

Spawning substrate shift associated with the evolution of a female sexual characteristic in a family of fishes

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Because ‘primary’ sexual characteristics (i.e. those directly associated with reproduction) can be extremely variable, evolve quickly, and can be impacted by both natural and sexual selection, they are often considered excellent model systems in which to study evolution. Here, we explore the evolution of the anal sheath, a trait hypothesized to facilitate the release and proper placement of eggs on the spawning substrate, and its relationship to spawning habitat and maximum body size in a family of fish (Fundulidae). In addition to using phylogenetically informed statistics to determine the role of preferred spawning habitat and maximum body size on the evolution of anal sheath length, we reconstruct the evolutionary history of the anal sheath and preferred spawning habitat. We then test for significant phylogenetic signal and evolutionary rate shifts in the size of the anal sheath and the preferred spawning habitat. Our results indicate that preferred spawning habitat, and not maximum body length, significantly influences anal sheath size, which is associated with a significant phylogenetic signal, and an evolutionary rate similar to that of preferred spawning substrate. We discuss these results in terms of potential evolutionary mechanisms driving anal sheath length.

ADDITIONAL KEYWORDS: evolutionary rate – *Fundulus* – phylogenetic signal – sexual characteristic – urogenital pouch.

INTRODUCTION

Among species there exists an abundance of morphological variation at essentially every level of biological organization from individual tissues (Donoghue *et al.*, 2006) to whole bodies (Harmon *et al.*, 2010). This is perhaps more evident in ‘primary’ sexual characteristics (i.e. those directly associated with reproduction, such as genitalia and gametes) than in any other feature associated with an organism’s morphology. For instance, in ducks, penises can take on a multitude of spiral forms, as a result of extreme sexual antagonism (bends in the vagina that prevent forced copulations from males) imposed by female genitalia (Brennan *et al.*, 2010). Because primary sexual characteristics can be extremely variable (Arnqvist, 1998), evolve relatively fast (Eberhard, 2010), and can be driven by both natural and sexual selection (Leonard & Córdoba-Aguilar, 2010), they are

often considered excellent model systems in which to study evolution. Numerous studies have examined morphological variation associated with male primary sexual characteristics (Waage, 1979; Hosken & Ward, 2001; Baryshnikov *et al.*, 2003; Hosken *et al.*, 2019). However, as discussed by Ah-King *et al.* (2014), studies related to assessing variation in the primary sexual characteristics of females have received significantly less attention. Characterizing variation in the primary sexual characteristics of females is important because these characteristics have strong fitness implications, such as determining which sperm ultimately fertilizes the egg (Galicia-Mendoza & Cordero, 2008) and where and how eggs are deposited (Stamp, 1980).

In the evolution of primary sexual traits among species, natural and sexual selection are probably key drivers of variation (Richmond *et al.*, 2016). However, trait evolution can also be impacted by factors other than selection. Phylogenetic effects, where similar trait values are due to shared ancestry (Hansen, 1997; Hansen *et al.*, 2008) (which is often measured in terms of phylogenetic signal), can also influence trait evolution. As a result, it is important to consider this

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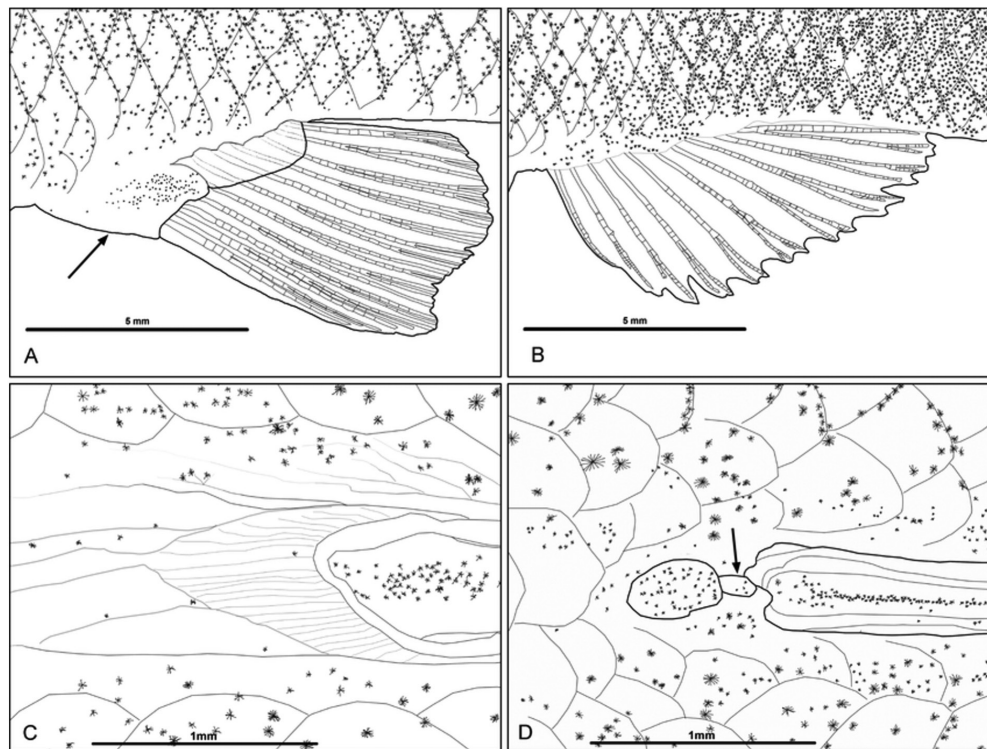
factor when identifying potential selection pressures associated with trait evolution.

Variation in the evolutionary rate across a phylogeny may further complicate our understanding of the mechanisms that have contributed to the evolution of primary sexual traits. For instance, changes in evolutionary rate on a phylogeny can highlight widening or narrowing of available niches (Collar *et al.*, 2010), changes in genetic variance/covariance within a lineage (Pitchers *et al.*, 2014) or strong stabilizing selection (Lemos *et al.*, 2005). Changes in evolutionary rate are often mistakenly inferred from differing levels of phylogenetic signal (non-independence among species due to common ancestry) (Bozinovic *et al.*, 2007; Revell *et al.*, 2008). This interpretation, however, can be problematic. At best, the relationship between phylogenetic signal and evolutionary rate is complex, and at worse, absent altogether (Revell *et al.*, 2008). As such, it is important to explicitly test for changes in rates of evolution and phylogenetic signal when attempting to identify processes involved with the evolution of primary sexual traits.

The family Fundulidae (Jordan and Gilbert, 1882) (killifish, topminnows and studfish found in North and Central America) offers a promising system

in which to determine the mechanisms that underlie the evolution of primary sexual characteristics in females, while taking into account potential compounding factors (e.g. phylogenetic signal) that might also influence the evolution of traits. Within the family, females of many species often possess an anal sheath during the breeding season (sometimes called a ‘urogenital pouch’; Wiley, 1986). This anal sheath is a small flap of tissue that covers part of the first few rays in the anal fin (Fig. 1) (Lopez *et al.*, 2010). While no mechanistic studies have been performed to determine, experimentally, its function, observations have led to it being described as acting to facilitate the release and proper placement of eggs prior to external fertilization by males (Foster, 1967; Thompson, 1999).

Preferred spawning habitat (i.e. the habitat that eggs are most often laid in or on) can affect the size of the structures involved in laying eggs. In the leaf beetle, for instance, ovipositor length is hypothesized to reflect local adaptation to host plants (Sota *et al.*, 2007). Similarly, the relationship between trait and spawning habitat might have been strong enough to contribute to postzygotic isolation between two pine sawfly species (Bendall *et al.*, 2017). For *Fundulus*, all of the species that live in high-salinity niches appear



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Figure 1. Typical anal sheath morphology. A, lateral view of the female anal sheath; B, lateral view of the anal fin of a male, which lacks the anal sheath; C, ventral view of the anal sheath (note how the genital papilla is covered by the anal sheath); and D, ventral view of male, which lacks the anal sheath. Figure reproduced with permission from Lopez *et al.* (2010).

to have moderate to large anal sheaths (Ghedotti & Davis, 2013). Because high-salinity environments tend to have looser substrates relative to lower salinity niches which are often more vegetated, spawning substrate may ultimately be the driving force behind anal sheath size (Ghedotti & Davis, 2013). However, this hypothesis has not been tested empirically using phylogenetic comparative methods.

Variation in the direction and/or strength of natural and/or sexual selection within a species can influence not only the size of a primary sexual trait, but also the relationship between the size of the trait and body size. Thus, understanding variation among species in sexual characteristics in relation to body size (in light of among-species variation in natural history) may allude to important mechanisms underlying the evolution of primary sexual traits. For instance, if a trait is disproportionately large in larger species relative to smaller species or disproportionately large in small species relative to large species, then this might suggest that historical selection favouring larger trait size has been stronger within larger or smaller species, respectively (Frankino *et al.*, 2005). Because body size is often a target of selection directly (Siepielski *et al.*, 2019) and may be strongly correlated with trait size, phenotypic response to selection acting on the size of a trait may be constrained or facilitated by the correlation that that trait shares with body size (Hallgrímsson *et al.*, 2019). Thus, understanding the evolution of primary sexual traits is not complete without understanding how its relationship to body size changes across species' divergence.

The main goal of this study was to determine if body size and preferred spawning substrate have had a significant influence on anal sheath size evolution in Fundulidae using phylogenetically informed statistics. In addition to testing this hypothesis, we assess phylogenetic signal and evolutionary rate shifts in spawning substrate and anal sheath size. We find that anal sheath size is significantly predicted by preferred spawning substrate, but not maximum body length, that there is a significant phylogenetic signal associated with anal sheath size but not spawning habitat, and that there is little evidence of an evolutionary rate shift for either trait.

MATERIAL AND METHODS

DATA SOURCES

For each species, we collected information related to maximum total length (TL; mm), preferred spawning substrate and anal sheath size. Preferred spawning substrate and anal sheath size data were extracted from Ghedotti & Davis (2013), the only complete

published account of the presence and size of the anal sheath in Fundulidae. For preferred spawning substrate, each species was given a score of either '0' if they spawned on branching macrophytes, algae or woody aquatic substrate (hereafter referred to simply as 'vegetation') or '1' if they spawned on loose substrate (i.e. sand or rock). Anal sheath size was also assigned to each species as a discrete variable. Species were assigned a value of '0' if their anal sheath was less than 1/10 the length of their first anal-fin ray or '1' if their anal sheath was longer than 1/10 the length of their first anal-fin ray. Throughout the remainder of the paper, we refer to species that were assigned a value of '1' as species with 'large' anal sheaths, and those that were assigned a value of '0' as species with 'small' anal sheaths. Maximum TL was obtained from a variety of sources that are listed in Table 1. Although we also collected data on average total body length for each species, we present data (and resulting models) related to maximum TL only because it was highly positively correlated with average total body length ($R^2 = 0.55$, $F_{(1,25)} = 32.5$, $P < 0.0001$).

PHYLOGENY

Using the RNA-sequencing data and evolutionary partitioning strategy established by Rodgers *et al.* (2018), we re-created the most recent and inclusive phylogeny for Fundulidae species. In our phylogenetic comparative analyses, we used a pruned version of this phylogeny, which included 26 species. Species were excluded from the phylogeny, and subsequent analysis, if they lacked data on preferred spawning habitat, maximum body size and/or anal sheath size, or if species exhibited polymorphic trait states. For instance, *Fundulus xenicus* was excluded from our analysis because it has been documented as spawning on both aquatic vegetation and loose substrate (Ghedotti & Davis, 2013). The pruned phylogeny was ultimately used to test whether anal sheath size was significantly influenced by maximum body size and preferred spawning habitat, if there was phylogenetic signal associated with preferred spawning habitat or anal sheath size, and if there is evidence of an evolutionary rate shift for either trait.

PHYLOGENETIC COMPARATIVE MODELS

To determine the evolutionary history of anal sheath length and preferred spawning habitat, respectively, we used the rerooting method of ancestral state reconstruction proposed by Yang *et al.* (1995) in phytools v.0.6–99 (Revell *et al.*, 2012) within R studio v.1.2.1335 (R Core Team, 2019). This approach used a maximum-likelihood method and data at the tips of the phylogeny to reconstruct ancestral states at the node.

Table 1. Anal sheath, spawning substrate and maximum body size (TL; mm) data for Fundulid species

Species	Anal sheath size	Spawning substrate	Maximum TL (mm)	Source for TL
<i>Fundulus catenatus</i>	0	1	200	Huber (1996)
<i>F. chrysotus</i>	0	0	85	Huber (1996)
<i>F. cingulatus</i>	0	0	78	Page & Burr (1991)
<i>F. confluentus</i>	0	0	80	Huber (1996)
<i>F. diaphanus</i>	0	0	130	Page & Burr (1991)
<i>F. dispar</i>	0	0	60	Huber (1996)
<i>F. grandis</i>	1	0	180	Ghedotti & Davis (2013)
<i>F. heteroclitus</i>	1	0	150	Page & Burr (1991)
<i>F. julisia</i>	0	0	52	Goldsworthy & Bettoli (2006)
<i>F. kansae</i>	1	1	100	Hassan-Williams <i>et al.</i> (2007)
<i>F. lineolatus</i>	0	0	84	Page & Burr (1991)
<i>F. luciae</i>	0	0	50	Huber (1996)
<i>F. majalis</i>	1	1	180	Robins & Ray (1986)
<i>F. notatus</i>	0	0	80	Huber (1996)
<i>F. nottii</i>	0	0	60	Huber (1996)
<i>F. olivaceus</i>	0	0	80	Huber (1996)
<i>F. parvapinnis</i>	1	1	108	Miller & Lea (1972)
<i>F. pulvereus</i>	0	0	65	Page & Burr (1991)
<i>F. rubrifrons</i>	0	0	78	Page & Burr (1991)
<i>F. seminolis</i>	0	1	125	Hugg (1996)
<i>F. sciadicus</i>	0	0	90	Huber (1996)
<i>F. similis</i>	1	1	120	Page & Burr (1991)
<i>F. stellifer</i>	0	0	96	Edberg & Powers (2010)
<i>F. zebrinus</i>	1	1	100	Shute & Allen (1980)
<i>Lucania goodei</i>	0	0	50	Mahardia <i>et al.</i> (2020)
<i>L. parva</i>	0	0	62	Gunter (1950)

Anal sheath and spawning substrate data were obtained from Ghedotti & Davis (2013). With respect to anal sheath length, each species was scored either '0' (short) if their anal sheath was shorter than 1/10th the size of the first anal fin ray or '1' (long) if their anal sheath was longer than 1/10th the size of the first anal-fin ray. For spawning substrate, each species was scored either '0' if they spawn on branching or fibrous aquatic substrates or '1' if they spawn on loose media, such as sand, soil or rocks. Maximum TL (mm) data for unsexed individuals were obtained from a variety of sources, as listed.

We used two transition models (SYM and ARD) when estimating the ancestral states at each node (Hueit *et al.*, 2018). The SYM model assumed that transitions between states (0 to 1 and vice versa) occurred with equal probability across the tree, while the ARD model allowed these rates to vary (Cunningham *et al.*, 1998). The model with the lowest log-likelihood was chosen as the best model.

Following ancestral state reconstructions, we accessed phylogenetic signal associated with our binary data for anal sheath length and preferred spawning substrate (Hypothesis 1 for anal sheath size and spawning habitat in Table 2). Phylogenetic signal is a measure of how much species' relatedness contributes to among-species variation in the trait (i.e. degree of phylogenetic autocorrelation). We used the method developed by Maddison & Slatkin (1991) and code written by Enrico Rezende (Saito *et al.*, 2016) to calculate phylogenetic signal within R studio v.1.2.1335 (R Core Team, 2019). Using parsimony, this method estimates the minimum number of transitions between traits across the phylogeny. It then reshuffles

the character states at the tips and calculates this statistic again. It repeats this process 999 times and then generates a distribution. The null hypothesis of no phylogenetic signal is rejected if the observed minimum number of transitions falls in the critical region of 0.05. We report the observed number of character changes obtained from a parsimony reconstruction (Δ_{obs}) and minimum, median and maximum number of character state changes obtained from random sampling (Δ_{min} , Δ_{med} and Δ_{max} , respectively; Table 2).

To determine if there was an evolutionary rate shift in anal sheath size or preferred spawning substrate (Hypothesis 2 for anal sheath size and spawning habitat in Table 2) in Fundulidae, we reconstructed the evolution of both variables, respectively, using the R package (R Core Team, 2019) corHMM (Beaulieu & O'Meara, 2016). The corHMM function within this package permits a binary trait to evolve under different transition rate matrices, which describe the rate at which a continuous Markov chain moves between two states (0 and 1) across a phylogeny (Anderson & Wiens, 2017). In our procedure, we

Table 2. Results from the test of phylogenetic signal and evolutionary rate shift in anal sheath size and spawning habitat

Trait	Hypothesis	Model	AICc	Parameter(s)	P-value	LRT	Reference
Anal sheath size	1. Phylogenetic signal small or large anal sheath	Phylogenetic signal (parsimony)	–	$\Delta_{\text{obs}} = 4$ $\Delta_{\text{min}} = 3$ $\Delta_{\text{med}} = 6$ $\Delta_{\text{max}} = 7$	$P = 0.018$	–	Maddison & Slatkin (1991); Saito <i>et al.</i> (2016)
	2. Evolutionary rate shift	Single rate model	0.00	$\sigma^2 = 17.5$	–		Beaulieu & O'Meara (2016)
		Multi-rate model	2.05	σ_1^2 (0 to 1) = 17.2 σ_2^2 (1 to 0) = 0	–	$\chi^2 = 0.31$, df = 1, $P = 0.58$	Beaulieu & O'Meara (2016)
Spawning habitat	1. Phylogenetic signal vegetated or loose spawning habitat	Phylogenetic signal (parsimony)	–	$\Delta_{\text{obs}} = 5$ $\Delta_{\text{min}} = 3$ $\Delta_{\text{med}} = 6$ $\Delta_{\text{max}} = 7$	$P = 0.17$	–	Maddison & Slatkin (1991); Saito <i>et al.</i> (2016)
	2. Evolutionary rate shift	Single rate model	0.00	$\sigma^2 = 23.5$	–		Beaulieu & O'Meara (2016)
		Multi-rate model	2.24	σ_1^2 (0 to 1) = 50.3 σ_2^2 (1 to 0) = 26.0	–	$\chi^2 = 0.12$, df = 1, $P = 0.58$	Beaulieu & O'Meara (2016)

Anal sheath size was scored either '0' (short anal sheath; less than 1/10th the length of first anal-fin ray) or '1' (long anal-fin sheath; greater than 1/10th the length of first anal-fin ray) based on Ghedotti & Davis (2013). Data related to spawning habitat were also obtained from Ghedotti & Davis (2013). Species were scored '0' if they spawn primarily on branching vegetation, and '1' if they spawn on loose substrate (i.e. sand). For each model, we report the relative Akaike's information criterion adjusted for low sample size (AICc) or P -value, the parameters estimated by the model, results from a likelihood ratio test (LRT) between a single rate and multi-rate model of evolution, and references for the statistical test. The following is a list of parameters estimated from the phylogenetic comparative models: observed character state changes obtained from parsimony reconstruction (Δ_{obs}), minimum, median and maximum number of character state changes obtained from random sampling (Δ_{min} , Δ_{med} and Δ_{max} , respectively), and evolutionary rate (σ^2). Models that produced significant results ($\alpha < 0.05$) are shown in bold.

competed two models against one another. The first model allowed shifts between trait states to occur at the same rate across the phylogeny. The second model allowed evolutionary shifts between trait states to differ across the phylogeny. We then compared the two models using likelihood ratio tests (LRTs) in the function `lrttest` in R studio v.1.2.1335 (R Core Team, 2019). For the LRT, we estimated χ^2 as twice the difference in log likelihoods between the two models. For this test, the degrees of freedom were set to that of the most complicated model.

We used phylogenetic logistic regression for binary dependent variables as described by Ives & Garland (2010) to determine if maximum TL and/or spawning substrate significantly predicted whether a species possesses an anal sheath (Table 3). The phylogenetic logistic regression produced by Ives & Garland (2010) allows binary response variables to evolve via a Brownian motion process between 0 and 1 across a phylogeny as a function of some independent variable. We compared the results of the phylogenetically informed logistic regression to one in which phylogenetic autocorrelation was not taken into account (i.e. one in which a 'star' phylogeny was used). The best model was taken as the one with the lowest

relative AICc (Akaike's Information Criterion adjusted for small sample sizes) score. From both regressions, we report estimated slopes, standard errors (SE), z -values and P -values. For the phylogenetic logistic regression, we also report an estimation of phylogenetic signal (α), which details how much shared ancestry is influencing among-species variation among traits. Large values of α imply rapid transitions between trait states across the evolutionary divergence of species, and fewer similarities between related species (i.e. lower levels of phylogenetic signal) (Hansen *et al.*, 2008).

It is important to note that the phylogenetic comparative models used in this study cannot rule out the role of sexual selection in driving the evolution of the anal sheath. As such, we use the general term 'selection' instead of 'natural selection' or 'sexual selection' throughout the remainder of the article.

RESULTS

PHYLOGENETIC COMPARATIVE MODELS

Ancestral state reconstructions of anal sheath length using identical forward and reverse rate (SYM) and all rates different (ARD) transition models produced

extremely similar results (log-likelihood = -10.8 vs. -10.68, respectively). As a result, in favour of conciseness, we only display the results from the SYM model (Fig. 2). Under both models, the common ancestors to *Fundulus kansae* and *F. zebrinus*, *F. similis* and *F. majalis*, and *F. grandis* and *F. heteroclitus* were predicted to possess a ‘large’

anal sheath, while ancestors to the remainder of the clades were predicted to have ‘small’ anal sheaths. The ancestral state reconstruction also revealed three transitions from ‘small’ to ‘large’ anal sheaths concentrated near the tips of the phylogeny with the common ancestor to the family probably possessing a small anal sheath (Fig. 2A). Likewise, ancestral

Table 3. Results from the phylogenetic logistic regressions (Ives & Garland, 2010) to determine if anal sheath size was significantly influenced by spawning substrate or total length (TL; mm)

Model		Dependent	Estimate	SE	z-value	P-value	
‘Star’	-	0.00	Intercept	-4.17	1.80	-2.31	0.02
			Substrate	2.46	1.19	2.07	0.04
			Max TL	0.02	0.02	1.38	0.17
Phyloglm	36.9	1.70	Intercept	-4.17	2.00	-2.07	0.04
			Substrate	2.47	1.28	1.92	0.05
			Max TL	0.02	0.02	1.59	0.11

Two models were run to test this hypothesis: one in which the effect of phylogenetic relatedness was not included (this is equivalent to running an analysis on a ‘star’ phylogeny) and one in which phylogeny was included (‘phyloglm’). For each model, we report the relative Akaike’s information criterion adjusted for low sample size (AICc), estimates, standard error (SE), z-value and P-value. An alpha (α) value was estimated in the ‘phyloglm’ model and reflects the amount that phylogenetic signal (i.e. non-independence due to shared ancestry) influences relationships among the traits. The best model was taken as the one with the lowest relative AICc score. Significant effects ($\alpha < 0.05$) are given in bold for both models.

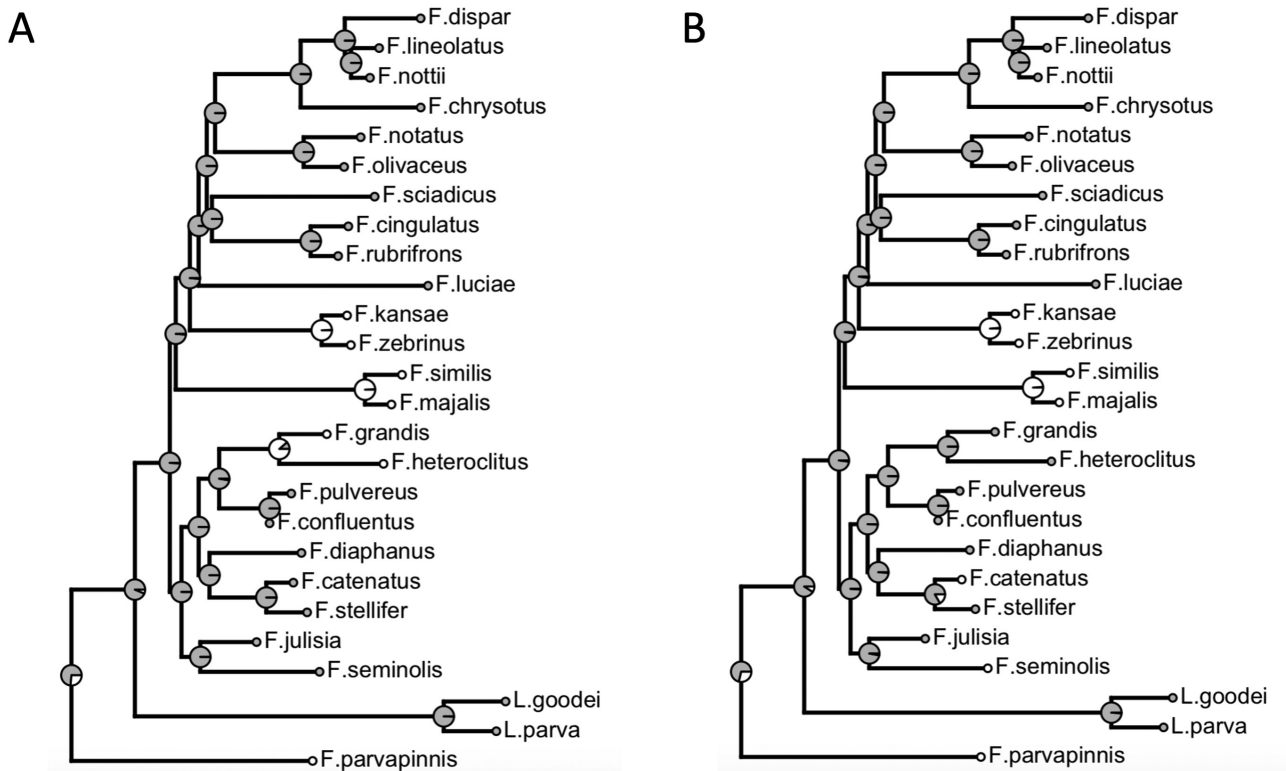


Figure 2. Ancestral state reconstructions for (A) anal sheath length (‘Small’ in grey or ‘Large’ in white), and (B) spawning habitat (‘Vegetation’ in grey or ‘Loose substrate’ in white). For each reconstruction, we used the partitioning strategy of Rodgers *et al.* (2018) and the rerooting method proposed by Yang *et al.* (1995) under two transition models: identical forward and reverse rates (SYM). Both transition models produced identical results. For conciseness, we include only the result from the SYM transition. Each pie chart shows the likelihood of the most recent common ancestor possessing either trait value.

state reconstructions of preferred spawning substrate produced nearly identical results for both the SYM and the ARD transition models (log-likelihood = -13.1 vs. -13.0, respectively). Under both models, the common ancestors to *F. kansae* and *F. zebrinus*, and *F. similis* and *F. majalis* were predicted to spawn on loose substrate while the common ancestors to the remainder of the clades were predicted to spawn on vegetation. The ancestral state reconstruction revealed two transitions from spawning on vegetation to loose substrate in the family with the common ancestor predicted to have spawned on vegetation (Fig. 2B).

We found significant phylogenetic signal associated with anal sheath size, but not preferred spawning habitat (Hypothesis 1 for anal sheath size in Table 2). We did not find evidence of an evolutionary rate shift for anal sheath size or preferred spawning habitat in this group, as the models with a single rate outperformed the model with two rates based on the relative AICc value ('Evolutionary rate shift' models in Table 2). Under the phylogenetic logistic regression, we found that the model using the 'star' phylogeny (the model that did not take phylogenetic relatedness into account) outcompeted the phylogenetic (i.e. 'phyloglm') model according to the relative AICc (Table 3). In this model, substrate had a significant effect on anal sheath size with loose substrate spawners having longer anal sheaths. In the same model, maximum total length did not have a significant effect on anal sheath size.

DISCUSSION

Based on our results, the common ancestor to Fundulidae possessed a small anal sheath (i.e. less than 1/10 the size of the first anal-fin ray) and spawned on vegetation. In this family, there have been three independent transitions between a small to a large anal sheath, and two transitions between spawning on vegetation to loose substrate. Preferred spawning substrate, which did not exhibit an evolutionary rate shift or strong phylogenetic signal, significantly predicted anal sheath size, while maximum total length did not. Spawners on loose substrate exhibited a significantly longer anal sheath than spawners on vegetation.

In many taxa, improper egg placement is a major contributor to egg and larval mortality (Simpson *et al.*, 2016). Eggs will be distributed in areas where threats (biotic or abiotic) are minimized (Claramunt *et al.*, 2005; Welsh & Fuller, 2011; DeSana *et al.*, 2020). Although the function of the anal sheath in Fundulidae remains unknown, it has been hypothesized to be important in the targeted deposition of eggs (Foster, 1967; Thompson, 1999). By providing individuals more control over

where their eggs are deposited, the anal sheath may improve spawning success. Perhaps, a major threat to proper egg deposition in fundulid species that occupy open areas with loose substrate comes from water currents. Water velocity influences egg placement and the survival of egg and young organisms in a variety of aquatic taxa, including insects (Reich & Downes, 2003), frogs (Kupferberg, 2011) and fish (Gauthey *et al.*, 2017). Vegetation creates drag in water currents and acts to slow water movement (Asaeda *et al.*, 2005; Henry *et al.*, 2015). Without the buffering effect of vegetation, stronger currents are likely to occur in more open areas. These currents are likely to disrupt species during egg deposition by pushing the egg away from the spawning site once it is released from the urogenital pore. The anal sheath may act as a shield to allow eggs to be placed within the loose substrate before the currents take hold of them. In this way, selection may have favoured longer anal sheaths in those species that prefer sandier substrates.

Phylogenetic signal reflects shared variation among species due to common ancestry (Molina-Venegas & Rodríguez, 2017). Evidence of this phenomenon has often been used to imply constraints on evolution imposed by common genetic, functional or developmental pathways shared by related species (Fraser *et al.*, 2018). Conclusions revolving around phylogenetic niche conservatism (shared variation among closely related species due to shared habitats) are equally common when significant phylogenetic signal is discovered (Kubota *et al.*, 2017; Barros *et al.*, 2020). In our study, we did not find significant phylogenetic signal associated with spawning habitat, suggesting that more related species did not share similar preferences toward a certain spawning habitat significantly more often than more distantly related species. Contrary to preferred spawning substrate, however, we did find a significant phylogenetic signal associated with anal sheath size (Hypothesis 1 for anal sheath size in Table 2). As such, these results, combined with an overall slower rate of evolution for anal sheath size relative to shared spawning habitat (σ^2 in single rate models for anal sheath size and spawning habitat in Table 2), may suggest greater constraints in the evolution of anal sheath size relative to spawning substrate and not shared variation due to phylogenetic niche conservatism. Indeed, the evolution of anal sheath length, like other morphological traits, is influenced by genetic, functional and developmental factors that may limit phenotypic responses to selection (Martínez-Abadías *et al.*, 2009; Styga *et al.*, 2018). The preference for a specific breeding habitat, on the other hand, may be more contingent on ecological pressures, such as the level of intra-/inter-specific competition, which may change more readily through time (Dammerman *et al.*, 2019).

Despite significant phylogenetic signal in the anal sheath alone, in our model system, spawning substrate appears to be still a major driver in the evolution of anal sheath length. However, this result should be treated with caution considering within-species variation in spawning habitat and anal sheath size may have introduced some bias into our phylogenetic comparative model (Losos, 2008). For example, although *F. heteroclitus* was categorized as preferring to spawn on vegetation in our study, individuals in some populations have been shown to adhere their eggs to other surfaces, including sand, mollusc shells and mud depending on availability (Able, 1984). In addition, perhaps the size of the anal sheath may be dependent on the time of season at which specimens were collected (Lopez *et al.*, 2010). Considering that Ghedotti *et al.* (2013) used museum specimens collected from a wide variety of locals, and presumably from across the breeding and non-breeding seasons (appendix I of Ghedotti *et al.* (2013)), it is difficult to determine the role that seasonality played in the estimation of a single anal sheath length for each species. Suffice to say, further work is needed to account for within-species variation in anal sheath size and preferred spawning habitat when assessing phylogenetic signal in these traits.

Selection on egg size might have driven the evolution of anal sheath size. In libellulid dragonflies, for instance, Koch & Suhling (2005) found that species with a greater egg width also had a longer oviposition period and a lower egg release rate, suggesting that larger eggs need more time to pass through the female's ovipositor. The average egg size produced by females of a certain species may, in part, reflect historical selection posed by numerous environmental factors, including predation (Belk, 1977), thermoregulation (Rhymer, 1988) and parental care (Summers *et al.*, 2006), experienced within populations of that species. Amid these variable selection regimes, and the evolution of different egg sizes, anal sheath size may have also evolved to facilitate the manipulation of eggs of corresponding sizes. Thus, selection on egg size could have driven the evolution of anal sheath size in fundulids. Evidence that egg size drives changes in reproductive morphology has been found in other species. For instance, selection for smaller eggs may have contributed to reproductive tracts with smaller diameters, and overall smaller body sizes, in painted turtles (Congdon & Gibbons, 1990).

A potential trade-off between swimming performance and egg placement may also explain why vegetation spawners lack long anal sheaths. Unsteady swimming is especially important in areas with dense aquatic structure because fish must manoeuvre among numerous obstacles (Larouche *et al.*, 2020). During unsteady swimming,

fish are especially unstable in the longitudinal axis (i.e. roll axis) (Standen & Lauder, 2007). Along with the dorsal fin, the anal fin helps to minimize roll and increase stability. It is possible that the anal sheath may stiffen the anal fin, thereby decreasing its ability to minimize roll. If this is the case, then selection should probably favour a large anal sheath when unsteady swimming performance is less critical. Indeed, in open environments, endurance (i.e. steady) swimming is likely to be favoured by natural selection at the cost of unsteady swimming (Langerhans, 2009), and as such the anal sheath may not be as costly in these areas. Future studies should focus on detailing covariation among swimming performance, anal sheath size and spawning habitat in fundulid species to determine if the length of the anal sheath may be negatively correlated with swimming performance, but only in specific environments.

Although selection imposed by spawning habitat is likely to be a major source of among-species variation in anal sheath size in Fundulidae, the relationship that anal sheath size shares with body size may also help to explain sources of variation among species. In this study, we did not find a significant relationship between anal sheath size and maximum total length. However, because anal sheath size was assigned relative to the size of the first anal fin ray, which probably increased with body size, this result should be taken with caution. Future studies should acquire anal sheath lengths independent of the length of the anal-fin ray and pair this information with a measure of body size on a per-individual basis across multiple species. This would give us a better understanding of possible constraints imposed on the length of the anal sheath by body size. Selection pressures that drive and maintain morphological variation among species, and often species diversity in general, are continuing to change in response to human-induced disturbance (Jiang *et al.*, 2020). As a result, biologists will be forced to combine their knowledge of important historical selection pressures (as well as constraints) to evolution if we are to understand how traits will respond to this change. This may be especially important in primary sexual traits, which are often intricately linked to fitness.

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SHARED DATA

Data and R script are available from Mendeley Data (Styga & Welsh, 2021).