

## RESEARCH ARTICLE

# They like to move it (move it): walking kinematics of balitorid loaches of Thailand

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## ABSTRACT

Balitorid loaches are a family of fishes that exhibit morphological adaptations to living in fast flowing water, including an enlarged sacral rib that creates a 'hip'-like skeletal connection between the pelvis and the axial skeleton. The presence of this sacral rib, the robustness of which varies across the family, is hypothesized to facilitate terrestrial locomotion seen in the family. Terrestrial locomotion in balitorids is unlike that of any known fish: the locomotion resembles that of terrestrial tetrapods. Emergence and convergence of terrestrial locomotion from water to land has been studied in fossils; however, studying balitorid walking provides a present-day natural laboratory to examine the convergent evolution of walking movements. We tested the hypothesis that balitorid species with more robust connections between the pelvic and axial skeleton (M3 morphotype) are more effective at walking than species with reduced connectivity (M1 morphotype). We predicted that robust connections would facilitate travel per step and increase mass support during movement. We collected high-speed video of walking in seven balitorid species to analyze kinematic variables. The connection between internal anatomy and locomotion on land are revealed herein with digitized video analysis,  $\mu$ CT scans, and in the context of the phylogenetic history of this family of fishes. Our species sampling covered the extremes of previously identified sacral rib morphotypes, M1 and M3. Although we hypothesized the robustness of the sacral rib to have a strong influence on walking performance, there was not a large reduction in walking ability in the species with the least modified rib (M1). Instead, walking kinematics varied between the two balitorid subfamilies with a generally more 'walk-like' behavior in the Balitorinae and more 'swim-like' behavior in the Homalopteroidinae. The type of terrestrial locomotion displayed in balitorids is unique among living fishes and aids in our understanding of the extent to which a sacral connection facilitates terrestrial walking.

**KEY WORDS:** Terrestrial excursion, Rheophilic, Fish locomotion, Hillstream loaches

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## INTRODUCTION

A major demand of terrestrial locomotion is that animals must support their own mass against the force of gravity; this is highly different from aquatic locomotion, in which the mass of the organism is supported by the buoyant forces of water (Denny, 1993; Turko and Wright, 2015). Although fishes are aquatic, terrestrial excursions, or purposeful temporary forays into terrestrial environments, are known to occur in several lineages.

Among the approximately 25,000 species of teleost fishes, 40 families from 17 orders have evolved varying forms of terrestrial excursions (Ord and Cooke, 2016; Wright and Turko, 2016). Some amphibious fishes have evolved a number of morphological and behavioral traits that facilitate moving out of water to escape predation, find food or new habitats, find mates, lay eggs, or, as is the case in the Pacific leaping blenny (*Alticus arnoldorum*), to spend the large majority of their lives out of water (Blob et al., 2006; Gibb et al., 2013; Hsieh, 2010; Michel et al., 2016; Ord and Cooke, 2016; Soares and Bierman, 2013; Wright and Turko, 2016).

Forms of terrestrial locomotion during these outings onto land vary in longevity and locomotor strategy. The simplest locomotion strategy observed during terrestrial excursions in fishes is modified undulatory swimming behaviors without specialized anatomy, as seen in eels (Gillis, 1998) and California grunions, *Leuresthes* spp. (Martin et al., 2004; Muench, 1941). During terrestrial locomotion, bichirs (*Polypterus*) augment lateral undulation of their elongate body with lifting of the pectoral fins (Standen et al., 2014, 2016), mudskippers use the pectoral fins as crutches to lift the body and swing forward (Kawano and Blob, 2013; Pace and Gibb, 2009), some species of blenny hop or jump (Hsieh, 2010), and walking catfishes, *Clarias* (Clariidae), use modified pectoral-fin spines as struts (Johnels, 1957; Van Oosterhout et al., 2009). Another form of terrestrial locomotion is observed in vertical climbers, including the waterfall climbing gobies of Hawaii, which use oral and pelvic suction created with modified structures (*Sicyopterus stimpsoni*) or sporadic, rapid axial undulation (*Awaous guamensis* and *Lentipes concolor*) to climb from their saltwater habitats as juveniles to freshwater habitats where they live as adults (Blob et al., 2006; Schoenfuss and Blob, 2003). There is also a recently described locomotor strategy, reffling, seen in the neotropical armored catfishes (Loricariidae), in which the fishes use asymmetric patterns of axial and appendage-based locomotion (Bressman et al., 2021).

Although the species listed above are successful in traversing the terrestrial environment, they are not performing a walking gait similar to that of terrestrial tetrapods. They are also missing a key feature that facilitates terrestrial walking: a robust, weight-bearing connection between the pelvic appendages and the axial skeleton via a sacrum or sacral attachment (Ahlberg, 2019; Clack, 2009; King et al., 2011). Terrestrial tetrapodal locomotion includes both symmetrical (i.e. walk and trot) and asymmetrical (i.e. gallop and

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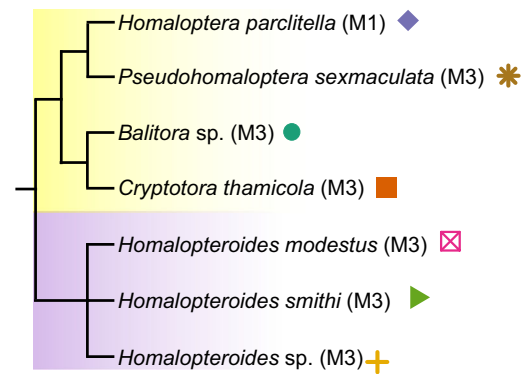
bound) gait patterns (Hildebrand, 1980). Differentiation of gait patterns requires partitioning step cycles of the limbs into stance phase, when the limb is in contact with the ground, and swing phase, when the limb is in an aerial phase (Hildebrand, 1980). The first record of a fish using a tetrapod-like gait in a terrestrial environment without the assistance of a buoyant medium was only recently published. In 2016, Flammang et al. (2016) found that the balitorid hillstream loach, *Cryptotora thamicola* (Balitoridae: Cypriniformes), is capable of walking with a salamander-like, lateral sequence diagonal couplets (LSDC) gait. The walking gait observed in *C. thamicola* is possible in part owing to morphological modifications of the pelvic girdle, including a robust skeletal connection between the pelvis and the axial skeleton via the sacral rib. This sacral rib support transfers forces from the hind appendages through the axial skeleton, allowing the fishes to support their mass out of the water. The expanded sacral rib of balitorid loaches is one of the morphological adaptations that allow them to maintain their position and generate forces to oppose being washed downstream in the fast flowing rivers and streams that they inhabit (Ahlberg, 2019; Beamish et al., 2008; Chang, 1945; Hora, 1930; Lujan and Conway, 2015). Further work on the morphology of other species in the family led to the delimitation of three distinct morphotypes (Crawford et al., 2020). These morphotypes (M1, M2 and M3) have increasing degrees of skeletal modification in the pelvic region: a long, narrow sacral rib (M1), a thickened, slightly curved sacral rib (M2), and a robust sacral rib with a flared crest (M3). In all three morphotypes, the distal end of the sacral rib inserts at the lateral foramen of the pelvic plate (Crawford et al., 2020). The connection between the pelvis and the axial skeleton via this insertion of the sacral rib is expected to enable the ability of these fishes to perform terrestrial walking. The varying extent of the rib thickness and the presence of the crest (and thus increased area for muscle attachment) are expected to support more tetrapod-like walking and more effective movement (i.e. greater distance traveled per step).

This work describes the kinematics of terrestrial walking in a group of fishes with morphology converging on tetrapodal characters crucial to supporting the vertebrate body against gravity and facilitating terrestrial walking. The three morphotypes described above were found to be spread throughout the balitorid family with low phylogenetic signal for the associated traits (Crawford et al., 2020). Given the variation in pelvic morphology, we chose to study whether the differences have implications for walking ability without the support of water and to analyze how well walking is accomplished by fishes in the two extremes of the three morphotypes. Our objective was to test the hypothesis that fishes with more robust connections between the pelvis and axial skeleton (M3) have better walking performance (i.e. travel further per step and move in a steadier manner), and fishes with reduced connectivity (M1) have lower performance (i.e. less forward movement per stride and more scurrying movements).

## MATERIALS AND METHODS

### Specimen collection

Specimens were collected in central Thailand in January 2020 (Table S1) using multiple methods including dip netting, cast netting and moving rocks to loosen fishes holding on to the rocky substrate. Species collected in the field for filming included *Cryptotora thamicola* (Kottelat, 1988), *Homalopteroides modestus* (Vinciguerra, 1890), *Homalopteroides smithi* (Hora, 1930), *Homalopteroides* sp., *Balitora* sp. and *Pseudohomaloptera sexmaculata* (Fowler, 1934). Individuals of another species,



**Fig. 1. Simplified phylogeny of study species.** Phylogeny follows Crawford et al. (2020), showing separation of Balitorinae (yellow) and Homalopteroidinae (purple). Color and symbol shape will continue through other figures. Morphotypes determined from Crawford et al. (2020) are indicated for each species.

*Homaloptera parclitella* (Tan and Ng, 2005), were purchased from the Wet Spot Tropical Fish (Portland, OR, USA) for laboratory-based video analysis. Fish in the laboratory were housed in three 10-gallon (=37.85 liter) tanks (four fish per tank), lined with rocky substrate and fitted with high turnover rate filters. Fish were fed live blackworms and sinking algae wafers daily. Work on live fish was conducted in accordance with NJIT/Rutgers University IACUC 17-058. The species used in this study cover the two subfamilies of Balitoridae, Balitorinae and Homalopteroidinae (Randall and Page, 2015) (Fig. 1), and two of the three morphotypes determined by Crawford et al. (2020). M1 was represented by *H. parclitella*, and M3 was represented by *C. thamicola*, *Balitora* sp. and *P. sexmaculata* from Balitorinae, and *H. modestus*, *H. smithi* and *Homalopteroides* sp. from Homalopteroidinae.

Field collection and filming on location in Thailand were conducted with permission issued to L.M.P. and Z.S.R. by the National Research Council of Thailand (permit 11880). We also thank the federal and other governmental agencies in Thailand for granting permission to A.S. and S. Tongnunui at the Kanchanaburi Campus of Mahidol University to collect fishes and conduct fieldwork in Thailand.

### Video collection

Filming was performed at collection sites where possible, and fishes were kept in buckets between capture and filming, which was completed for most within 3 h of collection. When filming could not occur immediately, water was aerated by portable aerators, and filming was completed within 24 h; this extended time period between capture and filming only occurred for fish collected in Ratchaburi (Table S1). A total of 22 individuals across all species were filmed in the field (Table S1). Two Edgertronic SC1 high-speed cameras (Sanstreak Corp., San Jose, CA, USA), each with a 105 mm Nikon lens, were used to collect videos of fishes walking on an acrylic trackway. Cameras were synchronized, and video was captured in lateral and ventral views at 500 frames s<sup>-1</sup>. During filming, two small LED lights were used for illumination when low light conditions made it difficult to clearly see the fish in the cameras. Fishes were filmed walking on an acrylic trackway with holes at the ends to allow water to drip out, thus maintaining a wet environment for walking while avoiding swimming behaviors supported by the presence of deep water. Individual fish required different levels of stimulus to elicit walking behavior; stimuli included simply placing the fish on the acrylic trackway, dribbling

water ahead of them, or coaxing with nudges to the caudal fin using the water dropper. Between filming, fish were placed back in water to recuperate for several minutes before the next walking trial.

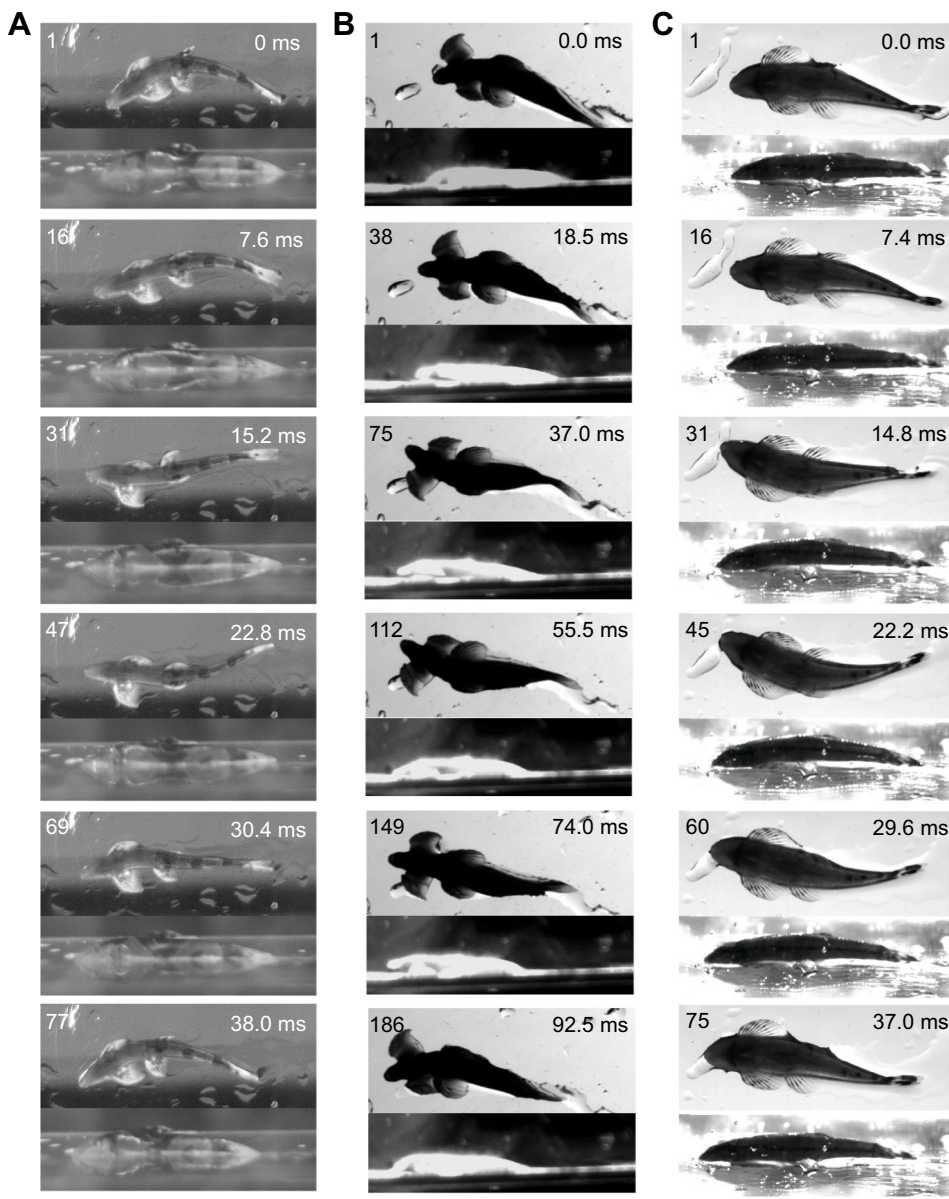
Post filming, specimens were euthanized in MS-222, labeled, fixed in 10% formalin, and later transferred to 70% ethanol for storage and accession into museum collections (Table S1). Representative species from each field collection site were  $\mu$ CT scanned with the GE Phoenix v|tome|x M scanner (GE Measurement and Control, Boston, MA, USA) at the University of Florida Nanoscale Research Facility for skeletal comparisons (Fig. S2).

Laboratory filming was performed using two Phantom Miro M110 cameras (Vision Research, Wayne, NJ, USA) recording at 500 frames  $s^{-1}$  in both lateral and ventral views. The ventral view was accomplished using a mirror at a 45 deg angle. The filming setup was illuminated with overhead lighting and an additional Dracast LED500 Bi-Color light (Dracast, San Jose, CA, USA) as needed. As with field filming, different stimuli were used to elicit walking behaviors. Between filming trials, specimens were kept in aerated containers. After filming, fish were returned to their

respective tanks where they were maintained for later studies. During field and lab filming, calibration videos were collected before and after each filming session using a custom-built Lego™ model for 3D digitization of videos.

### Video analysis

In the ventral view, 29 points were digitized on each individual (Fig. S1) using DeepLabCut (Mathis et al., 2018; Nath et al., 2019). Locations of digitized points were selected to include the individual fin movements and the movement of the midline of the fish. Step cycle measurements began with the start of stance phase of the left pectoral fin (Fig. 2). Walking bouts were analyzed if they included at least three consecutive steps with the fish moving in a straight direction. Videos where the individual turned or did not have at least three consecutive steps were not analyzed. The number of videos analyzed per species ranged from 14 to 24 (*Balitora* sp.,  $N=5$  individuals, 23 videos; *C. thamicola*,  $N=2$ , 22 videos; *H. parclitella*,  $N=5$ , 24 videos; *H. modestus*,  $N=3$ , 15 videos; *H.s smithi*,  $N=4$ , 14 videos; *Homalopteroides* sp.,  $N=3$ , 16 videos; and



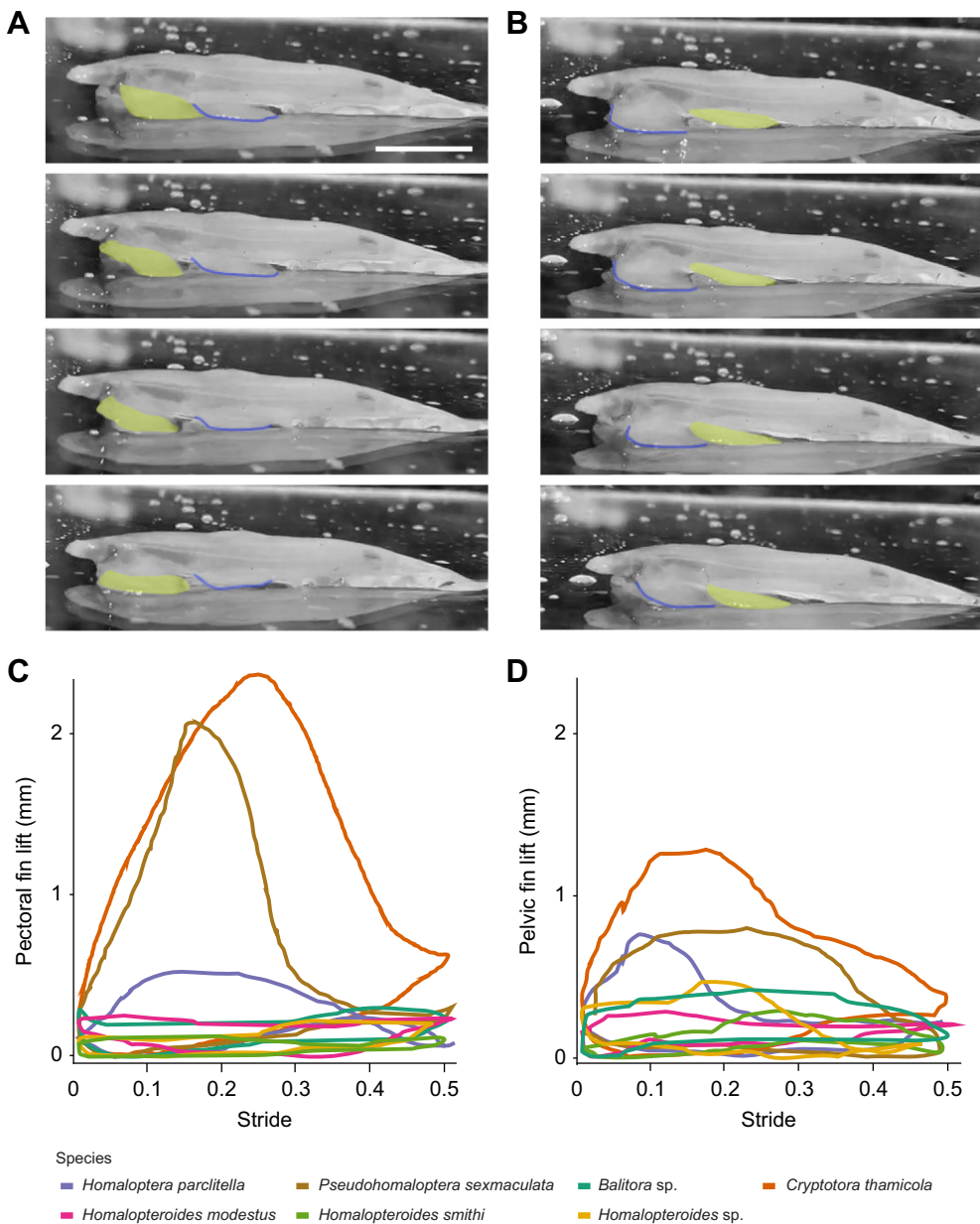
**Fig. 2. Representative sequences of fish walking on an acrylic trackway.** (A) *Homaloptera parclitella*, morphotype 1 (Flammang Lab/NJIT); (B) *Cryptotora thamicola*, Balitorinae, morphotype 3 (MARNM 7413), and (C) *Homalopteroides modestus*, Homalopteroidinae, morphotype 3 (UF 245290). Panels are in sequence from top to bottom and represent one stride sequence at 0, 20, 40, 60, 80 and 100%; frame number (top left) and time sequence (top right) are noted on each image. In each figure pair, the top image is the ventral view and bottom is the lateral view. Videos were filmed at 500 frames  $s^{-1}$ . Museum abbreviations: Maejo Aquatic Resources Natural Museum, MARNM; University of Florida, UF; New Jersey Institute of Technology, NJIT. Scale bars=10 mm.

*P. sexmaculata*,  $N=5$ , 22 videos). The number of individuals recorded was dependent on what was allowable under our research permits and how common the species was in the area; for example, *C. thamicola* is a very rare subterranean species, limiting our interactions with that taxon.

### Kinematic variables

We analyzed vertical lift of fins in swing phase, range of fin extension (in degrees), curvature of midline, torso length, speed of walking, step overlap, appendicular duty factor and diagonality. Vertical lift of fins was determined from 3D traces of the anterior edge of the pectoral and pelvic fins. Analysis of the 3D movement was completed using the DLTdv8 application (Hedrick, 2008) in MATLAB (ver. 2020a, MathWorks, Natick, MA, USA) with 3D calibration from a custom Lego™ brick structure. Range of fin extension was measured as the change in degree of the angle formed by the tip of the first pectoral fin ray and the anterior and posterior ends of the pectoral girdle, repeating this set of points for the pelvic

fins (Fig. S1). Curvature of the midline was calculated as the sum of the absolute value of the radius of curvature at each point along the midline and the mean maximum curvature for each step cycle was compared among species. Radius of curvature was calculated using the localCurvature() function in the EBImage package in R (Pau et al., 2010). The tail–hip insertion curvature correlation (THICC) was developed to compare the proportional length of the tail (points 22–29, Fig. S1) among the fish to the maximum curvature; tail length is measured as the proportion of total length starting at the anterior edge of the pelvis to the posterior tip of the tail. Speed of walking was calculated in three formats: body lengths per stride, body lengths per second and strides per second. Speed of movement was calculated using the CrudeSpd() function in the R package ‘Kraken’ (<https://github.com/MorphoFun/kraken>). Step overlap was calculated as the percent of the stride where both pectoral and both pelvic fins were in stance phase at the same time. Duty factor was calculated for each fin in each step cycle and differences among fins were tested for



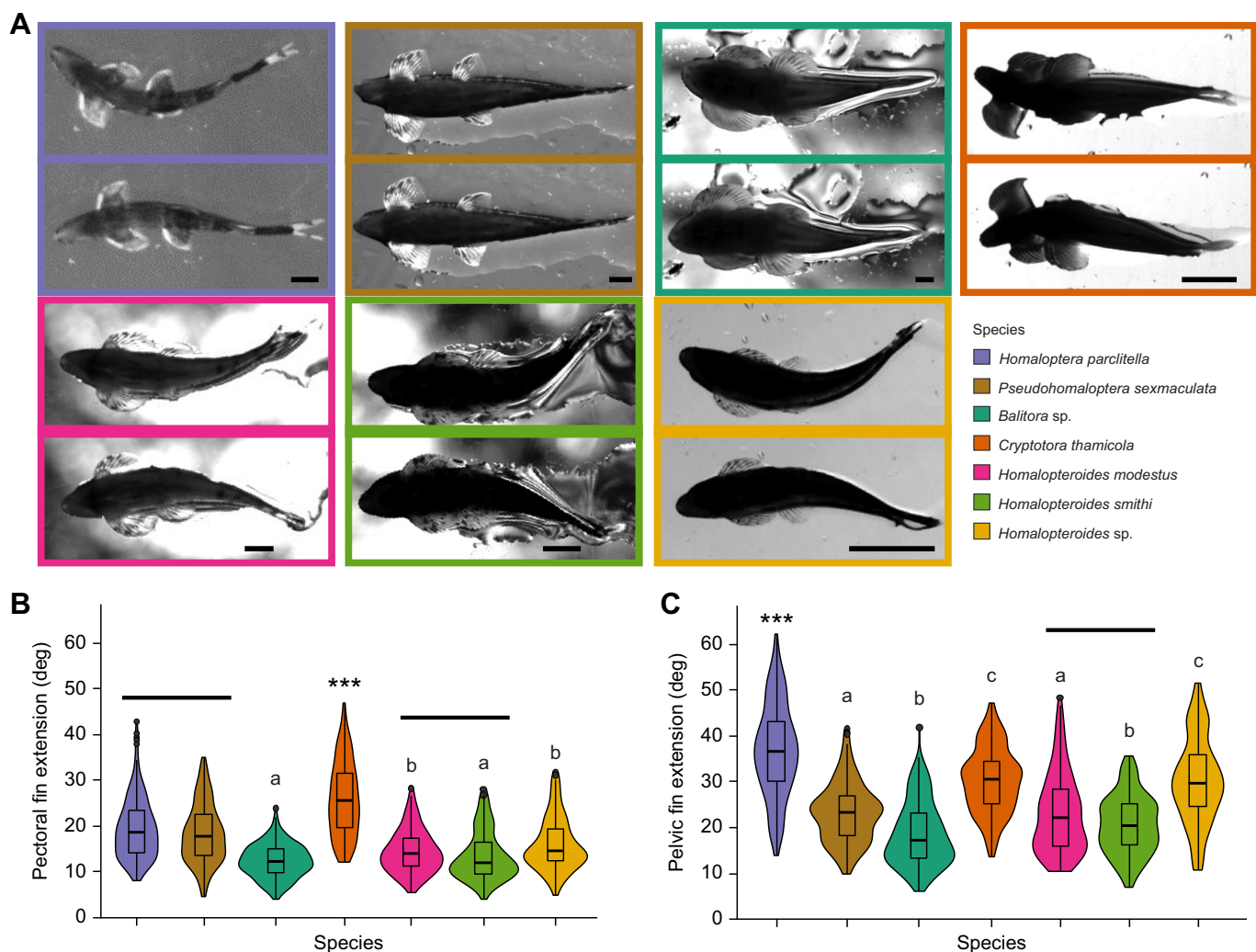
using an ANOVA/Tukey's HSD comparison. In balitorids, the body is not consistently lifted off the substrate, and for the majority of walking cycles, only the head was lifted, thus, we will refer to duty factor as appendicular duty factor to distinguish from tetrapodal walking where the body is lifted clear of the substrate and supported by the limb(s) in stance phase. Gait was also analyzed for diagonality, or the proportion of a step cycle between the placement of the hind fin and the sequential placement of the fore fin on the same side (Cartmill et al., 2002; Hildebrand, 1980; Lemelin and Cartmill, 2010; Nyakatura et al., 2014). Differences among kinematic variables between species were analyzed with ANOVA/Tukey's HSD comparisons. Analyses were performed in R v. 4.0 (<http://www.R-project.org/>). Values for each variable were measured per step cycle.

Initial aims of this work were to compare walking performance between all morphotypes. Owing to sampling limits in the field, we were only able to obtain one representative from M1. Because our comparisons were constrained, our overall generalizations have been minimized; broader conclusions would require data from more species in M1 from both subfamilies and representatives from M2 to compare against the two extreme morphotypes collected in this study.

## RESULTS

Although all species studied exhibited a diagonal-couplets walking pattern, movements were along a spectrum ranging from patterns more similar to swimming behaviors performed on land to those that more closely resembled terrestrial tetrapodal walking. Contrary to our prediction that the walking performance would align with morphotypes, the trend we observed tended to show most similar walking within subfamilies.

The first variable studied, vertical fin lift, exhibited large differences among species. The trace of the anterior tip of the fins in 3D illustrates the variation in step movement and the amount of lift during the swing phase (Fig. 3A). In *Homalopteroides* and *Balitora* sp., the fishes slid the fins along the substrate without visible lift to clear the surface, presenting more of a rowing shape in the pectoral and pelvic fin traces (Fig. 3B,C). Contrastingly, in *H. parclitella* (M1), *P. sexmaculata* (M3) and *C. thamicola* (M3), the pectoral and pelvic fins left the surface during swing phase. In *H. parclitella* and *C. thamicola*, there was also consistent lifting of the front portion of the body, with the head region being fully lifted from the substrate during most step cycles. In all species that exhibited clear vertical lifting of the fins, except for *H. parclitella*, there was more lift in the pectoral fin during swing phase and less lift

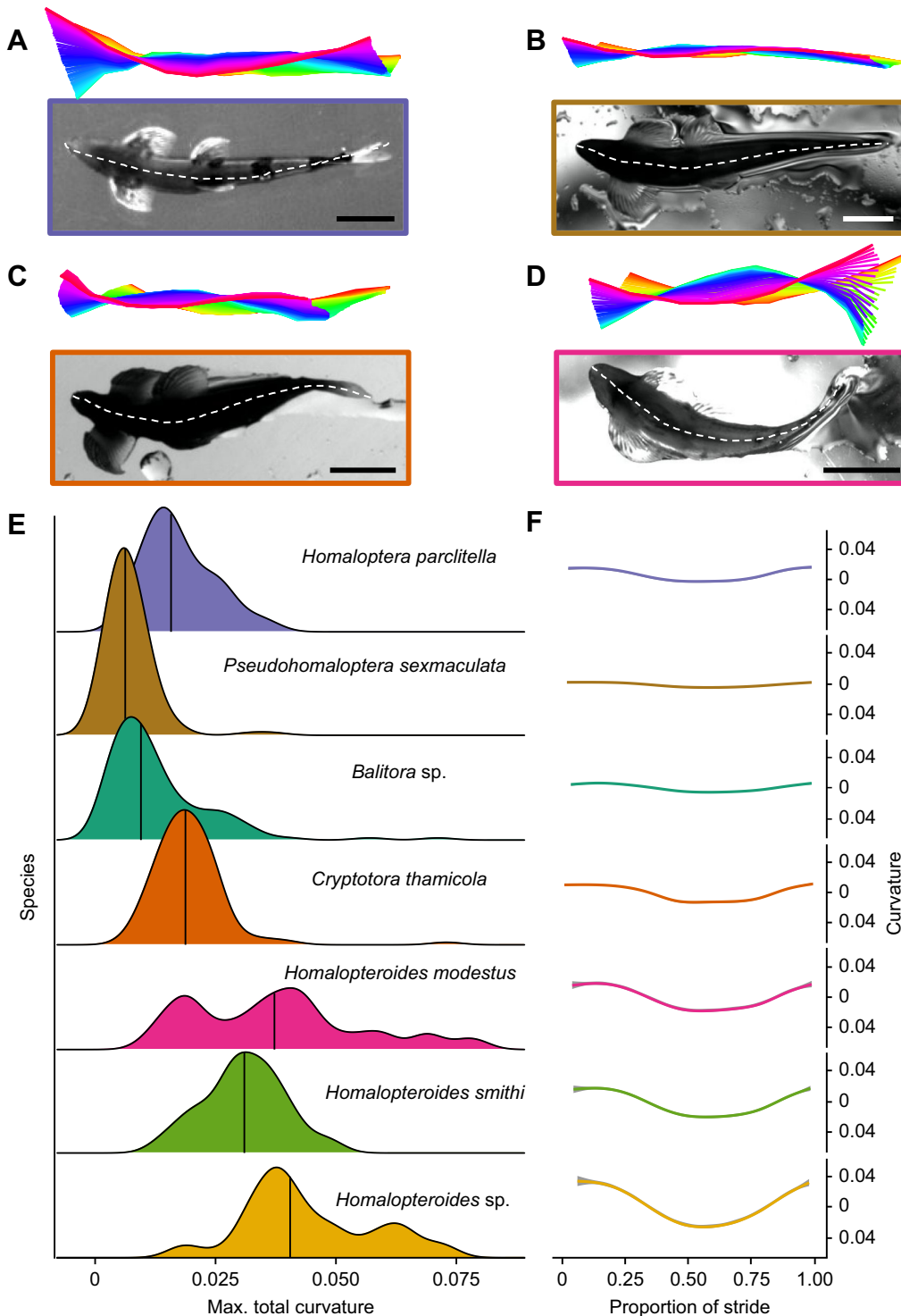


**Fig. 4. Range of fin extension during walking.** (A) Representative frames of videos from each species showing maximum and minimum fin extension. Violin plots of fin extension range (deg) for (B) pectoral and (C) pelvic fins. Horizontal bars denote no significant difference between species below bar. There are no significant differences between species marked with same lowercase letter; \*\*\* $P < 0.000$ , scale bars = 10 mm.

in the pelvic fin swing phase. *Homaloptera parclitella* had greater lift in the pelvic fin than in the pectoral fin; however, the duration of the lift was shorter, and the pelvic fin slid along the surface for most of the swing phase.

There was a varied range of extension for the fins across species and among individuals for different steps (Fig. 4). In some species, there were steps with a clear lifting of the pectoral and pelvic fins during the swing phase, while in others there was more often a sliding of the fins forward in both the pectoral and pelvic fins. In *C. thamicola*, there was a greater lifting of the fins in addition to a

large movement of the anterior body in the z direction (i.e. lifting the head and pectoral girdle vertically off the acrylic surface). The different species showed differences in pelvic fin range of motion during walk cycles. *Pseudohomaloptera sexmaculata*, *Balitora* sp. and *H. smithi*, all M3s, did not bring their pelvic fins completely under the body and maintained them laterally extended. Other species brought their pelvic fins completely under the body before bringing them forward again for the next step. The range of extension in the fins is greater in the pelvic fins for all species. *Cryptotora thamicola* had the greatest range of extension of the pectoral fins.



**Fig. 5. Midline curvature of balitorid walking patterns.** Representative frame and midline traces through a single step cycle of (A) *Homaloptera parclitella* (Flammang Lab/NJIT), (B) *Pseudohomaloptera sexmaculata* (UF 245546), (C) *Cryptotora thamicola* (MARNM 7413) and (D) *Homalopteroides modestus* (UF 245290), with still frames from the same video, white dotted lines trace the midline. (E) Frequency distribution plots of total body maximum curvature in each step cycle showing the lowest curvature in *Pseudohomaloptera sexmaculata*, mid-range curvature in *Homaloptera parclitella* and *Cryptotora thamicola*, and the greatest curvature in Homalopteroidinae. (F) Mean body curvature over stride cycle for each species with gray bands indicating 99% confidence interval. In A–D, midline traces begin at red for the first frame and over time travel through the rainbow pattern with the first frame at the bottom of the stacked lines and the last frame at the top. Ridge height indicates the proportion of steps at that curvature. Scale bars=10 mm.

Curvature of the midline showed a different trend between the M3 species in the two subfamilies, with Balitorinae having lower maximum curvature ( $0.014 \pm 0.001$  CI) than Homalopteroidinae ( $0.037 \pm 0.002$  CI). Within Balitorinae, there was further delineation among the species, with *H. parclitella* and *C. thamicola* having slightly greater maximum curvature ( $0.019 \pm 0.001$  CI) than *Balitora* sp. and *P. sexmaculata* ( $0.010 \pm 0.001$  CI). The Balitorinae had lower maximum curvature and a smaller range of curvature values (Fig. 5A–C,E,F; Fig. S3). The Homalopteroidinae showed high curvature throughout the body during a step cycle, creating nearly a half circle with the body during most steps along with a broad range of curvature throughout the step cycle and between steps (Fig. 5D–F; Fig. S3). Curvature along the body was greatest near the pelvis (points 23–27, Fig. S1) tapering off closer to the cranial and caudal ends of the fish (Fig. S4) in all species except *P. sexmaculata*, which had consistently low mean maximum curvature throughout the body.

The curvature pattern within Balitorinae (greater mean maximum curvature of *C. thamicola* and *H. parclitella*) was unexpected, and further inquiry led to a correlation with the body proportions, particularly with comparisons of the tail proportions (THICC; Fig. 6). The proportion of the total body length represented by the tail (points 22–29 in Fig. S1) was not significantly different among the three *Homalopteroides* species, *Balitora* sp. or *P. sexmaculata* ( $P > 0.65$ ) averaging 63% total body length; conversely, *H. parclitella* and *C. thamicola* averaged 60% and 70%, respectively (Fig. 6), significantly different from all other species ( $P < 0.004$ ).

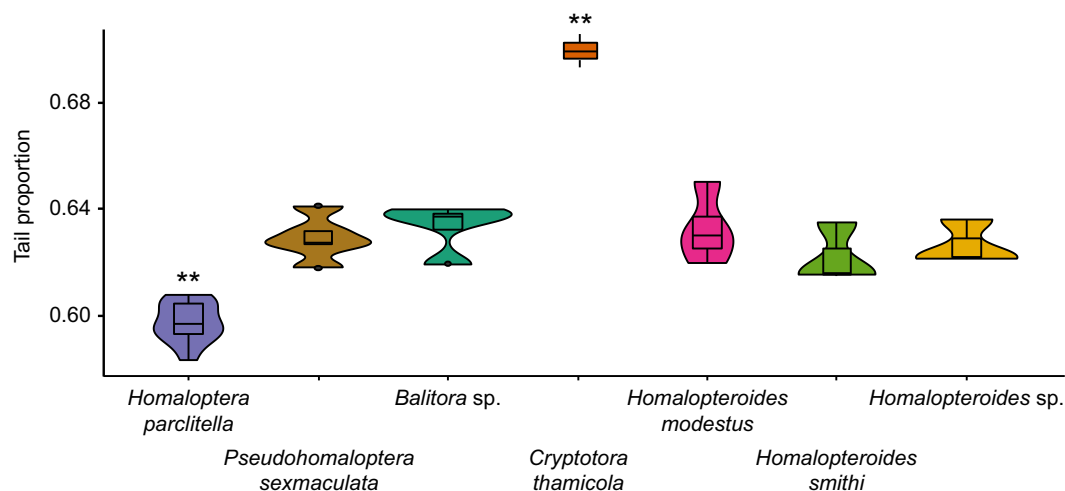
Walking velocity, compared as body lengths per second, body lengths per stride and strides per second, varied among species (Fig. 7). Body length per second showed differences between the two subfamilies, with the Homalopteroidinae moving, on average, faster than the Balitorinae; however, this does not fully describe all of the differences observed. Using body lengths per stride and strides per second aids in investigating the variation in walking. One major difference was shown by the body lengths per stride (Fig. 7B), in which *C. thamicola* was traveling significantly farther per stride, indicating potentially more force generated per stride resulting in more effective forward travel. The other species were not significantly different from one another in body lengths per stride.

Movement per second was another source of variation among species, with the Homalopteroidinae exhibiting increased strides per second and thus moving further per second, but with shorter distance traveled in body lengths. *Cryptotora thamicola* had the fewest strides per second coupled with the furthest distance traveled per stride.

The stride overlap, or percent of the stride in which either both fore or both hind fins are in stance phase, followed the trend of more similar values within the subfamilies (Fig. 8). The species of Homalopteroidinae were not significantly different from one another (3.78% mean overlap) and had lower overlap than the Balitorinae species (6.09%). Within Balitorinae, there was a significant difference between *H. parclitella* (M1, 5.31%) and *C. thamicola* (M3, 6.45%;  $P < 0.05$ ). *Homaloptera parclitella* was also not significantly different from *H. modestus*, which had the greatest overlap of the Homalopteroidinae (4.22%).

All measured appendicular duty factors, the proportion of the step cycle in which the fin is in stance phase, are within the walking proportion of over 50% (53–57%) for each fin. In all species there was no significant difference between the appendicular duty factors of the fore and hind fins or left and right; thus, the appendicular duty factors of each fin were combined for comparisons between species. Similar to the stride overlap, the appendicular duty factor was slightly lower in the Homalopteroidinae subfamily (54%) and greater in the Balitorinae (56%, Fig. 9). *Cryptotora thamicola* had a significantly greater appendicular duty factor (57%,  $P < 0.0001$ ) than all other species studied here, and *Homalopteroides* sp. had a significantly smaller mean appendicular duty factor than all other species (53%,  $P < 0.0001$ ).

Diagonality, which is the percentage of the step cycle by which the hind finfall precedes the ipsilateral fore finfall, was found to overlap values for diagonal sequence diagonal couplets (DSDC) and LSDC gaits for all species (Fig. 9). For all species except for *H. parclitella* and *C. thamicola*, the majority of steps analyzed were DSDC, with diagonality values above 0.5 (Fig. 9). In *H. parclitella* and *C. thamicola*, the mean diagonality was not significantly different from 0.5 ( $z$ -statistic =  $-0.186$  and  $-1.462$ , respectively, with  $P > 0.1$  for both species); thus, these two species may fluctuate more readily between the two phase patterns. Finfall patterns in all *Homalopteroides* species as well as in *Balitora* sp. and



**Fig. 6.** Violin plots of the proportional length of the tail (pelvis to tail tip) across species. *Cryptotora thamicola* and *Homaloptera parclitella* show a significantly different relationship for the tail–hip insertion curvature correlation (THICC) than the other species,  $**P < 0.01$  for all comparisons to *Cryptotora thamicola* or *Homaloptera parclitella*.

*P. sexmaculata* followed the DSDC more frequently, with the fore fin on the opposite side being the next fin placement after the hind fin placement.

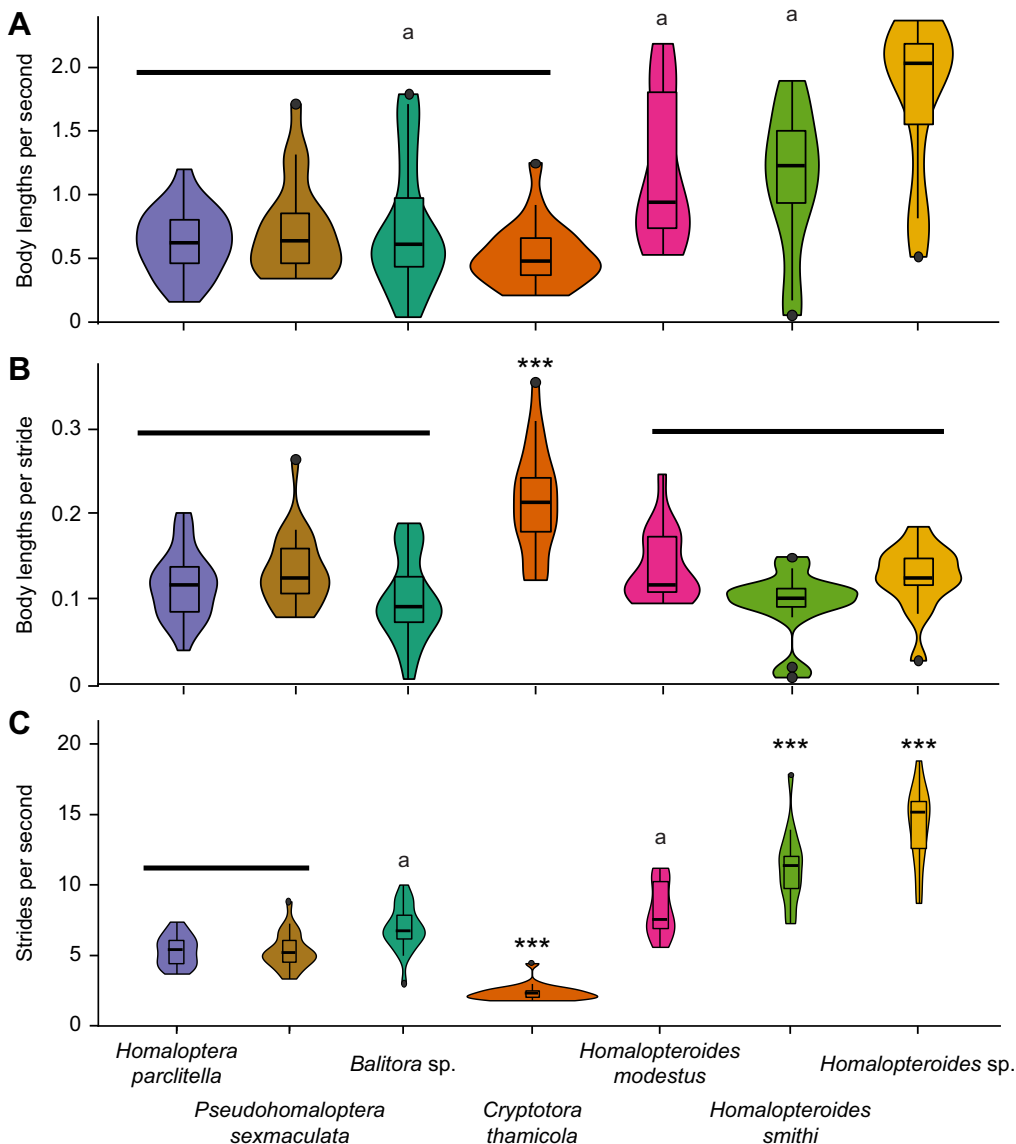
With variation in appendicular duty factor, step overlap and walking speed (strides per second), a trend of increased appendicular duty factor and stride overlap was seen in correlation with decreased strides per second (Fig. 10). With a longer and slower push-off of fishes with a larger appendicular duty factor and step overlap (i.e. *C. thamicola*) compared with those with faster and shorter strides (i.e. *Homalopteroides* sp.), there is a difference in walking tempo: more of a methodical and slower tempo in the Balitorinae and a scurrying movement in Homalopteroidinae.

## DISCUSSION

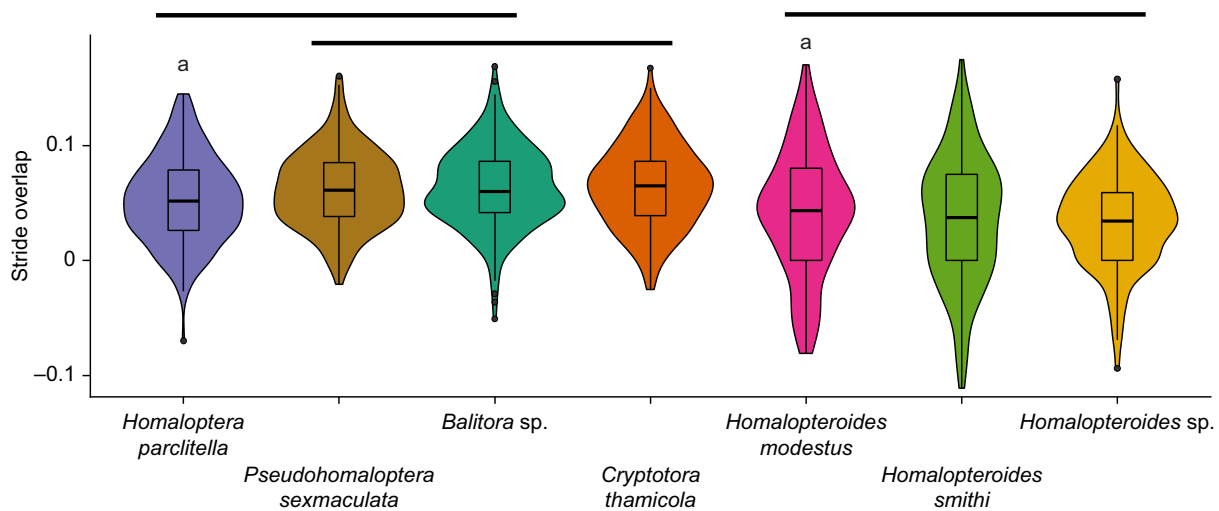
Terrestrial locomotion is found across the teleost tree of life through a range of behavioral and morphological adaptations. Here, we have expanded upon the kinematic analyses from Flammang et al. (2016) and present balitorid walking behaviors that are similar to walking patterns seen in terrestrial tetrapods. The species studied here represent the two extremes of the three morphotypes in the balitorid family (Crawford et al., 2020). Crawford et al. (2020) addressed

the variation in structural morphology within the lineage and hypothesized that the morphological differences may support variation in walking ability in this family. This family is the only known group of fishes to perform terrestrial walking behaviors similar to those seen in terrestrial tetrapods, which offers a unique opportunity to investigate morphological requirements for this convergent behavior.

Although there are differences among species in the amount of vertical lift of the pectoral and pelvic fins (Fig. 3), we show here that individuals in this family are capable of performing a walking gait with a distinct stance and swing phase. The Balitorinae are capable of lifting the fins during swing phase, while *Homalopteroides* species complete the swing phase by sliding the fins along the surface without visible clearance between the acrylic platform and the underside of the fins. In the pectoral fins, the vertical lift is greater in the species that also have a larger range of extension in the fin movement. The vertical lift of the pelvic fins was less than that of the pectoral fins for all species. Reduced clearance of the substrate during swing phase can be seen in some terrestrial tetrapods as well; for example, in high-stepping alligators, the hindlimbs were often not lifted enough for the toes to clear the substrate, leading to foot



**Fig. 7. Violin plot comparisons of walking speed and movement among balitorid species.** (A) Body lengths per second, (B) body lengths per stride, and (C) strides per second. Horizontal bars denote no significant difference between species below bar. Species with the same letter are not significantly different from one another; \*\*\* $P < 0.000$ .



**Fig. 8. Violin plot of stride overlap between left and right fins, pairing fore and hind fins.** Plotted are the overlap per step cycle of each species. Horizontal bars denote no significant difference between species below bar. Species with the same letter are not significantly different from one another.

dragging more often in the hindlimbs than the forelimbs (Wiley et al., 2004). In alligators, it must be noted, the hindfoot is longer than the forefoot, which would require the alligator to lift this limb substantially higher than the forelimb for complete clearance of the substrate. In comparison, balitorids have much larger pectoral fins than pelvic fins in both Homalopteroidinae and Balitorinae, with the pectoral fins ranging from 9% to 37% longer than the pelvic fins (Randall and Page, 2012, 2014, 2015; Randall and Riggs, 2015).

The variation in maximum midline curvature was a surprising finding. We expected to see reduced body curvature in the M3 morphotypes as compared with the M1, with the expectation that a more swimming-like behavior is exhibited by fishes with a less robust sacral rib connection. Instead, we found increased curvature in the Homalopteroidinae and reduced curvature in the Balitorinae. The reduced curvature in the Balitorinae illustrates a difference in propulsion. The Balitorinae are mainly using appendicular movements to propel the body forward whereas the Homalopteroidinae are utilizing a swimming-like undulatory movement with a lot of tail motion to propel themselves forward. The two groupings of curvature within the Balitorinae, the minimal curvature in *Balitora* sp. and *P. sexmaculata* compared with the increased curvature in *C. thamicola* and *H. parclitella*, may be related to the body proportions (Fig. 6). The deviations from the tail length being, on average, 63% of the total body length for all species studied here other than *C. thamicola* and *H. parclitella*, may be related to the different motion in the movements.

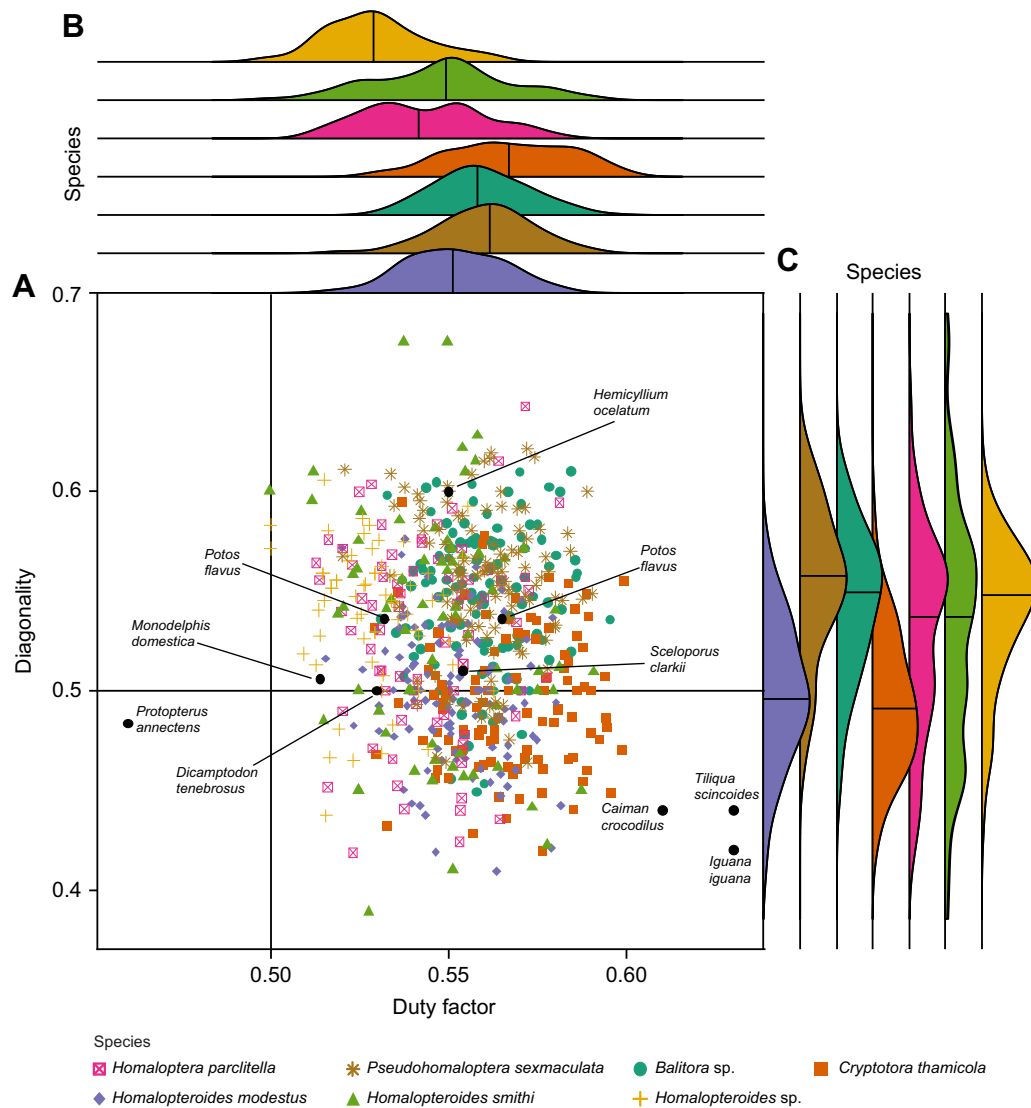
The speed of steps and distance traveled per step consistently shows a stark difference between *C. thamicola* and the other species. *Cryptotora thamicola* has slower and more controlled steps, each of which propels it forward a greater distance with each stride. Among the other species, the body lengths per stride are not different; however, between the other members of Balitorinae and the Homalopteroidinae, there is an increase in the strides per second. These faster strides in the Homalopteroidinae lead to a scurrying motion with the fins being slid along the surface very quickly; conversely, the Balitorinae exhibit a more controlled speed.

The mean stride overlap is generally lower in the Homalopteroidinae, although *H. parclitella* is not significantly different from *H. modestus*. There is also a greater range of stride overlap in *Homalopteroides* species, perhaps also found in other members of Homalopteroidinae. Species with lower stride overlap

also have increased stride speed and reduced appendicular duty factors. Species with greater overlap spend more time in a steadier stance (more than two fins on the substrate). This reduced overlap coupled with increased speed is likely helping the fishes maintain forward motion with a reduced area of support.

Across all species studied here, the gait phases were not significantly different between fore and hind fins (Fig. 9). The appendicular duty factors of all species studied here range from 53 to 57%, which are all lower proportions than seen in walking gaits of terrestrial tetrapods. In salamanders, duty factors have been found to range from 68 to 77% in various studies (Ashley-Ross and Bechtel, 2004; Ashley-Ross et al., 2009; Kawano and Blob, 2013). In other sprawling tetrapods, including the American alligator, walking duty factors have been recorded at 66% (Baier and Gatesy, 2013). The fishes in the present study are performing a walking gait pattern, and some species consistently exhibit lifting of the fins during swing phase; however, the body is not being lifted from the acrylic surface in all species. This lack of clearance is likely a major influence in why the balitorids have duty factors much lower than those of any tetrapods, and makes direct functional comparisons of duty factors between balitorids and tetrapods difficult. There are points where the head and anterior region up to the pectoral fins is lifted from the acrylic surface; however, there is never a time during walking on flat surfaces that the body is lifted clear of the surface. Lack of clearance between the ground and the entire midline of the body means that a fish is not balancing its mass completely on its fins and is supporting itself, at least in part, with the portion of its body remaining in contact with the ground. Although the fishes are not lifting their entire mass off the platform, they are capable of lifting their head, and for short periods of time, they were seen raising their entire body off the surface; however, this behavior was only observed during trials of inclined walking (Crawford, 2021). Future work investigating force production during walking will support a greater understanding of what these fish are doing during their walking behaviors.

The diagonality of the different species covered both lateral sequence and diagonal sequence phase relationships, ranging from 44.9 to 54.5% of the step cycle. In both DSDC and LSDC, the fins are diagonally coupled (left fore and right hind fin move in near synchrony); however, the difference is that in DSDC, the hind limb of the diagonal couplet touches down first and in LSDC gait the fore limb touches down first. With this change in footfall order,

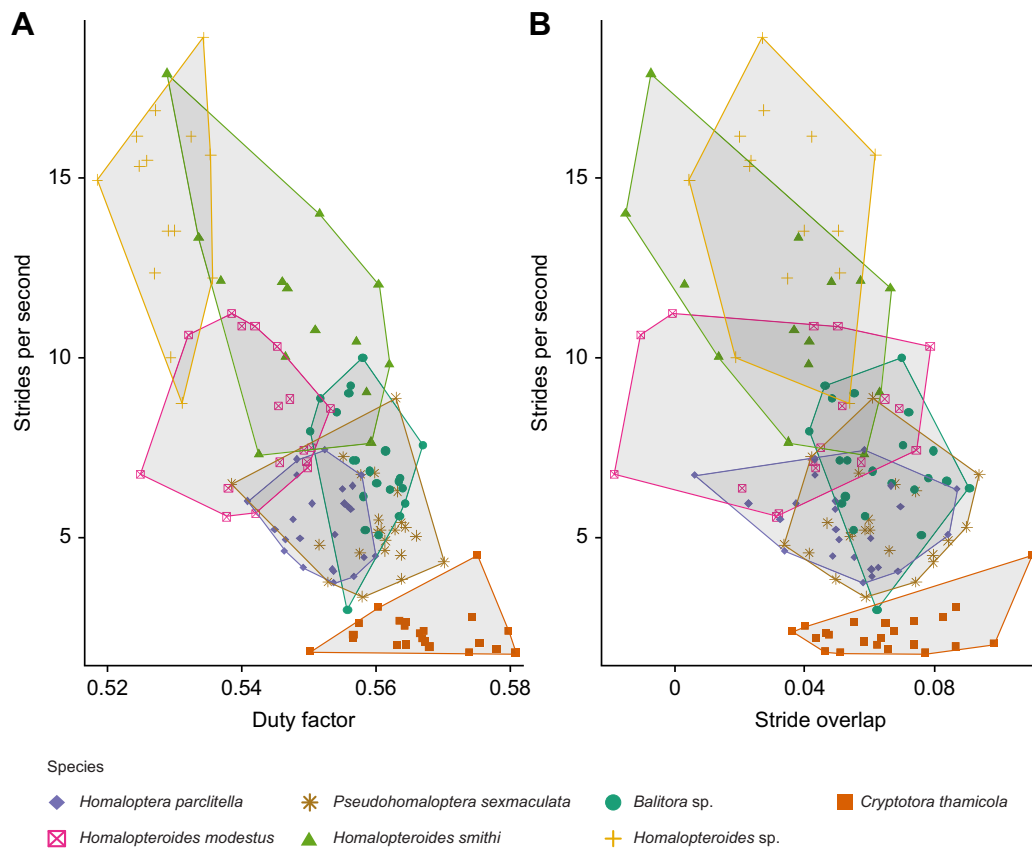


**Fig. 9. Comparisons of walking gait parameters.** Bivariate plot (A) of appendicular duty factor and diagonality of balitorid species walking with comparison points for other species (black). Duty factor below 50% is running and above 50% is walking. Diagonality between 25% and 50% is a lateral sequence gait (LSDC) and between 50% and 75% is a diagonal sequence gait (DSL). Ridgeplots of the density of the appendicular duty factor (B) and diagonality (C) with lines showing median value for each balitorid species. Gait examples are from published literature (black points): *Protopterus annectens* (West African lungfish), King et al. (2011); *Monodelphis domestica* (gray short-tailed opossum), Parchman et al. (2003); *Dicamptodon tenebrosus* (coastal giant salamander), Ashley-Ross (1994); *Hemicyllium ocellatum* (epaulette shark), Pridmore (1994); *Sceloporus clarkii* (Clark's spiny lizard), Reilly and Delancey (1997); *Potos flavus* (kinkajou), Lemelin and Cartmill (2010); *Caiman crocodilus* (spectacled caiman), *Iguana iguana* (green iguana) and *Tiliqua scincoides* (blue-tongued skink), Nyakatura et al. (2019).

terrestrial tetrapods using DSDC are considered less stable because the polygon of support (the area of the body that is supported by the limbs in stance phase) is smaller than in the LSDC gait. The dragging belly and use of the tail likely remove the comparison of stability and may explain why we find all species studied using both LSDC and DSDC finfall patterns. Although the DSDC gait observed in all seven species is not commonly seen in terrestrial tetrapods, it has been seen in the epaulette shark, *Hemicyllium ocellatum*, during terrestrial and submerged walking (Pridmore, 1994), which also maintains near continuous contact of the tail with the substrate. A DSDC gait with a duty factor of over 0.5 can also be described as a walking trot. The walking trot has been observed in high-stepping alligators, which also support some of their mass with their tail while performing this gait (Reilly and Elias, 1998; Willey et al., 2004). A DSDC gait is seen in the California newt, *Taricha torosa*, when walking under water and an LSLC gait when walking on

land; however, underwater walking includes a suspension period owing to the buoyant forces of water (Ashley-Ross and Bechtel, 2004). In arboreal primates, the diagonal sequence gaits have been suggested to allow the forelimb to test the stability of the next step before placing weight on something that may give way (Lemelin et al., 2003). As with the duty factor comparisons, further investigation into the importance of differences in diagonality will be supported by data on force production during walking and comparisons of this between walking bouts presenting DSDC and LSDC gaits.

*Cryptotora thamicola* is the only species of balitorid observed in the field to regularly leave the water to travel over rocks and up waterfalls (Flammang et al., 2016; Kottelat, 1988; Trajano et al., 2002; personal observation, Z.S.R., P.B.H., P.C. and A.S.). The other balitorid species studied here are not often seen exiting the aquatic environment, although they have the capability of traveling short distances on land. Additionally, *Homalopteroides* sp. has been



**Fig. 10. Interaction between appendicular duty factor and stride overlap with strides per second.** (A) Duty factor. (B) Stride overlap. In all species, the trend is slower walking speed with an increase in appendicular duty factor and stride overlap. Polygons show outline points for each species.

observed leaving the water during the rainy season in northeastern Thailand (Watcharapong Hongjamrassilp, personal communication with Z.S.R.). Other balitorids have not been documented in the field to leave the water but are known in the pet trade to climb out of tanks without secure lids (commonly noted in online fish forums) and were regularly seen climbing the sides of buckets during field collections for this study and during previous collection trips by Z.S.R., L.M.P. and A.S.).

Although we found differences among walking performance in the species and morphotype representatives analyzed here, we do not have a conclusive determination of the factors leading to these differences. The unexpected ability of *H. parclitella* (our M1 representative) to walk at comparable performance to species with more robust sacral ribs (M3) indicates that the mere existence of this skeletal connection facilitates walking. Variation in walking performance may be related to a combination of shared ancestry, ecology or other morphological structures not explored here, and additional studies are necessary to reach such conclusions. Further studies into the habitat and ecology of these fishes may help explain why there are differences in the walking performance. Additionally, XROMM (X-ray reconstruction of moving morphology; Brainerd et al., 2010) studies would be beneficial to furthering our understanding of how the skeletal structures are moving during the walking behaviors. Additional coverage for both subfamilies will illuminate variation in walking performance both within Homalopteroidinae and across the family by including coverage of representatives exhibiting M2 and more individuals with M1. Investigating energy expenditure during walking in the different morphotypes would allow comparison of walking efficiency in balitorids.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: C.H.C., B.E.F.; Methodology: C.H.C., P.B.H., A.B.K., B.E.F.; Formal analysis: C.H.C., B.E.F.; Investigation: C.H.C., A.W., P.B.H., Z.S.R., C.C., A.B.K., H.E.A., A.S., L.M.P., P.C., B.E.F.; Resources: A.S., L.M.P., P.C., B.E.F.; Data curation: C.H.C., Z.S.R.; Writing - original draft: C.H.C., B.E.F.; Writing - review & editing: C.H.C., A.W., P.B.H., Z.S.R., C.C., A.B.K., H.E.A., L.M.P., P.C., B.E.F.; Visualization: C.H.C.; Supervision: B.E.F.; Funding acquisition: L.M.P., P.C., B.E.F.

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#### Data availability

The CT scan data that support the findings of this study are openly available at [www.morphosource.org](http://www.morphosource.org), reference numbers: M159105, M159132, M159141, M159143, M159262, M159124 and M097019.

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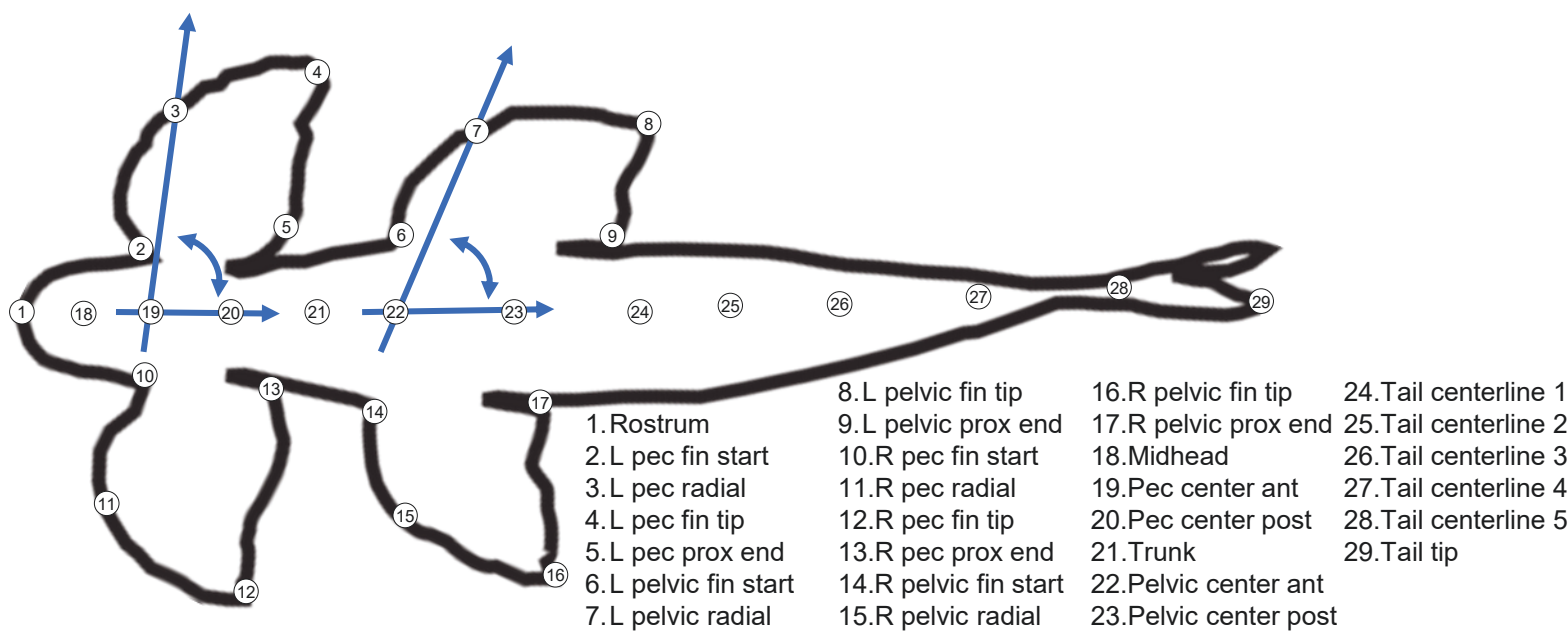
**Table S1.** Species and Filming Locality with Summary of Mean Kinematics Variables Measured from Walking Balitorids

| Variable                               | <i>Homaloptera parclitella</i> (n=5) | <i>Pseudohomaloptera sexmaculata</i> (n=5) |                                        | <i>Balitora</i> sp (n=5)                  | <i>Cryptotora thamicola</i> (n=2)    | <i>Homalopteroides modestus</i> (n=4)  |                                           | <i>Homalopteroides smithi</i> (n=3)    | <i>Homalopteroides</i> sp (n=3) |
|----------------------------------------|--------------------------------------|--------------------------------------------|----------------------------------------|-------------------------------------------|--------------------------------------|----------------------------------------|-------------------------------------------|----------------------------------------|---------------------------------|
| Locality                               | Laboratory                           | Tha Kha-nun, Thong Pha Phum, Kanchanaburi  | Prang Phe, Sangkhla Buri, Kanchanaburi | Tha Kha-nun, Thong Pha Phum, Kanchanaburi | Pang Mapha, Pang Mapha, Mae Hong Son | Lin Thin, Thong Pha Phum, Kanchanaburi | Huai Kayeng, Thong Pha Phum, Kanchanaburi | Lin Thin, Thong Pha Phum, Kanchanaburi | Kaem On, Chom Bueng, Ratchaburi |
| Museum ID                              | Flammang Lab Loach2                  | UF 245546                                  | UF 245427                              | UF 245561                                 | MARNM 7413                           | UF 245299                              | UF 245290                                 | UF 245298                              | UF 245539                       |
| Stride Length (BL)                     | 0.116                                | 0.132                                      |                                        | 0.100                                     | 0.214                                | 0.137                                  |                                           | 0.094                                  | 0.125                           |
| Cycle Duration (s)                     | 0.195                                | 0.192                                      |                                        | 0.146                                     | 0.440                                | 0.126                                  |                                           | 0.093                                  | 0.073                           |
| Body Lengths S <sup>-1</sup> (BL)      | 0.634                                | 0.734                                      |                                        | 0.744                                     | 0.525                                | 1.184                                  |                                           | 1.117                                  | 1.764                           |
| Appendicular Duty Factor               | 0.552                                | 0.560                                      |                                        | 0.559                                     | 0.567                                | 0.544                                  |                                           | 0.548                                  | 0.530                           |
| Diagonality                            | 0.449                                | 0.553                                      |                                        | 0.545                                     | 0.495                                | 0.527                                  |                                           | 0.529                                  | 0.540                           |
| Stride overlap                         | 0.053                                | 0.062                                      |                                        | 0.064                                     | 0.065                                | 0.042                                  |                                           | 0.036                                  | 0.035                           |
| Pectoral fin extension range (degrees) | 19.48                                | 18.244                                     |                                        | 12.305                                    | 25.831                               | 14.474                                 |                                           | 13.493                                 | 16.059                          |
| Pelvic fin extension range (degrees)   | 36.890                               | 23.250                                     |                                        | 18.581                                    | 30.397                               | 23.103                                 |                                           | 20.694                                 | 30.281                          |
| Maximum Curvature                      | 0.0173                               | 0.007                                      |                                        | 0.0128                                    | 0.0194                               | 0.0388                                 |                                           | 0.0312                                 | 0.044                           |

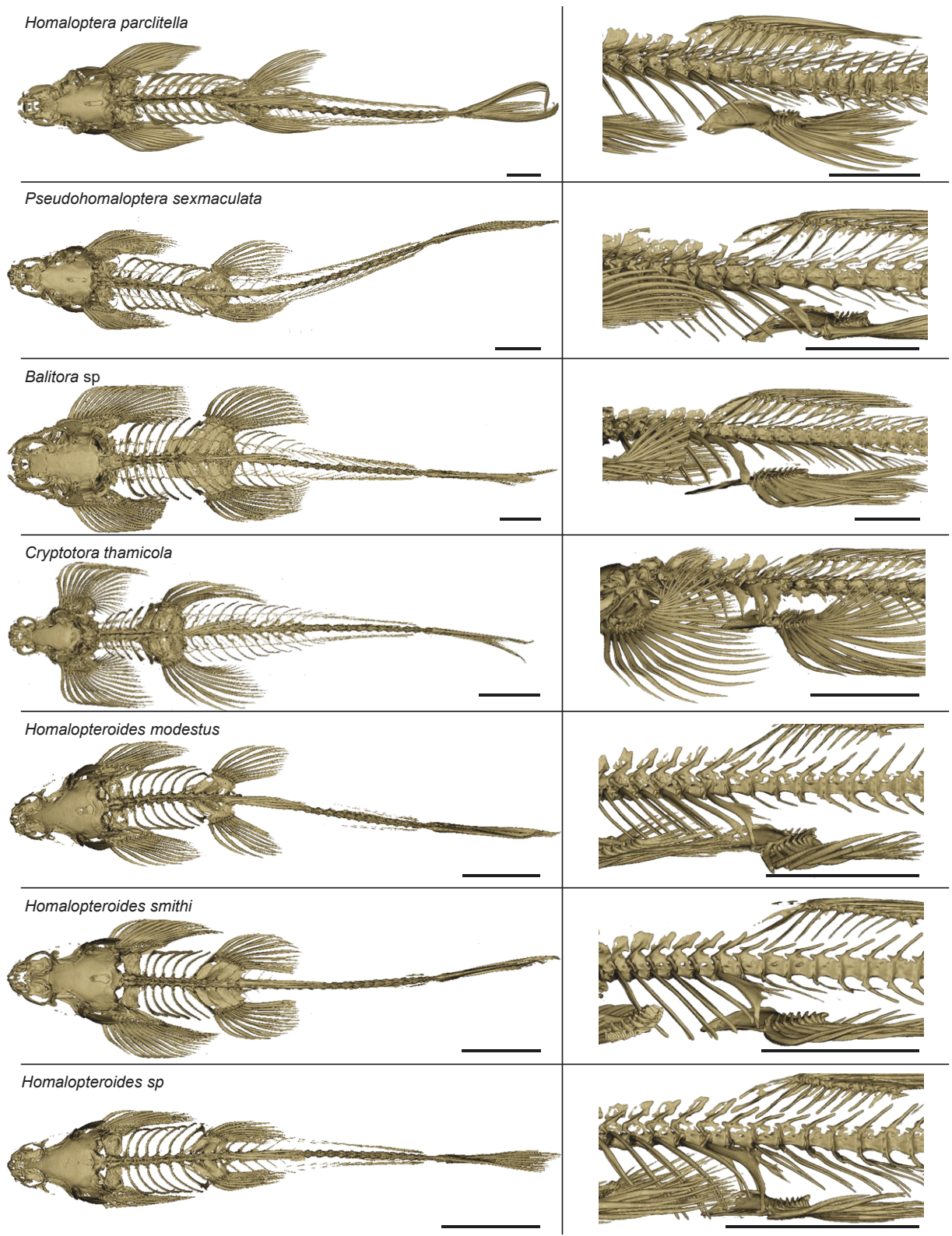
|                         |        |        |        |        |        |        |        |
|-------------------------|--------|--------|--------|--------|--------|--------|--------|
| Minimum Curvature       | 0.0015 | 0.0015 | 0.0013 | 0.0009 | 0.0035 | 0.001  | 0.0019 |
| Curvature Range         | 0.0158 | 0.0055 | 0.0115 | 0.0185 | 0.0353 | 0.0302 | 0.0421 |
| Trunk Maximum Curvature | 0.0179 | 0.0079 | 0.0125 | 0.0201 | 0.0354 | 0.0295 | 0.0422 |
| Trunk Minimum Curvature | 0.0013 | 0.0011 | 0.0010 | 0.0006 | 0.0031 | 0.0009 | 0.0018 |
| Trunk Curvature Range   | 0.0166 | 0.0068 | 0.0115 | 0.0195 | 0.0323 | 0.0286 | 0.0404 |
| Tail Maximum Curvature  | 0.0169 | 0.0068 | 0.0135 | 0.0193 | 0.0443 | 0.0345 | 0.0477 |
| Tail Minimum Curvature  | 0.0013 | 0.0011 | 0.0010 | 0.0006 | 0.0031 | 0.0009 | 0.0018 |
| Tail Curvature Range    | 0.0156 | 0.0057 | 0.0125 | 0.0188 | 0.0412 | 0.0336 | 0.0459 |

**Table S2.** Anova results for kinematic variables comparing across species.

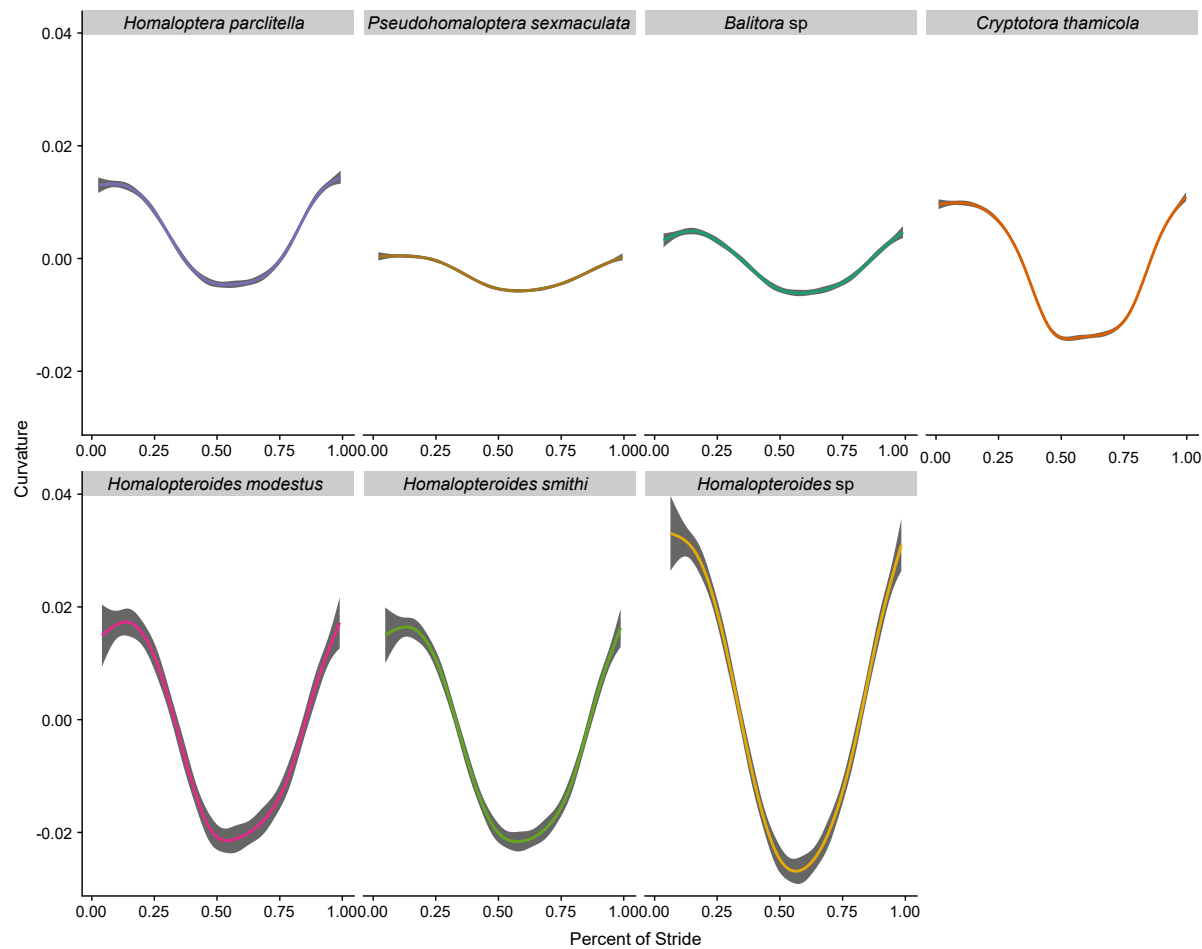
| Variable                                 | df | F     | P        |
|------------------------------------------|----|-------|----------|
| Body Lengths per second                  | 6  | 15.7  | 1.89E-13 |
| Body Lengths per stride                  | 6  | 15.54 | 2.44E-13 |
| Strides per second                       | 6  | 92.15 | <2e-16   |
| Tail-Hip Insertion Curvature Correlation | 6  | 27.22 | 4.94E-10 |
| Mean Maximum Curvature                   | 6  | 149.5 | <2e-16   |
| Step Overlap                             | 6  | 17.82 | <2e-16   |
| Appendicular Duty Factor                 | 6  | 110.4 | <2e-16   |
| Diagonality                              | 6  | 18.61 | <2e-16   |
| Pectoral fin extension range (degrees)   | 6  | 123.6 | <2e-16   |
| Pelvic fin extension range (degrees)     | 6  | 143.4 | <2e-16   |



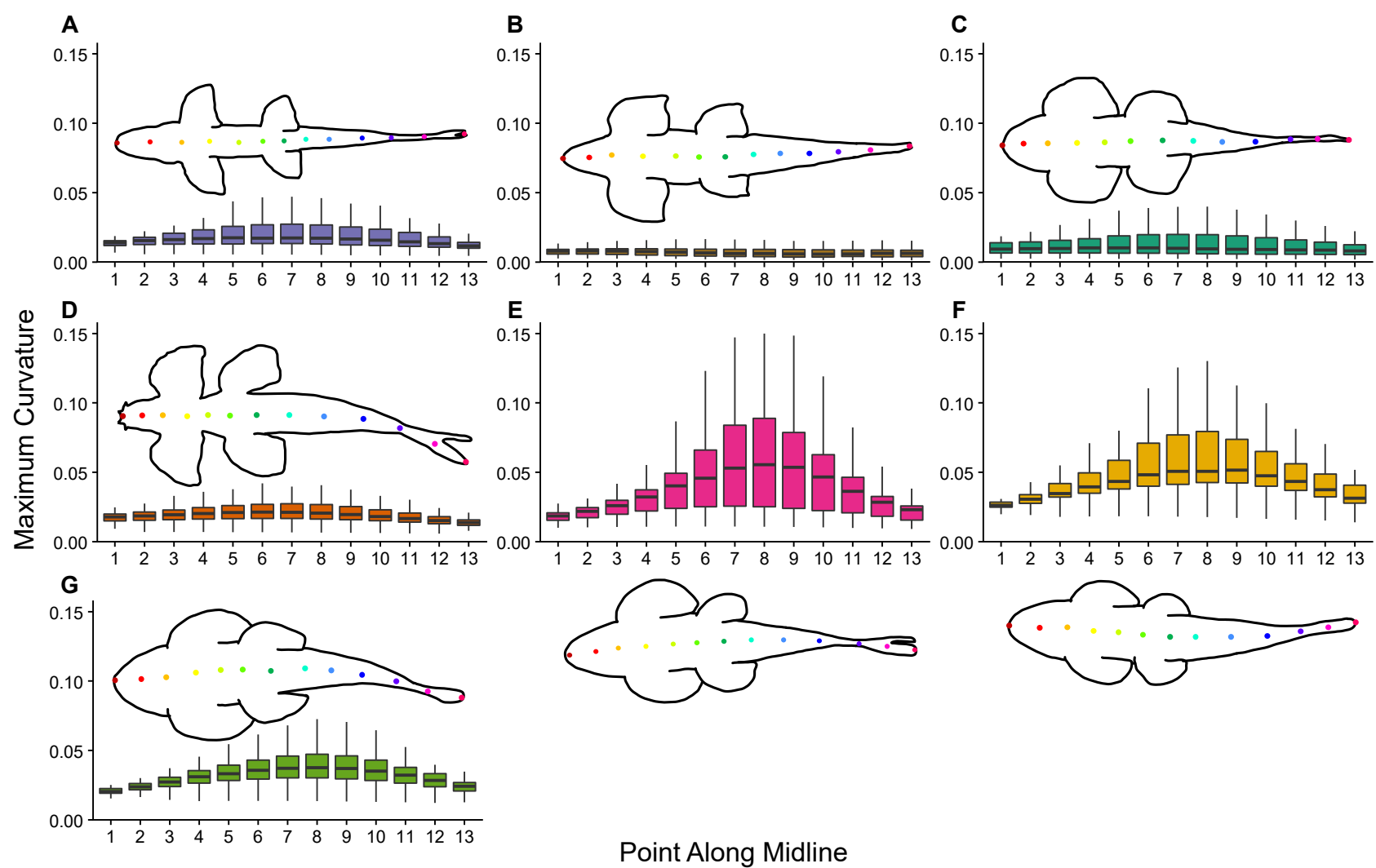
**Fig. S1.** Map of 29 digitized points used for analyses. Blue outlines illustrate angle measurements for pectoral and pelvic fin extension, points plotted on a line drawing of *Cryptotora thamicola*.



**Fig. S2. Reconstructions of  $\mu$ CT scans of the species studied.** Left panel shows whole body dorsal view and right panel highlights pelvic region. Scale bars = 10 mm.



**Fig. S3. Mean midline curvature over stride cycle separated by species:**  
A) *Homaloptera parclitella*, B) *Pseudohomaloptera sexmaculata*, C) *Balitora* sp.,  
D) *Cryptotora thamicola*, E) *Homalopteroides modestus*, E) *Homalopteroides smithi*, and  
F) *Homalopteroides* sp.. Each line shows the change in mean curvature over a full stride cycle with the grey band highlighting the 99% confidence interval.



**Fig. S4. Boxplots of mean maximum curvature of each species.** X-axis indicates the thirteen points down the midline with accompanying species outlines with the location of the 13 points illustrated. A) *Homaloptera parclitella*, B) *Pseudohomaloptera sexmaculata*, C) *Balitora* sp., D) *Cryptotora thamicola*, E) *Homalopteroides modestus*, F) *Homalopteroides* sp., and G) *Homalopteroides smithi*.