RESEARCH ARTICLE

Walking on smooth and rough ground: activity and timing of the claw retractor muscle in the beetle *Pachnoda marginata peregrina* (Coleoptera, Scarabaeidae)

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SUMMARY

The activity pattern of the claw retractor muscle of *Pachnoda marginata peregrina* beetles was examined in this study. We found this muscle to be located in the tibia, without a femoral part, as is the case in other insects. Electromyograms of the muscle revealed a rather similar activity pattern during beetle locomotion on rough and smooth substrates. We recorded units with small and large amplitude, with the smaller unit being active during almost the entire stance phase, and the larger unit active roughly in the first half of stance. Small but significant differences were found in the precise onset and end of activity. Both small and large units began their activity earlier on the rough surface. Although there was no difference at the end of activity in the small unit between surfaces, the large unit ended its activity significantly earlier on the rough substrate. The spike frequencies on both surfaces were also significantly different for small and large units. The small unit showed a higher spike frequency on the smooth surface, while the large unit had a higher spike frequency on the rough surface. From our experiments, we conclude that the muscle is controlled by the same basic activity pattern on different surfaces, with some adjustments that are due to sensory feedback. The adjustments cause differences in onset and end of activity, as well as in spike frequency of the involved muscle units.

INTRODUCTION

Insects are among the most successful animal groups that are adapted to terrestrial life, considering the number of species and their worldwide distribution. Terrestrial life makes numerous demands on an organism, including the ability to walk and climb. Hence, insect walking has received much attention in biological research. Different aspects have been studied: walking patterns in different situations (e.g. Wilson, 1966), sensory control of leg movements (e.g. Bässler, 1977), muscle innervation (e.g. Godden, 1972; Walther, 1980) and the motor pattern of several leg muscles during walking (e.g. Delcomyn, 1973; Delcomyn and Usherwood, 1973). The latter subject provides important general information about locomotion, as the leg muscles produce the driving force necessary for limb movement.

In horizontal forward walking, the step of an insect, as of any other legged animal, consists of two phases. The first is the stance phase, when the tarsus is in contact with the ground and the leg produces a propulsive force for the animal. During the second phase, the swing phase, the leg is moved in an anterior direction through the air until the foot is set onto the ground for the next stance phase. These two phases are controlled by different sets of muscles responsible for movements of the leg segments. The activity of some of these muscles has been studied in connection with insect walking. In particular, the flexor and the extensor of the tibia and several coxal muscles have been the subject of various studies (Delcomyn, 1973; Delcomyn and Usherwood, 1973; Duch and Pflüger, 1995; Krauthamer and Fourtner, 1978; Laurent and Hustert, 1988; Rosenbaum et al., 2010; Sponberg and Full, 2008; Watson and Ritzmann, 1998).

The site where the muscle force is transferred to the ground is the tarsus and pretarsus. Here the contact with the substrate is established with specialized attachment structures located on the insect's foot. Among these are the claws on the pretarsus, which exist in almost all adult insects (Chapman, 1998), and the pad-like structures responsible for adhesion on different substrates. These structures are either smooth or hairy (Gorb and Beutel, 2001) and are complemented by an adhesive fluid (e.g. Drechsler and Federle, 2006; Gorb, 2001; Vötsch et al., 2002).

Because the activity of muscles controlling both the tarsus and pretarsus has only rarely been studied (Bußhardt et al., 2011; Laurent and Hustert, 1988; Radnikow and Bässler, 1991), the activation pattern of the attachment structures during different behavioral situations, such as walking or clinging to substrates in different body positions, remains largely unknown. In a previous study, we examined the claw retractor muscle (m. retractor unguis) of two stick insect species while the animals were standing on a platform in horizontal, vertical or inverted positions (Bußhardt et al., 2011). We showed that tonic muscle units were active all the time, without significant differences for different body positions. Only during transitions from one position to another was an additional phasic unit active. In spite of the great importance of this muscle in securing substrate contact during locomotion and in releasing the tarsus from the ground, only very few studies have examined this muscle in walking insects. Previous authors have shown that the claw retractor muscle is not just responsible for claw engagement on a rough surface. Its functions also include stiffening of the tarsus (Frantsevich and Gorb, 2004), preventing spontaneous detachment of tarsal

attachment structures (Endlein and Federle, 2008; Federle et al., 2001; Radnikow and Bässler, 1991) and supporting detachment events in flies (Niederegger and Gorb, 2003). All insects studied so far have this muscle that deploys the claws and, because its tendon runs through the entire tarsus, also stiffens and bends the tarsus. In combination with other tarsal muscles, it is therefore responsible for bringing tarsal and pretarsal attachment structures in contact with the ground.

To understand the functional mechanism of the tarsal attachment systems of insects, detailed knowledge about the involved devices and their morphology is important. Among these devices are attachment pads, which have been investigated, for example, in cockroaches (Arnold, 1974; Clemente and Federle, 2008), flies (Bauchhenß, 1979; Langer et al., 2004), hymenopterans (Endlein and Federle, 2008; Frantsevich and Gorb, 2004), locusts (Perez Goodwyn et al., 2006), earwigs (Haas and Gorb, 2004), beetles (Bullock and Federle, 2011; Stork, 1980; Voigt et al., 2008) and stick insects (Bullock et al., 2008; Bußhardt et al., 2012; Clemente et al., 2010). However, not all insects have such attachment pads; some rely solely on their claws for attachment on rough substrates, for example Odonata and Zoraptera (Beutel and Gorb, 2001; Beutel and Gorb, 2006). Zoraptera live in the soil and wood and it is therefore easy to understand that they do not need attachment pads specialized for smooth surfaces. Odonata, as a rare example of flying insects without any additional attachment structures to the claws, represent rather a ground plan of the Pterygota. Thus, the role of the claws becomes even more important, as they are the only structure the animals rely on in securing ground contact and producing propulsive forces for locomotion.

The role of claws in attachment on different flat substrates with different microroughness was previously studied in the African rose chafer, *Pachnoda marginata* (Dai et al., 2002), and the migratory locust *Locusta migratoria manilensis* (Wang et al., 2011). Both studies indicate a strong involvement of claws in attaching to certain surface roughnesses. However, the muscular control of claws on different substrate roughnesses remains unknown. The present study is a first trial to explore the activity of the claw retractor muscles that control the tarsal and pretarsal movements during locomotion on different substrates. We investigated its temporal activity pattern in the beetle *Pachnoda marginata peregrina* (Coleoptera, Scarabaeidae) during tethered walking on rough and smooth surfaces. To compare the degree of activity between the different situations, we also measured the spike frequencies of the different detected muscle units.

MATERIALS AND METHODS
Animals
Male and female *Pachnoda marginata peregrina* Kolbe 1906 (Coleoptera, Scarabaeidae), taken from our laboratory colony at the Zoological Institute of the University of Kiel, were used in all experiments. The animals were kept at room temperature and fed with various fruits. Experiments were performed with the hind legs, and only some electromyograms (EMGs) were additionally performed with the front legs.

Anatomy
The legs were cut off the beetles, which were previously fixed in Bouin's solution (picric acid 0.9%, formaldehyde 9%, acetic acid 5%) for 24–96 h and stored in 70% ethanol. The preparations were performed under a Leica M205A stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a camera, in order to reconstruct the muscle arrangement within the leg. For this

purpose, we carefully removed the cuticle from the dorsal and anterior sides of the leg using a sharp scalpel, and exposed the leg musculature. The muscle and tendon arrangement was sketched according to the images made with the microscope camera and digitized (Fig. 1). We also investigated unfixed legs to reveal the function of the muscles we found in the legs. Therefore, the muscles and the corresponding tendons were exposed. By pulling the tendons and observing the ensuing tarsal movement, their function was disclosed.

Electromyographic recordings
We performed EMG recordings of the claw retractor muscle while the beetles were walking on rough and smooth surfaces. For the rough surface, we chose a Styrofoam ball with a diameter of 9 cm mounted on a rigid pivot as a substrate for walking. On this substrate, the animals were easily able to find a grip. The ball was suspended and free to rotate about one axis. The smooth surface was a smooth epoxy resin plate (Spurr, 1969). On this surface, the beetles were not able to produce propulsive forces. The claws slipped and therefore the animals maintained their position even on a stationary substrate.

For the recordings of the claw retractor muscle activity, the animals were fixed above the Styrofoam ball or the smooth surface. This was done with a clip glued on the elytra of the beetles with a wax/colophony mixture (Fig. 2). The clip was held by a clamp that was mounted on a vertically adjustable stand. In this configuration, the legs were free to move and the beetles could be placed at the appropriate height and position above the surface. Thus, the animals were tethered and their movement was restricted to one direction, but forward walking was unrestrained. We chose this setup because in this way the beetles could walk forward and remain in focus for the high-speed video camera recordings for several minutes (see below). If the animals did not begin to walk spontaneously, walking movements were elicited by a gentle touch on the abdomen with a brush.

We used copper wire with a diameter of 50 μm as electrodes. After drilling small holes into the cuticle with pointed insect needles, two wires were inserted into the muscle located in the tibia, from the dorsal side near the femoro-tibial joint (Fig. 1), for differential recordings. The electrodes were fixed to the tibia with a molten wax/colophony mixture. Because the second tarsal muscle, the promotor muscle, is situated more distally in the tibia, the danger of crosstalk from the promotor muscle could be excluded at the chosen insertion site. The electrode wire was insulated except for the tips. The two wires were glued together with the wax/colophony mixture in order to minimize potential noise. The ends of the wires were soldered to a shielded cable, which was connected to an amplifier system DA100C that led to the data acquisition tool MP100 (both from BIOPAC Systems, Goleta, CA, USA). We used Acq*knowledge* 3.7.3 software (BIOPAC Systems) for EMG recordings and imported the data into Spike2 6.10 software (Cambridge Electronic Design Limited, Cambridge, UK) for further analyses. The EMG recordings were performed with a sampling frequency of 1 kHz. Filter settings of the amplifier system were 5 kHz low-pass and 0.05 Hz high-pass. Additional software filtering was performed with Acq*knowledge* software to smooth data with a 50 Hz band-stop filter and a secondary high-pass filter with a 5 Hz cut-off frequency.

Statistics
Statistical analyses were accomplished with SigmaPlot for Windows (Version 10.0, Systat Software, San Jose, CA, USA). As nearly all data were non-normally distributed, comparisons of two groups were
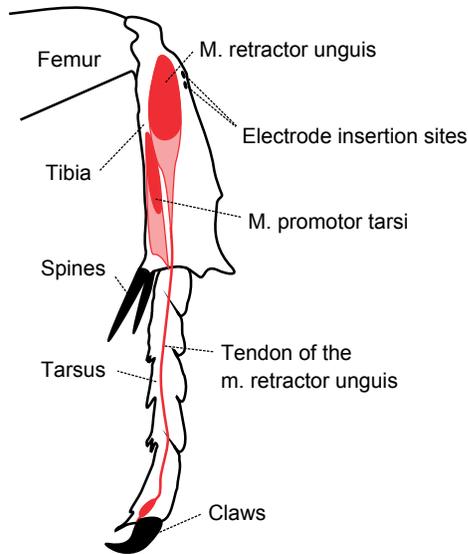
Fig. 1. Hind leg of the beetle *Pachnoda marginata peregrina*. Tarsus-controlling muscles, located in the tibia, are shown in red. Electromyography experiments were performed with the claw retractor muscle (musculus retractor unguis). The sites where the electrodes were inserted are marked with black ovals.

performed with the Mann–Whitney rank sum test. The box plots, used for data presentation, always show the median, the inter-quartile range, i.e. the 25th and 75th percentiles, and the 10th and 90th percentile limits (denoted by whiskers). In the phase diagrams, the horizontal bars are defined by the median values for the beginning and end of the muscle activity. The antennae mark the 10th percentile of the beginning and the 90th percentile of the end value.

Video recordings
Videos of the walking beetles were recorded using a Photron FASTCAM SA1.1 camera (Photron USA, San Diego, CA, USA) at 50 frames s^{-1} for the experiments on the Styrofoam ball and 125 frames s^{-1} on the smooth surface. The camera was synchronized electronically with the BIOPAC MP100 system, used for the EMG recordings. Hence, the EMGs and video recordings started simultaneously. The videos were saved on disk with the software Photron FASTCAM Viewer 3 and were analysed frame by frame with QuickTime Player 7.6.6 (Apple, Cupertino, CA, USA).

RESULTS
Anatomy
We studied the anatomy of the hind leg muscles controlling the tarsus and pretarsus in the beetle *P. m. peregrina*. We found the femur to be almost completely filled out with the flexor and extensor muscles of the tibia, with the flexor lying in the ventral and the extensor in the dorsal part of the femur. Two muscles are located in the tibia (Fig. 1). One of them was found to move the tarsus as a whole to its extreme anterior position about the tibio-tarsal joint (the tarsal promotor muscle: musculus promotor tarsi). We did not find an antagonistic muscle to the promotor. The second muscle located in the tibia was the claw retractor muscle (musculus retractor unguis), also having no antagonist. This muscle bends and stiffens the tarsus, and it spreads the claws and bends them ventrally towards the ground. Unlike the femur, the tibia is not completely filled out with muscles; its dorsal part remains rather empty. The claw retractor starts at the proximal end of the tibia, originating

laterally from the tibial exoskeleton. The muscle length corresponds approximately to two-thirds of the tibia length. The tarsal promotor is shorter than the retractor muscle and is shifted towards the distal part of the tibia, where it inserts at the posterior side of the tibial exoskeleton. From the point where the two muscles begin to overlap, the retractor muscle lies dorsal to the promotor. Hence, in the distal half of the tibia, the retractor muscle lies closer to the dorsal side of the tibia. The retractor muscle ends up in a tendon running through the distal part of the tibia and subsequently through all tarsal segments towards the pretarsus (Fig. 1), where it is connected to the unguitractor. The other side of the unguitractor itself is connected to the claws through membranous cuticle.

We briefly compared our findings with those from the other two leg pairs. Muscle arrangement in the middle leg was qualitatively the same as in the hind leg, while in the front leg we found a difference. There, the size, position and function of the claw retractor muscle is similar to the other two leg pairs, but the second muscle inserts at the anterior side and acts as a remotor of the tarsus, not as a promotor as in the middle and hind legs.

Analysis of walking movements
In animals walking on the rough substrate, clear stance and swing phases were observed. We defined the first ground contact of claws as the beginning of the stance phase. In most analyzed steps, the claws were the only part of the tarsus that touched the ground. During the stance phase, the leg was then moved backwards relative to the body. The end of the stance phase was characterized by the claws being lifted off the ground. In the swing phase, the leg was then moved through the air to its extreme anterior position to begin a new stance phase.

On the smooth substrate, leg movements were not as uniform as on the rough surface, because claws failed to interlock with the substrate. We did not observe as many clear step cycles as on the rough substrate. Leg movements consisted rather of grasping attempts, corresponding to the stance phase, and 'back swing' movements, corresponding to the swing phase. There, the tarsus was brought into the distal or most anterior position for a new grasping attempt. These movements were sometimes conducted through the air, and sometimes the leg was pulled over the ground without completely losing contact with the substrate. We analyzed the recorded video sequences frame by frame to estimate the beginning and the end of the stance phase or ground contact. Single video frames of both walking conditions are shown in Fig. 2.

Recordings of the muscle activity
We measured the activity of the claw retractor muscle in the hind leg during walking on rough and smooth substrates. In all recordings, we found the claw retractor muscle to be active during ground contact, i.e. during the stance phase of each step. On the smooth substrate, we also measured muscle activity in the front leg and compared it with that of the hind leg.

Walking on the rough substrate
From EMGs taken during beetle locomotion on the rough substrate, we identified one muscle unit with a small amplitude and, in most recordings, one muscle unit with a large amplitude. Sometimes two large units were observed, depending on both the insertion site of the electrodes and the quality of the EMG signal. Also, another very small unit was sometimes visible in the recordings, but when it was present, it was hardly distinguishable from the noise. As we did not reliably record more than one small and one large unit, we took only these two for further analysis.
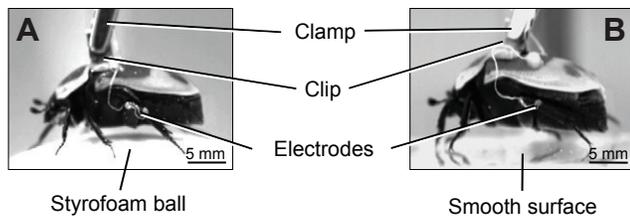
Fig. 2. Single frames of the video recordings of both walking conditions during the experiment. (A) Beetle walking on the rough Styrofoam ball. (B) Beetle walking on the smooth epoxy surface. Electrode wires are attached to the tibia and on the elytra to relieve the strain from the insertion sites, when there is a pull on the copper wires.

The small unit was active nearly all the time during the stance phase. The large unit was active mainly in the first half of the stance phase (Fig. 3A). The muscle unit activity is presented in phase diagrams normalized to the duration of the stance phase, where 0% corresponds to the beginning of the stance phase and 100% corresponds to its end. With this method of data visualization, we can show the duration of muscle activity during ground contact of the corresponding leg. Unless otherwise noted, in all of the following comparisons, the Mann–Whitney rank sum test was used. Box plots show the median and the 25th (Q_1) and the 75th quartiles (Q_3), and whiskers denote the 10th and 90th percentiles. Horizontal bars in the phase diagrams are defined by the median and the 10th percentile of the beginning of muscle activity, and the median and the 90th percentile of the end of muscle activity, respectively. The data in percent always refer to the stance phase taken as 100%.

Although the beginning of the muscle unit activity was very similar between the small (median: −5.9%, Q_1: −11.6%, Q_3: −0.8%) and the large units (median: −6.9%, Q_1: −11.9%, Q_3: −3.0%), the difference was still significant (U=91542.5, P=0.029). Both started to fire slightly before ground contact by the claws. The end of muscle unit activity differed much more between small and large units. The small unit was active up to 92.5% of the stance phase (Q_1: 85.3%, Q_3: 97.7%), whereas the large unit activity ended at 39.3% (Q_1: 18.5%, Q_3: 67.3%), a significant difference (U=152921.0, P<0.001). It should be noted that the large unit showed a much higher variability at the end of its activity than did the small unit.

We also measured the spike frequencies of small and large units. The spike frequency was significantly higher in the small unit than in the large one (small, median=29.195 Hz, Q_1=17.526 Hz, Q_3=36.059 Hz; large, median=19.981 Hz, Q_1=15.085 Hz, Q_3=25.288 Hz; U=92963.0, P<0.001). On the rough substrate, n=457 hind leg steps from N=5 animals were analyzed.

Walking on the smooth substrate

On the smooth surface, beetles did not accomplish a real stance phase as they were not able to find any grip on the substrate. Rather, movements here were unsuccessful attempts to grasp. During the swing phase on the smooth substrate, both the tarsus and claws did not always lose contact with the substrate. The anterior (forward) movements of the leg during the swing phase were only sometimes carried out in the air; often the leg was drawn along the substrate.

The EMG recordings taken during beetles walking on the smooth surface also revealed muscle units with small and large amplitudes, similar to those on the Styrofoam ball. We again analyzed one small and one large unit, according to the evaluation method above. The small unit started to burst at 0.1% in relation to the beginning of the stance phase (Q_1: −2.8%, Q_3: 2.3%). The onset of the large unit activity was at 3.6% of the beginning of the stance phase (Q_1: 0.8%,

Q_3: 32.1%), with a very large variation of data. The beginning of spike discharges was significantly different between the small and large units (U=116,376.5, P<0.001). The small muscle unit activity lasted up to 91.8% of the stance phase (Q_1: 85.9%, Q_3: 95.8%), whereas the large unit ended its activity at 54.5% (Q_1: 24.1%, Q_3: 81.0%), again with a much higher variability in the large unit. There was a statistically significant difference in median values of spike discharge termination between units (U=19,263.5, P<0.001).

The spike frequency of the small unit was 34.874 Hz (Q_1: 28.673 Hz, Q_3: 43.138 Hz) and that of the large unit was 15.697 Hz (Q_1: 10.572 Hz, Q_3: 22.943 Hz), showing a significant difference (U=16,197.0, P<0.001). On the smooth surface, we analyzed n=346 hind leg steps from N=4 animals.

Our observations of the animals walking on the smooth surface showed that hind legs tried to grasp less frequently than did the front and middle legs. This was most likely due to the fact that the front and middle legs were not able to produce a propulsive forward movement. As they failed, the beetle tried again to find a grip with the more anterior leg pairs instead of using the hind legs. Nevertheless, we recorded a sufficient number of hind leg movements for analysis. However, to test whether different leg pairs have a different employment of the claw retractor muscle, we also recorded EMGs of the retractor unguis in the front legs on the smooth surface. Here we found that the small muscle unit started firing at −5.1% in relation to the beginning of the stance phase (Q_1: −13.4%, Q_3: 2.7%), starting significantly earlier than the small muscle unit of the hind leg retractor (0.1%; for the quartile values of the hind leg, see above) (U=59,623.0, P<0.001). The end of small unit activity in the front leg was at 78.3% (Q_1: 62.2%, Q_3: 88.6%), which was again significantly earlier than in the hind leg (91.8%, U=38,169.0, P<0.001). The large unit of the front leg started its activity at 1.7% of the beginning of the stance phase (Q_1: −5.1%, Q_3: 11.4%). This was also significantly earlier than the hind leg unit, which started at 3.6% (U=65,968.0, P<0.001). The large unit of the front leg was active up to 53.9% of the stance phase (Q_1: 34.1%, Q_3: 67.4%), which was not significantly different to that of the hind leg (54.5%, U=52,401.0, P=0.536; Fig. 4A).

Comparison of the spike frequencies between front and hind legs revealed no significant difference. The small unit showed a median spike frequency of 35.069 Hz for the front leg (Q_1: 27.017 Hz, Q_3: 46.085 Hz) and 34.874 Hz for the hind leg (U=74,462.0, P=0.643; Fig. 4B). The median spike frequency of the large unit in the front leg amounted to 16.190 Hz (Q_1: 11.902 Hz, Q_3: 23.476 Hz), whereas that in the hind leg was 15.697 Hz (U=46,758.0, P=0.142; Fig. 4C).

From the comparison between the front and hind legs, we can draw the conclusion that the pattern of muscle activity is rather similar in both leg pairs. We found differences in the onset and end of the small muscle activity, and also for the beginning of activity of the large units, with the front leg activity starting and ending earlier in each respective case. However, no differences were found for the end of large unit activity and for small as well as large unit spike frequencies. The general activity pattern, thus, looked very similar in both legs, indicating that both leg pairs employ the same mechanism of tarsus control. We can therefore conclude that tarsal muscles behave similarly in all leg pairs, and that the hind legs perform the same movements as do the front legs, despite their lower rate of usage. Hence, the comparison of hind leg activity between the Styrofoam ball and the smooth surface is reasonable.

Comparison of muscle activity patterns during walking on rough and smooth substrates

We compared the activity of the claw retractor in the hind legs of beetles while the animals were walking either on the Styrofoam ball
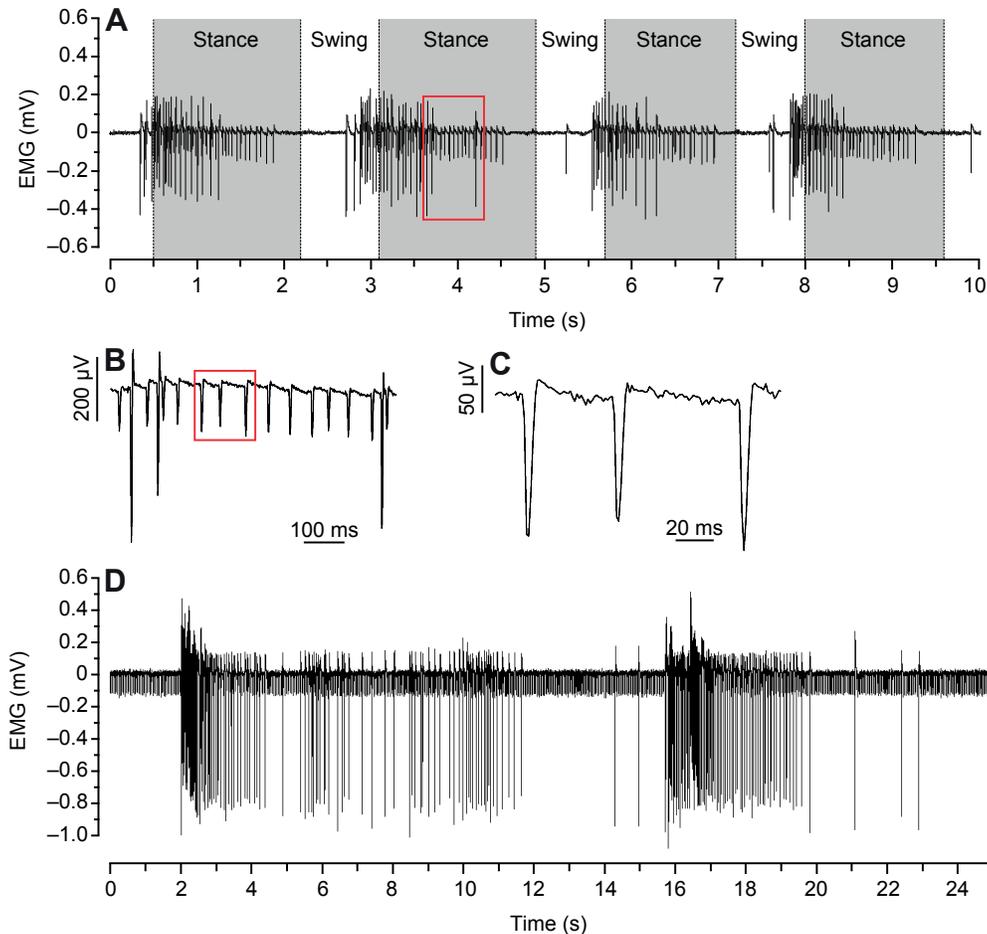
Fig. 3. (A) Sample of a typical electromyogram (EMG) recording of the claw retractor muscle. A stepping sequence of four stance phases (gray areas) and the three interjacent swing phases (white areas) on the Styrofoam ball is shown. Note the beginning of the muscle activity before the beginning of the stance phase, and the ending of the muscle activity before the end of the stance phase. The unit with larger amplitude was active at the beginning of the stance phase, while the smaller unit was active during almost the entire stance phase. (B) An enlargement of the area in the red frame in A. (C) An enlargement of the area in the red frame in B. (D) Reflex activation of the claw retractor muscle after touching the tarsus with a brush. A strong burst of the large muscle unit occurred immediately after touching the tarsus, followed by spike series with a lesser frequency for several seconds. The small unit seemed to remain unaffected or was sometimes even silenced for some seconds (not shown). The tarsus was touched twice in this example, right before the beginning of the large bursts.

or on the smooth stiff polymeric surface. The phase diagram shows a relatively similar pattern for both surfaces (Fig. 5A). Nevertheless, the comparison revealed significant differences. The beginning of the small muscle unit activity was earlier on the Styrofoam than on the smooth surface (U=46,025.0, P<0.001), whereas the end of small unit activity was not different on the different surfaces (U=86,506.0, P=0.068). The large unit again started to burst significantly earlier on the Styrofoam (U=150590.0, P<0.001), and the end of activity was also significantly earlier on the Styrofoam (U=93,938.0, P<0.001; Fig. 5A).

Comparison of spike frequencies revealed a significantly higher frequency of the small unit on the smooth surface (U=41,138.0, P<0.001; Fig. 5B). For the large unit, the difference in spike frequencies was also significant, but with a higher frequency on the Styrofoam (U=55,788.5, P<0.001; Fig. 5C).

We also recorded reflex activation of the claw retractor muscle in still (standing) animals after touching the tarsus with a brush (Fig. 3D). Here, a strong burst of the large muscle unit was obvious, followed by spikes series with a lower frequency for several seconds. The smaller units seemed to remain unaffected, or even silenced for a few seconds in some cases (not shown). These data were not analyzed quantitatively.

Stance duration

Additionally, the duration of the stance phase was measured in the different conditions, i.e. hind leg on the Styrofoam ball, and hind leg and front leg on the smooth substrate. A comparison of the median values of stance duration showed no difference between the Styrofoam ball (2.0 s, Q_1: 1.3 s, Q_3: 3.2 s) and the

smooth substrate (2.4 s, Q_1: 1.2 s, Q_3: 4.4 s) in the hind leg. Stance duration of the front leg on the smooth substrate, however, was significantly shorter than both hind leg conditions (0.9 s, Q_1: 0.5 s, Q_3: 1.6 s; P<0.05; Fig. 6A). In Fig. 6B–G we illustrate how the stance duration of the single steps was distributed over the relative time in the stepping cycle. All single measurements of beginning and end of spike discharge are plotted against stance duration for both units in all three conditions. The large variance in end of activity in the large unit, especially of the hind leg, can easily be seen here in the distribution of the single data points in Fig. 6C,E.

DISCUSSION
Anatomy

Our anatomy data show that, in contrast to stick insects (Radnikow and Bässler, 1991), locusts (Laurent and Hustert, 1988) and honey bees (Snodgrass, 1956), the claw retractor muscle of *P. m. peregrina* consists of only one part. The muscle is situated in the tibia and has no antagonist. Its tendon runs through the tibio-tarsal joint and the whole tarsus to the pretarsus, where it inserts at the unguitractor. Hence, as in the insects mentioned above, in *P. m. peregrina* the claw retractor muscle works against elastic structures as it is described in the tibio-tarsal joint in the stick insect (Walther, 1969) or the cockroach (Neff et al., 2000). These structures cause the tarsus to return to its initial position, when the muscle relaxes. In *P. m. peregrina*, they may contain resilin, as in the tibio-tarsal and the tarso-pretarsal joints of the cockroach, where this elastic protein inherits the antagonistic function to the claw retractor muscle (Frazier et al., 1999; Neff et al., 2000).
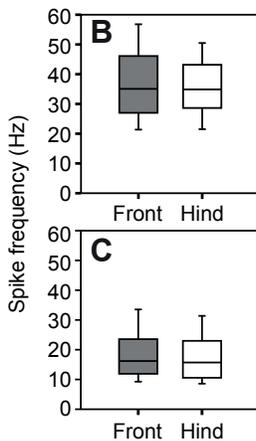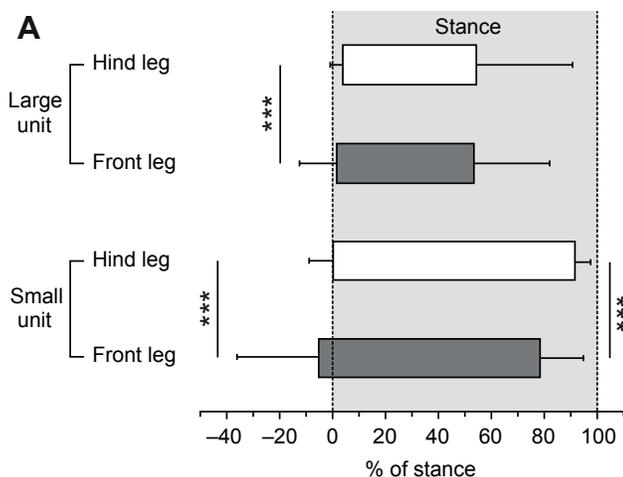
Fig. 4. Comparison of the claw retractor muscle activity in the front and hind leg on the smooth surface. (A) For both muscle units we found a significantly earlier onset of activity in the front leg. The end of activity was significantly earlier in the front leg only for the small unit. No differences were found in spike frequencies between the legs for both small (B) and large (C) units.

A similar retractor unguis arrangement as in our studied beetles was found in the hind leg of the larvae of the water bug *Corixa geoffroyi*. Here, the claw retractor muscle also consists of only one tibial part (Weber, 1930). In the middle leg of *C. geoffroyi* larvae, Weber described the presence of an unusual pretarsal extensor muscle as an antagonist to the common claw retractor. The middle legs of the larvae of the underwater *C. geoffroyi* bear extremely large claws and are specialized for anchoring the animals to the ground. This is why these animals probably need better articulation potential of the claws than other insects, and therefore have a second claw muscle.

It is very unusual that leg muscles, other than the claw retractor, exist without an antagonistic part; most joint-moving systems consist of an arrangement of several muscles responsible for opposing movements of a joint. Among these are, for example, the levator and depressor of the tarsus, the flexor and extensor of the tibia, the levator and depressor of the femur, and the promotor and remotor of the coxa (e.g. Snodgrass, 1935). A rare example of a leg muscle without an antagonist is the tarsal depressor of aphids and coccids (Weber, 1930). This muscle is counterbalanced only by the opposing force of the substrate and the elastic structures of the tibio-tarsal joint. In the tibia of *P. m. peregrina* we found a similar situation, where we discovered only one other tarsal muscle besides the claw retractor, thus without antagonist. In the hind and middle legs, the muscle was found to move the tarsus in the anterior direction (m. promotor tarsi), whereas in the front leg, the muscle works in the opposite direction, thus causing a posterior movement of the tarsus (m. remotor tarsi). Hence, we can assume that this muscle must also work against elastic structures, moving the tarsus to its initial position, when the muscle is relaxed. These findings are supported by the tarsal position in ablated legs. In hind and middle legs, the tarsus assumed an extreme posterior position, probably caused by the elastic forces acting against the promotor muscle. The opposite was found in the front legs, where the tarsus rested in an extreme anterior position in amputated legs, corresponding to the elastic forces acting against the remotor muscle.

Leg movements and muscle activity

On the Styrofoam ball, beetles walked properly. Their walking pattern could be clearly divided into stance and swing phases. On the smooth surface, in contrast, the walking pattern was not as explicitly discernible as it was on the Styrofoam ball. Beetles tried to walk, but since they failed to find a grip on the substrate, stepping cycles could only occasionally be performed. Thus, the question was whether the claw controlling muscle behaves differently in these different situations. On one substrate, we have the situation that claws can easily interlock with the substrate and, therefore, provide
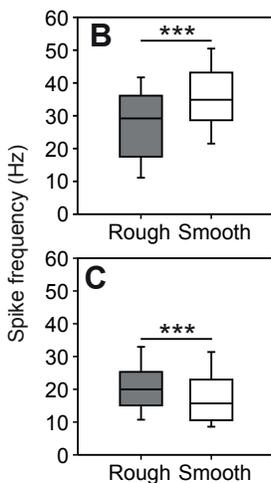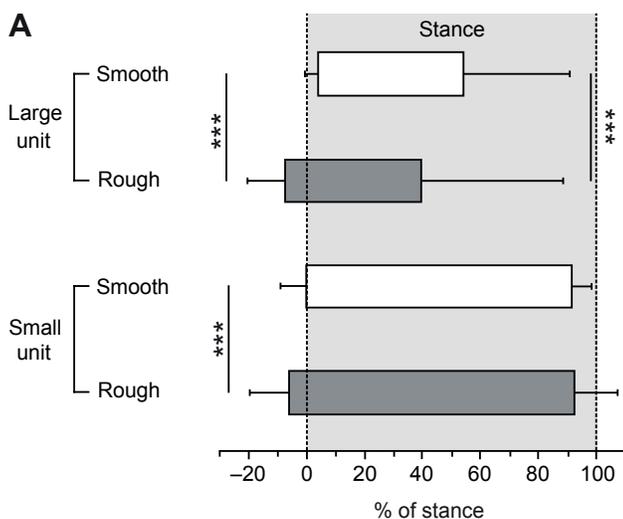

Fig. 5. Comparison of the claw retractor muscle activity in the hind leg on rough and smooth surfaces. (A) Both units started their activity significantly earlier on the rough surface than on the smooth surface. Additionally, the end of the activity in the large unit was significantly later on the smooth surface, whereas there was no difference in the end of activity in the small unit. Spike frequency of the small unit (B) was significantly higher on the smooth surface, while the spike frequency of the large unit (C) was higher on the rough surface.
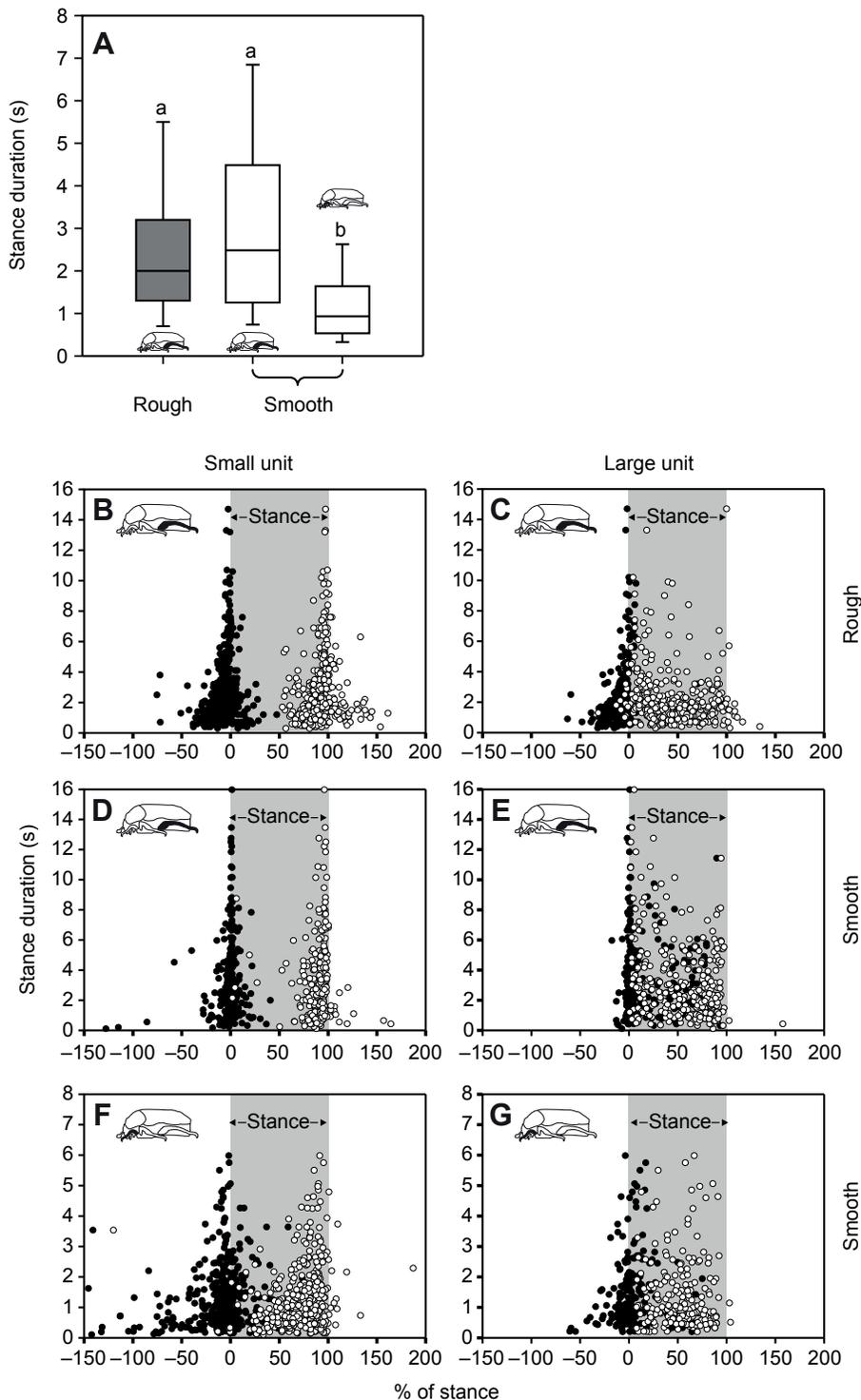
Fig. 6. (A) Comparison of stance duration in the three different configurations: hind leg on the rough surface; hind leg on the smooth surface; and front leg on the smooth surface. Stance duration of the hind leg was not different between the rough (2.0 s) and smooth (2.4 s) surfaces; however, stance duration of the front leg on the smooth surface was significantly shorter (0.9 s) than that of the hind leg. Panels B–G show single measurements of beginning and end of spike discharge of the small (B,D,F) and the large units (C,E,G). The values of the hind leg on the rough Styrofoam ball (B,C) and on the smooth surface (D,E) as well as the values of the front leg on the smooth surface (F,G) are shown. The pronounced variation in ending of large unit activity in the hind leg can be recognized (C,E), especially if compared with the small unit (B,D).

the friction necessary for the leg to produce a propulsive movement. On the other substrate, the claws try to hook into the substrate, but on the smooth stiff surface, they cannot interlock with absent surface asperities. One might assume that the claw retractor muscle behaves differently in slipping (stiff smooth substrate) and interlocking (soft corrugated substrate).

Our analysis of the muscle activity shows rather similar temporal muscle activity patterns on both surfaces. We found the muscle to be active during the stance phase, and to be mostly inactive during the swing phase. On both surfaces, the muscle activity consists of

units with small and large amplitude. The smaller unit is active during almost the entire stance phase, whereas the larger one is limited roughly to the first half of the stance phase. This shows that both the overall muscle activity and the pattern of tarsal movements are approximately the same on both substrates.

Nonetheless, there were differences in the precise onset and end of activity in both the small and large muscle units. For both units, we found an earlier beginning of activity on the rough ball. Here, the small unit started slightly before the beginning of the stance phase, which is also known for other leg insect muscles active during

the stance phase, like the depressor trochanteris both in the stick insect (Rosenbaum et al., 2010) and in the cockroach (Delcomyn and Usherwood, 1973; Watson and Ritzmann, 1998). This kind of muscle activation can be interpreted as a preparation for the following leg or tarsal movement. The end of the small unit activity, however, was not significantly different between the two substrates.

The differences, though, were much more expressed for the large unit. Here, the activity again began earlier on the rough surface, also starting before the beginning of stance. However, on the smooth surface, activity began in the large unit after the beginning of the stance phase, and therefore significantly later than on the rough surface. The earlier onset of muscle activity on the rough surface can be interpreted in the following way. The ability to walk on the rough surface, resulting in a regular walking pattern, elicits intrasegmental and intersegmental sensory information transfer between the legs. That is, legs, which are currently contacting the ground, obtain the information that their claws are able to interlock with or penetrate the surface. Such sensory information might be gathered by campaniform sensillae, as has been shown for the cockroach (Zill et al., 2010). This information is then transferred to the other legs, which are in the swing phase at the same moment. With this information, they can be prepared in time for their stance phase with some kind of preceding muscle tension and be ready to penetrate the surface or interlock with its asperities as soon as the claws touch the ground. Such information transfer between different legs is a common principle of adjusting the momentary walking pattern in insects (Brunn and Dean, 1994; Cruse, 1990; Dean and Wendler, 1983; Dürr et al., 2004; Stein et al., 2006). As such information from sensory organs is presumably lacking during the stance phase on the smooth surface, legs in the swing phase are not prepared for an immediate interlocking once they obtain ground contact. Rather, they accomplish a sliding movement with the claws across the surface and try to find grip, with a claw flexion that sets on later than if the claws had immediate interlocking. The end of the activity is rather similar for the small muscle unit on both the rough (end at 92.5% of the stance phase) and smooth surfaces (end at 91.8% of the stance phase). However, the large unit activity ends significantly later on the smooth (at 54.5%) than on the rough (at 39.3%) surface. This difference might be due to the short and successful action of interlocking on the Styrofoam ball. The interlocking movement is fast and strong on the rough substrate, and thus a shorter activity of the large and probably stronger muscle unit is necessary. In contrast, on the smooth and stiff substrate, the claws are not able to interlock with the ground and therefore the activity of the large muscle unit lasts longer. This longer-lasting force is probably maintained as long as the beetle tries to find an interlocking site. The end of activity at 54.5% of the stance phase on the smooth surface therefore reflects the end of unsuccessful trials to grip the ground. The small unit activity that lasted almost until the end of the stance phase is possibly needed to maintain a certain stiffness of the tarsus (Frantsevich and Gorb, 2004), in order to maintain the body posture and to prevent early detachment (Endlein and Federle, 2008; Federle et al., 2001; Radnikow and Bässler, 1991).

The fact that both units ended their activity before reaching 100% of the stance phase can be explained by the preparation for the following swing phase. By ending the small muscle unit activity at approximately 90% of the stance phase, the muscle has already lost its tonus and the tarsus its stiffness. The floppy tarsus can therefore be more easily detached from the substrate at the beginning of the swing phase. Such behavior is known for other stance phase muscles, such as the posterior rotators and the adductor of the coxa

(Duch and Pflüger, 1995) or the retractor unguis (Laurent and Hustert, 1988) in the locust.

Spike frequencies

Our analysis revealed significantly higher spike frequencies of the small unit on the smooth surface (34.8 Hz) than on the rough one (29.2 Hz). Higher spike frequencies of the small muscle unit are an indication of stronger muscle tension, and therefore of the increased stiffness of the tarsal chain. Because on the smooth substrate the claws cannot be interlocked with the ground immediately at the beginning of the stance phase, as they do on the rough substrate, they are pulled across the surface and remain prepared for interlocking at any time. Hence, the tarsus needs a strong stiffness with which to resist any unpredictable obstacles that may be encountered during the sliding movement, and to use the claws for interlocking. Furthermore, as the claws do not encounter a resisting force on the smooth ground, the putative interlocking mechanism at the unguitractor (Gorb, 1996; Seifert and Heinzeller, 1989) is not able to additionally support the stiffness of the tarsus. The whole stability of the tarsal chain thus has to be provided by the muscle force. On the rough substrate, this continuous muscle tension is obviously not needed to such a strong extent, because claws find their interlocking sites right at the beginning of the stance phase. The necessary tarsus stiffness is then achieved at a lower contribution of the muscle activity.

The spike frequencies of the large unit were also significantly different between both surfaces (rough: 19.981 Hz; smooth: 15.697 Hz). The higher frequency on the rough surface might be explained by the interlocking of the claws with the substrate immediately after touching the ground. There, a fast, strong movement was necessary to penetrate the surface. This was not the case on the smooth surface, where no penetration was possible and hence the large unit was recruited to a lesser extent. The surface texture and/or material properties of both substrates might elicit different motor responses of the animal locomotory system *via* sensory feedback. This hypothesis is supported by the longer-lasting large unit activity of the muscle on the smooth surface, which can be an indicator of a longer activity in a standby condition, when the muscle is prepared to interlock with the substrate. We can thus conclude that on the rough surface with a high probability of finding a grip, the large muscle unit provides a fast and strong movement for penetrating the rough surface until the claws are properly engaged with the substrate. The searching movement for such a site on the smooth surface takes longer but needs less force because no grip or penetration occurs.

Stance duration

Stance duration is known to be a good indicator of the walking rate as, at increased walking rates, normally it is mainly the stance duration that is shortened, while the swing duration remains much more constant (Bowerman, 1977; Duch and Pflüger, 1995). We therefore measured stance duration during the three EMG measurements: hind leg on rough substrate, front leg on rough substrate and front leg on smooth substrate. The stance duration of the walking sequences in the hind leg EMG measurements on both substrates turned out to be the same. Thus, the differences in timing of muscle activity and spike frequency of the involved muscle units cannot be attributed to different walking rates, but must be ascribed to the different substrates.

We have to consider that the beetles were not completely free to walk in our experiments. The animals were tethered to a clamp, and their body position could be adjusted in height, so that the distance

between the body and the substrate resembled natural conditions. The beetles were able to produce walking movements with their legs without difficulty. But we have to note that their body weight was suspended by the fixture. It is known that supporting the body weight can alter the motor output quantitatively by reducing the spike frequency in the burst of a given motor unit (Delcomyn, 1973). The measured spike frequencies in this study might therefore not quantitatively reflect natural walking conditions. But Delcomyn (Delcomyn, 1973) also showed that other parameters, such as burst duration, are hardly affected at all. Hence, temporal muscle activity, which is the most important result here, should not differ from that in freely walking animals. As we compared the motor activity of a muscle between two different substrates with beetles fixed exactly in the same conditions, we can compare these two conditions without losing qualitative information. Besides, the body mass of the beetles was $1.152 \pm 0.182\,$g ($N=18$), which resulted in a normal force of $11.3\,$mN. However, the forces that *P. m. peregrina* beetles are able to exert with the claws of a single leg amount to up to $200\,$mN (Dai et al., 2002). The total force of all legs was calculated to be ~$550\,$mN. This force is larger by a factor of 48 compared with the forces that are necessary to support the body weight. Thus, the fact that the beetles were suspended in our experiments can be almost completely neglected with regard to ground reaction forces of the claws. For these two reasons, we are confident that our experimental setup was suitable for our purposes and that our results can be considered as a valuable contribution to the understanding of locomotion and attachment control in insects.

Conclusions

Our analysis of the activity of the claw retractor muscle in the beetle *P. m. peregrina* on two different substrates revealed a relatively similar muscle activity pattern on both surfaces in different legs. This means that this muscle has the same basic nervous control in different leg pairs and on different substrates. The differences were found only in the precise onset and end of activity of the muscle units, as well as in spike frequencies between smooth and rough surfaces. This shows that the basic control pattern is adjusted through the sensory feedback of tarsal and pretarsal sensory organs. The two substrates used in the study presumably caused different sensory feedback because of their influence on the beetle's gripping ability. Such adjustment is also known for locomotory programs of other leg joints (for reviews, see Büschges, 2005; Büschges et al., 2008), but our study is the first report on the muscle control of pretarsal attachment devices.

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