Differential control of temporal and spatial aspects of cockroach leg coordination

E. Couzin-Fuchs\textsuperscript{a,b,1,2}, O. Gal\textsuperscript{a,1}, P. Holmes\textsuperscript{b,c}, A. Ayali\textsuperscript{a,d,*}

\textsuperscript{a} Department of Zoology, Tel Aviv University, Tel Aviv 6997801, Israel
\textsuperscript{b} Department of Mechanical and Aerospace Engineering, Princeton University, Princeton, NJ 08544, USA
\textsuperscript{c} Program in Applied and Computational Mathematics and Princeton Neuroscience Institute, Princeton University, Princeton, NJ 08544, USA
\textsuperscript{d} Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel

\textsuperscript{*}Corresponding author.
E-mail address: ayali@post.tau.ac.il (A. Ayali).
\textsuperscript{1} Equal contribution.

\textsuperscript{2} Current address: Department of Biology, Neurobiology, University of Konstanz, Universitätstraße 10, 78457 Konstanz, Germany.

\textbf{Article info}

Article history:
Received 19 February 2015
Received in revised form 12 June 2015
Accepted 14 June 2015
Available online 15 June 2015

\textbf{Keywords:}
Periplaneta americana
Proprioceptor
Chordotonal organs
Pymentrozine
Spatial and temporal coordination

\textbf{Abstract}

Ensembles of neuronal networks and sensory pathways participate in controlling the kinematic and dynamic parameters of animal movement necessary to achieve motor coordination. Determining the relative contribution of proprioceptive feedback is essential for understanding how animals sustain stable, coordinated locomotion in complex natural environments. Here, we focus on the role of chordotonal organs (COs), proprioceptors found in insect legs, in the spatial and temporal regulation of walking. We compare gait parameters of intact cockroaches (\textit{Periplaneta americana}) and sensory-impaired ones, injected with pymetrozine, a chemical previously shown to abolish CO function in locusts. We verify that afferent CO activity in pymetrozine-treated cockroaches is inhibited, and analyze the effect of this sensory deprivation on inter-leg coordination. We find significant changes in tarsi placement and leg path trajectories after pymetrozine treatment. Leg touchdown accuracy, measured from relative tarsi positions of adjacent legs, is reduced in treated animals. Interestingly, despite poorer spatial coordination in both stance and swing, temporal properties of the gait remain largely the same as in the intact preparations, apart from changes in ipsilateral phase differences between front and middle legs. These findings provide insights into the role of COs in insect gait control and establish pymetrozine as a useful tool for further studies of insect locomotion.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The American cockroach \textit{Periplaneta americana} (henceforth: the cockroach) is renowned for its fast and stable walking. Cockroaches commonly walk in a stereotypical double-tripod gait, in which the front and rear legs on one side of the body move in phase with the middle leg on the other side and in anti-phase with their contralateral partners, forming two alternating tripods. This gait is used over different substrates, in varied environments, and throughout most of the speed range (\textit{Delcomyn, 1971; Full and Tu, 1991}; above 10 steps/s according to \textit{Spirito and Mushrush, 1979}).

It is well established that sensory mechanisms play an instrumental role in locomotor control, affecting leg movements and gait patterns induced by central pattern-generating neuronal networks (CPGs) (\textit{See review of early work in \textit{Pearson, 1995}; also see Borgmann et al., 2009; Zill et al., 2009; Puhl and Mesce, 2010; Gorelkin et al., 2013; Ayali et al., 2015, in press; Borgmann and Büschges, 2015}). Insect legs contain various types of sensory organs and receptors, including mechanoreceptors involved in proprioception (\textit{Wilson, 1965; Mücke, 1991; Keil, 1997}). Previous studies collectively suggest that the relative contribution and importance of sensory inputs varies among different insects and across different behavioral contexts. For example, \textit{Mendes et al. (2013)} show that inactivation of sensory neurons in fruit fly legs degrades step precision, especially in slow walking, but has little effect on inter-leg coordination and specifically on the ability to execute a tripod gait. Moreover, studies in stick insects show that intersegmental activity and coordination depend on local load signals (\textit{Borgmann et al., 2009, 2011}). Previous studies in cockroaches have revealed that walking-like motor patterns can be elicited from the thoracic motor centers in the absence of sensory feedback (\textit{Pearson and Iles, 1970, 1973; Fuchs et al., 2011}). These patterns are, however, highly variable, and further studies, using semi-intact or intact preparations, have shown that sensory feedback can substantially improve coordination (\textit{Fuchs et al., 2012})

\url{http://dx.doi.org/10.1016/j.jinsphys.2015.06.007}
0022-1910/© 2015 Elsevier Ltd. All rights reserved.
Couzin-Fuchs et al., 2015 see recent review in Ayali et al., 2015). For example, Fuchs et al. (2012) showed that externally-imposed step-like movements of one leg could entail the motor pattern and reinforce coordination between motor neurons in other thoracic ganglia.

Among the sensory mechanisms that have been identified as providing information necessary for stepping are inputs related to contact with the substrate, loading/unloading, and leg position or velocity. Load-sensing mechanisms appear to play an important role in intersegmental coordination during cockroach walking. Zill et al. (2009) demonstrated that sensory activity of tibial campaniform sensilla in a middle leg closely followed stance onset of the ipsilateral hind leg, suggesting that the mechanical action of hind-leg loading facilitates the onset of swing in the middle leg through local reflexes, presumably via decrease in middle leg loads.

Position-related signals may also be important, both in controlling single legs, and in coordinating legs during walking. In particular, the chordotonal organs (COs), spanning each leg joint, function as stretch receptors and encode states of leg joints (Hofmann et al., 1985; Field and Matheson, 1998). Thoroughly studied is the metathoracic femoral chordotonal organ of the locust, which has been reported to respond to tibial position, velocity, or acceleration, or to combinations of these parameters (including the identification of the neurons sensitive to each type of stimuli; e.g. Field and Burrows, 1982; Matheson 1990a,b). Furthermore, Page and Matheson (2009) demonstrated that disruption (surgical shortening) of COs affects aimed leg movement. Further examples include displacements applied to cockroach claws in restrained preparations that activate tarso-pretarsal COs encoding angles of the most distal leg joints, thereby eliciting reflex activation of the tibial extensor muscle and a crossed extensor reflex in the contralateral leg (Larsen et al. 1997). Mendes et al. (2013) also directly investigated the effects of inactivation of leg COs in mutant fly lines, and reported several locomotion defects including an increase in the step length and a larger variability in footprint clustering. Both swing and stance duration increased, resulting in longer stepping periods, but these authors did not explicitly quantify interleg timing or phase differences. Another well-studied example from the stick insect is mediated by femoral COs attached to the femur-tibia (FTi) joint that generate local reflexes in a context-dependent manner, producing resistance reflexes at rest but assisting movements during walking (Büschges and El Manira, 1998; Hellekes et al., 2012).

Of special interest is the use of the insecticide pymetrozine as a tool in the study of the effects of proprioceptive feedback in insect locomotion. Pymetrozine was reported to selectively eliminate the function of COs in locust, stick insect, and cockroach (Kaufmann et al. 2004; Ausborn et al., 2005). In the migratory locust (Locusta migratoria), the chemical was reported to affect COs alone and no other sensory organs in the leg. According to Ausborn et al. (2005),”interneurons, motoneurons and central motor control circuitry in general did not noticeably respond to the insecticide”, nor did it significantly affect any neuromuscular components. A conspicuous effect on posture was also reported in the locust. In treated insects the body was not partially supported above the substrate (see Section 2.3), but rather rested on the floor with both hind legs extended and levated (Kaufmann et al. 2004; Ausborn et al., 2005). Stick insects showed similar responses to pymetrozine, although they were slower to develop. In particular, the effect on the resistance reflex was a reduction in the response amplitude with no change in the response phase (Ausborn et al., 2005). Kaufmann et al. (2004) offer a brief note suggesting that pymetrozine-treated cockroaches exhibited behavioral changes similar to those described for the insecticide pymetrozine-treated locusts and cockroaches, we did not observe lifting and stretching of the hindlegs in cockroaches (demonstrated by the treated locusts).

In this paper, we utilize the well-established effects of pymetrozine on COs described above. We continue to investigate sensory contributions to coordination, by pharmacologically reducing leg proprioception and studying its effects on locomotion in otherwise intact insects. The cockroach model is of special interest in this respect due to its fast and stable gait on the one hand, and to the recognized complex role of sensory feedback, on the other (Ayali et al., 2015, in press). We hypothesized that sensory-impaired cockroaches will demonstrate alterations in both temporal and spatial aspects of their walking gaits, expecting the latter to be more pronounced because temporal coordination is thought to be under more dominant control of the central pattern generating networks. Following electrophysiological validation of the effects of pymetrozine, we compared gait characteristics before and after treatment in the same animals, tethered and walking on a slippery surface, using video analysis and leg tracking techniques. Spatial and temporal properties of gaits were also compared with those of freely-walking preparations and animals receiving a control treatment.

2. Methods

Experiments were conducted on healthy and intact adult cockroaches (P. americana) obtained from our colony at the Department of Zoology, Tel Aviv University. Animals were kept at a controlled temperature of 30°C and 35–60% humidity, in a 60 L container with cardboard shelters, fed with dry cat food (LaCat, BioPet Israel) and water ad libitum.

2.1. Pymetrozine treatment

Pymetrozine Pestanal® (Sigma–Aldrich) in dry crystalline form was first dissolved in DMSO (dimethylsulfoxide) to create a stock solution, and then diluted with cockroach saline (NaCl: 214 mM; CaCl: 9 mM; KCl: 3.1 mM; TES: 10 mM; pH: 7.2) to a final concentration of 10⁻³ M. The target dose for application was 0.5 μg of dry pymetrozine per 1 g of body mass (Ausborn et al., 2005). For example: a cockroach that weighs 3 g would be injected with 7 μL of 10⁻³ M solution containing approximately 1.5 μg of pymetrozine. A vehicle solution was created by diluting DMSO in cockroach saline 1:100, to serve as control. Pymetrozine (or vehicle solution) was applied by injection between the first and second abdominal segments, using a Hamilton micro-syringe.

2.2. Neurophysiological procedures

Cockroaches were decapitated and pinned onto a cork platform, ventral side up, with their front and middle legs cut off. The hind legs were fixed to the platform with the FTi joints at an angle of 90°, which approximates mid-range of movement at the FTi (as in Brodhuber and Fournier, 1983). A window was then cut into the femur of the hind leg to reveal the apodeme of the femoral chordotonal organ (FeCO), which was attached via a custom-made clamp to a micromanipulator (Narishige, Japan). The clamp was positioned so as to stretch and release the FeCO apodeme in its natural orientation, and attached to a force transducer (Harvard APP Ltd., Holliston, MA, USA) to monitor the mechanical stimulations.

To access afferents from the FeCO, a rectangular section of cuticle was removed at the distal part of the coxa and trochanter to reveal sensory branches of nerve 5 arising from the COs at the proximal part of the femur. To isolate the sensory responses, nerve 5 was cut near the thorax-coxa joint. In a second set of
preparations we recorded activity from nerve 3B, which innervates the extensor tibiae, accessing it through a section cut in the ventral side of the thorax. In both sets of preparations, we used bipolar silver hook electrodes for extracellular recordings while stretching and relaxing the FeCO apodeme, before and after pymetrozine treatment.

The response to FeCO stimulations was monitored by extracellular recordings from sensory afferents in four animals and from motor units controlling tibial extension in four different animals. In all tested preparations, we first observed responses to FeCO stimulations (i.e., increase in afferent activity and the presence of resistance reflexes) before pymetrozine injection during four stimulation sequences, each of five repetitive stimuli at circa 0.5 Hz, separated by intervals of at least 5 min. Then pymetrozine was injected and 30 min later, activity in response to stimulation sequences was monitored in a similar manner. Pre-injection activity and responses were practically unchanged for at least two hours in a preparation that was monitored for a longer period. The clamp displacement was monitored by the output of the force transducer to indicate stimuli onsets and durations, and to verify that displacement magnitudes were comparable in all experiments. In a preliminary set of trials, responses to different levels of clamp displacements were tested and the one selected for all experiments was estimated to be of 1.5 mm.

All electrophysiological recordings were amplified through a 4-channel differential amplifier (Model 1700, A-M systems, WA, USA), digitized by an A-D converter (Digidata 1200, Molecular Devices, CA, USA) using Axoscope PC software (Molecular Devices, CA, USA) at 10,000 Hz, and analyzed off-line with DataView software (W.J. Heitler, University of St. Andrews, UK).

2.3. Tethered walking setup

Cockroaches were briefly anesthetized in CO₂, their wings removed at the base, and a small magnet glued to their pronotum. Animals were attached via the magnet to a lifting arm, and then lowered onto a flat, horizontal glass surface covered by a thin layer of glycerol, until standing on the glass in a natural walking posture (but supporting only part of their weight). Lighting was placed at either side of the tethered preparation (turned off between trials to avoid overheating the animals). A high-speed camera (Prosilica GT, Allied Vision Technology, Strafordt, Germany) was positioned below the glass surface, monitoring the tethered cockroach from its ventral side at an acquisition rate of 200–320 frames/s. Data were recorded with StreamPix5 video software (Norpix Inc., Quebec, Canada) for later offline analysis. After a short acclimatization period, 2–4 sequences of tethered walking (8–15 steps each) were recorded. If needed, animals were induced to walk by gentle irritation of the cerci or rear abdomen. Next, the cockroaches were injected with pymetrozine (or vehicle solution for control) and left to rest in dark conditions for 30 min, before recording 2–4 additional sequences of tethered walking.

Recorded images were analyzed offline using custom tracking software developed in Matlab (Mathworks Inc., MA, USA) for automated digitization of tarsi and body positions, body orientation, and computation of tarsi positions in body coordinates, hereafter “tarsi coordinates” (for details see Couzin-Fuchs et al., 2015).

2.4. Analyses of spatial and temporal kinematics

Leg tarsi tip coordinates were used to analyze the preparations’ leg kinematics. Stance was defined as starting at the tarsus tip’s anterior extreme position (AEP) and ending at the posterior extreme position (PEP); and swing as starting at the PEP and ending at the AEP (Cruse, 1976; Graham, 1985); stance and swing together constitute the step cycle. To quantify the spatial and temporal properties of leg movements we used the following metrics:

- **Phase**: Time lag between the AEP time of a leg divided by the step cycle duration of that leg.
- **Phase difference**: Time lag between the time of AEP of two selected legs divided by the step cycle duration of the first.
- **Stance deviation**: Euclidean distances were computed between digitized points on a leg’s tarsus tip trajectory during stance though each step and the extrapolated point with the same phase for the trajectory averaged over the walking sequence. These distances were then summed over three walking sequences and their mean squares computed for each preparation. Note that this differs from the stance linearity index of Mendes et al. (2013), which is based on deviations from a piecewise linear “5-point smoothed line” calculated for each stride.
- **Swing deviation**: Defined as above, but for swing trajectories.
- **AEP clustering**: Euclidean distances between all AEP locations and the AEP location averaged over a walking sequence were computed for each leg and these distances were averaged over three walking sequences for each preparation.
- **PEP clustering**: Defined as above, but for PEP locations. These are the same as footprint clustering in Mendes et al. (2013).
- **Metachronal Lag**: Time interval between a hind leg swing onset and the next swing onset of the ipsilateral foreleg (following Graham, 1972; Mendes et al., 2013).
- **Spatial and temporal coordination precision indices**: Deviations in Euclidean distances and temporal differences between a leg’s AEP and the PEP of the leg in front of it were computed. For each walking sequence the average spatial and temporal differences were calculated for each leg pair and the deviations from the corresponding averages were used to produce a distribution of deviations for each experimental condition.
- **Step Length**: Distance on the longitudinal axis between a leg’s AEP and PEP. Data show average step lengths calculated for each leg and each walking sequence separately, plotted vs. stepping frequency of that sequence.
- **Stance and Swing durations**: Time intervals between each leg’s AEP and PEP for each stepping sequence.
- **Duty cycle**: Ratio between stance duration and cycle (stance + swing) duration.

In the figures that follow all distances are given in body lengths (b.l.) to normalize for insect size, and times are normalized by stepping cycle duration (c.d.). The supplementary figure shows stance and swing durations (in msec) and duty cycles plotted vs. stepping frequency.

2.5. Analysis of free-walking traces

Walking gaits of tethered preparations were also compared with those of freely-walking insects. To obtain the latter, seven cockroaches were released to run along a 600 × 70 mm Plexiglass tunnel, while the camera placed below captured their motion at 350 fps. Images were then analyzed to detect locations of all tarsi tips and body centroids, and tarsi trajectories relative to the body center were used to analyze temporal parameters, as for the tethered sequences. For details on the free walking setup and image digitization see Couzin-Fuchs et al., 2015.

3. Results

3.1. The effects of pymetrozine on cockroach leg chordotonal organs

Before investigating the effects of pymetrozine injection on walking, we studied its physiological influence on cockroach leg FeCos in order to compare this with the effects previously
the time-frame of our experiments. The resistance response in the meta-thoracic FeCOs evoked by stretching and relaxing the FeCO apodeme, as well as monitoring FeCO-induced motor resistance responses.

Fig. 1A shows a typical recording made from nerve 5 at the coxa-trochanter joint, after cutting the nerve proximal to the electrodes. The FeCO apodeme was mechanically stretched and released with natural amplitude and orientation. As can be seen, in the control recordings, afferent activity reliably followed FeCO lengthening but response was practically absent when tested 30 min after pymetrozine injection. Similar responses were seen in all tested preparations (n = 4). No time-dependent failures of the FeCO response were observed in untreated preparations in the time-frame of our experiments.

The resistance response in the tibial extensor motor neurons was also investigated. Before treatment, the FeCO-induced motor response evoked by stretch or relaxation of the FeCO typically resulted in activation or deactivation, respectively, of fast and slow extensor tibiae motor neurons (FETi and SETi, mostly the latter) and common inhibitor units, which can be monitored from the meta-thoracic nerve 3B (Pringle, 1939; Brodfuehrer and Fourtner, 1983); see Fig. 1B (upper panel). After confirming a consistent response in a series of stimulations, the cockroach was injected with pymetrozine. This resulted in a complete failure to evoke the response or to demonstrate any FeCO-induced effect: responses were replaced by tonic nerve firing regardless of the FeCO state, as seen in Fig. 1B (lower panel). Average (± stdev) spike densities of large units observed at the motor nerve containing the tibiae motor neurons before pymetrozine injection were 0.59 ± 0.51 and 14.89 ± 3.14 spikes/s in the relaxed and stretched conditions, while after injection values were 9.10 ± 4.97 and 8.51 ± 2.86 spikes/s in the two conditions respectively. These results demonstrate that the dose of pymetrozine used in the present study affects cockroaches in a similar way to that reported for locusts (Ausborn et al., 2005), and, more importantly, suggests that the pymetrozine-treated cockroaches are largely deprived of functional proprioceptive sensory feedback arising from leg COs.

3.2. Effects of pymetrozine on cockroach leg kinematics

To investigate the functional consequences of the reduction of proprioceptive signals, we first compared the stepping frequency of treated and control animals during tethered walking on a slippery surface. Eight animals were treated with pymetrozine, and seven received vehicle solution as control. For each animal, 2–4 sequences of repetitive stepping, each containing at least eight steps were recorded, before and 30 min after treatment, using a high-speed camera (see supplementary videos). Tarsi locations were detected and leg kinematics of recorded sequences were comparatively analyzed for 46 sequences from pymetrozine-treated and for 40 from control animals. Step frequencies ranged from 5 Hz to 22 Hz, within the range of free-walking step frequencies in which the tripod gait is predominant (Delcomyn, 1971). Mean stepping frequency and estimated forward speed (computed as the average stance length of all legs multiplied by the stepping frequency) were calculated for each animal and the ratios of stepping frequencies and estimated speeds after/before treatment were compared. Mean frequencies decreased slightly and mean stance lengths and speeds increased slightly after treatment, but no significant differences in frequency or speed were found between the two experimental groups (p = 0.3854 and p = 0.4958; Mann–Whitney U test, see supplementary figure for details), providing no evidence for an effect of pymetrozine on stepping frequency or walking speed. Similarly, no differences were found in stance duration, swing duration, and duty cycle in stepping sequences before and after pymetrozine application and in their dependence on stepping frequency (supplementary figure).

3.2.1. Spatial metrics of leg trajectories

We next analyzed the treatment effects using the spatial metrics as detailed in Section 2.4. Fig. 2A shows examples of tarsi trajectories obtained from a single preparation during one stepping sequence before pymetrozine injection, and one after, illustrating substantial differences in both stance (inner arcs of traces) and
swing (outer arcs). Including all tested preparations, no significant differences were found in mean step lengths (see supplementary figure for details), but step variability appears to dramatically increase upon Pymetrozine treatment. Fig. 2B–E summarize data from all walking sequences in control and pymetrozine-treated animals. Panels B and C show differences between stance and swing deviations from control for each leg in body length units. Box heights in all panels denote individual leg differences (after/before injection) averaged over all control preparations (left on each panel) and for pymetrozine-treated preparations (right on each panel). Panels D and E show AEP and PEP clustering metrics for individual legs, presented in the same format.

Fig. 2. Spatial parameters. A. Representative plots of tarsus trajectories during sequences of tethered walking as imaged from below, before and after pymetrozine injection; stepping frequencies were 16.8 and 16.5 Hz respectively. Tarsi locations are normalized to body length (b.l.) and center of mass is at (0,0). Trajectories during stance and swing phases are shown separately for clarity. Cockroach silhouette is shown to give general relative position of head and body. B–E. Treatment effects on spatial precision of leg trajectories. Stance and swing deviations (panels B and C) and AEP and PEP clustering (panels C and E) were calculated as described in Section 2.4. Plotted values are differences from control, expressed in b.l., between deviations of sequences before and after treatment in the same preparation, shown for control (vehicle only) and pymetrozine-treated groups. Positive values show increase in variability, hence decrease in precision. Error bars denote standard error of the mean. Asterisks indicate statistical significance between the pymetrozine and control group using Friedman’s Two-way analysis of variance (*p < 0.05 and **p < 0.01). In panels B–E, 0 denotes averages over all legs in control sequences.
3.2.2. Temporal parameters and coordination precision

We next compared temporal coordination among pairs of legs in the pymetrozine-treated animals to control animals and also to data obtained from freely running intact animals (see Section 2.5). We first calculated metachronal lag as described in Section 2.4, and plotted it as a function of hind-leg period. This metric characterizes a key temporal feature of the gait (following Graham, 1972; Mendes et al., 2013, 2014). Fig. 3A reveals that metachronal lags closely follow the hind-leg periods, as is typical for the tripod gait (Graham, 1972), and suggests that there are only minor differences among the three groups.

Leg coordination is often investigated in terms of timing or phase differences among leg pairs (Delcomyn, 1971; Graham, 1972; Mendes et al. 2013; Couzin-Fuchs et al., 2015). Histograms of phase differences between AEPs of the front right leg (R1) and all other legs (R2...L3) are plotted in Fig. 3B. Again, data are presented for free-walking, control, and pymetrozine-treated cockroaches. Phase differences in all three groups are again consistent with a tripod gait, with phase differences between adjacent contralateral and ipsilateral leg pairs of approximately 0.5, and between ipsilateral front and hind legs of approximately 0 (see also supplementary videos). However, although the distributions displayed by all three groups have mean values typical of tripod gaits, significant differences exist between four of the control and pymetrozine-treated groups. In order to better understand this, we compared phase differences between all adjacent leg pairs (nearest neighbors) in control and pymetrozine-treated preparations (parametric two-way ANOVA test for circular data). The results, displayed in Table 1, show that only the ipsilateral front-middle leg phase differences are significantly affected; differences between adjacent middle-hind ipsilateral and all contralateral pairs are insignificant, indicating that differences between longer range contralateral pairs (such as R1–L2 or R1–L3 in Fig. 3B) result from differences in the ipsilateral connections (R1–R2). As was previously shown by Tryba and Ritzmann (2000), gait timing remains similar in free-walking and untreated tethered preparations. Pymetrozine-induced reduction of feedback from COs resulted in significant changes in phase differences only between front and middle ipsilateral legs and not contralaterally.

Finally, spatial and temporal inter-leg coordination were studied independently, using the coordination precision indexes defined in Section 2.4. These metrics quantify deviations from precise foot placement, and provide additional details beyond the metachronal lag data of Fig. 3A, which display temporal

---

**Fig. 3.** A. Temporal parameters. Duration of the metachronal lag (time difference between sequential swing onsets from ipsilateral hind-to forelegs; see cockroach cartoon on the right) as a function of hindleg period for free-walking (Free Run), in comparison to control and pymetrozine-treated (both tethered, after vehicle or pymetrozine injection). Regression lines (Metachronal Lag = Period) correspond to a perfect tripod gaits. B. Histograms of phase differences between leg pairs (R1 and all other legs; see cockroach cartoons on the right). Data for all panels were taken from all steps in all tested sequences of free-walking animals (n = 7), control and pymetrozine-treated animals (n = 7 and n = 8 respectively). Note that ranges of abscissae are plotted so that histograms are centered both for approximately in phase (0) and antiphase (0.5) pairs. Significance levels of pairwise comparisons of phase difference distributions using ANOVA for circular data test are marked by asterisks *p* < 0.05.
coordination only between ipsilateral hind and front legs (in deviations from the regression lines). Spatial indices are shown in Fig. 4A and temporal indices in Fig. 4B. As in Fig. 2, spatial deviations are normalized to body length (b.l.); and temporal deviations are normalized to stepping cycle duration (c.d.). The results confirm that, despite the significant decrease in spatial coordination between all ipsilateral leg pairs resulting from CO inactivation, temporal coordination is practically unaffected (p < 0.01 or p < 0.005, non-parametric Kruskal–Wallis test).

4. Discussion

In this study we examined the role of proprioceptive feedback in cockroach locomotion by pharmacologically hampering sensory organs that normally provide information on position and velocity of leg joints. Specifically, we have shown that pymetrozine-induced elimination of sensory inputs from chordotonal organs (COs) significantly degrades spatial properties of leg coordination, but has little effect on leg timing or interleg phase differences during straight tethered walking. Untrained animals, tethered and walking on a slippery surface, stepped in a prototypical double tripod gait with distinctive phasing among the legs in terms of time and position, similar to those of intact free-walking preparations. Pymetrozine treatment increased the variability of spatial trajectories of individual legs and decreased their targeting accuracy, quantified via relative foothold positions of neighboring legs, but had little effect on interleg phase relationships. Pymetrozine has proven to be a useful tool in the study of the effects of proprioceptive feedback in insect locomotion.

The effects of pymetrozine reported here are in general agreement with previous findings in locust, stick insect, and cockroach (Kaufmann et al., 2004; Ausborn et al., 2005), as summarized in the Introduction. In contrast to the observation of Kaufmann et al. (2004) regarding pymetrozine-induced behavioral changes in cockroaches, in our experiments treated cockroaches walked using all six legs and no hind leg levation was observed (see supplementary videos). While we cannot account for this discrepancy, our tethered preparations allowed us for the first time to thoroughly investigate the effects of the confirmed pymetrozine-induced sensory deprivation on walking kinematics, including temporal and spatial characteristics, in the cockroach, a quintessential “walking machine”.

Our general findings in the cockroach are consistent with the recent study of sensory-deprived Drosophila melanogaster (Mendes et al., 2013), in which inactivation of proprioceptive pathways in the flies’ legs led to deficient step precision, while inter-leg coordination and the ability to execute a tripod gait were only slightly compromised. Mendes et al. (2013) used the nanchung genetic line of flies, in which COs are inactivated in all legs, and the 540×> TNT line, in which all leg proprioceptors, including hair plates, campaniform sensilla and mechanosensory bristles are inactivated. They found decreases in spatial step precision similar to our results, but slower walking speeds, and noted that “gait parameters and interleg swing phases were largely normal” but without explicitly quantifying interleg timing or phase differences. They suggest that central coupling among segmental subunits of the CPG largely preserves interleg coordination, and speculate that flies resort to a CPG-based coordination when all legs are equally impaired. This does not exclude the possibility that proprioception plays an important role at yet lower speeds.

Research on stick insects has shown that a leg’s touch-down location is targeted towards the stance position of the more anterior leg, especially in the case of the hind and middle legs (Cruse, 1979; Wosnitza et al., 2013). Information on the anterior leg’s posture and position is mediated through local leg proprioceptors including coxal and trochanteral hair plates and the femoral COs (Cruse et al., 1984), transferred via interneurons passing through the ipsilateral connective (Brunn and Dean, 1994). Similar targetting behavior, especially between the hind and middle legs, was exhibited in our cockroach preparations and was degraded by pymetrozine treatment, consistent with the involvement of COs in signaling foothold positions. Interestingly, corresponding times of leg touch-downs relative to the PEP of the neighboring anterior leg appeared unaffected, although their relative positions did change.

It is widely accepted that many among the plethora of mechanoreceptors found both internally and externally in leg joints, could assist in coordination through signals arriving at different phases during the stepping cycle. How these diverse inputs are integrated and complemented, which work in parallel and which are range fractionated (i.e., operating at different phases of the walking cycle) is still largely unknown. Leg COs have been shown to provide information on the position, velocity, acceleration and direction of movement of leg segments and of the entire leg. Regarding other mechanoreceptors which may be instrumental in the control of walking, we should note that the insects in our tethered setting did not entirely bear their own weight. This implies that inputs related to load, mediated by campaniform sensilla, which are known to be important for local leg reflexes (e.g., Zill and Moran, 1981a,b) and also affect the CPG (Zill et al., 2004), were also limited. This condition applied, however, to both our control and pymetrozine-treated groups.

Requirements for sensory information change depending on behavioral contexts and the environment in which they are performed. In another leading model of rhythmic behavior – locust flight, it was found that although the rhythmic motor pattern is generated by the CPG (Wilson, 1961), a multitude of sensory inputs are crucial to maintain ongoing behaviors (e.g., stretch receptors, Burrows, 1975, tegula, Fischer and Ebert, 1999; wind sensitive hairs, Tyer et al., 1979; visual inputs, Taylor, 1981a,b). Further demands arise from the speed of locomotion. Slow walking, with frequent halts, relies on static stability via multi-leg ground contact and reaction forces supporting the body, but as speed increases the body’s center of mass can escape the support of stance feet and aerial phases may develop (Full and Tu, 1991; Ting et al., 1994). In these regimes, gaits must be dynamically stable. In the case of cockroaches there is experimental evidence (Jindrich and Full, 2002), supported by mathematical models (Kukillaya and Holmes, 2009; Kukillaya et al., 2009), that initial recoveries from large perturbations are due to mechanical reaction forces. While monosynaptic reflexes can elicit changes in motor neuron outputs within 10–15 ms (Höltje and Hustert, 2003), muscle forces take considerably longer to develop, so we expect proprioceptive feedback to be less dominant in fast running. Our results confirm that, while inactivation of CO sensors increases spatial variability of leg motions, it has little effect on interleg timing.

Table 1

<table>
<thead>
<tr>
<th>Leg pair</th>
<th>Free vs. before</th>
<th>Before vs. after</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1–L1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2–L2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3–L3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1–R2</td>
<td></td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>R2–R3</td>
<td></td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>L1–L2</td>
<td></td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>L2–L3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4A and temporal indices in Fig. 4B. As in Fig. 2, spatial deviations are normalized to body length (b.l.); and temporal deviations are normalized to stepping cycle duration (c.d.). The results confirm that, despite the significant decrease in spatial coordination between all ipsilateral leg pairs resulting from CO inactivation, temporal coordination is practically unaffected (p < 0.01 or p < 0.005, non-parametric Kruskal–Wallis test).
and adjacent leg phase relationships, supporting the conjecture of Mendes et al. (2013) that the CPG dominates the latter behavior.

The current findings add specificity to the intriguing concept of differential control of temporal and spatial aspects of leg coordination in insects. They provide a further step in characterizing the multiple effects of specific sense organs on the complex walking behavior of the cockroach (cf. Ayali et al., 2015). A more complete view of the relative role of sensory control in insect (and other animal) locomotion will likely arise from comparative studies, using different preparations, in different behavioral contexts, in combination with mathematical modeling as well as computer simulations and mechatronic realizations in legged robots.

Fig. 4. Nondimensional spatial (A) and temporal (B) deviations from average successive foot placement in terms of differences between a leg’s AEP and the PEP of its ipsilateral foreleg (see cockroach cartoon within each panel). Deviations are given in body lengths (b.l) and cycle periods (c.d.). Histograms show distributions for control animals (blue) and pymetrozine-treated animals (red). Asterisks indicate treatment effect significance, using non-parametric Kruskal–Wallis test, **p < 0.01, ***p < 0.005. Area plots are shown, rather than bar histograms, to allow better illustration of data congruence and differences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Acknowledgments

This work was supported by the US-Israel Binational Science Foundation (Grant number 2011059), and the US National Science Foundation NSF-CRCNS (Grant number DMS-1430077).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.06.007.

References

Mendes, C.S., Bartos, I., Akay, T., Märka, S., Mann, R.S., 2013. Quantification of gait parameters in freely walking wild type and sensory deprived Drosophila melanogaster. elife 2, 1–24.