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The biomechanical and neural control of hydrostatic limb movements in *Manduca sexta*

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Summary

Caterpillars are ecologically successful soft-bodied climbers. They are able to grip tightly to foliage using cuticular hooks at the tips of specialized abdominal limbs called prolegs. The neural control of proleg retraction has been examined in some detail but little is known about how prolegs extend and adduct. This is of particular interest because there are no extensor muscles or any obvious mechanisms for directing hydraulic flow into the proleg. In restrained tobacco hornworms (*Manduca sexta*), adduction can be evoked by stimulating mechanosensory hairs on the medial surface of the proleg. 3-D kinematics show that extension and adduction occur simultaneously through an unfolding of membrane between the pseudo segments. Hemolymph pressure pulses are not necessary to extend the proleg; instead, the pressure at the base of the proleg decreases before adduction and increases before retraction. It is proposed that these pressure changes are caused by muscles that stiffen and relax the body wall during cycles of retraction and adduction. Electromyographic recordings show that relaxation of the principal planta retractor muscle is essential for normal adduction. Extracellular nerve and muscle recordings in reduced preparations show that medial hair stimulation of one proleg can strongly and bilaterally excite motoneurons controlling the ventral internal lateral muscles of all the proleg-bearing segments. Ablation, nerve section and electromyographic experiments show that this muscle is not essential for adduction in restrained larvae but that it is coactive with the retractors and may be responsible for stiffening the body wall during proleg movements.

Key words: caterpillar, proleg adduction, muscle, nerve, planta retractor muscle, motor inhibition, *Manduca sexta*, tobacco hornworm.

Introduction

Although hard skeletons are abundant throughout the animal kingdom, they are not the predominant body form. Most invertebrates are soft-bodied and use combinations of fluid (hydrostatic) and tissue pressure (muscular hydrostats) to provide stiffness against which muscles can work. This includes the larval stages of the holometabolous insects such as flies, beetles, butterflies and moths. The contribution of these insects alone makes soft-bodied crawling an extremely common form of terrestrial locomotion. Such movement is particularly interesting from a neural control perspective. With no easily defined joints, each muscle contraction has the potential to move the body in any plane and to cause crumpling, rotation and complex bending. Soft-bodied creatures must have evolved neural control strategies to deal with this increased range of possible movements.

Studies of hydrostatic locomotion have concentrated on swimming, burrowing (Trueman, 1975) and telescopic crawling in legless species such as worms (Quillin, 1998, 1999). Others have examined the movements of myostatic tissues such as elephant trunks, vertebrate tongues and octopus arms (Gutfreund et al., 1998; Matzner et al., 2000; Wilson et al., 1991). There is also considerable interest in the hydrostatic components of spider leg joints (Sensenig and Shultz, 2003). However, legged crawling by terrestrial soft-bodied animals such as caterpillars is particularly interesting and has been the subject of both kinematic and energetic studies (Barth, 1937; Brackenbury, 1996, 1997, 1999; Casey, 1991). These animals can climb in a complex three-dimensional environment and exhibit remarkable static and dynamic stability by virtue of their "*swinging, discrete, big-footed*" gait (Yim, 1994). Because of this stability, crawling insects do not need widely spaced articulated legs and have a small frontal area to body size ratio. Caterpillars, in particular, maintain a tight grip on the substrate using cuticular hooks (crochets) at the tip (planta) of the abdominal prolegs. In the tobacco hornworm, *Manduca sexta*, this grip is released at the start of the proleg swing phase through the activation of a 'retractor' muscle attached to the planta (Belanger et al., 2000).

What is not known is how the prolegs re-extend and grip the substrate again. There are no specific extensor muscles, so the leg must extend passively by cuticle elasticity or hydrostatic pressure. The control must be local since proleg extension is not always accompanied by segment shortening,

nor does segment compression always lead to proleg extension. The following studies examine the extension and adduction movements of the proleg in detail and show that biomechanical and neural mechanisms work in close coordination to correctly grasp the substrate. A noteworthy finding is that motor inhibition is a key element for active grasping rather than proleg eversion through an increase in hydrostatic pressure.

Materials and methods

Experimental animals

Manduca sexta sexta Linnaeus 1764 larvae were reared on artificial diet (Bell and Joachim, 1978) on a 17 h:7 h L:D cycle at 27°C. First or second day 5th instars of both sexes were used for experiments. Paired prolegs are present on mid-abdominal segments 3–6 (designated A3–A6) and on the more specialized terminal segments (also called anal claspers). The experiments reported here were on prolegs in segments A3–A6 only.

Sensory-evoked adduction

Although the proleg withdrawal response is most easily evoked in isolated abdomens (Weeks and Jacobs, 1987), the proleg extension and adduction reflex is more reliable in intact larvae (S.M. and B.A.T., unpublished observations). Animals were anesthetized by chilling on ice for at least 30 min and then restrained by attaching their dorsal surface to a flat or slightly convex surface with Vetbond™ adhesive (3M Corp., St Paul, MN, USA). At least 30 min after recovery from anesthesia, the different groups of sensory hairs (plant hairs, PH; medial hairs, MH; and ventromedial hairs, VMH) were manually stimulated by brief (1 s) deflection with a cactus spine or pin. Responses were videotaped using an S-VHS-resolution camcorder (Canon ES-400) from both a lateral and ventral view. The recorded behavioral responses were scored for at least five animals. In some preparations, the MHs or VMHs were removed at their socket using a razor blade while the larva was anesthetized. Animals were then allowed to recover for 1 h before behavioral testing.

Kinematics

Proleg movements were tracked in three dimensions using a custom-built 3-D motion capture system. Larvae were suspended dorsal surface uppermost attached to a convex surface so that the prolegs were visible. Fluorescent polymer microspheres of different sizes (22, 48 or 169 μm; Duke Scientific, Palo Alto, CA, USA) were placed onto the cuticle at the attachment points of specific muscles and at other landmarks on the proleg. The movements of these points were recorded under ultraviolet illumination (Model B, 100 W, long wavelength; Blak-Ray, Upland, CA, USA) using two digital camcorders (Canon ZR10) fitted with Hoya green (X1) filters (Edmund Optics Inc., Barrington, NJ, USA). The cameras were mounted on positioners placed at the same height, angled approximately 90° to one another and 45° to the longitudinal plane of the larva. The recordings on each camera were synchronized using a green light-emitting diode (LED) in the field of view, manually activated to flash (~20 ms) at the start of an event. The two video recordings were transferred to a Windows-based Pentium III PC through an IEE1394 (‘Firewire’) interface. The positions of the microspheres were mapped using APAS software (Advanced Performance Analysis System; Ariel Dynamics, Inc., San Diego, CA, USA) with semi-automatic point tracking. Three-dimensional reconstructions were calculated using a direct linear transform calibrated for each preparation from at least 18 non-coplanar points. The maximum time resolution was 16.7 ms (NTSC video field rate) but, for very slow movements, the points were digitized every 5–10 video fields.

Nerve recordings from isolated nerve cord/proleg preparations

An incision was made along the dorsal cuticle of the anesthetized larvae and the gut was removed. The larvae were pinned dorsal side up to Sylgard plates and bathed in cold Miyazaki saline (Trimmer and Weeks, 1989). The lateral branch of the ventral nerve (VN_L) in A4 was dissected out towards the proleg retractor muscles and cut distally. The remaining ventral nerves of ganglion A4 were kept intact. The abdominal portion of the nerve cord, along with the proleg in A4, was then removed and placed into a Sylgard recording dish. A suction electrode was placed onto the cut end of VN_L to record spontaneous and evoked activity. Similarly, *en passant* recordings were made from branches of the left A4 dorsal nerve (DN). In some preparations, two suction electrodes were used to record from pairs of dorsal nerves simultaneously.

In some experiments, active motoneurons were identified by severing connectives. In these preparations, only ganglia A4, A5 and A6 were isolated together with the proleg in A5. Recordings were made from DN in A5 before and after the A4–A5 connective was cut. All signals were amplified with cut-off filters of 10 Hz and 10 kHz (model 1700; A-M Systems Inc., Carlsborg, WA, USA).

Muscle recordings in reduced (‘flatterpillar’) preparations

After removing the gut, the larvae were pinned out in saline with the nerve cord and muscles exposed dorsally. To gain access to MHs on the left-side proleg, the muscles, cuticle and right-side proleg were removed from one right-side body segment. The remaining ganglia, nerves and muscles were intact. A suction electrode was used to record excitatory junction potentials (EJPs) from muscles innervated by the posterior branch of the dorsal nerve (DN_P). These recordings were digitized (EGAA software; RC Electronics, Santa Barbara, CA, USA) and viewed in Sigma Plot (SPSS Inc., Chicago, IL, USA). Periods of MH stimulation were recorded with a manually activated event marker. EJPs were counted by threshold detection using DataView software (W. Heitler, University of St Andrews, Scotland, UK).

Muscle and nerve ablation

Ventral muscles were severed in chilled, anesthetized

animals using micro-dissecting scissors on one side of the A4 segment (*N*=4), both the ipsilateral and contralateral sides of the A4 segment (*N*=6) and the ipsilateral sides on the A4 and A5 segments (*N*=4). In separate experiments, micro-dissecting scissors were used to sever the dorsal nerve on one side of the A4, A5 or A6 segment (*N*=4), the ipsilateral and contralateral DNs in the A6 segment (*N*=3) or the ipsilateral DNs in the A5 and A6 segment (*N*=3). Vetbond was used to seal the wounds. After 24 h, the adduction response was tested by stimulation of the MHs with fine forceps while mounted on their dorsal side. The location and extent of muscle and nerve damage was assessed by dissection.

Pressure measurements

Most recordings reported here were carried out with a saline-filled polyethylene catheter (0.28 mm×0.061 mm×0.011 mm, 42 cm long) inserted at the subcoxa and body wall junction in A4 of an anesthetized animal and held in place with Vetbond. The catheter was connected to a mineral oil-filled polyethylene tube (0.83 mm×1.6 mm×0.2 mm, 35 cm long) and a solid-state pressure sensor (PX170 or PX40; Omega Engineering Inc., Stamford, CT, USA). In an attempt to increase the resolution of local pressure changes, we also used an implantable solid-state Mikro-Tip sensor (tip diameter 0.47 mm; model SPR 671; Millar Instruments, Houston, TX, USA). This was inserted through a small incision in the cuticle and allowed to seal in place by hemolymph coagulation. Although less sensitive to movement artifacts (‘catheter whip’), recordings from the solid-state sensor were similar to those of the remote catheter sensors. When both sensors were implanted at the same location and the caterpillar squeezed repeatedly, the remote sensor had a negligible response lag (28 ms by cross-correlation). Signals were amplified (Brownlee Precision Co., San Jose, CA, USA) and digitized at 1–10 kHz using WinDaq Software (Dataq Instruments Inc., Akron, OH, USA). The dorsal side of the caterpillar was glued to a flat surface, and proleg movements were captured on videotape using an S-VHS-resolution camcorder (Canon ES-400). The video and pressure recordings were synchronized using a voltage pulse that also triggered an LED in the camera’s field of view. The timing and amount of proleg movement were monitored by measuring the distance between the left and right crochets on each body segment in a single two-dimensonal view using APAS. The phase relationships of proleg movements and pressure changes were estimated from the peak lag calculated by cross-correlation using MatLab (Mathworks, Inc., Natick, MA, USA). Because the sampling frequency of the movement data and pressure records was different, correlations were carried out using a discrete cross-correlation function that employs binning and a ‘z’ transform. This function was kindly provided by Dr E. Ofek, School of Physics & Astronomy, Tel-Aviv University, Israel. Both sensors were calibrated with a head of water, and the values converted to Pa.

Electromyography

Bipolar electrodes were made from 50 μm-diameter Formvar-insulated Nichrome wire (AM Systems). The electrode was inserted through the cuticle into the origin of the principal planta retractor muscle (PPRM) or the ventral internal lateral muscle (VIL) in anesthetized animals on a chilled metal block. A silver ground electrode, 75 μm in diameter, was inserted into the body cavity in the terminal segment. The animal was epoxyed (Pacer Technology, Rancho Cucamonga, CA, USA) to a flat surface and videotaped using a digital video camcorder (Canon ZR10). The EMG signal was amplified (AM Systems, model 1700) and digitized at 10 kHz using WinDaq software. After 30 min of recovery, adduction and retraction were stimulated *via* pin manipulation of the PHs and MHs.

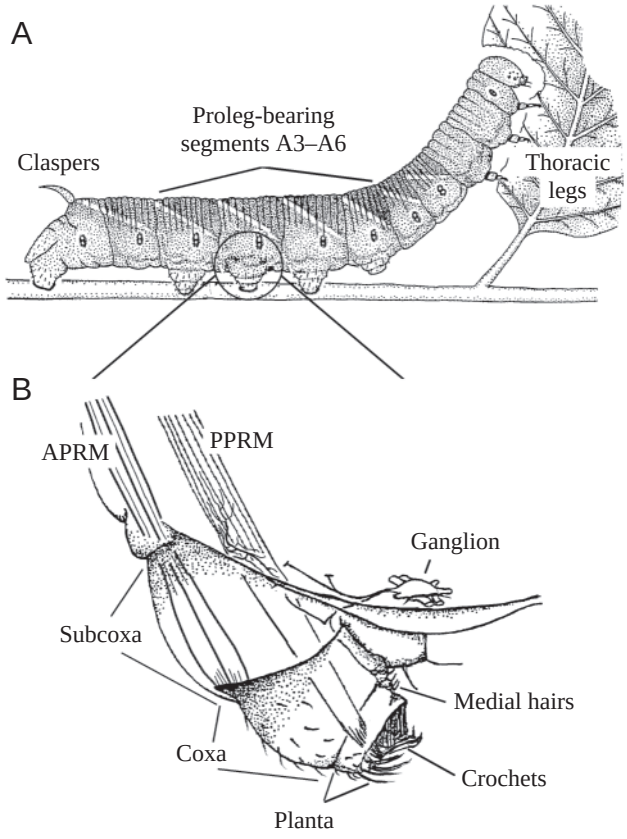


Fig. 1. Anatomy of a *Manduca* proleg. (A) A late 5th instar larva is illustrated from the right-hand side. Prolegs are found on abdominal segments 3–6 (A3–A6). The specialized legs on the most posterior segments are called claspers. (B) A single right-hand side proleg is illustrated in a three-quarter frontal view and is representative of each proleg on body segments A3–A6. The pseudo segments, subcoxa and coxa are named by analogy with segments in the articulated thoracic legs. The tip of the proleg is called the planta and it carries the curved cuticular hooks (crochets) used for gripping. The principal and accessory planta retractor muscles (PPRM and APRM, respectively) have their origin on the lateral body wall near the spiracle (not illustrated) and insert at the planta and coxa–subcoxa boundary, respectively. The medial hairs are located along the inner surface of the proleg.

Results

Anatomy of the proleg

Prolegs are found on abdominal segments 3–6 (A3–A6), and a more specialized pair called the claspers or terminal prolegs are found on the most posterior body segment (Fig. 1A). Unlike the thoracic legs, the prolegs are not obviously segmented. However, early anatomical studies (Snodgrass, 1952) and recent results in molecular development (Suzuki and Palopoli, 2001) suggest that each proleg contains distinct proximal to distal divisions. The most basal region where the proleg joins the ventral body wall will be referred to as the subcoxa (Fig. 1B). The primary compartment of the proleg has been termed the coxapodite and, by homology with the thoracic legs, will be called the coxa. The tip of the proleg (the planta) is lobular and made of relatively soft and opaque cuticle with heavily sclerotized cuticular hooks (crochets) attached to its most distal margin. The crochets curve towards the ventral midline and each is arranged at a slight angle to its neighbor, forming a semi-circular array. Connections between leg divisions are visible as external folding points (see Snodgrass, 1952; Hinton, 1955).

Two main muscles insert into the proleg: the principal planta retractor (PPRM), with its insertion point at the lateral edge of the planta, and the accessory planta retractor (APRM), which inserted more proximally and laterally on the wall of the coxa–planta boundary. Both muscles originate from apodemes high up on the lateral body wall posterior to the spiracle (Weeks and Truman, 1984). During crawling, contraction of PPRM disengages the crochets from the substrate (Belanger et al., 2000). Stronger contraction of PPRM during the proleg withdrawal reflex retracts the crochets further into the inverted

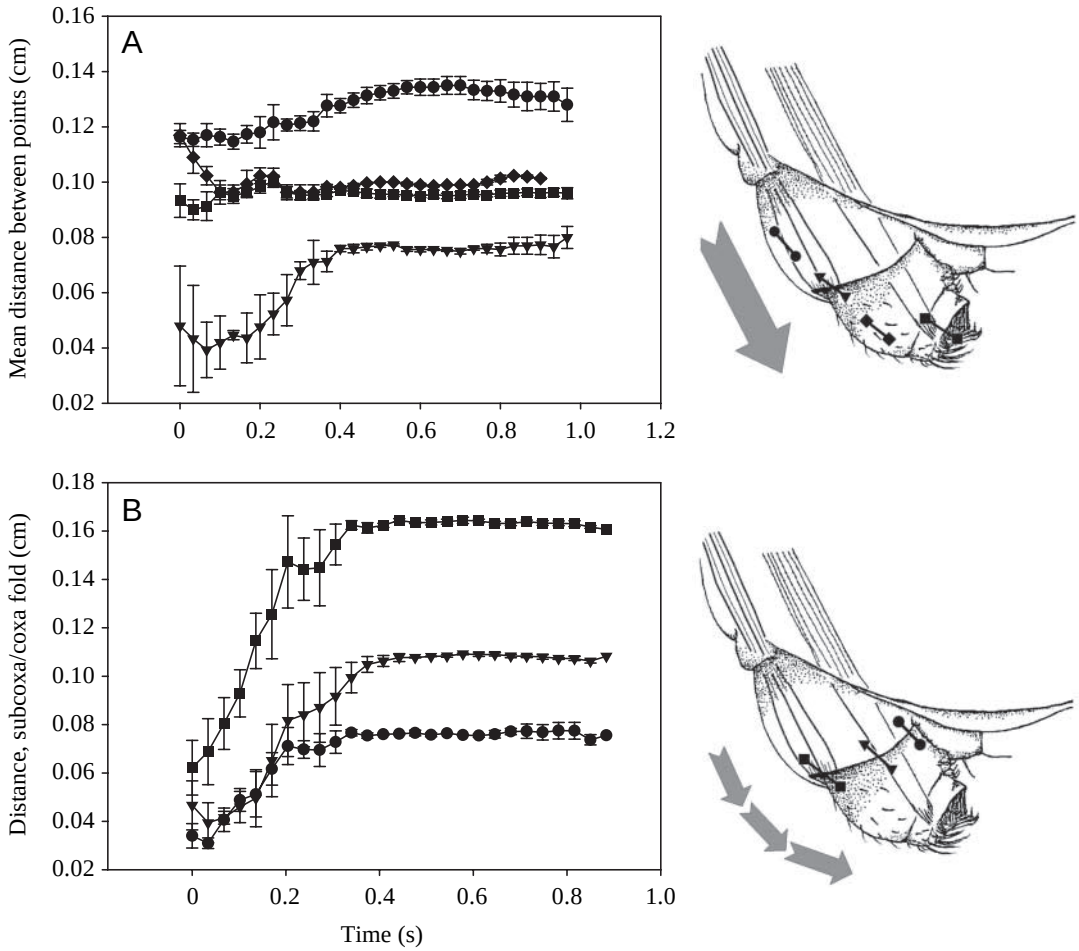


Fig. 2. Kinematics of adduction. (A) Markers were placed to span the cuticle of the subcoxa (circles), coxa (diamonds), planta (squares) and subcoxa–coxa membrane (triangles). These were tracked in three dimensions during adduction and the distance between them calculated to estimate the main site of extension. Most of the total extension results from an increase in the spacing across the subcoxa–coxa membrane with a small contribution from cuticle stretching in the subcoxa. (B) In a related experiment, markers were placed at lateral (squares), central (triangles) and medial (circles) positions spanning the subcoxa–coxal membrane and their spacing tracked during adduction. Both the rate and extent of membrane stretching are greatest at the lateral margin and decrease towards the medial surface causing adduction. In all cases, extension and adduction are coincident. In each figure, the results are the mean length changes (± S.E.M.) from three discrete adductions in one larva. Data from each adduction were aligned relative to the transition point from decreasing to increasing length at the subcoxa–coxal membrane but were otherwise not normalized. Different larvae were used for A and B and each panel is representative of nine adductions in three larvae.

planta region (Weeks and Jacobs, 1987). APRM is also active during crawling and complete proleg withdrawal. A number of small muscles are attached close to the rim of the subcoxa (see Weeks and Ernst-Utzschneider, 1989).

Kinematics of adduction

When the prolegs grasp an object at the ventral midline, the movement consists of a relatively smooth eversion of the leg and its simultaneous adduction. Most of the initial eversion is generated by an increase in the size of the coxal segment, with smaller contributions from the proximal part of the planta. This increase in length is accomplished primarily through an unfolding of the crease between the subcoxa and coxa and a smaller extension of the coxal cuticle itself (Fig. 2A). The relative contribution of these mechanisms is variable and may depend on the hydrated state of the caterpillar and its resting body pressure. Adduction occurs because the lateral coxal wall expands more than the medial wall, thereby rotating the planta medially (Fig. 2B). The final grasping movement involves an eversion of the planta, which inflates medially and fans out into a broad lobe along the rostral–caudal axis. At this point, the crochets appear to erect as they meet at the midline. The whole movement is variable in duration but typically takes between 0.3 and 0.4 s.

Stimulation of adduction

It has been proposed that adduction is elicited when the large identified ventromedial hairs (VMHs) are bent (Levine et al., 1985). We found that careful stimulation of VMHs alone in one body segment [avoiding the nearby medial hairs (MHs)] rarely initiated adduction. Furthermore, removing the VMHs had no detectable effect on crawling, grasping or adduction. Instead, adduction could be evoked more reliably by stroking the MHs on the inside surface of the proleg (see also Peterson and Weeks, 1988). Touching MHs on one proleg generally evoked adduction of both prolegs in a body segment, although a single proleg could be extended on its own. Most commonly, a single touch to MHs in one segment evoked both bilateral and multisegmental adduction that spread anteriorly and posteriorly. These responses could be elicited in segments with the VMH

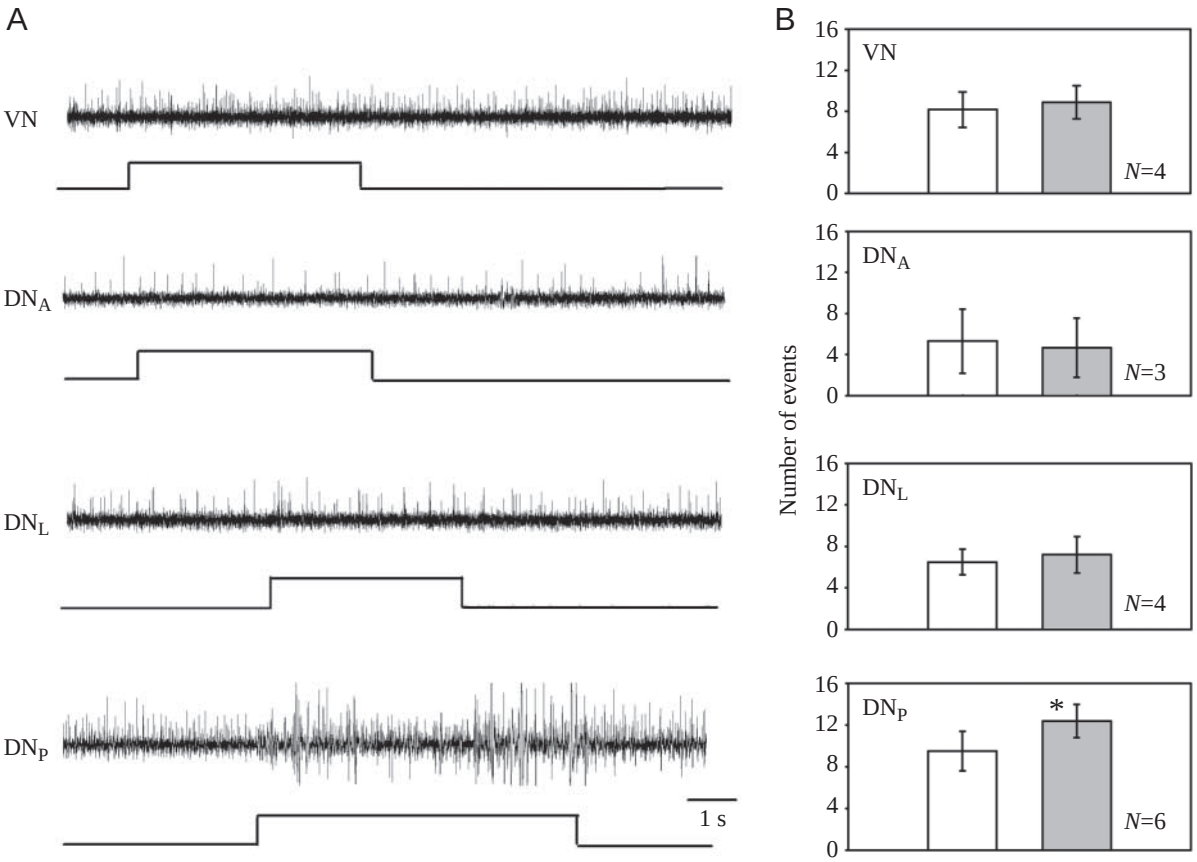


Fig. 3. Identification of medial hair (MH)-activated motor activity. (A) Whole nerve cords from the thoracic to the terminal ganglion were removed along with the attached A4 proleg. Recordings were made from nerves contralateral to the removed proleg. The dorsal nerve (DN) was dissected such that the anterior (DN_A), lateral (DN_L) and posterior (DN_P) branches were distinct, and extracellular recordings were made from each branch. The ventral nerve (VN) was not dissected into separate branches. Representative traces are shown of the spike activity before, during and after MH stimulation, which is indicated by the marker trace below each record. (B) Histograms showing mean responses of each nerve branch to an MH stimulus. The number of distinct events was counted for 1 s before the stimulus (open bars) and for 1 s beginning 1 s after the start of an MH stimulus (shaded bars). Each bar is the mean (± S.E.M.) of 3–6 preparations. Asterisks indicate significant differences (paired *t*-tests, $P<0.05$).

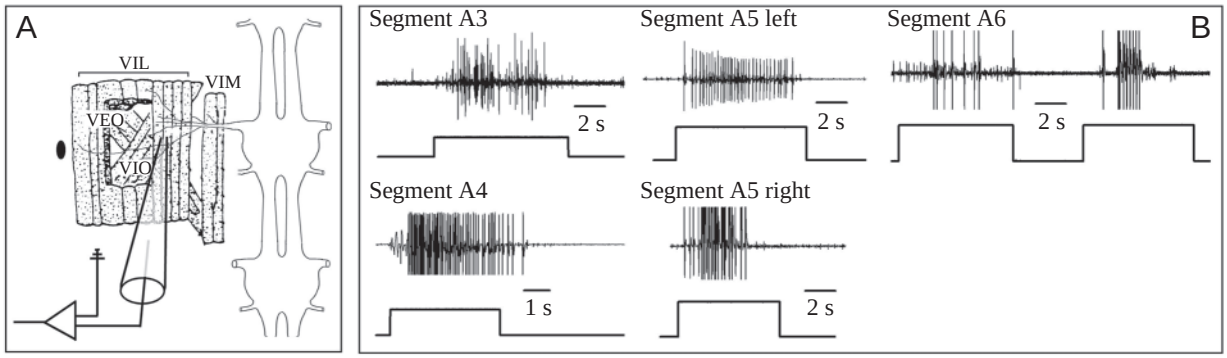
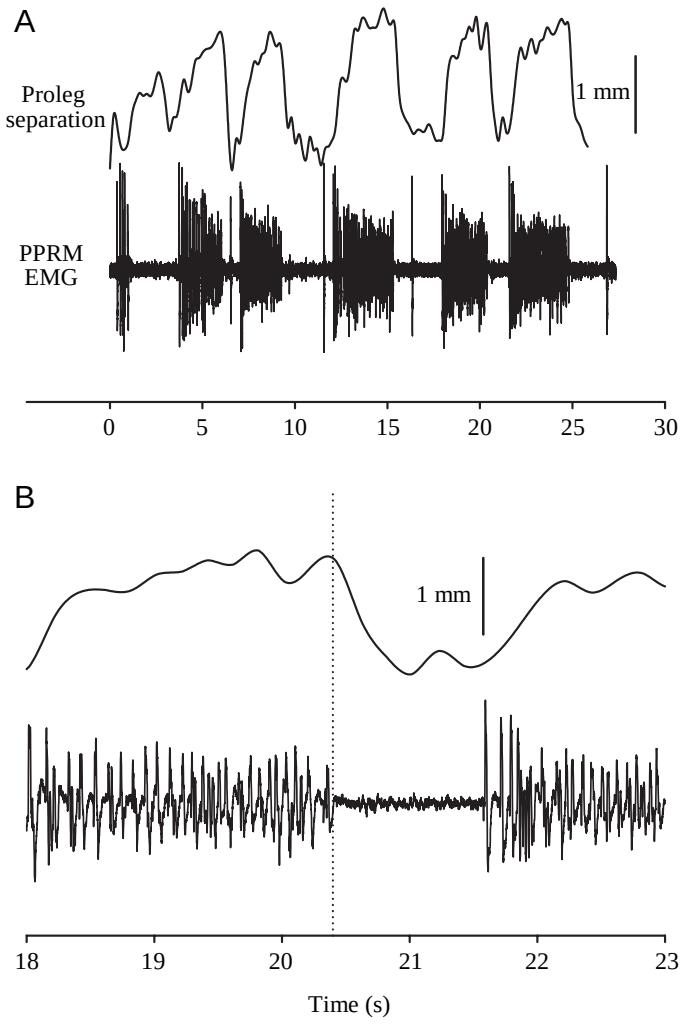


Fig. 4. Medial hair (MH) stimulation excites VIL bilaterally and multisegmentally. (A) The preparation consisted of a whole animal with the gut removed and pinned to display the ventral muscles and nerve cord. A suction electrode was placed on muscle fibers of VIL. For illustrative purposes, the size of the nerve cord has been exaggerated relative to the segmental muscles. (B) Representative traces are shown from extracellular recordings taken from VIL in segments A3 (N=3), A4 (N=10), A5 and A6 (N=3) (left side) and A5 (right side, N=2). Stimulus bars below the recordings show the duration of mechanical MH stimulation on the left side A4 proleg.

removed but not when the MHs were cut (although adduction in these segments was normal when evoked by stimulating MHs in other segments). Adduction can also be stimulated by lightly blowing air over the intact insect, although we have not determined which sensory hairs are involved in this response.



Adduction cannot be elicited reliably in isolated abdomens or in larvae with the abdominal connectives severed anterior to the stimulated proleg. This implies either that essential adduction circuitry is present in anterior ganglia or that adduction is ‘gated’ by descending information. Cutting the connectives between the subesophageal and thoracic ganglia does not prevent adduction (although it eliminates crawling), but cuts between A2 and A3 eliminate adduction in all the prolegs.

Motoneurons involved in adduction

Although adduction requires most of the abdominal and thoracic nerve cord to be intact, MH stimulation will stimulate motoneuron activity in chains of isolated abdominal ganglia and in flatterpillar preparations. In both of these preparations, recordings were made from the ventral nerve (VN) and branches of the dorsal nerve (DN) in different body segments while stimulating MHs on the left proleg in segment A4. MH stimulation had no effect on the anterior and lateral branches of the dorsal nerve, but activity in the ipsilateral VN and the DN_P were both increased by MH stimulation (Fig. 3; P=0.0017, N=4; P=0.019, N=6, respectively). The effect on

Fig. 5. Proleg movements are highly correlated with the electromyogram (EMG) activity of the principal planta retractor muscle (PPRM). (A) Spontaneous proleg movements were monitored in a restrained larva by measuring the separation of the right and left plantas in one segment. Upward deflections are retractions with the peaks representing complete withdrawal and the troughs full adduction. The activity of PPRM on one side of the same segment was monitored with a bipolar electrode inserted into the origin on the body wall. Each retraction is concurrent with a burst of activity in PPR. One exception to this finding (arrow) is shown at the beginning of the trace and this corresponds to the unilateral withdrawal of the opposite leg. (B) An expanded part of the record shown in A to illustrate the start of adduction corresponds to the end of a burst of activity in PPRM. These responses are typical of several hundred cycles of both spontaneous and evoked proleg movements in six preparations.

VN activity was weak and not closely time-locked to the stimulus. By contrast, the increase in DN_P activity was robust and coincident with the duration of the stimulus. Spike analysis (separated by amplitude alone) of DN_P activity suggested that all the MH-evoked activity was accounted for by one or two large amplitude units.

The DN_P in A5 contains the axons of nine motoneurons [VIL neurons 1 and 2, ventral external oblique (VEO) neurons 1 and 2, the ventral internal oblique (VIO) neuron, the ventral internal medial (VIM) neuron, neuron 28, and two unidentified neurons, all with their cell bodies in A4] and one ventral unpaired midline neuron (with its cell body in A5) (Levine and Truman, 1985; Taylor and Truman, 1974). Severing the connective between A4 and A5 abolished MH-evoked activity in the DN of segment A5. This implies that MH sensory input activates one of the descending motoneurons in A4.

Using a flaterpillar preparation, recordings from each of the muscles VIM, VEO, VIO and VIL showed that only VIL is reliably excited by MH stimulation (Fig. 4). The activation of VIL was bilateral, persisted throughout the MH stimulation (mean EJP frequency: spontaneous, 1.10±0.25 Hz; evoked, 5.81±0.54 Hz, N=27) and could be detected in other segments (A3 mean EJP frequency: spontaneous, 3.93±0.55 Hz; evoked, 6.29±0.40 Hz, N=24) (A5 mean EJP frequency: spontaneous, 3.59±0.53 Hz; evoked, 4.67±0.35 Hz; N=14) (A6 mean EJP frequency: spontaneous, 0.94±0.42 Hz; evoked, 4.81±1.29 Hz; N=7). In 22 out of 35 recordings from VIL, the EJPs could be sorted into two amplitude groups that might correspond to the activity of the two VIL motoneurons. The EJP activity was increased by MH stimulation regardless of its amplitude. Although the timing of A4 MH deflection could not be controlled precisely, the responses of VIL in body segments A3–A6 were initiated within 0.5–1.2 s of one another.

Electromyography

During cycles of proleg retraction and adduction, the activity of PPRM was strongly correlated with the prolegs separating and moving away from the midline. Withdrawal movements began immediately at the onset of EMG activity in PPRM, and adduction began precisely when EMG burst activity ceased (Fig. 5). This relationship was consistent in both spontaneous and evoked movements. The activity of ventral muscles (recordings were made close to VIL) was usually coincident with proleg activity (Fig. 6) but there were periods during which EMG bursts in VIL did not correspond to proleg movements and some retractions during which VIL was not active.

Muscle ablation and nerve section

Activity in VIL and other large segmental ventral muscles is implicated in proleg movements, but damage to these muscles in one body segment did not block adduction or retraction (Table 1). The section of the dorsal nerve through which the axon of VIL projects also failed to block adduction responses. Although it would be informative to cut selected branches of the ventral nerve (e.g. VNA_P, which innervates the MHs and VMHs; Trimmer and Weeks, 1993), we have not yet managed to perform this surgery without damaging the cuticle and muscles at the base of the proleg.

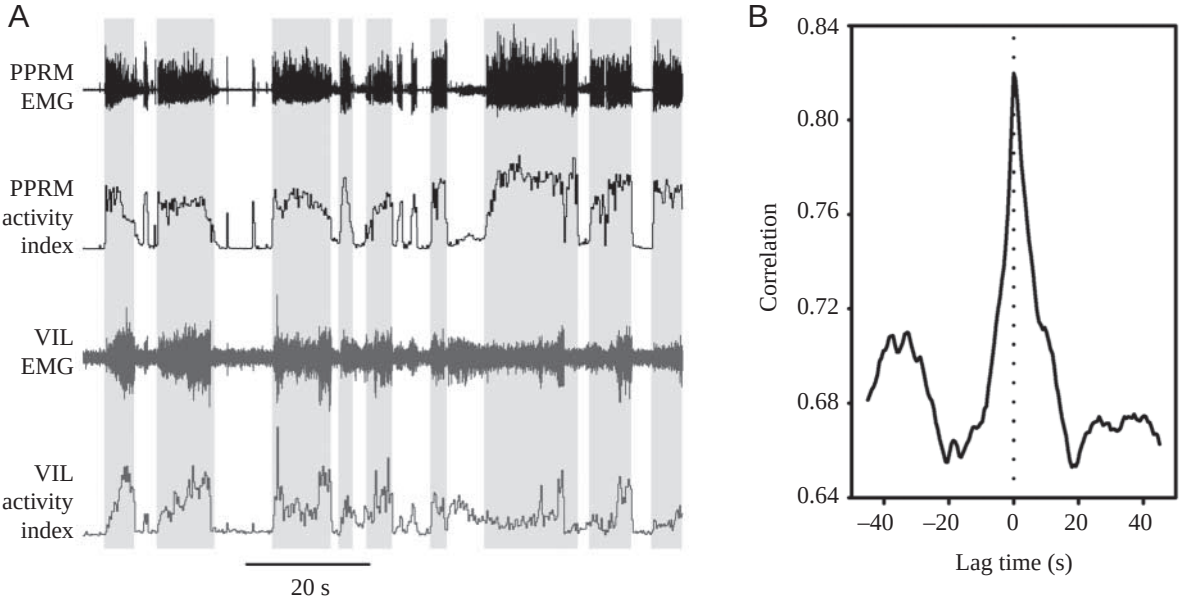


Fig. 6. The activity of the principal planta retractor muscle (PPRM) is highly correlated to that of the ventral muscles. (A) Electromyogram (EMG) recordings from the origin of PPRM and the insertion point of VIL in abdominal segment A5 were made in a restrained larva. Cycles of retraction (gray regions) and adduction were evoked by mechanically stimulating the planta hairs and the medial hairs, respectively. The activity index for each muscle was calculated by demeaning and rectifying the EMG (no smoothing), then integrating the voltage using a 200 ms bin. In most cases, activity in the two muscles was coincident but not identical. (B) A cross-correlation analysis (MatLab, function xcorr) of the activity index for each muscle shows that they are highly correlated with little or no response lag.

Table 1. *Adduction response in animals with muscular and nervous damage*

Lesion	*N*	Survival	Normal adduction
Ventral muscles – unilateral[1]	8	8	8
Ventral muscles – bilateral[2]	4	3	2
Ventral muscles – unilateral and multisegmental[3]	4	3	3
Dorsal nerve cut – unilateral	4	4	4
Dorsal nerve cut – bilateral	3	3	3
Dorsal nerve cut – unilateral and multisegmental	3	3	3

[1]25–90% muscle disruption; [2]60–80% muscle disruption; [3]30–60% muscle disruption. Ventral muscles, including VIL, ventral external oblique (VEO), ventral internal oblique (VIO), ventral internal medial (VIM) and ventral external medial (VEM), in the A4 and A5 segments were damaged between 25% and 90% using fine dissecting scissors. The dorsal nerve in the A5 and A6 segments was severed in separate experimental trials. Animals were left to recover for 24 h before their adduction response was tested. To test for the ability to adduct, animals were placed on their dorsal side, and medial hairs were stimulated with fine forceps. Stimulation occurred at least five times per animal. Normal responses were those in which 80% of medial hair stimulation reliably evoked adduction.

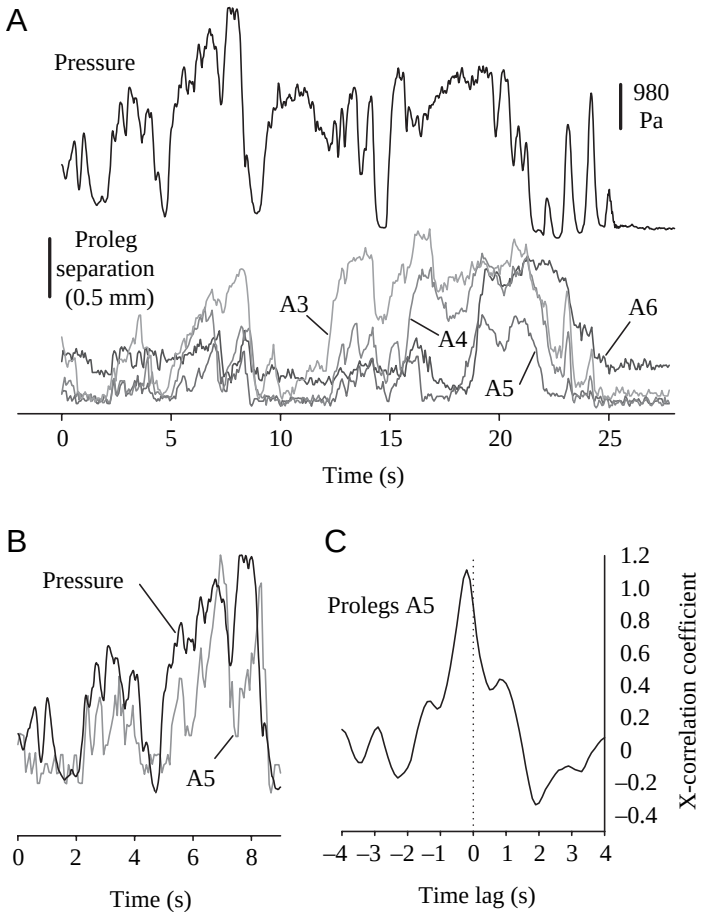


Fig. 7. Body pressure changes in response to adduction and retraction. (A) Pressure changes were measured at the base of the subcoxa in segment A4 (upper trace) while monitoring the separation of the prolegs in all body segments (lower traces). An upward deflection indicates an increase in pressure and retraction of the prolegs. Proleg movements tend to occur concurrently in different body segments. The pressure scale bar corresponds to 980 Pa (10 cmH_2O). (B) The first 9 s of data for the pressure and prolegs in A5 are shown on an expanded scale. The magnitude of the pressure trace has been rescaled to demonstrate the close relationship between pressure changes and proleg movements. (C) A cross-correlation plot of movements and pressure change in A5. A peak at the dotted line would indicate exact coincidence of movement and pressure. Here, the peak lags behind, showing that the pressure pulse precedes the proleg movement by 200 ms. This correlation is very similar for the other prolegs at the start of the recording but the relationship breaks down completely for the rest of the recording (see text).

Pressure recordings

In unrestrained larvae, the internal pressure changes were complex and dominated by large peaks corresponding to gross body movements. Because cycles of proleg retraction and adduction were synchronized to these crawling movements, specific pressure changes related to proleg control could not always be detected. However, in restrained larvae, some pressure changes closely matched overall proleg movements (Fig. 7A). These changes varied between 1500 and 7900 Pa depending on the number of prolegs moving simultaneously. A cross-correlation analysis of these pressure changes with proleg movements showed that proleg retraction was preceded by an increase in pressure with a lead time of 0.1–1 s (Fig. 7B). A drop in pressure usually preceded adduction. In some recordings, the prolegs at the recording site (A4) were glued together to prevent them retracting. When a single pair of prolegs moved in another segment, a small pressure pulse could often be detected preceding the movement.

Discussion

Caterpillars are among the most successful arboreal herbivores and are one of the few groups of soft-bodied terrestrial animals that can climb. This ability is largely attributable to the specialized abdominal prolegs, which grip the surface tightly but can be released quickly during locomotion. The results presented here suggest that gripping is achieved primarily through the arrangement of passive biomechanical elements and muscles that indirectly control adduction.

Movements of the proleg

Because caterpillars do not have a hard skeleton, they cannot use levers to extend or direct the positions of their limbs. It has generally been assumed that prolegs are extended by pressure, but movements of the crochets towards the midline have not been directly examined. The kinematics of adduction in intact caterpillars reveal that extension and adduction are not distinct

from one another but proceed in a smooth movement with no discontinuity in any of the three planes. Adduction occurs through a larger increase in the length of the lateral margin relative to the medial surface, which causes a rotation around the anterior–posterior axis. Most of this extension involves an unfolding of the membrane between the coxa and the subcoxa, with a smaller contribution from expansion of the subcoxal cuticle. This differential stretching probably results from differences in the local cuticle stiffness. However, it is unlikely that the physical properties of the proleg cuticle are sufficient to direct adduction. When pairs of prolegs are removed and mounted on a syringe barrel, they inflate and deflate with changes in saline pressure but they do not adduct in a natural way (N.P. and A.T., unpublished observations). This highlights the importance of maintaining the appropriate cuticle geometry and suggests that either passive tension provided by retractor muscles or active tension in the ventral muscles (see below) is essential for normal gripping.

Neural control

Previous research has shown that activation of the mechanosensory planta hairs can stimulate proleg withdrawal through the activation of PPRM and APRM (Weeks and Jacobs, 1987). This reflex is context-sensitive; it is inhibited during the stance phase of crawling, habituated by repeated stimuli (Weil and Weeks, 1996) and sensitized by noxious stimuli (Walters et al., 2001). Unlike retraction, adduction is not reliably evoked in isolated abdomens or in reduced preparations. Furthermore, because proleg extension is the 'default' state (the prolegs are extended in anesthetized or resting larvae), adduction could be viewed as the cessation of retraction. However, from a behavioral and experimental perspective, adduction is a distinct process. For example, during the swing phase of crawling, the prolegs do not truly retract but instead shorten from a stretched state back to their resting length (Belanger et al., 2000; Belanger and Trimmer, 2000). Hence, adduction during the stance phase cannot be defined as the end of retraction.

Because adduction is very hard to see in freely moving animals, we have used restrained larvae with their ventral surface uppermost. In this situation, gripping can be initiated by placing a small probe along the midline. This stimulus causes the proleg to extend and adduct whether it is fully or partially retracted. As described previously, adduction is stimulated mainly by proleg MHs (Peterson and Weeks, 1988) and we have found that it does not require the VMHs. To try and identify changes in neural activity that accompany adduction, we examined the effects of MH stimulation on different nerves in semi-intact preparations. The strongest and most consistent effect of MH stimulation was the bilateral activation of VIL in all the proleg-bearing segments, which closely matches the recruitment of prolegs during normal adduction. Analysis of VIL EJPs suggests that both motoneurons are activated by MH stimulation. Because neither the MH projections nor the VIL dendritic arbors cross the midline, the activation of contralateral prolegs must involve interneurons. VIL is a large, wide muscle extending from the anterior ventral apodeme to a similar position at the posterior margin of the same segment (Levine and Truman, 1985). The contraction of VIL would be expected to shorten or stiffen a large region of the body wall ventral to the spiracle.

In intact larvae, EMG recordings showed that both evoked and spontaneous adduction occur at the end of a burst of activity in PPRM. As discussed below, the prolegs cannot be extended during strong contractions of PPRM. Because movements of the MHs can initiate adduction, it was expected that MH stimulation would inhibit the proleg retractor motoneurons. However, in semi-intact flaterpillar preparations, activity in the lateral branch of the ventral nerve, which carries these axons, was not reduced but sometimes increased by MH stimulation. This result is consistent with previous findings using isolated proleg/ganglion preparations in which activation of the sensory branches of the ventral nerve (including VNA_P, which innervates the MHs) excited the retractor motoneuron principal planta retractor (Trimmer and Weeks, 1993).

These apparently contradictory findings probably reflect major differences in the way information is processed in intact insects and reduced preparations and they highlight the need for high-resolution EMG recordings in freely moving larvae. Unlike other model systems such as locusts and cockroaches, *Manduca* muscles are not innervated by fast and slow motoneurons, nor do they have common inhibitors. This simplicity should help in the interpretation of EMG activity and in relating it to the role of individual muscles in normal movement.

The role of pressure and muscle activation

Anatomically, *Manduca* differs from both classical hydrostats and muscular hydrostats. Classical hydrostats such as the mollusks *Lingula anatina* and *Donax serra* use fluid-filled appendages to burrow and provide muscular antagonism (Trueman and Brown, 1985; Trueman and Wong, 1987). Likewise, sea anemones are able to modify their size by exploiting the inherent elasticity of their hydrostatic skeleton (Truman, 1992). In muscular hydrostats, the pressure tissues are largely compartmentalized and packed with muscles (Trueman and Clarke, 1988) that can be selectively activated to control local pressure changes (Nishikawa et al., 1999). This type of skeletal system provides support for appendages ranging from squid tentacles to frog tongues and can be used for precise movements (Kier and Curtin, 2002; Nishikawa et al., 1999). *Manduca* can be viewed as a combination of these two hydrostatic systems, with segments functionally partitioned by muscle and with hemolymph that can move between compartments.

The results reported here show that proleg extension occurs when the retractors are inactive and that adduction results from a differential unfolding of the intersegmental membrane on the lateral and medial surfaces. Although this arrangement is simple, there are important aspects that have not previously been noted. First, although it is possible that weak MH

excitation of PPR (Trimmer and Weeks, 1993) could stiffen the medial plane of the proleg and force the leg to adduct, this activity is not evident in restrained larvae. Second, pressure pulses are not used to help extend the proleg. Instead, hemolymph pressure often rises before retraction and usually falls before extension. These fluctuations are probably caused by motor activity in muscles that stiffen the body wall and assist in directing movements. Our physiology recordings suggest that VIL is one of these muscles because it is activated by MH stimulation. However, damage to VIL and other body wall muscles does not prevent adduction so it is clear that local stiffening is not essential for movements of the proleg. By bracing the ventral–lateral wall, the contraction of PPRM and APRM retracts the proleg instead of buckling the upper attachment point.

The increased hemolymph pressure caused by tension in body wall muscles is unlikely to influence retraction. We have found that the prolegs can be retracted fully even when the hemolymph pressure is increased to 10 times its normal level by saline injection. It is also clear that basal body pressure is sufficient to extend the proleg. From Pascal's principal relating force, displacement and area in connected fluid-filled compartments, a shortening of the body will extend the smaller-diameter proleg by a much larger distance. Assuming a body radius of 0.5 cm with the gut occupying 36% of the cross-sectional area (both measurements from magnetic resonance imaging of a 5th instar larva), a proleg radius of 0.125 cm and a constant pressure of 2 kPa, a proleg could be extended by 0.5 cm for a 500 μm shortening of the body. This calculation does not take into account other geometric or pressure changes but it demonstrates that a very small amount of body shortening can account for full proleg extension. Because the proleg radius is about a quarter of that of the body, tension in the limb walls will be about a quarter of that in the body at the same pressure. This probably explains why extension occurs mainly through unfolding of intersegmental membranes. Unless the proleg cuticle is considerably less stiff than the body wall, the normal hydrostatic pressure that maintains turgor will not be sufficient to expand the proleg cuticle itself.

An important aspect that has not been addressed in these studies is the force of adduction and gripping by the prolegs. It is quite likely that restrained and supported larvae do not grip in the same way that freely moving larvae do. Despite the strong MH activation of VIL, the nerve sectioning and muscle ablation experiments imply that adduction does not require active contraction of ventral muscles in restrained larvae. However, we have not yet been able to measure the proleg grip force in normal and surgically altered animals. It is possible that muscles such as VIL are more important when the larva needs to support itself and that they are recruited to generate the normal adduction force. We are currently exploring these possibilities using custom-designed force sensors and multi-site EMG recordings in freely moving larvae.

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