



Tardigrades exhibit robust interlimb coordination across walking speeds and terrains

Jasmine A. Nirody^{a,b,1}, Lisset A. Duran^c, Deborah Johnston^d, and Daniel J. Cohen^{e,1}

^aCenter for Studies in Physics and Biology, Rockefeller University, New York, NY 10065; ^bAll Souls College, University of Oxford, Oxford OX1 4AL, United Kingdom; ^cDepartment of Molecular Biology, Princeton University, Princeton, NJ 08540; ^dUniversity of Rochester, Rochester, NY 14627; and ^eDepartment of Mechanical and Aerospace Engineering, Princeton University, Princeton, NJ 08540

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Tardigrades must negotiate heterogeneous, fluctuating environments and accordingly utilize locomotive strategies capable of dealing with variable terrain. We analyze the kinematics and interleg coordination of freely walking tardigrades (species: *Hypsibius exemplaris*). We find that tardigrade walking replicates several key features of walking in insects despite disparities in size, skeleton, and habitat. To test the effect of environmental changes on tardigrade locomotor control circuits we measure kinematics and interleg coordination during walking on two substrates of different stiffnesses. We find that the phase offset between contralateral leg pairs is flexible, while ipsilateral coordination is preserved across environmental conditions. This mirrors similar results in insects and crustaceans. We propose that these functional similarities in walking coordination between tardigrades and arthropods is either due to a generalized locomotor control circuit common to panarthropods or to independent convergence onto an optimal strategy for robust multilegged control in small animals with simple circuitry. Our results highlight the value of tardigrades as a comparative system toward understanding the mechanisms—neural and/or mechanical—underlying coordination in panarthropod locomotion.

tardigrade | locomotion | biomechanics | biophysics | panarthropods

The vast majority of animals need to move to survive. Tardigrades, though famed for their slow and unwieldy gait, are no exception. One of the smallest legged animals, tardigrades rely on their locomotive abilities to escape from predators and to find food and mates (1, 2). The first observations of tardigrades in the 18th century centered around their distinctive gait: They were described, in quick succession, as “water bears” (3) and “*il tardigrado*” (4) due to their slow, lumbering style of walking. Beyond these initial characterizations, however, not much is known about how tardigrades move about in their environment. In more recent years, their ability to withstand environmental extremes by entering a dormant state called a “tun” has garnered significant attention (5–7). However, this survival strategy is reliant on the animal’s ability to adeptly navigate its surroundings: Tun formation requires slow and controlled dehydration, and drying out too rapidly can be fatal. This makes moving in dramatically fluctuating microenvironments an important behavioral factor in successful dehydration and rehydration (8). Unlike other fundamental rhythmic motor programs (e.g., heartbeat or respiration), locomotion needs to be flexible and responsive to environmental stimuli. Terrestrial tardigrades in particular must traverse a complicated three-dimensional environment comprising a wide range of terrain types. Furthermore, different behavioral goals call for different walking speeds, ranging from slow exploratory walking to swift running for escape maneuvers (9). As such, locomotor output must be tuned to both speed and substrate. Such tuning can be achieved via adjusting the kinematics of single legs (e.g., stepping frequency or step length) but often also results in changes in temporal coordination between legs.

In some animals, these changes occur in the form of transitions between discrete gaits: For instance, a horse switches from walking to trotting to galloping as it goes from slow to intermediate to high speeds (10, 11). Alternatively, stepping patterns can lie along a continuum of interleg coordination patterns (ICPs). This may indicate that a single control circuit may suffice to generate all observed ICPs—that is, there need not be separate dedicated controllers for each “gait.” Excitingly, recent analyses have suggested the existence of such a continuum in walking insects (12–15).

Are there fundamental principles behind the generation of such a continuum that can be generalized beyond insects to describe walking in other legged panarthropods? Morphological similarities in the underlying neural structure have been noted between tardigrades and arthropods, supporting a sister-group relationship between these taxa (16). However, given the large disparities in size, skeletal morphology, and environment between arthropods and tardigrades, it is unclear if these morphological parallels translate to similarities in whole-organism performance between tardigrades and arthropods.

For instance, tardigrades, with body lengths down to just several hundred micrometers, exist at a scale where most other organisms have opted for locomotive modes other than walking. To what extent does their scale and aquatic environment affect their interleg coordination and biomechanical strategy in comparison to larger legged organisms? Furthermore, little is known

Significance

As perhaps the smallest legged animal and one of the only known soft-bodied walkers, tardigrades possess a uniquely versatile set of locomotor tools. Tardigrades have evolved to move through a vast array of environments—freshwater, marine, and terrestrial—using a strongly conserved body plan. We characterize tardigrade interleg coordination patterns using high-speed video of animals walking on gel substrates. Tardigrades utilize a tetrapod-like stepping pattern remarkably similar to that observed in insects, despite significant disparities in size and skeletal structure between the two groups. We find that tardigrades adapt their locomotion to a “galloping” coordination pattern when walking on softer substrates. This strategy has also been observed in arthropods to move efficiently on flowing or granular substrates.

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¹To whom correspondence may be addressed. Email: jasmine.nirody@all-souls.ox.ac.uk or danielcohen@princeton.edu.

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about how the control of locomotion in soft-bodied animals differs from those with rigid skeletons. Most soft animals are legless (e.g., nematodes or the larval stages of insects like *Drosophila*), and the lack of discrete contact points with the substrate makes it difficult to identify changes in the timing of ground interactions (17). Deeper evaluation in Onychophorans (velvet worms) (18), and in the larval stages of holometabolous insect orders which have developed leg-like appendages called “prolegs” on their abdomens [e.g., beetles (19), sawflies, moths, and butterflies (20–22)], has indicated that soft-bodied locomotion is less regular than that observed in jointed arthropods. It is unclear if this increased variability is due to differences in underlying neural connectivity or is simply a consequence of increased degrees of freedom associated with controlling lobopodal locomotion.

Here, we utilize the framework developed from studies of insect walking to compare the biomechanical strategies used by the eutardigrade *Hypsibius exemplaris* with those described in arthropods. Is walking in tardigrades described by a single continuum of ICs? What is the relationship between the structure of the tardigrade neural system and organismal function during locomotion? We characterize the kinematics of how tardigrades generate a range of forward walking speeds, as well as how their strategies adjust given variation in environmental properties like substrate stiffness. Surprisingly, we find marked similarities in coordination between tardigrades and arthropods during spontaneous planar walking (12–15, 23, 24), suggesting that either 1) there exists a common circuit for the control of forward walking in panarthropods or 2) that tardigrades and arthropods independently converged upon a similar set of coordination strategies for locomotion despite striking differences in size, skeletal morphology, and ecology. Our findings emphasize the value of the tardigrade as a compelling comparative system to study the evolution and mechanisms of legged locomotion. Additionally, characterizing the similarities and differences between tardigrade locomotion and that of other legged organisms may also inform the development of both soft robotics and microscale technologies.

Results

Overview of Tardigrade Leg and Claw Morphology. We quantify spontaneous planar walking in the eutardigrade *H. exemplaris* (see *Materials and Methods*). All known tardigrade species have four pairs of legs, with the fourth leg pair oriented posteriorly (Fig. 14). Structural studies have demonstrated that while all four legs are similar in structure and show serial homologies, the number of leg muscles decreases moving anterior to posterior, with the fourth leg pair having the fewest and most divergent musculature (25). Several kinematic parameters of the fourth leg pair show larger variation and lower dependence on walking speed relative to that in the first three pairs (*SI Appendix, Fig. S1–S3*). This is in accordance with the hypothesis that the posterior legs of tardigrades are used primarily for grasping rather than propulsion in forward locomotion (26). Despite observations of a monotonic decrease in number and monotonic increase in branching of musculature from the anterior to posterior segments in tardigrades (25), we find no significant differences in kinematic parameters or stepping precision among the first three leg pairs, suggesting that the first three leg pairs function equivalently for forward planar walking.

We observe that tardigrades had considerable difficulty achieving persistent locomotion on standard, polished glass slides (*Movie S1*). Without claw engagement, animals appear unable to successfully move forward; we find a higher probability of productive directed movement on roughened glass substrates. This reliance on substrate engagement may be because, as soft-bodied animals—and unlike arthropods with rigid exoskeletons—tardigrades utilize a tension-based

biomechanical strategy similar to that observed in caterpillars (27, 28). Detailed analysis of the ground reaction forces during a stride will further elucidate this relationship; we leave this for a future study. To more effectively capture their native environment and support claw–ground engagement we perform all core characterizations on soft, polyacrylimide gels engineered to have a stiffness of 50 kPa, where we could clearly observe claw engagement causing gel deformation (*Movies S2 and S3 and SI Appendix, Fig. S4*).

Tardigrade Kinematics across Walking Speeds. In our experiments, tardigrades walk with an average speed of $163.0 \pm 49.9 \mu\text{m/s}$ ($N = 23$ animals: 0.48 ± 0.11 body lengths per s; range: 79.1 to 263.5 $\mu\text{m/s}$). To initially describe the walking behavior we examine the relationship between several kinematic parameters and speed (Fig. 2 and *SI Appendix, Fig. S1*). A stride for each leg comprises a “swing” phase, in which the leg is lifted and takes a step, and a “stance” phase, in which the leg is in contact with the ground. Step amplitude, defined as the distance between the posterior extreme position (PEP) measured at liftoff of a leg at the start of a swing and the anterior extreme position (AEP) measured at touchdown of the same leg at the end of swing, increases with forward walking speed (Fig. 24). Stride period decreases with walking speed, plateauing at walking speeds of approximately $v > 100 \mu\text{m/s}$ (Fig. 2B). As speed increases, stance duration is modulated strongly while swing duration remains relatively constant (Fig. 2C). Both stance duration and stride period show a hyperbolic relationship with speed, as observed in insects [*Drosophila* (13), stick insect *Carausius morosus* (29), and desert ant *Cataglyphis fortis* (30)]. In line with studies in arthropods, we find that swing duration is coordinated with stride period ($\rho = 0.54$, $P < 0.001$).

This relative modulation is cleanly characterized by changes in the duty factor, the proportion of a gait cycle spent in stance phase. Duty factor changes smoothly with walking speed across the majority of arthropods, which is consistent with observations that arthropod stepping patterns lie along a continuum. Tardigrades, like all arthropod species surveyed, show a smooth relationship between duty factor and forward walking speed (Fig. 3), suggesting that they similarly do not display discrete gaits but continuously transition between ICs. Faster walking speeds generally result in stepping patterns with lower duty factors—i.e., legs spend proportionally less time on the ground the faster the animal is moving. Species that do not utilize a wide range of stepping patterns therefore do not show significant changes in duty factor over observed speeds. For instance, the adult stick insect *C. morosus* is a slow, careful walker and overwhelmingly favors a stable tetrapodal coordination (in which four legs are kept on the ground at any given time), maintaining a near-constant duty factor across its small range of natural walking speeds (Fig. 3, *Inset, purple*). The tardigrade *H. exemplaris*, which has to navigate similarly complex terrain (albeit at a very different length scale), displays a relatively weak correlation between duty factor and speed in ($\rho = -0.33$, $P < 0.001$). This may suggest that living in and moving through variable environments results in a preference for a consistent, stable stepping pattern over the ability to walk at a wide range of speeds.

Smooth Transitions between Stepping Patterns. As previously noted, stepping patterns in hexapods are often grouped into three canonical “gaits” (Fig. 1D). In tripod gait, two sets of three limbs swing together; in tetrapod gaits, three groups of two limbs swing together; in wave gait, each limb swings alone (13). Our experiments show that *H. exemplaris* primarily prefer a tetrapod-like stepping pattern across speeds; an exemplary gait cycle is shown in Fig. 1B. We note that tardigrade stepping patterns deviate from the “canonical” tetrapod characterized in stick insect

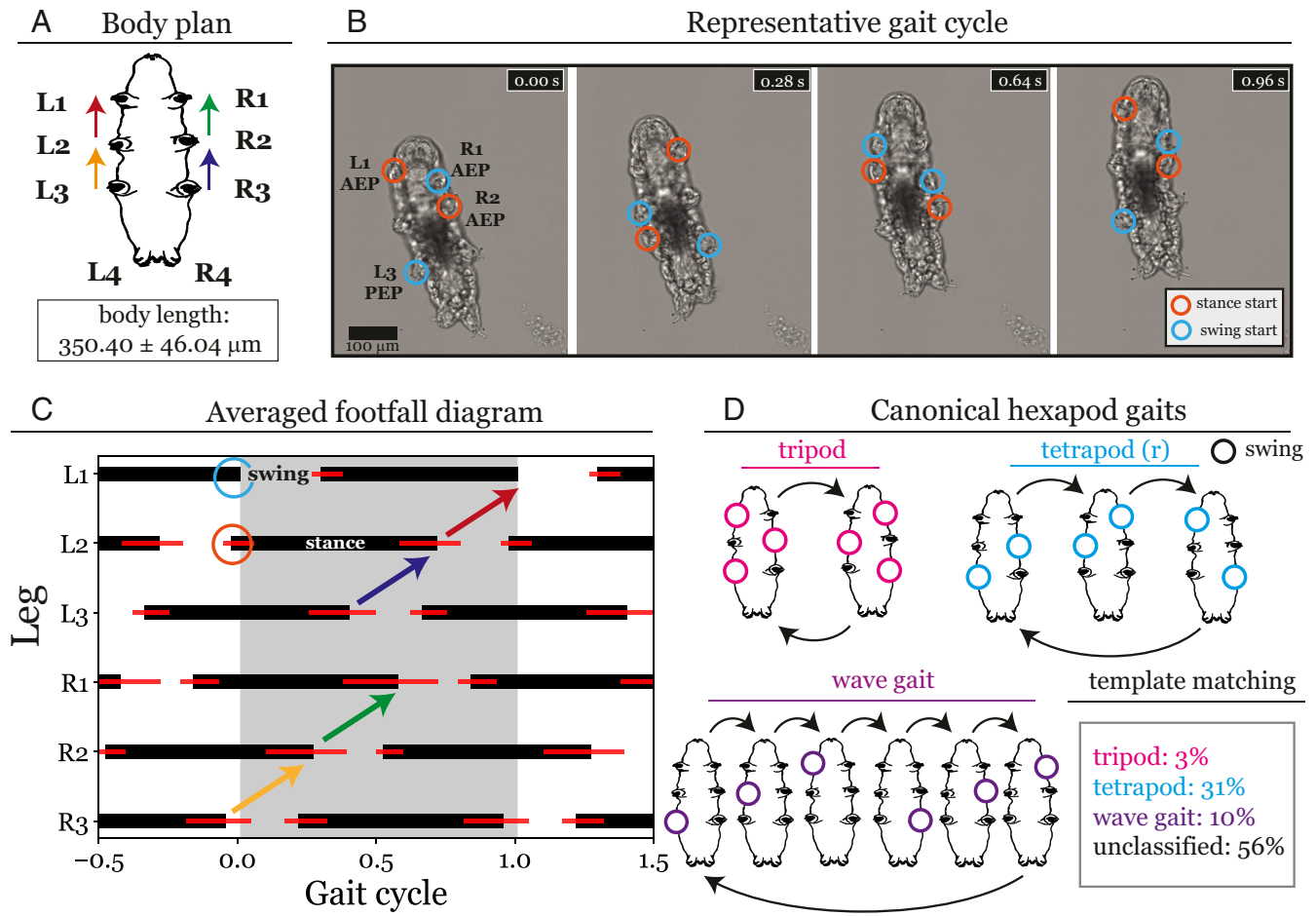


Fig. 1. Overview of tardigrade kinematics. (A) Tardigrade body plan. Colored arrows denote interleg relationships as in the podogram shown in C. Note that kinematic data for the reduced back legs (L4, R4) are not shown here (see *SI Appendix*). Mean \pm SD body length (measured from the tip of the nose to the attachment of the back leg pair) is provided for animals pooled between 50-kPa ($N = 23$) and 10-kPa ($N = 20$) conditions. (B) An exemplary stepping cycle comprising one swing and stance phase for all legs. (C) Podogram shows the average temporal sequence of ground contacts for legs L1 to L3 (left, anterior to posterior) and R1 to R3 (right, anterior to posterior). Values are normalized to cycle period of the left front leg L1, shown within the gray shaded region (0.0 to 1.0). Extrapolated sequences for previous $[-0.5, 0)$ and subsequent $(1, 1.5)$ periods are shown outside the shaded area. A total of $n = 122$ cycles (here, we define “cycle” as a sequence containing one full stride from each leg) from $N = 23$ animals are shown. Mean \pm SD is depicted; SD is indicated by red lines. Colored arrows highlight posterior-to-anterior propagation of ipsilateral swing events; color scheme for legs is as shown in tardigrade schematic drawing in A. (D) Schematic of canonical hexapod stepping patterns: tripod, tetrapod, and wave gait. In tripod, three limbs swing simultaneously; in tetrapod, two limbs swing simultaneously; in (pentapodal) wave gait each limb swings individually. The transitions between configurations are shown to reflect the posterior-to-anterior propagation of ipsilateral swing events (as in C). Gait template matching, a technique which classifies each stride to a “canonical” pattern of phase offsets for swing initiations (see *SI Appendix, Table S1*), fails to classify 56% of strides. This is largely because while the majority of strides are “tetrapod-like,” limbs that are expected to swing simultaneously instead swing with a slight temporal offset so that antiphase contralateral coordination is maintained.

walking (24, 31); as such, we refer to the coordination patterns observed in tardigrades as “tetrapod-like.” This is consistent with our finding that tardigrade duty factor maintains the expected value for tetrapod-like coordinations (duty factor = 0.67) at all observed speeds (Fig. 3).

As previously mentioned, there is mounting evidence that stepping patterns in insects (and perhaps, more generally) do not correspond to distinct gaits but instead form a speed-dependent continuum of ICPs (13–15). Tardigrades, similarly, do not adhere to cleanly prescribed canonical gaits. Classification using gait template matching results in 56% of tardigrade strides remaining “unclassified” and noncanonical, even after allowing for behavioral variance and tracking discrepancies (Fig. 1D). In accordance with our initial observations, ~70% of classified strides are sufficiently aligned with “idealized” tetrapod coordination (Fig. 1D and see *Materials and Methods*); this alignment increases with walking speed (*SI Appendix, Fig. S5*).

To determine how this spectrum of gaits arises, we considered that studies in *Drosophila* have indicated that tuning a single parameter, stance duration, can generate the spectrum of observed walking patterns (15). Here, we find that tardigrades show a smooth, continuous relationship in both stance duration (Fig. 2C) and duty factor (Fig. 3) with speed, supporting our hypothesized lack of distinct gaits and lack of discrete transitions between them.

Simple, Local Coordination Rules Explain Tardigrade ICPs. To better understand what kind of controller is responsible for tardigrade locomotion patterns we turn to studies demonstrating that a small set of simple “coordination rules” is sufficient to generate the continuum of observed insect ICPs during planar walking (32). These locally distributed rules describe how a leg affects the likelihood of the initiation of a swing event in an anterior or contralateral neighboring leg (23, 24). Rule 1 states that a leg’s

Kinematic parameters and forward speed

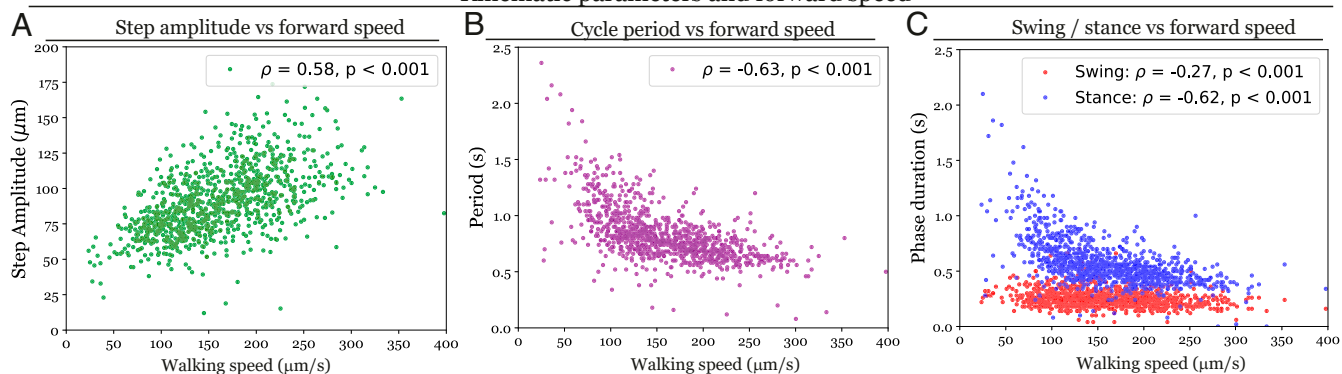


Fig. 2. Leg stepping parameters relative to forward speed. Each point represents a stride (stance + swing) for an individual leg; $n = 989$ strides are shown. Data for the first three leg pairs are pooled; individual fits for each leg pair are provided in *SI Appendix, Fig. S1*. Stride length (A) smoothly increases and period (B) smoothly decreases with walking speed, suggesting that tardigrades modulate both stride length and stepping frequency to increase forward speed. (C) Each step is composed of a swing (leg lifted) and stance (leg on the ground) period; swing duration stays roughly constant with speed, while stance duration is modulated, decreasing with increasing speed.

stance-to-swing transition is suppressed while its neighbor is in swing, while Rule 2 states that the likelihood of liftoff increases once the neighboring leg touches down. Both rules have been shown to be stronger between ipsilateral rather than between contralateral leg pairs (24, 32).

A unifying hypothesis for the observed continuum of stepping patterns (15) and decentralized control of insect walking (24, 32) is rooted in the structure of the arthropod nervous system. As anatomical studies have highlighted the similarities between the tardigrade and arthropod nervous systems (16, 33), we hypothesize that stepping patterns in tardigrades should exemplify several key attributes of arthropod ICPS. Each pair of legs—front, middle, and hind leg pairs in arthropods—is controlled by its own ganglion of the ventral nerve cord (VNC), each of which consists of two right and left hemiganglia, which control the corresponding legs. The three segmental ganglia are linked by longitudinal connectives (33). A simple hypothesis consists of mutual inhibitory coupling between each right and left hemiganglion and a posterior-to-anterior inhibitory coupling between the longitudinal commissures connecting ipsilateral ganglia (15). This is analogous to Rule 1 (Fig. 4A and *SI Appendix, Fig. S6C*). Indeed, our analysis of interleg coordination compellingly suggests that both Rule 1 and Rule 2 are active between ipsilateral

leg pairs in tardigrades. In accordance with Rule 1, our data show that the likelihood of a swing initiation is nearly zero after its posterior ipsilateral neighbor lifts off (Fig. 4A and *SI Appendix, Fig. S6C*). This likelihood sharply rises after the time since its posterior ipsilateral neighbor's swing initiation surpasses the sample average swing duration ($\langle t_{\text{swing}} \rangle = 0.18$ (normalized to one cycle length; $n = 654$ strides, $N = 23$ animals)). To check compliance with Rule 2, we examine the likelihood of a leg lifting off into swing phase after its posterior ipsilateral neighbor completes its swing and touches down (Fig. 4B). As predicted, we find that this probability rises sharply immediately after touchdown of the ipsilaterally posterior leg.

Compliance with these rules in tardigrades results in a back-to-front wave of swing initiations across walking speeds (Fig. 1C), a pattern observed in a broad range of arthropod taxa (12–15, 23, 24, 34). The average ipsilateral offset in our data, $\phi_I = 0.36 \pm 0.17$ ($n = 654$ strides, $N = 23$ animals), is approximately as predicted for the phase difference between ipsilateral leg pairs in tetrapodal leg coordinations (ipsilateral phase offset for an “ideal” tetrapod: $\phi_I = \frac{1}{3}$). Phase offsets are equivalent between all ipsilateral leg pairs (Fig. 4C). Leg pairs maintain this offset across walking speeds; coordination is more variable at very low speeds, as was found in previous studies (13, 15).

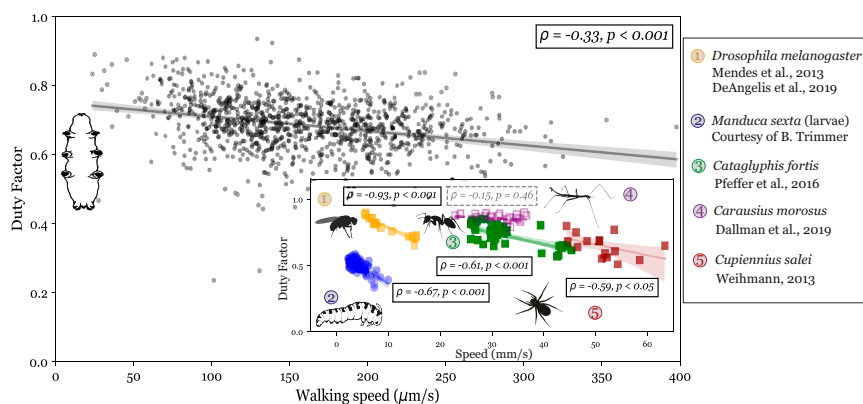


Fig. 3. Comparison of duty factor vs. forward speed across Panarthropoda. Tardigrades maintain a relatively constant duty factor across all observed forward walking speeds; we find a weak negative correlation between duty factor and walking speed ($\rho = -0.33$, $P < 0.001$). As in Fig. 2, each point represents a stride for a single leg; a total of $n = 989$ strides are shown. (Inset) The duty factor vs. walking speed relationship for several arthropod species during slow walking. All species except the stick insect (inset gray points; data from ref. 29) show significant inverse relationships between forward speed and duty factor. Linear regression fits are shown as solid lines alongside 95% confidence intervals; fits for which $P > 0.05$ are shown as dotted lines.

Inter-leg ‘rules’: temporal coordination

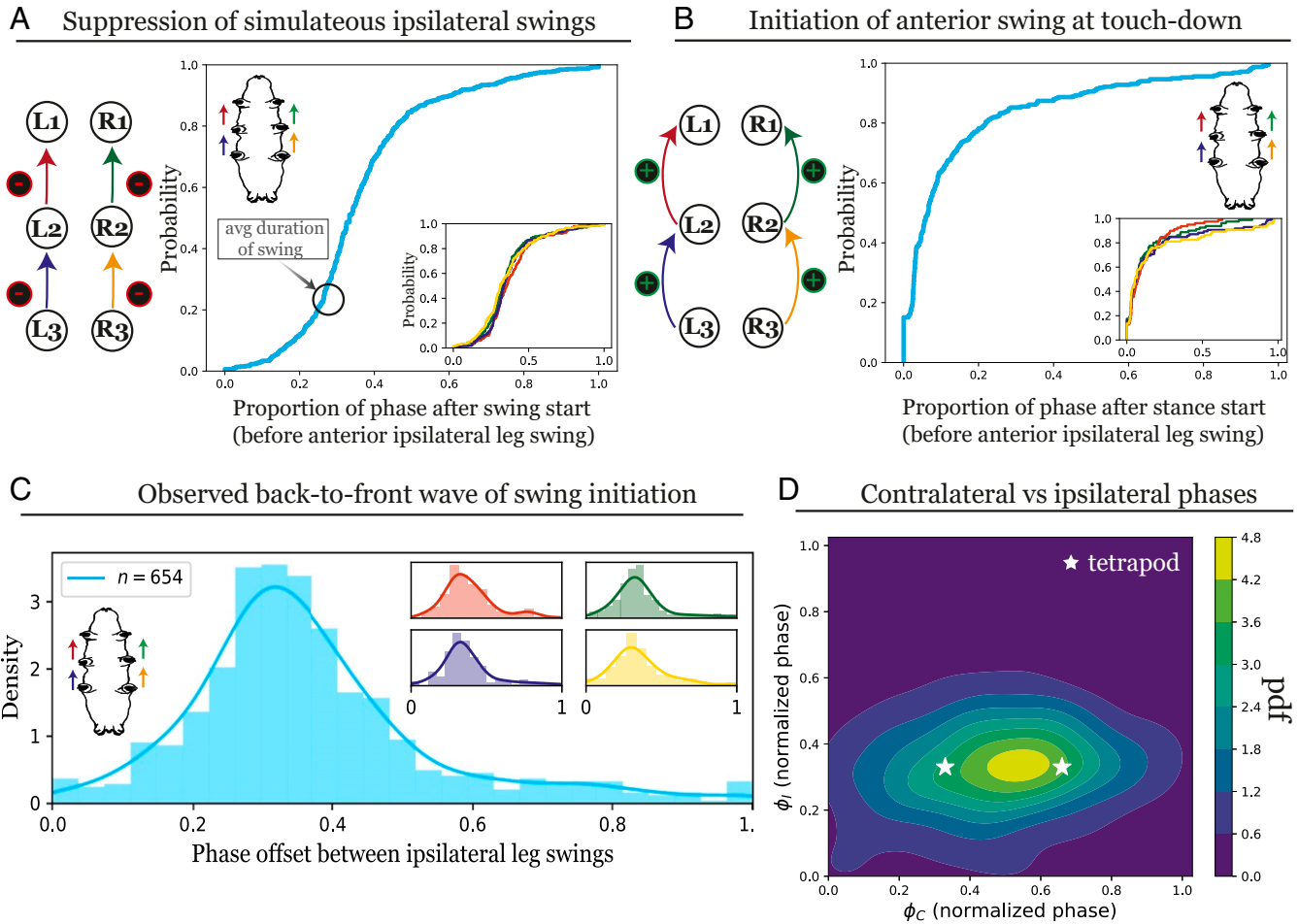


Fig. 4. Temporal coordination between ipsilateral leg pairs. Plots represent data pooled from all leg pairs; a total of $n = 654$ strides are shown. (*Insets*) Pairwise interleg relationships, with color scheme as demarcated by the arrows on tardigrade schematics. Cumulative distribution functions show that leg swings are (A) suppressed immediately following the swing initiation of the posterior ipsilateral neighbor and (B) initiated after the posterior ipsilateral leg has touched down into stance. (C) Histogram and probability density of observed phase offset between ipsilateral legs show that tardigrades maintain a posterior-to-anterior wave with a phase difference $\phi_l = \frac{1}{3}$ between swing onsets. Similar observations have been made in several arthropods (13, 14, 23). Pairwise comparison using the Kolmogorov–Smirnov test found no significant differences between any leg pairs, after controlling for multiple testing. (D) Joint distribution of the phase difference between contralateral (ϕ_c) and ipsilateral (ϕ_l) leg pairs. Preferred coordination shows characteristic phase differences different from those observed in “idealized” tetrapod. Notably, while tardigrades prefer the expected $\phi_l = \frac{1}{3}$, contralateral leg pairs display antiphase coordination $\phi_c = \frac{1}{2}$. pdf, probability density function.

Interleg coupling in the context of these rules is significantly weaker (if active at all) between contralateral leg pairs (*SI Appendix, Fig. S6C*). Our data find that all contralateral leg pairs show an average antiphase preference. Such a value may arise from a bimodal distribution with twin peaks at $\phi_c = \frac{1}{3}$ and $\phi_c = \frac{2}{3}$, as might be expected from the two idealized tetrapod patterns (Fig. 1D). However, we find contralateral phase differences to be normally distributed about 0.5 (Fig. 4D and *SI Appendix, Fig. S6B*). This relationship arises from a slight cross-body offset in swing initiations, such that limbs that would be expected to swing simultaneously are slightly offset in their liftoff time. Interestingly, previous work in *Drosophila* has also reported antiphase contralateral coordination across forward walking speeds (13–15); though it has not yet been explicitly tested, this pattern may be common to tetrapod-like stepping patterns across arthropods.

Changes in Limb Kinematics and ICPs with Substrate Stiffness. Natural terrain is rarely homogeneous, and legged animals often need to cope with environmental inconsistencies such as changes in

substrate roughness (35–37) or stiffness (22). These irregularities often constrain organismal performance and can result in constraints on walking speed as well as changes in limb kinematics. Limnoterrestrial tardigrades, like *H. exemplaris*, walk along soft, uneven plant matter. Having established earlier that claw engagement is essential for *H. exemplaris* and that hard, flat surfaces posed a particular challenge, we next explore the importance of substrate stiffness, hypothesizing that particularly soft substrates may pose problems to proper claw engagement (27, 28).

To assess the role of stiffness, we compare tardigrade locomotion on our standard, 50-kPa gels to that on 10-kPa gels (see *Materials and Methods*). Tardigrade walking speed on the soft gels decreases nearly twofold relative to performance on the stiff gels ($160.5 \pm 57.8 \mu\text{m/s}$ on the 50-kPa substrate with $N_{50\text{kPa}} = 23$ animals vs. $91.0 \pm 32.0 \mu\text{m/s}$ on the 10-kPa substrate with $N_{10\text{kPa}} = 20$ animals; see *SI Appendix, Fig. S4*). Because tardigrades do not always walk at steady speeds, we compare walking speed distributions on 50-kPa and 10-kPa substrates to

rule out the apparent slower speed coming from an increase in stop-start motion. However, speeds on both substrates are distributed unimodally, suggesting that the change in walking speed with substrate stiffness is due to a shift toward lower preferred walking speeds rather than more frequent acceleration and deceleration. More specifically, the observed reduction in speed is achieved largely through changes in stride period rather than step length; within each stride, the stance duration varies significantly between conditions, while swing duration does not (Fig. 5A). As such, higher duty factors are associated with stepping patterns on the 10-kPa substrate (SI Appendix, Table S2), perhaps indicating greater locomotive effort necessitating longer ground contact times.

We also explore how substrate stiffness modulated specific interleg control rules and find that interleg phase relationships are mostly robust to changes in substrate stiffness (Fig. 5B). The preferred stepping patterns on the 10-kPa substrate maintain several key features to that observed on the 50-kPa substrate: 1) ipsilateral swing events proceed in a posterior-to-anterior fashion; 2) adjacent ipsilateral legs show a preferred phase difference $\phi_I \approx \frac{1}{3}$; and 3) contralateral leg pairs show a preference for antiphase coordination $\phi_C \approx \frac{1}{2}$. However, we find a smaller second peak in the distribution of ipsilateral phase differences at $\phi_I \approx \frac{2}{3}$ of strides taken on the 10-kPa substrate (Fig. 5B and C). Closer analysis reveals that this second peak arises due to the posteriormost leg pair showing in-phase rather than antiphase

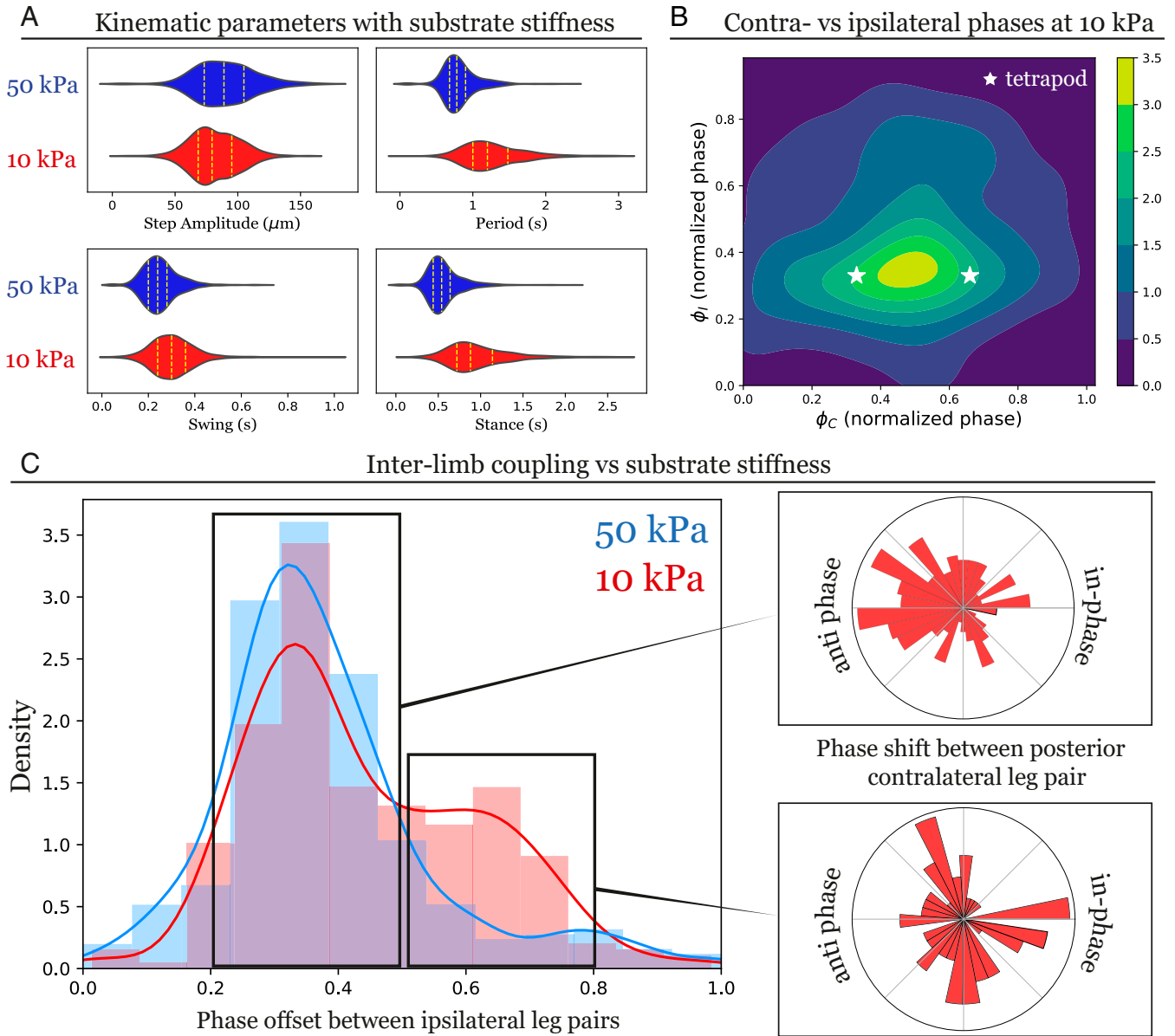


Fig. 5. Effects of substrate stiffness on walking kinematics and interleg coordination. (A) Tardigrades adjust period (Top Right) and stance duration (Bottom Right) with changes in substrate stiffness. Distributions for step amplitude (Top Left) and swing duration (Bottom Left) are largely unchanged between stiffness conditions. (B) Joint distribution of the phase difference between contralateral (ϕ_C) and ipsilateral (ϕ_I) leg pairs on the 10-kPa substrate. As on the 50-kPa substrate, tardigrades prefer an ipsilateral phase difference $\phi_I \approx \frac{1}{3}$ but display antiphase contralateral coordination $\phi_C \approx \frac{1}{2}$. Larger variance in ipsilateral phase is apparent than on the 50-kPa substrate. (C) Comparison of the marginal ipsilateral phase difference distributions on 50-kPa and 10-kPa substrate shows an additional peak at $\phi_I = \frac{2}{3}$ on the 10-kPa substrate (red). This arises because of transient in-phase stepping by the neighboring posterior leg pair (Bottom Inset), deviating from the preferred antiphase (Top Inset).

coordination (Fig. 5C). These alternate stepping patterns arise when a leg pair steps in phase ($\phi_C = 0$), and consequently, the leg pair in front of it may either adopt an 1) in-phase contralateral coordination to maintain a constant ipsilateral phase difference $\phi_I = \frac{1}{3}$ or an 2) antiphase contralateral coordination to “reset” to the preferred tetrapod-like pattern, which leads to one side showing an ipsilateral difference of $\phi_I = \frac{1}{3}$ and the other side showing a phase difference $\phi_I = \frac{2}{3}$. The latter strategy is preferred, with in-phase contralateral stepping patterns being largely transient; however, we did observe rare instances of a sustained “galloping” gait as would result from the former strategy (Movie S4).

Discussion

In addition to their famed resilience under conditions of extreme stress, tardigrades also display a remarkable robustness in their day-to-day activities. Tardigrade morphology is strongly conserved across species that live in and move through a large range of habitats, including marine, freshwater, and limnoterrestrial environments. Here, we characterize how kinematics and interleg coordination in the limnoterrestrial tardigrade *H. exemplaris* adapt to changes in walking speed and substrate properties. We find that tardigrade stepping patterns change smoothly with walking speed, rather than displaying sharp transitions between discrete “gaits.” Further, the observed patterns reproduce key features in the spectrum of ICPs characterized in various insect species, namely 1) a posterior-to-anterior wave of ipsilateral swing initiations across all walking speeds and 2) a general preference for antiphase coordination between contralateral legs. More generally, we find that the observed spectrum of tardigrade ICPs emerges naturally from a surprisingly small set of local coordination rules derived from behavioral observations in insect species (24).

These functional parallels are particularly striking given the large disparities in size, skeletal morphology, and environmental between tardigrades and insects. Tardigrades are several orders of magnitude smaller than stick insects, and yet “rules” derived from behavioral studies in *C. morosus* sufficiently and accurately describe walking patterns in *H. exemplaris* (23, 24). Whether these commonalities arise from shared ancestral structures remains an exciting open question (16, 33, 38).

Some support for the hypothesis of a shared circuit between arthropods and tardigrades comes from anatomic similarities in underlying neural circuitry. A simple model for locomotor control in insects was proposed based on studies in walking *Drosophila* and is built upon the anatomy of the VNC. This model supposes a posterior-to-anterior inhibitory coupling between ipsilateral neuropil, as well as mutual inhibitory coupling between contralateral neuropil of the VNC. There are various striking similarities in tardigrade and arthropod VNC structure: 1) The VNC comprises three segmented ganglia, each associated with a walking leg pair, and 2) each ganglion is divided into left and right hemiganglia linked by contralateral projections. In contrast, the closely related velvet worms—which, like tardigrades, are among the only extant soft-bodied walkers—have two laterally located nonsegmented ganglia (16, 33). This disparity in underlying neuroanatomy may account for the differences between onychophoran gaits and those observed in tardigrades and arthropods (18). As such, comparative analyses making use of VNC structure indicate a sister grouping between tardigrades and arthropods (16, 33).

It is an intriguing hypothesis that there may exist a common simple locomotor circuit underlying walking in panarthropod species, which has been modified along certain clades due to specific pressures on organismal performance [e.g., in-phase contralateral coordination in crayfish swimmerets (39)]. An example of one such modification is the modulation of inter-

intra-segmental connections in response to changes in the environment, e.g., varying surface stiffness. The behavioral flexibility observed in arthropod walking is partially ascribed to the modular nature of segmented body plans. Varying of substrate stiffness is likely salient for tardigrades because they lack a rigid skeleton, and accordingly may utilize a biomechanical strategy distinct from most adult insects that relies crucially on environmental stiffness (27, 28). Analyses in the soft-bodied hawk moth larvae found that *Manduca sexta* caterpillars did indeed sense changes in substrate stiffness and adjusted their stepping patterns to accommodate these changes (22). Studies in several insect species suggest that ipsilateral (intersegmental) synchronization dominates interleg coupling patterns, while contralateral (intra-segmental) coupling is adjustable (32, 36, 40). Consistent with these observations, tardigrades show changes in their contralateral phase offset but largely preserve ipsilateral coordination on soft substrates. We observe both transient strides in which contralateral limbs step in phase, as well as occasional sustained in-phase contralateral coordination resulting in a “bounding” or “galloping” gait. Interestingly, several species of dung beetle in the genus *Pachysoma* have also been observed to maintain a galloping gait across shifting sands (40).

However, alternative analyses—including molecular analyses (41) and comparative studies of brain structure (38)—group onychophorans together with arthropods, an interpretation which suggests that functional analogs between tardigrade and arthropod walking have independently evolved. In this case, the similarities in underlying circuitry controlling tardigrade and insect walking may not be ancestral. It may be that the observed set of coordination patterns, which only require a single simple controller, might be preferable in small animals with small circuits for limb control (15). Parallel convergence onto similar interleg coordination strategies by tardigrades and arthropods is intriguing given their varied ecology, disparities in size, and difference in skeletal structure between the two groups and can provide significant insight into general design principles for efficient and robust control of multilegged locomotion.

Previous studies have also proposed that the magnitude and robustness of static stability during walking may affect preference for certain ICPs (14). In line with these predictions, we find that tardigrades maintain the stability of their center of mass while walking (~97% of frames show stable configurations; see *SI Appendix*, Fig. S7). It is unclear, however, whether stability or the need to stay upright would be a concern for tardigrades, which are far smaller than stick insects or even fruit flies and walk underwater with the assistance of buoyancy. However, while falling down may not cause the tardigrade harm, lack of stability during walking may still hurt the performance and fitness even of small animals, for instance by impeding its progress across the substrate during foraging, avoiding predators, or finding mates. Further, in limnoterrestrial tardigrades, processive locomotion is vital to the organism’s ability to navigate a heterogeneously moist environment and avoid rapid dehydration. Alternatively, the functional commonalities between tardigrades and arthropods may arise from a shared ancestral circuit preceding the miniaturization of the tardigrade body plan. In this case, the observed stepping patterns in tardigrades may be the remnants of the stability concerns of a larger ancestor.

A more definitive distinction between these scenarios will require deeper functional studies combined with molecular, phylogenetic, and anatomical analyses and is beyond the scope of this study. We note that this work focuses on analyzing spontaneous walking in *H. exemplaris* and that our data are accordingly constrained both in number of species considered and in the range of walking speeds observed. Future work should expand upon both of these parameters, both by surveying walking dynamics across Tardigrada (keeping in mind the wide

ecological range of the phylum) and by perturbation or manipulation experiments that may expand the observed range of walking speeds.

Tardigrade walking also poses several fundamental mechanistic questions. Tardigrades are among the smallest legged animals, and, given their ecological success, investigations into their biomechanical strategy provide valuable insight into the scaling of efficient polypedal walking in various ecological conditions. For instance, the common preference for tetrapod-like coordination in both tardigrades and far larger species like the stick insect points to the selective importance of static stability in species that regularly navigate variable, three-dimensional terrain (24, 31). Similarly, the shift toward in-phase contralateral stepping on soft substrates in tardigrades is mirrored in the evolved “galloping” gait of desert-dwelling beetles orders of magnitude larger in size (40). This common strategy may reflect an energetic benefit to in-phase contralateral coordination on unstable or shifting terrain that holds across a remarkably large range of length scales. Furthermore, tardigrades are one of the only soft-bodied animals that walk using “true” legs, which allows for cleaner characterization of coordination and kinematic strategy than is possible in soft animals that crawl without discrete ground contacts. To this end, our findings here highlight the value of tardigrades both as a comparative system toward understanding the mechanisms underlying coordination in panarthropod locomotion and as an organismal system uniquely positioned to inform our understanding of the design and control of small, soft-bodied locomotive systems, from organisms to robots.

Materials and Methods

Tardigrade Husbandry. Specimens of the eutardigrade *H. exemplaris* strain Z151 were purchased from Carolina Biological Supply. Tardigrades were maintained in Chalkey’s medium (42). Animals were fed *Chlorococum* sp. algae; algae was purchased from Carolina Biological Supply and maintained in Bold’s basal medium. Cultures were maintained at 20 to 25°C under a 16-h light/8-h dark cycle.

Experimental Procedure and Imaging. We studied tardigrades walking on polyacrylamide gels of stiffnesses ~50 kPa and 10 kPa. An initial gel precursor solution (40% acrylamide and 2% bis-acrylamide) was diluted in 1 mL of Milli-Q purified water according to measurements made in ref. 43: 8%/0.4% acrylamide/bis-acrylamide for 50-kPa gel substrate and 8%/0.1% acrylamide/bis-acrylamide for 10-kPa gel substrate. Solutions were degassed before gelation. To initiate gelation, 5 μ L of 100 mg/mL ammonium persulfate was added to 1 mL of diluted precursor solution, followed by 0.5 μ L of N,N,N',N'-tetramethyl-ethylenediamine accelerator. After being mixed by gentle pipetting, 20 μ L of the gel solution was added to a glass-bottom dish and flattened with an 18-mm round coverslip. Glass-bottom dishes were treated with Bind-silane solution, washed with ethanol, and air-dried before addition of solution. Gels were polymerized at room temperature (23°C) for 30 min, after which the coverslip was removed carefully from the gel surface using tweezers. Further details on preparation and stiffness measurements of polyacrylamide gel substrate are provided in ref. 43.

Using a pipette, 3 mL of tardigrade-containing solution was removed from culture. This solution was filtered using 100 μ m mesh to remove algae, and 400 μ L of filtered tardigrade culture was added onto the gel substrate for imaging. Tardigrades were allowed to settle for 10 min, after which an additional 3 mL of spring water was added to ensure tardigrades were kept submerged throughout imaging. Animals were imaged using differential interface contrast microscopy on an inverted microscope (Nikon Ti2, 20 \times /0.75 objective or Zeiss Primovert, 10 \times /0.3 for locomotion on pure glass surfaces). Videos from the ventral view of the animal were recorded at 60 frames per second using a Basler acA2040-90um monochrome complementary metal-oxide-semiconductor (CMOS) camera. Several videos were obtained for each animal, such that 5 to 10 complete strides for each tardigrade could be extracted.

Kinematic and Statistical Analysis. Tardigrades were recorded walking spontaneously across gel substrates. We recorded trials of straight walking containing at least two complete cycles per leg. For each animal, between 5 and 15 complete cycles were recorded ($N=23$ animals on 50-kPa gel

substrate, $N=20$ animals on 10-kPa gel substrate, and $N=4$ animals on glass). Recorded videos from the ventral view were then evaluated frame-by-frame semiautomatically.

Exact time and location of leg liftoff (swing initiation) and touchdown (stance initiation) events, as well as frame-by-frame position of head, center of mass (COM), and tail, were visually determined and tracked using the ManualTracking plugin in ImageJ (44). Positions for head, COM, and tail were defined by the tip of the tardigrade’s nose, the midpoint between the second leg pair, and the point of attachment of the back leg pair to the tardigrade’s body, respectively. Tracking of leg kinematics was only done for videos taken on gel substrates; only COM position with time was recorded for glass trials. All measurements were made in ground-fixed coordinates. Data obtained in this manner were then processed using Python. All raw and processed data, as well as analysis code, are available at <https://github.com/jnirody/waterbears>.

A stride period for an individual leg was defined as the time difference between two consecutive liftoff events. Each stride comprises a stance and a swing. The durations of swing and stance were calculated as the time difference between a liftoff event and the subsequent touchdown (swing) or the time difference between a touchdown and the subsequent liftoff (swing). Temporal coordination of leg pairs was determined using the relative timings of swing onsets.

Step amplitude for an individual leg was determined as distance between the point of liftoff (PEP) and the location of the subsequent touchdown (AEP). As in ref. 13, we use step amplitude instead of stride length, which is calculated instead as the distance between two consecutive AEPs. This is because stride length is more affected than step amplitude by factors unrelated to active changes by the animal (e.g., slipping or the effects of a softer substrate). In contrast, step amplitude is more tied to an active change in the tardigrade’s kinematics.

Walking speed was calculated as the change in the measured position of the COM. The walking speed associated with a particular stride (e.g., as in Fig. 2 or *SI Appendix, Fig. S4 B and C*) was calculated as follows. We first determined the relevant time period (in the case of a stride period, this would be the time difference between two liftoff events). We then used the displacement of the COM between these two points to calculate an averaged speed over that interval. If walking speed was considered alone (e.g., as in *SI Appendix, Fig. S4A*), speed was averaged in the same manner over nonoverlapping 60-frame (1-s) windows.

Correlation coefficients between variables were calculated over the entire observed walking speed range; we used Spearman ρ to determine correlation due to the nonlinear relationships between several of our kinematic variables. Data in most plots presented were pooled across walking legs (the first three leg pairs). Leg-separated data are presented in *SI Appendix, Fig. S1*. Kolmogorov–Smirnov tests showed no significant differences in any kinematic parameter between legs after correction for multiple testing. Regression fit lines (e.g., in *SI Appendix, Fig. S1*) and density distribution fits to histogram data (e.g., in Fig. 5C and *SI Appendix, Fig. S2*) were computed using the Python package seaborn (45) and are intended to guide the eye. Joint distributions of phase angles (e.g., Figs. 3D and Fig. 4B and *SI Appendix, Fig. S6*) were computed using a kernel density estimate using Gaussian kernels; we note that this may have resulted in slight oversmoothing for the $\phi_C-\phi_I$ diagram on the 10-kPa substrate (Fig. 5B). To this end, the marginal distribution for ipsilateral phase differences is provided in Fig. 5C. All fits were computed using the Python package scipy stats (46).

Comparison of Duty Factor vs. Speed Relationship across Arthropod Species.

Duty factor is defined as the proportion of time spent in stance phase; specifically, for each stride, it was calculated as the ratio of the stance duration to the stride period. All statistics were computed as described above for other kinematic parameters. Duty factors for other arthropod species were extracted from published articles as cited. For some articles, tabular data were not available; in these cases, data were extracted from paper figures using the R package digitize (47). Kinematic data for *M. sexta* caterpillars was provided courtesy of Barry Trimmer. All numerical data used to make Fig. 3 are available at <https://github.com/jnirody/waterbears>.

Interleg Coordination and Gait Template Matching. For gait template matching we utilize a framework similar to that described in ref. 31. A gait was assigned to a block of time in which each walking leg completed one full stride; the boundaries of this period are from the earliest swing onset to the latest stance onset. To determine the assigned gait, phase relationships were calculated as the onset of swing relative to the stepping period of the right hind leg (R3). *SI Appendix, Table S1* shows the idealized step pattern

for each canonical gait; note that “tetrapod” actually comprises two mirror-image coordination patterns, denoted as “tetrapod 1” and “tetrapod 2.” Because perfectly synchronous swing movements were exceedingly rare in our data, and to account for human error in tracking, we tolerate a deviation from ideal swing relationships by ± 0.12 . When assigning a gait, we allowed one erroneous step for a single leg per full cycle. This is equivalent to the allowances given to stick insects in ref. 31.

Although we use the terminology often seen in the literature referring to the described canonical gaits for hexapodal walking, we note that this is purely for descriptive purposes. Insect (and likely panarthropod) walking is better represented by a continuum of ICPs rather than by distinct, discrete gaits. This is emphasized in our results by the fact that 56% of strides were unable to be classified into one of these three categories using the generous gait template matching framework described above.

Therefore, in order to more accurately describe the walking patterns observed during our experiments, we focused on measuring pairwise inter-leg coordination, particularly between ipsilateral and contralateral neighbors. We define an ipsilateral leg pair as two neighboring legs on the same side (e.g., R1 and R2) and contralateral leg pairs as two legs directly opposite each other (e.g., L2 and R2). Phase differences between leg pairs are denoted throughout as ϕ_i between ipsilateral leg pairs and ϕ_C between contralateral leg pairs. The leg within the pair which swings first within a

full cycle (comprising swing events of all six legs, as described above) is considered the reference leg; the phase offset is normalized with respect to its stride period. For example, let us consider the ipsilateral leg pairing (R1, R2); in our data the posterior leg is always the reference leg in ipsilateral leg pairings. Consecutive swing initiations of R2 demarcate the boundaries of the period $(t_0, t_1]$. Then, if R1 swings at time t_s , the phase difference ϕ_1^{R2-R1} is given by

$$\phi_1^{R2-R1} = \frac{(t_s - t_0)}{(t_1 - t_0)} \quad [1]$$

Phase differences between contralateral leg pairs are calculated equivalently.

Data Availability. Code and text data have been deposited in GitHub (<https://github.com/jnirody/waterbears/>).

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