

RESEARCH ARTICLE

Single perturbations cause sustained changes in searching behavior in stick insects

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SUMMARY

Stick insects (*Cuniculina impigra*) possessing only a single front leg perform untargeted stereotypical cyclic searching movements with that leg when it loses contact with the ground. When encountering an object, the animals grasp it. We hypothesized that removal of the object immediately after contact with the leg's tibia would result in a change in searching strategy, i.e. searching movements confined to the former location of the object to regain contact. In our experimental setup, searching movements were restricted to upward and downward movements. After removal of the object, searching movements were continued. However, in post-contact searching, two movement parameters were usually changed: (1) average positions of searching movements were shifted towards the former position of the object; and (2) movement amplitudes were considerably smaller and accompanied by a decrease in cycle period. This confinement of searching movements to the location of contact was interpreted as targeting behavior. All parameters regained initial values after approximately 6 s. Changes in position and amplitudes were independently controlled. Neither of the changes was under visual control, but rather depended on the presence of the trochanteral hairplate, a sensory organ that measures the coxa–trochanter joint position. Changes in average leg position were linked to changes in the ratio of electrical activity in the levator and depressor trochanteris muscles, which were based on altered activity in both or either one of the muscles. Our data demonstrate a switch in a simple behavior that is under local sensory control and may utilize a form of short-term memory.

Key words: adaptation, invertebrate, searching, sensory–motor control, targeting.

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INTRODUCTION

Animals that move through the environment constantly need to adapt their movements to the environmental constraints. In vertebrates and invertebrates, tactile cues, besides input from other senses, play a prominent role in the exploration of the environment (Prescott et al., 2011). Rodents, for example, use whiskers to palpate objects (Diamond et al., 2008). During locomotion, many insects constantly use antennal exploration to gain information about their environment [cockroach (Camhi and Johnson, 1999; Harley et al., 2009; Baba et al., 2010); cricket (Honegger et al., 1990; Horseman et al., 1997); stick insect (Dürr et al., 2001; Schütz and Dürr, 2011)]. Insects also perform leg searching movements when losing ground contact, e.g. when approaching a gap. Such leg searching movements have been reported for stick insects (Dürr, 2001; Bläsing and Cruse, 2004a; Bläsing and Cruse, 2004b), locusts (Pearson and Franklin, 1984), cockroaches (Delcomyn, 1987) and fruit flies (Pick and Strauss, 2005).

Clearly, limb movements of insects can be targeted. These movements may be directed towards non-transient stimuli, e.g. cricket antennal movements that follow a visual cue (Honegger, 1981), but also towards transient stimuli. For example, stick insects target their front legs towards the location of preceding antennal contact (Schütz and Dürr, 2011). In some cases, targeted movements outlast the stimuli. For example, locusts perform targeted hindleg scratching movements that outlast the tactile stimulus on their wing (Matheson, 1997; Matheson, 1998). In locusts walking on a ladder, front leg placement is under visual control (Niven et al., 2010). However, when a rung has been relocated during a front leg's swing

phase, searching movements are performed at the original location of a rung. Because of this observation and the absence of immediate modification of the step, a memory of the rung position has been suggested (Niven et al., 2010). Inspired by these experiments, we became interested in the mechanisms employed by a sensory–motor system to adapt to such an unexpected event. A stick insect searching for foothold in a bush may touch a leaf or a twig that moves out of reach after the first contact, e.g. because of the elasticity of the material. We hypothesized that a change in searching strategy, i.e. searching movements confined to the former location of the object to regain contact, would result after such an event. Such change in searching strategy could employ a short-term memory in the range of seconds, as inferred from rung-searching locusts. Therefore, we aim to study how searching movements of a stick insect's leg are changed in response to immediate removal of an object that was in contact with the leg.

A stick insect possessing only one front leg performs searching movements with that leg upon loss of ground contact (Karg et al., 1991; Bässler, 1993). These rhythmic stereotypical leg movements consist of several cycles; their trajectories have been described (Karg et al., 1991; Dürr, 2001). When touching an object with the tibia during a downward or upward searching movement, stereotypical grasping movements are performed to hook the object with the claw (Bässler et al., 1991).

The present work demonstrates that stick insects show targeted searching movements after immediate removal of an object that was touched by the tibia. In targeted searching, two movement parameters are changed. First, the average position of searching

movements is shifted towards the former position of the object. Second, movement amplitudes are considerably smaller, accompanied by a decrease in cycle period. All parameters regain initial values after approximately 6 s. Changes in leg levator and depressor muscle activity and the relevance of several sensory organs for the control of these movements are analyzed.

MATERIALS AND METHODS

Animals

Experiments were carried out on adult female stick insects *Cuniculina impigra* Brunner von Wattenwyl 1907 from the colony maintained at the Zoological Institute, Biocenter Cologne. Animals were kept at constant temperature (22–24°C), 60% humidity and under a 12 h:12 h light:dark cycle. Experiments were performed at room temperature (20–24°C) and under dimmed light conditions.

Preparation

For experiments, all legs except the left prothoracic leg were cut off mid-coxa. Animals were mounted dorsal side up with insect pins or dental cement (Prottemp II, 3M ESPE, Seefeld, Germany) on a foam platform, the coxa of the remaining leg being located on the edge of the platform. The leg was fixed at an angle of 90 deg with respect to the body axis by applying dental cement to the thorax–coxa (ThC) joint. Movements of all other leg joints [coxa–trochanter (CTr), femur–tibia (FTi), tibia–tarsus (TTa) and tarsal joints] were not restricted, thus the animal could freely move its leg in the vertical plane. Animals were not able to touch the ground.

For recordings of levator and depressor trochanteris muscle activity, two copper wires (57 µm, isolated except for the tip) were inserted into each muscle through small holes in the dorsal and ventral posterior coxa of the front leg before fixation of the coxa (Rosenbaum et al., 2010).

For experiments regarding the influence of vision, the animals' view was either blocked by a black paperboard between head and leg or their eyes were covered with black ink. In some experiments, the trochanteral hairplate (trHP) (Wendler, 1964) was shaved off with a razor blade (Akay et al., 2001). Success of the ablation was verified by means of a scanning electron microscope (Quanta FEG 250 ESEM, FEI) after the experiments. The influence of tibial and tarsal sensory information (tactile hairs, campaniform sensilla) was eliminated by cutting the leg distal to the FTi joint. The tibial stump was hollowed out with an insect pin to prevent signals from campaniform sensilla groups 6A and 6B (Zill et al., 2011), which are located just distal to the FTi joint. The tibia was replaced by a wooden stick of appropriate length and mass to serve as a prosthesis. Values for length and mass had been obtained beforehand from mean values of several animals.

Experimental setting

Searching movements were elicited by slightly touching the animal at the abdomen with a paintbrush ('tickling') or by a puff of air directed at the antennae or abdomen. A metal stick with a tip made of fibreglass was used as an obstacle to be introduced into the plane of leg movement. The stick was mounted to a micromanipulator, aligned in parallel to the animal and could be quickly moved forward by approximately 1 cm. The stick was always touched by the most distal third of the tibia. The stick was located caudal to the intact foreleg, and thus was moved into the plane of movement from behind. A coil spring enabled a fast retraction of the stick as soon as the animal had touched it. The stick was black with a small red marker (fluorescent pigment, Dr Kremer Farbmühle, Aichstetten, Germany) at the very tip to make it detectable during video analysis

of the leg's movements. In random order, the stick was introduced into the plane of leg movements in one of four different positions [henceforth 'position of object' (PO); see Fig. 1A].

Data recording

Video

Leg movements were recorded from a frontal view at a frame rate of 50 Hz (AVT Marlin, Allied Vision Technologies, Stadroda, Germany) and stored on a computer using firmware (AVT ActiveCam). If electromyogram (EMG) signals were obtained simultaneously, then both film and EMGs were recorded using Spike2 software (Version 5.20, CED, Cambridge, UK). Yellow fluorescent markers (fluorescent pigment, Dr Kremer Farbmühle, Aichstetten, Germany) were applied to the leg (Fig. 1A) to facilitate the analysis of leg movements. Pigment fluorescence was evoked by LED illumination ($\lambda=395$ nm) and filtered by a high-pass filter ($\lambda>575$ nm) mounted on the camera lens.

Electromyograms

EMG signals were amplified (custom-built amplifier, model 102 Electronics workshop, Zoological Institute, University of Cologne, Cologne, Germany) 3000–10,000 times according to signal amplitude and filtered (lowcut 100 Hz, highcut 2 kHz). The data were digitized with a rate of 12.5 kHz (Micro 1401k II, CED) and stored using Spike 2 (Version 5.20, CED).

Data analysis

Video

Movements were tracked using WinAnalyze (Version 2.2 2D, Mikromak, Berlin, Germany) or MATLAB (Version 7.11, MathWorks, Natick, MA, USA) using a custom-written program (StickAnalyze, written by Dr Till Bockemühl, Zoological Institute, University of Cologne, Cologne, Germany). As the size of animals varied, positions of the leg were not expressed in coordinates but as an angle, α , which was formed by a straight line from the coxa to the distal end of the tibia and a horizontal that was set by the coxa and a reference marker to the right side of the animal (Fig. 1C). Positions above the horizontal were defined as positive values of α , positions below the horizontal as negative values. When considering positions of the CTr and FTi joints, we used angles β and γ , respectively. The coordination of the CTr and FTi joints during searching movements is very stereotyped; movements in both joints are generally coupled (Fig. 1B; see Results). Because of this coupling, the same α is generally defined by very similar β and γ values in each animal and the distal tibia repeatedly moves on very similar trajectories during consecutive searching cycles (Fig. 1A). Therefore, α sufficiently describes the position of the most distal third of the tibia, which was the location of contact with the stick.

Calculation of average leg position before and after contact with the object

For each experiment, average leg position as the average α was calculated for four consecutive searching cycles directly before and after leg contact with the object. α values were obtained for each video frame in four cycles. For example, four searching cycles in 2.4 s give 120 α data points. The average of these gives the average leg position. For each experiment, the pooled data points obtained from four cycles before and after leg contact with the object were used to test for statistical significance of differences of the means (Mann–Whitney *U*-test, $\alpha=0.05$).

Amplitudes were calculated for half-cycles of searching movements. Thus, eight values obtained from four cycles before

touching the object were pooled and compared with eight values after touching (Mann–Whitney U -test, $\alpha=0.05$). No pause occurred within each of the four searching cycles and animals were not ‘tickled’ along the way. Animals were only included in analyses if at least one experiment was performed with the PO above the horizontal (0, +15 and +25 deg) and one with PO=−40 deg. If the distance from the PO to average leg position before touching was <10 deg, the experiment was excluded from the statistical analysis of the change of average leg position, but included in all other analyses. Statistical analyses were conducted in SPSS (IBM, Armonk, NY, USA) and MATLAB. Curves were fitted to the amplitude and average leg position data sets, respectively, in MATLAB. Data points directly after touch were left out of the fit if the maximum deviation of values from control occurred in the second cycle instead of the first after touch (amplitudes: one data point in 7 of 23 cases; average leg position: three data points in 3 of 21 cases). The mean angular speed was calculated by averaging the absolute values of the first derivation of α from one minimum of the leg position to the next minimum.

Electromyograms

EMG recordings were analyzed in Spike 2 using the ‘bursts’ script for evaluation of burst duration. Cycles were measured from the onset of levator muscle activity to the next onset of levator muscle activity. A custom-written script was used for calculation of integrals of electrical muscle activity. To obtain the ratio of levator to depressor trochanteris muscle activity, the sum of levator integrals of four searching cycles was divided by the sum of depressor integrals of four searching cycles both before and after touching the object. The resulting ratios were normalized to the ratio before touching the object. Figures were created in Origin (Version 6.0, Microcal Software, Northampton, MA, USA) or MATLAB and modified in Corel Draw (Version 13, Corel, Ottawa, CA).

A single experiment consisted of four searching cycles before touching the object and four cycles after touching the object. Throughout the text, N denotes the number of animals and n denotes the number of experiments.

RESULTS

When slightly touched on the abdomen or activated by a puff of air, stick insects performed stereotypic searching movements in the vertical plane with their foreleg (Fig. 1A,B) (Karg et al., 1991). Searching movements covered a wide range of 75 ± 17 deg (measured as α ; see Fig. 1C; $N=14$, $n=76$, ~300 cycles) and were centered on a rather stable average leg position. As described by Karg et al. (Karg et al., 1991), downward movements started with depression of the CTr joint whereas the FTi joint remained fully extended or was finishing its extension movement from the previous searching cycle. Approximately halfway down the searching range, CTr movements slowed down whereas the FTi joint started to flex (Fig. 1A, black lines). During upward movements, the FTi joint was extended together with a simultaneous or immediately following elevation of the CTr joint (Fig. 1A, magenta lines). The coordination of both joints was highly consistent throughout consecutive cycles of searching movements, as can be seen when the FTi angle (γ) is plotted against the CTr angle (β), which results in closely matching trajectories (Fig. 1B).

Qualitative analysis of searching movements

To determine whether searching movements are modified after touching an object with the leg, after four cycles of undisturbed searching movements, a stick was moved into the plane of movement

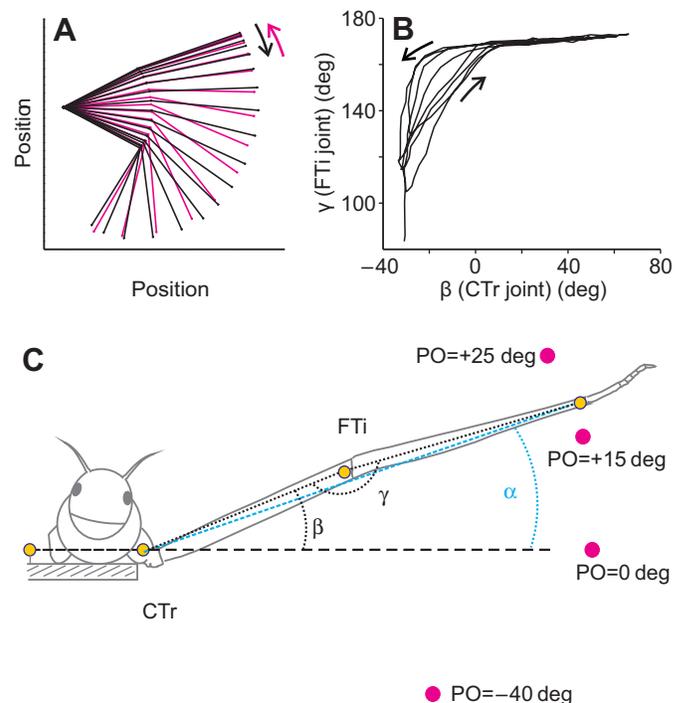


Fig. 1. (A) Schematic of leg kinematics during downward (black) and upward (magenta) movement of an undisturbed searching cycle. (B) Movements of the femur-tibia (FTi) joint plotted against movements of the coxa-trochanter (CTr) joint for several cycles of searching movements. Arrows indicate upward and downward movements of the leg. Coordination of both joints movements are very stereotyped. (C) Schematic drawing of the experimental setup. The leg is shown from the camera's perspective. Magenta circles indicate the four different positions of the object (POs); yellow circles denote markers. Angle α gives the position of the distal tibia relative to the body, angle β gives the position of the coxa-trochanter joint and angle γ gives the position of the femur-tibia joint. Modified from von Uckermann and Büschges (von Uckermann and Büschges, 2009).

from behind such that the animal would eventually touch it with its distal tibia. Immediately after contact, the stick was retracted and searching movements were observed. The stick was alternately placed in one of four positions (see Fig. 1C). Upon touching the object, generally two parameters of the searching movements changed. Fig. 2 shows these changes in searching movements of a single animal for all four POs. Searching trajectories are plotted as angles (α) over time.

First, upon touching the object, the average leg position was shifted towards the PO. It was shifted upward if the PO was located above the average leg position before touching (Fig. 2A–C) and downward if the PO was located below (Fig. 2D). Subsequently, searching movements were gradually shifted back towards the initial average leg position. Second, upon touching the object, the amplitude of searching movements was instantly decreased and gradually increased again. The combined occurrence of these changes may be interpreted as a targeted searching movement that wanes over time. Henceforth, movements that shifted towards POs and that were reduced in amplitude will be named targeted searching movements. Movements that showed changes in only one or none of the two parameters were considered non-targeted.

The mean angular speed of leg searching cycles did not change after touching the object (Fig. 3A). Single experiments (colored lines) could show a decrease or increase in speed; however, these changes

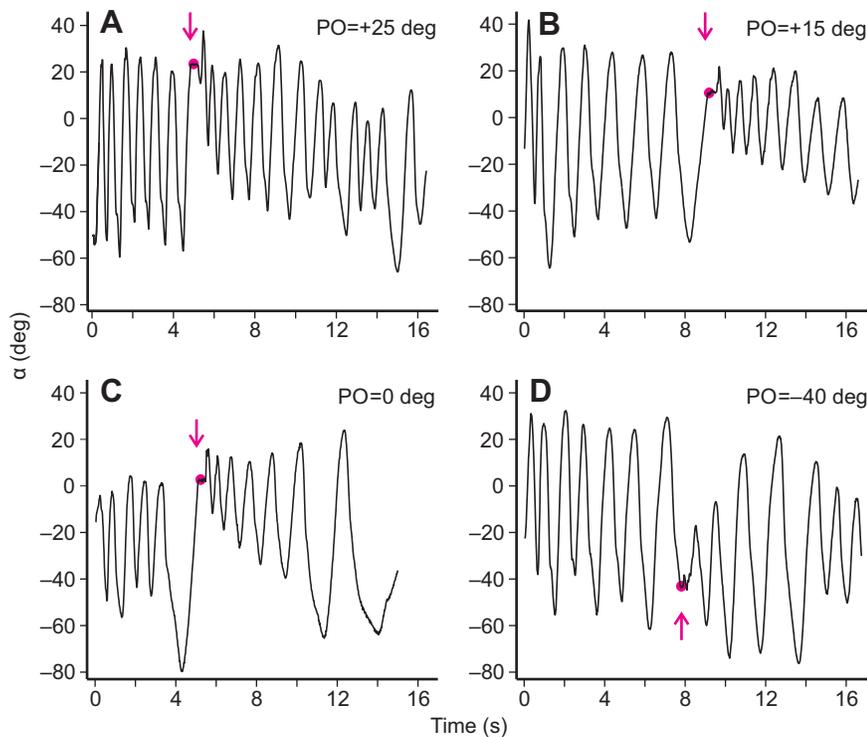


Fig. 2. Searching movements plotted as angle α over time. All four experiments are recorded from the same animal and examples are shown for each of the four different POs used: +25 deg (A), +15 deg (B), 0 deg (C) and -40 deg (D). Magenta arrows indicate the point in time when the object was touched, and magenta circles indicate the PO. The large amplitude before contact in C is an outlier with no relation to the subsequent contact.

were not systematic, as average data (black line) were not different from control values (Fig. 3A). As a consequence of the unchanged angular speed and the decrease in amplitude, the cycle duration of searching movements instantly decreased upon touching an object and subsequently increased again (Fig. 3B). Fig. 3 shows the results for targeted searching movements at a PO of +15 deg ($N=9$, $n=15$). Curve progression was similar for non-targeted searching movements as long as the amplitude of movements decreased ($N=4$, $n=5$; not shown). If the amplitude was not decreased, both angular speed and cycle duration tended to increase ($N=2$, $n=2$).

Despite their overall stereotypy, searching movements in different experiments displayed a considerable amount of variability regarding initial average leg position and size of amplitude (compare cycles of undisturbed searching movements in Fig. 4). Also, average leg position and amplitude of searching movements could be altered independently of each other. Fig. 4A shows an experiment in which the amplitude changed but average leg position remained the same, whereas in Fig. 4B only the average leg position was changed.

Quantitative analysis of searching movements

For a quantitative measure of the observed changes, we tested for differences in average leg position and amplitude between four cycles directly before and after touching the object. The results show that the average leg position was significantly shifted towards the PO in most cases for all four POs (73–91% of experiments, Mann–Whitney U -test, $P<0.05$; Fig. 5A). Only rarely was the average leg position significantly shifted away from the PO (gray bars) or not significantly shifted at all. Also, the amplitude of leg movements was decreased significantly in the majority of experiments for POs of +25, +15 and 0 deg (84–100% of experiments, $P<0.05$; Fig. 5B, black bars). When disturbed at PO=-40 deg, the amplitude decreased significantly in 48% of experiments ($P<0.05$).

A more comprehensive view on leg searching movements is provided in Fig. 5C, which displays the frequency of appearance of

different combinations of changes in average position and changes in amplitude for the different POs. Targeted searching movements, that is a shift of average leg position towards the PO and at the same time a reduction of amplitude, occurred in the majority of experiments (60–91%) for POs of +25, +15 and 0 deg (upper left quadrant of Fig. 5C). Only for a PO of -40 deg was a targeted response not the predominant behavior, as the amplitudes of movements were often not decreased significantly (magenta columns in upper and lower left quadrants in Fig. 5C). Searching movements that, upon touching the object, were not shifted towards the PO and did not show decreased amplitudes only occurred in 5% of experiments (lower right quadrant).

Visual information was not necessary for the generation of targeted searching. In experiments performed with blindfolded animals ($N=8$, $n=76$), both average leg position and amplitude changed in the same way as in sighted animals (Fig. 5D). Evaluation of the frequencies of different combinations of changes in both parameters resulted in an average percentage of targeted responses (58%; Fig. 5D) very similar to that in sighted animals (60%; Fig. 5C).

In animals that responded with targeted searching movements to touching the object, the magnitudes of shifts were highly correlated with the distance between the PO and the average leg position before touching the object ($R^2=0.916$, $P<0.001$; Fig. 6A). This high correlation indicates the shift towards PO as a flexible situation dependent adaptive behavior. On average, leg positions were shifted by $62\pm 23\%$.

For amplitudes, the magnitudes of changes were moderately correlated with the size of the amplitude before touch ($R^2=0.609$, $P<0.001$; Fig. 6B). On average, amplitudes changed by $38\pm 14\%$.

Contribution of CTr and FTi joints to targeted searching movements

So far, leg positions have been described by a single angle, α . The coordination of the CTr and FTi joints during searching movements is very stereotyped, movements in both joints are generally coupled (Fig. 1B). Thus, α is a sufficiently precise single parametric

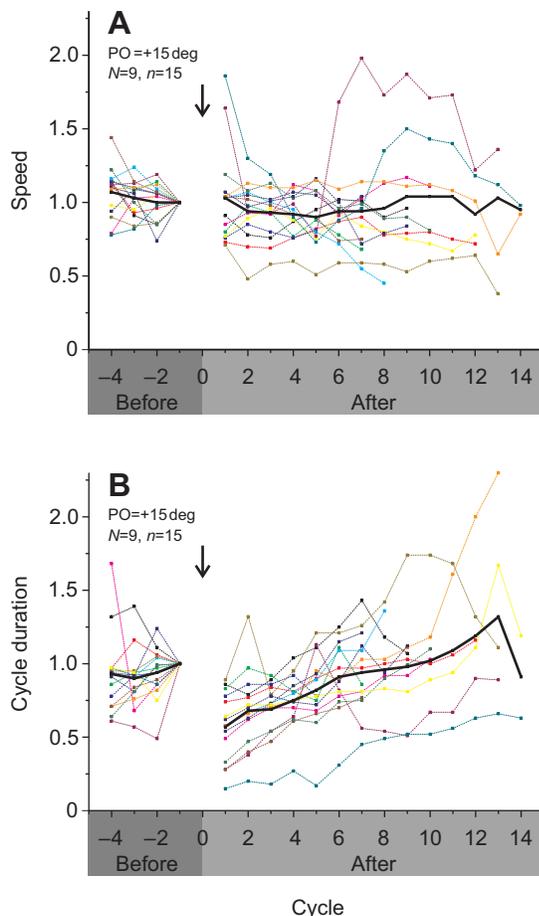


Fig. 3. Mean angular speed (A) and cycle duration (B) of searching movements plotted over cycle number. Values are normalized to the last cycle before touching the object. Cycles before touch (–4 to –1) are highlighted in dark gray on the x-axis; cycles after touch (cycle 1 to end) are highlighted in light gray. Colored curves depict single experiments; black curves show mean values. Arrows indicate the point in time when the object was touched. *N*, number of animals; *n* number of experiments.

measure of the overall leg movements to demonstrate the targeted response that animals perform upon touching an object. However, α does not describe how each of the two involved joints, CTr and FTi, contribute to the leg trajectories during searching movements and especially to the changes in searching movements upon touching an object. Thus, we plotted angle γ (FTi joint) against angle β (CTr joint); four examples from two animals are shown in Fig. 7.

As can be seen in Fig. 7, upon contact with the object, the movements of both joints were changed regarding their center and amplitude. The extent to which they contributed to the leg's new trajectory mainly depended on the PO: upon contact in an upper position, changes in α were mainly due to a decrease in FTi joint movements (Fig. 7Ai,Bi). Maximal extension of the FTi joint was almost unchanged. Changes of CTr joint movements were not as pronounced, but contributed to the change in α . Upon contact in a lower position (Fig. 7Aii,Bii), it was mainly the amplitudes of CTr joint movements that were decreased and centered around a new position, whereas movements of the FTi joint remained relatively unchanged compared with contact in upper positions. However, the relative contribution of each joint to a change in searching movements varied from animal to animal (compare Fig. 7A and 7B).

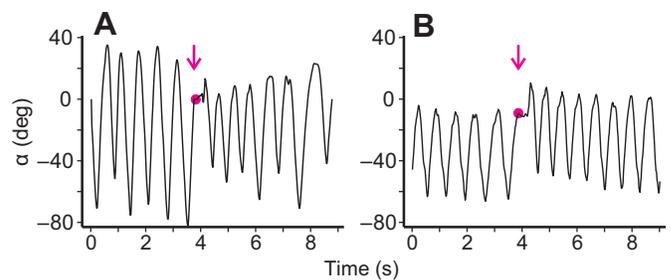


Fig. 4. Average leg position and movement amplitude may change independently after contact with the object. Searching movements plotted as angle α over time for two experiments with a PO of 0 deg. Magenta arrows indicate the point in time when the object was touched, and magenta circles indicate the PO. (A) Amplitudes change but average leg position remains constant. (B) Average leg position changes but amplitudes remain constant.

Fig. 7 also shows that even upon contact with an object, the stereotyped coordination pattern of both joints basically did not change. Rather, leg movements were confined to a section of the previous working range of searching movements while retaining the inter-joint coordination of the respective section. We never observed that amplitudes (α) were decreased by increased movements at the CTr joint and compensating increases in movements at the FTi joint. While the point of contact with the object did not change from animal to animal and a single animal's joint coordination was rather stereotypic, joint angles β and γ could vary among animals for a given α (compare Fig. 7Aii and 7Bii).

Trochanteral muscle activity underlying targeted searching movements

In order to analyze the muscle activity underlying the observed changes in searching movements, EMGs of levator and depressor trochanteris were recorded. The antagonists showed alternating bursting activity correlated with femoral levation and depression (Fig. 8, top two traces). Rectification and low-pass filtering (smoothing with a time constant of 0.05 s) of EMG recordings (RS-EMG) (Gabriel et al., 2003) give an approximation of the overall excitation of the respective muscles (Fig. 8, middle two traces). Changes in the integrals of RS-EMGs (gray areas below the curve) of the levator and depressor muscle were accompanied by shifts of average leg position (cyan line) upon touching the object. Upon contact, mainly depressor activity decreased, thereby causing an increase in the ratio (from 1 to 6) of levator to depressor activity (Fig. 8A). This relative increase in levator activity was accompanied by an upward shift of the average position of the femur (β , cyan line) and the entire leg (α , cyan line) (Fig. 8A, bottom two traces). A decrease in the ratio (from 1 to 0.003) of levator to depressor activity was accompanied by a downward shift of the average position of the femur and the entire leg (Fig. 8B). Such changes in activity were observed in 17 out of 18 experiments. The change in ratio was due to either altered activity in both the levator and depressor trochanteris or changes in activity in only one of the muscles. In some cases femoral levation and depression was accompanied by activity in only one of the antagonists (Fig. 8B, second, fourth and sixth traces from the top).

Role of sensory signals in targeted searching movements

As shown in Fig. 5, visual information is not necessary to perform a targeted response. Therefore, the execution of this response is very

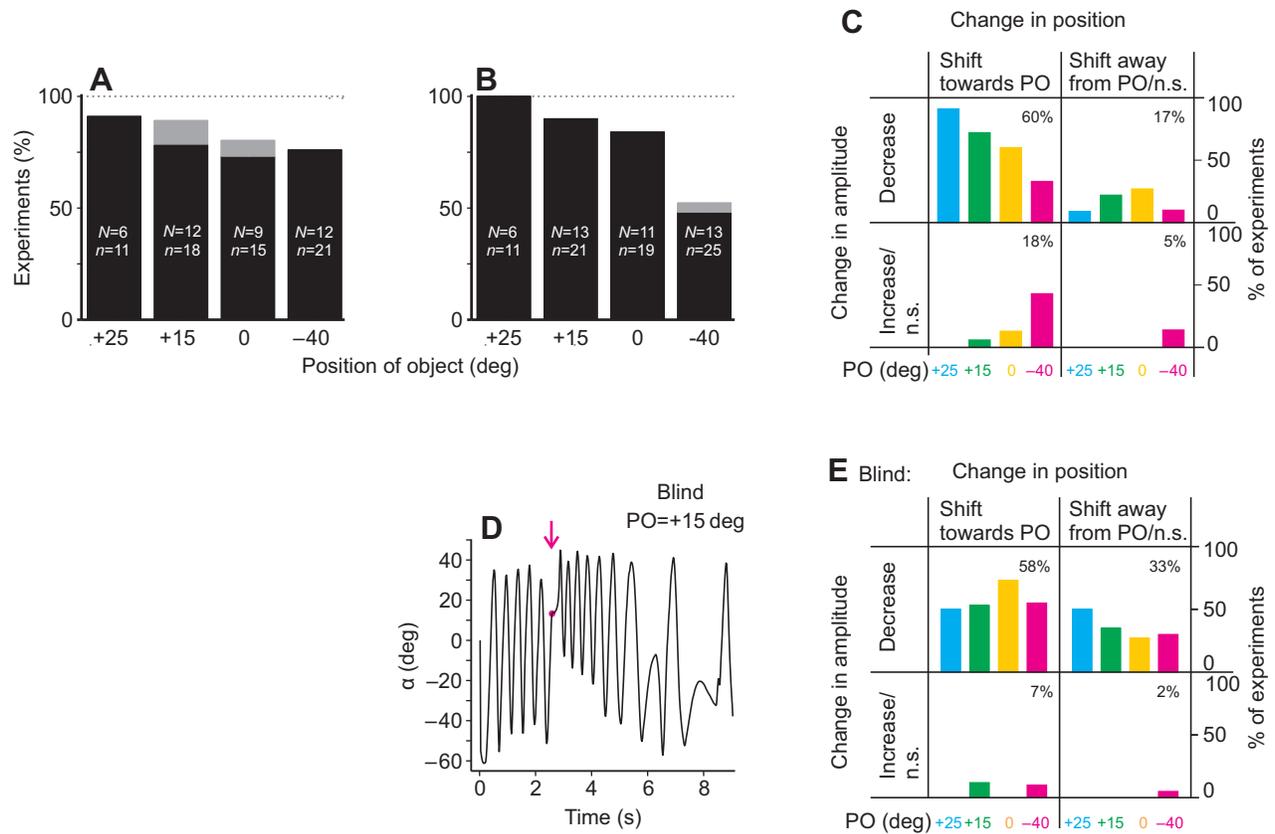


Fig. 5. Changes in average leg position and movement amplitude upon touching the object. (A) Change in position. Black bars, percentage of experiments in which average leg position shifted significantly towards the PO; gray bars, percentage of experiments in which average leg position shifted significantly away from the PO. Experiments are separated according to the respective POs of +25, +15, 0 or -40 deg. Non-significant changes are indicated by gaps between bars and the 100% line. (B) Change in amplitude. Black bars, percentage of experiments in which amplitudes significantly decreased; gray bars, percentage of experiments in which amplitudes significantly increased. (C) Frequency of appearance of different combinations of changes in average position and changes in amplitude for the different POs. POs are indicated by color. Left quadrants: average leg position was significantly shifted towards PO. Right quadrants: average leg position was significantly shifted away from PO or not significantly (n.s.) shifted. Upper quadrants: amplitudes decreased significantly. Lower quadrants: amplitudes increased significantly or did not change significantly. Numbers in quadrants give the mean relative frequency of the combined occurrence of a change in position and in amplitude. PO +25 deg: $N=6$ animals, $n=11$ experiments; PO +15 deg: $N=12$, $n=18$; PO 0 deg: $N=9$, $n=15$; PO -40 deg: $N=12$, $n=21$. (D) Searching movements of a blindfolded animal plotted as angle α over time for an experiment with a PO of +15 deg. (E) Experiments with blindfolded animals grouped according to the combination of changes in leg position and amplitude after contact with the object. Other details as in C. PO 25 deg: $N=3$, $n=8$; PO 15 deg: $N=7$, $n=17$; PO 0 deg: $N=6$, $n=15$; PO -40 deg: $N=7$, $n=20$.

likely to be based on information from leg sensory organs. In this case, the animal may use different sensory organs for two purposes: firstly, to sense contact with the object and secondly, to determine the actual position of the leg at the same time and thus gain information on the position of the object. Therefore, we decided to prevent any input from sensory organs on the tibia that might code the contact with the object. In a second set of experiments, we ablated the trHP (=Wendler's Bf1) (Wendler, 1964), which is known as an essential sensory organ in the coxa-trochanter control loop (Schmitz, 1986a; Schmitz, 1986b) (see Fig. 9Bi).

To prevent sensory information from the tibia and the tarsus, the tibia was cut distal to the FTi joint (Bässler et al., 1991), which abolished the tibial and tarsal tactile hairs and the tarsal campaniform sensilla, amongst others. The tibial stump was hollowed out to exclude influences of campaniform sensilla groups 6A and 6B (Zill et al., 2011), which are located on the proximal tibia. The missing tibia was replaced by a wooden stick (Fig. 9Ai). With this 'peg leg', animals were still able to show a targeted response upon touching the object in the same manner as with the intact leg. The percentage of targeted responses was 55% (Fig. 9Aii) and thus only slightly

less than in intact animals (60%; Fig. 5C). The magnitude of shifts of average leg position was correlated with the distance between the PO and the average leg position before touching the object ($R^2=0.793$, $P<0.001$); the magnitude of changes of amplitude was correlated, but again more weakly, with the amplitude before touching the object ($R^2=0.587$, $P<0.001$). The coordination of CTr and FTi joint movements remained the same as in animals with an intact leg (not shown). However, some animals (20% of experiments) did not respond to touching the object at all but instead quit making searching movements (Fig. 9Aii, shaded bar), a behavior that was not displayed by animals with an intact leg.

Following ablation of the trHP, animals performed searching movements with increased amplitude [mean amplitude 105 ± 22 deg instead of 85 ± 17 deg with intact leg; $N=4$, $n=16$ (intact), $n=31$ (trHP ablated)] but coordination of CTr and FTi joint movements remained the same as with the intact leg. The percentage of experiments in which a targeted response was exhibited decreased considerably from 58 to 21% after ablation of the trHP (Fig. 9Bii, black bars). This decrease was mainly due to animals failing to reduce the amplitude of movements (66% of experiments, Fig. 9Bii, light and

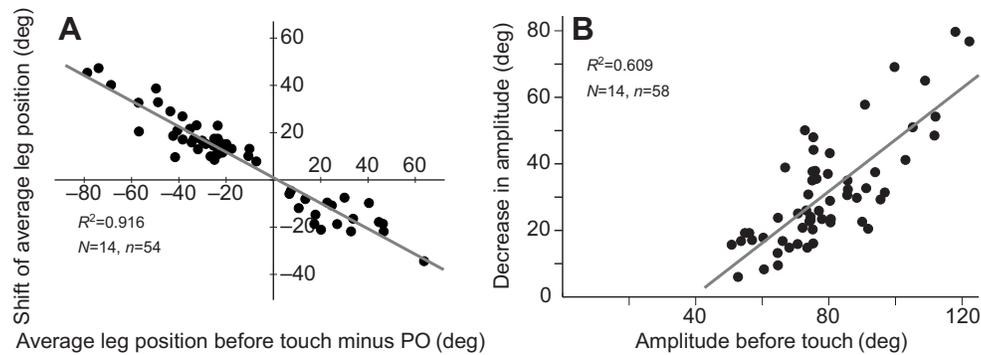


Fig. 6. Extent of changes in average leg position and movement amplitude. (A) The magnitude of shifts in average leg position depends on the difference from the PO to the average leg position before touch. Each data point denotes the extent of shift of leg position for one experiment. Data points in the upper left (lower right) quadrant come from experiments in which the PO was located above (below) the average leg position before touch. (B) Magnitude of decrease in movement amplitude depends on amplitude before touch. The regression line is drawn in gray. *N*, number of animals; *n*, number of experiments.

medium gray bars; Fig. 9Biii), whereas the average leg position was not shifted towards the PO in 46% of experiments (Fig. 9Bii, light and dark gray bars). Noticeably, in some trials (17%), animals neither decreased amplitude nor shifted average leg position (Fig. 9Biv).

In animals that showed a decrease in amplitude, the range and mean of the reduction (16 to 60 deg, mean=33 deg) was similar to that of the controls (12 to 76 deg, mean=34 deg). However, in contrast to the intact animals, the size of reduction was not correlated with the size of the amplitude before touching the object ($R^2=0.058$, versus $R^2=0.657$ with intact trHP). The extent of shifts of average leg position still correlated with the distance between PO and the average leg position before touching the object ($R^2=0.644$, $P<0.001$), albeit the correlation was weaker.

Thus, ablation of tibial and tarsal sensory organs decreased the number of experiments in which animals continued their searching movements upon touching the object, and ablation of trHP heavily impaired the accuracy of the targeted response.

Persistency of changed searching movements

We were interested in the time it takes for altered searching movements to be restored to initial values. Therefore, initial values were calculated for both parameters (average leg position and amplitude) from the four cycles (eight half-cycles resulting in eight data points) before touching the object (solid cyan lines in Fig. 10). For cycles after touch, average leg position and amplitudes of

movements were calculated per half-cycle and plotted over time. A curve (see below) was fitted to each resulting data set (solid dark blue lines in Fig. 10). Prediction bounds (dotted lines) were adjusted such that they contained 90% of the data points.

We evaluated 23 experiments from five animals. Amplitudes were evaluated for all 23 experiments; the average leg position was evaluated for only 21 experiments as a result of exceptionally large variations in leg position before touching the object in two experiments. In 91% of experiments evaluated regarding the amplitude and 81% regarding average leg positions, exponential fits yielded R^2 -values larger than 0.8 or resulted in the best R^2 -values (as compared with linear, power or logarithmic fits). However, in several cases the data could be well fitted ($R^2\geq 0.8$) by more than one function type. In 36% of cases the best fit was yielded by either power, linear or logarithmic curves. Nevertheless, in all 44 evaluated cases we used exponential fits to determine the duration of the changes.

The time from removal of the object (magenta arrow, Fig. 10A) to the intersection of the initial value fit and the exponential fit (arrows 't', Fig. 10A) was interpreted as the time required to restore initial values. In approximately half of the experiments, the exponential fit closely approached the initial value but did not intersect. In those cases, a different approach was used: the duration of change was defined as terminated when the exponential fit had achieved 99% of its final value, thus the slope was close to zero

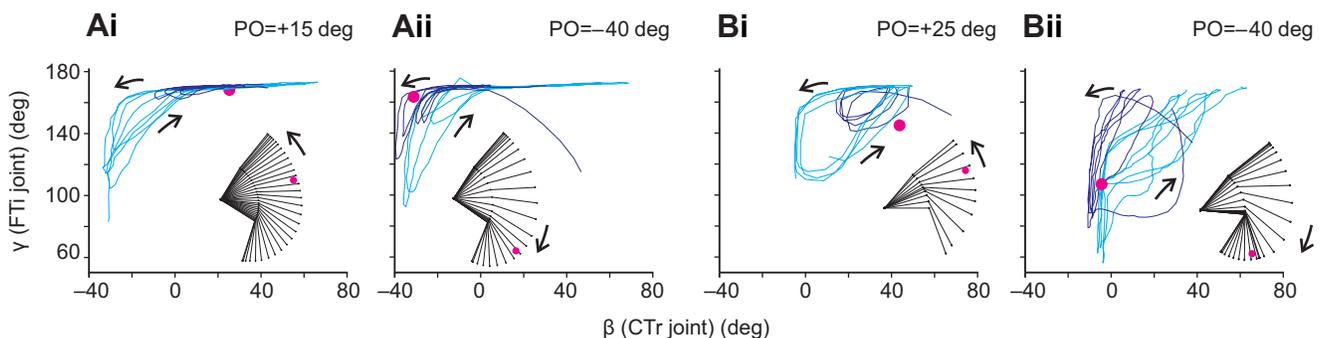


Fig. 7. Searching movements plotted as γ (FTi angle) against β (CTr angle). Cyan trajectories depict four cycles of searching movements before contact with the object; dark blue trajectories depict four cycles after contact. Magenta circles indicate leg joint angles when objects were touched (and thus the PO). Black lines show leg kinematics of half a cycle of undisturbed searching movements of the respective experiment. (A) Experiments from the same animal, with POs of +15 deg (Ai) and -40 deg (Aii). (B) Experiments from another animal, with POs of +25 deg (Bi) and -40 deg (Bii).

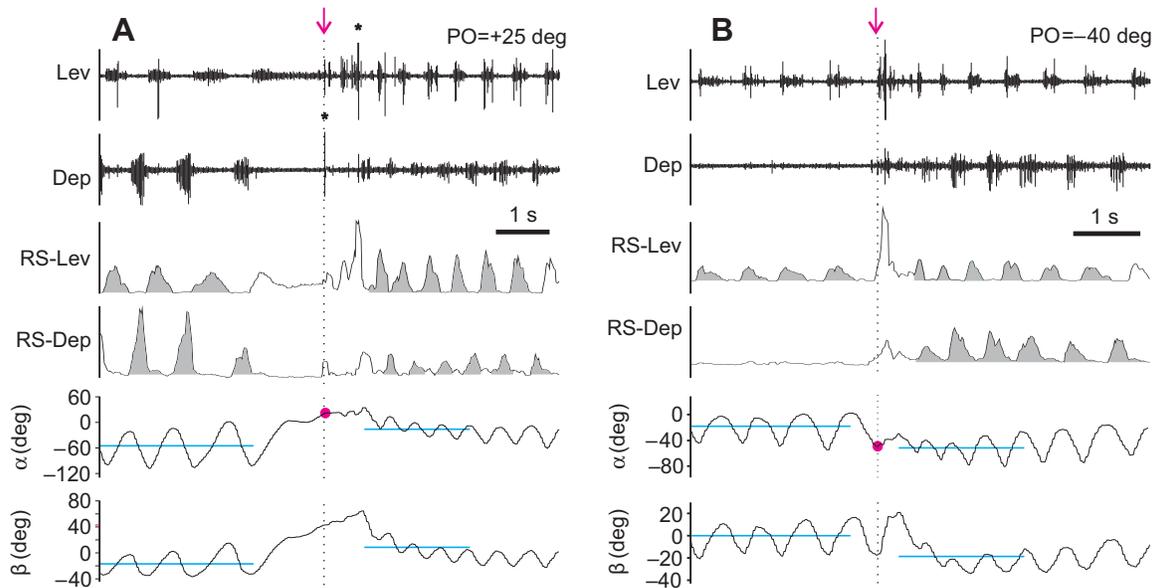


Fig. 8. Activity in levator and depressor trochanteris muscles in experiments with a PO above (+25 deg; A) and below (-40 deg; B) the average leg position, shown as original EMGs (two top traces) and after rectification and smoothing (RS-EMG; two middle traces). Asterisks mark two muscle potentials that have been clipped. Gray areas indicate integrals of RS-EMGs. Lower two traces show the position of the entire leg as angle α , and the position of the femur as angle β (CTr joint). Cyan lines indicate average leg position and femur position before and after touching the object. Please note that four cycles before touch were included in analysis but only three cycles are shown. Magenta arrows indicate the time point of touch, and POs are marked by magenta circles.

(arrow 't' in upper trace, Fig. 10B). Both approaches resulted in similar durations. In seven experiments, initial amplitudes or average leg positions were not regained, that is, even prediction bounds did not intersect. These cases were excluded from data evaluation. For amplitudes, it took 6 ± 2.9 s to be restored to initial values, and for average leg positions it took 6 ± 4.0 s. However, as is obvious from Fig. 10A, the duration required to regain the initial average leg position or amplitude could differ even in the same experiment.

DISCUSSION

In this study, we have shown that stick insects performing untargeted searching movements with a leg execute a targeted response upon a one-time contact of the leg's tibia with an object that is immediately removed after contact. The targeted response consists of two components: searching movements are confined to the former PO by (1) a shift in average leg position and (2) a decrease in movement amplitude. Both changes appear to be independent of each other. Average position and amplitude generally regain initial values after approximately 6 s. We interpret this data as a targeted response that wanes over time. Visual sensory information is not necessary to control this behavior. In contrast, sensory information on the position of the CTr joint is essential for the targeted response. The decreased amplitude of searching movements after contact coincides with an increased frequency of these movements. The shift of average leg position towards the PO is accompanied by a change in the levator to depressor trochanteris muscle electrical activity ratio, which is due to alterations in both or either one of the muscle activities.

In accordance with previous results (Karg et al., 1991; Dürr, 2001), we observed undisturbed searching movements of the intact leg to be very stereotyped and composed of the same sequence of events in time and space as described by Karg et al. (Karg et al., 1991). When the trHP, which measures the position of the CTr joint (Schmitz, 1986a; Schmitz, 1986b), was ablated, the amplitudes of undisturbed searching movements were larger compared with searching with the intact leg. This finding is in agreement with the

results of Wendler (Wendler, 1964), which showed that walking animals lifted their legs higher during swing phase if the trHP was ablated. We did not observe a change in inter-joint coordination upon ablation of trHP as was described by Karg et al. (Karg et al., 1991). In line with our results, Akay et al. (Akay et al., 2001) also found no change in inter-joint coordination upon ablation of the trHP. The ability of the trHP to affect inter-joint coordination might depend on the leg's position, as legs were fixed to different angles to the body axis [60 deg in experiments by Karg et al. (Karg et al., 1991), but 90 deg in experiments by Akay et al. (Akay et al., 2001) and in our experiments].

The initial reaction upon touching an object was to grasp for it. This grasping behavior, which was described by Bässler et al. (Bässler et al., 1991), demonstrates that the animals are not avoiding the object but instead are attracted to it. In our experiments the animals could not grasp the object because it was removed, but instead they displayed the targeted response.

Targeted movements

Shift in average leg position

To determine the PO, the animals might use a sensory signal indicating the leg's contact with the object as a trigger to 'read out' information on the leg's position. Removal of distal sense organs by substitution of the tibia by a 'peg leg' did not affect the ability to perform a targeted response. Therefore, tibial tactile hairs or tibial campaniform sensilla (CS), sense organs in the cuticle that detect forces as cuticular strains (Pringle, 1938a; Pringle, 1938b; Zill et al., 2004), are not crucial for triggering the targeted response. Instead, the contact signal is likely to be provided by coxal or trochanteral CS. In that line, Bässler et al. (Bässler et al., 1991) report that the typical leg movements preceding grasping behavior are still performed after substitution of the tibia.

The trHP appears to be an important sensor that provides position information, as ablation of trHP decreased the percentage of shift towards the PO from ~ 75 to 54% (Fig. 9Bii). This view is in

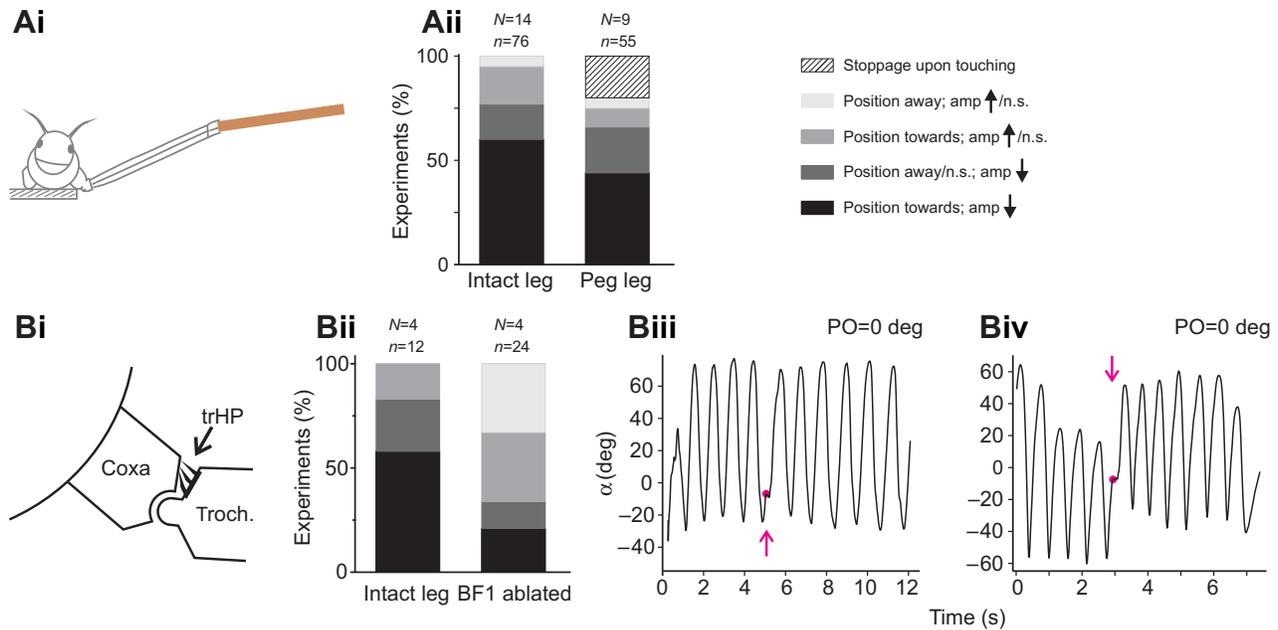


Fig. 9. Impact of sensory organs on the change of searching movements upon touch. (Ai) Schematic of preparation for 'peg leg' experiments. The tibia was substituted by a wooden stick. Figure modified from von Uckermann and Büschges (von Uckermann and Büschges, 2009). (Aii) Quantitative comparison of data from experiments with intact legs and 'peg legs'. Black bars depict the percentage of experiments in which a targeted reaction (position shift towards PO, amplitude decrease) was shown upon touch. Dark gray bars show the percentage of experiments in which only amplitudes were decreased. Medium gray bars show the percentage of experiments in which only the average leg position was shifted towards the PO. Light gray bars indicate the percentage of experiments in which average leg position was not shifted towards the PO nor were amplitudes decreased. Hatched bars depict experiments in which animals stop searching movements. n.s., not significant; amp↓, significant decrease in amplitude; amp↑, significant increase in amplitude. (Bi) Schematic of the trochanteral hairplate (trHP). Ablation of the trHP largely impairs amplitude control and shifting of average leg position towards the PO. Modified from Wendler (Wendler, 1964). (Bii) Quantitative comparison of data from experiments with intact trHP and data from experiments with ablated trHP. (Biii,iv) Searching movements plotted as angle α over time for experiments with ablated trHP.

accordance with Schmitz (Schmitz, 1986a; Schmitz, 1986b), who furnished evidence that the trHP is the only feedback transducer in the CTr control loop, and with Cruse et al. (Cruse et al., 1984), who showed that ablation of trHP leads to false estimation of a leg's position in inter-leg targeting. However, other sense organs might also contribute to mediation of the targeted response, as ablation of trHP did not always inhibit the ability to display a shift towards the PO and also to decrease the amplitude. Such sense organs are coxal strand receptors cxtSR1 and cxtSR2 (Bräunig, 1982a; Bräunig, 1982b; Schöwerling, 1991) or coxal muscle receptor organs, as shown for *Locusta* (Bräunig, 1982a). Yet, according to Schmitz (Schmitz, 1986b), a contribution of internal sense organs to the CTr loop would depend on an intact trHP.

The contribution of the CTr joint to the shift in average leg position appears to be based on a shift in the ratio of EMG activity in the levator and depressor trochanteris muscles. For example, an increase in levator activity, a decrease in depressor activity or a change in both muscles could contribute to an upward shift. All such changes were observed. This fits an observation made by Bässler et al. (Bässler et al., 1991), who states that levator and depressor trochanteris muscle activity is highly variable although the underlying movement is stereotypic.

An alternative mechanism for a shift in average leg position could be a 'catch-like' effect in the levator or depressor muscle. This effect describes the phenomenon that a short, high-frequency discharge of a motoneuron leads to a long-lasting increase in force production of a muscle that is maintained at a low frequency of motoneuron discharge. The effect has been described for crustacean muscles (Blaschko et al., 1931; Günzel and Rathmayer,

1994), locust tibial muscles (Wilson and Larimer, 1968) and the mesothoracic extensor tibiae in the stick insect *Carausius morosus* (Guschlbauer, 2009). If trochanteral muscles of stick insects possess catch-like properties, such an effect might hold the leg in the region of the PO. However, this explanation is a less likely alternative because we did not observe high-frequency discharges in EMGs. Furthermore, a catch-like effect perhaps triggered by a CS discharge is likely to interfere with grasping behavior and searching movements.

The average position of the CTr joint (angle β) can be set and rhythmic searching movements can be performed by activation of a single muscle (Fig. 8B). In the example shown, rhythmic levator activity was working against passive forces of the depressor muscle that is not activated. As the depressor trochanteris muscle is innervated by only two excitatory motoneurons (Schmitz, 1986b; Goldammer et al., 2012), it is unlikely that there were EMG spikes in some remote part of the muscle that were not picked up by the electrodes. Comparable to CTr joint movements in the stick insect, rhythmic movements of the FTi joint in the locust hind leg may be controlled by the flexor or extensor tibiae muscles working against passive forces in the antagonist that is not activated by motoneurons (Berkowitz and Laurent, 1996; Page et al., 2008).

Do passive forces determine the average leg position? Generally, small limbs assume gravity-independent rest positions without activity in leg motor neurons (Hooper et al., 2009; Ache and Matheson, 2012). The gravity-independent position of a joint depends on the passive forces of the antagonistic muscles (Hooper et al., 2009). However, it is unlikely that passive forces determine the average leg position after contact, as these positions change

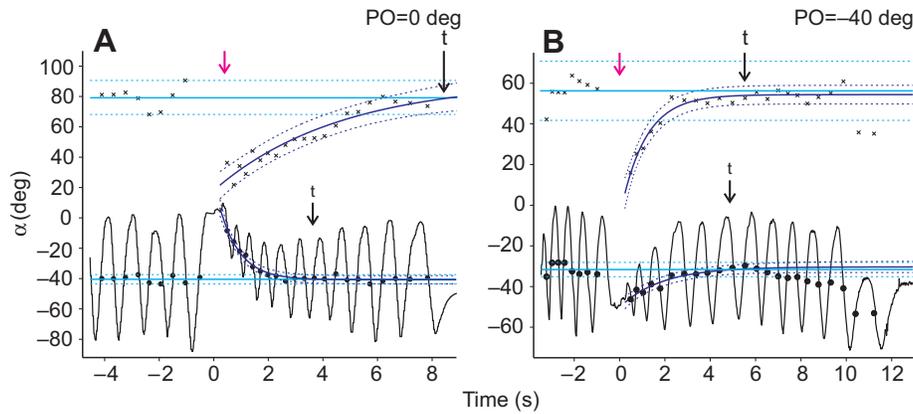


Fig. 10. Restoration of changes in searching movements upon touching the object at POs of 0 deg (A) and -40 deg (B). Black curves depict searching cycles throughout an experiment, plotted as angle α over time. Magenta arrows indicate the point in time when the object was removed after touch and searching movements were resumed. Black circles give average leg positions for half-cycles of searching movements; black crosses give mean amplitudes of half-cycles of searching movements. Initial mean values of average leg position and amplitudes before touch are displayed as cyan lines. Dark blue lines indicate best fits to average leg position and amplitude data after touch, showing the restoration of average leg position and amplitude; dotted cyan and dark blue lines give corresponding prediction bounds. Distances between magenta arrow and the two arrows marked 't' indicate recovery time for amplitude (upper) and average leg position (lower).

depending on object position and therefore differ from relatively fixed 'intrinsic' resting positions of a joint.

Decrease in amplitude and increase in frequency

The second component of the targeted response consists of a decrease in movement amplitude accompanied by an increase in movement frequency. Both changes largely depend on a functional trHP, as after ablation the percentage of responses that showed both reduced amplitude and an increase in searching movement frequency dropped from ~83 to 34%. Thus, information about contact with the object, possibly provided by CS on proximal leg segments, without information from the trHP is not sufficient to reliably induce the observed changes in amplitude and frequency.

In the stick insect, stimulation of the trHP by elevation of the leg activates depressor motoneurons (Schmitz, 1986b). In our ablation experiments, this influence is missing and this might contribute to the loss of the control of amplitudes. Thus, it appears that the trHP contributes to setting the movement amplitude and frequency of the pattern-generating networks that control the CTr and FTi joints during searching. Such sensory influences on the timing and magnitude of motor activity have been shown for other movement, position and load sensors in the walking system of the stick insect (Ritzmann and Büschges, 2007).

Comparison with other systems and considerations of possible underlying cellular mechanisms

At approximately 6 s, the targeted response far outlasts the duration of the given stimulus, and the underlying mechanism might be regarded as a simple form of short-term memory as the behavior refers to an already vanished stimulus. The behavior is reminiscent of effects shown in insects for which a 'positional memory' has been suggested. For example, walking fruit flies show a persistence of orientation for approximately 8 s toward a landmark that disappeared during the fly's approach (Strauss and Pichler, 1998). In walking fruit flies, the width of a gap that is being approached appears to be stored for a short time ('subsecond memory') to be read out upon tactile contact with the gap (Pick and Strauss, 2005). And ladder-walking locusts appear to memorize a rung's position, as dislocation of a rung during the legs' swing phase did not lead to modification of the step but evoked

persistent searching movements in the former rung position (Niven et al., 2010). In contrast to our experiments, the former examples of short-term memory involved visual input. Grooming movements of a locust hind leg that are targeted towards a stimulus side on a wing involve a short-term positional memory similar to the searching movements in our experiments because grooming movements may outlast electrical or mechanical stimuli (Matheson, 1997; Matheson, 1998). Because of the short-term 'learning' period and a similar time span of memory in the range of seconds, the underlying neuronal mechanisms might be similar. These mechanisms are very likely to be different from those involved in leg position learning in cockroaches and locusts, which are based on repetitive aversive stimuli, and memory in these experiments may be retained for hours (Horridge, 1962; Stowe and Leader, 1975; Hoyle, 1979).

The means by which information is 'stored' for a time period of a few seconds might be a result of the intrinsic membrane properties of participating neurons. Such properties can cause long-lasting effects in neurons that alter their firing activity upon preceding excitation. Properties include plateau-like properties based on diverse inward currents (Marder, 1991; Major and Tank, 2004), slow K^+ -channel kinetics (Turrigiano et al., 1996; Marder et al., 1996), slow post-inhibitory rebound mechanisms (Goaillard et al., 2010) and after-hyperpolarization depending on Na^+/K^+ -pump activity (Pulver and Griffith, 2010). As time constants of these cellular 'memory' mechanisms correspond to the recovery time constants, it is worthwhile to look for such a mechanism in those premotor interneurons that control leg movements. As quite a few of those neurons have been identified (Büschges et al., 1994; Büschges, 1995; Kittmann et al., 1996; von Uckermann and Büschges, 2009), the stick insect searching leg preparation is well suited for unraveling the mechanisms of short-term memory in a motor system.

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