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

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Abstract

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 other than the previously shown modification in flexor (stance) MN activity (Gabriel and Büschges 2007). However, the pause between stance and swing motoneuron activity at the transition from stance to swing phase and stepping velocity are correlated. This pause becomes shorter with increasing speed and completely disappears 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.

Keywords: locomotion, control of speed, walking,
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 not only affects the coordination and direction of locomotion but also locomotor speed. Compared to 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 brainstem that excite the spinal 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 (Sirotta 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 (Sillar and Roberts 1993; Roberts et al. 1998) 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: Wendler 1964; Graham 1972; Graham and Cruse 1981) 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; review 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 while 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). However, recently, also a decrease in swing duration has 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 et al. 2007; Büschges and Gruhn 2008), very little is known on 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) have shown that stance phase motor neuron activity is responsible for stepping velocity, but that mechanisms for altering the velocity become effective only during an already ongoing stance phase. How exactly, however, the motor neurons and their activity patterns are affected in the course of changes in walking speed, in particular 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 (Hooper et al. 2007; Guschlbauer et al. 2007; Blümel et al. 2007; Hooper et al. 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.
Materials and 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 blackberry leaves *ad libitum* and kept at a 12h:12h 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 pro- 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 (Marquardt 1940; Graham 1985) 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 (Marquardt 1940; Graham, 1985), 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, Tamm, Germany).
To penetrate the ganglion more easily with intracellular electrodes, two approaches were taken. The ganglion sheath was either softened by quickly removing the saline from the body cavity and then treating the ganglion sheath with crystals of a proteolytic enzyme (Pronase, Merck, Darmstadt, Germany) for 60-90 s. Alternatively, 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, while 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 is 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 0.05s in our analyses.

Behavioral Experiments

For the behavioral experiments, intact animals were glued (ProTempII, ESPE, see above) ventral side down onto a balsa stick that was thinner than the width of the insect (3x5x100 mm, W x H x 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 and was adjusted to a position approximately 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 that was used to verify touch down and lift off positions for single legs has been 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 appx. 435.8 cStokes 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 monitor also 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 in order 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 either elicited 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 (diameter 130 mm, Marata screens, 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 sec 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, Stadtroda, Germany). The legs were marked at the distal end of the femur and the tibia with orange and yellow fluorescent pigments (gold-orange, catalogue #56200, and yellow, catalogue #56150, Dr. Georg Kremer Farbmühle, Aichstetten, Germany) which were dissolved in two-component glue (ProTempII, ESPE, see above). 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 LED arrays (12 V AC/DC, Conrad Electronic, 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, Vers.1.9, Mikromak service, Berlin, Germany). Figures were prepared with Origin (Vers. 6.1, Origin Lab Corp., Northampton, MA, USA) and Photoshop software (Vers.6.0, Adobe Systems Inc., San Jose, CA, USA).

**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 second 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 more than 3 data points existed (in almost all cases the analysis was based on 15-35 data points). Statistical testing was done with Origin (Vers. 6.1, see above).

The inter-leg 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 (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 extracellular and intracellular recordings as well as 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, resp.) 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 that is 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 1A, 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 (1/interspike interval, middle trace). Regression analysis showed no significant correlation of maximum FETi spike frequency with maximum stepping velocity in 13 out of 15 experiments (Fig. 1B). Similarly, mean spike frequency was not significantly correlated with mean stepping velocity in 10 out of 15 experiments (Fig. 1B). 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 while 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\text{max} = maximum instantaneous spike frequency, FETi\text{first} = first FETi spike, and Tr\text{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\text{max}- FETi\text{first}, hence 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\text{max}-Tr\text{max}, hence 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. 1C 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\text{max}) and the first FETi spike (t_{p2-t_{p1}}) was plotted against mean velocity. Figure 1D shows the results of the regression analyses for one experiment. The regression line for t_{p1} (light grey dotted line) showed a negative slope and was not significantly related to mean velocity. The regression line for t_{p2} (dark grey dashed line) showed a negative slope, but was not significantly related to mean velocity, either. 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 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 eleven out of fifteen experiments. However, t_{p2-t_{p1}} and mean belt velocity were significantly correlated in ten out of the fifteen 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 was observed in the course of this study.

Inter-leg influence in stepping velocity
From the above 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 one is mechanical coupling between the legs through the ground. A second one 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 2A 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=0.05s). During straight walking, the retractor coxae muscle acts as a stance phase muscle, while the retractor 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 respective step cycle period (Fig. 2A). The two integrals were then
plotted against each other. Figure 2B shows this plot for protractor (gray) and retractor MNs (black) in the experiment shown in figure 2A. 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 velocity and protractor MN activity was found in only four, 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 to 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 2C 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=0.05s). 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. 2C). Figure 2D shows a plot of mean stance velocity against mean activity of protractor (gray) and retractor (black) MNs for the experiment shown in figure 2C. 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 out of seven experiments a correlation between retractor MN activity and front leg stepping velocity was found, while protractor MN activity was correlated with front leg stepping velocity in only two out 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 due to 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. 3A) and contralateral (N=8, n=37; Fig. 3B) 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. 3C,D, light gray), the phasic component (dark gray), and both components together with respect to a putative dependence on the velocity of the stepping front leg. Figures 3C and D show the regression analyses for one 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.3C,D). In four out 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 5 of 8 animals (data not shown). This was equally true for stepping sequences that started autonomously as well as for those which were 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 where 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 4A 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 8 animals and for 248-390 steps per leg. The average stepping velocity for the front legs was 42.8 mm s⁻¹ (n=739; s.d. 11.9), for the middle legs 32.5 mm s⁻¹ (n=754; s.d. 9.9) and for the hind legs 31.4 mm s⁻¹ (n=501; s.d. 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. 4B). 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 0.92s (±0.29) to 1.25s (±0.46) and due to longer step lengths in the front legs, while the hind legs generally performed fewer steps and therefore also had longer cycle periods between 1.34s (±0.46) and 1.39s (±0.54). We tested whether the stepping velocities of neighboring ipsi- or contralateral legs were correlated with each other. Figure 4Ci-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. 4D, 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 results the question arose, as to the extent to which stepping velocity influences inter-leg information transfer from a stepping leg to its neighbors. Firstly, it is well known that neural inter-leg information transfer contributes to the coordination of stepping between insect legs. 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 Graham 1985; Cruse 1990).
Secondly, Borgmann et al. (2009) have 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 5A 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 = 0.05s) 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 5B (left) shows the regression analysis of mean front leg stance velocity against mean pro-meso neuronal activity in all 5 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 out 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. 5B right) was observed in only 2 out of five animals. In addition, both neck connectives showed significant increases in mean activity together with increases in stepping velocity (N=4; Fig. 5C). In summary, neural activity in the connectives was modulated with front leg stance velocity (see also Borgmann et al. 2009). From these observed correlations, however, no conclusions can be drawn at this point as 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 does either increase the firing frequency of specific neurons or activates new neurons that therefore contribute to the observed effect on the overall connective activity.

Are there, however, conditions under which the stick insect walking system uses the information on stepping velocity present in the connectives and coordinates stepping velocity between the legs neuronally? 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 6A 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. 6B). 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 at least 12 out of 18 cases (Fig. 6Ci-iii and 6D). 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 (Wendler 1964; Gabriel and Büschges 2007). In the present study we further investigated changes at the neuronal and behavioral level 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 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, upon 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 towards faster speeds while 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 investigated, whether also 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 ten out of fifteen 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 unlikely (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: inter-leg 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 the line with earlier results by Foth and Bässler (1985a,b), who have shown 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 central pattern generating networks and feedback from leg sensors (for summary see Büschges and Gruhn 2008).
As stated above, no correlation existed between the stepping velocity in the front leg and motoneuronal activity in the next posterior segment, neither in animals that initiated stepping autonomously nor 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 are not 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 inter-leg information as such are not 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 (Graham and Cruse 1981; Cruse and Epstein 1982; Epstein and Graham 1983; Cruse and Schwarze 1988; Gruhn et al. 2009). This supports the findings from our electrophysiological experiments and indicates that there is indeed very little neuronal inter-leg 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 (Hooper et al. 2007, Guschlbauer et al. 2007, Blümel et al., 2007, Hooper et al. 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.
We have been able to identify single units projecting in both directions. The quality of our recordings has not permitted us a detailed analysis of interganglionic connective activity on the single neuron level, though. One of the reasons is most likely the large number of approx. 2000 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 coordinate the stepping velocities of its legs neuronally. 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 figures 4Ci and 6Ci, 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, 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 mesothoracic motor neuron activity in deafferented 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 inter-leg 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 (Watson and Burrows 1983; Laurent and Burrows 1988; Laurent and Burrows 1989a,b), 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 coworkers which point towards co-activating influences between
neighboring stick insect legs (e.g. Cruse and Saxler 1980; Cruse 1985b). 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
1985b). This mechanism enables the animal to increase the total force propelling the body.
Interestingly, just as in our results, the co-activating 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
inter-leg 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, i.e. 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 anatomical 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, do 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 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 (Matsushima and Grillner 1992; Grillner and Wallén 2002; Grillner et al. 2007; Hocker et al. 2000; Friesen and Kristan 2007). 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 is 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 co-workers (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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Figure Legends

**Fig.1:** Extensor 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 FETi activity, while SETi and 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 time scale, with the corresponding extensor MN activity (nerve recording) and instantaneous FETi spike frequency to illustrate the definitions of \( t_{p1} \) (FETi first to ISF\(_{\text{max}}\)), \( t_{p2} \) (Tr\(_{\text{max}}\) to ISF\(_{\text{max}}\)), and \( t_{p2}-t_{p1} \) (Tr\(_{\text{max}}\) 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 \( p>0.05 \).

**Fig.2:** Inter-leg 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 3\(^{rd}\) and 5\(^{th}\) trace 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 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.
Fig. 3: Inter-leg 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: 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 (red). No correlation was observed. C: Intracellular recording of a mesothoracic MN during stepping of the contralateral front-leg. Membrane potential showed phasic modulation. D: Regression analysis of MN activity against mean front leg stance velocity. Separate calculations were performed as in B. No correlation was observed.

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 30s 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. C-i-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, resp. In figure Ci, the data points for Exp.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 is given in parentheses.

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 trace are rectified and smoothed (T=0.05s) 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 four of five experiments. The neuronal activity in the meso-meta connective was correlated with front leg mean stance velocity in two of five 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 eight experiments. Asterisks mark level of significance: *** p<0.001.

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 30s sequence, with tactile stimuli given at t=6s, t=13s and t=22s (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, resp. In figure Ci, the data points for Exp.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 is given in parentheses.
**A**

- Stance velocity [mm/s] vs. time [s]
- Front legs: black circles
- Middle legs: white squares
- Hind legs: black crosses

**B**

- Bar chart of percentage:
  - Front legs
  - Middle legs
  - Hind legs

**Ci**

- Line graphs:
  - Exp. 1*
  - Exp. 2*
  - Exp. 3*

**D**

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