



SYMPOSIUM

Genes, Morphology, Performance, and Fitness: Quantifying Organismal Performance to Understand Adaptive Evolution

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Synopsis To understand the complexities of morphological evolution, we must understand the relationships between genes, morphology, performance, and fitness in complex traits. Genomicists have made tremendous progress in finding the genetic basis of many phenotypes, including a myriad of morphological characters. Similarly, field biologists have greatly advanced our understanding of the relationship between performance and fitness in natural populations. However, the connection from morphology to performance has primarily been studied at the interspecific level, meaning that in most cases we lack a mechanistic understanding of how evolutionarily relevant variation among individuals affects organismal performance. Therefore, functional morphologists need methods that will allow for the analysis of fine-grained intraspecific variation in order to close the path from genes to fitness. We suggest three methodological areas that we believe are well suited for this research program and provide examples of how each can be applied within fish model systems to build our understanding of microevolutionary processes. Specifically, we believe that structural equation modeling, biological robotics, and simultaneous multi-modal functional data acquisition will open up fruitful collaborations among biomechanists, evolutionary biologists, and field biologists. It is only through the combined efforts of all three fields that we will understand the connection between evolution (acting at the level of genes) and natural selection (acting on fitness).

Introduction

Forty years ago, following a symposium on snake adaptive evolution, Stevan Arnold published the seminal paper “Morphology, performance, and fitness” (Arnold 1983). In this paper, he established the idea that organismal performance is the mechanism by which selection acts on morphology, thereby developing a novel mechanistic structure through which researchers could understand and quantitatively study the role of morphological innovations in adaptive evolution. Arnold’s emphasis on quantifying “performance,” the execution of a task, has proven to be highly influential. Among other things, it has directed the attention of comparative biologists to

the importance of quantifying the effects of trait variation on the ability of an organism to complete necessary tasks within its environment. Arnold used snake swallowing ability as an example of performance in his 1983 paper, but since then researchers have used a wide variety of performance metrics such as maximal running speed, prey capture success rate, and maximal escape velocity. More recently, as we have entered the modern genomic era, the focus has expanded beyond Arnold’s schema to include a genetic perspective (Sanger and Rajakumar 2019; Bomblies and Peichel 2022). For example, studies have begun examining the adaptive value of individual loci, thereby enabling us to gather

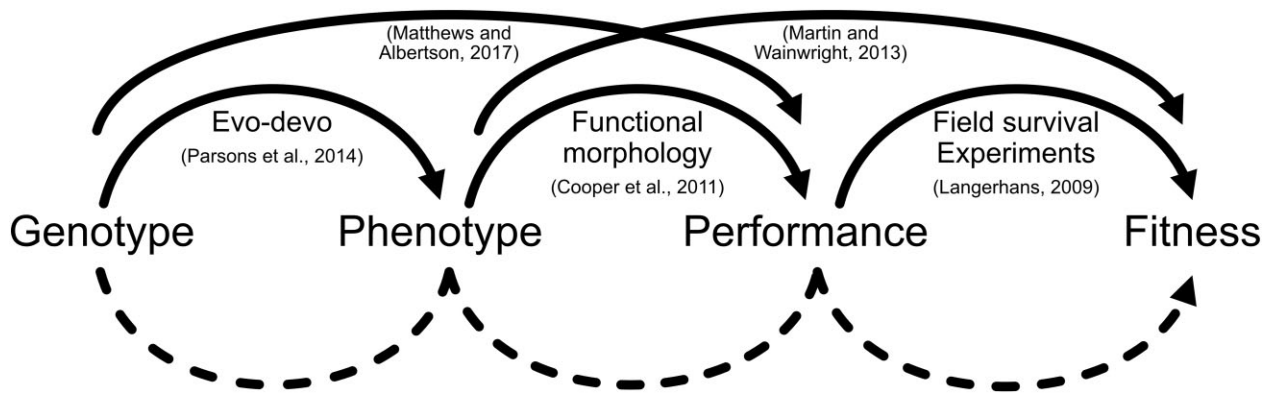


Fig. 1 Genotype is connected to fitness through phenotype (such as morphology) and performance. Several research areas correspond to portions of this path, but studies that simultaneously examine all four elements (dashed line) are rare and restricted to functionally simple systems. Examples of studies that address particular connections are given for each path.

data directly connecting evolution (acting at the level of the genome) to natural selection (acting at the level of fitness).

Many studies have attempted to show that a locus is adaptive based on the presumed functional implications of the associated phenotype or by using population genetics to find statistical signatures of selection (Nielsen 2005; Horscroft et al. 2019). However, neither of these approaches proves adaptive value, even when used in tandem (Nielsen 2009). The only way to directly show how a gene affects fitness is to directly connect the two by integrating across multiple levels of biological organization (Dalziel et al. 2009). While the exact mapping of gene to fitness will vary based on the study system (Dalziel et al. 2009; Barrett and Hoekstra 2011; Irschick et al. 2013), by adding genotype to the beginning of Arnold's schema we obtain the full research program needed to study adaptation experimentally: genotype → morphology → performance → fitness (Fig. 1).

This paradigm has been successfully applied in several systems to show the adaptive value of various traits (table 1 in Barrett and Hoekstra 2011; Blob et al. 2023). Some studies have gone as far as documenting how allele frequencies change in response to selective pressures, thus serving as direct examples of evolution happening in the wild. However, most of these studies have been done in systems for which there is a relatively simple relationship between phenotype and performance (Irschick et al. 2013; Bomblies and Peichel 2022). Using such examples of traits that map one-to-one from phenotype to fitness, meaning that a single phenotypic measurement can predict performance and fitness in a given environment, makes it significantly easier to connect phenotype to fitness (Irschick et al. 2013). Unfortunately, few traits operate on such simple terms (Wainwright 2007; Higham et al. 2021) and therefore there is much to be learned about how

genes connect to fitness in the vast majority of phenotypes for which the map of phenotype to fitness is more complicated.

Development of functional traits

Evolutionary developmental biology, “evo-devo,” is the study of variation during development and how this variation leads to diverse evolutionary endpoints (Fig. 1). There has been a recent push in evo-devo to incorporate functional models in our understanding of how evolution proceeds, giving more attention to concepts such as modularity and plasticity (Breuker et al. 2006; Sanger and Rajakumar 2019). This integration of fields has led to the discovery of the genetic basis of many traits implicated in adaptive divergence (Albertson et al. 2003; Albertson and Kocher 2006; Parsons et al. 2014, 2016; Jamniczky et al. 2015) and an expanded ability to recreate evolutionary variation through genetic and developmental manipulations (Parsons et al. 2014; Martin et al. 2016; Klaassen et al. 2018; Livraghi et al. 2018; Wucherpfennig et al. 2019; Clark et al. 2022; Urban et al. 2022). Notably, we have learned that many complex traits are controlled by a few loci of large effect and many loci of small effect (Orr 2005; Young et al. 2014; Kratochwil and Meyer 2015; Peichel and Marques 2017; Schluter et al. 2021). This means that the genetic basis of complex traits is highly complex but ultimately tractable, and it highlights the fact that the functional effects of individual loci are likely to be small and methods to quantify performance variation need to be further developed to incorporate increased sensitivity and precision.

In addition, because selection acts on the phenotype instead of directly on the genotype (Lande and Arnold 1983; Irschick et al. 2008), it is important to consider the functional consequences of variation at

higher developmental levels such as genetic pathways and hormonal cascades. Each of these represents an exciting alternative focus for functional studies of evolutionarily relevant variation since they carry the potential for larger phenotypic effect sizes than would be expected from individual alleles. For example, experimental modulation of canonical Wnt signaling in cichlid fish has been shown to be sufficient for species to phenocopy the cranial morphology of ecologically divergent clades (Parsons et al. 2014; Powder et al. 2015). Similarly, variation in thyroid hormone is sufficient to alter fin morphology (Hu et al. 2020), body stiffness (Parikh et al. 2022), swimming kinematics (Parikh et al. 2022), craniofacial morphology (Keer et al. 2019, 2022), and feeding kinematics and performance in zebrafish (McMenamin et al. 2017; Galindo et al. 2019; Conith et al. 2022). Since a great deal of evolution is driven by recombination of standing variation in the genome, it is possible for multiple alleles affecting the same pathway or hormone to act in concert and elicit a larger response than they would individually. Therefore, studying these higher levels of organization is evolutionarily relevant and may better reflect the functional changes that selection is acting on.

By and large, the functional effects of morphological variation are studied at the adult stage, but both the developmental acquisition of form and the selective pressures acting on these traits occur at many stages throughout ontogeny. Thus, it is necessary to identify and quantify meaningful function at relevant stages of development, which can vary dramatically with life-history strategy. For example, in organisms adopting a precocial onset of locomotor capacity, we see that due to disproportionate changes in muscle growth, escape performance peaks at the juvenile stage before decreasing into adulthood (Carrier 1983, 1996; Gibb et al. 2006; Dial et al. 2016). Thus, the genetic and hormonal basis of muscle growth is likely most relevant at juvenile, not adult stages in precocial species. In contrast, organisms that assume an altricial life-history strategy, where adults are responsible for protecting the developing young, more often display continuous growth of the locomotor anatomy throughout ontogeny (Dial and Carrier 2012; Dial et al. 2012). Continuous growth of morphological elements indicates that the trait of interest has experienced substantial change between juvenile and adult stages, possibly even entering novel morphospace in the adult (Dial and Carrier 2012). Thus, connecting trait-specific variation to performance throughout ontogeny is complicated by rapidly changing morphology and by diverse life-history strategies, but provides necessary context of the selective background on which functional mechanisms evolved.

Functional morphology of complex traits

The relationship between morphology and function (Fig. 1) can vary in complexity from relatively simple relationships like fish lower jaw lever ratios, determining the speed and force of jaw motion (Thompson et al. 2017), to highly complex ones such as fish's cranial kinesis during prey capture and transport (Olsen et al. 2019, 2020). While simple traits are rare in nature and therefore not widely representative of morphological evolution (Irschick et al. 2013), excessive trait complexity can render a system intractable for high-throughput functional analysis. Therefore, it is necessary to study traits that are approachably complex.

One such class of traits that have been well studied are four-bar linkages, termed “many-to-one” mechanisms because many different phenotypes can lead to the same functional output (Wainwright et al. 2005). Although individual morphological elements in these systems can have high mechanical sensitivity and evolve similarly to one-to-one systems (Anderson and Patek 2015; Muñoz et al. 2017, 2018; Muñoz 2019), the relative functional importance of each individual element tends to be reduced as mechanical complexity increases (Alfaro et al. 2004; Collar and Wainwright 2006; Thompson et al. 2017). This means that the evolution of these systems is less predictable as selection for mechanical output is weaker on the individual elements and because there are multiple solutions to the same functional needs (Anderson and Patek 2015; Higham et al. 2016; Thompson et al. 2017; Muñoz 2019). The morphological evolution of a complex trait is therefore more dependent on genetic drift and the developmental underpinnings of the trait (Kingsolver and Huey 2003; Dalziel et al. 2009; Bright et al. 2016; Muñoz 2019; Sanger and Rajakumar 2019). However, this does not imply that the evolutionary outcomes are not adaptive in complex traits. Rather, it means that in order to understand the evolution of complex traits, we must build mechanistic models that incorporate as much contextual information as possible (Koehl 1996; Higham et al. 2021).

Fitness measurements

One key aspect of connecting evolution to natural selection is understanding how variation in performance affects an organism's fitness (Fig. 1). Although measurements of fitness are largely outside the scope of this perspective, we note that experimental methods for ascertaining fitness in the field have greatly improved since Arnold's (1983) paper. Modern studies often use growth, survival, or reproductive success to represent fitness, and connect these metrics to function or functional morphology (Svensson and Calsbeek 2012; Martin and Wainwright 2013; Arnegard et al.

2014; Laughlin and Messier 2015; Martin and Gould 2020; Patton et al. 2022). This has been enabled, in part, by the continued advancement of inexpensive genetic testing that can establish pedigrees among field populations (López-Sepulcre et al. 2013; Reznick et al. 2019) and the increased usage of field-based adaptive landscapes (Martin and Wainwright 2013; Martin and Gould 2020). Therefore, although these studies are often difficult and labor-intensive, it is currently feasible to experimentally measure fitness in wild organisms.

Choice of model organism

Model systems are often chosen for their tractability in a particular field, meaning that selecting a model organism can be challenging for highly integrative research programs such as the one introduced by Arnold (1983). In addition to the experimental tractability of the organism, it is also important to choose a clade that has previously been well studied. One consistent feature of the research program discussed here (Fig. 1) is that biological context is needed at each step to help guide our questions and to differentiate signal from noise. For example, evolutionary context is needed to understand which traits are likely to be under selection; developmental context is essential to understand the potential effects of any genomic change; life-history context is necessary to understand how selective pressures change over ontogeny; behavioral context is needed to understand how morphology affects performance; and environmental context is vital to understand how performance affects the fitness of an individual. Additionally, when dealing with multivariate data, it is important to avoid multiple comparisons (also known as “p-hacking” or “researcher degrees of freedom”), which can be accomplished by using prior knowledge to pre-form hypotheses and statistical models (Gelman and Loken 2014). Therefore, the best way to successfully connect genotype to fitness through morphology and performance is to do so in a system that has been well studied in a wide variety of fields.

We believe that fish represent an excellent model for these highly integrative studies. Fish have long been recognized for their research potential and incredible diversity (Herre 1922), and many of the canonical model systems in organismic and evolutionary biology already come from piscine clades. To name just a few, zebrafish are a preeminent model of genetics and development (Driever et al. 1994; Meyers 2018); stickleback and cichlids are major models of evo-devo and adaptive radiation (Kocher 2004; Cresko et al. 2006; Salzburger 2018; Reid et al. 2021); wrasses, sunfish, cichlids, and sticklebacks are all important models of locomotor and feeding functional morphology (Wainwright 1996; Lauder

2015; Wainwright and Longo 2017); and guppies, stickleback, and pupfish are all tracked in the wild to determine fitness (Martin and Wainwright 2013; Arnegard et al. 2014; Reznick and Travis 2019; Schluter et al. 2021). Additionally, zebrafish are one of the seven major model organisms recognized in the NIH Alliance of Genome Resources (Bradford et al. 2022). Finally, fish ecology is well studied because of both biological and economic importance. Therefore, fish are uniquely situated as a clade that has ample background knowledge and experimental resources to accomplish the research program outlined in Fig. 1. While there are many other indispensable model systems that this research program could be applied to, here we focus our discussion on its application to studies of fish genetics, morphology, performance, and fitness.

Linking evolutionary developmental biology to performance

Many methods that have been established in functional morphology are intended to measure the relatively large differences seen in interspecific comparisons. These studies tend to examine large-scale differences between clades, for example, differences in body shape relating to swimming ecology (Claverie and Wainwright 2014; Friedman et al. 2020, 2021; Larouche et al. 2020). Given the relatively large magnitude of effect sizes in these comparisons, data noise can be high without affecting the statistical significance of the results. However, advancing our understanding of the relationship between fitness and genotype in animal evolution hinges on being able to measure the functional implications of smaller-scale *intraspecific* variation. Some studies have begun to address such questions (reviewed in Higham et al. 2016), although few of these examples have been linked back to genomic mechanisms. Indeed, past attempts to incorporate a mechanistic understanding of the adaptive value of individual loci have been hindered by technical limitations (Dalziel et al. 2009). And while novel methods have been utilized to attain high-resolution functional measurements in the context of ecology and ontogeny (Herrel and Gibb 2006; Gemmill et al. 2014; Higham et al. 2019; Dial and Lauder 2020; Friedman et al. 2020; Olsen et al. 2020; Ferry and Higham 2022; Garner et al. 2022; Lee et al. 2022), this approach has not been extended to the functional significance of genomic variation.

Altogether, we believe that evolutionary biologists currently have at hand the necessary methodologies needed to find the genetic basis of complex traits and to understand how functional variation leads to changes in individual fitness. Therefore, the primary remaining hurdle to fully connecting genotype to fitness in

complex systems (Fig. 1) is the development and utilization of new methods to allow functional measurements of the complex phenotypes underlying the microevolutionary variation that has been uncovered by evolutionary developmental biologists. Only with this advancement will we be able to quantify their adaptive value and thereby expand our understanding of evolutionary dynamics to a much broader swath of phenotypes. Here, we present three examples of how such an approach might work when studying fish, emphasizing their potential to improve the accuracy and precision of performance measurements in response to genetic and developmental manipulations. We believe that if functional morphologists and biomechanists can continue to develop novel methodologies to allow measurement of intraspecific performance variation, we can fulfill the promise of the Arnold (1983) research plan to understand the adaptive evolution of morphology and functional traits.

Methods for intraspecific performance analysis

Since the publication of the Arnold (1983) paper, tremendous technical advances have occurred in the quantification of organismal biomechanics, function, and performance that promise to greatly enhance our ability to measure how both individual traits and combinations of phenotypic traits are used by animals. The advent of high-speed digital video, miniature accelerometer tags, animal-mounted cameras, remote field recording capability for sound and video, and the ability to quantify air- and water-flow patterns for quantification of feeding and locomotor performance all enable us now to measure performance metrics that were previously inaccessible. Additionally, new statistical, analytical, and computational approaches are now available that were not available in 1983 to researchers interested in measuring performance. Here, we present three methodological frameworks that we have used to study locomotion and feeding in fish. Additionally, we show that in some cases several of these methods can be combined to glean an even deeper understanding of intraspecific performance variation. We believe that methods such as these are well poised to allow for the large sample sizes and high precision that will be needed to detect the functional effects of genetic and developmental variation.

Structural equation modeling

When Arnold (1983) proposed studying performance as the key link between phenotype and fitness, he suggested path analysis as a quantitative framework with

which to formulate adaptive questions. This method, and the more generalized form known as structural equation modeling (SEM), provides a statistical framework with which an entire hierarchical structure connecting many variables can be simultaneously evaluated. Through this, SEM is able to make causal inferences more confidently, which is extremely valuable when estimating the adaptive value of a trait. Evolutionary biologists were initially eager to utilize SEM, but its use has been relatively scarce since then. Meanwhile, the fields of ecology and molecular biology have continued to use these methods to great effect (Kaplan and Phillips 2006; Langerhans 2009; Eisenhauer et al. 2015; Fan et al. 2016; Igolkina and Samsonova 2018; Grotzinger et al. 2019).

Functional morphology and biomechanics are based on the idea that biological systems can be explained using classical mechanics and first principles of physics. This means that a hierarchical understanding of the relationship between interacting biological structures is an implicit part of the field. Therefore, we believe that SEM is a natural statistical framework to utilize in these fields. We suggest two main advantages of the technique that are particularly apt for the biomechanical analysis of intraspecific variation and present an example of how SEM can yield insight into the relationship between form and function.

First, SEM allows the use of intermediate variables, either in series or in parallel, to help find correlations that would otherwise not be observable. Unlike classical multiple-regression methods, SEM allows one to integrate their knowledge of the mechanistic relationship among all the variables in the statistical model. This means that variation which other methods would only see as noise is instead accounted for by these intermediate variables, giving us stronger estimates of statistical effects that would otherwise be obscured. A second major advantage of SEM is the ability to include latent variables, variables that are inferred from other measured variables. Oftentimes functional morphologists have a strong mechanistic understanding of a given system but are unable to measure all of the components. By measuring several proxy variables, we are able to model unmeasured traits within a larger model of the whole system. In addition to estimating traits that are difficult to measure experimentally, latent variables can be used to estimate more abstract behavioral traits that are expected to affect performance. For instance, it is well established that feeding performance in fish decreases as they reach satiation. Unfortunately, motivation is not a discretely measurable trait that is easily included in many statistical models. By measuring related variables such as the amount of food already eaten and time to

approach the food, motivation can be estimated and included in SEM analyses.

The hierarchical nature of SEM, while carrying many advantages, also brings an increased risk of statistical multiple comparison problems. As the number of variables in a model increases, the possible number of path topologies increases exponentially. Therefore, even a well-intentioned researcher who views the data before defining the structure of their model will likely be able to find a significant effect. The best way to avoid such a type I error is to use prior knowledge of the focal organism to create hypothesized structural models and then to test only these models after collecting the data. This approach ensures that the research question is well grounded in the functional, ecological, and evolutionary history of the organism, and again highlights the importance of utilizing a study system that has been well studied to minimize the chance of mistaking random statistical significance for biological significance.

Here, we demonstrate the efficacy of this method using a study on the effect of immune responses on escape performance in threespine stickleback (*Gasterosteus aculeatus*; Matthews et al. 2023). Freshwater stickleback are often infected with a tapeworm parasite that can be lethal. While many populations have an immune response in which they fibrose the tissues in their coelom, some populations actively suppress this response (Vrtílek and Bolnick 2021; Weber et al. 2022). This led us to ask whether there is a locomotor cost associated with the fibrosis immune response that could help explain the suppressed response. We hypothesized that inducing fibrosis would affect escape performance by changing the animal's behavior and their body stiffness (Fig. 2). Then, we induced fibrosis in experimental fish with an immune adjuvant and measured the severity of fibrosis, body stiffness, and body curvature during an escape. We also measured both linear and angular escape velocities to represent escape performance. We compared these values to fish that were injected with saline as a control.

We first attempted to connect fibrosis severity to escape performance using multivariate linear regressions. These models indicated that fibrosis does not significantly predict either linear ($P = 0.851$) or angular ($P = 0.509$) escape velocities. However, when we conduct SEM analysis with the same variables (Fig. 2), we find that there is a significant effect of fibrosis on linear velocity during an escape behavior. Interestingly, we found that fish with fibrosis have improved linear escape performance compared to control fish. Furthermore, we can separate the causal paths from fibrosis to performance to learn that passive body stiffness only plays a small part in determining escape performance. Instead,

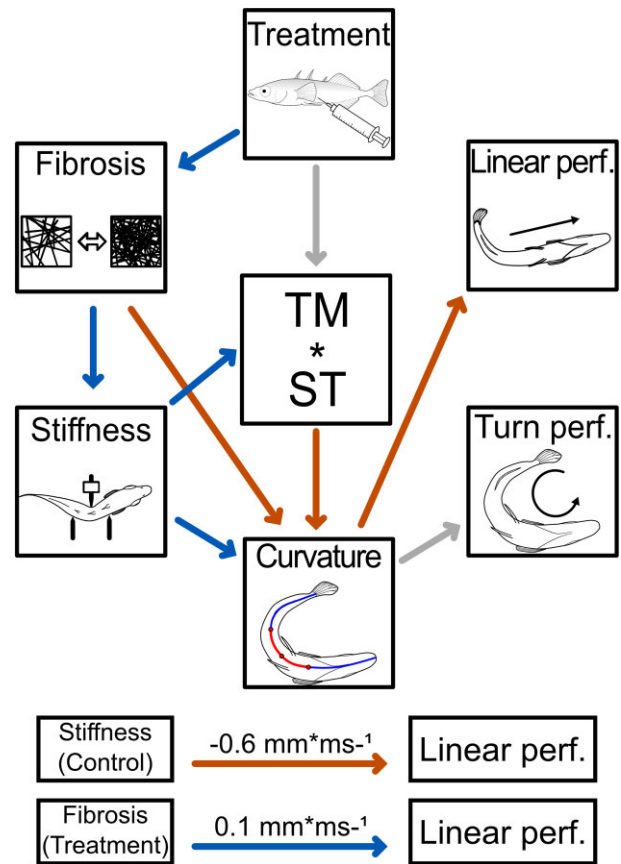


Fig. 2 A structural equation model used to find the effect of fibrosis on C-start escape performance in stickleback fish, both in terms of angular velocity at the beginning of the escape and linear velocity at the end of the escape. We hypothesize that fibrosis affects escape performance by changing body curvature, both through increased body stiffness and through behavioral changes (represented as a direct arrow from fibrosis to curvature). Additionally, the effect of stiffness on body curvature was regulated by an interaction with the treatment, represented by the box labeled “TM*ST.” We found no effect of this treatment on angular velocity but did find that control fish displayed variation in body stiffness that accounted for as much as a -0.6 mm ms^{-1} change in linear velocity. Conflictingly, fish with induced fibrosis had an increase of 0.1 mm ms^{-1} on average, despite having stiffer bodies overall. Blue arrows represent positive effects and orange arrows represent negative effects. Data from Matthews et al. (2023).

we found that other behavioral mechanisms such as active modulation of body stiffness cause the increased performance. This example shows how SEM allows us to build in a mechanistic understanding of the relationship between our variables to uncover an effect that would be unmeasurable with any single regression.

Biological robotics

One technical approach that was not available 40 years ago (Arnold 1983) for evaluating performance and testing hypotheses about the relationship between mor-

phology and performance is the use of robotic models of organisms [recently discussed in the context of comparative biology by [Lauder \(2022\)](#)]. Organismal phenotypes are complexly interconnected and altering an individual element tends to affect many other components. Due to pleiotropic genetic effects during development and the mechanical connections among the components of complex biochemical systems in organisms, it is difficult if not impossible to isolate and manipulate an individual element in an organism to determine its effect on performance. For example, if we are interested in the performance effects of changes in fish body stiffness during ontogeny or differences in body stiffness among fish species, it is effectively impossible to experimentally alter stiffness without simultaneously changing many other aspects of the phenotype and altering connections among elements. Comparative phylogenetic studies of body stiffness do not resolve this problem, as they are confounded by the many other uncontrolled changes that accompany evolutionary diversification.

The use of robotic systems has been proposed as a comparative method that can be used to both tease apart otherwise correlated elements and to directly measure critical performance variables such as the efficiency and energy consumption of alternative morphologies ([Lauder 2022](#)). Robotic systems allow the experimenter to modify only the trait of interest (impossible in living animals), and to propose hypotheses about complex phenotypic systems that can then be tested in living organisms, an approach termed “robotics inspired biology” ([Gravish and Lauder 2018](#)). Furthermore, using robotic models allows the direct measurement of energy use, efficiency, and key performance metrics such as the forces produced by moving mechanical systems.

One recent example that illustrates this approach is the use of mechanical models of the fish caudal region that vary in stiffness at the base of the tail ([Matthews et al. 2022](#)). By using multi-material 3D printing to produce physical models of the tail region of fish that vary in stiffness and then actuating these models with a robotic system in which we could measure swimming forces and efficiency, it was possible to demonstrate the complexity of the interaction between stiffness and swimming performance; there was no one stiffness that maximized two key performance metrics (efficiency and thrust, [Fig. 3](#)). Each was maximized at different combinations of movement and stiffness. Given the complexity of organismal mechanics and interactions among components in the phenotype, we can expect that there may be many such tradeoffs between performance metrics ([Careau and Wilson 2017](#)), and it may not be possible to simultaneously maximize multiple performance variables. Such tradeoffs are extremely

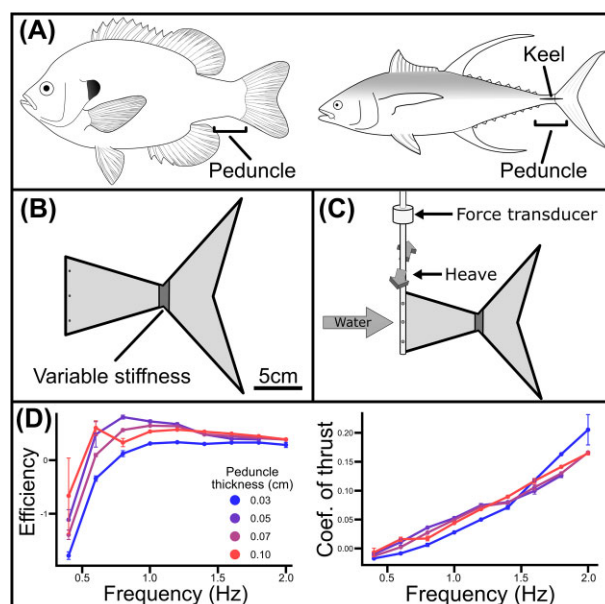


Fig. 3 Robotic methods allow us to measure small changes in performance by granting fine scale control over the motion patterns of our models and allowing us to record the resulting differences with high-resolution force transducers. In this example, natural variation in stiffness of the caudal peduncle in fish (A) is recreated in a simple biomimetic model (B), then actuated using a robotic apparatus (C). This allows small differences in performance metrics such as efficiency and thrust to be measured and compared across different models at various swimming speeds, demonstrating that there is no one set of parameters that maximizes all performance metrics (D). Data from [Matthews et al. \(2022\)](#).

challenging to demonstrate in living animals but can be investigated and tested directly using mechanical models ([Fig. 3](#)). Results such as this one that are obtained using robotic systems show how complex interactions between phenotypic elements can be thereby accentuating how challenging it is to tease apart causal relationships between genotype, phenotype, and performance.

Simultaneous multi-modal data collection

Many integrative studies of organismal performance are conducted by independently measuring separate levels of biological organization (e.g., morphology, kinematics, and performance), then combining them during the analysis. This has the advantage that methods from individual fields can be used as-is but also creates a situation in which the correlation between each level has not been directly measured. It would often be preferable to measure multiple performance metrics simultaneously, though this can be technically challenging.

For example, we know that during suction feeding in fish, the kinematics of the jaws are highly consequential in determining feeding performance. Performance, in turn, is often measured as suction pressure or the result-

ing flow velocity (Ferry-Graham and Wainwright 2002; Higham et al. 2006; Wainwright et al. 2007; Holzman et al. 2012; Matthews and Albertson 2017). Both of these performance metrics can be measured using particle image velocimetry (PIV), a method in which neutrally buoyant particles are suspended in the water, illuminated, and then tracked in the resulting videos. While there are multiple ways to illuminate the particles, including a laser sheet (Drucker and Lauder 1999), infrared backlighting (Lee et al. 2022), or fiber optic visible lights (Dial and Lauder 2020), all of these methods require overexposure of the particles in order for them to be analyzed. Unfortunately, this often overexposes the fish as well, making it impossible to track jaw kinematics during the same feeding strike. Setting up a second camera next to the first would not be an option because the accurate measurement of distances, and therefore velocities, requires videos to be captured exactly in the plane of the feeding event. It is therefore impossible with traditional methods to simultaneously measure both kinematics and performance in such a system. While it can be useful to measure the kinematics and performance of the organism independently, the two cannot be correlated unless we know which kinematic value pairs with each performance outcome.

One way to avoid this conflict is to record through a semi-silvered mirror, also known as a beam splitting mirror or a one-way mirror. A semi-silvered mirror is a coated piece of glass that reflects some of the light that reaches it and transmits the rest of the light. The exact ratio of reflectance to transmission depends on the coating, but often ranges from 30:70 to 50:50. We can take advantage of this by recording both pathways that the light takes, with one camera recording the reflected light and the other recording the transmitted light (Fig. 4). We therefore have two cameras recording the exact same scene and can control the exposure of each independently. For instance, we can overexpose one camera to record PIV data and lower the exposure on the other camera to record jaw kinematics during fish feeding (Fig. 4). If the experimenter wishes to capture different views without changing the shutter speed, aperture, or ISO, then they can alternatively place an IR-pass filter on one camera and an IR-cut filter on the other camera. Then, they can separately adjust the amount of visible light and IR light to achieve the appropriate exposure for each camera. While this is only one example of how multiple data types can be collected simultaneously, we believe that the use of multi-modal data collection will be valuable in many study systems, and that biomechanists should prioritize methods that allow for such data collection efforts.

The ability to simultaneously record multiple data types and combine them in a single SEM analysis opens

the door for studies on the smaller effects that would be expected from evolutionarily relevant changes in development. However, successfully separating these small effects from the noise associated with measurement error and behavioral variation also requires large datasets. Functional morphology has classically been a field of small sample sizes in part due to limitations imposed by the unfortunate overlap of uncooperative animals and the many technical challenges of quantifying organismal function. Luckily, in the modern age of digital cameras and ample digital storage, this barrier has fallen. Yet the sheer effort required to digitize videos still prevented many studies from attaining large sample sizes. However, there has recently been a push for the use of deep learning in studies of organismal performance and behavior that may well remove this final hurdle (Mathis et al. 2018; Lauer et al. 2022; Pereira et al. 2022). For example, an ongoing behavioral and kinematic study of fish negotiating complex flows utilizes digital video recordings of 12 fish under three experimental conditions at 10 Hz for 10-min bouts, totaling 216,000 frames to digitize. By training a deep learning software, such as DeepLabCut (DLC; Mathis et al. 2018) or SLEAP (Pereira et al. 2022), to track three morphological features of interest for each individual (Fig. 5A) on a subset of the dataset (1000 frames total; Fig. 5B), we are able to obtain 648,000 sets of spatial coordinates—a number of points unfeasible to track by hand. We thereby generated a dataset with both high temporal resolution and a long duration (Fig. 5C), allowing for a more complete description of swimming behavior than was previously practically achievable.

A case study: performance effects of a developmental manipulations

To illustrate how it is possible to combine some of the methods discussed above to answer questions about the adaptive value of single developmental pathways, we integrate past results of the genetic basis of development in fish with semi-silver mirror video recording, deep learning video analysis, and structural equation modeling to measure the functional implications of modulation in the canonical Wnt signaling pathway. Wnt signaling is a wide-reaching and context-dependent developmental transduction pathway that plays a myriad of roles in both embryos and adult vertebrates, notably including the determination of cell fate and morphogenesis (Moon et al. 2004; Milat and Ng 2009). Alterations in this pathway are thought to be an important mechanism by which fish have diverged from a generalist ancestor feeding in the midwater into a benthic feeding specialist (Parsons et al. 2014). Wnt signaling is a strong candidate for functional analy-

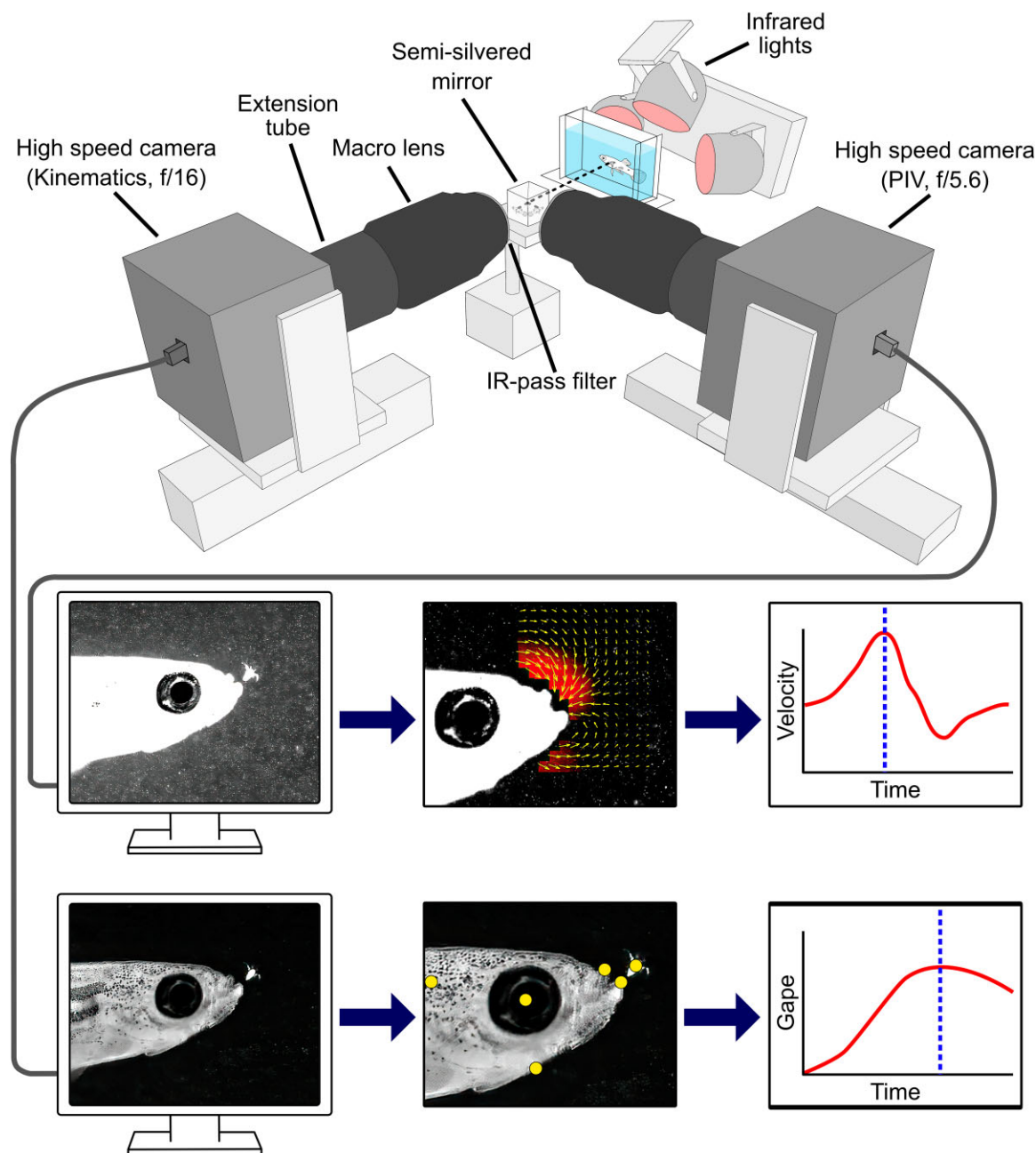


Fig. 4 Filming through a semi-silvered mirror allows two cameras to simultaneously record the exact same scene. Changing the exposure between the two cameras allows for different types of data to be recorded in two synchronized videos. In this case, one camera has a higher exposure and thereby visualizes algae particles suspended in the water. These particles can be tracked, converted to a velocity field, and the water velocity over time calculated (upper row of panels). The other camera (lower row of panels) has a lower exposure allowing machine learning algorithms to track landmarks on the head throughout the video. These landmarks can then be used to calculate feeding kinematic variables.

sis since upregulation and downregulation is sufficient to phenocopy morphologies seen in the alternate ecology, implying that this developmental mechanism controls a significant portion of adaptive craniofacial variation (Parsons et al. 2014). Since fish feeding mechanisms are well-studied models of complex biomechanical systems (Westneat 1990; Wainwright et al. 2007; Irschick et al. 2013), we are able to hypothesize a mech-

anistic path to connect all levels from development to performance.

To test the functional implications of modulation to the Wnt signaling pathway, we raised zebrafish (*Danio rerio*) in the presence of small molecules that upregulate (LiCl) or downregulate (IWR-1) the pathway. Following established methods (Parsons et al. 2014; Powder et al. 2015), we raised the fish under these conditions for

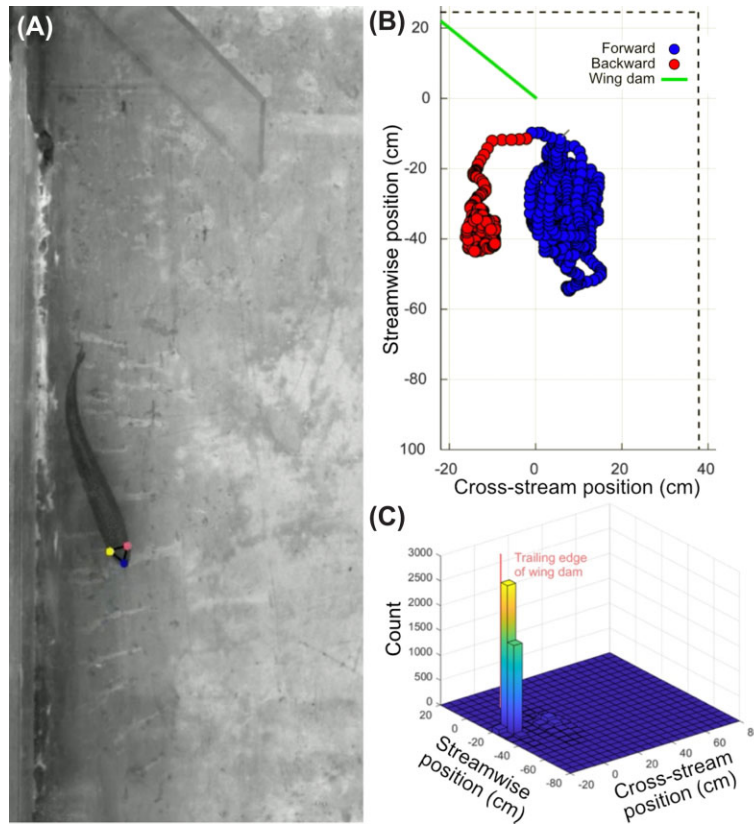


Fig. 5 (A) Deep learning program DeepLabCut (DLC) was trained to digitize the snout tip and eyes of individual fish, then successfully tracked 648,000 points in an ongoing behavioral study on swimming in complex flows. (B) DLC generates an output of x - y coordinates for each point tracked for each frame, which are displayed relative to the study's filming chamber and show head orientation (total points 6000). (C) The study used the positional data to generate a spatial time budget for each of several individuals across several experimental conditions—an experimental design that would not have been feasible if not for deep learning digitizing programs.

35 days, then fed them live *Artemia* in front of a semi-silvered mirror filming apparatus to capture both kinematics and feeding performance (Fig. 4). We then euthanized the fish, photographed them, and conducted 2D geometric morphometric analysis (Fig. 6A and B) using the StereoMorph and GeoMorph packages in R (Olsen and Westneat 2015; Baken et al. 2021; Adams et al. 2022). We analyzed jaw kinematics by tracking 5 points on the head through the entire prey capture strike (Fig. 6C) using the SLEAP deep-learning software (Pereira et al. 2022). These points were analyzed with a custom script in R to track jaw protrusion, hyoid depression, gape, head elevation, as well as the time that it took from the beginning of the strike to reach the maximum value of each kinematic trait. We then analyzed the water flow velocity produced during suction feeding using particle image velocimetry data using the DaVis analysis software (v. 7.3, LaVision Inc.). This provided the maximum fluid velocity in front of the fish's mouth during the suction feeding event, our measure of feeding performance (Fig. 6D). Finally, we combined all of these data into an SEM model based on our understanding of feeding mechanics (Fig. 7) and included a latent

variable that represents feeding effort based on times to peak kinematics. This SEM model therefore connected development \rightarrow morphology \rightarrow kinematics \rightarrow performance.

Through this experiment, we were able to find a direct effect of developmental modulation on feeding performance. Specifically, downregulation of Wnt signaling through an IWR-1 treatment decreased feeding performance (Fig. 7A). Notably, head elevation was not affected by either treatment. The fact that this important aspect of cranial kinesis in suction feeding was able to function normally indicates that integration and control of the feeding mechanism in developmentally manipulated fish was largely intact, and that decreased feeding performance was not caused by a complete disruption of this mechanism. Additionally, by examining the separate paths through which the treatment connects to feeding performance (Fig. 7B, blue and orange), we are able to find the specific functional mechanisms by which this developmental manipulation alters performance. Namely, the decrease in performance is driven primarily by a decrease in jaw protrusion (Fig. 7B, blue arrows), with a smaller portion of the change

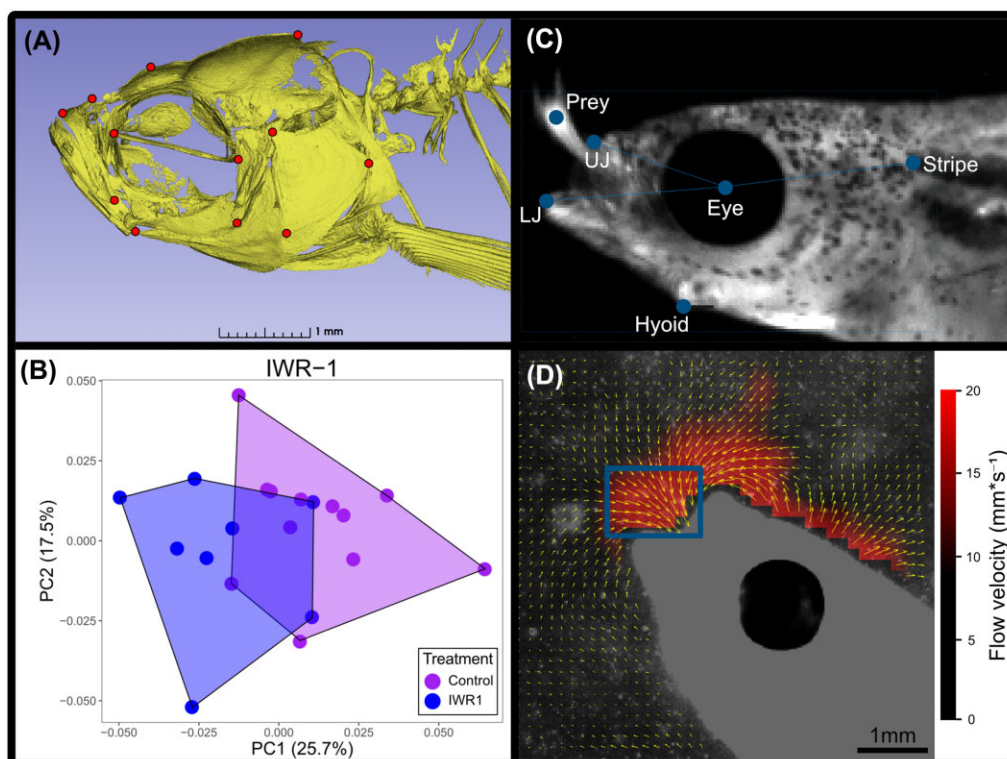


Fig. 6 A variety of methods were used to analyze developmentally modulated zebrafish. (A) Each fish was μ -CT scanned and then a screenshot was captured from a lateral view. A total of 12 landmarks were placed on each skull using Stereomorph (V1.6.7). (B) Landmark data were processed using a generalized procrustes analysis in Geomorph (V4.0.4). The allometric scaling component was removed from the data, which was then subjected to a principal components analysis. This analysis revealed that IWR-1 treated fish ($Wnt \downarrow$) were morphologically distinct from control fish. (C) SLEAP software was used to track 6 points in the kinematics video during each recorded feeding strike. UJ—upper jaw. LJ—lower jaw. (D) PIV videos of water flow velocity were processed in DaVis software to quantify water flow in front of the mouth (red color indicates maximum velocities). The blue box represents the area from which the maximum value was recorded to represent peak performance in the given frame.

attributable to decreased gape (Fig. 7B, orange arrows). Conversely, upregulation of Wnt signaling with LiCl did not affect feeding performance. This is perhaps not surprising because increased Wnt signaling is associated with diminished jaw protrusion in cichlids (Parsons et al. 2014) and juvenile zebrafish already have minimal jaw protrusion (Galindo et al. 2019). We believe that this example clearly demonstrates the power of using this methodological combination to not only find the functional implications of developmental variation, but also identify individual causal mechanisms by which performance is affected. Comparable methods could similarly be used in other systems to more precisely measure the performance effects of genetic and developmental variation, allowing us to better understand the mechanisms driving fitness differences between individuals or populations.

Conclusion

Arnold in 1983 focused the attention of evolutionary biologists on the value of integrating performance into

studies of adaptation and also introduced the idea of path analysis to partition the effect of different variables in the analysis of morphology, performance, and fitness. In reconsidering the Arnold (1983) research program 40 years later amid the post-genomics era (Richardson and Stevens 2015), we find that his framework has not been fully extended to include genetics (Fig. 1; Irschick et al. 2013) despite significant advancements in our understanding of the genetic basis of adaptation (Kratochwil and Meyer 2015; Bomblies and Peichel 2022). Additionally, the ability to use genetic manipulation as well as developmental and hormonal modulation to recreate evolutionary variation in the lab carries the promise of tractable models on which functional effects can be tested. We believe that the last step necessary to realize this integrative framework is the creation of a new suite of methods in functional morphology that allows for high-accuracy and high-precision measurements of animal performance. Here, we have provided examples of methods that we are using to achieve this goal in the hopes that they will spur further method development and inspire a new generation of functional

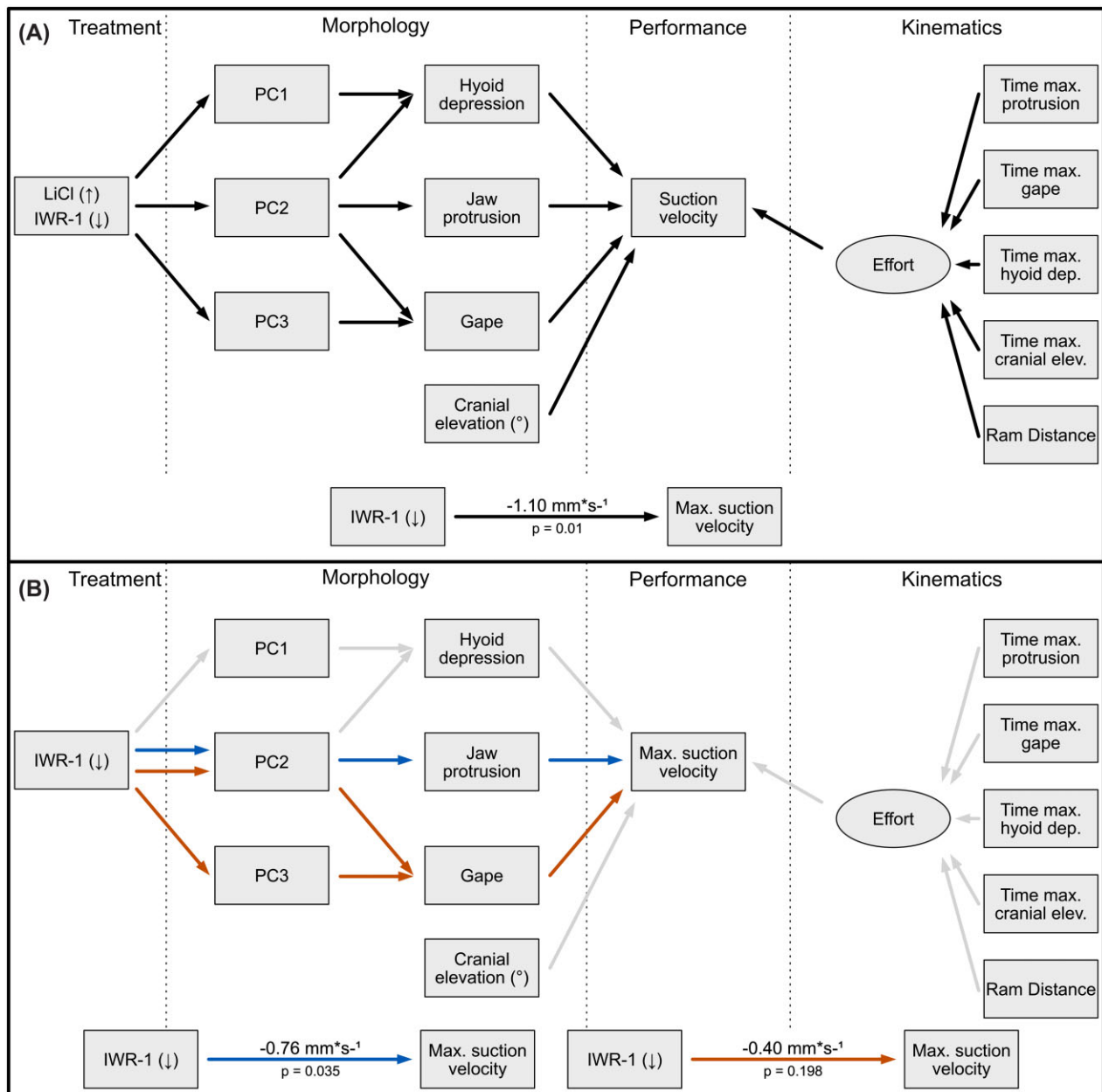


Fig. 7 Structural equation modeling allows for complex mechanistic models to be created, in this case linking developmental modulation to fish suction feeding performance. (A) Here, we give an example in which we create a model connecting development to static morphology, static morphology to feeding morphology (e.g., jaw protrusion), and feeding morphology to suction feeding performance. We also include a latent variable (oval) that represents effort, which is modeled based on several kinematic traits since slow feeding strikes are more representative of low effort than of limited ability. The total effect of downregulating Wnt signaling with IWR-1 treatment is to decrease feeding performance by 1.10 mm s^{-1} , from a mean performance of 23.2 mm s^{-1} in control fish. (B) Indirect effects can be used to understand the mechanism by which developmental manipulation affects performance. We find that IWR-1 primarily decreases feeding performance through decreased jaw protrusion (blue arrows, -0.76 mm s^{-1}), while there is a smaller effect through gape (orange arrows, -0.40 mm s^{-1}). These indirect effects are composed of the individual regressions between each variable, which have been omitted for clarity.

morphologists to frame their work in a microevolutionary context.

We highlight the use of robotics as a tool in comparative biology and the simultaneous collection of diverse datasets to allow for mechanistic analysis through

structural equation models. Furthermore, we suggest that these methods can be combined with experimental genetic methods such as CRISPR and small molecule developmental manipulation (e.g., Figs. 6 and 7) in order to test the functional implications of evolutionar-

ily relevant variation in fish. Additionally, other groups are developing new methods that can similarly be integrated with evolutionary research to apply this framework in a greater variety of model systems. We believe that the continued development of X-ray motion capture (Brainerd et al. 2010) allowing for highly accurate 3D spatial tracking and future developments in field tagging technologies carry particular promise as new approaches to quantifying organismal performance.

Here, we have primarily focused on the connection between genes and performance, but as Arnold pointed out, field measurements of fitness are a necessary step to understand evolutionary outcomes. We believe that the best approach is an extension of the one proposed by Arnold (1983), in which laboratory experiments are used to connect genotype → morphology → performance and separate field experiments are used to connect performance → fitness. Although it is outside the scope of this paper, there has been considerable advancement in field methodologies that could be applied to this research program. For example, there has been great progress in the ability to experimentally measure adaptive landscapes (Martin and Wainwright 2013; Arnegard et al. 2014; Polly et al. 2016; Martin and Gould 2020; Patton et al. 2022), conduct field genealogical analysis (López-Sepulcre et al. 2013; Reznick et al. 2019), and replicate natural conditions with mesocosm experiments and ecological transplants (Reznick et al. 1990; Harmon et al. 2009; Bassar et al. 2010; Arnegard et al. 2014; Travis et al. 2014; Moser et al. 2016). It will be necessary to apply these methods to any performance variation that is found to be under genetic control in order to complete the path from genes to fitness (Fig. 1).

While much of the presented work is ongoing, we are confident that these examples demonstrate that functional morphology can be used to examine intraspecific traits with relatively small variance in the context of development and genetics. As we continue this work, we hope that other groups working on a diverse set of organisms will join us to integrate biomechanics and functional morphology into the post-genomic era. We believe that such collaborative efforts promise to bring considerable advances in our understanding of the complexity of organismal design and adaptation.

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Conflicts of Interest

The authors report no conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author, D.G.M., upon reasonable request.

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