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RESEARCH ARTICLE

Burrowing by small polychaetes – mechanics, behavior and muscle structure of *Capitella* sp.

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ABSTRACT

Worms of different sizes extend burrows through muddy sediments by fracture, applying dorso-ventral forces that are amplified at the crack tip. Smaller worms displace sediments less than larger worms and therefore are limited in how much force they can apply to burrow walls. We hypothesized that small worms would exhibit a transition in burrowing mechanics, specifically a lower limit in body size for the ability to burrow by fracture, corresponding with an ontogenetic transition in muscle morphology. Kinematics of burrowing in a mud analog, external morphology and muscle arrangement were examined in juveniles and adults of the small polychaete *Capitella* sp. We found that it moves by peristalsis, and no obvious differences were observed among worms of different sizes; even very small juveniles were able to burrow through a clear mud analog by fracture. Interestingly, we found that in addition to longitudinal and circular muscles needed for peristaltic movements, left- and right-handed helical muscles wrap around the thorax of worms of all sizes. We suggest that in small worms helical muscles may function to supplement forces generated by longitudinal muscles and to maintain hydrostatic pressure, enabling higher forces to be exerted on the crack wall. Further research is needed, however, to determine whether surficial sediments inhabited by small worms fail by fracture or plastically deform under forces of the magnitudes applied by *Capitella* sp.

KEY WORDS: Helical muscles, Peristalsis, Crack propagation, Functional morphology, Hydrostatic locomotion, Confocal laser scanning microscopy

INTRODUCTION

Burrowing infauna are morphologically diverse and range in body size over several orders of magnitude. Macrofauna initially populate muddy sediments as larvae or juveniles of similar size to the surrounding sediment grains but grow to a size for which the sediment can be considered as a bulk material or continuum of small particles (Dorgan et al., 2006). Not only do body sizes relative to sediment grains change ontogenetically but also the mechanics of burrowing through muds depend on body size (Che and Dorgan, 2010).

Mechanics of burrowing in muddy sediments

Muddy sediments respond to burrowing organisms as elastic solids through which burrowers move by propagating cracks (Dorgan et al., 2005). Stresses applied dorso-ventrally to burrow walls are amplified at the tip of the burrow and lead to anterior or lateral crack propagation if the critical stress intensity factor, the parameter used

to quantify fracture toughness of the sediment, is exceeded (Dorgan et al., 2007). This fracture releases stored elastic energy generated by forces acting on spatial scales much larger than single grains. Mechanical properties depend on adhesive and cohesive forces of the organic material that surrounds sediment grains (Dorgan et al., 2006) affecting kinematics and behaviors of burrowers (Dorgan et al., 2008). Burrowers using this mechanism correspondingly have wedge-shaped morphologies (Dorgan et al., 2007).

The mechanics of burrowing, and consequently the behaviors of burrowing animals, depend on body size (Che and Dorgan, 2010; Kurth and Kier, 2014; Quillin, 1999). From fracture mechanics theory, the work a worm needs to do to burrow in muddy sediments consists of two components, both affected by body size – the work necessary to extend the crack a certain distance, which depends on the fracture toughness of the sediment (the energy required to create new crack surfaces), and the work to deform the sediment to create space for the worm's body, which depends on the stiffness of the burrowing medium (Dorgan et al., 2008). Smaller worms apply smaller forces to the crack walls and may therefore be limited in their ability to exceed the critical stress intensity factor and burrow by fracture (Dorgan et al., 2008). In contrast, larger worms are predicted to be limited by the elastic work necessary to deform the sediment dorsally and ventrally to make space for the worm's body (Dorgan et al., 2008). Small specimens of the cirratulid polychaete *Cirriformia moorei* use their hydrostatic skeletons to increase the relative thickness of their thoraxes and show larger amplitudes of the peristaltic wave than larger worms, behaviors consistent with burrowing in a medium with high fracture toughness (Che and Dorgan, 2010). That small burrowing worms exhibit modified behaviors and body shapes is consistent with predictions from fracture mechanics theory. These predictions can be extended to suggest that there may be a minimum size for burrowing by fracture below which even modified behaviors cannot apply enough force to extend the burrow by fracture.

Smaller worms have been shown to use their hydrostatic skeletons to make their anterior ends thicker and blunter, facilitating burrow extension by fracture (Che and Dorgan, 2010). Additionally, smaller worms using pharynx eversions for crack propagation are expected to show relatively more cycles with pharynx eversions than large specimens, because it increases body thickness near the crack tip, facilitating crack extension (see Che and Dorgan, 2010). It is also plausible that ontogenetic changes in morphological shape or muscle structure, rather than or in addition to behaviors, may occur. The earthworm *Lumbricus terrestris*, one of the best known worms, showed small but significant deviations from isometric growth (Kurth and Kier, 2014). Previous work by Quillin (1998) had suggested that earthworms grow isometrically by maintaining a constant length-to-thickness ratio of the segments and increasing segment size rather than adding new segments over much of ontogeny. Investigating a greater size range revealed, however, that *L. terrestris* shows morphological differences as it grows that are consistent with the fracture theory, growing relatively longer and

thinner (Kurth and Kier, 2014). Relative cross-sectional areas of the longitudinal musculature were significantly greater and circular musculature was significantly smaller in smaller worms in contrast to expected geometric similarity (Kurth and Kier, 2014). These findings are consistent with the allometry of forces applied by burrowing earthworms; smaller earthworms exert relatively higher radial forces with longitudinal muscles (Quillin, 2000). Fracture mechanics theory predicts that smaller worms exert larger radial forces, which is supported by greater changes in thorax thickness for smaller than for larger burrowing worms (Che and Dorgan, 2010).

Worms too small to crack muddy sediments may use habitat selection to avoid the need to fracture, with small worms inhabiting and perhaps limited to less compacted surface sediments. The polychaete *Armandia brevis* uses body undulations to burrow in surface sediments, movements inconsistent with burrowing by fracture (Dorgan et al., 2013). Dorgan et al. (2013) suggested that the surface layer of marine sediments may comprise loosely compacted aggregates that are more granular and less cohesive than the subsurface muds in which linear elastic fracture occurs. Worms burrowed through a transparent granular material by plastically rearranging grains (Dorgan et al., 2013). It is plausible that other small worms may also rearrange unconsolidated surface sediment to plastically deform rather than fracturing it.

Here, we used the small-bodied, burrowing polychaete *Capitella* sp. to explore the hypothesis that a minimum body size exists below which burrowing by crack propagation is not feasible. Specifically, we examined burrowing behaviors and morphologies across body sizes of juveniles and adults to look for a transition point that may indicate a minimum body size required for burrowing by fracture. We explored four hypotheses (H1 to H4) to relate the range of body sizes of the *Capitella* sp. used in our study to a potential minimum body size for burrowing by fracture (Fig. 1). (H1) The range of *Capitella* sp. tested here includes a minimum size below which cracking the sediment is not feasible. In this case, some of the worms tested would be too small to be able to burrow by fracture whereas larger worms would be able to crack the sediment. We hypothesize that a transition in body shape, muscle morphology and/or burrowing behavior occurs that distinguishes worms too

small to burrow by fracture from worms that do burrow by fracture. (H2) *Capitella* sp. exhibits an ontogenetic gradient in geometry, behavior and morphology, consistent with the prediction from fracture mechanics that small worms are thicker, blunter wedges as shown for *C. moorei* by Che and Dorgan (2010). Smaller worms are predicted to have a relatively blunter thorax, more frequent pharynx eversions and a higher relative change in thorax length while burrowing than larger worms, enabling small specimens to apply enough force to fracture the sediment. (H3) All worms examined are able to burrow by fracture, but kinematics do not show as strong a dependence on body size as observed for *C. moorei*. Rather, *Capitella* sp. of all sizes exhibit some additional mechanism for burrowing by fracture. We specifically explored helical muscles as a structure to facilitate burrowing by fracture for small worms (see 'Helical muscles', below). (H4) All worms tested are too small to burrow by fracture, indicating that the minimum size for burrowing by fracture falls between the maximum for *Capitella* sp. and the minimum size of the cirratulids studied by Che and Dorgan (2010) or shows species-specific differences.

Helical muscles

In hydrostatic skeletons, two muscle layers running in different directions enclose a fluid cavity and act antagonistically (Kier, 2012). The body wall of most annelids comprises an outer layer of circular muscles and an inner layer of longitudinal muscles (Tzetlin and Filippova, 2005). In addition to the circular and longitudinal muscles, contracting alternately during peristalsis, *Capitella* sp. possesses helical muscles that wrap around the thorax in left- and right-handed helices, forming a lattice (Seaver et al., 2005). Those muscles clearly differ in position and orientation from the 'oblique muscles' that extend from the lateral to the ventral body wall through the coelomic cavity, e.g. in opheliid polychaetes (Law et al., 2014).

Helical muscles also occur in muscular hydrostats such as tongues, elephant trunks or appendages of cephalopod mollusks (Kier and Smith, 1985; Kier, 2012). Contraction of only right- or left-handed helical muscles results in torsion or twisting around the longitudinal axis (Kier, 2012). The muscle bands are usually located near the surface to increase the torsional moment (Kier, 2012).
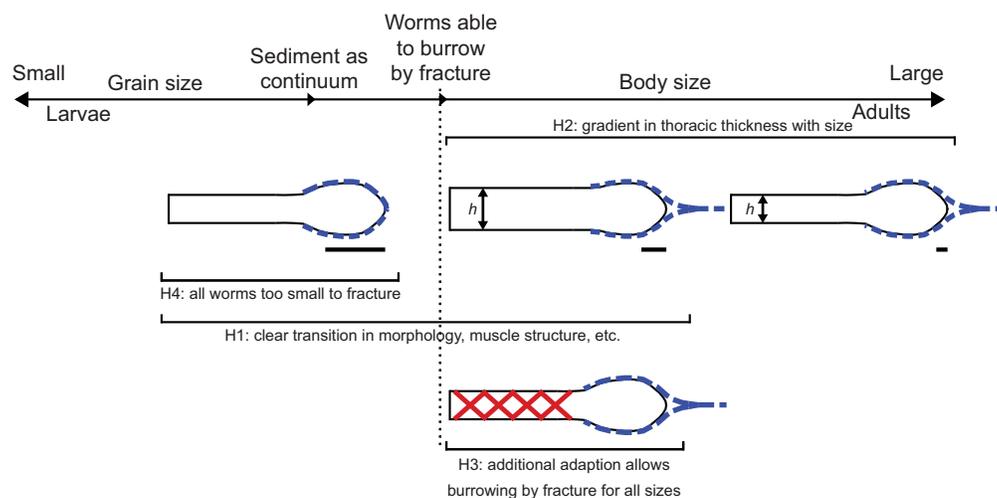

Fig. 1. Schematic diagram of the four hypotheses. (H1) A clear transition in behavior or morphology (e.g. thoracic shape or muscle structure) distinguishes large worms that are able to burrow by fracture (pointed, dashed blue lines outline crack wall) from small worms that are not (curved, dashed blue lines outline walls of burrow formed by plastic deformation). A scale bar of constant size (black line) illustrates the differences in body size of the illustrated worms.
(H2) Decreasing relative thickness (*h*) of the body and increasing bluntness of the anterior with increasing size facilitates fracture by small worms, consistent with the theory of fracture mechanics. (H3) An additional structure (e.g. helical muscle bands, red lines) enables worms over the entire examined size range to burrow. (H4) All worms examined are too small to crack the sediment.

Periodic twisting behavior has been described for other polychaetes, e.g. the orbiniid *Leitoscoloplos pugettensis* (Francoeur and Dorgan, 2014), although the musculature driving the twisting behavior of *L. pugettensis* has not been explored.

Connective tissue fibers can also form arrays of left- and right-handed helices around hydrostatic skeletons. These fibers are inextensible but allow changes in shape depending on hydrostatic pressure and fiber angle (Kier, 2012). When a helix with a fiber angle θ>54°44′ (54.73 deg) is placed under tension, the body elongates and θ decreases towards 54°44′ (Kier and Smith, 1985). In early tail-bud embryos of frogs, a sheath of helical, inextensible fibers with θ>54°44′ limits the change of shape during osmotic inflation in early morphogenesis, causing the notochord to straighten and elongate (Koehl et al., 2000). The burrowing caecilian (limbless amphibian) *Dermophis mexicanus* possesses a crossed-helical array of tendons around its body cavity that are connected to oblique body wall muscles (O'Reilly et al., 1997). Placed under tension by contracted muscles, the array of tendons with θ>54°44′ increases the hydrostatic pressure and elongates the body (O'Reilly et al., 1997). This elongation contributes to a forward-directed force twice as high as the maximum forward force that snakes possessing only circular muscles exert (O'Reilly et al., 1997).

To determine whether helical muscles in *Capitella* sp. are contracted on alternate sides to achieve twisting or simultaneously either to elongate or shorten the body, we observed burrowing behavior for evidence of twisting and also measured helical muscle angle using confocal laser scanning microscopy (cLSM). Whereas twisting of the ovoid body shape of *L. pugettensis* facilitates burrowing by fracture by enabling greater displacement of the burrow walls (Francoeur and Dorgan, 2014), twisting of the more cylindrical body of *Capitella* sp. would be unlikely to facilitate burrowing by fracture. If *Capitella* sp. uses the helical muscles similarly to the limbless caecilians (O'Reilly et al., 1997) to increase the forward force, helical muscle angles would be greater than 54°44′ from the longitudinal axis. However, small burrowing worms are limited by fracture toughness of the sediment, more specifically, by the distance they are able to displace sediment. Therefore, angles of muscle strands are expected to be lower in smaller worms, supplementing longitudinal muscles in expanding the worm to apply radial forces, facilitating fracture of the sediment.

This study relates morphology, muscle structure and burrowing behavior to body size to address the question of whether small polychaetes are able to burrow by crack propagation through muddy sediments and, if so, which features enable fracturing. In addition, this investigation increases the very small number of polychaete species for which the kinematics of burrowing by fracture has been examined.

RESULTS

Preliminary observations indicated that *Capitella* sp. of all sizes had some difficulty burrowing in gelatin, used in previous burrowing investigations (Che and Dorgan, 2010; Dorgan et al., 2005, 2007) as an analog for mud based on similar mechanical properties (same ratio of fracture toughness to stiffness) (Johnson et al., 2002). While the low rate of burrowing success indicated that the size range of worms used here may have been close to a minimum for burrowing by fracture in an elastic medium, attempts to relate body size to burrowing ability in gelatin were complicated by uncertainty of whether worms that did not burrow were unable or simply unwilling. Worms of a range of sizes, however, burrowed readily in a gel with lower fracture toughness and the same stiffness as gelatin, so this analog material was used for kinematic experiments. Whereas fracture toughness and stiffness are roughly linear in natural muds, considerable variability is found in both variables (Johnson et al., 2002); thus, our 25% decrease in stiffness likely remains within the range of natural sediment properties.

External morphology

Among 45 *Capitella* sp. individuals measured to test for geometric similarity, there was a nearly threefold difference in thoracic length. The widths of segments 1, 2, 5, 9 and 10 were measured and the width of segment 2, w_2, showed the highest correlation with thoracic length and was therefore used as the metric of body size for the rest of the analysis (Fig. 2). Regression slopes did not vary significantly among different segments (ANCOVA: no significant interaction, $P=0.91$) indicating that *Capitella* sp. grows isometrically (Fig. 3; data in supplementary material Table S1). We predicted from fracture mechanics theory that smaller worms would have thicker, blunter anteriors, but the slightly lower slope of the relationship between the width of segment 1 and thoracic length compared with that for other segments was not statistically significant.

Burrowing behavior

Videos of *Capitella* sp. burrowing in a clear mud analog (agarose–gelatin gel) showed that *Capitella* sp. burrows by retrograde peristalsis (a wave of contraction travels opposite to the direction of movement) and everts its pharynx while burrowing. All 13 worms for which 20 or more cycles could be analyzed showed cycles with pharynx eversion and cycles with no eversion. No twisting was observed in any of the 20 burrowing individuals.

Burrowing cycles are distinguished as alternating forward and smaller backward movements (Fig. 4; supplementary material Movie 1), similar to peristaltic burrowing by *C. moorei* (Che and Dorgan, 2010). Thoracic length showed a similar pattern to distance traveled (with coinciding local maxima), with a worm elongating to extend the burrow anteriorly (Fig. 4). Thorax length increased as the worm moved forward and peaked at the maximum distance traveled, then reached a minimum when or just after the worm slipped back to a minimum distance traveled. Some worms exhibited a regular pattern of burrowing with clear maximum and minimum thoracic lengths in each cycle and similar cycle periods (Fig. 4A), whereas others (generally those moving more slowly) exhibited greater variability in cycle period and movement patterns (Fig. 4B). Pharynx eversions in all 13 worms examined occurred as the worm slipped back and the thorax length decreased (Fig. 4), indicating that pharynx eversion does not extend the crack anteriorly; rather, it is used for lateral crack expansion. Worms exhibited some variability in pharynx eversion behavior,
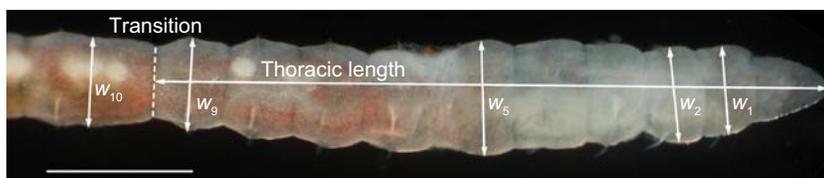

Fig. 2. Dorsal view of a representative specimen of *Capitella* sp. The widths (*w*) of segments 1, 2, 5, 9 (the last thoracic segment) and 10 (the first abdominal segment) and thoracic length (~1/4 to 1/3 of entire body length) are shown. The thoracic–abdominal transition is indicated with a dashed line. The region anterior to segment 1 includes the prostomium and peristomium. Scale bar, 0.5 mm.
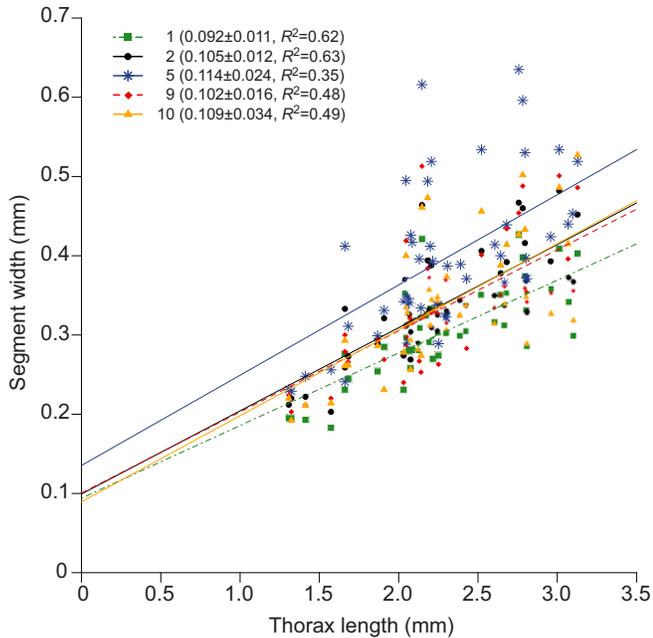
Fig. 3. Geometric similarity of *Capitella* sp. Widths of segments 1, 2, 5, 9 and 10 plotted as a function of thoracic length of anesthetized *Capitella* sp. with best-fitted linear regression lines (slope±s.e., and R^2, $P<0.001$ for all; $N=45$). Segment 10 is the first abdominal segment.

with pharynges everted partially to fully in different cycles (see supplementary material Movies 1 and 2 – pharynx eversions are visible as lighter body parts appearing lateral and/or anterior of the head). We did not quantify the extent of pharynx eversion.

None of the observed burrowing parameters, including the percentage of cycles with pharynx eversion, the maximum, minimum and difference in thoracic length, relative cycle distance and cycle period, correlate significantly with body size (Table 1, Fig. 5; data in supplementary material Table S2). *Capitella* sp. everts its pharynx in 30±12% (mean±s.d., $N=13$ individual worms) of the cycles independent of body size (Fig. 5A). Neither maximum nor minimum thorax length (as a percentage of thorax length in relaxed posture) while burrowing showed a significant correlation with body size (Fig. 5B). While burrowing, *Capitella* sp. stretches its thorax to 118±5% (mean±s.d.) of the minimum thoracic length. Relative cycle distance (Fig. 5C) and cycle period (Fig. 5D) did not change in relation to body size.

Muscle structure

Muscle morphology, examined using cLSM, was qualitatively similar for all worms, with no obvious differences in the relative size of longitudinal and circular muscles among worms of different sizes. Directly beneath the epidermis is a layer of circular muscles, beneath which bands of longitudinal muscle appear. Helical muscle strands lie between circular and longitudinal muscle layers in the anterior of the worm and are oriented in both right-handed and left-handed helices (Fig. 6). These helical muscles are fairly evenly spaced, forming a very regular lattice (especially apparent in Fig. 6B). The angle of helical muscle strands θ (mean of 6–20 muscle strands or fiber bundles per worm) of 45.3±10.8 deg (mean± s.d.; $N=9$ individual worms) is not correlated with body size (Fig. 7A; data in supplementary material Table S3) ($P=0.85$). This mean angle is significantly lower than 54°44′ (=54.73 deg), although for two of the nine individual worms, θ did not differ significantly from 54.73 deg (two-sided *t*-test, $P<0.05$; Fig. 7A).
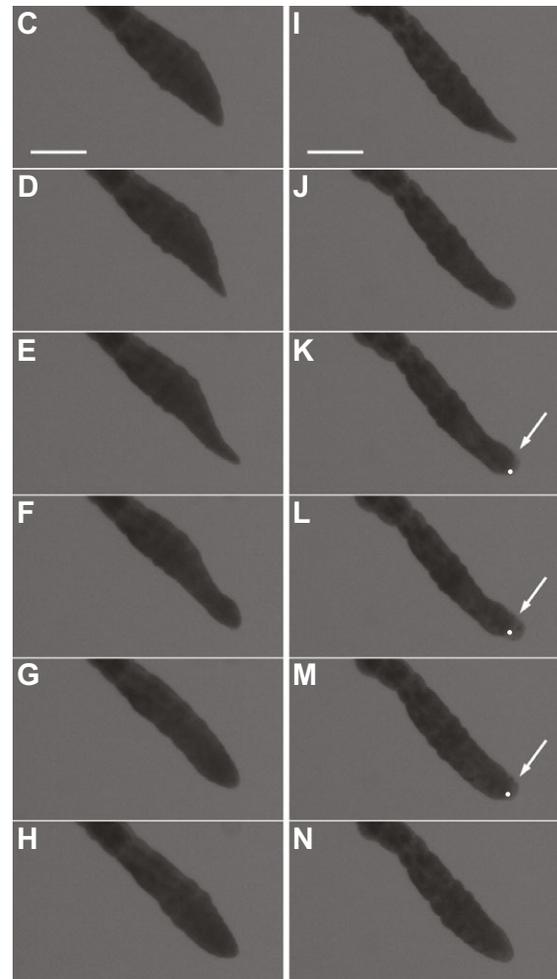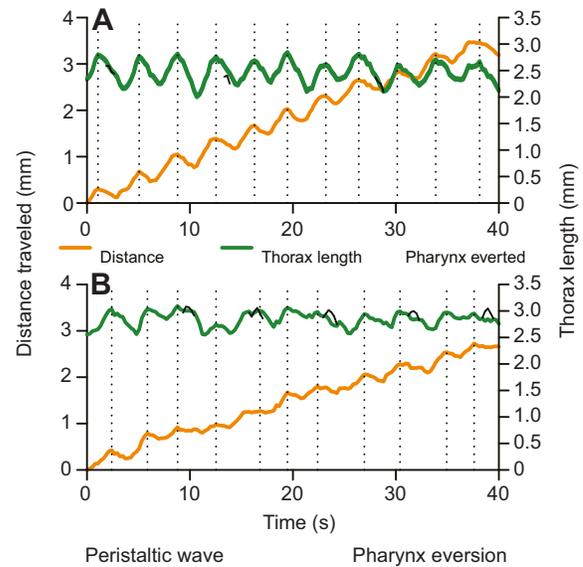

Fig. 4. Burrowing behavior. (A,B) Distance traveled (starting at 0) and thorax length while burrowing in a mud analog (gelatin–agarose) plotted as a function of time for two representative worms. (A) $w_2=0.394$ mm; (B) $w_2=0.541$ mm. Vertical lines mark local maxima of thorax length. (C–H) Sequence of images showing a peristaltic wave (same worm as in B and supplementary material Movie 1). (I–N) Sequence of images showing a pharynx eversion (same worm as in B and supplementary material Movie 1) with the tip of the head shown as a white dot and arrows indicating the everted pharynx. Less prominent pharynx eversions can be seen in supplementary material Movie 2. Scale bars, 0.5 mm.

Table 1. Predicted relationships between burrowing parameters and body size based on kinematic similarity and fracture mechanics compared with results of the burrowing experiment with respective statistics (cf. Fig. 5)

Parameter	Prediction		Justification for fracture mechanics prediction	Results	Statistics
	Kinematic similarity	Fracture mechanics			
Normalized minimum thorax length	=	+	A shorter and thus wider thorax would displace the burrow walls, increasing the force exerted and facilitating crack propagation	n.c.	P=0.075 R^2=0.185 N=18
Normalized maximum thorax length	=	= (?)	Smaller worms are predicted to have shorter thoracic lengths (above) but also greater variability in thorax length, potentially resulting in similar maximum length	n.c.	P=0.084 R^2=0.175 N=18
% Difference in thorax length	=	−	Greater variation in length would accompany greater variation in thickness, which facilitates fracture for small worms	n.c.	P=0.51 R^2=0.027 N=18
Cycle period	=	=	Variability in timing of burrowing cycles is unlikely to affect fracture	n.c.	P=0.73 R^2=0.008 N=18
Normalized cycle distance	=	+	Increased thickness and muscle mass facilitates fracture	n.c.	P=0.791 R^2=0.009 N=10
Cycles with pharynx eversion	=	−	The everted pharynx can increase forces exerted on the burrow walls, facilitating fracture	n.c.	P=0.42 R^2=0.060 N=13

Prediction values are based on kinematic similarity and fracture mechanics. +, increase with increasing body size; −, decrease with increasing body size; =, no change with body size.
Results are based on the burrowing experiment. n.c., no correlation with size.
Statistics are given for linear regression with body size. N, number of worms analyzed for each parameter. The size range of worms examined was 0.27 mm $\leq$ w_2 $\leq$ 0.54 mm (where w_2 is the width of segment 2) for all parameters except cycles with pharynx eversion, for which the size range was 0.20 mm $\leq$ w_2 $\leq$ 0.54 mm.

The number of helical muscle strands in each segment decreases from the anterior towards the transition between the thorax and abdomen, but shows high variability (Fig. 7B). No helical muscles were found in the abdominal regions of any worms examined. We were unable to determine from cLSM images the number of segments that each helical muscle strand spans.

DISCUSSION

Theory indicates that a minimum size for burrowing exists, but predicting an exact minimum size for burrowing is complicated by the ability of soft-bodied animals such as worms to change body shape. We hypothesized that the size range of worms examined may include this minimum size for burrowing by fracture. Bigger *Capitella* sp. specimens were able to burrow in gelatin but did not do so readily, which suggests that this size range may be close to but not below the theoretical minimum size for burrowing by fracture. Worms of all sizes, from the smallest juveniles in our culture (with nine thoracic segments) to reproductive adults, burrowed in the clear mud analog with lower fracture toughness, indicating that the minimum size, if it exists, must be smaller than the examined size range. This minimum size from our culture (0.203 mm) coincides with the smallest size class (0.2–0.25 mm thoracic width) included in a population study of *Capitella* cf. *capitata* in Tomoe Cove, Japan (Tsutsumi, 1987). This minimum size is considerably smaller than the *C. moorei* analyzed by Che and Dorgan (2010) with segment widths (setiger 8) of 1–3 mm. We caution, though, that by altering the properties of the burrowing medium (lower fracture toughness), we likely also lowered the minimum body size for which burrowing by fracture is feasible, perhaps enabling small specimens to burrow that would not be capable of fracture in their natural environment. We hypothesized that a transition in behavior and muscle morphology at a given size would be apparent (H1), but our data showed no transitions in behavior, muscle structure or morphology. We cannot eliminate the possibility that a transition below this size range occurs in other species. That worms seemed to be burrowing

near the limit of their physical ability in the modified gel indicates that it is a good model system for testing the remaining hypotheses.

We also hypothesized that small worms burrowing by fracture would exhibit anterior allometry and ontogenetic changes in burrowing behavior consistent with the theory of fracture mechanics (H2). Linear correlations between segment widths and thoracic lengths reveal that *Capitella* sp. grows isometrically from juvenile to adult in the examined size range. The anterior-most segment does exhibit a slightly lower regression slope, however, consistent with the statistically significant allometry shown for earthworms that spanned a much larger range in body size than our *Capitella* sp. (see Kurth and Kier, 2014). In contrast to our expectations that body size would be more important for smaller worms, there seemed to be less dependence of burrowing parameters on body size for the smaller *Capitella* sp. than for *C. moorei*, for which a similar range of relative sizes were compared (Che and Dorgan, 2010). Whereas smaller *C. moorei* exhibited relatively thicker anterior regions than larger worms, consistent with predictions from fracture mechanics (Che and Dorgan, 2010), thorax lengths of *Capitella* sp. were not shorter for smaller worms (Table 1, Fig. 5B). Similarly, the thickness of smaller *C. moorei* varied more over a burrowing cycle than that of larger worms (Che and Dorgan, 2010), whereas no relationship between the difference in thorax length and body size was apparent for *Capitella* sp. (Table 1, Fig. 5B). We expected more cycles with pharynx eversions for smaller specimens (Table 1), enabling them to exert higher forces on the crack walls, but our data do not indicate any relationship with size. However, unlike *Alitta virens*, which extends the burrow with its everted pharynx (Dorgan et al., 2007), pharynx eversion by *Capitella* sp. does not extend the burrow anteriorly. It may contribute to lateral crack extension (crack edges were difficult to see around these small worms), but this is predicted to be less size dependent than anterior extension – lateral crack extension reduces the elastic restoring force compressing worms dorso-ventrally, which is more important for larger worms (Che and Dorgan, 2010;
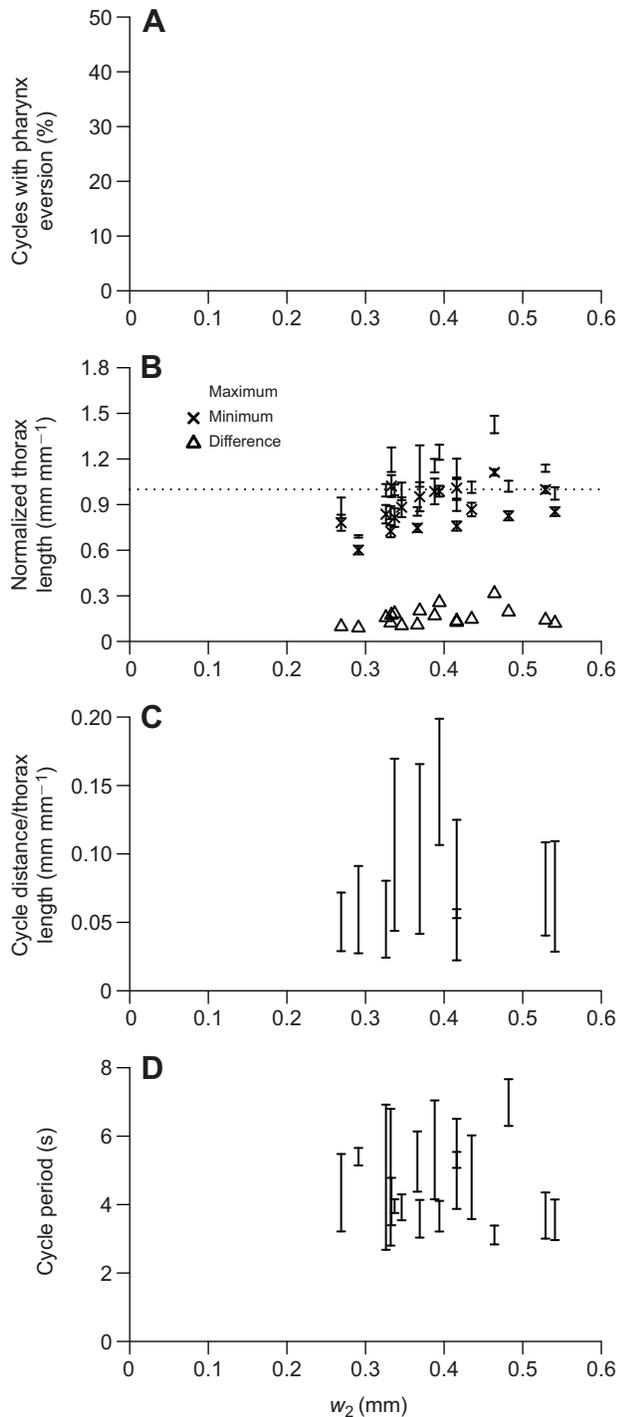
Fig. 5. Burrowing parameters plotted as a function of body size (w_2).
(A) Percentage of cycles with pharynx eversion (N=13). (B) Maximum and minimum thorax length (means±s.d.) and difference between maximum and minimum thorax length normalized by relaxed thorax length while burrowing (N=18). Horizontal dotted line at 1 indicates thorax length equal to relaxed thorax length. (C) Cycle distance normalized by relaxed thorax length (means±s.d.) (N=9). (D) Cycle period (means±s.d.) (N=18).

Fig. 6. Confocal images of the body wall musculature of *Capitella* sp. stained with phalloidin. (A) Anterior two segments (w_2=0.453 mm). (B) Anterior three segments (w_2=0.259 mm). (C) Segments 4–6 (w_2=0.273 mm). (D) Segments 4 and 5 (w_2=0.371 mm). CM, circular muscle; HM, helical muscle; LM, longitudinal muscle; θ, helical muscle angle. All worms are positioned with their anteriors to the left. Scale bars, 100 μm.

Dorgan et al., 2008). Also, head geometry while burrowing has not been analyzed for *Capitella* sp. Worms can use a wider body and pointed head to focus stress and extend a very narrow crack that is then extended laterally on alternate sides and/or in small increments (Che and Dorgan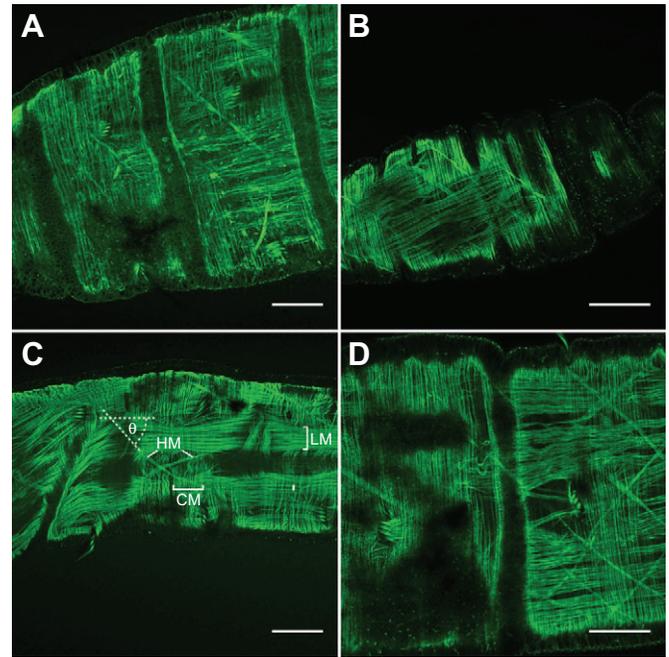, 2010). Although all of our worms were collected from the same site and cultured in the lab, *Capitella* sp. is part of a species complex (Grassle and Grassle, 1976), and we cannot discount the possibility that our specimens may belong to more than one species, which might contribute to greater variability in morphology and behavior.

Helical muscles as a functional structure enabling small polychaetes to burrow

We found neither a transition in burrowing mechanisms nor a gradient in body thickness, muscle morphology or body shape consistent with predictions by fracture theory for small worms burrowing by fracture. The decreasing number of helical muscles from the anterior of the worms towards the transition between the thorax and abdomen, however, is consistent with a function of these muscles in burrowing, as posterior segments are likely less important for burrowing than anterior segments (e.g. Seymour, 1969). *Capitella* sp. exhibited no twisting behavior while burrowing that would be consistent with a torsion or twisting function of helical muscles, as in squid arms (Kier, 2012) or the behavior of the polychaete *L. pugettensis* (Francoeur and Dorgan, 2014). We cannot, however, eliminate the possibility that *Capitella* sp. does use twisting movements in its natural, more heterogeneous, environment. The absence of helical muscles in any other polychaete yet studied is also consistent with, albeit weak support for, the hypothesis that helical muscles serve as an additional structure enabling small polychaetes to burrow by fracture.

The helical muscles of *Capitella* sp. are oriented at θ=45.3± 10.8 deg (mean±s.d.) from the longitudinal axis, which is significantly lower than 54.73 deg. This more longitudinal orientation means that the helical muscle bands likely supplement longitudinal muscles in shortening and fattening the body. Simultaneous contraction of helical muscle with longitudinal muscle (Fig. 8A,B) would increase thoracic thickness and the
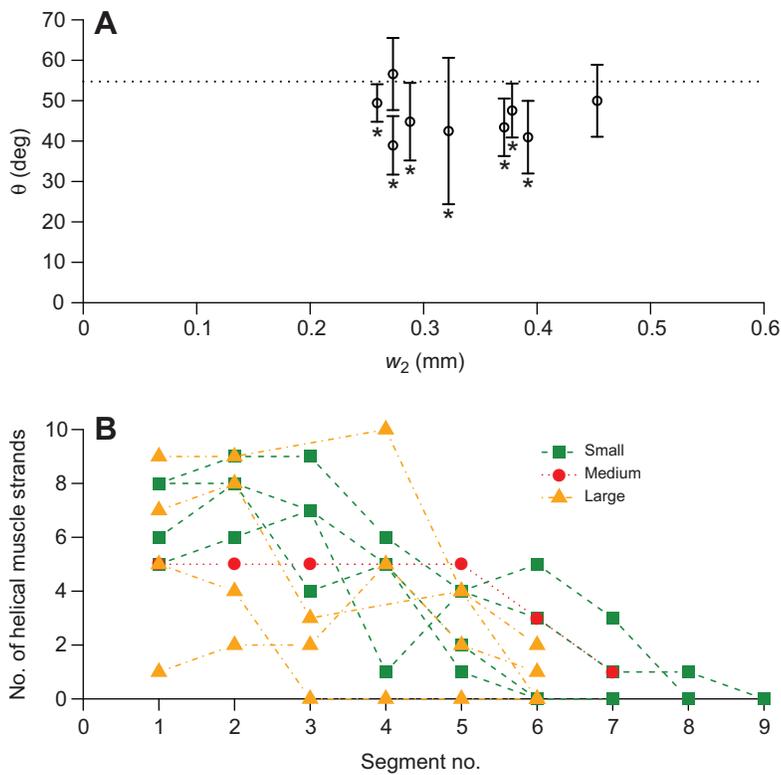
Fig. 7. Helical muscle bands of *Capitella* sp. (A) Angle θ of helical muscle bands (means±s.d., *N*=9). Specimens with asterisks differ significantly from 54°44′ (54.73 deg, dotted line). (B) Number of helical muscle strands (both left- and right-handed orientations) visible in each segment for different size classes (small<0.290 mm *N*=4, medium=0.322 mm *N*=1, large>0.370 mm *N*=4). Note that only fibers in the ventral or dorsal side were counted; the total number would be twice as high.

forces exerted on the crack walls, facilitating burrow extension by fracture. Helical inextensible tendons in the burrowing caecilians oriented at a higher θ of 60 deg from the longitudinal axis support transversely oriented muscles in decreasing diameter and generating forward thrust in the burrowing direction (O'Reilly et al., 1997). If helical muscles of *Capitella* sp. instead were oriented at θ>54.73 deg, contraction would supplement circular muscle to elongate the body (Fig. 8D).

Helical muscles oriented at θ=54.73 deg would be consistent with limiting shape changes and stiffening the hydrostatic skeleton, as observed for inextensible helical connective tissue around frog notochords and nemerteans (Clark and Cowey, 1958; Koehl et al., 2000). This function does not seem to be consistent with the peristaltic behavior of *Capitella* sp. Especially for a small worm trying to exert large forces onto the crack wall, limited shape change would restrict forces, and limit rather than enhance smaller worms' ability to fracture the sediment. Finally, helical muscle bands are an unlikely substitute for inextensible fibers used to control shape changes as muscle is energetically expensive.

Helical muscles oriented at θ=54.73 deg in relaxed animals can, however, be interpreted as facilitating peristaltic movements. When segments shorten, the angle of the helical muscle increases above 54.73 deg so that subsequent contraction of helical muscle elongates the segment. Similarly, elongated segments have helical muscles that are oriented more longitudinally and would therefore supplement longitudinal muscles in shortening the body. This fluctuation in fiber angle is amplified in muscle compared with inextensible fibers as contraction of helical muscle shortens and relaxation extends the length of the muscle fiber. Thus, whereas a hydrostat lined with helical inextensible fibers would experience a loss of turgor pressure accompanying an increase in fiber angle from <54.73 deg to 54.73 deg, contraction of helical muscle would shorten the total fiber length and potentially maintain a rigid body shape. Helical muscles that oscillate in fiber angle around 54.73 deg

(Fig. 8C) could supplement the forces exerted by alternating contraction of both circular and longitudinal muscles during peristaltic movements. This potential, novel function of helical muscles would be expected in worms of intermediate body size for which enhanced radial expansion would facilitate cracking sediments and elongation may facilitate larger steps in each burrowing cycle. The use of one additional muscle type to supplement two other muscles types could potentially save expensive muscle mass. During peristalsis, helical muscles would theoretically be contracted until reaching 54.73 deg, then would relax and elongate (Fig. 8C). For *Capitella* sp., we calculated that a decrease of thoracic length of 12.5% (12.5±13%, mean±s.d., *N*=18) relative to the relaxed length of specimens examined with cLSM would increase the helical muscle angle from 45.3 to 49.5 deg, with a maximum (at +1 s.d.) of 52.5 deg, still not exceeding 54.73 deg. That the fibers are much thinner than longitudinal muscles and oriented such that only part of their force is aligned longitudinally (Fig. 6) suggests that the overall contribution of helical muscle to the radial expansion force for *Capitella* sp. may be small and therefore less important than the potential for helical muscles to fine-tune the turgor pressure of the hydrostatic skeleton and maintain body rigidity over an entire peristaltic cycle.

While the anterior position and orientation of helical muscles is consistent with the idea of helical muscles being a supplementary structure for small worms to burrow by fracture, the question of whether helical muscles are indeed limited to small-bodied polychaetes and whether the fiber angle differs in larger worms remains unanswered. Helical muscles seem to be unusual among polychaetes, having been explicitly described only in *Capitella* sp. (Seaver et al., 2005). But polychaete musculature has been primarily investigated using dissection and histology methods (Mettam, 1971; Purschke and Müller, 2006; Storch, 1968; Tzetlin et al., 2002a,b) that have some limitations in finding weakly developed muscles (Tzetlin and Filippova, 2005). Moreover, most tissue
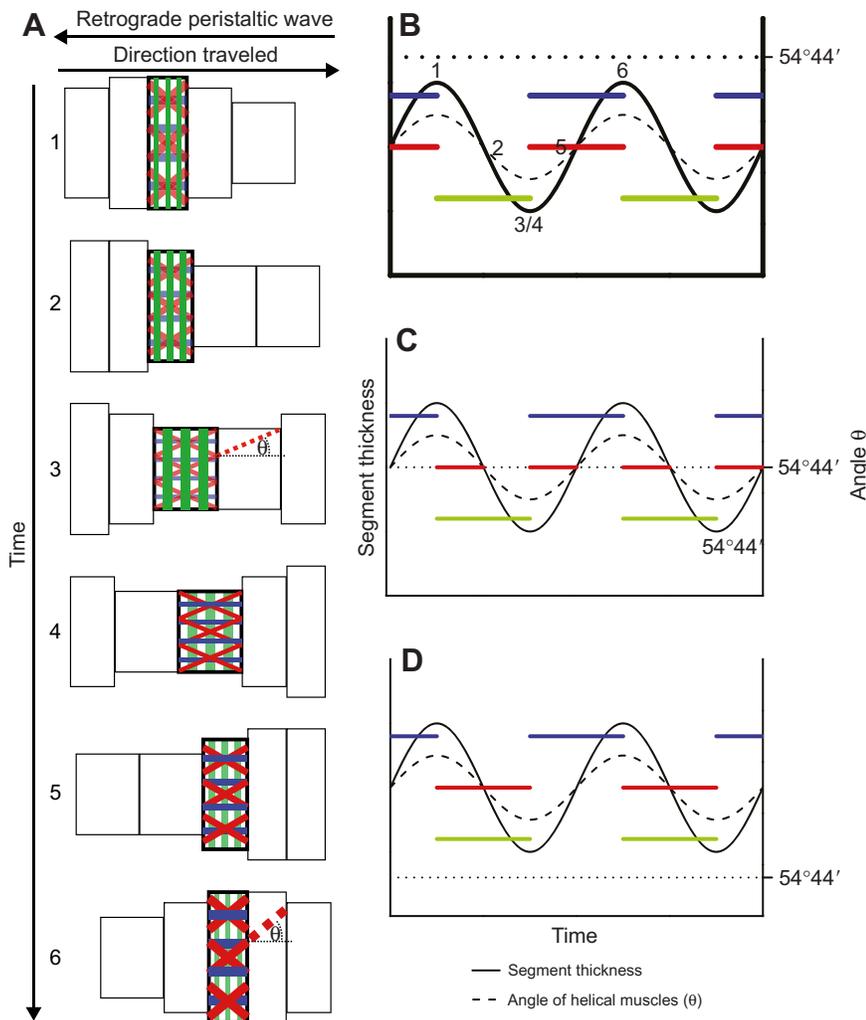
Fig. 8. Schematic diagram of the suggested functions of helical muscle bands while burrowing. (A) Retrograde peristaltic wave with shape change of segments. Contracting muscles (blue, longitudinal muscle bands; red, helical muscle bands; green, circular muscle bands) are opaque and overlay more transparent relaxed muscles. Muscle bands are shown thicker when contracted and thinner when elongated. Over one cycle, (1) the thick and short segment anchors the body in the sediment, and circular muscles (green) start contracting. (2) Contracting circular muscles elongate the segment until it is long and thin. (3) Helical muscle angle θ decreases as the segment elongates. (4) Longitudinal muscles (blue) supplemented by helical muscles (red) begin contracting, shortening and dilating the segment (5), until it reaches the initial shape (6). (B) Segment thickness and fiber angle θ during peristalsis plotted over time with muscle contraction shown as colored lines for helical muscle angle of *Captella* sp. θ<54°44′ (54.73 deg). The six time periods for the segment illustrated in A are indicated. (C) Predicted pattern of muscle contraction during peristalsis if fiber angle were close to 54°44′ and oscillated higher in amplitude when the segment was dilated and lower when the segment was elongated. Note that the *y*-axis showing θ has changed. Helical muscle close to 54°73′ could alternate between supporting circular and longitudinal muscle bands over a cycle of peristalsis. (D) Predicted pattern of muscle contraction if θ>54°44′. Helical muscles would supplement circular muscles to elongate segments and would contract simultaneously. θ=54°44′ is shown as a horizontal dotted line in B–D.

studies use cross-sections or sagittal sections in which helical muscles are not evident without elaborate reconstruction over multiple sections. In cross-sections of a worm, transverse muscle bundles would be in the section plane so could be recognized easily. A helical muscle bundle, however, would be difficult to distinguish from a longitudinal muscle bundle, unless the elliptical shape of the oblique could be discerned. Recent application of cLSM (e.g. Tzetlin et al., 2002a,b) enables visualization of even small muscular structures (Tzetlin et al., 2002a,b). cLSM, however, is limited by light penetration through tissue to small-bodied animals, and only a few species have been studied in detail, e.g. *Magelona* cf. *mirabilis* and *Prionospio cirrifera* (Filippova et al., 2005), *Nerilla antennata*, *Nerillidium* sp. and *Trochonerilla mobilis* (Müller and Worsaae, 2006). We were able to find helical muscles in preliminary investigations of *Capitella* sp. using traditional histological methods, but only in a few sagittal sections through the body wall and after knowing of their existence through cLSM. These very thin diagonal muscles would have been challenging to interpret as helical without first observing them with the cLSM.

Our findings highlight a potential novel role of helical muscles in peristaltic burrowing and raise the question of whether these muscles are indeed a mechanism for enhancing burrowing ability for small worms. Investigation of the body wall musculature of burrowers of various sizes is needed to determine whether helical muscles are limited to small polychaetes or are found in peristaltic

burrowers of all sizes. Large worms would be expected either to lack helical muscles or to possess muscle bands oriented at or greater than 54.73 deg. Peristaltic burrowers exhibiting relatively large changes in body shape could use helical muscle oscillating around 54.73 deg to supplement both circular and longitudinal muscles and maintain hydrostatic pressure throughout the peristaltic cycle. Because behaviors of even very small worms are consistent with the mechanism of burrowing by fracture in sediment, the need for better understanding of the mechanical responses of sediments on these small scales is fundamental.

MATERIALS AND METHODS
Animals
Individuals of the polychaete *Capitella* sp. were collected from the upper 2–3 cm of intertidal mud near the Cabrillo Marine Aquarium, Los Angeles, CA, USA. *Capitella* sp. is very abundant and easy to maintain in cultures, enabling use of a size range from juveniles to adults. Specimens were maintained at 15°C in containers with sieved, frozen and thawed sediments from the collection site. Sediment was changed weekly and ground fish food (Tetramin, Tetra, Melle, Germany) was added.

External morphology
External morphology was investigated by measuring length and width of several segments along the worm to determine whether body shape changes as the worms grow. Individuals of *Capitella* sp. (thorax length 1.31–3.82 mm, w_2 0.203–0.482 mm) were sieved out of the mud; 45 specimens

were relaxed in a 7% magnesium chloride solution for 10 min. Relaxation of the worms appeared to change body shape very little but ensured that body shapes were consistent (Fig. 2). Photographs of those 45 specimens were taken with a Canon EOS Rebel T2i digital SLR (Canon Inc., Tokyo, Japan) camera mounted to the Leica MZ95 dissecting microscope (Leica Camera AG, Solms, Germany). ImageJ (version 1.45s, W. S. Rasband, ImageJ, National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/) was used to measure thorax lengths and segment widths from photographs. Widths of thoracic segments 1, 2 and 5 as well as the last segment of the thorax (segment 9) and the first segment of the abdomen (segment 10) were measured.

Segment width was plotted against thorax length to test for geometric similarity. Linear regressions were calculated for the width of each measured segment as a function of thorax length and an ANCOVA was performed in R (R Core Team, 2014) to test for difference of slopes. The segment whose width showed the strongest relationship with thorax length was chosen as the metric of body size for ontogenetic analysis.

Burrowing medium

Following methods from Dorgan et al. (2008), we developed a combination of gelatin and agarose that matched the stiffness of gelatin but with a lower fracture toughness. We tested several concentrations of gelatin and agarose and evaluated them based on measurements of stiffness and observations of worms burrowing (with qualitative assessment of 'willingness' to burrow).

To measure stiffness of different burrowing media, a custom-made force–displacement device was used. A 6 mm diameter ball-bearing was attached to a rigid probe and extended using a linear actuator (L12-P, Firgelli Technologies Inc., Victoria, BC, Canada) connected to a PC with an actuator control board (Firgelli Technologies Inc.). An in-line force sensor (Futek LSB200 JR S-beam load cell, Futek Advanced Sensor Technology, Inc., Irvine, CA, USA) measured force and was connected to a PC using the Futek USB210 connector. Both the actuator and force sensor were controlled using custom-written LabView (v8.2, National Instruments, Austin, TX, USA) software. The ball-bearing was lowered to contact the surface of the gel, then lowered either 4 or 5 mm with the actuator, held for >5 s to obtain an average force measurement, then raised. Force–displacement data were analyzed with Matlab (R2012a; The MathWorks, Inc., Natick, MA, USA). The finite element program franc2d (Cornell Fracture Group: www.cfg.cornell.edu) was used to convert the force divided by distance value to stiffness (see Dorgan et al., 2008).

To create a burrowing medium with lower fracture toughness but similar stiffness to gelatin (and therefore a lower ratio of fracture toughness to stiffness than natural sediments), we first determined the concentration of agarose that yielded a gel with comparable stiffness to gelatin, then mixed the gelatin and agarose in different proportions and assessed the willingness of worms to burrow. Natural sediments vary considerably in fracture toughness and stiffness, although the ratio of the two is roughly similar (Barry et al., 2013); gel mimics have lower values of stiffness and fracture toughness but similar ratios to natural sediments (Dorgan et al., 2008). Different concentrations of agarose (Apex Bioresearch Products, Inc., North Liberty, IA, USA) in seawater were boiled and cooled overnight, and stiffness was compared with that of gelatin (28.35 g l^{-1}, stiffness of 7100± 372 Pa, mean±s.d.) (Natural Foods Inc., Toledo, OH, USA) used in previous experiments (Dorgan et al., 2008). The concentration of 2.5 g l^{-1} agarose in seawater showed similar stiffness to gelatin. Next, the 2.5 g l^{-1} agarose was mixed with gelatin in two different proportions: 50% gelatin with 50% agarose and 75% agarose with 25% gelatin. Agarose and gelatin were boiled separately, mixed together, stirred well and stored at 4°C overnight. The concentration of agarose was adjusted slightly to keep the stiffness similar to that of gelatin alone. The 75% agarose and 25% gelatin mixture (39.60 g l^{-1} gelatin in seawater and 3.50 g l^{-1} agarose in seawater) exhibits a stiffness of 6272±676 Pa (mean±s.d.) and was chosen because more worms burrowed in this medium.

Experimental setup

The experimental setup followed methods from Che and Dorgan (2010). A clear glass aquarium with inside dimensions 0.072 m wide×0.072 m deep×0.10 m high was filled with either gelatin or gelatin–agarose. The glass container was placed between a Porta-trace 25×30 cm light table (Gagne, Inc., Johnson City, NY, USA) and a CCD videocamera (Basler A622f, Exton, PA, USA) with a 6× close-focus zoom lens with fixed focal length (Edmund Optics no. 52-274, Barrington, NJ, USA), and a 2× C-Mount Lens Extender, which increased the magnification, enabling better visualization of the small worms. Experiments were conducted in a cold room at 15°C. Worms of varied size were sieved out of the mud, left in seawater until they were free of sediments stuck to them with mucus, then put in a crack just large enough to fit the entire worm that was made with forceps in the surface of the gelatin–agarose. Videos of burrowing worms were acquired using LabView (version 7.1.1) at 7.5 frames s^{-1}. The camera or aquarium was moved to keep the worm in focus, ensuring a constant distance between the worm and camera for calibration. The distance was adjusted when the worm burrowed out of focus, and the camera was moved vertically when the worm reached the upper or lower edge of the camera's field of view. Worms that burrowed (20 individuals) were anesthetized in 7% MgCl$_2$, photographed for later measurement of thorax length and segment width, then preserved in 95% ethanol and stored at 4°C.

Video analysis

In previous analyses of burrowing kinematics of worms in gelatin, dorsal and lateral view videos were analyzed separately for width and thickness, respectively (Che and Dorgan, 2010; Dorgan et al., 2007). The small size of *Capitella* sp., relatively cylindrical morphology and very small lateral parapodia that were not visible in videos made distinguishing between dorsal and lateral views of burrowing *Capitella* sp. challenging. The transition between thorax and abdomen, however, could be seen clearly in videos as a visible constriction in the intersegmental zone with different patterns of peristalsis in the thoracic and abdominal regions. Hence, the thorax length over the burrowing cycle could be measured. Because the segments are separated by septa, and the internal fluid is incompressible, the thorax has a constant volume and the thorax length is negatively correlated with width and thickness as the worm changes shape.

Video segments in which worms were burrowing in focus and in the field of view of the camera were subsampled using LabView. For each specimen, one sequence was chosen for analysis in which the worm burrowed the straightest with head to thoracic-abdominal transition in line in a plane normal to the camera's line of sight and where at least four cycles were recorded. Only video segments with a visible transition were analyzed (*N*=18). Video segments were resolved into frames using VirtualDub (version 1.9.11, Avery Lee, http://www.virtualdub.org). Stacks were loaded into ImageJ and the positions of the transition, anterior tip of the head and the pharynx when everted were tracked in every other frame using the Manual Tracking plugin (Fabrice Cordelieres, Institut Curie, Orsay, France). Thorax length was calculated from the positions of the head and thoracic-abdominal transition with up to three points in between, depending on the curvature of the worm's thorax, and was plotted over time. Burrowing cycles were distinguished using the plot of thorax length as a function of time. One cycle extends between two successive minimum thoracic lengths. Thorax length was used as the indicator for the burrowing cycle rather than distance traveled as it reflects peristalsis whereas distance traveled represents the result of this movement.

Minimum and maximum thorax length as well as mean cycle period were calculated for each individual (*N*=18). Minimum and maximum thorax length was divided by relaxed thorax length (measured from anesthetized worms after burrowing) to acquire relative values. The percentage difference in thorax length while burrowing was calculated (*N*=18) as the difference between maximum and minimum thorax length while burrowing, normalized by minimum thorax length. Mean cycle distance was analyzed for worms that burrowed along a straight line (*N*=10). The position of the tip of the head at minimum thorax length of sequential cycles was determined and linear distance calculated. Mean cycle distance for each individual was divided by relaxed thorax length to obtain relative cycle distance (*N*=10). For the six out of 10 worms that everted their pharynges during the analyzed sequence (all 10 having everted their pharynges at some time during burrowing), we also plotted thorax length and distance traveled using the anterior tip of the everted pharynx.

Relationships between body size and several kinematic parameters were assessed and compared with predictions based on a null hypothesis of

kinematic similarity, or no effect of body size on burrowing, and an alternative hypothesis based on predictions from fracture mechanics (Table 1). From fracture mechanics theory, smaller *Capitella* sp. specimens are expected to be relatively blunter and thicker and show greater variation in thickness than larger worms (Che and Dorgan, 2010). Regression analyses were conducted using R with α=0.05 (see Table 1).

Preliminary observations showed that *Capitella* sp. uses pharynx eversion in some, but not all, burrowing cycles. The percentage of burrowing cycles with pharynx eversions was calculated for worms for which at least 20 cycles could be acquired (26–237 cycles for each worm, N=13). The null hypothesis that there is no correlation between the percentage of pharynx eversion and body size was tested using R, linear regression with a significance level of α=0.05.

cLSM

To investigate muscle structure, specimens of *Capitella* sp. were kept in seawater overnight or until their guts were empty, then fixed and muscles stained with phalloidin, which binds to F-actin. A confocal laser scanning microscope was used to visualize the body wall musculature. Body wall musculature of worms of varied sizes was qualitatively assessed for differences in the relative size of circular and longitudinal muscles and any other differences in muscle morphology. Numbers and angles of helical muscle strands were measured.

Specimens of *Capitella* sp. were starved in a container without sediment for at least 24 h until no sediment grains were visible in the worm's gut. Worms were photographed after relaxing for about 10 min in $MgCl_2$ to determine size. The staining procedure was adapted from Hochberg (2005) and Müller and Worsaae (2006). Worms were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.15 mol l^{-1} phosphate buffer (PBS) with 12% sucrose. Fixation lasted 2 h at room temperature (30 min initially, then 90 min rotating) followed by three 10 min glycine washes [50 mmol l^{-1} glycine (Sigma-Aldrich Co., St Louis, MO, USA) in 1×PBS] and three 10 min 1×PBS washes. After 1 h in PBT ([PBS containing 0.1% Triton X-100 (Electron Microscopy Sciences)] specimens were stained in 2 U ml^{-1} Alexa Fluor 488 phalloidin (Life Technologies, Eugene, OR, USA; 200 U ml^{-1} stock solution diluted as 5 μl stock solution in 500 μl 1×PBS) in the dark for 50 min and then twice rinsed in 1×PBS. Fixed and stained *Capitella* sp. worms were mounted on slides in Citifluor AF1 (Ted Pella Inc., Redding, CA, USA). Depending on the size of the worm, one or two layers of double-sided tape (thickness 88.9 μm; Scotch, 3M Corporation, St Paul, MN, USA) were used to create a space between the slide and coverslip to avoid over-compressing the worms. Using a Zeiss laser scanning microscope 700 (Zeiss, Oberkochen, Germany; 20× objective, with Observer 21 Axio camera HRM), the structure of the body wall musculature of nine *Capitella* sp. worms was examined. The worms ranged from 1.66 to 3.48 mm in thorax length and 0.26 to 0.45 mm in width of the second segment (w_2) and were scanned through the body wall towards the gut. Settings were adjusted using Zen 2010 (Zeiss) software. The first channel was set for the Alexa Fluor 488 phalloidin at an excitation wavelength of 488 nm and an emission wavelength of 519 nm.

Angles of helical muscles, θ (from the longitudinal axis), were measured at an intersection of two muscle strands when intersections were present. Otherwise, the angle between the helical muscle and the intersegmental lines was measured, assuming lines to be perpendicular to the longitudinal axis. A one-sample, two-sided *t*-test was applied for each worm (6≤N≥27) and for the means of all worms (N=9) testing for equality with 54.73 deg (P=0.05). Mean angle was plotted as a function of body size and regression analysis conducted using R (α=0.05). In addition, every left- and right-handed muscle strand was counted in each segment. The total number of muscle strands per segment was analyzed as a function of body size.

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