Effect of craniofacial genotype on the relationship between morphology and feeding performance in cichlid fishes

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The relationship between morphology and performance is complex, but important for understanding the adaptive nature of morphological variation. Recent studies have sought to better understand this system by illuminating the interconnectedness of different functional systems; however, the role of genetics is often overlooked. In this study, we attempt to gain insights into this relationship by examining the effect of genotypic variation at putative craniofacial loci on the relationship between morphology and feeding performance in cichlids. We studied two morphologically disparate species, as well as a morphologically intermediate hybrid population. We assessed feeding performance, jaw protrusion, and general facial morphology for each fish. We also genotyped hybrid animals at six previously identified craniofacial loci. Cichlid species were found to differ in facial geometry, kinematic morphology, and performance. Significant correlations were also noted between these variables; however, the explanatory power of facial geometry in predicting performance was relatively poor. Notably, when hybrids were grouped by genotype, the relationship between shape and performance improved. This relationship was especially robust in animals with the specialist allele at *sox9b*, a well-characterized regulator of craniofacial development. These data suggest a novel role for genotype in influencing complex relationships between form and function.

KEY WORDS: Adaptive radiation, evolutionary genetics, functional morphology, *sox9*, suction feeding.

To fully understand the course of evolution by natural selection, it is necessary to understand the effect that intraspecific variation has on the fitness of each individual. In particular, morphological variation often plays an important role in determining an animal's fitness, as the morphology of a structure dictates how it can be used. However, the relationship between morphology and performance is often complicated and highly dependent on context (Koehl 1996). As a result, there are many instances where a change in morphology does not directly cause a corresponding change in performance (Koehl 1996). Given a constant context and optimal behavior, the relationship between form and function can be characterized in one of two ways. The most simple relationship is one-to-one mapping, where each level of performance is associated with a specific morphology (Dean et al. 2007; Cooper and Westneat 2009). Alternatively, in complex mechanical systems morphology and performance can be decoupled. With many-to-one mapping, performance may be the same in animals with different morphologies (Alfaro et al. 2005; Wainwright et al. 2005; Bellwood et al. 2006; Collar et al. 2006; Vanhooydonck et al. 2006; Kolmann et al. 2015). This often happens as a result of balanced changes in two or more characters such that the kinematics of the mechanical system are unaffected. Not all systems are capable of many-to-one mapping, since there must be at least three elements in the system for the necessary tradeoffs to occur, but given the complexity of most biological systems, many-to-one mapping is likely to be pervasive (Collar et al. 2006).

One functional system that has been particularly well studied is the skull of neo-teleosts, specifically in relation to suction feeding performance. Unlike tetrapod skulls, neo-teleost skulls are composed of many unfused and loosely articulated skeletal elements that produce complex and dynamic movements. An example of this is jaw protrusion and how it is used to quickly expand the buccal cavity and approach prey during suction feeding (van Leeuwen 1984; Holzman et al. 2008). Although many

morphological characters have been implicated as determinants of suction feeding success, the system is too complicated to be explained by morphology alone (Wainwright et al. 2007; Holzman et al. 2008). To more thoroughly describe the kinematics of jaw movement, biologists have often turned to engineering principals. One major focal point has been four bar linkage mechanisms, which can be used to predict the efficiency of oral jaw opening and protrusion (Westneat 1994). Four bar linkage systems provide a classic example of many-to-one mapping (Wainwright et al. 2005), as there are many different combinations of linkage element lengths that will give the same displacement advantage of jaw opening (i.e., kinematic transmission). At the population level this is one of several mechanisms that allow for increased evolutionary potential, because it enables variation in different skeletal elements of the head to arise due to other selective pressures without having a deleterious effect on suction feeding performance. Thus, the relationship between form and function is explicitly linked to organismal survival and trait evolvability, but it is inherently difficult to predict.

For this reason, an integrative approach to studying the morphology-performance-fitness axis has been proposed wherein multiple functional systems are considered (Kane and Higham 2015). While the integration of multiple functional contexts undoubtedly has the potential to increase our understanding of the origins and maintenance of adaptive morphological variation (as suggested by Kane and Higham 2015), we propose to extend this integrative approach to consider genetic contexts. In particular we ask the question of whether genotype might influence the widely explored relationship between form and function (e.g., Arnold 1983; Shaffer and Lauder 1985; Wainwright et al. 1991; Waltzek and Wainwright 2003). Operationally, an extension of this research paradigm to include genotype (i.e., genotype-morphology-kinematics-performance-fitness) is not trivial. This is because most functional model systems are not genetically tractable, and traditional laboratory models do not exhibit especially dynamic performances. East African cichlid fishes however offer an appealing balance between functional diversity and experimental tractability, and represent a good model system in which to integrate the two.

East African cichlids are a group of fish that are known and studied for their rapid adaptive radiation, a process that has divided them into an estimated 2000 species in three large lakes (and many smaller lakes and rivers) over the last ∼20 million years (Kocher 2004). Since these fish largely process food with their pharyngeal jaws, their oral jaws are free to diverge into many forms, leading to extensive functional diversity in modern species (Liem 1973). In many cases, this divergence has taken place along a bentho-pelagic morphological axis, leading to functional specificity across an array of foraging niches (Cooper et al. 2010; Hulsey et al. 2013). Although many cichlids are obligate

specialists within a foraging niche, most species persist as ecological generalists capable of exploiting multiple different resources. While many fish are capable of altering their feeding mode (Robinson et al. 1993), their performance at any one task may not be optimal due to mechanical tradeoffs in bone structure and jaw musculature (Meyer 1989; Huckins 1997; Mittelbach et al. 1999; Albertson and Kocher 2006). Here we focus on this "modulatory multiplicity" (*sensu* Liem 1979) in terms of suction feeding. In the context of cichlid adaptive radiations, the ability to gather food from the water column enables species to capitalize on periodic algal blooms, which is a resource that even obligate benthic foraging fish will take advantage of when available (Liem 1980; Mckaye and Marsh 1983).

The evolutionary history of cichlids has also facilitated their tractability as a genetic model. For instance, the relatively recent speciation of many East African cichlids means that hybridization is often possible in the lab, which allows for the generation of genetic mapping pedigrees with which to explore genotype-phenotype relationships. A large and accumulating number of genetic and genomic resources have arisen from this experimental attribute (collated at http://cichlid.umd.edu/CGCindex.html). As a result much has been learned over the past 10+ years about the genetic basis of various craniofacial traits (Albertson et al. 2003, 2005, 2014; Albertson and Kocher 2006; Hu and Albertson 2014; Powder et al. 2014; Schneider et al. 2014; Parsons et al. 2015), including the identification of several candidate genes.

In this study, we took advantage of the experimental tractability of cichlids to integrate craniofacial genetics with the relationship between facial morphology and suction feeding performance. Using two morphologically disparate cichlid species, as well as intermediate hybrids, we examined the relationships between facial geometry, kinematic morphology, and performance as well as the influence of craniofacial genotype on these interactions. While we expected there to be substantial differences between cichlid species and their hybrids, more surprising trends emerged once we parsed the data by genotype at known craniofacial loci. These results validate the assertion that genotype is an important predictor of the form-function relationship, and have important implications for how this relationship might evolve over time.

Methods
ANIMAL CARE
We studied two morphologically distinct species of Lake Malawi cichlids at opposite ends of the benthic-pelagic morphological axis, *Labeotropheus* fullebornii (LF), an algae scraping specialist, and *Maylandia* zebra (MZ), a generalist but one of the only rock-dwelling species to routinely feed via suction feeding. We also examined F_5 hybrids between the benthic specialist (LF)

and a morphologically intermediate species, *Tropheops sp.* "Red Cheek" (TRC). While TRC is not one of the pure species examined, it is intermediate to LF and MZ in terms of skull and jaw shape (Albertson 2008; Parsons et al. 2014), and trends toward MZ in many morphological traits (Cooper et al. 2010; Parsons et al. 2011, 2014). TRC is naturally an algae scraper, but it feeds in a very different manner to LF (Ribbink et al. 1983), and is a member of an ecologically and morphologically diverse species complex that includes species that sift and suction feed on loose debris in sediment rich habitats (Ribbink et al. 1983; Albertson 2008). TRC are highly territorial and do not acclimate to tanks of small volumes. They are therefore poorly suited for these experiments. However, this species has also been shown to segregate (and even be fixed for) MZ alleles at key craniofacial loci (Roberts et al. 2011; Albertson et al. 2014; Hu and Albertson 2014; Parsons et al. 2014, 2015). Thus, MZ is a suitable stand-in for TRC, and our hybrid population is suitable for studying the mechanisms that underlie variation in foraging performance in species along the benthic-pelagic ecomorphological continuum (Parsons et al. 2014). Based on similar body size (values reported below), we selected 25 hybrids for our study as well as 8 LF and 8 MZ. Two weeks before each set of feeding trials, we moved eight focal animals of the same species into 37 liter tanks. Each tank was divided into two halves with a perforated barrier, and one fish was housed in each half. Animals were fed *Daphnia sp.* daily for one week to allow them to acclimate to their new environment and food source. After the acclimation period, food was withheld from the fish for one week to insure maximal effort during feeding trials.

HIGH SPEED VIDEO AND DIGITIZATION

To measure feeding performance, suction feeding strikes were elicited by placing live *Daphnia sp.* in the open water column in front of each fish. *Daphnia,* instead of a more elusive prey species, were chosen for this study so that LF, a highly specialized benthic forager, would be able to successfully capture the prey. To ensure that MZ would exert maximum effort when feeding on *Daphnia*, we compared jaw protrusion in size-matched MZ fed either *Daphnia sp* or a more evasive prey item (i.e., juvenile zebrafish, ~1 cm). The extent of protrusion measured from videos did not differ between prey items confirming that our choice of prey did not significantly decrease effort in more capable suction feeders. This observation is also consistent with MZ's natural diet, which rarely includes elusive prey and mainly involves loose algae and other nonevasive planktonic prey (Ribbink et al. 1983). Prior to each trial, *Daphnia* were hand selected by size to minimize hydrodynamic differences between prey items across all feeding sequences. Strikes were recorded using a Photron high speed video system (500–1000 frames per second, 1/500-1/1500 shutter speed, f.32-f.22 aperture), mounted to a tripod. We calibrated

each set of videos using a ruler held in the plane of the feeding strike. After recording all the strikes, we removed any videos in which the animal did not strike perpendicular to the camera or in which the prey went out of view during the strike. This left us with 3–8 videos per LF (mean of 6), 6–10 videos per MZ (mean of 7), and 17–35 videos per hybrid (mean of 27). In each of these strikes, we digitized the position of the prey from the time that suction feeding began until the prey completely entered the fish's mouth using a custom Matlab script. The linear displacement of the prey between each set of consecutive frames was divided by the frame rate to give the velocity of the prey between those two frames. The maximum induced velocity of the prey over the whole strike was recorded for each suction event. This value was used to measure the suction feeding performance of the fish, as the velocity of the prey is indicative of the efficacy of suction feeding and is a direct result of the drag, pressure gradient, and acceleration reaction force created by the fish (Wainwright et al. 2007). This metric of performance is particularly effective for high throughput analysis but is susceptible to variation due to behavioral and nonstructural variables such as the position of the prey relative to the fish's mouth. However, this variation is ecologically relevant and so the metric reflects in situ prey capture ability instead of a theoretical predatory capacity. Because we seek to better understand the relationship between genotype and performance, large sample sizes are important, and thus we focus on an approach that balances an ability to measure animals both accurately and in a high throughput manner. For each animal, we used the highest maximum induced prey velocity measured in any one strike to represent the maximum suction forces that a fish was capable of achieving.

MORPHOMETRICS

Once we completed filming each set of eight animals, we anesthetized individuals with a nonlethal dose of Tricaine mesylate (MS-222) and took two lateral view photographs. In the first picture we did not manipulate the fish's jaws, allowing us to measure facial morphology and standard length (MZ: $6.15 \pm .64$cm, LF: $6.19 \pm .57$ cm, hybrid: $6.21 \pm .46$ cm). For the second picture, we used forceps to fully extend the upper jaw. This picture was used to determine jaw protrusion, a kinematically relevant trait, which was measured as the length from the flap of skin that covers the premaxillary head of the maxilla to the tip of the premaxilla (Fig. 2). Although this is not a direct measure of kinematics, it does reflect a dynamic morphological system, separating it from facial morphology and making it more kinematic in nature. We then used geometric morphometrics to measure general facial morphology in all of the fish (following Cooper et al. 2010). Using unmanipulated pictures, we digitally landmarked 11 locations on each fish (James Rohlf TPS suite) (Fig. S1). Several Matlab extensions were used to standardize the landmarks to a common

Table 1. Genotype results of our putative craniofacial loci: Hybrids were genotyped at six genes known to be important in craniofacial development.

Gene	Morphological correlate	Supporting literature	Genotype: LF/LF	LF/MZ	MZ/MZ
ptch1	Retroarticular process (in-lever of jaw opening)	Roberts et al. 2011, Hu and Albertson 2014	0	0	25
bmp4	Coronoid process (in-lever of jaw closing)	Albertson et al. 2005, Powder et al. 2014	23	1	1
lbh	Coronoid process (in-lever of jaw closing)	Powder et al. 2014	24	0	1
hsp47	Face shape	Parsons et al. 2015	0	17	8
runx2	Face shape	Parsons et al. 2015	14	11	0
sox9b	Mandible length and width (out-lever of jaw opening)	Albertson et al. 2014, in preparation	15	9	1

Hybrid genotypes only segregated at three loci (*hsp47*, *runx2*, and *sox9b*).

scale and remove allometry from the dataset (i.e., CoordGen6, Standardize6). Principal component analysis scores were calculated in PCAGen6 and used to produce deformation grids of principle component axis 1. PCA scores were recorded for all axes that explained more than 10% of the morphological variation (Table 2). To understand the morphological variation amongst the hybrids, we reran this analysis using only landmarks from hybrid animals. We refer to PC values measured across all animals as general PC and those measured only in the hybrids as hybrid PC.

GENOTYPING

We selected six genes that have previously been shown to be associated with variation in functionally relevant facial phenotypes and that segregate between LF and TRC/MZ (Table 1). Each of these has either been experimentally shown to control phenotype or has been identified as a strong candidate with QTL and F_{ST} outlier data (i.e., *bmp4*–Albertson et al. 2005 and Powder et al. 2014; *lbh* – Powder et al. 2014; *ptch1* – Roberts et al. 2011 and Hu and Albertson 2014; *hsp47*, *runx2* – Parsons et al. 2015; *sox9b* – Albertson et al. 2014 and in preparation). We extracted gDNA from fin clips taken from each hybrid while the fish was under anesthesia. PCR was used to amplify the focal allele, and the product was genotyped using either a digestion enzyme or direct sequencing.

ANALYSIS

Before any comparisons were made, we corrected jaw protrusion and performance for size by fitting a linear regression between the relevant trait and standard length across all animals. The residuals of these models were used as the size corrected values. We then compared phenotype, kinematic morphology, and performance between our focal species by fitting one-way ANOVA models comparing species with size-adjusted PC values, jaw protrusion

residuals, and maximum induced prey velocity residuals. A Tukey honest significant differences post-hoc analysis was run on these models to reveal pairwise comparisons between species. We next examined whether facial geometry and kinematic morphology were correlated as well as whether either of them predicted performance by fitting linear models between each pair of general PC1 scores, jaw protrusion residuals, and maximum induced prey velocity residuals. To fully investigate the relationship between morphology and performance, we also fit linear models between performance residuals and the scores from each PC axis. We further examined the effects of morphology and performance by using a partial correlation analysis to explore the relationship of each variable with performance independently of the other variable.

To examine the effect of genotype, we continued our analysis only using hybrid data. A linear regression model was fit between hybrid PC1 and maximum induced prey velocity as well as between jaw protrusion residuals and maximum induced prey velocity residuals. Within each of these comparisons, we then fit new linear regressions to each set of animals with a shared single locus genotype. This was repeated for hybrid PC axes that explained >10% of the variance in facial geometry, allowing us to broadly compare the relationship between function and morphology across two genotypes at each locus. To show that increased R^2 values within single genotype regressions were not simply an artefact of excluding certain individuals, we ran a repeated permutation analysis. To do this, we randomly assigned animals to one of two genotypes 50,000 times, and recorded the distribution of R^2 values. The two genotypes were always assigned so that our measured genotype frequencies were maintained. We then found the percentile of the R^2 value from each genotype specific regression compared to our repeated permutation distribution. This value represented the likelihood that the strength of the genotype's effect on the relationship between morphology and performance
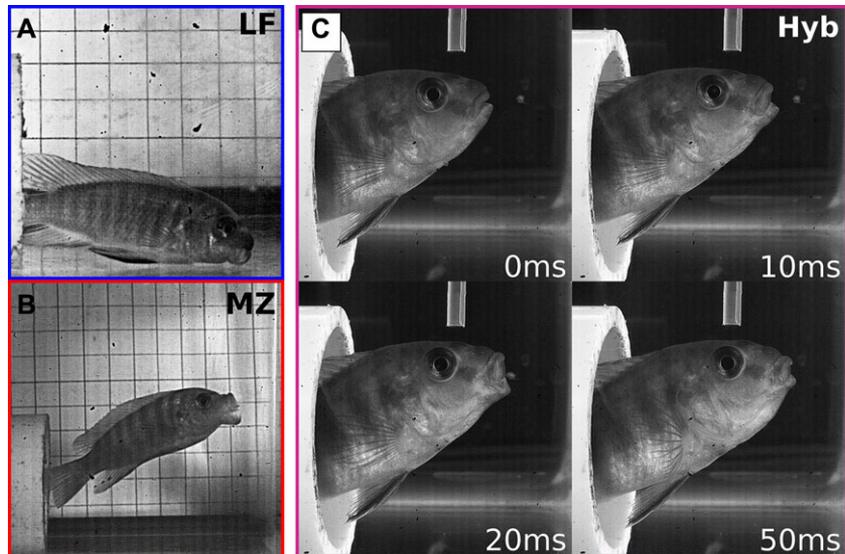
Figure 1. Fish from each group differed qualitatively in their feeding strikes. (A) LFs were generally prone to feeding near the bottom, and their jaws often protruded downwards. (B) MZ individuals were more often observed feeding limnetically, and appeared to have a more forward facing gape. (C) Hybrids also fed more often in the open water column with their jaws protruding forward. However, the gape of their mouth appeared to be smaller than in MZs.

was a result of random sampling. Finally, we fit two more linear regressions between general PC1 score and maximum induced prey velocity, one within only the MZs and one within only LFs. The LF-specific regression was refit after excluding an outlier. All analyses were run in R.

Results

CRANIOFACIAL FORM AND FUNCTION IN CICHLID FISHES

All three groups of fish in our study performed suction feeding strikes in a similar manner, by rapidly dropping the lower jaw and extending the premaxilla. While there was variation in this action between individual animals within species, there were also differences between groups in terms of the degree and direction of jaw protrusion as well as willingness to feed limnetically (Fig. 1). All three groups of fish displayed both limnetic and benthic strikes, however MZs and hybrids were generally more willing to feed limnetically, and LF preferred to feed benthically. This is not surprising, given that LF is an obligate benthic forager (Ribbink et al. 1983; Konings 2001) with a highly specialized trophic morphology to accommodate this task (Albertson and Kocher 2001; Cooper et al. 2010, 2011). Our measurements of facial morphology, kinematic morphology, and performance also yielded quantitative differences between groups. Namely, facial morphology and protrusion residuals were different between all three groups, while maximum suction velocity residuals were only significantly different between MZ and the other two groups (Fig. 2). Despite the lack of a significant

difference between the maximum suction velocity of hybrids and LF, the mean velocity of the hybrids was higher than the mean value in LFs. In all three variables, MZs had the highest mean value.

Linear regressions revealed that both our measures of facial morphology and of kinematic morphology (i.e., protrusion) predicted variation in suction feeding performance across all species. However, protrusion residuals were a much better predictor than general PC1 scores, as protrusion explained 46.6% of variation in velocity while PC1 scores only explained 19.5% of the variation (Fig. 3). No other PC axis was significantly correlated with prey velocity (Table 2), however this does not rule out the effects of other unexplored aspects of morphology. The effects of general PC1 and protrusion are not completely discrete however as there was a significant correlation between PC1 and protrusion ($R^2 = .352$). Partial correlation analysis supports this assertion. When controlling for protrusion, the relationship between PC1 and performance becomes nonsignificant ($P = .72$). This result is intuitive, as craniofacial shape variation is known to influence protrusion in acanthomorph fishes (e.g., Westneat 1990; Wainwright et al. 2004; Cooper and Westneat 2009; Cooper et al. 2016). Since much of the variation explained by PC1 involves traits directly involved in protrusion (e.g., jaw length and orientation, Fig. 2A), removing these aspects of shape results in a highly reduced degree of variability that was no longer correlated with performance. Alternatively, when PC1 is controlled for, protrusion maintains its correlation with performance ($P < .001$). This too makes sense, as protrusion is influenced by factors other than shape (e.g., Rice et al. 2008; Wainwright et al. 2015).
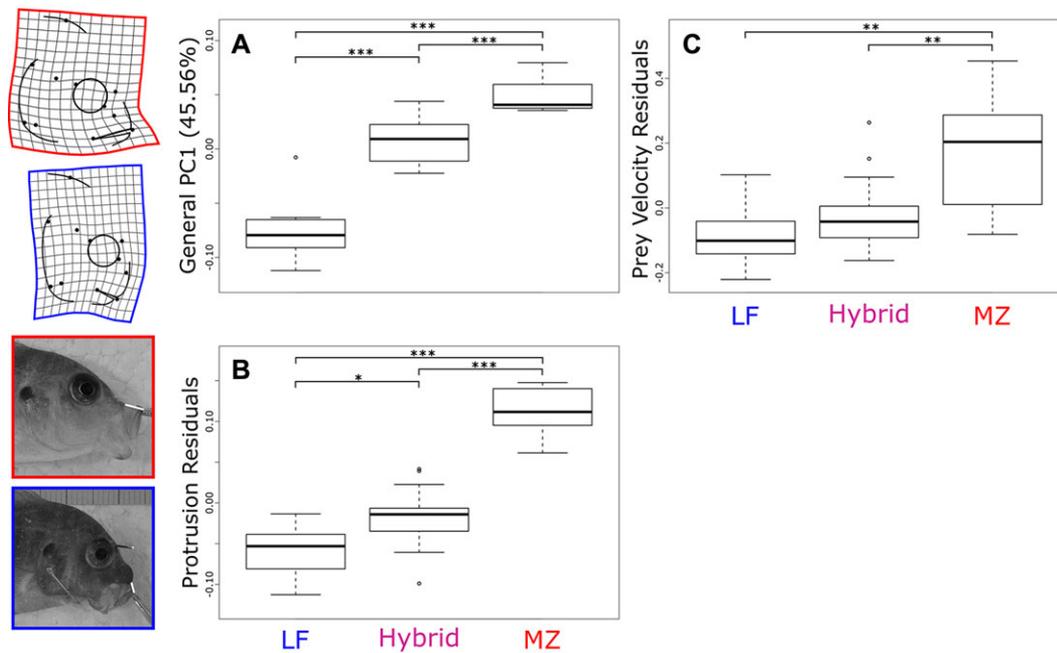
Figure 2. The three groups of fish examined in our study were quantitatively different in their facial morphology, kinematic morphology, and performance. (A) The general morphology of the face as measured by a principal component analysis (PCA) of 11 landmarks was significantly different between all species ($P < .001$ in all cases). Outlined in red is a deformation grid illustrating the shape of the face of animals with the highest measured PC1 values (i.e., MZ). In blue is a deformation grid illustrating the shape of animals with the lowest PC1 value (i.e., LF). (B) Protrusion, a suction feeding kinematic morphological trait, was also significantly different between all three groups when corrected for standard length ($P < .001$ in MZ-LF and MZ-Hybrid. $P = .011$ in Hybrid-LF). Protrusion was measured as the distance from the distal tip of the premaxilla when extended to the flap of skin that covers the premaxillary head of the maxilla. (C) Suction feeding performance as measured by the maximum induced prey velocity across all feeding strikes in any given animal was significantly different between MZ and both other groups when corrected for standard length ($P = .002$ in MZ-LF and $P = .003$ in MZ-Hybrid). Asterisks indicate Tukey's HSD significance ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$).

FORM-FUNCTION RELATIONSHIP IN HYBRIDS: A ROLE FOR CRANIOFACIAL GENOTYPE

We next sought to assess the effects of genotype on the relationship between shape and performance at previously identified craniofacial loci. Of the six loci at which we genotyped hybrid animals, three were either completely or nearly fixed for one genotype. For the linked loci *bmp4* and *lbh,* animals were fixed for the LF allele, whereas at *ptch1* they were fixed for the MZ allele. This mix of genotypes across loci is consistent with hybrid animals having largely intermediate skull shapes, kinematic morphology, and performance. The other three loci, *hsp47*, *runx2*, and *sox9b*, were found to segregate in the hybrids, although in general only two genotypes were represented in the hybrid population at each locus (Table 1). This genetic variation allowed us to assess whether genotype influenced the relationship between morphology and performance by comparing this relationship between animals with different genotypes at each locus. Note that the PC axis for hybrids is different from that depicted in Figure 2 (i.e., derived from all groups). Hybrid animals with positive PC1 scores possessed on average longer jaws (LMs 3 and 4), smaller opercles (LM 9), and shorter supraocciptial crests (LM 11). Animals

with negative scores possessed shorter jaws, deeper opercles, and deeper supraocciptial crests (Fig. 4A). Linear regressions across all of the hybrids revealed that prey suction velocity was correlated with hybrid PC1 values, albeit weakly (Fig. 4BI, $R^2 = .213$, $P = .031$). A stronger relationship emerged when animals were grouped by craniofacial genotype (Fig. 4BII–IV). While genotype at *hsp47* did not alter the relationship between performance and shape, genotype at both *runx2* and *sox9b* did. Specifically, the R^2 value increased markedly in LF homozygotes at *runx2* ($R^2 = .376$, $P = .026$) and *sox9b* ($R^2 = .456$, $P = .011$). Alternatively, there was no relationship between induced prey velocity and shape in heterozygous animals at either locus. Similar to our broader analysis, no other PC axis was strongly correlated with performance in the hybrids or any genotypic subset of hybrids (Table 2). Our permutation analysis suggested that the increased R^2 value when categorizing individuals by *runx2* genotype could be explained by the random grouping of individuals (i.e., the R^2 of .376 is at the 51.36 percentile of possible R^2 values). Alternatively, at *sox9b* the permutation test suggested that the increased R^2 value in LF allele homozygous hybrids is less likely to be caused by the random grouping of individuals (i.e., the R^2 of .456 is at the
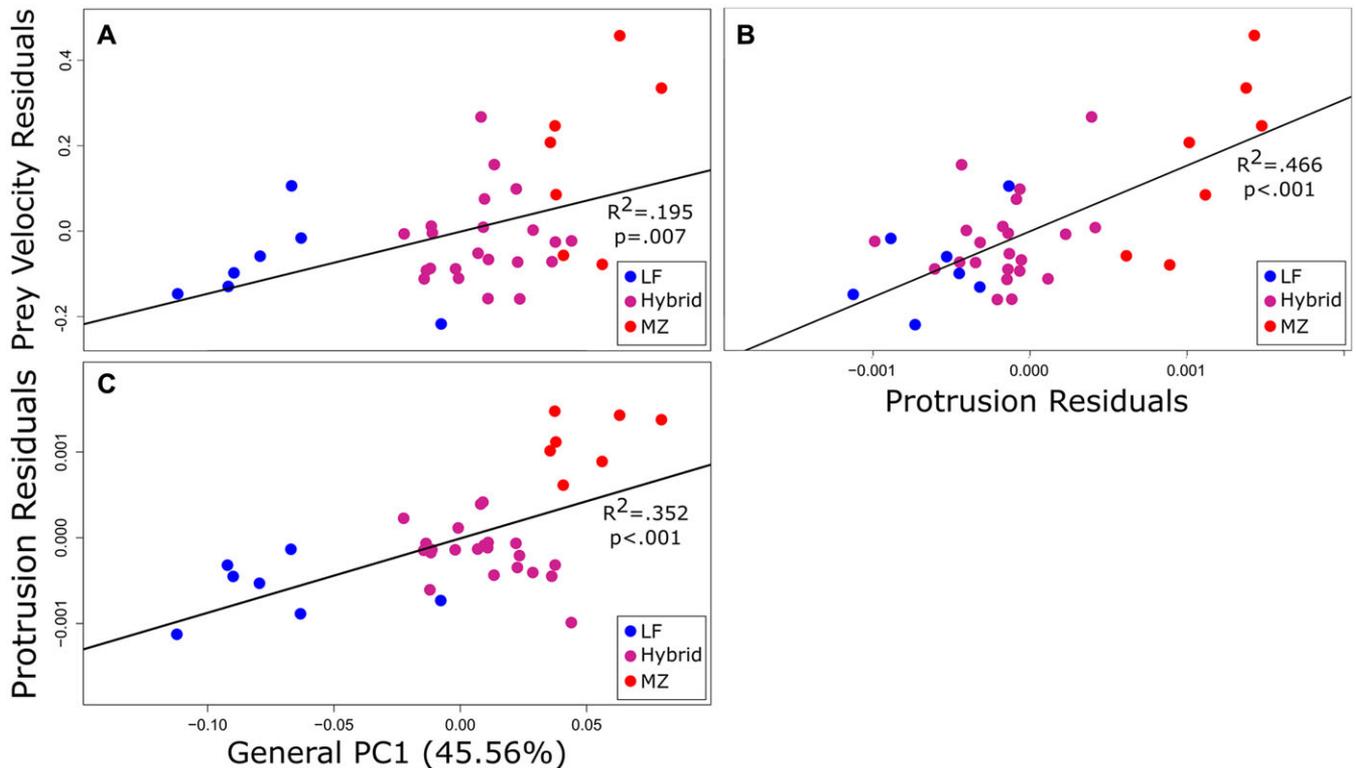
Figure 3. Our measurements of facial morphology, kinematic morphology, and performance were linearly correlated across all three groups of fish. All variables are the same as described in Figure 2. (A) Performance is significantly correlated with facial morphology, however there is relatively little explanatory power in the relationship ($R^2 = .195$). (B) Kinematic morphology is a much better predictor of performance ($R^2 = .466$). (C) The correlation between facial morphology and kinematic morphology is significant, and also has relatively good predictive power ($R^2 = .352$). All regressions are from linear models fit in R.

73.55 percentile of possible R^2 values). Thus, at both *runx2* and *sox9b,* the LF haplotype appears to influence the relationship between foraging performance and craniofacial shape, and the trend is especially robust at *sox9b.*

FORM-FUNCTION RELATIONSHIP WITHIN SPECIES

Based on the trend in hybrid animals segregating LF and MZ alleles, we expected that pure LF and MZ, which should be fixed for alternate alleles at most craniofacial loci, will show differences in the strength of the relationship between induced prey velocity and shape. As predicted, MZ showed no relationship between facial morphology and maximum suction velocity ($R^2 = .191$, $P = .327$). LF also showed no relationship between these two variables when all animals are included. However, when considering this group alone, we noted an extreme outlier in terms of craniofacial shape (Fig. 3A and C; Fig. 5B). Its shape was well within the distribution of the hybrid population, and was as close to the MZ distribution as it was to the other LF. Given the propensity of cichlids to hybridize in the wild and the ongoing segregation of polymorphisms within and between species, this morphological observation raises the very reasonable possibility that this animal is segregating a disproportionate number of MZ alleles

(unfortunately parental animals were fixed before DNA could be extracted). If we exclude this statistical outlier in facial morphology, a very strong trend is observed, which is consistent with the hybrid data ($R^2 = .687$, $P = .041$). Within LF, the correlation suggests that suction performance decreases with more extreme benthic morphologies (i.e., negative PC1 scores), and increases with more generalized morphologies (i.e., increasingly positive PC1 scores). Even if we include the outlier in our consideration of the data, the trend suggests that with more generalized morphologies performance may be high or low, but performance is consistently low with a more specialized morphology.

Discussion
EVALUATING PERFORMANCE ACROSS SPECIES

Across all three groups, we found that the relationship between our kinematically related variable, jaw protrusion, and performance was fairly strong. This makes sense in a many-to-one system, as protrusion is an emergent property of various facial linkages and therefore already reflects some morphological tradeoffs. While behavioral variation is relevant to suction feeding performance (Holzman et al. 2008), our measurement did not incorporate this, allowing us to more directly characterize the morphological

Table 2. Form-function relationship across genotypes. *P*-values reveal that only the first principal component axis of both general and hybrid PCA significantly predicts prey velocity residuals.

Species group	PC1 (45.6%)	PC2 (13.6%)	PC3 (11.1%)
All	0.007	0.728	0.187
LF	0.041	0.177	0.461
MZ	0.327	0.26	0.564

	HybPC1 (25.3%)	HybPC2 (19.0%)	HybPC3 (13.6%)	HybPC4 (12.5%)	HybPC5 (10.2%)
Hyb-LF	0.011	0.47	0.053[*]	0.743	0.327
Hyb-Het	0.582	0.504	0.486	0.679	0.095

Each principal component axis is labeled with the percentage of morphological variation that it explains. The next axis in both general and hybrid PCAs explained much less variation than the previous axis and in both cases was below 10% (general = 5.0%, hybrid = 5.8%), so all subsequent axes were excluded. Segregation in hybrid animals is based on genotype at *sox9b*. [*]Although this *P*-value is close to significance, we did not explore this axis because it likely would not hold up with the permutation test, and also because hybrid PC3 explains much less variation than hybrid PC1.

constraints placed on the system. There was also a significant relationship between morphology and performance where animals possessing longer, terminally oriented jaws and a more shallow facial profile (General PC1+) exhibited on average higher prey velocity values. Other axes of morphological variation did not predict performance in our study (Table 2). Notably, our partial correlation analysis suggests that the effect of morphology on performance appears to depend on protrusion, which underscores the idea that basic shape does not directly influence performance, but rather acts through more kinematically related valuables such as protrusion (e.g., Bergmann and McElroy 2014, and references therein).
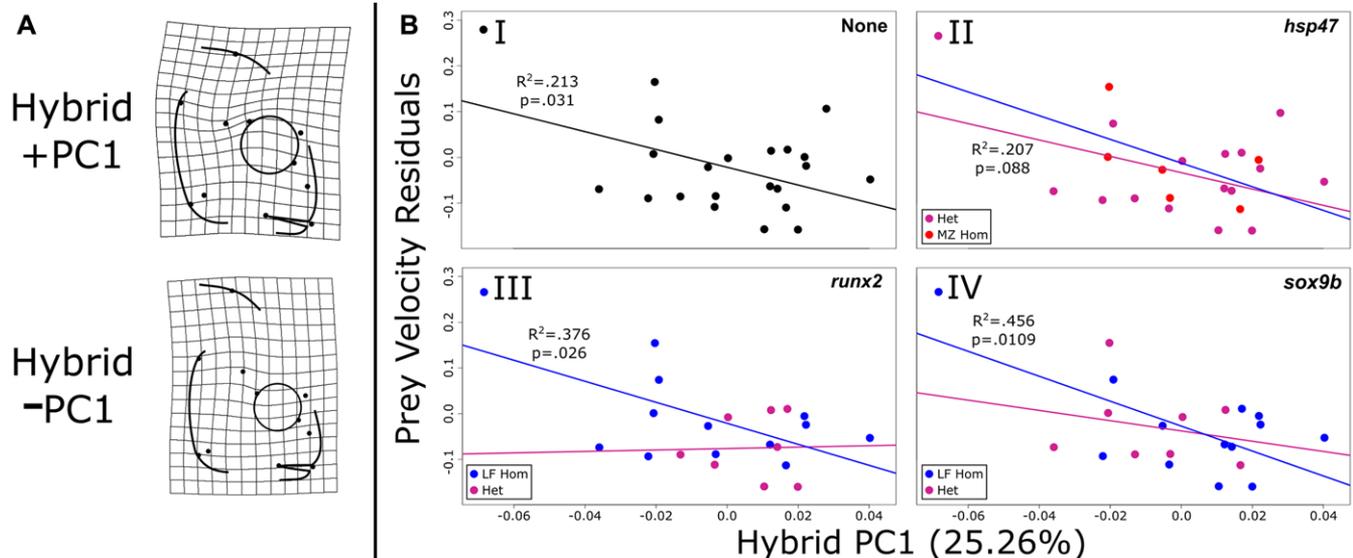
Figure 4. Within hybrid animals the correlation between facial morphology and performance is strongest in animals that are homozygous for the LF allele at certain craniofacial loci. (A) Deformation grids demonstrate the morphology of fish at either extreme of hybrid PC1 values. Note that animals with extreme negative PC values possess relatively small mouths and high supraoccipital crests, two traits predicted to enhance suction feeding. (B, I) Facial morphology versus feeding performance without accounting for genotype. (B, II) Hybrids were only found to be heterozygous or homozygous for the MZ allele at *hsp47*, and both groups lack a significant correlation between morphology and performance. (B, III) At the *runx2* locus hybrids were found to be either heterozygous or homozygous for the LF allele. In heterozygous animals, facial morphology, and performance were not correlated. However, in homozygous animals there was a stronger correlation ($P = .026$, $R^2 = .376$) than the trend without accounting for genotype. Permutation analysis suggested that this could be an artifact of subsampling. (B, IV) Hybrids were either heterozygous or homozygous for the LF allele at *sox9b*. Again, heterozygous hybrids exhibited no correlation between facial morphology and performance. However, homozygous hybrids had a much stronger correlation ($P = .0109$, $R^2 = .456$) than a regression without genotype. Permutation analysis suggests that this is not an artifact of subsampling.
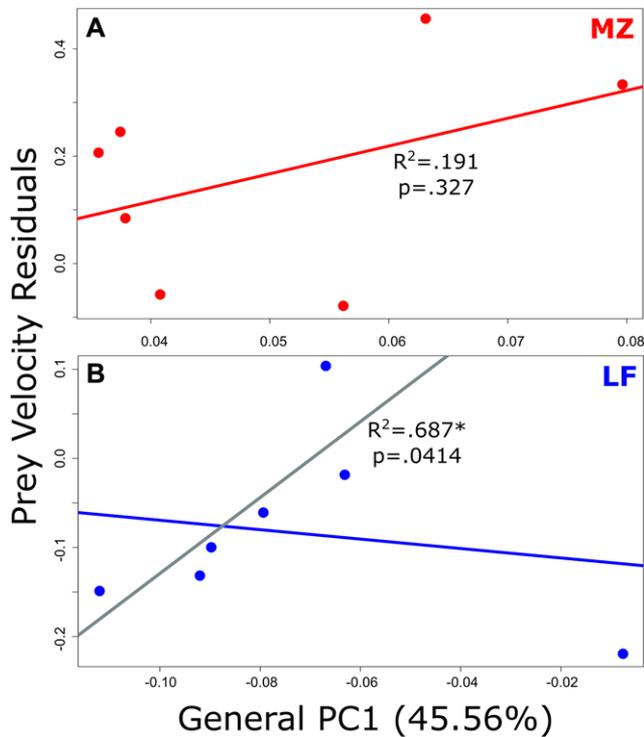
Figure 5. Correlations between facial morphology and performance in MZ and LF follow the same trend as in the hybrids. (A) There is no trend between facial morphology and performance in MZ. (B) When an outlier for facial morphology is removed, there is a strong correlation between morphology and performance in LF ($P = .0414$, $R^2 = .687$). These trends are consistent with the observation that only hybrids segregating LF alleles at craniofacial loci exhibited a correlation between form and function.

EVALUATING PERFORMANCE IN HYBRIDS

This study is unique in that we evaluated suction performance in morphologically intermediate hybrid cichlids in addition to two trophically diverse species. In doing so, we were able to create a continuum of values in all of our measurements from morphology to performance. This allowed us to more reliably investigate any correlations existing between these variables. Notably, when evaluated alone, hybrid morphology exhibited the same degree of predictive power for performance (i.e., Fig. 3A, 21.3%) compare to the correlation that included all three groups (i.e., Fig. 2A, 19.5%). This consistency is especially notable given that PC1 described different aspects of variation across all species, compared to within hybrids. Across all species, PC1 explained variation in jaw length and orientation, as well as head depth and profile. Greater prey velocity was associated with animals with terminally oriented mouths and longer, more streamlined heads/jaws. This axis of variation is consistent with previous studies in cichlids (Albertson et al. 2003; Cooper et al. 2010; 2011). Among hybrids, PC1 described different aspects of morphological variation. While all animals exhibited relatively steeply descending

skulls and subterminally directed mouths, differences were noted in jaw length, as well as depth of the opercle and supraoccipital crest. Animals with smaller jaws and deeper opercles and crests were found to induce higher prey velocities, which is consistent with the literature. Smaller mouths are expected to induce stronger flow during suction feeding (Liem 1991 and references therein). In addition, deeper supraoccipital crests should provide greater surface area for the epaxial musculature, which is important for head-lifting during suction feeding (Liem 1980). Finally, opercle depth represents the in-put link of the opercle four-bar linkage model (Westneat 1990). Variation in this system has been shown to be associated with ecological shifts among cichlid species (Hu and Albertson 2014), and increasing the length of the input link alone is sufficient to increase the predicted kinematic transmission of the system (Hu and Albertson, unpubl. data). In both analyses of the morphology-performance relationship, the predictive power was low. However, in hybrids we found that morphology became a much stronger predictor of performance when genotype was considered. In particular, hybrids with the LF/LF genotype at *sox9b* provided a robust example of genotype influencing the relationship between morphology and performance.

PUTATIVE ROLES FOR SOX9 IN BONE DEVELOPMENT, FORM, AND FUNCTION

The *sox9b* locus was examined in this study because it exhibits a strong signature of divergence when species with alternate jaw morphologies are compared (i.e., SNPs with outlier F_{ST} values > 0.95, see Table S1 in Albertson et al. 2014), and it colocalizes to QTL for variation in jaw length and width (Albertson, in prep.). The combination of these two analyses strongly implicates *sox9b* in mediating variation in cichlid jaw shape (Parsons and Albertson 2013). How it does so remains to be examined. *Sox9* is known to be a transcription factor that controls craniofacial development at multiple stages including neural crest cell induction, cartilage morphogenesis, skeletal polarity, and bone development (Yan et al. 2002, 2005; Mori-Akiyama et al. 2003; Lee and Saint-Jeannet 2011; Le Pabic et al. 2014). In humans, mutations in and around this gene lead to various craniofacial defects (Gordon et al. 2014). In rodents it has been shown to regulate the size of the coronoid process (Anthwal et al. 2015), and in birds it helps control the size and shape of Meckel's cartilage, which eventually becomes the beak (Eames and Schneider 2008). Thus, roles for *sox9* in shaping the lower jaw are extensive and conserved across vertebrates. Our data suggest further that shape changes induced by variation at *sox9b* may be adaptive by influencing the relationship between form and function.

Although our data cannot provide a specific mechanism through which *sox9b* might exert such an effect, there are several possibilities based on work in other systems. The most obvious explanation would be a morphological change linked to *sox9b*,

which in turn influenced performance. While our analysis did not reveal any such correlation, the change could have been subtle and/or unmeasured. For instance, based on work in rodents, *sox9* has been shown to mediate cartilage free bone growth in response to muscle usage (Anthwal et al. 2015). In particular *sox9*-induced ossification was noted around points of muscle insertion. Based on the molecular crosstalk between muscle and bone (Blitz et al. 2009), this local effect on bone development could lead to muscle hypertrophy, and ultimately to greater force transduction. One could envision this process occurring across the feeding apparatus in cichlids, and in particular within and around bony processes important for suction feeding, such as the retroarticular process (e.g., Westneat 1990; Roberts et al. 2011) and supraoccipital crest (Liem 1980). The effect would be relatively subtle shifts in morphology, but correspondingly large shifts in performance. Such a global effect on skeletal morphology could also serve to change the degree of phenotypic integration across the skull. If the LF *sox9b* genotype, for example, leads to greater mechanical-load induced mineralization and subsequent muscle hypertrophy, the integrated phenotypic response across the skull could result in a higher degree of one-to-one mapping of form to function. Alternatively, if the MZ allele leads to a less sensitive bone response, the potential for many-to-one mapping should be greater. While these scenarios remain speculative, they are consistent with known roles for *sox9*, as well as differential bone sensitivities in cichlids with alternative craniofacial genotypes (Parsons et al. 2016). Thus, we consider these to be plausible and testable predictions.

Although no other locus yielded such a strong trend, other analyses indicated that animals with more LF alleles at craniofacial loci displayed a more significant and predictive trend. This was also true in both purebred species, with MZs displaying no trend at all and LF displaying an exceptionally strong trend once an obvious outlier was removed. These data suggest that the evolutionary origin of a craniofacial allele can influence the relationship between morphology and performance, and that few genotypic changes can be the difference between intraspecific one-to-one mapping and intraspecific many-to-one mapping. Recent work in fish suction feeding functional morphology has generally focused on creating more complex models to more accurately explain the relationship between morphology and performance in this system (e.g., Kane and Higham 2015). Our results suggest that relatively simple genetic considerations can also be insightful, and moving forward argue for a more extensive genome-wide approach to linking genotype to performance.

ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

In addition to providing a more powerful predictive model of performance based on morphology, our data have several ecological and evolutionary implications. For example, they underscore the idea that a species displaying many-to-one mapping should exhibit greater evolvability than a species with one-to-one mapping in the same functional system (Alfaro et al. 2005). In cichlids, a generalist like MZ that exhibits many-to-one mapping should be more capable of adapting its morphology, both evolutionarily and plastically, in response to other selective pressures without negatively impacting suction feeding performance. Alternatively, since LF seems to possess a more genetically mandated one-to-one mapped morphology, it is expected that any change in facial morphology could negatively affect its suction feeding performance. If we put our results in the context of each species' ecological role, the generalist allele (i.e., MZ) serves to maintain performance despite other morphological variation, while the specialist allele (i.e., LF) corrals that species into its niche. This fits in the theoretical framework that generalists should be more flexible in their use of complex functional systems (Williams 1986; Sanderson 1991; Nemeth 1997; Wainwright et al. 2001; Lawton et al. 2012; Kane and Higham 2015), and hints at a possible mechanism for this idea.

In addition to changing selective pressures at an organismal level, this genotypic effect on the relationship between morphology and performance could also change the magnitude of selection on other regions of the genome. For instance, if suction feeding performance is dependent on facial morphology in animals with the LF halpotype at *sox9b*, then other unlinked craniofacial loci should face relatively stronger selective pressures in populations with a higher frequency of this allele. Alternatively, in populations where the MZ allele is at a high frequency, other craniofacial genes would not be expected to face strong selective pressure because feeding performance is less correlated with morphology. In this way, a single locus genotype has the potential to dramatically alter selection on several other unlinked loci, which in turn could have a pronounced impact on multilocus evolution. It is worth noting that this is not a hypothetical scenario, as many Lake Malawi cichlid lineages (e.g., many *Tropheops* species) with more generalized feeding morphologies are segregating both LF and MZ alleles at different frequencies (Roberts et al. 2011; Hu and Albertson 2014; Powder et al. 2014).

In conclusion, we acknowledge the exquisitely complex relationship between morphology and performance, but suggest that through the incorporation of genetic data we might be able to gain a deeper understanding for how this relationship arises over development and evolves over time. This role of genotype in functional morphology appears to be novel, and we believe such work warrants further exploration.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Animals were landmarked at 11 craniofacial landmarks following Cooper et al. 2010. These landmarks were used for principal component analyses. Points correspond to (1) posterior curvature of the interopercle, (2) posterior curvature of the preopercle, (3) posterior tip of the maxilla, (4) anterior tip of the premaxilla, (5) nares, (6,8) opposite points on the orbital bone bisecting the eye horizontally, (7) closest point of the facial profile to the eye, (9) point of articulation between the opercle and neurocranium, (10) posterior curvature of the opercle, and (11) insertion point of the first dorsal fin ray. Image is a hybrid animal.