# Leg Coordination during Walking in Insects

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# Contents

# 1. Zusammenfassung

Um eine erfolgreiche Fortbewegung gewährleisten zu können, müssen Bewegungen kontinuierlich an die Bedingungen der Umgebung angepasst werden. Eine sinnvolle räumliche und zeitliche Koordination von verschiedenen Körperteilen ist hierfür notwendig. Bisher ist nicht bekannt, wie neuronale Strukturen diese sinnvollen Anpassungen verwirklichen. Der genaue Beitrag von Nervensystem, Muskulatur und mechanischen Randbedingungen ist unklar. Durch die Verwendung von Präparationen, mit denen spezielle Formen adaptiven Verhaltens unter Bedingungen untersucht werden können, die gezielt externe Einflüsse wie z. B. die mechanische Kopplung der Beine oder Unterschiede in der Körpermasse ausschließen, können Rückschlüsse auf die Organisation der jeweils zugrunde liegenden neuronalen Strukturen gezogen werden.

In der vorliegenden Arbeit werden vier Publikationen vorgestellt, die jeweils Hinweise auf Mechanismen der zeitlichen oder räumlichen Koordination der Beinbewegungen bei der Stabheuschrecke *Carausius morosus* oder der Fruchtfliege *Drosophila melanogaster* unter verschiedenen Versuchsbedingungen geben. Zunächst wurden zustandsabhängige, lokale koordinierende Mechanismen untersucht. Anhand von elektromyographischen Messungen wurden die drei wichtigsten antagonistischen Beinmuskelpaare in der vorwärts und rückwärts laufenden Stabheuschrecke untersucht. Es wird deutlich, dass sich beim Wechsel der Laufrichtung nur die Aktivität des proximalsten Beingelenks ändert. Dies ist ein Beleg für die modulare Organisation der neuronalen Netze, die für die Bewegung der einzelnen Beine zuständig sind.

Der zweite Abschnitt beschäftigt sich mit Mechanismen, die die Fortbewegungsgeschwindigkeit der einzelnen Beine und die Koordination der Geschwindigkeit zwischen den verschiedenen Beinen bei der Stabheuschrecke beeinflussen. Elektrophysiologische und Verhaltensexperimente mit dem intakten Tier oder reduzierten

Präparaten wurden angewendet. Es wurden Zusammenhänge untersucht zwischen der Geschwindigkeit eines schreitenden Beins und der neuronalen Aktivität im benachbarten Ganglion, sowie Korrelationen zwischen den Geschwindigkeiten verschiedener Beine während Läufen mit kontinuierlicher Geschwindigkeit oder mit deutlichen Beschleunigungen. Es konnte gezeigt werden, dass die Schreitgeschwindigkeit eines Beines weder in der Aktivität der Motoneurone anderer Beine noch in deren Schreitgeschwindigkeit widergespiegelt wird. Nur bei einer Zunahme der Fortbewegungsgeschwindigkeit konnte eine Korrelation zwischen den Schreitgeschwindigkeiten verschiedener Beine gefunden werden.

Im Anschluss zeigt die Untersuchung der Veränderungen in der zeitlichen Koordination der Beine während verschiedenen Fortbewegungsgeschwindigkeiten, dass das Lokomotionssystem von *Drosophila* einen breiten Bereich an Geschwindigkeiten abdecken kann und dabei sehr ähnlichen Regeln folgt, wie das Lokomotionssystem der Stabheuschrecke. Die Laufgeschwindigkeit wird durch Veränderungen in der Stemmphasendauer variiert, während Schwingphasendauer und Schrittweite nahezu unverändert bleiben. Änderungen in der Koordination der Beine sind graduell und systematisch mit der Fortbewegungsgeschwindigkeit und können gravierenden biomechanischen Änderungen, wie etwa der Amputation eines Beines, angepasst werden.

Im letzten Abschnitt war es das Ziel, die Rolle der neuronalen Mechanismen bei der Orientierung und räumlichen Koordination der Aufsetzpositionen der Beine bei der Stabheuschrecke zu verstehen. Die Positionierung der Mittel- und Hinterbeine wurde in Bezug auf die Position ihres entsprechenden anterioren Nachbarbeins bei zwei verschiedenen Aktivitätszuständen untersucht. Es wurden segment- und zustandsabhängige Unterschiede in der Zielgenauigkeit von Mittel- und Hinterbeinen gezeigt. Dies weist auf Unterschiede in den zugrunde liegenden neuronalen Strukturen der verschiedenen Segmente, sowie die Bedeutung der Bewegung im Ziel-Bein für die Verarbeitung der Positionsinformation hin.

Zusammenfassend können aus den Arbeiten gemeinsame Gesetzmäßigkeiten für die Beinkoordination wie z. B. Ähnlichkeiten zwischen verschiedenen Organismen und segment- oder zustandsabhängige Modifikationen im Fortbewegungssystem abgeleitet werden. Diese können als Beleg für die starke Anpassungsfähigkeit und die modulare Struktur der zugrunde liegenden neuronalen Strukturen angesehen werden.

# 2. Abstract

Locomotion depends on constant adaptation to different requirements of the environment. An appropriate temporal and spatial coordination of multiple body parts is necessary to achieve stable and adapted behavior. To date, it is unclear how the underlying neuronal structures can achieve these meaningful adaptations. The specific roles of the nervous system, muscles and mechanical constrains are not known. By using preparations in which special forms of adaptations are considered under experimental conditions that selectively exclude external influences, like mechanical interactions through the ground or differences in body mass, one can draw conclusions about the organization of the respective underlying neuronal structures.

In the present thesis, four different publications are introduced, focusing on mechanisms of temporal or spatial coordination of leg movements in the stick insect *Carausius morosus* and the fruit fly *Drosophila melanogaster* in different experimental paradigms. First of all, state dependent local coordinating mechanisms were analyzed. Electromyographic measurements of the three major antagonistic leg muscle pairs of the forward and backward walking stick insect were evaluated. It became evident that only the motor activity of the most proximal leg joint is changed when walking direction is changed from forward to backward. This demonstrates that the neuronal networks driving movement in each individual leg seem to be organized in a modular fashion.

In the second part, mechanisms that influence movement speed of the individual leg and coordination of speed between the different legs of the stick insect come into focus. Electrophysiological and behavioral experiments with the intact and reduced stick insect were used to examine relationships between the velocity of a stepping front leg and neuronal activity in the mesothoracic segment, as well as correlations between the

stepping velocities of different legs during walks with constant velocity or with distinct accelerations. It was shown that stepping velocity of single legs were not reflected in motoneuron activity or stepping velocity of another leg. Only when an increase in walking speed was induced, clear correlations in the stepping velocities of the individual legs were found.

Subsequently, the analysis of changes in temporal leg coordination during different walking speeds in the fruit fly revealed that the locomotor system of *Drosophila* can cover a broad range of walking speeds and seems to follow very similar rules as the locomotor system of the stick insect. Walking speed is controlled by modifying stance duration, whereas swing duration and step amplitude remain largely constant. Changes in inter-leg coordination are gradual and systematical with regard to walking speed and can be adapted to major biomechanical changes, like the amputation of one leg.

In the final part, the aim was to understand the role of neuronal mechanisms for the orientation and spatial coordination of foot placement in the stick insect. Placement of middle and hind legs with respect to the position of their respective rostrally neighboring leg were analyzed under two different conditions. Segment and state dependent differences in the aiming accuracy of the middle and hind legs could be shown. This indicates differences in the underlying neuronal structures in the different segments and the importance of movement in the target leg for the processing of the position information.

Taken together, common principles in inter-leg coordination were found, comprising similarities between different organisms and segment specific or state dependent modifications in the walking system. These common principlesc can be interpreted as evidence for a highly adaptive and modular design of the underlying neuronal structures.

# 3. Introduction

If animals want to navigate through any kind of environment, they need to constantly adapt their motor output to produce appropriate temporal and spatial coordination of their movements. During evolution different species have developed different ways of locomotion, but regardless whether swimming, crawling, flying, or walking, all ways of locomotion have to meet the same prerequisites. Locomotion always emerges from a complex interplay of the activities of nervous system, muscles, and sense organs with the environment (Orlovsky et al. 1999). During locomotion, antagonistic muscles of specialized body parts have to be activated in recurrent patterns of coordinated, rhythmical contractions. These contractions move the body, multi-jointed limbs or other appendages. For locomotion a complex rhythmic motor pattern has to be generated, coordinated intersegmentally and adapted to the environment the animal locomotes in.

Terrestrial vertebrates and invertebrates have developed different numbers of multi-jointed limbs, ranging from two in humans to up to 750 in myriapods. The cyclic pattern of a walking leg consists of two phases: stance (power stroke) and swing (return stroke). During stance phase the leg is on the ground to produce propulsion of the animal while during swing phase the leg is moved to the starting position of the next stance. The movements of the legs have to be not only coordinated intra- and intersegmentally, but also adapted to different walking terrains, body postures and behavioral situations to allow speed changes, reversed walking direction, and goal-directed locomotion. Especially, when navigating through an uneven terrain or when slow explorative walking has to be changed into a fast escape run, the temporal and spatial adaptations in the movement of the limbs are drastic. For example when an animal has to escape a predator or cross terrain without cover, it has to distinctly increase its movement speed. Legged animals can achieve a change in walking speed by changing cycle period or stride length. Changes in cycle period are found in some vertebrates, insects, or crustaceans,

and are usually achieved by modifying stance duration, whereas swing duration remains largely unchanged (cat: Halbertsma 1983; dog: Maes et al. 2008; stick insect: Wendler 1964; locust: Burns 1973; lobster: Clarac & Chasserat 1983; Chasserat & Clarac 1983; reviewed in Orlovsky et al. 1999). However, a decrease in swing duration has also been reported as a means to decrease cycle period in alligators (Reilly & Elias 1998), mice (Herbin et al. 2004, 2008), horses (Robilliard et al. 2007), and elephants (Hutchinson et al. 2006).

A well established system for slow walking behavior is the stick insect. The simply organized and easily accessible nervous system of the stick insects shows only a comparatively narrow behavioral repertoire. In their natural habitat, stick insects walk and climb on the bushes they feed on. They have six multisegmented legs that have to be coordinated properly to achieve a stable locomotor pattern. The insect leg consists of five main segments: the coxa, the trochanter, the femur, the tibia, and the segmented tarsus. In the stick insect *Carausius morosus* the trochanter is fused with the femur and hence, in this organism, leg movements are mainly controlled by muscles of the thorax-coxa (ThC) joint, the coxa-trochanter (CTr) joint and the femur-tibia (FTi) joint. The muscles of the ThC joint move the leg forwards through activity of the *protractor coxae* muscle and backwards through that of the *retractor coxae* muscle. The *levator* and *depressor trochanteris* muscles lift and lower the leg through the CTr joint and the flexion and extension of the FTi joint is mediated by the *flexor* and *extensor tibiae* muscles (Graham & Epstein, 1985). These antagonistic muscle pairs are active in alternation during the generation of a step but very little is known about the timing of leg muscle activity during walking of the intact animal (Epstein & Graham 1983; Graham & Epstein 1985). However, to understand how sensory input induces the transitions between the different phases of a step, it is necessary to know the exact timing of muscle activities (Büschges & Gruhn 2008). With detailed knowledge of the muscle activity during straight walking it is possible to interpret the alterations in muscle activity that occur during alterations and adaptations in walking, e.g. changes in walking direction (Cruse et al. 2009; Gruhn et al. 2009a; Mu & Ritzmann 2005; Ridgel et al. 2007; Akay et al. 2007) or changes in walking speed (Gruhn et al. 2009b).

The leg of an insect is equipped with different sense organs like femoral and trochanteral campaniform sensilla, which provide information about load or forces (Tatar 1976; Bässler 1977; Hofmann & Bässler 1982; Akay et al. 2004), hair plates and hair rows, which measure the relative position of leg segments (Wendler 1964; Tatar 1976; Bässler 1977), and the femoral chordotonal organ, which measures angle and movement of the FTi joint (Borchardt 1927; Bässler 1965, 1967; Füller & Ernst 1973). Sensory feedback from these sense organs contributes both to coordination of motor activity of the single stepping leg (Büschges et al. 2008) as well as to intersegmental coordination between legs (Dürr et al. 2004). Behavioral studies have led to the proposition of a set of coordination rules, which suggest that signals from these sense organs contribute to the coordination between legs (Cruse 1990; Dürr et al. 2004). Furthermore, studies with reduced mechanical interaction between the legs have demonstrated the importance of intersegmental neural pathways (Graham & Cruse 1981; Cruse & Epstein 1982; Gruhn et al. 2006, Gruhn et al. 2009a). Further evidence confirmed the importance of central inter-segmental neural pathways for the coordination of local networks controlling walking movements in the cockroach *Periplaneta americana* (Pearson & Iles 1973), the locust *Schistocerca americana* (Ryckebusch & Laurent 1993) and *Manduca sexta* (Johnston & Levine, 2002). However, different studies have also demonstrated the role of local sensory feedback in establishing inter-leg coordination, e.g. in the hawk moth (Johnston & Levine 1996; 2002) and the stick insect *C. morosus* (Borgmann et al. 2009; Büschges et al.

1995). While it is clear that during normal walking both mechanical and neural coupling between individual legs play important roles, their specific contribution for the generation of leg coordination is not clear.

Even though sensorimotor control of walking in general is fairly well understood in the stick insect (Büschges et al. 2007; for review see Büschges & Gruhn 2008), very little is known about the neural mechanisms underlying fast specific adaptations of the walking pattern that are necessary, for example, to change the walking speed. Bender and coworkers (2010) identified brain structures in the central complex of cockroaches, which are involved in the control of locomotor speed. Foth and Bässler (1985a,b) showed that the cycle period of all six legs adjusts to whole number ratios in a situation in which five legs are stepping on a passive treadmill, while a single hind leg is stepping on a separate treadmill with a given speed. However, it is unclear if this is due to a control of stepping velocity commonly shared between the legs, as it might as well be a consequence of coordinating influences between the legs. Gabriel and Büschges (2007) could show in the single middle leg preparation of the stick insect, that stance phase motor neuron activity is responsible for stepping velocity. They also discovered that mechanisms for altering the velocity become effective only during an already ongoing stance phase. However, exactly how the motor neurons and their activity patterns are affected in the course of changes in walking speed is still largely unresolved. One mechanism for velocity adjustments without neural origin, which should not be neglected are muscle characteristics, especially the force–velocity relation (Blümel et al. 2007; Guschlbauer et al. 2007; Hooper et al. 2007, 2009). Forces generated by the stepping front legs could be transferred to the posterior legs by altering the forces acting on them and their muscles as a result of mechanical coupling through the ground. This might in turn change the muscle contraction velocity, as predicted by the force-velocity curve of the respective muscles. To exclude these mechanical properties, experimenters have used preparations with mechanically uncoupled legs. This can be achieved by using single leg preparations (Bässler 1993; Fischer et al. 2001), isolated nerve cords (Bässler & Wegener 1983; Büschges et al. 1995) or a slippery surface setup (Graham & Cruse 1981; Cruse & Epstein 1982; Gruhn et al. 2006). The slippery surface setup reliably removes effects of ground contact-mediated mechanics and hence facilitates the study of the neuronal control of leg movements.

Changes in walking speed usually also entail changes in the coordination between several or all legs. Depending on the movement speed, quadrupeds like cats, dogs or horses, for instance, often use specific gaits (Alexander 1989). Leg coordination is changed from slow to fast speeds using walking and pace gaits at slow speeds, trotting gaits at intermediate speeds and gallop at high speeds to select the energetically optimal gait at a given speed (Hoyt and Taylor 1981). The temporal coordination of the front and hindlegs changes from anti-phase in walking to nearly in-phase during gallop (Orlovsky et al. 1999). It has been found that the mechanism underlying speed changes varies with the gait (dogs: Maes et al. 2008; cats: Halbertsma 1983; Yakovenko et al. 2005; reviewed in Orlovsky et al. 1999; mice: Herbin et al. 2004, 2006; and elephants: Hutchinson et al. 2006). During walking and trot, speed is increased by a decrease in cycle period, whereas during gallop, speed is increased by an increasing stride length.

At first glance, in hexapods, i.e. insects, the situation appears to be comparable as they also show different preferred patterns of intersegmental coordination during different walking speeds. During very slow walking a coordination pattern called wave gait is generated. This gait is characterized by a metachronal wave from

back to front along each side of the body, while at least five legs are always in stance phase (Hughes 1952). When the walking speed increases, the coordination of the legs is changed in a way that the number of legs that are simultaneously on the ground is reduced, i.e. the number of legs that perform a swing phase are increased at the same time. At medium speeds, primarily the so called tetrapod coordination occurs. This coordination is characterized by the fact that four legs perform a stance phase while a diagonal, contralateral pair of legs is simultaneously in swing (Burns 1973; Graham 1972; Hughes 1952; Spirito & Mushrush 1979; Wendler, 1964, 1966). The tripod coordination, with three legs in stance while the three remaining legs are in swing, prevails at high speeds (Bender et al. 2011; Delcomyn 1971; Graham 1985).

However, while quadrupeds show a distinct, discontinuous, and speed dependent switch between two patterns of inter-leg coordination, this is not the case in invertebrates. Invertebrates appear to display a speed-dependent continuum of inter-leg coordination and the specific patterns together with intermediate forms of coordination are part of this continuum. By simply modifying stance duration insects can seamlessly transition between tetrapod and tripod coordination without changing the locomotion speed (Cruse 1990; Graham 1985; Wendler 1966). Many insect species like stick insects (*C. morosus*), cockroaches (*P. americana*), ants (*Cataglyphis*, *Formica*, *Lasius* and *Myrmica*), and fruit flies (*Drosophila melanogaster*) are known to use tripod coordination during fast locomotion, while at lower speeds, leg coordination becomes much more variable, even approaching tetrapod coordination (Wendler 1964; Graham 1972; Bender et al. 2011; Strauss & Heisenberg 1990; Zollikofer 1994). Although it is the current notion that invertebrates show a speed-dependent continuum of interleg coordination, this idea is yet unproven because it has never been shown to be present in a single species.

One aspect why the neural mechanisms underlying inter-leg coordination could not be analyzed in more detail is the fact that insect species at given developmental stages (Graham, 1985) often show a rather narrow range of preferred walking speeds. For example, under natural conditions cockroaches mostly use tripod coordination (Bender et al., 2011) although they can use the full range of inter-leg coordination from metachronal wave gait to tripod coordination (Hughes, 1952). Adult stick insects almost exclusively use tetrapod coordination during level walking, while at high speeds they also use tripod coordination (Graham, 1972, Grabowska et al. 2012). Although tripod coordination is less frequent in adult stick insects, the much smaller larval stages tend to use tripod coordination much more frequently (Graham, 1972). As a consequence, only small ranges of walking speeds could be investigated reliably in the species studied so far. However, as the inter-leg coordination is often used as indicator of how the neural mechanisms generating walking behavior may be structured (Zollikofer, 1994) it is crucial to capture a large range of walking speeds in a single species.

To capture such a large range of walking speeds in a single species one could either use species that show a broad range of walking speeds, or use genetically different strains of the same species. Both is possible in the fruit fly *Drosophila melanogaster* which already shows a broad range of walking speeds in the wild type and additionally numerous transgenic organisms are available, which show altered walking behavior. Previous studies on *Drosophila* have already analyzed inter-leg coordination (Strauss & Heisenberg, 1990; 1993) and global parameters of locomotor activities (Martin, 2004; Martin et al., 1999) in the two wild-type strains *Canton-S* and *Berlin*. In addition, one neuromodulator that is implicated to have an effect on the higher-level control of locomotor activity of insects is octopamine (Brembs et al., 2007; Gal & Libersat, 2008; 2010).

Therefore, by choosing *Drosophila* strains with reduced levels of this biogenic amine, one could extend the range of observable walking speeds to lower values. The two mutant *Drosophila* strains, *white*$^{1118}$ and $w^{1118}$, $Tbh^{nM18}$ meet these conditions. $w^{1118}$ flies have reduced levels of octopamine (Sitaraman et al., 2008), while $w^{1118}$, $Tbh^{nM18}$ lacks this neuromodulator altogether (Monastirioti et al., 1996). As an extensive amount of transgenic flies have a $w^{1118}$ background, characterization of this strain is also necessary as control for future studies with other transgenic strains of *Drosophila*.

However, not only the temporal coordination of the legs and the muscles within is necessary to locomote, also spatial coordination has to be achieved. Especially when moving through an unpredictable environment this is crucial to reliably find foothold. In several species, it is known that targeting of leg movements is primarily mediated by visual information (e.g. human: Mohagheghi et al. 2004, Patla & Vickers 2003; cat: McVea & Pearson 2007, McVea et al. 2009, Wilkinson & Sherk 2005, fruit fly: Pick & Strauss 2005, Triphan et al 2010; locust: Niven et al. 2010). However, how do animals find appropriate foothold when visual information is not available? In the same study as mentioned above, Niven et al. (2010) also observed that placement of the middle leg in locusts was not visually guided. Information on where to place the middle legs has therefore to be acquired differently. Being a nocturnal animal, the stick insect *Carausius morosus* primarily relies on mechanosensory information from the antennae to guide its front legs towards an appropriate foothold and does not use vision for this purpose (Bläsing & Cruse 2004, Dürr 2001, Schütz & Dürr 2011). How it guides its hind legs towards an appropriate foothold has also been the focus of several investigations (e.g. Cruse 1979, Cruse et al. 1984, Dean 1984 & 1989, Dean & Wendler 1983). From work on the stick insect it is known that proprioceptive inputs of several sensory structures in the leg influence the protraction endpoint of all legs (Wendler 1964; Bässler 1977; Dean and Wendler 1983). This implies that the nervous system has information about the position of all legs and integrates it at all times during walking to target the tarsi. This information can be provided by several kinds of sense organs and different animals use different sources. Cats, for example, use information from muscle receptors and cutaneous receptors in the skin from different joints to be integrated and reliably represent the position of the limb relative to the body in the dorsal root ganglia (Stein et al. 2004). The stick insect uses information from hair rows and hair fields on the coxa to measure the position of the leg parallel to the body axis (Cruse et al. 1984, Dean & Wendler 1983) and the femoral chordotonal organ to measure the position perpendicular to the body axis (Cruse et al. 1984). However, it is still unclear how information from sense organs of different legs is integrated to achieve appropriate spatial coordination. Three types of interneurons are known that each signal the angle of one single leg joint and hence together are able to encode the tarsus position (Brunn & Dean 1994). And, at least for the middle leg, this information is transmitted caudally via the ipsilateral connective (Dean 1989). The touchdown position of the hind leg depends on the position of the standing middle leg (Cruse 1979), but it is not known how stick insects guide their middle legs towards an appropriate foothold, e.g. if they use position information from the front legs. In addition, many studies have shown that the behavioral state of the animal is important for the effectiveness of sensory input on the motoneurons (e.g. Hellekes et al. 2012; for review, see, e.g., Büschges & El Manira 1998, Clarac et al. 2000, Duysens et al. 2000, Pearson 1993) but it is not known to what extend movement of the anterior leg influences the targeting accuracy of the middle or hind leg. Until now the question has also been neglected as to what extend targeting behavior might be a result of limb joint constraints or mechanical coupling via the ground or if it is an effect that actually arises only from properties of the neuronal system.

In the present thesis, I will present evidence for several mechanisms of temporal and spatial coordination of leg movements in the stick insect *Carausius morosus* and the fruit fly *Drosophila melanogaster* during different experimental paradigms. Starting with local coordinating mechanisms of antagonistic muscle pairs within the individual leg, I will continue with mechanisms that influence movement speed of the individual leg and coordination of speed between the different legs. Then, I will analyze how changes in walking speed are implemented in the fruit fly and compare those with the stick insect. Finally, I will focus on spatial leg coordination of the stepping stick insect. The four parts of the thesis each consist of one publication.

The first publication (Rosenbaum et al. 2010) compares the activity of the three major muscle pairs of the stick insect middle leg between straight forward and backward walking. It shows that the timing of *protractor* and *retractor* is inverted while timing of the other muscle pairs remains largely unchanged. In this study, the slippery surface setup is used together with electromyographic measurements to investigate the activity of *protractor* and *retractor coxae*, *levator* and *depressor trochanteris* and *flexor* and *extensor tibiae* of the stick insect *C. morosus*. The measurements were evaluated with respect to touchdown and liftoff.

The second publication (Gruhn et al. 2009) gives evidence for a neural control mechanism to change stepping speed. In this study, electrophysiological and behavioral experiments with the intact and reduced stick insect (*Carausius morosus*) where used. Extra- and intracellular recordings in single-leg stick insect preparations where used to examine relationships between the velocity of a stepping front leg and the motoneuronal activity in the ipsi- or contralateral mesothoracic *protractor* and *retractor*, as well as *flexor* and *extensor* MNs. I performed experiments with intact stick insects tethered above a slippery surface to effectively remove mechanical coupling through the ground and to elucidate correlations between the stepping velocities of different legs during walks with constant a velocity or with distinct accelerations.

The third publication (Wosnitza et al. 2013) shows that *Drosophila* changes the coordination of its legs gradually and systematically with walking speed and can adapt its coordination to major biomechanical changes in its walking apparatus. In this study, I used four different *Drosophila* strains in order to capture as large a range of walking speeds as possible in a single species. The two wild-type strains *Canton-S* and *Berlin* represented the typical behavior in the wild. In addition, two mutant *Drosophila* strains, $white^{1118}$ and $w^{1118}$, $Tbh^{nM18}$ where used to extend the range of observable walking speeds to lower values. Furthermore, in some individuals of the wild-type strain *Canton-S*, I removed one of the hindlegs to analyze if *Drosophila* is capable of adapting to major biomechanical changes in its walking apparatus.

The fourth and final publication of my thesis (Wosnitza et al, in prep.) gives evidence for differences in the targeting accuracy of the middle and hind leg of *Carausius morosus* in first steps of a sequence and during continuous walks. I investigated the placement of middle and hind legs in the stick insect *C. morosus* in a slippery surface setup to understand how important neuronal mechanisms are for the orientation and spatial coordination of foot placement without visual guidance. I measured the targeting accuracy of the middle leg towards the front leg and the targeting accuracy of the hind leg towards the middle leg and compared their performance with each other under two behavioral conditions. First, targeting was investigated in the resting animal when the anterior leg was standing on one of seven defined positions. Second, to identify to which extend the state of the animal influences the targeting accuracy, I also looked for dependencies of the touchdown position on the position of the rostrally adjacent leg during continuous walks.

# 4. Published Studies

## 4.1 Activity Patterns and Timing of Muscle Activity in the Forward Walking and Backward Walking Stick Insect *Carausius morosus*

Philipp Rosenbaum, Anne Wosnitza, Ansgar Büschges, Matthias Gruhn

Published in Journal of Neurophysiology (104(3):1681-1695, 2010)

Für diese Publikation habe ich die Versuchsserien und das Konzept der Arbeit zusammen mit den Koautoren entwickelt. Ich habe die Versuche gemeinsam mit Philipp Rosenbaum durchgeführt (Vorwärtslaufen – Rosenbaum; Rückwärtslaufen – Wosnitza), was auch für die Datenauswertung gilt. Die Ergebnisse wurden gemeinsam diskutiert. Ich habe alle Abbildungen fertig gestellt sowie gemeinsam mit Philipp Rosenbaum die ersten Versionen der Methoden, Ergebnis- und Diskussionsteile der Arbeit entworfen.

# Activity Patterns and Timing of Muscle Activity in the Forward Walking and Backward Walking Stick Insect *Carausius morosus*

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**Rosenbaum P, Wosnitza A, Büschges A, Gruhn M.** Activity patterns and timing of muscle activity in the forward walking and backward walking stick insect *Carausius morosus*. *J Neurophysiol* 104: 1681–1695, 2010. First published July 28, 2010; doi:10.1152/jn.00362.2010. Understanding how animals control locomotion in different behaviors requires understanding both the kinematics of leg movements and the neural activity underlying these movements. Stick insect leg kinematics differ in forward and backward walking. Describing leg muscle activity in these behaviors is a first step toward understanding the neuronal basis for these differences. We report here the phasing of EMG activities and latencies of first spikes relative to precise electrical measurements of middle leg tarsus touchdown and liftoff of three pairs (*protractor/retractor coxae, levator/depressor trochanteris, extensor/flexor tibiae*) of stick insect middle leg antagonistic muscles that play central roles in generating leg movements during forward and backward straight walking. Forward walking stance phase muscle (depressor, flexor, and retractor) activities were tightly coupled to touchdown, beginning on average 93 ms prior to and 9 and 35 ms after touchdown, respectively. Forward walking swing phase muscle (levator, extensor, and protractor) activities were less tightly coupled to liftoff, beginning on average 100, 67, and 37 ms before liftoff, respectively. In backward walking the protractor/retractor muscles reversed their phasing compared with forward walking, with the retractor being active during swing and the protractor during stance. Comparison of intact animal and reduced two- and one-middle-leg preparations during forward straight walking showed only small alterations in overall EMG activity but changes in first spike latencies in most muscles. Changing body height, most likely due to changes in leg joint loading, altered the intensity, but not the timing, of depressor muscle activity.

## INTRODUCTION

Freely behaving animals often display much more complex locomotor outputs than those observed in reduced preparations because of the needs to respond to environmental contingencies and to produce goal-directed movements. Despite considerable work devoted to understanding how this behavioral plasticity arises (humans: Lamb and Yang 2000; van Deursen et al. 1998; salamander: Ashley-Ross and Lauder 1997; fish: Orger et al. 2008; lamprey: Islam et al. 2006; fruit fly: Frye and Dickinson 2004a,b; cockroach: Watson et al. 2002a,b; stick insect: Dürr and Ebeling 2005; Gruhn et al. 2009a,b), we are still only beginning to understand the underlying mechanisms on the neuronal level (Akay et al. 2007; Pick and Strauss 2005; Ridgel and Ritzmann 2005; Ridgel et al. 2007; Schaefer and Ritzmann 2001).

For the stick insect *Carausius morosus* substantial knowledge exists about leg kinematics during adaptive locomotor behaviors such as different walking directions, turning, and gap climbing (Blaesing and Cruse 2004; Cruse 1976a; Cruse et al. 2009; Dürr and Ebeling 2005; Gruhn et al. 2009b; Jander 1985). Substantial information also exists about the central neural mechanisms that generate the locomotor output (for review, see Bässler and Büschges 1998; Büschges 2005; Büschges and Gruhn 2008), although very little is known about the timing of leg muscle activity during walking (Epstein and Graham 1983; Graham and Epstein 1985). This information is important because *1*) the exact timing of muscle activities during swing-to-stance transitions is needed to assess how sensory input induces them (Büschges and Gruhn 2008) and *2*) detailed knowledge of straight walking muscle activity is required to correctly interpret the alterations in muscle activity that occur during locomotor output changes such as turns (Cruse et al. 2009; Gruhn et al. 2009a; Mu and Ritzmann 2005; Ridgel et al. 2007), changes in walking speed (Gruhn et al. 2009b), and switches between tunneling and climbing (Harley et al. 2009) and forward and backward walking (Akay et al. 2007).

In the present study we set out to bridge this gap in knowledge by recording stick insect leg muscle activity during forward and backward walking. Studying neuronal or muscular activity in behaving animals requires recording techniques that do not unduly interfere with animal movement. Two techniques that have been successfully applied to relatively large animals are to use implantable electrodes and then to transmit the data along a long tether (Böhm et al. 1997; Clarac et al. 1987; Duch and Pflüger 1995; Gruhn and Rathmayer 2002) or using telemetric devices (Fischer et al. 1996; Hama et al. 2007; Kutsch et al. 1993; Tsuchida et al. 2004; Wang et al. 2008). These methods are difficult to apply to small animals such as stick insects because smaller animals become easily entangled in the long, often heavy tethers and the weight of telemetric devices is often very large. One solution to this problem is to conduct experiments with tethered animals on a slippery surface, where the animal is free to walk but the body nonetheless stays stationary. This has been used to study escape responses (Camhi and Levy 1988; Camhi and Nolen 1981), turning (Gruhn et al. 2009a; Tryba and Ritzmann 2000a,b), backward walking (Graham and Epstein 1985) , and changes in velocity (Gruhn et al. 2009b). It also allows easy combination of intra- and extracellular physiology with kinematics analyses, particularly when coupled with our electronic measurement of tarsus ground contact (Gruhn et al. 2006). Furthermore, this approach allows one to study the neuronal control of a leg movement without the effects of ground contact mediated mechanics

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because the respective interleg interactions through the surface are not present and the moving legs therefore exert no force on the body.

We have used the slippery surface setup to investigate the activity of the three major muscle pairs of the stick insect middle leg with respect to touchdown and liftoff during forward and backward straight walking as a first form of adaptive behavior.

## METHODS

### *Animals*

All experiments were performed on adult female stick insects (*Carausius morosus*). Animals were reared in the animal facility of the institute in a 12-h/12-h light/dark cycle at 20–22°C and were fed with blackberry leaves (*Rubus fructiosus*) without restriction.

### *Experimental setup*

In all experiments, animals walked on a 13.5 × 13.5 cm polished nickel-coated brass plate divided into two halves. To allow unimpeded walking under tethered conditions and remove mechanical coupling between the legs, the plate was covered with a lubricant composed of 95% glycerin, 5% saturated NaCl, and a small amount of electrode cream (Marquette Hellige, Freiburg, Germany). This created a slippery surface and also allowed recording of tarsal contact by electric current flow during ground contact (Gruhn et al. 2006). The animal was glued ventral side down on an 80 × 3 mm (length × width) balsa rod using dental cement (ProTempII, ESPE, Seefeld, Germany) so the legs and head protruded from the rod and all joints were unrestrained. Animal height above the substrate was adjustable, but was typically 10 mm. Experiments were performed in a darkened Faraday cage at room temperature.

Walking was elicited by projecting a progressive striped pattern (pattern wave length 21°) onto two 13.5-cm diameter round glass screens (Scharstein 1989) placed at right angles to each other and at a 45° angle to the walking surface, about 6–7 cm away from the eyes of the animal. Reflections on the polished brass plate further increased the field of view. Alternatively, a single white stripe on dark background (toward which the animals orient with straight walking sequences) was placed in front of the animal. If the animal did not begin locomotion spontaneously, walking was elicited by light brush strokes to the abdomen. Backward walking was elicited by gentle pulls on the antenna (Graham and Epstein 1985).

### *Electrophysiology*

Muscle activity (electromyogram [EMG]) was recorded using two twisted, coated copper wires (OD: 57 or 49 $\mu$m) placed in each muscle about 1 mm apart and held in place with dental cement (ProTempII, ESPE) or tissue adhesive (Vetbond; 3M, St. Paul, MN). Figure 1*A* shows the approximate sites for the EMG wire placement in the cuticle of the leg and thorax. All recordings were differentially amplified. The EMG signal was preamplified 100-fold (electronics workshop, Zoological Institute, Cologne, Germany), band-pass filtered (100 to 2,000 Hz), when necessary further amplified 10- to 1,000-fold, and imported into Spike2 (version 5.05, CED, Cambridge, UK) through an AD converter (Micro 1401k II; CED). A reference electrode was placed in the abdomen of the stick insect.

In most experiments, two antagonistic joint muscles were recorded simultaneously. *Protractor coxae* and *retractor coxae* EMGs were recorded in the thorax, *depressor trochanteris* and *levator trochanteris* in the coxa, and *extensor tibiae* and *flexor tibiae* in the femur. In two experiments three muscles, in three experiments four muscles, and in one all six muscles were recorded from simultaneously. These experiments gave the same results as the others.

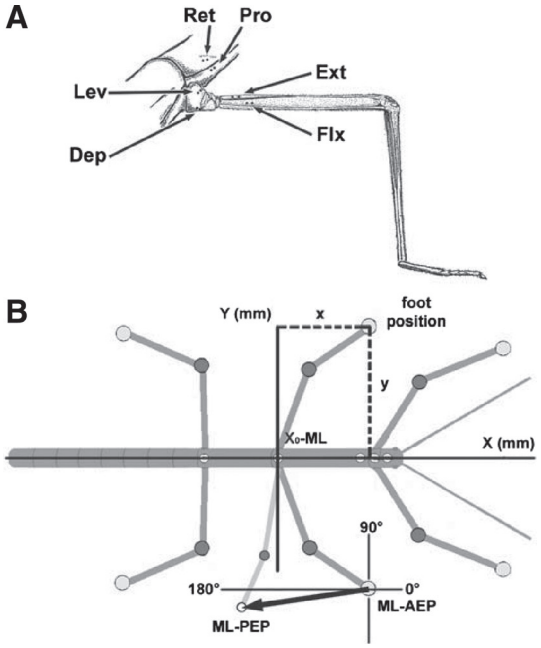


FIG. 1. *A*: drawing of the stick insect middle leg and the adjacent mesothorax with the approximate placement sites for the electromyographic (EMG) electrodes for recordings of the main leg muscles. Pro, *protractor coxae*; Ret, *retractor coxae*; Ext, *extensor tibiae*; Flx, *flexor tibiae*; Lev, *levator trochanteris*; Dep, *depressor trochanteris*. *B*: schematic drawing of the stick insect with the tracked reference points for the analysis of leg kinematics marked as gray dots. *x*-values are always points along the length of the animal, whereas *y*-values mark points perpendicular to the animal. The $x_0$-value was set at the level of the middle leg coxa to give a clear reference point. As an example for the determination of the step length vector and its direction, the right middle leg is drawn at 2 arbitrary positions, one anterior extreme position (ML–AEP) and one posterior extreme position (ML–PEP). The vectors for all steps connecting the 2 positions, normalized to the origin in the AEP, gave direction in degrees and step length in millimeters. The 0–180° axis was always parallel to the body axis and crossed the AEP, 90° always points toward the animal perpendicularly.

### *Recording tarsal contact*

To determine the exact moment of the switch between stance and swing we used middle leg tarsal contact as a switch to open and close an electric circuit (Gruhn et al. 2006). Briefly, we used a 2- to 4-mV amplitude square-wave signal generated with a pulse generator (Model MS501, electronics workshop; Zoological Institute) and applied to one half of the slippery surface and a lock-in amplifier (electronics workshop; Zoological Institute) as a reference signal. We tied a copper wire (OD: 49 $\mu$m) with its insulation removed at the tip around the tibia of the leg being monitored and connected it to the lock-in amplifier with an alligator clip. The resistance between the cuticle and copper wire was reduced with a drop of electrode cream (Marquette Hellige) placed at the area of contact, allowing a 2- to 4-nA current to pass through tarsus and tibia. During stance, current flowed from the plate through tarsus and tibia into the copper wire, but during swing, when the leg was in the air, the circuit was disconnected. Amplifier output was fed into a CED AD converter and digitized using Spike2.

Due to the low-pass filter properties of the lock-in amplifier and the gradual liftoff/touchdown of the tarsus, the signal was not exactly square. We therefore used thresholds set close to the transition point to define the timing of tarsal contact and manually checked each event. Touchdowns could be determined at a resolution of $<$1 ms.

Liftoff transitions were less steep and more delayed because of delayed tearing of the lubricant from the tarsus due to a capillary action and occasional upward movements of the leg during stance without complete liftoff. To have comparable liftoff times in all experiments we therefore always defined liftoff as the time point with the steepest ascending slope.

### *Optical recording and digital analysis of leg movements*

Optical recordings of forward and backward walking were performed and analyzed as in Gruhn et al. (2009a). In brief, we recorded walking sequences with a high-speed video camera (Marlin F-033C; Allied Visions Technologies, Stadtroda, Germany) that was externally triggered at 100 fps. Insect head, thorax, and legs were marked with fluorescent pigments (Dr. Kremer Farbmühle, Aichstetten, Germany) mixed with dental cement. During the recording of walking sequences, the animal was illuminated with blue light-emitting diode arrays (12 V AC/DC; Conrad Electronic, Berlin). The video files were analyzed using motion-tracking software (WINanalyze 1.9; Mikromak Service, Berlin). AEP describes the anterior extreme position of the leg at touchdown, whereas PEP is the posterior extreme position at liftoff. In forward stepping AEP in stance is anterior to PEP, whereas in backward stepping AEP in stance is posterior to PEP. AEP and PEP values are always given in millimeters in the form *xx.x*; *yy.y* ($SD_x$; $SD_y$); *x*-values are given with respect to the length of the animal, a virtual 0 line being drawn across the animal at the level of the coxa. Positive and negative *x*-values indicate points anterior and posterior to the coxa, respectively; *y*-values are given with respect to the axis perpendicular to the length of the animal. Larger *y*-values denote more distal points, smaller values more central points. Figure 1*B* shows a schematic drawing of the stick insect with the tracked reference points for the analysis of leg kinematics marked as gray dots. As an example for the step length vector determination and its direction, the right middle leg is drawn at two fictive positions, one anterior (ML–AEP) and one posterior (ML–PEP). The vectors for all steps connecting the two positions, normalized to the origin in the AEP, gave direction in degrees and step length in millimeters. The 0–180° axis was always parallel to the body axis and crossed the AEP; 90° always points inside perpendicularly. The simultaneous recordings of the EMG trace and the camera trigger and tarsal contact signals allowed frame-by-frame correlation of filmed movement and EMG and tarsal contact traces. In calculating middle leg movement vectors all steps were transposed to reflect walking as a right leg regardless of which leg was being recorded from.

### *Data analysis and figure preparation*

Leg positions were measured with their *x* and *y* coordinates. Care was taken to choose animals of the same size and leg lengths. The number of animals used for a given condition (*N*) and the number of steps evaluated (*n*) are given in the figures. The sample size for the kinematics analysis of straight forward walks was $N = 5$ ($n = 125$), for backward walks $N = 3$ ($n = 83$).

Cycle period was calculated from touchdown to touchdown, as determined from the tarsal contact trace. For comparisons of EMG activity of the six different muscles between intact forward and backward walking and between intact and reduced forward stepping preparations, EMG traces were rectified and smoothed ($\tau = 50$ ms) and each single data point of each step was exported in Excel (Microsoft, Redmond, WA) to allow averaging. In each step the minimum muscle activity was set to zero and the maximum to one. In several cases, weak cross talk from the antagonist muscle was removed mathematically using the EMG trace from the antagonist: the activity of the EMG in the antagonist was triggered to the same point in time as that of the EMG in the agonist (i.e., liftoff or touchdown of the tarsal contact trace, common for both EMGs) and exported in the same way as before. Then its minimum activity was set to 0, but its maximum to an arbitrary value of 0.5, due to the smaller size of the antagonist signal in the agonist EMG. The normalized activity of the antagonistic muscle was then subtracted from the corresponding value of the muscle under investigation (see Supplemental Fig. S1).[1]

First spike latencies with respect to liftoff or touchdown were calculated relative to the tarsal contact signal (see preceding text). The absolute latency was then normalized with respect to the corresponding step cycle and averaged for the plot in Fig. 11*C*. Average swing/stance phase duration was calculated from each evaluated step from liftoff to touchdown for swing and from touchdown to liftoff for stance.

All angles were analyzed using the Watson–Williams test, the circular analogue of the two-sample *t*-test (Matlab, circular statistics toolbox; Berens 2009). Circular variance of vector angles was tested using the variance test in the same toolbox (Matlab, circular statistics toolbox; Berens 2009). For all other statistical analyses, a nonparametric Wilcoxon *U* test (Matlab, Statistics toolbox; The MathWorks, Natick, MA) was used, except for the comparison of integrals of depressor activity, where a standard Student's *t*-test was used. Statistical significance was assumed at values of $P < 0.01$. Figures were prepared in Origin 6.1 (OriginLab, Northampton, MA) and Photoshop 6.0 (Adobe Systems, San Jose, CA).

## RESULTS

Understanding how animals adapt their motor behavior to changing environmental conditions requires measuring limb kinematics and muscle activity in different behaviors. We have shown elsewhere that stick insect leg kinematics differ in straight and curve walking and examined the effect of reducing leg number on these changes (Gruhn et al. 2009a). Here we compare middle leg kinematics during forward and backward walking in intact animals and then examine muscle activity in these two behaviors in the intact and reduced preparations.

### *Kinematics of straight forward versus backward walking in the middle leg*

Figure 2*A* shows a schematic drawing of the stick insect with marked anterior and posterior extreme positions (AEP and PEP plus SD) of the right middle leg in forward and backward straight walking. The data for forward walking (gray) were taken from Gruhn et al. (2009a). AEP is defined as tarsus position at touchdown and PEP as tarsus position at liftoff, always with respect to the direction in which the animal moves. During forward walking (FW), the leg is moved anteriorly during swing and posteriorly during stance. This order of leg movements is reversed during backward walking (BW). In backward walking each step's AEP is therefore more caudal along the long axis of the animal than the PEP. Forward steps were significantly longer (mean step length FW: 16.2 ± 5.4 mm; BW 9.9 ± 4.7 mm, $P < 0.0001$) and their movement direction was on average more parallel to the body length axis than were backward steps (Fig. 1*A*). To compare movement vector angles, we mirror-imaged the forward step movement angles in Fig. 2*B* along the horizontal axis. The resulting mean angles of forward (8.8 ± 17.3°, gray) and backward (36.1 ± 20.3°, black) steps are shown in Fig. 2*D* and differed significantly ($P < 0.0001$) from each other. The variability between the movement vector angles of single steps is similar in both directions and spans angles over a range of 83° during FW and 88.5° during BW between the respective extremes. Mean touchdown position along the transverse axis was significantly

[1] The online version of this article contains supplemental data.

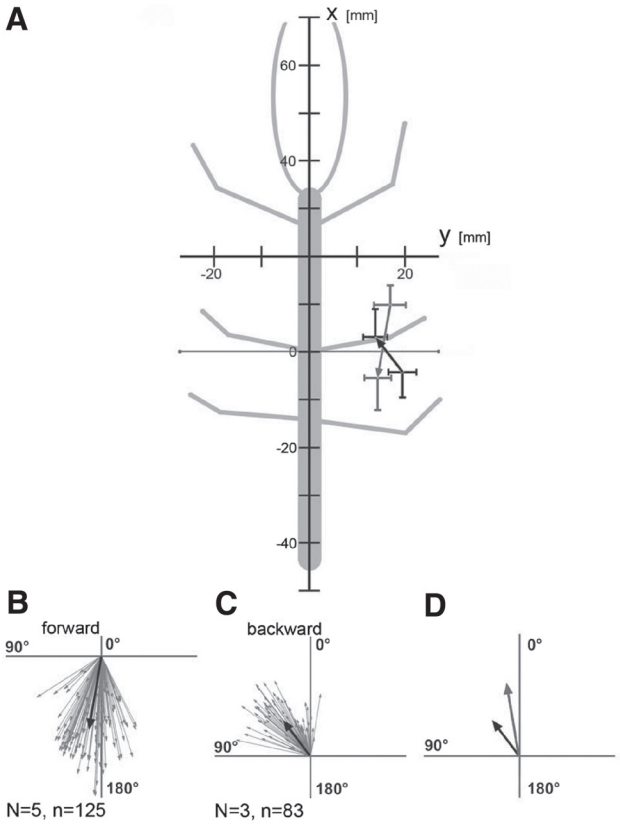


FIG. 2. Kinematics of a forward and backward walking stick insect middle leg on a slippery surface. *A*: schematic drawing of a stick insect with the mean anterior extreme position (AEP) and posterior extreme position (PEP) values (and SD error bars) of the right middle leg for forward (gray) and backward (black) walking. Note that for the backward walking animal the AEP is posterior to the PEP; the gray line marks the $X_0$-value for the middle leg. *B* and *C*: step-to-step variability in angle and length of stance movement of forward (*B*) and backward (*C*) steps normalized to touchdown position (AEP); the average stepping vector is drawn in black in both cases. *D*: average stepping vectors for forward (gray) and backward (black) walking from *B* and *C*; the average vector for forward walking from *B* was mirrored across the horizontal plane for easier comparison. $N$ = animal number, $n$ = step number.

closer to the midline in FW versus BW ($y$-positions: $AEP_{FW}$ 16.9 ± 3.3 mm; $AEP_{BW}$ 19.6 ± 2.9 mm, $P < 0.0001$), but mean liftoff position was not significantly different ($y$-positions: $PEP_{FW}$ 14.4 ± 2.9 mm; $PEP_{BW}$ 13.8 ± 2.6 mm, $P = 0.32$). However, because during backward walking the movement is more inward directed in each step, the PEP is generally reached after a shorter step length (Fig. 2, *B–D*). Taken together, these data show that in intact animals middle leg backward stepping is not simply reversed forward walking, but is instead altered to having shorter and more inward directed steps, albeit with a similar degree of variability as seen for forward stepping.

### *Stance duration alone determines cycle period*

In stick insects walking on nonslippery surfaces, in which the different legs are coupled mechanically through the ground on which the animal walks, step cycle period depends on stance duration (Graham 1972, 1985; Wendler 1964). We tested whether this relationship is also present in slippery surface forward and backward walking and, to test for interleg interactions, in animals with reduced leg numbers (only the two middle legs or only one single middle leg). In all these cases, the cycle period varied linearly with stance duration but did not depend on swing duration, which was essentially constant at all cycle periods (Fig. 3).

### *Phasing of leg muscle activity*

EMG recordings of various leg muscles during walking have been made (Fischer et al. 2001; Graham and Epstein 1985), but with few exceptions, only the activities of single muscle pairs were recorded (Bässler 1993; Cruse and Pflüger 1981; Epstein and Graham 1983). In addition, the timing reference for the beginning or end of muscle activity relative to step cycle, if present at all, was not precise. To remedy this lack we made comprehensive paired EMG recordings of all three major muscle pairs controlling leg movements: the *protractor/retractor coxae*, *levator/depressor trochanteris*, and the *extensor/flexor tibiae* muscles at a time during both forward and backward walking.

Figure 4 shows the activity of the muscles of the most proximal leg joint, the thorax–coxa joint, the *protractor* and *retractor coxae* muscles, which serve to protract and retract the leg, respectively. The traces in Fig. 4*A* show raw EMG activity, those in Fig. 4*B* rectified and smoothed ($\tau$ = 50 ms) activity, and those in Fig. 4*C* mean rectified activity from one stepping sequence (*gray trace*) and from five animals (*black trace*). In forward walking protractor activity began before the liftoff of the leg, reached its main activity during swing, and then decreased toward the end of swing. In backward walking the protractor was barely active during swing but began at the transition between swing and stance and reached peak activity

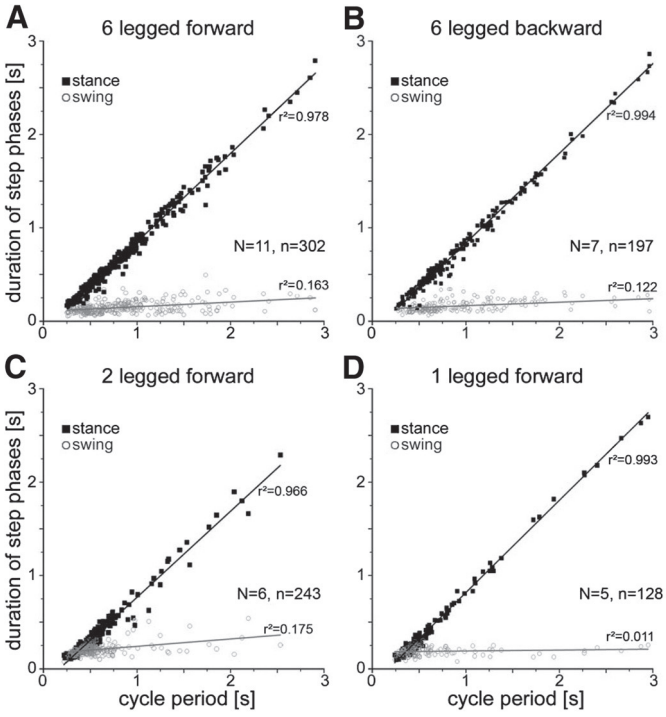


FIG. 3. Cycle period depends on stance, not swing, duration. Gray circles represent swing phase, filled black boxes stance. *A*: straight forward walking, 6-legged animal. *B*: backward walking, 6-legged animal. *C*: straight forward walking, 2-legged (only middle legs) animal. *D*: one-legged (middle leg) animal. $N$ = animal number, $n$ = step number.

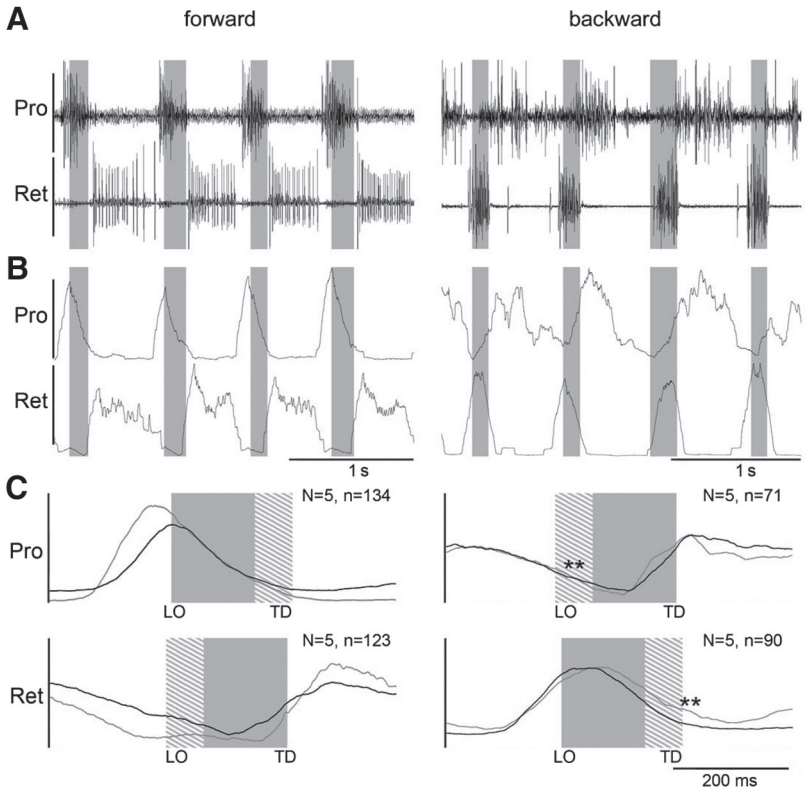


FIG. 4. Right middle leg protractor and retractor EMG recordings during forward (*left column*) and backward (*right column*) walking on a slippery surface. Gray boxes mark swing phase. *A*: raw EMG recordings. *B*: rectified and smoothed traces of EMGs in *A*. *C*: mean rectified and smoothed traces of recordings (gray) from one and from 5 animals (black). Gray boxes mark the average swing duration; shaded area shows swing duration SD. Double asterisks mark where cross talk from the retractor was removed mathematically from the protractor traces. $N$ = animal number, $n$ = step number.

about 100 ms into stance. This activity pattern was the same for the retractor muscle except that it showed stance activity during forward walking and swing activity during backward walking.

Figure 5 shows the activity of the muscles of the next most distal leg joint, the coxa–trochanter joint, the *depressor trochanteris* and *levator trochanteris* muscles, which serve to depress and lift the leg, respectively. The traces in Fig. 5*A* again show raw EMG activity, those in Fig. 5*B* rectified and smoothed ($\tau$ = 50 ms) activity, and those in Fig. 5*C* mean rectified muscle activity from one stepping sequence (*gray trace*) and from five animals (*black trace*). Depressor activity

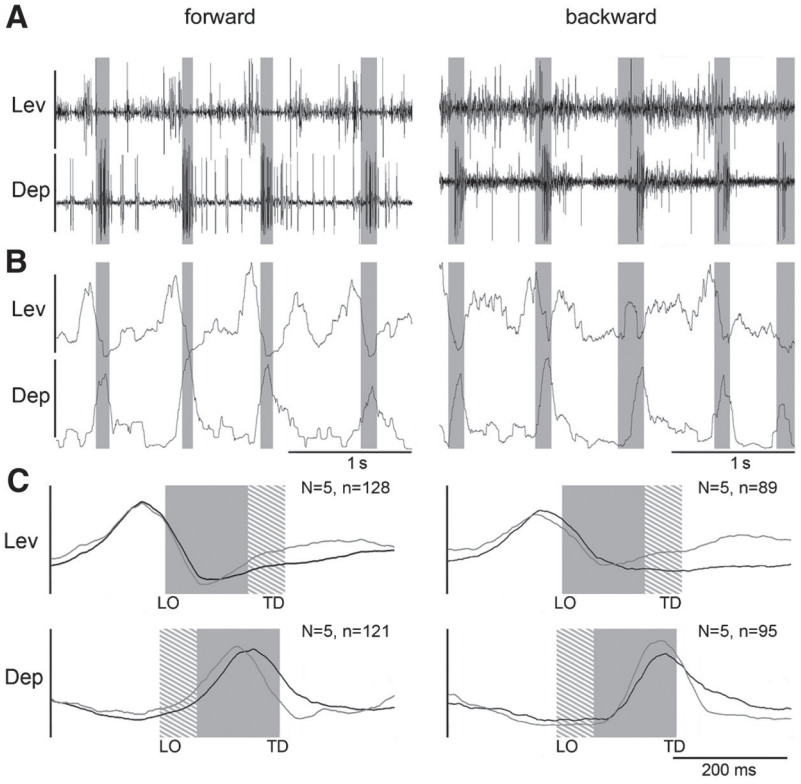


FIG. 5. Right middle leg levator and depressor activity during forward (*left column*) and backward (*right column*) walking on a slippery surface. Gray boxes mark swing phase. *A*: raw EMG recordings. *B*: rectified and smoothed traces of EMGs in *A*. *C*: mean rectified and smoothed traces of recordings from one (gray) and from 5 animals (black). Gray boxes mark the average swing duration; shaded area shows swing duration SD. $N$ = animal number, $n$ = step number.

began very shortly after swing beginning, was active throughout swing, and declined shortly after stance beginning. Provided the animal was maintained at a constant height about the substrate (see following text), depressor activity was the same in forward and backward walking. Moderate levator muscle activity was present at the beginning and middle of stance, with a substantial peak of activity occurring just before the stance to swing transition. Levator activity decreased and reached a minimum shortly after swing beginning. As with the depressor, levator activity was the same in forward and backward walking.

The last muscles analyzed (Fig. 6) were the *extensor tibiae* and *flexor tibiae* muscles, which move the femur–tibia joint and extend and flex the tibia, respectively. The traces in Fig. 6*A* again show raw EMG activity, those in Fig. 6*B* rectified and smoothed ($\tau$ = 50 ms) activity, and those in Fig. 6*C* mean rectified muscle activity from one stepping sequence (*gray trace*) and from five animals (*black trace*). Peak extensor activity occurred around liftoff in forward and in backward walking, whereas flexor activity peaked during the first half of stance in forward and backward walking.

### *Latency of muscle timing during forward and backward walking*

Reliably comparing muscle activity in different walking directions and across preparations requires determining the exact timing of muscle activity within the step cycle. Swing to stance and stance to swing transitions are two such points. Figures 7 and 8 show first spike latencies relative to these points for all six muscles in forward and backward walking, respectively, from five animals each. The gray areas mark mean swing duration averaged across all steps and animals.

The protractor, levator, and extensor muscles move the middle leg forward, up, and extend the femur–tibia joint. During forward walking these movements occur during swing. We therefore measured the first spikes in these muscles relative to liftoff (Fig. 7, *A*, *C*, and *E*). Activity occurred earliest in the levator (mean first muscle potential 99.9 ± 64.2 ms before liftoff), followed by the extensor (66.9 ± 47.3 ms) and then the protractor (36.5 ± 36.3 ms). The retractor, depressor, and flexor muscles move the leg backward, down, and flex the femur–tibia joint. During forward walking these movements occur during stance. We therefore measured the first spikes in these muscles relative to touchdown (Fig. 7, *B*, *D*, and *F*). Activity occurred earliest in the depressor with the mean first muscle potential 93.1 ± 33.9 ms before touchdown, 22% into the swing phase. The first flexor activity occurred next with mean first muscle potential 9.0 ± 13.3 ms after touchdown. Single first spikes occurred just before touchdown, confirming previous findings for the timing of this muscle (Gruhn et al. 2006). First retractor activity was more variable, with mean first muscle potential 34.6 ± 33.6 ms after touchdown and first activity occurring ≤50 ms before touchdown. The joint activation sequence in swing is thus the same as that for stance, i.e., first coxa–trochanter, then femur–tibia, and finally thorax–coxa. The high SD values result from the high variability in the stepping of the stick insect on the slippery surface. Walking sequences with many consecutive straight forward steps do not occur often and every step has a slightly different direction and stance duration.

As was shown earlier in the kinematics and EMG data, in backward walking protractor and retractor timing is the reverse of that in forward walking. To continue to compare the timing of functional swing and stance muscles in the two walking

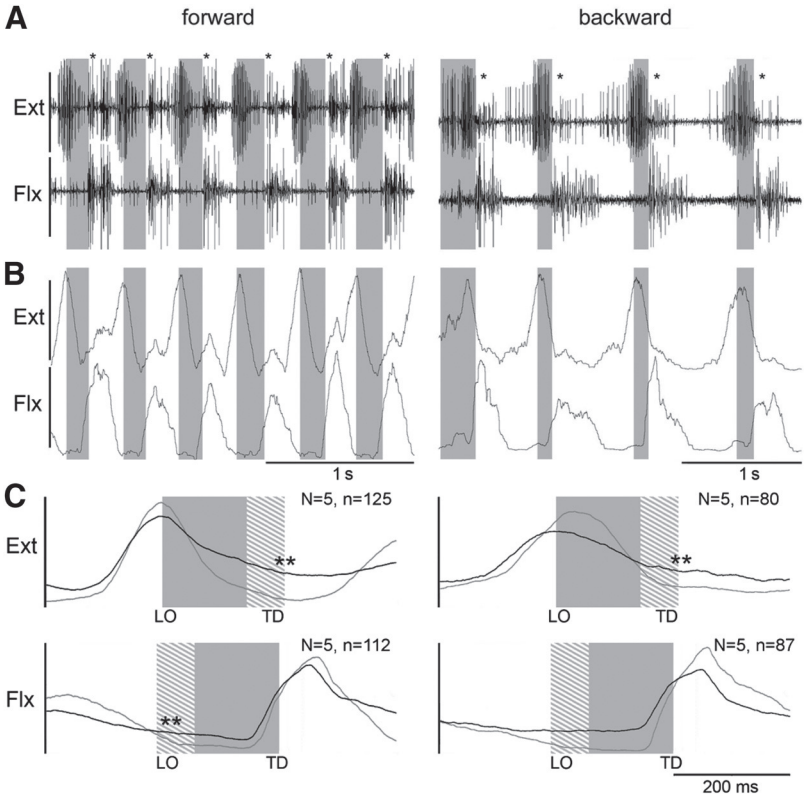


FIG. 6. Right middle leg extensor and flexor activity during forward (*left column*) and backward (*right column*) walking on a slippery surface. Gray boxes mark swing phase. *A*: raw EMG recordings; asterisks mark cross talk from the flexor in the extensor trace. *B*: rectified and smoothed traces of EMGs in *A*. *C*: mean rectified and smoothed traces of recordings from one (gray) and from 5 animals (black). Gray boxes mark the average swing duration; shaded area shows swing duration SD. Double asterisks mark where cross talk from the antagonist muscle was removed mathematically. $N$ = animal number, $n$ = step number.

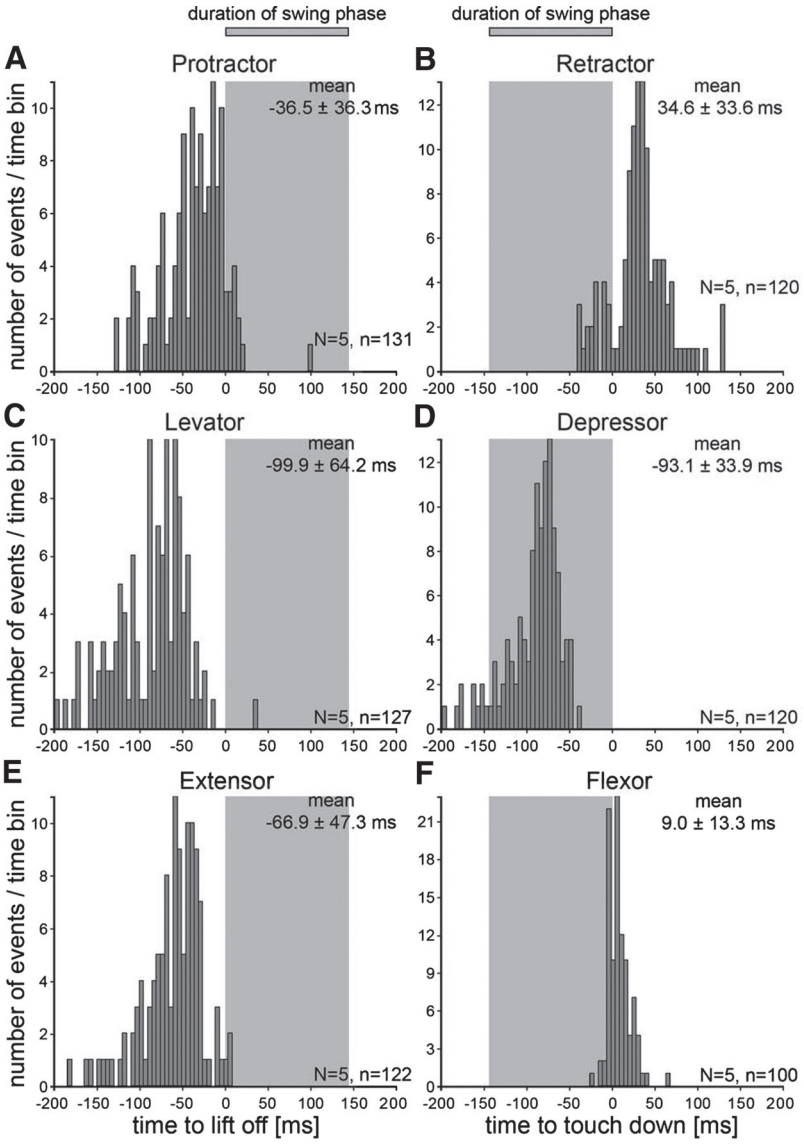


FIG. 7. Histograms of the latency distribution of the first muscle potentials in the EMG traces of the 6 analyzed leg muscles during forward walking. Timing values of the first spikes in protractor, levator, and extensor were measured with respect to the time of liftoff. Retractor, depressor, and flexor activity spikes were measured with respect to leg touchdown. Gray boxes mark the average swing phase length. Average latency of the first spike is given with SD. $N$ = animal number, $n$ = step number.

directions, in backward walking sequences we therefore referenced retractor activity to liftoff and protractor activity to touchdown, but continued to reference the activity of the other muscles as before (Fig. 8, *A–F*). Sequence of levator and extensor activation as well as the latencies for the first muscle potential (100.2 ± 60.5 ms, Fig. 8*C*; 56.8 ± 48.0 ms, Fig. 8*E*, respectively) were the same as in forward walking ($P_{Lev}$ = 0.98; $P_{Ext}$ = 0.31). During backward walking the retractor activated 18.5 ± 36.5 ms before liftoff (Fig. 8*A*), dramatically different from this muscle's activation in forward walking (Fig. 7*B*), but barely not significantly different from the timing of the functionally analogous protractor during forward walking ($P$ = 0.012) (Fig. 7*A*).

Except for the difference mentioned earlier that the protractor is a stance phase muscle in backward walks, the timing and activation sequence of the functional stance phase muscles were also similar in forward and backward walking. The depressor again activated first (Fig. 8*D*), although only halfway through swing at 64.9 ± 25.1 ms before touchdown, significantly later than that in forward walking ($P < 0.0001$). The protractor and flexor activated next at almost the same time: 10.9 ± 34.4 and 5.3 ± 28.6 ms after touchdown (Fig. 8, *B* and *F*). Flexor timing did not differ significantly from that in forward walking ($P$ = 0.12). Despite their large SDs, protractor timing in backward walking (10.9 ± 34.4 ms) and retractor timing in forward walking (34.6 ± 33.6 ms) did differ significantly ($P < 0.0001$).

In summary, these data show that *1*) only the muscles controlling the thorax–coxa joint showed large changes when walking direction changed, and *2*) with respect to liftoff and touchdown, the timing of functionally analogous muscles in swing and stance is almost the same in both directions.

### *Muscle activity in reduced preparations*

Many studies on stick insect walking have been conducted in preparations with reduced leg number (e.g., Akay et al. 2001, 2004; Fischer et al. 2001; Gabriel and Büschges 2007; Gabriel et al. 2003; von Uckermann and Büschges 2009). Because these preparations lack interleg sensory interactions, it is important to test whether data from such experiments are applicable to intact animals. Leg kinematics in straight forward

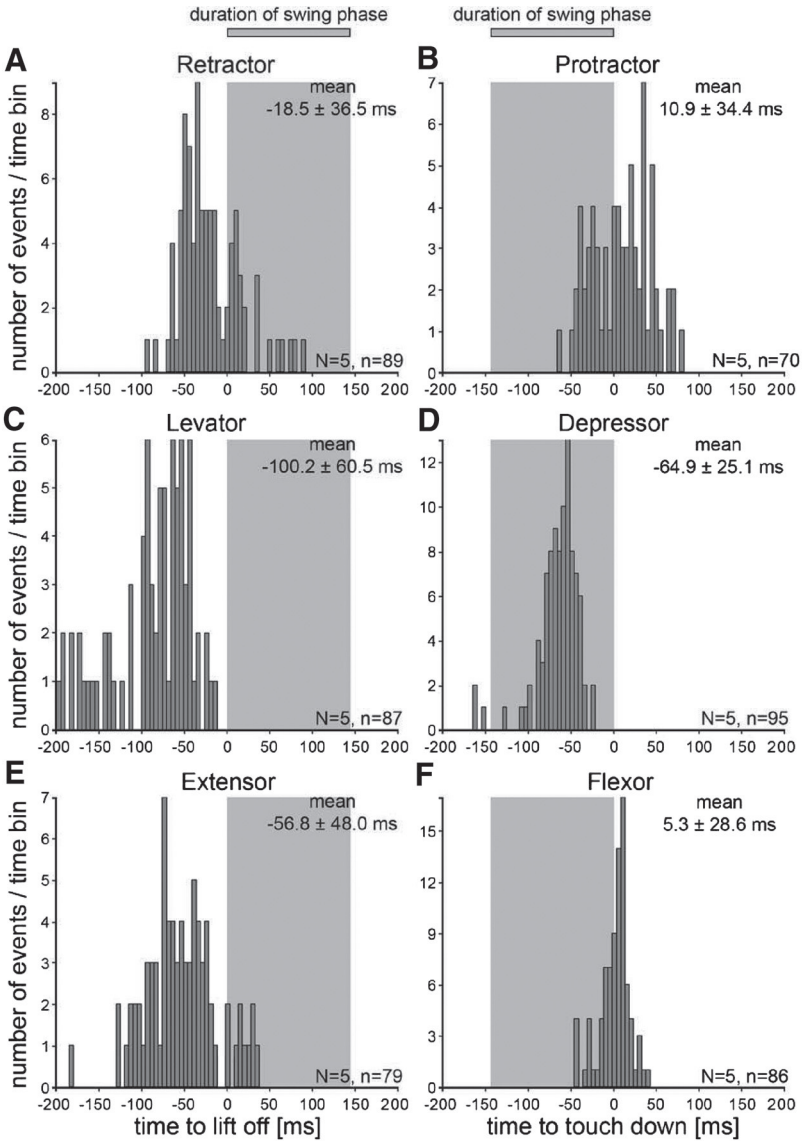


FIG. 8. Histograms of the latency distribution of the first muscle potentials in the EMG traces of the 6 analyzed leg muscles during backward walking. Timing values of the first spikes in retractor, levator, and extensor were measured with respect to the time of liftoff. Protractor, depressor, and flexor activity spikes were measured with respect to leg touchdown. Gray boxes mark the average swing phase length. Average latency of the first spike is given with SD. $N$ = animal number, $n$ = step number.

walking and turning change only little in reduced preparations (Gruhn et al. 2009a), but muscle activity in reduced preparations has not been measured. We therefore next compared forward walking muscle activity in intact and two-legged (2L) and one-legged (1L) animals.

Figure 9 shows mean rectified and smoothed extensor and flexor EMGs from 112 to 125 steps from five different animals for each leg number condition. Extensor activity began about 100 ms before the stance–swing phase transition, peaked between the stance–swing transition and the first third of swing, and lasted throughout swing. Flexor activity was also similar in all leg number conditions. It started at the beginning of stance and the greatest activity occurred during the first 100 ms of stance. Similar data were found for the levator/depressor and protractor/retractor. In no case were major differences in EMG activity of the three antagonistic muscle pairs found between the intact, 2L, or 1L preparations (data not shown).

Removal of four or five legs to produce 2- or 1-middle legged preparations, however, did alter the timing of first muscle activity in all three muscle pairs, at least under some reduced leg number conditions. The first swing phase muscle to be activated, the levator, was on average activated 92.8 ± 99.4 ms before liftoff in the 2L preparation and 88.4 ± 37 ms before liftoff in the 1L preparation. For both preparations this time was not significantly ($P_{2L} = 0.15$, $P_{1L} = 0.61$) later than that in intact animals; neither were the values for the 1L and 2L preparations significantly different from each other ($P = 0.38$). The second muscle to be activated in swing, the extensor, activated significantly later in both reduced preparations than that in intact animals, with first activity occurring on average 29.7 ± 38.9 ms (2L) and 33.2 ± 27.9 ms (1L) before liftoff ($P < 0.0001$). The timing of the first extensor spike in these two reduced preparations, on the other hand, did not differ significantly from each other ($P = 0.47$). The third muscle activated in swing, the protractor, activated 19.9 ± 32.5 ms before liftoff in the 2L preparation, significantly later than that in intact animals ($P = 0.0005$) (1L preparations were not investigated in this muscle).

All stance muscles showed small changes in activation timing. The depressor activated slightly but significantly earlier than that in intact animals in both the 2L (99.6 ± 24 ms, $P < 0.0001$) and 1L (123.7 ± 32.5 ms, $P < 0.0001$) preparations.

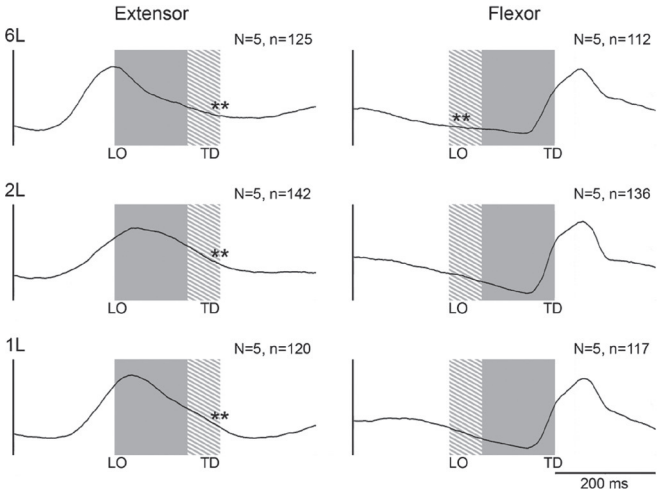


FIG. 9. Averaged, rectified, smoothed, and normalized middle leg extensor and flexor EMGs in intact, 2-legged, and single-leg preparations during forward walking. Double asterisks mark traces where cross talk from antagonist muscle was removed mathematically. Gray boxes show mean swing duration; shaded areas swing duration SD. $N$ = animal number, $n$ = step number. LO, liftoff; TD, touchdown.

The flexor activated at statistically equivalent times in both reduced preparations (2L, 16 ± 12.5 ms; 1L, 15.9 ± 9.5 ms, $P = 0.9$), with both preparations also differing significantly from intact animals ($P < 0.0001$). In the 2L preparation the retractor showed a large and significant change in the timing of first activity (10.2 ± 34.5 ms after touchdown, $P < 0.0001$) compared with that in intact animals (1L preparations were not investigated in this muscle).

The data show that middle leg muscle activity changes only slightly in reduced preparations. Nonetheless, the presence of clear changes in the latencies of most muscles indicates that interleg sensory input does contribute to the timing of middle leg swing and stance muscle activation.

*Depressor muscle activity depends on animal height*

Depressor activity is strongly influenced by movement, strain, and load-related inputs from the trochanteral hair plate (Cruse et al. 1993; Schmitz 1986a,b), campaniform sensilla (Borgmann et al. 2005), and from the femoral chordotonal organ (Hess and Büschges 1997, 1999). In our experimental setup the animals were attached to a small wooden dowel held at a fixed height above the slippery surface. The animals therefore could not regulate their height and thus did not experience the changes in leg loading that would occur in completely free walking. We therefore tested the effect of decreased and increased load by lifting or lowering the tethered animal during walking sequences and comparing the depressor activity under these conditions. Figure 10, *A–C* shows middle leg *depressor trochanteris* and *levator trochanteris* recordings from a single stick insect while the animal walked at 10 (the physiological walking height and height of all other experiments shown here), 13, and 7 mm above the slippery surface. The normalized rectified and smoothed depressor activities from all recorded steps of all animals under the different conditions are shown in Fig. 10*D*. Increasing walking height from 10 to 13 mm (Fig. 10*B*) had little effect on the depressor activity (Fig. 10*A*). At both heights the depressor was mainly active during the second half of swing, although in three of six animals, as in this example, slightly fewer depressor spikes occurred in stance at a height of 13 mm. Levator activity showed no detectable changes in activity. At a height of 7 mm the depressor was active not only in the second half of swing

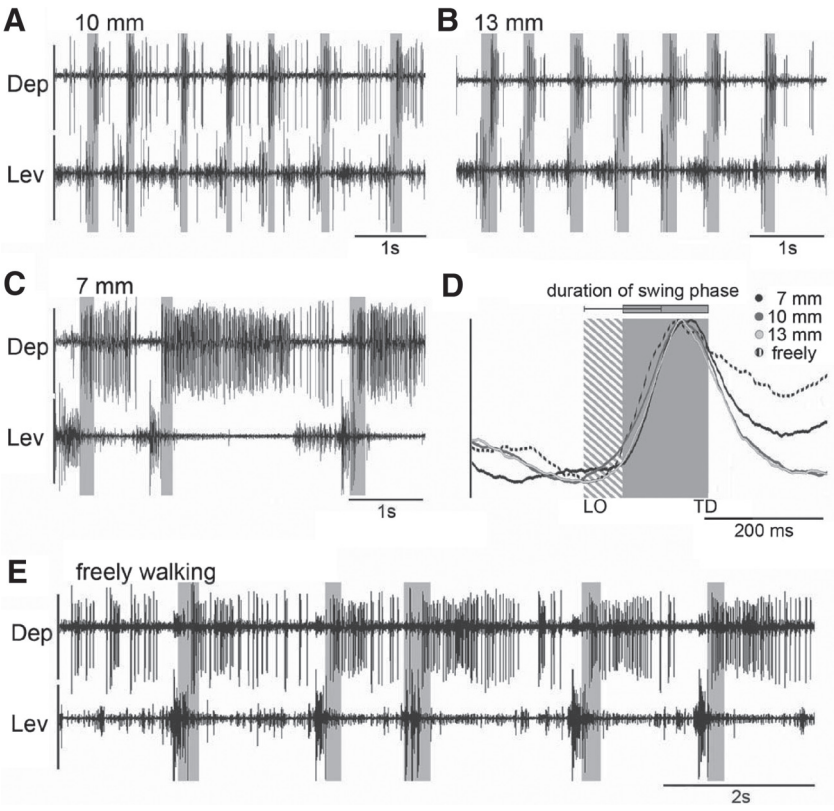


FIG. 10. Middle leg depressor and levator in forward walking, intact animals fixed at different walking heights. *A*: 10 mm. *B*: 13 mm. *C*: 7 mm. *D*: shows averaged, rectified, and smoothed depressor EMG traces at 7 (black, $N = 6$, $n = 178$), 10 (dark gray, $N = 6$, $n = 221$), and 13 (light gray, $N = 6$, $n = 184$) mm and from a freely walking animal (stippled, $N = 1$, $n = 34$). *E*: middle leg depressor and levator EMG activity in forward free walking. Gray boxes mark swing duration,

but also throughout two thirds of stance, continuing until levator activity began. Since the depressor activity was very similar during swing, we compared the integrals under the rectified and smoothed EMG traces after touchdown from 178 to 221 steps from six animals at all three heights. Four of six animals showed higher mean depressor activity during stance at 7 mm compared with the other two conditions. Even with all animals at this condition pooled together, average depressor activity was significantly greater at 7 mm than that in the other two situations ($P < 0.0001$), whereas the activity was the same in the animals at 10 and 13 mm height ($P = 0.49$). These differences in depressor activity at different animal heights made it very important to measure depressor activity in freely walking animals that could control their own body height. We therefore dismounted one stick insect from the wood dowel after first recording depressor and levator activity under tethered conditions at different heights. The glycerin was then wiped from the slippery surface and the still completely wired animal was allowed to walk freely on the surface while we continued to record stepping tarsal contact (Fig. 10*E*). Under these conditions, depressor EMG activity did not start at a different time from the values at all heights seen in the tethered animal. The stippled trace of rectified and smoothed average EMG activity in this animal (Fig. 10*D*) shows the similarity in mean rectified activity for the freely walking animal and the averaged six animals fixed at 7 mm. The time course of depressor activity pattern, however, was very similar to that seen in tethered walking at 7 mm height (Fig. 10*C*), i.e., depressor activity lasted long into stance. This suggests that, when the animal has to control its own height during walking, the depressor acts not only to lower the leg to the ground (swing activity), but also acts during stance to help carry the animal's weight and keep it at a specific height above the ground.

## DISCUSSION

### *Kinematics/cycle period*

Multiple studies have investigated insect forward and curve walking (walking and turns on solid substrate: Cruse 1976b; Cruse et al. 2009; Jindrich and Full 1999; Ridgel et al. 2007; Rosano and Webb 2007; Strauss and Heisenberg 1990; Wendler 1966; Zollikofer 1994a; Zolotov et al. 1975; air-cushioned Styrofoam ball: Dürr and Ebeling 2005; Frantsevich and Mokrushov 1980; Jander 1982; slippery surface: Camhi and Nolen 1981; Gruhn et al. 2009a; Mu and Ritzmann 2005; Ridgel et al. 2007). Backward walking has been investigated in crustacea (Ayers and Davis 1977a; Chasserat and Clarac 1980), scorpions and spiders (Bowerman 1981), and stick insects (Graham and Epstein 1985). However, this quite early stick insect work was only qualitative and did not have precise measurements of stance–swing transitions.

The data presented here confirm observations by Graham and Epstein (1985) that stick insects can perform coordinated backward walks on a slippery surface. Our data show that forward and backward steps are equally variable but backward steps are significantly shorter and therefore more inward directed than forward steps. Touchdown positions were only slightly different in the transverse axis to the animal ($y$), and liftoff positions were unchanged, in forward and backward walking. This observation is consistent with the activity of the femur–tibia joint muscles, especially the flexor, being very similar in forward and backward walking because it is flexor activity that determines $y$-position at stance end. It should be noted, though, that backward stepping was elicited by a continuous pull on the antennae, whereas forward walking was undisturbed. This may be an additional reason for some of the observed changes in vector length and direction.

Cycle period depended only on stance duration in both forward and backward slippery surface walking. This has been long known for stick insects walking forward on nonslippery substrates (Graham 1972, 1985; Wendler 1964), although it was unclear whether this relationship held for forward walks on a slippery surface or for backward walks. Our findings confirm previous results (Büschges et al. 1995; Gabriel and Büschges 2007; Gruhn et al. 2009b) showing that in pharmacologically activated neuron preparations only stance motor neuron firing duration, and in single leg preparations only leg stance duration, vary with cycle period. We have not tested whether the time of onset of muscle bursting changes with the rate of walking. Such dependence has been shown in a previous study of muscle activities in cockroach (e.g., Delcomyn 1989), where the phase of the onset of bursts in a leg stance phase muscle shifted with respect to the neighboring leg when walking speed increased and bursting was initiated earlier during rapid walking. However, on the level of the single leg, such a shift has been shown to occur in flexor motor neurons in the stick insect middle leg, albeit not with respect to a neighboring leg (Gabriel and Büschges 2007). It is therefore not unlikely that shifts in the muscle activity onset of stance-related muscles in the stick insect exist with respect to neighboring legs.

### *Muscle activity and latencies in forward versus backward walking*

Recent work on stick insect muscles (Guschlbauer et al. 2007; Hooper et al. 2007, 2009) highlights the slow responses of these muscles to neural input and thus the importance of direct measurement of muscle activation in describing how neural activity generates behavior in this system. Our EMG recordings of the six main middle leg muscles showed that only the muscles controlling the thorax–coxa joint (protractor, retractor) had large changes in activity when stick insects reversed walking direction (Fig. 11). Figure 11*A* shows the average onsets of muscle activities of the functional swing phase muscles, timed to liftoff, and Fig. 11*B* the average onsets of activities of the functional stance phase muscles timed to touchdown, in both walking directions with their respective SDs. The levator and extensor muscles are always functional swing muscles and the depressor and flexor always stance muscles. The protractor and retractor muscles, alternatively, switched from being (respectively) functional swing/stance muscles to being stance/swing muscles when walking direction reversed (Fig. 11*C*; see also Fig. 11*A*, which shows, in addition to swing onsets, the average end of retractor activity during stance in forward walking and the average end of protractor activity during stance in backward walking). The activity of these muscles is thus determined by each muscle's function in the behavior that is being produced. Provided this switch is noted, the activation sequence of functional swing and stance

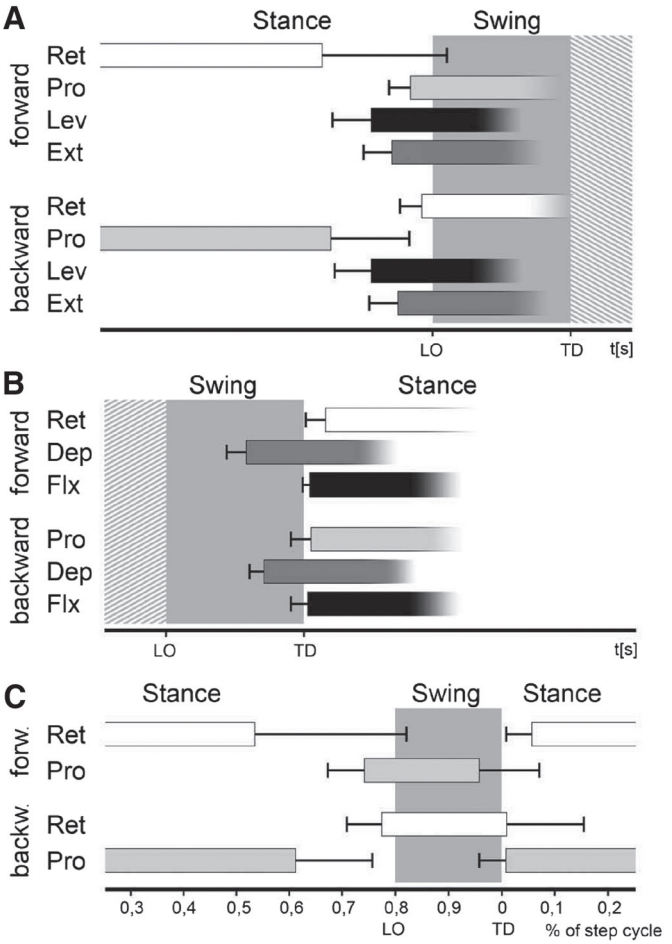


FIG. 11. Summary of right middle leg muscle timing in forward and backward walking in intact tethered animals walking on a slippery surface. *A*: comparison of the average beginning of activity in the protractor (fwd), retractor (bwd), levator (fwd and bwd), and extensor (fwd and bwd), and the average end of activity in the retractor (fwd) and protractor (bwd) with respect to liftoff (stance end). *B*: comparison of the average beginning of activity in the retractor (fwd), protractor (bwd), depressor (fwd and bwd), and flexor (fwd and bwd) muscles with respect to touchdown (stance beginning). Gray areas mark swing duration; shaded areas SD of swing duration. *C*: relative timing and duration of retractor and protractor activity in forward and backward walking animals with respect to mean cycle period calculated from first and last spikes and corresponding cycle periods of each step (swing marked with gray rectangle); error bars mark SD.

muscles is the same in forward and in backward walking: *1*) all swing muscles are activated before liftoff, levator first, extensor second, and protractor (forward walking) or retractor (backward walking) third; *2*) in stance the depressor is activated first (during swing) followed by the flexor at or shortly after touchdown and then the retractor (forward walking) or protractor (backward walking).

A general finding was that first activity timing was relatively imprecise for most muscles with SDs ranging from 13.3 (flexor in forward walking) to 64.2 ms (levator in forward walking). One reason for high levator and extensor variability could be that these muscles are timed to liftoff and measurement of the liftoff signal can be less precise than that of the touchdown signal. However, pro- and retractor muscle timing variability was similar in forward and backward walking despite different reference points being used in the two walking directions. Furthermore, the flexor shows much higher variability in backward (28.6.4 ms) (reference point touchdown) than that in forward (13.3 ms) walking and the opposite is the case for the depressor. These results suggest that variability differences do not result from a lack of precision in determining liftoff or touchdown times, but are instead true differences in motor patterning.

Another interesting observation in this context was the early activation of swing muscles before the actual kinematically observed onset of the swing movement and the activation of stance muscles before or around touchdown and therefore before the kinematically observed stance movement. This finding can be explained with the muscle properties reported for stick insects and smaller animals in general (Guschlbauer et al. 2007; Hooper et al. 2007, 2009). With decreasing diameter of a given muscle, the forces needed to overcome the passive forces of its antagonist become so large that muscle activity has to start well before an observed movement that is caused by the muscle contraction (Hooper et al. 2009). In this case, as opposed to that in large animals such as cat or human, the role of gravity in moving a limb becomes negligible and thus the early onset of muscle activity in levator and depressor is needed to counteract the respective antagonist before movement can begin.

Previous work has shown that signals from movement and load sensors are important for inducing stance–swing and swing–stance transitions during walking (Büschges and Gruhn 2008; Büschges et al. 2008; Cruse et al. 2004). In most of this work, however, motor activity timing was inferred from leg kinematics, which means that the measurement of sensory input timing with respect to motor output was imprecise. It is therefore useful to compare our precisely measured muscle activities and these previous data.

*1* Tibia extension signals arising from the femur–tibia joint have been identified as a trigger for depressor activity in swing (Bucher et al. 2003; Hess and Büschges 1999). The depressor activation times we measure, well into swing (93 ms prior to touchdown), and at a time when the middle leg tibia is known to be well extended (e.g., von Uckermann and Büschges 2009), are consistent with this conclusion (Fig. 11, *A* and *B*).

*2* Leg loading, as occurs after touchdown, has been reported to initiate retractor activity (in forward walking), as a result of signals from the trochanteral campaniform sensilla (Akay et al. 2004, 2007), and flexor activity, as a result of signals from the femoral campaniform sensilla (Akay et al. 2001). Although the mean activation times we measure for these muscles agree with this hypothesis, first activation of both muscles was either prior to touchdown or so shortly after that it is difficult to imagine load signals from trochanteral or femoral campaniform sensilla alone to activate either muscle at stance onset.

*3* Load signals arising from the trochanteral campaniform sensilla (Akay et al. 2007) have been reported to support ongoing depressor and retractor activity during stance (Bässler 1967, 1972; Schmitz 1986a). This matches the finding that depressor activity was increased and prolonged when the animal was lowered to the surface or walked freely on the slippery surface.

*4* Our data show that during swing the levator is activated first, then the extensor, and finally the protractor (Fig. 11*A*). This sequence matches the predicted effects of known sensory influences: leg unloading signaled by the femoral and trochanteral campaniform sensilla activates extensor (Akay et al.

2001) and protractor (Akay et al. 2004, 2007) motoneurons. The observed latency of about 40 ms between levator activation and activation of the other two swing muscles is ample time for sensory activation of the latter.

*5* The only muscle for which no sensory input possibly responsible for its initial activation has been identified is the levator. It is likely, however, that position signals from the coxa and unloading signals contribute to its activation (Cruse 1985). Potential additional sensory signals contributing to levator activation remain to be identified.

In summary, our precisely measured data are generally consistent with prior interpretations of the role of sensory feedback in inducing step phase transitions. The only exceptions are the flexor and retractor (forward walking) and protractor (backward walking) muscles, which appear to activate too soon for the putative sensory triggering input to actually induce the activations.

### *Implications of pro- and retractor switch in forward and backward walking*

The finding that the timing of the pro- and the retractor muscles, which control the thorax–coxa joint, switched independently of that of the muscles for the other joints, and that it depended on the functional role of the muscle rather than on the muscle itself, raises questions about the neuronal control of forward and backward walking. How does the nervous system alter the control for the joint network to reverse the motor pattern and how is the similar timing of muscle activity achieved? This includes the question on how cycle period continues to depend on stance duration in backward walking, even though under this condition the stance phase muscle at the thorax–coxa joint, and only at this joint, switches.

One explanation for the retractor–protractor switch is that the phase coupling between the thorax–coxa joint pattern generator and the pattern generators of the other joints, the coxa–trochanter and femur–tibia joints, is altered centrally so that the thorax–coxa central pattern generator's "protractor motor neuron driving interneurons" fire during stance in backward walking. An input with this effect has not been identified, nor would this mechanism explain why cycle period continues to depend on stance duration in backward walking. That is, if in forward walking the thorax–coxa central pattern generator's cycle period depends on "retractor interneuron" burst duration, it is unclear why switching the pattern generator's phase relative to the coxa–trochanter and femur–tibia central pattern generators would change the dependence of the pattern generator on retractor interneuron burst duration. This could be explained if this dependence is not associated with retractor (forward walking) and protractor (backward walking) activity duration, but only with flexor and depressor durations (note that in Fig. 3 whole leg stance vs. phase durations, not individual joint movement durations, were measured and that our experiments did not test for the effects of independently altering the durations of individual joint movements). Flexor activity does indeed play an important role in determining cycle period (Gabriel and Büschges 2007). It is thus possible that the retractor–protractor switch does not pose a difficulty for understanding the dependence of cycle period on stance duration because thorax–coxa joint activity is simply not a part of this process.

Another possibility is that the synaptic drive of the thorax–coxa pattern generator to the coxal motoneurons reverses in backward walking. This mechanism would explain why cycle period continues to depend on stance duration in backward walking in that in both cases pattern generator cycle period would continue to depend on the burst durations of the same set of interneurons, with these interneurons driving different motor neurons in forward and backward walking, but nonetheless always stance phase motor neurons.

This change in central drive could be assisted by altering the effects of leg-derived sensory input (reviewed in Büschges and Gruhn 2008) such that these changes result in the observed switch. In this case, for example, parallel pathways from load sensors such as the trochanteral campaniform sensilla to the premotor interneurons and the two motor neuron pools of retractor and protractor could be weighted differently during forward and backward walking and thereby reverse the effect of the sensory signal. Similar mechanisms have been demonstrated to alter several sensorimotor processes (Büschges and El Manira 1998; Clarac et al. 2000) and, indeed, Akay et al. (2007) previously showed that trochanteral campaniform sensilla activity initiates retractor muscle during forward stepping, but protractor muscle during backward stepping.

### *Interleg influences on muscle activity in the forward walking animal*

Reducing leg number caused only relatively small alterations in overall leg muscle activity but shifted the average latency of the first muscle spikes in the pro-/retractor, in the extensor and the flexor muscles, and the depressor, whereas no effect was seen on levator activity. These data are consistent with kinematics analyses of straight walking and turning stick insects showing that single legs produced leg movements with changes in the precise leg positioning when the number of legs was reduced (Gruhn et al. 2009a). These changes are also in line with considerable evidence suggesting that interleg influences could play a prominent role in shaping leg motor output (Borgmann et al. 2007, 2009; Ludwar et al. 2005). In our data the greatest changes in forward walking between intact and reduced animals were in the extensor and retractor muscles, where the onset of activity shifted by about 25–30 ms toward liftoff and thus activation started later than that in the intact animal. In the case of the flexor, the effect was a shift of 5–10 ms, yet this muscle was also activated significantly later in the reduced preparations. Interestingly, the effect of the reduction was not always a delay in the activation, but in the case of the retractor and the depressor muscle a significantly earlier activation, demonstrating that the sensory intersegmental effects influence the timing in both directions and, in the case of the depressor, even the ablation of the contralateral leg and the lack of its sensory input may have an effect on timing. The activity of the abovecited muscles may be especially influenced by interleg influences because they largely determine the leg's anterior and posterior extreme positions and stance and swing phase duration, all extremely important components of interleg coordination (Cruse 1990; Cruse et al. 1998). The origins of the sensory input from the neighboring legs exerting these influences is unclear, although Ludwar et al. (2005) demonstrated that flexion signals from the front leg femoral chordotonal organ can facilitate middle leg retractor activity and also contralateral influences have been

shown to exist, although not for the depressor muscle (Stein et al. 2006).

*Local influence on depressor muscle activity*

When the animals were tethered ≥1 cm above the slippery surface, depressor activity strongly decreased very early in stance, but in animals tethered at a lower height, depressor activity continued throughout the greater part of stance. Increased depressor duration was also seen in freely walking animals. These data suggest that sensory input in freely walking animals prolongs depressor activity so that, in addition to lowering the leg at stance end, the muscle also helps to support the animal during stance. Work in stick insect and other insects suggests that the sense organs most likely responsible for this effect are, again, the campaniform sensilla, the same organs involved in switching protractor to retractor activity at touchdown (see preceding text and Akay et al. 2004, 2007). However, the role of campaniform sensilla in the magnitude control of motor neuron activity in stick insects is much less understood. Cruse et al. (1993) previously demonstrated in double-treadwheel experiments that stick insects walking with small distances between body and wheel push the wheel away from the body, resulting in increased depressor activity. In cockroach different subgroups of tibial campaniform sensilla react to increases or decreases in body load (Noah et al. 2004; Zill et al. 2009) and fire prolonged spike trains when legs actively support the body. Increased load also increases cockroach trochanteral extensor motoneuron firing, the functional analog of stick insect depressor motoneurons (Quimby et al. 2006). These and our data suggest that local mechanisms controlling depressor activity are a major component of the support of body load and maintenance of body height.

*Conclusions*

We have described the activity and timing of all major middle leg muscles during forward and backward walking in the tethered stick insect. As the animal switched from forward to backward walking the major observed change was that the functional stance muscle of the thorax–coxa joint switched from retractor to protractor, with both muscles showing the same activity times when serving as stance muscles. These findings demonstrate again the modular structure of the neuronal networks driving leg movement. They also suggest potential ways in which the CNS controls adaptive walking behaviors and how sensory input may be differentially modulated, depending on behavioral task. With these data at hand it will now be possible to study the effect of selective manipulation of single-sense organs on these now well-defined behaviors.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

## 4.2 Control of Stepping Velocity in the Stick Insect *Carausius morosus*

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Anne Wosnitza, Ansgar Büschges, Anke Borgmann

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Zu dieser Publikation habe ich sehr wichtige Ergebnisse beigetragen. Ich habe die Versuche zu den Abbildungen vier und sechs und die jeweiligen Datenauswertungen durchgeführt, sowie die Abbildungen erstellt. Ich habe die Daten zusammen mit den Koautoren diskutiert und an der Texterstellung mitgearbeitet.

# Control of Stepping Velocity in the Stick Insect *Carausius morosus*

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**Gruhn M, von Uckermann G, Westmark S, Wosnitza A, Büschges A, Borgmann A.** Control of stepping velocity in the stick insect *Carausius morosus*. *J Neurophysiol* 102: 1180–1192, 2009. First published June 17, 2009; doi:10.1152/jn.00257.2009. We performed electrophysiological and behavioral experiments in single-leg preparations and intact animals of the stick insect *Carausius morosus* to understand mechanisms underlying the control of walking speed. At the level of the single leg, we found no significant correlation between stepping velocity and spike frequency of motor neurons (MNs) other than the previously shown modification in flexor (stance) MN activity. However, pauses between stance and swing motoneuron activity at the transition from stance to swing phase and stepping velocity are correlated. Pauses become shorter with increasing speed and completely disappear during fast stepping sequences. By means of extra- and intracellular recordings in single-leg stick insect preparations we found no systematic relationship between the velocity of a stepping front leg and the motoneuronal activity in the ipsi- or contralateral mesothoracic protractor and retractor, as well as flexor and extensor MNs. The observations on the lack of coordination of stepping velocity between legs in single-leg preparations were confirmed in behavioral experiments with intact stick insects tethered above a slippery surface, thereby effectively removing mechanical coupling through the ground. In this situation, there were again no systematic correlations between the stepping velocities of different legs, despite the finding that an increase in stepping velocity in a single front leg is correlated with a general increase in nerve activity in all connectives between the subesophageal and all thoracic ganglia. However, when the tethered animal increased walking speed due to a short tactile stimulus, provoking an escape-like response, stepping velocities of ipsilateral legs were found to be correlated for several steps. These results indicate that there is no permanent coordination of stepping velocities between legs, but that such coordination can be activated under certain circumstances.

## INTRODUCTION

Locomotion results from a complex interplay between neural network activity, muscle activity, and sensory feedback about the self-generated movement as well as the environment. Proper locomotion requires a constant adjustment of the locomotor pattern to the changing surroundings. This affects not only the coordination and direction of locomotion but also the locomotor speed. Compared with walking, swimming and crawling largely result from an undulatory wave of the body and movement speed is altered by altering the frequency of the rhythmically moving tail, fin, or body. Neuronally, this can be achieved for example by altering the tonic excitatory drive from reticulospinal neurons in the brain stem that excite the spinal central pattern-generating (CPG) networks, as is the case in the lamprey (Buchanan et al. 1987; reviewed in Grillner et al. 1998). The greater the tonic excitatory drive to the CPG interneurons, the faster the networks oscillate (Orlovsky et al. 1999). With increasing drive not only the frequency, but to some extent also the magnitude of motor neuron activation and muscle contractions increases (Sirota et al. 2000), which in a freely moving animal would consequently lead to an increase in the swimming velocity. Results on fictive swimming in the *Xenopus* embryo (Roberts et al. 1998; Sillar and Roberts 1993) and the marine mollusk *Clione* (Satterlie 1993; reviewed in Orlovsky et al. 1999) point in a similar direction.

In a walking animal, a change in walking speed is achieved by a change in cycle period (lobster: Clarac and Chasserat 1986; stick insect: Graham 1972; Graham and Cruse 1981; Wendler 1964) or stride length. Often, a change in walking speed is also accompanied by a gait change. In many quadrupeds, for example, an increase in speed is accompanied by a change from walk to trot and further to gallop, in insects from a tetrapod to a tripod gait (Graham 1985). In dogs (Maes et al. 2008), cats (Halbertsma 1983; Yakovenko et al. 2005; reviewed in Orlovsky et al. 1999), mice (Herbin et al. 2004, 2006), and elephants (Hutchinson et al. 2006) it has been found that the mechanism underlying speed change varies with the gait. In these animals, during walking and trot, speed is increased by a decrease in cycle period, whereas during gallop, speed is increased by an increasing stride length.

At the level of individual legs, the changes in cycle period found in some vertebrates, insects, or crustaceans are usually achieved by modifying stance duration, whereas swing duration remains largely unchanged (cat: Halbertsma 1983; dog: Maes et al. 2008; stick insect: Wendler 1964; locust: Burns 1973; lobster: Clarac and Chasserat 1983a,b; reviewed in Orlovsky et al. 1999). Recently, however, a decrease in swing duration has also been reported as a means to decrease cycle period in alligators (Reilly and Elias 1998), mice (Herbin et al. 2004, 2007), horses (Robilliard et al. 2007), and elephants (Hutchinson et al. 2006).

Even though sensorimotor control of walking in general is fairly well understood in the stick insect (Büschges and Gruhn 2008; Büschges et al. 2007), very little is known concerning the neural mechanisms underlying changes in walking speed. Foth and Bässler (1985a,b) showed that in a situation in which five legs are stepping on a passive treadmill, while a single hind leg is stepping on a separate treadmill with a given speed, the cycle period of the five legs and that of the hind leg adjust to whole number ratios. This might be due to coordinating influences between the legs but could also be a consequence of a commonly shared control of stepping velocity. In the single middle leg preparation, Gabriel and Büschges (2007) showed that stance phase motor neuron activity is responsible for stepping velocity, but that mechanisms for altering the velocity

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become effective only during an already ongoing stance phase; however, exactly how the motor neurons and their activity patterns are affected in the course of changes in walking speed—particularly in a walking animal in vivo—is still largely unresolved.

Recent results on stick insect muscle characteristics, especially the force–velocity relation, suggest a reasonable mechanism for a velocity adjustment without neural origin (Blümel et al. 2007; Guschlbauer et al. 2007; Hooper et al. 2007, 2009). If the forward-stepping front legs alter their stepping speed, this change could be transferred to the posterior legs by altering the forces on them and their muscles due to mechanical coupling. This might in turn change the muscle contraction velocity, as predicted by the force–velocity curve of the respective muscles. In the study presented here, we used electrophysiology in behavioral experiments with the intact and reduced stick insect (*Carausius morosus*), to investigate on different levels of the stick insect walking system whether there exists evidence for a neural control mechanism to change stepping speed.

## METHODS

All experiments were performed at room temperature (18–24°C) on adult female stick insects of the species *Carausius morosus* (Brunner 1908) that were raised on unrestricted access to blackberry leaves and kept at a 12-h:12-h light:dark cycle.

### *Electrophysiological recordings*

Depending on the preparation, all legs except a single front or single middle leg were amputated at mid-coxa (Fischer et al. 2001). The animal was then fixed with dental cement (two-component glue; Protemp II, ESPE, Seefeld, Germany), dorsal side up, on a foam platform. The dorsal side of the thorax was opened, the gut moved aside, and connective tissue carefully removed to expose the connectives or the mesothoracic ganglion and respective leg nerves for extracellular recording. In the single middle leg preparation, protraction and retraction of the remaining middle leg were prevented mechanically with dental cement applied to the coxa and by severing lateral nerves nl2 and nl5 (Graham 1985; Marquardt 1940), which contains coxal protractor and retractor motor neurons (MNs), respectively. In all other cases the mesothoracic ganglion was completely deafferented prior to all extracellular or intracellular recordings by cutting or crushing the lateral nerves ipsilateral and contralateral to the recording site to exclude local sensory input, and the body cavity was filled with saline (Weidler and Diecke 1969).

In the recordings from the single front-leg preparation, mesothoracic nerve activity was recorded extracellularly from the following leg nerves (Graham 1985; Marquardt 1940), using monopolar hook electrodes (modified after Schmitz et al. 1991): leg nerve nl2; leg nerve nl5; and the main leg nerve, ncr, which contains the flexor tibiae motoneurons. Furthermore, the activity of connectives was recorded extracellularly from the pro-meso, meso-meta, and the neck connectives between the subesophageal ganglion and the prothoracic ganglion. Activity of identified MNs was recorded intracellularly from their neuropilar arborizations in the mesothoracic ganglion as described previously (Westmark 2007). In short, the mesothoracic ganglion was placed on a wax-covered steel platform and pinned down with cactus spines (*Nopalea dejecta*). Recordings were made using thin-walled glass microelectrodes (GC100TF-10; Harvard Apparatus, Edenbridge, UK), filled either with a solution of 3 mol/l potassium acetate with 0.1 mol/l KCl or with a solution of 1.5 mol/l potassium acetate and 1.5 mol/l KCl (electrode resistance, 15–25 MΩ). Recordings were made from the neuropil region of the mesothoracic ganglion ipsi- or contralateral to the walking front leg. Signals were amplified by means of an SEC-10 L amplifier (npi Elektronik, Tamm, Germany). To penetrate the ganglion more easily with intracellular electrodes, two approaches were taken. The ganglion sheath was softened by quickly removing the saline from the body cavity and then either treating the ganglion sheath with crystals of a proteolytic enzyme (Pronase, Merck, Darmstadt, Germany) for 60–90 s or the ganglion sheath of the segment in focus was removed mechanically with a pair of fine scissors.

In all experiments with a one-leg preparation, animals walked on passive, light-weight, low-friction treadmills (Bässler 1993; Gabriel et al. 2003). A DC motor attached to the treadmill measured treadmill velocity. The animal accelerated the treadmill during the stance phase. The treadmill velocity therefore indicates step stance phase. The start of the velocity increase was defined as stance beginning. The last maximum in the velocity trace before velocity began its decrease to zero was defined as stance end. Maximum velocity was defined as the maximum of the tachometer trace for a given step, whereas average velocity was calculated by the integral under the tachometer trace during the stance phase divided by stance duration.

In some analyses extracellular recordings were rectified and smoothed. The smoothing was performed with the Spike2 smoothing function. The waveform was smoothed by calculating for each sample point the average value of the input data points from time $t - T$ to $t + T$ seconds. $T$ was 0.05 s in our analyses.

### *Behavioral experiments*

For the behavioral experiments, intact animals were glued (ProTempII, ESPE), ventral side down, onto a balsa stick that was thinner than the width of the insect ($3 \times 5 \times 100$ mm [W × H × L]). The head and legs protruded from the front and side of the stick to allow their free movement. The area around the coxae of all legs and the major part of the abdomen were left free of glue. The balsa stick was inserted into a brass tube that was connected to a micromanipulator, adjusted to a position about 8–15 mm above a slippery surface, which corresponds to the height during free walking. The slippery surface on which the animals walked and the electrical measurement of tarsal contact used to verify touchdown and liftoff positions for single legs were previously described in detail in Gruhn et al. (2006). Slipperiness and simultaneous conductivity were conveyed through a glycerin/saturated NaCl-solution mix at a ratio of 95:5 (viscosity, ~435.8 centistokes, as determined through use of a table in Römpp 1966), which was applied with a soft cloth to ensure an almost even distribution of a very thin film. Small artifacts at contact of each leg allowed us to also monitor the legs that were not directly connected to the two lock-in amplifiers. A very small signal voltage (2–4 mV) and an amplifier with high-input resistance (1 MΩ) were chosen to avoid affecting the walking behavior of the animal. This allowed us to keep the current passing through tarsus and tibia between 2 and 4 nA.

Walking episodes were elicited either as optomotor responses as described previously (Gruhn et al. 2006) or by placing a bar of 1.5-cm width as an attractor in front of the animal. Moving stripes were projected onto two glass screens (Marata screens; diameter, 130 mm; Linos Photonics, Göttingen, Germany) in front of the animal, positioned left and right of the head at right angles to each other, and at a distance of 70 mm from the eyes. Forward walking was induced by a progressive pattern on both screens with stripes moving outward. The experiments were set up in a darkened Faraday cage and performed in a darkened room at 22–24°C. Acceleration of the legs was induced by a brush stroke to the abdomen (Bässler and Wegener 1983). The striped pattern was kept moving until the animal stopped walking or until after 30 s of continuous recordings.

### *Optical recording and digital analysis of leg movements*

Walking sequences were recorded from above with a high-speed video camera (Marlin F-033C; Allied Vision Technologies, Stadtroda,

Germany) at 100 fps. The camera was externally triggered and pictures were fed into a PC through a FireWire interface and then assembled into a video (*.avi) file ("fire-package" software; Allied Vision Technologies). The legs were marked at the distal end of the femur and the tibia with orange and yellow fluorescent pigments (gold-orange, catalog No. 56200; yellow, catalog No. 56150; Dr. Georg Kremer Farbmühle, Aichstetten, Germany) that were dissolved in two-component glue (ProTempII, ESPE). Additional markers, pigments dissolved in a shellac/alcohol solution, were set at the center of the thorax between the pro-, meso-, and metathoracic legs, as well as at the end of the prothoracic segment and in the middle of the head. During the recording of walking sequences, the animal was illuminated with blue light-emitting diode arrays (12 V AC/DC; Conrad Electronic, Hirschau, Germany). A yellow filter in front of the camera lens was used to suppress the short wavelength of the activation light to increase contrast for the video recordings. The video files were analyzed using motion-tracking software (WINanalyze, v. 1.9, Mikromak Service, Berlin). Figures were prepared with Origin (v. 6.1, Origin Lab, Northampton, MA) and Photoshop software (v. 6.0, Adobe Systems, San Jose, CA).

### *Statistical analysis*

In text and figures, $N$ is always the number of experiments and $n$ is the sample size (number of stepping sequences or number of steps depending on the experiment).

For the evaluation of walking sequences in the slippery surface experiments, the sequences were subdivided into bins of 1-s duration. Every step was associated with a bin depending on the starting point of its stance phase. The velocities of steps falling into one bin were matched with each other and evaluated. Regression analysis was done for all leg pairs for which >3 data points existed (in almost all cases the analysis was based on 15–35 data points). Statistical testing was done with Origin (v. 6.1).

The interleg influence of the stepping velocity of the single front leg on retractor, protractor, and flexor MN activity was evaluated by regression analysis as well. The integral of the MN activity normalized by step-cycle length was plotted against mean stance phase velocity (integral under tachometer trace during stance normalized by stance duration). The regression analysis was done with MATLAB 7.0 (The MathWorks).

Significance levels marked with asterisks are as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

## RESULTS

We investigated how changes in walking speed were reflected on different levels of the stick insect walking system. For this purpose, we used not only extracellular and intracellular recordings but also behavioral experiments to study how velocity changes affected activity in the connectives, in MNs and of the whole intact animal, and what this reveals about mechanisms for the control of stepping velocity.

### *Control of stepping velocity in the single middle leg*

In the single middle-leg preparation, leg swing is determined by extensor tibiae MN activity (summary in Bässler et al. 2007), whereas flexor MNs are active during stance. A recent study has shown that changes in stepping velocity in this preparation are correlated with changes in flexor MN activity of the same leg (Gabriel and Büschges 2007). At the same time, no alterations were found in mean activity, or most hyperpolarized and least hyperpolarized potentials (trough and peak potentials, respectively) of tibial extensor motoneurons with respect to stepping velocity. However, whether other specific timing parameters of extensor MN activity change with locomotor speed remains unknown. This might apply in particular to the time course of motor activity generated at the transition from stance to swing (Fischer et al. 2001). We therefore extended the study by Gabriel and Büschges (2007) in the single middle-leg preparation under the same conditions and analyzed the relationship between instantaneous fast extensor tibiae (FETi) spike frequency and stance phase velocity, which was registered with a treadmill tachometer as belt velocity.

Figure 1*A* shows an episode from a typical stepping sequence with treadmill belt velocity and extensor MN activity (SETi and FETi), monitored by means of a nerve recording. In addition, FETi spike activity is shown as instantaneous spike frequency (ISF; 1/interspike interval, middle trace). Regression analysis showed no significant correlation of maximum FETi spike frequency with maximum stepping velocity in 13 of 15 experiments (Fig. 1*B*). Similarly, mean spike frequency was not significantly correlated with mean stepping velocity in 10 of 15 experiments (Fig. 1*B*). In only one of 15 experiments a significant correlation existed between mean and maximum FETi spike frequency and stepping velocity. This indicates that stepping velocity solely results from flexor MN activity, as previously shown (Gabriel and Büschges 2007), and is independent of swing phase, i.e., extensor MN activity. This complements the earlier finding that an increase of locomotor speed in the stick insect is mainly achieved by a decrease in stance phase duration, whereas swing phase duration remains relatively constant (Wendler 1964).

In a next step, we analyzed the time course of the stance-to-swing transition, asking whether mean belt velocity influences the subsequent swing activation, measured as time-to-peak of the instantaneous FETi spike frequency (with $ISF_{max}$ = maximum instantaneous spike frequency, $FETi_{first}$ = first FETi spike, and $Tr_{max}$ = treadmill trace maximum). The time-to-peak of the instantaneous FETi spike frequency ($t_p$) was given two possible definitions: first, as the time from the time of the first FETi spike, defined as the onset of swing phase, until the peak frequency is reached ($ISF_{max}$ to $FETi_{first}$, henceforth $t_{p1}$); second, as the time from the end of stance phase, i.e., time of the last maximum in the treadmill trace, until the peak frequency is reached ($ISF_{max}$ to $Tr_{max}$, henceforth $t_{p2}$). A schematic drawing of one single step together with the extensor MN activity from the extracellular nerve recording and the instantaneous FETi spike frequency is shown in Fig. 1*C* to illustrate the definitions of $t_{p1}$, $t_{p2}$, and $t_{p2}$–$t_{p1}$. Both $t_{p1}$ and $t_{p2}$ were plotted against mean velocity to analyze the possible correlation between the activation strength of the swing phase motor output and the stepping velocity of the previous step. In addition, the time between stance end ($Tr_{max}$) and the first FETi spike ($t_{p2}$–$t_{p1}$) was plotted against mean velocity. Figure 1*D* shows the results of the regression analyses for one experiment. The regression line for $t_{p1}$ (light gray dotted line) showed a negative slope and was not significantly related to mean velocity. The regression line for $t_{p2}$ (dark gray dashed line) showed a negative slope, but also was not significantly related to mean velocity. The linear fit of $t_{p2}$–$t_{p1}$ plotted versus mean velocity, however, resulted in a significant correlation with a regression line of negative slope [black solid line (*)]. This result from one experiment reflects the outcome of all 15

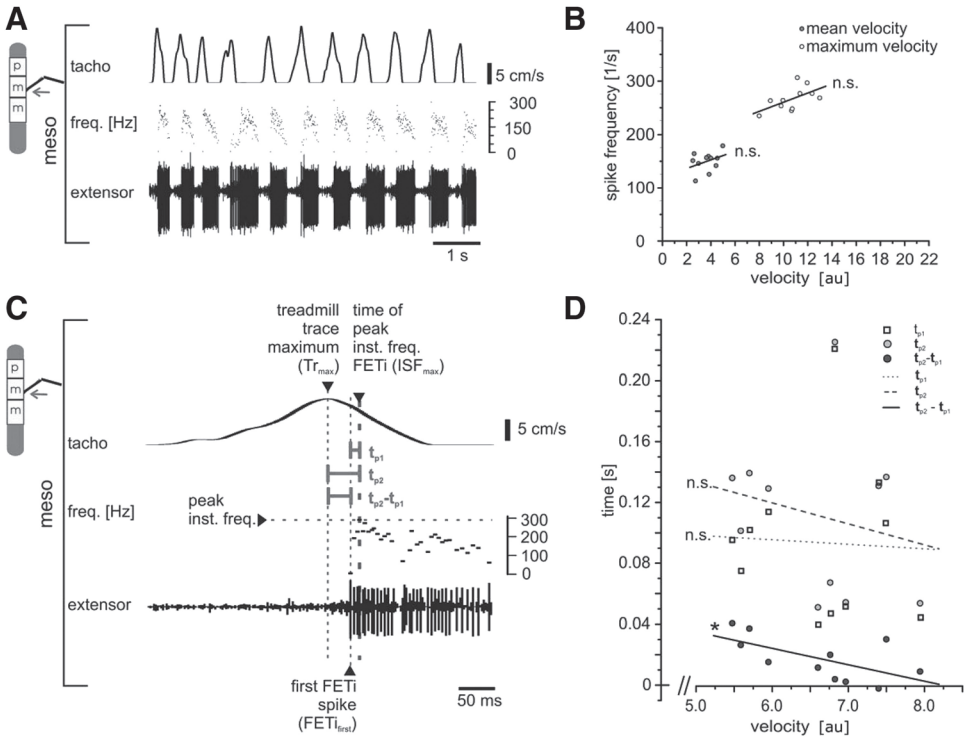



FIG. 1. Extensor motor neuron (MN) activity and stepping velocity. *A*: episode from a typical stepping sequence in the middle leg, shown with treadmill belt velocity (tacho) and extensor MN activity from the nerve recording. Large bouts of activity show instantaneous fast extensor tibiae (FETi) activity, whereas instantaneous slow extensor tibiae (SETi) and common inhibitor (CI) activity are not distinguishable at this time resolution. FETi activity is also shown as instantaneous spike frequency (freq). *B*: regression analysis of maximum FETi spike frequency with maximum stepping velocity (open circles) and mean spike frequency with mean stepping velocity (filled circles) from the stepping sequence shown in *A*. *C*: single-step tachometer trace at an increased timescale, with the corresponding extensor MN activity (nerve recording) and instantaneous FETi spike frequency to illustrate the definitions of $t_{p1}$ ($FETi_{first}$ to $ISF_{max}$), $t_{p2}$ ($Tr_{max}$ to $ISF_{max}$), and $t_{p2}$–$t_{p1}$ ($Tr_{max}$ to $FETi_{first}$). *D*: example of a regression analysis from one experiment (with $n = 11$ steps analyzed) with $t_{p1}$, $t_{p2}$, and $t_{p2}$–$t_{p1}$ plotted against mean belt velocity. $t_{p1}$ [open boxes, dotted line (n.s.)] and $t_{p2}$ [gray circles, dashed line (n.s.)] did not, but $t_{p2}$–$t_{p1}$ [filled circles, solid line (*)] did result in a significant correlation with mean velocity. Level of significance: $*P < 0.05$; not significant, $P > 0.05$.

experiments analyzed. In all these experiments, no significant correlation was found between the FETi time-to-peak-activity, measured from the beginning of the swing phase ($t_{p1}$), and mean belt velocity. Nor did we find a significant correlation between the FETi time-to-peak-activity measured from the end of the stance phase ($t_{p2}$) and the mean belt velocity in 11 of 15 experiments. However, $t_{p2}$–$t_{p1}$ and mean belt velocity were significantly correlated in 10 of the 15 experiments. This means that the pause between stance and swing phase activity becomes shorter with increasing speed and completely disappears during fast stepping sequences, as observed in the course of this study.

### *Interleg influence in stepping velocity*

From the preceding analysis it became clear that only stance-phase–related aspects of the single-leg motor output for stepping are modified with alterations in stepping velocity. However, how does the intact, six-legged animal modify its stepping velocity? Three possibilities for how alterations in stepping velocity of a six-legged insect are brought about are conceivable, which need not be mutually exclusive. The first possibility is mechanical coupling between the legs through the ground; a second is that the activity of stepping legs influences their adjacent legs neuronally; and a third possibility is that the stepping velocity of each leg is controlled individually by descending information. Mechanical coupling can be eliminated either by removing all but one leg and investigating its influence on neighboring legs or by letting the animal walk above a slippery surface. In the next experiments, we used these two strategies to eliminate mechanical coupling and to investigate the neuronal contribution to coordinating stepping velocity between the six legs.

The influence of a single leg, especially a stepping single front leg, on MN pools in other legs has been investigated extensively in the stick insect by extracellular and intracellular recordings. These investigations have shown that a general tonic increase in the activity of protractor, retractor, and flexor MNs in all hemisegments can be observed together with the stepping sequence of a single front leg (Fig. 2; Borgmann et al. 2007; Ludwar et al. 2005a,b). In addition to this increase in activity, front-leg stepping induces alternating activity in antagonistic MN pools of the ipsilateral mesothoracic hemiganglion, which is coupled to the steps in the front leg (Borgmann et al. 2007; Ludwar et al. 2005a).

By using this latter strategy and removing all legs but the stepping front leg, we first studied a potential correlation between front-leg stepping velocity and the activity in MN pools of the ipsilateral middle leg. We used regression analysis to find such a potential correlation during the respective front-leg step cycle. Figure 2*A* shows a front-leg stepping sequence and simultaneous extracellular recordings of ipsilateral mesothoracic protractor and retractor MNs with the rectified and smoothed traces underneath for further analysis ($T = 0.05$ s). During straight walking, the retractor coxae muscle acts as a stance phase muscle, whereas the protractor coxae muscle moves the leg anteriorly during swing. The mean treadmill velocity was determined by the integral under the tachometer trace during the stance phase, normalized to respective stance duration. As a correlate of the mean neuronal activity in the extracellular nerve recording, we used the integral under the rectified and smoothed recording for each step cycle, normalized by the respective step-cycle period (Fig. 2*A*). The two integrals were then plotted against each other. Figure 2*B* shows this plot for protractor (gray) and retractor MNs (black) in the experiment shown in Fig. 2*A*. In this case no significant correlation existed between mean stance velocity and ipsilateral mesothoracic retractor or protractor MN activity. Out of 11 experiments, a significant correlation between mean stance

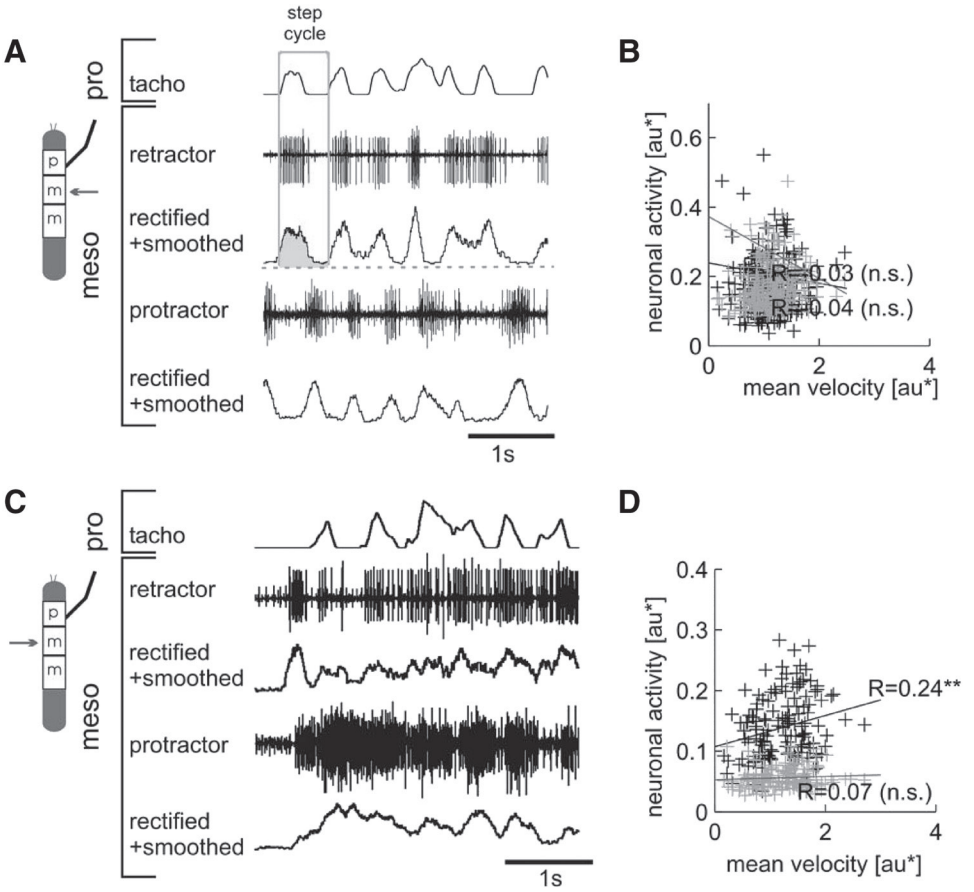



FIG. 2. Interleg influence of front-leg stepping velocity: extracellular recordings. *A*: extracellular recordings of mesothoracic protractor and retractor MNs during stepping of the ipsilateral front leg. Protractor and retractor MN activity alternated and was in phase with front-leg stepping. The *3rd* and *5th traces* are rectified and smoothed ($T$ = 0.05 s) transforms of the corresponding extracellular recordings of protractor and retractor MNs. *B*: regression analysis of motoneuron activity against mean front-leg stance velocity for protractor (gray crosses) and retractor MNs (black crosses). Motoneuron activity was calculated as the integral under the rectified and smoothed recording trace (see shaded area in *A*) normalized by step-cycle period. No correlation was observed. *C*: extracellular recordings of mesothoracic protractor and retractor MNs during stepping of the contralateral front leg. Front-leg stepping induced a general increase, but not rhythmicity, in protractor and retractor MNs. Order of the traces is the same as that in *A*. *D*: regression analysis of motoneuron activity against mean front-leg stance velocity for protractor (gray crosses) and retractor MNs (black crosses). Retractor MN activity varied significantly with mean front-leg stance velocity in this experiment. Protractor MN activity showed no significant correlation with mean front-leg stance velocity.

velocity and protractor MN activity was found in only 4, a correlation with retractor MN activity in only one experiment. In none of the experiments were both protractor and retractor MN activity correlated with front-leg stepping velocity. In summary, we did not detect a systematic relationship between the motoneuronal activity in deafferented ipsilateral mesothoracic protractor and retractor MNs and front-leg stance velocity. We also checked for a velocity dependence of the instantaneous frequency of the mesothoracic retractor MNs, which are active in stance and, again, found no velocity dependence on front-leg stepping.

We found similar results for the activity of motoneurons located contralateral to the stepping front leg. We recorded from pro- and retractor MN pools of the contralateral mesothoracic hemiganglion, which display tonic activity under these conditions (Borgmann et al. 2007). In analogy to the experiments on the ipsilateral side, we again used a regression analysis to test for a potential correlation between front-leg stepping velocity and the motoneuronal activity during the respective front-leg step cycle. Figure 2*C* shows a front-leg stepping sequence and simultaneous extracellular recordings of contralateral mesothoracic protractor and retractor MNs with the rectified and smoothed traces underneath for further analysis ($T$ = 0.05 s). Again, the integrals under the tachometer trace and the rectified and smoothed traces from the extracellular recordings were used as correlates of the stepping velocity and mean activities of protractor and retractor MNs (Fig. 2*C*). Figure 2*D* shows a plot of mean stance velocity against mean activity of protractor (gray) and retractor (black) MNs for the experiment shown in Fig. 2*C*. In this case a linear relationship existed between stepping velocity and mean MN activity for retractor MNs but not for protractor MNs. In total, in four of seven experiments a correlation between retractor MN activity and front-leg stepping velocity was found, whereas protractor MN activity was correlated with front-leg stepping velocity in only two of seven experiments. In only one experiment were both protractor and retractor MN activity correlated with front-leg stepping velocity. In summary, again, we did not detect a systematic relationship between the motoneuronal activity in contralateral mesothoracic protractor and retractor MNs and front-leg stepping velocity.

Some of the variability seen in the results might be explained by the fact that we performed extracellular recordings and that a potential velocity dependence might have been masked by a variation in the number of motor units firing or the quality of the recording. Since flexor motor neuron activity is known to change at different walking speeds, as shown by Gabriel and Büschges (2007), we chose to record intracellularly from ipsilateral ($N$ = 6, $n$ = 25; Fig. 3*A*) and contralateral ($N$ = 8, $n$ = 37; Fig. 3*B*) mesothoracic flexor MNs during single front-leg stepping. As reported previously, flexor motoneurons in the deafferented mesothoracic segment are tonically depolarized during front-leg stepping, with some rhythmic modulation riding on top of this depolarization that is correlated with the cycle period of the stepping front leg (Büschges et al. 2004; Ludwar et al. 2005b). We analyzed the amplitude of the tonic depolarizing component (Fig. 3*A*, dark gray), the phasic component (light gray), and both components together with respect to a putative dependence on the velocity of the stepping front leg. Figure 3, *C* and *D* shows the regression analyses for one

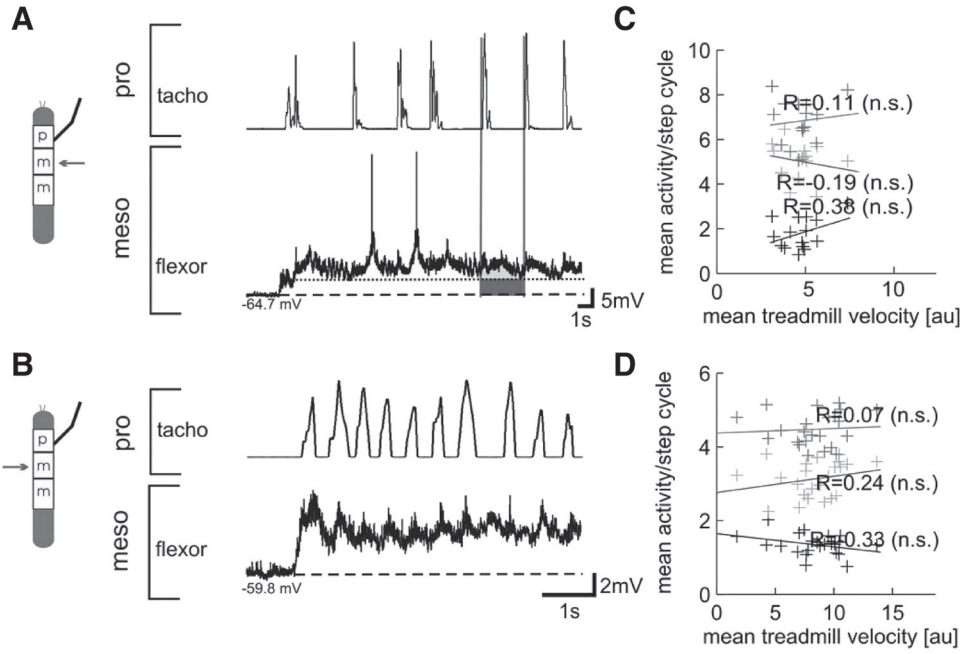



FIG. 3. Interleg influence of front-leg stepping velocity: intracellular recordings. *A*: intracellular recording of a mesothoracic flexor MN during stepping of the ipsilateral front leg. Membrane potential of the MN showed phasic modulation coupled to front-leg stepping. These were composed of a tonic component (dark gray) and a phasic component (light gray). *B*: intracellular recording of a mesothoracic MN during stepping of the contralateral front leg. Membrane potential showed phasic modulation. *C*: regression analysis of motoneuron activity against mean front-leg stance velocity. MN activity was calculated as the integral between recording trace and resting potential before the beginning of the stepping sequence, normalized by step-cycle period. Separate calculations were performed for the phasic component (black), the tonic component (gray), and the combined integral (dark gray). No correlation was observed. *D*: regression analysis of MN activity against mean front-leg stance velocity. Separate calculations were performed as in *B*. No correlation was observed.

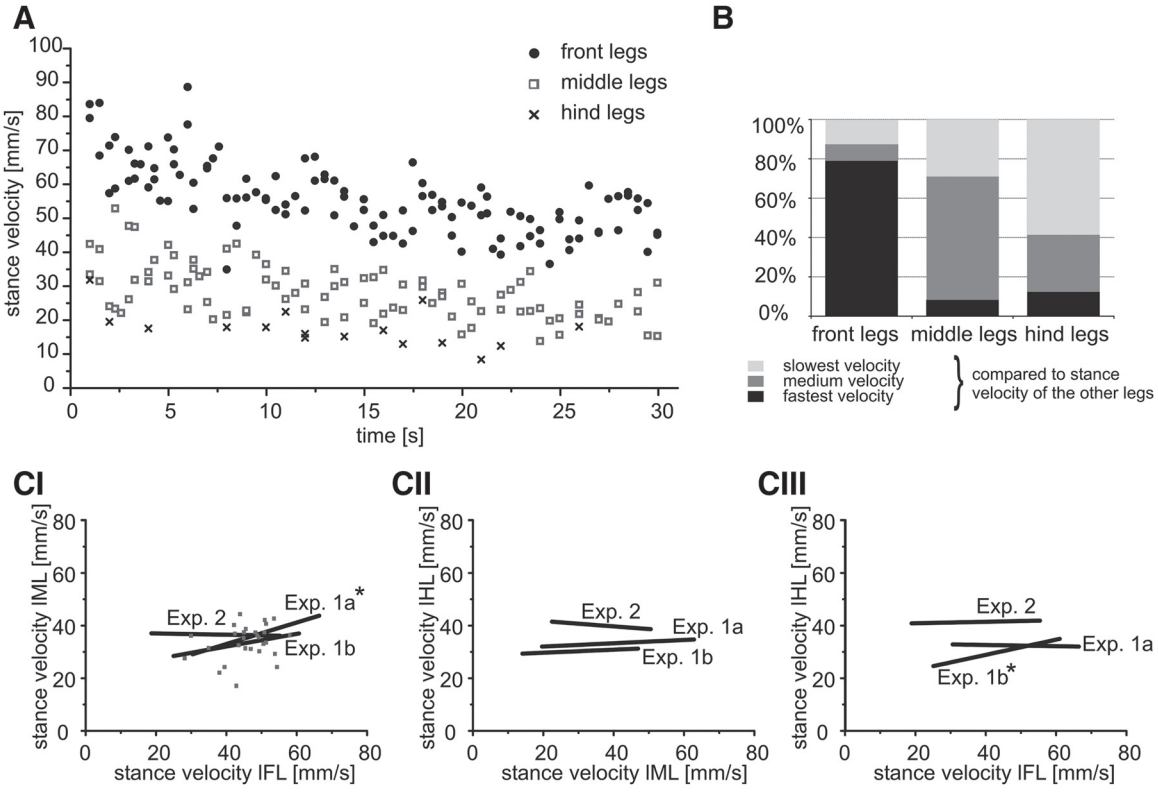
ipsilateral and one contralateral experiment each. None of the components was systematically correlated with stepping velocity of the front leg, neither for the ipsilateral nor for the contralateral mesothoracic segment (Fig. 3, *C* and *D*). In four of six animals we found no significant correlation between front-leg stepping velocity and the tonic or the phasic membrane potential modulation or both together in recordings from ipsilateral flexor motor neurons (data not shown). On the contralateral side, no correlation was detectable between the tonic, the phasic membrane potential modulation in the MNs, or both, and the mean front-leg stance velocity in five of eight animals (data not shown). This was equally true for stepping sequences that started autonomously as well as for those elicited by tactile stimulation.

*Walking and acceleration in the six-legged, intact animal*

In the preceding paragraph, we reported a lack of systematic influence of front-leg stepping velocity in the single-leg preparation on the neuronal activity of motor neurons of the neighboring legs. From this it is conceivable that each leg of a stepping stick insect regulates its own stepping velocity independently of the other legs and that entrainment to a common speed in the intact animal is achieved by coupling the legs through their contact with the substrate. Based on this assumption, one should expect that a six-legged stick insect, walking under conditions in which an entrainment of stepping speed through the passive displacement of the legs is prevented, would fail to show correlated stepping velocities of its six legs. To test this hypothesis, we used the second strategy to eliminate mechanical coupling between the legs. In the slippery surface setup, as implemented by Gruhn et al. (2006, 2009), an intact animal is tethered above the surface and can walk without mechanical coupling between the legs due to the slipperiness of the surface.

Figure 4*A* shows the stepping velocities of the front (closed circles), middle (open squares), and hind legs (crosses) of a typical straight-walking sequence on the slippery surface, without prior tactile stimulation. We compared the stepping velocities of all legs from 12 straight-walking sequences in eight animals and for 248–390 steps per leg. The average stepping velocity for the front legs was 42.8 mm $s^{-1}$ ($n = 739$; SD 11.9), for the middle legs 32.5 mm $s^{-1}$ ($n = 754$; SD 9.9), and for the hind legs 31.4 mm $s^{-1}$ ($n = 501$; SD 11.0). The range of speeds that we observed for the front legs was between 11.4 and 98.1 mm $s^{-1}$, for the middle legs between 9.9 and 105.2 mm $s^{-1}$, and that for the hind legs between 6.7 and 104.8 mm $s^{-1}$. In most walking sequences, one of the front legs displayed the highest stepping speed (79.2%) and the hind legs had the lowest stepping velocity (58.3%; Fig. 4*B*). However, despite the observed gradient from front to hind legs, in 20.8% of the sequences we also observed middle (8.3%) or hind legs (12.5%) to be the fastest stepping legs in a given walking sequence. The cycle periods of the front and middle legs were similar due to similar numbers of steps, ranging from 0.92 ± 0.29 to 1.25 ± 0.46 s and due to longer step lengths in the front legs, whereas the hind legs generally performed fewer steps and therefore also had longer cycle periods between 1.34 ± 0.46 and 1.39 ± 0.54 s. We tested whether the stepping velocities of neighboring ipsi- or contralateral legs were correlated with each other. Figure 4*C*, *i–iii* shows the relationships between stepping velocities of the different ipsilateral legs to each other, with the first leg always plotted on the *x*-axis (ipsi- to contralateral legs not shown). As one can see in the table in Fig. 4*D*, there is no systematic relationship between the stepping velocities of any two legs despite occasional significant correlations between the stepping velocities of single-leg pairs in the different walking sequences. Thus there is no evidence that a general neuronal control of stepping velocities for all stepping legs of a walking stick insect exists.

From the above-cited results the question arose with respect to the extent to which stepping velocity influences interleg information transfer from a stepping leg to its neighbors. First, it is well known that neural interleg information transfer contributes to the coordination of stepping between insect legs.




D

| R | rFL - rML | rML - rHL | rFL - r HL | lFL - lML | lML - lHL | lFL - lHL | rFL - lFL | rML - lML | rHL - lHL |
|---|---|---|---|---|---|---|---|---|---|
| Exp. 1a | 0,56 (23) | -0,43 (15) | -0,29 (17) | 0,49 (30) | 0,08 (25) | -0,04 (25) | 0,08 (27) | 0,42 (25) | 0,05 (15) |
| Exp. 1b | 0,08 (23) | 0,14 (22) | -0,21 (19) | 0,26 (28) | 0,07 (26) | 0,39 (26) | 0,32 (25) | 0,35 (27) | 0,12 (22) |
| Exp. 2 | 0,44 (60) | 0,19 (51) | 0,51 (50) | -0,03 (29) | -0,04 (26) | 0,01 (28) | 0,5 (34) | -0,2 (36) | -0,11 (27) |

FIG. 4. Analysis of stepping velocities in the intact tethered stick insect, walking steadily on the slippery surface. *A*: example of the stance velocities of all front (filled circles), middle (empty boxes), and hind legs (crosses) in a 30-s sequence. *B*: percentage of fastest velocity, medium velocity, and slowest velocity steps per leg type from 12 walking sequences in 8 animals, showing that the front legs most often expressed the fastest stepping velocity. *Ci–iii*: correlation analyses of 3 examples each, for stepping velocities between ipsilateral leg pairs. IFL, IML, and IHL: ipsilateral front leg, middle leg, and hind leg, respectively. In *Ci*, the data points for *experiment* 1b were added as an example. *D*: table with the correlation coefficients for all leg combinations tested, including the examples in *C*; gray background marks significant correlations. No systematic correlations were found between any given leg pair. $n$ = number of steps evaluated, given in parentheses.

This becomes particularly obvious when they walk on a slippery surface, i.e., when mechanical coupling between the legs is reduced or completely removed (Epstein and Graham 1983; Graham and Cruse 1981; for reviews see Cruse 1990; Graham 1985). Second, Borgmann et al. (2009) have recently shown that the activity of fibers projecting through the connectives between thoracic segments is modulated phasically, with stepping movements of a single stepping front leg.

We therefore recorded the extracellular nerve activity from the pro-to-meso-, the meso-to-metathoracic, and the neck connective during single front-leg stepping. Figure 5*A* shows an extracellular recording from the ipsilateral pro-meso- and the meso-metathoracic connectives during a front-leg stepping sequence together with the tachometer trace of the stepping front leg. In addition to the original recordings, rectified and smoothed ($T$ = 0.05 s) traces of the nerve activities are shown. When the front leg was at rest, a certain level of tonic activity was present in the connectives. With the start of a front-leg stepping sequence, neuronal activity in all three [pro-meso ($N$ = 5), meso-meta ($N$ = 5), both neck ($N$ = 4)] connectives increased and was phasically modulated. This is particularly apparent in the rectified and smoothed traces. Borgmann et al. (2009) have already shown that these modulations are correlated to the front-leg step cycle. We further investigated whether this increase in neural activity in the connectives also showed a dependence on front-leg stepping velocity. Figure 5*B* (*left*) shows the regression analysis of mean front-leg stance velocity against mean pro-meso neuronal activity in all five animals. The mean neuronal activity was estimated by the integral under the rectified and smoothed recording of the respective step cycle, divided by step-cycle period. For clarity, only the regression lines are shown. In four of five experiments, an increase in front-leg stance velocity was associated with an increase in the overall activity in the recorded connective. A similar, significant correlation in the meso-meta connective (Fig. 5*B*, *right*) was observed in only two of five animals. In addition, both neck connectives showed significant increases in mean activity together with increases in stepping velocity ($N$ = 4; Fig. 5*C*). In summary, neural activity in the connectives was modulated with front-leg stance velocity (see also Borgmann et

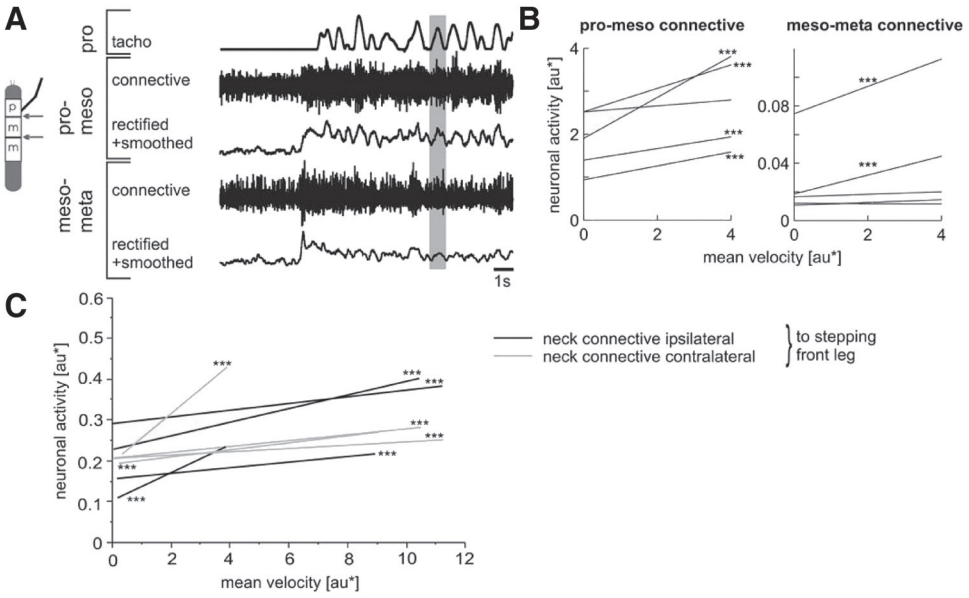


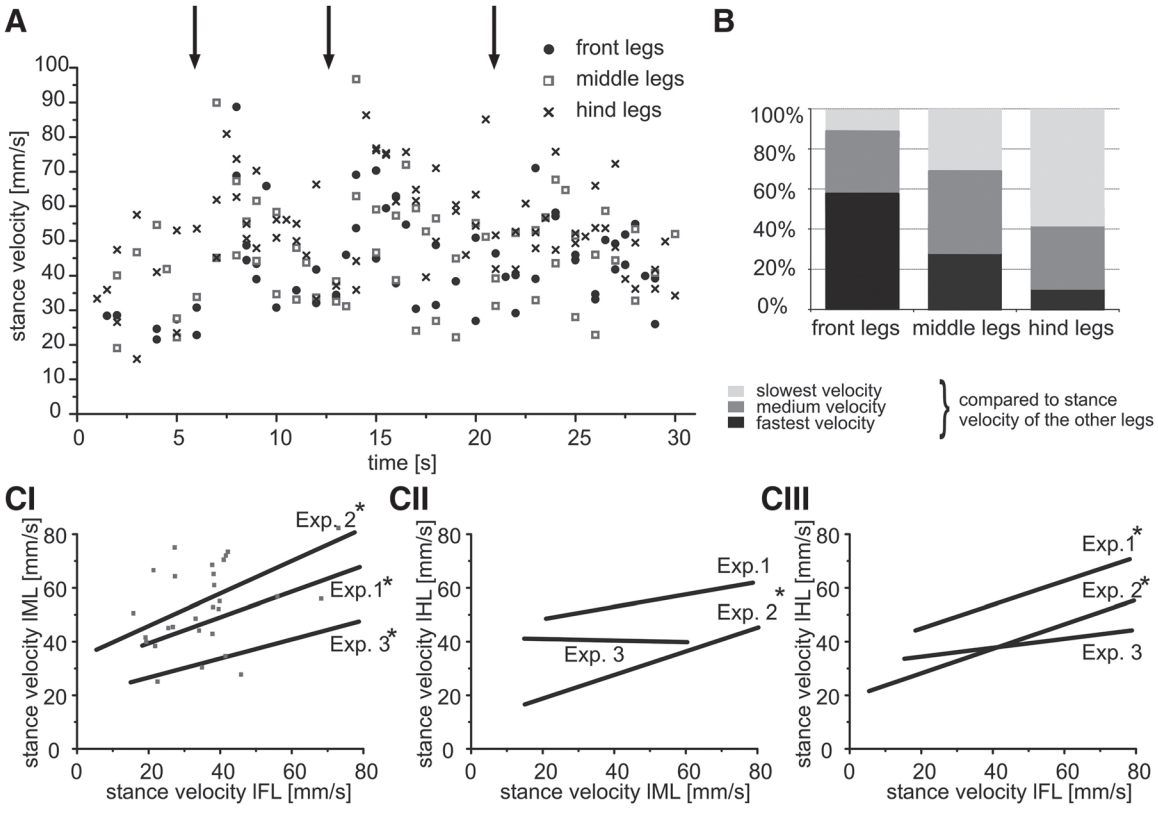

FIG. 5. Analysis of neuronal activity in interganglionic connectives. *A*: extracellular recordings of the pro-meso and meso-meta connectives during a front-leg stepping sequence. The *3rd* and *5th traces* are rectified and smoothed ($T = 0.05$ s) transforms of the corresponding extracellular recordings from the pro-meso (*2nd trace*) and meso-meta (*4th trace*) connectives, respectively. Neuronal activity in both connectives was modulated with front-leg stepping. The shaded box marks a single front-leg step cycle. *B*: regression analyses of neuronal activity against mean front-leg stance velocity for the pro-meso (*left*) and meso-meta connectives (*right*). The neuronal activity in the pro-meso connective was correlated with mean front-leg stance velocity in 4 of 5 experiments. The neuronal activity in the meso-meta connective was correlated with front-leg mean stance velocity in 2 of 5 experiments. Asterisks mark level of significance: $***P < 0.001$. *C*: regression analysis of neuronal activity against mean stance velocity for the subesophageal (SOG)-pro connective ipsilateral (black) and contralateral (gray) to the stepping front leg. The neuronal activity in the SOG-pro connectives was correlated with mean stance velocity in the front leg for all 8 experiments. Asterisks mark level of significance: $***P < 0.001$.

al. 2009). From these observed correlations, however, no conclusions can be drawn at this point with respect to the direction of information flow or the type of information exchanged between the ganglia. The observations do show, however, that an increase in stepping velocity either increases the firing frequency of specific neurons or activates new neurons that thus contribute to the observed effect on the overall connective activity.

Are there conditions, however, under which the stick insect walking system uses the information on stepping velocity present in the connectives and neuronally coordinates stepping velocity between the legs? One such situation could be acceleration, elicited in cases of escape-like walking sequences in response to a tactile stimulus to the abdomen. One could conceive that under this condition there is a general command that speeds up the single legs and couples their stepping velocities to one another. We measured the stepping speeds of all legs in 18 walking sequences of $N = 7$ animals during regular straight-walking sequences and then stimulated the animal with a slight brush stroke to the abdomen to elicit acceleration during walking. Figure 6*A* shows the stepping velocities of the front (closed circles), middle (open squares), and hind legs (crosses) during such a walking sequence. The arrows mark the time of the tactile stimulus and the resulting acceleration in all legs is clearly visible. In this case we could observe that during the time of acceleration and the subsequent deceleration, front, middle, and hind legs could again all be the fastest stepping legs of the animal, with the middle and hind legs showing a higher proportion of fastest velocity than before stimulation (FL, 58.3%; ML, 27.8%; HL, 13.9%; Fig. 6*B*). All leg pairs showed a marked reduction in the cycle periods, with the front legs showing the shortest and the hind legs having the longest cycle periods. In the accelerating animals, we could observe a significant correlation between the stepping velocities of the front legs, the middle legs, and the front and middle legs in ≥12 of 18 cases (Fig. 6, *C*, *i–iii* and *D*). Interestingly, no correlation was observed between the stepping velocities of the hind legs and inconsistent correlations were found between hind legs and the other legs. These results show that there are indeed conditions under which the nervous system is capable of neuronally coordinating stepping speeds between the anterior legs of the animal. The mechanism for this increased neuronal control, however, still needs to be elucidated.

## DISCUSSION

Previous studies have shown that in the stick insect, walking speed is dependent on changes in cycle period and stance phase motor output (Gabriel and Büschges 2007; Wendler 1964). In the present study we further investigated changes at the neuronal and behavioral levels that accompany alterations in walking speed in the stick insect. First, we provide additional evidence, based on a detailed analysis of the time course of the stance-to-swing transition, that swing phase motor activity is not being modified in conjunction with changes in walking speed. Only the latency between the end of stance phase motor activity and onset of swing phase motor activity was found to be reduced with increasing stepping velocity. Second, using extra- and intracellular recordings from middle-leg motoneurons in animals stepping with a single front leg, we found that alterations in its stepping velocity were not reflected in motoneuron activity of the caudal thoracic segment, either in the extracellularly recorded activity of motoneuron pools or in single intracellularly recorded motoneurons. Third, studying




D

| R | rFL - rML | rML - rHL | rFL - r HL | IFL - IML | IML - IHL | IFL - IHL | rFL - IFL | rML - IML | rHL - IHL |
|---|---|---|---|---|---|---|---|---|---|
| Exp. 1 | 0,72 (13) | 0,16 (19) | 0,02 (19) | 0,49 (30) | 0,32 (33) | 0,58 (31) | 0,65 (22) | 0,82 (20) | 0,33 (29) |
| Exp.2 | 0,85 (32) | 0,77 (25) | 0,71 (24) | 0,6 (35) | 0,63 (35) | 0,65 (25) | 0,68 (30) | 0,42 (41) | 0,39 (25) |
| Exp.3 | 0,27 (23) | 0,65 (20) | 0,4 (18) | 0,63 (30) | -0,03 (29) | 0,38 (27) | 0,53 (27) | 0,55 (28) | 0,32 (20) |

FIG. 6. Analysis of stepping velocities in the intact tethered stick insect during episodes of acceleration on a slippery surface. *A*: example of the stance velocities of all front (filled circles), middle (empty boxes), and hind legs (crosses) in a 30-s sequence, with tactile stimuli given at $t = 6$ s, $t = 13$ s, and $t = 22$ s (arrows). *B*: percentage of fastest velocity, medium velocity, and slowest velocity steps per leg type from 18 walking sequences in 7 animals, showing that the majority of fastest stepping velocities were executed by the front legs. *Ci–iii*: correlation analyses of 3 examples, each, for stepping velocities between ipsilateral leg pairs. IFL, IML, and IHL: ipsilateral front leg, middle leg, and hind leg, respectively. In *Ci*, the data points for *experiment* 2 were added as an example. *D*: table with the correlation coefficients for all leg combinations tested, including the examples in *C*; gray background marks significant correlations. The stepping velocities between the ipsilateral front and middle legs, and contralateral front legs as well as contralateral middle legs were found to be highly correlated. $n$ = number of steps evaluated, given in parentheses.

the intact, six-legged animal, walking on a slippery surface, we found no systematic correlations between the stepping velocities of the different legs in the steadily walking animal, although a gradient with decreasing stepping velocities from front to hind legs was observed. However, on acceleration and subsequent deceleration, induced by tactile stimulation of the abdomen, stepping velocities for neighboring front and middle legs were systematically correlated with each other.

### *Middle-leg stepping velocity: the role of swing phase motoneuron activity*

In the six-legged stick insect, changes in walking speed are known to be associated with alterations in the stance phase, which shortens toward faster speeds whereas the stride amplitude remains unaffected (Wendler 1964). The same can be said for the single stepping leg of the stick insect, as reported by Gabriel and Büschges (2007), or the equivalent stance phase motor neurons in the cockroach (Watson and Ritzmann 1998a,b). When we also investigated whether extensor, that is, swing motoneurons of a stepping middle leg contribute to changes in stepping velocity, we found stepping speed not to be correlated with any characteristics of extensor motoneuron activity such as spike frequency. Instead, a correlation between stepping velocity and the timing of the extensor burst onset became apparent. In 10 of 15 experiments, the time between stance end and the onset of swing activity (pause at the transition from stance to swing) showed a significant negative correlation with mean stepping velocity. The pause between stance and swing phase activity became shorter with increasing speed and completely disappeared during fast stepping sequences. This result is reminiscent of the shortening of transition between coxa–femur flexion and extension by the aid of fast depressor coxae spikes at the transition from swing to stance in fast-running cockroaches (Watson and Ritzmann 1998b), but differs from earlier findings by Fischer et al.

(2001), who described the duration of the pauses in the stepping cycle of the single stick insect middle leg to be independent of cycle period. However, these earlier results are based on measurements using an earlier version of the treadmill, which had much larger friction and therefore allowed only slow velocities (Gabriel et al. 2003). Mechanical influences through the treadmill used are therefore likely (for discussion see Gabriel et al. 2003). Our results show that there are no velocity-dependent alterations in swing activity or stance-to-swing transition, at least not in the way that the activation strength of stance would influence the subsequent activation strength of swing. That is further supported by the finding that stepping velocity was independent of extensor MN activity, as found in the analysis of FETi spike frequency. This again suggests that the hypothesis formulated by Cruse (2002) on the influence of a given stance phase on the subsequent swing phase does not apply for the control of a single stepping leg (see also Gabriel and Büschges 2007).

*Front-leg stepping velocity: interleg influences of front-leg stepping*

We then investigated the possibilities by which stepping velocity between legs may be controlled. In a first strategy, we used the single-leg preparation, thereby effectively eliminating all but the neuronal coupling between the legs. When a single front leg was stepping on a treadband, we observed that its stepping velocity was not correlated with motoneuronal activity of *protractor* and *retractor coxae* or *flexor tibiae* MNs of the ipsilateral and contralateral middle legs. This was obvious for both the extracellular recordings of coxal motoneuron pools and the intracellular recordings of individual flexor MNs in the deafferented mesothoracic segment. Such a finding is in line with earlier results by Foth and Bässler (1985a,b) who showed that each stick insect leg is capable of generating functional stepping movements, even when individual legs are generating different cycle periods for stepping movements. In addition, it is well known that generation of stepping in single legs of the stick insect relies heavily on the interplay between the activity of CPG networks and feedback from leg sensors (for summary see Büschges and Gruhn 2008).

As stated earlier, no correlation existed between the stepping velocity in the front leg and motoneuronal activity in the next posterior segment, either in animals that initiated stepping autonomously or in animals that were given a tactile stimulus to walk. This indicates that the cycle period of the functional stepping motor output of a single segment and its leg do not constitute a sufficient determinant for the stepping velocity of the other segments of the stick insect walking system. This may be an indication that alterations in motoneuronal activity as a result of interleg information as such do not constitute the critical factor that determines stepping velocity of the different legs in the stick insect. Instead, changes in stepping velocity may result from a more complex interplay between descending signals from the brain, from rostral segments, the local pattern-generating networks, and local sensory information. Therefore taken together, our results again corroborate evidence for the modular organization of the stick insect walking system (for review see Bässler and Büschges 1998; Büschges et al. 2008).

*Single-leg stepping velocities in the six-legged walking animal*

In a second strategy, we used the slippery surface setup, thereby effectively eliminating the mechanical coupling between the legs through the ground. An analysis of stepping velocities of all given pairs of legs in six-legged, intact animals that walked on a slippery surface failed to reveal systematic correlations—even though stepping movements of the legs were coordinated as reported previously (Cruse and Epstein 1982; Cruse and Schwarze 1988; Epstein and Graham 1983; Graham and Cruse 1981; Gruhn et al. 2009).

This supports the findings from our electrophysiological experiments and indicates that there is indeed very little neuronal interleg influence with respect to stepping velocity. This strongly suggests that there is also no continuous common neuronal control of stepping velocity for all six legs in the walking stick insect. Recent results on stick insect muscle characteristics, especially the force–velocity relation, suggest a reasonable biomechanical contribution for a velocity adjustment without neural origin (Blümel et al. 2007; Guschlbauer et al. 2007; Hooper et al. 2007, 2009). If the forward-stepping front legs alter their stepping speed, this change could be transferred to the posterior legs by altering the forces on them and their muscles due to mechanical coupling. This might in turn change the muscle contraction velocity, as predicted by the force–velocity curve of the respective muscles.

However, interestingly, recordings of the activity of intersegmentally projecting fibers in the thoracic connectives revealed that their activity does reflect stepping velocity (Fig. 5). We have been able to identify single units projecting in both directions. The quality of our recordings, however, has not permitted us a detailed analysis of interganglionic connective activity on the single-neuron level. One of the reasons is most likely the large number of about 2,000 axons that project through the connectives (Leslie 1973). A new series of experiments using elaborate single-unit isolation from multiunit connective recordings such as in Brunner et al. (1990) and Brunner and Koch (1991) is planned.

Theoretically, the walking system could use the information present in the connectives to neuronally coordinate the stepping velocities of its legs. However, only under conditions when the experimental animal was induced to modify its stepping velocity after we applied a tactile stimulus were we able to detect significant systematic correlations in stepping velocities between legs. Such correlations in stepping speeds were present between ipsilateral front and middle legs as well as contralateral front and middle legs. The distribution of data points for two walking sequences, exemplified in Figs. 4*Ci* and 6*Ci*, also shows that the correlation present in the accelerating animal is not simply due to a correlation of the highest velocity steps through reaching maximum speed in those legs. The number of steps that showed a strong increase in stepping velocity after a single tickle to the abdomen was usually no more than three or four. Therefore it appears, indeed, that coordination in general is improved after tactile stimulation also at lower stepping speeds. On the other hand, even under tactile stimulation, no systematic correlation was detected between hind-leg stepping velocity and that of front or middle legs. This is reminiscent of the lack of correlation between the front-leg stepping velocity and deafferented mesothoracic mo-

tor neuron activity in the single front-leg preparation. One can conclude that stronger neuronal coupling can be elicited by an appropriate sensory, e.g., tactile input, but that this stronger coupling is limited to selected leg pairs only and, in addition, might depend on local sensory feedback. This strongly suggests that neuronal interleg coordination of stepping velocity in the stick insect walking system is limited to specific behavioral conditions.

For the stick insect, little knowledge exists on the origin and destination of intersegmentally projecting neurons and nothing is known about neurons that might be responsible for conveying velocity information between the legs. In a few studies, origin and destination of specific intersegmental interneurons have been identified in the locust (Laurent and Burrows 1988, 1989a,b; Watson and Burrows 1983), but here again, nothing is known as to the transmission of velocity information. A potential neuronal pathway that may be involved in coordinating stepping velocities under certain conditions can be deduced from previous data by Cruse and colleagues, which point toward coactivating influences between neighboring stick insect legs (e.g., Cruse 1985; Cruse and Saxler 1980). They found that artificially interrupting or slowing down the stance phase of one leg in the walking stick insect leads to a simultaneous increase in the force developed by the other legs in stance (Cruse 1985). This mechanism enables the animal to increase the total force propelling the body. Interestingly, just as in our results, the coactivating effect on the hind legs reported by Cruse was much smaller than that in the other legs. It is quite conceivable that this effect, and the interleg influences presented here share common neuronal pathways, which are activated only upon the need for common action between the legs of the animal.

Another result from the present study is that the stepping velocities of the front, middle, and hind legs of the intact animals walking on the slippery surface were significantly different from each other. The highest velocities were in 79% of the cases generated by the front legs and the slowest most often by the hind legs. This observation was true for undisturbed, steady walks and a similar tendency was seen in those walks during which stepping velocity was increased in response to tactile stimulation of the abdomen, eliciting an escape-like locomotor behavior in the stick insect. Such a finding is in accordance with reports that the front legs take a leading role in forward stepping (Borgmann et al. 2007; Rosano and Webb 2007). Since stepping velocity was evaluated as stance progression per time, one could argue that morphological or geometrical differences between the legs (such as leg length) were responsible for the observed differences in stepping velocities. However, the cycle periods of stepping movements for front and middle legs were similar during steady walking and shorter in the front legs during acceleration, despite the fact that the front legs are the longest and the middle legs the shortest legs in *C. morosus*. This suggests that anatomic constraints did not adversely affect our measurements and that the neural networks generating the stepping movements of the individual legs have different default operating frequencies. The front legs, which are functionally the leading legs in many locomotor situations, also generate the fastest stepping velocities and have the shortest cycle periods. This also appears reasonable, given the natural habitat in which stick insects live, where they have to climb bushes to reach food sources.

The gradient in stepping velocities in the stick insect walking on the slippery surface bears similarities to that of other locomotor systems that consist of chains of pattern generators or oscillators. Experimental and simulation studies in the lamprey spinal network for swimming or the leech network for swimming, for example, have created the notion that in case of weakly coupled oscillators the leading kernel exerts its influence via a faster cycle period (Friesen and Kristan 2007; Grillner and Wallén 2002; Grillner et al. 2007; Hocker et al. 2000; Matsushima and Grillner 1992). Similar weak interactions between the thoracic segments may be involved in velocity control between legs in the stick insect, but are then complemented and entrained through the mechanical interaction between the legs during normal walking conditions. Such influences have recently been demonstrated (Borgmann et al. 2009) and are also known to exist in lamprey (McClellan 1990) and the leech (Yu et al. 1999).

### *Conclusion*

What can we learn from the above-described data about the neuronal control of stepping velocity in the stick insect? Although in the walking six-legged stick insect stepping activities of individual legs are permanently coordinated, the stepping performance for each individual leg appears not to be commonly controlled. In the absence of mechanical coupling between the different legs, the only general principle is a gradient in stepping velocities from front to back, with the front legs stepping fastest and the hind legs stepping more slowly. An increasing stepping velocity in a single front leg is clearly correlated with an increase in activity of the intersegmental axons in the connectives between the thoracic ganglia. However, in steady-walking conditions this information does not appear to be used to coordinate stepping velocities between legs. This result corroborates the notion formulated by Cruse and colleagues (e.g., Schmitz et al. 2008) that the stick insect walking system is operated by "decentralized neural control" due to the decisive role of sensory feedback in single-leg control. It is only under special conditions—such as a simulated form of "escape" run through tactile stimulation—that neuronal coupling between legs in the stick insect is increased and becomes apparent to show correlations between stepping velocities. This finding asks for an in-depth mechanistic explanation at the neuronal level of the stick insect walking system that needs to be elucidated in future studies.

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GRANTS

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## 4.3 Inter-leg Coordination in the Control of Walking Speed in *Drosophila*

Anne Wosnitza, Till Bockemühl, Michael Dübbert, Henrike Scholz, Ansgar Büschges

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Für diese Publikation habe ich die Versuche gemeinsam mit Till Bockemühl und Ansgar Büschges konzipiert. Ich habe alle Versuche durchgeführt und die Datenauswertung zusammen mit Till Bockemühl vorgenommen. Die Ergebnisse wurden von mir, Till Bockemühl und Ansgar Büschges diskutiert. Für das Manuskript, inkl. Abbildungen, habe ich die erste Version der Methoden und Ergebnisse erstellt und die Arbeit dann in Zusammenarbeit mit Till Bockemühl und Ansgar Büschges fertiggestellt.

## RESEARCH ARTICLE

# Inter-leg coordination in the control of walking speed in *Drosophila*

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**SUMMARY**

**Legged locomotion is the most common behavior of terrestrial animals and it is assumed to have become highly optimized during evolution. Quadrupeds, for instance, use distinct gaits that are optimal with regard to metabolic cost and have characteristic kinematic features and patterns of inter-leg coordination. In insects, the situation is not as clear. In general, insects are able to alter inter-leg coordination systematically with locomotion speed, producing a continuum of movement patterns. This notion, however, is based on the study of several insect species, which differ greatly in size and mass. Each of these species tends to walk at a rather narrow range of speeds. We have addressed these issues by examining four strains of *Drosophila*, which are similar in size and mass, but tend to walk at different speed ranges. Our data suggest that *Drosophila* controls its walking speed almost exclusively *via* step frequency. At high walking speeds, we invariably found tripod coordination patterns, the quality of which increased with speed as indicated by a simple measure of tripod coordination strength (TCS). At low speeds, we also observed tetrapod coordination and wave gait-like walking patterns. These findings not only suggest a systematic speed dependence of inter-leg movement patterns but also imply that inter-leg coordination is flexible. This was further supported by amputation experiments in which we examined walking behavior in animals after the removal of a hindleg. These animals show immediate adaptations in body posture, leg kinematics and inter-leg coordination, thereby maintaining their ability to walk.**

Key words: walking, motor control, sensory feedback, inter-leg coordination.

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## INTRODUCTION

In terrestrial animals, legged locomotion is a behavior that is highly optimized (Alexander, 1989). It is also flexible and can be adapted to the external environment and to specific behavioral goals. The locomotor apparatus often has to be used on a variety of substrates such as level surfaces, twigs in a bush or ragged cliffs. Furthermore, the locomotor output can change from slow explorative walking to swift running when it becomes necessary to escape a predator or cross terrain without cover.

Frequently, changes in locomotor output are not restricted to the movements of single legs but also entail changes in the temporal coordination between several or all legs. Many quadrupeds, like cats, dogs or horses, for instance, use specific gaits depending on their movement speed (Alexander, 1989). In these animals, leg coordination changes from walking and pace gaits at slow speeds to trotting gaits at intermediate speeds and, eventually, to gallop at high speeds. The coordination of the frontlegs and hindlegs changes from anti-phase in walking to nearly in-phase during gallop (Orlovsky et al., 1999). The transition from one gait to another is discontinuous and it can be shown that quadrupeds select the energetically optimal gait at a given speed (Hoyt and Taylor, 1981).

In hexapods, i.e. insects, the situation appears, at first glance, to be comparable. However, different patterns of leg coordination can occur. These patterns are typically characterized by the number of legs that are on the substrate during stance. Very slow-walking insects, for example, generate a metachronal wave of leg movements along each side of the body sequentially from back to front while at least five legs are always in stance phase, a coordination pattern called wave gait (Hughes, 1952). For faster walking speeds, coordination is modified accompanied by an apparent reduction in the number of legs that are on the ground simultaneously. At medium speeds, the number of legs is reduced to four, termed tetrapod coordination (Burns, 1973; Graham, 1972; Hughes, 1952; Spirito and Mushrush, 1979; Wendler, 1964; Wendler, 1966), and at high speeds to three, called tripod coordination (Bender et al., 2011; Delcomyn, 1971; Graham, 1985). Interestingly, bipedal anti-phase coordination of insect hindlegs has been reported for the cockroach, *Periplaneta americana*, during top speed running (Full and Tu, 1991). In this situation, the anterior part of the animal is lifted and the front and middle legs no longer touch the ground.

While in quadrupeds the switch between two patterns of inter-leg coordination, or gaits, is distinct and dependent on speed, studies in invertebrates indicate that specific patterns of coordination are part of a larger and speed-dependent continuum and that intermediate forms of coordination exist. In the same speed range, insects can use either tetrapod or tripod coordination, seamlessly transitioning from one to the other by modifying stance duration (Cruse, 1990; Graham, 1985; Wendler, 1966). Several genera of ants (*Cataglyphis*, *Formica*, *Lasius* and *Myrmica*), cockroaches (*P. americana*), fruit flies (*Drosophila melanogaster*) and stick insects (*Carausius morosus*) are known to use tripod coordination during fast locomotion, while at lower speeds leg coordination becomes much more variable, approaching tetrapod coordination (Wendler, 1964; Graham, 1972; Bender et al., 2011; Strauss and Heisenberg, 1990; Zollikofer, 1994).

How is inter-leg coordination achieved? Behavioral studies on four-, six- and eight-legged animals have suggested that sensory signals which reflect the movements of individual legs contribute to the coordination between legs, thereby generating an emergent set of coordination rules (Cruse, 1990; Dürr et al., 2004). Furthermore, the importance of intersegmental neural pathways has also been shown based on studies that reduce or eliminate the mechanical interaction between legs (Graham and Cruse, 1981; Cruse and Epstein, 1982; Gruhn et al., 2006). In normal walking situations, the coordination rules arise from the interplay of mechanical and neural coupling between individual legs during walking. While it is clear that both mechanical and neural influences play important roles, their specific contribution for the generation of leg coordination patterns is not clear, yet. In contrast, there is evidence confirming the importance of central inter-segmental neural pathways for the coordination of local networks controlling leg movements in insect walking, for example. This has been shown for the cockroach *P. americana* (Pearson and Iles, 1973), the locust *Schistocerca americana* (Ryckebusch and Laurent, 1993) and the hawk moth *Manduca sexta* (Johnston and Levine, 2002). However, studies have shown the role of local sensory feedback in establishing inter-leg coordination, e.g. in the hawk moth (Johnston and Levine, 1996; Johnston and Levine, 2002) and the stick insect *C. morosus* (Borgmann et al., 2009; Büschges et al., 1995).

One aspect that has so far hindered further elucidation of the neural mechanisms underlying inter-leg coordination is the fact that insect species at given developmental stages (Graham, 1985) often show a rather narrow range of preferred walking speeds. For example, while it is known that cockroaches can use the full range of inter-leg coordination from metachronal wave gait, in which only one leg is in swing phase at any given time, to tripod coordination (Hughes, 1952), under natural conditions they mostly use tripod coordination (Bender et al., 2011). Adult stick insects also show a preference for a particular coordination pattern. They almost exclusively use tetrapod coordination during level walking, while at high speeds they also use tripod coordination (Graham, 1972). In adult stick insects, tripod coordination is less frequent, though; larval stages tend to use tripod coordination much more frequently (Graham, 1972) but are also much smaller. As a consequence, in the insect groups studied so far only a rather limited continuum of walking speeds could be investigated reliably. This is all the more unsatisfactory as the specifics of inter-leg coordination are often used as important indicators of how the neural mechanisms generating walking behavior are structured (Zollikofer, 1994). It is therefore crucial to determine the full possible range of walking speeds with regard to inter-leg coordination.

In the present study, we used four different *Drosophila* strains in order to address this issue and capture as large a range of walking speeds as possible in a single species. The two wild-type strains *Canton-S* ($wt^{CS}$) and *Berlin* ($wt^{Berlin}$) represented the typical behavior in the wild. These two strains have previously been used in studies on inter-leg coordination (Strauss and Heisenberg, 1990; Strauss and Heisenberg, 1993) and global parameters of locomotor activities (Martin, 2004; Martin et al., 1999). In addition, we selected two mutant *Drosophila* strains, *white*$^{1118}$ ($w^{1118}$) and $w^{1118}$, $Tbh^{nM18}$ to extend the range of observable walking speeds to lower values. $w^{1118}$ flies have reduced levels of octopamine (Sitaraman et al., 2008), while $w^{1118}$, $Tbh^{nM18}$ lacks this biogenic amine altogether (Monastirioti et al., 1996). Octopamine is implicated in the high-level control of locomotor activity (Brembs et al., 2007; Gal and Libersat, 2008; Gal and Libersat, 2010) and, as we show here, a reduced level or absence of octopamine seems to induce lower walking speeds in *Drosophila*. Furthermore, the results we present here for $w^{1118}$ flies can also serve as a control for future studies in *Drosophila*, as an extensive amount of transgenic flies have a $w^{1118}$ background. As we show, there are important differences between wild-type flies and $w^{1118}$, and this might be important for the interpretation of behavioral studies based on transgenic strains.

We show that under relatively unconstrained conditions, individuals of different *Drosophila* strains cover a broad range of speeds during walking. We found that leg coordination patterns change gradually and systematically with walking speed. This suggests that the neural controllers responsible for inter-leg coordination are able to generate a marked flexibility with respect to walking behavior. Furthermore, removing one of the hindlegs revealed that *Drosophila* is capable of adapting its leg coordination immediately, thereby maintaining the ability to propel itself forward even after major biomechanical changes in its walking apparatus.

## MATERIALS AND METHODS

### Fly strains and breeding

Flies were raised at 25°C and 60% humidity on a 12 h/12 h light/dark cycle and maintained on standard medium containing cornmeal, molasses, yeast and agar. For the experiments presented here, we used the following *Drosophila melanogaster* strains: wild-type Canton-S ($wt^{CS}$), wild-type Berlin ($wt^{Berlin}$), $w^{1118}$, and $w^{1118}$, $Tbh^{nM18}$ (Monastirioti et al., 1996). Flies were kindly provided by Dr M. Leptin ($wt^{CS}$), Dr R. Strauss ($wt^{Berlin}$) and Dr H. Scholz ($w^{1118}$ and $w^{1118}$, $Tbh^{nM18}$).

### Experimental procedure

For all experiments, 5 day old males were used. At least 2 h prior to an experiment, flies were cold anesthetized and put into isolation tubes without food but with water. One fly at a time was then transferred from its isolation tube into the experimental setup were it walked spontaneously back and forth on a 5 mm wide transparent walkway (Fig. 1A). Wings were left intact; therefore, to prevent escape by flight, the walkway was enclosed on all sides with acrylic glass. Furthermore, the inner walls of the enclosure were covered with a layer of Fluon (AGC Chemicals Europe, Thornton Cleveleys, UK), which prevented the flies from scaling the walls. To allow for video recordings, a small area (20 mm) on one side of the walkway was kept free of Fluon. Beneath this area, we attached a glass prism providing a ventral view of the walkway. Thus, using a single camera we were able to simultaneously record a lateral (Fig. 1B) and a ventral view (Fig. 1C) of the walking fly. Video recordings were taken with a high-speed digital camera (AOS S-PRI High Speed Color 5.2, AOS Technologies AG, Baden Daettwil, Switzerland) at 500 frames $s^{-1}$, with a shutter time of 200 µs. The setup was illuminated with infrared LEDs (λ=880 nm). The LEDs were externally synchronized to the shutter of the camera in order to provide maximum illumination during the time the camera shutter was open. The camera was controlled *via* AOS Imaging Studio v3 (AOS Technologies AG). After each set of experiments, a 10 mm wide marker was recorded with the same settings. This marker was then used to calibrate the analyzed videos.

For the amputation experiments, flies were cold anesthetized followed by the removal of one of the hindlegs at the midpoint of the femur, leaving only a stump consisting of the coxa, trochanter and part of the femur. Flies were then moved to isolation tubes and subsequently treated as described above for the intact animals.

To determine the average mass of the flies, between 9 and 35 flies (3–7 days old) of each sex and strain were collected into separate 1.5 ml plastic tubes (Table 1). The tubes including the flies were

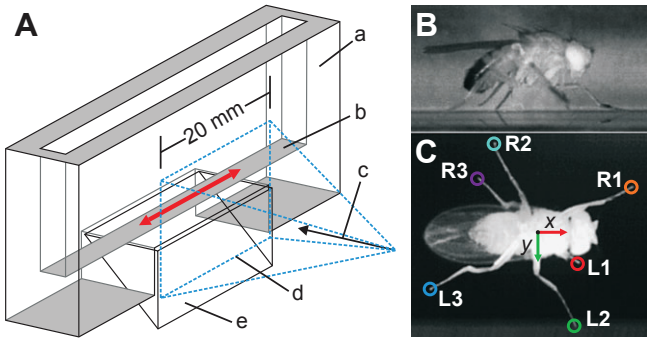


Fig. 1. (A) Schematic diagram of the experimental setup. Flies walked spontaneously back and forth on a walkway as indicated by the red arrow. Walks were recorded through a 20 mm wide window simultaneously from one side and from below (a: acrylic glass, coated on the inside with a layer of Fluon to prevent the flies from scaling the glass; b: 5 mm wide transparent walkway; c: camera viewpoint; d: camera field of view, free of Fluon; e: glass prism, providing a ventral view of the walkway). (B) Exemplary lateral view of a male *Drosophila*, wild-type $wt^{CS}$ strain, during one of the recorded walks. (C) Ventral view of the same fly in the same video frame. The tips of the tarsi are marked with colored circles (R1, R2, R3: right front leg, middle leg and hindleg; L1, L2, L3: left front leg, middle leg and hindleg). Red and green arrows indicate the origin and orientation of the body coordinate system.

then weighed, the mass of the empty tube was subtracted, and the mass of a single fly was calculated. In addition, the body length of each fly recorded during the behavioral experiments was determined by marking the base of the antennae and the tip of the abdomen in the ventral view of the fly, using the same software as for video analysis (ProAnalyst, XCitex, Inc., Cambridge, MA, USA).

### Data analysis

During experiments, flies walked spontaneously back and forth on the walkway. We recorded straight walks containing 5–12 complete step cycles per leg. The recorded videos were then evaluated frame-by-frame in a semi-automatic fashion. Body position and axes were determined automatically with ProAnalyst (XCitex, Inc.). The exact times of tarsal lift-off and touchdown events were visually determined in the lateral view of the fly, while the associated tarsus positions were visually determined in the ventral view. Data obtained in this manner were then further processed in MATLAB (MathWorks, Inc., Natick, MA, USA).

The durations of swing and stance phases were calculated as the difference between the time of lift-off and subsequent touchdown of the same leg (swing) or *vice versa* (stance). One cycle period was defined as the time difference between two consecutive lift-off events of the same leg. Onset of swing was used as the reference time for the analysis of temporal coordination of all legs. In trials with intact animals, the reference leg was always the front leg that completed the most cycles during a given trial. In trials with animals lacking one hindleg, the reference leg was always the front leg contralateral to the lesioned side. Results from the phase analysis of trials in which the right front leg was the reference leg were then flipped in order to combine the results with those in which the left front leg was the reference leg. The CircStat Toolbox for MATLAB was used for phase analyses and the corresponding plots (Berens, 2009).

Table 1. *Drosophila* strains

| | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Mass (mg) | *N* | Size (mm) | *N* | Mass (mg) | *N* |
| $wt^{CS}$ | 0.70 | 29 | 2.06±0.08 | 6 | 1.17 | 29 |
| $wt^{Berlin}$ | 0.86 | 22 | 2.12±0.01 | 3 | 1.32 | 22 |
| $w^{1118}$ | 0.70 | 27 | 2.09±0.08 | 5 | 1.05 | 35 |
| $w^{1118}$, $TbH^{nM18}$ | 0.71 | 12 | 2.07±0.03 | 5 | 1.21 | 9 |

All positional information with regard to tarsal touchdown and lift-off was transformed into the body-centered *xy*-coordinate system (see also Fig. 1C). Furthermore, in order to compensate for small variations in body size, these body-centered data were then normalized to the respective body length of the fly. Based on these data, we calculated stance trajectories in the body-centered *xy*-coordinate system (Fig. 2B). Step amplitude of a particular step was determined as the distance between the posterior extreme position (PEP) of the tarsus at lift-off and the subsequent touchdown at the anterior extreme position (AEP) in body-centered coordinates. It should be noted that we used step amplitude instead of stride length, which is defined as the distance between two consecutive touchdown positions in floor-fixed coordinates. Stride length is not independent of movement speed and might change even without active changes in the walking motor pattern. This is not true for step amplitude. A change in this measure always necessitates a change in the motor output. Although the two measures are closely related, step amplitude is much more informative when one is interested in kinematic changes the animal has to actively make.

Based on the ventral view, walking speed was calculated for each frame in a trial as the change in position of the fly's body relative to the ground. The resulting speed profile was smoothed with a gliding average of 5 frame width. Based on this complete speed profile, the walking speed associated with a particular swing phase, as used in Fig. 3B,D, for instance, was calculated as follows: we first determined the time interval between the onset and offset of the swing phase and found the section of the complete speed profile associated with this interval. We averaged the speed profile within the interval to obtain a single average speed value. This average speed value was then used as the walking speed associated with a particular swing phase.

### Coordination patterns

In hexapod walking, the literature typically distinguishes between three different coordination patterns: tripod coordination, tetrapod coordination and wave gait. The mere existence of these categories implies three distinct gaits, and, in fact, these coordination patterns have often been used synonymously with gaits. The literature, however, also implies that there is a speed-dependent continuum between these prominent patterns (Wendler, 1964; Graham, 1972). Therefore, because they are established, we use these terms; however, we do so in a purely descriptive manner and refer to coordination patterns rather than gaits.

In order to describe the walking patterns that occurred during the recorded trials, we classify these as tripod, tetrapod or undefined coordination according to the following considerations. Tripod coordination is described as the alternating movement of two distinct groups of legs (Hughes, 1952; Wilson, 1966). These tripod groups consist of an ipsilateral front leg and hindleg, and a contralateral middle leg (L1, L3, R2, and R1, R3, L2, respectively). Tripod coordination is typically found in fast-moving animals and therefore constitutes the extreme case at the highest end of the aforementioned speed-dependent continuum. In its ideal form, tripod coordination is characterized as the simultaneous lift-off and touchdown of all legs in one tripod group, while the legs associated with the other tripod group are on the ground. However, using this strict definition of tripod coordination is

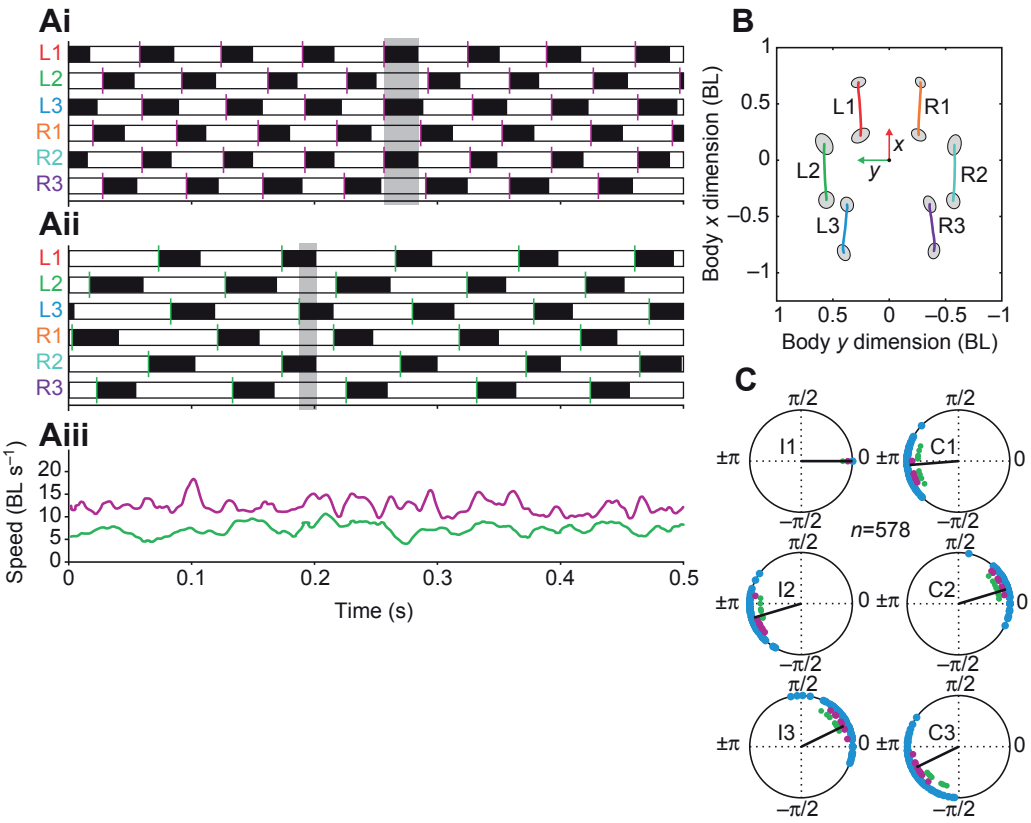


Fig. 2. Walking parameters of $wt^{CS}$. (Ai) Footfall pattern of all six legs during 0.5 s of one faster trial and (Aii) 0.5 s of one slower trial, and (Aiii) walking speed (BL, body lengths) of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (R1, R2, R3: right front leg, middle leg and hindleg; L1, L2, L3: left front leg, middle leg and hindleg). Black bars indicate swing phase, white bars indicate stance phase; magenta lines indicate the onset and end of complete step cycles in the faster trial, green bars indicate those in the slower trial. Shaded areas highlight coordination patterns of interest (see Results). (B) Average stance trajectories of all legs from all trials in relative body coordinates. (C) Phase plots of swing onset of all legs with respect to the left front leg (I, ipsilateral; C, contralateral; blue: data from all trials; magenta: data from Ai; green: data from Aii; black line: mean vector – length indicates variance). *n*, number of trials.

problematic, for two main reasons. Firstly, in this strict sense, tripod coordination occurs very rarely; even during highly coordinated walking, random fluctuations or small systematic shifts in the phase relationships between legs of one tripod group tend to persist (Bender et al., 2011); in addition, it is known that in most insects the legs of one tripod group are not completely in phase (Hughes, 1952). Secondly, concentrating on this narrow aspect of inter-leg coordination potentially diverts attention from other interesting coordination patterns that do not happen to fall under the tripod definition but might nevertheless be highly regular. In order to address this conceptual problem, we used a more flexible description of tripod coordination: we defined a particular walking pattern as tripod coordination when, during one step, the swing phases of all legs associated with a tripod group concurrently overlapped for at least one frame of recorded video. Here, this is equivalent to 2 ms; for comparison, typical swing durations observed during experiments were in the range 20–40 ms. In addition, once a walking pattern was defined as tripod, we determined the tripod coordination strength (TCS), which we obtained as follows. First, we calculated the time from the earliest swing onset to the latest swing termination. This gave us time $t_1$, during which at least one of the three legs was in swing phase. Then we determined time $t_2$, during which all three legs were in swing phase at the same time. The ratio $t_2/t_1$ then described the TCS. A TCS of 1 indicated perfect tripod coordination; it approached 0 when the temporal relationship of swing phases shifted to other coordination patterns. Tetrapod coordination is defined as a walking pattern in which exactly two legs are lifted off the ground at a particular time (Graham, 1985; Hughes, 1952). Therefore, a walking pattern was defined as tetrapod when, during one step, the swing phases of exactly two legs overlapped for at least one frame of recorded video. Tetrapod coordination constitutes a further special case within the continuum of coordination and is generally associated with intermediate walking speeds. Finally, when a step was neither tripod nor tetrapod we classified it as undefined. This category is largely identical to what is usually called wave gait, although this was not explicitly tested. It should be noted that we used this classification schema on a step-by-step basis; each step was evaluated separately and could be classified as tripod, tetrapod or undefined, but never as two of these. Although tripod coordination was predominantly found at high speeds, tetrapod coordination was most frequently found at intermediate speeds, and undefined coordination was most common at low speeds, the classification was completely independent from the walking speed during a particular step; each coordination class could have occurred at any speed.

## RESULTS

The four different strains of *Drosophila* studied here were similar in size and mass (Table 1). The body lengths of males ranged from 2.06 to 2.12 mm, and their mass ranged from 0.70 to 0.86 mg. In general, the mass of females was higher, ranging from 1.05 to 1.32 mg. Males of the strains $wt^{CS}$, $w^{1118}$ and $w^{1118}$, $Tbh^{nM18}$ were almost identical in size and mass, while $wt^{Berlin}$ males were slightly larger (5%) and on average 20% heavier. The same was true for females of $wt^{Berlin}$. In order to minimize potential age- or sex-related influences on walking behavior, we selected 5 day old males for the present study.

### Wild-type $wt^{CS}$

In the first set of experiments, we studied leg kinematics and inter-leg coordination in $wt^{CS}$ during spontaneous walking. Generally, animals generated walking sequences that were straight with features that were in accordance with previously published findings (Strauss and Heisenberg, 1990; Strauss and Heisenberg, 1993). Legs were coordinated in tripod fashion, as exemplified in the trial displayed in Fig. 2Ai (highlighted area). The features of all further recorded trials of $wt^{CS}$ were qualitatively similar to the one shown in Fig. 2Ai. Movement speed was always relatively constant during each trial;

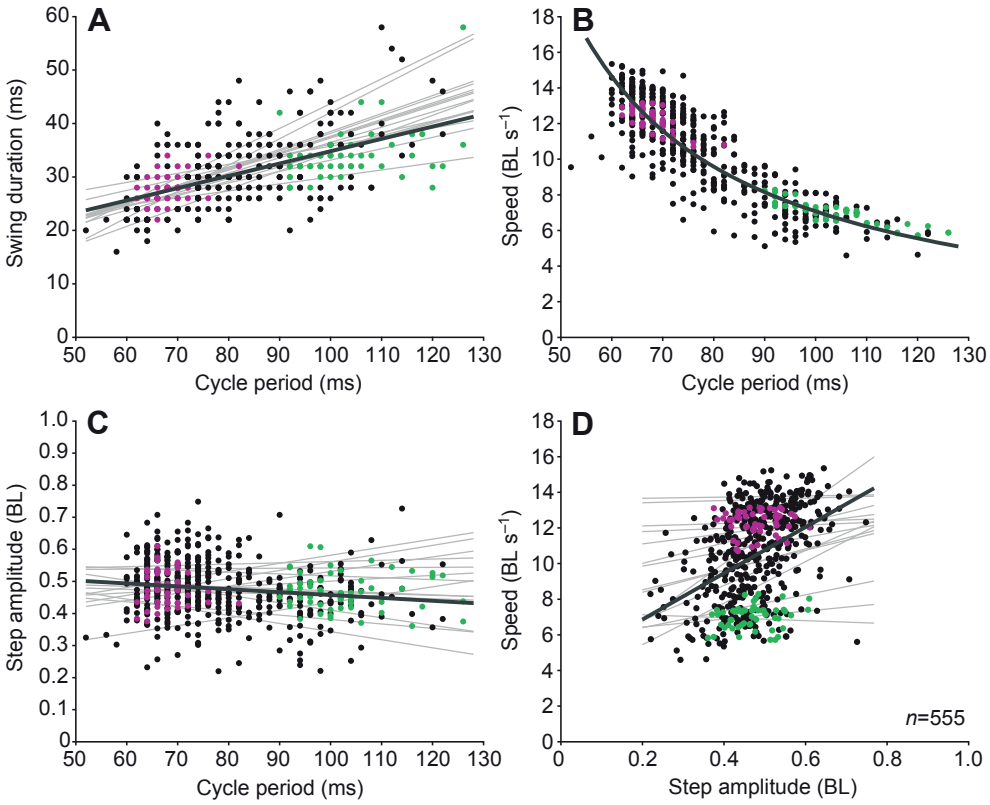


Fig. 3. Evaluation of leg stepping parameters of $wt^{CS}$. (A) Swing duration as a function of cycle period (black: data from all trials; magenta: data from trial in Fig. 2Ai; green: data from trial in Fig. 2Aii). (B) Walking speed as a function of cycle period (same color coding as in A). (C) Step amplitude as a function of cycle period (same color coding as in A). (D) Walking speed as a function of step amplitude (same color coding as in A). Each panel contains a regression line for the complete data set (black) as well as several further regression lines (gray), each of which is associated with one trial ($n$=15).

in the sequence shown in Fig. 2Ai, for instance, movement speed was approximately 13 body lengths per second (BL $s^{-1}$) on average. However, over all trials, average walking speed ranged from 5 to 16 BL $s^{-1}$. This was equivalent to absolute values of 11–32 mm $s^{-1}$ (6 individuals, 555 steps). Average stance phase trajectories of all six legs were relatively straight and almost parallel to the longitudinal body axis (Fig. 2B). The length of stance trajectories was similar for all legs and in the range of half the body length. With regard to temporal coordination, each of the three leg pairs showed anti-phase swing activity on average (Fig. 2C). Legs were generally coordinated in tripod fashion; however, the front leg of a tripod group tended to initiate swing phase first, followed by the middle leg with a phase shift of approximately 15 deg. The middle leg was in turn followed by the hindleg with a further phase shift of 15 deg (Fig. 2C).

Tripod coordination was more variable only during particularly slow walking sequences. An example of this is shown in Fig. 2Aii. Here, a section of 0.5 s from one of the slower trials in $wt^{CS}$ is shown (approximately 7 BL $s^{-1}$ on average). However, even during these slowest walking sequences, coordination was still tripod, according to our conservative definition (see highlighted area in Fig. 2Aii), and phase relationships were similar to those of the faster trials (Fig. 2C, green points). In contrast to a tripod group, in which the temporal succession of swing onset was directed posteriorly, the order of swing onsets on each body side was always directed anteriorly, beginning with the hindleg, followed by the middle leg and finally the front leg, after which the next series started again with the hindleg.

As it is known that insects walking in tripod coordination adapt swing duration depending on step cycle period (Graham, 1985), we examined this relationship for $wt^{CS}$. We found that swing duration indeed moderately correlated with cycle period (Fig. 3A); this was true for the complete data set (Fig. 3A, black regression line, coefficient of determination $R^2$=0.37), as well as for individual trials (Fig. 3A, gray regression lines). Another parameter that more strongly depended on cycle period was walking speed; we modeled this dependence as a hyperbolic relationship over the complete range of cycle periods (Fig. 3B, black line, pseudo $R^2$=0.76). At the same time, cycle period did not correlate with step amplitude (Fig. 3C, black regression line, $R^2$=0.03). Although step amplitude contributes weakly to walking speed when we examine the complete range of step amplitudes (Fig. 3D, black regression line, $R^2$=0.16), this relationship cannot be shown reliably for individual trials (Fig. 3D, gray regression lines).

### Wild-type $wt^{Berlin}$

We then collected data for the $wt^{Berlin}$ strain (Fig. 4). Similar to $wt^{CS}$ flies, $wt^{Berlin}$ flies almost exclusively used tripod coordination during all recorded trials. As an example of comparatively strict tripod leg coordination in this strain, Fig. 4Ai shows a 0.5 s long section of a fast walking trial. Overall, average walking speed ranged from 5 to 15 BL $s^{-1}$, which was equivalent to absolute speeds of 11–34 mm $s^{-1}$ (3 individuals, 403 steps). Stance trajectories in $wt^{Berlin}$ were on average straight and almost parallel to the longitudinal body axis (Fig. 4B). Each of the three leg pairs showed clear anti-phase swing activity during tripod coordination (Fig. 4Ai, highlighted area; Fig. 4C, magenta points for the sequence shown in Fig. 4Ai). Analogous to $wt^{CS}$, we found that the front legs of a tripod group initiated swing first, followed by the middle legs, which in turn were followed by the hindlegs (Fig. 4C, blue data points). Only during very slow walking sequences did tripod coordination become more variable and we also found intermittent tetrapod coordination (Fig. 4Aii, highlighted area); this was also reflected in the phase relationship, which started to deviate in a more pronounced way from the typical tripod pattern (Fig. 4C, green data points). These shifts to tetrapod coordination were, however, rare. The succession of swing onset on each body side was always directed

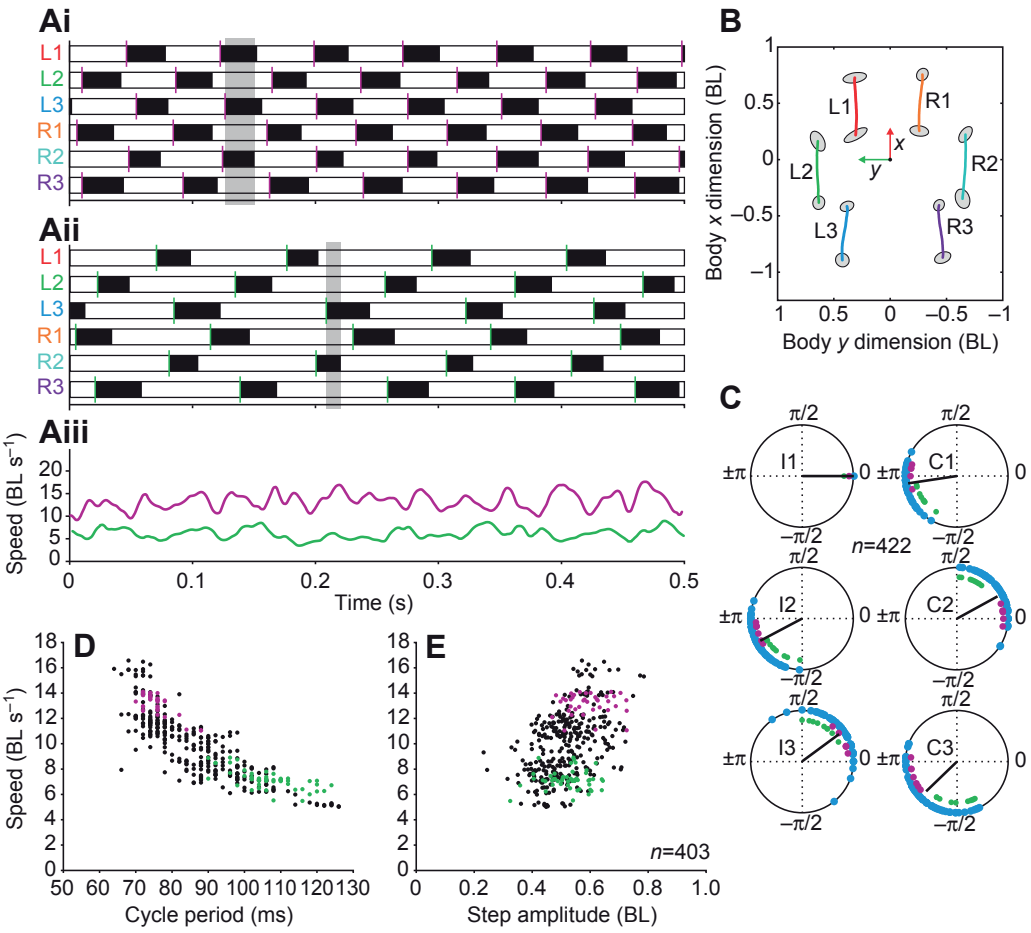


Fig. 4. Walking parameters of wild-type strain $wt^{Berlin}$. (Ai) Footfall pattern of all six legs during 0.5 s of one faster trial and (Aii) 0.5 s of one slower trial, and (Aiii) walking speed of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (R1, R2, R3: right front leg, middle leg and hindleg; L1, L2, L3: left front leg, middle leg and hindleg). Black bars indicate swing phase, white bars indicate stance phase; magenta lines indicate the onset and end of complete step cycles in the faster trial, green bars indicate those in the slower trial. Shaded areas highlight coordination patterns of interest (see Results). (B) Average stance trajectories of all legs of all trials in relative body coordinates. (C) Phase plots of swing onset of all legs with respect to the left front leg (I, ipsilateral; C, contralateral; blue: data from all trials; magenta: data from Ai; green: data from Aii; black line: mean vector – length indicates variance). (D) Cycle period as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii). (E) Step amplitude as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii).

anteriorly. Analogous to that in $wt^{CS}$, walking speed in $wt^{Berlin}$ was clearly correlated with cycle period (Fig. 4D), while it did not depend on step amplitude (Fig. 4E).

### Mutant strain $w^{1118}$

In the third set of experiments, we analyzed walking in $w^{1118}$ flies (Fig. 5). The total range of walking speeds in this strain was similar to that of $wt^{CS}$ and $wt^{Berlin}$ flies, with values ranging from 2 to 15 BL s$^{-1}$, i.e. from 4 to 31 mm s$^{-1}$ (5 individuals, 695 steps), as exemplified for a single trial in Fig. 5Ai. However, $w^{1118}$ flies walked at lower speeds more frequently. In general, speed appeared to be somewhat more variable within single walking sequences compared with that for $wt^{CS}$ and $wt^{Berlin}$ (cf. Fig. 2A, Fig. 4A, Fig. 5A). Stance trajectories were parallel to the longitudinal body axis for all three pairs of legs. On average, step amplitude was slightly shorter than 0.5 BL and was thus shorter than for the other two strains (Fig. 5B). Individuals of $w^{1118}$ often used tripod coordination (e.g. Fig. 5Ai, highlighted area), although the variability of inter-leg coordination seemed to be relatively high (blue points in Fig. 5C; 5 individuals, 713 steps). Nevertheless, according to our conservative definition, inter-leg coordination was still tripod on average (black lines in Fig. 5C). This variability can partially be attributed to the fact that at lower speeds animals no longer used tripod coordination but instead used tetrapod coordination (Fig. 5Aii, *) or even wave gait-like coordination (Fig. 5Aii, **). Similar to $wt^{CS}$ and $wt^{Berlin}$ flies, average swing phase onset of $w^{1118}$ posterior legs in a tripod group trailed front legs (Fig. 5C, magenta points for the trial in Fig. 5Ai, blue points for all data). Still, even in the slowest trial, the succession of swing phase onsets on a body side was directed anteriorly. The walking speed of $w^{1118}$ flies was strongly correlated with cycle period (Fig. 5D). We found only a weak correlation between walking speed and step amplitude (Fig. 5E, $R^2$=0.17).

### Mutant strain $w^{1118}$, $Tbh^{nM18}$

The octopaminergic neurotransmitter system has been implicated in the regulation of walking in stick insects, cockroaches and crabs. $w^{1118}$, $Tbh^{nM18}$ mutants lacking the enzyme tyramine β-hydroxylase necessary for the conversion of tyramine into octopamine have deficiencies in locomotor performance compared with wild-type flies (Brembs et al., 2007; Scholz, 2005). We found that this offered the chance to extend the range of movement speeds studied here to even lower values. For $w^{1118}$, $Tbh^{nM18}$ flies, movement speed ranged from 3 to 14 mm s$^{-1}$ (5 individuals, 681 steps), i.e. from 1.5 to 7 BL s$^{-1}$. $w^{1118}$, $Tbh^{nM18}$ flies only rarely walked at higher speeds, as exemplified for a single trial in Fig. 6Ai (see highlighted area for an instance of tripod coordination). Again, average stance trajectories were parallel to the longitudinal body axis and were slightly shorter than those in the $w^{1118}$ strain (Fig. 6B). However, average phase relationships of swing onset were no longer typical for tripod coordination: for example, phase values for R1, L2 and R3 relative to L1 were 175, 120 and 140 deg, respectively. Phase plots show a substantial variability of inter-leg coordination (Fig. 6C; magenta points for the sequence shown in Fig. 6Ai, blue points for all steps; 5 individuals, 713 steps). At low speeds (<5 BL s$^{-1}$), $w^{1118}$, $Tbh^{nM18}$ flies often used tetrapod coordination; during the slowest trials (2–3 BL s$^{-1}$), coordination resembled wave gait (Fig. 6Aii: see highlighted areas: *tetrapod; **wave gait-like coordination; Fig. 6C, green points). Analogous to the other strains examined here, the

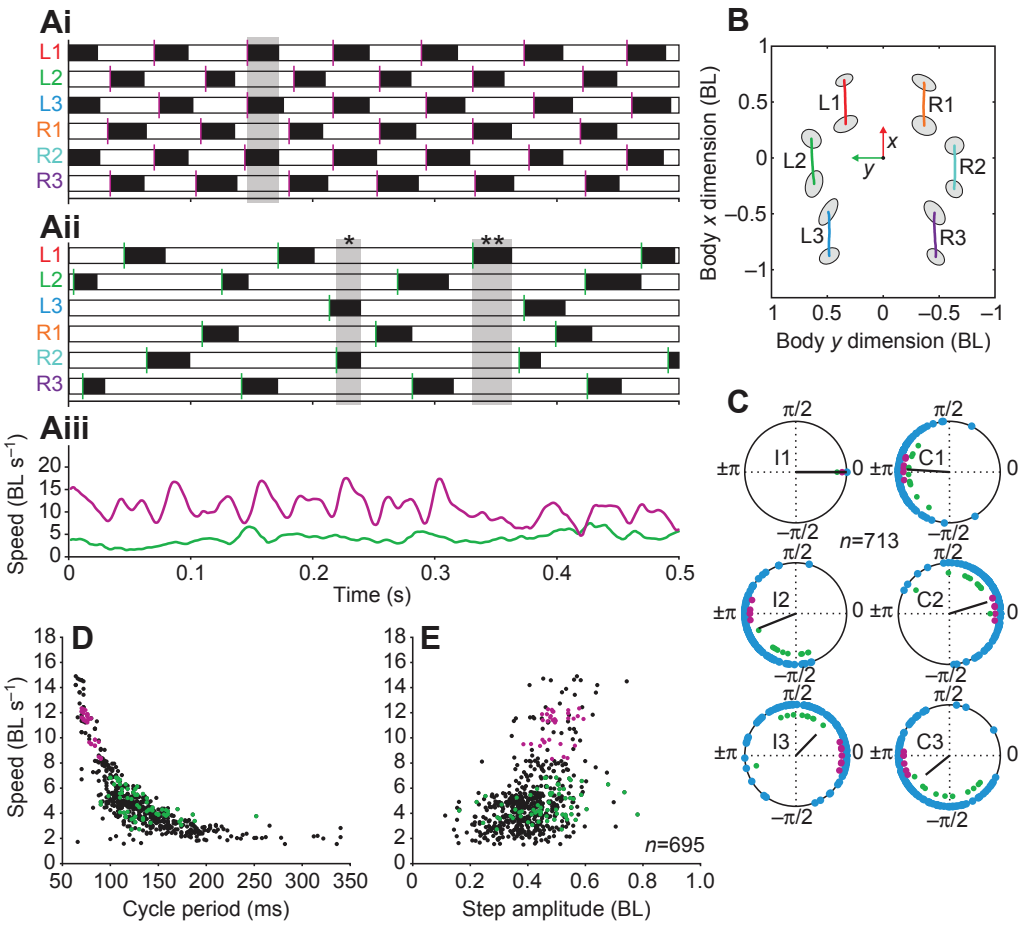


Fig. 5. Walking parameters of mutant strain $w^{1118}$. (Ai) Footfall pattern of all six legs during 0.5 s of one faster trial and (Aii) 0.5 s of one slower trial, and (iii) walking speed of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (R1, R2, R3: right front leg, middle leg and hindleg; L1, L2, L3: left front leg, middle leg and hindleg). Black bars indicate swing phase, white bars indicate stance phase; magenta lines indicate onset and end of complete step cycles in the faster trial, green bars indicate those in the slower trial. Shaded areas highlight coordination patterns of interest (see Results). (B) Average stance trajectories of all legs of all trials in relative body coordinates. (C) Phase plots of swing onset of all legs with respect to the left front leg (I, ipsilateral; C, contralateral; blue: data from all trials; magenta: data from Ai; green: data from Aii; black line: mean vector – length indicates variance). (D) Cycle period as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii). (E) Step amplitude as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii).

succession of swing onset on each body side was directed anteriorly. Only small deviations from this pattern could be observed during very slow trials (cf. third swing of R3 in Fig. 6Aii). Again, walking speed in $w^{1118}$, $Tbh^{nM18}$ was strongly correlated with cycle period (Fig. 6D). The correlation between walking speed and step amplitude was weak (Fig. 6E, $R^2$=0.15).

### Inter-leg coordination depends on movement speed

While all strains used tripod coordination during fast walking, at lower speeds inter-leg coordination became more variable or changed to other patterns such as tetrapod coordination. Based on this observation, we wanted to know whether inter-leg coordination depends systematically on walking speed. Therefore, we first determined the relative frequency of occurrence of tripod, tetrapod and undefined coordination in all four fly strains. We found that $wt^{CS}$ and $wt^{Berlin}$ flies almost exclusively used tripod coordination, while in $w^{1118}$ and $w^{1118}$, $Tbh^{nM18}$ flies tetrapod and undefined coordination patterns represented almost one-third of all patterns (Fig. 7A). When we pooled the data from all strains and plotted the relative frequency of occurrence of coordination types in three different speed ranges we found that tetrapod and undefined coordination patterns occurred almost exclusively at speeds below 5 BL s$^{-1}$ (Fig. 7B). Because we chose a rather conservative tripod definition, we frequently found this coordination type in all four strains. To further flesh out the relationship between tripod coordination and walking speed, we examined the TCS as a function of speed in all four strains (Fig. 7C–F). Fig. 7G shows five exemplary footfall patterns illustrating TCS ranging from 0.8 to 0.1. Generally, in all four strains TCS was variable, but depended systematically on movement speed. While we did not expect TCS to reach 1.0 because of the aforementioned phase lags within a tripod group, at speeds higher than 10 BL s$^{-1}$ it reached maximal values of up to 0.85 (see Fig. 7G). Below 10 BL s$^{-1}$,TCS ranged from 0.02 to 0.8. In general, at speeds higher than 10 BL s$^{-1}$, inter-leg coordination was tripod. Its variability increased noticeably towards lower speeds, as indicated by lower TCS values. In the range of low walking speeds (<10 BL s$^{-1}$), *Drosophila* seems to be able to also use tetrapod coordination or even wave gait.

### Inter-leg coordination changes after the loss of one hindleg

The results presented here suggest that *Drosophila*'s walking system does not generate a fixed motor output. Instead, it seems to be able to flexibly produce inter-leg coordination patterns that change in a systematic and gradual fashion with walking speed. At very slow walking speeds, *Drosophila* uses wave gait; with an increase in speed, inter-leg coordination then transitions to tetrapod and finally becomes tripod at the highest speeds. In order to further study the basis of this apparent flexibility, in a final set of experiments we examined walking in $wt^{CS}$ flies shortly after the removal of one hindleg (Fig. 8). The loss of a leg drastically changes the body geometry and if the animal wants to continue walking successfully it has to adapt its movement pattern to this new geometry. One necessary prerequisite for such an adaptation is that sensory information originating in the legs is taken into account by the neural system that generates walking behavior.

We observed five changes in the walking behavior of flies after the loss of one hindleg: (i) $wt^{CS}$ flies with a missing hindleg walked on average slower than intact animals of the same strain (Fig. 8A;

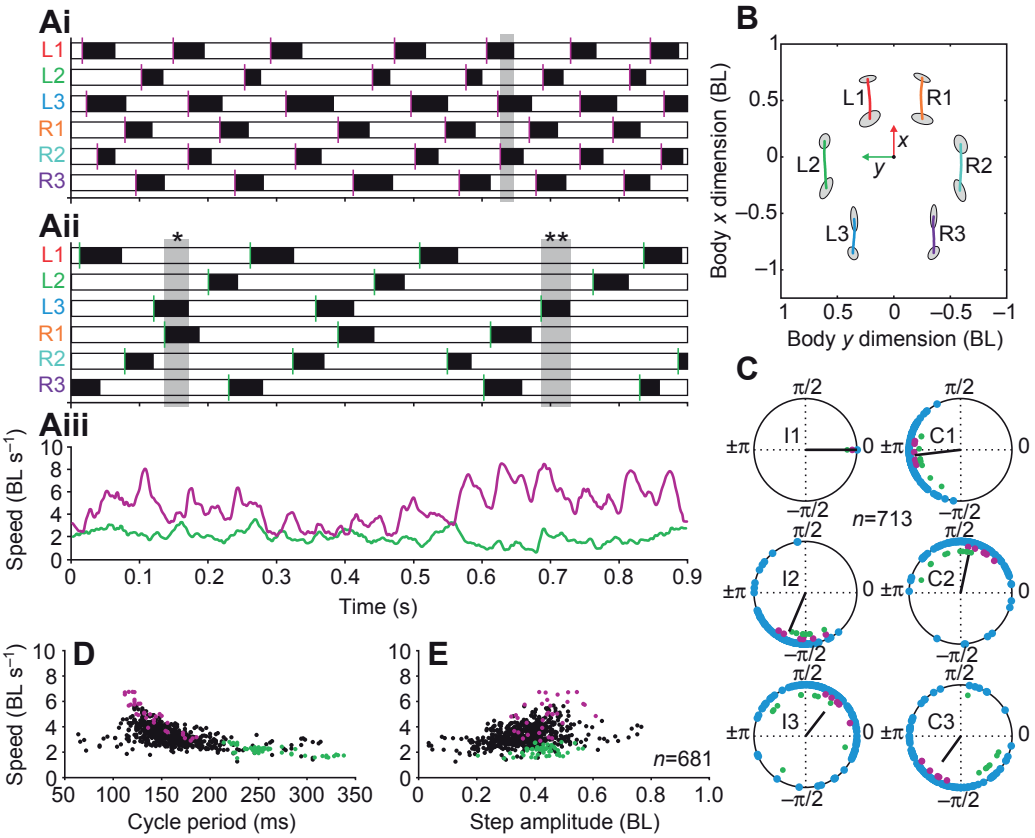


Fig. 6. Walking parameters of mutant strain $w^{1118}$, $Tbh^{nM18}$. (Ai) Footfall pattern of all six legs during 0.5 s of one faster trial and (Aii) 0.5 s of one slower trial, and (iii) walking speed of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (R1, R2, R3: right front leg, middle leg and hindleg; L1, L2, L3: left front leg, middle leg and hindleg). Black bars indicate swing phase, white bars indicate stance phase; magenta lines indicate onset and end of complete step cycles in the faster trial, green bars indicate those in the slower trial. Shaded areas highlight coordination patterns of interest (see Results). (B) Average stance trajectories of all legs of all trials in relative body coordinates. (C) Phase plots of swing onset of all legs with respect to the left front leg (blue: data from all trials; magenta: data from Ai; green: data from Aii; black line: mean vector – length indicates variance). (D) Cycle period as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii). (E) Step amplitude as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii).

cf. Fig. 2A). Average walking speeds ranged from 1 to 13 BL s$^{-1}$, which is equivalent to approximately 2–26 mm s$^{-1}$ (5 individuals, 664 steps), compared with a range of 4 to 18 BL s$^{-1}$ in intact animals. (ii) The shape of stance trajectories changed after amputation of one hindleg and showed distinct curvature. (iii) In all legs, AEPs and PEPs changed within the body coordinate system (Fig. 8B). Generally, we found an outward shift of AEPs and PEPs. In addition, especially in the remaining middle legs and hindlegs, these positions were also shifted caudally. (iv) The average stance trajectories of the remaining hindleg and of both middle legs became noticeably longer. Stance trajectory length increased in the remaining hindleg from 0.43 to 0.47 BL, and in the middle leg contralateral to the lesion from 0.50 to 0.53 BL. The most noticeable increase was found in the middle leg ipsilateral to the lesion. Here, average stance trajectory length increased from 0.50 to 0.60 BL. (v) Phase relationships of both the contralateral middle leg and the remaining hindleg were altered. The hindleg contralateral to the lesion (leg I3) was, on average, no longer in phase with the ipsilateral middle leg (C2); it increased its phase with regard to I3 to 0.85 rad (Fig. 8C) as compared with the intact animal in which the phase of C2 with regard to I3 was 0.16 on average (Fig. 2C). Furthermore, the contralateral middle leg showed an increase in phase with regard to the contralateral front leg (Fig. 8C; cf. Fig. 2C). As a consequence, generally three to four legs were simultaneously on the ground. Slow-walking individuals used either tetrapod or wave gait coordination (Fig. 8Aii). The correlation between walking speed and cycle period was still present though, and step amplitude was not correlated with speed (Fig. 8D,E).

## DISCUSSION

We have shown that the walking system of *Drosophila* is able to generate a broad range of locomotion speeds and different strains walked at preferred parts of this complete range. $wt^{CS}$ flies tended to walk faster than both $wt^{Berlin}$ and $w^{1118}$ individuals. Mutant $w^{1118}$, $Tbh^{nM18}$ individuals walked at the lowest speeds. At high speeds, all individuals walked in tripod coordination. With decreasing walking speed, TCS decreased as well (Fig. 7C–F) and animals also used tetrapod coordination more frequently (Fig. 7B). Finally, at very low speeds, walking was often accomplished by simultaneous stance phases of five legs while only a single leg was in swing phase at a time. These findings imply that *Drosophila*'s walking behavior is more flexible than previously thought (Strauss and Heisenberg, 1990): there are no clearly separable gaits and, more specifically, the neural controller producing inter-leg coordination is not restricted to a fixed tripod pattern.

This notion is substantiated by amputation experiments, in which we examined the walking behavior of animals after the loss of one hindleg. These experiments were carried out with individuals of $wt^{CS}$, which is the strain that showed the most robust tripod coordination when intact. Removal of a hindleg in these flies resulted in an immediate reorganization of overall posture, single leg kinematics and inter-leg coordination: the legs of the animals were positioned in a broader frame, the stance trajectories of the remaining middle legs and hindlegs were elongated while the phase of these legs was increased.

### Changes in inter-leg coordination related to walking speed

In the first part of the present study we analyzed walking in the *Drosophila* strains $wt^{CS}$ and $wt^{Berlin}$ as well as the mutant strains $w^{1118}$ and $w^{1118}$, $Tbh^{nM18}$ with respect to single leg kinematics and inter-leg coordination. Walking speed differed noticeably between strains, with that of $wt^{CS}$ and $wt^{Berlin}$ ranging from 5 to 16 BL s$^{-1}$ (11–32 mm s$^{-1}$), $w^{1118}$ speed ranging from 2 to 15 BL s$^{-1}$ (3.5–31 mm s$^{-1}$), and $w^{1118}$, $Tbh^{nM18}$ speed ranging from 1.5 to

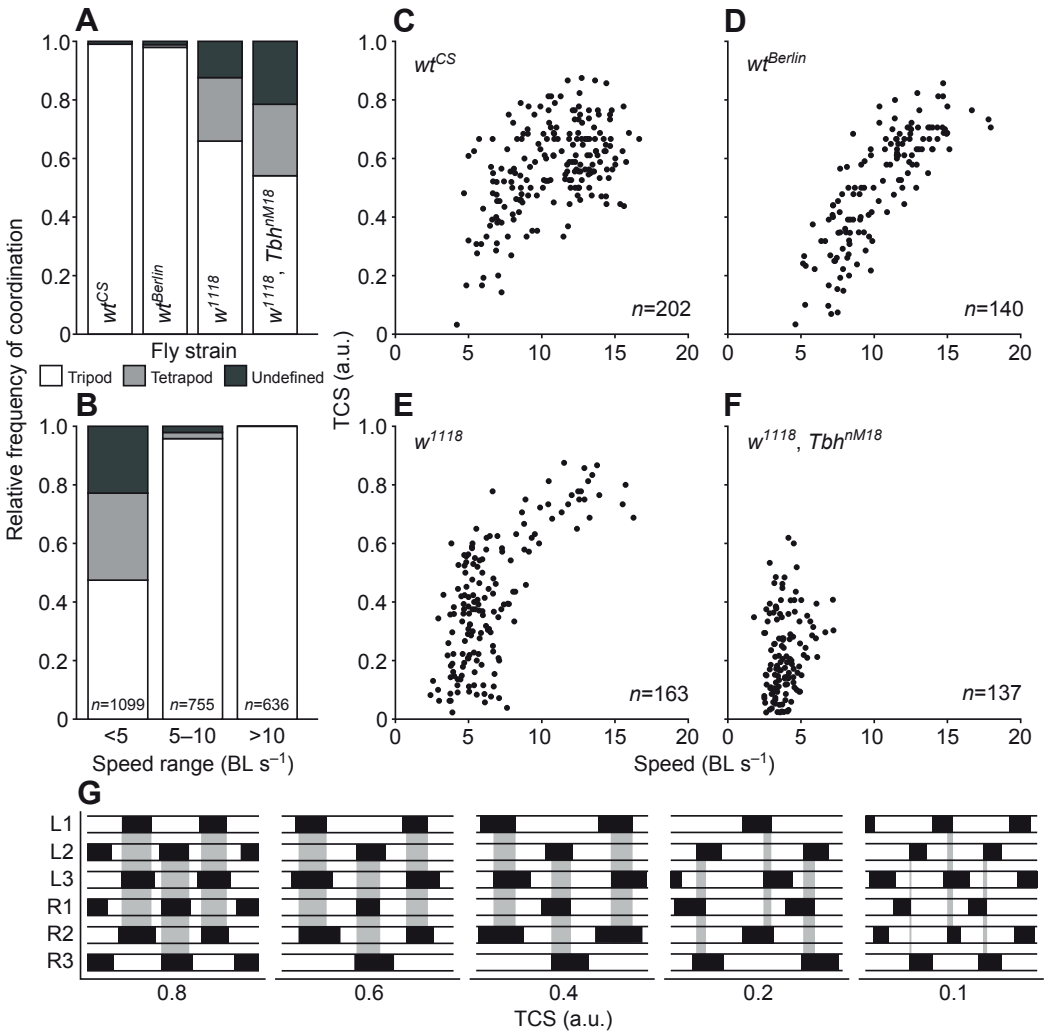


Fig. 7. Analysis of inter-leg coordination. (A) Relative frequency of tripod, tetrapod and undefined coordination in the four different strains (for definition of coordination types see Materials and methods). (B) Relative frequency of tripod, tetrapod and undefined coordination at slow (<5 BL $s^{-1}$), medium (5–10 BL $s^{-1}$) and high walking speeds (>10 BL $s^{-1}$). (C–F) Tripod coordination strength (TCS, for definition see Materials and methods) as a function of walking speed for the different strains: C, $wt^{CS}$; D, $wt^{Berlin}$; E, $w^{1118}$; and F, $w^{1118}$, $Tbh^{nM18}$. (G) Five exemplary footfall patterns with a TCS of 0.8, 0.6, 0.4, 0.2 and 0.1 taken from footfall patterns of five different flies. Shaded areas highlight the concurrent overlap of swing phases in the legs of one tripod group.

7 BL $s^{-1}$ (3–14 mm $s^{-1}$). For the strains $wt^{CS}$ and $wt^{Berlin}$, the reported average walking speeds in the literature range from 2.2 and 2–3 mm $s^{-1}$ (Serway et al., 2009) to 15 and 21 mm $s^{-1}$ (Poeck et al., 2008; Strauss and Heisenberg, 1993), respectively. Average walking speed for $w^{1118}$ was reported to be approximately 2 mm $s^{-1}$ and for $w^{1118}$, $Tbh^{nM18}$ it was 4 mm $s^{-1}$ (Scholz, 2005). More detailed data concerning the range of walking speeds are only available for the strain $wt^{Berlin}$, for which speeds of 12–40 mm $s^{-1}$ were found (Strauss and Heisenberg, 1990). These values correspond with our data in which we found only slightly lower speeds for $wt^{Berlin}$ (11–34 mm $s^{-1}$). It should be noted, though, that we used a different behavioral protocol from that in previous studies. Some of these used Buridan's paradigm (Bülthoff et al., 1982; Götz, 1980) to elicit straight walks on level ground (Poeck et al., 2008; Serway et al., 2009; Strauss and Heisenberg, 1990; Strauss and Heisenberg, 1993), while others studied walking in *Drosophila* under ambient light conditions without the presentation of visual cues (Scholz, 2005; Wolf et al., 2002).

For all strains examined here, we found that walking speed is controlled *via* changes in step cycle period and stance duration. Over the complete range of walking speeds we found only moderate changes with regard to swing duration, and no systematic modification of step amplitude could be detected. This complements and extends a previous study in which *Drosophila* altered not only its cycle period but also its stride length over the range of walking speeds (Strauss and Heisenberg, 1990). These authors, however, examined stride length, while the present study focused on step amplitude (see also Materials and methods). The findings presented here do not contradict the previous ones; here, however, we wanted to dissociate the effect body translation during swing phases has on stride length from actual adaptations in leg kinematics during a step cycle. As a consequence, our findings indicate that *Drosophila* controls walking speed solely by adjusting step cycle period while it keeps step amplitude mostly constant.

Strauss and Heisenberg reported that *Drosophila* uses tripod coordination for a large part of the observed speed range (Strauss and Heisenberg, 1990). They found tetrapod coordination only during 'deceleration episodes prior to turns or to a complete stop'. In general, we can confirm these findings. However, in the present study $wt^{Berlin}$ flies also spontaneously generated relatively slow walking bouts. In these trials we found that inter-leg coordination deviated from a strong tripod pattern, as indicated by low TCS values. Comparing this result with the data for $wt^{CS}$ and $w^{1118}$ revealed that this change in coordination is indeed systematically found when *Drosophila* walks more slowly. At walking speeds higher than 10 BL $s^{-1}$, inter-leg coordination was always tripod. At lower speeds, TCS decreased and within this speed domain we also observed tetrapod coordination. This analysis suggests that the kinematics of the movement pattern generally change systematically and continuously with walking speed.

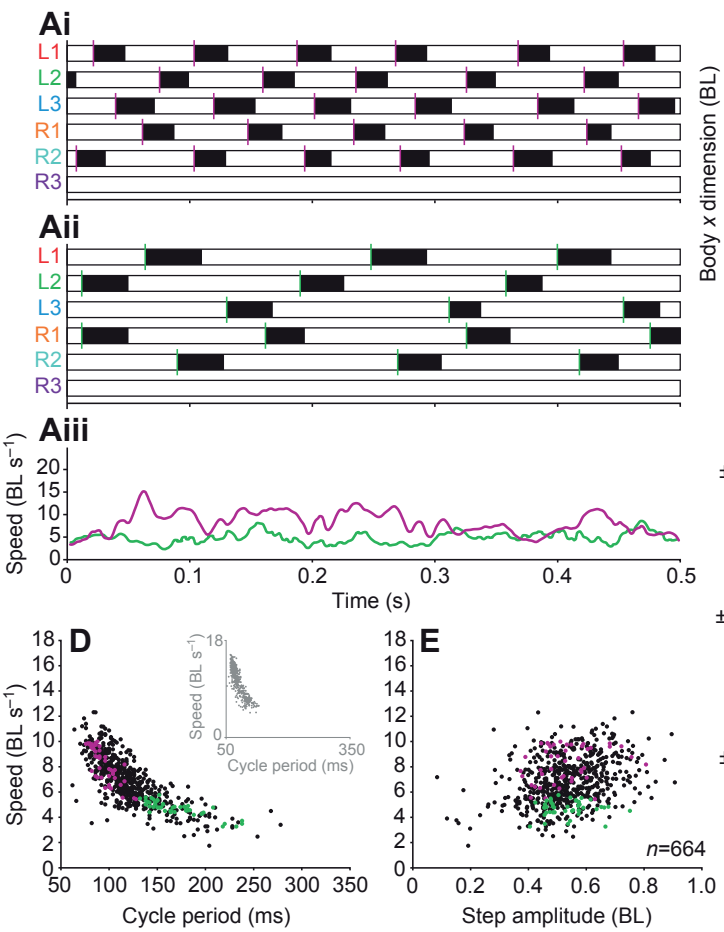


Fig. 8. Walking parameters of $wt^{CS}$ after removal of one hindleg. (Ai) Footfall pattern of all six legs during 0.5 s of one faster trial and (Aii) 0.5 s of one slower trial, and (Aiii) walking speed of the body during the 0.5 s of the trials shown in Ai (magenta graph) and Aii (green graph) (R1, R2, R3: right front leg, middle leg and hindleg; L1, L2, L3: left front leg, middle leg and hindleg). Black bars indicate swing phase, white bars indicate stance phase; magenta lines indicate onset and end of complete step cycles in the faster trial, green bars indicate those in the slower trial. (B) Average stance trajectories of all legs of all trials in relative body coordinates. Black arrows indicate shifts of the anterior extreme position (AEP) and posterior extreme position (PEP) (cf. Fig. 2B). (C) Phase plots of swing onset of all legs with respect to the left front leg (blue: data from all trials; magenta: data from Ai; green: data from Aii; black line: mean vector – length indicates variance). (D) Cycle period as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii). For comparison, gray inset shows rescaled data from Fig. 3B. (E) Step amplitude as a function of walking speed (black: data from all trials; magenta: data from Ai; green: data from Aii).

It is important to emphasize what a decrease in TCS means with regard to inter-leg coordination: a TCS of 0.5 means that the swing phases of the legs associated with a tripod group overlap 50% of the time during which any of these legs move. For a TCS of 0.4 this decreases to 40%; however, this also means that for 60% of the time these legs are not in swing phase simultaneously. In other words, during this time four or five legs are on the ground. This time only increases with a further decrease in TCS. Consequently, although low TCS levels still indicate tripod coordination (according to our conservative definition), swing phase overlap in these cases might be more consistent with coordination patterns that conventionally have been associated with tetrapod coordination. In addition, examination of the two mutant strains $w^{1118}$ and $w^{1118}$; $Tbh^{nM18}$ shows that at very low walking speeds *Drosophila* no longer uses tetrapod coordination and instead coordinates its legs in a pattern that resembles wave gait, a pattern first described for larger insects (Hughes, 1952; Wilson, 1966).

Interestingly, it appears that inter-leg coordination in *Drosophila* reflects all possible coordination patterns known in insects. Studies on inter-leg coordination in other, much larger insects, including cockroaches and beetles (Hughes, 1952), or grasshoppers (Burns, 1973), showed that inter-leg coordination is tripod only at high walking speeds and short cycle periods. At lower speeds, inter-leg coordination becomes increasingly variable, including tetrapod walking patterns. However, in these studies the examined species often differed noticeably in size and mass. Burns, for instance, studied two orthopteran species, locusts and grasshoppers, which differed in size by a factor of two (Burns, 1973). With respect to a systematic analysis of inter-leg coordination and walking speed, previous insights were derived from studies on ants (Zollikofer, 1994), cockroaches (Delcomyn, 1971) and stick insects (Graham, 1985; Wendler, 1966). Freely walking ants predominantly use tripod coordination in a speed range between 5 and 32 BL s$^{-1}$; no data, however, are available for slower walking speeds. Unrestrained cockroaches walk at speeds in the range 1–20 BL s$^{-1}$ (Bender et al., 2011; Delcomyn, 1971) and it has been reported that tripod coordination is present across a broad range of speeds, i.e. above 1.2 BL s$^{-1}$. However, inter-leg coordination in cockroaches becomes more variable with slower speeds. Delcomyn (Delcomyn, 1971) used the term ‘uncoupled alternating triangle’ for the increasing variability in tripod coordination occurring at slow speeds (Kozacik, 1981). Bender and co-workers (Bender et al., 2011) also reported clear changes in inter-leg coordination related to walking speed. They proposed the term ambling gait for inter-leg coordination that is found during slow walking. It is important to note that although cockroaches tend to move the legs of a tripod group simultaneously at low speeds, the coordination pattern becomes much more variable and there does not seem to be a fixed coupling anymore. When adult stick insects walk on a level surface they mostly do so at speeds well below 1 BL s$^{-1}$; in this situation, their preferred inter-leg coordination is tetrapod (Cruse et al., 2009; Graham, 1972). At higher speeds, sequences of tripod coordination can also be observed (Graham, 1972). Our results on *Drosophila* show two things: firstly, as has been found in the stick insect, inter-leg coordination in *Drosophila* is not fixed, but changes systematically and gradually as a function of walking speed over a broad speed range; secondly, below walking speeds of 5–6 BL s$^{-1}$, *Drosophila* seems to be able to choose which coordination type it uses and can walk in tripod, tetrapod or even wave gait-like inter-leg coordination. Importantly, we found that

swing duration was uncorrelated with walking speed. These findings have implications for the organization of the neural structure controlling walking in *Drosophila*: there is no justification for the hypothesis that there is a specific neural tripod generator in *Drosophila*.

This conclusion is corroborated by the changes observed in inter-leg coordination following the loss of one hindleg in $wt^{CS}$, which is the strain that had the most robust tripod coordination pattern. We found that inter-leg coordination as well as stance kinematics changed after the loss of one hindleg (Fig. 8). In the present study, compensatory changes were observed on two different levels: temporal and kinematic. With regard to temporal coordination, the stepping activity of the remaining legs, specifically the contralateral middle leg and hindleg, was modified such that the absence of support from the missing hindleg was compensated for. Swing phase activity in the contralateral hindleg and middle leg was delayed compared with that in an intact animal. Kinematic changes entail an extended stance trajectory in the ipsilateral middle leg and a general outward shift of AEPs and PEPs, i.e. overall, the animal adopts a broader posture. In particular, this outward shift suggests an overall compensatory modification of body posture. In addition, the extended stance trajectory of the ipsilateral middle leg nicely corresponds to Cruse's coordination rule 1 (Cruse et al., 1998; Dürr et al., 2004). This rule ensures that a leg in swing phase inhibits the transition to swing phase in an anterior neighbor. As the amputated leg in the present study can be interpreted as being locked in swing phase, this would explain the extended stance phase in the ipsilateral middle leg. These findings are interesting as they provide evidence for cooperative interactions (neural and mechanical) between the legs in the generation of propulsion and posture. Similar changes in inter-leg coordination after the loss of one leg have been reported for stick insects (Bässler, 1972; Graham, 1977) and cockroaches (Delcomyn, 1991; Hughes, 1957). Hughes (Hughes, 1957), for instance, found that upon removal of one hindleg in cockroaches, the other legs had extended stance trajectories and the stance trajectories were shifted outward. Our results also parallel findings reported by Delcomyn (Delcomyn, 1991), who showed that inter-leg coordination during walking became more variable after the loss of one hindleg (compare Fig. 2C with Fig. 8C). We note, however, that the postural adaptations we observed, especially the broader placement of the tarsi, might at least in part be due to a relative increase in load, a consequence of the loss of muscle force available to the animal.

Based on the changes in inter-leg coordination with regard to walking speed and upon removal of one leg, we conclude that the neural control system for walking in *Drosophila* allows for a modular control of single-leg stepping in which individual legs are largely independent of each other and are only loosely coupled. We hypothesize that the neural control system for walking in *Drosophila* is similar to that in fast-walking insects, like ants and cockroaches, as well as to that found in insects like the stick insect. The behavior of *Drosophila* agrees well with that found in stick insects (see Introduction). Behavioral studies in stick insects suggest that inter-leg coordination is the result of the interplay of individual leg controllers based on specific rules (Cruse, 1990; Dürr et al., 2004). Although not (yet) studied in *Drosophila*, it is quite conceivable that the 'coordination rules 1–3', as proposed by Cruse (Dürr et al., 2004), would suffice to generate the walking behavior observed here. However, the fact that the output of any locomotor system is shaped by the complex interaction between neural and mechanical influences needs to be taken into account. In order to further substantiate how *Drosophila*'s walking system compares to that of other insects it will be necessary to distinguish between the level of neural control and the level of mechanical coupling. Experimental paradigms for insect locomotion are available that allow for this dissection, e.g. slippery surfaces that reduce or even remove mechanical coupling between the legs (Graham and Cruse, 1981; Gruhn et al., 2006).

Another interesting aspect of the present study is the results for the two mutant strains $w^{1118}$ and $w^{1118}$, $Tbh^{nM18}$. Both of these strains exhibited walking speeds that were lower than those of the two wild-type strains, a fact that allowed us to extend the range of speeds that we investigated. Walking speed in $w^{1118}$, $Tbh^{nM18}$ was lower than that in $w^{1118}$. It is quite conceivable that $w^{1118}$ flies walk slower because of visual impairment (Kalmus, 1943). The even lower speed range used by $w^{1118}$, $Tbh^{nM18}$ can likely be attributed to the fact that $w^{1118}$, $Tbh^{nM18}$ lacks octopamine (Monastirioti et al., 1996), a biogenic amine that plays an important role during various locomotor behaviors in invertebrates. It is known to influence the initiation and maintenance of flight (Brembs et al., 2007) and pre-flight jumps in *Drosophila* (Zumstein et al., 2004), and is also implicated as a modulator of walking behavior in cockroaches, for example (Gal and Libersat, 2008; Gal and Libersat, 2010). Interestingly, in all of these studies octopamine appears to selectively influence high-level aspects of locomotion, while more low-level aspects, such as leg kinematics, for instance, remain unaffected. Although the present study did not focus specifically on the effects of octopamine, our data support the findings of these previous works. Individuals of the $w^{1118}$, $Tbh^{nM18}$ strain walked noticeably slower and less frequently, while inter-leg coordination and kinematics seemed to be very similar to those of $w^{1118}$. It is important to note that these low octopamine levels might only explain reduced walking speed in $w^{1118}$, $Tbh^{nM18}$. While $w^{1118}$ also has reduced levels of other biogenic amines like dopamine and serotonin (Sitaraman et al., 2008), its octopamine levels are similar to wild-type or are only very slightly reduced (Sitaraman et al., 2008; Yarali et al., 2009). Modifying octopamine levels might be useful in future studies in order to specifically modulate the walking behavior in *Drosophila* mainly with regard to movement speed.

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## 4.4 Segment-specific and state-dependent targeting accuracy of the stick insect

Anne Wosnitza, Jennifer Engelen, Matthias Gruhn

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Für diese Publikation habe ich das Konzept des Projekts mit Matthias Gruhn zusammen entworfen. Ich habe die Hälfte der Versuche durchgeführt und alle Daten alleine ausgewertet. Ich habe die Ergebnisse mit Matthias Gruhn diskutiert und alle Abbildungen entworfen und erstellt. Ein Textvorschlag für das Manuskript wurde von mir erstellt und dann gemeinsam mit Matthias Gruhn zum endgültigen Manuskript fertiggestellt. Der geplante Termin für die Einreichung zur Publikation ist Ende Mai 2013.

# Segment-specific and state-dependent targeting accuracy of the stick insect

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*Running title:* targeting of stick insect legs

*Keywords:* walking, motor control, sensory feedback, intersegmental coordination

## ABSTRACT

In their natural habitat, stick insects (*Carausius morosus*) live in bushes and trees and climb on their branches. Previous work (e.g. Cruse 1979) suggested that stick insects perform targeting movements with their hind legs to find support more easily. Based on such behavioral experiments, it has been assumed that the animals use position information from posterior extreme positions of the middle legs to control the anterior extreme position of the ipsilateral hind legs. Here we address the question whether this targeting is also present in the middle legs towards the ipsilateral front legs. Furthermore we wanted to learn if targeting is still present when influences of mechanical coupling through the ground are removed. If targeting is present under these conditions, this would emphasize the role of underlying neuronal mechanisms. We used a slippery surface setup (Graham & Cruse, 1981; Gruhn et al. 2006) to provide a walking situation with strongly reduced mechanical influences between the legs. First, we studied whether targeting occurred in hind- or middle legs during walking on the slippery surface, when the rostral neighboring leg, i.e. either middle- or front leg, was placed at defined positions relative to the body. Targeting precision during the first step of a sequence of steps of the ipsilateral posterior leg was analyzed for dependency on the targeted position. Under these conditions, the touchdown positions of the hind legs show correlation parallel and perpendicular to the body axis. Between the front and middle legs, only weak correlation exists, and only in parallel to the body axis. Secondly, we looked for evidence of leg targeting in animals walking continuously on the slippery surface. Targeting accuracy of middle and hind legs parallel to the body axis was barely changed. However targeting became significantly more accurate perpendicular to the body axis. Our results suggest that a neural mechanism exists for controlling the anterior extreme position of the posterior leg but that the strength of this mechanism is segment-specific as well as dependent on the behavioral context in which it is used.

## INTRODUCTION

If terrestrial animals want to walk through any kind of environment, they need to know how to move their legs to reliably find foothold. This information becomes particularly relevant when navigating through an unknown or irregular terrain. For cats and humans it is known that targeting of leg movements is primarily mediated by visual information and this information for the correct placement is captured on average two steps ahead (cat: McVea & Pearson 2007, McVea et al. 2009, Wilkinson & Sherk 2005, human: Mohagheghi et al. 2004, Patla & Vickers 2003). Likewise Niven et al. (2010) could show that locusts visually target their front legs towards the position of a ladder rug and information about the position of the rug is acquired before leg swing is initiated. However, targeted leg movement not only implies that the control system has information about the environment, but also on the actual leg position. This information can be provided by several kinds of sense organs. Cats, for example, use information from muscle receptors and cutaneous receptors in the skin that matches sensory information from different joints to reliably represent the position of the limb relative to the body in the dorsal root ganglia (Stein et al. 2004). This information is also transferred to area 5 in the posterior parietal cortex to be integrated with memorized visual information to perform appropriate leg movements (McVea et al. 2009). From there information is transferred back to local networks in the spinal cord where the final motoneuron activity in generated (for review, see, e.g., Grillner & Jessell 2009, Kiehn et al. 2010).

However, how do animals find appropriate foothold when visual information is not available? In the same study as mentioned above, Niven et al. (2010) also observed that placement of the middle leg in locusts was not visually guided. Information about where to place the middle legs has therefore to be acquired differently. And in fact, from work on stick insects it is known that proprioceptive inputs of several sensory structures in the leg influence the protraction endpoint of all legs (Wendler 1964; Bässler 1977; Dean & Wendler 1983).

In its natural habitat, the stick insect *Carausius morosus* lives in a complex three dimensional maze of twigs and leaves, and hence has to constantly adapt its walking behavior. As a nocturnal animal *C. morosus* primarily relies on mechanosensory information from the antennae to guide its front legs towards an appropriate foothold and does not use vision for this purpose (Dürr 2001, Bläsing & Cruse 2004, Schütz & Dürr 2011). How it guides its hind legs towards an appropriate foothold has also been the focus of several earlier investigations (e.g. Cruse 1979, Cruse et al. 1984, Dean 1984 & 1989, Dean & Wendler 1983). In these it has been shown that the touchdown position of the hind leg, in particular, parallel and perpendicular to the body axis, depends on the position of the standing middle leg (Cruse

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1979). The sense organs that appear to be primarily responsible for targeting parallel to the body axis are hair rows and hair fields on the coxa (Cruse et al. 1984, Dean & Wendler 1983). Perpendicular to the body axis it seems to be primarily information from the femoral chordotonal organ that is needed for targeted movements of the hind leg (Cruse et al. 1984). Brunn and Dean (1994) described three interneurons, each signaling the angle of one single leg joint and hence together able to encode the tarsus position. Information about the position of the middle leg is transmitted via the ipsilateral connective (Dean 1989). However, it is still unclear how information from sense organs that detect angular positions and velocities of joints is incorporated into a reference frame for motor control. Aside from this, there are several other pieces of the picture that are still missing. For instance, it is not known how stick insects guide their middle legs towards an appropriate foothold, e.g. if they use position information from the front legs. In addition, many studies have shown that the behavioral state of the animal is important for the effectiveness of sensory input on the motoneurons (for review, see, e.g., Büschges & El Manira 1998, Clarac et al. 2000, Duysens et al. 2000, Pearson 1993) but it is not known to what extend movement of the anterior leg influences the targeting accuracy of the middle or hind leg and at which time point the information used for targeting is sampled. Furthermore, neglected in all the existing studies, has been the question to what extend targeting behavior might be a result of limb joint constraints or mechanical coupling via the ground or if it is an effect that actually arises only from properties of the neuronal system.

To understand how important neuronal mechanisms are for the orientation and spatial coordination of foot placement without visual guidance, we investigated the placement of middle and hind legs in the stick insect *Carausius morosus* in a slippery surface setup. By tethering the animal above a slippery surface we could reliably remove mechanical coupling of leg movements through the ground (Gruhn et al. 2006). If targeting is present under these conditions, this would emphasize the role of underlying neuronal mechanisms. We measured the targeting accuracy of the middle leg towards the front leg and the targeting accuracy of the hind leg towards the middle leg, and compared their performance with each other to find out if there were segment-specific differences. In addition, we analyzed targeting of both legs under two behavioral conditions to identify to which extend targeting accuracy is state-dependent: first, with the anterior leg standing on one of seven defined positions, similar to the experimental protocol of Cruse (1979), and second, during continuous walks.

# Materials and Methods

### ANIMALS

All experiments were performed on adult female stick insects (*Carausius morosus*). Animals were reared in the animal facility of the institute in a 12-h/12-h light/dark cycle at 23–25°C and were fed with blackberry leaves (*Rubus fructiosus*) *ad libitum*.

### EXPERIMENTAL SETUP

In all experiments, animals walked on a 13.5 x 13.5 cm polished nickel-coated brass plate. To allow unimpeded walking under tethered conditions and minimize mechanical coupling between the legs, the plate was covered with a lubricant composed of 95 % glycerin, 5 % saturated NaCl. The animal was glued ventral side down on a 3 x 5 x 100 mm [W x H x L] balsa rod using dental cement (ProTempII, ESPE, Seefeld, Germany) so the legs and head protruded from the rod and all joints were unrestrained. Animal height above the substrate was adjustable, but was typically set to 10 mm. Experiments were performed in a darkened Faraday cage at room temperature.

In the continuous walking sequences, walking was elicited by projecting a progressive striped pattern (pattern wave length 21 °) onto two 13.5² cm diameter round glass screens (Scharstein 1989) placed at right angles to each other and at a 45 ° angle to the walking surface, about 6-7 cm away from the eyes of the animal. Reflections on the polished brass plate further increased the field of view. Alternatively, a single white stripe on dark background (toward which the animals orient with straight walking sequences) was placed in front of the animal. If the animal did not begin locomotion spontaneously, walking was elicited by light brush strokes to the abdomen. In all sequences with the previously positioned, standing anterior leg, stepping of the posterior leg was also elicited by light brush strokes to the abdomen.

To move the anterior leg to a specific position, we used a small cardboard platform with a particularly rough surface. This small platform was attached to a brass tube which was connected to a micromanipulator. Exact positioning of the anterior leg was achieved by carefully placing the tarsus of the leg onto the small platform and then moving the platform to one of seven aiming positions. The location of these positions was defined by the central position (No. 5). This central position is directly underneath the femur-tibia joint when the tibia is perpendicular to the surface and the femur is perpendicular to the body. The other six tested positions were arranged around position No. 5 as following: positions 1 and 2 are 5 mm farther posterior while positions 8 and 9 are 5mm farther anterior. Positions 1 and 4 are 5 mm farther proximal while positions 6 and 9 are 5 mm farther lateral. The standing position of the anterior leg was randomly changed to a different position after each step of the posterior leg.

### OPTICAL RECORDING AND DIGITAL ANALYSIS OF LEG MOVEMENTS

Optical recordings were performed and analyzed as in Gruhn et al. (2009a). In brief, we recorded walking sequences with a high-speed video camera (Marlin F-033C; Allied Visions Technologies, Stadtroda, Germany) that was externally triggered at 100 fps. Insect head, thorax, and legs were marked with fluorescent pigments (Dr. Kremer Farbmühle, Aichstetten, Germany) mixed with dental cement. During the recording of walking sequences, the animal was illuminated with blue light-emitting diode arrays (12 V AC/DC; Conrad Electronic, Berlin). The video files were analyzed using motion-tracking software (WINanalyze 1.9; Mikromak Service, Berlin). AEP describes the

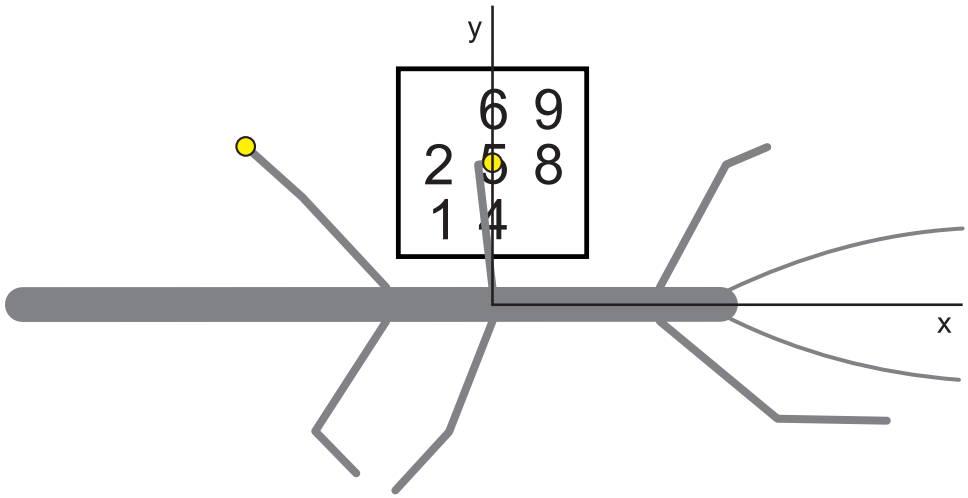


***Figure 1:*** *Schematic drawing of the stick insect with coordinate system and marked standing positions of the front leg.*

anterior extreme position of the leg at touchdown, whereas PEP is the posterior extreme position at liftoff. Position values are always given in millimeters in the form xx.x; yy.y (SDx; SDy). A virtual 0 line was drawn across the animal at the level of the coxa of the anterior leg. Positive and negative x-values indicate points anterior and posterior to this coxa, respectively; y-values are given with respect to the axis perpendicular to the length of the animal. Larger y-values denote more distal points, smaller values more central points. Figure 1 shows a schematic drawing of the stick insect with the tracked reference points for the analysis of leg kinematics marked as yellow dots and the standing positions of the anterior leg. All steps were transposed to reflect walking as a left leg regardless of which leg was being recorded.

## DATA ANALYSIS AND FIGURE PREPARATION

Leg positions were measured with their x and y coordinates in mm. Care was taken to choose animals of the same size and leg lengths. The number of animals used for a given condition (N) and the number of steps evaluated (n) are given in the figures. The sample size for the kinematic analysis of continuous walks was N = 8, for the standing front leg or middle leg it was N = 6, respectively.

For statistical analyses, Mann-Whitney U test, Hotellings $T^2$ test and Pearson's correlation test were used (Matlab, Statistics toolbox; The MathWorks, Natick, MA). Statistical significance was assumed at values of $P < 0.01$.

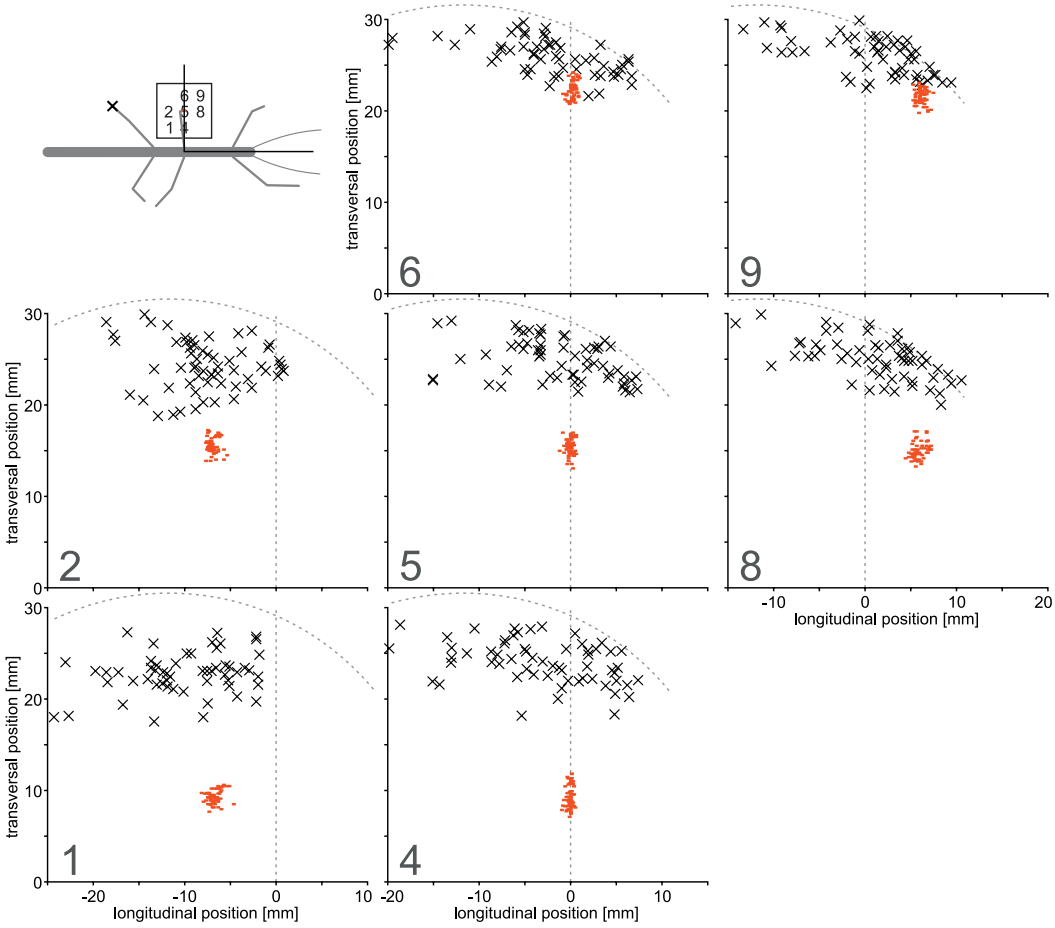


***Figure 2:*** *Standing positions of the middle leg (red dots) and touchdown positions of the stepping hind leg (black crosses) on the slippery surface. Each sub-plot shows data belonging to one of the seven standing positions of the middle leg. The vertical dotted line marks the position of the middle leg coxa which is located at zero on the x-axis. The dotted half circle depicts the calculated average maximum range of fully stretched hind legs.*

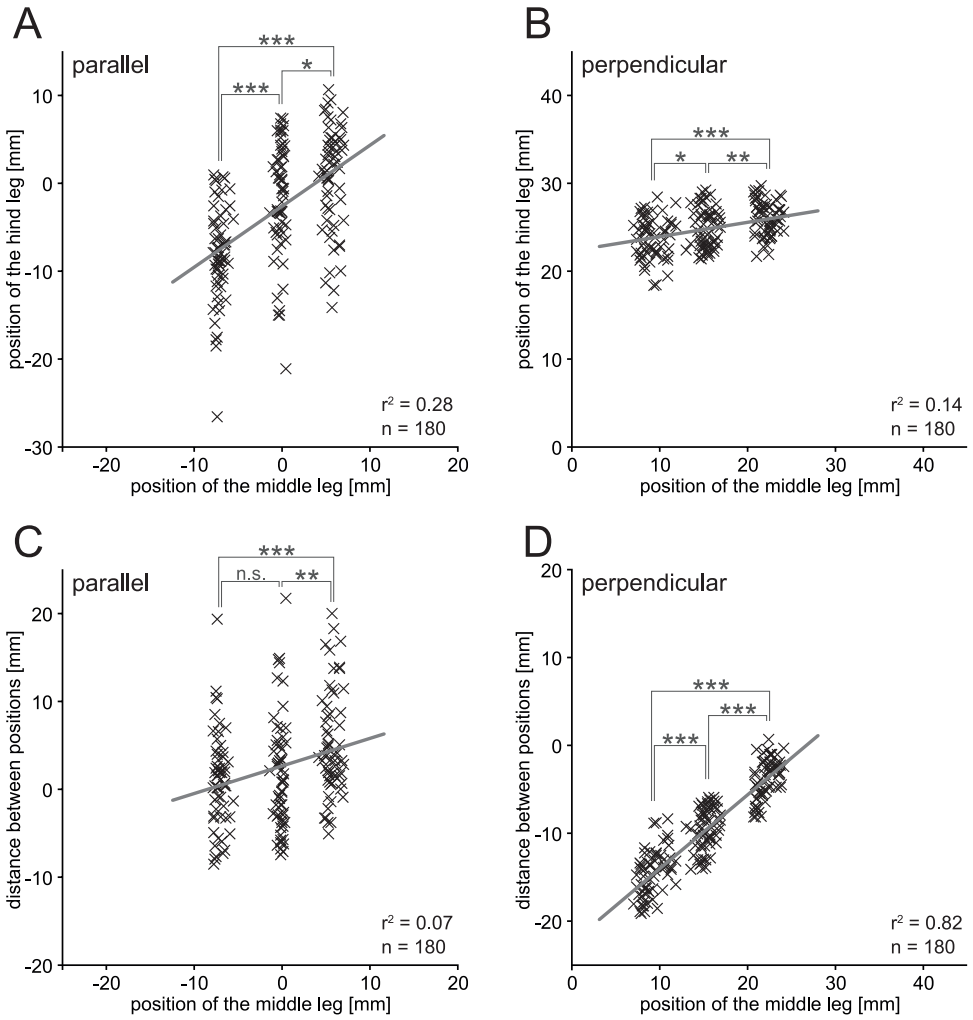


***Figure 3:*** *Scatterplot of the middle leg standing positions against the touchdown positions (**A** and **B**) and distances (**C** and **D**) of the ipsilateral hind leg. Separated into the component parallel (**A** and **C**) and perpendicular (**B** and **D**) to the body axis. Also linear correlation and test upon significant differences between the groups of data using the Man-Whitney-U-test. Plotted are pairs of data that belong to middle leg standing positions which only differ in the considered coordinate. In figure **A** and **C** these are positions two, five, and eight. In **B** and **D** these are positions four, five and six.*

# Results

## TARGETING ACCURACY OF THE HIND LEG TOWARDS THE MIDDLE LEG

First, we analyzed whether the hind legs of Carausius target the position of the ipsilateral middle leg during walking, when the mechanical coupling through the ground is reduced. We tethered the animals above a slippery surface and placed one middle leg onto one of seven pre-defined standing positions. Each position was used ten times in a randomized succession. Walking of the animal was initiated by a brush stroke to the abdomen, and the position of touchdown of the first step by the hind leg was recorded. The middle leg had to keep the defined position until the hind leg had finished its swing phase and touched the ground again. Sequences in which the middle leg moved before this moment were not evaluated.

The plot with the positions of the standing middle leg and the respective touchdown position of the stepping ipsilateral hind leg (Fig. 2) shows that all seven positions of the middle leg are within reach of the hind leg (dotted half circle depicts calculated average maximum range of fully stretched hind legs). The touchdown position of the hind leg was often anterior to the position of the middle leg coxa (vertical dotted line). Only when the middle leg was standing at positions one or two, did the hind leg rarely touch the ground anteriorly to the middle leg coxa. This could have been due to mechanical constrains through the standing middle leg blocking hind leg movement. Taking the position of the middle leg coxa (dotted vertical line) as a reference, it becomes apparent that the touchdown positions of the hind leg were more anterior for farther anterior standing position of the middle leg. The same was true for the distribution perpendicular to the body axis. When the middle leg was standing more laterally, the touchdown position of the hind leg was on average also more laterally.

To test these qualitative observations, we first tested if the target (middle leg) and the touchdown (hind leg) positions, either parallel (Fig. 3A) or perpendicular to the body axis (Fig. 3B) were significantly different from one another using the Man-Whitney-U-test. Significant differences between the three groups of data are one prerequisite for linear correlation. We then looked for linear correlation between the data groups. We performed a pair wise analysis of data that belonged to middle leg standing positions that only differed along one of the two axes. In both cases, we tested 180 pairs of positions each.

Although the distribution of the touchdown positions for the three middle leg positions along the body axis (two, five, and eight) was relatively big, they were nevertheless significantly different from one another (Fig. 3A; $p_{P2\text{-}P5} < 0.0001$; $p_{P5\text{-}P8} = 0.0255$; $p_{P2\text{-}P8} < 0.0001$). To identify a linear correlation parallel

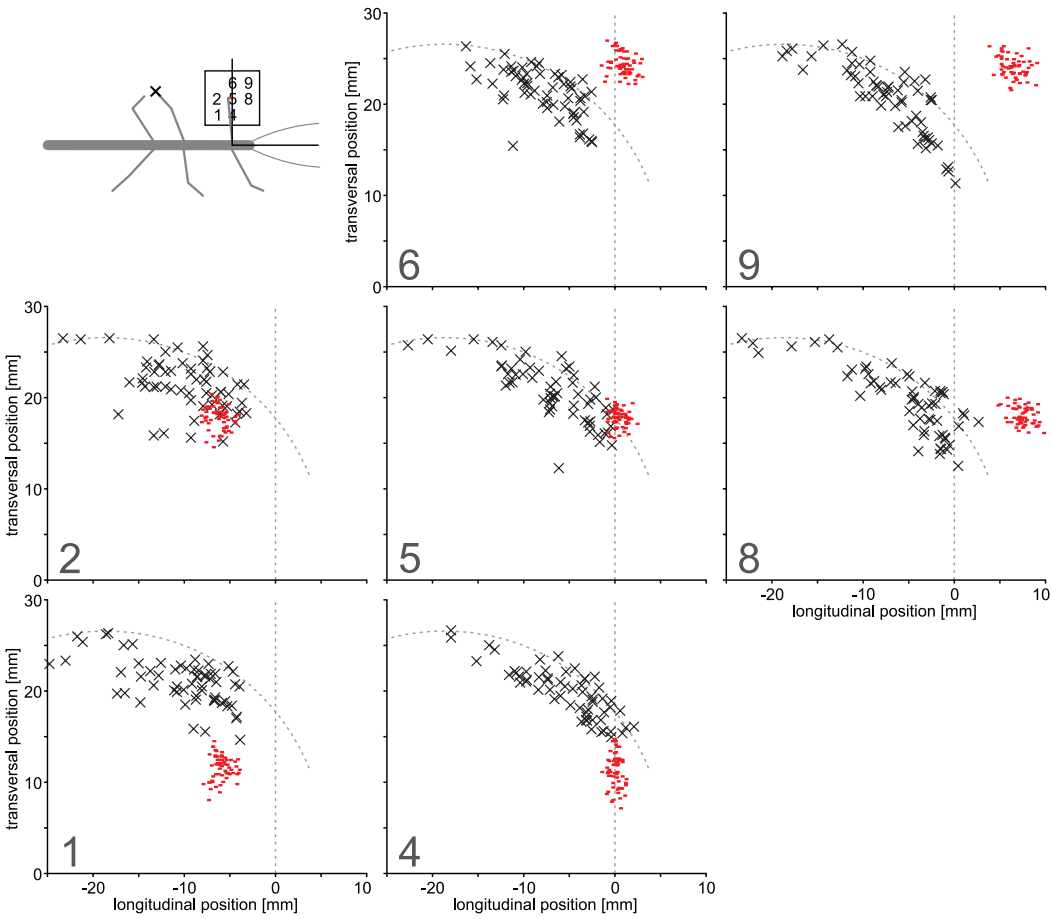


***Figure 4:*** *Standing positions of the front leg (red dots) and touchdown positions of the middle leg (black crosses) on the slippery surface. Each sub-plot shows data from one of the seven positions of the standing front leg. The vertical dotted line marks the level of the front leg coxa which is located at zero on the x-axis. The dotted half circle depicts the calculated average maximum reach of fully stretched middle legs.*

to the body axis we used data that belonged to these standing positions. With a coefficient of determination of $r^2_x = 0.28$, a linear correlation parallel to the body axis can be assumed. On average, the x-coordinate of the touchdown position of the hind leg increased with increasing x-coordinate of the standing middle leg ($X_{P2}$ = -7.99mm ± 5.34; $X_{P5}$ = -1.78mm ± 6.29; $X_{P8}$ = 0.72mm ± 5.84). To test for a possible correlation perpendicular to the body axis, we used middle leg positions four, five, and six (Fig. 3B). Although the mean values of these three data groups did not differ much ($Y_{P4}$ = 23.76mm ± 2.31; $Y_{P5}$ = 24.89mm ± 2.23; $Y_{P6}$ = 26.00mm ± 1.91) they were still significantly different from each other ($p_{P4\text{-}P5}$ = 0.0154; $p_{P5\text{-}P6}$ = 0.0058; $p_{P4\text{-}P6}$ < 0.0001) as a result of their small variability. The linear correlation along this axis was smaller but still present ($r^2_y = 0.14$).

We also calculated the distances between the standing position of the middle leg and the touchdown position of the hind leg parallel (Fig. 3C) and perpendicular to the body axis (Fig. 3D). These values were plotted against the standing position of the middle leg. We calculated the mean values of the groups, tested for significant differences between the groups and for linear correlation. The comparison of these values helps to estimate the targeting accuracy of the hind leg. If, on average, the distance between middle and hind leg stayed the same or were not significantly different for differing middle leg standing positions, one could assume targeting by the hind leg. Should instead the distance between the two positions become systematically bigger with a more anteriorly or distally standing middle leg, respectively, this would indicate weak or no targeting by the hind leg. On average, the distances parallel to the body axis between middle leg standing position and hind leg touchdown position increased only slightly between positions two to five ($X\text{-}dist_{P2}$ = 0.94mm ± 5.25; $X\text{-}dist_{P5}$ = 1.63mm ± 6.26). The distances between middle and hind leg at positions two and five were not significantly different to each other ($p_{P2\text{-}P5}$ = 0.6612), while the distance at position eight was significantly bigger than those at positions two and five ($X\text{-}dist_{P8}$ = 5.00mm ± 5.89; $p_{P5\text{-}P8}$ = 0.0024; $p_{P2\text{-}P8}$ = 0.0002). There was almost no correlation between the standing positions of the middle leg and the distances to the touch down position of the hind leg along the body axis ($r^2_x = 0.07$), again supporting targeting of the hind leg towards the standing position of the middle leg parallel to the body axis. The differences between the average distances between hind leg touchdown and the standing middle leg at the three considered standing positions perpendicular to the body axis, on the other hand grew bigger (Fig. 3D). From one standing position to the next, the average distance increased by about five millimeters each ($Y\text{-}dist_{P4}$ = -14.53mm ± 2.64; $Y\text{-}dist_{P5}$ = -9.53mm ± 2.40; $Y\text{-}dist_{P6}$ = -3.75mm ± 2.25) and the distances between the different positions were significantly different from one another ($p_{P4\text{-}P5}$ < 0.0001; $p_{P5\text{-}P6}$ < 0.0001; $p_{P4\text{-}P6}$ < 0.0001). Because of the small variability within the groups and the big systematic increase of the mean values, the linear correlation between these standing positions and the distances was strong ($r^2_y = 0.82$), which suggests no or only minor targeting of the hind leg towards the standing position of the middle leg perpendicular to the body axis.

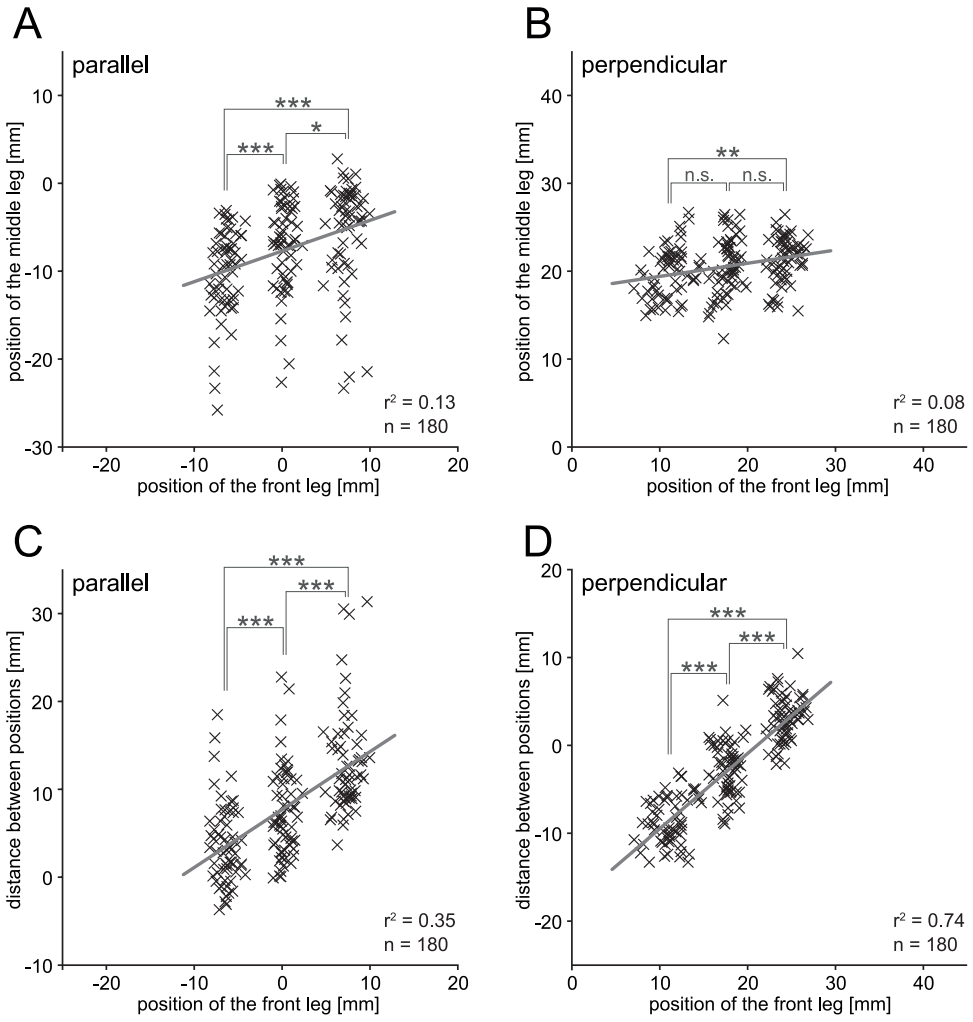


***Figure 5:*** *Scatter plot of the front leg standing positions against the touchdown positions of (**A** and **B**) and distances from (**C** and **D**) the ipsilateral middle leg. Separated into the components parallel (**A** and **C**) and perpendicular (**B** and **D**) to the body axis. Each panel also shows linear correlation and test upon significant differences between the groups of data using the Man-Whitney-U-test. Plotted are pairs of data that belong to front leg standing positions which only differ in the considered axis. In figure **A** and **C** these are positions two, five, and eight. In **B** and **D** these are positions four, five and six.*

## TARGETING ACCURACY OF THE MIDDLE LEG TOWARDS THE STANDING FRONT LEG

We performed the same experiments with the standing front and stepping middle leg to test the targeting accuracy of the middle leg towards the front leg. A plot of the seven different standing positions of the front leg and the respective touchdown positions of the stepping ipsilateral middle leg (Fig. 4) shows that the touchdown of the middle leg usually occurred close to its maximum reach (dotted semi circle). The middle leg only rarely had its touchdown anterior of the front leg coxa (vertical dotted line). The front leg positions six, eight, and nine were even outside the dotted semi circle, hence out of reach for the middle leg. To identify a potential systematic dependence between the touchdown position of the middle leg and position of the standing front leg, we plotted the positions against each other and tested for linear correlation parallel (Fig. 5A) and perpendicular to the body axis (Fig. 5B). For this purpose, we again used pairs of data from front leg positions that only differed along one of the two axes.

To identify a potential correlation parallel to the body axis we used 180 step pairs from the positions two, five, and eight of the standing front leg (Fig. 5A). The coefficient of determination ($r^2_x = 0.13$) indicates only a small linear correlation parallel to the body axis. On average the x-coordinate of the touchdown position increased with increasing x-coordinate of the standing front leg ($X_{P2}$ = -10.13mm ± 4.84; $X_{P5}$ = -7.00mm ± 4.97; $X_{P8}$ = -5.52mm ± 5.78). Although the scatter of touchdown positions along the body axis was relatively large, the touchdown positions for different target positions were significantly different from one another (Fig. 5A; $p_{P2\text{-}P5} = 0.0002$; $p_{P5\text{-}P8} = 0.0280$; $p_{P2\text{-}P8} < 0.0001$). To test whether a correlation existed perpendicular to the body axis, we used positions four, five, and six (Fig. 5B). Here only a slight increase in the mean values of three data groups ($Y_{P4}$ = 19.82mm ± 2.79; $Y_{P5}$ = 20.62mm ± 3.19; $Y_{P6}$ = 21.29mm ± 2.60) was found, and these differences were not significant ($p_{P4\text{-}P5} = 0.1333$; $p_{P5\text{-}P6} = 0.1825$; $p_{P4\text{-}P6} = 0.0022$). A linear correlation along this axis could also not be detected ($r^2_y = 0.08$).

We then calculated the distances between the position of the standing front leg and the touchdown position of the middle leg parallel (Fig. 5C) and perpendicular to the body axis (Fig. 5D). A comparison of these values should yield an estimate for the targeting accuracy of the middle leg towards the front leg. If on average the distance between front and middle leg stayed constant or were not significantly different between different front leg standing positions, one could assume targeting. If, however, the distance between the two positions became systematically bigger with a more anteriorly or distally standing front leg, respectively, this would indicate only weak if any targeting of the middle leg. We plotted the distance values against the

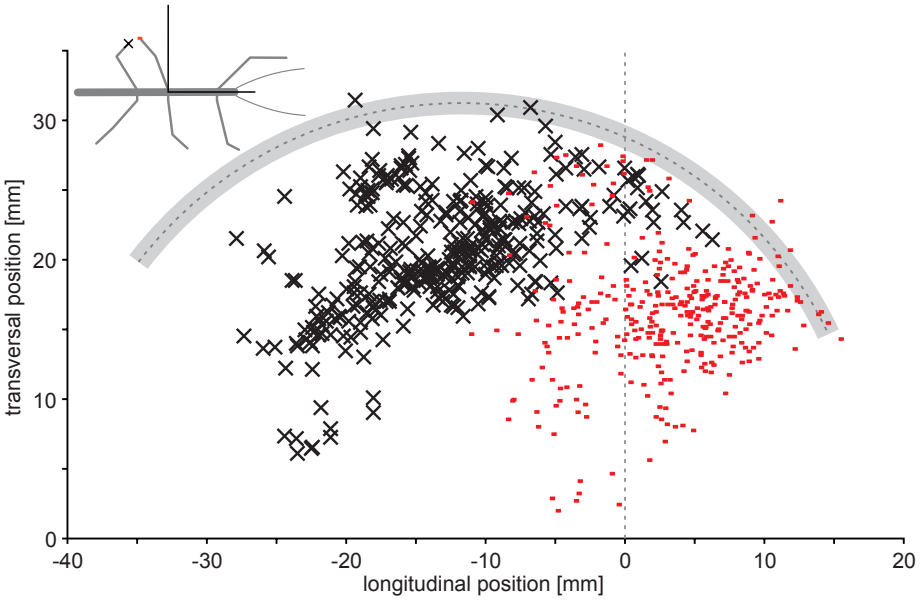


***Figure 6:*** *Scatter plot of middle and hind leg positions during walks on the slippery surface. The red dots represent the positions of the middle leg at the time of the liftoff of the hind leg. The black crosses show the subsequent touchdown position of the hind leg. The vertical dotted line marks the zero on the x axis and also the position of the coxa of the middle leg. The dotted half circle depicts calculated average maximum range of fully stretched hind legs with its standard deviation (grey area).*

position of the standing front leg, calculated the mean distance for each group, and tested significant differences and for linear correlation between the groups. Although the touchdown positions of the middle leg were on average more anterior when the front leg was standing on a more anterior position (Fig. 5A), the distance between middle leg and front leg tarsus parallel to the body axis also increased significantly from positions two through eight ($X\text{-}dist_{P2}$ = 3.74mm ± 4.57; $X\text{-}dist_{P5}$ = 7.33mm ± 4.98; $X\text{-}dist_{P8}$ = 12.96mm ± 5.83; $p_{P2\text{-}P5}$ = 0.0001; $p_{P5\text{-}P8}$ < 0.0001; $p_{P2\text{-}P8}$ < 0.0001). The difference between the distances at positions five and eight is particularly big. This might be caused by the fact that the middle leg was still anatomically able to reach position five, while this was not possible for position eight. We found a linear correlation between the position of the standing front leg and the distances to the middle leg touchdown parallel to the body axis ($r^2_x$ = 0.35), which is again indicative of only weak targeting of the middle leg towards the standing position of the front leg in this direction. As the touchdown positions of the middle leg were not found to differ significantly with a more distally positioned front leg, it was to be expected, that this distance between middle and front leg would differ between the standing positions perpendicular to the body axis (Fig. 5D). In fact, the average distances increased from one standing position to the next significantly by about five millimeters ($Y\text{-}dist_{P4}$ = -8.60mm ± 2.68; $Y\text{-}dist_{P5}$ = -2.80mm ± 2.93; $Y\text{-}dist_{P6}$ = 3.07mm ± 2.57; $p_{P4\text{-}P5}$ < 0.0001; $p_{P5\text{-}P6}$ < 0.0001; $p_{P4\text{-}P6}$ < 0.0001). The resulting strong linear correlation between the standing positions of the front leg and the distances to the middle leg touchdown position perpendicular to the body axis ($r^2_y$ = 0.74) again suggests no or only minor targeting of the middle leg towards the front leg in this axis.

## TARGETING ACCURACY IN THE TETHERED WALKING ANIMAL

The experimental situation with a standing anterior leg corresponds to a situation where the animal starts locomotion after standing still, but this is a special case that may have limited relevance for the freely locomoting animal. Therefore, we also analyzed the targeting precision of the hind legs onto the middle leg during walking sequences on the slippery surface. The animal was again tethered above the slippery surface as before, but this time the middle or front legs were not placed on one of the defined positions but moved freely. The position of the posterior leg used for the analysis, was again its touchdown position. However, since it is not known at what time during the step cycle of the posterior leg its touchdown position is determined, we tested if we could see a correlation of this touchdown position with the position of the anterior leg at three different time points during its step cycle: 1. The position of the anterior leg at the time when the posterior leg finished its swing phase and touches the ground (comparable to the control with a standing anterior leg, only without pre-defined positions). 2. The position of the anterior leg at the time when the posterior leg was lifted off the ground and began its swing phase. 3. The liftoff position of the anterior leg directly following liftoff in the posterior leg.

We calculated the coefficients of determination for each of these three combinations, and, to ensure that the results were not caused by noise, we also calculated the coefficients of determination between the touchdown positions of the posterior leg and a set of random variables. The random variables had the same distribution as the real data (front leg: X between -10.20 and 28.68mm; Y between 0.52 und 31.18mm; middle leg: X between -11.15 und 15.41mm; Y between 2.02 und 28.26mm). Table 1 lists the coefficients of determination of the linear regressions and the corresponding numbers of data pairs. All linear regressions of the real data are significantly different from zero ($P < 0.001$), while the linear regressions with the random variables are not significantly different from zero ($P > 0.05$). Also, all coefficients of determination of the real data are bigger than the values for the used random variables. In both directions the strongest linear correlation was found between the touchdown position of the posterior leg and the position of the anterior leg at the time of liftoff of the posterior leg. This was the case for the middle as well as for the hind leg as posterior leg. For all further evaluations of targeting during walking, we therefore

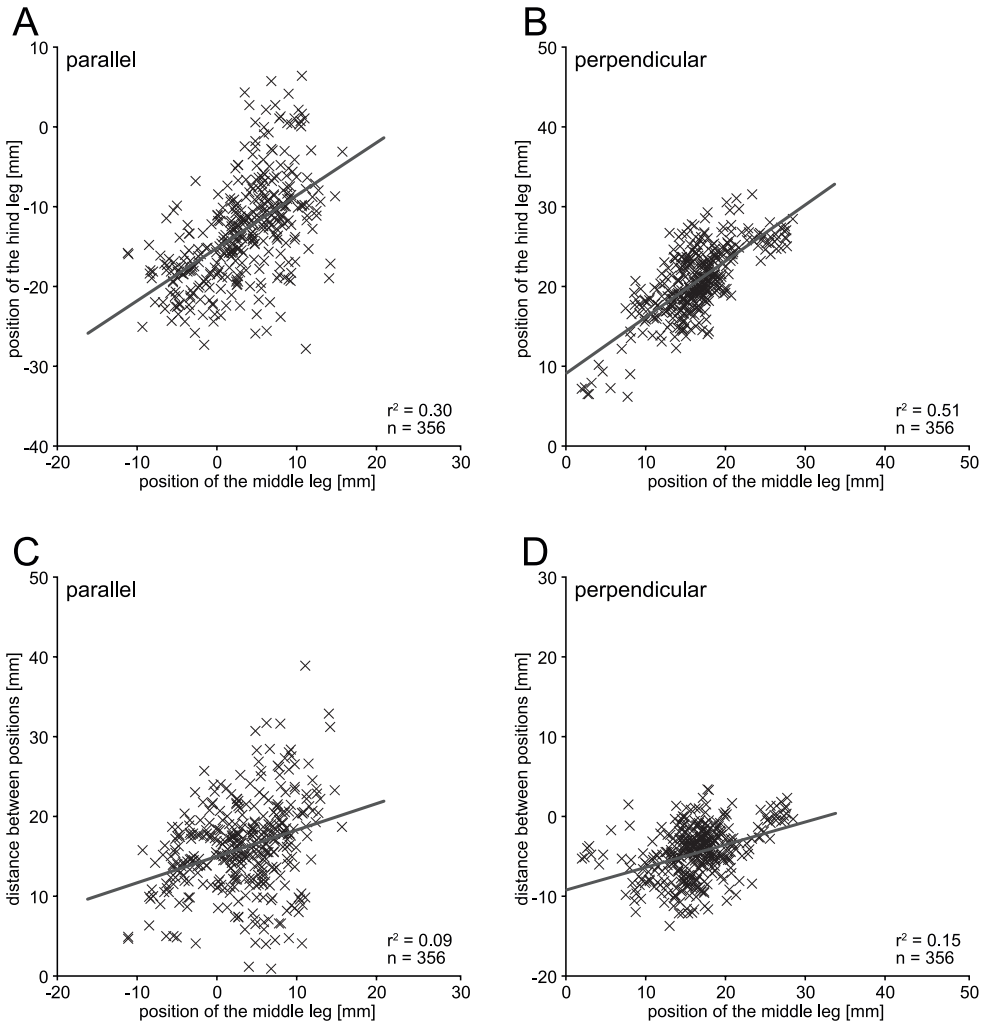


***Figure 7:*** *Scatter plot with test upon linear correlation of the positions of the middle leg at the time of the liftoff of the hind leg against the subsequent touchdown position of the hind leg (**A** und **B**) and against the distance between middle and hind leg (**C** und **D**), respectively. Separated into the components parallel (**A** und **C**) and perpendicular (**B** und **D**) to the body axis.*

used the position of the anterior leg at the time of the liftoff of its respective posterior leg. We determined all liftoff and touchdown events of the posterior leg. For all touchdown events of the hind leg, we identified the position of the hind leg. We then identified the position of the anterior leg for all liftoff events of the posterior leg. If the anterior leg was performing a swing phase at that time point, then no position could be identified and the corresponding touchdown position of the posterior leg was removed from the dataset.

All data pairs from the hind and middle leg are plotted in figure 6. Most of the time, the touchdown positions of the hind leg were posterior of the middle leg coxa (dotted vertical line), but occasional stepping to more anteriorly located positions occurred. The mean values and the overall scatter of the touchdown positions of the hind leg perpendicular to the body axis were similar to those of the hind leg touchdown positions in all experiments with predefined standing positions of the middle leg (Y = 20.6mm ± 4.33; see for comparison Fig. 2), but were slightly shifted caudally (X = -13.05mm ± 6.43; see for comparison Fig. 2). Since the reference positions of the middle leg were taken at the time of liftoff in the hind leg, the middle leg had not completed its stance phase and thus had not reached its PEP, yet. Therefore the middle leg positions are comparably far rostral, and distances between them and the hind leg touchdown positions were larger than for the standing middle leg. Under tethered walking conditions, the touchdown positions of the hind legs were mostly distributed posterior of the middle leg positions with an average distance along the length of the animal of X-dist = 16.08 mm (SD = 5.67) while the lateral distribution of the two data groups was similar with an average distance of Y-dist = -4.39 mm (SD = 3.28; XY-dist = 17.2mm ± 4.99). As a result of the length of the hind leg most of the middle leg positions were within the reach of the hind leg (dotted semi circle depicts calculated average maximum reach of fully stretched hind legs; grey area represents the standard deviation). We tested for linear correlation of the hind and middle leg positions and distances parallel and perpendicular to the body axis (Fig. 7). For positions parallel to the body axis (Fig. 7A) the coefficient of determination ($r^2_x = 0.30$) was similar to the results with standing middle leg and targeting hind leg (cf. Fig. 3A). A much stronger linear correlation was found for the positions perpendicular to the body axis $r^2_y = 0.51$ (Fig. 7B). Only very weak linear correlations were found for the distances between the two positions either parallel (Fig. 7C; $r^2_x = 0.09$) or perpendicular (Fig. 7D; $r^2_y = 0.15$) to the body axis. Altogether it appears that the state of activity of the middle leg has a strong influence on the targeting accuracy. Aiming precision of the hind leg towards the middle leg perpendicular to the body axis is only present when the animal locomotes steadily, while no additional improvement was found for the aiming precision along the body axis.

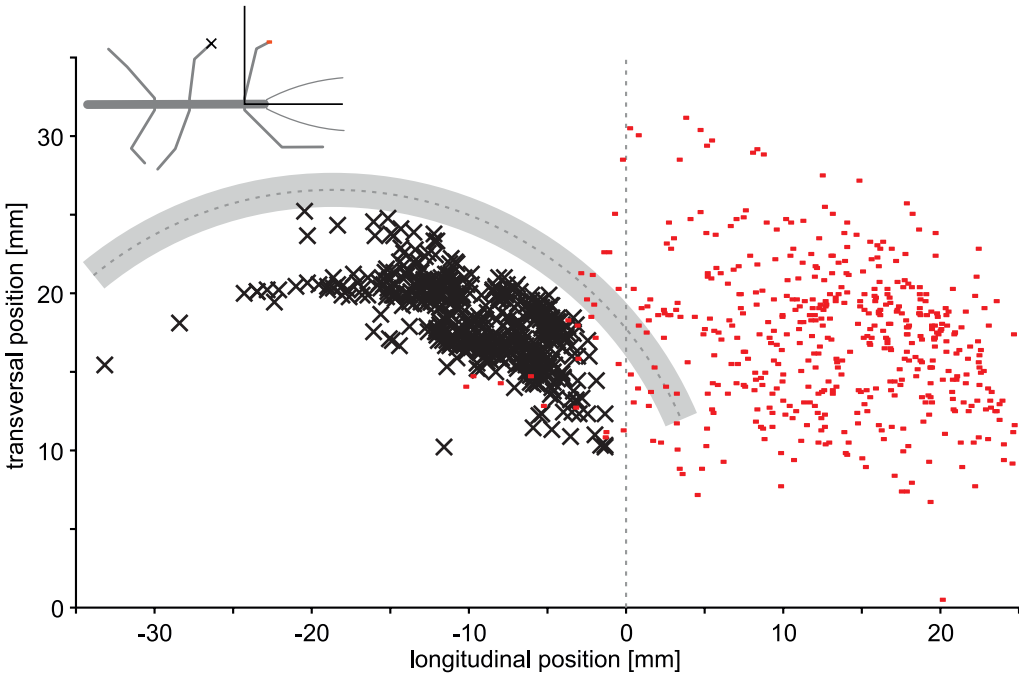


***Figure 8:*** *Scatter plot of the positions of the front leg and middle leg on the slippery surface. The red dots represent the positions of the front leg at the time of the liftoff of the middle leg. The black crosses show the subsequent touchdown position of the middle leg. The vertical dotted line marks the zero on the x axis, and also the position of the coxa of the front leg. The dotted half circle depicts calculated average maximum range of fully stretched middle legs with its standard deviation (grey area).*

To find out if the aiming precision of the middle leg onto the front leg is also changed during regular walking, we repeated this analysis for the middle and front legs under tethered walking conditions. We determined all liftoff and touchdown events of the middle leg. For all touchdown events of the middle leg, we also identified its position. As previously done for the hind and middle legs, we also identified the position of the front leg for all liftoff events in the middle leg. If the front leg was performing a swing phase at the time, then no position could be identified and the corresponding touchdown position of the middle leg was not included in the analysis. The scatter plot of all data pairs is shown in figure 8 and reveals that the majority of touchdown positions of the middle leg were close to the legs maximum reach (dotted semi circle depicts calculated average maximum range of fully stretched middle legs; grey area represents the standard deviation). There were no touchdown positions of the middle leg anterior of the coxa of the front leg (vertical dotted line). This overall distribution of the touchdown positions (mean values: X = -9.30mm ± 4.43; Y = 18.21mm ± 2.51) was similar to that of the touchdown positions that were measured with standing front leg (cf. Fig. 4). Interestingly, the spread among touchdown positions of the middle leg was much smaller than that among the touchdown positions of the hind leg (cf. Fig. 6). As the reference positions of the front leg were taken at the time of the liftoff of the middle leg, the front leg had not finished its stance phase, yet, and hence not reached its posterior extreme position. As a result, the front leg positions are all relatively far anterior and in most cases even out of reach for the middle leg (dotted semi circle). Therefore, at least parallel to the body axis, we did not expect small distances between the middle and front legs, and there was indeed only a very small overlap in the spread of the middle and front leg positions parallel to the body axis. On average the touchdown positions of the middle leg were located posterior of the front leg positions (X-dist = 22.66mm ± 6.31) while the lateral distribution of the two data groups was similar (Y-dist = -1.36mm ± 4.27; XY-dist = 23.13mm ± 6.18). Nevertheless a systematical dependency between the positions is theoretically possible. We therefore tested for linear correlation of the middle and front leg positions and the distances between them, parallel and perpendicular to the body axis (Fig. 9). With a coefficient of determination of $r^2_x = 0.27$ one can assume a linear correlation between the positions of middle and front leg along the body axis. This coefficient of determination was in the same range as that for the standing middle and targeting hind leg (cf. Fig 3A) and about twice as strong as the coefficient of determination of the standing front and targeting middle leg (cf. Fig 5A). Perpendicular to the body axis, there was only a slight linear correlation between the positions of the middle and front leg (Fig. 9B; $r^2_y = 0.18$), but this was still more than twice as strong than that between standing front and targeting middle leg (cf. Fig 5B). The distances between the touchdown position of the middle leg and the position of the front leg at middle leg liftoff showed a strong linear correlation parallel (Fig. 9C; $r^2_x = 0.639$) as well as perpendicular to the body axis (Fig. 9D; $r^2_y = 0.717$). Overall these results indicate targeting of the middle leg to the position of the moving front leg along the body axis and at least a slight targeting perpendicular to the body axis. Altogether, similar to the findings for the hind to middle leg movement, the targeting accuracy of the middle to the front leg appears to improve once the animal locomotes steadily.

One can summarize that, in general, the hind leg appears to show more precision than the middle leg in finding its anterior neighbor under both conditions, and that movement of the respective anterior leg seems to be of importance for the accuracy of the targeting movement perpendicular to the body axis.

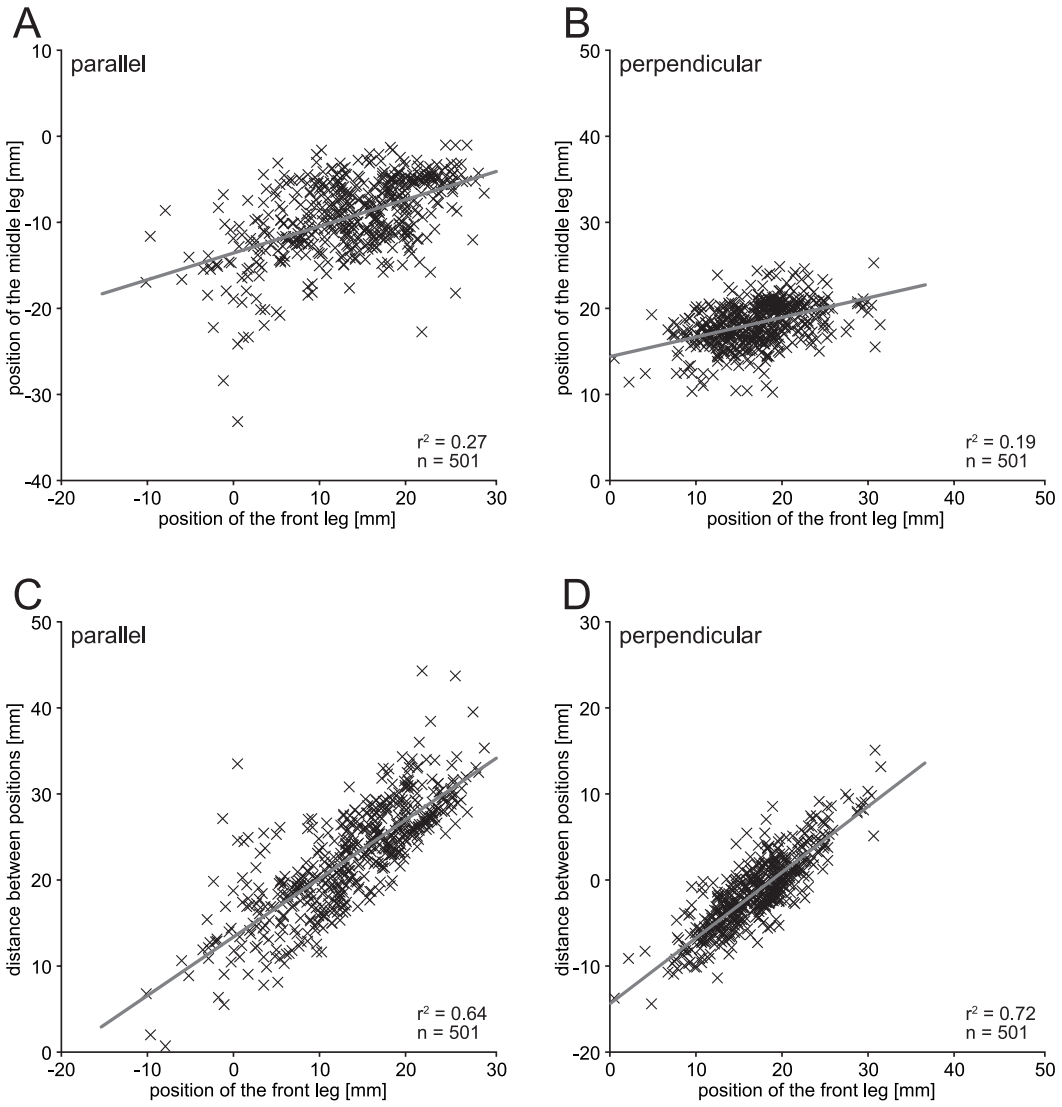


***Figure 9:*** *Scatter plot with test upon linear correlation of the positions of the front leg at the time of the liftoff of the middle leg against the subsequent touchdown position of the middle leg (**A** und **B**) and against the distance between front and middle leg (**C** und **D**), respectively. Separated into the component parallel (**A** und **C**) and perpendicular (**B** und **D**) to the body axis.*

# Discussion

We have investigated the aiming accuracy of middle and hind legs of stick insects on a slippery surface. With our analyses we could demonstrate that targeted leg movements towards their rostral neighboring leg can occur under certain conditions, even without mechanical coupling through the ground, but that this ability is not equally strong between the hind and the middle legs. While targeting from hind to middle leg seems present when the animal starts to walk, it seems largely absent from middle to front leg under these conditions.

On the other hand, in walking animals, targeting of both legs towards their rostral neighbor improves and shows clear correlations of the touchdown positions of hind and middle legs with the target position of their rostral neighbor in parallel to the body axis and a clear improvement of targeting perpendicular to the body axis.

### TARGETING ACCURACY WITHOUT MECHANICAL COUPLING

In earlier investigations it had been shown that stick insects can perform targeted movements with their hind legs and that the touchdown position of the hind leg depends on the position of the standing middle leg when the rest of the legs are walking on the same treadwheel (Cruse 1979). This approach, however, did not allow separating the contribution of passive mechanical interaction between the leg movements and active neuronal processes to the targeting mechanism. The use of slippery surface setups allows the removal of mechanical interaction between or passive movements of the legs due to coupling through the ground and thereby investigate the neuronal contribution to a given behavior. Previous studies using animals tethered above a slippery surface setup could show that stick insects are able to perform normal walking movements under this condition (Epstein & Graham 1983, Graham & Cruse 1981, Graham & Epstein 1985, Gruhn et al. 2006 & 2009). However information about targeting movements of the legs was not only very thin but also contradictory. While Graham & Cruse (1981) postulated targeting of the legs based on the distribution of touchdown and liftoff positions of ipsilaterally neighboring legs, Epstein & Graham (1983) claimed that they could not observe targeting behavior during their experiments with walking stick insects. By specifically analyzing the linear correlation of corresponding pairs of touchdown and liftoff positions of stick insects tethered above a slippery surface setup, we could now confirm the hypo-

thesis of Graham & Cruse and prove that stick insects actually can perform targeted leg movements towards their anteriorly neighboring leg in the absence of mechanical coupling through the ground. This can be interpreted as evidence for the existence of a neuronal mechanism that is involved in spatial coordination of leg movements.

### COMPARISON OF TARGETING ACCURACY OF HIND AND MIDDLE LEGS

By comparing the targeting accuracy of the hind towards the middle legs with the targeting accuracy of the middle towards the front legs we could show that the precision of the hind leg targeting was distinctly more accurate than targeting of the middle leg. In fact, when the front leg was standing and the middle leg performed its first step of the walking sequence, this step forwards can hardly be called targeted at all (see results above). This is a novel result because none of the previous studies investigating targeting behavior of stick insects (e.g. Cruse 1979, Cruse et al. 1984, Dean 1984, Dean & Wendler 1983) measured the accuracy of the middle leg foot placement towards its ipsilateral front leg to compare it with the targeting accuracy of the hind leg. It was merely assumed from comparing distances between average touchdown and liftoff positions of neighboring legs (Cruse 1976) that the hind legs showed better targeting than the middle legs (Cruse 1979). With our results we could now confirm this assumption. However, it is still unclear why the targeting of the hind leg is more accurate. In addition, it is interesting that targeting perpendicular to the body axis was virtually non-existent in both legs, unlike in earlier studies. However, in these previous studies at least the targeting hind leg was either standing (Cruse 1979; Cruse et al. 1984) or moving (Dean & Wendler 1983; Dean 1984) on a treadwheel thereby possibly pre-defining the axis of movement perpendicular to the body. In addition, the position analyses were performed between the touchdown position of the hind leg and the position of the middle leg at the same time which, as will be discussed below, may not be the best choice for the moving animal.

The induction of the first step by a light touch to the abdomen was the same between activating either the hind or the middle leg to perform its first step and thus seems unlikely to be the reason for the difference. However, one explanation for the distinctly better targeting of the hind legs compared to the targeting accuracy of the middle legs could be based on simple anatomical constrains for the middle legs. The middle leg is the shortest leg of the stick insect (Cruse 1979) and is anatomically not capable of reaching all posterior extreme positions of the front leg, while the distinctly longer hind leg (Cruse 1979) is anatomically capable of reaching almost every posterior position that the middle leg can take up. This may however only be relevant at the beginning of a movement when the body is not simultaneously displaced forwards by the movement of several legs at the same time.

The reason for the better targeting performance by the hind legs may be that the center of mass of the stick insect is located close to and posterior of the coxae of the hind legs (Cruse 1976). It might therefore be of greater importance for the stability of the animal to reliably find foothold with the hind than with the middle legs and as a consequence sensory processing of information on the target leg's location in the resting animal may be different between meso- and the metathoracic segment. So far, no direct evidence exists to support this hypothesis for the case of targeting. However, Hellekes et al. (2012) have shown that there is segment specific differential processing of sensory information from the femoral chordotonal organ (fCO), which signals the femur-tibia joint angle, and which could also be integrated with other known sensory signals to yield distance information to a neighboring leg. F urther implications of this differential processing will be discussed below.

### COMPARISON OF TARGETING QUALITY BETWEEN STANDING AND MOVING TARGET LEG

Interestingly, targeting performance improved when the animal was moving as compared to when the animal was stationary. We found this to be true for the middle leg targeting the front leg parallel to the body axis, as well as for hind and middle legs targeting perpendicular to the body axis. This suggests that movement or activity in the target leg seems to be of importance of for the targeting precision.

It is currently unknown, at what time or at what position of the target leg the targeting information is read out in order to produce aimed movements by the targeting leg. For exact targeting, the animal would have to know the position of the target leg at the targeting legs touchdown, which, during walking, is not a trivial task for the animal. This is due to the fact that the target position has to be read out while the target leg is still moving backwards towards this position. Thus the animal would have to be able to extrapolate the probable target position. However, the time of readout can be assumed to be within a time frame that allows the nervous system to process the information and for the targeting leg to actually produce a movement so that is has not become obsolete by the forward movement of the animal.

By calculating conduction times, one can get a rough estimate for the minimal time span that is necessary for this information transfer. First, the position information from the sense organs of the targeted leg has to be transmitted to the local thoracic ganglion and from data in the locust it can be assumed that it takes about 2 ms for the first spikes to travel from the sense organ to interneurons within its own hemiganglion (Höltje & Hustert 2003). The information then has to travel into the ganglion in the neighboring segment. Hardly any direct connections from sensory neurons into neighboring segments have been demonstrated, yet (Hustert 1978), but with connective lengths averaging about 17 mm between pro- and mesothorax and 10 mm between meso- and methathorax (Cruse 1976), and conductance velocities within the connective of about 2-2.8 mm/s (Brunner et al. 1990) one can assume at least another 4-9 ms until the first spikes reach the neighboring ganglion. The transfer of this information into a targeted movement of the leg takes time as well, because, depending on how far distally in the leg the innervated muscle is, it takes about 1-5 ms for the motor-

| Positions of the anterior leg at the time of the... | ML - VL | | | HL - ML | | |
|---|---|---|---|---|---|---|
| | $r^2_X$ | $r^2_Y$ | n | $r^2_X$ | $r^2_Y$ | n |
| AEP of the posterior leg | 0.186 | 0.146 | 494 | 0.154 | 0.305 | 216 |
| last PE P of the posterior leg | 0.270 | 0.185 | 501 | 0.296 | 0.514 | 356 |
| next PEP of the anterior leg | 0.196 | 0.056 | 494 | 0.075 | 0.223 | 216 |
| random variables | 0.016 | 0.033 | 501 | -0.007 | 0.095 | 356 |

***Table 1:*** *Coefficients of determination of the linear regressions of touchdown positions of the posterior leg against the position of the anterior leg at three different time points and against a set of random variables. All linear regressions of the real data are significantly different from zero ($P < 0.001$). The linear regressions with the random variables are not significantly different from zero ($P > 0.05$).*

neuron spikes to travel from the hemiganglion to the neuromuscular end plate (Höltje & Hustert 2003). Finally, the muscle needs another 5-20 ms to build up the muscle tension needed for the movement of the leg (Höltje & Hustert 2003; Hooper et al. 2009). It is unclear how many synapses and interneurons have to be passed before the information reaches the motoneurons of the targeting leg, but both intersegmental as well as local interneurons have been described to take part in the targeting process (Brunn & Dean 1994). Altogether, in the most conservative estimate and without considering synaptic transmission, it would take at least 12 ms to process and target a measured leg position. Most likely this takes more time. Schütz & Dürr (2011), for example, could show that after antennal contact with an object, re-targeting of an ongoing swing movement by the front leg occurs after about 40ms. That is why the position information has to be collected and read out before the targeting leg finishes its swing phase.

Taking the above considerations into account, the position of the target leg at the time when the targeting leg finishes its swing phase and touches the ground or even the posterior extreme position of the target leg leave not enough time for processing and could only have a good correlation with the touchdown position if one assumes a perfect prediction of this position by the animal. Indeed, the coefficients of determination were very weak (see Table 1). Since we did not know the exact point in time that is used by the animal, we therefore chose the position of the target leg at the time when the targeting leg is lifted off the ground and begins its swing phase. This is well above the range reported by Schütz and Dürr (2011), and hence leaves enough time (on average 141 ± 57ms Rosenbaum et al. 2010, unpublished results) for the neuromuscular system to transmit and process the information. However, we cannot exclude that the time point at which the placement of the foot is actually decided lies further in the future, as has been reported for vertebrates that use visual and mechanosensory information to guide leg trajectories during walking (cat: McVea & Pearson 2007, McVea et al. 2009, Wilkinson & Sherk 2005, human: Mohagheghi et al. 2004, Patla & Vickers 2002). In the case of humans wanting to place their foot at a specific target position, it has been reported that they fixate on this position on average two steps ahead, and at least 800–1,000 ms before the limb is placed on the target area (Patla & Vickers 2002).

The questions that arise now are why targeting of the hind and the middle leg generally improved during walking, why this is not the case for the hind leg in parallel to the body axis, and what the underlying neuronal mechanisms could be. It is known that sensory information signaling leg angles is integrated by intersegmental and local interneurons to provide the targeting information for the hind leg (Brunn & Dean 1994). Primarily responsible for the targeting accuracy perpendicular to the body axis is the fCO which measures the angle between femur and tibia (Bässler 1977, Cruse et al. 1984). Processing of fCO activity changes between standing and walking animals (Bässler 1974, 1976, 1988; Stein et al. 2006; Hellekes et al. 2012). In addition, it is also known that fCO signals from an anterior leg in the actively stepping animal affect the next posterior leg (Ludwar et al. 2005; Stein et al 2006). So far no interneurons have been described that receive solely position information from the fCO. Most of the interneurons receive a combination of movement velocity and acceleration information from the femoral chordotonal organ (Büschges 1989, Brunn & Dean 1994). Altogether, these findings make it very plausible that fCO signals from the anterior leg may only be processed to help targeting the posterior leg to its anterior neighbor perpendicular to the body axis, if the animal is actually walking.

Targeting of the hind leg in parallel to the body axis, seems to be primarily controlled by coxal hair rows and hair fields which measure the position of the coxa and pro- and retraction movements of the leg (Bässler 1977, Dean & Wendler 1983, Cruse et al. 1984). So far, no data exist on state-dependent or thoracic-segment-dependent processing of this type of sensory information, however, it is again known from the fCO, that its signals are processed differentially in the different thoracic segments (Hellekes et al. 2012). Therefore, state-dependent facilitation of the sensory signal does seem to be a plausible explanation for the improved targeting from the middle to the front leg parallel to the body axis in the walking animal. At the same time, a different segment-specific processing in the metathorax may be responsible for a lack of this effect in hind to middle leg targeting.

Interestingly, this state-dependent influence of sensory input on the spatial coordination between the legs also matches the description of movement-induced temporal coordination in

the stepping stick insect by Borgmann et al. (2009) and it's improvement with acceleration (Gruhn et al. 2009). And the fact that these influences may not be equally strong between different thoracic segments is in accordance with Grabowska et al. (2012), who could show, that temporal coupling of the front with either middle or hind legs during walking is also much weaker than temporal coupling between middle and hind legs.

In conclusion, our data, together with findings of previous studies, support a model in which middle and hind legs can aim towards their anterior neighbor either when performing a first step or during steady walking. However, the fact that the correlations are not always very strong, especially for the first step in the standing animal, suggests that processing of the relevant sensory information is differentially achieved in middle and hind legs as the hind leg is more accurate than the middle leg in finding its anterior neighbor under both conditions. The fact that movement of the animal strongly improves targeting accuracy suggests that processing of information on leg position to produce spatial coordination in the stick insect is state-dependent and segment-specific and supports previous findings of state-dependent and segment-specific processing of sensory information for temporal coordination.

# 5. Discussion

The four presented studies give evidence for several mechanisms of temporal and spatial coordination of leg movements in the stick insect *Carausius morosus* and the fruit fly *Drosophila melanogaster* during different experimental paradigms. They start with local coordinating mechanisms of antagonistic muscle pairs within the individual leg, and continue with mechanisms that influence movement speed of the individual leg and coordination of speed between the different legs. Then an analysis of how changes in walking speed are implemented in the fruit fly and its comparison with the stick insect is presented. And finally, the focus shifts slightly onto spatial coordination of the legs of the stepping stick insect.

In the first study (Rosenbaum et al. 2010), the timing and activity of leg muscles of the three main joints in the stepping middle leg of the forward and backward walking stick insect was analyzed. The results provide evidence that between the two walking directions solely motor activity of the most proximal leg joint is changed, while timing and activity of the muscles controlling the distal leg joints is virtually identical. So when walking direction is reversed, the functional stance muscle of the thorax-coxa-joint is switched from *retractor* to *protractor*, with both muscles showing the same timing of activity when serving as stance muscles.

The second study (Gruhn et al. 2009) investigated changes that accompany alterations in walking speed in the stick insect at the neuronal and behavioral level. It could be shown that swing phase motor activity is not changed with changes in walking speed. With increasing stepping velocity, the latency between the end of stance phase motor activity and onset of swing phase motor activity was found to be reduced. Alterations in stepping velocity of a single front leg were not reflected in motoneuron activity of the mesothoracic segment, neither in the extracellularly recorded activity of motoneuron pools nor in single intracellularly recorded

motoneurons. During steady walking of the intact, six-legged animal, on a slippery surface there was no correlation between stepping velocities of the individual legs. However, when an increase in walking speed was induced, clear correlation in the stepping velocities of the individual legs was found.

The results of the third study (Wosnitza et al. 2013) did not only prove that *Drosophila* can cover a broad range of walking speeds, but also that *Drosophila* increases its walking speed in a very similar way as most other animals do, i.e. by modifying stance duration, whereas swing duration and step amplitude remain largely unchanged. The temporal coordination between the legs changes gradually and systematically with walking speed without discrete gait changes, and *Drosophila* is able to acutely adapt its leg coordination to major biomechanical changes in its walking apparatus like the loss of one of the hind legs.

The fourth study (Wosnitza et al. in prep.) investigated the placement of middle and hind legs in the stick insect *Carausius morosus* in a slippery surface setup to understand the importance of neuronal mechanisms for spatial coordination of foot placement without visual guidance. Evidence is presented that middle and hind legs of *C. morosus* can target their anterior neighbor in first steps of a sequence and during continuous walks. However, under both conditions the hind leg is more accurate than the middle leg in finding its anterior neighbor. Especially for the first step in the standing animal, the correlations are generally not very strong and movement of the respective anterior leg seems to be of importance for the accuracy of the targeting movement perpendicular to the body axis.

All four studies have dealt with temporal or spatial coordination during walking in insects. Common principles in inter-leg coordination like similarities between different organisms and segment-specific or state-dependent modifications in the walking system were found. They can be interpreted as evidence for a highly adaptive and modular design of the underlying neuronal structures.

## 5.1 Similarities between *Carausius* and *Drosophila*

In several vertebrates, insects, or crustaceans changes in walking speed are usually achieved by modifying cycle period through changes in stance duration, whereas swing duration and step amplitude remain largely unchanged (cat: Halbertsma 1983; dog: Maes et al. 2008; stick insect: Wendler 1964; locust: Burns 1973; lobster: Clarac & Chasserat 1983; Chasserat & Clarac 1983; reviewed in Orlovsky et al. 1999). In stick insects walking forward on non-slippery substrates cycle period depends solely on stance duration (Wendler 1964; Graham 1972; 1985). For the fruit fly *Drosophila*, previous studies have found that walking speed is changed not only by changing cycle period but also by changing stride length (Strauss and Heisenberg 1990). However, stride length is defined as the distance between two consecutive touchdown positions in floor-fixed coordinates, and hence it is not independent of movement speed and might change even without active changes in the walking motor pattern. By transferring all positions into a body centered coordinate system in the present study, and repeating the analysis with step amplitude, it could be shown that the dependency of walking speed on step amplitude was most likely an effect of body translation during swing phases (Wosnitza et al. 2013). This means that *Drosophila* changes its walking speed in the same way as other insects do, i.e. by modifying cycle period via changes in stance duration, whereas swing duration and step amplitude remain constant (e.g. stick insect: Wendler 1964; locust: Burns 1973).

Many insect species like stick insects (*Carausius morosus*), cockroaches (*P. americana*), and ants (*Cataglyphis, Formica*, *Lasius* and *Myrmica*) are known to use tripod coordination during fast locomotion, while leg coordination at lower speeds becomes much more variable, approaching tetrapod and even wave gait coordination (Wendler 1964; Graham 1972; Bender et al. 2011; Strauss & Heisenberg 1990; Zollikofer 1994). It has been proposed that invertebrates use a speed-dependent continuum of interleg coordination and the specific patterns together with intermediate forms of coordination are part of this continuum. Adult stick insects are known to preferably walk at slow speeds and then mostly display tetrapod coordination (Cruse et al. 2009; Graham, 1972), but faster sequences with tripod coordination have also been observed (Graham, 1972). Furthermore, it is known that stick insects can seamlessly transition between tetrapod and tripod coordination without changing the locomotion speed by simply modifying stance duration (Cruse 1990; Graham 1985; Wendler 1966). Until recently it has been the common notion that *Drosophila* mainly uses tripod coordination for a large part of the observed speed range (Strauss & Heisenberg 1990). However, in the present study (Wosnitza et al., 2013), it could be shown that *Drosophila* also spontaneously generated relatively slow walking bouts where inter-leg coordination deviated from a strict tripod pattern. At the highest walking speeds, inter-leg coordination was always tripod. With decreasing speed, the accuracy of the tripod coordination systematically decreased, and within a slower speed domain also tetrapod coordination could be observed. In addition, at very low walking speeds, *Drosophila* no longer uses tetrapod but instead shows wave gait coordination, a pattern first described for larger insects (Hughes 1952; Wilson 1966). It appears that inter-leg coordination in *Drosophila* reflects all possible coordination patterns known in insects. As in the stick insect, inter-leg coordination in *Drosophila* is not fixed, but changes systematically and gradually as a function of walking speed over a broad speed range. And at slow walking speeds, *Drosophila* seems to be able to choose which coordination type it uses and can walk with tripod, tetrapod, or even wave gait-like inter-leg coordination.

Sensory feedback from several leg sensory organs contributes to coordination of motor activity of the single stepping leg of the stick insect (Büschges et al. 2008). A set of coordination rules has been proposed as a result of behavioral studies in the stick insect, which have suggested that signals from these sense organs also contribute to the coordination between legs (Cruse 1990; Dürr et al. 2004). Kinematic analyses of walking stick insects after the removal of single legs revealed temporal and spatial modifications in the stepping activity of the remaining legs (Bässler 1972; Graham 1977). Ipsilaterally adjacent legs showed extended stance trajectories, which were most likely caused by increased load and the simultaneous inhibition of swing phase initiation (coordination rule 1; Cruse et al. 1998; Dürr et al. 2004). Similar effects could be observed in *Drosophila* after removing one of the hind legs (Wosnitza et al. 2013). Under these conditions, *Drosophila* displayed immediate changes in inter-leg coordination and stance kinematics. The stepping activity of the remaining legs, specifically the contralateral middle and hind legs, was modified such that the now absent support of the missing hind leg was compensated. The timing of swing phases in these legs was delayed as compared to the intact animal. Additionally, the ipsilateral middle leg showed a distinctly extended stance trajectory, and in all legs, touchdown and liftoff positions were shifted outwards so that the animal adopted a broader posture. Especially this outward shift suggests an overall compensatory modification of body posture, although it might also be due to a relative increase in load, a consequence of the loss of muscle force available to the animal. These findings are interesting as they provide evidence for cooperative neuronal and

mechanical interactions between the legs in the generation of propulsion and posture. They support the hypothesis that the neural control system for walking in *Drosophila* is similar to that in other insects, like ants and cockroaches, and agrees well with results from stick insects. Although not yet studied in *Drosophila*, it is quite conceivable that the ‚coordination rules 1-3', as proposed by Cruse (Cruse 1990) and based on behavioral studies in the stick insect, would suffice to generate the observed walking behavior in the fruit fly.

Taken together, these results substantiate the assumption that the walking system of *Drosophila* indeed is very similar to that of other insects like the stick insect. This provides the opportunity to benefit from the different advantages those different animal systems can offer. For example the nervous system of the stick insects is rather large and easily accessible, and hence allows for intra- and extracellular electrophysiological measurements. On the other hand, *Drosophila* allows the use of a broad spectrum of molecular and neurogenetic tools which might provide insights into different aspects of neural network organization and function. The combination of physiological and neurogenetic approaches facilitates the investigation of quetions in motor control that cannot be resolved using either approach alone.

## 5.2 State dependent differences in coordination

When a stick insect changes its walking direction from forward to backward walking, it has to distinctly change the kinematics of its legs. The touchdown and liftoff positions are reversed, and hence the movement of the leg joints and the activity of the leg muscles have to be adjusted. The kinematic comparison of forward and backward steps has revealed that backward steps are equally variable but significantly shorter and more inward directed than forward steps (Rosenbaum et al. 2010). In the transverse axis to the animal, liftoff positions are unchanged and touchdown positions are only slightly different. Previous observations of stick insects performing coordinated backwards walks on a slippery surface (Graham & Epstein 1985) could be confirmed. It could be shown that cycle period depends solely on stance duration in both forward and backward slippery surface walking, which corroborates results from stick insects walking forward on non-slippery substrates (Wendler 1964; Graham 1972; 1985). The EMG recordings of the six main middle leg muscles showed that *levator* and *extensor* muscles are functional swing muscles and *depressor* and *flexor* stance muscles under both conditions while the muscles controlling the thorax-coxa joint (ThC; protractor, retractor) switched their function when walking direction was reversed (Rosenbaum et al. 2010). Even the succession of activation of the muscles is the same under both conditions. The functional stance muscles are always activated with *depressor* being activated first and well before touchdown, followed by the *flexor* at or shortly after touchdown, and then the *retractor* (in forward walking) or *protractor* (in backward walking). The functional swing muscles are all activated before liftoff in the order of first *levator*, then *extensor*, and finally *protractor* (in forward walking) or *retractor* (in backward walking). The fact that only the timing of the *pro-* and the *retractor* muscles is inverted, while the activities of all other muscles remain unchanged, raises questions about the neuronal control of forward and backward walking. It has been known for a long time that each joint of the stick insect leg is controlled by its own pattern generating network (Büschges et al. 1995). Based on this knowledge, a theoretical study by Tóth and coworkers (2012) has suggested a model in which movement of each leg joint is controlled by its own pattern generator together with an additional layer of interneurons. In the model, this additional layer of interneurons can redistribute the output of the central pattern generator to the motoneurons and hence is able to instantaneously switch the timing of the

*pro-* and *retractor* muscles, independently of the other muscles. Such a mechanism, if present in the stick insect, could also explain the fact that cycle period of the backward walking animal continues to depend on stance duration because cycle period would continue to depend in the same way on the burst durations of the same set of pattern generating neurons. State-dependent alterations in the effects of sensory input from the leg might assist this change in central drive (reviewed in Büschges & Gruhn 2008). It is known that influence of several sensorimotor processes change between forward and backward walking (Büschges & El Manira 1998; Clarac et al. 2000; Hellekes et al 2012). Akay and coworkers (2007) have directly shown that input from trochanteral campaniform sensilla inhibits retractor motoneuron activity during backward walking, but promotes it during forward walking. This influence is strong enough that rhythmic stimulation of campaniform sensilla can entrain motor activity in the ThC joint in both walking directions. However, not only sensory signals from the respective leg influence the timing of the first muscle spikes. By reducing the number of legs, changes in the average latency of the *protractor*, *retractor*, *extensor*, *flexor*, and *depressor* could be produced. Several other studies have given considerable evidence that suggests that inter-leg influences play a prominent role in shaping leg motor output (Ludwar et al. 2005, Borgmann et al. 2007, 2009).

Only little is known about the origin and destination of intersegmentally projecting neurons, and nothing is known about neurons that convey velocity information between the legs. In a few studies, origin and destination of specific intersegmental interneurons have been identified in the locust (Watson & Burrows 1983; Laurent & Burrows 1988, 1989a,b), but again, it is unclear if also velocity information is transmitted. In the stick insect, the rectified cumulative activity in the thoracic connectives is correlated with the stepping velocity of a single front leg (Gruhn et al. 2009b), and theoretically, the walking system could use this information to neuronally coordinate the stepping velocities of all legs. Interestingly, when the intact stick insect performs continuous walking sequences on the slippery surface no correlations between the stance velocities of any pair of legs could be detected. However, under conditions, when a change in stepping velocity was induced, significant systematic correlations in stepping velocities between legs could be detected. This leads to the conclusion that neuronal coupling can be strengthened by an appropriate sensory, e.g. tactile input, and in addition might depend on local sensory feedback. Yet, it also strongly suggests that neuronal coordination of stepping velocity in the stick insect walking system is limited to specific behavioral conditions.

Just like the correlation of stance velocities, also the aiming accuracy of the legs has been found to be state dependent (Wosnitza et al. in prep.). For the aiming accuracy to improve, however, it is not acceleration but steady walking, compared to a first step after standing, that seems to be the relevant parameter. When the stick insect performs a first step, the targeting accuracy of both legs perpendicular to the body axis is only weak and improves significantly during continuous walks. This suggests that movement or activity in the target leg seems to be of importance for the targeting precision. One reason for this improvement might be the known state dependency of sensory processing. Primarily responsible for the targeting accuracy perpendicular to the body axis is the femoral chordotonal organ (fCO) which measures the angle between femur and tibia (Bässler 1977, Cruse et al. 1984). Processing of signals from the fCO is different between standing and walking animals (Bässler 1974, 1976, 1988; Stein et al. 2006; Hellekes et al. 2012). So far no interneurons have been described that receive solely position information from the fCO. Instead, most of the interneurons receive a combination of movement velocity and acceleration information from the fCO (Büschges 1989,

Brunn & Dean 1994). In the case of the targeting of one leg towards its anterior neighbor, these findings make it very plausible that fCO signals from the anterior leg may only be processed to help targeting the posterior leg perpendicular to the body axis, if the animal is actually walking.

Interestingly, this state-dependent influence of sensory input on the spatial coordination between the legs also matches the description of movement-induced temporal coordination in the stepping stick insect (Borgmann et al. 2009) and it's improvement with acceleration (Gruhn et al 2009b). And taken together these results indicate that many aspects of the locomotor system are state dependent and highly adaptive. Future studies will have to take this into account, especially when generalizations are made based upon results from reduced preparations.

## 5.3 Segment specific differences in coordination

When the stick insect performs continuous walking sequences on the slippery surface, its front, middle and hind legs display significantly different average stepping velocities. No correlations between the stance velocities of any pair of legs could be detected (Gruhn et al. 2009b). In many locomotor situations the front legs are functionally the leading legs (Borgmann et al. 2007; Rosano & Webb 2007), and they frequently displayed the fastest stepping velocities and shortest cycle periods whereas the hind legs displayed the slowest velocities. This gradient in stepping velocities bears similarities to other locomotor systems that consist of chains of pattern generators or oscillators. Studies in lamprey and leech networks for swimming hypothesized that the leading kernel of the weakly coupled oscillators exerts its influence via a faster cycle period (Matsushima & Grillner 1992; Grillner & Wallén 2002; Grillner et al. 2007; Hocker et al. 2000; Friesen & Kristan 2007). Similar weak interactions between the thoracic segments may be involved in velocity control between legs in the stick insect, but during normal walking conditions they are complemented and entrained through the mechanical interaction between the legs. Such influences have been demonstrated (Borgmann et al. 2009) and are also known to exist in lamprey (McClellan 1990) and leech (Yu et al. 1999). The correlations of stepping speeds that can be observed upon acceleration in the stick insect were found between ipsilateral and contralateral front and middle legs. At the same time the hind legs did not show significant correlations in stepping velocity to any other leg (Gruhn et al. 2009b). Previous studies have shown a coordination of the force generated by different legs that were simultaneously in stance, when the animal had to increase the total force to propel the body (Cruse 1985b). Interestingly, also in this case, the co-activating effect on the hind legs was much smaller than on the other legs. One can conclude that the stronger neuronal coupling that can be elicited by an appropriate sensory input may be limited to selected leg pairs, and in addition might depend on differences in local sensory feedback.

The aiming accuracy of middle and hind legs of stick insects can be observed under certain conditions, even without mechanical coupling through the ground. However, this ability seems to be not equally strongly distributed between hind and middle legs. While targeting from hind to middle leg seems to be present when the animal starts to walk, it seems largely absent from middle to front leg under these conditions. When the animal is walking, targeting accuracy of both legs improves and shows clear correlations of the touchdown positions (Wosnitza et al. in prep). Interestingly, the improvement in hind leg targeting accuracy is only perpendicular to the body, while in the middle leg the accuracy of targeting in both directions, i.e. parallel and

perpendicular to the body is improved. Under both conditions the targeting accuracy of the hind legs was distinctly more accurate than targeting of the middle leg. In fact, when the front leg was standing and the middle leg performed its first step of the walking sequence, this step forwards can hardly be called targeted at all. One explanation for the distinctly better targeting of the hind legs compared to the targeting accuracy of the middle legs could be based on simple anatomical constrains for the middle legs. The middle leg is the shortest leg of the stick insect (Cruse 1979) and is anatomically not capable of reaching all positions of the front leg, while the distinctly longer hind leg (Cruse 1979) is anatomically capable of reaching almost every position that the middle leg can take up. This may however only be relevant at the beginning of a movement when the body is not simultaneously displaced forwards by the movement of several legs at the same time. Another reason for the better targeting performance by the hind legs may be that the center of mass of the stick insect is located close to and posterior of the coxae of the hind legs (Cruse 1976). It might therefore be of greater importance for the stability of the animal to reliably find foothold with the hind than with the middle legs and as a consequence sensory processing of information on the target leg's location may be different between meso- and the metathoracic segment. So far, no direct neurophysiological evidence exists to support this hypothesis for the case of targeting, but the reported segment specific differences fit to findings of Hellekes and coworkers (2012). These have shown that there is segment specific differential processing of sensory information from the fCO, which signals the femur-tibia joint angle. And taken together all these results indicate that there are several segment specific differences in the stick insect locomotor system. Future studies will therefore have to be careful about extensions of hypotheses on the complete walking system based upon results from only one segment.

## 5.4 Conclusion

Many of the results presented in this thesis and in the work referenced here point towards a modular structure of the insect locomotor system. The fact that *Drosophila* can change its inter-leg coordination seamlessly between tetrapod and tripod coordination implies that no specific tripod generator is present in the neural structure controlling walking in *Drosophila*. And further evidence for the highly adaptive control of single-leg stepping in which individual legs are largely independent of each other are the observed changes in inter-leg coordination upon removal of one leg. Additionally, the result that motor output of a single segment is not a sufficient determinant for the stepping velocity of any other thoracic segment under steady walking conditions, point towards a modular organization of the stick insect walking system. Finally, the finding that only the motor activity of the most proximal leg joint is changed when walking direction is changed from forward to backward again supports existing knowledge that even the neuronal networks driving movement in each individual leg are organized in a modular structure. Therefore all these results again corroborate evidence for the modular organization of the entire stick insect walking system (for review see: Bässler & Büschges 1998; Büschges et al. 2008).

Further interesting findings of the publications are the state dependent and segment specific differences in temporal and spatial coordination. Similar results have been found in other studies that could demonstrate movement-induced temporal coordination in the stepping stick insect (Borgmann et al. 2009) or segment and state dependent differential processing of sensory information from the fCO (Hellekes et al. 2012). Taken together these results indicate that many aspects of the locomotor system are segment specific, state

dependent, and highly adaptive. Yet, it is still largely unknown how the nervous system can achieve this flexibility. The pathways by which sensory information is processed or transferred to neighboring segments are still largely unresolved and we only begin to understand the contribution of individual sensory structures to specific regulatory systems. Future studies will profit from the combination of physiological and neurogenetic approaches to investigate issues in motor control that cannot be resolved using either discipline alone.

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# 7. Teilpublikationen

**Research Articles:**

**Wosnitza A, Bockemühl T, Dübbert M, Scholz H, Büschges A** (2013). Inter-leg coordination in the control of walking speed in *Drosophila*. *J Exp Biol* 216(3):480-491.

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**Poster Abstracts:**

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# Beteiligung an den publizierten Studien

Ich habe zu den vorliegenden publizierten Arbeiten substantiell wie folgt beigetragen:

**1. Rosenbaum P*, Wosnitza A*, Büschges A, Gruhn M**

* gemeinsame Erstautorenschaft

Journal of Neurophysiology 104(3):1681-1695 (2010)

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Journal of Neurophysiology 102(2):1180-1192 (2009)

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Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Ansgar Büschges betreut worden.

Köln, den 13.05.2013

Anne Wosnitza