	21	0,59	6	0,14
	8	33	0,52	12	0,15
	9	13	0,83	5	0,3
8	1	6	1,05	0	
	2	9	1,2	0	
	3	5	0,93	0	
	4	5	0,93	0	
	5	3	1,25	0	
	6	3	1,55	0	
	7	4	0,62	0	
	8	4	0,43	3	
	9	2		4	0,86
	10	4	0,63	0	
9	1	26	0,83	0	
	2	18	0,7	0	
	3	10	0,9	6	0,59
	4	5	0,2	17	0,56
	5	14	0,64	4	0,56
	6	5	0,2	18	0,71
	7	4	0,2	12	0,55
	8	8	0,55	0	

Table A.2: *Parameters of front and ipsilateral middle leg steps for stepping sequences of experiment seven to nine of twelve experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
10	1	3	1,13	0	
	2	7	0,82	0	
	3	7	0,92	0	
11	1	8	0,47	6	0,54
	2	7	1	0	
	3	5	1,19	0	
	4	4	1,17	0	
	5	3	1,8	0	
	6	5	1,3	0	
	7	22	0,94	9	0,3
	8	8	1,1	5	0,48
	9	5	2	0	
	10	12	0,5	7	0,34
	11	7	0,47	4	0,42
	12	10	0,5	2	
	13	3	0,5	3	
12	1	15	0,63	0	
	2	5	0,48	0	
	3	10	0,44	0	

Table A.3: *Parameters of front and ipsilateral middle leg steps for stepping sequences of experiment ten to twelve of twelve experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
3	1	0		4	0,59
	2	13	0,44	6	0,42
	3	15	0,53	5	0,5
	4	9	0,42	4	0,5
	5	11	0,54	4	0,42
4	1	7	0,59	0	
	2	8	0,49	5	0,45
	3	6	0,59	0	
	4	8	0,43	5	0,43
	5	3	0,63	6	0,47
	6	9	0,42	4	0,52
	7	8	0,41	7	0,92
	8	11	0,61	9	0,61
	9	6	0,31	12	0,8
	10	14	0,33	33	0,57
	11	9	0,64	9	0,49
5	1	6	0,36	0	
	2	3	0,58	0	
	3	5	0,35	0	
	4	3	0,37	3	0,7
	5	7	0,31	0	
	6	8	0,56	0	
	7	7	0,47	0	
	8	9	0,63	0	
	9	6	0,73	0	
	10	6	0,5	0	
	11	5	0,65	0	
	12	7	0,66	0	
	13	11	0,72	0	

Table A.4: *Parameters of front and ipsilateral hind leg steps for stepping sequences of experiment three to five of nine experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
6	1	10	0,75	8	0,59
	2	4	0,62	3	0,42
	3	10	0,38	21	0,84
	4	2		5	0,49
	5	3	0,93	0	
	6	0		4	1,56
	7	3	1,1	0	
	8	6	0,37	10	1,12
	9	0		4	1,87
	10	21	0,46	26	0,72
7	1	9	0,95	0	
	2	21	0,61	0	
	3	5	1,1	0	
	4	13	0,69	0	
	5	5	1,18	0	
	6	8	0,73	0	
	7	5	0,91	0	
	8	8	0,87	0	
	9	11	1,08	0	
	10	17	0,72	0	
	11	7	0,58	0	

Table A.5: *Parameters of front and ipsilateral hind leg steps for stepping sequences of experiment six and seven of nine experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
8	1	12	0,62	0	
	2	5	0,9	0	
	3	7	1,04	0	
	4	5	0,42	0	
	5	4	0,35	0	
	6	8	0,42	0	
	7	4	1,38	0	
	8	4	1,21	0	
	9	5	0,62	0	
	10	17	0,81	0	
	11	21	0,67	0	
	12	8	0,77	0	
	13	3	1,27	0	
	14	16	0,97	0	
	15	5	0,95	0	
	16	14	1,1	0	
	17	11	1,28	0	
	18	13	0,81	0	
	19	7	0,41	0	
	20	5	0,46	0	
	21	5	0,48	0	
	22	4	0,63	0	
	23	6	0,5	0	
	24	3	0,63	0	
	25	8	0,59	0	

Table A.6: *Parameters of front and ipsilateral hind leg steps for stepping sequences of experiment eight of nine experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
9	1	17	0,94	4	1,64
	2	4	1,36	0	
	3	4	1,64	0	
	4	7	0,96	0	
	5	19	1,1	0	
	6	10	0,88	8	0,78
	7	4	1,87	0	
	8	0		7	0,68
	9	9	1,29	0	
	10	5	1,64	0	
	11	7	1,32	0	
	12	0		8	0,77
	13	4	0,77	0	

Table A.7: *Parameters of front and ipsilateral hind leg steps for stepping sequences of experiment nine of nine experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
2	1	5	1,14	5	1,22
	2	3	3,1	0	
	3	14	2,1	13	1,8
	4	0		3	2,13
	5	6	2,6	0	
	6	8	1,6	0	
	7	3	2,3	0	
	8	25	1	0	
	9	8	1	0	
	10	4	1,4	0	
	11	17	0,92	0	
	12	3	0,45	0	
3	1	9	0,77	0	
	2	6	1,15	0	
	3	11	0,79	0	
	4	8	0,56	5	0,84
	5	3	0,59	0	
	6	3	0,53	0	
	7	0		3	1,96
	8	3	1,37	4	1,25
	9	5	0,76	11	1,69
4	1	0		5	0,59
	2	12	1,13	9	0,68
	3	0		3	1,01
	4	0		5	0,59
	5	0		12	0,61
	6	0		5	0,61

Table A.8: *Parameters of front and contralateral middle leg steps for stepping sequences of experiment two to four of nine experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
5	1	24	0,84	3	0,56
	2	93	0,84	2	0,26
	3	6	0,3	3	0,82
	4	14	0,63	2	0,64
	5	8	0,37	3	0,27
	6	7	0,38	3	0,42
	7	7	0,56	2	0,47
	8	2	0,85	0	
	9	7	0,46	2	0,24
6	1	4	2,44	0	
	2	4	1,25	0	
	3	15	1,32	0	
	4	8	2,28	0	
	5	10	1,92	0	
	6	9	1,99	2	0,34
	7	4	0,7	0	
	8	3	1,62	0	
	9	0		2	1,89
	10	3	2,82	0	
	11	8	1,4	4	1
7	1	5	0,73	0	
	2	25	1,15	11	0,68
	3	23	0,97	9	0,48
	4	10	1	12	1,16
	5	14	0,83	17	1

Table A.9: *Parameters of front and contralateral middle leg steps for stepping sequences of experiment five to seven of nine experiments.*

experiment	stepping sequence	front leg steps	mean frequency	middle leg steps	mean frequency
8	1	7	0,7	19	1,06
	2	0		3	1,71
	3	0		4	1,15
	4	0		7	1,31
	5	0		12	0,97
	6	12	0,98	15	0,91
	7	0		6	1,61
	8	4	1,1	5	1,73
	9	3	2,47	6	1,89
	10	8	0,87	0	
9	1	5	2,32	3	1,37
	2	10	1,13	6	0,68
	3	3	2,4	0	
	4	5	1,66	4	0,96
	5	12	1,12	8	0,67

Table A.10: *Parameters of front and contralateral middle leg steps for stepping sequences of experiment eight and nine y of nine experiments.*

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