

RESEARCH ARTICLE

Speed-dependent interplay between local pattern-generating activity and sensory signals during walking in *Drosophila*

Volker Berendes¹, Sasha N. Zill², Ansgar Büschges^{1,*} and Till Bockemühl^{1,*,‡}**ABSTRACT**

In insects, the coordinated motor output required for walking is based on the interaction between local pattern-generating networks providing basic rhythmicity and leg sensory signals, which modulate this output on a cycle-to-cycle basis. How this interplay changes speed-dependently and thereby gives rise to the different coordination patterns observed at different speeds is not sufficiently understood. Here, we used amputation to reduce sensory signals in single legs and decouple them mechanically during walking in *Drosophila*. This allowed for the dissociation between locally generated motor output in the stump and coordinating influences from intact legs. Leg stumps were still rhythmically active during walking. Although the oscillatory frequency in intact legs was dependent on walking speed, stumps showed a high and relatively constant oscillation frequency at all walking speeds. At low walking speeds we found no strict cycle-to-cycle coupling between stumps and intact legs. In contrast, at high walking speeds stump oscillations were strongly coupled to the movement of intact legs on a one-to-one basis. Although during slow walking there was no preferred phase between stumps and intact legs, we nevertheless found a preferred time interval between touch-down or lift-off events in intact legs and levation or depression of stumps. Based on these findings, we hypothesize that, as in other insects, walking speed in *Drosophila* is predominantly controlled by indirect mechanisms and that direct modulation of basic pattern-generating circuits plays a subsidiary role. Furthermore, inter-leg coordination strength seems to be speed-dependent and greater coordination is evident at higher walking speeds.

KEY WORDS: Motor control, Locomotion, Insect walking, Sensory feedback, Inter-leg coordination

INTRODUCTION

Insects have to coordinate the rhythmic motor activity of their six legs during walking to produce a movement pattern that can reliably propel the animal forward. On the lowest behavioral level, this requires that each leg joint generates a motor output that results in coordinated return and power strokes (intra-leg coordination). On an intermediate level, all six legs have to be coordinated with each other (inter-leg coordination). On the highest level, the walking pattern has to be adaptable with regard to walking speed and direction. Currently, it is often assumed that the motor output

produced during walking in insects depends on three interdependent processes.

Intra-leg coordination is thought to result from interplay between the activity of central pattern generators (CPGs) and sensory signals originating in the legs (Büschges et al., 1995). CPGs provide rhythmic activity, which is modulated on a cycle-to-cycle basis by afferent signals providing information about the current state of the legs. There is evidence that intra-leg coupling between CPGs responsible for the movements of individual joints is mainly established via sensory signals (Bässler and Büschges, 1998; Akay et al., 2001; Büschges, 2005; Büschges and Gruhn, 2007). Inter-leg coordination has mainly been investigated in behavioral studies in the stick insect and cockroach (e.g. Foth and Bässler, 1985a,b; Delcomyn, 1991a,b). These studies suggest a set of behavioral rules governing inter-leg coordination (Cruse, 1990) based on sensory signals that are active locally between legs. Forces exerted upon the substrate may also strongly influence inter-leg coordination (Zill et al., 2009). Finally, it is hypothesized that the activation of locomotor CPGs is controlled by descending signals whose magnitudes determine walking speed (Orlovsky et al., 1999). In insects, these signals probably originate in the sub-esophageal ganglion (Graham, 1979; Kien, 1983; Kien and Williams, 1983; Ridgel and Ritzmann, 2005). The question of whether they target CPGs directly has not been addressed conclusively; there is evidence, however, that they influence the processing of sensory feedback (Sauer et al., 1997; Gabriel and Büschges, 2007).

Temporal coordination between all six legs depends on walking speed (Wendler, 1964; Wilson, 1966; Graham, 1972; Gruhn et al., 2009; Wosnitza et al., 2013). During slow walking, insects use a coordination pattern called wave gait (Hughes, 1952). At intermediate speeds, insects use tetrapod coordination (Graham, 1972), and at high speeds, they use tripod coordination (Graham, 1972; Strauss and Heisenberg, 1990; Wosnitza et al., 2013; Wahl et al., 2015). An increase in walking speed is accompanied by a decrease in power stroke duration, while return stroke duration is kept relatively constant (e.g. Wosnitza et al., 2013). Furthermore, behavioral studies suggest that two neighboring legs are never in return stroke simultaneously (Cruse, 1990; Dürr et al., 2004). Therefore, at high walking speeds, return strokes have to be timed precisely so that they coincide with the short power strokes of neighboring legs.

To investigate the roles of these processes, it is necessary to measure them independently during walking. A method of differentiating more clearly between local, pattern-generating activity and sensory influences is leg amputation. Amputation removes sensory structures, thus reducing intra-leg sensory influences. The remaining stump cannot reach the ground anymore, which eliminates mechanical influences and load. Studies in the cockroach (Hughes, 1957; Delcomyn, 1991a,b; Noah et al., 2004) and the stick insect (Wendler, 1964; Graham, 1977) have shown that animals adapt leg coordination after leg amputation. These studies also report that stumps continue

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List of abbreviations

AEP	anterior extreme position
BL	body length
CPG	central pattern generator
DEP	dorsal extreme position
PEP	posterior extreme position
PP	polypropylene
VEP	ventral extreme position

oscillatory movements during walking. These oscillations are usually interpreted to be mainly driven by local pattern-generating circuits, owing to the reduction of intra-leg sensory signals. Previously, we used leg amputation in *Drosophila* to investigate its effect on coordination (Wosnitza et al., 2013), but did not analyze the residual stump activity.

Drosophila is a vigorous walker with a large range of walking speeds (Strauss and Heisenberg, 1990; Mendes et al., 2013; Wosnitza et al., 2013). Here, we used leg amputations in *Drosophila* to investigate the roles that local pattern-generating networks and inter-leg sensory signals play in the generation of motor activity during walking. We analyzed the kinematics and coordination of single front, middle and hind leg stumps with respect to ipsilateral intact legs and how this dependence is influenced by walking speed. We investigated how the frequency of stump oscillations depends on walking speed, i.e. whether local rhythm generation is modulated by descending signals. A change in the stump oscillation frequency would indicate that descending signals might target rhythm-generating networks directly. We also analyzed coordination between stumps and ipsilateral intact legs. This addresses the question of how inter-leg coordination is achieved with regard to local pattern-generating activity. Furthermore, it is known that coordination during walking in *Drosophila* becomes more precise as walking speed increases (Wosnitza et al., 2013). How this is achieved is unknown; therefore, we analyzed phase relations and absolute temporal coordination between intact legs and stumps to characterize the role of speed-dependent coupling between pattern-generating networks.

Leg stumps were always rhythmically active during walking. Movement periods of all stumps were relatively constant over the

whole walking speed range and on the order of approximately 100 ms; in intact legs, this value was only reached during fast walking. This independence suggests that descending control of walking speed does not target CPGs directly but might act via modification of sensory signals. Second, the high natural frequency of the rhythm-generating networks facilitates coordination at high walking speeds. This is supported by our finding that stumps became strictly coordinated with the activity in intact legs at high speeds. In contrast, during slow walking, stumps oscillated faster than intact legs and cycle-to-cycle coordination was absent; however, we still found preferred time intervals between lift-off events in intact legs and onset of levection or depression in the stumps. This indicates that inter-leg coordination signals are also active during slow walking; these signals seem to be more effective at high speeds though, when oscillation frequencies are similar and entrainment can therefore occur more easily (Kuramoto, 1984; Pikovsky et al., 2003).

MATERIALS AND METHODS**Animals**

We used 4- to 6-day-old males of the *Drosophila melanogaster* wild-type strain Canton-S (*wt^{CS}*). Flies were raised on standard medium with cornmeal, yeast, agar and molasses. Flies were kept at 23 to 25°C and 60% humidity on a 12 h:12 h light:dark cycle.

Experimental setup

Flies were kept isolated for 1 to 2 h before an experiment. During that time, flies were kept without food, but had access to wet filter paper providing water. We used either animals in which all six legs were intact (Fig. 1B) or ones in which we amputated one leg (right front, middle or hind leg) at the proximal half of the femur (Fig. 1C–E). The remaining stump could not reach the ground even if it was extended downward (Fig. 2B). Legs were removed with micro-scissors during cold anesthesia; isolation and food deprivation was identical to conditions for intact flies.

Before experiments, flies were cold-anesthetized again and placed in a groove in a cooled aluminum block. Using a dissecting microscope, a copper wire (diameter 0.15 mm) was attached to the dorsal side of the thorax with light-curing glue

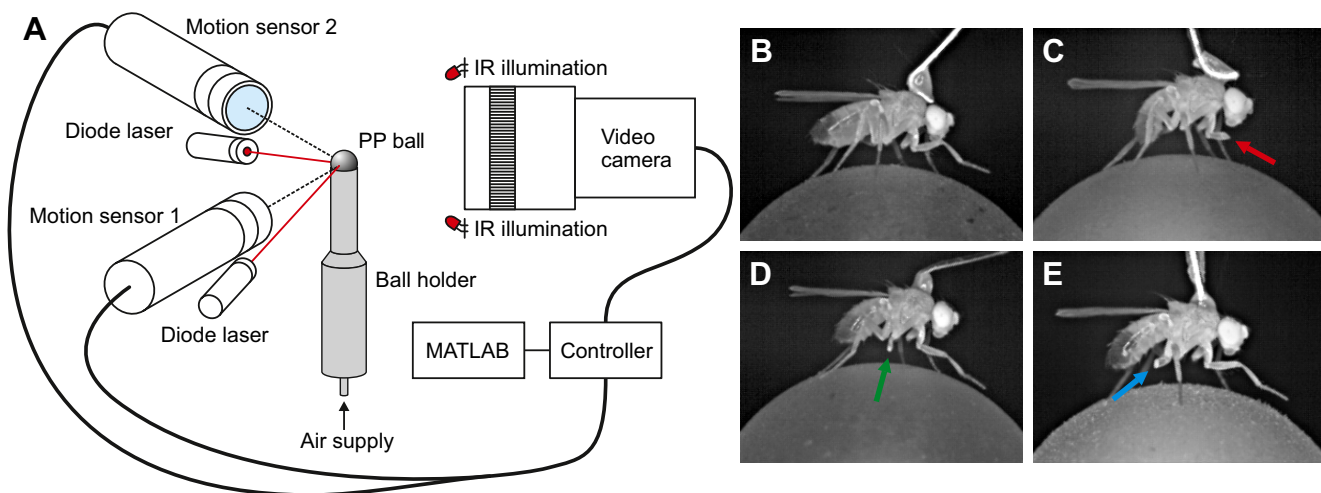


Fig. 1. Experimental setup. (A) Schematic of the experimental setup. Flies were tethered, placed on an air-supported 6 mm polypropylene (PP) ball, and recorded with a high-speed video camera at 500 Hz from the right side. A custom-built pulsed IR-LED ring around the camera lens illuminated the fly. Movements of the ball were measured by two optical sensors with a method similar to the one described previously (Seelig et al., 2010). We either used intact flies (B) or front, middle or hind leg amputees (C, D and E, respectively).

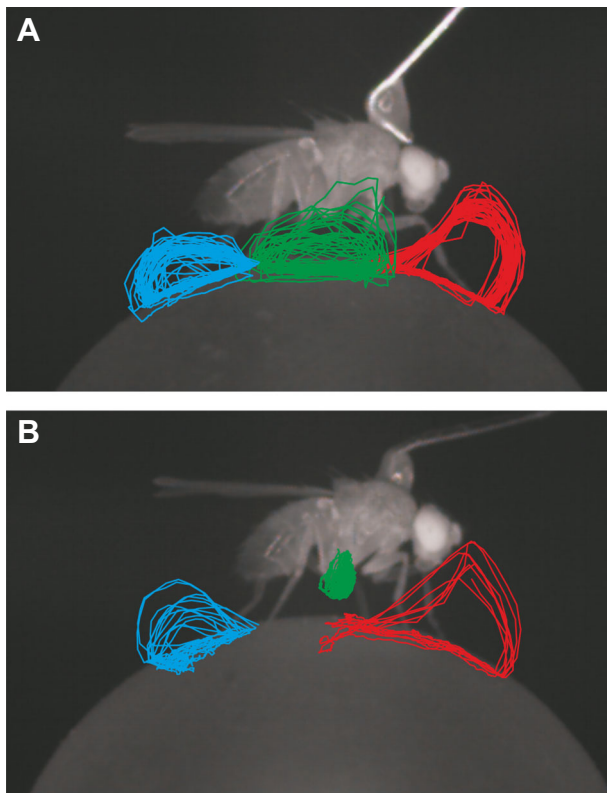


Fig. 2. Exemplary trajectories of tarsi and the stump tip. Here, this is shown for an intact fly (A) and a middle leg amputee (B), respectively. Trajectories were measured during single trials and superimposed on one frame of the corresponding video [red: front leg trajectory, green: middle leg (A) or stump (B), blue: hind leg].

(ESPE Sinfony, 3M ESPE AG, Seefeld, Germany) cured with a curing light (wavelength ~ 460 nm). The copper wire was then inserted into a holder attached to a 3D-micromanipulator and flies were positioned atop an air-suspended polypropylene (PP) ball (diameter: 6 mm, Spherotech GmbH, Germany). The wire was rigid relative to the fly's weight and the forces it could exert on the ball. Consequently, the orientation and ground clearance of the fly with regard to the ball was constant during the experiment.

Fig. 1A shows a schematic of the setup. A high-speed camera (AOS S-PRI High Speed Color 5.2, AOS Technologies, Baden Daettwil, Switzerland) placed to the right of the fly recorded its behavior (500 Hz, spatial resolution: $17\ \mu\text{m}$). The camera was operated by AOS Imaging Studio V3 (AOS Technologies, Baden Daettwil, Switzerland). A custom-built infrared-LED ring (wavelength: 880 nm, Electronics workshop, Zoological Institute, University of Cologne) positioned around the camera lens provided illumination for the camera (shutter speed: $200\ \mu\text{s}$). LEDs were synchronized with the camera and were active only during image acquisition.

Rotation of the ball around its three axes was measured by two optical sensors according to a previously described method (Seelig et al., 2010). We used two optical sensors (ADNS-9500, Avago Technologies, San Jose, CA, USA) to measure optic flow on the ball's surface. Acquisition of these data was synchronized to video acquisition. Each sensor measured the 2-D movement velocity of the ball's surface at its equator at 50 Hz. Combining these values, we reconstructed the movement velocities of the ball's three rotational axes. Based on these velocities we calculated the fly's virtual walking trajectories and its speed. Low-level control of the

optical sensors and synchronization to the camera was implemented with custom-made hardware (Electronics workshop, Zoological Institute, University of Cologne); high-level control was implemented with custom-written software in MATLAB 2011b (The MathWorks, Natick, MA, USA).

Data acquisition

Each trial was visually inspected for straightness based on the reconstructed virtual walking trajectory. Trials with noticeable curvature were discarded. For the intact legs and the stumps ipsilateral to the camera (right body side; legs R1, R2, and R3), positions of the tarsi and stump tips, respectively, were annotated manually in each video frame (Fig. 3). Additionally, we determined the times at which touch-down and lift-off occurred. The anterior extreme position (AEP) and posterior extreme position (PEP) were defined as the first or last positions, respectively, at which the leg had contact with the substrate. Return stroke was defined as the part of a step cycle between a PEP and the subsequent AEP; the time between PEP and AEP was defined as return stroke duration. Power stroke was defined as the part of a step cycle between an AEP and the subsequent PEP; the time between AEP and PEP was defined as power stroke duration. We defined a step cycle as the movement of a leg between two consecutive PEPs; the associated time interval was defined as a step's period (Fig. 4). For leg stumps, touch-down and lift-off cannot be defined; therefore, instead of AEP and PEP, we determined the ventral and dorsal extreme positions (VEP and DEP) for each complete stump oscillation as the minima (VEP) and maxima (DEP) of the vertical component of the stump movement (Fig. 3C,D). Stump movement also had a protraction and retraction component; however, its amplitude was small and we therefore focused on levation and depression. The time interval between two DEPs was defined as a stump oscillation's period (Fig. 4). For the detection of maxima and minima we used the MATLAB function 'peakfinder' (MATLAB File Exchange). Walking speed associated with a step or stump oscillation was calculated as the average walking speed throughout the analyzed step or stump oscillation and was converted from mm s^{-1} to body lengths s^{-1} (BL s^{-1}) based on the known diameter of the PP ball visible in the video and the fly's apparent size.

Tracking was done using ProAnalyst Software (XCitex, Cambridge, MA, USA). The tracking data were imported to MATLAB and analyzed with custom functions. Figures and graphs were created in MATLAB 2011b, Origin (OriginLab Corporation, Northampton, MA, USA) and CorelDRAW (Corel Corporation, Ottawa, ON, Canada).

Inter-leg coordination and coordination strength

As a measure of inter-leg coordination we used the phase between an ipsilateral reference leg and a dependent leg (Fig. 5). We calculated the reference leg's instantaneous phase as a value increasing linearly from 0 to 1 between consecutive PEP events. Phase was then calculated as the reference phase at the time of a dependent leg's PEPs. We denoted the phase between two legs with $X>Y$; with X as the dependent leg and Y the reference leg. A phase relationship of 0 indicated in-phase activity. Because PEP was undefined for stumps, we substituted DEP in phase analyses involving the stump. DEP was more sharply defined in the stump and therefore provided more accurate timing information than VEP. To indicate the presence of a stump we used an additional 's'; for instance, R2s>R3 describes the phase of DEP events in the R2 stump in reference to R3.

Phase relationships were relatively variable on a cycle-to-cycle basis (Fig. 5, gray dots). To show speed-dependent trends more

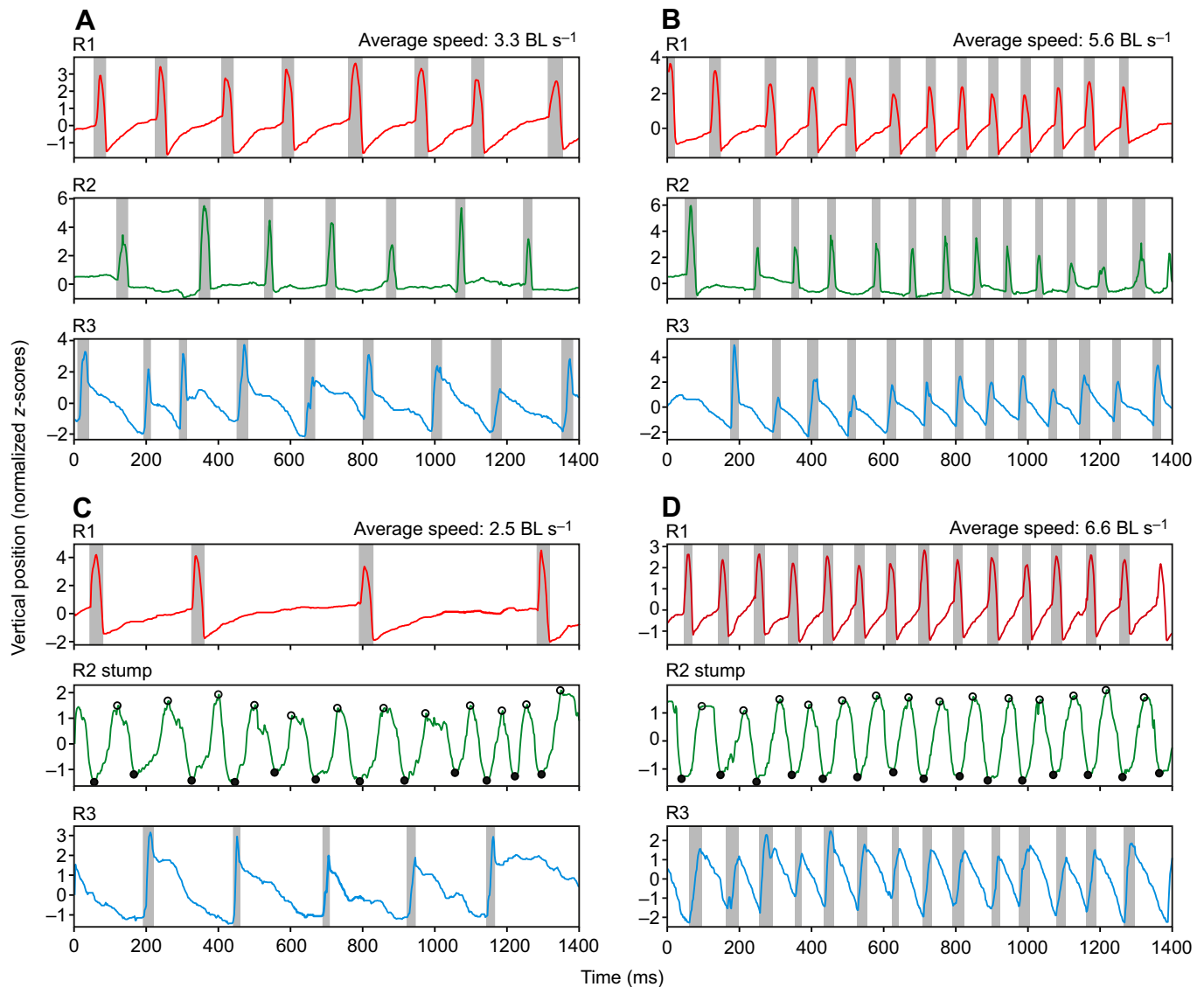


Fig. 3. Exemplary vertical movement of tarsi and stumps over time. This is shown for representative slow (A,C) and fast (B,D) walking sequences in an intact fly (A,B) and a middle leg amputee (C,D). Gray areas indicate return strokes in intact legs. Filled circles indicate ventral extreme positions (VEPs); open circles indicate dorsal extreme positions (DEPs) for stumps. To improve comparability, trajectories were normalized to their z-scores. Legs and stumps are color coded (red: front leg, green: middle leg or stump, blue: hind leg). In intact animals (A,B) all legs have a very similar stepping frequency; we found this for low (A) and high (B) walking speeds. (C) In the amputee, during slow walking the stump oscillates with a higher frequency than the intact legs, i.e. on a cycle-to-cycle basis its movement is not coupled to the movement of the intact legs. This frequency, however, is independent of walking speed. This can be observed in the fast-walking amputee (D). Here, the stump frequency is comparable to the frequency during slow walking as shown in C. The frequency in the intact legs is similar to that in the stump, however, and a cycle-to-cycle coupling can be observed.

clearly we used a gliding circular average with a step size of 1 on the data for individual steps and stump oscillations. We averaged 15 consecutive speed-sorted steps or stump oscillations to obtain an average phase for this sub-sample. The corresponding walking speed was calculated as the average walking speed in this sample. We did this for the complete data set; data averaged in this way are superimposed on the data for individual steps or stump oscillations (Fig. 5).

Coordination strength for a particular walking speed was calculated based on the variance in phase at this speed; low variability thereby corresponded to high coordination strength, and vice versa. To determine the coordination strength at a particular speed we used the same sub-samples of 15 consecutive steps or stump oscillations that were the basis for the analysis of phase (see

previous paragraph and Fig. 5) and determined its normalized circular variance. This variance was subtracted from 1, resulting in values between 0, indicating maximum variability and low coordination strength, and 1, indicating maximum coordination strength (Fig. 6). All phase data (Figs 5, 6) were processed with the CircStat Toolbox in MATLAB (Berens, 2009).

To further characterize the temporal relationship between intact legs and stump activity, we calculated the absolute time interval between PEPs in intact reference legs and DEPs and VEPs in stumps (Fig. 7, see also Fig. S6). We determined the times of PEPs in an intact leg and then calculated the time to the first subsequent DEP or VEP in the observed stump; any further DEPs or VEPs that occurred after this and before the next reference PEP were ignored for this analysis. To investigate the effect of walking speed on this, we

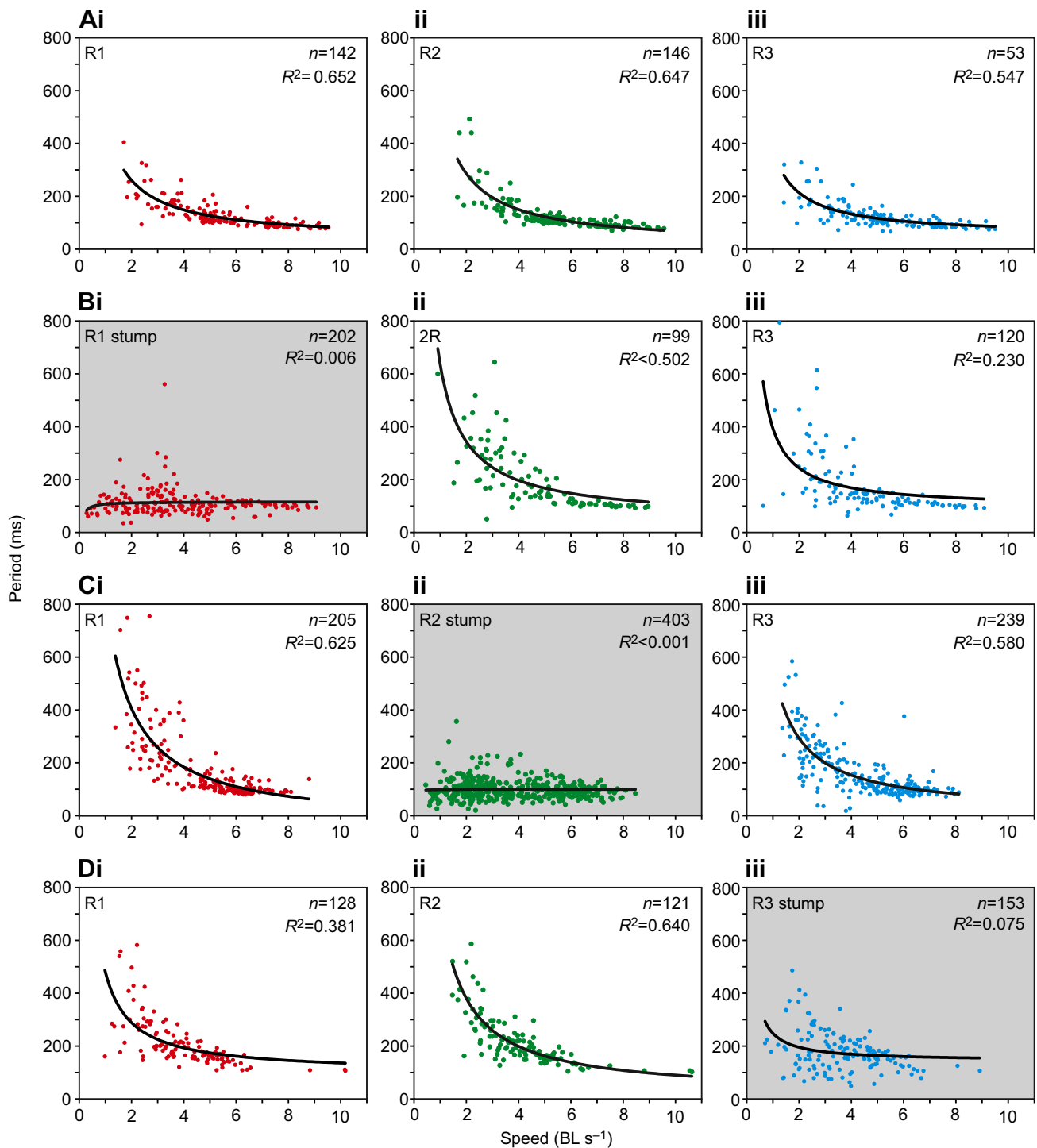


Fig. 4. Movement periods as a function of walking speed. This is shown for intact flies (Ai–iii, number of animals $N=7$), front leg amputees (Bi–iii, $N=7$), middle leg amputees (Ci–iii, $N=10$) and hind leg amputees (Di–iii, $N=7$). Each data point corresponds to one step in intact legs or one complete stump oscillation. Red scatter plots indicate data for front legs or front leg stumps (Bi), green indicates middle legs or middle leg stumps (Cii), and blue indicates hind legs or hind leg stumps (Diii). Stump data are additionally highlighted by gray backgrounds. Data in each panel were fitted with an exponential fit. In contrast to intact legs, the stump data show no clear exponential correlation between walking speed and movement period (Bi, Cii and Diii).

divided the data into two subsets: the first subset refers to time intervals at walking speeds lower than or equal to 5 BL s^{-1} (green circles, Fig. 7), the second refers to time intervals at speeds higher than 5 BL s^{-1} (red circles, Fig. 7). We also analyzed the complete stump movement aligned to PEP events in the remaining intact legs (Figs S2–S4). For this we extracted the vertical movement of the

respective stump from 100 ms before to 100 ms after a reference PEP. We then subdivided these time courses into those that occurred at slow walking (lower than or equal to 5 BL s^{-1}) and those that occurred during fast walking (higher than 5 BL s^{-1}). The respective time courses for each analysis were averaged and the standard deviation was calculated over the complete 200 ms.

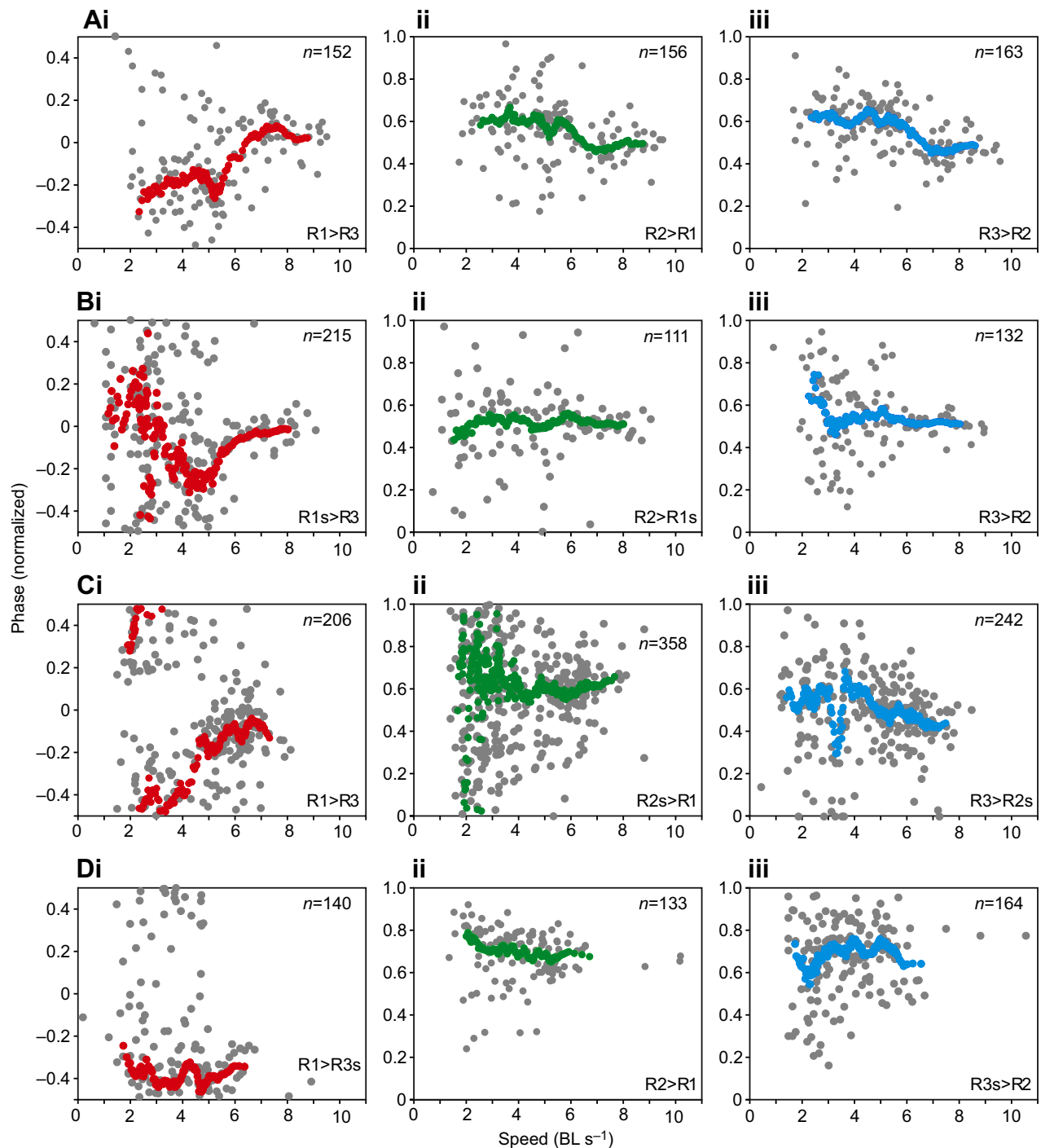


Fig. 5. Phase differences between reference legs and dependent legs as a function of walking speed. Data are shown for intact flies (Ai–iii), front leg amputees (Bi–iii), middle leg amputees (Ci–iii) and hind leg amputees (Di–iii). Each panel contains data for one pair of reference and dependent legs. For instance, R1>R3 indicates the right hind leg as the reference leg and the right front leg as the dependent leg. Stumps are indicated by an additional 's', e.g. R1s denotes the front leg stump. Phases are normalized to values between 0 and 1 or -0.5 and 0.5 . Each gray circle represents a single step of a dependent leg; colored circles show the gliding circular average of the underlying individual steps (width of 15 data points).

RESULTS

Intact flies

We recorded walking sequences of seven intact animals in 10 trials. Average walking speeds per trial ranged from 2.2 to 7.5 BL s^{-1} (4.1 to 17.9 mm s^{-1}). Trials consisted of five to 31 steps of R1, five to 31 steps of R2 and five to 33 steps of R3. In total, we analyzed 142 steps of R1, 146 steps of R2 and 153 steps of R3.

Average step periods were 132 ms for R1, 129 ms for R2 and 123 ms for R3. Step periods of all legs decreased with increasing speed (Fig. 4). This follows from geometrical reasons, when we assume that step amplitude and return stroke duration are constant. All legs in an intact animal performed the same number of steps during a given time (Fig. 3A,B). This is consistent with a metachronal wave traveling from the hind leg to the front leg.

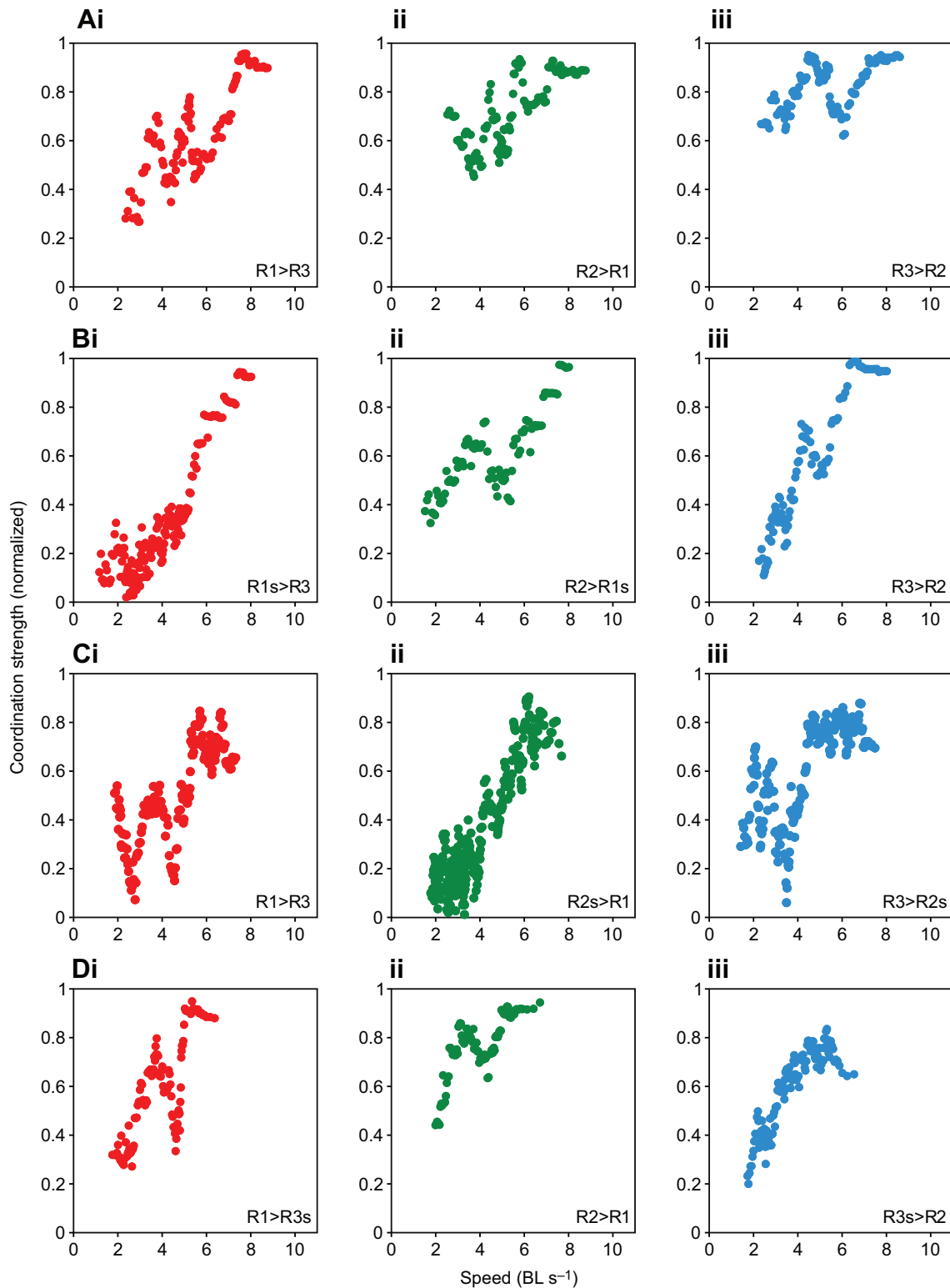


Fig. 6. Coordination strength between reference legs and dependent legs as function of walking speed. Data shown here are based on the average data shown in Fig. 5. For each average value based on 15 individual steps in Fig. 5 we calculated the corresponding circular variance and subtracted this value from 1. The resulting values can adopt values between 0 and 1. A value of 0 thereby indicates maximum circular variability, i.e. no preferred phase relation and low coordination strength; a value of 1 indicates minimum circular variability, i.e. a strictly preferred phase and high coordination strength. In analogy to Fig. 5, data are shown for intact flies (Ai–iii), front leg amputees (Bi–iii), middle leg amputees (Ci–iii) and hind leg amputees (Di–iii).

Although we did not explicitly quantify the consistency of metachronal waves, qualitative observations confirmed that most of the walking activity is consistent with metachronal waves (see Fig. 3A, 400 to 1400 ms). Return stroke durations were largely constant for all legs; average return stroke durations were 34 ms

(s.d.=8 ms) for R1, 25 ms (s.d.=6 ms) for R2 and 28 ms (s.d.=6 ms) for R3. Interestingly, the interval between an AEP in R3 and the subsequent PEP in R2 was largely constant over the complete walking speed range (approximately 25 ms). This corresponds fairly well to gait 2 (a coordination pattern similar to tetrapod coordination) described by Graham (1972) for the stick insect.

Phase between R1 and R3 tended to be variable at speeds between 1.5 and 5 BL s⁻¹. It had values of approximately 0 at speeds between 5 and 10 BL s⁻¹, indicating simultaneous return stroke onset (Fig. 5Ai). Phase values between R2 and R1 (Fig. 5Aii) and R3 and R2 (Fig. 5Aiii) were similar and had values of approximately 0.5, indicating anti-phase return stroke activity. All legs tended to become more coordinated with increasing speed (Fig. 6Ai–iii). Coordination strength between R1 and R3 increased from approximately 0.3 at a speed of 2 BL s⁻¹ to almost 1 at a speed of 8 BL s⁻¹. Coordination strength between R2 and R1 had a value of approximately 0.7 at a speed of 2 BL s⁻¹ and increased to 0.9 at approximately 6 BL s⁻¹ (Fig. 6Aii). Coordination strength between R3 and R2 had values of approximately 0.7 at 2 BL s⁻¹ and increased to values of 0.9 for speeds above 8 BL s⁻¹ (Fig. 6Aiii).

R1 amputees

We recorded walking sequences of seven front leg amputees in 12 trials with average speeds per trial between 2.2 and 7.3 BL s⁻¹ (4.7 and 15.7 mm s⁻¹). Trials contained nine to 28 oscillations of the R1 stump, two to 28 steps of R2 and three to 28 steps of R3. We analyzed 202 oscillations of the front leg stump (R1s), 99 steps of R2 and 120 steps R3.

The average step period of R2 was 201 ms (s.d.=118 ms) and for R3 it was 175 ms (s.d.=114 ms), while the average oscillation period of the R1 stump was 106 ms (s.d.=32 ms). Periods of the intact legs R2 and R3 decreased with increases in walking speed (Fig. 4Bii,iii). Oscillation period of the R1 stump did not show such a dependence; here, we found average values of approximately 100 ms for the complete range of walking speeds ($R^2=0.006$). Consequently, during slow walking we observed multiple oscillations of the stump during single steps in intact legs (see Fig. 3C). This is similar to findings that were reported for the cockroach (Delcomyn, 1991a,b; Noah et al., 2004). In intact legs of the R1 amputees, return stroke durations were largely constant over the complete speed range; return stroke durations for R2 were on average 31 ms (s.d.=8 ms) and for R3 were 32 ms (s.d.=9 ms).

Between walking speeds of 1.5 and 5 BL s⁻¹, phase between the R1 stump and R3 was more variable than in intact animals. However, average phase also approached 0 between 5 and 8 BL s⁻¹ (Fig. 5Bi). Coordination strength was almost 0 at very low walking speeds of approximately 1.5 BL s⁻¹, reached values of 0.5 at around 5 BL s⁻¹, and increased further to high values of almost 1 at approximately 8 BL s⁻¹. Coordination strength between R2 and the R1 stump increased over the whole speed range from approximately 0.3 to almost 1. Interestingly, the phase relation between these legs did not show major differences when compared with those in intact animals. In contrast, the phase between the intact legs R3 and R2 at speeds between 0 and 5 BL s⁻¹ is much more variable than in intact animals. For the speed range above 5 BL s⁻¹ the phase converged to 0.5 (Fig. 5Biii); coordination strength increased in the range between 2 and 6 BL s⁻¹ from approximately 0.2 to almost 1 (Fig. 6Biii).

The analysis of temporal delay between R2 and R3 and the stump shows that, during fast walking, there is a preferred time interval between PEPs in the intact legs and events in the stump (Fig. 7A, red circles). This time interval is either approximately 0 or 100 ms (R2-VEP and R3-DEP) or approximately 50 ms (R2-DEP and R3-VEP).

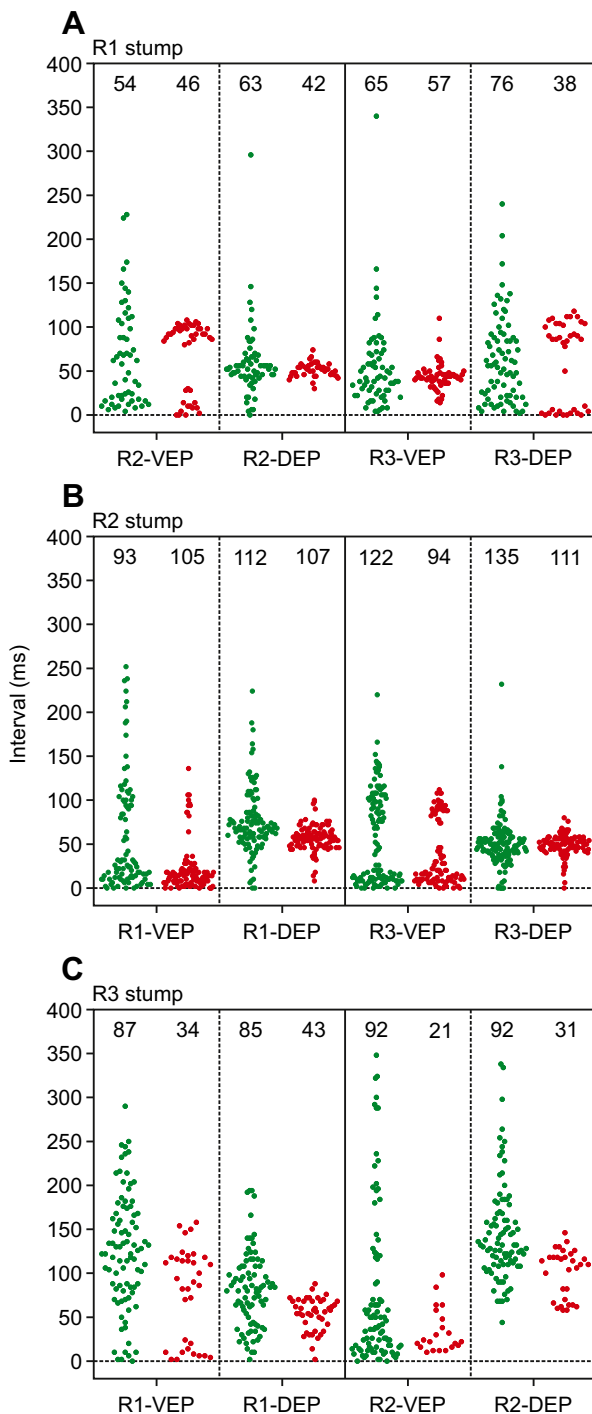


Fig. 7. Time intervals between posterior extreme positions (PEPs) in intact legs and VEPs and DEPs in stumps. Data are shown for (A) front leg amputees, (B) middle leg amputees and (C) hind leg amputees. Each data point refers to the first VEP or DEP event in a stump following a PEP in an intact leg; for instance, R2-VEP in A refers to the time that elapsed between a PEP in the middle leg (R2) and the next VEP in the front leg stump. Data have been subdivided into those associated with a walking speed below 5 BL s⁻¹ (green circles) and those associated with walking speeds above 5 BL s⁻¹ (red circles).

During slow walking (Fig. 7A, green circles), most of this strict temporal coordination vanished (R2-VEP, R3-VEP and R3-DEP); this is consistent with our finding that at these speeds there was no cycle-to-cycle coupling between the intact legs and the stump. However, we found a preferred temporal delay of approximately 50 ms between PEP events in the middle leg and subsequent DEP events in the front leg stump (Fig. 7A, R2-DEP, green circles) even during slow walking. The strong coupling at high walking speeds was substantiated when we temporally aligned the stump movements to PEP events in the intact legs (Fig. S2). Here, we found that at high walking speeds, the stump's movements coincided systematically with PEPs in the intact legs (Fig. S2B,D). At low speeds this was less pronounced (Fig. S2A,C). At high walking speeds, the onset of stump levation was approximately 5 ms before PEP was reached in the middle leg (Fig. S2B); the onset of stump depression was approximately 5 ms before PEP is reached in the hind leg.

R2 amputees

For middle leg amputees, we obtained data from 10 animals during 12 trials. The mean speed of the recorded walking sequences differed between 1.8 and 6.4 BL s⁻¹ (3.7 to 13.8 mm s⁻¹). Trials consisted of four to 26 steps of R1, 11 to 38 oscillations of the R2 stump and six to 26 steps of R3. We recorded 205 steps of R1, 403 oscillations of R2 and 239 steps of R3. Average step periods were 175 ms (s.d.=127 ms) for R1 and 161 ms (s.d.=96 ms) for R3. The R2 stump oscillated with an average period of 99 ms (s.d.=37 ms).

The step period of the intact legs R1 and R3 decreased with increasing speed ($R^2=0.63$ and 0.58 , respectively; see Fig. 4Ci,ii), whereas the oscillation period of the R2 stump did not show such a decrease ($R^2<0.01$; Fig. 4Cii). Comparable to the findings for the R1 stump, the oscillation periods of the R2 stump were independent of walking speed, with periods around 100 ms. During slow walking, we regularly observed multiple stump oscillations during single steps of intact legs (Fig. 3C). In fast walking animals the number of stump oscillations converges to the number of steps in the intact legs (see Fig. 3D). Again, return stroke durations in the intact legs were largely constant. In R1 return stroke duration was on average 29 ms (s.d.=6 ms), in R3 return stroke duration was 27 ms (s.d.=20 ms). The large standard deviation of return stroke durations in R3 was due to a small number of relatively short steps (see also Fig. 4Ciii).

Phase showed higher variability compared with intact animals, R1 amputees and R3 amputees (Fig. 5A,B,D). The mean phase between R1 and R3 varied around 0.5 in a speed range of approximately 1.5 to 5 BL s⁻¹. For speeds above 5 BL s⁻¹, mean phase approached 0. Coordination strength rose to values of approximately 0.7 at speeds of 5 to 8 BL s⁻¹ (Fig. 6Ci). Phase between the R2 stump and R1 were even more variable, with no preferred value at speeds between 1.5 and 5 BL s⁻¹. For speeds above 5 BL s⁻¹, the variability decreased and phase converged to approximately 0.6 (Fig. 5Cii); coordination strength increased linearly from 0.1 to 0.8 over the observed speed range (Fig. 6Cii). The phase analysis between R3 and the R2 stump showed average phases between 0.25 and 0.75 at walking speeds between 1.5 and 5 BL s⁻¹ (Fig. 5Ciii); at walking speeds above 5 BL s⁻¹ the values approached approximately 0.4. However, coordination strength did not show a constant increase with speed but varied between 0.6 and 0.7 (Fig. 6Ciii).

Analysis of the temporal delay between the intact legs (R1 and R3) and the stump (R2) showed that during fast walking, the situation was similar to the front leg amputees (Fig. 7B, red circles).

Because of relatively strict stump coordination at this speed, we found preferred temporal intervals between PEPs in intact legs and the stump of either 0 and 100 ms (R1-VEP and R3-VEP), respectively, or approximately 50 ms (R1-DEP and R3-DEP). At low speeds (Fig. 7B, green circles), the variance of these intervals was higher but they still clustered around the values observed during fast walking. This was also reflected in the average stump movements around the times of PEP in the intact legs (Fig. S3). For middle leg amputees, stump movements were clearly aligned with regard to PEPs in the intact legs. At high walking speeds (Fig. S3B,D) this alignment was stronger than at low speeds (Fig. S3A,C). Consistent for all of these average movements was the fact that the onset of levation in the stump occurred approximately 10 ms after PEP in an intact leg was reached.

R3 amputees

In hind leg amputees we recorded walking sequences of seven animals during 13 trials. The average speed in these trials was between 2.0 and 8.7 BL s⁻¹ (4.6 and 20.5 mm s⁻¹). Trials consisted of four to 16 steps of R1, three to 16 steps of R2 and two to 19 oscillations of the R3 stump. We recorded 128 steps of the right front leg (R1), 121 steps of the right middle leg (R2) and 153 oscillations of the right hind leg stump (R3).

Average step periods were 213 ms (s.d.=95 ms) for R1 and 226 ms (s.d.=110 ms) for R2, while the R3 stump oscillated with an average period of 117 ms (s.d.=77 ms). Step period for both intact legs R1 ($R^2=0.50$; Fig. 4Di) and R2 ($R^2=0.66$; Fig. 4Dii) decreased with increasing speed. Oscillatory periods in the stump did not depend on speed in the same way as intact hind legs did; however, it did not show the same clear independence of walking speed as the front and middle leg stumps, either ($R^2=0.14$; Fig. 4Diii). However, even in R3 stumps we regularly observed multiple oscillations during single steps of the intact legs during slow walking. Return stroke durations in intact legs in R3 amputees were largely constant. For R1, the average return stroke duration was 38 ms (s.d.=6 ms) and for R2 it was 24 ms (s.d.=6 ms).

Phase in R3 amputees was less variable than in all other experiments, as indicated by the distribution of mean phases (Fig. 5D). Phase between R1 and R3 ranged from -0.5 to -0.25 (Fig. 5Di). Phase values between R2 and R1 were almost constant at approximately 0.75 for the whole range of walking speeds (Fig. 5Dii). In both cases, coordination strength generally increased with speed (Fig. 6Di,ii). The average phase between the R3 stump and R2 ranged from 0.5 to 0.75 (Fig. 5Diii). Here, coordination strength generally also increased with walking speed (Fig. 6Diii).

Compared with the findings for front and middle leg amputees, analysis of the temporal delay between R1 or R2 and the hind leg stump revealed a different picture (Fig. 7C). During fast walking (Fig. 7C, red circles), the time intervals between PEPs in the intact legs and events in the stump were more variable even though the tendency to cluster at approximately 0 and 100 ms (R1-VEP, R2-VEP, R2-DEP) or 50 ms (R1-DEP) persisted. During slow walking, this variability was higher (Fig. 7C, green circles); compared with data from the middle leg amputees, the tendency for preferred intervals was lower. When we analyzed complete stump movements around the times of PEPs in the intact legs (Fig. S4) we found that at high walking speeds movements of the stump are systematically aligned with movements of the intact legs (Fig. S4B,D). This alignment is weaker during slow walking (Fig. S4A,C). At high walking speeds, onset of stump levation occurred approximately 10 ms before PEP in a front leg (Fig. S4B) and approximately 20 to 30 ms after PEP in the middle leg.

DISCUSSION

Here, we investigated the activity of leg stumps in tethered fruit flies during walking behavior on a ball. We analyzed its temporal and kinematic characteristics as well as its coordination with ipsilateral intact legs (Figs 1, 2). After single-leg amputation, *Drosophila* is still able to walk coordinately. Speed is reduced in amputees, but we still observed a large range of walking speeds. Stumps were active during walking and oscillated rhythmically up and down (Figs 2B, 3).

Compared with intact legs, the oscillatory frequency in stumps was often much higher (Fig. 3C). Importantly, on average it was constant over the observed range of walking speeds (Fig. 4). This contrasted with intact legs, for which we found a correlation between walking speed and stepping frequency. This correlation has been observed in previous studies (e.g. Wosnitza et al., 2013) and is a relatively strict invariant; absence of this correlation is, therefore, noteworthy. The average oscillatory frequency of the stumps was largely independent of the intact legs during slow walking. In contrast, during fast walking, stump oscillations were coupled to those of the intact legs and we often found synchrony between them (Figs 5, 6). Over the complete range of walking speeds we found a correlation of walking speed and coordination strength between intact legs and stumps; at high speeds coordination strength between stumps and intact legs was similar to the coordination strength between intact legs (Fig. 6, but see Fig. S5). Finally, although at low speeds we did not find a cycle-to-cycle coupling between stumps and intact legs, we observed a preferred temporal delay between PEP events in intact legs and the first subsequent DEPs and VEPs in the stump (Fig. 7, see also Figs S2–S4).

Rhythmic stump activity

After amputation, stumps oscillated with an almost constant frequency during walking. This frequency was largely independent of the stepping frequency of intact legs or walking speed. Here, we discuss two alternatives concerning the origin of this rhythmic activity, although these alternatives are not mutually exclusive. Stump oscillations could be primarily driven by local pattern-generating networks situated in the associated hemiganglion, potentially in concert with feedback from still-functional afferents. Alternatively, rhythmic inter-segmental influences of sensory signals from intact legs might give rise to stump oscillations. The second alternative can be largely ruled out by the findings presented here. If stump oscillations were solely driven by sensory events in intact legs, then we would have observed a correlation between stump and intact leg frequency. This was not the case. Our observations are, however, consistent with independent oscillations whose cycle-to-cycle specifics can be modulated by inter-leg sensory information.

Presumably, intra-leg sensory and mechanical influences are reduced after amputation. Sensory structures distal to the lesion are absent and cannot provide information (see Bässler, 1983). Campaniform sensilla on the coxa and trochanter are probably silent because of missing ground contact; campaniform sensilla in insects are activated only when the leg is loaded or when movements are resisted (Zill et al., 2009, 2011, 2013). Consequently, most of the intra-leg sensory signals that are known to dominate motor output during walking can probably be ruled out as playing a role in oscillatory stump movements. This is supported by the observation that a leg stump can be used coordinately if ground contact is re-established (Noah et al., 2004).

Several sense organs probably remain intact in the proximal segments, however. Several types of receptors, e.g. hair plates and rows (Schmitz, 1986; Dean and Schmitz, 1992), or multipolar

stretch receptors (Schmitz and Schöwerling, 1992), monitor movements of the coxo-trochanteral and thoraco-coxal joints (see also Burrows, 1996; Field and Matheson, 1998). These sense organs could signal movements and provide information used in walking. Movements of the stump toward levation of the coxa-trochanter joint can be detected by the trochanteral hair plate. In cockroaches and stick insects this hair plate is known to excite depressor motor neurons (Wong and Pearson, 1976; Schmitz, 1986). This connection is thought to potentially contribute to the onset of depressor activity at the start of the power stroke (but see Akay et al., 2001). In the stump, movement of the leg toward extreme levation could generate depressor firing that would produce rapid movement toward maximal depression. This could be detected by sensory structures, such as the levator stretch receptor and coxal chordotonal organ (Bräunig et al., 1981; Schmitz and Schöwerling, 1992), which have been shown to activate levator motor neurons and could contribute to the termination of the power stroke. Thus, the receptors remaining in the stump could generate sequential activation of motor neurons resulting in alternating movements. The latencies to activation of the sensory signals would be of uniform duration due to the minimal inertia of the leg stump, which is supported by the finding that the stump's movement rate remains constant.

In other insects, e.g. in the hawk moth (Johnston and Levine, 1996), locust (Ryckebusch and Laurent, 1993) or stick insect (Büschges et al., 1995), the existence of local CPG networks for rhythmic motor activity of the legs is well established. For the stick insect it has been shown that there are independent CPG networks for each leg joint. These findings, however, are based on reduced preparations. Because of the presence of remaining proximal sensory structures, we argue that the stump oscillations observed here probably result from interplay of local CPG activity and intra-leg sensory signals originating in still-intact leg segments; we refer to this combined activity as local rhythm or pattern generation.

Descending control of walking speed

Assuming that stump oscillations are largely based on interactions of sensory feedback and local rhythm-generating networks, we hypothesize that a central descending influence controlling walking in *Drosophila* might have a bipartite influence. The first part might be a qualitative influence that activates local rhythm-generation in the stump. Importantly, this influence would only induce, but not modulate, motor output. Thus, descending signals would activate thoracic networks, which, in turn, drive leg muscles, resulting in rhythmic movement with a constant frequency. This is complementary to previous studies that suggest the existence of a central mechanism that influences the state of the animal by inducing qualitative changes not only in local CPGs (Büschges et al., 2004) but also in how sensory information is processed (Bässler and Büschges, 1998). Anecdotal observations support this idea; in several instances we observed stump oscillations without overt walking. The animal still seemed active although no stepping was observed in the intact legs. Simultaneously, the stump oscillated up and down, performing searching-like movements (Berg et al., 2015), indicating that local pattern-generating networks were active (Fig. S1).

The finding that the oscillatory frequency of the stumps was largely constant holds for all legs. How can the animal change its walking speed at all if the stump frequencies are constant but, at the same time, changes in walking speed are mainly controlled via stepping frequency (Wosnitza et al., 2013)? This suggests a second, quantitative influence of the descending signal, which must modulate the stepping frequency. In contrast to the first influence, which might act as a switch and target rhythm-generating networks

directly, the second influence might have an indirect effect. We hypothesize that this might happen via modulation of sensory information, especially associated with the power stroke; only during stance phase can a leg influence the overall walking speed. A target for such an influence might be sensory information provided by load sensors, i.e. campaniform sensilla, which are normally active at the transition from return to power stroke and during power stroke (Zill et al., 2009), signaling touch-down and ground contact. In leg stumps these signals are probably absent, either because campaniform sensilla were removed during amputation or are never stimulated owing to absence of ground contact. This is supported by a previous study in the stick insect, where changes in the synaptic drive to power stroke motor neurons become correlated to walking speed only after onset of the power stroke (Gabriel and Büschges, 2007). Alternatively, motor output might be controlled by the descending drive only during the power stroke.

Speed dependence of coordination strength

Fig. 5 shows considerable variability in the phase between intact legs and stumps. Nevertheless, our results show a speed-dependent increase in coordination strength between movements of intact legs and stumps. This might be explained by considering the frequencies of the oscillatory systems investigated here. During slow walking, the frequency difference between an intact leg and the stump is large. Weakly coupled oscillators with very different frequencies cannot entrain each other in a 1:1 ratio (Kuramoto, 1984; Pikovsky et al., 2003). This might be the reason why there is no preferred phase between the stump and either intact leg (Fig. 3C). However, an increase in walking speed is accompanied by an increase in the stepping frequency in intact legs; once the frequencies of the stump and intact legs are similar, 1:1 entrainment occurs (Fig. 3D).

We indeed found that coordination strength between stumps and intact legs increases with walking speed in all leg stumps (Fig. 6). We also found similar coordination strengths for amputees at stepping frequencies that were close to the stumps' natural frequency (approximately 10 Hz). Finally, during slow walking, the cycle-to-cycle coupling between stumps and intact legs is lost and, consequently, there is no preferred phase (Figs 5, 6). However, we observed a preferred delay between PEPs in intact legs and subsequent DEPs or VEPs in the stump even at low speeds (Fig. 7). This suggests that there is information transfer between sensory events in intact legs and the stump and is consistent with the idea that the legs are driven by weakly coupled oscillators that entrain in a 1:1 ratio only at similar frequencies.

The high natural frequency of stump oscillations might be explained as an adaptation to the requirements of fast walking. A leg can only execute its return stroke during the power strokes of its neighboring legs (Cruse, 1990; Dürr et al., 2004). Power strokes become shorter with increasing walking speed, as shown for *Drosophila* and other insects (Wendler, 1964; Wosnitza et al., 2013). Consequently, the timing of return stroke execution is particularly important during fast walking; here, the time in which a return stroke can be executed, i.e. during the power strokes of neighboring legs, is short and there is a smaller margin of error. Hence, neural signals mediating coordination have to be more effective during fast walking. A natural oscillatory frequency of a local rhythm generator that is similar to the stepping frequency at high speeds would facilitate this. It might be informative to examine this issue in insects that often walk fast, e.g. the desert ant (Zollikofer, 1994; Wahl et al., 2015) or the cockroach (Full and Tu, 1990, 1991). Alternatively, stump oscillations might resemble searching movements which are suppressed when the leg is touching the

ground (Berg et al., 2015). In the stump, i.e. with the permanent absence of ground contact, searching is continuously activated and can then subsequently be modulated or even entrained by sensory events in the intact legs. Similar to the data for *Drosophila* presented here, searching movements in stick insects tend to have comparatively high frequencies (Dürr, 2001; Bläsing, 2006).

New insights and open questions

Previous studies reported stump oscillations or muscle activity in otherwise intact walking insects (Hughes, 1957; Delcomyn, 1988, 1991a,b; von Buddenbrock, 1921; Wendler, 1966; Graham, 1977; Bässler et al., 1987; Grabowska et al., 2012). These studies report multiple stump oscillations during single steps in intact legs but were often only qualitative and focused on effects of amputations on the remaining intact legs. Here, we quantified stump oscillations in the fruit fly in the front, middle and hind legs, with a particular focus on walking speed dependence. We analyzed the stumps' temporal activity and their coordination with intact legs. The present study also tried to differentiate between local CPG-like activity and inter-segmental sensory influence during walking. One aspect that has not been addressed here is the influence of contralateral legs on the stump. Coordinating influences are also active between contralateral legs (Cruse, 1990; Dürr et al., 2004) and the stump might be influenced by them. Although this influence cannot be excluded, it has also been suggested that ipsilateral legs are coordinated more strongly than contralateral legs (Wilson, 1966). Potential effects should therefore be observable most strongly in ipsilateral legs.

During fast walking, stumps became highly coordinated with intact legs. Two processes might be responsible: central coupling of the rhythm-generating networks or indirect coupling via sensory information. It is likely that at least one of these influences plays a role for entrainment of the stump. We cannot distinguish between these alternatives, however, because both motor activity and, consequently, sensory signals are correlated with the rhythmic activity of the pattern-generating networks.

Our findings are consistent with a bipartite effect of descending signals, but it is unclear how this is implemented neurally. We would like to propose two alternatives. The descending drive controlling initiation of walking and speed could be one global signal originating in higher centers that is split up into its two putative components locally in the ganglia. Alternatively, there might be two anatomically different descending channels that influence CPG activity and walking speed independently from each other; this solution has been implemented technically in the Walknet (Schilling, et al., 2013). Although the second alternative is probably more complex, recent findings support it. Octopamine-deficient *Drosophila* mutants walk coordinately, but do so at lower average speeds (Wosnitza et al., 2013). This is consistent with the hypothesis that descending modulation of sensory influences required for higher walking speeds is absent in these mutants and might therefore be dependent on octopamine.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

V.B., S.N.Z., A.B. and T.B. conceived the study. V.B. and T.B. designed and carried out the experiments. V.B. and T.B. carried out the data analysis. V.B., S.N.Z., A.B.

and T.B. contributed to preparation of the manuscript. All authors read and approved the final manuscript.

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Supplementary information

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