



Are extreme anatomical modifications required for fish to move effectively on land?

Comparative anatomy of the posterior axial skeleton in the Cyprinodontiformes.

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Abstract: Many teleost fishes with no apparent modifications for life on land are able to produce effective terrestrial locomotor behaviors, including a ballistic behavior called the “tail-flip” jump. Cyprinodontiformes (killifishes, Teleostei: Atherinomorpha) that live at the water’s edge vary in morphology and inclination to emerge onto land. Do fish with an amphibious predisposition have extensive modification of the propulsive region of the body when compared to fully-aquatic relatives? We quantified body shape and anatomy of

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the caudal peduncle and tail (the propulsive organ on land and in water) in eleven cyprinodontiform species and two outgroup taxa (Atherinomorpha). We hypothesized that amphibious species would have longer, “shallower” bodies (larger body fineness ratios), deeper (proportionally larger) caudal peduncles, and more robust bones in the tail fin (larger ossified area of the hypural/epural bones) to facilitate locomotor movements on land. We found no evidence of convergence in body shape or skeletal anatomy among species known to make voluntary sojourns onto land. In fact, deep-bodied species, shallow-bodied species, and species with intermediate morphologies all are able to emerge from the water and move on land. It is possible that there are as-yet-undocumented subtle soft-tissue (muscle, tendon, ligament) modifications that enhance terrestrial locomotor performance in species known to spend large periods of time on land. However, it is also possible that extreme anatomical changes are not required for aquatic cyprinodontiform species to produce effective locomotor movements when they emerge out of the water and move across the land.

Keywords: amphibious fishes, terrestrial locomotion, environmental transition, biomechanics of locomotion, vertebral morphology, neural and hemal spine, caudal peduncle

Introduction:

Fishes that regularly emerge onto land are thought to possess characteristic morphologies (*sensu* Pace and Gibb, 2014): very elongate bodies (eels; Anguillidae), robust appendages (mudskippers; Oxudercinae), or some combination of these features (walking catfishes; Clariidae). However, fish species with no obvious adaptations to facilitate movement on land can still produce effective terrestrial locomotor behaviors. Indeed, the Cyprinodontiformes (killifishes and livebearers) is a large and diverse order of freshwater and intertidal fishes that contains species with no obvious morphological adaptations for terrestrial locomotion, yet many taxa within this group are reported to emerge voluntarily onto land (Bayliss, 1982; Barber et al. 2002; Turko and Wright, 2015). Many of these cyprinodontiform species have been described moving on land using a terrestrial movement that can displace an individual fish over multiple body lengths as the result of a single ballistic leap — the “tail-flip” jump (Mast, 1915; Gibb et al. 2011; Minicozzi, 2017).

The tail-flip jump was first described over 100 years ago (Mast, 1915) for individuals of an intertidal killifish species (*Fundulus majalis*) that were observed moving out of shallow tide-pools, across a sandy bank, and into the ocean. This behavior has more recently been observed in several teleost lineages and quantified using high-speed imaging (Gibb et al. 2011; Minicozzi, 2017). The tail-flip jump (Fig. 1, Video 1) is produced when a fish rests with the lateral aspect of its body in contact with the ground and then elevates its anterior body and head up, off of the ground. While keeping the head elevated, the fish

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accelerates the anterior body toward the posterior body — until it achieves a C-shaped body-bend, with the posterior-most region of the body (caudal peduncle and tail fin) remaining in direct contact with the ground. At this point, the fish straightens the axial body and pushes off the ground to launch into a posteriorly-oriented, ballistic-flight trajectory (Gibb et al., 2011).

The order Cyprinodontiformes is an excellent group in which to consider the evolution of amphibious behavior because species within the group demonstrate variation in inclination to leave the water and in terrestrial locomotor ability (Minicozzi, 2017).

Kryptolebias marmoratus, the mangrove “rivulus,” can live out of the water for two months (Davis et al., 1990) and moves more than eight body lengths as the result of a single tail-flip jump (Brunt et al., 2016). The mosquitofish, *Gambusia affinis*, voluntarily leaps onto land to evade aquatic predators (Bayliss, 1982) and can tail-flip jump to move five body lengths or more (Gibb et al. 2011). In contrast, our preliminary data for *Cyprinodon variagatus*, the sheepshead minnow, suggest that individuals of this species are unable to jump when stranded on land (pers. obs.) and, despite decades of use as a model research animal, this species has never been reported to voluntarily leave the water. In this study, we examine the caudal anatomy of representative cyprinodontiform taxa to test hypotheses about form-function relationships in fish species that live at the water’s edge.

Recent comparative analyses of external body shape in terrestrial-jumping teleosts that are highly modified for life on land, including mudskippers (Perciformes: Gobiidae:

Oxudercinae: *Periophthalmus spp.*) and intertidal blennies (Perciformes: Blenniidae: *Alticus spp.*), yielded the observation that fishes prone to moving over land via jumping behaviors tended to have more elongate, less fusiform bodies, relative to fully aquatic fishes (Gibb et al., 2013). Based on these findings, we hypothesize that cyprinodontiform fishes that voluntarily leave the water will have longer, “shallower” bodies and a higher fineness ratio (body length:body width), relative to cyprinodontiform species that do not make voluntary excursions onto land.

In addition, because the caudal peduncle and tail fin are the body surfaces in contact with the ground just before fish enter the “launch phase” of a tail-flip jump, we hypothesize that cyprinodontiform species with a greater predisposition for terrestrial movement will have a proportionally “deeper” (larger in the dorso-ventral axis) skeleton in the caudal region to facilitate increased contact with the substrate during terrestrial locomotor behaviors. We further posit that fishes that emerge onto land as a part of their life history will exhibit modification of the morphology of the axial skeleton in the posterior region of the body. We predict that the morphology of the spines that extend from the vertebral centra to the dorsal and ventral margins of the body (neural and hemal spines) will change in length and orientation (angle of spine with centrum) to generate variation in the proportional (relative) size of the caudal peduncle.

Finally, because the caudal region (peduncle and tail fin) of species that emerge onto land is in direct contact with the ground and used to propel the fish against the forces of

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gravity, we expect these species to exhibit increased ossification of the skeleton in the base of the tail fin. Increased ossification should occur because (1) denser, stronger bone will increase the safety factor preventing physical damage to the bone (Kawano et al., 2015) and (2) bone will become more heavily ossified in response to the insertion of more forceful muscles (Benjamin et al., 2006). Therefore, we hypothesize that fishes that emerge onto land as a normal part of their life history will have more “robust” (greater ossification) tail elements (measured as the total ossified area and number of the hypural and epiural elements in direct contact with the caudal fin rays; see Costa, 2012), relative to cyprinodontiform taxa that do not naturally make terrestrial excursions.

To test these hypotheses about adaptations for terrestrial movement in the Cyprinodontiformes, we obtained museum specimens representing eleven cyprinodontiform species (in nine genera) from five families (Atherinomorpha: Cyprinodontiformes, Fig. 2) with different reported degrees of voluntary terrestriality (Table 1). Because individuals representing these species have been observed voluntarily emerging onto land, we considered seven of these eleven cyprinodontiform species to display amphibious behavior as part of their life history. However, these seven species display a broad range of amphibious inclination. *Kryptolebias marmoratus*, for example, is widely considered to be a truly amphibious species because it can respire on land and remain out of water for weeks at a time (Davis et al., 1990; Wright, 2012). *Gambusia affinis*, *Fundulus majalis* and *Fundulus heteroclitus*, in contrast, make only brief sojourns onto land,

but can determine their position on land using environmental cues and use this information to navigate back to the water (Mast, 1915; Boumis et al., 2014; Bressman et al., 2016). Individuals of *Lucania parva*, the rainwater killifish, will leap out of the water and onto terrestrial vegetation to avoid predators (Baylis, 1982), but nothing further is known about their terrestrial abilities. Cyprinodontiform species that appear to have limited terrestrial abilities include *Jordanella floridae* and *Fundulopanchax gardneri*: *Fundulopanchax* individuals have only been reported to jump of the water when held in captivity (Turko and Wright, 2015) and *Jordanella* individuals may bury themselves in the substrate to become dormant (pseudo-estivation, Barber, 2002) in response to environmental stress. Two cyprinodontiform species that have never been reported to emerge onto land are also considered in our analysis: *Cyprinodon varigatus* and *Fundulus bermudae*. Our observations of *Cyprinodon varigatus* in the laboratory environment suggest it *cannot* move effectively on land (pers. obs.). Finally, to place this analysis in a comparative context, we considered the caudal anatomy of two species representing outgroup genera for the Cyprinodontiformes, following relationships established in recent multigene teleost phylogenies (Rabosky et al., 2013; Pohl et al., 2015): *Psuedomugil* (Atherinomorpha: Pseudomugilidae) and *Oryzias* (Atherinomorpha: Adrianichthyidae).

With these thirteen species, we asked the following questions: (1) does body shape, as measured by body fineness and size of the caudal peduncle, differ among the eleven cyprinodontiform taxa considered here? In addition, if there is variation in body shape

among taxa, which characteristics of the axial skeleton change as body proportions vary? We quantified shape of the posterior vertebral elements by measuring the angle and length of the neural and hemal spines that project dorsally and ventrally from the vertebral centra to define the distal margins of the body and asked (2) how does internal skeletal morphology vary to generate variation in external body morphology and (3) when multiple shape parameters are considered, are more amphibious cyprinodontiform taxa more similar in body shape to one another than to other taxa? Finally, given that the tail is responsible for generating ground-reaction forces to propel the fish away from the ground during the launch phase of the tail-flip jump, (4) does the degree ossification or the total number of bones present at the base of the tail fin vary with amphibious habit among cyprinodontiform taxa?

Materials and Methods:

Species Examined

Adult individuals *Gambusia affinis* (n=12), *Poecilia mexicana* (n=9), *Jordanella floridae* (n=5), *Kryptolebias marmoratus* (n=6), *Fundulus majalis* (n=2, CU 77022 and CU 79296), *Fundulus bermudae* (n=4, USNM 112083), *Fundulus heteroclitus* (n=4), *Cyprinodon variegatus* (n=5, USNM 124665), *Fundulopanchax gardneri* (n=5, CU 47357), *Austrolebias luteoflammulatus* (n=5, USNM 389719), *Lucania parva* (n=3, CU 43775), *Oryzias celebensis*

(n=5, USNM 316513), and *Pseudomugil signifer* (n=4) were obtained from the pet trade, ponds monitored by Arizona Game and Fish, an established lab colony (Ryan Earley, University of Alabama), or catalogued museum specimens from Cornell University and the National Museum of Natural History (Table 1). When it was possible to identify fish by sex using skeletal anatomy, both male and female individuals of a given species were included in shape analyses.

Species were selected for analysis based on specimen availability, but also with the goal of representing both taxonomic diversity within the order and variation in inclination to leave the water. Amphibious behaviors in the wild (as observed by researchers in the field) and terrestrial locomotor behaviors reported for these species on land (from either lab or field observations) were obtained from the literature (Table 1). Taxa with documented amphibious behaviors are distributed across the cyprinodontiform phylogeny (Table 1 and Fig. 2).

Morphology

Standard length, maximum body depth (greatest dorsal-ventral distance along the anterior-posterior body axis), and minimum peduncle depth (smallest dorsal-ventral distance found along the posterior-most axial body region) were measured for each individual representing every species. Standard length was divided by maximum body depth to quantify body fineness (body length: body depth, where higher numbers are

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shallower, more elongate bodies) and minimum peduncle depth was divided by standard length to obtain relative peduncle size for each specimen's peduncle (peduncle ratio, or peduncle depth: body length, where larger numbers are deeper peduncles). To quantify *change* in body depth along the anterior-to-posterior body axis, the relative size of the peduncle was subtracted from the inverse of body fineness (converted so that larger numbers will indicate a greater decrease in height of the body along the anterior to posterior axis). Thus, as quantified by the change body depth, a fish species with a large difference between maximum and minimum body depth will have a more "fat-bodied" shape that is "deep" in the middle and "shallow" near the tail.

To measure the skeletal elements that comprise the posterior third of the body across taxa, soft tissues were cleared and cartilage and bones were stained with alcian blue and alizarin red (respectively) following a protocol by Song and Parenti (1995); however, some species obtained from museums arrived as cleared and stained specimens. To quantify the relationship between vertebral morphology and peduncle shape, we measured neural and hemal spine angles (the angle a given spine creates with the midline of the vertebral column) and the neural and hemal spine lengths for the last twelve caudal vertebrae (the twelve vertebrae most proximal to the hypural plate, see Fig. 3) using the cleared and stained specimens. Neural and hemal spine lengths were measured as straight-line distances from the base (where they originate from the centra) to the distal tip of each

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spine; spine length was divided by the standard length of the specimen to quantify relative spine length.

In this study, we sought to determine which aspects of vertebral morphology are associated with variation in depth of the caudal peduncle among taxa. To this end, we measured the neural and hemal spine angles and the relative spine length (spine length: body length) of vertebra number 8 or 9, whichever corresponded to the shallowest region of the peduncle. We then examined the relationship between these two variables and peduncle size among species.

To quantify how variation in spine angles and length along the body of a fish can generate variation in shape among taxa, we plotted relative spine length and angle for spines 1 – 8 or 9 (because spines 9/10 – 12 are components of the hypural plate of the caudal fin they were excluded from this analysis). Using these values, we calculated the slope for a line describing the change in angle from anterior to posterior and a second slope quantifying the change in spine length from anterior to posterior. The slope of these lines provides two key parameters: the directionality of the relationship (that is, if spine angle or length is *increasing* or *decreasing* along the body) and the magnitude of the change (where *larger slopes* correspond to *greater changes* in spine angle or length along the body). Thus, a slope value indicates the rate of change per vertebrae over the caudal-most nine vertebrae; for example, a slope of -2 for spine angle documents an average decrease of 2° from vertebra to vertebra as you approach the hypural plate.

We quantified skeletal anatomy of the tail by measuring the number of elements and degree of ossification of the bones associated with the hypural plate — as well as any other ossified structures that interdigitate with the caudal fin rays (Fig. 4). We counted the number of elements associated with the caudal fin rays and then measured the total ossified area of each element using NIH Image-J (ver 1.51j8). The measured areas of ossification for all elements were then summed to obtain the total area of ossification in the tail for each species. *Fundulopanchax gardneri* was excluded from this analysis because we were unable to obtain sufficient museum specimens with intact hypural plates. The two outgroup species (*Oryzias* and *Pseudomugil*) were also excluded because their tail anatomy is distinct from that of cyprinodontiform fishes. Finally, the square-root of total area of ossification was divided by standard length to yield a size-adjusted measure of tail fin ossification for each species.

Statistical Analyses

To analyze the relationship between spine angle, spine length, and peduncle depth, we performed a linear regression for spine angle and the (relative) spine length versus (relative) peduncle size across all species. We evaluated the resulting slope with respect to the null hypothesis of zero slope (that is, no relationship between spine angle or length with peduncle depth), with the *a priori* expectation that both angle and length could potentially be altered during development to generate the observed anatomical variation in

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peduncle depth. In this analysis, a positive slope would indicate a positive correlation in spine length or angle with size of the peduncle.

To generate a multivariate description of shape, a Principal Component Analysis (PCA) with varimax rotation was performed using SPSS (ver. 24) on a matrix of eleven morphological variables that quantified body shape and vertebral morphology. PCA was used to reduce the eleven variables into a few variables that summarize overall variation in shape among all individuals used in the study. Variables with loadings 0.60 or greater were assigned to components and components were retained if they had eigenvalues greater than one. Shape components (PC1, PC2, etc.) were then interpreted if they described variation among species (interspecific variation), as opposed to variation in morphology within species (intraspecific variation).

We also examined the morphological data using comparative methods that take into account the potential for statistical non-independence of traits among taxa due to shared ancestry. For this analysis, we pared down a recently published, time-calibrated phylogeny (Rabosky et al., 2013) to focus on the species included in our data set; this allowed us to retain ten species for a phylogenetic analysis of morphological traits quantifying the caudal anatomy of these taxa (Figure 2). Using the ‘phytools’ package (Revell, 2012) in R, we performed a phylogenetic principal components analysis (PCA) with the same 11 morphological traits as our previous “non-phylogenetic” PCA. We also replicated the regressions between morphological characteristics within a phylogenetic framework using

phylogenetic generalized least squares (PGLS; implemented using the ‘nlme’ package in R).

PGLS is an OLS regression weighted by phylogeny, and we assume the underlying correlation in the residuals follows a Brownian-motion structure.

Results:

Body Shape

The thirteen species considered here display variation in body fineness, from “fat-bodied” fishes (characterized by smaller body fineness ratios), such as *Cyprinodon*, to shallow-bodied fishes with a more uniform height along the body (characterized by larger fineness ratios), such as *Kryptolebias*. In most instances, an increase in body fineness is accompanied by a decrease in the proportional depth of the caudal peduncle ($r=0.548$, $p<0.001$); that is, fishes with “shallow” bodies also have “shallow” peduncles, even after size correction (Fig 5). Fish species known to emerge onto land demonstrated a variety of body shapes as measured by body fineness and peduncle size (Fig. 1 and Fig. 5).

Skeletal Anatomy and Peduncle Shape

Because the spine angle and relative spine length for vertebra number 8 and 9 were not different for the neural and hemal spines (t -test $p=0.91$ and $p=0.44$, respectively), only hemal spine data are illustrated here. Analysis of the vertebral elements of the posterior

body suggests that a change in spine length (but not angle) is associated with a change in external peduncle morphology. When spine angle is regressed on peduncle size, there is no significant relationship ($r=0.21$, $p=0.11$, Figure 6A); however, there is a significant relationship ($r=0.84$, $p<0.001$) between relative length of the vertebral spines and peduncle size (Figure 6B). In addition, when considered within a phylogenetic framework (phylogenetic generalized least squares), spine angle did not demonstrate a significant relationship with peduncle size, while spine length did ($p=0.147$ and $p>0.001$, supplemental figures 1 and 2). Thus, the relationship between spine length and caudal peduncle shape is supported by both phylogenetically-corrected and non-corrected analyses, while the relationship between spine angle and peduncle size is supported by neither. In fact, no matter how deep the caudal peduncle is, all species considered here are characterized by neural and hemal spine angles in the peduncle of $\sim 40^\circ$.

Multivariate Analysis of Body Shape

Principal Components Analysis (PCA) was used to produce a multivariate description of overall body shape for all individuals of all species using the eleven morphological variables that quantified overall body shape and skeletal anatomy of the caudal region. Of these eleven variables, seven loaded on the first two principal components (PC1 and PC2, which together quantified 50% of the variance, see Table 2) and these two components outlined a “morphospace” that described variation in anatomy among taxa (Fig. 7). The

third and fourth principal components (PC3 and PC4) had Eigenvalues greater than one, but were not interpreted because they quantified variance within species, as opposed to variation among species. We had anticipated that species with a documented inclination to leave the water and emerge onto land would cluster together in PCA morphospace as a result of selective pressures on these taxa to improve locomotor performance on land. However, the species cluster into morphospaces that do not appear to be related to documented terrestrial inclination or ability (Fig. 7).

1. Specimens of *Cyprinodon* and *Jordanella* (Cyprinodontidae) occupy the upper right quadrant (positive values for PC1 and PC2). These species are deep bodied (small number for body fineness), have a large change in body depth from anterior to posterior (peduncle is shallow by comparison with the deep anterior body), and a large change in spine length along the anterior-to-posterior axis of the body. In cyprinodontid species (pupfishes), the characteristic “fat-bodied” shape appears to be generated primarily by changes in vertebral *spine length* along the body (high scores for PC2).
2. Specimens of *Gambusia* and *Poecilia* (Poeciliidae) cluster together in the lower right quadrant (positive for PC1 and negative for PC2). These species have a deeper caudal peduncle with proportionally longer vertebral spines, a small change in spine angle from anterior to posterior, and a less dramatic change in body shape along the anterior-to-posterior axis. These data suggest that the fairly uniform body-shape

and proportionally larger peduncle of the poeciliids is generated by longer vertebral spines in the posterior-most region of the axial skeleton (high scores on PC1).

3. Nine of the thirteen species (five different teleost families) fall in or near the lower left quadrant (negative for both PC1 and PC2): the two outgroup species (*Oryzias* and *Pseudomugil*), the Fundulidae (*Lucania* and three species of *Fundulus*), Rivulidae (*Kryptolebias* and *Austrolebias*) and the one species representing Nothobranchiidae (*Fundulopanchax*). Although most of these species are not closely related, they all display relatively little change in body shape from anterior to posterior and shallow peduncles. The shallow peduncle exhibited by many of these species (low scores on PC 1) appears to be generated by moderate changes in vertebral spine length along the anterior-to-posterior axis, as opposed to the extreme changes of cyprinodontids.

Phylogenetically-corrected analyses also supported the hypothesis that shared ancestry accounts for most of the variation in body shape and vertebral anatomy observed among taxa. Correspondingly, because amphibious species are present in all occupied quadrants of the morphospace (supplemental figure 3), the phylogenetic analysis suggests there is no morphological convergence among species with more amphibious habits.

In summary, fish known to make voluntary terrestrial excursions are never seen to cluster in body/vertebral anatomy morphospace. Rather, fish with documented amphibious habits can be found in three of four quadrants of the morphospace created in the multivariate analysis of shape and caudal anatomy. Although there *is* variation in

skeletal anatomy that is associated with variation in body shape, the handful of species with distinctive body shapes are very closely related to one another.

Bones of the Tail Fin

To determine if amphibious and non-amphibious species differ in the anatomy of the bones that comprise the base of the tail-fin, we quantified the number of bony elements in the tail that interdigitate with the caudal fin rays and the total area of ossification for these bony elements. Generally, species with more elements at the base of the tail fin have a greater ossified area ($r=0.476, p=0.002$). In contrast with our *a priori* expectations, there is no relationship between the number of elements forming the base of the tail fin and documented amphibious behavior. There are species known to voluntarily emerge onto land only occasionally that possess many bony elements in the tail (e.g., *Fundulus heteroclitus*, Fig. 8A) and species that spend long periods of time on land that possess relatively few bony elements (*Kryptolebias*, Fig. 8A). When corrected for phylogeny, there was no significant relationship between the number of elements and the proportion of ossified area ($p=0.14$ supplemental figure 4), suggesting that the relationship between number of elements and total ossified area is driven by shared ancestry rather than selection to enhance locomotor performance in a new environment. Surprisingly, amphibious fishes generally had a proportionally *smaller* ossified area within the tail, relative to species presumed to be fully aquatic. In fact, overall size of the caudal peduncle

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appears to be the best predictor of ossification, where deeper caudal peduncles have proportionally more ossification in the bones at the base of the tail, even after size is taken into account ($r=0.91$, $p<0.001$, Figure 8B). When data on peduncle size were corrected for phylogeny, there was still a significant relationship between the peduncle ratio and ossified area ($p<0.001$ supplemental figure 5).

Discussion:

Because certain body shape parameters have been hypothesized to enhance locomotor performance for fishes that move on land, our *a priori* prediction was that cyprinodontiform species that exhibit terrestrial locomotor behaviors would possess long, shallow bodies with proportionally deeper, more robust caudal peduncles. However, we found that cyprinodontiform species reported to move on land possess a variety of body shapes. In fact, even when multiple variables used to quantify body shape and vertebral anatomy are considered within a phylogenetic context, it is evident that species with deep bodies and shallow peduncles (e.g., *Jordanella*), species with shallow bodies and deep peduncles (e.g., *Gambusia*), and species with shallow bodies and shallow peduncles (e.g., *Kryptolebias*) are all capable of moving effectively on land (Barber, 2002; Baylis, 1982; Gibb et al. 2011; Brunt et al., 2016). In addition, species that will not jump when manually stranded on land in the laboratory, such as *Cyprinodon variegatus* (pers obs), are not morphologically distinct from a close relative (*Jordanella*) known to voluntarily emerge onto land and capable of producing a tail-flip jump (Barber, 2002; Minicozzi, 2017).

In the atherinomorph species considered here, we observed a previously undocumented morphological pattern: variation in caudal peduncle depth is associated with variation vertebral spine *length* and not with variation in spine *angle*. Despite significant variation in peduncle depth, spine angle is consistently ~40° across taxa. Perhaps atherinomorph taxa all have vertebral spine angles of ~40° due to shared ancestry — although it is also possible that there is a developmental limitation or functional constraint as to how peduncles are constructed. Indeed, relatively little diversity has been observed in the peduncle region in a wide variety of teleost taxa (Flammang, 2014). However, in a remarkable example of convergence, both scombrids (Scombridae; Collette, 1978, Westneat et al., 1993) and the Colorado River bonytail, *Gila elegans* (Cyprinidae; Moran et al., 2016), exhibit highly modified vertebral spine angles of <13°. Thus, high-performance swimmers have evolved extreme peduncle morphologies, but the peduncles of atherinomorph fishes that move on land are not distinguishable from those of their aquatic relatives.

Ashley Ross and collaborators (2014) recently suggested that a teleost species with longer vertebral spines in the caudal region will possess spines that span a greater number of vertebral joints, relative to a species with shorter vertebral spines. These researchers also posited that spines that span more vertebral joints will store greater amounts of elastic energy when they are deformed during body bending, relative to species with spines that span fewer joints (Ashley-Ross et al, 2014). Thus, variation in spine morphology and,

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by extension from the model, variation in the elastic energy stored in deformed spines, could potentially explain the inability of some teleost species inability to produce effective jumps when on land, relative to the proficient tail-flip jumping behavior exhibited by other species. However, a preliminary study comparing elastic energy storage/recovery in amphibious *Kryptolebias* vs. fully-aquatic *Micropterus salmoides* (Perciformes: Centrarchidae) failed to support this hypothesis (Ashley-Ross et al, 2014). Our data for spine angle/length in atherinomorph fishes also fails to demonstrate any consistent association between vertebral spine morphology and terrestrial inclination.

Based on variation in hypural plate anatomy described by Costa (2012), we originally hypothesized that species with life histories characterized by predictable bouts of emergence onto land would consistently possess large, highly-ossified hypural plates that are composed of few bony elements. However, we found that increased ossified area of the tail is positively associated with a larger peduncle depth, where deeper peduncles tend to have more bones forming the base of the tail fin and a greater total ossified area. It is possible that fishes with larger caudal peduncles require more bones in the tail and greater amount of ossification to “fill” the area created where a deep caudal peduncle abuts the fin rays. In contrast, the species for which voluntary terrestrial behaviors are documented tend to have a *smaller* ossified area in the tail fin, relative to fully aquatic species. Perhaps there is an advantage to applying the force transmitted from the body to the tail across a

small area to produce “focused” ground reaction forces at a particular position on the substrate during the launch phase of the tail-flip jump.

Although it is clear from this study that cyprinodontiform species with diverse body shapes can emerge onto land and move about effectively, it is also possible that subtle, as-yet-undocumented, differences in body shape facilitate improved performance in more amphibious taxa. Undoubtedly the selective pressures on *Kryptolebias*, a species that can spend two months on land at a time (Davis et al., 1990), are quite different from those on *Gambusia*, a species that only leaves the water only occasionally (Baylis, 1982). Do subtle morphological differences — variation in parameters not measured here — exist among cyprinodontiform taxa with greater (or lesser) terrestrial locomotor abilities?

One possibility is that “terrestrial specialist” species have a redistribution of the mass of muscle (and associated connective tissues) along the body. In most teleosts, the bulk of the fast-glycolytic (“white”) muscle is located in the anterior region of the body, and it is this muscle that powers the tail-flip jump (Perlman and Ashley-Ross, 2016). A redistribution of fast-glycolytic muscle mass would allow a jumping fish to more easily accelerate the head up, away from the substrate (because a lower-mass anterior body has less inertia), and provide additional muscle cross-sectional area to the posterior region, where it can be used to produce ground reaction forces during the critical launch phase of the jump. Future work examining the distribution of mass along the body, in concert with laboratory jumping performance studies, may be key to teasing apart how fish anatomy

changes in response to the selective pressures associated with making periodic excursions on land.

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Video Legend:

Video 1. Example of a tail-flip jump to move on land

Figure Legends:

Figure 1. The tail-flip jump is a ballistic movement used by small-bodied teleosts to move on land (Gibb et al, 2011). An individual fish (*Jordanella floridae*) begins a tail-flip jump by lying with its lateral surface in contact with the ground (0 ms). During the first state of the behavior, the head is lifted up, off the ground (14.4 ms), the body bends away from the ground, and the head+anterior body are accelerated toward the caudal peduncle+tail fin. At the end of this stage, the fish is bent into a C-shape, with the head and anterior body positioned directly above the tail fin (41.6 ms). In the second stage of the behavior, the fish straightens the axial body and pushes off the substrate with the tail fin (62.4 ms); this movement produces the ground reaction forces that launch the fish into ballistic flight (88.0 ms). During the third stage, or flight phase, the fish moves through the air to subsequently land multiple body lengths away from its initial position (113.6 ms). Images depicted here were recorded using a high-speed digital imaging system (see Minicozzi, 2017), imported into Adobe Photoshop, cropped and adjusted to enhance brightness and contrast and then labeled to indicate elapsed time (in milliseconds) for the behavior.

Figure 2. Simplified phylogeny based on recent molecular work (Rabosky et al., 2013; Pohl et al., 2015) depicting hypothesized relationships among select cyprinodontiform and outgroup genera considered in this study. Genera containing species known to exhibit

voluntary amphibious behaviors are illustrated in green; this designation is based on field (Baber et al., 202; Baylis, 1982; Bressman et al., 2016; Halpin and Martin, 1999) observations or the voluntary behaviors of captive fish (Turko and Wright, 2015). See text for additional details on the natural history of the species used in this study.

Figure 3. Vertebral spine length, angle of the spine relative to the midline of the vertebral column, number of elements that interdigitate with the caudal (tail) fin rays, and the total area of ossification of the bones at the base of the caudal fin were measured from cleared and stained (see Song and Parenti, 1995) specimens using the program NIH Image-J. Abbreviations are as follows: "A" - area of the hypural plate, a large ossified elements that interdigitates with the caudal fin rays; elements above and below the hypural plate were also measured to obtain total ossified area. "L" - length of the hemal spine projecting away from the vertebra. "Theta" - the angle formed by the projection of the hemal spine away from the midline of the vertebral column. Vertebra are numbered from 1 to 12, with 12 being the vertebral element closest to the hypural plate of the tail fin. See text for details.

Figure 4. Cyprinodontiform species vary in the number of bony elements at the base of the caudal (tail) fin and in the length of the neural and hemal spines of the vertebrae; however, the angle of the neural and hemal spines is consistently ~40° in all taxa. Digital photographs were taken of cleared and stained (red for bone, blue for cartilage, see Song

and Parenti, 1995) specimens and images uploaded to Adobe Photoshop. In Photoshop, images were cropped and brightness and contrast manipulated to enhance image quality. Nine study species are depicted here: A) *Pseudomugil* (outgroup), B) *Gambusia*, C) *Poecilia*, D) *Fundulus* [*F. bermudae*], E) *Lucania*, F) *Jordanella*, G) *Kryptolebias*, H) *Austrolebias*, and I) *Fundulopanchax*.

Figure 5. There is a negative association between body fineness (length:width) and relative peduncle size in cyprinodontiform species and their relatives ($r=0.548, p<0.001$), such that species with more elongate bodies tend to have proportionally shallower caudal peduncles, and vice versa. Species known to exhibit voluntary amphibious behaviors are illustrated in green; this designation is based on field (Baber et al., 202; Baylis, 1982; Bressman et al., 2016; Halpin and Martin, 1999) observations or the voluntary behaviors of captive fish (Turko and Wright, 2015). See text for additional details on the natural history of the species used in this study.

Figure 6. (A) Vertebral spine angle (hemal spine angle illustrated here) does not predict peduncle size (depth) in cyprinodontiform species and their relatives ($r=0.21, p=0.11$). (B) However, there is a positive relationship between peduncle depth and length of the hemal spines ($r=0.84, p<0.001$), even after both variables have been corrected for body size. Species known to exhibit voluntary amphibious behaviors are illustrated in green; this

designation is based on field (Baber et al., 202; Baylis, 1982; Bressman et al., 2016; Halpin and Martin, 1999) observations or the voluntary behaviors of captive fish (Turko and Wright, 2015). See text for additional details on the natural history of the species used in this study.

Figure 7. Principal Components Analysis of eleven morphological variables yields two factors that describe 50% of the variance (Table 2) and separate taxa into different morphospaces. Upper right quadrant: two species from the Cyprinodontidae have a “fat-bodied” morphology, with a deep body anteriorly, a caudal peduncle that is intermediate in size, and a marked decrease in body depth from anterior to posterior along the body. Lower right quadrant: two species from the Poeciliidae have shallow bodies, with proportionally larger (deeper) caudal peduncles and a less dramatic change in body depth from anterior to posterior. Lower left quadrant: most of the species considered in this analysis have a proportionally shallow body and shallow peduncle, with a minimal change in body depth from anterior to posterior. Species known to exhibit voluntary amphibious behaviors are illustrated in green; this designation is based on field (Baber et al., 202; Baylis, 1982; Bressman et al., 2016; Halpin and Martin, 1999) observations or the voluntary behaviors of captive fish (Turko and Wright, 2015). See text for additional details on the natural history of the species used in this study.

Figure 8. (A) There is a significant relationship between the area of ossification at the base of the caudal fin and number of elements that interdigitate with the fin rays ($R^2=0.223$);

Table 1. Atherinomorph species categorized by family, size range, sample size, and known terrestrial ability.

| Species | Family | SL (mm) | n | Terrestrial | Citation | Source of Specimens |
|--------------------------------------|-------------------|------------|----|-------------|------------------|------------------------|
| <i>Oryzias celebensis</i> | Adrianichthyidae* | 32 - 37 | 5 | Unknown | - | NMNH |
| <i>Pseudomugil signifer</i> | Pseudomugilidae* | 25 - 27 | 4 | Unknown | - | WC |
| | | 21 - | | | Baylis, 1982 | WC |
| <i>Gambusia affinis</i> | Poeciliidae | 33 | 12 | Field | | |
| <i>Poecilia mexicana</i> | Poeciliidae | 33 - 37 | 9 | Unknown | - | WC |
| | | 113 - | | | Mast, 1915 | CU |
| <i>Fundulus majalis</i> | Fundulidae | 119 | 2 | Field | | |
| <i>Fundulus bermudae</i> | Fundulidae | 37 - 54 | 4 | Unknown | - | NMNH |
| <i>Fundulus heteroclitus</i> | Fundulidae | 53 - 62 | 5 | Field | Halpin, 1999 | WFU |
| | | 33 - | | | Bayliss, 1982 | CU |
| <i>Lucania parva</i> | Fundulidae | 37 | 3 | Field | | |
| <i>Cyprinodon variegatus</i> | Cyprinodontidae | 44 - 53 | 5 | Unknown | - | NMNH |
| <i>Jordanella floridae</i> | Cyprinodontidae | 35 - 37 | 5 | Field | Barber, 2002 | WC |
| <i>Kryptolebias marmoratus</i> | Rivulidae | 24 - 28 | 6 | Field | Davis, 1990 | UA |
| <i>Austrolebias luteoflammulatus</i> | Rivulidae | 30 - 39 | 5 | Field | | NMNH |
| <i>Fundulopanchax gardneri</i> | Nothobranchiidae | 36 - 53 | 5 | Unknown | - | |
| | | | | Laboratory | Turko, 2015 | CU |

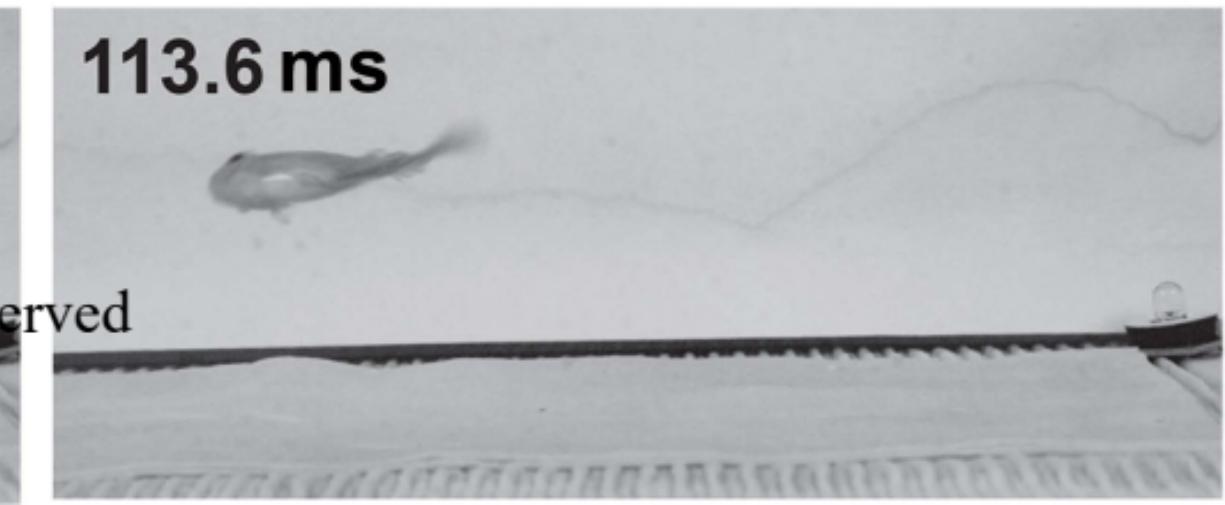
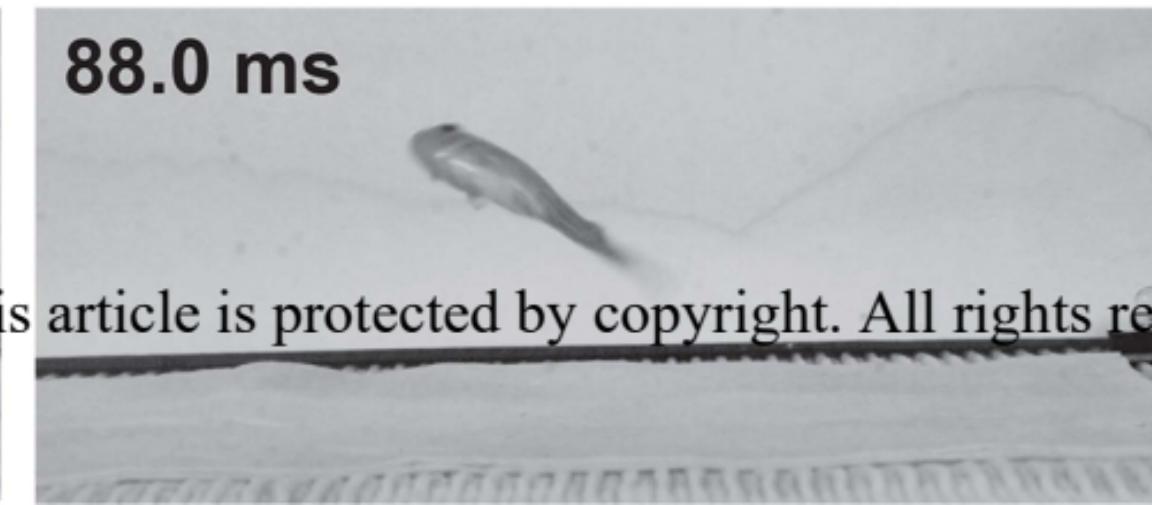
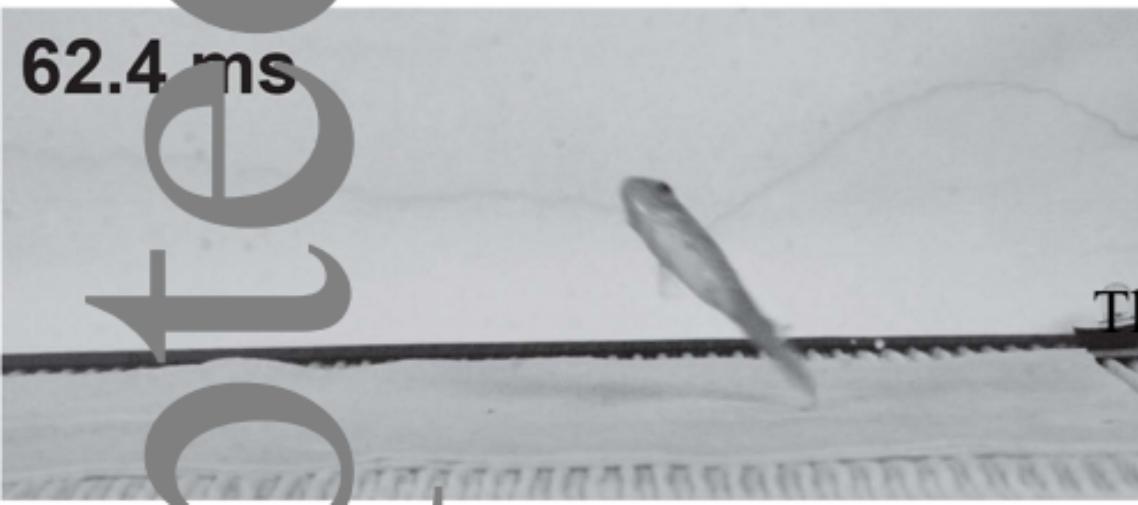
* Denotes non-cyprinodontiform, outgroup species.

Field observations are from studies that observed voluntary emersion in the wild and Laboratory observations are voluntary emersion of captive held fish.

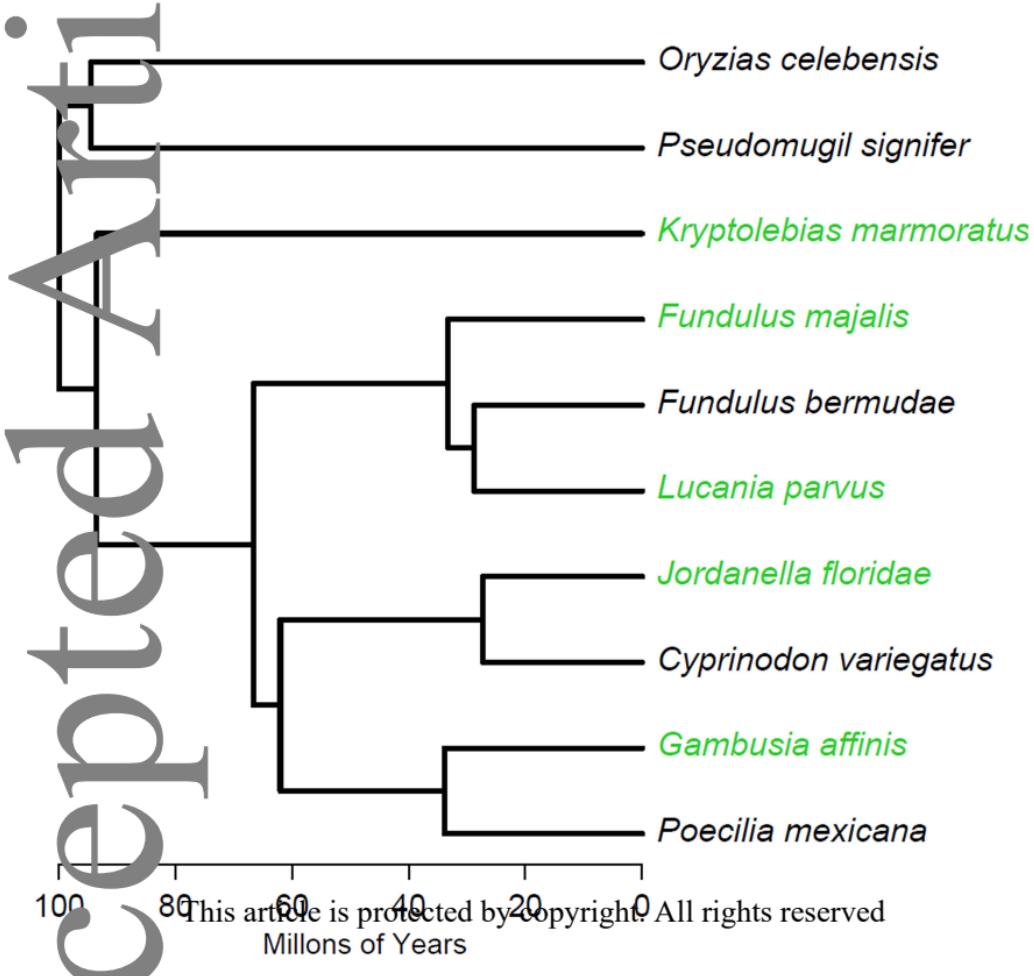
NMNH indicates specimens obtained from the National Museum of Natural History (Smithsonian), WC indicates specimens that were wild caught and held at Northern Arizona University. CU indicates specimens obtained from the Cornell University Museum of Vertebrates. WFU indicates specimens obtained from Wake Forest University, and UA indicates specimens obtained from University of Alabama.

Table 2. PC loadings for eleven morphological variables considered in this study.

| Variable | PC1 | PC2 | PC3 | PC4 |
|---------------------------|--------|--------|--------|-------|
| % Variance | 29.60% | 21.10% | 18.90% | 9.70% |
| Hemal Spine Length Ratio | 0.894 | | | |
| Neural Spine Length Slope | 0.873 | | | |
| Peduncle Ratio | 0.83 | | | |
| Neural Spine Angle Slope | 0.65 | | | |
| Change in Body Depth | | 0.923 | | |
| Fineness Ratio | | -0.892 | | |
| Hemal Spine Length Slope | | -0.663 | | |
| Hemal Spine Angle Slope | | | 0.903 | |
| Hemal Spine Angle | | | 0.8 | |
| Neural Spine Length Ratio | | | | 0.811 |
| Neural Spine Angle | | | | 0.649 |

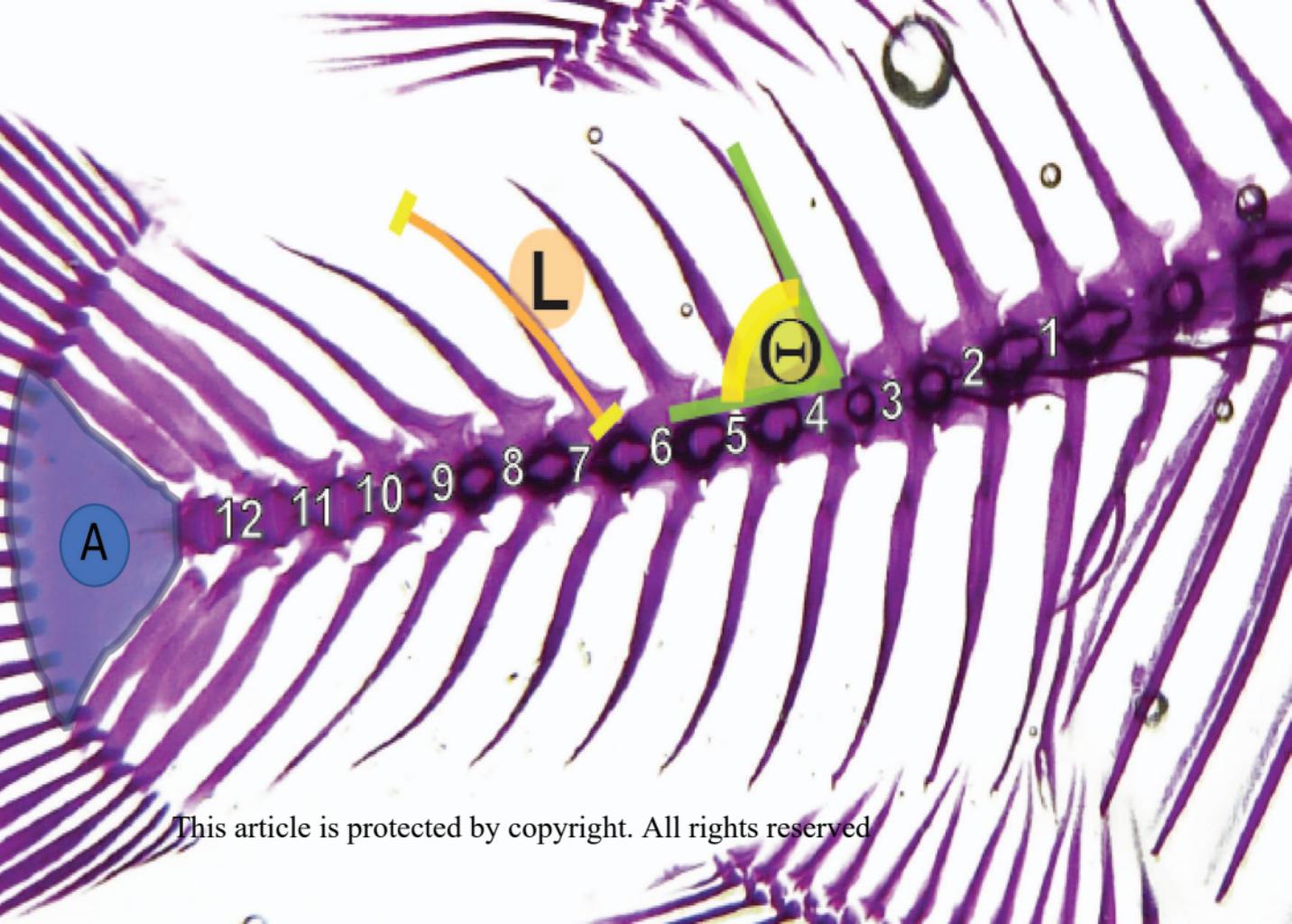


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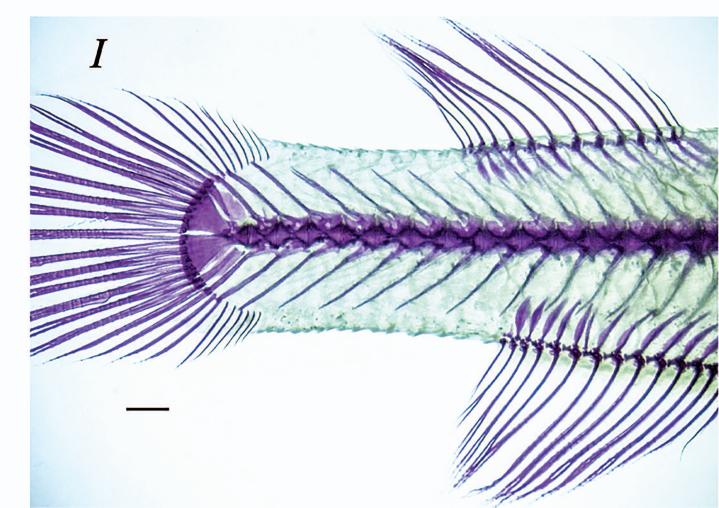
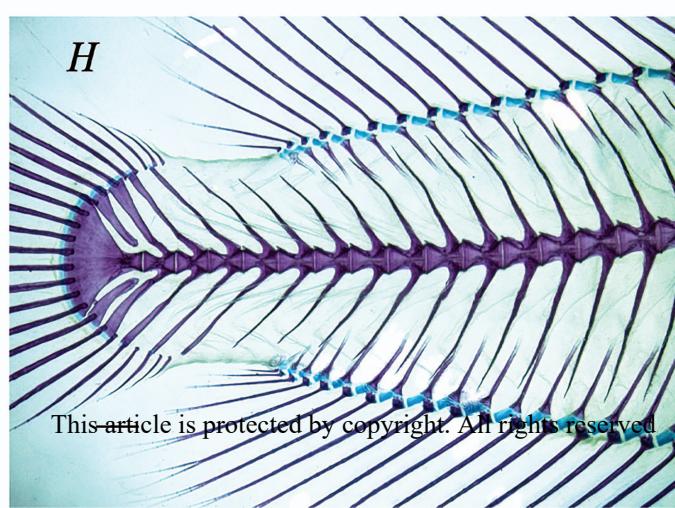
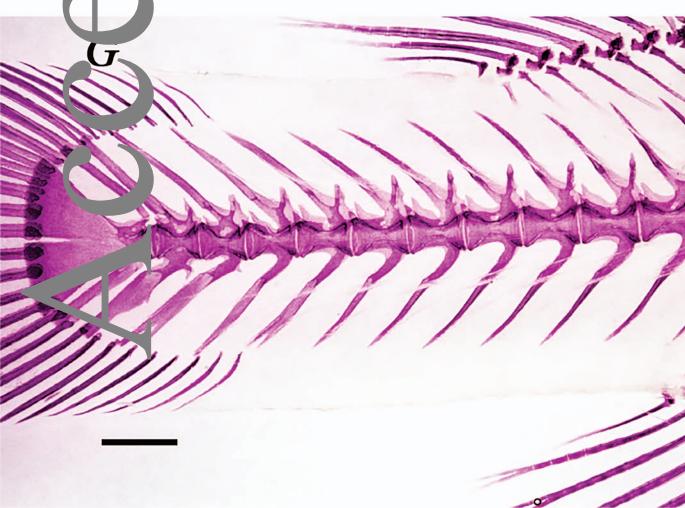
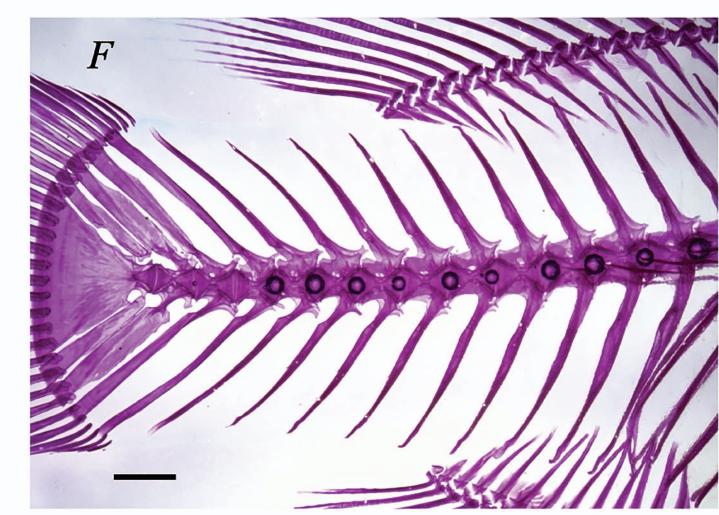
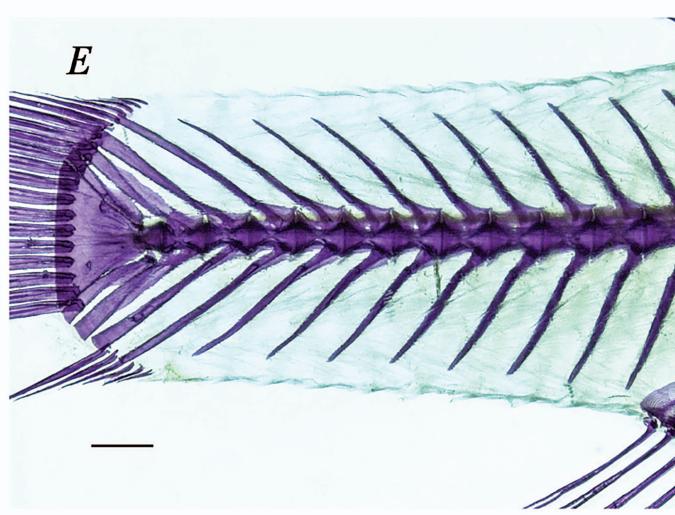
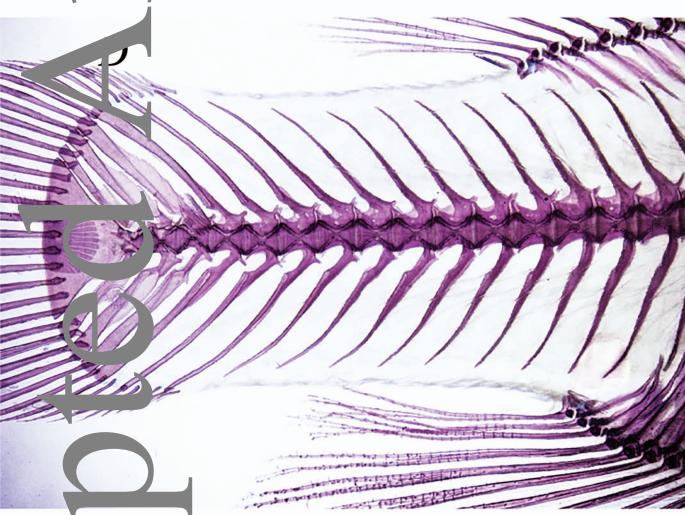
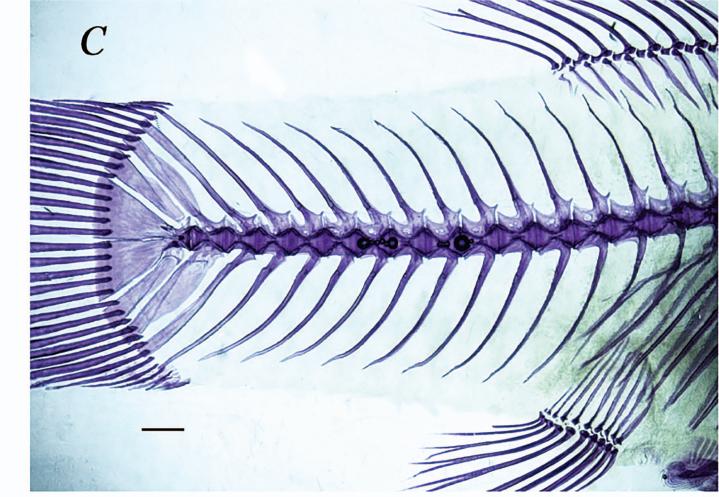
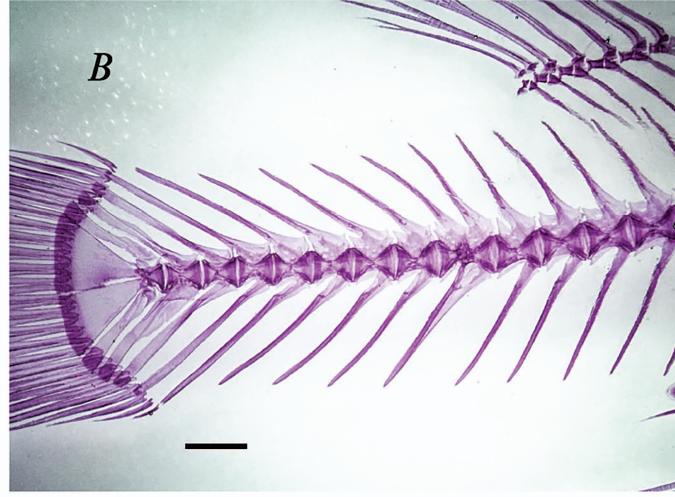
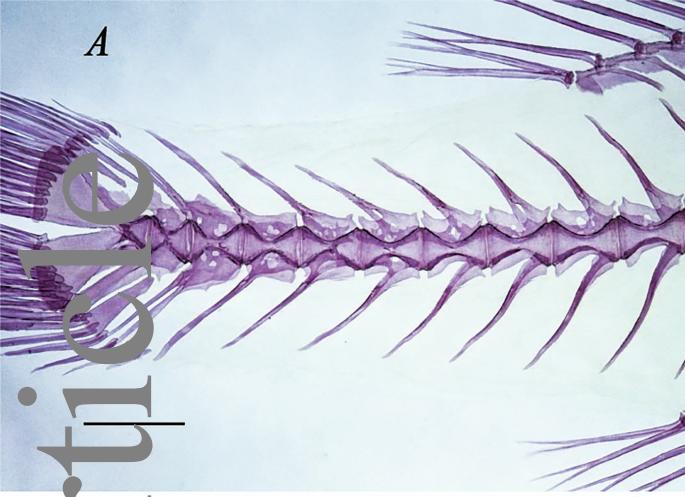
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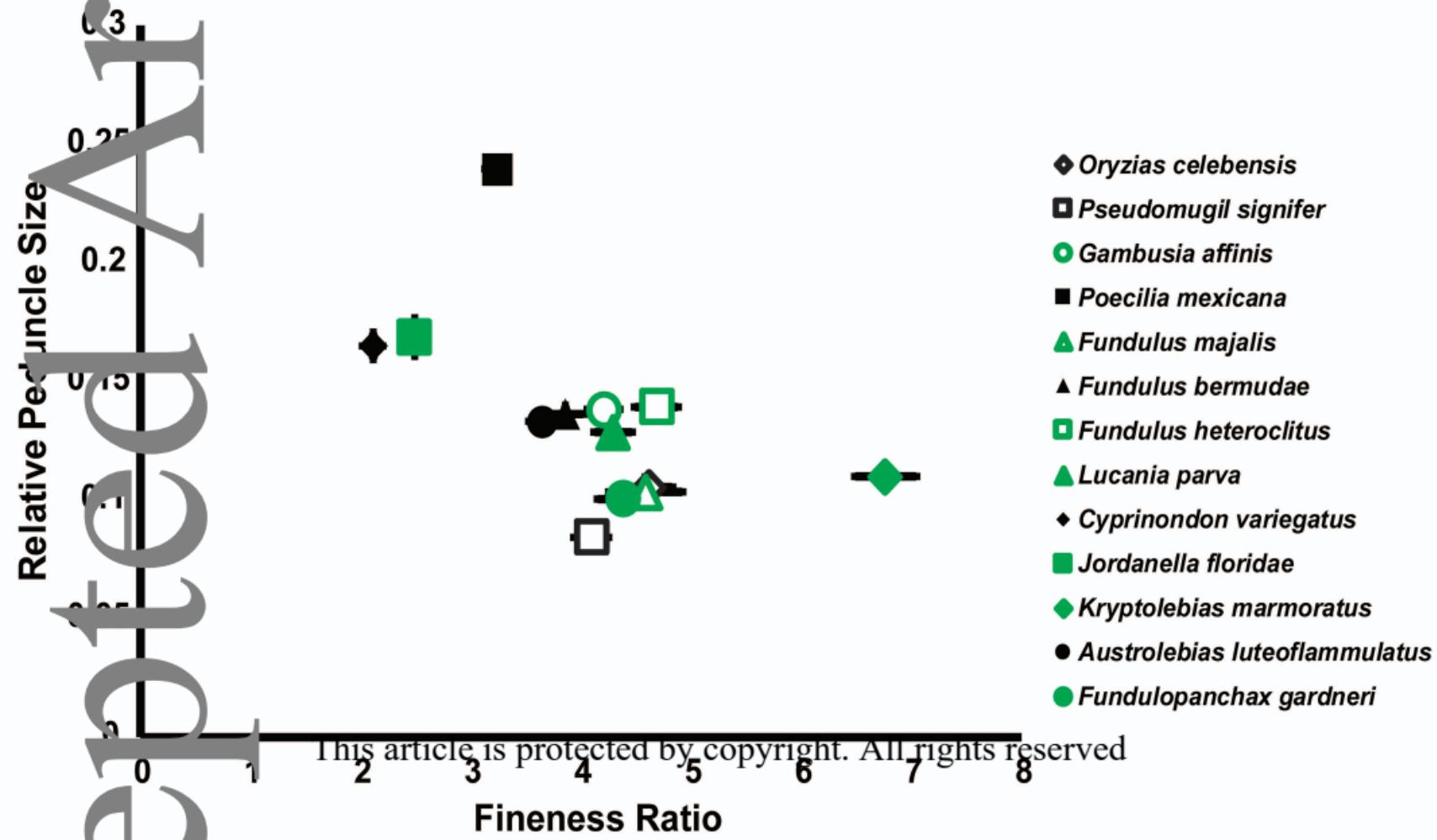
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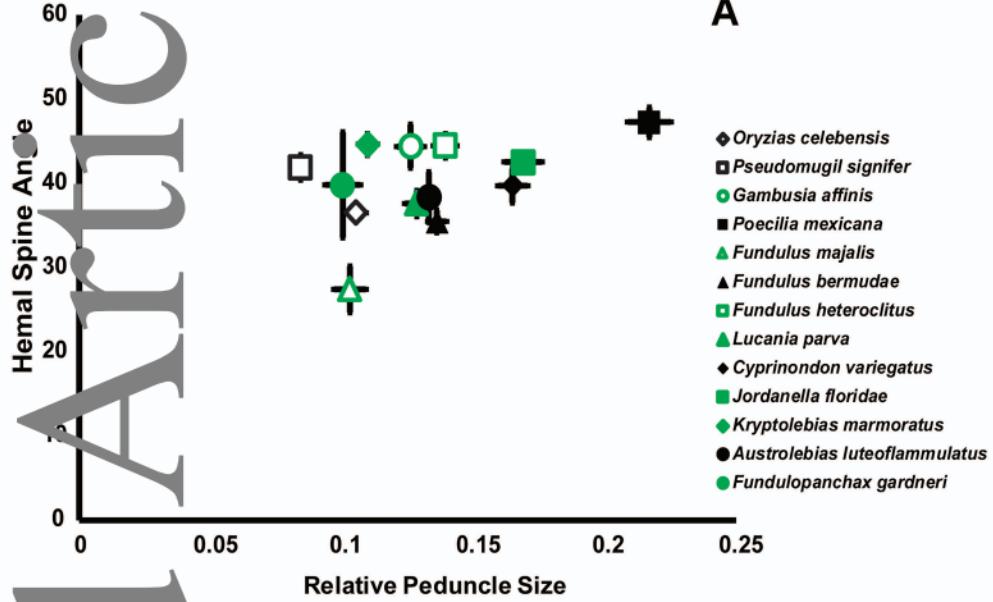
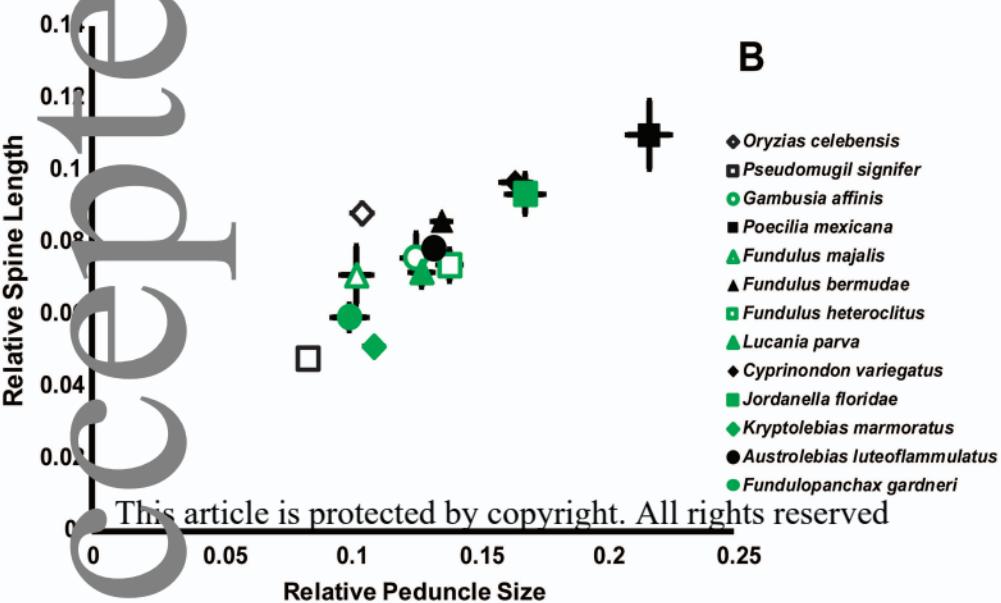


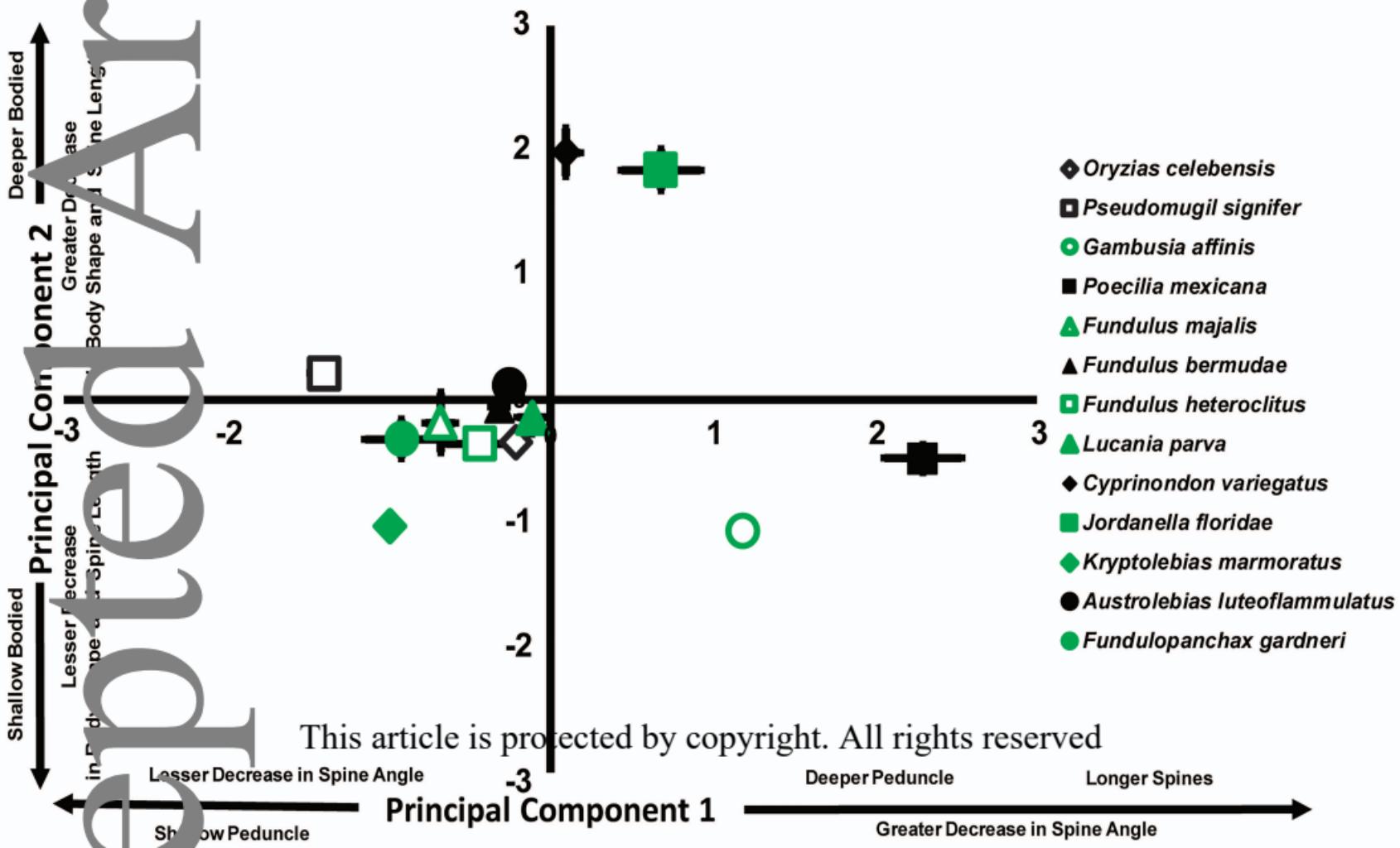
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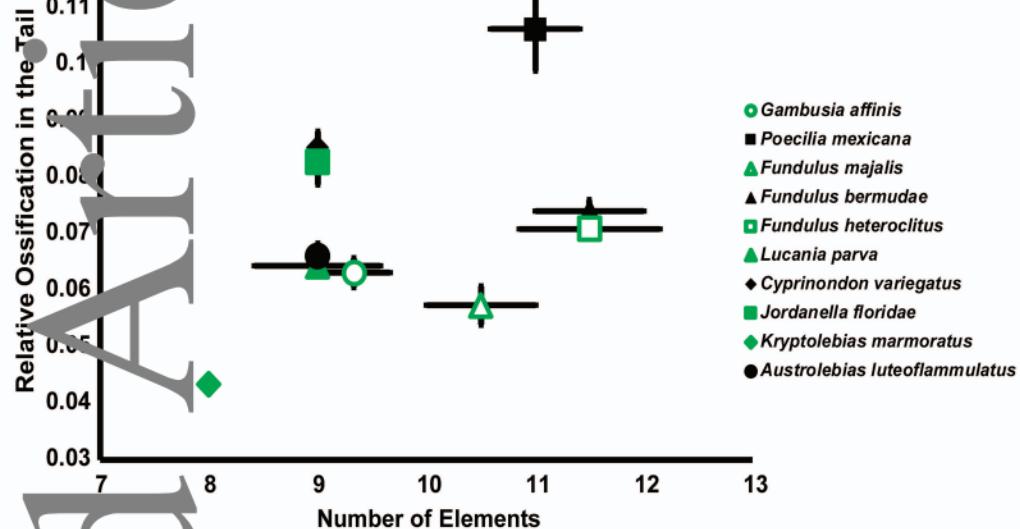


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