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ABSTRACT

THESE FISH WERE MADE FOR WALKING: MORPHOLOGY AND WALKING KINEMATICS IN BALITORID LOACHES

by Callie Hendricks Crawford

Terrestrial excursions have been observed in multiple lineages of marine and freshwater fishes. These ventures into the terrestrial environment may be used when fish are searching out new habitat during drought, escaping predation, laying eggs, or seeking food sources. The physiological demands for life under water and on land are vastly different and require different functional adaptations. Fish with terrestrial excursions must be capable of dealing with the stresses of both aquatic and terrestrial environments for varying periods of time. To deal with these stresses, amphibious fishes exhibit many morphological and behavioral adaptations. These adaptations have led to a range of locomotor strategies when traversing the terrestrial environment. Importantly, a broader understanding of the ecomorphology and biomechanics of terrestrial excursions in living fishes will aid in interpretation of both fossil fish and early tetrapod anatomy and trackways.

The rheophilic hillstream loaches (Balitoridae) possess a pelvic morphology which are attributed to adaptations for life in rapidly flowing water. The unique connectivity of the pelvic plate to the vertebral column via a sacral rib, and the relative size and shape of the sacral rib, fall within a spectrum of three discrete morphotypes. These morphotypes, determined through skeletal morphology and compared phylogenomically, are correlated with patterns observed in the pelvic muscle morphology of these fishes and are expected to provide a mechanical advantage for generating force against the ground.

The skeletal connection via the sacral rib in balitorid loaches is hypothesized to facilitate terrestrial locomotion observed in the family. Field and laboratory-collected high-speed video is used to analyze terrestrial walking kinematics in seven balitorid species representing both subfamilies and two of the three morphotypes. Contrary to the hypothesis that robustness of the sacral rib would strongly influence walking performance, there is not a large reduction in walking ability in the Morphotype 1 representative. Major differences in walking kinematics distinguish the two balitorid subfamilies; with a generally greater walking performance in Balitorinae and reduced capability in the Homalopteroidinae representatives. The connection between internal anatomy and locomotion on land are explored with digitized video analysis, µCT scans, and in the context of the phylogenetic history of this family of fishes.

The unexpected result of *Homaloptera parclitella* being capable of walking with comparable performance to other balitorids prompted further exploration into the walking mechanics in this species. To assess changes in terrestrial walking gait, walking kinematics are explored at experimental inclines (0°, 15°, 30°, and 45°) along with electromyography (EMG) during flat walking. *H. parclitella* is capable of walking at inclines without much change to the distance traveled over time. Additionally, another walking behavior is described; termed high walking, where the fish lifts its entire body off the substrate and carries its mass on its fins.

THESE FISH WERE MADE FOR WALKING: MORPHOLOGY AND WALKING KINEMATICS IN BALITORID LOACHES

by Callie Hendricks Crawford

A Dissertation Submitted to the Faculty of New Jersey Institute of Technology and Rutgers, The State University of New Jersey - Newark in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

Federated Biological Sciences Department

May 2021

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APPROVAL PAGE

THESE FISH WERE MADE FOR WALKING: MORPHOLOGY AND WALKING KINEMATICS IN BALITORID LOACHES

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CHAPTER 1

TERRESTRIAL EXCURSIONS BY TELEOST FISHES

1.1 Introduction

As of 2021, there are over 35,000 described species of fishes (Fricke et al., 2021). While the majority of these fishes spend their entire lives underwater, terrestrial locomotion has evolved separately in multiple lineages and extant fishes exhibit many morphological and behavioral characteristics which allow for moving out of water (Kawano and Blob, 2013; King et al., 2011; Standen et al., 2014; Wright and Turko, 2016). Finding new food resources, habitats, finding mates, laying eggs, dispersal, and escaping predation are all potential reasons a fish may leave its aquatic or marine environment (Blob et al., 2006; Bressman et al., 2018; Gibb et al., 2013; Lindsey, 1979; Michel et al., 2016; Ord and Cooke, 2016; Soares and Bierman, 2013; Van Wassenbergh, 2013; Wright and Turko, 2016).

Excursions into the terrestrial environment have been observed as purposeful or accidental and the ability to return directly back to the water is greater in those which have purposefully left the water (Gibb et al., 2013). Species with purposeful sojourns into terrestrial environments often have modified locomotion strategies for traversing land. The simplest locomotion strategy observed during terrestrial excursions in fishes is modified swimming without exceptional modification to the anatomy (i.e., eels, killifishes, and pricklebacks (Clardy, 2012; Gibb et al., 2011; Gibb et al., 2013; Gillis, 1998)) whereas at the other end of the spectrum, fish have developed specialized behavioral and anatomical modification to facilitate terrestrial locomotion (i.e., *Polypterus*, lungfish, mudskippers, and some loaches) (Flammang et al., 2016; Kawano

and Blob, 2013; King et al., 2011; Pace and Gibb, 2009; Standen et al., 2014). Life underwater and in air requires very different means in order to deal with the many physical and physiological challenges that change between the two environments. These physiological challenges include respiration, osmoregulation, and thermoregulation, in addition to the different effects both media have on the senses, including vision, olfaction, and mechanoreception (Clardy, 2012; Sayer, 2005). In the aquatic environment, water creates a buoyant environment which removes the requirement of weight bearing in fully submerged fishes, on land, this support is gone and the fish must work against gravity in order to move (Clack, 2012; Denny, 1993; Graham, 1997).

1.2 Reasons for Terrestrial Excursions

1.2.1 New or More Desirable Habitats

Some species which are not voluntarily amphibious may enter the terrestrial environment when their aquatic home becomes undesirable. Reasons for leaving to find more suitable habitats include reducing competition, hypoxic conditions, or drying of the habitat. When small pools become too crowded, climbing perch have been observed traveling to new pools with smaller populations and reduced competition for resources (Sayer and Davenport, 1991). Reduced oxygen levels or increased hydrogen sulfide levels have been seen to cause fish, including various species of killifish, to leave to find pools with better gas levels (Turko and Wright, 2015). Changes in gas content of pools are often linked to lowering water levels where fish and other organisms in the pools create a buildup of the byproducts of respiration. Some fish must be seasonally amphibious when their pools dry out completely. For example, lungfish survive drought conditions by either traveling to deeper pools or aestivating, where they cocoon themselves in a mucus layer (Wright and Turko, 2016).

1.2.2 Reproduction

Multiple lineages of teleost fishes leave the aquatic or marine environment for some aspect of reproduction, such as finding a mate or laying eggs. Some fishes, such as the grunion, only enter the terrestrial environment in order to spawn (Martin et al., 2004; Muench, 1941). These beach-spawners come ashore during spring tides to lay eggs in the subtidal zone and only remain out of water for a few minutes for females to lay eggs and males to fertilize those eggs, although they are able to move about on land (Martin et al., 2004). Many of the waterfall climbing fish known must make terrestrial excursions to get to their breeding habitat (Blob et al., 2006; Carvajal-Quintero et al., 2015). In these fishes, the immature individuals must travel from their natal pools up waterfalls to the pools where the mature fish are found. These small fish climb, either by using oral and pelvic suction, or by wriggling their bodies in order to use a powerful push up the waterfall. Some species of mudskipper have been observed completing mating displays out of water, even though they reproduce in the water (Sayer and Davenport, 1991). Eels, which reproduce in marine environments are regularly seen traveling across barriers to get to their reproductive habitat (Gillis, 1998; Sayer and Davenport, 1991).

1.2.3 Predation

Fish which enter terrestrial environments in order to feed must be able to find food and consume it. Water and air provide different challenges for chemoreception, audition, and vision, leading to fish needing adaptations in order to find their prey outside of their normal habitat. Additionally, as fish are often ram or suction feeders, functions that are

not possible in air without adaptive behaviors, these obstacles must be overcome. Some fishes are able to adjust their stance in order to close down around prey. Mudskippers use a combination of pivoting on their pectoral fins while using their protrusible jaws to engulf their prey (Michel et al., 2014). Fish that eat invertebrate prey, including ants and termites, leave the water for prey capture but often return to water to consume the prey (Michel et al., 2014; Turko and Wright, 2015).

1.2.4 Predatory Escape

The majority of predators for fish are in their same marine or aquatic habitats and some amphibious fishes will use the terrestrial environment to escape predation. Some species which are found to travel on land for predatory escape do not have respiratory adaptations and are only able to remain on land for a short period of time before they would otherwise desiccate or asphyxiate (Gibb et al., 2011). Turko and Wright (2015) summarize many instances of predatory escape in different groups of fishes, some will go onto the banks of ponds to escape their pursuers while others will use lily pads as refuge from larger fish. Although the terrestrial excursions may be a strategy to evade predators in the water, entering the terrestrial environment may lead to increased predation risk from avian and other vertebrate predators. Unlike fish leaving pools in order to find areas with reduced competition, those fish species which leave their natural habitat to escape predators have not been observed entering new habitats, they return to their original pools after a relatively short amount of time (Sayer and Davenport, 1991).

1.3 Locomotor Strategies of Fishes on Land

The ability to traverse the terrestrial environment for any of the previously mentioned reasons, in addition to others not explored here, is one of the most important and defining

features of amphibious fishes. Extensive work has been done to study terrestrial locomotion across different levels of specialization in morphology and behavior. Locomotor strategies can be broken down into three categories determined by the basis of the locomotion: axial, appendicular, and axial-appendicular-based locomotion (Pace and Gibb, 2014). Within these groupings, terrestrial locomotion can be further differentiated by the type of movement and movement patterns (Table 1). There is great variation among the groups of fish making terrestrial excursions and these modifications can be both behavioral and morphological. Terrestrial strategies for locomotion used by aquatic fishes range from modified swimming as seen in eels (Gillis, 1998; Redmann et al., 2020), killifishes (Gibb et al., 2011) and pricklebacks (Clardy, 2012); crutching or dragging with the pectoral fins as in *Polypterus* (Standen et al., 2014) and mudskippers (Kawano and Blob, 2013; Pace and Gibb, 2009; Wicaksono et al., 2018); alternating pelvic fin movements to push the body along as seen in lungfishes (King et al., 2011); to modified fins acting as suction disks for climbing in some Hawaiian gobies (Blob et al., 2006). The locomotion strategies listed before do not comprise walking, only one species is known to truly walk out of water, the waterfall cave-fish, Cryptotora thanicola (Flammang et al., 2016).

1.3.1 Swimming on Land

As the least morphologically modified approach to terrestrial locomotion, swimming on land uses body undulations similar to what is seen in swimming fishes (Clardy, 2012; Gibb et al., 2011; Gillis, 1998). Swimming on land is mainly a behavioral adaptation, without morphological changes to allow for the movements. Some catfish have spines on their pectoral fins to help gain better resistance against the ground while slithering and

undulating their bodies to travel to a new body of water (Johnels, 1957; Lindsey, 1979),

but many of these fishes simply replicate underwater movements on land.

| Family | Species | Propulsion | Movement | References |
|-----------------|-----------------------------------|------------------------------------|---|---|
| Lepidosirenidae | Protopterus annectens | Axial- Appendicular | Pelvic fin driven | King et al., 2011 |
| Polypteridae | Polypterus senegalus | Axial- Appendicular | Pectoral lift and lateral undulation | Standen, 2016 |
| Anguillidae | Anguilla rostrata | Axial | Lateral Undulation | Gillis, 1988, Redmann et al., 2020 |
| Stichaidae | Xiphister mucosus | Axial | Lateral Undulation | Clardy, 2012 |
| Poeciliidae | Gambusia affinis | Axial | C-Start | Gibb et al., 2011 |
| Blennidae | Praealticus labrovittatus | Axial | C-Start | Hsieh, 2010 |
| Blennidae | Alticus arnoldorum | Axial + appendicular support | C-Start- Tail twisting, hopping, porpoising | Hsieh, 2010 |
| Cottidae | Oligocottus maculosus | Axial- Appendicular | Rotate about pectoral fins | Bressman et al., 2018 |
| Clariidae | Clarias spp., | Axial- Appendicular | Pectoral spines for leverage | Pace and Gibb, 2010, Johnels, 1957, Bressman et al., 2018 |
| Gobiidae | Periophthalmus argentilineatus | Appendicular | Crutching | Pace and Gibb, 2014, Kawano and Blob, 2013 |
| Gobiidae | Periophthalmus variabilis | Appendicular | Crutching and climbing | Wicaksono et al., 2016; Wicaksono et al., 2018 |
| Gobiidae | Sicyopterus stimpsoni | Axial- Appendicular | Climbing- alternating oral and pelvic sucker attachment- inching | Blob et al., 2006; Schoenfuss and Blob, 2003, Blob et al., 2019 |
| Gobiidae | Lentipes concolor | Axial- Appendicular | Climbing- Undulation powerbursts | Blob et al., 2006; Schoenfuss and Blob, 2003, Blob et al., 2019 |
| Balitoridae | Homalopteroidinae | Axial- Appendicular | LSDC/DSDC with increased axial undulation | Crawford et al (in prep) |
| Balitoridae | Balitorinae | Axial- Appendicular | LSDC/DSDC with low axial undulation | Flammang et al., 2016 and Crawford et al (in prep) |

Table 1.1. Example Strategies for Terrestrial Locomotion in Fishes.

American eels, *Anguilla rostrata*, increase their frequency of undulations and have more equal waves throughout the body during terrestrial locomotion. Although they move their bodies faster, the distance and speed at which they travel on land is less than that observed in water (Gillis, 1998). Pricklebacks, like eels, swim in an undulatory manner. On land, these fish also employ undulation, and although they move at a similar speed, the undulations occur at a reduced frequency on land (Clardy, 2012). Although swimming on land does not require modified morphology, only changes in behavior in order to allow for movement in the terrestrial environment, further adaptations, including morphological changes, are required for other modes of terrestrial locomotion.

1.3.2 Jumping

Other fish, like the killifish, *Gambusia affinis*, are able to flip their bodies with precision in order to reach a specific destination (Gibb et al., 2011). Terrestrial jumping in fishes is often produced through modifications of the C-start escape response in which the head is bent back towards the tail while the fish is on its side then pushes off the substrate with the tail (Gibb et al., 2011). When jumping on land, these fish can sometimes reach large heights even without known adapted morphological features. Terrestrial movements in these fish are often for evading predators and these fish do not have respiratory adaptations to allow prolonged periods of time on land.

Another form of jumping seen in some blennies (Blenniidae) involves the tail being bent anteriorly towards the head, with the tail then being either placed ventral side down (amphibious blennies, *Praealticus labrovittatus*) or twisted so that the lateral surface is against the substrate (*Alticus arnoldorum*) and then pushed against the substrate to propel the fish forward (Hsieh, 2010). The Pacific leaping blenny, *Alticus arnoldorum*,

spends almost its entire life out of water, above the intertidal zone waterline where it feeds on algae and defends a territory (Hsieh, 2010; Ord and Hsieh, 2011). In addition to its leaping behavior, the leaping blenny is often observed climbing rocks and hopping, the latter is kinematically similar to jumping behavior although at lower velocity and not used during escape response (Hsieh, 2010).

1.3.3 Crutching or Pushing with the Pectoral Fins

Some species of fish which travel onto land use a crutching or dragging motion with the pectoral fins as in mudskippers (Kawano and Blob, 2013; Pace and Gibb, 2009) when they enter the terrestrial environment. The ability to crutch (movements similar to humans using crutches) is heavily dependent on the increase in robustness in the pectoral girdle and fins. In many teleost fishes, the pectoral fins are up on the sides of the fish and are used for propulsion or agility around their environment, not for weight bearing. Crutching-like behavior has been hypothesized to be a potential locomotion strategy utilized by the early tetrapod, *Ichthyostega* (Nyakatura et al., 2014; Pierce et al., 2012; Pierce et al., 2013).

Mudskippers use their paired pectoral fins to crutch simultaneously and the pelvic fins, which are also more anteriorly located, are used as part of a tripod-like system to maintain balance. Crutching is a means of slowly traversing the terrestrial environment and is commonly seen in mudskippers, although these fish can also jump if a quick escape is needed (Kawano, 2014). In one species, *Boleophthalmus boddarti*, the pelvic fins are fused and allow the mudskipper to climb vertically up trees (Wicaksono et al., 2016).

In *Polypterus*, the pectoral movement is mainly used to anchor the anterior body of the fish against the substrate and then the tail end pushes the fish forward (Standen et al., 2014). Along with lifting the anterior body, *Polypterus* laterally undulate their elongate body to travel out of water (Standen et al., 2014; Standen et al., 2016). *Polypterus* has been used to study the developmental plasticity of fishes on land, showing both morphological and kinematic variation in fishes raised on land as opposed to those in water (Standen et al., 2014).

1.3.4 Pushing with Pelvic Fins

Instead of using the pectoral fins as the means of terrestrial locomotion, some species use alternating pelvic fin movements to push the body along as seen in lungfishes. The African lungfish, *Protopterus annectens*, is often seen pushing its body along flat substrates by either simultaneously or asynchronously moving its pelvic fins (King et al., 2011). King *et al* (2011) noted that there was often a large amount of slippage during terrestrial locomotion in lungfish, likely impacted by the fish not reducing the anterior body friction by lifting up on their pectoral fins. Lungfish were also observed to not use its pectoral fins for propulsion, but instead seemed to use these fins for balance and support and sometimes not using those fins at all during terrestrial locomotion (King et al., 2011).

1.3.5 Climbing

Another morphological change that allows for terrestrial locomotion is the modification of fins to act as suction disks for vertical climbing, as seen in Hawaiian waterfall climbing gobies (Blob et al., 2006) and Loricariid catfishes (Carvajal-Quintero et al., 2015). Climbing fish are often seen climbing waterfalls and have the respiratory benefit

of still being wet, however, they must work against both gravity and the flow of the water coming down the waterfall. The Loricariid catfish observed climbing waterfalls uses a combination of its mouth and modified pectoral and pelvic fins to climb (Carvajal-Quintero et al., 2015). The juvenile waterfall climbing gobies of Hawaii use a few different methods for getting to their adult habitat at the top of the waterfalls. Some species use oral and pelvic suction created with modified structures (*Sicyopterus stimpsoni*) or sporadic, rapid axial undulation (*Awaous guamensis* and *Lentipes concolor*) to climb from juvenile habitats to adult habitats (Blob et al., 2006; Schoenfuss and Blob, 2003).

1.3.6 Walking

Although the previously mentioned locomotion strategies produce effective terrestrial locomotion, none of the previously discussed fishes walks with a true walking gait on land. Walking in terrestrial tetrapods is described as a symmetrical gait pattern in which stance phase for each of the four limbs is greater than 50% of the total step cycle, which results in no aerial phase, or a point in the step cycle where the animal is completely off the ground. (Hildebrand, 1980).

Work published in 2016 on the walking behavior observed in the walking cave fish, *Cryptotora thamicola*, described this fish as performing a lateral sequence diagonal couplets gait, a walking pattern previously described as exclusive to tetrapods (Clack, 2012; Flammang et al., 2016). This was the first example of behavioral and morphological adaptation in an extant fish that converge on tetrapodal walking behavior and morphology. Unlike in most other fishes where the pelvic bones are suspended in a muscular sling or loosely attached to the pectoral girdle anteriorly, *Cryptotora* has a

pelvic girdle with a large, broad puboischiadic plate that directly articulates with an enlarged sacral rib. This fish also has unique morphology in its vertebral column where it has large anterior and posterior zygapophyses and broad neural spines, both of which are features associated with terrestrial organisms (Flammang et al., 2016). The robust pelvic girdle and enlarged processes on the vertebral column play a large part in the ability of the fish to carry itself in a walking manner without water to assist against gravity. The sacral rib fulfills a key feature which facilitated the evolution of terrestrial walking: a robust, weight bearing connection between the pelvic appendages and the axial skeleton (Ahlberg, 2019; Clack, 2009; King et al., 2011).

Further work by Crawford et al. (2020; in prep) on the morphology and kinematics of other balitorid species suggests a further separation within the walkers with a range of swimming-like walking to more tetrapod-like walking. In the fish with movements more similar to swimmers (members of the Homalopteroidinae subfamily), the axial body has increased curvature and the forward propulsion is reduced per stride. Conversely, the fish which show movements more similar to tetrapodal walkers (members of the Balitorinae subfamily) have reduced axial bending and increased forward propulsion via appendicular movements.

1.4 Stepping Through Time

In the Devonian period, around 365 million years ago, a transition occurred in which the fish ancestor to tetrapods ventured onto land, paving the way for the diversity we see today in amphibians, reptiles, birds, and mammals (King et al., 2011; Pierce et al., 2013). This transition required many morphological changes, along with changes in behavior and physiology, to facilitate life on land. While the fish ancestors to tetrapods evolved at

this time, these 'fishapods' were still largely aquatic and lacked a robust pelvis to support their weight, and primarily used their forelimbs to lift their heads out of the water (e.g., *Tiktaalik*; Shubin et al., 2014) or for using a crutching behavior on land (e.g., *Ichthyostega*; Pierce et al., 2012). Studying fossil species and the form and function of extant species can enable inferences of possible evolutionary trajectories. The fin-to-limb transition was a pivotal point in the evolution of terrestriality. Although this transition has been studied extensively, limited fossil evidence and a focus on anterior structures has produced little information on how the pelvis evolved as a functional requirement for terrestrial walking.

Terrestrial excursions in fishes are not solely linked to the fin-to-limb transition; fishes outside of the sarcopterygian lineage which led to tetrapods also made terrestrial excursions as noted here. Additionally, trackways from the Valencia Slate Formation in Ireland predate the Devonian origin of tetrapods (Clack, 2012) and may have been made by a walking fish as the trackways are similar to the pattern created by *Cryptotora* (Flammang et al., 2016). Some non-amphibious fishes which exhibit locomotion strategies described as walking, walk underwater using their pectoral and pelvic fins (e.g., frogfish; Edwards 1989); however, in submerged walking the bodyweight is supported by the fluid around the organism instead of the fins as in terrestrial locomotion. Walking patterns are even older than tetrapodal walking and are seen in elasmobranchs such as epaulette sharks (Goto et al., 1999; Pridmore, 1995). The motor neurons responsible for walking patterns were recently found to be present in skates, indicating that the neuronal requirements for limb control and appendicular-based locomotion were present before terrestrialization (Jung et al., 2018).

Terrestriality in extant fishes is seen in many lineages, both marine and aquatic and in both Sarcopterygians and Actinopterygians, over 25 different genera across the phylogeny of teleosts contain representatives that traverse onto land (Gibb et al., 2013; Sayer and Davenport, 1991; Wright and Turko, 2016). Excursions into the terrestrial environment can be either purposeful or accidental, and depending on which group each species is in, it may or may not have evolved various behavioral or morphological traits to get back into the aquatic or marine environment. Species which accidentally find themselves on land will often use locomotor strategies similar to those they use under water in order to try to get back to their preferred environment (Gibb et al., 2013). Those which purposefully enter the terrestrial environment for any of the previously mentioned reasons, often have more advanced methods of returning to the water or for traversing the terrestrial environment (Gibb et al., 2013; Graham, 1997; Sayer, 2005; Sayer and Davenport, 1991). Although the majority of fishes discussed here are primarily aquatic with terrestrial excursions, some species, including multiple species of blennies, spend the vast majority of their time out of water and in the terrestrial environment (Gibb et al., 2013; Hsieh, 2010; Ord and Hsieh, 2011).

Fishes which spend time in the aquatic and terrestrial environments must be able to function under very different conditions depending on which environment they are inhabiting at a given time. Along the spectrum of terrestrial locomotion strategies in fishes is a range of undulatory locomotion, bipedal, quadrupedal, suction-based, and combinations of different strategies. The morphological and functional requirements of the different strategies vary, yet all must be able to transmit forces against the ground to counter the gravitational force which they do not experience when submerged. Further

understanding the variation in how different fishes traverse terrestrial environments has the potential to help increase our knowledge of how extinct species may have moved both above and below water and the functional limits of terrestrial locomotion.

CHAPTER 2

SKELETAL AND MUSCULAR PELVIC MORPHOLOGY OF HILLSTREAM LOACHES (CYPRINIFORMES: BALITORIDAE)

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2.1. Introduction

The hillstream loaches, Balitoridae (Cypriniformes), are a family of 101 species of morphologically diverse rheophilic freshwater fishes inhabiting south and southeast Asia (Fricke et al., 2021; Kottelat, 2012; Nelson et al., 2016). Species in this family are characterized by a dorsoventrally flattened body, enlarged pelvic basipterygium, and expanded pectoral and pelvic fins that are ventrally located (Figure 2.1) (De Meyer and Geerinckx, 2014; Hora, 1932; Nelson et al., 2016). In the majority of extant teleost fishes, the pelvic fins are positioned abdominally in earlier diverging groups, with more recent lineages having the fins located more anteriorly and attached to the pectoral girdle (Yamanoue et al., 2010). In the balitorids, there is a skeletal connection between the pelvic plate (basipterygium) and the vertebral column via a modified rib and its distal ligament (Chang, 1945; Sawada, 1982; Saxena and Chandy, 1966).

The morphology of these loaches may be an adaptation for life in their fastflowing environment. The hypertrophied ossification observed in the modified pleural rib, referred to here as a sacral rib, is likely an adaptation that allows the fish to transmit counterforces against the substrate to hold against the flow of fast moving water (Ahlberg, 2019; Chang, 1945). The hyperossification and connection between the basipterygium and axial skeleton in these fishes is reminiscent of the sacrum in terrestrial tetrapods (Flammang et al., 2016). This structural connection between the axial and appendicular skeleton in tetrapods was important for the evolution of terrestrial walking (Lebedev, 1997). The dorsoventral body compression seen in these fishes, along with the horizontal placement of their broad pelvic and pectoral fins, allows for increased contact with the substrate to support station-holding in fast water (Chang, 1945; Lujan and Conway, 2015; Sawada, 1982). Adhesive pads are also present on the leading pectoral and pelvic fin rays formed from thickened subepidermal connective tissue on the ventral side of the fin, and possess keratinized unculi (Chang, 1945; Conway et al., 2012; Hora, 1930; Sawada, 1982; Saxena and Chandy, 1966). Such pads have been recorded in taxa of four rheophilic Ostariophysi orders (Gonorynchiformes, Cypriniformes, Characiformes, and Siluriformes) (Conway et al., 2012).

Recently, the cave-obligate balitorid loach, *Cryptotora thamicola*, was shown to walk with a salamander-like lateral-sequence diagonal-couplets (LSDC) gait (Flammang et al., 2016). The walking behavior recorded in *C. thamicola* is facilitated by morphological features converging on terrestrial tetrapod synapomorphies, including robust pectoral and pelvic girdles, a connection of the pelvic girdle to the vertebral column via a fused sacral rib, broad neural spines, and zygapophyses connecting serial vertebrae. We hypothesize that the features supporting terrestrial locomotion are also features that support life in fast moving water and are present to varying degrees throughout Balitoridae.

Previous work separating balitorids and their sister family Gastromyzontidae based on morphology has led to the characterization of morphotypes based on the number of simple pelvic radials (Hora, 1930) and basipterygium shape, most notably the presence of lateral foramina (Balitoridae) or posterolateral horns (Gastromyzontidae) as the point of connection between the sacral rib and the pelvic plate (Sawada, 1982). Herein, we describe the skeletal and muscular pelvic fin modifications found throughout balitorid loaches and identify three discrete subgroups based on morphological features that are functionally important for walking behavior. In addition, we present here a novel molecular phylogeny that includes *Cryptotora thamicola*, providing a framework for the comparison of these morphotypes from a phylogenomic perspective.



Figure 2.1. Anatomical key to study region from a μ CT scan of *Cryptotora thamicola* (MARNM 6183), (A) complete skeleton from the dorsal view, (B) lateral, and (C) dorsal (left) and ventral (right) view of the study region (anterior to top); intermuscular bone (light purple); pelvic radials (dark blue); basipterygium (tan); ribs (light blue) sacral ribs (dark purple). Scale bars = 2.5 mm.
2.2. Materials and Methods

2.2.1 Species and Specimens

This study follows Kottelat (2012) and Tan and Armbruster (2018) in the classification of Balitoridae. We used a broad sampling of natural history museum specimens for this work, representing 29 species and 14 of 16 balitorid genera for the skeletal work. From the skeletal observations, one species per morphotype was analyzed for muscle morphology. The outgroup comparison for skeletal and muscular morphology was *Carassius auratus* (Cyprinidae). Specimens were borrowed from the American Museum of Natural History (AMNH), the Academy of Natural Sciences of Drexel University (ANSP), the California Academy of Sciences (CAS), the Florida Museum of Natural History (UF), Maejo Aquatic Resources Natural Museum, Maejo University, Nong Han (MARNM), the Smithsonian National Museum of Natural History (USNM), and the Zoological Reference Collection at the Lee Kong Chian Natural History Museum (ZRC) (Table 2.1).

2.2.2 µCT Scanning, Staining, and Segmentation

To visualize skeletal and muscular morphology, we collected Computed Microtomographic (μCT) scans of all loaned species and, when permitted by collections staff and curators, stained specimens in phosphotungstic acid (PTA) to increase radiopacity of muscle. We μCT scanned museum specimens (Table 2.1) using a Bruker SkyScan 1275 at the New Jersey Institute of Technology Otto York Bioimaging facility, a Bruker SkyScan 1275 at Microphotonics (Allentown, PA), a GE Phoenix v|tome|x M at the American Museum of Natural History, GE Phoenix v|tome|x M scanner (GE Measurement & Control, Boston, USA) at the University of Florida's Nanoscale

Research Facility, and a Nikon XTH 225 ST at Duke University. The scanner settings varied due to specifications of the different types of scanners and are available in Appendix A, Table A.1. Specimens for this study were μ CT scanned at voxel sizes ranging from 10 μ m-26 μ m.

After the initial scan for skeletal morphology, specimens were stained in 3% PTA solution in 70% ethanol for two weeks to allow for full penetration of the stain. PTA readily stains tissues with high protein and collagen content, including muscles and ligaments; however, it does not stain cartilage (Descamps et al., 2014; Metscher, 2009). Staining with PTA causes considerably less specimen shrinkage than has been seen with iodine staining, (Buytaert et al., 2014), which we verified through comparison to original scans, and does not visibly discolor specimens. After staining, we scanned the specimens again at settings appropriate for stained material.

Scan data were reconstructed using reconstruction software accompanying the different scanners, following manufacturer guidelines for appropriate reconstructions. We then used FIJI (Fiji is just ImageJ, http://fiji.sc) and DataViewer (Bruker, Belgium) to crop datasets for visualization and segmentation in Mimics Segmentation Software Research Suite v20.0 (Materialise, Belgium). The digital dissections segmented from the scans were used to separate the species into morphotypes based on the shape of the sacral rib, its connection to the basipterygium, and the shape of the basipterygium.

| Taxon | Museum/Institution | Specimen Number | Morphotype |
|-----------------------------|--------------------|--------------------|------------|
| Carassius auratus | Flammang Lab/NJIT | N/A | Т |
| Ghatsa montana | CAS | SU39871 | M1 |
| Homaloptera bilineata | USNM | 378394 | M1 |
| Homaloptera ogilviei | USNM | 288431 | M1 |
| Homaloptera orthogoniata | Flammang Lab/NJIT | N/A | M1 |
| Homaloptera parclitella | Flammang Lab/NJIT | N/A | M1 |
| Homalopterula vanderbilti | ANSP | 68689 | M1 |
| Neohomaloptera johorensis | UF | 166089 | M1 |
| Balitoropsis zollingeri | UF | 235547 | M2 |
| Hemimyzon formosanus | USNM | 161711 | M2 |
| Hemimyzon taitungensis | USNM | 300711 | M2 |
| Homalopteroides nebulosus | UF | I235748 | M2 |
| Homalopteroides rupicola | CAS | 231726 | M2 |
| Homalopterula gymnogaster | USNM | 409946 | M2 |
| Homalopterula heterolepis | AMNH | 9263 | M2 |
| Homalopterula ripleyi | USNM | 390014 | M2 |
| Jinshaia abbreviata | ANSP | 185166 | M2 |
| Lepturichthys fimbriatus | ANSP | 185165 | M2 |
| Sinogastromyzon puliensis | UF | 185384 | M2 |
| Balitora burmanica | USNM | 44808 | M3 |
| <i>Balitora</i> sp | ANSP | 179834 | M3 |
| Balitoropsis ophiolepis | UF | 166109 | M3 |
| Bhavania australis | USNM | 165107 | M3 |
| Cryptotora thamicola | MARNM | 6183 | M3 |
| Hemimyzon macroptera | USNM | 293925 | M3 |
| Homalopteroides smithi | UF | 172923 | M3 |
| Homalopteroides stephensoni | ZRC | FIS51741 | M3 |
| Homalopteroides tweediei | Flammang Lab/NJIT | N/A | M3 |
| Homalopteroides weberi | USNM | 393729 | M3 |
| Pseudohomaloptera leonardi | UF | 183398 | M3 |

Table 2.1. List of study taxa with associated museum and specimen numbers used in this study with the designated morphotype determined with μ CT scan segmentations

Note: Museum abbreviations: AMNH, American Museum of Natural History; ANSP, Academy of Natural Sciences of Drexel University; CAS, California Academy of Sciences; MARNM, Maejo Aquatic Resources Natural Museum, Maejo University, Nong Han; UF, Florida Museum of Natural History; USNM, Smithsonian Institute; and ZRC, Zoological Reference Collection at the Lee Kong Chian Natural History Museum.

2.2.3 Shape Analysis

Rib shape of 29 balitorid species and one outgroup (*Carassius auratus*, Cyprinidae) was analyzed using the Elliptical Fourier Descriptors (EFD) approach (Kuhl and Giardina, 1982) in order to analyze two-dimensional (2D) rib shape changes among the three morphotypes. 2D images were taken of the three-dimensional (3D) segmented models from the right sacral rib of each species in the study. The rib models were oriented perpendicularly to the screen to capture their overall shape. The 2D images were converted to grayscale bitmaps in Fiji (Schindelin et al., 2012) for outline analysis and converted to chain code using the SHAPE 1.3 program (Iwata and Ukai, 2002). Chain codes along the perimeter of each rib shape were used to create a harmonic series using 80 harmonics within SHAPE 1.3. The principal component analysis (PCA) was completed using PrinComp, another program within the SHAPE 1.3 software. The PC scores from SHAPE 1.3 were used to visualize the variance within and between the morphotypes. One specimen for each species was included in the shape analyses. To quantitatively test the morphotypes delimited from the shape data of the analyzed species, Linear Discriminant Analysis was run using the *lda* function in the R-package MASS (Ripley and Venables, 2002). The confusion matrix function was employed to compare our classification of morphotype with that predicted using the LDA.

2.2.4 PCSA

Within Mimics Segmentation Software, the μ CT scan data of the PTA-stained specimens were used for muscle analysis. Fiber lengths were calculated by measuring the length of the fiber bundles and taking the average over 3-10 bundles, with more bundles measured whenever possible. The physiological cross-sectional area (PCSA) of each muscle was

calculated as muscle volume/fiber length and then normalized. The fiber lengths and PCSA were normalized to the total fish volume measured from Mimics using $V^{1/3}$ and $V^{2/3}$, respectively. Normalized PCSAs were plotted against normalized fiber lengths to generate a functional morphospace of the pelvic fin muscles. This morphospace creates a visualization of the trade-offs between muscle force (PCSA) and range of muscle shortening (fiber length) (Allen et al., 2010; Dickson and Pierce, 2019; Lieber, 2002). Following Allen et al. (2010), the morphospace can be broken into quadrants with the upper left being "force-specialized" muscles with large forces and small extension ranges; upper right are "powerful" muscles with large force and large extension; and finally the lower left are "generalized" muscles with low force and small extension.

2.2.5 Phylogenomics

For our molecular phylogeny, we sampled across seven families of loaches (Cypriniformes) with members of the Vaillantellidae used as the outgroup (Appendix A.2 Table A.2.1, N = 62). Samples were chosen due to changing taxonomic classification among the loach families (Kottelat, 2012; Randall and Page, 2015; Šlechtová et al., 2007; Tan and Armbruster, 2018). We used ultraconserved element (UCE) loci as a reduced representation genomic dataset to reconstruct the evolutionary relationships. These loci have both areas of high conservation, allowing for comparisons across species, and also flanking regions that contain genetic variability to allow for identification of diversity (Faircloth et al., 2012). Our molecular methods and bioinformatics processing were identical to those in Hart et al. (2020; detailed in Supplementary Material 2). We partitioned the data using Sliding-Window Site Characteristic based on site entropies

(Tagliacollo and Lanfear, 2018) in PartitionFinder2 (v.2.1.1; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017) on CIPRES Gateway. We reconstructed relationships using Maximum Likelihood (RAxML-HPC2 on XSEDE v.8.2.10; Stamatakis, 2014) and a coalescent species tree method (SVDQuartets in PAUP* v.4.0a; PAUP*: Swofford, 2002; SVDQuartets: Chifman & Kubatko, 2014), both using a concatenated dataset of 75% completeness (N = 411 loci). We matched morphotypes from our skeletal morphology portion onto the multispecies coalescent tree to visualize the distribution of morphotypes in a phylogenetic context. Collaboration on this project was preceded by independent studies of morphology and molecular phylogenetics. Due to the nature of the work required for these different approaches (e.g., formalin-fixed specimens can be used for morphological work, but not always for a molecular approach), it was not possible to get permission to stain and scan all species sampled for the phylogeny nor obtain tissue samples from all species scanned.

We tested for phylogenetic signal in our discrete character of morphotypes using the *phylo.signal.disc* function in R (v.4.0) (Bush et al., 2016) following pruning our tree for only species that overlapped in both morphological and molecular datasets. We also pruned the tree to include a single tip (individual) per species so as not to bias the distribution of morphotypes. Phylogenetic signal is the notion that closely related species resemble each other more so than they resemble randomly chosen species from the phylogeny (Blomberg et al., 2003; Münkemüller et al., 2012). The *phylo.signal.disc* function uses the Maddison and Slatkin (1991) method in which the number of minimum observed evolutionary transitions at nodes is compared to the distribution of transitions from a null model. If the observed number is significantly less than the median from the

null distribution, a significant *p*-value is inferred. We performed 999 randomizations. To examine if our small sample size for M1 (N=2) affected our phylogenetic signal results, we analyzed the data with and without M1 specimens.



Figure 2.2 Representation of variation in balitorid pelvic morphology shown from (A) lateral view, (B) dorsal view, (C) close-up view of sacral rib with other ribs removed, and (D) dorsal view of the basipterygium and pelvic fin rays. Typical fish morphology, *Carassius auratus* (Flammang Lab); Morphotype 1, *Neohomaloptera johorensis* (UF 166089); Morphotype 2, *Homalopterula vanderbilti* (ANSP 68689); and Morphotype 3, *Cryptotora thamicola* (MARNM 6183). Intermuscular bone (light purple); pelvic radials (dark blue); basipterygium (tan); ribs (light blue) sacral ribs (dark purple).

2.3. Results

2.3.1 Skeletal Morphology

We found a broad spectrum of pelvic morphology in the balitorids studied here; this variation was separated into three pelvic morphotypes determined from μ CT reconstructions of the skeletal structures in the pelvic region (Figure 2.2). The structures we used to distinguish the morphotypes were the enlargement or elongation of the sacral rib, the curvature of the sacral rib, the presence of a flared lateral edge of the sacral rib as previously described in *Cryptotora thamicola* (Flammang et al., 2016), the extent of the connection to the basipterygium, and the shape of the puboischiadic plate, or basipterygium (puboischiadic plate in Flammang et al., 2016).

2.3.1.1 Cyprinid Outgroup. In the general teleost outgroup used here for skeletal comparisons, *Carassius auratus* (Figure 2.2, row 1), the vertebrae lacked zygapophyses or similar bony connections between serial neighbors. Thoracic ribs were long and tapered, attached to the anteroventrally-positioned parapophysis and diapophysis, and extended ventrally. Importantly, the ribs did not attach to the basipterygium; the pelvic fin bones hang in a muscular sling in the ventral body, as is considered the more ancestral condition (Yamanoue et al., 2010). The basipterygium is long and narrow, and the two bilateral halves joined only at the anterior symphysis and posteriorly between the fin rays; the central halves of the pelvis did not meet at the midline. There were no lateral foramina in the basipterygium. The posterior processes of the basipterygium were long and narrow and did not connect at the midline but instead tapered caudally and laterally.

2.3.1.2 Balitorid Morphotype 1. Of the 29 balitorids scanned for this study, seven fit into Morphotype 1 (M1; Figure 2.2, row 2): *Ghatsa montana, Homaloptera bilineata,*

Homaloptera ogilviei, Homaloptera orthogoniata, Homaloptera parclitella,

Homalopterula vanderbilti, and *Neohomaloptera johorensis* (Table 2.1). Of the three morphotypes, M1 fishes were the most similar to the typical teleost anatomy. Thoracic ribs were attached to the anteroventrally-positioned parapophysis and more dorsally located diapophysis and extend ventrally. The attachment area of the rib to the vertebrae was smallest, and the angle of this attachment was shallowest compared to the other two morphotypes. On the anterodorsal aspect of the thoracic vertebral centra of all species were small bilateral anteriorly facing articular facets, or zygapophyses. Posteriorly facing articular facets were located at the posterior end of the thoracic vertebral centra on all species except for *Homaloptera bilineata* and *Neohomaloptera johorensis*. In M1, the anterior and posterior zygapophyses or the vertebra supporting the sacral rib were significantly smaller than those of M2 and M3, averaging 12.53% (n=7, SD=0.06) and 5.19% (n=7, SD=0.03) of the vertebral length, respectively. Zygapophyses were not observed on caudal vertebrae, i.e., those caudal to the sacral vertebra.

The intermuscular bones of fishes in morphotype 1 were simple and had low visibility in some μ CT scans (*Neohomaloptera johorensis*, Figure 2.2, light purple). One rib, described here as the sacral rib (after Flammang et al., 2016) (Figure 2.2, dark purple) extended distally through the lateral foramen of the basipterygium. The sacral rib in M1 fishes was morphologically similar to the thoracic ribs preceding it, with the exclusion of its proximity to the basipterygium. The basipterygium averaged 72.60% as wide as it was long (n=7, SD=0.15) and was roughly diamond-shaped, with the widest aspect at the anterior attachment of the fin rays. The bilateral halves of the basipterygium were joined at the midline, creating a dome that extended dorsally with a wide ventral concavity;

however, the lateral aspects of basipterygium were flat with a large lateral foramen on each side, just anterior to the pelvic fin. The bilateral posterior processes of the basipterygium were thin and tapered toward each other.

2.3.1.3 Balitorid Morphotype 2. Morphotype two (M2) exhibited intermediate rib morphology (Table 2.1; Figure 2.2, row 3) and was comprised of eleven of the 29 studied species (*Balitoropsis zollingeri, Hemimyzon formosanus, Hemimyzon taitungensis, Homalopterula ripleyi, Homalopteroides nebulosus, Homalopteroides rupicola, Homalopterula gymnogaster, Homalopterula heterolepis, Jinshaia abbreviata, Lepturichthys fimbriata,* and *Sinogastromyzon puliensis*). Thoracic ribs, including the sacral rib, were attached to vertebral centra with a larger contact area than in M1 fishes: the parapophysis was in the typical anteroventral position but the diapophysis was dorsal and more posterior, approximately mid-centra.

The anterior and posterior zygapophyses in M2 fishes were significantly larger than those of M1 and smaller than those in M3, averaging 19.44% (n=11, SD=0.05) and 8.60% (n=11, SD=0.03) of the vertebral length, respectively. The sacral rib was distinguishable from the distally tapered thoracic ribs preceding it by having a broad distal end, which was anchored through the lateral foramen of the basipterygium via a distal ligament. Basipterygium width was 92.67% of its length (n=11, SD=0.14), and the central dome formed by the fusion of the bilateral halves was not as high and curved as in M1 fishes, and in the two *Hemimyzon*, was nearly flat. The posterior processes of the basipterygium in nearly all species (*Homalopteroides nebulosus* had posterior processes resembling those in M1) were only about half as long as those observed in M1 fishes and ended with a blunt taper caudally, as opposed to a long point.

2.3.1.4 Balitorid Morphotype 3. Eleven species were categorized into a third morphotype (M3; Table 2.1; Figure 2.2, row 4), which included species with the most extreme differences from the typical teleost pelvic morphology (*Balitora burmanica, Balitora* sp., *Balitoropsis ophiolepis, Bhavania australis, Cryptotora thamicola, Hemimyzon macropterus, Homalopteroides smithi, Homalopteroides stephensoni, Homalopteroides tweediei, Homalopteroides weberi*, and *Pseudohomaloptera leonardi*). Thoracic ribs in M3 fishes had the largest vertebral contact area as compared to the other morphotypes and the cyprinid outgroup, and the angle of the contact area was closest to vertical, extending from the anteroventral parapophysis to the diapophysis located at the base of the neural spine. Zygapophyses were robust and had articulating facets significantly larger than those seen in M1 and M2, with anterior zygapophyses averaging 23.75% (n=11, SD=0.04) and posterior zygapophyses averaging 12.67% (n=11, SD=0.05) of the vertebral length.

The first caudal vertebra, directly following the vertebrae supporting the sacral ribs, had anterior zygapophyses articulating with the sacral vertebrae and reduced posterior zygapophyses. The sacral rib was more robust than the thoracic ribs preceding it, thicker throughout its length, and extended in a large flared crest near its midpoint. The distal end of the sacral rib was firmly attached to basipterygium at the lateral foramina via ligamentous attachment (Figure 2.8). Similar to M2, the basipterygium was nearly as wide as it was long, with average width 96.54% of length (n=11, SD=0.12), and the central region of the plate, where the two halves joined at the midline, was the least domed of the balitorid fishes and the lateral edges of the basipterygium anterior to the fin rays were curved dorsally. Posterior processes of the basipterygium were on average

smaller than those in the other two morphotypes, with some lacking these processes completely. While the intermuscular bones in other morphotypes, as compared to outgroup teleosts, were largely unremarkable, in M3 fishes they were very thick and often attached to the lateral aspect of the vertebral centra. Morphotypes 1 and 2 did not show a consistent pattern in neural spine shape. However, in M3, the neural spines anterior to the dorsal fin were broadened, with the extent varying from only slightly, about 25% of the spine height in *Homalopteroides smithi*, to 100% of the spine height in *Balitora burmanica* and *Balitoropsis ophiolepis*.



Figure 2.3 Visualization of the rib shape variation explained by PCs 1-5 at ± 2 standard deviations and the mean shape for each with overlay at left and the percent of shape variance explained given for each PC.

2.3.2 Shape variation and PCA

Shape variation using Elliptical Fourier Analysis of rib shape outlines accounts for 82.06% of the variance observed in morphotype variation of rib shape within the first three principal component axes (Figure 2.3). PC1 describes 47.56% of the shape variation in the thickness of the rib with the low values showing long and narrow ribs, and the higher values showing stockier, thickened ribs with the thickened crest distinctive of M3 (Figure 2.3, top row). PC2 (Figure 2.3, second row) describes 22.45% of the shape variation with low values indicating ribs with less curvature and a more flattened shape and high values indicating ribs with highly arched shape and an increased area below the rib with increased curvature. PC3 describes 12.05% of the variation in the shape of the rib near the attachment to the vertebrae and the mediolateral location of the crest relative to the rib attachment site to the vertebra (Figure 2.3, third row). Other PC axes illustrate variation in the location of the crest and size of the vertebrae attachment (Figure 2.3, PC 4-5, and Appendix A.1 Table A.1.2).

The morphospace determined by the shape analysis using Elliptical Fourier Analyses indicates distinct spatial separations among the three morphotypes (Figure 2.4). Results of the LDA explain 89.81% and 10.19% of the among-group variation in LD1 and LD2 (Appendix A.1 Figure 2.1), respectively, with a resubstitution accuracy of 1.0 and a jackknifed (leave-one-out) accuracy of 0.857. The reduced accuracy of the jackknifed LDA will likely impact species which are either on the extremes of the shape variation (outliers) or are on the edge of the boundaries of two morphotypes. PC1 shows significant differences between the balitorid morphotypes (p<0.002 for all morphotype comparisons except for M1 against typical teleost rib morphology) with greater overlap



Figure 2.4 Results of the principal components analyses of rib shape using Elliptical Fourier Analyses for inferring morphotypes of balitorid loaches. (A) Morphospace represented by the bivariate graph of PC1 and PC2 scores; (B) morphospace represented by the bivariate graph of PC1 and PC3 scores; (C) morphospace represented by the bivariate graph of PC2 and PC3 scores; (D) 3D morphospace represented by the 3D plot of PC1, PC2, and PC3 with ellipses representing two standard deviations from the mean shape. See Appendix A.1 Table 4 for identification of numbered species in A-C.

among morphotype space observed in PCs 2 and 3. Along PC1 (47.56%), M1 is grouped towards the end of the axis representing long and narrow sacral ribs and small vertebral attachment area, M2 in the center with thicker ribs and intermediate attachment area, and

M3 at the other end of the axis representing enlarged ribs and a flared crest (Figure 2.3, top row and Figure 2.4). For the PC2 axis (22.45%) M3 species were evenly distributed along the axis of the principal component, including species with ribs of both minimal curvature (*Bhavania australis*, Figure 2.4, #6) and tight curvature (*Hemimyzon macropterus*, Figure 2.4, #13). M2 has a more constrained range along PC2 with the greatest difference between *Homalopteroides rupicola* (Figure 2.4, #20) and *Homalopterula ripleyi* (Figure 2.4, #26). Along PC2, M1 is even more restricted to the central area of space with curvature closer to the mean shape (Figure 2.4) of all species analyzed. Most of the PC3 axis (12.05%) for M3 defines the location of the flared crest of the rib with the two extremes within M3 illustrated by *Balitoropsis ophiolepis* (Figure 2.4, #3) and *Homalopteroides smithi* (Figure 2.4, #21). M1 variation along PC3 shows the location of the major bend in the rib along the mediolateral axis.

2.3.3 Muscle Morphology

The axial body wall muscles (Figure 2.5, grey) exhibited the typical fish w-shaped myomere configuration and its two hypaxial subdivisions, the obliquus superioris and obliquus inferioris (Winterbottom 1973), were readily distinguished in the µCT scans. The obliquus superioris muscle fibers were oriented anterodorsally to posterioventrally whereas the obliquus inferioris muscle fibers were oriented anteroventrally to posteriodorsally. In M2 and M3, the distal end of the sacral rib passes superficial to the obliquus before inserting into the basipterygium (Figure 2.5A); this lateral supraposition is not as pronounced as in M1. In our example of a typical teleost, this opening is not visible, and the ribs do not pass through the body wall to reach the basipterygium; instead they are deep to the obliquus superioris (Fig 5A, grey). In contrast to the teleost outgroup,

which had small infracarinalis muscles, fishes in all three balitorid morphotypes had thick infracarinalis anterior and infracarinalis medius muscles, which were connected by a thin ligament along the ventral side of the pelvis (Figure 2.5A, pink). The extensor proprius (Figure 2.5, green), which is part of the muscular sling holding the pelvis in place in typical teleosts, was not found in any of the balitorids stained with PTA.

We found large arrector muscles in all three morphotypes, although both the arrector dorsalis (Figure 2.5, fuchsia) and arrector ventralis (Figure 2.5, orange) have a greater physiological cross-sectional area in M2 and M3 than in M1 or in the typical teleost. The arrector dorsalis originates at the dorsolateral edge of the basipterygium following the anterolateral edge of the basipterygium and inserts on the first fin ray.

In M1, the adductor superficialis (Figure 2.5, purple) extended anteriorly on the lateral edge whereas M2 had some, although less of an extension anteriorly compared to the placement of the adductor profundus (Figure 2.5, yellow). Adductor profundus origin placement on the basipterygium was near the midline of the basipterygium in M1 and M2; however, in M3, the origin was more lateral (Figure 2.5C). We found that the adductor superficialis and adductor profundus tightly follow the curvature of the posterior edge of the basipterygium in all morphotypes. In M3, the dorsal pelvic muscles do not meet at the midline as seen in the other morphotypes and in other fishes. The extensor proprius (Figure 2.5, green), which is part of the muscular sling holding the pelvis in place in typical teleosts, was not found in any of the balitorids stained with PTA.

With the broadening of the basipterygium from M1 through M3, there is a shallower angle of the muscle fibers, with the adductor and abductor muscles of M1 being steepest, those of M2 having intermediate muscle fiber angles and those of M3



Figure 2.5 Pelvic girdle musculature from CT scans with PTA staining. Bony structures of the basipterygium and fin rays are inserted from CT scans of the same specimens prior to staining. Typical fish morphology, *Carassius auratus* (Flammang Lab); Morphotype 1, *Homaloptera ogilviei* (USNM 288431); Morphotype 2, *Homalopterula ripleyi* (USNM 390014); and Morphotype 3, *Balitora* sp. (ANSP 179834). (A) Lateral view shows the pelvis with the axial muscles and the sacral rib (red), (B) ventral and (C) dorsal views show segmented pelvic muscles (anterior to the top). Axial muscles (gray); infracarinalis (pink) abductor superficialis (blue); abductor profundus (turquoise); adductor profundus (yellow); adductor superficialis (purple); arrector dorsalis (fuchsia); arrector ventralis (orange); extensor proprius (green); and bone (tan), sacral rib insertion (black dotted outline). Scale bars. Scale bars = 2.5 mm.

closest to horizontal. The abductor profundus in the balitorid fishes fills in the area

underneath the concavity of the basipterygium leading to an increase in muscle volume in M2 and M3 which have a larger concavity volume (Figure 2.5B, turquoise).

2.3.4 PCSA

The physiological cross-sectional area (PCSA) of the pelvic muscles of balitorids is overall greater when compared to the outgroup teleost, *Carassius auratus* (Figure 2.6 and Appendix A.1 Table 3). Among the balitorid fishes, M1 had pelvic muscle PCSAs most similar to that of a typical fish, whereas M2 and M3 had greater PCSAs indicative of increased potential maximum force production within these muscles. Overall, PCSA of all pelvic muscles in M1 indicate a lower capacity for force production than all pelvic muscles in M2 and M3. Fiber length range varied between the morphotypes as well, with M2 exhibiting the greatest variability in fiber lengths and M3 fiber length falling in the median among the fishes analyzed. M3 had the largest PCSA of all muscles except for the adductor superficialis, which was greatest in M2. The abductor and adductor muscles of M1 had low PCSA and short fiber lengths, indicating smaller maximum force production and limited range of movement while the arrector muscles had longer fiber lengths and thus a potential for greater range of motion. The large PCSA of the abductor profundus in M2 and M3, nearly three times that calculated for the same muscle in M1, indicates an increase in the potential maximum force exerted by the muscle and thus potentially an increased ability to position their fins under the body in support of their body for locomotion. Although the PCSA of all muscles are smaller in M1 than M2 or M3, the extent of the difference varies among the muscles (Figure 2.6B). Most muscles in M1 show a PCSA 34.15% that of the PCSA in M2 or M3, however, the superficial abductor muscle in M1 is similar in PCSA as found in M2 (89.82%) and in the superficial

adductor, the PSCA of M1 is about 70.22% of that in M2 or M3.





2.3.5 Phylogenomic Relationships

The multispecies coalescent tree and the Maximum Likelihood phylogenetic reconstruction (Figure 2.7 and Appendix A.2 Figures A.1 & A.2) had identical topologies, and we matched the morphotypes to their position on the multispecies coalescent tree (Figure 2.7). Balitoridae is resolved as monophyletic and sister to a clade consisting of Gastromyzontidae and Serpenticobitidae. This clade is sister to the stone loach family Nemacheilidae. We recovered many of the same relationships (Figure 2.7 and Appendix A.2 Figure 2.1) within Balitoridae as the molecular phylogeny presented in Randall and Page (2015). We have expanded the phylogeny from Randall and Page (2015) by including Cryptotora and Neohomaloptera. Within Balitoridae two subfamilies have strong support: Homalopteroidinae Randall and Page 2015 (Homalopteroides, Homalopterula, and Neohomaloptera) and Balitorinae Swainson 1839 (Balitora, Hemimyzon, Sinogastromyzon, Cryptotora, Homaloptera, Balitoropsis, and *Pseudohomaloptera*). Within Balitorinae, two major clades are resolved: a strongly supported clade consisting of *Cryptotora* as the sister group to *Sinogastromyzon*, *Hemimyzon*, and *Balitora*, and a weakly supported clade consisting of *Pseudohomaloptera* as the sister group to *Balitoropsis* plus *Homaloptera*.

Although there are some sampling differences between our morphological and molecular datasets, all three morphotypes are included within the two subfamilies. The morphotypes are not monophyletic (Figure 2.7). The clade containing *Cryptotora*, *Balitora*, *Hemimyzon*, and *Sinogastromyzon* includes M2 and M3, while the clade containing *Homaloptera*, *Balitoropsis*, and *Pseudohomaloptera* contains all three morphotypes. The clade containing *Homalopteroides* includes M2 and M3, while the

clade containing *Neohomaloptera* and *Homalopterula* includes M1 and M2. Two pairs of sister species share different morphotypes: *Balitoropsis zollingeri* and *B. ophiolepis* (M2 and M3, respectively) and *Homalopteroides tweediei and H. nebulosus* (M3 and M2, respectively). One of four species of *Homalopterula* (*Homalopterula vanderbilti*, not sampled in Figure 2.7) grouped into M1 while the other three were M2 and two of five species of *Homalopteroides* grouped in M2 while the other four were in M3. We did not find significant phylogenetic signal in the distribution of morphotypes both with and without the M1 specimens (with M1, p = 0.365, without M1, p = 0.314). We found 7.00 observed evolutionary transitions with a randomization median of 8.00.



Figure 2.7 SVDQuartets tree of Balitoridae with boxes highlighting known rib morphotypes, Morphotype 1, yellow; Morphotype 2, blue; and Morphotype 3, green. Species without color coding have not been analyzed for morphotype. A) Balitorinae and B) Homalopteroidinae.

2.4. Discussion

Terrestrial excursions by fishes are observed throughout the teleost tree of life and include varying forms of locomotion (Wright and Turko, 2016). Methods used to move across terrestrial environments range from simply modified swimming, undulating or flipping the body as seen in seen in eels (Gillis, 1998), sticklebacks (Clardy, 2012), and killifishes (Gibb et al., 2011; Gibb et al., 2013); moving on land by crutching as in mudskippers (Kawano and Blob, 2013; Pace and Gibb, 2009) or pectoral fin-driven forward propulsion with undulation of the posterior body as in *Polypterus* (Standen et al., 2014); to using alternating pelvic fin movements to generate forward momentum, as seen in lungfishes (King et al., 2011). The balitorid *Cryptotora thanicola* walks with a lateralsequence-diagonal-couplets (LSDC) gait similar to that observed in salamanders, which is a unique form of terrestrial locomotion among fishes (Flammang et al., 2016). This locomotion is likely due to the fact that *Cryptotora thamicola* shares several morphological features with terrestrial tetrapods, including a robust pelvic girdle rigidly attached to the axial skeleton via a sacral rib, broad neural spines, and zygapophyses connecting serial vertebrae (Flammang et al., 2016).

2.4.1 Balitorid Pelvic Skeleton

Unlike typical teleost fishes in which the bones of the pelvic fins are either suspended in a muscular sling or anteriorly attached to the pectoral girdle (Stiassny and Moore, 1992; Yamanoue et al., 2010), the bones of the pelvic fins in the balitorid species studied here (representing 14 of 16 genera) are connected to the axial skeleton via an elongated and/or enlarged sacral rib. Enlargement of the rib associated with the basipterygium is seen in Balitoridae and has also been reported in its sister family Gastromyzontidae (Conway,

2011; Sawada, 1982 [recognized as the balitorid subfamily, Gastromyzontinae]). In a morphological phylogenetic analysis, Conway (2011) reported an enlarged rib associated with the basipterygium as a character (117) that supports the monophyly of Balitoridae (recognizing Gastromyzontidae as a subfamily of Balitoridae). This character was also recognized as being independently gained in the family Psilorhynchidae, and the taxon *Garra dembeensis* (Conway, 2011), both found in fast-flowing riverine habitats similar to hill-stream loaches.

Mapping the morphotypes to the UCE phylogeny (Figure 2.7) shows that sacral rib shape varies along balitorid phylogenetic relationships with all three morphotypes showing up in both subfamilies Balitorinae and Homalopteroidinae. The two species from M1 (Homaloptera ogilviei and Neohomaloptera johorensis) are found in two different subfamilies whereas M2 and M3 are dispersed throughout Balitoridae. Based on the current sampling in this study, with the exception of *Homaloptera*, the groupings of described morphotypes M1-M3 do not reflect current phylogenetic relationships at the generic level (when more than two species were sampled within a genus) (Figure 2.7). Three of the genera studied (Homalopterula, Homalopteroides and Balitoropsis) (Table 2.1 and Figure 2.7) have species separating into different morphotypes. It is worth noting that no genus encompassed both M1 and M3, however, suggesting that while the morphological differences may reflect a spectrum, variation was consistently directional, as observed through the PCA results (Figure 2.4). More morphological and molecular samples of Balitoridae, Gastromyzontidae, Serpenticobitidae, and Barbuccidae are needed in order to perform a robust character ancestral state reconstruction and assess if

an enlargement of the rib associated with the basipterygium is a character that unites Balitoridae (*sensu lato* of Tan & Armbruster, 2018).



Figure 2.8 Lateral and dorsal views of the distal ligament connecting the sacral rib to the lateral foramina of the basipterygium. Morphotype 1, *Homaloptera ogilviei* (USNM 288431); Morphotype 2, *Homalopterula ripleyi* (USNM 390014); and Morphotype 3, *Balitora* sp. (ANSP 179834); ligament (transparent purple); bone (tan), scale bars = 0.5 mm.

The rib shape variation among the three morphotypes (Figures. 2.2-2.4) is largely explained (PC1, 47.56%) by the differences in thickness of the sacral rib and the presence or absence of an enlarged crest. Thicker ribs connecting the pelvis to the vertebrae allow

for a greater transmission of forces from the pelvis to the axial skeleton, and support of the weight of the fish when out of the water (as reported for *Cryptotora* in Flammang et al., 2016) or counteracting a strong current. Increased robustness of the pelvis and its connection to the vertebral column was important in the evolution of terrestrial walking in tetrapods (King et al., 2011; Lebedev, 1997) as was the development of stronger pelvic musculature (Cole et al., 2011). These thicker ribs have a larger cross-sectional area, leading to increased strength in the bone (Hyman and Wake, 1992) and an increased force resistance (Blob and Biewener, 1999). The flared crest seen in M3 offers an increased surface area for muscle attachment at the pelvis. This flared crest is most often observed on the lateral portion of the rib at the point where the rib turns down toward the basipterygium although one species, Balitoropsis ophiolepis (Figure 2.4, #3), has a more medial crest. Increased contact area via enlarged fin rays and the dorsoventrally flattened body increases the frictional surface and promotes adhesion (Chang, 1945; Hora, 1930; Sawada, 1982). From the linear discriminant analysis, the morphotype of species not yet analyzed here could be predicted with high accuracy (0.857 from the jackknifed analysis, 1.0 from the resubstitution analysis) as accessibility to specimens and scanning facilities increases. Species which fall near the bounds of the morphotypes or have more extreme shape variations will be more difficult to place into the discrete morphotypes.

The pre- and post-zygapophyses seen in varying degrees in M1 and present in M2 and M3 have been recorded in highly rheophilic species (He et al., 1999; Lujan and Conway, 2015; Sawada, 1982). Reinforcement of the vertebral column via processes between serial vertebrae is seen in different groups of rheophilic fishes and theorized to be specialized for this habitat (Lujan and Conway, 2015). In addition to the zygapophyses

strengthening the axial skeleton, broadening of the neural spines was seen in one species of M1 (*Homaloptera orthogoniata*) three species of M2 (*Balitoropsis zollingeri*, *Hemimyzon formosanus*, and *Hemimyzon taitungensis*) and in all M3 species. This broadening of the neural spines, like the enlargement of a flared crest in the sacral rib, increases surface area for muscle attachment; in tetrapods broad neural spines support a system of ligaments that reinforce the stiffness of the axial skeleton and help counteract the effect of gravity on the abdomen.

2.4.2 Balitorid Pelvic Musculature

In the pelvic muscles of the fishes studied here, as the basipterygium becomes broader compared to its length, we see more shallow fiber angles in the adductor and abductor muscles. This change in fiber angle and the increased size of the muscles increase the maximum force of the contraction of these muscles that may help the pelvic fins adhere to the substrate and keep the fish from being carried downstream (Chang, 1945). The absence of the extensor proprius in balitorids is unsurprising as it is often absent in benthic fishes (Stiassny and Moore, 1992; Winterbottom, 1973; Yamanoue et al., 2010).

In M3, the dorsal pelvic muscles do not appear to meet at the midline as seen in M1, M2, and typical fishes (represented here as *C. auratus*). This could be from inconsistent staining in the individuals examined and increased coverage of individuals is necessary to determine if this is real or an artifact. Nonetheless, the normalized muscle volume and PCSAs are still greater for nearly all of the muscles in M3.

Higher PCSA values indicate a greater capacity for force in fishes with enlarged sacral ribs (M2 and M3). In M3, all muscles, except for the adductor superficialis, had the greatest PCSA values, whereas *Carassius auratus*, representing typical fish morphology,

had the lowest PCSA for all muscles. Moving from a typical teleost to M1, M2, then M3, PCSA increased as expected with increased area of the basipterygium and, thus, more space for muscle attachment. The abductor profundus (Fig 5B, turquoise) has the largest PCSA in M2 and M3, largely due to increased volume of muscle originating from the basipterygium concavity. The increased force capacity in M2 and M3 may indicate an increased ability to hold place in fast-flowing water and is presumed to indicate an increased ability to perform walking behaviors. The morphospace of the potential muscle force (PCSA) and range of extension in the muscles (fiber length) (Figure 2.6) illustrate the tradeoffs between these two metrics of muscle architecture and function. The long fiber lengths and relatively low PCSA values for the arrector muscles in all four species studied allows for larger movements of the first fin ray but lower power producing the movement. The deep adductor and abductor muscles of M2 and M3 have the largest PCSA values of all muscles measured and all have moderate fiber lengths, these muscles generate the most power of the pelvic muscles with the highest PCSA in the deep abductors, which may be important for gripping to the substrate with the fin-rays- and would facilitate positioning the fins under the body in support of walking.

The sacral rib is held securely in place by a ligament encapsulating the distal end of the rib and connecting it to the basipterygium within the lateral foramen. In addition to increasing the radiopacity of the musculature, PTA staining highlighted the connective ligament holding the enlarged sacral rib in place in all three morphotypes (Figure 2.8). This ligament was also found during dissection of specimens from M1 (*Homaloptera parclitella*) and M3 (*Homalopteroides tweediei*). In the dissections, the ligament firmly held the rib in place at the lateral edge of the lateral foramen of the basipterygium. The

ligament was larger in M3 than in M1, encapsulating a larger proportion of the distal end of the rib. In addition, the distal end of the sacral rib in M3 is larger and rounded, as opposed to coming to a tapered point as in the M1 fish. The increased size of the ligament reduces the amount of movement possible at the distal end of the rib and likely increases the stability of the rib-basipterygium connection. The ligament connecting the sacral rib and the basipterygium has been noted before and was presumed to support the enlarged basipterygium, helping to maintain the large ventral surface of the pelvic region (Chang, 1945; Sawada, 1982).

2.4.3 Ecology and Phylogeny of Balitorid Morphotypes

The present study examined the skeletal morphology of a broad sampling of balitorid fishes which resulted in the delimitation of three morphotypes. The structures that support the different morphotypes are expected to have major implications for the biomechanics of the terrestrial locomotion behaviors observed in this family. Testing for phylogenetic signal indicates that the morphotype groupings are not congruent with evolutionary relationships. From the lack of phylogenetic signal, we can conclude that the variation in pelvic structures in balitorid loaches are independent adaptations in response to a rheophilic environment (Lujan and Conway, 2015).

In addition to the selection for enlarged sacral ribs and connectivity between the pelvis and the axial skeleton, the wide range of pelvic morphology seen within the family may indicate adaptive phenotypic plasticity; however, determining this requires more study. Phenotypic plasticity, or changes in an organism as a result of interactions with its environment, can lead to the evolution of adaptations and specializations (Pigliucci et al., 2006; West-Eberhard, 1989). Phenotypic plasticity in teleost skeletal and muscular

structures has been observed in response to changes in loading systems on fin structures, muscular structures, feeding morphology, and the development of intermuscular bones (Danos and Ward, 2012; Hegrenes, 2001; McFarlane et al., 2019; Standen et al., 2014). Unfortunately, while the fishes here are all classified as hill-stream loaches, at this time there are no published details regarding their habitats through ontogeny or over time that would allow for further investigation into the environmental forces with which these fishes interact.

Although balitorids are well documented in museum collections and some species are commonly collected for the pet trade, little is known about the details of their various habitats, particularly how physical parameters of the habitat change between the wet and dry season. These loaches are known to be from fast-flowing rivers and streams in eleven countries (Cambodia, China, India, Indonesia (Borneo, Java, and Sumatra), Laos, Malaysia (peninsular and Borneo), Myanmar, Nepal, Taiwan, Thailand, and Vietnam) and are often found on rocky substrates, but the flow rates and physical properties of their habitats are not well known (Alfred, 1969; Dudgeon, 2000; Kottelat, 2012; Trajano et al., 2002). Alfred (1969) remarked on the substrate and water velocity preference for some species on the Malay peninsula, and his findings of velocity preference align with our morphotype findings with M1 (*Homaloptera ogilviei* and *Homaloptera orthogoniata*) preferring lower velocities, and M2 and M3 (Balitoropsis zollingeri and *Pseudohomaloptera leonardi*, respectively) preferring higher velocities. More recently, a study on the physical parameters of balitorid and nemacheilid loaches in central Thailand found that water velocity was not significantly different in habitats occupied by the species studied (Beamish et al., 2008); however, that study included many nemacheilids

which do not possess the enlarged sacral rib and may have impacted the findings. A more inclusive review of water flow rates and substrate structure of balitorid habitats will greatly aid in our understanding of the ecology of these fishes.

A strong hypothesis for evolutionary relationships is critical for accurate comparative study (Garland et al., 2005; Sanford et al., 2002), and we have reconstructed our own evolutionary hypothesis of balitorids using phylogenomic data. This is the first investigation to include the cave inhabiting *Cryptotora thamicola* and recover it as belonging to the subfamily Balitorinae. Further work into the phylogenetic relationships and the biomechanics of the unique walking behavior observed in these fishes will provide an opportunity to increase our knowledge of morphological evolution in balitorids.

CHAPTER 3

THEY LIKE TO MOVE IT (MOVE IT): WALKING KINEMATICS OF BALITORID LOACHES OF WESTERN THAILAND

3.1. Introduction

A major demand of terrestrial locomotion is that animals must support their own mass against the forces of gravity which 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 the terrestrial environment are known to occur throughout the group.

Among the approximately 35,000 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 (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* (Martin et al., 2004b; Muench, 1941). During terrestrial locomotion, Bichirs (*Polypterus*) augment lateral undulation of their elongate body with lifting of the pectoral fins (Standen et al.,

2014; Standen et al., 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). Although these species 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 which 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 bound) gait patterns (Hildebrand, 1980). Differentiation of gait patterns requires partitioning step cycles of the limbs into stance, when the limb is in contact with the ground, and swing phase, when the limb is in an aerial phase (Hildebrand, 1980). Here, we focus on the walking gait, a symmetrical gait in which stance phase for each of the four limbs is greater than 50% of the total step cycle, resulting in no aerial phase—a point in the step cycle where the animal is completely off the ground.

In 2016, researchers found that the balitorid loach, *Cryptotora thamicola* (Balitoridae), is capable of walking with a salamander-like, lateral-sequence-diagonal-

couplets (LSDC) gait (Flammang et al., 2016). This was the first recording of a fish using a tetrapod-like gait in a terrestrial environment without the assistance of a buoyant medium. The walking gait observed in C. thanicola is possible in part, due 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 fish to support its mass out of the water. Further work on the morphology of other species in the family led to the delimitation of three distinct morphotypes of varying degrees of morphological modification (Crawford et al., 2020). These morphotypes (M1, M2, and M3) have increasing degrees of skeletal modification in the pelvic region: long, narrow sacral rib (M1); a thickened, slightly-curved sacral rib (M2); and a robust, crested 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 fish 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.

The three morphotypes were found to be spread throughout the balitorid family with low phylogenetic signal for the trait (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 within the three different morphotypes. We hypothesized 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 more stable manner), and fishes with reduced connectivity (M1) have lower performance (i.e., less forward movement per stride and more scurrying movements). The objective of this study is to test this hypothesis.

3.2. Materials and Methods

3.2.1 Specimen Collection

Specimens were collected in central Thailand in January 2020 (Table S1) using multiple methods including dip netting, electrofishing, cast netting, and moving rocks to loosen fish holding on to the rocky substrate. Targeted localities were part of ongoing studies in the area, collected under the Thailand Research Administration Division permit number 4660 (issued to Dr. Lawrence Page, Zachary Randall, and David Boyd at the Florida Museum of Natural History at the University of Florida). 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, Homaloptera parclitella (Tan and Ng, 2005) were purchased from the Wet Spot Tropical Fish (Portland, Oregon) for lab-based video analysis. Fish in the lab were housed in three 10-gallon tanks (four fish per tank), lined with rocky substrate, and fitted with high turnover rate filters. Fish were fed live blackworms and singling 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) (Figure 3.1), and two of the

three morphotypes determined by Crawford et al. (2020). Morphotype 1 (M1) is represented by *Homaloptera parclitella* and morphotype 3 (M3) is represented by *Cryptotora thamicola, Balitora* sp., and *Pseudohomaloptera sexmaculata* from Balitorinae and *Homalopteroides modestus, Homalopteroides smithi*, and *Homalopteroides* sp., from Homalopteroidinae.





3.2.2 Video Collection

For field collected species, filming was performed at collection sites where possible, and fishes were kept in buckets between capture and filming which was completed for most within three hours of collection. When filming could not occur immediately, water was aerated by portable aerators and filming was completed within 24 hours; this extended time period between capture and filming only occurred for fish collected in Ratchaburi (Table B.1). A total of 22 individuals across all species were filmed in the field (Table B.1). 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 per second. 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 an environment for walking and avoiding swimming behaviors supported by the presence of 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 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 accessioning into museum collections (Table A1). Representative species from each field collection site were μ CT scanned with the GE Phoenix v|tome|x M scanner (GE Measurement and Control, Boston, MA) at the University of Florida Nanoscale Research Facility for skeletal comparisons (Figure A2).

Lab filming was performed using two Phantom Miro M110 cameras (Vision Research, Wayne, NJ) recording at 500 frames per second in both lateral and ventral views. The ventral view was accomplished using a mirror at a 45° angle. The filming setup was illuminated with overhead lighting and an additional Dracast LED500 Bi-Color light (Dracast, San Jose, California) 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 LegoTM model for 3D digitization of videos.

3.2.3 Video Analysis

In the ventral view, 29 points were digitized on each individual (Figure B.1) 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 cycles measurements began with the start of stance phase of the left pectoral fin (Figure 3.2). 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 14-24 (*Balitora* sp., N=5, 23 videos; *Cryptotora thamicola*, N=2, 22 videos; *Homaloptera parclitella*, N=5, 24 videos; *Homalopteroides modestus*, N=3, 15 videos; *Homalopteroides smithi*, N=4, 14 videos; *Homalopteroides* sp, N=3, 16 videos; and *Pseudohomaloptera 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, *Cryptotora* is a very rare subterranean species limiting our interactions with that taxon.



Figure 3.2 Representative sequences (continued on next page.)

Figure 3.2 Representative sequences of A) *Homaloptera parclitella*, Morphotype 1 (Flammang Lab/NJIT), B) *Cryptotora thamicola*, Balitorinae Morphotype 3 (MARNM7413), and C) *Homalopteroides modestus*, Homalopteroidinae Morphotype 3 (UF245290) walking on an acrylic trackway. 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 filmed at 500 fps. Museum abbreviations: Maejo Aquatic Resources Natural Museum, MARNM; University of Florida, UF; New Jersey Institute of Technology, NJIT. Scale bars = 10 mm.

3.2.4 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, 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) with 3D calibration from a custom LegoTM 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 (Figure B.1). 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 proportional length of the tail (points 22-29, Figure B.1) among the fish to the maximum curvature; tail length is measured as the proportion of total length starting at the pelvis and moving to the 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-HSD comparison. Gaits were 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-HSD comparisons. Analyses were performed in R v. 4.0 (http://www.R-project.org/). Values for each variable were measured per step cycle.

3.3 Results

Although all species studied exhibited a diagonal-couplets walking pattern, movements were along a spectrum ranging from patterns more similar to swimming on land to those which more closely resembled terrestrial tetrapodal walking on the other. 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 vast 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 (Figure 3.3). In the Homalopteroidinae and in *Balitora* sp., the fish slid the fins along the substrate without visible lift to clear the surface, presenting more of a rowing shape in the pectoral and



Figure 3.3 Representative frames from a video of *Cryptotora thamicola* with the A) pectoral fin B) and pelvic fin highlighted showing the fin of interest (shaded in chartreuse) and the fin in stance (outlined in blue). Traces representing the travel of the anterior tip of the C) pectoral and D) pelvic fin through one step. In C and D, greater y indicates increased lift during swing phase. Direction of travel is to the left. Color coding for traces follows other figures. Scale bar = 10 mm.

pelvic fin traces (Figure 3.3). Contrastingly, in *Homaloptera parclitella* (M1), *Cryptotora thamicola* (M3), and *Pseudohomaloptera sexmaculata* (M3), we see clearance of the substrate in the pectoral and pelvic fins during swing phase. In *Homaloptera parclitella* and *Cryptotora thamicola*, we also see 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 which exhibit clear vertical lifting of the fins, except for *Homaloptera parclitella*, there is more lift in the pectoral fin during swing phase and less lift in the pelvic fin swing phase. *Homaloptera parclitella* has greater lift in the pelvic fin than the pectoral fin; however, the duration of the lift is shorter, and the pelvic fin is slid along the surface for most of the swing phase.

There is a varied range of extension for the fins across species and among individuals for different steps (Figure 3.4). In some species, there are steps with a clear lifting of the pectoral and pelvic fins during the swing phase while in others there is more often a sliding of the fins forward in both the pectoral and pelvic fins. In *Cryptotora* we see a greater lifting of the fins in addition to a large movement of the anterior body in the *z* direction: 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, Balitora*, and *Homalopteroides smithi*, all M3s, do not bring their pelvic fins completely under the body and maintain them laterally extended. Other species bring their pelvic fins completely underneath the body before they bring them forward again for the next step. The range of extension in the fins is greater in the pelvic fins for all species. *Cryptotora* has the greatest range of extension of the pectoral fins.



Figure 3.4 Representative frames of videos from each species showing maximum and minimum fin extension (A). Violin plots of fin extension range (degrees) for pectoral (B) and pelvic (C) fins. Horizontal bars denote no significant difference between species below bar. \dagger , $\dagger\dagger$, and $\dagger\dagger\dagger$ indicate no significant difference species marked with same symbol, *** p< 0.000, scale bars = 10 mm.

Curvature of the midline shows a different trend between the M3 species in the two subfamilies with Balitorinae having lower maximum curvature (0.014 ± 0.001 CI) than Homalopteroidinae (0.037 ± 0.002 CI). Within Balitorinae, there is further delineation among the species with *Homaloptera parclitella* and *Cryptotora thamicola* having slightly greater maximum curvature (0.019 ± 0.001 CI) than *Balitora* sp. and *Pseudohomaloptera parclitella* (0.010 ± 0.001 CI). The Balitorinae have lower maximum curvature and a smaller range of curvature values (Figure 3.5A-C, E-F). The Homalopteroidinae show 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



Figure 3.5 Midline curvature of balitorid walking patterns (continued on next page.)

Figure 3.5 Midline curvature of balitorid walking patterns. Representative traces of the midline through step cycles of A) *Homaloptera parclitella* (Flammang Lab/NJIT), B) *Pseudohomaloptera sexmaculata*, (UF245546), C) *Cryptotora thamicola* (MARNM7413), and D) *Homalopteroides modestus* (UF245290), 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 Homaloptera parclitella for each species with grey bands indicating 99% confidence interval. In A-C, 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.

throughout the step cycle and between steps (Figure 3.5D-F). Curvature along the body was greatest near the pelvis (points 23-27, Figure B.1) tapering off closer to the cranial and caudal ends of the fish (Figure. B.4) in all species except *Pseudohomaloptera* which had consistently low mean maximum curvature throughout the body.

The curvature pattern within Balitorinae (greater mean maximum curvature of *Cryptotora thamicola* and *Homaloptera parclitella*) was unexpected and further inquiry led to a correlation with the body proportions, particularly with comparisons of the tail proportions (THICC; Figure 3.6). The proportion of the total body length represented by the tail (points 22-29 in Figure 3.3) was not significantly different among the three *Homalopteroides* species, *Balitora* sp, or *Pseudohomaloptera parclitella* and *Cryptotora thamicola* averaged 60% and 70% respectively (Figure 3.6), significantly different from all other species (p<0.004).

Walking velocity, compared as body lengths per second, body lengths per time, and strides per second, varied among species (Figure 3.7). Body length per second shows differences between the two subfamilies, with the Homalopteroidinae moving, on



Figure 3.6 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*.

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 we see is shown by the body lengths per stride (Figure 3.7 B), in which *Cryptotora thamicola* is traveling significantly farther per stride, in terms of body lengths, indicating potentially more force generated per stride resulting in more efficient forward travel. The other species are 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 move further per second but with shorter distance traveled in body lengths. *Cryptotora* has the fewest strides per second coupled with the furthest distance per stride.



Figure 3.7 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. † indicates no significant difference between marked species, *** p< 0.000.

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 (Figure 3.8). The Homalopteroidinae were not significantly different from one another (3.78% mean overlap) and had lower overlap than the Balitorinae (6.09%). Within Balitorinae, there was a significant difference between *Homaloptera parclitella* (M1, 5.31%) and *Cryptotora thamicola* (M3, 6.45%), p < 0.05. *Homaloptera parclitella* was also not significantly different from *Homalopteroides modestus*, which had the greatest overlap of the Homalopteroidinae (4.22%).



Figure 3.8 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 denotes no significant difference between species below bar. † indicates no significant difference between *Homaloptera parclitella* and *Homalopteroides modestus*.

All measured duty factors, the proportion of the step cycle 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 duty factors of the fore and hind fins or left and right, thus, the duty factors of each fin were combined for comparisons between species. Similar to the stride overlap, the duty factor is slightly lower in the Homalopteroidinae subfamily (54%) and greater in the Balitoridae (56%, Figure 3.9). *Cryptotora thamicola* has a significantly greater duty factor (57%, p<0.0001) than all other species studied here and *Homalopteroides* sp. had a significantly smaller mean duty factor than all other species (0.53%, 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 (DSLC) and Lateral Sequence Diagonal Couplets (LSDC) gaits for all species (Table 3.1). Diagonality values above 50% reflect a DSDC gait while values below 50% indicate a LSDC gait. For all species, except for *Homaloptera parclitella* and *Cryptotora thamicola*, the majority of steps analyzed were DSDC with diagonality values above 0.5 (Figure 3.9). In *Homaloptera parclitella* and *Cryptotora 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 fish may fluctuate more readily between the two-phase patterns. Finfall patterns in all *Homalopteroides* species as well as in *Balitora* sp. and *Pseudohomaloptera sexmaculata* followed the DSDC more frequently with the fore fin on the opposite side being the next fin placement after the hind fin

With variation in duty factor, step overlap, and walking speed (strides per second), a trend of increased duty factor and stride overlap was seen in correlation with decreased strides per second (Figure 3.10). With longer and slower push off of fish with larger duty factor and step overlap (i.e., *Cryptotora thamicola*) compared to those with faster and shorter strides (i.e., *Homalopteroides sp.*), we see a difference in walking tempo, more of a methodical and slower tempo in the Balitorinae and a scurrying movement in Homalopteroidinae.



Figure 3.9 Bivariate plot (A) of 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 (DSLC). Ridgeplots of the density of the duty factor (B) and diagonality (C) with lines showing median value for each balitorid species. Gait examples 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 et al., 1995; *Hemicyllium ocelatum* (epaulette shark), Pridmore 1994; *Sceloporus clarkii* (Clark's spiny lizard), Reilly & Delancey, 1996; *Potos flavus* (kinkajou), Lemelin and Carmill, 2011; *Caiman crocodilus* (spectacled caiman), *Iguana iguana* (green iguana), and *Tiliqua scincoides* (bluetongued skink), Nyakatura, 2019.

3.4. Discussion

Terrestrial locomotion is accomplished across the teleost tree of life through a range of behavioral and morphological adaptations. Here we expand upon the kinematic analyses of Flammang et al. (2016) and have presented the tetrapod-like terrestrial walking exhibited by species in the balitorids. The species studied here represent the two extremes of the three morphotypes in the balitorid family (Crawford et al., 2020).

Although there are differences among species in the amount of vertical lift of the pectoral and pelvic fins (Figure 3.3), 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* spp 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 which also have a larger range of extension in the fin movement. The vertical lift of the pelvic fins was less than the pectoral fins for all species. Reduced clearance of the substrate during swing phase can be seen in other terrestrial tetrapod walking organisms 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 dragging more often in the hindlimbs than the forelimbs (Willey et al., 2004).

The variation in maximum midline curvature was a surprising finding. We expected to see reduced body curvature in the M3 morphotypes as compared to the M1 with the hypothesis that a more swimming-like behavior is exhibited by fish 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 while 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* and *Pseudohomaloptera sexmaculata* compared to the increased curvature in *Cryptotora thamicola* and *Homaloptera parclitella*, may be related to the body proportions (Figure 3.6). The deviations from the tail length being, on average, 63% of the total body length, may be related to the different motion in the movements.



Figure 3.10 Interaction between duty factor and stride overlap with strides per second. In all species, the trend is slower walking speed with an increase in duty factor and stride overlap. Polygons show outline points for each species.

The speed of steps and distance traveled per step consistently shows a stark difference between *Cryptotora 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.

| Species | Duty Factor (average ± SD) | Diagonality (average \pm SD) | Ν | DSDC | LSDC |
|----------------------------------|-------------------------------|--------------------------------|-----|------|------|
| Homaloptera parclitella | 0.552 ± 0.019 | 0.449 ± 0.034 | 111 | 49 | 62 |
| Pseudohomaloptera sexmaculata | 0.560 ± 0.019 | 0.553 ± 0.037 | 117 | 102 | 15 |
| Balitora sp. | 0.559 ± 0.019 | 0.545 ± 0.035 | 128 | 110 | 18 |
| Cryptotora thamicola | 0.567 ± 0.023 | 0.495 ± 0.035 | 108 | 42 | 66 |
| Homalopteroides modestus | 0.544 ± 0.025 | 0.527 ± 0.049 | 72 | 49 | 23 |
| Homalopteroides smithi | 0.548 ± 0.025 | 0.529 ± 0.059 | 72 | 46 | 26 |
| Homalopteroides .sp. | 0.530 ± 0.020 | 0.540 ± 0.035 | 64 | 54 | 10 |

Table 3.1 Gait parameters of walking balitorids. Duty factor and diagonality of analyzed step cycles

Note: All step cycles are above 0.50 duty factor, indicating walking gaits for all trials in all species. N is the total number of step cycles, the number of step cycles above 0.50 diagonality are listed as Diagonal Sequence Lateral Couplets (DSDC) and the number of step cycles at or below 0.50 diagonality are listed as Lateral Sequence Diagonal Couplets (LDSC).

The mean stride overlap is generally lower in the Homalopteroidinae subfamily, although *Homaloptera parclitella* is not significantly different from *Homalopteroides modestus*. There is also a greater range of stride overlap in *Homalopteroides* spp.,

perhaps also found in other members of subfamily Homalopteroidinae. Species with lower stride overlap also have increased stride speed and reduced 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 fish maintain balance with a less stable stance.

Across all species studied here, the gait phases were not significantly different between fore and hind fins (Figure 3.9). The duty factors of all species studied here range from 53-57% which are all lower proportions than seen in walking gaits of terrestrial tetrapods. In salamanders, duty factors have been found to have a duty factor range of 68-77% 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). Although these fish are performing a walking gait pattern, and some species consistently exhibit lifting of the fins during swing phase, the body is not consistently being lifted from the acrylic surface in all species. 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 fish are not continually lifting their mass off the platform, they are capable of lifting their head and, for short periods of time, do raise their entire body off the surface (unpublished data).

The diagonality of the different species covered both lateral sequence and diagonal sequence phase relationships, ranging from 44.9%-54.5% of the step cycle. DSDC are considered less stable because the polygon of support (the area of the body

which is supported by the limbs in stance phase) is smaller than in the LSDC gaits. The dragging belly and use of the tail may make up for the reduced stability of the diagonal sequence gait (Hildebrand, 1980; Nyakatura et al., 2014).

Although the DSDC gait observed in all seven species is considered less stable than a LSDC gait, the continuous or near continuous contact of the tail increases the stability as seen in the epaulette shark, Hemiscyllium ocellatum (Pridmore, 1995). 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 diagonal sequence diagonal couplets gait is also seen in the California or orange bellied newt, Taricha torosa, when walking under water and a lateral sequence lateral couplets gait when walking on land; however, underwater walking includes a suspension period due 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). Outside of *Cryptotora thamicola*, the balitorids studied here are not often seen exiting the aquatic environment although they have the capability of traveling short distances on land and *Homalopteroides* sp. has been observed leaving the water during the rainy season in southeast Thailand (personal observation, Z. S. Randall). Observing the diagonal sequence gait in these fish may be an indicator here of walking out of the water being less "normal" for these species in DSDC gaits while *Cryptotora* is more stable and walks in its natural habitat.

Balitorids inhabit fast flowing rivers and streams in south and southeast Asian and have morphological adaptations which allow them to maintain their position and generate forces to oppose being washed downstream (Ahlberg, 2019; Beamish et al., 2008; Hora, 1930; Lujan and Conway, 2015). These benthic fishes are rarely seen swimming in the water column, instead, they primarily use their enlarged paired fins for locomotion and typically do not use their caudal fin when moving along the substrate (Chang, 1945). *Cryptotora thamicola* is the only species of the balitorids 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, pers. observation). 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 regularly climb the sides of buckets when collected in the field (pers obs.). Although the large majority (6 of 7) of the species studied here had the same morphotype (M3) with an enlarged, arched, and crested sacral rib, we were able to compare them to one species with a thin sacral rib (M1). The ability of *Homaloptera parclitella* (our Morphotype 1 representative) to walk at comparable performance to species with more robust sacral ribs (Morphotype 3) indicates that the mere existence of this skeletal connection facilitates walking.

While 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. Variation in walking performance may be related to a combination of shared ancestry, ecology, or rib morphology and additional studies are necessary to reach such conclusions. Further studies into the habitat and ecology of theses 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 the Homalopteroidinae will illuminate variation in walking performance within this subfamily along with coverage of representatives exhibiting Morphotype 2 and more individuals with Morphotype 1. Another future angle to investigate is energy expenditure during walking in the different morphotypes which would allow another angle of comparing walking efficiency in balitorids.

CHAPTER 4

WALKING BY FISHES THAT SHOULDN'T: THE CASE OF THE RED LIZARD LOACH (HOMALOPTERA PARCLITELLA)

4.1 Introduction

Balitorid loaches are found in fast flowing hillstreams of south and southeast Asia (Alfred, 1969; Kottelat, 2012; Nelson et al., 2016). Species in this family exhibit hyperossification of the sacral rib which acts as a skeletal connection between the pelvis plate and the axial skeleton (Chang, 1945; Crawford et al., 2020; Flammang et al., 2016; Hora, 1930; Sawada, 1982). A functionally similar connection and the muscular adaptations coinciding with it, were important for the evolution of terrestrial walking (King et al., 2011; Lebedev, 1997). This skeletal connection is one of the morphological adaptations of hillstream loaches which facilitate the transmission of counterforces against the high water flow and allow the fishes to maintain their position and keep from being washed downstream (Ahlberg, 2019; Beamish et al., 2008; Hora, 1930; Lujan and Conway, 2015). The degree of hyperossification and the robustness of the sacral connection seen in balitorids varies across the family and has been separated into three morphotypes (M1, M2, M3). The three morphotypes can be separated based on the relative size and shape of the sacral rib which inserts at the lateral foramen of the pelvic plate: an elongated yet narrow sacral rib; a thicker, slightly-curved sacral rib; and a robust sacral rib with a flared crest on the lateral edge.

Flammang et al., (2016) studied the skeletal morphology and walking behavior of the balitorid loach, *Cryptotora thamicola* (Morphotype 3) and found that the fish could walk with a tetrapod-like gait on level and angled (up to vertical) surfaces. The walking

gait was facilitated in part, by its robust sacral rib. Further exploration of the walking kinematics of a sample of balitorid loaches found that species in both the Homalopteroidinae and Balitorinae subfamily are capable of performing a walking gait with the Balitorinae being capable of propelling their bodies forward primarily using appendicular motion and the Homalopteroidinae using more axial bending for forward propulsion (Crawford et al., *in prep*).

Homaloptera parclitella (Tan and Ng, 2005), a balitorid hillstream loach in the balitorinae subfamily, found Malaysia and southern Thailand has Morphotype 1, in which the sacral rib is elongated and inserts into the lateral foramen of the pelvic plate (Crawford et al., 2020). The skeletal connection, although not as robust as that found in other members of the family, still facilitates terrestrial walking in balitorid loaches. Due to the narrow sacral rib, *H. parclitella* was hypothesized to have reduced walking capabilities compared to other balitorid species with greater robustness in the sacral connection. Unexpectedly, our recent work on the walking kinematics of balitorids showed that *H. parclitella* is capable of walking in a manner similar to other species in the same subfamily which have a more robust sacral connection (i.e., *Pseudohomaloptera sexmaculata* and *Cryptotora thamicola;* Crawford et al., 2020 and Crawford et al., *in prep*).

Herein, we investigate how a fish with such a thin sacral rib is capable of walking and test how the walking kinematics change, if at all, when the fish is walking up an incline. In addition to the incline walking, this work provides a baseline understanding of the appendicular and axial muscle activity during terrestrial walking in a primarily aquatic fish.

4.2 Materials and Methods

4.2.1 Fish

Twelve individuals of *Homaloptera parclitella* were purchased from the Wet Spot Tropical Fish (Portland, Oregon). The individuals had total lengths ranging between 48.91 mm to 67.76 mm (mean: 59.55 mm). The fish were kept in three 10-gallon tanks (four fish per tank) with high turnover rate filters and rocky substrate and fed live blackworms and sinking algae wafers. The tanks were located in a light controlled room on a 12-hour light 12-hour dark cycle. Studies were conducted in accordance with NJIT/Rutgers University IACUC 17-058.

4.2.2 Video Collection

During filming, all fish were removed from the 10-gallon tank and placed in holding tanks in the filming room with bubblers and heaters to maintain suitable temperatures. Fish were photographed and identified by size and markings along the left and right side to identify individuals through trials. Filming was performed using two synchronized Phantom Miro M110 cameras (Vision Research, Wayne, NJ) recording at 500 frames per second in both lateral and ventral views; ventral view was recorded using a mirror angled at 45°. An external trigger was designed to trigger an LED light visible in both camera views and provide a 5-volt pulse to the amplifier to allow for synchronization of the video and electromyography (EMG) data. The filming setup was illuminated with overhead lighting and an additional Dracast LED500 Bi-Color light (Dracast, San Jose, California) as needed. Windows were blocked to avoid changes in lighting throughout the day.

Fish were encouraged to walk by dribbling water ahead of them or coaxing with nudges to the caudal fin with water dropper, forceps, or a researcher's hand; individuals required different stimuli to prompt walking behaviors. Between filming trials, fish were kept in an aerated container to recuperate before the next walking bout. After filming flat and incline trials, fish were returned to their respective tanks where they were maintained for later components of the study. Fish used in the electromyography portion of the study were later euthanized in MS-222, labeled, and fixed in 10% formalin, and later transferred to 70% ethanol for dissection and electrode placement verification. The different walking trials (flat walking, the three incline levels and EMG data collection) were completed on different days to allow fish to avoid overtiring the fish. Calibration videos were collected before and after each filming session using a custom-built LegoTM model.

4.2.3 Walking Platforms

Fish were filmed walking on an acrylic trackway at 0°, 15°, 30°, and 45° angles. The flat trackway was used for filming the fish walking freely and while collecting the EMG data. A separate trackway was designed for filming the fish walking at inclines which could be adjusted for different angles in 5° increments up to 60°, however, fish were not capable of walking above 45° during trials. On days when the angle walking trials were filmed, each fish was walked at 45°, then 30° and finally ending with 15° angles. This process allowed each fish to rest between incline trials and allowed for calibration videos to be taken before and after the incline was adjusted.

4.2.4 EMG Data Collection and Analysis

EMG data were recorded during flat walking in seven individuals. Loaches were selected for these trials based on previous walking performance and size. Buffered tricaine methanesulfonate (MS222) at a concentration of 150 mg/L was used to anesthetize the loaches and oxygenated water was used to ventilate the fish through the use of a peristaltic pump during electrode insertion, following procedures from previous studies (Flammang and Lauder, 2008; Horner and Jayne, 2008; Jayne and Lauder, 1993). Surgery for electrode implantation took between 15 and 20 minutes. After implantation, fish were placed in oxygenated fresh water and allowed to fully recuperate for three times the duration of anesthetization before any walking trials were started; recuperation time ranged from 32 minutes to 1-hour 15.

EMG electrodes were custom made from 0.05 mm diameter bifilar Teflon-coated steel wire (California Fine Wire Co., Grover Beach, CA). The wires were split along 0.5-0.75 mm along their length and about 0.25 mm of the tip of one wire was trimmed to avoid crosstalk between the wires. 0.25-0.5 mm of the insulation of each wire was stripped and the two wires were bent back to create a hook shape. In two of the larger fish, ten electrodes were implanted, one each in the ventral and dorsal muscle of each fin and two axial (one in the torso and one at the tail. In the subsequent five fish used for EMG, the number of electrodes was reduced due to the size of the fish and six electrodes were surgically implanted subcutaneously into the pectoral and pelvic fin flexors or extensors and the left axial myomeres at the trunk and tail of each fish. With the reduced number of electrodes were not implanted into the flexor and extensor muscles on

both the left and right side. Instead, each fish was implanted with both axial electrodes (torso and tail) on the left side in addition to left extensors and right flexors on both pelvic and pectoral fins or right extensors and left flexors on both pelvic and pectoral fins (Figure C.2). EMG signals from the implanted electrodes will be amplified using a 16-channel extracellular differential AC amplifier (model 3500; A-M Systems, Carlsborg, WA) and recorded using an ADInstruments PowerLab/16SP analog-to-digital converter and LabChart 8 software (ADInstruments Inc., Colorado Springs, CO). Electromyographic signals were amplified by a factor of 5000 using Grass model P511 K amplifiers with high- and low-bandpass filter settings of 100 Hz and 30 Hz, respectively, with a 60 Hz notch filter.

4.2.5 Video Analysis

In the ventral view, 29 points were digitized for each individual (Figure C.1) using DeepLabCut (Mathis et al., 2018; Nath et al., 2019). Point locations were selected to obtain coverage of the midline of the fish and the movements of the individual fins. The beginning of stance phase of front left fin was used as the starting point for each step cycle and the cycle ended when the left pectoral fin ended its swing phase. Three consecutive step cycles were required for the inclusion of a walking bout and the fish needed to be traveling in a straight line. In the trials for flat walking, videos were included if the fish walking in any direction, for the trials at 15°, 30°, and 45° angles trials were only included if the fish walked directly up the incline or straight across the faceplate. The total number of videos analyzed per individual ranged from 5-11 across all trials (Table C.2).

4.2.6 Kinematic Variables

For each trial, we analyzed the range of fin extension (in degrees), curvature, speed of walking, step overlap, duty factor, and diagonality. Range of fin extension was measured as the change in degree of the angle formed by the tip of the first fin ray and the anterior and posterior ends of the girdles, calculated separately for the pectoral and pelvic fins. Body curvature was calculated following Brainerd and Patek (1998), calculating a curvature coefficient as the minimum distance between the anterior end of the pectoral girdle and the caudal peduncle (bent vertebral length between points 19 and 28 in Figure C.1) divided by the maximum distance between these two points (straight vertebral length). This curvature coefficient measures the ratio of the maximal to minimal bending in the vertebral column, yielding an inverse measurement of the vertebral curvature. We calculated the walking speed in three formats, body lengths per stride, body lengths per second, and strides per second. The CrudeSpd() function in the R package 'Kraken' (https://github.com/MorphoFun/kraken) was used for walking speed calculations. Step overlap was calculated as the percent of the stride during which both fore fins or both hind fins were in contact with the substrate at the same time. Duty factor, the proportion of the step cycle during which the fin is in stance phase, was calculated for each fin and differences among fins were tested for using an ANOVA/Tukey-HSD comparison. The 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) was also calculated for both sides. ANOVA/Tukey-HSD comparisons were run to test for

differences among kinematic variables between trials. Analyses were performed in R v. 4.0 (http://www.R-project.org/).



Figure 4.1 Range of fin extension and retraction. Representative frames of videos from each incline showing maximum and minimum fin extension (A). Violin plots of fin extension range (degrees) for pectoral (B) and pelvic (C) fins. Pectoral range at 30° is significantly greater than 0°, but no significance is seen across incline levels. The horizontal bar denotes no significant difference in pelvic fin range across incline levels. ** p < 0.01, *** p < 0.001, scale bars = 10 mm.

4.3 Results

4.3.1 Kinematic Analysis

Not all individuals were capable of walking at all inclines. Only two individuals were able to walk at 45°, seven individuals walked at 30°, eight walked at 15° and 10 walked on the flat platform. A small increase in incline angle does not have a significant impact in the walking pattern observed in *Homaloptera parclitella*. Greater increases in the incline at which the fish were walked, however, did have an impact on some of the walking kinematic variables studied here.

The range of extension (Figure 4.1) in the pectoral fins did increase with steeper inclines with a significant increase at 30° from flat (0° mean: 20.17° and 30° mean: 22.03°, p < 0.05). There was no significant difference between the pectoral fin extension of the three incline levels, however, the fish with EMG electrodes implanted had a significantly greater pectoral fin range of extension (p<0.02 for all comparison). In the pelvic fins, there is a significant increase in all angled walks compared to the flat walking (p < 0.001) and no significant difference among the incline walks. The fish with electrodes implanted had significantly reduced range of extension in the pelvic fins (mean: 28.24°, p< 0.0001 for all comparisons).

The curvature of the fish increases with increasing inclines (Figure 4.2). At 15° , the curvature coefficient is not significantly different from flat walking, nor is the EMG significantly different from flat (p< 0.001). There is a steady and significant increase in curvature (reduced curvature coefficient) as the incline continues to increase (Figure 4.2E). The three incline levels are significantly different from one another and 30° and 45° are significantly different from flat walking. Even at the highest incline, the fish are

capable of walking with low curvature, although the mean curvature coefficient is significantly reduced (0.89 ± 0.008 SE).



Figure 4.2 Midline curvature for inclines and EMG trials. Representative traces of the midline through step cycles of *Homaloptera parclitella* (Flammang Lab/NJIT) walking at A) 0°, B) 15°, C) 30°, and D) 45° with still frames from the same videos, white dotted lines trace the midline. E) Frequency distribution plots of curvature coefficient in each step cycle, smaller values indicate greater curvature. No significant difference between EMG (flat) and 0° and 15°. Scale bars = 10 mm.

Walking velocity, compared across the walking trials as body lengths per second, body lengths per stride, and strides per second (Figure 4.3), were consistent for flat, 15° and 30° with no significant difference found for any comparisons. There was a significant decrease in body lengths per stride and body lengths per second at 45° (p<0.0001), however, there was not a significant difference for the strides per second at 45°.

Additionally, there was no significant difference between the mean values for the EMG trials and the flat, 15°, or 30° for the body lengths per stride or body lengths per second. There was a significant decrease in the strides per second for the EMG trials with the mean EMG strides per second at 4.65 whereas all other walking trials were above 5.0 strides per second.



Figure 4.3 Violin plot comparisons of walking speed and movement for the different inclines and EMG trials. A) Body lengths per second, B) body lengths per stride, and C) strides per second. Horizontal bar denotes no significant difference between 45° and EMG trial in strides per second and † indicates significant reduction at 45° (A and B) and at EMG (C).

The percent of the stride in which both fore or both hind fins are in contact with the substrate at the same time, or the stride overlap, followed a similar trend as the curvature coefficient (Figure 4.4). The flat, 15°, and EMG walking trials were not significantly different from one another (0° mean: 5.5% 15° mean: 5.3, and EMG mean: 5.6%, p > 0.90). The 30° and 45° walking trials had mean stride overlaps significantly greater than the other trials and significantly different from one another (30° mean: 7.6% and 45° mean: 9.4%, p < 0.02).



Figure 4.4 Violin plot of stride overlap between left and right fins, pairing fore and hind fins. Plotted are the overlap per step cycle of each trial. Horizontal bar denotes no significant difference between trials below bar. † indicates no significant difference between 0° and EMG trial. 30° and 45° trials were significantly greater than other trials.

The duty factor (Figure 5A-B), the proportion of the step cycle during which the fin is in contact with the substrate, were all above the cutoff for a walking gait, 50% (mean range: 55.1%-59.3%). As seen in the stride overlap, flat, 15°, and EMG walking trials were not significantly different (55.1%-55.5%). The other incline walks were significantly different from one another and from flat, 15°, and EMG duty factors (p < 0.0001 for all comparison).



Figure 4.5 Bivariate plot (A) of duty factor and diagonality of for the different walking trials. 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 (DSLC). Ridgeplots of the density of the duty factor (B) and diagonality (C) with lines showing median value for each balitorid species.

The diagonality (Figure 4.5A, C), or the percentage of the step cycle by which the hind finfall precedes the ipsilateral fore finfall had a mean for all walking trials above 50% (50.4%-53.9%) which indicates an average Diagonal Sequence Diagonal Couplets (DSDC) gait. All walking trials also had diagonality values that also crossed below 50%

which indicates a Lateral Sequence Diagonal Couplets (LSDC) gait, suggesting variability in the diagonality during walking for this species. The greatest diagonality was seen in the EMG walking trials, however this was not significantly greater than the 45° walking trials. While there is not a significant difference between flat and 30° walk diagonality, there was a significant increase in diagonality at 15° compared to flat walking (p<0.002).

4.3.2 Electromyography

The implantation of the electrodes did not significantly change the curvature coefficient, duty factor, stride overlap, or the body lengths per stride from those values for fish walking at 0° without electrodes being implants. There was, however, a significant reduction in the strides per second (EMG mean: 5.65, 0° mean: 5.57, p < 0.001, Figure 4.4) and a significant increase in the pectoral fin extension (EMG mean: 24.9°, 0° mean: 20.2° , p < 0.001, Figure 4.1A) and a significance decrease in the pelvic fin extension (EMG mean: 28.3, 0° mean: 36.1°, p < 0.001, Figure 2B). The preliminary EMG output indicate that the fish are activating their extensor and flexor muscles for 50-70% of the walk cycle, stiffening the fins and not only activating the muscles during elevation and depression of the fins (Figure 4.6).



Figure 4.6 Example EMG output of walking *Homaloptera parclitella* showing muscle activation in dorsal and ventral muscles of all four fins and left axial epaxial and left caudal muscles. Arrows and fish outline indicate direction of motion. Color coding for electrode placement: Left dorsal pectoral, green; left pelvic dorsal, purple; right pectoral ventral, blue; right pelvic ventral, orange; left axial epaxial, black; and left tail epaxial, grey.

4.3.3 High Walking

In two videos at the 15° incline, one of the individuals performed a walking behavior we describe as high-walking (Figure 4.7A, e.g., 1.40 s). During high walking, the fish lifted its body completely off the substrate and traveled on the fin tips. In high-walking, the tail
is lifted clear of the substrate at times, although it is in contact with the substrate the majority of the time (Figure 4.7 B-D). The duty factor during high walking was inconsistent (Figure 4.7C-D), with fins in stance phase from 20% to 100% of the step cycle (mean: 52%). In the instance where the fin was down 100% of the step cycle, the fin remained on the substrate into the next step (Figure 4.7C). The high walking produced significantly greater strides per second (mean: 8.72 ± 0.51 SE), body lengths per stride (mean: 0.37 ± 0.03 SE), and body lengths per second (mean: 1.70 ± 0.16 SE), compared to all other walking trials.



Figure 4.7 High walking in *H parclitella*. A) Representative frames of high-walking shown in the lateral view. B-D) Gait diagrams of three instances of step cycles illustrating inconsistent duty factor for all fins and varying tail-substrate contact timing. Scale bar = 10 mm.

4.4 Discussion

Among the balitorid loaches there is variation in the robustness of the rib connecting the axial skeleton and pelvic plate (Crawford et al., 2020). *Homaloptera parclitella* is one of the species with a thin sacral rib creating that connection which led to the prediction that this species would not be a very capable walker. Previous work on the walking kinematics of a range of morphologically varied balitorid loaches (Crawford et al., *in prep*) found that *H. parclitella* is capable of walking in a way similar to other species in the family that possess a more robust sacral connection. To build on the previous work comparing walking across species, we explored the ability of *H. parclitella* to walk up inclines and studied muscle activation during flat walking.

For most of the kinematic variables analyzed here, there is not a significant difference in walking at 0° and 15°, however, once we increase the incline to 30°, there begins to be a significant change in some of the measured variables. Both duty factor and step overlap increase significantly at 30° and 45° inclines and the increased contact with the substrate may help lessen the probability of slippage when walking. It is necessary to note that some of the differences observed may be attributable to the unequal number of individuals tested at each phase of the trials. The low number of walking bouts at 45° are potentially impacting the results presented here.

The mean range of the pectoral fins, although significantly different between the 30° and flat walking and between the EMG trials and all other trials, only range from 20.17° to 24.93° whereas the pelvic fins range from means of 28.25° to 46.75°. The reduced range of the pectoral fins is likely related to the constraint put on forward extension by

the pectoral girdle. The pelvic girdle, conversely, is more loosely attached via the sacral rib and thus is less constrained.

The increasing stride overlap coupled with the increasing duty factor at the higher inclines is likely due to the fish needing to maintain more contact with the substrate in order to keep from sliding backwards down the inclined platform. The inclusion of both LSDC and DSDC gait patterns is not surprising as all balitorids studied in our previous work were found to use both gait patterns while walking on a flat acrylic platform (Crawford et al., *in prep*). Most terrestrial tetrapods walk with a LSDC gait, DSDC gaits are primarily used by arboreal primates (Lemelin et al., 2003). However, the DSDC gait is also observed in high stepping alligators (Willey et al., 2004) and during underwater walking, with the support of a buoyant environment, in the epaulette shark (Pridmore, 1995) and the orange bellied newt (Ashley-Ross and Bechtel, 2004).

There was a significant decrease in body lengths per stride and per second at the 45° incline, however stride frequency was not reduced, this is counter to what has been observed in the American eel, *Anguilla rostrata*, which was found to reduce wave frequency at higher inclines (Redmann et al., 2020). The reduced wave frequency in *A. rostrata* is inferred to be a way the eels avoid slipping when traversing an incline (Redmann et al., 2020), while we do not see a frequency reduction in *H. parclitella*, the increased duty factor and stride overlap, as noted above, may be how this fish mitigates slippage.

The EMG data are relatively preliminary and definitive conclusions will require more trials. With the data presented here, we can infer that the fish are using their pectoral and pelvic adductors and abductors during more of the walk cycle than solely

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during the fin elevation and depression in the shift between stance and swing phases. This continuous activation of the muscles may be related to their function in holding tight to the substrate and keeping the fish in place against the high flow rivers and streams in which they live. The antagonistic contractions in the dorsal and ventral muscles of the fins are important in stiffening the flexible fin rays (Alben et al., 2007). With near continuous activation of antagonistic muscles, the relative intensity of the muscle activation is important in the ability of the fish to stiffen the fin rays and still complete the walking movements (Dickinson et al., 2000). This continuous activation is also likely influencing the ability of these fish to walk on the tips of their fins during high-walking.

The majority of walking bouts during all trials had the fish maintaining their midline and tail on the substrate, however, the high stepping walking bouts indicate that these fish are capably of lifting their entire body off the substrate. During these high stepping walks, the tail is in contact with the substrate for the majority of the walk, but the fish is seen supporting its mass entirely on the fins for a short time, before returning to the more common walking pattern with the ventral aspect of the fin in contact with the substrate.

Although *Homaloptera parclitella* has a thin rib connecting its pelvic plat to the vertebral column, it is still capable of performing a walking behavior both on flat and inclined terrain. The ability of this fish to walk with comparable performance to other fish in the family which exhibit a more robust sacral rib connecting the pelvic girdle and axial skeleton (Crawford et al., *in prep*), suggests that merely having this connection has a large impact on the ability to support the body against gravitational forces. A more robust sacral rib likely increases performance in more difficult terrain, such as inclines. Further work on other species comprising the other two morphotypes will further

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elucidate the influence of the variation in hyperossification of the rib on walking capabilities.

Two characteristics of terrestrial tetrapodal locomotion are a lateral-sequence diagonal couplets (LSDC) gait (Clack, 2012) and a robust, weight bearing connection between the pelvic appendages and the axial skeleton via a sacrum or sacral attachment capable of supporting the posterior body (Ahlberg, 2019; Clack, 2009; King et al., 2011). This work indicates that the skeletal connection does not need to be robust to support walking, especially in animals which are belly draggers and not lifting the body off the substrate. Although the balitorid loaches are not repeating the fin-to-limb transition, they are working against the same physical forces that needed to be overcome by early tetrapods and may help in understanding the potential locomotion strategies of the transitional species along the transition to tetrapods.

APPENDIX A

SUPPLEMENTARY MATERIAL FOR MORPHOLOGICAL AND MOLECULAR DATA

Supplemental material pertaining to Chapter 2: Skeletal and Muscular Pelvic Morphology of Hillstream Loaches (Cypriniformes: Balitoridae). Supplementary material I supports morphological variability and Supplementary Material II supports the molecular component of Chapter 2.

A.1 Supplementary Material 1

Table A.1.1 CT Scanners Used for Data Collection with Ranges of Relevant Settings Used for Stained and/or Unstained Scan DataCollection

| Saannan | Min | Max | Min | Max | Min | Max | Filton | Stained/ |
|---------------------|-----|-----|-----|-----|----------|----------|-----------|-------------|
| Scanner | kV | kV | uA | uA | Exposure | Exposure | rnter | Unstained |
| Nikon XTH 225 ST | 120 | 120 | 100 | 104 | 500 ms | 500 ms | No filter | Unstained |
| Bruker Skyscan 1275 | 60 | 65 | 123 | 166 | 60 ms | 75 ms | 1mm Al | Unstained |
| Bruker Skyscan 1275 | 65 | 92 | 105 | 140 | 45 ms | 80 ms | 1mm Al | PTA Stained |
| GE Phoenix | 55 | 90 | 130 | 200 | 200 ms | 500 ms | No filter | Unstained |

| | Eigenvalue | Proportion (%) | Cumulative (%) |
|------|------------|----------------|----------------|
| PC1 | 8.55E-03 | 47.56 | 47.56 |
| PC2 | 4.03E-03 | 22.45 | 70.01 |
| PC3 | 2.17E-03 | 12.05 | 82.06 |
| PC4 | 1.14E-03 | 6.33 | 88.39 |
| PC5 | 5.67E-04 | 3.16 | 91.55 |
| PC6 | 4.42E-04 | 2.46 | 94.01 |
| PC7 | 2.68E-04 | 1.49 | 95.50 |
| PC8 | 1.71E-04 | 0.95 | 96.45 |
| PC9 | 1.30E-04 | 0.72 | 97.17 |
| PC10 | 1.02E-04 | 0.57 | 97.74 |
| PC11 | 9.50E-05 | 0.53 | 98.27 |
| PC12 | 7.59E-05 | 0.42 | 98.69 |
| PC13 | 6.68E-05 | 0.37 | 99.06 |
| PC14 | 4.34E-05 | 0.24 | 99.30 |
| PC15 | 2.31E-05 | 0.13 | 99.43 |
| PC16 | 2.07E-05 | 0.11 | 99.55 |
| PC17 | 1.80E-05 | 0.10 | 99.65 |
| PC18 | 1.23E-05 | 0.07 | 99.72 |
| PC19 | 1.00E-05 | 0.06 | 99.77 |
| PC20 | 9.68E-06 | 0.05 | 99.83 |
| PC21 | 7.56E-06 | 0.04 | 99.87 |
| PC22 | 7.01E-06 | 0.04 | 99.91 |
| PC23 | 4.77E-06 | 0.03 | 99.93 |
| PC24 | 3.91E-06 | 0.02 | 99.96 |
| PC25 | 3.34E-06 | 0.02 | 99.97 |
| PC26 | 2.21E-06 | 0.01 | 99.99 |
| PC27 | 1.41E-06 | 0.01 | 99.99 |
| PC28 | 1.09E-06 | 0.01 | 100.00 |

Table A.1.2 Variance Explained by Each Principal Component from Shape 1.3 Output

| Muscle | Action | Outgroup | M1 | M2 | M3 |
|---------------------------|-------------------|-------------|----------|----------|----------|
| Abductor superficialis | Fin abduction | 0.000745795 | 0.002338 | 0.002603 | 0.005178 |
| Abductor profundus | Fin abduction | 0.001785994 | 0.005247 | 0.018322 | 0.018962 |
| Adductor superficialis | Fin adduction | 0.000589665 | 0.005942 | 0.008688 | 0.008248 |
| Adductor profundus | Fin adduction | 0.002676218 | 0.004206 | 0.009895 | 0.011952 |
| Arrector dorsalis | Fin adduction | 0.000647063 | 0.001316 | 0.002616 | 0.005893 |
| Arrector ventralis | Fin abduction | 0.000276075 | 0.001229 | 0.002871 | 0.005161 |
| Extensor proprius | Downward movement | 0.000255525 | NA | NA | NA |

Table A.1.3 Comparison of Normalized Physiological Cross-Sectional Area AmongMuscles of Different Morphotypes

Note: Typical fish morphology, *Carassius auratus* (Flammang Lab); Morphotype 1, *Homaloptera ogilviei* (USNM 288431); Morphotype 2, *Homalopterula ripleyi* (USNM 390014); and Morphotype 3, *Balitora* sp. (ANSP 179834).

| Taxon | Museum/ Institution | Specimen Number | Morphotype | Figure 2.3 # | |
|-----------------------------|------------------------|--------------------|------------|-----------------|--|
| Carassius auratus | Flammang Lab/NJIT | N/A | Т | 7 | |
| Ghatsa montana | CAS | SU39871 | M1 | 11 | |
| Homaloptera bilineata | USNM | 378394 | M1 | 15 | |
| Homaloptera orthogoniata | Flammang Lab/NJIT | N/A | M1 | 18 | |
| Homaloptera parclitella | Flammang Lab/NJIT | N/A | M1 | 35 | |
| Neohomaloptera johorensis | UF | 166089 | M1 | 30 | |
| Homalopterula vanderbilti | ANSP | 68689 | M1 | 28 | |
| Balitoropsis zollingeri | UF | 235547 | M2 | 4 | |
| Hemimyzon formosanus | USNM | 161711 | M2 | 12 | |
| Hemimyzon taitungensis | USNM | 300711 | M2 | 14 | |
| Homalopteroides nebulosus | UF | I235748 | M2 | 19 | |
| Homalopteroides rupicola | CAS | 231726 | M2 | 20 | |
| Homalopterula gymnogaster | USNM | 409946 | M2 | 25 | |
| Homalopterula heterolepis | AMNH | 9263 | M2 | 16 | |
| Homalopterula ripleyi | USNM | 390014 | M2 | 26 | |
| Jinshaia abbreviata | ANSP | 185166 | M2 | 32 | |
| Lepturichthys fimbriatus | ANSP | 185165 | M2 | 33 | |
| Sinogastromyzon puliensis | UF | 185384 | M2 | 36 | |
| Balitora burmanica | USNM | 44808 | M3 | 1 | |
| Balitora sp. | ANSP | 179834 | M3 | 2 | |
| Balitoropsis ophiolepis | UF | 166109 | M3 | 3 | |
| Bhavania australis | USNM | 165107 | M3 | 6 | |
| Cryptotora thamicola | MARNM | 6183 | M3 | 9 | |
| Hemimyzon macroptera | USNM | 293925 | M3 | 13 | |
| Homalopteroides smithi | UF | 172923 | M3 | 21 | |
| Homalopteroides stephensoni | ZRC | FIS51741 | M3 | 22 | |
| Homalopteroides tweediei | Flammang Lab/NJIT | N/A | M3 | 23 | |
| Homalopteroides weberi | USNM | 393729 | M3 | 24 | |
| Pseudohomaloptera leonardi | UF | 183398 | M3 | 31 | |

Table A.1.4 Specimens Used in PCA Of Shape Variation with Identifying NumbersUsed In Figure 3



Figure A.1.1 Linear Discriminant Analyses (A) Histogram of LD1 discrimination of balitorid morphotypes and (B) LDA biplot of LD1 (89.81%) and LD2 (10.19%) of the three balitorid morphotypes.

A.2 Supplementary Material 2

Phylogenomic data analysis led by P. B. Hart.

Phylogenomic Materials and Methods

Molecular Labwork

We collected and extracted whole genomic DNA from fin and tissue clips in 95% ethanol using Qiagen DNEasy Blood and Tissue kits (Appendix A.1 Table 1). DNA random shearing to lengths of ~600 base pairs was performed with the Episonic Multi-Functional Bioprocessor. Library preparation was performed with the HyperPrep Kit and hybrid enrichment was performed with the Acanthomorph target capture kit, targeting 1,314 ultraconserved element (UCE) loci (Arbor Biosciences, 2,600 probes; McGee et al., 2016). Quantities for the reactions of library preparation were scaled by half. Manufacturers protocols were followed for all other aspects. Raw sequence reads were created using the Illumina HiSeq3000-PE150 platform at the Oklahoma Medical Research Foundation. We sequenced for ~30X coverage.

Bioinformatics Processing

The Phyluce pipeline (v.1.5) was used for processing and cleaning of reads (Faircloth, 2016). Trimming of adapters from the reads was performed using *trimmomatic* (Bolger et al., 2014) within the *illumiprocessor* function. We used the ABySS assembler (v.1.9.0) (Simpson et al., 2009) on the Lousisiana State University High Performance Computing cluster SuperMike-II using a kmer value of 55. UCE contigs were extracted with the commands *phyluce_assembly_match_contigs_to_probes*, *phyluce_assembly_get_match_counts* and *phyluce_assembly_get_fastas_from_match_counts*. We aligned the UCE loci using *phyluce_align_seqcap_align*. We created a 75% complete datamatrix (equating to 411 UCE loci) using the *phyluce align get only loci with min taxa* function.

We partitioned the data with Sliding-Window Site Characteristic using site entropies, the best-performing UCE-specific partitioning method described by Tagliacollo and Lanfear (2018). The output of Sliding-Window Site Characteristic using site entropies is multiple data blocks for each UCE locus (i.e., right flanking region, center, left flanking region). We used PartitionFinder2 on XSEDE (v.2.1.1; Lanfear et al., 2017) on the CIPRES Science Gateway (v.3.3; https://www.phylo.org/) to group the data blocks from the UCE-specific partitioning method into similar data blocks, estimating the best partitioning scheme while ensuring we are not over-partitioning.

Supplementary Material 2

| Catalog Number | UCE ID | Sequence ID | Family | Genus | Species | Tissue Vial Number |
|-------------------|-----------|------------------|-------------|-----------------|-------------|-----------------------|
| UF 183865 | BaBur01 | 004_Ba_bur_01 | Balitoridae | Balitora | burmanica | 2012-0242 |
| UF 188372 | BaBur02 | 005_Ba_bur_02 | Balitoridae | Balitora | burmanica | ICH-00016 |
| ANSP 179834 | BaCfMer01 | 006_Ba_mer_01 | Balitoridae | Balitora | sp. | 333 |
| ANSP 178677 | BaSpNAN01 | 007_Ba_mer_02 | Balitoridae | Balitora | sp. | 352 |
| UF 237571 | BaCfMer02 | 008_Ba_mer_03 | Balitoridae | Balitora | sp. | 2015-0395 |
| UF 237572 | BaSpMAE01 | 009_Ba_sp_MAE_01 | Balitoridae | Balitora | sp. | 2015-0476 |
| UF 188275 | BaSpNAN02 | 010_Ba_sp_NAN_01 | Balitoridae | Balitora | sp. | ICH-00094 |
| UF 166109 | BsisOph01 | 011_Bsis_oph_01 | Balitoridae | Balitoropsis | ophiolepis | 2006-0588 |
| UF 161715 | BsisZol01 | 012_Bsis_zol_01 | Balitoridae | Balitoropsis | zollingeri | 2005-0962 |
| UF 235547 | BsisZol02 | 013_Bsis_zol_02X | Balitoridae | Balitoropsis | zollingeri | 2012-0587 |
| MARNM 6183 | Crypt01 | 154_Crypt01 | Balitoridae | Cryptotora | thamicola | Crypt01 |
| UF 188164 | HdesLin01 | 018_Hdes_lin_01 | Balitoridae | Homalopteroides | lineatus | 2015-0128 |
| UF 181080 | HdesMod01 | 019_Hdes_mod_01 | Balitoridae | Homalopteroides | modestus | 2011-0230 |
| UF 183825 | HdesMod02 | 020_Hdes_mod_02 | Balitoridae | Homalopteroides | modestus | 2012-0085 |
| UF 235748 | HdesNeb01 | 021_Hdes_neb_01 | Balitoridae | Homalopteroides | nebulosus | 2012-0608 |
| CAS 231726 | HdesRup01 | 022_Hdes_rup_01 | Balitoridae | Homalopteroides | rupicola | DAN09- 182.04 |
| UF 172923 | HdesSmi01 | 023_Hdes_smi_01 | Balitoridae | Homalopteroides | smithi | 2008-0562 |
| UF 235717 | HdesSmi02 | 024_Hdes_smi_02 | Balitoridae | Homalopteroides | smithi | 2014-0267 |
| ZRC THH07-14 | HdesSte01 | 025_Hdes_ste_01X | Balitoridae | Homalopteroides | stephensoni | ZRC09 |
| ZRC THH09- 107 | HdesTwe01 | 026_Hdes_twe_01 | Balitoridae | Homalopteroides | tweediei | ZRC06 |

Table A.2.1 Tissue Specimens and Associated Repository and Collection Identifications Used for the UCE Dataset (N =62)

| Table A.2.1 Con | tinued | | | | | |
|-----------------|-----------|------------------|-------------|-------------------|-------------|-----------------------|
| Catalog Number | UCE ID | Sequence ID | Family | Genus | Species | Tissue Vial Number |
| UF 161619 | HdesWas01 | 027_Hdes_was_01 | Balitoridae | Homalopteroides | wassinkii | 2005-0975 |
| USNM 393671 | HdesWeb01 | 028_Hdes_web_01 | Balitoridae | Homalopteroides | weberi | E97 |
| UF 185370 | HemiFor01 | 029_Hemi_for_01 | Balitoridae | Hemimyzon | formosanus | 2012-0357 |
| UF 185378 | HemiFor02 | 030_Hemi_for_02 | Balitoridae | Hemimyzon | formosanus | 2012-0225 |
| UF 169906 | HeraCon01 | 031_Hera_con_01 | Balitoridae | Homaloptera | confuzona | 2007-1185 |
| UF 166096 | HeraOce01 | 032_Hera_oce_01 | Balitoridae | Homaloptera | ocellata | 2006-0609 |
| UF 161719 | HeraOce02 | 033_Hera_oce_02 | Balitoridae | Homaloptera | ocellata | 2005-0943 |
| UF 161716 | HeraOgi01 | 034_Hera_ogi_01 | Balitoridae | Homaloptera | ogilviei | 2005-0963 |
| UF 235716 | HeraOgi02 | 035_Hera_ogi_02 | Balitoridae | Homaloptera | ogilviei | 2014-0238 |
| USNM 409946 | HulaGym01 | 036_Hula_gym_01X | Balitoridae | Homalopterula | gymnogaster | D24 |
| UF 185031 | HulaGym02 | 037_Hula_gym_02 | Balitoridae | Homalopterula | gymnogaster | SN25 |
| USNM 390061 | HulaHet01 | 160_Hula_het_01 | Balitoridae | Homalopterula | heterolepis | A89 |
| USNM 390011 | HulaRip02 | 039_Hula_rip_02 | Balitoridae | Homalopterula | heterolepis | C35 |
| USNM 390013 | HulaRip01 | 038_Hula_rip_01X | Balitoridae | Homalopterula | ripleyi | A14 |
| UF 166089 | NeohJoh01 | 042_Neoh_joh_02 | Balitoridae | Neohomaloptera | johorensis | 2006-0651 |
| UF 235746 | PseuLeo01 | 047_Pseu_leo_01 | Balitoridae | Pseudohomaloptera | leonardi | 2012-0597 |
| UF 188618 | PseuLeo02 | 048_Pseu_leo_02 | Balitoridae | Pseudohomaloptera | leonardi | ICH-00298 |
| UF 183398 | PseuSex02 | 050_Pseu_sex_02 | Balitoridae | Pseudohomaloptera | leonardi | 2012-0340 |
| UF 183334 | PseuSex01 | 049_Pseu_sex_01 | Balitoridae | Pseudohomaloptera | sexmaculata | 2012-0039 |
| UF 185384 | SinPul01 | 055_Sin_pul_01 | Balitoridae | Sinogastromyzon | puliensis | 2012-0361 |
| UF 237561 | SynHel01 | 056_Syn_hel_01 | Botiidae | Syncrossus | helodes | 2015-0084 |
| UF 237558 | YasMor01 | 060_Yas_mor_01 | Botiidae | Yasuhikotakia | morleti | 2015-0478 |
| UF 188109 | AcaSp01 | 001_Aca_sp_01 | Cobitidae | Acantopsis | rungthipae | 2015-0001 |
| UF 185368 | CobSin01 | 014_Cob_sin_01 | Cobitidae | Cobitis | sinensis | 2012-0377 |

| Table A.2.1 Cont | inued | | | | | |
|------------------|-----------|-----------------|-------------------|---------------------|-----------------|-----------------------|
| Catalog Number | UCE ID | Sequence ID | Family | Genus | Species | Tissue Vial Number |
| UF 237389 | LepBer01 | 040_Lep_ber_01 | Cobitidae | Lepidocephalichthys | berdmorei | 2015-0110 |
| UF 237405 | PanAng01 | 044_Pan_ang_01 | Cobitidae | Pangio | anguillaris | 2015-0162 |
| UF 185397 | AnNor01 | 002_An_nor_01 | Gastromyzontidae | Annamia | normani | 2012-0687 |
| UF 185479 | AnNor02 | 003_An_nor_02 | Gastromyzontidae | Annamia | normani | 2012-0672 |
| UF 185369 | ForLac01 | 015_For_lac_01 | Gastromyzontidae | Formosania | lacustre | 2012-0205 |
| UF 185385 | ForLac02 | 016_For_lac_02 | Gastromyzontidae | Formosania | lacustre | 2012-0204 |
| USNM 409794 | GasSp01 | 017_Gas_sp_01 | Gastromyzontidae | Gastromyzon | sp. | H40 |
| UF 185476 | SewElo01 | 053_Sew_elo_01 | Gastromyzontidae | Sewellia | elongata | 2012-0668 |
| UF 185488 | SewElo02 | 054_Sew_elo_02 | Gastromyzontidae | Sewellia | elongata | 2012-0682 |
| UF 191060 | YaoPac01 | 058_Yao_pac_01 | Gastromyzontidae | Yaoshania | pachychilus | ICH-00344 |
| UF 191060 | YaoPac02 | 059_Yao_pac_02 | Gastromyzontidae | Yaoshania | pachychilus | ICH-00345 |
| UF 237559 | NemBin01 | 041_Nem_tin_01 | Nemacheilidae | Nemacheilus | binotatus | 2015-0268 |
| UF 188371 | NeonLab01 | 043_Neon_lab_01 | Nemacheilidae | Neonemacheilus | labeosus | ICH-00017 |
| UF 188053 | ParNSp01 | 045_Par_n_sp_01 | Nemacheilidae | Paracanthocobitis | nigrolineata | 2015-0384 |
| UF 188066 | PhyPseu01 | 046_Phy_pseu_01 | Nemacheilidae | Physoschistura | pseudobrunneana | 2015-0112 |
| UF 188063 | SchiAur01 | 051_Schi_aur_01 | Nemacheilidae | Schistura | aurantiaca | 2015-0405 |
| UF 190429 | SerpZon01 | 052_Serp_zon_01 | Serpenticobitidae | Serpenticobitis | zonata | ICH-01353 |
| UF 236153 | VailMaa01 | 057_Vail_maa_01 | Vaillantellidae | Vaillantella | maassi | 2012-0596 |

Note: Tissues were provided by: Academy of Natural Sciences of Drexel University (ANSP), California Academy of Sciences (CAS), Maejo Aquatic Resources Natural Museum (MARNM), University of Florida (UF), and Lee Kong Chian Natural History Museum (ZRC). Locality information is available through iDigBio, VertNet, or the Florida Museum of Natural History Ichthyology Collection Specify Database (http://specifyportal.flmnh.ufl.edu/fishes/).

Supplementary Material 2

Figures



Figure A.2.1 Coalescent species tree of loaches created with 75% complete data matrix of UCE loci. Node values indicate bootstrap support. Scale bar units are number of substitutions per site. Analyses completed by P.B. Hart.



Homaloptera ogilviei 01 Homaloptera ogilviei 02 Balitoropsis zollingeri 01 Balitoropsis zollingeri 02 Balitoropsis ophiolepis 01 Pseudohomaloptera leonardi 01 Pseudohomaloptera leonardi Pseudohomaloptera leonardi 02 Pseudohomaloptera sexmaculata 01 Balitora sp. 01 Balitora sp. 01 Balitora sp. 01 Balitora sp. 02 Balitora sp. 03 Balitora burmanica 01 Balitora burmanica 02 Bailtora burmanica 02 Hemimyzon formosanus 02 Hemimyzon formosanus 01 Sinogastromyzon puliensis 01 Cryptotora thamicola 01 Homalopteroidea smithi 01 Homalopteroidea smithi 01 Homalopteroides smithi 02 Homalopteroides lineatus 01 Homalopteroides modestus 01 Homalopteroides modestus 02 Homalopteroides rupicola 01 Homalopteroides tweediei 01 Homalopteroides nebulosus 01 Homalopteroides vessinkii 01 Homalopteroides stephensoni 01 Homalopteroides weberi 01 Homalopterula heterolepis 01 Homalopterula heterolepis 02 Homalopterula ripleyi 01 Homalopterula gymnogaster 01 Homalopterula gymnogaster 02 Neohomaloptera johorensis 01 Formosania lacustre 01 Formosania lacustre 01 Yaoshania pachychilus 01 Yaoshania pachychilus 02 Sewellia elongata 01 Sewellia elongata 02 Annamia normani 01 Annamia normani 02 Gastromyzon sp. 01 Serpenticobitis zonata 01 Physoschistura pseudobrunneana 01 Schistura aurantiaca 01 Neonemacheilus labeosus 01 Paracanthocobitis nigrolineata 01 Nemacheilus binotatus 01 Lepidocephalichthys berdmorei 01 Pangio anguillaris 01 Acantopsis rungthipae 01 Cobitis sinensis 01 Syncrossus helodes 01 Yasuhikotakia morleti 01 Vaillantella maassi 01

Figure A.2.2 Maximum likelihood phylogeny of loaches created with 75% complete data matrix of UCE loci. Node values indicate bootstrap support. Scale bar units are number of substitutions per site. Analyses completed by P.B. Hart.

APPENDIX B

SUPPLEMENTARY MATERIAL FOR KINEMATIC DATA

Supplemental material pertaining to Chapter 3: They Like to Move it (Move it): Walking

Kinematics of Balitorid Loaches of Western Thailand

| Table B.1 | Species Filmed | on Location | in Central | Thailand |
|-----------|----------------|-------------|------------|----------|
|-----------|----------------|-------------|------------|----------|

| Taxon | Ν | Letter | Province | Museum ID |
|----------------------------------|---|------------------|--|------------|
| Cryptotora thamicola | 2 | A, B | Pang Mapha, Pang Mapha District, Mae Hong Son, Thailand | MARNM 7413 |
| Homalopteroides sp | 3 | C, D, E | Kaem On, Chom Bueng District, Ratchaburi, Thailand | UF 245539 |
| Homalopteroides modestus | 3 | F, J, K | Lin Thin, Thong Pha Phum District, Kanchanaburi | UF 245299 |
| Homalopteroides smithi | 3 | G, H, I | Lin Thin, Thong Pha Phum District, Kanchanaburi | UF 245298 |
| <i>Balitora</i> sp | 5 | L, M, O, P, Q | Tha Kha-nun, Thong Pha Phum District, Kanchanaburi | UF 245561 |
| Pseudohomaloptera sexmaculata | 1 | N | Tha Kha-nun, Thong Pha Phum District, Kanchanaburi | UF 245546 |
| Homalopteroides modestus | 1 | R | Huai Kayeng, Thong Pha Phum District, Kanchanaburi | UF 245290 |
| Pseudohomaloptera sexmaculata | 4 | T, U, V, W | Prang Phe, Sangkhla Buri District, Kanchanaburi, Thailand | UF 245427 |

| Variable | Homaloptera parclitella | Pseudohomaloptera sexmaculata | Balitora sp | Cryptotora thamicola | Homalopteroides modestus | Homalopteroides smithi | Homalopteroides sp |
|---|----------------------------|----------------------------------|----------------|-------------------------|-----------------------------|---------------------------|-----------------------|
| Stride Length (%BL) | 0.1158 | 0.1321 | 0.0998 | 0.2144 | 0.1374 | 0.0940 | 0.1248 |
| Cycle Duration (s) | 0.193 | 0.196 | 0.153 | 0.440 | 0.127 | 0.093 | 0.073 |
| Duty Factor (%) | 0.552 | 0.560 | 0.559 | 0.567 | 0.544 | 0.548 | 0.530 |
| Stride overlap (%) | 0.053 | 0.062 | 0.064 | 0.065 | 0.042 | 0.036 | 0.035 |
| Pectoral fin extension range (degrees) | 19.480 | 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 | 0.0070 | 0.0136 | 0.0203 | 0.0406 | 0.0312 | 0.0440 |
| Minimum Curvature | 0.0015 | 0.0015 | 0.0014 | 0.0008 | 0.0035 | 0.0010 | 0.0019 |
| Curvature Range | 0.0158 | 0.0054 | 0.0122 | 0.0194 | 0.0371 | 0.0302 | 0.0420 |
| Trunk Maximum Curvature | 0.0179 | 0.0079 | 0.0127 | 0.0201 | 0.0358 | 0.0295 | 0.0402 |
| Trunk Minimum Curvature | 0.0013 | 0.0011 | 0.0011 | 0.0006 | 0.0030 | 0.0009 | 0.0015 |
| Trunk Curvature Range | 0.0166 | 0.0068 | 0.0116 | 0.0196 | 0.0328 | 0.0286 | 0.0386 |
| Tail Maximum Curvature | 0.0175 | 0.0061 | 0.0120 | 0.0189 | 0.0341 | 0.0276 | 0.0350 |
| Tail Minimum Curvature | 0.0018 | 0.0023 | 0.0016 | 0.0012 | 0.0037 | 0.0010 | 0.0018 |
| Tail Curvature Range | 0.0157 | 0.0038 | 0.0104 | 0.0177 | 0.0304 | 0.0266 | 0.0333 |

Table B.2 Summary of Mean Kinematics Variables Measured from Walking Balitorids



Figure B.1 Map of 29 digitized points used for analyses with blue outlines of angle measurements for pectoral and pelvic fin extension, points plotted on a line drawing of *Cryptotora thamicola*.



Figure B.2 Reconstructions of μ CT scans of the species studied shown in whole body dorsal view (left) and pelvic region (right). Scale bars = 10 mm.







Figure B.4 Boxplots of mean maximum curvature of each species at the thirteen points down the midline accompanied by 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*.

APPENDIX C

SUPPLEMENTARY MATERIAL FOR KINEMATIC DATA

Supplemental material pertaining to Chapter 4: Walking by Fishes That Shouldn't: The

Case of The Red Lizard Loach (*Homaloptera parclitella*)

| Individual | 0° | 15° | 30° | 45° | EMG | Total |
|------------|----|-----|-----|-----|-----|-------|
| AA | 4 | 4 | 4 | 0 | 0 | 12 |
| BB | 4 | 4 | 4 | 4 | 0 | 16 |
| CC | 3 | 3 | 3 | 0 | 1 | 10 |
| DD | 0 | 0 | 0 | 0 | 3 | 3 |
| EE | 3 | 0 | 0 | 0 | 11 | 14 |
| FF | 5 | 0 | 0 | 0 | 3 | 8 |
| GG | 3 | 3 | 0 | 0 | 1 | 7 |
| HH | 0 | 0 | 0 | 0 | 5 | 5 |
| II | 7 | 3 | 2 | 0 | 4 | 16 |
| JJ | 4 | 3 | 3 | 3 | 0 | 13 |
| KK | 3 | 3 | 3 | 0 | 0 | 9 |
| LL | 6 | 2 | 1 | 0 | 0 | 9 |

 Table C.1
 Videos Analyzed Per Individual Fish Delimited by Walking Trial



Figure C.1 Map of 29 digitized points used for analyses on *Homaloptera parclitella* with blue outlines of angle measurements for pectoral and pelvic fin extension.



Figure C.2 The three arrangements of electrode placements in *H. Parclitella*. A) Electrodes placed in the right extensors and left flexors; B) electrodes placed in right flexors and left extensors; and C) electrodes in extensors and flexors of all fins. In all three arrangements the axial electrodes, torso and tail, are inserted on the left side.

APPENDIX D

INFORMATION PERTAINING TO AUTHORSHIP, ACKNOWLEDGEMENTS, AND FUNDING OF DISSERTATION CHAPTERS

D.1 Information pertaining to authorship, acknowledgements, and funding for Chapter 1: Terrestrial Excursions in Teleost Fishes

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Author Contributions

CHC and BEF conceived of and designed the study and wrote the initial manuscript draft. CHC and ZSR µCT scanned material and CHC segmented all scan data used. PBH completed molecular work and analyses for the UCE phylogeny. LMP and PC were involved in data collection and analysis. AS was involved in collection of *Cryptotora* specimens. All authors were involved in revisions and approved the final manuscript.

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Data Accessibility Statement

The CT scan data that support the findings of this study are available at www.morphosource.org, reference numbers M13737, M14876, M15050, M15107, M15110, M15116, M15120, M15126, M15127, M15164, M15493, M19663, M22168, M53469, M53472, M53476, M53479, M53481, M53484, M53485, M53485, M53489, M53490, M53491, M53492, M53493, M53494, M53495, M53497, and M53498. Raw sequence data for ultraconserved elements will be accessible on the Sequence Read Archive from the National Center for Biotechnology Information. Molecular specimen accession and catalog numbers are available in the Supplementary Material.

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National Science Foundation, Grant/Award Number: 1839915; American Museum of Natural History (Lerner- Gray Grant for Marine Research); American Society of Ichthyologists and Herpetologists (Raney Fund Award); Duke University (Research Triangle Nanotechnology Network Free-Use); Sigma Xi (Grants in Aid of Research) **D.2** Information pertaining to planned authorship, acknowledgements, and funding for Chapter 3: They Like to Move it (Move it): Walking Kinematics of Balitorid Loaches of Western Thailand

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