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Report

Visceral-Locomotory Pistoning in Crawling Caterpillars

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Summary

Animals with an open coelom do not fully constrain internal tissues [1–3], and changes in tissue or organ position during body movements cannot be readily discerned from outside of the body. This complicates modeling of soft-bodied locomotion, because it obscures potentially important changes in the center of mass as a result of internal tissue movements [4, 5]. We used phase-contrast synchrotron X-ray imaging [6–10] and transmission light microscopy to directly visualize internal soft-tissue movements in freely crawling caterpillars. Here we report a novel visceral-locomotory piston in crawling *Manduca sexta* larvae, in which the gut slides forward in advance of surrounding tissues. The initiation of gut sliding is synchronous with the start of the terminal prolegs' swing phase, suggesting that the animal's center of mass advances forward during the midabdominal prolegs' stance phase and is therefore decoupled from visible translations of the body. Based on synchrotron X-ray data and transmission light microscopy results, we present evidence for a two-body mechanical system with a nonlinear elastic gut that changes size and translates between the anterior and posterior of the animal. The proposed two-body system—the container and the contained—is unlike any form of legged locomotion previously reported and represents a new feature in our emerging understanding of crawling.

Results and Discussion

We used synchrotron X-rays generated by the Advanced Photon Source at Argonne National Laboratory in a phase-contrast configuration [6–10] to visualize internal tissue movements in crawling hawkmoth caterpillars (*Manduca sexta*, Figure 1A; see also Movie S1 available online). The most prominent internal features were tracheae, gas-filled tubes that supply all tissues in the body with oxygen and vent carbon dioxide to the exterior via spiracular openings in the body wall (Figure 1A). Tracheae appeared most clearly in X-rays because of the large difference in density at the air-to-tissue interface and the edge-enhancement effects of phase-contrast imaging [6], making them reliable internal markers (Figures 1B and 1C). Many tracheae connect to muscles that insert into the body wall, whereas others connect directly to the gut (Figure S1). This arrangement of trachea has also been noted in caterpillars of other species with a comparatively transparent body wall [11–13]. By simultaneously tracking these different trachea, we could determine the timing of movements of internal tissues during crawling.

We quantified the relative timing of gut, body wall, and proleg movements during individual crawls using synchronized X-ray and visible light videos (Movie S2). Each crawl began with a step by the terminal prolegs (TP) (Figure 1A), followed by an anterograde wave of overlapping contractions and abdominal proleg steps in successive segments (Movie S1) [14, 15]. Remarkably, at the start of each crawl, the gut in midbody segments moved in advance of the body wall and prior to the proleg swing phases. This can be seen in the sixth abdominal segment (A6), where the gut-associated tracheae moved out of phase with the A6 proleg and body wall (Figure 1D). This difference was even more pronounced in the more anterior fourth abdominal segment (A4), where the delay between gut procession and body wall movement was as long as 1 s in a 4 s crawl cycle (Figure 1E). Thus, the midgut typically advanced an entire step forward before the body wall caught up with the gut at the start of the next abdominal proleg stance phase. Because the visible-light videos included the entire body of the animal, we were able to determine that these locally decoupled gut movements were synchronous with movements of the terminal segment (Figures 1D and 1E).

To confirm these results throughout the entire gut and to better establish the source of these gut movements, we used transmission light microscopy to analyze gut movements in *Manduca* hatchlings, which are translucent and crawl similarly to later instars (Figure 2A). By tracking internal or external markers relative to reference points at each end of the animal (Figure 2B), we could correlate their movements to those of the gut and body wall (e.g., Figures 2C and 2D, respectively). The movement profiles of the internal and external markers differed significantly (Table 1). Internal marker movements throughout the body correlated positively with those of the TP and head (Rayleigh's test for uniformity: correlation with head movements, $\overline{B} = 31.89^\circ \pm 2.87^\circ$, $n = 19$, $R = 18.59$, $p < 0.0005$; correlation with TP movements, $\overline{B} = 19.94^\circ \pm 3.81^\circ$, $n = 19$, $R = 18.30$, $p < 0.0005$). In contrast, external body wall movements did not correlate positively with those of the terminal prolegs and even correlated negatively with terminal proleg movements during TP stance phase, because the crawl wave propelling the external markers moved faster than either reference point (Table 1).

Although correlations of gut markers with the reference points were positive, they were not constant throughout the body but were instead position dependent (Figure 2E). Generally, posterior gut markers correlated more strongly with terminal proleg movements than did anterior gut markers (linear regression, $\beta' = -32.5^\circ$/body length (BL), $R^2 = 0.378$, $F_{17,1} = 10.335$, $p = 0.005$), and anterior gut markers correlated more strongly with head movements than did more posterior gut markers (linear regression, $\beta' = 24.7^\circ$/BL, $R^2 = 0.381$, $F_{17,1} = 10.447$, $p < 0.005$). These correlations indicate that the gut

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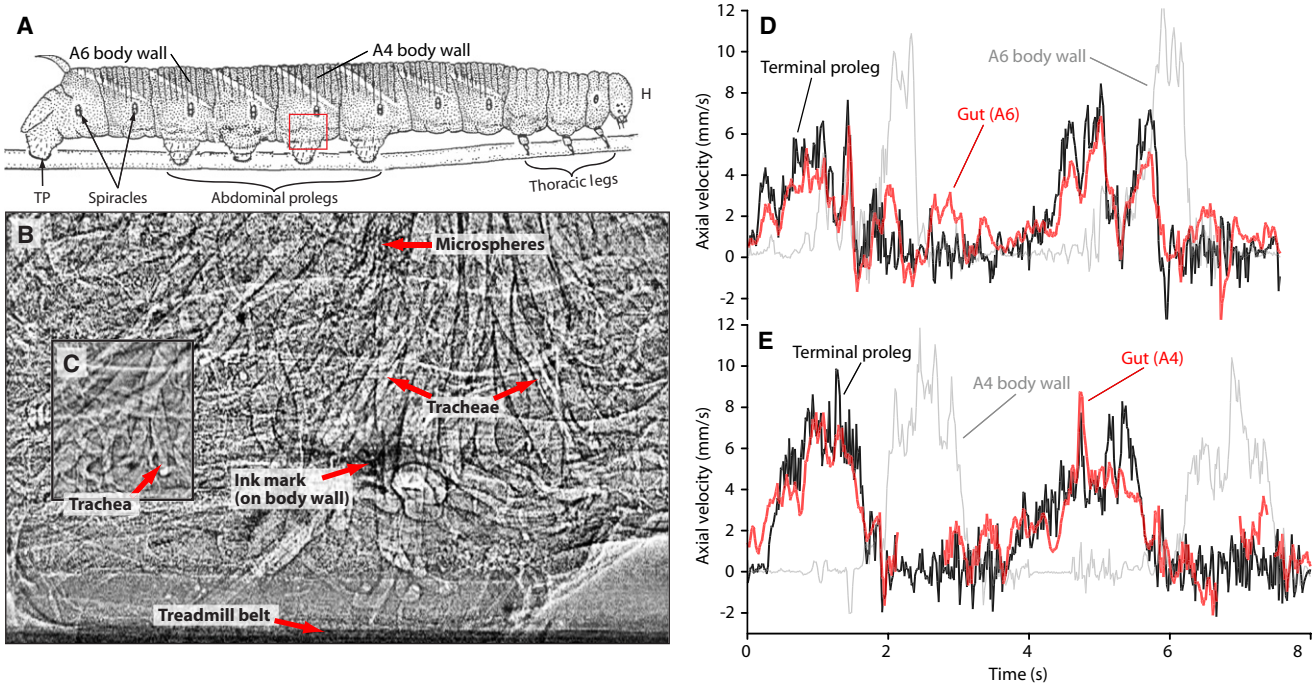

Figure 1. The Gut Moves out of Phase with the Surrounding Body Wall and Prolegs but Moves Simultaneously with Forward Movement of the Terminal Prologs

(A) Major external and internal features of *Manduca sexta*. The body is divided into the head (H), three thoracic segments (T1–T3), seven midbody abdominal segments (A1–A7), and the most posterior, terminal segment (TS). Segments A3–A6 each have a pair of prolegs. TS is specialized compared to the other abdominal segments, with different musculature and its own terminal (or anal) prolegs (TP). Most X-ray imaging was carried out on the prolegs and segments labeled A3 though TS. The boxed region is shown in the contrast-enhanced X-ray image in (B). See also Movie S1 for an example of animal crawling.

(B) This image of a region of the fourth abdominal segment of a fifth-instar larva was taken from a video sequence during crawling. The prominent tubular structures are tracheae. Also visible are folds in the body wall and experimentally applied contrast agents such as microspheres (injected into the hemocoel) and opaque ink (applied to the outside body wall). See also Figure S1.

(C) The inset shows a region of the X-ray image after frame averaging to reveal tracheal locations more clearly. For video processing details, see the Supplemental Experimental Procedures.

(D and E) Gut movements from the perspective of the sixth abdominal segment (A6, D) and the fourth abdominal segment (A4, E). The axial velocity of the gut, terminal proleg, and local body segments (A6 and A4, respectively) were tracked throughout a crawl. Gut movements are shown as a heavy red line, and those of the terminal proleg are shown as a black line. The local body wall velocity is traced as a gray line. The gut consistently moved forward at the start of a crawl cycle, concomitantly with terminal proleg movement but out of phase with the local body wall and proleg movement. Gaps in gut velocity tracking were due to the occasional disappearance of tracking points. See also Movie S2 for an example of X-ray imaging.

was differentially displaced throughout the body, effectively shortening and lengthening throughout the duration of each crawl cycle.

This is, to our knowledge, the first report of an animal locomotory system with decoupled viscera. Although internal tissue movements caused by locomotion have been identified in many organisms, including mammals and birds [5, 16, 17], the unusual phenomenon of visceral-locomotory pistoning that we describe here is not generated by cyclic inertial forces from the locomotion itself, as in previous reports. In fact, most caterpillars move so slowly that they can stop and restart during any part of their crawl cycle without major changes in the subsequent kinematics (i.e., they are quasistatic) [15, 18]. Rather, the unique phenomenon of gut sliding is made possible by the convergence of several factors: (1) *Manduca sexta* crawl axially, such that longitudinal movement of organs is in the direction of motion, (2) *Manduca sexta* are not septate or otherwise divided into separate compartments, so fluid and tissues can be displaced from one body segment to another, and (3) caterpillars are highly deformable and lack an axial skeleton. In animals with a stiff skeleton, organ movement can be constrained by anchoring soft tissues directly or indirectly to nearby rigid structures. In contrast, the larval gut is a tube—functionally divided into a crop, midgut, and hindgut—suspended between the mouth and rectum [2]. The midgut of a continuously feeding caterpillar is typically full, with the crop acting as a temporary storage area for food matter before digestion in the midgut. Therefore, the full crop and midgut represent a mostly solid mass that is relatively free to slide, shorten, and lengthen axially.

The movements of the hatchling gut are correlated with those of the anterior and posterior parts of the animal rather than the body wall, validating X-ray evidence for a gut decoupled from the body wall. Additionally, the position dependence of marker correlation offers a clue to the mechanical properties of this system. Because points within the gut move at different rates relative to each other, we hypothesize that the gut behaves mechanically as a nonlinear elastic structure that translates, shortens, and lengthens within the confines of the body, moved by forces exerted from the anterior and posterior ends of the animal. In light of the presence and timing of these gut movements, we propose a new mechanism for crawling in *Manduca sexta* (Figure 3A). The gut can be described as a cylindrical, nonlinear elastic mass that extends from the terminal segment to the head. The caterpillar body wall and musculature constitute a soft shell around the

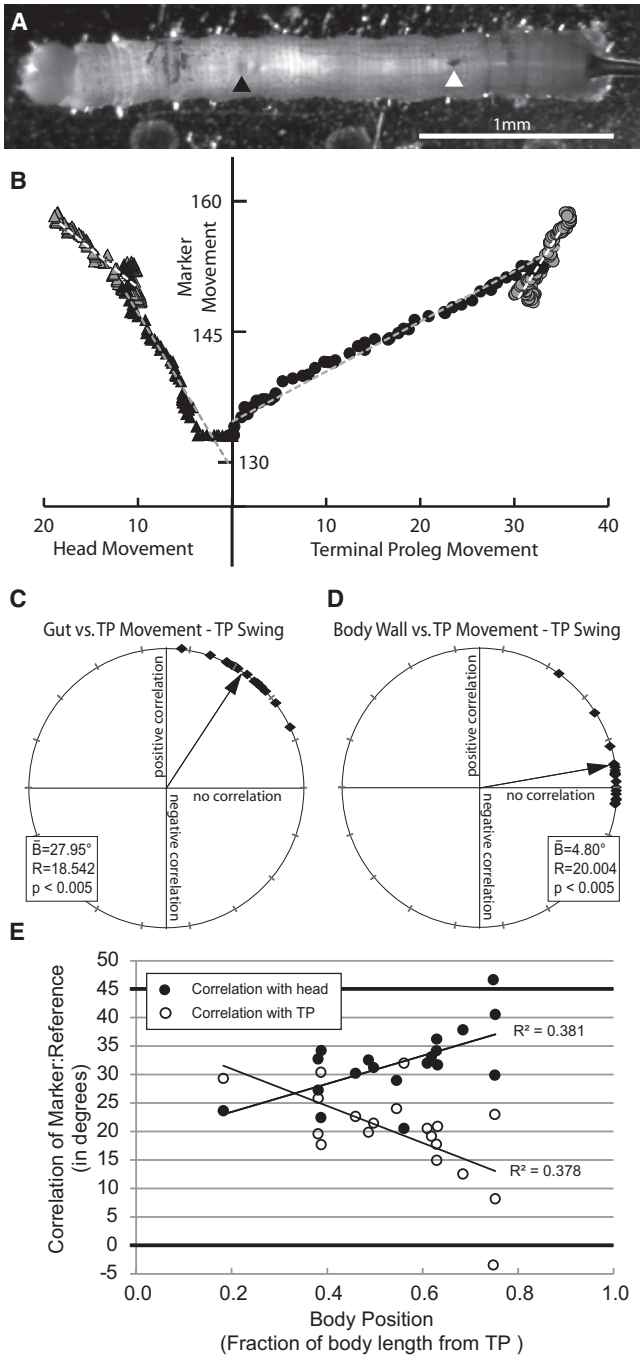

Figure 2. Gut Movements in Crawling First-Instar *Manduca sexta*, as Measured by Transmission Light Microscopy

(A) First-instar *Manduca sexta* first-day hatchlings were placed on a microscope slide cover printed with regularly spaced black dots and allowed to crawl freely. Points on the interior (black arrowhead) and exterior (white arrowhead) of the animal's body were tracked using kinematic software (see Supplemental Experimental Procedures).

(B) In this representative example, marker movements were regressed against TP movements (circles) and head movements (triangles) throughout the entire crawl or during each of the TP swing (black markers) and stance (gray markers) phases. Linear regression results for separate swing and stance phase analysis are shown here as dashed lines.

(C) The aggregated regression coefficients (transformed to the circular variable B; see Supplemental Experimental Procedures for details) were tested for dispersion by Rayleigh's R [40]. Representative example of internal markers during TP swing phase, n = 19.

(D) Like (C), but with external markers during TP swing phase, n = 19. All aggregated results and statistical analyses are summarized in Table 1.

(E)Correlation of hatchling gut movement with movement of head and terminal prolegs is body-position dependent. Correlation was determined by regressing gut marker displacement throughout the duration of a crawl against that of a reference point, either the TP or the head (See Body-Position Dependence in the Supplemental Experimental Procedures). Heavy horizontal lines emphasize correlation values of 0° (no interaction) and 45° (perfect positive correlation). Each circle represents one gut marker tracked throughout a single crawl: filled circles show correlation with the head, and empty circles show correlation with the terminal prolegs. n = 19 markers in 10 animals.

gut, the cuticle resembling a woven tube [19, 20] and the musculature resembling a complicated, repeating series of primarily longitudinal and oblique muscles [21–23]. Within the crawling caterpillar's body wall, the gut shortens and slides forward during the terminal proleg swing phase, resulting in a shift forward of the animal's center of mass (CoM). The combined mass of the gut and terminal segment constitutes a large proportion of the animal's total mass—the full midgut alone represents one-third of a new fifth-instar larva's total mass—so this CoM shift is substantially greater than would be expected from external observation. Because the terminal proleg swing phase is advanced relative to the midabdominal segments, this movement by the CoM occurs before the rest of the body is shifted forward, an important distinction from general biomechanical templates (such as the SLIP model [24]) applicable to legged locomotion in a variety of animals [25, 26]. Rather, these results support a template in which a deformable gut is nonelastically connected to the front and back of the animal and effectively disconnected from much of the remaining propulsive musculature (Figure 3B). This caterpillar is a two-body system—the container and the contained—in which propulsion of the internal tissues is decoupled from grip and propulsion of the body wall [18, 27], contributing to the considerable freedom of movement observed in these soft-bodied crawlers.

Our discovery that the caterpillar midgut moves between body segments during crawling prompts a new question: Is this visceral pistoning merely a by-product of the forces generated during crawling, or does it also confer an evolutionary advantage to the caterpillar? Previous research on inertial visceral movements in running vertebrates suggested a respiratory advantage to synchronizing breathing and tissue movements [4, 5]. Animals demonstrating such timing are not limited in locomotory performance by restricted respiratory function during vigorous activity, as are many reptiles [16]. In addition, arm swinging by walking humans, a repeated motion coupled through elastic linkages (i.e., the trunk and shoulders), increases stability by dampening torso and head rotation [28], stimulates locomotory activity through recruitment of lower-limb motor units [29], and reduces the overall metabolic cost of walking [30]. A similar advantage may be conferred to the caterpillar by gut sliding. Because the larval stage of *Manduca* is focused on eating and growing, its body should be structured to permit rapid growth and uninterrupted digestion. By freeing the gut from local perturbations caused by the locomotory compressive wave of crawling, visceral-locomotory pistoning may facilitate a wider expression of movements and behaviors while minimizing mechanical impacts on the digestive system (for example, anterograde crawling waves interacting or interfering with the retrograde peristalsis of digestion [31]).

Table 1. Correlation of Marker Movements with Head and Terminal Proleg Position

			Correlation (in Degrees)					Watson-Williams Test		
	TP Phase	Marker Location	Mean	95% CI	n	Rayleigh's R	p Value	df	F	p Value
Correlation with TP	All	Internal	19.94	3.81	19	18.30	<0.00005	32	27.066	<0.00005
		External	−9.86	12.44	15	11.20	0.0001			
	Swing	Internal	23.30	3.69	17	16.47	<0.00005	30	96.708	<0.00005
		External	−12.77	7.04	15	13.68	<0.00005			
	Stance	Internal	21.59	5.31	16	15.08	<0.00005	31	44.774	<0.00005
		External	−33.27	15.23	17	10.45	0.0010			
Correlation with head	All	Internal	31.89	2.87	19	18.59	<0.00005	36	20.526	0.0001
		External	45.32	8.80	19	17.61	<0.00005			
	Swing	Internal	31.53	3.22	18	17.54	<0.00005	37	67.705	<0.00005
		External	48.69	2.86	21	20.50	<0.00005			
	Stance	Internal	32.12	4.25	19	18.14	<0.00005	38	22.058	<0.00005
		External	52.94	8.60	21	17.67	<0.00005			

Interactions are expressed as degrees of correlation between internal marks and either the terminal prolegs (TP) or the head, where a correlation of 0° = no interaction and 45° = perfect positive correlation (see Aggregating Sample Interactions in the Supplemental Experimental Procedures), with relative motion determined throughout the entire step or during just the TP swing or stance phases. Mean degree of correlation is expressed with 95% confidence intervals (CI), and significance of the mean is tested using Rayleigh's R [40]. Internal and external points are compared separately using the Watson-Williams test for two circular samples to demonstrate differences in interaction with the body. df denotes degrees of freedom.

Regardless of whether an evolutionary advantage exists, the presence of gut sliding during crawling means that the material properties of the gut, and especially its elasticity and response to compressive and tensile forces, may be important features of caterpillar biomechanics and control of locomotion. The presence of these gut movements in hatchlings and large fifth-instar larvae suggests that size is not limiting for visceral-locomotory pistoning. We predict that this mechanism is applicable to caterpillars with different body morphologies [32–34] and that similar internal tissue movements may be present in other soft-bodied organisms with an open coelem, such as the leech and some oligochaetes [35], but not in highly muscularized structures, such as the elephant trunk or human tongue [36]. These findings also have significance beyond animal locomotion, because they are already contributing to the design and development of deployable, maneuverable, and orientation-independent soft material robots [37]. Given the ubiquity of studies on the biomechanics of the skeleton and of soft tissues anchored to it in research on mammalian (and, more specifically, human) gait and posture, these findings may prompt a reexamination of the potential role of soft tissues in the biomechanical performance of animals with stiff skeletons.

Experimental Procedures

Animals

Larvae of the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae, L.) from a colony at Tufts University were raised in a 17 hr:7 hr light:dark cycle at 27°C on an artificial diet [38].

Synchrotron X-Ray Visualization

Caterpillars at all developmental stages were transported by commercial jet to Argonne National Laboratory. The X-ray source was the Advanced Photon Source, beamline 32-ID-C, at Argonne National Laboratory, as described previously [6, 7]. A scintillator positioned 80 cm behind the crawling caterpillars converted X-rays to visible light, which was imaged onto a Cohu 2700 camera via a Mitutoyo 2× microscope objective and tube lens. X-ray video data were recorded on miniDV digital tape with an image size of 720 × 480 pixels in 8-bit monochrome at 29.97 frames per second, and video data from standard cameras were recorded on site on recordable DVDs in MPEG-2 format at 29.97 frames per second.

Video processing was performed using VirtualDub video editing software (http://www.virtualdub.org/ by A. Lee). Because of poor visual contrast in digitized recordings, we enhanced all X-ray videos with a windowed histogram equalizer filter (http://www.neuron2.net/ by D. Graft). Only scenes of continuous, straight-line crawling by fifth-instar larvae were used for kinematic and temporal analysis. Representative crawls were analyzed using kinematic software, either with Ariel Performance Analysis System (Ariel Dynamics) or DLTdv3 (T.L. Hedrick, University of North Carolina at Chapel Hill) [39].

Hatchling Analysis

Male and female first-day hatchlings selected for translucence crawled on a glass microscope slide and glass coverslip printed with regularly spaced dots. Images were magnified by dissection microscope, recorded by video camera, and then captured to computer. Videos were screened for bouts of straight-line crawling. Individual crawls that were at neither the beginning nor the end of a bout of crawling were randomly chosen for further analysis.

Hatchling movements were digitized using DLTdv3 [39]. For each crawl, at least one but no more than three internal points contrasting with the transmitted light were identified within the body and tracked. At least one but no more than two marks on the external surface of the abdomen of the animal were tracked as controls, as were reference points at the tip of the head and the TP. The axial positions of the head and TP were used as reference points for body position, and the initial position of TP was selected as the origin of the system. To determine relative gut movement, we tracked marker progress relative to TP progress and head progress. Regressing either one against animal length resulted in a coefficient (β) representing the ratio of marker progress to reference progress, with positive correlation at $\beta = 1$ and no correlation at $\beta = 0$.

To aggregate interaction coefficients, we analyzed the random circular variable B, where $B = \tan^{-1}(\beta)$ with range $B \in [-\pi, \pi]$. Dispersion of B and significance of the mean $\bar{B}$ were tested using Rayleigh's R, and aggregate differences between samples were tested by the Watson-Williams test for distance between circular samples [40]. To determine body-position dependence, we regressed marker/reference interaction coefficients (β) against relative marker position within the body. The resulting regression coefficient (β') represented the change in marker/reference interaction over the length of the animal (i.e., β/BL).

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, one figure, and three movies and can be found with this article online at doi:10.1016/j.cub.2010.06.059.

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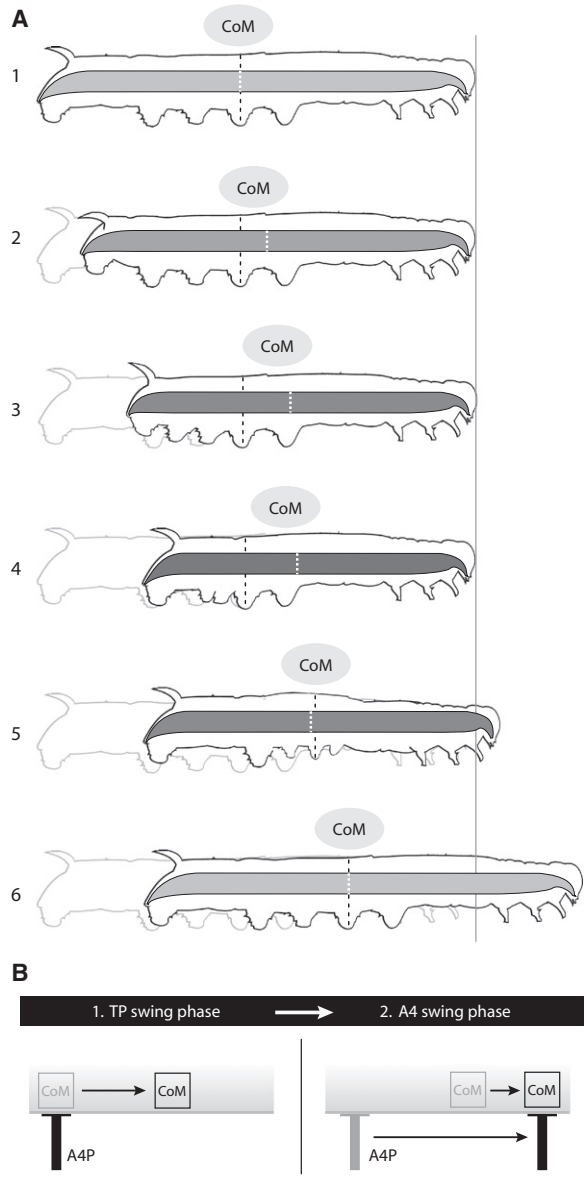

Figure 3. Proposed Mechanism of Gut Sliding in Crawling Caterpillars

(A) The light outline represents the initial position of the *Manduca* at the start of a crawl, and the black dashed line represents the position of the fourth abdominal segment (A4) at various points during a crawl. (1) The *Manduca* center of mass (CoM), estimated from the external dimensions, is initially close to segment A4 and the resting A4 segment of the gut (marked with a white dashed line throughout). (2) As the caterpillar picks up its TP, the gut shortens and slides forward, causing the CoM to move forward. (3) This forward gut movement and accompanying CoM translation continues during the sixth and fifth abdominal proleg swing phases, whereas A4 remains stationary. (4) At the end of the terminal proleg swing phase, the initial A4 region of the gut and the CoM have moved anterior to A4. (5) During the A4 proleg swing phase, the CoM again advances as the body wall catches up with the displaced gut. (6) By the end of the third abdominal proleg swing phase, the gut has returned to its original position within the body, as has the CoM.

(B) A simple biomechanical template of this process. At left, a hypothetical CoM moves anteriorly over the stationary fourth abdominal segment prolegs (A4P) during the TP swing phase. During the A4 proleg swing phase, illustrated at right, the CoM continues to shift as the A4 proleg returns to its original position relative to the gut.

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