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RESEARCH ARTICLE

Stepping pattern changes in the caterpillar *Manduca sexta*: the effects of orientation and substrate

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ABSTRACT

Most animals can successfully travel across cluttered, uneven environments and cope with enormous changes in surface friction, deformability and stability. However, the mechanisms used to achieve such remarkable adaptability and robustness are not fully understood. Even more limited is the understanding of how soft, deformable animals such as tobacco hornworm *Manduca sexta* (caterpillars) can control their movements as they navigate surfaces that have varying stiffness and are oriented at different angles. To fill this gap, we analyzed the stepping patterns of caterpillars crawling on two different types of substrate (stiff and soft) and in three different orientations (horizontal and upward/downward vertical). Our results show that caterpillars adopt different stepping patterns (i.e. different sequences of transition between the swing and stance phases of prolegs in different body segments) based on substrate stiffness and orientation. These changes in stepping pattern occur more frequently in the upward vertical orientation. The results of this study suggest that caterpillars can detect differences in the material properties of the substrate on which they crawl and adjust their behavior to match those properties.

KEY WORDS: Gait, Proleg, Locomotion, Climbing, Motor control

INTRODUCTION

Most legged terrestrial animals change their gait (i.e. stepping pattern) to accommodate different substrates, carrying loads, speeds and inclines. For articulated animals such as vertebrates and adult insects, changes in gait (e.g. from walking to running or trotting) can be identified by looking at the sequence and timing of leg movements or the force profiles generated as the legs contact the ground (Alexander and Jayes, 1983, 1980). Stepping patterns often change in response to sensory feedback from interactions with the environment, as such feedback alters the underlying motor coordination. For example, when humans detect a large change in contact friction by stepping on oil or ice, they quickly adjust their posture, stance duration and push-off forces (Redfern et al., 2001; Weerdesteyn et al., 2018). In a similar way, animals alter their gait in response to changes in the stiffness (Ferris et al., 1999) or tendency to yield of the substrate on which they move as, for example, when running on sand (Li et al., 2013).

These adaptive locomotive mechanisms are particularly well understood in terrestrial animals with a stiff, articulated skeleton (Cruse et al., 2004; Dürr et al., 2017). Soft, deformable animals face very different biomechanical challenges compared with articulated animals and, thus, adopt substantially different locomotion strategies. Experimentally, in soft animals, the absence of discrete contact points with the substrate makes it very challenging to measure ground reaction forces or identify changes in the timing of ground interactions (Trueman, 1975). Therefore, gait switching in soft animals can only be identified by large-scale alterations in the coordination of body movements. For example, changes in the undulation pattern of annelids (e.g. *Caenorhabditis elegans*) when moving from aquatic to more solid environments have been described as separate gaits (Gray and Lissmann, 1964), although they might also be considered as different modes of locomotion (swimming versus crawling).

Locomotion in soft insects has been extensively studied in larval Diptera (Berrigan and Pepin, 1995; Berrigan and Lighton, 1993; Heckscher et al., 2012) but there is no direct evidence of gait adaptations in response to different substrates. Indeed, most studies involving *Drosophila* have leveraged the animal's genetic tractability to analyze the neural circuits controlling body movements and sensing (Clark et al., 2018; Kohsaka et al., 2017). One study reported escape responses such as rearing and rolling (Ohyama et al., 2015), while another found that *Drosophila* explores the substrate by moving forward and backward and by changing its tendency to turn (Berni, 2015). *Drosophila*'s crawling speed and inter-segmental delay were also found to be influenced by mechanosensing neurons (Caldwell et al., 2003; Hughes and Thomas, 2007; Song et al., 2007) and external temperature (Bellemer, 2015). Nevertheless, very limited information is available on how *Drosophila* adapts its crawling to different substrates or orientations. In one study, mass behavior screening methods (Günther et al., 2016) correlated the genetic background, age and substrate to the overall speed of larval locomotion (Del Pino et al., 2012), but the substrate was found to have little effect on a wide range of locomotion parameters (Aleman-Meza et al., 2015). As such, although many of the neurons involved in coordinating crawling have been identified – and the functional connections between them characterized – gait adaptation in response to varying substrate properties has yet to be conclusively established in *Drosophila*.

In contrast to the peristaltic movements of annelids and the burrowing movements of *Drosophila*, caterpillars and many soft larval insects need to negotiate complex three-dimensional structures, such as when climbing plants. As a result, to grip firmly onto leaves and stems, and yet still be capable of moving from place to place, these larvae have developed additional leg-like structures (prolegs) on their abdomen. These abdominal legs are found in major holometabolous insect orders such as Coleoptera (beetles), some Hymenoptera (e.g. sawfly larvae) and most Lepidoptera (caterpillars, the larval stages of moths and butterflies) (Hinton, 1955). Locomotion in caterpillars is generally

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described as either inching or crawling (van Griethuijsen and Trimmer, 2014): inching is characterized by sequences of long steps, often involving the whole body, in which anterior and posterior contact points alternate their swing phase (Trimmer and Lin, 2014); in contrast, crawling is characterized by a forward-moving abdominal wave (posterior-to-anterior movement of successive body segments that shorten and typically lift upward) that progresses as the prolegs successively release their grip, move through a swing phase to a new forward position, and then re-attach to the substrate.

Of the two locomotion modalities, crawling is believed to be the ancestral mode of caterpillar locomotion, while inching is believed to have evolved from crawling in some caterpillar species along with a reduction in the number or function of mid-body prolegs (Manton, 1952; Nagy and Grbić, 1999). As crawling caterpillars move in complex three-dimensional environments such as plants and trees, the wide range of biomechanical challenges they are presented with are unlikely to be addressed by a single gait or stepping pattern. However, the variety of crawling patterns used by caterpillars and the conditions that lead to changes in gait have not been examined in previous studies (Belanger and Trimmer, 2000; Brackenbury, 1996, 1997, 1999; Casey, 1991; Lin and Trimmer, 2010b; Snodgrass, 1961; van Griethuijsen and Trimmer, 2009, 2010, 2014; Vaughan et al., 2018). Accordingly, this study aimed to identify and evaluate changes in the sequence of proleg movements of caterpillars crawling in different orientations and on substrates of different stiffness.

MATERIALS AND METHODS

Animals

Experiments were done on 5th instar *Manduca sexta* (Linnaeus 1763) caterpillars from a colony at Tufts University, Medford, MA, USA. The caterpillars were reared on an artificial diet on a fixed 17 h:7 h light:dark cycle at 27°C (Bell and Joachim, 1978).

Stepping pattern analysis

A treadmill (Fig. 1A) was 3D printed using a fused deposition modeling printer (Dimension 1200, Stratasys) and mounted so that the crawling substrate (i.e. the treadmill belt) could be rotated by any angle (Fig. 1B). The effect of orientation on crawling was tested at three different angles: (1) horizontal, i.e. 0 deg rotation (Fig. 1A), (2) upward vertical, i.e. 90 deg rotation; and (3) downward vertical, i.e. −90 deg rotation (Fig. 1B).

The treadmill was rotated only during crawling to maintain the position of the crawling animals at the center of the treadmill belt. Exceedingly slow/fast crawls were discarded as studying the effect of speed on stepping patterns was not one of the goals of the current paper.

To test the effect of substrate stiffness on gait, two treadmill belts (both 5 mm in diameter) were used: a custom-made belt made of platinum cure liquid silicone (Dragon Skin® 20) and an off-the-shelf belt made of nitrile rubber (Buna-N O-ring), a copolymer of acrylonitrile and butadiene. Based on their shore A durometer hardness (20 for Dragon Skin® 20 versus 70 for Buna-N), tensile strength at break (3.79 MPa versus 14.00 MPa) and elongation at break (620% versus 400%), the Dragon Skin® belt was considered as a soft substrate and the Buna-N belt as a stiff substrate

The movements of the animals on the treadmill were recorded using a digital video camera (Casio EXZ R400) at 15 frames s^{-1}. The videos were analyzed with the software VirtualDub (v1.10.4, by Avery Lee) which allowed the identification of the start of swing phase (defined as the first frame when the upward motion of each proleg could be detected; Fig. 1C) and the end of swing phase (defined as the first frame when the proleg motion could no longer be detected; Fig. 1C) of each proleg in abdominal segments A6–A3 with a single frame resolution. The stepping patterns were identified as in a previous study (Metallo and Trimmer, 2015). Accordingly, the most commonly occurring stepping pattern, named the progressive (p) pattern, was considered as having two main phases: (1) each proleg is lifted in succession, from A6 to A3; (2) each proleg is brought back down to contact the substrate in the same order, from A6 to A3 (Fig. 1D). Patterns that differed from the progressive pattern were called non-progressive (np) and further classified as np1, np2 and np3 depending on the sequence of up and down proleg movements that characterize them (Fig. 1D). To simplify the following description, we refer to each of these stepping patterns as different 'gaits'.

For each caterpillar, six experimental conditions were considered using the two substrates (soft, stiff) and the three orientations (horizontal, upward/downward vertical) described above. For each experimental condition (substrate×orientation), 27 crawls per animal were randomly selected. The start and end points of a single crawl were defined as the start of swing phase in the A6 proleg and the end of swing phase in the A3 proleg, respectively.

Six kinematic variables were analyzed (Fig. 1E): (1) proleg stepping pattern, defined as the order in which each proleg (A6, A5, A4, A3) lifts off and re-contacts the substrate (Fig. 1D); (2) crawl cycle duration, defined as elapsed time between A6 lift-off and A3 re-contact with the substrate; (3) proleg swing duration (SwN), defined as elapsed time between lift-off and re-contact with the substrate for a single proleg; (4) proleg swing delay (SwY), defined as elapsed time between A6 lift-off and proleg (A5, A4, A3) lift-off; (5) proleg stance delay (StY), defined as elapsed time between proleg A6 lift-off and proleg (A5, A4, A3) touch down, corresponding to the crawl duration for segment A3 and SwN for segment A6; and (6) inter-proleg delay (IPY), defined as elapsed time between lift-off in adjacent prolegs. All kinematic variables were analyzed after expressing them as a proportion of the crawl duration, except for the initial analysis of the proleg swing duration.

Statistical analysis

The effect of different substrates in the same orientation and different orientations on the same substrate (i.e. substrate+orientation+substrate:orientation to include additive and interactive effects) on the kinematic variables that described SwN, StY, SwY and IPY was tested for statistical significance using two-way MANOVA Type I sum of squares (using class manova, requiring packages car and MASS, stats v3.6.0; https://CRAN.R-project.org/package=car, https://CRAN.R-project.org/package=MASS). A multivariate analysis comprising linear discriminant analyses was performed to identify the kinematic variables that differed significantly with orientation and substrate. To further evaluate the effects of orientation and substrate on the identified kinematic variables, linear mixed models and univariate ANOVA were performed (lme4::lm and car::anova; https://CRAN.R-project.org/package=lme4, https://CRAN.R-project.org/package=car). Differences in the kinematic variables across the prolegs were analyzed using one-way ANOVA, multiple comparison Tukey HSD, and Wilcoxon signed rank test for paired comparisons. All analyses were conducted using the free statistical software R (http://www.R-project.org/).

Three caterpillars were used to perform 486 trials for the three orientations and two substrates. To account for individual-animal effects, a 3-way interaction of the trials on substrate, orientation and individual caterpillar differences was also tested (Kinematic response

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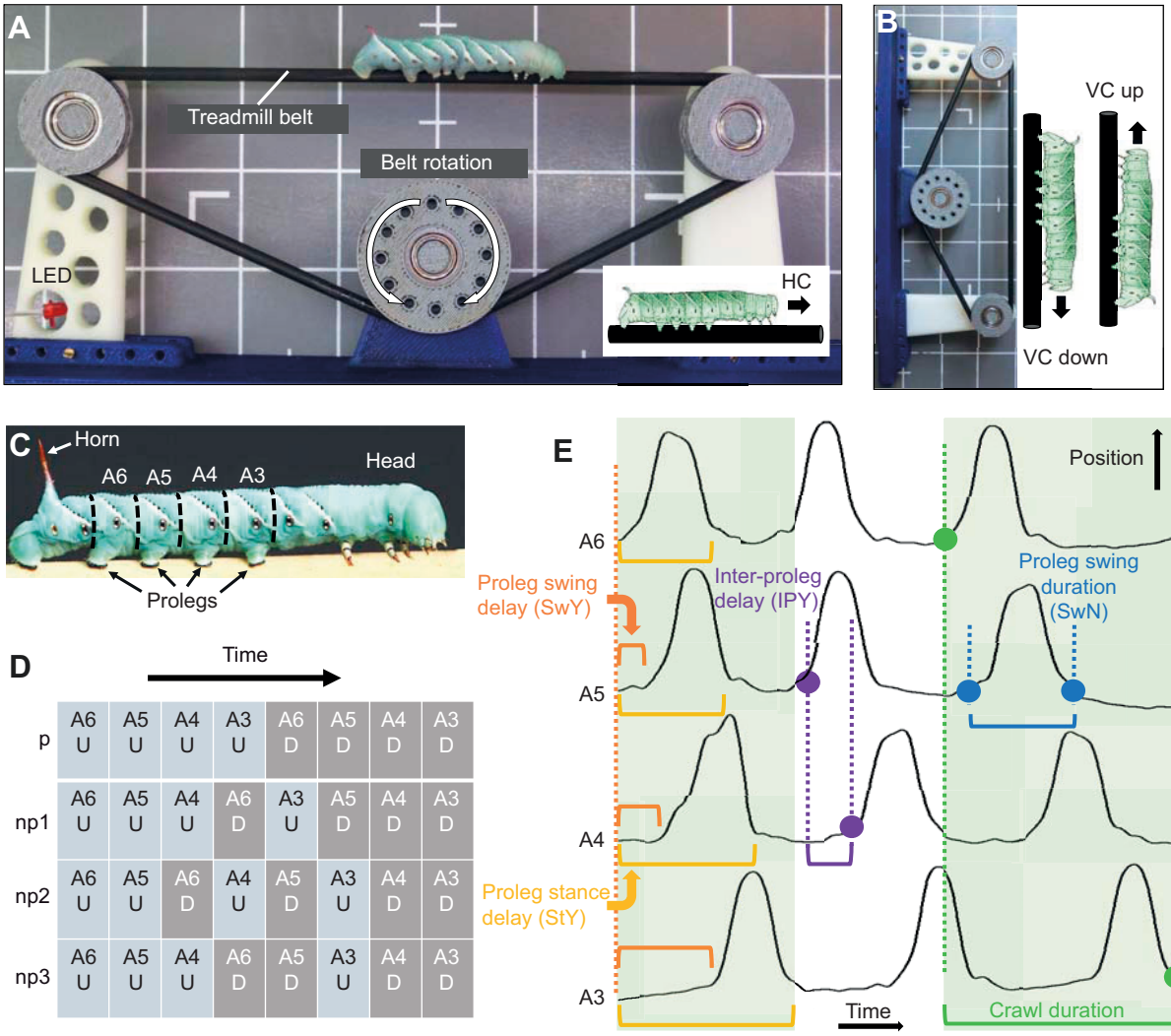

Fig. 1. Recording and quantifying *Manduca sexta* stepping patterns. (A) *Manduca sexta* movements were recorded on a treadmill belt so that crawls could be maintained in the field of view. All animals were tested on belts of two different stiffness. HC, horizontal crawl. (B) The treadmill could be rotated so that the animals could crawl vertically (VC, vertical crawl) up and down. (C) The timing of proleg steps was recorded in each of the mid-abdominal body segments (A6–A3). (D) Different gaits were identified by the relative timing of proleg swing phases. Progressive (p) patterns were defined as a continuous sequence of proleg lift off (up, U) and re-contact with the substrate (down, D) in successive prolegs. Gaits that differed from this sequence were termed non-progressive (np) and had three slightly different variations (np1, np2 and np3). (E) A sketch of three crawls in a representative crawling movement (position versus time), illustrating the five kinematic variables considered in the study: crawl duration (shaded areas) was defined as the time from A6 up to A3 down; proleg swing delay (SwY) was defined as the time from the start of a crawl to the onset of swing in each segment; proleg stance delay (StY) was defined as the time from the start of crawl to the end of swing in each segment, inter-proleg delay (IPY) was defined as the time between the onset of swing in successive prolegs; and proleg swing duration (SwN) was defined as the time between swing onset (U) and re-contact (D) for each proleg.

variables~Substrate×Orientation×Animal; this includes their additive and interactive effects on the kinematics). While the effects of individual animals were significant, there were larger, more significant fixed effects due to orientation and substrate. This suggested that the effects of orientation and substrate would persist even when averaged across animals. Therefore, the 486 trials across the three animals were averaged to evaluate the larger trends of orientation, substrate and their interaction on the gait of the caterpillar.

RESULTS

Stepping patterns

An initial analysis of the proleg stepping patterns was carried out by manually identifying the relative timing of proleg movements across all orientations and substrates. On the stiff substrate and in the horizontal orientation, approximately 80% of the crawls were found to be progressive (Fig. 2A). The only observed non-progressive pattern was np1, which differs from the progressive pattern in that A6 touches the substrate before A3 is lifted (Fig. 1D). On the stiff substrate, the progressive pattern occurred approximately 50% of the time in the upward vertical orientation and only 25% of the time in the downward vertical orientation, where most patterns became non-progressive (Fig. 2A). This shift from progressive to non-progressive patterns in the downward vertical orientation was even more marked on soft substrates as no progressive pattern was observed (Fig. 2B).

Because of the relatively small number of observed np2 and np3 crawls, an analysis was carried out by comparing progressive patterns with the all non-progressive patterns for all crawls combined. Overall, regardless of orientation, non-progressive crawls increased from 49% on the stiff substrate to 82% on the

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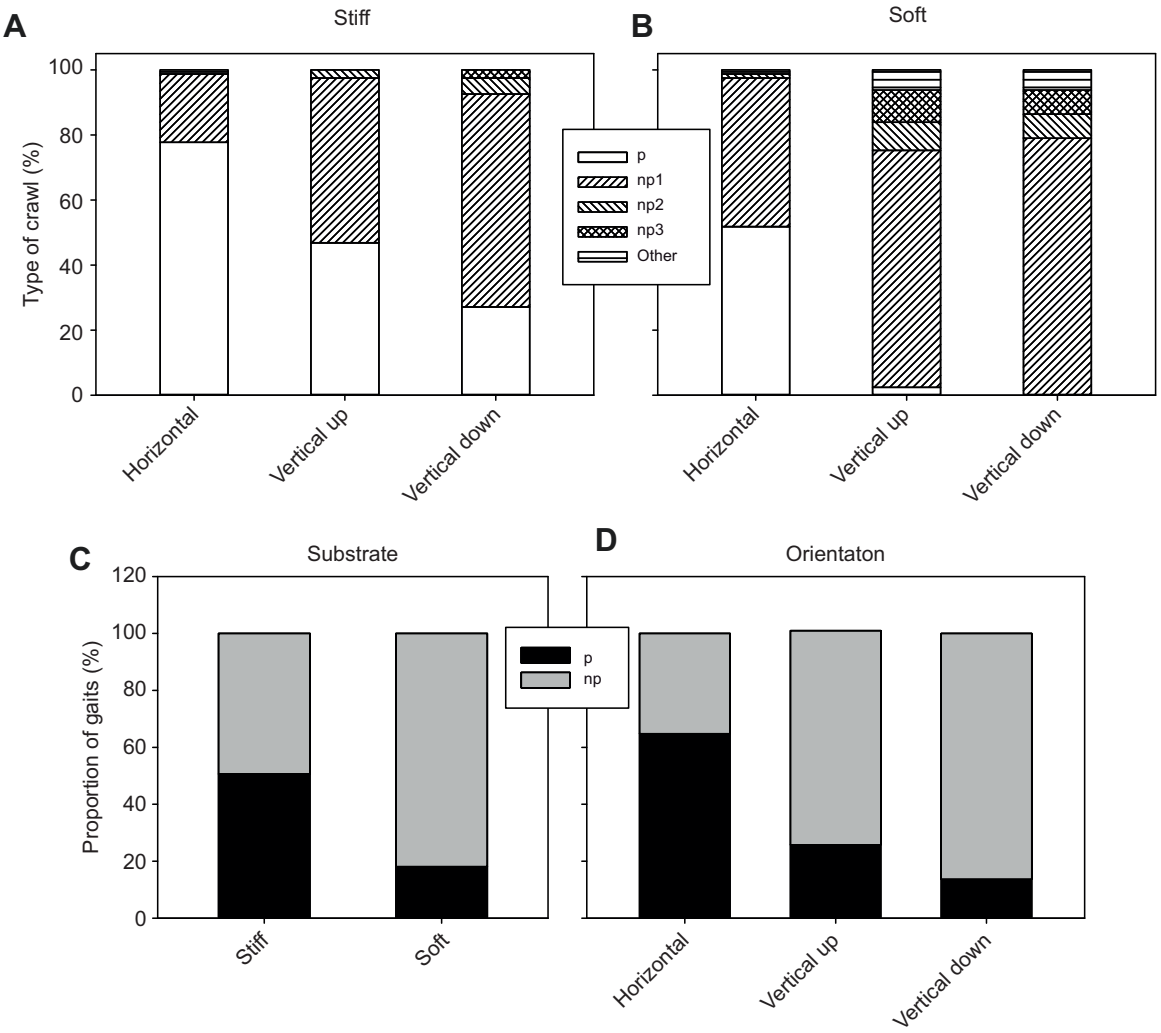

Fig. 2. The likelihood of different gaits depends on the substrate and orientation. (A) On the stiff substrate, during horizontal crawling, the majority of crawls were progressive (p) but alternative gaits (np1–3) became increasingly common while crawling vertically, particularly in the downward direction. (B) On the soft substrate, progressive patterns occur approximately 50% of the time in the horizontal orientation but are rarely, or never, observed during vertical crawling in either direction. (C) These relationships were more clearly seen when non-progressive (np) patterns were counted together and compared by substrate regardless of orientation. Non-progressive patterns were most common on the soft substrate. (D) Similarly, the proportion of non-progressive patterns increased during upward/downward vertical crawling regardless of substrate type.

soft substrate (Fig. 2C). Further, regardless of substrate type, the proportion of non-progressive crawls increased from 35% in the horizontal orientation to 76–86% in the vertical orientation (Fig. 2D).

Crawl and proleg swing duration

While the overall duration of crawling was extremely variable (Fig. 3), there was a statistically significant difference in crawl cycle duration due to orientation (2-way MANOVA; effect of orientation alone; $F_{2,486}$=9.33, P<0.0001) and by an interaction of substrate and orientation (2-way MANOVA; effect of orientation:substrate; $F_{2,486}$=35.04, P<0.0001) but not due to substrate alone (2-way MANOVA; effect of substrate alone; $F_{2,486}$=3.68, P=0.056). The crawl cycle duration was longest for the combination of horizontal orientation and stiff substrate (3.54±0.08 s) and shortest for the combination of upward vertical orientation and stiff substrate (3.00± 0.08 s, P<0.0001, *post hoc* Tukey HSD). The duration of the swing phase of each proleg was strongly correlated with the overall crawl duration (linear regression: A3 R^2=0.67, A4 R^2=0.75, A5 R^2=0.79, A6 R^2=0.71). Across all experimental conditions, the swing duration was shortest in A5 (median, 1.37 s, versus A6 Z=−6.48, versus A4 Z=8.37, versus A3 Z=8.18, all P<0.001, Wilcoxon signed rank test). No double steps were observed in the proleg stepping sequences.

Kinematic variables

Given the large variation in crawl duration, the measured crawl kinematic variables (SwN, SwY, StY and IPY) were normalized for statistical analysis by expressing them as a percentage of the crawl duration. This allowed us to compare changes in the relative timing of proleg movements across all 486 crawls. Across all the trials, SwN was approximately 50% of the crawl duration (means±s.e.m., A6 49.64±0.44%, A5 46.96±0.34%, A4 49.51±0.43%, A3 51.07± 0.44%) but was significantly shorter for the A5 proleg and significantly longer for prolegs in segment A3 (one-way ANOVA of means; $F_{3,1943}$=17.72, P<0.001; mean A5 SwN versus means of all other groups: P<0.001; mean A3 SwN versus means of all other groups: P<0.055). Overall, SwY was approximately evenly spaced

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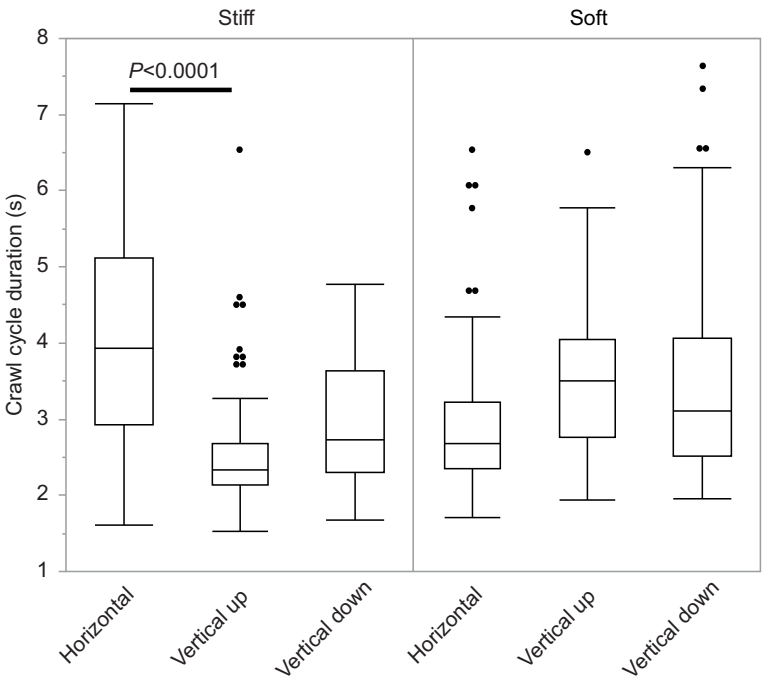

Fig. 3. Crawl cycle duration is context dependent. Crawl cycle duration (measured from A6 up to A3 down) was highly variable but statistically different based on orientation and the interaction of substrate and orientation (see Results for statistical details). Cycle duration was longest for horizontal orientation and stiff substrate and shortest for upward vertical orientation and stiff substrate.

across successive proleg steps (A5 17.73±0.27%, A4 33.22±0.34%, A3 51.32±0.43%), as was StY (A6 49.65±0.44%, A5 64.66±0.39% and A4 82.91±0.73%), but significant differences emerged when these parameters were analyzed by gait type (see below). Across all the crawls, IPY was also relatively consistent between the different prolegs (A6–A5 29.69±0.32%, A5–A4 31.35±0.34%, A4–A3 30.66±0.39%) but, again, significant differences emerged when these parameters were analyzed by gait type (see below).

As an initial unbiased test of the effects of substrate and orientation on locomotion, statistical comparisons were made using all four kinematic variables but without grouping them into identified gait patterns (progressive, non-progressive). Horizontal crawling was significantly dependent on the substrate ($F_{1,149}$=3.231, P=0.0004). Vertical climbing also significantly depended on the substrate ($F_{1,308}$=9.023, P<0.0001) and there was a statistically significant effect of direction (up or down), but this was not as important as the substrate ($F_{1,308}$=2.482, P<0.001). When the vertical climbing data were separated by substrate type, it was found that on soft substrates, vertical crawling was not affected by the direction (up or down, $F_{1,149}$=0.894, P=0.555) but on stiff substrates there was a significant effect of direction ($F_{1,149}$=4.072, P<0.0001).

Identification of gaits

These results showed that the stiffness of the substrate and orientation had a significant effect on the timing of the proleg steps. To examine how the stepping cycle varied between progressive and non-progressive patterns, the normalized kinematic variables for each stepping pattern were compared on each combination of substrate and orientation. SwN was significantly shorter for non-progressive gaits than for progressive gaits for all the prolegs regardless of substrate and orientation except for downward vertical crawls, where there was no difference (Fig. 4A,B). StY for proleg A6 (which is equivalent to the SwN for A6) and A5 was generally shorter for np gaits than p gaits but did not change consistently for proleg A4 (Fig. 4C,D).

IPY was also significantly shorter for non-progressive gaits than for progressive gaits regardless of orientation or substrate (Fig. 5A, B). SwY of prolegs A4 and A3 was generally longer for non-progressive gaits than for progressive gaits (A4 non-progressive 34.2±0.41%, progressive 31.3±0.58%; A3 non-progressive 54.02±0.46%, progressive 46.21±0.76%) for all substrates and orientations but did not vary consistently between gaits for proleg A5 (A5 non-progressive 17.64±0.30%, progressive 17.90±0.52%) (Fig. 5C,D).

The differences in kinematic variables observed in the present study are consistent with the previously identified gait differences. As illustrated in Table 1, the sequence of proleg lift-off (up) and re-contact with the substrate (down) predicted for progressive and non-progressive patterns was found to correlate with the measured changes in SwY and StY for specific prolegs, confirming the existence of alternative gaits.

DISCUSSION

The environmental skeleton strategy

Most legged animals with stiff skeletons can be modelled as a mass bouncing on a spring-loaded inverted pendulum (Dickinson et al., 2000; Full and Tu, 1991). Although modifications are needed to account for specific gaits and species, this model provides a basic framework to explain how legs mediate a cyclic exchange between kinetic energy, elastic storage and potential energy. In contrast, in *M. sexta*, previous kinematic studies have shown that proleg stepping is not related to any of the existing biomechanical models of legged locomotion (Trimmer and Issberner, 2007). Instead, *M. sexta* prolegs act as support struts that grip and release the substrate as they are carried along by an anterograde body movement (Trimmer and Issberner, 2007). In particular, the posterior prolegs resist forward progression for part of each cycle to create a drag component and place the body in tension and the three pairs of thoracic legs effectively act as a single contact point and are not essential for producing normal crawling movements (Lin and Trimmer, 2010a,b). By using the environment as a skeleton, caterpillars can apply compressive forces to the substrate while keeping their body relatively soft. Recordings of kinematics and ground reaction forces show that *M. sexta* uses a similar biomechanical strategy for both horizontal and

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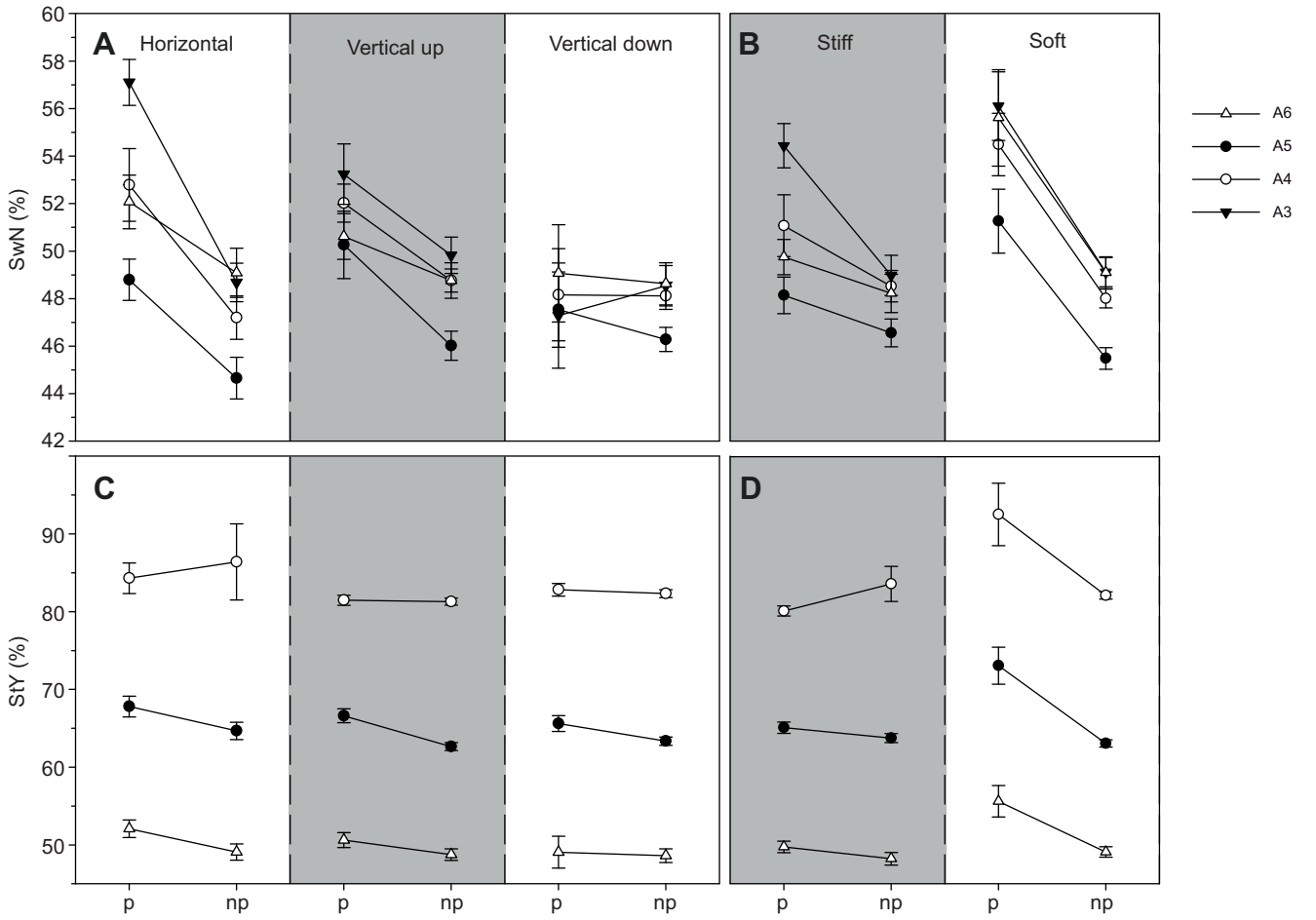

Fig. 4. Comparison of proleg swing duration and stance delay in progressive and non-progressive gaits. (A) The duration of swing (SwN) for each segmental proleg (A6–A3) is shown for horizontal and upward/downward vertical crawling for progressive (p) and non-progressive (np) patterns regardless of substrate. (B) SwN on stiff and soft substrates is shown for progressive and non-progressive patterns regardless of orientation. (C) The delay between the start of a crawl and stance onset (StY) is shown for prolegs A5–A3 for horizontal and upward/downward vertical crawling for progressive and non-progressive patterns. (D) StY on stiff and soft substrates is shown for progressive and non-progressive patterns regardless of orientation. All kinematic variables are expressed as a percentage of the crawl duration.

upward vertical crawling (van Griethuijsen and Trimmer, 2009; Vaughan et al., 2018).

Caterpillars can alter their stepping pattern

A previous study reported that *M. sexta* are capable of crawling horizontally and vertically on stiff substrates without major changes in their biomechanical strategy (van Griethuijsen and Trimmer, 2009). However, the present study uncovered that *M. sexta* can alter the relative timing of proleg steps.

We have analyzed these patterns by treating them as different gaits, but this does not mean that *M. sexta* controls its movements by selecting between fixed motor patterns. It is much more likely that the stepping patterns we describe represent a continuum of possible sequences. Stepping patterns in other insect species can be broadly variable without necessarily dividing into distinct gaits (DeAngelis et al., 2019; Dürr et al., 2019; Hughes, 1952). Furthermore, EMG recordings made from *M. sexta* abdominal muscles during crawling provide little evidence for strongly coupled coordination between motor pools, or for gaits dominated by central pattern generators. Longitudinal muscle activation during crawling occurs in prolonged bursts in different segments that overlap one another for the majority of a crawling cycle (Simon et al., 2010a). While there are phase delays between the onset and cessation of activity in successive segments, these vary during each stepping cycle, suggesting that tight coordination of segment shortening is not necessary for normal crawling (Metallo and Trimmer, 2015).

Although crawling-like patterns of motor activity have been generated pharmacologically in isolated *M. sexta* nerve cords (Johnston et al., 1999; Johnston and Levine, 1996), these patterns are much slower than any natural crawl and they vary considerably over time. Our own experiments have failed to produce sustained well-organized fictive crawling in isolated nerve cords. Presumably, the neural connections that produce such patterns require a normal sensory context to generate appropriately coordinated motor activity. Given that caterpillar crawling is relatively slow, it is likely that sensory feedback is sufficient to coordinate and modify stepping patterns to accommodate different substrates and obstacles.

Although *M. sexta* prolegs do not actively propel locomotion, they are critical for normal crawling by controlling grip timing. In contrast to the prolonged activation of abdominal muscles, proleg grip is controlled by brief bursts of retractor muscle activation in a precise sequence (Belanger and Trimmer, 2000; Mukherjee et al., 2018). Although we have not systematically studied alterations in central motor pattern retractor muscle activation in the context of

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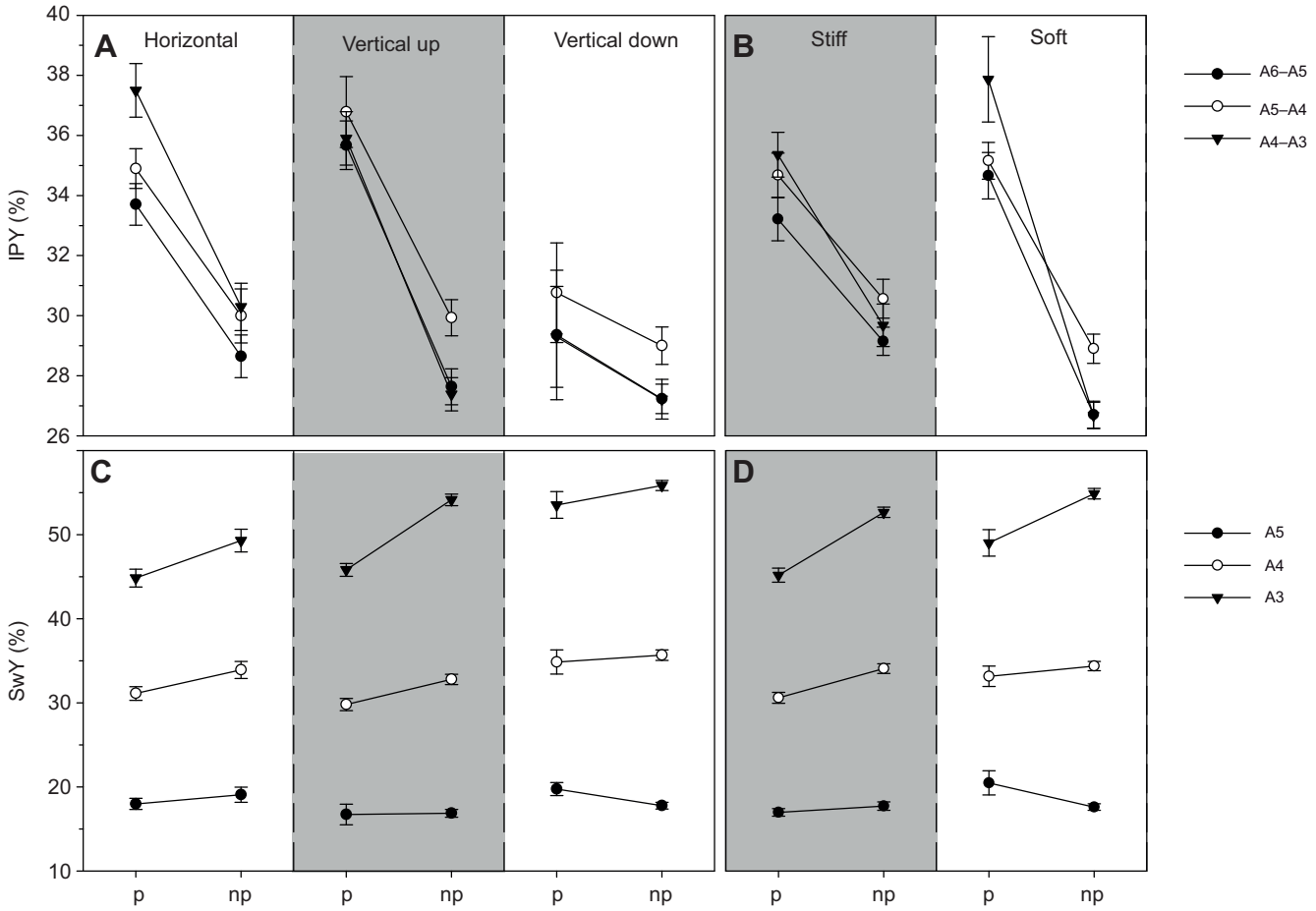

Fig. 5. Comparison of inter-proleg delay and proleg swing delay in progressive and non-progressive gaits. (A) The inter-proleg swing delay (IPY) between successive prolegs (A6–A3) is shown for horizontal and upward/downward vertical crawling for progressive (p) and non-progressive (np) patterns regardless of substrate. (B) IPY on stiff and soft substrates is shown for progressive and non-progressive patterns regardless of orientation. (C) The delay between the start of a crawl and the start of swing (SwY) is shown for prolegs A5–A3 for horizontal and upward/downward vertical crawling for progressive and non-progressive patterns. (D) SwY on stiff and soft substrates is shown for progressive and non-progressive patterns regardless of orientation. All kinematic variables are expressed as a percentage of the crawl duration.

changing substrates, the timing and duration of motor spikes to the principal planta retractor muscle (PPRM) changes slightly in response to proleg loading (Mukherjee et al., 2018). The changing stepping pattern seen in the current study suggests that crawling on different substrates will also involve changes in the timing of PPRM activation.

Although small, changes in the stepping patterns could be regarded as alternate gaits given that, while rare during horizontal crawling on stiff substrates, they become much more common during vertical crawling, particularly on the soft substrate, suggesting that they serve to compensate for changes in body loading and grip. The analysis of the kinematic variables related to

Table 1. Comparison of changes in kinematic variables with those predicted from the stepping patterns

Parameter	Proleg swing/stance delay		
SwY	SwY A5 (A6 up to A5 up)	SwY A4 (A6 up to A4 up)	SwY A3 (A6 up to A3 up)
Predicted change in parameter, np vs p	Unchanged	Increased for np2	Increased for all np gaits
Value np vs p	17.4 vs 17.2	33.7 vs 30.7	53.97 vs 46.3
Wilcoxon statistic	Z=–0.17	Z=–4.74	Z=–9.82
Probability value	P=0.685, n.s.	P<0.0001	P<0.0001
Parameter StY	StY A6=SwN A6 (A6 up to A6 down)	StY A5 (A6 up to A5 down)	StY A4 (A6 up to A4 down)
Predicted change in parameter, np vs p	Decreased for all np gaits	Decreased for np2,3	Unchanged
Value np vs p	49.1 vs 51.6	63.8 vs 66.4	82.2 vs 81.9
Wilcoxon statistic	Z=3.26	Z=4.76	Z=–0.69
Probability value	P<0.0011	P<0.0001	P=0.490, n.s.

The predictions are based on the stepping patterns shown in Fig. 1D comparing the proleg swing delay (SwY) and proleg stance delay (StY) for non-progressive (np) and progressive (p) gaits.
The test rows show the median values for np versus p gaits, the Wilcoxon Z statistic and the probability value (P). The predicted effects on these parameters match the experimental results in all cases. n.s., not significant.

M. sexta's step timing identified changes that correlate with these alternative gaits. The same kinematic variables were found to be influenced by both substrate and orientation, confirming that environmental factors affect the likelihood of a gait.

Interestingly, non-progressive stepping patterns were observed with increased frequency as the animals transitioned from the horizontal to the vertical orientation. Such non-progressive patterns are generated when the start of the A4 and A3 proleg swing phases is delayed (relative to the beginning of a crawl cycle) and the delay between the proleg lift-off in each segment decreases, resulting in a decrease in the relative duration of the proleg swing phase. Together, these changes alter the sequence of movements in the middle of a crawl – and thus produce a non-progressive pattern – but have little impact on the first two proleg movements (A6 up and A5 up) and the last two proleg movements (A4 down and A3 down). Nevertheless, the shorter swing phase and the longer stance phase that characterize non-progressive stepping patterns in the middle of a crawl will result in longer periods of contact with the substrate. This increase in the duration and number of segments gripping the surface might help caterpillars increase their stability and could therefore explain why non-progressive patterns are much more likely during vertical crawling and on soft substrates. Remarkably, during downward vertical crawling, the swing phase was found to be short even for the small number of observed progressive crawls (Fig. 4A), suggesting that shortening the swing phase is an important adjustment to orientation. Apart from an increase in stability, it is not clear what other biomechanical advantages arise from switching from progressive to non-progressive patterns. The change in swing timing is expected to affect the compression and re-extension phases of the body wall, which will also alter mass distribution. However, *M. sexta* crawling is slow and quasi-static, so inertial effects are relatively small (Trimmer and Issberner, 2007) and shifts in mass distribution are likely to be small compared with the effects of gravity.

Although the initiation of proleg swing phase is controlled primarily by the activation of the PPRM, other mechanical factors can play a role in releasing grip and reattaching to the substrate. Kinematic and electromyographic recordings from the prolegs show that crochet release is facilitated by swelling of the planta, potentially caused by forward movements of fluid and tissues (Mukherjee et al., 2018; Simon et al., 2010b). Therefore, it is possible that the gait change seen on vertical and soft substrates is a mechanical effect caused by changes in gravitational loading and grip strength, rather than by alterations in a central motor pattern. Withdrawal of the prolegs is context dependent (Belanger et al., 2000) but it remains to be seen whether gait changes involve a shift in the activation of retractor muscles during upward vertical crawling.

Recordings from the dorsal internal muscle (DIM) of *M. sexta* A4 body segment have shown that there is a phase shift in the activity of one of the DIM motoneurons during upward vertical crawling and that this is even more marked in non-progressive patterns (Metallo and Trimmer, 2015). The overall effect is that, during upward vertical crawling, DIM activation is delayed, occurring at the end of the A4 swing phase (overlapping with A3 swing) rather than at the beginning of the A4 swing phase (overlapping A5 swing). However, this is unlikely to explain the gait change itself because it also occurs in both non-progressive and progressive stepping patterns during upward crawling. The timing shift is likely to be a response to the increased axial loading that occurs during upward vertical crawling. It has been proposed that A4 DIM changes its function in each orientation, contributing to increase the tension on posterior segments during horizontal crawls and assisting the anterior segments in lifting the body forward when crawling upward vertically (Metallo and Trimmer, 2015). However, this hypothesis has not been tested in other orientations (e.g. downward vertical).

Crawling, inching and motor pattern generation

Previous work on caterpillar locomotion has concentrated on major gaits such as inching and crawling or alternative movements such as ballistic rolling and lifeline climbing (Brackenbury, 1996, 1997, 1999). Nevertheless, little is known about the evolution or ecological significance of inching and crawling. Although inching has the longest step length and is expected to be faster (for a given cycle period), it places more demands on the gripping system as a single pair of prolegs must support the animal when the other legs are in swing phase, and the prolegs must oppose the lateral or backwards toppling moment as the body lifts a long way from the substrate. While crawling is believed to be the ancestral mode of caterpillar locomotion, in some species, inching is considered to have evolved from crawling along with a reduction in the number of mid-body prolegs (Manton, 1952; Nagy and Grbić, 1999). Despite this, one question has yet to be addressed: what is the role of the central motor programs in coordinating different gaits? We have previously proposed that inching evolved from crawling by eliminating mid-body gripping (Trimmer and Lin, 2014), and that this could be accomplished through a loss of mechanical function (e.g. mutations that prevent formation of the crochets; Suzuki and Palopoli, 2001; Xiang et al., 2011) or by changes in motor commands controlling the retractor muscles. As crawling motions can be elicited in some inching species by artificially preventing dorsal flexion (Huai-Ti Lin and B.A.T., unpublished observations), it is possible that the underlying motor programs differ only in the timing and location of substrate grip. In addition, some caterpillar species (e.g. cutworms and other members of the Noctuidae family) use inching in early instars but crawling in later instars and the size of their more anterior prolegs typically increases during the transition from early to later instars (Hinton, 1955). In the current study, caterpillars were found to be able to adopt alternative gaits without undergoing morphological changes or switching to inching. Instead, alternative gaits emerged as the orientation and mechanical properties of the substrate changed. Further research is warranted to assess whether changes in underlying motor programs are responsible for the observed alternative gaits – and, if so, how *M. sexta* sense and evaluate the mechanical properties of their environment.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.A.T.; Methodology: C.M.; Formal analysis: C.M., R.M., B.A.T.; Investigation: C.M.; Data curation: R.M., B.A.T.; Writing - original draft: C.M.; Writing - review & editing: C.M., R.M., B.A.T.; Supervision: B.A.T.; Project administration: B.A.T.; Funding acquisition: B.A.T.

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Supplementary information

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Movie 1

Movie 2

Movie 3