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Spatial co-ordination of foot contacts in unrestrained climbing insects

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ABSTRACT

Animals that live in a spatially complex environment such as the canopy of a tree, constantly need to find reliable foothold in three-dimensional (3D) space. In multi-legged animals, spatial co-ordination among legs is thought to improve efficiency of finding foothold by avoiding searching-movements in trailing legs. In stick insects, a 'targeting mechanism' has been described that guides foot-placement of hind- and middle legs according to the position of their leading ipsilateral leg. So far, this mechanism has been shown for standing and tethered walking animals on horizontal surfaces. Here, we investigate the efficiency of this mechanism in spatial limb co-ordination of unrestrained climbing animals. For this, we recorded whole-body kinematics of freely climbing stick insects and analysed foot placement in 3D space. We found that touch-down positions of adjacent legs were highly correlated in all three spatial dimensions, revealing 3D co-ordinate transfer among legs. Furthermore, targeting precision depended on the position of the leading leg. A second objective was to test the importance of sensory information transfer between legs. For this, we ablated a proprioceptive hair field signaling the levation of the leg. After ablation, the operated leg swung higher and performed unexpected searching movements. Furthermore, targeting of the ipsilateral trailing leg was less precise in anteroposterior and dorsoventral directions. Our results reveal that the targeting mechanism is used by unrestrained climbing stick insects in 3D space and that information from the trochanteral hair field is used in ipsilateral spatial co-ordination among legs.

KEY WORDS: Locomotion, Stick insect, Kinematics, Targeting, Climbing, Movement, Motion capture, Leg co-ordination

INTRODUCTION

Efficient locomotion in complex environments depends on finding appropriate foothold locations. In multi-legged locomotion, spatial co-ordination of limbs should benefit from sequential use of the same foothold by two or more legs, as it reduces the need for repetitive searching movements. Animals can determine foothold location from exteroceptive spatial cues such as vision or touch, or from proprioceptive spatial cues, e.g. by exchanging postural information among limbs. Here, we focus on the latter, providing a first data set on spatial co-ordination among limbs in unrestrained climbing insects, and identifying a proprioceptive cue involved in the underlying co-ordinate transfer.

For spatial co-ordination of legs, animals and humans continuously scan the environment with exteroceptors to obtain information about obstacle location. Humans visually locate obstacles in their path and adapt to them by adjusting step length, increasing leg clearance, changing direction, or simply by stopping (Patla, 1997). Visual information is also used by insects: locusts place their front legs accurately on rungs during ladder climbing (Niven et al., 2010), and horse-head grasshoppers use visual cues for targeting their front legs at the opposite side of a gap (Niven et al., 2012). For other animals, visual cues might be less useful because they are nocturnal or dwell in dark areas. Cockroaches use their antennae to follow along walls (Camhi and Johnson, 1999), to orient their body towards objects (Okada and Toh, 2006) and to climb across obstacles (Watson et al., 2002). Furthermore, cockroaches use antennal contacts to decide whether to tunnel under or climb across obstacles (Harley et al., 2009). Stick insects use their antennae to detect the far edge of a gap (Bläsing and Cruse, 2004b), to scan the edges of obstacles (Krause and Dürr, 2012), and to guide aimed reaching movements of a front leg (Schütz and Dürr, 2011).

Both mammals and insects perform stereotypic searching movements if a leg does not find ground contact (e.g. Gorassini et al., 1994; Dürr, 2001). Searching movements become very frequent when an insect crosses large gaps (Bläsing and Cruse, 2004b) or when it climbs over rungs (Niven et al., 2010). In principle, the necessity of searching movements may be reduced by information transfer among legs, thus allowing trailing legs to exploit successful footholds of leading legs. This is important for all multi-legged animals that may see neither the trailing legs nor the obstacles located in their path. Indeed, both mammals and insects use co-ordinate transfer to trailing legs. Cats remember the position of an obstacle and have a spatial representation of their own body and their immediate environment to solve this problem (McVea and Pearson, 2006). In stick insects, the hindlegs follow the position of the middle legs. The underlying 'targeting mechanism' makes the hindleg touch down close to the current position of the ipsilateral middle leg (Cruse, 1979). The same mechanism, though with lower precision, was found for the middle legs, which follow the position of the front legs (Dean and Wendler, 1983; Wosnitza et al., 2013). During visually induced curve walking, the targeting mechanism is affected differently, depending on whether it is an inner or an outer leg with respect to the curvature (Ebeling and Dürr, 2006). So far, this targeting mechanism was only characterized for tethered animals walking on horizontal surfaces (either a tread wheel or a slippery surface), whereas in nature, stick insects climb freely through complex environments. Targeting in the vertical direction has never been studied before. Thus the main objective of this study was to determine the accuracy and precision of spatial co-ordination among legs in unrestrained climbing animals, with a particular emphasis on differences in dorsoventral direction.

In insects, both temporal and spatial co-ordination among legs is known to depend on sensory feedback (Wendler, 1964; Cruse, 1990; Ritzmann and Büschges, 2007), involving information about limb posture (Wong and Pearson, 1976; Cruse et al., 1984), load

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(Borgmann et al., 2009; Zill et al., 2009; Zill et al., 2012) and ground contact (Zill et al., 2010; Theunissen and Dürr, 2013). Thus the second objective of this study was to reveal the contribution of a proprioceptive hair field on the accuracy of spatial co-ordination between middle and hindlegs. We focused on sensory hair fields because they provide postural information about the proximal joint angles of a leg (Wendler, 1964). Hair fields on the coxa measure the protraction/retraction angle of the thorax–coxa joint, whereas the trochanteral hair field measures the levation angle of the trochantero-femur (Wendler, 1964; Schmitz, 1986; Dean and Schmitz, 1992). Ablation of hair fields has been shown to affect not only the movement of the manipulated leg, but also the spatial co-ordination of neighboring legs (Dean and Wendler, 1983; Cruse et al., 1984; Dean and Schmitz, 1992). All of these experiments were done on tethered animals walking on horizontal surfaces, thus neglecting the third dimension of spatial co-ordination. As reasoned above, dorsoventral differences in limb posture should be important for spatial co-ordination during climbing. Here, we focused on the trochanteral hair field because it monitors levation of the leg which, in turn, affects dorsoventral foot placement.

With regard to our two objectives, we hypothesized that limb targeting is not specialized to planar walking but that it extends to the vertical direction. Moreover, we expected that the ablation of the trochanteral hair field would affect targeting precision and accuracy in the dorsoventral direction. In order to analyse the spatial co-ordination of the legs in three dimensions (3D), we used the same staircase setup as described in a previous study (Theunissen and Dürr, 2013), allowing us to vary the height of the stairs to be climbed. Body kinematics were recorded using a Vicon motion capture system, yielding sufficiently large sample sizes for separating two functionally distinct classes of steps, i.e. short and long steps (Theunissen and Dürr, 2013), and to conduct the analysis separately for different types of long steps, i.e. flat and climbing steps. For investigating the role of the trochanteral hair field, we analysed targeting of the middle and hindlegs in intact and manipulated animals.

RESULTS
Neighboring legs follow each other

Stick insects (*Carausius morosus*) readily walked along a 40 mm wide walkway and climbed stairs of different height without noticeable difficulty. We recorded the movements of the animals with a motion capture system that allowed us to reconstruct the whole-body kinematics. When the animals started to climb, body orientation changed, i.e. the body pitch angle increased by about 60 deg (Fig. 1A). This change in body orientation played a major role in the analysis of the spatial co-ordination, because we projected foot contact positions into body-fixed co-ordinates. Based on our earlier results (Theunissen and Dürr, 2013), we separated two functionally distinct classes of steps by their step length, i.e. the distance between lift-off and touch-down. The class boundary between long and short steps was set by the local minimum of the bimodal step length distribution. Since short steps are thought to correct for inappropriate foothold whereas long steps are associated with forward propulsion of the body, all further analysis was limited to the class of long steps. From the foot trajectories, two further step types could be separated in order to compare distinct locomotion episodes: flat steps and climbing steps (Fig. 1B,C). Flat steps lifted off and touched down on horizontal surfaces, whereas climbing steps touched down on the vertical surface of a stair. Generally, the trajectories of neighboring feet were very similar and touch-down positions were located close together (Fig. 1B,C). A notable
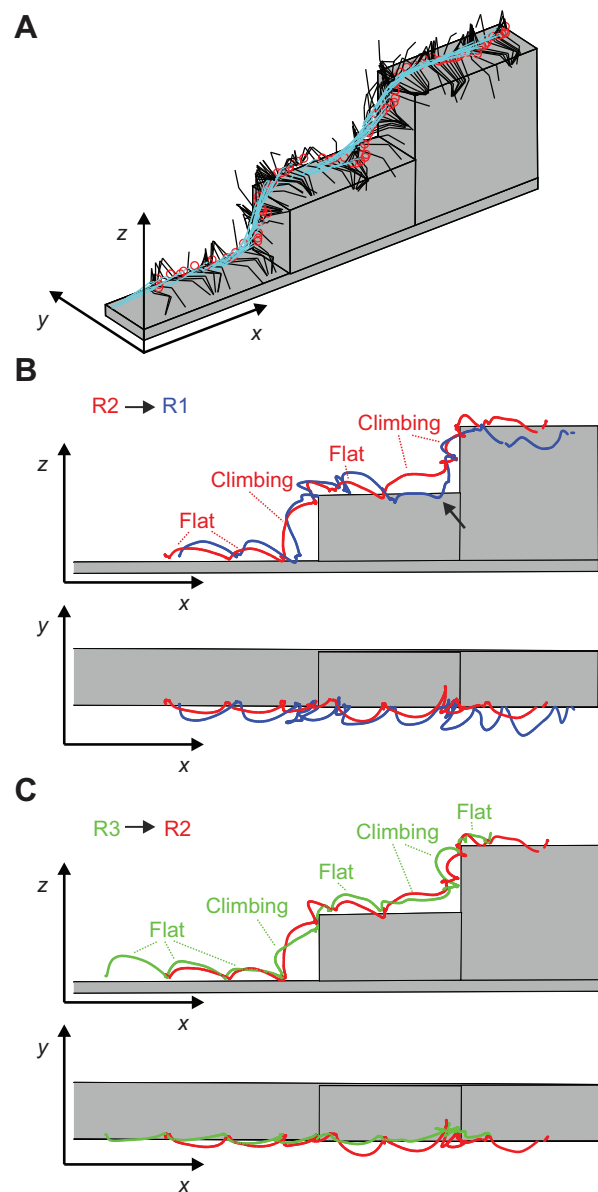

Fig. 1. Trajectories of adjacent feet are similar. (A) Representative climbing sequence with high stairs. The body axis (cyan), the head (red) and the front leg segments (black) are superimposed on the setup every 100 ms. Body orientation changed gradually during climbing. (B,C) Trajectories of the right front leg (B; blue, R1), the right middle leg (B,C; red, R2) and the right hindleg (C; green, R3) are shown in top and side views. Examples of flat and climbing steps were identified. The trajectories of adjacent feet resemble each other and mostly share common touch-down locations. A notable exception is pointed out by the black arrow (in B), where the middle leg (red) does not follow the low trajectory of the front leg (blue) but moves straight towards the next foothold of the front leg. Note that at the same point, the hindleg (in C) closely follows the middle leg. Co-ordinate systems have a length of 100 mm.

exception, where the touch-down location of the front leg that was not followed by the middle leg, can be observed in Fig. 1B (black arrow). Instead of a touch-down, the middle leg performed a prolonged swing movement to the next front leg touch-down position. This swing movement made an additional loop, which was replicated by the hindleg (Fig. 1C). Overall, hind- and middle leg trajectories appeared to be more similar than front and middle leg trajectories.
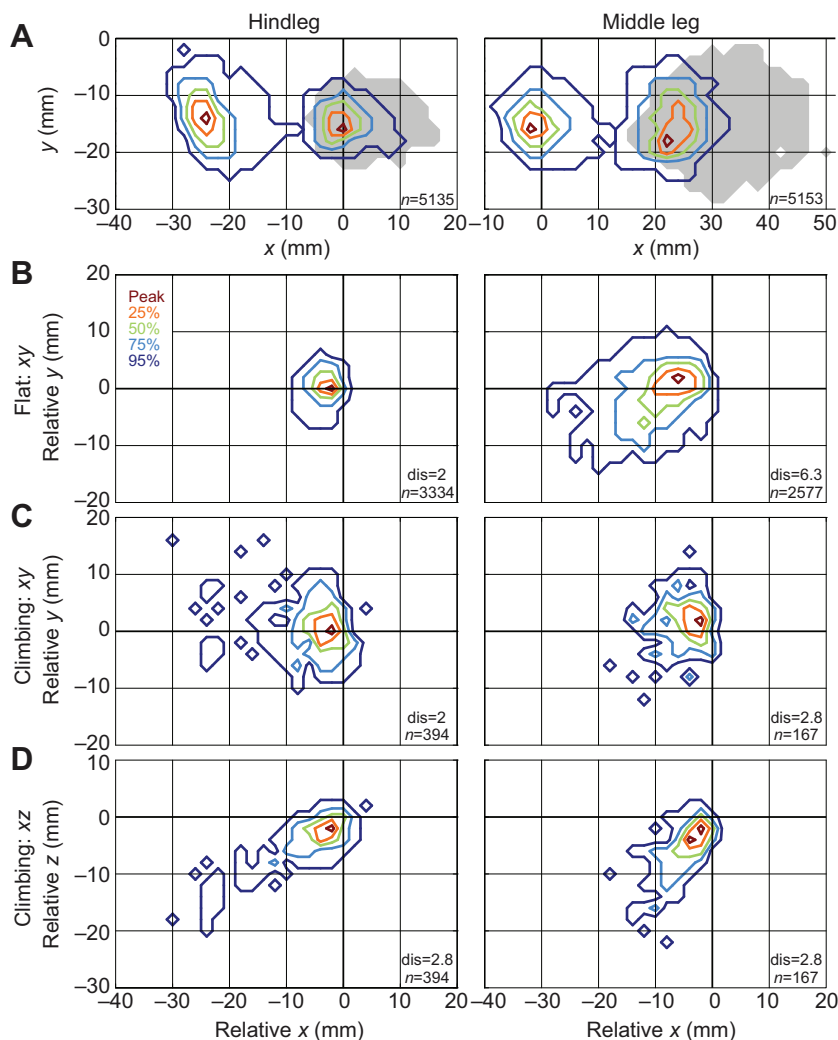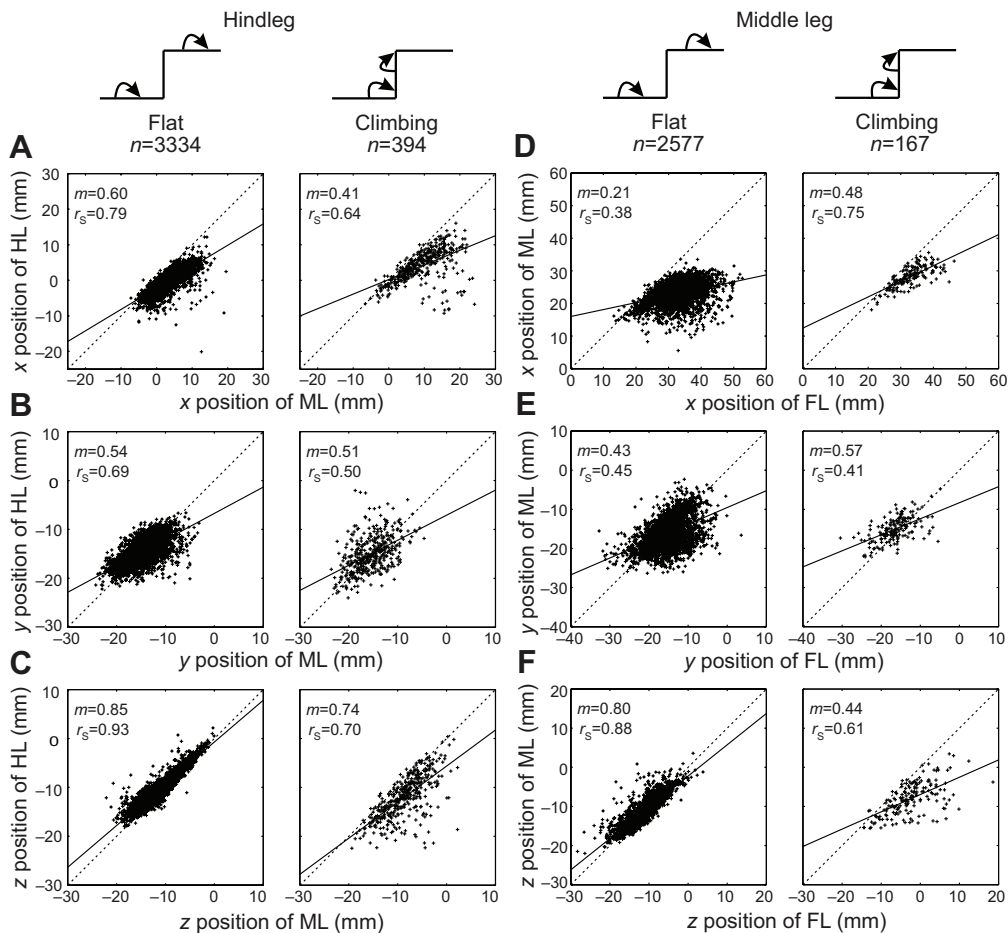
Fig. 2 Middle and hindlegs touch down close to their anterior neighbor. (A) Hind- and middle leg distributions of lift-off (left), touch-down (right) and target positions (gray) in body-fixed co-ordinates. The target positions were defined as the actual positions of the anterior leg, i.e. middle or front leg, when the posterior leg touched down. The gray area describes the 95th percentile of the target positions. (B–D) Distributions in body-fixed co-ordinates of hind- and middle leg touch-down positions relative to the target position (origin) given by the adjacent leg. Distributions are shown for flat steps in the horizontal plane (B) and for climbing steps in the horizontal (C) and the vertical plane (D). All 2D probability lines show the peak, the 25th, 50th, 75th and 95th percentiles. $N=9$ animals. The Euclidian distance of the maximum to the origin (dis) and the number of steps (n) are given in the lower right of each graph.

To analyse the spatial co-ordination in more detail, we calculated 2D probability distributions of lift-off and touch-down locations of the trailing leg, along with the corresponding target positions. All of these co-ordinates were measured in a body-fixed co-ordinate system. In Fig. 2A, target positions are shown by the gray area, delimiting the 95% of current positions of the ipsilateral leading leg. These positions were measured at the time when the trailing leg touched down (see supplementary material Fig. S1). The distributions of touch-down and target positions of hindlegs were narrower than the distribution of their lift-off positions (Fig. 2A). This was the opposite in middle legs. The similar sizes of touch-down and target areas suggest that the touch-down positions were adjusted to the target positions. However, in principle such similar area sizes could be a result of random distributions around a fixed point with low precision. To determine the accuracy of a putative targeting mechanism, the distance between adjacent legs needed to be measured. For this, we calculated the 2D probability distributions of touch-down positions with respect to the corresponding target position (origin, Fig. 2B–D).

Accuracy of spatial co-ordination among legs is high, precision depends on context
From Fig. 2B–D, we can retrieve the accuracy, i.e. distance-to-target, and the precision, i.e. spread of the distributions. Touch-down positions were centered very close to the target position.

Accordingly, accuracy was high in both flat steps and climbing steps. Judged from the distance of the maximum to the origin, accuracy was higher in hindlegs (flat: 2 mm; climbing xy/xz: 2/2.8 mm) than in middle legs (flat: 6.3 mm; climbing xy/xz: 2.8/2.8 mm). Furthermore, touch-down positions were bounded by the x-position of the leading leg. In hindlegs, targeting was most precise on horizontal surfaces, where the spread of distribution was smallest (Fig. 2B). During climbing, targeting was less precise, but the majority of the steps touched down less than 10 mm behind the target position (Fig. 2C,D). A comparison of the top view (xy) and the side view (xz) of the touch-down distributions of climbing steps indicated that hindleg targeting was more precise in dorsoventral than in mediolateral directions (Fig. 2C,D). During flat walking, middle leg targeting was less precise than hindleg targeting, as revealed by the wider spread of the distribution (Fig. 2B). During climbing, the spreads of the corresponding distributions were very similar within the 75% percentile range. Beyond the 75% percentile range the spread of the distributions differed most strongly between flat and climbing steps, revealing a decrease in precision in hindlegs and an increase in precision in middle legs (compare Fig. 2B and 2C). As a consequence, the average Euclidian distance between hind- and middle legs was significantly larger in climbing than in flat steps (flat: 4.21±2.45 mm; climbing: 7.53±5.81 mm; Wilcoxon signed rank: $P<0.005$, $N=9$), whereas between middle and front legs it was

Fig. 3. Touch-down positions of adjacent legs are highly correlated. (A–C) Correlations between hindleg (HL) and middle leg (ML) positions of flat steps, which touched down on horizontal surfaces and climbing steps, which touched down on a vertical surface. (D–F) Correlations between ML and front legs (FL). All correlations were highly significant ($P<0.001$). The slopes of the regression lines (m) and Spearmans's rho (r_s) are given in the top left of each plot. The dashed bisecting line has a slope of 1.

significantly lower (flat: 10.65±5.64 mm; climbing: 8.69±5.09 mm; Wilcoxon signed rank: $P<0.05$, $N=9$). Thus precision of spatial co-ordination among legs is context dependent.

Touch-down positions are highly correlated

Although trailing legs touched down close to the position of their adjacent leading leg, we cannot yet conclude that they actively followed this position. For example, nearby touch-down locations between ipsilateral legs could have resulted from mechanical limitations. This is why we calculated correlations between the positions of hind- and middle legs (Fig. 3A–C) and between middle and front legs (Fig. 3D–F) separately for each direction (x, anteroposterior; y, mediolateral; z, dorsoventral). Because flat steps were characterized by both legs being located on the same horizontal surface, a high correlation in the dorsoventral direction for flat steps was obvious (Fig. 3C,F). Note, however, that the slope was less than 1 because the inclined body axis caused smaller dorsoventral distances for posterior than for anterior foot locations on the same planar surface.

First, we concentrated on hindleg targeting, where all correlations were highly significant for each animal (Table 1; Spearman's rank correlation, $P<0.001$), and all linear regression lines had positive slopes ranging from 0.41 to 0.85 (Fig. 3A–C). The strength of the correlations (Spearman's rho, r_s) depended on the direction. Whereas r_s was high in anteroposterior and dorsoventral directions, it was lower in the mediolateral direction. Apart from the slope, the location of the points with respect to the bisecting line also depended on the direction. In the anteroposterior direction, a systematical shift below the midline indicated that hindlegs most

often touched down behind the middle legs. In contrast, the distributions were centered on the bisecting line in the mediolateral direction (Fig. 3B). In the dorsoventral direction, the location of the points depended on the step type. During climbing, the hindleg touched down below the position of the middle leg, i.e. most points lay below the bisecting line (Fig. 3C).

The high correlation of climbing steps in the dorsoventral direction ($r_s=0.70$) and the relatively steep slope of the regression line ($m=0.74$) reveal that the hindlegs followed the position of the middle legs in 3D space (Fig. 3C). Intriguingly, during climbing both the correlation coefficient and the slope were even higher in the dorsoventral direction than in the other two directions. In general, there was a tendency for stronger correlations and steeper slopes for flat than for climbing steps. The latter seemed to be more influenced by outliers.

Middle leg targeting was more variable than hindleg targeting, but middle leg touch-down positions were still significantly correlated with their target positions (Fig. 3C–F; Spearman's rank correlation, $P<0.001$). In contrast to the hindlegs, the correlation of flat steps was stronger in the mediolateral (Fig. 3E; $r_s=0.45$) than the anteroposterior direction (Fig. 3D; $r_s=0.38$). This changed during climbing, where the correlation coefficient in the anteroposterior direction almost doubled, whereas it remained virtually unchanged in the mediolateral direction. Most interestingly, the correlation in the dorsoventral direction was not only significant, but also strong during climbing (Fig. 3F; $r_s=0.61$). This reveals that the middle legs adjusted their dorsoventral foot position to the position of the front legs and that targeting of the middle legs works in 3D space, too, though less efficiently than in hindlegs.

Table 1. Correlations between hind- and middle leg positions for each animal

Animal	Condition	n steps	x		y		z	
			r_S	P	r_S	P	r_S	P
1	Flat	378	0.78	<0.001	0.70	<0.001	0.96	<0.001
	Climbing	72	0.44	<0.001	0.47	<0.001	0.52	<0.001
2	Flat	228	0.66	<0.001	0.47	<0.001	0.93	<0.001
	Climbing	39	0.78	<0.001	0.47	0.003	0.72	<0.001
3	Flat	250	0.87	<0.001	0.77	<0.001	0.89	<0.001
	Climbing	38	0.83	<0.001	0.71	<0.001	0.73	<0.001
4	Flat	259	0.83	<0.001	0.50	<0.001	0.85	<0.001
	Climbing	52	0.49	<0.001	0.57	<0.001	0.67	<0.001
6	Flat	532	0.83	<0.001	0.61	<0.001	0.94	<0.001
	Climbing	43	0.38	0.012	0.45	0.003	0.73	<0.001
7	Flat	453	0.70	<0.001	0.74	<0.001	0.96	<0.001
	Climbing	25	0.86	<0.001	0.49	0.014	0.84	<0.001
9	Flat	435	0.67	<0.001	0.59	<0.001	0.89	<0.001
	Climbing	53	0.72	<0.001	0.39	0.004	0.84	<0.001
11	Flat	395	0.85	<0.001	0.52	<0.001	0.96	<0.001
	Climbing	28	0.57	0.002	0.55	0.003	0.68	<0.001
12	Flat	404	0.74	<0.001	0.81	<0.001	0.95	<0.001
	Climbing	44	0.69	<0.001	0.59	<0.001	0.91	<0.001

The correlations between hind- and middle leg positions were calculated for each animal for each direction. 'Animal' indicates the number of the animal in the experiments. 'Condition' separates between flat and climbing steps. The number of steps is given by *n* and the corresponding r_S (Spearman's rho) and the *P*-value are given for each direction (*x*, *y*, *z*).

Spatial congruence

To test whether the accuracy of the targeting mechanism of the hindlegs was spatially congruent or depended on the position of the middle leg (target), we subdivided the target positions of flat and climbing steps into nine groups. For this we separated the distribution according to its 33 and 66% quantiles. In particular, we were interested to see whether there were directional differences depending on the position of the leading leg. For each of the nine groups, we calculated the median target position and the corresponding median touch-down position of the hindleg. These positions, connected by the green line, are shown in Fig. 4. As a result of the subdivision by percentiles, the target positions always showed a rectangular three-by-three grid. The same grid was observed for the median hindleg foot positions for both flat and climbing steps in the top view (Fig. 4A). However, the target positions of climbing steps were distributed more widely than

those of flat steps. Moreover, for climbing steps the target positions were shifted anteriorly (Fig. 4A). Along the anteroposterior direction, distances between hind- and middle leg foot positions were larger, the more anterior the target was. This effect was more pronounced for climbing steps, potentially because the target positions were outside the working range of the trailing leg.

In the side view (Fig. 4B), the 3-by-3 grid was also perfectly represented by the hindleg positions of flat steps. As described for Fig. 3C, the apparent dorsal shift of the hindleg foot positions is due to the inclined body axis. In contrast, the grid was distorted in climbing steps, because of the most dorsal and anterior position (Fig. 4B). Note that the three most anterior target positions were located outside the working range of the hindleg, as indicated by the blue line. Still, this extreme location of the target position appeared to affect targeting much less in the median and ventral range than in
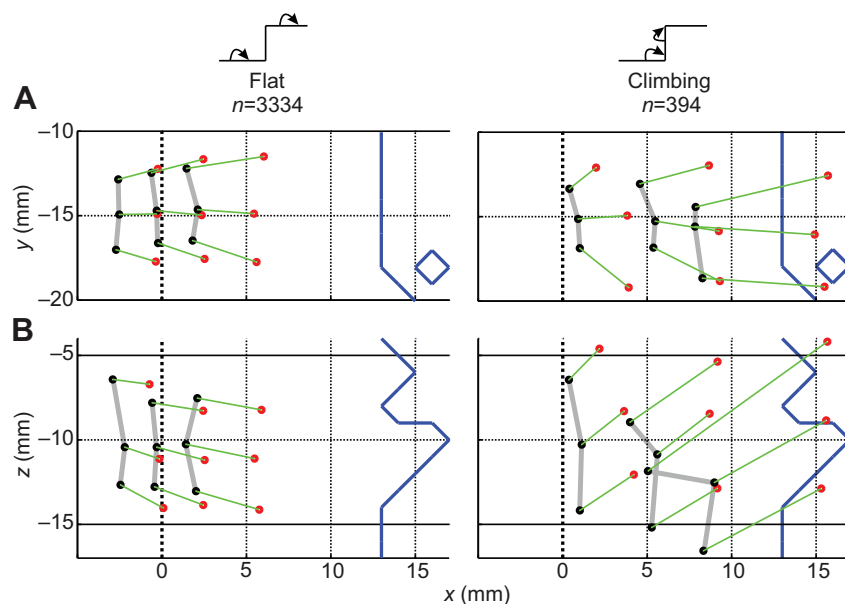

Fig. 4. Accuracy of hindleg targeting depends on the target position. (A) Top view. (B) Side view. Middle leg positions were separated into nine groups by the 33rd and 66th percentiles of each direction, respectively. The median positions of these groups (red) were connected by the green lines to the corresponding median touch-down positions of the hindlegs (black). The distance between these positions was larger, when the target was more anterior. To illustrate the differences between flat and climbing steps, hindleg touch-down positions were connected by gray lines. The blue lines show the maximum of the touch-down distributions. All positions were measured in body-fixed co-ordinates. The number of steps (*n*) varied between groups from 247 to 531 for flat steps and from 25 to 57 for climbing steps.

the dorsal range. The same tendency was observed for targeting in the median anteroposterior range (i.e. the second column of the grid), despite the fact that here the target positions were well inside the working range of the hindleg. These patterns show that touch-down positions of the hindlegs were adjusted to the position of the ipsilateral middle legs irrespective of whether the animal was flat walking or climbing. Furthermore, the accuracy of targeting depends on the location of target position. As a consequence, targeting is spatially congruent only when excluding the anterodorsal part of the legs' working range.

Manipulation of middle leg proprioception

Spatial correlation and congruence of the targeting mechanism requires information transfer from the leading to the trailing leg. For planar, tethered walking animals, it was shown that sensory hair fields at the coxa and the trochanter contribute to spatial co-ordination (Cruse et al., 1984; Dean and Schmitz, 1992). In order to analyse the influence of sensory feedback from hair fields on 3D-targeting, we manipulated middle leg proprioception and monitored its effect on spatial co-ordination of the legs. As the height of the foot is mainly influenced by the coxa–trochanter joint, which elevates the trochanterofemur (Wendler, 1964), we hypothesized that the trochanteral hair field might be involved in the information transfer about dorsoventral foot position. We reasoned that, after ablation, the trochanteral hair field could no longer measure the levation angle of the coxa–trochanter joint, thus affecting the dorsoventral accuracy of targeting. To control for the complete ablation of all hairs, we examined the coxa–trochanter joint with a scanning electron microscope (SEM) after the behavioral experiments were done (Fig. 5A,B). In all but one animal, the complete hair field was removed, without destroying the cuticle of the joint. In one animal, a few hairs were still visible under the SEM and we could not exclude that these hairs remained intact. Nevertheless, this animal showed the same behavioral effects as the

other four animals (probably because most hairs were ablated). This is why we included it in the analysis.

In all animals, the manipulated leg performed unexpected additional searching movements (Fig. 5C,D). These movements were characterized by a single circular trajectory of the foot at the end of a swing movement, without touching the ground (Fig. 5D, zoom). The similarity with searching movements described by Dürr (Dürr, 2001) suggested that these were searching movements. On the additional circular trajectory, the leg first moved up and backwards, before it moved down again. As a result of ablation, the number of occurrences of searching movements by the manipulated leg increased in all five animals by 9–38% (Fig. 6A). This behavioral change persisted from the day of ablation (Day 1) to the day after ablation (Day 2), and often caused the leg to touch down more anteriorly than in a regular swing movement. Since we could not find any behavioral differences between Day 1 and Day 2, we pooled those data for further analyses.

Apart from the terminal searching movements, the swing movements of the operated leg were also significantly higher after hair field ablation (Mann–Whitney U-test: $P<0.001$ for each animal; $n=180/479, 259/380, 173/343, 113/395, 189/450$; $z=-12.1, -12.0, -10.2, -5.8, -3.4$). The swing height was only affected in the operated leg, but not in any of the other five legs (Fig. 6B). In the operated leg, the median swing height increased in flat steps by 47% (from 10.5 to 15.4 mm) and in climbing steps by 29% (from 16.3 to 21.0 mm). Both the higher swing movements and the additional searching movements indicate that proprioceptive feedback is not only important for the spatial information transfer between legs, but also for controlling the swing movement of the leg itself.

With regard to spatial co-ordination of hind- and middle legs, ablation of the trochanteral hair field also had significant effects. For example, the Euclidian distance between the manipulated middle leg (leading leg) and its trailing hindleg increased significantly (U-test: $P<0.001$ for each animal; $n=389, 443, 332, 319, 403$; $z=-8.0, -3.5$,
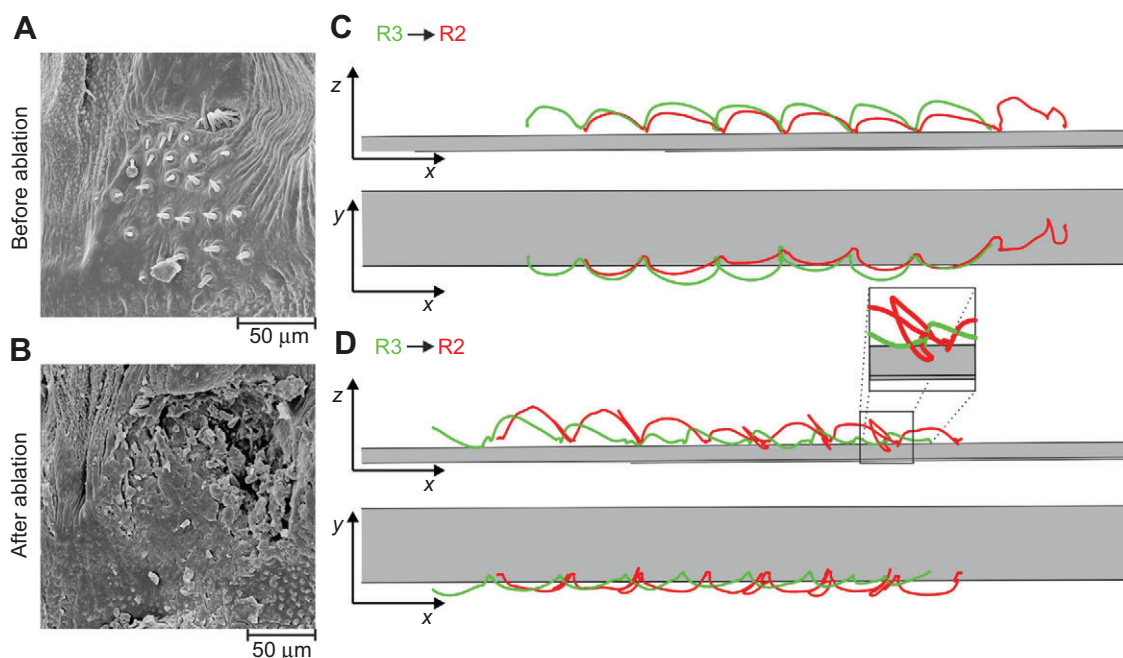

Fig. 5. After hair field ablation, the operated leg performs additional searching movements. (A) Intact trochanteral hair field of a right middle leg. (B) Cuticle at the trochanter after the hair field has been removed. (C,D) Trajectories of the leading right middle leg (red, R2) and its trailing right hindleg (green, R3) before (C) and after (D) ablation are shown in side and top view. One example of a typical searching movement is enlarged. Co-ordinate systems have a length of 50 mm.
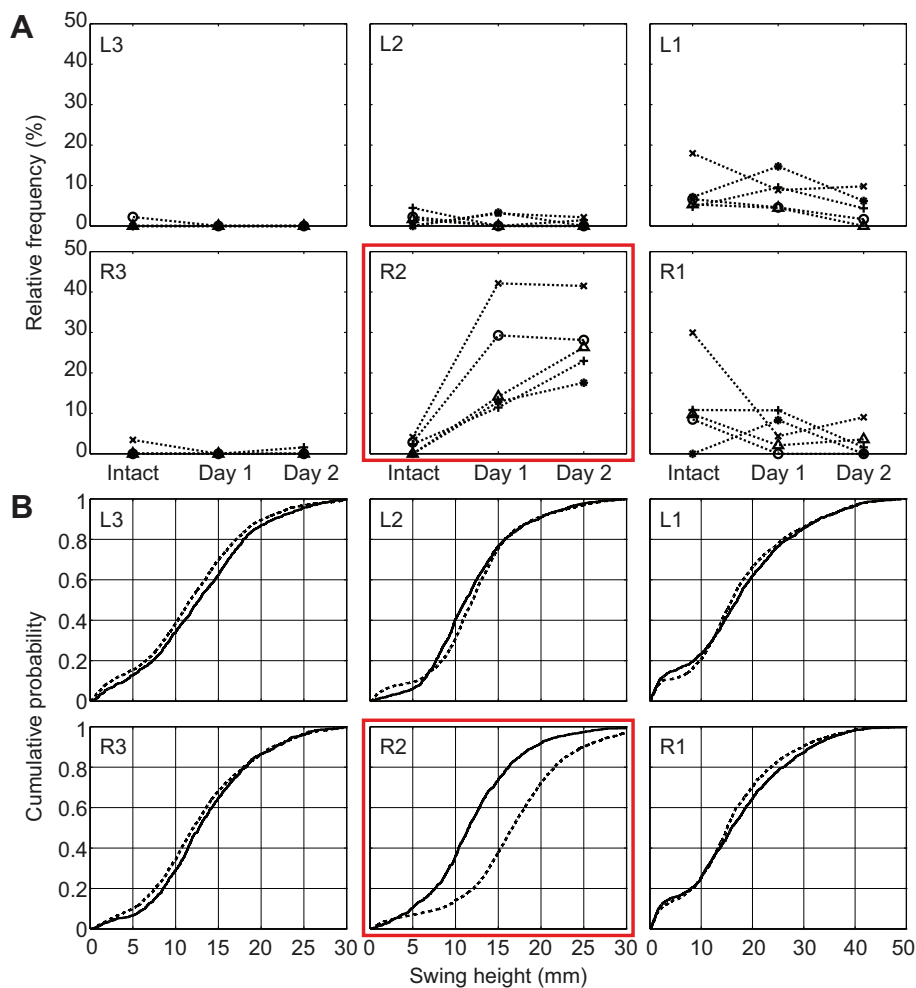
Fig. 6. Proportion of searching movements and swing height are increased after hair field ablation. (A) The mean proportion of searching movements was calculated for each leg (L1–L3 and R1–R3), and for each individual animal during flat walking trials before ablation (Intact), on the day of ablation (Day 1), and 1 day after (Day 2). In the manipulated leg (R2, red box) the proportion of searching movements was increased after ablation. (B) Cumulative probabilities of swing height for flat and climbing steps before (continuous line) and after (dashed line) trochanteral hair field ablation in the right middle leg. Only in the operated leg (R2), the distribution shifted towards larger swing heights.

−10.7, −4.1, −8.1). This increase did not result from the additional searching movements, because they were excluded from this analysis.

A second effect concerned the spatial correlations. Before ablation, the hindleg followed the middle leg in 3D space and spatial correlations were even a bit stronger than in the first experiment (Fig. 7A−C; Spearman's rank correlation, $P<0.001$ for all directions). After ablation, all spatial correlations remained significant ($P<0.001$), but the correlation coefficients diminished for anteroposterior and dorsoventral directions (Fig. 7D,F). In the anteroposterior direction, r_S was significantly reduced from 0.83 to 0.30 for flat steps (Fisher transformation, $P<0.001$) and from 0.58 to 0.16 for climbing steps (Fisher transformation, $P<0.001$). In the dorsoventral direction, spatial correlation had to remain very strong for flat steps. During climbing, the correlation in the dorsoventral direction was significantly weaker after ablation (r_S reduced from 0.79 to 0.64, Fisher transformation, $P<0.005$). There was no effect in the mediolateral direction, where r_S remained unchanged after ablation (Fig. 7E, Fisher transformation, $P>0.05$). The reduced correlations revealed that targeting was affected by hair field ablation.

With regard to spatial congruence, the effect of ablation appeared to be stronger in the parasagittal plane (xz plane; side view) than in the horizontal plane (xy plane; top view). When analysing the data in the same way as in Fig. 4, the median positions of the manipulated middle leg were found to be shifted anteriorly after ablation (red dots in Fig. 8), irrespective of the step type (flat or climbing). The trailing hindleg did not follow this shift. In contrast, after ablation all median

hindleg foot positions of flat steps were located posterior to the hindleg coxa, and accuracy was lower (i.e. green connecting lines were longer) than in the intact animal (Fig. 8A). During climbing, the target positions set by the leading middle leg were shifted anteriorly, laterally and dorsally after ablation (Fig. 8B,C). As in flat walking, the hindleg did not follow this shift (Fig. 8C) and accuracy was reduced after ablation. The effect was strongest in the dorsoventral direction, where in the intact animals only the most anterior target positions were not followed in a congruent manner (Fig. 8C; see also Fig. 4B). After ablation, the grid of median touch-down locations of the hindleg was strongly distorted, and the nine positions almost aligned on a single, curved line.

We can summarize that, apart from the effects on swing movements of the operated leg, ablation of the trochanteral hair field had two main effects: (1) the target positions of the leading leg were shifted (mainly anteriorly and dorsally); and (2) the opposite was found in the trailing hindleg, which touched down more posteriorly and more ventrally. This led to reduced spatial correlation and a loss of spatial congruence, as revealed by the distorted grid of median touch-down locations in Fig. 8. Finally, targeting in anteroposterior and dorsoventral directions was less accurate after ablation.

DISCUSSION

Spatial co-ordination of multiple legs can be an important means for improving locomotion efficiency by allowing a trailing leg to exploit the spatial information from its leading neighbor. To date, spatial co-ordination among legs in insects has only been studied in tethered
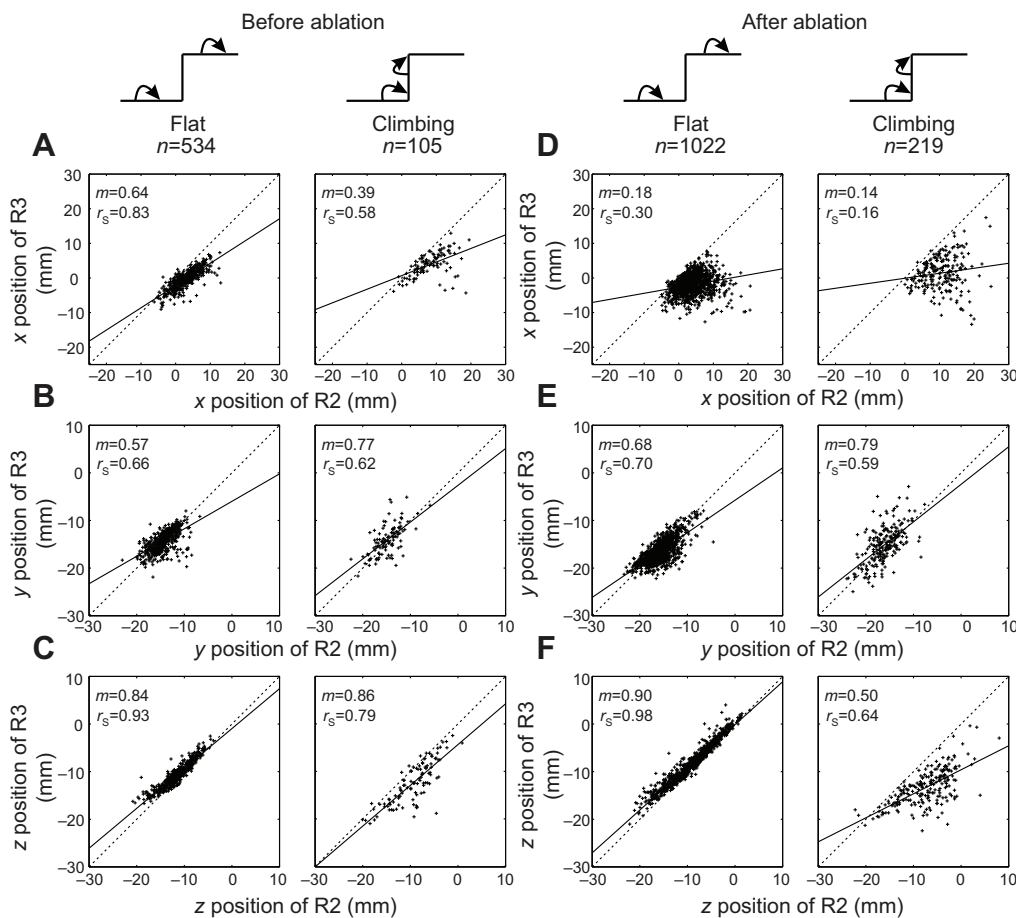
Fig. 7. Correlations between the position of the right hindleg and the right middle leg differ before and after hair field ablation in the right middle leg. Correlations are shown for flat and climbing steps in each direction: anteroposterior (A,D), mediolateral (B,E) and dorsoventral (C,F). The hair field ablation in the middle leg affected the correlation in anteroposterior and dorsoventral directions. The slopes of the regression lines (m) and Spearman's rho (r_s) are given in each plot. The dashed bisecting line has a slope of 1.

walking or standing animals. In order to provide better understanding of the relevance of spatial co-ordination in natural behavior, the main objective of this study was to determine the accuracy and precision of spatial co-ordination during unrestrained locomotion. We show that, in walking and climbing stick insects, both hind- and middle legs follow their leading neighbor in 3D space. Furthermore we show that the trochanteral hair field is involved in both the local control of swing movements and the transfer of spatial information to trailing legs.

Spatial co-ordination in 3D

Based on the 3D motion analysis of the entire insect body, we were able to analyse the probability distributions of foot placement during unrestrained walking and climbing. The resulting body-centered distributions showed that accuracy of foot placement is high and similar during flat walking and during climbing (Fig. 2). Significant correlations between touch-down positions of adjacent legs revealed that targeting works in the dorsoventral direction, too. As a consequence, the underlying mechanism takes account of all three dimensions (Fig. 3). Finally, the spatial congruence was maintained throughout large parts of the legs' working ranges. As a result, the spatial ordering of touch-down positions of a trailing leg copied that of leading legs (Fig. 4), except for the most dorsal and anterior target positions.

A targeting mechanism underlying spatial co-ordination in insects was first proposed by Cruse (Cruse, 1979), who found that the hindleg of a tethered standing stick insect follows the position of the middle leg within the horizontal plane. Later, targeting was shown for walking insects, too, both for hind- and middle legs (Dean and Wendler, 1983). Recently, state dependency was reported, based on the comparison of standing and stationary walking (Wosnitza et al., 2013). In comparison, we show that spatial co-ordination among legs persists in freely walking and climbing animals, even if body orientation varies considerably and each leg's contribution to propulsion and body support changes. Unrestrained locomotion increases the variability of step parameters and even reveals functionally distinct step classes (Theunissen and Dürr, 2013). Our analysis shows that spatial co-ordination remains spatially congruent throughout large areas of foot positions, proving that spatial correlation of tarsus positions is not a consequence of limited space, neither in range nor in dimensionality. The precision of hindleg touch-down, i.e. the width of the spatial distribution relative to the target position, suggests that most variability in spatial co-ordination is in the range of a tarsus length (ca. 5 mm). The high accuracy, i.e. short distance between target and touch-down positions, irrespective of walking and climbing steps, reveals that efficiency of spatial co-ordination is maintained during climbing. In hindlegs, precision is reduced during climbing. In part, this might be linked to increased variability in body orientation throughout single steps. Interestingly, precision of middle leg targeting improved during climbing, potentially hinting at a similar state dependency as was described for the onset of locomotion (Wosnitza et al., 2013).

The strong correlation of dorsoventral co-ordinates during climbing confirmed our hypothesis that spatial co-ordination indeed involves a 3D targeting mechanism. As it is known that reaching movements of front legs may be guided by touch events of the antennae (Schütz and Dürr, 2011), stick insects appear to pass on 3D spatial information across the entire body, from the antennae to the
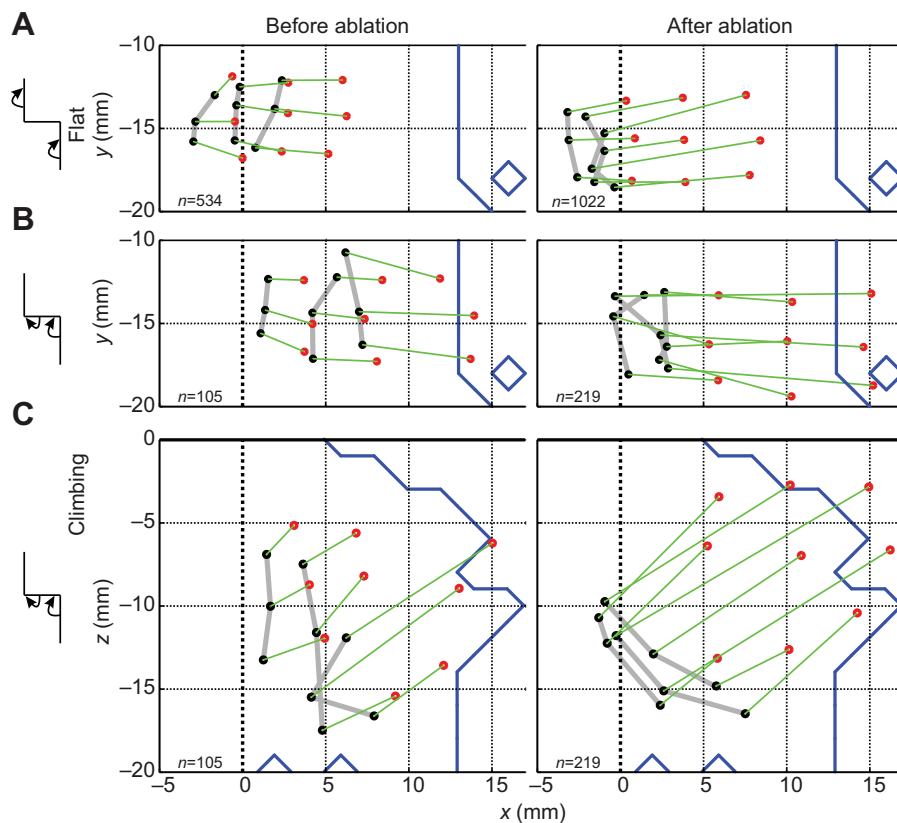
Fig. 8. Precision of hindleg targeting is decreased after ablation. (A,B) Top view. (C) Side view. Middle leg positions were separated into nine groups by the 33rd and 66th percentiles of each direction, respectively. The median positions of these groups (red) were connected by the green lines to the corresponding median touch-down positions of the hindlegs (black). After ablation, target positions were shifted anteriorly and targeting was distorted in the dorsoventral direction. The blue lines show the maximum of the touch-down distributions. The number of steps before ablation varied from 40 to 82 for flat steps, and from 6 to 17 for climbing steps. After ablation, the number varied from 89 to 140 for flat steps, and from 13 to 34 for climbing steps. The total number of steps (n) is given to the bottom left.

hindlegs (Ache and Dürr, 2013). Spatial co-ordination has also been described in locusts and horse-head grasshoppers, both of which adjust their front leg positions in 3D space according to visual cues (Niven et al., 2010; Niven et al., 2012). Humans, too, use visual feedback to adjust the height of the foot to the height of an obstacle (Patla and Rietdyk, 1993). In stick insects, visual feedback appears to play only a minor role in the spatial co-ordination of the legs (Dürr, 2001; Bläsing and Cruse, 2004a), though detailed studies on visual cues on foot placement are missing.

With regard to the idea that spatial information is passed on across the body, it is important to note that targeting is less precise in middle legs than hindlegs. In part, this may be due to the relatively large variability of front leg movements (Cruse, 1976; Theunissen and Dürr, 2013). Moreover, the long distance between the front leg and middle leg coxae (the mesothorax is the longest body segment), along with the front legs being longer than the middle legs, render middle leg targeting more 'difficult' than hindleg targeting: front leg foot positions are often out of reach for the middle leg (Fig. 2A: see overlap of touch-down and target areas). Our findings confirm results on tethered walking animals (Dean and Wendler, 1983; Wosnitza et al., 2013). In contrast to these earlier studies, our animals had to support their own body weight and could move freely. The only constraints were due to the surfaces of the setup, which inevitably formed a physical boundary to foot placement. As a consequence, dorsoventral correlation of foot positions had to be strong for flat steps. Mediolateral correlations were slightly influenced by the limited width of the walkway (40 mm). Moreover, anteroposterior correlations of climbing steps were affected by the vertical surfaces of the stairs. Note that these constraints are inevitable when studying natural locomotion, simply because the animal has to make contact with a surface. As yet, the persistence of correlations, but also the effect of proprioceptor ablations, cannot be explained by physical constraints of the setup.

Note that the strength of spatial correlation between neighboring legs (Fig. 3) would have been higher if the time of lift-off had been used as the reference time point, instead of the time of touch-down (see Materials and methods). Because using the earlier time point (lift-off of trailing leg) yielded stronger spatial correlations than using the later time point (touch-down of trailing leg), we can exclude that stick insects take into account the effect of their own forward movement in spatial co-ordination. If they had done so, one would have expected the opposite effect, i.e. using the later time point should have yielded stronger correlations.

Finally, accuracy of targeting depended on the position of the leading leg. During climbing, the middle leg positions were shifted anteriorly, and the hindleg did not fully compensate for this shift (Fig. 4). With respect to the mean length of the hindlegs (approximately 30 mm), target positions that were more distant than 20 mm from the coxa could only be reached with full or nearly full extension of the hindleg. This can explain the decrease in accuracy during climbing (Fig. 4). Despite the decrement in accuracy, spatial congruence was maintained except for the most dorsal and anterior target position.

Sensory control of foot placement

Our ablation experiment showed that spatial co-ordination in 3D depends on proprioceptive information about leg levation. After hair field ablation, the correlation in the anteroposterior direction was diminished (Fig. 7) and spatial congruence in the parasagittal (xz) plane almost vanished (Fig. 8). Given the large area covered by touch-down positions, this finding implies that the significant spatial correlations in intact animals were not caused by physical constraints by the setup, but that targeting is a sensory-guided mechanism.

Our results are consistent with the results by Cruse et al. (Cruse et al., 1984) who analysed the effects of different sensory hair fields on

horizontal targeting in tethered animals. When the two coxal and trochanteral hair fields were ablated together, the distance between hind- and middle leg positions was increased in the anteroposterior direction by ~4 mm, i.e. targeting accuracy decreased (Cruse et al., 1984). In contrast, ablation of the trochanteral hair field only caused an anteroposterior shift by ~1 mm, and the lateral direction was not affected at all (Cruse et al., 1984). This is in line with results by Dean and Wendler (Dean and Wendler, 1983) and Dean and Schmitz (Dean and Schmitz, 1992), showing that ablation of the coxal hair plates causes reduced spatial correlation between middle and hindlegs. Owing to these results, we focused on the role of the trochanteral hair field that monitors levation of the leg. We hypothesized that it is involved in spatial co-ordination in the dorsoventral direction.

In comparison with the former results, we found a slightly stronger effect of hair field ablation: for flat steps, the distance increased from 3.2±1.9 to 6.1±4.3 mm. This larger change and the associated high standard deviation after ablation may be explained by the larger variability (in 3D) of target positions than in earlier experiments; in the study by Cruse et al. (Cruse et al., 1984), animals were tethered and the target position was manually set to distinct positions.

Contrary to our hypothesis, ablation of the trochanteral hair field only slightly affected the dorsoventral correlation of foot positions. It was still stronger than the mediolateral correlation, which the ablation did not affect at all. The persistence of significant correlations suggests that sensory organs other than the trochanteral hair field (e.g. other hair fields or chordotonal organs) contribute to the spatial co-ordination in all directions. This is supported by studies showing that multiple hair field ablations cause stronger behavioral effects than single ones (Cruse et al., 1984; Krause et al., 2013).

In addition to the effect on spatial co-ordination among legs, we also found local effects on the manipulated leg itself. The ablation increased the frequency of searching movements in the manipulated leg (Fig. 6), an effect that has not been reported before. Searching movements are usually made after loss of ground contact, for example when stepping into a gap or across an edge (Dürr, 2001; Bläsing and Cruse, 2004a). They have been modeled as an integral property of the controller that generates aimed swing movements. According to this view, searching movements arise automatically if a swing movement is not terminated by ground contact (Dürr, 2001). This is analogous to the cyclic grooming movements of locusts that first aim at the touched site on the body surface, only to transition into a set of loops (Dürr and Matheson, 2003). Similarly, the stick insect *Medauroidea extradentata* (=*Cuniculina impigra*) shows aimed searching movements with its front legs that repetitively wave across the space where an object is touched. Interestingly, these movements are no more directed to the former object location after ablation of the trochanteral hair field (Berg et al., 2013). The increase in swing height after ablation of the trochanteral hair field (Fig. 6) can be explained by the lack of the sense organ measuring leg levation (Wendler, 1964). If not only the swing movement but also the set point of the searching movement was raised due to the ablation, one would expect a higher incidence rate of searching movements during walking. The effect would be as if the leg 'expected' to touch down earlier than it actually could, leading to the beginning of a searching movement just above the substrate.

We can conclude that proprioceptive feedback is essential for spatial co-ordination of foot placement in multi-legged locomotion. In combination with other results, it appears as if spatial co-ordinates are transferred along the body. The antennae transfer spatial near-range information about touch events to the front legs (Schütz and Dürr, 2011), the middle legs follow the front legs (Figs 2, 3; supplementary material Fig. S1) (Dean and Wendler, 1983), and the

hindlegs follow the middle legs in 3D space (Figs 2–4). Indeed, descending interneurons of the head ganglia were shown to encode antennal posture (Ache and Dürr, 2013) and local interneurons of the metathoracic ganglion encode the positions of middle leg tarsus (Brunn and Dean, 1994). This cascade of information transfer between adjacent limbs is likely to improve locomotion efficiency by helping each leg to find appropriate foothold in complex natural environments.

MATERIALS AND METHODS
Animals and experiments
Experiments were carried out on adult female stick insects (*Carausius morosus* de Sinéty 1901), from a parthenogenetic colony at Bielefeld University. Nine intact animals were used in the first experiment, and five in the second. Unrestrained animals walked on a horizontal footbridge (40×490 mm, polyvinyl chloride). The first experiment had four conditions: animals walking without stairs (control) and animals climbing over a set of two stairs of height (h): low (h=8 mm), middle (h=24 mm) and high (h=48 mm). The conditions were presented randomly for at least ten times each. In the second group of animals, the trochanteral hair field of the right middle leg was ablated with a razor blade. SEM confirmed whether the hairs under the joint (condyle) were completely removed (Fig. 5A,B). The ablation experiment was repeated with the conditions control and high. In order to monitor potential gradual effects during the recovery time, post-ablation measurements were taken on the day of ablation (Day 1) and on the day after ablation (Day 2). Note that ablated hairs do not grow back (Cruse et al., 1984).

Motion capture and reconstruction
Motion capture and kinematic reconstruction of the animals were described in detail by Theunissen and Dürr (Theunissen and Dürr, 2013). Data acquisition was done with a Vicon motion capture system (Vicon, Oxford, UK), consisting of eight cameras (Vicon T10). The system reached a spatial resolution of 0.1 mm and recorded the positions of retro-reflective markers with 200 Hz. A digital camera (Basler A602fc, Ahrensburg, Germany) with a near range zoom lens (Edmund Optics, Barrington, NJ, USA) recorded the right side view of the first stair with a spatial resolution of ~0.14 mm per pixel and a temporal resolution of 50 Hz. The markers of diameter 1.5 mm were glued with nail polish to the animal's body. Care was taken that the nail polish did not restrain the movement of the joints. Twelve markers were attached to the legs: one to each femur and one to each tibia. Three markers were attached to the metathorax. These markers were used to obtain the body position and orientation in space and to derive the body-fixed co-ordinate system (body-CS) (Theunissen and Dürr, 2013). Additional markers were attached to the prothorax and the head. In five animals, an additional marker was placed on the mesothorax, resulting in 18 markers. Except for the marker on the head, the same marker constellation was used in the ablation experiment. The software Nexus 1.4.1 (Vicon) was used to identify and label the markers. Once the markers were labeled, they were detected automatically throughout the trial with less than five gaps per 60 s in single marker trajectories. Gaps shorter than 200 ms (40 frames) were filled by use of an interpolation algorithm of the software Nexus.

To reconstruct the body of the animal, we needed to know the length of each segment and the position of every marker on the segments. This was measured from high-resolution photographs (0.02 mm per pixel) taken under a stereo lens (Olympus SZ61T, equipped with a Pixelink camera PL-B681CU). Since the length of the segments, the position of markers on each segment and the trajectories of the markers were known, the movements of the animals could be reconstructed.

The center of the border between the first and second abdominal segment (in *C. morosus* the first abdominal segment is fused with the metathorax) was considered as the origin for the body-CS. The *x*-axis of the body-CS was directed anteroposteriorly (positive anterior), the *y*-axis mediolaterally (positive left) and the *z*-axis dorsoventrally (positive upward).

Step detection
For the analysis of targeting, it was necessary to have a robust step detection mechanism. The tibia–tarsus joint was taken as foot position. Three

heuristics concerning foot velocity, duration and distance-to-setup were applied to decide whether a stance phase had occurred. If the velocity was less than 25 mm s^{-1} for at least eight continuous frames (40 ms), the foot was assumed to stand still. The mean foot position during this putative stance phase was calculated and foot contact was verified by the distance between the foot position and the surface of the setup. With a distance threshold of 10 mm we excluded very slow movements, during which the leg was held relatively still in the air. The apparently high threshold mainly accounted for the fact that the calculated foot position was an estimate of the tibia–tarsus joint position, and thus neglected the relatively long tarsus (average length, 5.2–5.7 mm).

To analyse spatial co-ordination of ipsilateral leg pairs, we took the touch-down event of the trailing leg as the reference time point (supplementary material Fig. S1). This reference time point was used to project foot contact positions of both the leading and the trailing leg into the same body-CS. This projection into body-fixed co-ordinates was essential for comparing flat with climbing steps, as it accounted for changes in body orientation. We could have also used the body-CS at the time of lift-off to project the positions into body-fixed co-ordinates. The difference between these alternatives is caused by the animal's forward movement during a swing movement. Using the lift-off time as a reference would have led to significantly stronger correlations (Wilcoxon signed rank test: $P<0.05$), but touch-down positions would have been unrealistic in that most of them would have been out of reach for the posterior leg.

Accuracy and precision of spatial co-ordination was defined in analogy to the terminology used for technical measurements (Joint Committee for Guides in Metrology, 2008). Assuming that spatial co-ordination is governed by a targeting mechanism that controls the touch-down position of the trailing leg according the lift-off position of the leading leg, we define 'accuracy' as distance between foot contacts and 'precision' as the spread of the distribution of touch-down locations.

In the present analysis we excluded short steps and focused on long steps only (Theunissen and Dürr, 2013). We further excluded steps that touched down on the side of the setup and those where the leading leg was not on the same surface or in swing phase at the reference point. Steps were divided into flat and climbing steps (Fig. 1). Flat steps lifted off and touched down on the same horizontal surface, whereas climbing steps touched down on the vertical surface of one of the stairs, lifting off either from a horizontal surface or from the vertical surface. Climbing steps, which lifted off from the vertical wall and touched down on a horizontal surface, were excluded, because the horizontal surface would have strongly affected foot placement in the dorsoventral direction. Steps from contralateral legs were pooled and represented as steps from right legs. In total, we analysed the following number of steps of intact animals: flat steps (middle legs: $n=2579$; hindlegs: $N=3334$); climbing steps (middle legs: $n=176$; hindlegs: $n=393$). Twelve trials used in this study have been made available in an open-access data publication (Theunissen et al., 2014a) as part of a collaborative project on improvement of availability and sharing of research data (Theunissen et al., 2014b). From the manipulated animals, we analysed spatial co-ordination before/after ablation: flat steps ($n=534/1026$) and climbing steps ($n=106/220$).

Statistics

Statistical tests were calculated using the Statistics Toolbox of MATLAB (MathWorks, Inc., Natick, MA, USA). Spearman's rank correlation coefficient was used to test the significance of correlations. Fisher transformation was used to compare correlations before and after ablation. The Mann–Whitney U-test (U-test) was used to compare the Euclidian distances between adjacent legs and to compare the swing heights before and after ablation.

Competing interests
The authors declare no competing financial interests.

Author contributions
L.M.T. carried out experiments, analysed data, interpreted the findings and wrote the manuscript. S.V. carried out ablation experiments and analysed data. V.D. designed experiments, supervised L.M.T. and S.V., interpreted findings and revised the manuscript.

Supplementary material
Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.108167/-/DC1

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