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RESEARCH ARTICLE

The neuromechanics of proleg grip release

Ritwika Mukherjee, Samuel Vaughn and Barry A. Trimmer*

ABSTRACT

Because soft animals are deformable, their locomotion is particularly affected by external forces and they are expected to face challenges controlling movements in different environments and orientations. We have used the caterpillar *Manduca sexta* to study neuromechanical strategies of soft-bodied scansorial locomotion. *Manduca* locomotion critically depends on the timing of proleg grip release, which is mediated by the principal planta retractor muscle and its single motoneuron, PPR. During upright crawling, PPR firing frequency increases approximately 0.6 s before grip release but during upside-down crawling, this activity begins significantly earlier, possibly pre-tensioning the muscle. Under different loading conditions the timing of PPR activity changes relative to the stance/swing cycle. PPR motor activity is greater during upside-down crawling but these frequency changes are too small to produce significant differences in muscle force. Detailed observation of the proleg tip show that it swells before the retractor muscle is activated. This small movement is correlated with the activation of more posterior body segments, suggesting that it results from indirect mechanical effects. The timing and direction of this proleg displacement implies that proleg grip release is a dynamic interplay of mechanics and active neural control.

KEY WORDS: Biomechanics, Caterpillar, *Manduca sexta*, Climbing, Soft bodied

INTRODUCTION

Locomotion by terrestrial animals requires direct contact with solid or granular surfaces which, in natural environments, are typically complex and cluttered. During horizontal walking and running on solid substrates, animals must compensate for perturbations caused by uneven terrain. This is generally accomplished by a combination of mechanical compliance and sensory feedback that modulates motor patterns. The importance of these different mechanisms depends on the environment, the animal speed and specifics of the gait. In some cases, animals run so quickly they effectively ignore local disturbances and rely upon the comparatively large inertial forces to carry them forward or stabilize (Full and Tu, 1991; Jindrich and Full, 2002). In other cases the inertial forces are relatively small and locomotion is slow, so sensory feedback is the primary mechanism for compensatory movements (Büschges, 2012).

Animals that climb have the additional challenge of resisting the effects of gravity pulling them away from the substrate. They overcome this by enhancing grip through interlocking hooks, strong bonds with the substrates using adhesives (Labonte et al., 2016) or suction, and active grasping by the limbs (Gorb, 2001; Endlein et al., 2017). Most climbing animals employ a combination of attachment mechanisms to cope with different surfaces and modes of locomotion (Labonte and Federle, 2015). Hooks are considered to be highly effective when they are matched with surface asperities on a similar scale (Dai et al., 2002; Asbeck et al., 2006), adhesives and suction are well suited to smooth surfaces, and active grasping is used when it enhances frictional forces between points on the substrate. Although these mechanisms provide static stability, animals need to move from place to place and so must also control grip release, a process that is not so well studied as attachment. Also, climbing does not take place in a single plane but involves a complex three-dimensional (3D) environment and interactions with uneven surfaces in different orientations. This combination of environmental complexity and continually changing grip makes the control of scansorial locomotion particularly challenging.

Caterpillars provide an excellent opportunity to understand the mechanisms underlying climbing. Most species are obligate leaf eaters and must move around on plants; in fact, they are some of the most prevalent herbivores on the planet. They cope with diverse physical barriers (e.g. plant hairs, prickly surfaces, glassy smooth surfaces), climb in all possible orientations, and have extremely effective static grip. In addition, they are a tractable model system because they are readily available, their movements are relatively easy to observe, and the neural control of their behavior can be studied at the level of single identified neurons (Metallo and Trimmer, 2015).

Caterpillars are also predominantly soft and operate at relatively low internal pressure so external forces such as gravity can deform their bodies. A model of the body bending stiffness has been constructed from tissue mechanical testing, pressurizing the insect and by modeling the anisotropic properties of the body wall and muscle (Lin et al., 2011). Treating *Manduca* as a hollow beam anchored at one end, the specific stiffness (body weight/tip deflection relative to body length) varies from less than 1 (a large resting caterpillar) to about 10 (a small caterpillar in whole body tetanus) at normal physiological pressures. This implies that a caterpillar weighing 2 g held at one end will 'flop' down approximately 5 cm under its own body weight. Small caterpillars are stiffer, but will sag about 1/10 of their length unless supported. This has major implications for the caterpillar crawling strategy and during climbing in varying orientations this gravitational force component changes and creates a new challenge for controlling movements. In previous work, measurements of the ground reaction forces during upright crawling show that for most of the step cycle *Manduca* maintain their body in tension so that compressive forces are carried by the substrate. This is called the environmental skeleton strategy (Lin and Trimmer, 2010a,b). Negative ground reaction forces normal to the substrate (i.e. 'pull-off' forces) were either absent or undetectably small, which implies that grip release is an extremely effective process. The control of grip release is important because the attachment of a single proleg can support the entire weight of a caterpillar and prevent locomotion. Caterpillars

Tufts University, Department of Biology, 200 Boston Avenue, Suite 2600, Medford, MA 02155, USA.

*Author for correspondence (barry.trimmer@tufts.edu)

B.A.T., 0000-0003-1782-7373

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can therefore serve as excellent models to learn about control principles for locomotion in animals and climbing machines with high degrees of freedom.

Caterpillar species can vary enormously in appearance because of elaborate protuberances and hair-like coverings, but all of them have soft, generally cylindrical bodies. In addition to three pairs of short articulated legs on the thorax, caterpillars have ventral gripping appendages called prolegs on their abdomen. Different species have a different number of prolegs (Angelini and Kaufman, 2005) and they vary in the structure of their gripping surfaces. These prolegs are critical for locomotion such as inching and crawling (van Griethuijsen and Trimmer, 2014) but they do not directly propel the caterpillar forward (Snodgrass, 1961; Lin and Trimmer, 2010a,b). Most large and thick caterpillars (such as *Manduca*) crawl with an anterograde wave of muscle activity passing from the terminal segment to the head, coordinated with proleg grip and release (Simon et al., 2010a; Metallo and Trimmer, 2015). In *Manduca*, each proleg grips the substrate during the stance phase and is lifted during the swing phase by the movement of the entire segment rather than shortening only the proleg (Belanger et al., 2000; Mezoff et al., 2004; Trimmer and Issberner, 2007).

The prolegs are bilateral, soft, retractable sac-like structures on the ventral abdominal surface. In *Manduca* (and many other crawling species) the prolegs are only found on abdominal segments 3 to 6 (A3–A6) and the terminal (anal) body segment (Fig. 1A). The proleg tip (the planta) has a medial-facing flexible membrane embedded with sclerotized curved hooks called crochets (Fig. 1B). The crochets can be partially withdrawn by contraction of a single muscle, the principal planta retractor muscle (PPRM). The PPRM has its origin near the posterior margin of the spiracle and inserts into the planta membrane slightly lateral to the crochets (Figs 1C and 2B,C). A second proleg retracting muscle, the accessory planta retractor muscle (APRM), also originates near the spiracle and inserts at the first infolding between the planta and the upper part of the proleg. Contraction of both proleg muscles results in planta retraction and abduction of the proleg (Weeks and Jacobs, 1987). Each PPRM is controlled by a single motoneuron (PPR) in the dorsal–lateral region of the corresponding segmental ganglion (Weeks and Truman, 1984a; Sandstrom and Weeks, 1991). When the motoneurons are inactive the muscles are relaxed and the planta is extended and adducted (Mezoff et al., 2004) with the crochets directed towards the midline to grip the substrate (Hinton, 1952). There are no antagonistic muscles; cessation of retractor neuron activity is sufficient for the prolegs to immediately extend through turgor pressure and tissue elasticity (Mezoff et al., 2004). Previous electromyography (EMG) studies have demonstrated that during normal crawling, the PPRM is activated in brief bursts that correlate with movements of the proleg. Although the timing of these bursts is tightly coupled to the phasing of the crawl, the duration and intensity (spike frequency) of this activity are not correlated to the muscle length changes (Belanger et al., 2000; Belanger and Trimmer, 2000). We hypothesized that the primary function of the activity of PPRM was to disengage the crochets.

This study aims to describe the process of proleg grip release and to determine how it is achieved under the different loading conditions that occur during upright and upside-down climbing. Our hypothesis was that proleg grip release is controlled by active neural control, and release from the substrate in the upside-down orientation requires an increase in the duration or firing frequency of the PPR, to compensate for the increased planta loading. Contrary to our hypothesis, we find that changes in neural activity controlling proleg retraction do not explain the robustness of grip release under different loads. Instead, release appears to be mechanically coupled to body movements.

MATERIALS AND METHODS

Animals

Second-day fifth instar larvae of tobacco hornworm *Manduca sexta* (Linnaeus 1763) with an average body length of 6 cm were used for the experiments. The animals were raised on an artificial diet at a constant temperature of 27°C in a light:dark cycle of 17 h:7 h while

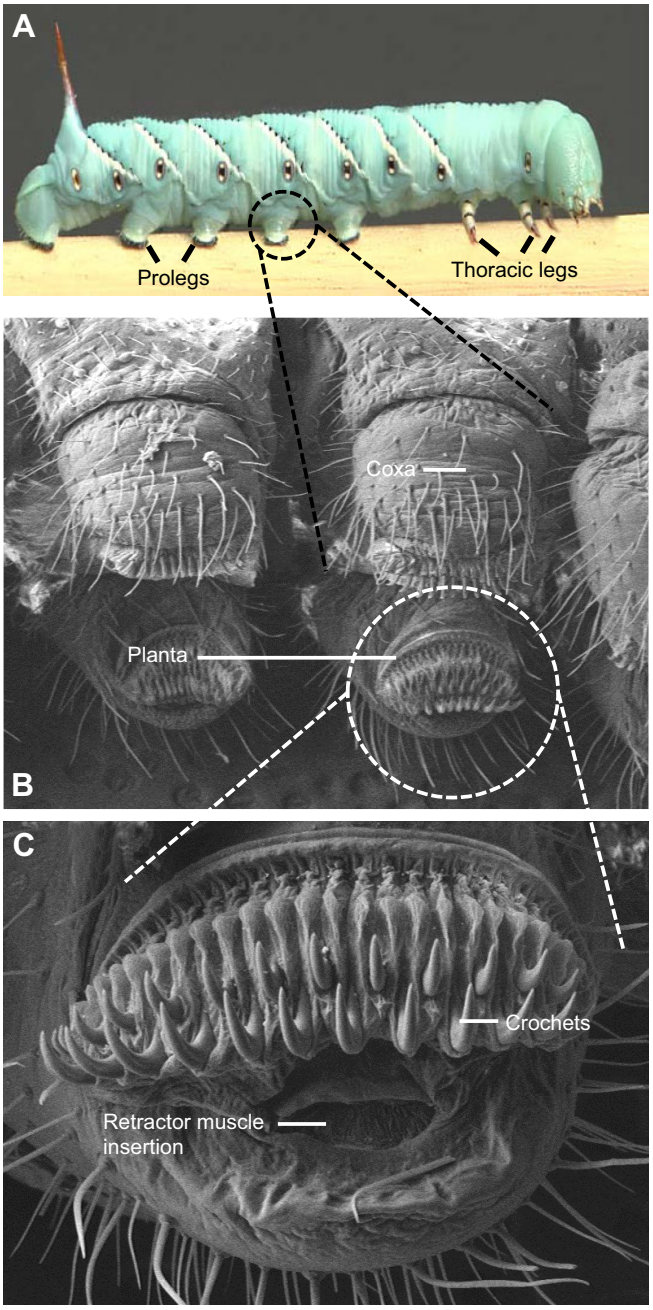

Fig. 1. The abdominal prolegs grip the substrate using sharp cuticular hooks. (A) In *Manduca*, the prolegs are only found on abdominal segments 3 to 6 (A3–A6) and the terminal body segment. (B) The medial view of a proleg tip (the planta) has a mediolateral-facing flexible membrane embedded with sclerotized curved hooks called crochets. (C) Medial view of the crochets: they can be partially withdrawn by contraction of a single muscle, the principal planta retractor muscle (PPRM).

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following the maintenance protocol as described by Bell and Joachim (Bell and Joachim, 1976). Before using them for the trials, the animals were encouraged to climb on a frame of differently oriented balsa wooden sticks of diameter 5 mm. All experiments were carried out at 27°C.

The experimental design

The neural control of proleg release was examined by comparing the activity of PPR during crawling in two different horizontal orientations, upright and upside-down (Fig. 2A). In both cases, the vertical component of the contact force balanced the opposing weight of the caterpillar. This makes the normal forces acting on the prolegs (N_1 and N_2) important variables. The normal forces acting on the proleg as it releases grip in either orientation are different. In one case, the substrate helps balance the weight, which is lacking in the upside-down orientation.

In addition to measuring the effect of orientation on activity of the retractor muscles, we added perturbation to the system by applying loads that pulled *Manduca* away from the substrate. During upright crawling, *Manduca* also experiences lateral toppling forces but we have not studied the effects these might have on proleg retraction.

Experiments

High-speed video-recording of grip release

Movements of the planta were recorded in a freely crawling caterpillar using a Phantom VEO 640L monochrome camera (Vision Research, Wayne, NJ, USA) at 1000 frames per second (2560×1600 pixels) and an InfiniProbe TS-160 macro objective lens (Infinity Photo-Optical Company, Boulder, CO, USA). The field of view (ventral aspect, approximately 6.8×4.3 mm) was calibrated using glass microspheres of known diameter: the calculated scale factor was 2.67 µm per pixel. Retraction was characterized by tracking selected parts of the planta membrane and crochets in successive frames at 1 ms intervals using Kinovea software (https://www.kinovea.org/).

During proleg stance phase (approximately 1 s duration prior to release), the planta membrane can be seen to partially withdraw without detaching the crochets. These movements were quantified at 5 ms intervals by measuring the average pixel intensity of the region around the insertion point. Small displacements of the substrate (caused by movements in other body segments) were stabilized by aligning image frames using the Image Align Plugin in ImageJ (https://imagej.nih.gov/ij/index.html). This ensured that the planta was always in the same position within the frame. The average pixel intensity in a background region was then subtracted frame-by-frame from the region of interest to compensate for changes in illumination or light reflections. The resultant changes in pixel intensity correlated very well with observed folding of the planta membrane.

3D motion capture of proleg retraction: tracking surface area of the proleg

Proleg retraction during natural crawling was recorded using four Basler 602f cameras at 200 frames per second, fitted with Computar Ganz 3.3X zoom lenses. Each lens was focused on a different but overlapping view of the proleg to ensure that tracking markers were visible in at least two planes simultaneously. The A4 proleg of an anesthetized animal (chilled in ice) was cleaned with acetone, and dried with compressed air before marking with 35 to 62 small ink dots to aid movement tracking. Although the exact number and location of dots were random, no animal was used without at least three locations at the base of the crochet hooks, and three locations on the very tip of the coxa. The planta was considered to be fully withdrawn when it no longer moved with respect to the rest of the proleg. Experiments were carried out after a recovery period of 1 h once the insects crawled on a wooden dowel, and each recording was a sequence of uninterrupted steps from the release of the A5 proleg to the full retraction of the A4 proleg. Recording and synchronization of the cameras was carried out using Vicon Motus 9.2 (Vicon Corporation, Denver, CO, USA). Recordings were exported to the Digitizing Tools suite of programs (DLTcal5 and DLTdv5) (Hedrick, 2008) for 3D reconstruction. We calibrated the recording volume by using a stack of four aligned pieces of acrylic (1.5 mm×3 mm×4 mm) each with 13 evenly spaced holes that formed a 52-point frame of known dimensions. DLTcal5 and DLTdv5 were used to reconstruct the 3D coordinates of each tracked marker. This software uses an 11 coefficient direct linear transform (DLT) to establish the location and orientation of each camera view in space. After coefficients were calculated, a reconstruction of the calibration frame was used as a control to ensure accuracy of the calibration. The parameters were then applied to each view of the proleg withdrawal and points were tracked through a combination of manual clicking and automatic pattern recognition in DLTdv5.

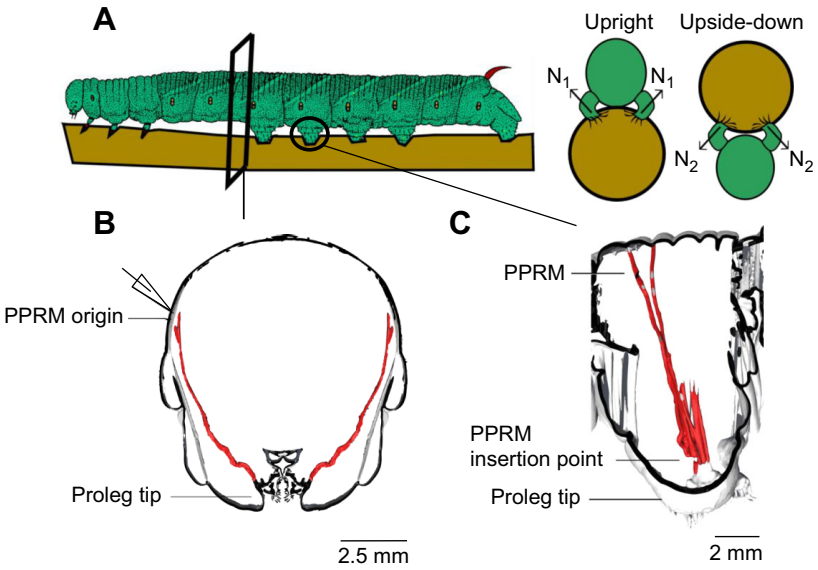

Fig. 2. *Manduca* can crawl in any orientation and grip is released by PPRM in each proleg. (A) Free body diagrams of a transverse subsection of *Manduca* when it is crawling upright and upside-down. When *Manduca* switches from crawling upright to upside down, forces on the crochets pulling the body away from the substrate will increase and torque applied laterally will decrease. (B) Drawing of the proleg in the transverse plane showing the origin of the PPRM and site of electrode placement (arrow). (C) Drawing of the proleg in the longitudinal plane showing the PPRM insertion point and the inner surface of the planta. B and C are drawings based on computerized tomography (CT) scans supplied by Anthony Scibelli, Tufts University and Dr Hitoshi Aonuma, Hokkaido University.

Raw locations of each point were filtered in DLTdv5 using a Monte Carlo approach to smooth out any digitization error. The filtered data were output to MATLAB 2010b (MathWorks, Natick, MA, USA) for analysis.

Electromyography recordings of natural crawling

Bipolar EMG electrodes were used to record from the PPRM of prolegs in segment A4 or A5 in the animals while they crawled on a treadmill. The treadmill was 3D-printed using a fused deposition modeling printer (Dimension 1200, Stratasys, Eden Prairie, MN, USA) (Metallo and Trimmer, 2015). It was used to maintain the insect in the field of view of the camera and to restrict electrode displacement. The treadmill belt was made of nitrile rubber bands with a cross-sectional diameter of 5 mm. The bipolar electrodes were fabricated by soldering a pair of intertwined 25 or 50 µm diameter Formvar-insulated Nichrome wires to adjacent terminals of a male three-pin connector. The other end of the wires were cleaned and cut at an angle to increase the surface area of contact. A surgical needle was used to make a small hole beside but slightly dorsal to the spiracle of an anesthetized animal, and the electrode was inserted through the cuticle into the PPRM, where it attaches to the body wall. A fine silver ground wire was inserted into the slightly cut horn and sealed with cyanoacrylate adhesive. The electrode wires were connected to the inputs of a differential AC amplifier (model 1700, A-M Systems, Sequim, WA, USA) that amplified the signals 1000-fold with cut-off filters at 10 Hz and 10 kHz. These signals were further amplified by a DC high gain/low noise filter and amplifier (model 210A instrumentation amplifier, Brownlee Precision, Neurophase, Santa Clara, CA, USA) set at a wide bandwidth filter on a 10-fold gain. The data were digitized at 40 kHz using a data acquisition system (PowerLab 26T; https://www.adinstruments.com/products/labchart). Because the PPRM is innervated by a single neuron, EMGs can be resolved into electrical spikes representing neuron activity (Weeks and Truman, 1984b). Each spike in the EMG traces was detected as it exceeded an amplitude threshold and the neuron activity was represented by the instantaneous frequency calculated as the reciprocal of the inter-spike interval. We compared the overall muscle activity between the different orientations by counting the spikes in every burst. In addition, we calculated the average spike frequency, i.e. count of the burst/duration of the activity (see 'Data analysis' section).

Kinematics and EMG recordings during natural crawling

Movements of the prolegs during crawling were recorded at 30 frames per second using a Logitech C920 HD Pro webcam. The proleg tip was tracked frame by frame throughout locomotion using Kinovea 8.5 video-editing software. A small LED in the field of view flashed sporadically and the LED driving voltage was captured with the EMG recordings to synchronize movements and muscle activity. A crawl was defined as an anterograde wave of successive proleg steps starting the moment the terminal proleg loses contact with the substrate (Trimmer and Issberner, 2007). Each proleg takes one step per crawl that consists of an extended stance phase and a brief (∼1 s) swing phase. The step duration is defined as the time from the onset of stance to the end of the following swing phase. This study concentrated on neural/muscle activity preceding and accompanying crochet release from the substrate.

Loading experiments

To directly test the effects of load on PPRM firing, weights were attached to the insects and crawling was compared between both orientations. A range of weights were tested to find a weight that added substantial load to the body and yet allowed the insect to crawl. Weights larger than approximately 0.3BW (where BW is the body weight of the insect) discouraged crawling behavior. It should be noted that the insect can lift much larger loads (at least 2BW), but this severely restricts locomotion. To evenly distribute the weight across the body, tiny loads were attached to the caterpillar at three positions around the body: around the thoracic segment T3, between A4 and A5, and around A7, using thin silicone bands (Biomedical Silicone Tubing, 0.012×0.025×0.0065 inches, catalogue no. 806100, A-M Systems). Five insects were used to do three sets of tests each: (1) upright crawling with weights mounted on a pulley above *Manduca* to pull the body away from the substrate; (2) upside-down crawling with weights pulling *Manduca* downwards; and (3) upside-down crawling with only the silicone bands strapped around the body to control for the effects of strapping. A force transducer (isometric force transducer, model 60-2996, Harvard Apparatus, South Natick, MA, USA) was used to ensure that the applied weight component was equal in both orientations.

Data analysis

3D motion capture analysis

Surface areas were calculated using the Delaunay function in MATLAB 2014b that fitted a triangular mesh over the data in the x–z plane. This was further extrapolated to the y-plane based on the y-values at each point. Because the number and location of points was different for each animal, comparisons of surface area changes were only made within single animal trials. The timings were normalized to the A5 release–A4 release interval and aligned to the time at which the proleg lifted from the substrate.

The path of each tracked point was calculated as a 3D path but during the initial stages of grip release there was relatively little movement along the direction of crawling (x-axis) so our analysis concentrated on movements in the y–z plane. The initial direction of movement in the transverse plane was calculated for tracked points corresponding to the subcoxa, coxa, planta and crochets. This direction was measured as the angle in the y–z plane between the starting position of a tracked point (defined as the axis origin in the y–z plane) and the average of its position 15–40 ms later. Horizontal movement away from the midline was 180 deg and that towards the midline (i.e. 0 and 360 deg) was always positive and greater than 90 deg (hence angles such as −10 and 10 deg were measured as 350 and 370 deg, respectively).

EMG signal analysis

Rapid spikes in the EMG recordings were used to estimate neuron spike activity before and during each step; only recordings that were free of movement-induced artifacts were selected. Because crawling of the caterpillar is considered quasi-static, there is no significant inertial component transferred across steps. Thus, successive steps were selected for signal processing. An average of 8–10 steps was analysed for seven insects in both orientations. Because of the high variability in the instantaneous spike frequency across steps and between behaviors, all comparisons between upright and upside-down crawling were made within an individual.

EMGs were recorded using LabChart 7Pro v7.3.4. The signals were filtered using a low-pass digital filter (the frequency cut-off varied with insect, but was in the range of 700–1000 Hz) and individual spikes detected using a voltage threshold. The instantaneous frequency (IF) of the spikes was plotted for the period before and during each step. The IF plots were normalized to the swing duration, averaged for every insect, and compared between

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the orientations (seven comparisons per orientation) with DataView (https://www.st-andrews.ac.uk/~wjh/dataview/), SigmaPlot 12.0 (https://systatsoftware.com/) and MATLAB R2014a. Because the PPRM is responsible for proleg retraction, the activity before the start of swing is critical. We analysed the muscle activity before proleg movement by creating two timespans: early (>600 ms before start of swing; −1300 to −600 ms) and late (<600 ms before start of swing; −600 to 0 ms), normalized to start of swing at 0. Next, the spikes per burst were counted and compared separately for both timespans. The shift in spike activity from one time period to the other was then represented as the ratio of early/late events before the start of swing (equal to early spike events/late spike events) allowing the change to be compared across all the experiments.

To confirm the identity of the recorded muscle, the position of the electrode was determined in a dissected preparation (Trimmer and Weeks, 1989).

RESULTS

Proleg movements during natural crawling

While gripping the substrate, the proleg extended and adducted with the sharp tips of the crochets engaging the substrate. The soft planta membrane distended and bulged outwards except for a shallow indentation at the insertion of PPRM (Fig. 3A). This region of the planta spontaneously pulled inwards (partial retraction) when PPRM was activated (Fig. 3C) but the crochets were not released unless there was a sustained contraction. During a complete detachment, the planta membrane was pulled inward progressively, with both medial and lateral margins of the indentation collapsing in tension. As the planta pulled inwards, the crochets were rotated and lifted away from the substrate. This phase of retraction started approximately 50 ms before the last crochet was released and it brought the crochets together as they withdrew into the main body of the proleg (Fig. 4).

Reconstruction of the proleg shape from 3D kinematic measurements showed that the visible planta surface area increased immediately (within 50 ms) before the start of swing or release from substrate (Fig. 5). Expansion of the planta was followed by its retraction as the surface area decreased substantially and the proleg was lifted off the substrate. The phase order of enlargement and shrinkage was consistent amongst all trials, and the final tracked surface area (proleg withdrawn) was always less than the surface area in stance phase. Although all parts of the planta and proleg were pulled dorsally and laterally once retraction was underway, tracking of points on the crochets, subcoxa, coxa and planta in the transverse plane revealed a consistent difference in the initial trajectory of the crochets. During the first part of their movement the crochets moved inward towards the midline. The rest of the proleg on average, moved outwards, away from the substrate (Fig. 6A). Crochet motion was significantly different from all other segments [ANOVA with Tukey's *post-hoc* test; treatment groups were independent with the 'crochets' group being significantly different from all the other groups; d.f. (between groups)=3, d.f. (residual)=132, F=24.501, P<0.001] (Fig. 6B).

Activity of the planta retractor neuron, PPR

The activation of PPRM always preceded crochet detachment and the onset of the proleg swing phase. This EMG activity persisted throughout the swing phase and ceased shortly before the onset of the next stance phase. The instantaneous spike frequency during the swing phase was highly variable and there was no

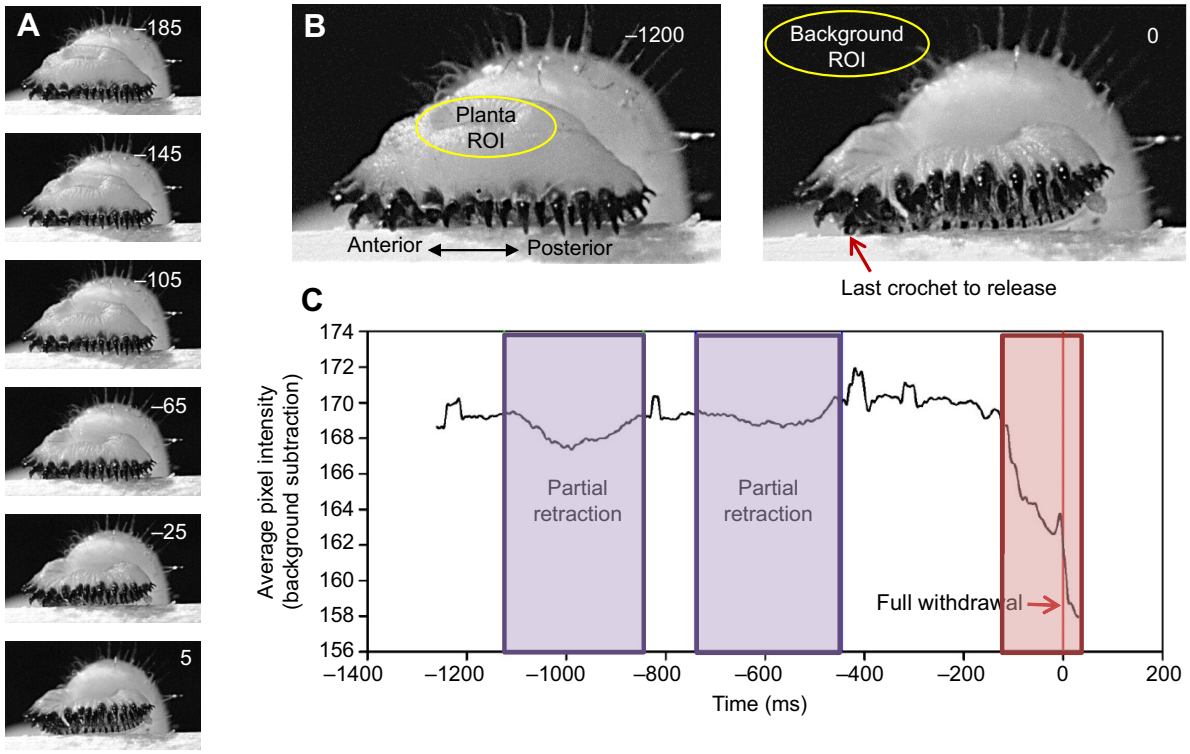

Fig. 3. Activity of PPRM deforms the planta membrane. (A) Vertical filmstrip of the proleg withdrawal of every 40 ms, from 185 ms before to 5 ms after full withdrawal. (B) Enlarged view of the first and last frames of the filmstrip. The region around the insertion point is planta region of interest (Planta ROI) and the background region of interest (Background ROI). (C) Retraction was characterized by tracking selected parts of the planta membrane and crochets in successive frames at 1 ms intervals. The purple shaded bars show partial retractions and the red shaded bar shows the change in pixel intensity during proleg withdrawal starting 185 ms before all the crochets release from the substrate.

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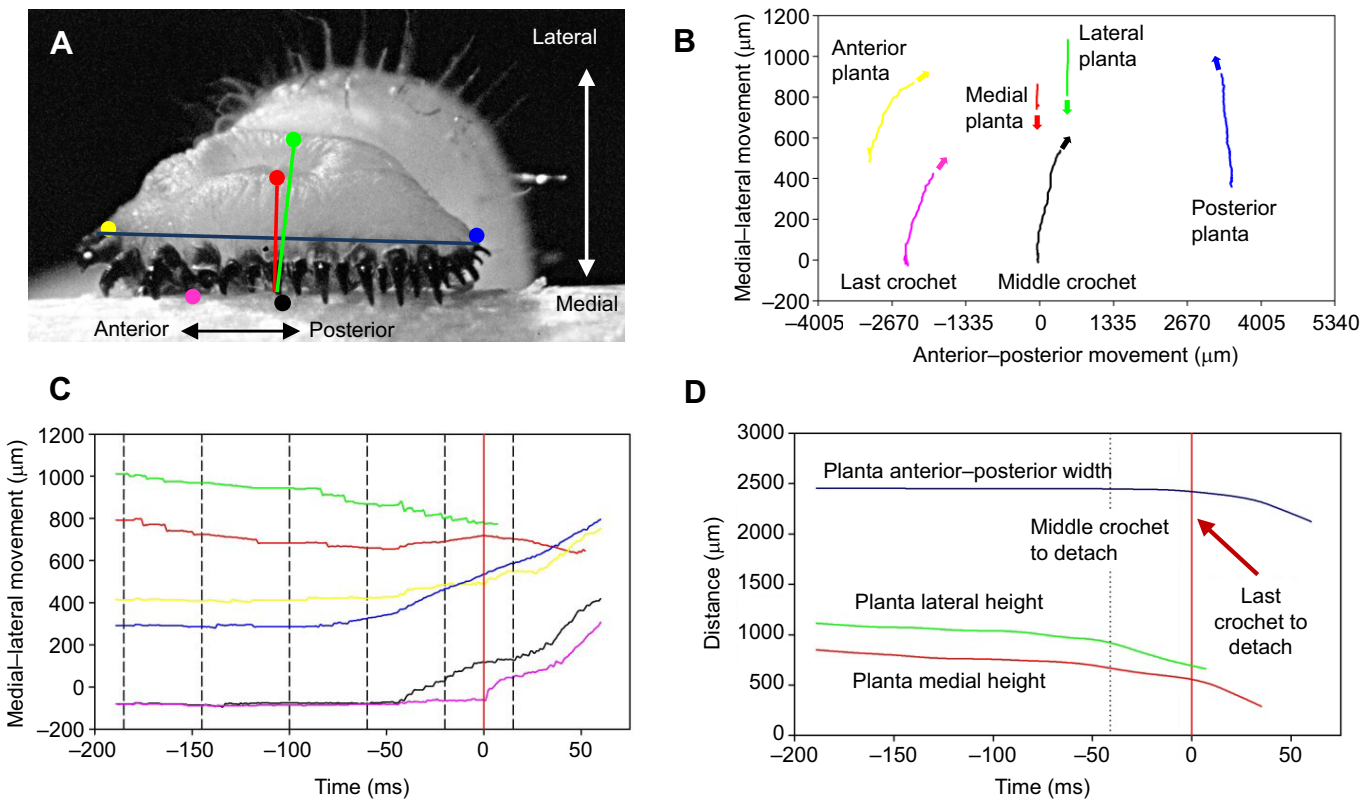

Fig. 4. Proleg grip release and movement of different parts and distances of the proleg in *M. sexta*. (A) The image is 185 ms before proleg withdrawal. The different parts of the proleg are marked as: green, lateral planta; blue, posterior planta; red, medial planta; yellow, anterior planta; blue line, planta anterior–posterior width; red line, planta medial height; green line, planta lateral height. (B) Proleg movement tracked from −200 to 50 ms where the proleg lifts off completely at 0 ms. The different colors are described by markers in A. The movement is along the anterior–posterior axis on the *x*-axis and the medial–lateral axis on the *y*-axis. Arrowheads in a path represent 1/200th of a second interval. (C) Medial–lateral movement tracked for the markers from −200 to 50 ms of proleg withdrawal, where the proleg lifts off at 0 ms. (D) Distances measured across the proleg are tracked across the entire duration. At 0 ms, the last crochet lifts off.

consistent pattern to the burst (Fig. 7) in either crawling orientation. During upright crawling, PPR activity began approximately 600 ms before the start of swing and the average spike frequency was 18.72±2.49 Hz during the whole burst of swing phase (n=47 steps in seven animals) (Fig. 8A, inset a). During upside-down crawling, PPR activity often began as much as 1.2 s before the onset of the swing phase and the average spike frequency was 16.73±2.37 Hz during the whole burst of swing phase (n= 52 steps in seven animals) (Fig. 8A, inset b). There was a significant difference in the average spike frequencies between both orientations (unpaired t-test: P<0.001). We tested for inter- and intra-individual variabilities by describing linear mixed models (ANOVA mixed model with two random effects: fixed differences among individuals and the possibility that orientation

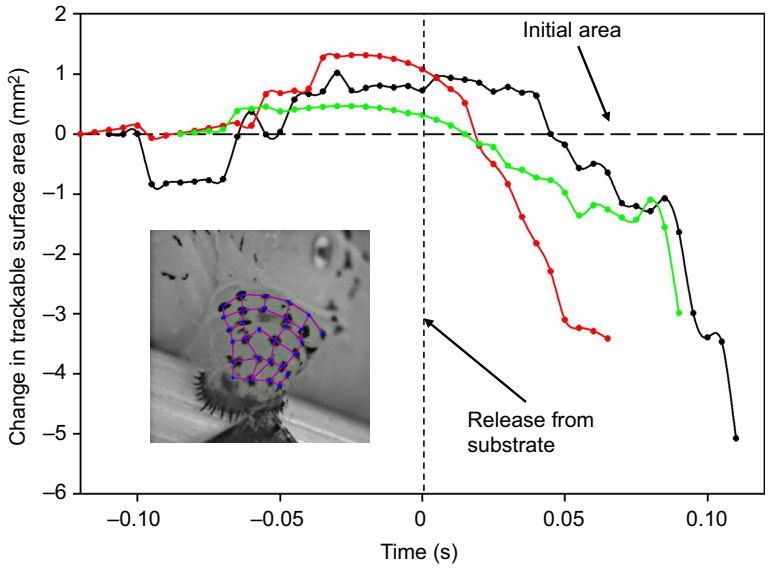

Fig. 5. The proleg surface area increases before crotchet retraction. Reconstruction of the proleg shape from 3D kinematic measurements shows that the visible planta surface area increases immediately (within 50 ms) before the start of swing. Each line represents the surface area of the planta for a single animal during and immediately before grip release. The expansion of the planta is followed by its retraction as the surface area decreases substantially and the proleg is lifted off the substrate. The inset marks the tracked points on the proleg.

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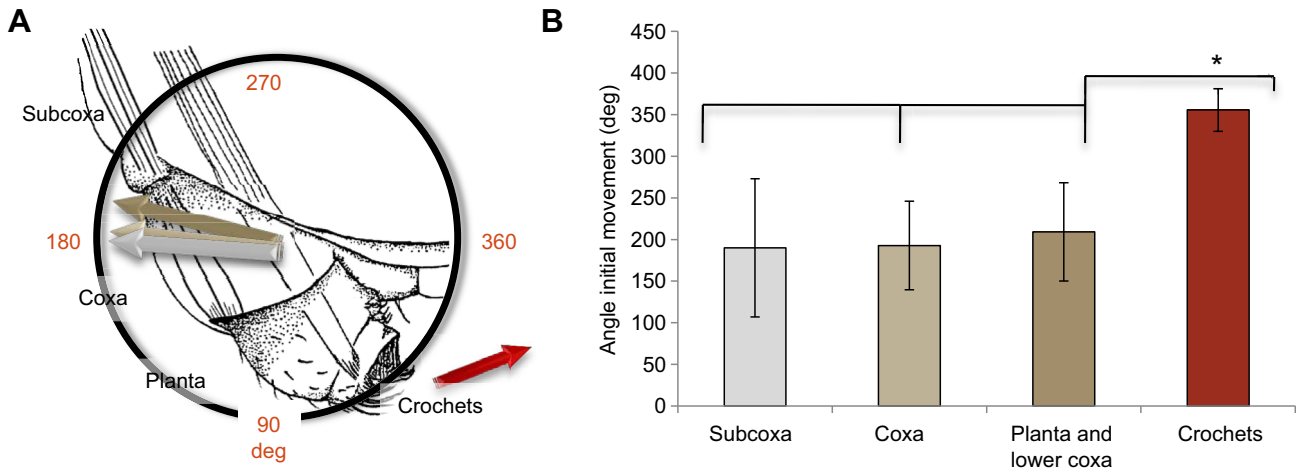

Fig. 6. The crochets initially move towards the midline to assist detachment. (A) Average initial angle of motion by the subcoxa, coxa, planta and crochets. This direction was measured as the angle in the *y*–*z* plane between the starting position of a tracked point (defined as the axis origin in the *y*–*z* plane) and the average of its position 15 to 40 ms later. Movement at 0 and 360 deg is movement towards the animal's midline and toward the substrate; 180 deg is movement laterally away from the midline. (B) Crochet hook motion is significantly different (*) from all other segments.

affects individuals differently: P=0.133; ANOVA mixed model with only one random effect of fixed differences among caterpillars: P=0.036; ANOVA mixed model with no random effects: P=0.046). The results show that the model with no random effects among caterpillars is a better model with the AIC (Akaike information criterion) value being the lowest.

This difference in the timing of PPRM activation was quantified by comparing the spike frequency in the early phase (>600 ms before start of swing) and the late phase (<600 ms before start of swing) during upright and upside-down crawling. The average count of spikes was significantly greater in the early phase of upside-down crawling (Mann–Whitney U-test; W=885, P=0.009, n_{upright}=47, $n_{\text{upside-down}}$=52 steps) than upright crawling (Fig. 8A). The data were also analysed by comparing the ratio of early/late firing events before start of the swing. The ratio was significantly higher for upside-down crawling (Mann–Whitney U-test; W=811.5, P=0.006, n_1=32, n_2=47 steps). However, the total number of spikes before grip release did not differ (Mann–Whitney U-test; W=4434.5, P=0.254, n_1=94, n_2=104 steps), demonstrating that PPRM activation occurred earlier but not more intensely during upside-down crawling.

When additional loads were attached to the caterpillar to pull its body away from the substrate, the overall firing frequency before swing phase increased regardless of orientation. The early/late ratio was significantly higher for the loaded conditions compared with the natural condition in both upright crawling (Mann–Whitney U-test; W=420, $P\leq$0.001, n_1=42, n_2=47 steps) and upside-down crawling (Mann–Whitney U-test; W=894.5, P=0.006, n_1=32, n_2=50 steps). For loaded animals, the early/late ratio was significantly higher during upside-down crawling than for upright crawling (Mann–Whitney U-test; W=646, P=0.002, n_1=42, n_2=50 steps) (Fig. 8B). There were negligible effects of the silicone bands because only strapping the insect body with the silicone tubes caused no significant change in the early/late events and was similar to natural upside-down crawling conditions (Mann–Whitney U-test; W=623.5, P=0.051, n_1=32, n_2=50). The same experiment could not be conducted for upright crawling because the tubes would obstruct the locomotion of the insect due to a lack of force pulling the tubes upwards.

DISCUSSION

Although proleg grip has been recognized as critical for locomotion in caterpillars (Barth, 1937; Hinton, 1955; Snodgrass, 1961), the mechanism of grip release during normal behavior has been difficult to discern. Proleg retraction can be stimulated by activating the planta hairs (Weeks and Jacobs, 1987) but this reflex is generally confined to the local body segments and does not need to be coordinated with ongoing movements. In fact, stimulated proleg retraction is inhibited during crawling and functions as an assistance reflex to avoid obstructions (Belanger et al., 2000; Belanger and Trimmer, 2000; Griethuijsen and Trimmer, 2010). The evoked full retraction reflex involves strong activation of PPRM preceding a slightly weaker activation of APRM (Weeks and Jacobs, 1987) and both muscles are active during the swing phase of normal crawling (Belanger et al., 2000; Belanger and Trimmer, 2000). Although we cannot rule out the possibility that APRM (or another nearby ventral muscle) contributes to proleg release, its insertion point (on the lateral wall of the coxa) and its delayed activation suggests that its primary role is to retract the proleg after the crochets have detached.

Here we have focused on the mechanism of proleg grip release during the initial phase when the PPRM becomes active. Although stimulus-evoked retraction of the proleg has been described generally (Weeks and Jacobs, 1987), the details of grip release during normal locomotion have not been described with high spatial and temporal resolution. Using a variable iris, internally focused macro lens system and a high-speed video-camera, we have now visualized movement of the planta membrane and crochets during normal crawling. We have used high-speed video-tracking to observe movements of the crochets and planta membrane, 3D motion capture to measure surface area of the proleg and movements of the crochets in the transverse plane, and EMG recordings of PPRM (representing spike activity in motoneuron PPR) to determine how grip is released.

Movements of the planta

During normal locomotion and throughout the stance period leading to proleg retraction, there are small spontaneous contractions of PPRM that are visible as transient withdrawals of the planta membrane at the PPRM insertion. Although these withdrawals can last 200 ms, the crochets are not released and they typically remain

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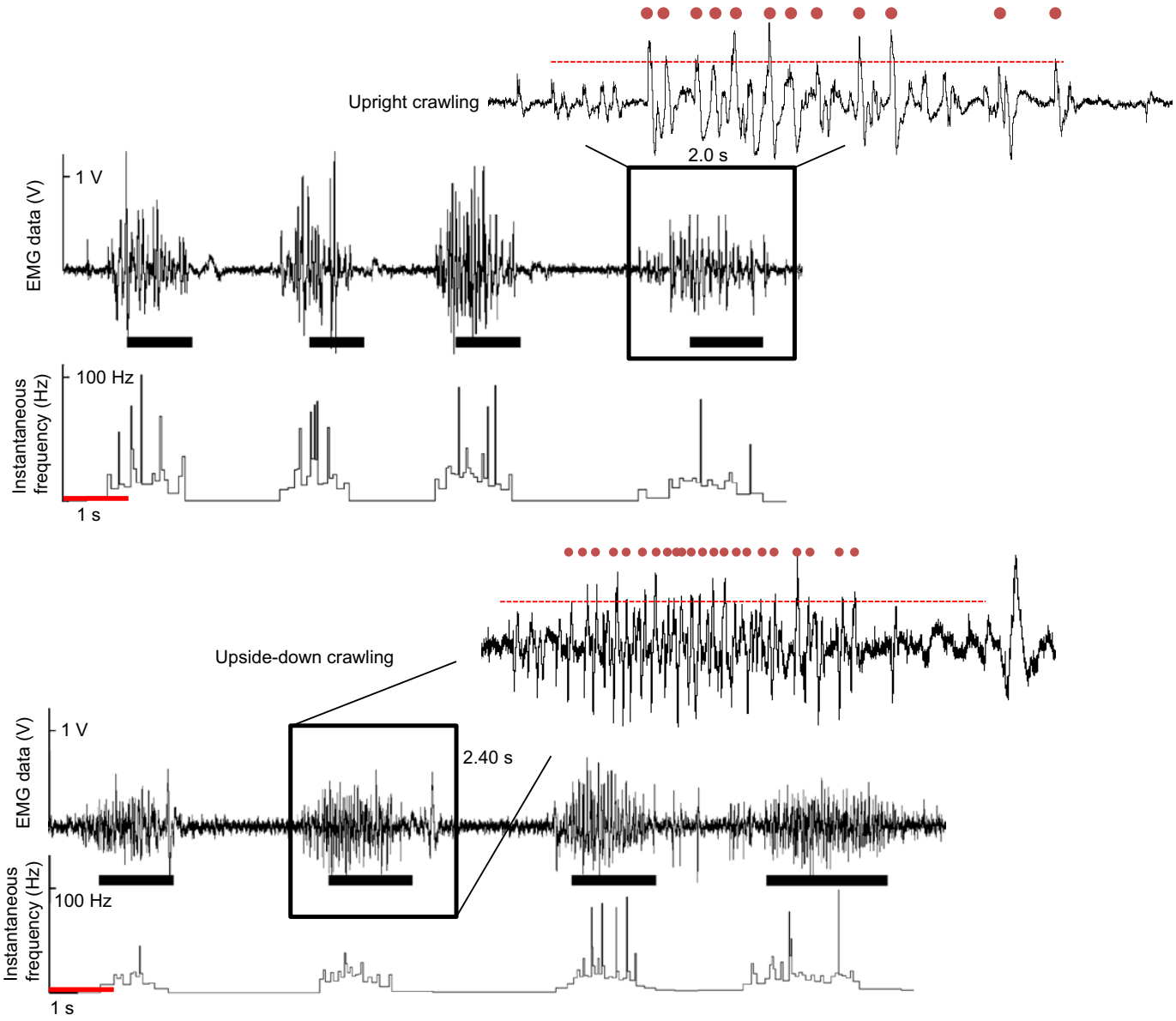

Fig. 7. Electromyography data of upright and upside-down swing phases with synchronized instantaneous frequency graphs. Black horizontal bars show the swing phases of the steps. The onset of activity in PPR precedes proleg swing phase and the average firing frequency is highly variable between steps and across orientations. The insets show a zoom-in view of one of the EMG recordings for both orientations. The durations of the recordings are given for both. The dashed red horizontal line shows the threshold amplitude for spike detection with the red dots annotating the events that were determined to be spikes. The red horizontal bar shows the time scale.

motionless. This suggests that, at rest, mechanical coupling between PPRM and the crochets is relatively weak; the planta membrane that surrounds the crochets is effectively slack. However, once PPRM activation is sustained the membrane pulls back from the body of each crochet and they begin to rotate away from the attachment point. During this process the 'cuticular ligaments' described by Barbier are clearly visible in the planta membrane (Barbier, 1985). These fibrous components of the planta extend radially from the PPRM insertion to the rim of the planta where the crochets are embedded. Presumably these fibers help to transmit force to the crochet and limit stretching of the membrane.

Shortly before detachment there are two previously unreported movements of the distal proleg that probably assist grip release. First, starting about 60 ms before release, the surface area at the tip of the proleg increases. It is unclear if the proleg swells by stretching the body wall or by unfolding the creases in the surface. The mechanism of this change in shape is also unknown but there are no muscles in the proleg that could directly expand the cuticle. We assume that hemolymph is forced into the proleg to cause the swelling. This effect is supported by pressure recordings made at the base of the prolegs in immobilized caterpillars, which show that retraction is accompanied by an increase in local internal pressure (the pressure falls again as the proleg is re-extended) (Mezoff et al., 2004). Interestingly, this swelling occurs at a time when more posterior segments are moving forwards and internal tissues, including the gut, are sliding forwards in advance of the proleg detachment (Simon et al., 2010a). This internal movement (visceral locomotory pistoning) could be responsible for the proleg swelling.

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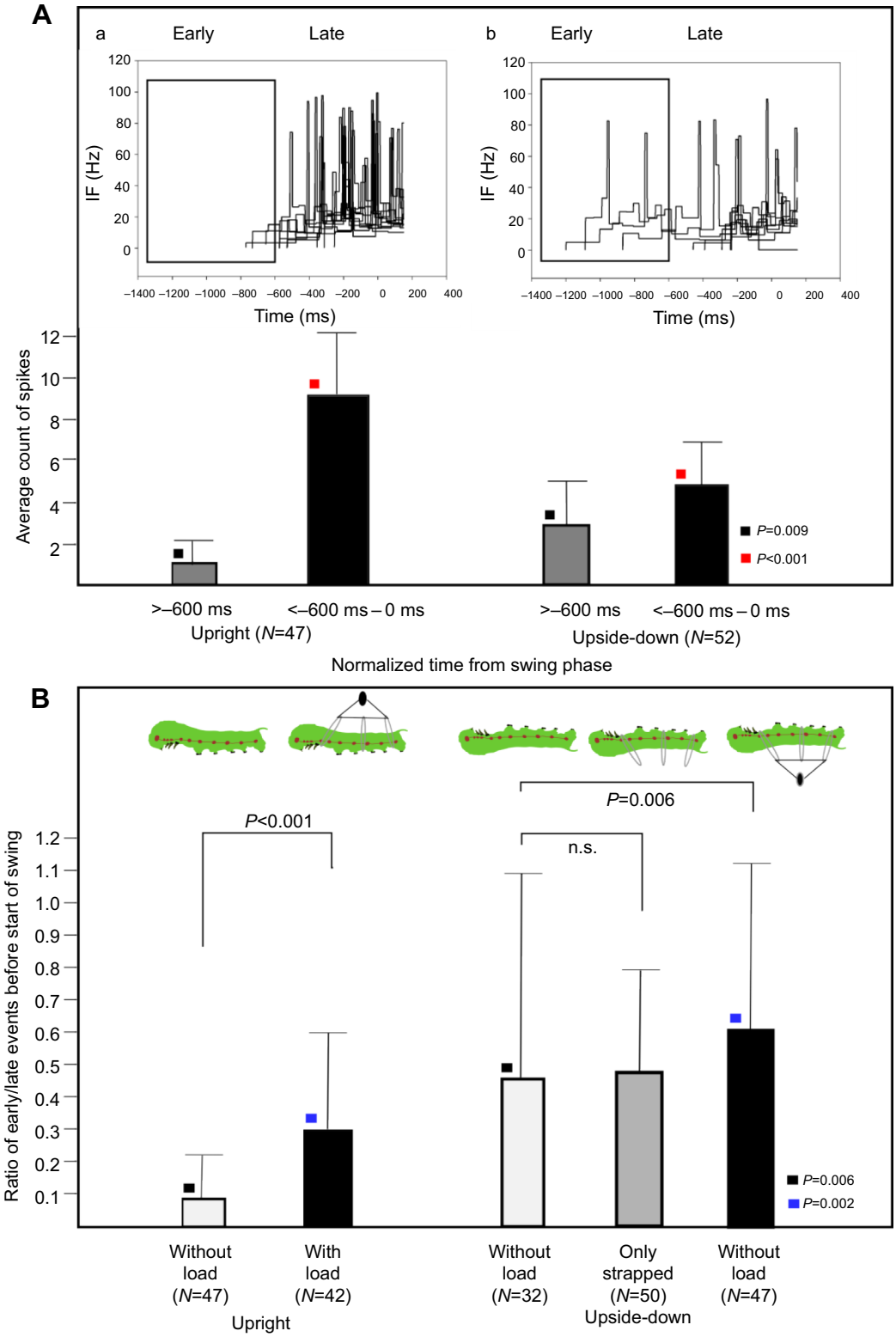

Fig. 8. PPRM is activated before crochet retraction and the timing is orientation, and load, dependent. (A) Comparison of average count of spikes for early and late timespans, where 0 ms is start of swing. Inset 'a': multiple IF curves of EMG data of 10 steps in one animal crawling upright show minimal activity in the early phase (black box). The PPR activity begins ~600 ms before the onset of swing (in late phase). Corresponding bar graphs show that spike frequency is significantly lower in the early phase. Inset 'b': multiple IF curves of EMG data of eight steps in the animal crawling upside-down show significant activity in the early phase (black box). Activity commences ~1 s before swing (in early phase). The bar graphs show increase in spike frequency in the early phase. (B) The ratio of early/late events before the start of swing (equal to early spike events/late spike events) were compared across different experimental conditions. The ratio was significantly higher for upside-down crawling. When loads are attached to *Manduca* to pull the body away from the substrate, the early/late ratio was significantly higher for the loaded conditions in both upright crawling and upside-down crawling. For loaded conditions, the early/late ratio was significantly higher during upside-down crawling. Only strapping the insect body with the silicone tubes had similar effects as natural upside-down crawling conditions. n.s., not significant.

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The second new observation is that the initial movement of the crochets is directed towards the midline rather than away from it. This movement is extremely small and brief but occurs simultaneously with proleg swelling. Because the crochets are engaged with the surface asperities (or embedded in a softer substrate), such movements would be expected to unload the tip of the crochet and allow it to be released from the surface. It is entirely possible that proleg swelling causes crochet unloading and that this constitutes a simple mechanical system to automatically coordinate grip release timing between different body segments.

Neural activity accompanying proleg release

The swing phase of the prolegs is always characterized by a burst of excitatory junction potentials (EJPs) in the PPRM (Belanger et al., 2000; Belanger and Trimmer, 2000) and during evoked proleg retraction (the withdrawal reflex) the onset of extension is tightly correlated with the end of the burst (Mezoff et al., 2004). During the swing phase the average EJP frequency of the PPRM is relatively low (approximately 16–19 Hz) and the firing pattern is highly irregular. The closely timed EJPs that give rise to intermittent spikes in the firing rate, both before and after release, are probably not generating large forces as larval *Manduca* muscles are slowly contracting synchronous muscles. They produce weak twitch forces (about 25 times smaller than adult flight muscles) at low frequencies (Rheuben and Kammer, 1980) and when stimulated to tetany at 20 Hz they develop peak force in about 2 s (Woods et al., 2008). Although tension during tetany increases with stimulation frequency up to approximately 90 Hz (Rheuben and Kammer, 1980), the low overall stimulation frequencies seen during crawling suggest that PPRM is operating well below its maximum force capability.

Here we show that the PPRM is activated in advance of the swing phase and the activation of PPRM is context dependent: when *Manduca* is crawling upside down, activation occurs earlier in the stepping cycle and the average frequency immediately preceding retraction is roughly halved. This can be re-stated to say that the total number of spikes preceding retraction is the same in both orientations but they occur over a longer period in the upside-down orientation.

When additional loads were attached to the caterpillar to pull its body away from the substrate, the overall firing frequency increased and the pre-release activity was phase-advanced regardless of orientation. Although the changes in frequency are very small and unlikely to generate significant changes in muscle force production (Rheuben and Kammer, 1980; Woods et al., 2008), the change in the timing of PPRM activation suggests that it is related to the force keeping the proleg in contact with the substrate (presumably at the tip of the crochet).

Neuromechanical control of grip release

In contrast to the long-lasting, multi-segmental bursts of longitudinal muscle activation that occur during crawling (Simon et al., 2010a; Metallo and Trimmer, 2015), activation of the PPRM is brief and highly correlated with grip release and the first part of the proleg swing phase (Belanger and Trimmer, 2000). The results presented here show that, on average, the timing of PPRM activation before grip release is affected by orientation and loading. This change in the pattern of firing suggests that *Manduca* can modify its motor output in response to orientation or loading. However, it is worth noting that these firing patterns are variable from step to step and from animal to animal; some upside-down steps do not include the early component. We propose that robust and highly regular grip release is achieved through a combination of neural and local mechanical control. In this scenario, each crawl cycle involves an anterograde wave of muscle activation that is accompanied by internal tissues and fluids sliding forwards (Simon et al., 2010b). There are no septa preventing hemolymph from flowing between segments and it is expected that fluid displaced by this visceral pistoning could be responsible for the observed proleg swelling. The swelling is correlated with a transient medial movement of the crochets which will unload the tip attachment and allow the crochets to retract. Activity in the PPRM thus serves two roles: early activity provides internal tension to resist premature unloading as the proleg swells; later, more intense activity provides the force needed to retract the crochets and withdraw the proleg.

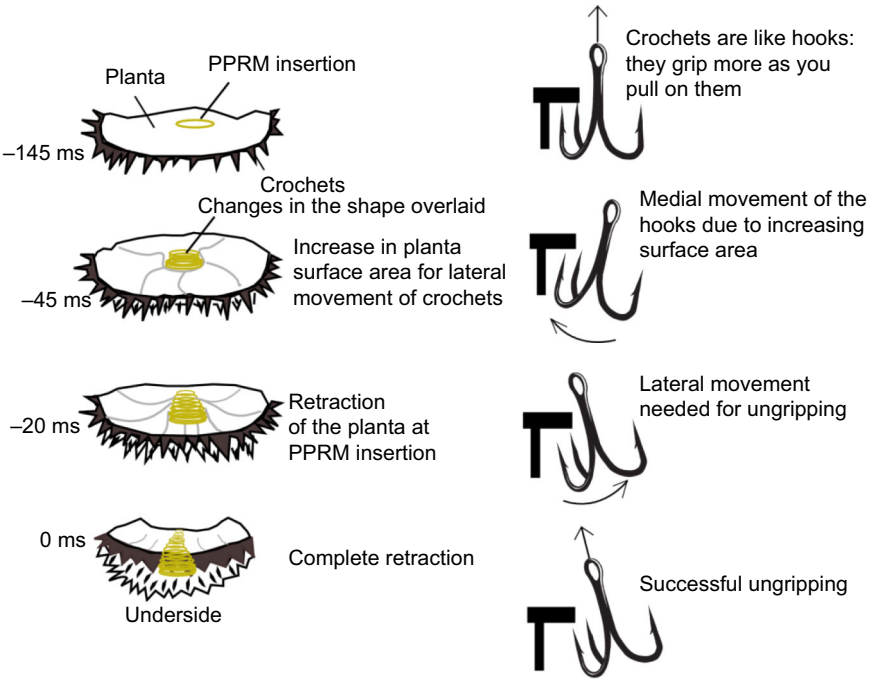

Fig. 9. The grappling hook analogy. Crochets act as grappling hooks. The swelling of the planta moves the crochets laterally so they unhook from the substrate before proleg retraction. This is a mechanical action that prepares the planta for subsequent retraction controlled by activity in PPR. Fluid deposition occurs in the planta immediately before proleg lift-off. 3D kinematic measurements show that the planta surface area increases immediately before the start of swing.

In this process, the crochets act like grappling hooks (Fig. 9). The gripping force exerted by a grappling hook increases with the applied load (up to the material limits of the surface or the hook itself) and release requires the hook to be unloaded or for the applied load to be re-orientated. In this analogy, the retractor muscles cannot simply pull the proleg upwards, this would cause the crochets to 'dig in' rather than release. Proleg swelling is a mechanism to unload or re-orient the applied forces to promote release. This proposed neuromechanical system is predicted to be highly adaptable. For example, activation of the PPRM could increase grip and this has been observed when attempting to evoke proleg withdrawal responses while *Manduca* is upside down on a branch. *Manduca* can also detach the crochets when it is not crawling and there is no anticipatory swelling of the proleg. In these cases, the PPRM is strongly activated (Belanger et al., 2000) to collapse the planta and effectively fold the grappling hooks away before retraction. We propose that the combination of proleg swelling and PPRM activation is a mechanism to more tightly couple grip release to the ongoing wave of locomotor muscle activity without increasing the precision of the afferent neural activity.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.M., B.A.T.; Methodology: R.M.; Validation: R.M.; Formal analysis: R.M., S.V., B.A.T.; Investigation: R.M., S.V.; Resources: B.A.T.; Writing - original draft: R.M.; Writing - review & editing: R.M., B.A.T.; Supervision: B.A.T.; Project administration: B.A.T.; Funding acquisition: B.A.T.

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