RESEARCH ARTICLE

Rapid adaptive evolution of scale-eating kinematics to a novel ecological niche

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ABSTRACT

The origins of novel trophic specialization, in which organisms begin to exploit resources for the first time, may be explained by shifts in behavior such as foraging preferences or feeding kinematics. One way to investigate behavioral mechanisms underlying ecological novelty is by comparing prey capture kinematics among species. We investigated the contribution of kinematics to the origins of a novel ecological niche for scale-eating within a microendemic adaptive radiation of pupfishes on San Salvador Island, Bahamas. We compared prey capture kinematics across three species of pupfish while they consumed shrimp and scales in the lab, and found that scale-eating pupfish exhibited peak gape sizes twice as large as in other species, but also attacked prey with a more obtuse angle between their lower jaw and suspensorium. We then investigated how this variation in feeding kinematics could explain scale-biting performance by measuring bite size (surface area removed) from standardized gelatin cubes. We found that a combination of larger peak gape and more obtuse lower jaw and suspensorium angles resulted in approximately 40% more surface area removed per strike, indicating that scale-eaters may reside on a performance optimum for scale biting. To test whether feeding performance could contribute to reproductive isolation between species, we also measured F1 hybrids and found that their kinematics and performance more closely resembled generalists, suggesting that F1 hybrids may have low fitness in the scale-eating niche. Ultimately, our results suggest that the evolution of strike kinematics in this radiation is an adaptation to the novel niche of scale eating.

KEY WORDS: Feeding kinematics, Novelty, Key innovation, Lepidophagy, Performance, Postzygotic isolation, Hybrid kinematics

INTRODUCTION

Determining how organisms use resources for the first time and occupy novel niches is an outstanding question in evolutionary ecology. Many changes accompany adaptation to a novel niche, and previous studies have investigated how shifts in behaviors (Bowman and Billeb, 1965; Tebbich et al., 2010; Curry and Anderson, 2012), morphologies (Ferry-Graham et al., 2001; Ferry-Graham, 2002; Hata et al., 2011; Davis et al., 2018), physiologies (Arias-Rodriguez et al., 2011; Tobler et al., 2015, 2018) and kinematics (Janovetz,

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2005; Patek et al., 2006; Cullen et al., 2013; McGee et al., 2013) can all facilitate this transition.

Studying shifts in kinematic traits – particularly those affecting prey capture and feeding – is especially promising, because they can provide biomechanical insights into the origins of novel trophic niches. For example, the wimple piranha (*Catoprion mento*) uses a ram attack coupled with a uniquely large gape angle to knock scales free from its prey (Janovetz, 2005); syngnathiform fishes specialize on evasive prey items using power-amplified jaws (Longo et al., 2018); and the Pacific leaping blenny (*Alticus arnoldorum*) is able to feed and reproduce on land by using unique axial tail twisting to improve propulsion and stability for greater jumping performance (Hsieh, 2010).

Differences in prey capture kinematics between species may also contribute to post-zygotic extrinsic reproductive isolation by reducing hybrid feeding performance (Higham et al., 2016), which may lead to speciation (Henning et al., 2017; Matthews and Albertson, 2017). For example, McGee et al. (2015) measured prey capture kinematics and performance in two sunfish species (Centrarchidae) and their naturally occurring hybrids. Hybrid sunfish displayed intermediate gape size compared with parental types and initiated strikes from an intermediate distance, yet their actual suction-feeding performance was less than predicted from these additive traits. Hybrid Lake Victoria cichlids (produced by crossing thick-lipped *Haplochromis chilotes* and thin-lipped *Pundamilia nyererei* parents) also exhibited lower foraging performance at removing prey from crevices compared with their parental species, most likely due to antagonistic pleiotropy and genetic correlations between head and lip morphology (Henning et al., 2017). Despite these findings, few studies investigate how hybrid kinematics affect the evolution of ecological novelty or explicitly relate kinematics to performance consequences.

Scale-eating (lepidophagy) provides an excellent opportunity for connecting a mechanistic understanding of feeding kinematics with adaptation to a novel trophic niche. It is a rare trophic niche that has convergently evolved at least 20 times in approximately 100 fish species out of over 35,000 (Sazima, 1983; Martin and Wainwright, 2013a; Kolmann et al., 2018). Current hypotheses for the origins of scale-eating vary, but they all propose that it may be related to shifts in behaviors related to foraging, such as shifts in aggression, shifts from algae grazing to scale eating, and even shifts from removing epibionts or ectoparasites to scale eating (Fryer et al., 1955; Greenwood, 1965; Sazima, 1983; St. John et al., 2018). This suggests that shifts in kinematics during feeding strikes may accompany the origins of scale eating. However, only a few studies have investigated the feeding kinematics and performance of scale-eating fishes. Janovetz (2005) measured feeding kinematics of *C. mento* while consuming: (1) free-floating scales, (2) whole fish and (3) scales off the sides of fish, and found that scale-eating kinematics were different from those used during suction feeding or biting. Interestingly, scale-eating attacks produced gape angles that

ranged from 30 to 100% larger than those produced from consuming free-floating scales or whole fish, respectively, suggesting that a larger gape is necessary for scale-eating. Furthermore, this variation in gape angle across food items was documented within individuals, indicating that scale-eating kinematics may be behaviorally mediated (Janovetz, 2005). Other studies have also documented a significant interaction between kinematic traits, behavior and morphology. For example, the Lake Tanganyikan scale-eating cichlids (*Perissodus microlepis*), which possess an asymmetric mouth morphology, are able to perform more successful scale-eating strikes using their dominant side (Takeuchi et al., 2012; Takeuchi and Oda 2017), and a similar behavioral laterality has been documented in a scale-eating characiform (*Exodon paradoxus*; Hata et al., 2011). While these studies provide valuable insights into scale-eating kinematics and performance, the lack of comparative data on the kinematics of closely related non-scale-eating species or hybrids has so far limited further investigations of the origins of scale eating.

The aim of our study was to fill the following knowledge gaps and shed light on the relationship between kinematic traits and occupation of a novel niche. First, comparisons of scale-eating kinematics across scale-eating and closely related non-scale-eating outgroup species is necessary for investigating the origins of ecological novelty. Without the comparative method it is impossible to determine which kinematic variables are unique or important for scale eating. Second, very few kinematic studies investigate hybrid kinematics despite the fact that hybridization is quite common, especially among species that diverged recently (Hubbs, 1955; Mayr, 1963; Arnold, 1992; Richards et al., 2019). Understanding hybrid kinematics, especially in the context of ecological novelty, is informative because: (1) impaired performance in hybrids is a form of extrinsic post-zygotic isolation between species (McGee et al., 2015; Higham et al., 2016) and (2) it can allow the decoupling of morphology, behavior and kinematics, making it easier to identify causative traits underlying performance (Holzman and Hulsey, 2017). Finally, few studies connect observed variation in kinematics to variation in whole organism feeding performance (but see: Svanbäck et al., 2002; Takeuchi et al., 2012; China et al., 2017; Sommerfeld and Holzman, 2019; Whitford et al., 2019). Making this connection is important because it can identify kinematic traits associated with performance tasks relevant to evolutionary fitness rather than simply describing phenotypic variation in kinematic traits, most of which may not be relevant to performance or fitness (Arnold, 1983; Hu et al., 2017).

The scale-eating pupfish (*Cyprinodon desquamator*) is an excellent organism to investigate the interaction of kinematics and ecological novelty for several reasons. First, the scale-eating pupfish evolved within a recent sympatric radiation of pupfishes on San Salvador Island, Bahamas. This radiation is endemic to a few hypersaline lakes on the island (Martin and Wainwright, 2013a; Martin et al., 2019), which were most likely dry during the last glacial maximum 10–15 kya (Hagey and Mylroie, 1995). Second, the radiation provides closely related sister taxa for kinematic comparison, including: (1) the scale-eating pupfish, (2) a generalist pupfish (*Cyprinodon variegatus*) and (3) the snail-eating pupfish (*Cyprinodon brontotheroides*). Phylogenetic evidence suggests that scale-eating pupfish form a clade across all lakes where they are found on San Salvador and that this clade is sister to a clade containing generalists and snail-eaters (Martin and Feinstein, 2014; Lencer et al., 2017), although gene flow is still ongoing among all three species (Richards and Martin, 2017). All three pupfish species can be crossed in the lab to measure the kinematics and performance of hybrid phenotypes.

The morphological similarities and differences between San Salvador pupfishes have also previously been described. Specifically, (1) like all cyprinodontiforms, pupfish species exhibit a vestigial ascending process of the premaxilla, allowing for independent movement of the upper and lower jaws during jaw protrusion (Hernandez et al., 2009, 2018), and (2) scale-eating pupfish have two-fold larger, supra-terminal oral jaws compared with the smaller, terminal jaws of the generalist or snail-eating pupfish (Martin and Wainwright, 2011, 2013a; Martin, 2016). Their divergent morphology, along with Janovetz's (2005) finding that scale-eating strikes by the lepidophagous piranha (*C. mento*) were associated with larger peak gapes, led us to predict that scale-eating pupfish should have larger gapes during scale-eating strikes compared with closely related species, and that this increased peak gape should result from a larger angle between the anterior tip of the premaxilla, the quadrate-articular joint, and the anterior tip of the dentary.

We investigated the interaction between kinematics and ecological novelty in pupfishes using high-speed videos of the feeding strikes of San Salvador generalist, snail-eating and scale-eating pupfishes, along with F1 hybrids. If shifts in kinematics are an evolutionary adaptation for the ecological novelty in this system, then scale-eaters may have divergent feeding kinematics compared with other species and may have greater feeding performance on scales. We tested this by: (1) comparing the feeding kinematics of scale-eating pupfish with other species during scale-eating and suction-feeding strikes, (2) investigating whether variation in kinematics was associated with bite performance (i.e. bite size) and (3) determining if F1 hybrid feeding kinematics differed from parental species.

Ultimately, we found that the feeding kinematics of scale-eating pupfish diverged from all other species and were not solely due to their increased oral jaw size. Instead, scale-eaters may be behaviorally mediating their feeding kinematics to optimize the surface area removed per strike, suggesting that scale-eater kinematics are a recent adaptation to scale eating.

MATERIALS AND METHODS
Collection and husbandry
We used seine nets to collect generalist [*Cyprinodon variegatus* (Lacépède 1803)], snail-eating (*Cyprinodon brontotheroides* Martin and Wainwright 2013) and scale-eating (*Cyprinodon desquamator* Martin and Wainwright 2013) pupfishes from Crescent Pond, Little Lake, and Osprey Lake on San Salvador Island, Bahamas in July 2017 and March 2018. Wild-caught fish were maintained in 37–75 liter mixed-sex stock tanks at a salinity of 5–10 ppt and temperatures of 23–27°C. While in stock tanks, fish were fed a diet of frozen bloodworms, frozen mysis shrimp and commercial pellet foods daily. In the lab, we crossed generalist and scale-eating pupfishes from both Little Lake and Crescent Pond to produce F1 hybrid offspring. Prior to filming, pupfishes were isolated in 2 liter tanks to maintain individual IDs throughout the study.

Feeding kinematics
We recorded pupfishes feeding on three different food items: frozen mysis shrimp (Mysida, Hikari Inc.), scales and standardized gelatin cubes (dimensions: 1.5×1.5×1.5 cm; Repashy Superfoods, Community Plus Omnivore Gel Premix; prepared following manufacturer's instructions). We measured feeding kinematics while fish consumed both shrimp and scales because it allowed us to ask whether: (1) scale-eating pupfish differed in their feeding kinematics compared with other groups; (2) the kinematics of

scale-eating strikes differed from those used during suction-feeding (e.g. shrimp); and (3) F1 hybrid feeding kinematics differed from their parental species. We explicitly examined F1 hybrid kinematics in this study because lowered hybrid feeding performance may contribute to reproductive isolation between species and may shed light on rapid adaptive diversification of this clade. We additionally measured feeding kinematics across all groups while fish consumed gelatin cubes to ask whether variation in kinematic traits affected feeding performance (i.e. bite size).

In the lab, fish freely consumed mysis shrimp, but we had to train all species to feed on scales from the sides of euthanized zebrafish (*Danio rerio*; stored frozen) and to feed from gelatin cubes (stored at 4°C). For training, we isolated each fish in a 2-liter plastic tank and presented a given food item (either euthanized zebrafish or gelatin cube) daily. If a pupfish began feeding on the item, it was left in the tank until the pupfish stopped feeding. If a pupfish did not begin feeding within 1 min, the food item was removed from the tank. Any pupfish that did not feed received a supplemental feeding of commercial pellet food (New Life Spectrum Thera-A, medium sinking pellets). If an individual did not feed on a training item for more than 2 days, we reduced supplemental feedings to once every 2 days to ensure that the fish was sufficiently motivated. Once pupfish reliably began feeding on either scales or gelatin cubes, we proceeded to film their feeding behaviors according to the filming protocol below. Fish were never trained on more than one item at a time, and we instead ensured that all filming was completed for a single food item before proceeding to train for the next item.

For all three food items, we used a Sony Cyber-shot DSC-RX10 III or Sony Cyber-shot DSC-RX100 IV 20.1 MP to obtain high-speed videos (480 frames per second) of foraging strikes. Illumination was provided by a dimmable bi-color 480 LED light (Neewer) positioned approximately 0.3 m from the filming tank. Pupfish were allowed to acclimate to the lighting before feeding commenced. Fish were considered acclimated when they moved around their tank freely (usually after ∼5 min). For scale eating, we used forceps to hold a euthanized zebrafish horizontally in the water column and perpendicular to the front of an individual. For mysis shrimp and gelatin cubes, we dropped the food item a few inches in front of an individual. All videos were taken from a lateral perspective. Once filming for one food item was completed, the process was repeated until we filmed each individual consuming all three food items.

Kinematic analyses

Videos were converted to image stacks and analyzed using the image processing software ImageJ (FIJI; Schindelin et al., 2012). To quantify feeding performance, we measured 10 kinematic trait metrics including (1) peak jaw protrusion, defined as the distance (mm) from the center of the orbit to the anterior tip of the premaxilla. (2) Time to peak jaw protrusion, defined as the time (s) from the start of an attack (defined as 20% of peak gape) to peak protrusion. (3) Peak gape, defined as the distance (mm) from the anterior tip of the premaxilla to the anterior tip of the dentary. (4) Time to peak gape, defined as the time (s) from the start of an attack at 20% of peak gape to peak gape. (5) Gape angle was the angle (degrees) produced at peak gape between the anterior tip of the premaxilla, the quadrate-articular joint, and the anterior tip of the dentary. (6) Lower jaw angle was the angle produced at peak gape between the lower jaw, the quadrate-articular joint, and the ventral surface of the fish beneath the suspensorium (Figs 1 and 2). (7) Time to impact was the time (s) from the start of an attack (20% peak gape) to first contact of the oral jaws with the prey item. (8) Time from peak gape to impact
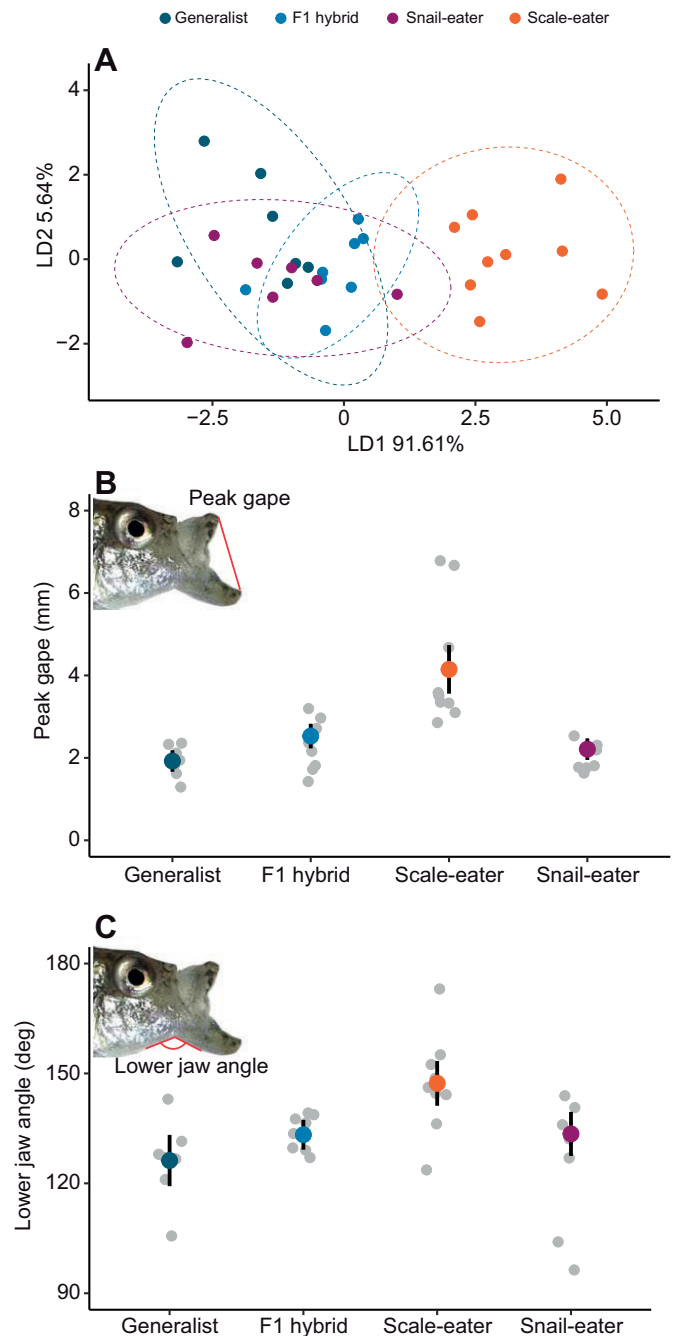

Fig. 1. Divergent feeding kinematics in scale-eating pupfish compared with other *Cyprinodon* pupfish species. (A) Biplot of discriminant axes 1 (LD1) and 2 (LD2) describing overall kinematic differences among pupfish groups. Generalist pupfish, *Cyprinodon variegatus*; snail-eaters, *Cyprinodon brontotheroides*; scale-eaters, *Cyprinodon desquamator*; F1 hybrids, generalist×scale-eater crosses. Ellipses represent 95% CIs. (B) Mean peak gape (mm) for each species with ±95% CIs calculated via bootstrapping (10,000 iterations). (C) Mean lower jaw angle at peak gape (mm) for each species with ±95% CIs calculated via bootstrapping (10,000 iterations).

was the difference between the time to impact (s) and the time to peak gape (s). (9) Starting distance from prey was the distance (mm) from the center of the orbit at the start of an attack to the center of the orbit at impact with prey item. Finally, (10) ram speed was the starting distance from prey at 20% of peak gape (m) divided by the time to impact (s). In addition to our kinematic metrics, we also measured body length and lower jaw length (Table S1) using images
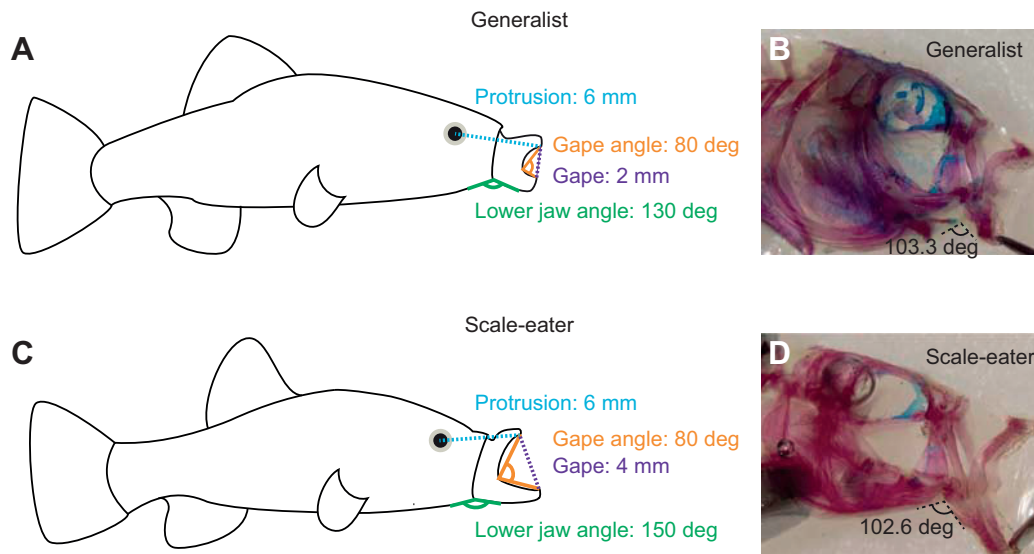
Fig. 2. The large jaws of scale-eating pupfish allow them to double their gape size and increase the angle between their lower jaw and suspensorium (lower jaw angle) while maintaining the same gape angle as other species during feeding strikes. (A) Hypothetical measurements of a generalist's protrusion distance, peak gape and lower jaw angle if they strike a food item with an 80 deg gape angle. (B) Lower jaw angle produced by maximum depression of a generalist's lower jaw on a cleared and Alizarin Red/Alcian Blue double-stained specimen. (C) Hypothetical measurements of a scale-eater's protrusion distance, peak gape and lower jaw angle if they strike a food item with an 80 deg gape angle. (D) Lower jaw angle produced by maximum depression of a scale-eater's lower jaw.

from the video. We calibrated each video using a grid, positioned at the back of the filming tank.

Measuring bite size

In order to relate variation in feeding kinematics to variation in bite size we recorded high-speed strikes on gelatin meal replacement for fish in the shape of a 1.5×1.5×1.5 cm cube. Upon filming a feeding strike on a single cube, we immediately removed the cube from the tank. The gel cube retains its shape in water and therefore allowed us to precisely photograph and measure the area removed by each bite. We used an Olympus Tough TG-5 camera to take photos of each lateral surface of the cube – ensuring that we had photographed the entire bite – and measured the total surface area removed ($pixels^2$) from the cube (Fig. 3B). We then standardized bite sizes across photos by calculating bite area as a proportion relative to a standardized grid present in each photo, and converting this proportional data into area (mm^2) by multiplying the proportion times the area of the grid ($573.12 \ mm^2$). One caveat is we did not measure the depth of the bite, which may be affected by additional kinematic variables during the strike. However, scale-eating attacks observed in the lab and field do not typically produce deep wounds in which bite depth would be relevant, thus we expect that surface area is the best proxy for scale-biting performance in this system. Although bites were removed from both the lateral surface and edge of the gelatin cubes during strikes, there was no significant difference in surface area removed (*t*-test, *P*=0.12).

Statistical analyses
Comparing strike kinematics

We collected and analyzed 101 feeding strikes from 31 individuals striking both shrimp and scales (7 generalists; 7 snail-eaters; 9 scale-eaters; 8 F1 hybrids). We used linear mixed models (LMMs) in the lme4 package (https://cran.r-project.org/web/packages/lme4/index.html) in R to determine if any of our kinematic metrics varied between species or food item. A mixed model approach is appropriate for these data, because it accounts for errors due to

repeated measures (Holzman et al., 2008; Holzman and Wainwright, 2009). In each model we included: (1) the kinematic metric as the response variable; (2) species designation, food item and their interaction as fixed effects; (3) individual fish IDs and population nested within species as random effects; and (3) log body size as a covariate (Table 1). Although we compared kinematic data across multiple species, very few genetic variants are fixed between species (<1000 SNPs out of 12 million) and generalists and molluscivores cluster by lake rather than by species (McGirr and Martin, 2016; Richards and Martin, 2017; J. A. McGirr and C.H.M., unpublished data). Thus, it is appropriate to analyze species differences at these recent timescales as population-scale data using mixed model analyses of independent populations (e.g. Hatfield and Schluter, 1999; McGee et al., 2013), rather than phylogenetic comparative methods.

We also performed a linear discriminant analysis (LDA) on the combined shrimp and scales kinematic data to reduce dimensionality and identify which kinematic metrics contributed most to differences between species (Table 2, Fig. 1A). We used a MANOVA and Wilks' λ to assess the significance of the LDA. We did not have enough degrees of freedom to perform these analyses with all of our kinematic variables, so we excluded time to peak protrusion and time to impact as they were highly correlated with time to peak gape (Table S2, r^2>0.85), and also excluded distance from prey as it was highly correlated with ram speed (Table S2, r^2=0.90). Our MANOVA ultimately included: (1) peak protrusion, peak gape, time to peak gape, gape angle, lower jaw angle, time from peak gape to impact, and ram speed as response variables; (2) species designation as a predictor variable; and (3) individual ID as a random effect.

Determining how kinematic variables affect bite performance

We collected and analyzed 31 strikes on cubes across all three species and F1 hybrids. We used generalized additive models (GAMs) from the mgcv package (https://cran.r-project.org/web/packages/mgcv/index.html; Wood, 2011) in R to investigate how
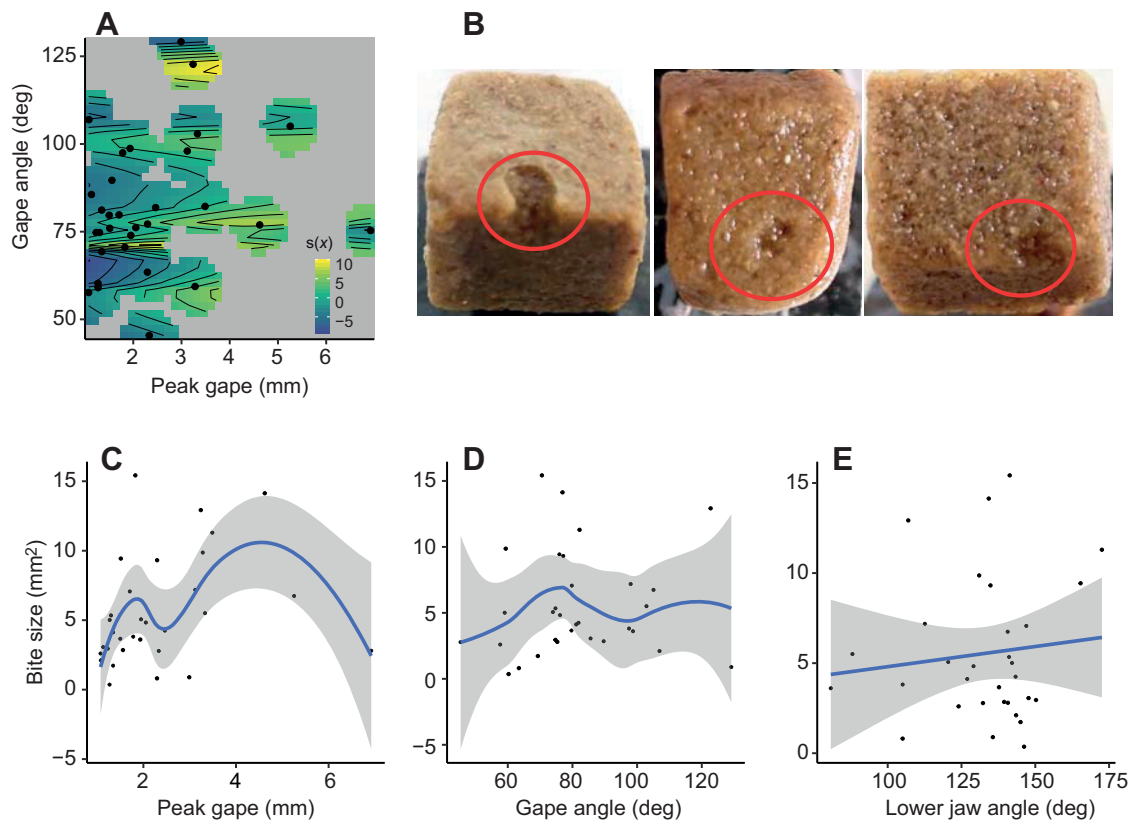
Fig. 3. The interaction of peak gape and gape angle may result in a performance optimum for scale biting. (A) Visualization of the two-way thin-plate spline from the best-fitting GAM model. Points represent raw data and colors represent relative bite sizes [s(x)] from a thin-plate spline fit to peak gape (mm) and gape angle (degrees). Estimates of the surface by the GAM model are only calculated in regions containing data. (B) Representative scale-eating bites taken out of gelatin cubes. Visualization of the relationship between bite size (surface area removed from the gelatin cube per strike) and (C) peak gape (mm), (D) gape angle and (E) lower jaw angle from the best-fitting GAM model. Points represent raw data from each strike and lines represent univariate splines (C,D) or a linear regression (E) fit to the data along with 95% CIs in gray.

peak gape, peak protrusion, gape angle and lower jaw angle affected bite size. We used GAMs for this analysis because they do not assume a linear relationship between performance (i.e. bite size) and our given kinematic variables, but instead can fit smoothing splines to the data to test for nonlinear associations. We used AIC scores to select our optimal model (Table 3). We started with the most complete model which included: (1) bite size as the response variable; (2) a spline modeling the interaction between two of our predictor variables; and (3) a single fixed effect. There were insufficient degrees of freedom to test all four terms at once in this model; therefore, we tested all combinations of this model with our four predictor variables (Table 3A). We also tested all nested versions of this complex model by: (1) removing the interaction term, but maintaining two splines and a fixed effect (Table 3B); (2) removing one spline and including three fixed effects (Table 3C); and finally, (3) by testing the model with all four variables as only fixed effects (Table 3D). Ultimately, our best supported model included bite size as the response variable, a thin-plate spline of the interaction between peak gape and gape angle and lower jaw angle as a fixed effect (ΔAIC of next best-fitting model=32.56).

Finally, we predicted the bite size for each fish from their peak gape and gape angle kinematic measurements using a machine-learning algorithm from the caret package (https://cran.r-project.org/web/packages/caret/index.html; Kuhn, 2008) using a spline-based method. Predictive modeling allowed us to address two problems from our original cube dataset and analysis. First, cubes are an ideal food item for connecting variation in kinematics to bite size (something that was very difficult to do with shrimp and zebrafish), but are ultimately an unnatural food item for fish, and their feeding strikes on cubes may not reflect feeding on natural prey. Predictive modeling allowed us to use kinematic data from strikes performed on zebrafish and shrimp to estimate bite sizes for each relevant prey item. Second, the cube dataset and analysis did not look for variation across species, and instead, explicitly connected variation in feeding kinematics (regardless of species) to bite size. Applying our predictive model to the shrimp and zebrafish dataset allowed us to gain additional insight into differences between species (Fig. 4).

We used a GAM model, estimating the effect of gape size and gape angle on the area removed from gelatin cubes, to predict bite performance (bite size) from the 101 feeding strikes on scales and mysis shrimp used in our previous analyses. Although we would not realistically expect suction feeding strikes on mysis shrimp to result in a bite per se, we found no difference in any kinematic traits between food items, and therefore used strikes on both scales and shrimp for this analysis.

Ideally, we would have used our best-fitting GAM model, which also included lower jaw angle as a fixed effect. However, the caret package currently only accepts two fixed effects, and lower jaw angle ultimately did not affect bite size (P=0.219). We trained the model using all strikes observed on gelatin cubes (31 strikes across all three species and F1 hybrids) and 10-fold cross-validations with three repeats as the resampling scheme. We tested the accuracy of this model by comparing fitted values from the model to observed

Table 1. Results of linear mixed models investigating variation in strike kinematic variables in *Cyprinodon* pupfish species

Response	Predictor	χ^2	d.f.	P
Peak protrusion (mm)	Species	4.01	3	0.26
	Food item	1.10	1	0.29
	log(Body length)	3.01	1	0.082
	Species×Food item	2.03	3	0.57
Time to peak protrusion (s)	Species	3.80	3	0.27
	Food item	0.73	1	0.39
	log(Body length)	1.02	1	0.31
	Species×Food item	4.03	3	0.26
Peak gape (mm)	**Species**	**23.13**	**3**	**3.8×10^{-5}**
	Food item	0.71	1	0.40
	log(Body length)	1.24	1	0.27
	Species×Food item	0.65	3	0.88
Time to peak gape (s)	Species	2.43	3	0.49
	Food item	0.57	1	0.45
	log(Body length)	2.80	1	0.17
	Species×Food item	1.87	3	0.60
Gape angle (deg)	Species	3.28	3	0.35
	Food item	0.032	1	0.86
	log(Body length)	1.01	1	0.32
	Species×Food item	3.43	3	0.33
Lower jaw angle (deg)	**Species**	**18.62**	**3**	**0.00033**
	Food item	0.0031	1	0.96
	log(Body length)	3.53	1	0.060
	Species×Food item	3.56	3	0.31
Time to impact (s)	Species	2.55	3	0.47
	Food item	2.05	1	0.15
	log(Body length)	1.40	1	0.24
	Species×Food item	4.69	3	0.20
Time from peak gape to impact (s)	Species	2.44	3	0.48
	Food Item	0.97	1	0.32
	log(Body length)	0.57	1	0.45
	Species×Food item	1.39	3	0.71
Starting distance from prey (mm)	Species	0.43	3	0.93
	Food Item	1.99	1	0.16
	log(Body length)	2.77	1	0.10
	Species×Food item	0.80	3	0.85
Ram speed (m s^{-1})	Species	3.25	3	0.35
	Food Item	3.75	1	0.053
	log(Body length)	1.55	1	0.21
	Species×Food item	2.02	3	0.57

Table shows results of linear mixed models investigating if strike kinematic variables vary among: (1) species (generalists, snail-eaters, scale-eaters or F1 hybrids), (2) food item (shrimp or scales) or (3) the interaction between the two. Significant predictors are indicated in bold.

values from the data set and found that our model was able to predict 46% of the variance in the gelatin-strike dataset (d.f.=1, F=25.06, P=2.5×10^{-5}, R^2=0.46). We then used this model to predict bite size from each scale-biting and suction-feeding strike based on the kinematic measurements alone. We used bootstrap resampling (10,000 iterations) to calculate mean bite size and 95% confidence intervals for each species.

Table 2. Results of a linear discriminant analysis for kinematic variables for strikes on shrimp and scales

Kinematic metric	LD1	LD2	LD3
Peak jaw protrusion (mm)	−0.082	−0.49	−0.065
Peak gape (mm)	1.55	0.39	−0.56
Time to peak gape (s)	−8.00	12.16	10.24
Gape angle (deg)	−0.032	−0.012	−0.033
Lower jaw angle (deg)	0.069	0.0029	0.022
Time to impact (s)	−9.85	31.32	−33.03
Ram speed (m s^{-1})	−7.98	17.27	10.67
Proportion of trace	0.92	0.056	0.028

Determining whether hybrid kinematics match additive predictions

We calculated the predicted values for peak gape, lower jaw angle and bite size for the scale-eater×generalist F1 hybrids under the hypothesis that these kinematic traits would be additive and therefore intermediate between generalist and scale-eater values. We used a one-sample *t*-test to test whether the observed values of the three traits (peak gape, lower jaw angle, predicted bite sizes) for F1 hybrids deviated from additive predictions.

RESULTS

Scale-eaters exhibited divergent feeding kinematics compared with other pupfishes

Scale-eaters exhibited divergent feeding kinematics, while consuming both shrimp and scales, compared with other species and groups (Fig. 1A). A MANOVA supported the significance of this discriminant analysis and found that species designation was a significant predictor of kinematics (Wilks' λ=0.13; F=3.05; d.f.=3; P=0.00036). Species significantly varied in their peak gape and lower jaw angles during feeding strikes – regardless of the food item – in a linear mixed model controlling for individual ID and body length (Table 1). This pattern was driven by scale-eaters who had peak gapes that were twice as large as other species, but also had lower jaw angles with their suspensorium that were 14% more obtuse than other species (Fig. 1B,C). Importantly, the scale-eaters' more acute angle of the jaw complex with respect to their body, along with their greatly enlarged oral jaws, allows them to have increased peak gape while maintaining the same gape angle as other species (Fig. 2). This may allow their upper jaws to more effectively 'rake' scales from the prey surface. Ram speed was the only kinematic variable that marginally varied between food items: strikes on shrimp were approximately 16% faster than those on scales (Table 1, Fig. S1; P=0.053).

Variation in strike kinematics affected bite size performance

GAM modeling indicated that the thin-plate spline of the interaction between peak gape and gape angle was significantly associated with bite size (*edf*=22.85, F=3.27, P=0.0391). However, the fixed effect of lower jaw angle was not significant (t=−1.37, P=0.219). Ultimately, this model explained 94.6% of the observed deviance in bite size, and suggests that large gapes of about 4–5 mm paired with gape angles of 80 deg are associated with larger bites (Fig. 3).

F1 hybrid kinematics are not strictly additive and more closely resemble generalist kinematics

F1 hybrid feeding kinematics differed from scale-eater kinematics (Tukey HSD, peak gape: P=1.2×10^{-6}, lower jaw angle: P=0.0090), but were not significantly different from generalist kinematics (Tukey's HSD, peak gape: P=0.21, lower jaw angle: P=0.37). Mean hybrid peak gape was 39% smaller than scale-eater peak gape and 32% larger than generalist peak gape (Fig. 1B). Similarly, mean hybrid lower jaw angle was 9.5% more acute than scale-eater peak lower jaw angle, and 5.6% more obtuse than the mean generalist lower jaw angle (Fig. 1C). F1 hybrids failed to match additive predictions of intermediate kinematics (i.e. the mean of the two parental species) for peak gape (*t*-test, μ=3.035, mean=2.52 mm, P=0.013), but did meet these predictions for lower jaw angle (*t*-test, μ=136.5, mean=133.92 deg, P=0.18). Our machine learning model also predicted that scale-eater kinematics would result in bite sizes that are approximately 40% larger than the predicted bites of the other species (Fig. 4). Estimates for F1 hybrid bite sizes were approximately 5% smaller than expected based on additive predictions (*t*-test, predicted=6.40 mm^2, observed=6.08 mm^2, P=0.49).

Table 3. Results of generalized additive model (GAM) comparisons using AIC score

	Model		ΔAIC
A	1	Area~s(Peak gape, Peak protrusion, bs='ts')+(Lower jaw angle)	38.76
	2	Area~s(Peak gape, Peak protrusion, bs='ts')+(Gape angle)	39.81
	3	**Area~s(Peak gape, Gape angle, bs='ts')+(Lower jaw angle)**	**0**
	4	Area~s(Peak gape, Gape angle, bs='ts')+(Peak protrusion)	44
	5	Area~s(Peak gape, Lower jaw angle, bs='ts')+(Peak protrusion)	42.86
	6	Area~s(Peak gape, Lower jaw angle, bs='ts')+(Gape angle)	43.97
	7	Area~s(Peak protrusion, Lower jaw angle, bs='ts')+(Peak gape)	41.75
	8	Area~s(Peak protrusion, Lower jaw angle, bs='ts')+(Gape angle)	44.55
	9	Area~s(Peak protrusion, Gape angle, bs='ts')+(Peak gape)	36.93
	10	Area~s(Peak protrusion, Gape angle, bs='ts')+(Lower jaw angle)	44.29
	11	Area~s(Gape angle, Lower jaw angle, bs='ts')+(Peak gape)	41.75
	12	Area~s(Gape angle, Lower jaw angle, bs='ts')+(Peak protrusion)	44.87
B	13	Area~s(Peak gape)+s(Peak protrusion)+(Lower jaw angle)	32.56
	14	Area~s(Peak gape)+s(Peak protrusion)+(Gape angle)	34.64
	15	Area~s(Peak gape)+s(Gape angle)+(Lower jaw angle)	37.35
	16	Area~s(Peak gape)+s(Gape angle)+(Peak protrusion)	34.64
	17	Area~s(Peak gape)+s(Lower jaw angle)+(Peak protrusion)	32.56
	18	Area~s(Peak gape)+s(Lower jaw angle)+(Gape angle)	34.88
	19	Area~s(Peak protrusion)+s(Lower jaw angle)+(Peak gape)	43.45
	20	Area~s(Peak protrusion)+s(Lower jaw angle)+(Gape angle)	47.74
	21	Area~s(Peak protrusion)+s(Gape angle)+(Peak gape)	44.15
	22	Area~s(Peak protrusion)+s(Gape angle)+(Lower jaw angle)	47.97
	23	Area~s(Gape angle)+s(Lower jaw angle)+(Peak gape)	45.07
	24	Area~s(Gape angle)+s(Lower jaw angle)+(Peak protrusion)	47.74
C	25	Area~s(Peak gape)+Peak protrusion+Lower jaw angle+Gape angle	33.8
	26	Area~s(Peak protrusion)+Peak gape+Lower jaw angle+Gape angle	45.45
	27	Area~s(Lower jaw angle)+Peak protrusion+Peak gape+Gape angle	45.45
	28	Area~s(Gape angle)+Peak protrusion+Peak gape+Lower jaw angle	45.45
D	29	Area~Peak gape+Peak protrusion+Lower jaw angle+Gape angle	45.45

The best-fitting model is indicated in bold. AIC, Akaike information criterion. s() is used to indicate spline-based smooths. bs='ts' indicates that a thin plate regression spline was used.

DISCUSSION

Scale-eating pupfish have divergent feeding kinematics

Scale-eating pupfish exhibited peak gapes that were twice as large as other groups, but simultaneously displayed gape angles that were not different from other groups, and lower jaw angles that were 12% more obtuse. Thus, scale-eaters kept their jaws less open during strikes compared with other species, resulting in smaller gape sizes than the maximum achievable gape given their morphology. These counterintuitive results only partially support our prediction that
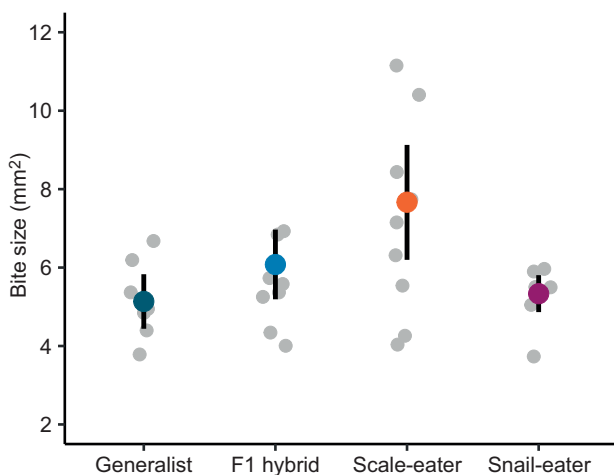

Fig. 4. Scale-eaters have larger predicted bite sizes compared with other *Cyprinodon* pupfish species. Predicted bite sizes for all strikes from each species using machine-learning optimization of GAM models. Gray points represent predicted bite sizes for individuals, color points represent means, and bars represent ±95% CIs calculated via bootstrapping (10,000 iterations).

scale-eaters should have larger peak gapes, similar to the findings of Janovetz (2005) for the scale-eating piranha. Increased gape size in scale-eating pupfish was not due to an increased gape angle as we predicted. Instead, scale-eaters appear to maintain the same gape angle of their oral jaws as in other species (~80 deg) and increased their lower jaw angle resulting in less open jaws during strikes. Morphologically, it appears that scale-eaters are not physically constrained from depressing their lower jaw much more than the observed 150 deg during strikes (Fig. 2D), indicating that their obtuse lower jaw angles are decreasing their physically obtainable maximum peak gape (Fig. 2). For example, if a scale-eater were to adopt a generalist lower jaw angle of 130 deg, they could increase their peak gape by about 8%. One possibility is that this more obtuse lower jaw angle is an artifact of filming scale-eating strikes in the lab. To investigate this, we analyzed four scale-eating strikes performed by wild scale-eaters observed in Crescent Pond, San Salvador Island, Bahamas [filmed using a Chronos camera (Kron Technologies, model 1.4, 16 GB memory, Color image sensor) with an f1.4 zoom lens in a custom underwater housing (Salty Surf, Inc. Krontech Chronos 1.4 housing with M80 flat port)] and compared their jaw angles with strikes measured in the lab. Wild strikes had an even more obtuse mean lower jaw angle of 168 deg, whereas scale-eating strikes in the lab had a mean lower jaw angle of 153 deg, suggesting that an obtuse lower jaw angle is also used during natural scale-eating strikes in hypersaline lakes on San Salvador Island.

Strike kinematics did not vary across prey items (Table 1), contrary to Janovetz (2005). In fact, the only kinematic variable that remotely varied between prey items was ram speed (Table 1, Fig. S1), but this may simply be due to the fact that sinking frozen shrimp were a moving target during feeding trials whereas

euthanized zebrafish were held stationary with forceps for scale-eating strikes. Alternatively, phenotypic plasticity due to rearing environment could produce a similar pattern; however, we did not observe any differences in strike kinematics between wild-caught and lab-reared fish.

Is jaw morphology solely responsible for kinematic variation?

The kinematic variables that varied the most between scale-eating and non-scale-eating pupfishes were peak gape and lower jaw angle – both of which are related to the size of the oral jaws. Previous work has documented that the oral jaws of scale-eating pupfish are two-fold larger than their sister species (Holtmeier, 2001; Martin and Wainwright, 2013a; Martin, 2016) and may be controlled by four moderate-effect quantitative trait loci (QTL) with all positive effects on jaw size, consistent with directional selection on this trait (Martin et al., 2017). It may be that increased oral jaw size is sufficient to create variation in feeding kinematics without an accompanying shift in behavior. Previous studies have documented how changes in morphology alone can alter feeding kinematics. For example, kinematic studies have found that the scaling of the lower jaw in bluegill (Wainwright and Shaw, 1999) and body size in largemouth bass (*Micropterus salmoides*; Richard and Wainwright, 1995) both significantly affected prey capture kinematics. Furthermore, Ferry-Graham et al. (2010) used the pike killifish (*Belonesox belizanus*) to show that simply doubling the length of the jaws significantly affected key kinematic variables such as peak gape size – even while keeping lower jaw angle constant. Simply stated, the key adaptation necessary for scale eating may be enlarged, supra-terminal jaws. If this hypothesis were true, we would expect that peak gape would increase with jaw size and that gape angle would increase with the shift from terminal to supra-terminal jaws, but all other kinematics variables would remain constant across species. Our results reject this hypothesis. Instead, scale-eaters maintain the gape angle observed in other species and increase their lower jaw angle with the suspensorium by 12 deg, resulting in a reduction in their potential peak gape size (Fig. 2). This suggests that scale-eaters have evolved more obtuse lower jaw angles during strikes to increase feeding performance (Figs 3 and 4). Another explanation for the obtuse lower jaw angles observed in scale-eaters may be related to the position of the lower jaw joint. In scale-eaters, the lower jaw joint is more ventral than it is in generalists and snail-eaters because of the supra-terminal position of the mouth. This positioning may physically constrain how acute the lower jaw angle can be, preventing scale-eaters from depressing their lower jaws past an angle of ~150 deg. However, this is highly unlikely because the lower jaws of cleared and Alizarin Red-stained scale-eating pupfish specimens can be depressed to angles as small as ~100 deg with the suspensorium (Fig. 2B). The jaws of cleared and stained generalists can be depressed to a similar angle (Fig. 2D). This strongly suggests that scale-eater morphology does not physically constrain them from opening their jaws even wider than is observed during strikes.

Scale-eating performance optimum

Scale-eaters may have reduced their lower jaw angles relative to other species in order to remain on a performance optimum for scale-eating. Our models of bite size supported this: peak gapes larger than approximately 4.5 mm counterintuitively resulted in smaller bite sizes (Fig. 3A,C). An enlarged lower jaw angle in scale-eating pupfish results in a lower jaw that points directly towards the prey during strikes – possibly resulting in greater stability for biting scales while retaining a large gape (Fig. 2). This large gape and

unique jaw alignment may allow scale-eaters to attack prey from a roughly perpendicular angle (as frequently observed during field observations), whereby they appear to wrap their large lower jaw under prey items and subsequently scraping scales from their sides using their independently protrusible upper jaws (also observed in a scale-eating characin: Hata et al., 2011). Interestingly, perpendicular angles of attack and large gapes are associated with scraping in benthic feeding fish (Van Wassenbergh et al., 2008; O'Neill and Gibb, 2013). In fact, two prominent hypotheses for the origins of scale-eating are that it arose from: (1) an algae-scraping ancestor or (2) an ancestor specializing on scraping parasites from other fish (Sazima, 1983).

One caveat for this hypothesis, however, is that our current performance estimates do not include all possible combinations of peak gape and lower jaw angle, and we have few observations of the largest peak gape sizes. Future work should estimate performance across multiple performance axes (e.g. Stayton, 2019; Keren et al., 2018 preprint, Dickson and Pierce, 2019), ideally using F2 hybrids. F2 hybrids are a useful tool for this type of experiment, as they are the first generation of offspring in which recombination among parental alleles can produce new combinations of kinematic, morphological and behavioral traits not observed in the F0 or F1 generations. Identifying and measuring other traits that may be important for scale eating, such as bite force, bite depth or endurance (which may affect prey acquisition), would also be informative.

Non-additive F1 hybrid feeding kinematics may contribute to reproductive isolation of scale-eaters

It is well documented that complex performance traits, such as feeding kinematics, are most likely controlled by numerous loci (i.e. polygenic), and can mostly be described as additive (reviewed in Sella and Barton, 2019). We therefore expected F1 hybrids to exhibit intermediate kinematics and performance relative to both parental species. Instead, we found that F1 hybrid kinematics more closely resembled generalists (Table 1; Fig. 1) suggesting that F1 hybrids may have higher performance in a generalist trophic niche.

Current evidence from field fitness experiments supports the idea that hybrid pupfish exhibit better performance in the generalist ecological niche compared with their performance in the scale-eater niche. One field experiment in these lakes measured hybrid fitness in the wild and found high mortality and low growth rates for hybrids most closely resembling the scale-eating phenotype (Martin and Wainwright, 2013b). Furthermore, for the few hybrids resembling scale-eaters which did survive, only 36% had recently consumed any scales compared with 92% of wild-caught scale-eaters (Martin and Wainwright, 2013a,b). Impaired hybrid performance in the scale-eating niche may contribute to extrinsic post-zygotic isolation between species (McGhee et al., 2007; McGee et al., 2013; Higham et al., 2016). Reproductive isolation may also evolve more quickly in species that occupy a more distant fitness peak with a larger fitness valley, such as the scale-eating pupfish, as a result of stronger selection against hybrids and reinforced pre-mating isolation (Martin and Feinstein, 2014). Thus, impaired hybrid scale-eating performance could also contribute to increased diversification rates through the mechanism of a wider fitness valley.

Reduced hybrid performance may also be due to the morphological differences between scale-eaters and generalists. As mentioned above, it is possible that a shift in morphology – such as enlarged oral jaws in scale-eaters – may alone be sufficient to change kinematic profiles. F1 hybrid kinematics clearly differed from scale-eater kinematics, but their jaw lengths were also significantly smaller than the jaws of scale-eaters (Tukey's HSD,

$P=5.21\times10^{-5}$). Furthermore, previous work has shown that F1 hybrid pupfish offspring (produced from generalist×scale-eater crosses) tend to develop along a more similar trajectory to their maternal parent (Holtmeier, 2001). This could indicate that F1 hybrid pupfish with scale-eating mothers are more likely to develop jaws resembling a purebred scale-eater, but may also retain their generalist-like kinematics. The resulting mismatch between morphology, kinematic traits and ecological niche may be driving low hybrid survival in the scale-eating niche and contributing to reproductive isolation between generalist and scale-eating pupfish species.

Conclusions

This study explicitly takes advantage of an adaptive radiation of *Cyprinodon* pupfishes to make comparisons of scale-eating kinematics across multiple species. This comparative approach allowed us to pinpoint traits that are important for scale-eating. Our results suggest that shifts in key kinematic traits may have preceded or facilitated the origin of scale eating in *Cyprinodon* pupfishes. Scale-eating pupfish exhibited peak gapes that were twice as large as other pupfish species, but simultaneously had lower jaw angles that were significantly more obtuse. We also directly connected variation in kinematic traits to feeding performance – a step that is rarely taken in kinematic studies. Surprisingly, we found that this unique combination of scale-eater kinematics may reside on a performance optimum, as large peak gapes and large lower jaw angles resulted in larger bite sizes. Impaired F1 hybrid kinematics and performance in the scale-eating niche also suggests that kinematic traits contribute to reproductive isolation of the scale-eating pupfish and the evolution of ecological novelty. Future work should investigate if other performance optima exist on the kinematic landscape and whether F2 hybrid fitness in the wild is reduced because of a mismatch between morphology and feeding kinematics.

Author contributions
Conceptualization: M.E.S.J., R.H., C.H.M.; Methodology: M.E.S.J., C.H.M.; Validation: C.H.M.; Formal analysis: M.E.S.J.; Investigation: M.E.S.J., C.H.M.; Resources: C.H.M.; Data curation: M.E.S.J., C.H.M.; Writing - original draft: M.E.S.J.; Writing - review & editing: M.E.S.J., R.H., C.H.M.; Visualization: M.E.S.J.; Supervision: C.H.M.; Project administration: C.H.M.; Funding acquisition: C.H.M.

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