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Spencer J. Ingley
Brigham Young University

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Repeated Trait Evolution Driven by Divergent Natural Selection at Early and Late Stages of Speciation

Spencer J. Ingley

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Jerald B. Johnson, Chair
Jack W. Sites, Jr.
John S. K. Kauwe
Byron J. Adams
Patrik Nosil

Department of Biology

Brigham Young University

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ABSTRACT

Repeated Trait Evolution Driven by Divergent Natural Selection at Early and Late Stages of Speciation

Spencer J. Ingley
Department of Biology, BYU
Doctor of Philosophy

Speciation – the process by which new species arise – is of fundamental importance in the biological sciences. The means by which new species arise, and the relationship among living species, has been a topic that has captivated both lay and scientific observers for centuries. In recent years, the study of speciation has enjoyed increased attention, resulting in significant advances in our understanding of how species form. Although our understanding of the processes that contribute to speciation has increased dramatically in recent years, our knowledge of how reproductive barriers accumulate as speciation proceeds is still limited. Thus, studies that evaluate trait divergence and its consequences at early verses late stages of divergence can provide valuable insight into the speciation process. Chapter 1 of my dissertation focuses on the role of animal personality in the speciation process. Animal personality – defined as consistent individual differences in behavioral tendencies – has been identified as a key player in several ecological and evolutionary processes, yet the role of personality in speciation remains unexplored. In this chapter I discuss the ways by which personality can contribute to a suite of reproductive barriers and drive the speciation process. Chapters 2 through 5 provide a case study evaluating how selection acts on traits at early and late stages of speciation, using the Neotropical Livebearing fish genus *Brachyrhaphis* as a model system. *Brachyrhaphis* is ideally suited for this research because several species pairs and population pairs within species occur in similarly divergent selective regimes. I first present results from a field demographic study that shows that the strength of divergent selection acting on life-history traits in populations from divergent predation environments diminishes as speciation proceeds. I then show that population pairs at different stages of divergence are evolving similar morphological patterns along parallel trajectories. At both early and late stages of divergence, populations from environments with dense predator populations have a body shape that appears to be optimized for burst-speed swimming, and important component of predator escape. In contrast, populations from environments lacking predators have a body shape optimized for endurance swimming ability, which is important in environments where competition for foods and mates is high. Next, I show that populations from divergent predation environments do indeed differ in their swimming abilities according to our predictions, reflecting a population level trade-off between burst and endurance swimming ability. Although population level trade-offs were strong, I found no evidence of within population level trade-offs, suggesting that populations have arrived at novel solutions to between population trade-offs that were not present within ancestral populations. Finally, I show that these specialized swimming modes are locally adaptive, and that divergent ecology selects against immigrants, effectively reducing gene flow between populations from divergent environments. Together, these studies provide a valuable glimpse into the repeatability and predictability of trait divergence at different stages of speciation.

Keywords: speciation, trait divergence, *Brachyrhaphis,* predation, natural selection

ACKNOWLEDGEMENTS

"If you want to go fast, go alone. If you want to go far, go together." So states an African proverb that has profoundly impacted the way I view my life, and the way I view my science. None of the work described in this dissertation would have been possible if I had gone it alone. For this reason I have a long list of faithful family, friends, colleagues, and mentors to acknowledge for their contributions – big and small – to what has become my dissertation.

First: my family. Starting as a young child my parents provided me with myriad opportunities to learn and to grow in diverse areas. My backyard and the lake that it bordered became a playground for learning, exploration, and adventure. I thank my parents for kindling in me a desire to explore, and for providing invaluable guidance, love, and support along the way. I thank them for teaching me of a wise and loving God, and encouraging me to study his countless and beautiful works. My dear wife has been my cornerstone since we were married and I can't thank her enough for her love and support, and for our two beautiful boys. They have helped me re-learn how to see the world with the enthusiasm of a child.

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None of this work would have been possible without the help of several generous funding sources. First, the BYU Department of Biology and BYU Graduate Studies have provided me with the means to eat and to study. A National Science Foundation Graduate Research Fellowship provided an invaluable source of support over three years, allowing me to travel to conferences, workshops, and the field. The Explorers Club, the National Geographic Society, the American Society of Naturalists, the American Museum of Natural History, and the Smithsonian Tropical Research Institute also provide crucial funding and support for my field expeditions. I thank all those who have worked long hours running these programs, and I thank the generous and numerous donors who make it all possible.

TABLE OF CONTENTS

CHAPTER

LIST OF TABLES

LIST OF FIGURES

Chapter 1

The Role of Personality in the Speciation Process

Spencer J. Ingley[1], and Jerald B. Johnson[1, 2]

[1] Department of Biology and [2] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602, USA

Corresponding author: Ingley, S.J. (sjingley@byu.edu)

Abstract

Although interest in the ecological and evolutionary implications of animal personality continues to grow, the role that personality plays in speciation has received only modest attention. Here we explore links between personality and speciation, and offer a framework for addressing some of this field's most interesting questions.

Introduction

Recognizing that individual animals can possess unique personalities has had a profound impact on several disciplines in ecology and evolutionary biology [1, 2]. Theoretical and empirical results demonstrate the importance of considering individual personality in fields as diverse as invasion and dispersal dynamics, social evolution, life history evolution, and ecological transmission dynamics (e.g., disease or information). Likewise, the past decade has also seen renewed interest in research focused on processes that contribute to speciation [3]. Key to this work has been the integration of traditional lines of inquiry, such as sexual and ecological isolation, with emerging fields, such as genomics. It is surprising, however, that researchers have almost entirely overlooked potential links between personality traits and speciation (but see [4]). Although the role of behavior in speciation is well established (e.g., sexual selection; [5]), how personality (defined as consistent individual differences in behavior over time and across contexts) contributes to speciation is poorly understood. Here, we consider the interplay between personality and the evolution of reproductive isolation, highlighting several promising areas for future research.

How Could Personality Affect Speciation?

Speciation research has typically focused on two major processes that contribute to reproductive isolation: (1) geographic isolation of incipient species, either by colonization or as the result of vicariant events; and (2) divergent natural selection. Here, we explore several ways that personality traits could influence speciation through these fundamental processes.

Personality and Speciation Via Peripheral Isolates

Colonization events can play an important role in speciation. Newly founded populations often do not represent the overall diversity of the source population. This is usually ascribed to random sampling error associated with the small sample size of the founding population. However, recent work suggests that personality could play a role in this process if dispersing individuals have different personality types than average individuals from the source population. For example, bold and aggressive individuals could be more likely to disperse and colonize a new area than their shy counterparts [2]**.** These differences in personality traits, coupled with different selective pressures in the new environment [2], could lead to evolutionary divergence ultimately leading to reproductive isolation. Empirical tests evaluating personality traits of more vagile individuals, or comparisons between source and sink populations, could valuable provide insight into the role of personality in classic speciation models (e.g. founder-flush dynamics).

Personality as a Neglected Trait in Selectively Divergent Populations

Populations of a given species that occur in different selective environments often diverge in multiple traits. Differences among populations in these traits have the potential to contribute to reproductive isolation. One likely form of ecologically driven reproductive isolation is immigrant inviability [6]. This occurs when an individual that immigrates into a habitat different from their native habitat suffers decreased fitness. Several traits can contribute to immigrant inviability, including predator escape ability, morphology, and cryptic coloration. The impact of personality traits on immigrant inviability is largely unknown. When personality traits are adaptive (e.g., reduced activity in the presence of a predator [7], or increased boldness/aggression in the presence of predators [8, 9]), selection should act against immigrants that possess the opposite, presumably maladaptive, personality types. For example, in a case where increased boldness and aggression increases survival in the presence of a predator (e.g., [3, 8]), less bold predator-naïve individuals could suffer reduced survival if they migrate into a high-predation environment. Selection against hybrid personality types could also lead to reproductive isolation via reinforcement if traits are genetically based and hybrids exhibit less-adaptive intermediate phenotypes than parental forms. Yet, how individuals with different personalities fare in new habitats remains largely unknown.

Personality and Non-Random Mating Within and Among Populations

Personality traits could play an important role in non-random mating. Any trait that diverges, either within or among populations, has the potential to contribute to speciation if individuals that possess the same traits mate assortatively. Divergence in courtship or mating preferences, and subsequent assortative mating, can result in reproductive isolation [6]. Sexual or behavioral isolation arises when individuals either do not prefer, or do not recognize, each other as potential mates [6]. This form of pre-mating isolation can result in significant levels of reproductive isolation, even in cases where other reproductive isolation mechanisms are lacking [6]. Personality traits could be important in this regard. If individuals with similar personalities are attracted to one another, or show an increase in fitness when they mate assortatively, this could lead to sexual isolation [9]. For example, individuals at different ends of the personality distribution (i.e., very bold vs. very shy) could prefer each other as mates because they perform together better as parents than they would with a mate with a different personality type (e.g., [10]). For example, this has been shown in the great tit (*Parus major*) where females that explore slowly are better able to exploit the resources available in a lower-quality habitat occupied by males that also explore slowly [10].

Personality can also affect divergence in mate preferences in cases where populations become locally adapted to different environments. Divergent selection on geographically isolated populations could result in the differential expression of personality traits among populations. Again, if individuals prefer mates with a similar personality type (i.e., assortative mating for personality), sexual isolation between individuals from divergent populations is likely to occur. This is expected when personality traits are adaptive in the native habitat (i.e., the adaptive nature of the personality trait is context dependent) and are genetically based, such that reinforcing selection may drive mating preference for individuals with adaptive personality types in order to avoid unfavorable hybridization. However, it is also possible that individuals with opposite personality traits could be favored if disassortative mating increases favorable genetic diversity. In summary, sexual isolation attributed to personality differences could act to drive reproductive isolation and divergence within populations, or to maintain adaptive divergence among populations from divergent selective regimes by driving assortative mating preferences. Further studies are needed to determine how commonly individuals use personality traits in choosing a mate, how often personality influences reproductive success [10], and what role personality could play in reinforcement of sexual isolation.

How Personality Can Affect Temporal and Habitat Reproductive Isolation

Individuals that are active at different times or that use different habitats or microhabitats could become reproductively isolated from one another due to a decrease in encounters [6]. This so called temporal or habitat reproductive isolation could be affected by individual personalities. If individuals with different personality types prefer to mate in spatially separated habitats or at different times of day, a reduction in mating encounters between dissimilar individuals should occur. For example, individuals that have bold personality types might be more likely to engage in conspicuous reproductive activities during times of high-predation risk compared to individuals with shy personality types. The fact that personalities represent consistent differences across time and contexts suggests that if, for example, a bold individual is likely to engage in conspicuous reproductive activities, it is also likely to engage in other conspicuous activities (e.g., foraging). These consistent behavioral differences over time and across contexts are likely to influence the frequency with which individuals with different personality types encounter one another. To date little work has explored the relationship between personality and temporal activity levels, and no work to our knowledge has explored the connection between personality-dependent differences in the timing of activity and reproductive isolation. Likewise, the potential implication of variable habitat use by individuals with different personalities on the evolution of reproductive isolation has not been investigated.

Conclusions and future directions

Here, we touch on several ways that personality could influence speciation. The paucity of controlled studies testing for causal links between personality and reproductive isolation points to the need for more deliberate work in this area. Several critical gaps exist in our current understanding of personality as it relates to speciation. First, our understanding of how personality can affect the evolution of traditional pre-mating reproductive isolation barriers (e.g., immigrant inviability and sexual or behavioral isolation) is limited to only a few case studies. Second, we know little about how personality is expressed in hybrids, and whether post-mating reproductive isolation barriers can be driven by maladaptive hybrid personality traits. Finally, although causal relationships between personality and dispersal tendencies have been established, little is known about how personality affects the formation of peripheral isolates and subsequent divergence and speciation within those peripheral isolates. Understanding the interplay between personality, ecology, and evolution offers exciting prospects for future speciation research.

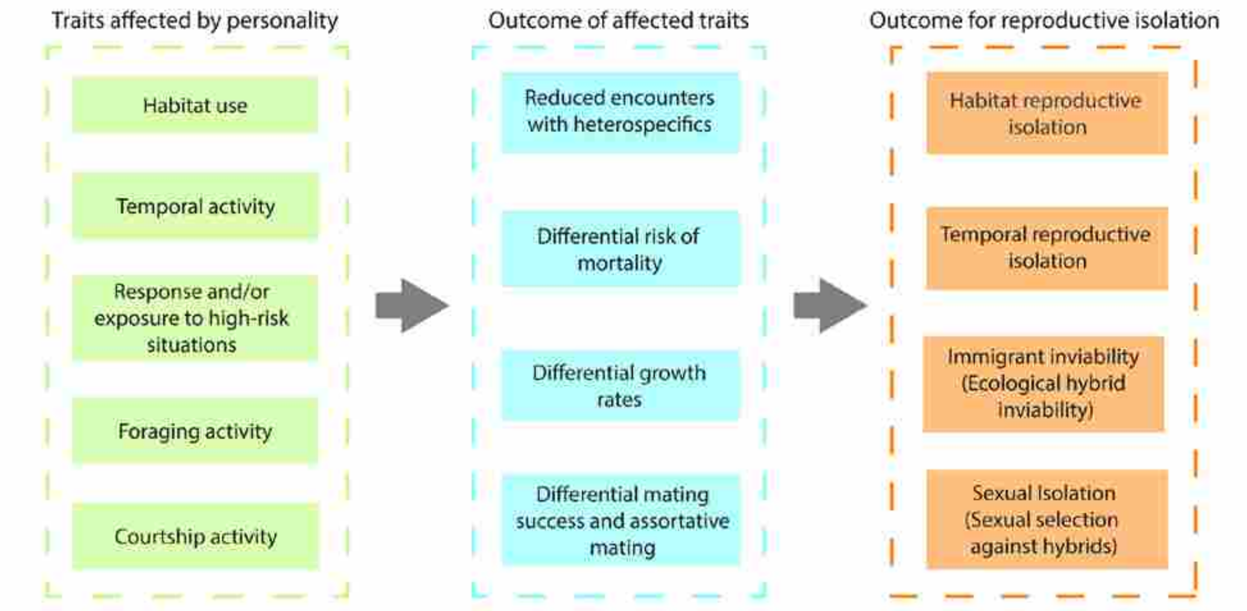

Figure 1.1. The role of personality in speciation.

Flow diagram indicating the potential role of personality in the evolution of reproductive isolation. Items contained in green boxes represent traits known to be affected by personality. Items contained in blue boxes represent expected outcomes of affected traits. The term 'heterospecifics' refers to individuals with different personality types. Items in orange boxes represent pre-zygotic and post-zygotic (contained within parentheses) reproductive isolating mechanisms that could be affected by personality. For the purposes of this diagram, hybrids are assumed to have intermediate personality phenotypes, although this is likely not always the case. Arrows represent the hypothesized connection between personality, traits of interest, and reproductive isolating mechanisms.

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Chapter 2

Strength of selection decreases following speciation in a Neotropical livebearing fish

Spencer J. Ingley[1,2] and Jerald B. Johnson[1, 3]

[1]Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT 84602

[2] Corresponding Author: E-mail: sjingley@byu.edu

[3] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602

Abstract

How selection acts to drive trait evolution at different stages of divergence is of fundamental importance in our understanding of the origins of biodiversity. Yet, most studies have focused on a single point along an evolutionary trajectory. Here, we provide a case study evaluating the strength of selection acting on life-history traits at early-versus-late stages of divergence in *Brachyrhaphis* fishes. We find that selection is much stronger at early stages of divergence, and that trait differences acquired early are maintained over time, despite weakening selective pressure.

Keywords

Matrix modeling; elasticity; mark-recapture; *Brachyrhaphis*; Fisher's fundamental theorem

Introduction

Comparative studies of recently diverged populations (i.e., nascent species) provide valuable insight into the forces that drive trait evolution and speciation [1]. Yet, they provide little information regarding the progression of trait evolution in later stages of divergence (e.g., post-speciation). Specifically, these studies fail to address how the strength of divergent selection changes at different stages of speciation, despite a long-standing theoretical framework suggesting that the strength of selection should change as diversification occurs [2]. For example, Fisher's fundamental theorem [2], for which evidence from the wild is rare [3], posits that selection strength should increase as variance in fitness increases. Thus, if recently diverged populations, which have yet to reach adaptive optima, have experienced more recent gene flow relative to more established species pairs, the strength of divergent selection acting on those populations should be higher than that experienced by more divergent and presumably better adapted species pairs.

Life-history traits (e.g., age/size at maturity) are of particular importance when considering how traits change in response to selection because they translate directly into population level demographic phenomena [4]. In addition, they are often subject to strong selection that can initially result in rapid evolutionary change [5]. Unfortunately, although variable life-histories among populations from different environments are often described (e.g., [6, 7]), the effects of this variation on population-level metrics, such as population growth rate (λ), and the strength of selection acting on these traits, are seldom addressed [8]. Even more rare are studies evaluating these processes at early-versus-late stages of evolutionary divergence.

Brachyrhaphis fishes (Poeciliidae) are a useful system for studying selection over time because this genus contains several *within* species population pairs and *between* sister species pairs that occur in similarly divergent selective regimes [9]. For example, populations of *B. rhabdophora* (*BRh*) occur in divergent predation environments throughout their range (e.g., fish predators present in some populations but absent in others), resulting in the evolution of divergent life-histories [7] and morphologies [10]. A strikingly similar pattern is observed *between* sister species *B. roseni* (*BR*) and *B. terrabensis* (*BT*) [10], which primarily occur in streams with predators present or absent, respectively [10]. This species pair has evolved similar patterns of morphological [10] and life-history (Belk et al., in review) divergence to those seen among populations of *BRh* from different predation environments, suggesting that each pair is found at different points along the same evolutionary trajectory [10]. Here, we use serial mark-recapture experiments (SMR) and population matrix-models (PMMs) to test if the strength of divergent selection on life-history traits is greater at early (e.g., within *BRh*) versus late (e.g., between *BR* and *BT*) stages of life-history divergence. We predict that, in accordance to Fisher's fundamental theorem [2], the strength of divergent selection will be greater between recently diverged populations of *BRh* than between more established sister species *BR* and *BT*.

Methods

Mark-recapture experiment

We conducted a SMR with *BR* (predators present) and *BT* (predators absent). To facilitate comparisons with previously published work on *BRh* (in which populations occur both with predators, 'Javilla' population, and without predators, 'Grande' population; [11]), and to allow us to compare patterns of selection at early-versus-late stages of divergence, we followed the methods of Johnson and Zuniga-Vega [11]. In short, we selected two sites, one with *BR* and one with *BT*, which consisted of relatively isolated pools within streams characterized by a pool-riffle-pool structure (electronic supplementary material; hereafter 'ESM'). For each location, we sampled the pool over the course of 1-2 hours until we were confident that we had captured most fish in the pool (at least 10 subsequent seine hauls with no captures). We anesthetized (using MS-222), measured, sexed, and marked each fish in the caudal region with a unique sub-cutaneous injection of latex paint (suspended in Ringer's solution), allowing us to recognize individual fish upon recapture. After marking, fish recovered in a poolside tank until day's end (4-6 hours), at which time they were released into their pool of origin. We returned four times to each site at one-week intervals and repeated the protocol, recording and measuring recaptures and marking newly captured fish. This resulted in individualized mark-recapture and growth histories for each fish over a 5-week period, allowing us to account for the impact of migration (in/out of the pool) and incomplete sampling on recapture rates, and track growth over the 5-

week period (ESM). In total, we marked 223 *BR* and 266 *BT*. Marking mortalities were extremely rare (<1%) and marked fish held under controlled conditions through the duration of the experiment kept their marks.

Mortality estimates

We analyzed recapture histories to estimate mortality rates using Program MARK [12]. Mortality rates are a critical input for PMMs, which we use to create elasticity estimates (i.e., an indicator of strength of selection). We assigned each fish to one of five ontogenetic stages (three non-reproductive and two reproductive stages; ESM). We tested 12 competing models that varied parameter constraints for mortality and recapture rates among the five stages, using AIC to select the best-fit model [13]. For both species, the top model for survivorship constrained mortality of large adults to be different from the remaining four stages (ESM), as was true for *BRh* [11]. We used model averaging to generate our final mortality estimates for each stage, thus taking into account the relative weight of each model and providing more robust estimates [13].

Demographic analyses

Using mortality estimates from MARK, we created PMMs [4] to estimate several demographic variables, including population growth rate (λ; ESM), sensitivities, and elasticities. These models use the following as inputs: stage specific mortality (from MARK), growth rates (i.e., transition rates among classes), and fecundity (estimated from life-history dissections; ESM). PMMs facilitate comparative evaluations of population dynamics using estimates of sensitivities (the effect on λ of changing each vital rate by the same magnitude [4, 14]) and elasticities of λ (the effect on λ of changing each vital rate by the same proportion [4, 14]). Elasticities allow for comparison among matrices derived from populations/species with divergent life-histories because they are standardized [4, 15], and represent a standardized estimate of the strength of selection acting on each component of the life-history matrix (e.g., stage specific survival, growth, and fecundity [14, 16]). Thus, comparing elasticities among populations allows us to identify the vital rates that are under the strongest selection at early (among *BRh* populations) versus late stages of divergence (between *BR* and *BT*). Finally, we conducted a permutation analysis following the methods of [11] to generate 95% confidence intervals (CI) for elasticity measures and overall population growth parameters (ESM). These CIs provided an estimate of significance when comparing ranges of elasticities for each vital rate and summed elasticities for each stage.

Results

Our SMR revealed that mortality rates were higher in *BR* than in *BT.* Furthermore, we found that large adult *BR* suffered the highest mortality (ESM). Despite differences in mortality rates, the 95% CIs for λ for each species overlapped and spanned 1 (i.e., stable population size; Table 2.1), indicating that population growth rates did not differ between species and were stable. Matrix elasticities revealed that selection acted similarly on both *BR* and *BT* (CIs overlapped for all summed stages; Table 2.1, Figures 2.1-2.2)*,* with strong selection on surviving and remaining in the first four stages. However, populations of *BRh* experienced divergent selection on both small juveniles and large adults (non-overlapping CIs for J1, minimal overlap for A2; Table 2.1; Figure 2.1). Overall selection on growth, fecundity, and stasis was similar in all populations (overlapping CIs; ESM), although stasis was under slightly stronger selection in *BR* and *BT* than in *BRh* (ESM).

Discussion

Our work provides additional evidence that predation environment is a ubiquitous driver of life-history trait divergence, a pattern found both within *Brachyrhaphis* and across poeciliids in general (e.g., [5, 6]). Indeed, our work and others' suggest that increased mortality rates, whether due to predation or abiotic stressors [17], consistently drive the evolution of predictable life-history adaptations. Furthermore, *Brachyrhaphis,* both at early and late stages of divergence, maintain similar population growth rates despite different mortality rates. When taken in context, these results highlight the conserved nature of not only life-history traits, but also growth rates of populations that occur in divergent predation environments [8]. In short, different populations solve the same demographic challenges using alternate life-history strategies.

Despite a long-standing theoretical framework, the way in which selection acts on recently diverged populations relative to established sister species remains poorly studied in the wild. Our study provides evidence that divergent selection on life-history traits can be stronger during early stages of divergence (i.e., between Javilla and Grande) relative to late stages of divergence (i.e., between *BR* and *BT*). Furthermore, despite weakening signatures of divergent selection, adaptive trait differences are maintained. This suggests that although strong selection might be required to drive divergence initially, more modest selective pressure is sufficient to maintain differences over time.

The observed differences in the strength of divergent selection at early-versus-late stages of divergence could be attributed to several processes. According to Fisher's fundamental theorem, divergent selection should increase in strength in proportion to variance in fitness [2]. Several lines of evidence suggest that variance in fitness could be higher in *BRh* relative to *BR* and *BT*. First, genetic divergence between Javilla and Grande is nearly an order of magnitude lower than between *BR* and *BT* [10]. Previous work suggests that low-predation populations of

BRh recently diverged from high-predation populations as they moved among drainages along the coast and subsequently expanded their ranges upstream to reaches less affected by predators [18]. This pattern suggests that Grande would have only recently become subjected to a selective regime divergent to that of Javilla, with little time to move towards adaptive optima. Furthermore, morphological traits [10] and life-history traits [7], which are likely tightly linked to fitness, show greater within-population variance in *BRh* relative to *BR* and *BT.* This suggests that a recent origin and the potential of ongoing gene flow could contribute to increase variance in fitness, providing more material upon which selection can act. In contrast, *BR* and *BT* are more likely to have neared their phenotypic optima some time ago. Given the depth of divergence between *BR* and *BT* [9, 10]*,* selection is more likely to have eroded additive genetic variance in these species relative to *BRh.* Thus, differential patterns of selection could have been dampened simply due to a lack of variance upon which selection can act [19]. That we note differences in strength of selection in early-versus-late stages of evolutionary divergence in *Brachyrhaphis* fishes suggests that a single snapshot in evolutionary time may often fail to capture the process by which evolutionary diversification occurs.

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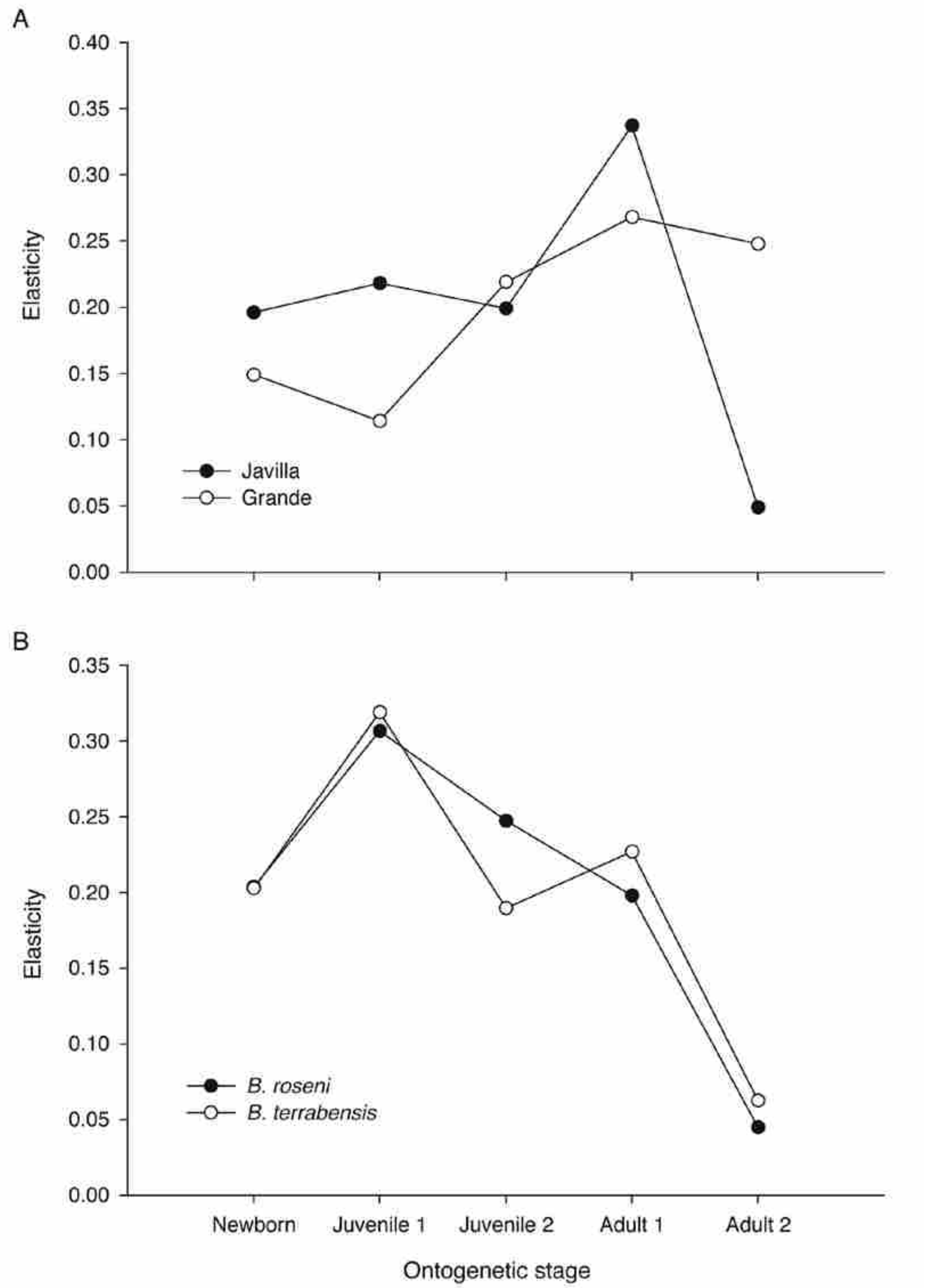

Figure 2.1: Summed elasticities for each life-history stage.

A) Javilla and Grande; and B) *BR* and *BT*. Elasticities represent strength of selection acting on each ontogenetic stage.

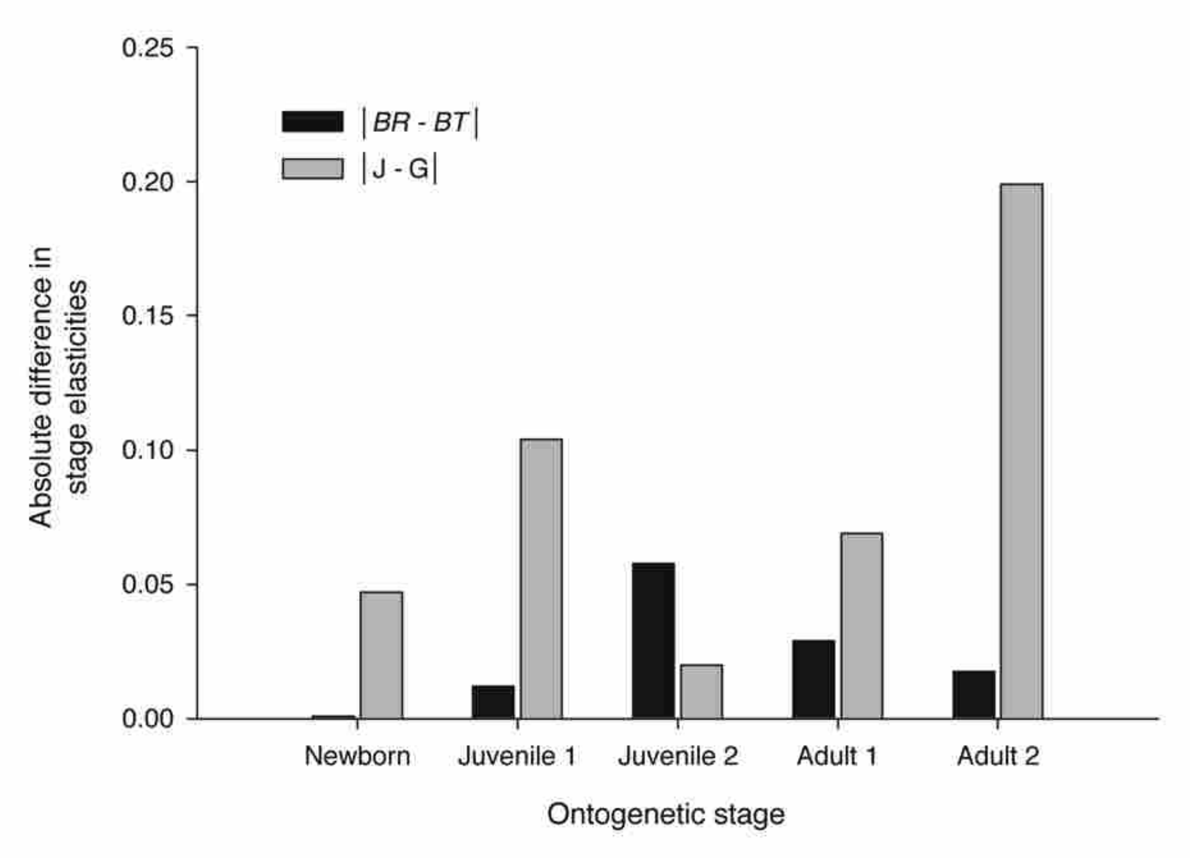

Figure 2.2: Absolute values of difference in elasticities.

Absolute values of differences in elasticities for each ontogenetic stage for *BR* – *BT* (black bars) and Javilla – Grande (J – G, gray bars). Larger values indicate stronger differences in selection on a given stage between populations.

Table 2.1: Elasticity matrices.

Elasticity matrices for *BT, BR,* Grande and Javilla (*BRh* data taken from [11]).

Elasticities	*B. terrabensis* (Predators absent)				
Life-history stage	Newborn	Juvenile 1	Juvenile 2	Adult 1	Adult 2
Newborn	**0.153**	0	0	0.043	0.011
Juvenile 1	0.054	**0.269**	0	0	0
Juvenile 2	0	0.054	**0.14**	0	0
Adult 1	0	0	0.054	**0.178**	0
Adult 2	0	0	0	0.011	0.033
Σ elasticities	0.208	0.323	0.194	0.232	0.043
Σ elasticities 95% CI	0.148-0.215	0.263-0.365	0.208-0.305	0.110-0.273	0.011-0.136
λ 95% CI	0.906 – 1.024				

Elasticities	*B. rhabdophora* Grande (Predators absent)				
Life-history stage	Newborn	Juvenile 1	Juvenile 2	Adult 1	Adult 2
Newborn	0.058	0	0	0.049	0.042
Juvenile 1	0.058	0.056	0	0	0
Juvenile 2	0.033	0.058	**0.128**	0	0
Adult 1	0	0	0.091	**0.177**	0
Adult 2	0	0	0	0.042	**0.206**
Σ elasticities	0.149	0.114	0.192	0.268	0.248
Σ elasticities 95% CI	0.128-0.176	0.096-0.146	0.174-0.274	0.186-0.372	0.138-0.371
λ 95% CI	0.960 – 1.404				

Table 2.1 continued

Elasticities	*B. roseni* (Predators present)				
Life-history stage	Newborn	Juvenile 1	Juvenile 2	Adult 1	Adult 2
Newborn	**0.132**	0	0	0.048	0.023
Juvenile 1	0.071	**0.235**	0	0	0
Juvenile 2	0	0.071	**0.176**	0	0
Adult 1	0	0	0.071	**0.127**	0
Adult 2	0	0	0	0.023	0.025
Σ elasticities	0.203	0.306	0.246	0.197	0.048
Σ elasticities 95% CI	0.165-0.245	0.235-0.378	0.197-0.305	0.159-0.241	0.008-0.124
λ 95% CI	0.938 – 1.050				

Elasticities	*B. rhabdophora* Javilla (Predators present)				
Life-history stage	Newborn	Juvenile 1	Juvenile 2	Adult 1	Adult 2
Newborn	0.084	0	0	0.093	0.019
Juvenile 1	**0.112**	**0.106**	0	0	0
Juvenile 2	0	**0.112**	0.087	0	0
Adult 1	0	0	**0.112**	**0.225**	0
Adult 2	0	0	0	0.019	0.03
Σ elasticities	0.196	0.218	0.199	0.337	0.049
Σ elasticities 95% CI	0.152-0.227	0.174-0.247	0.159-0.228	0.260-0.411	0.019-0.235
λ 95% CI	0.851 – 1.247				

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Supplemental material

Study populations and background

Brachyrhaphis roseni and *B. terrabensis* are sister species of small live-bearing fishes [1] that have similar distributions, occurring from southeastern Costa Rica to central Panama along the Pacific versant [2]. Although these species generally occur within the same drainages, *B. terrabensis* typically occupies higher elevation headwater streams, while *B. roseni* occupies lower elevation coastal streams [2]. Consequently, these species tend to occupy streams that differ in a number of ecological variables, the most pronounced of which are characterized by the presence or absence of the piscivorous predators (e.g., *Hoplias microlepis*). *B. terrabensis* occurs in streams that are primarily void of predators, while *B. roseni* co-occurs with numerous and abundant predators (e.g., *Hoplias microlepis*). This pattern is similar to that observed among populations *within* other poeciliid species [3-9], including the well-studied sister species to this species pair, *B. rhabdophora* [10-14], and has resulted in a suite of traits that have evolved in parallel in multiple independent lineages (e.g., life history [10] and morphology [15]). That these species are sister taxa, and the fact that they occur in divergent predation environments and display predictable patterns of life-history, morphological, and behavioral divergence, suggests that the selective forces driving divergence between populations of *B. rhabdophora* (i.e., predator vs. predator free environments) might also have driven divergence between *B. roseni* and *B. terrabensis*. This provides an opportunity to compare the evolutionary processes that are driving variation both within (recently diverged) and between species of *Brachyrhaphis* from opposing predation environments in two closely related evolutionary lineages.

In this study, we examined mortality rates from two genetically isolated populations of *B. roseni* and *B. terrabensis* from the Rio David drainages in western Panama (*B. roseni*: N 8.51785, W 82.41894; *B. terrabensis*: N 8.65029, W 82.512581). We closely followed methods of [14] to facilitate comparisons with *B. rhabdophora.* We collected fish from each population beginning mid-March 2013. The streams we examined were characteristic of the respective habitat of each species, and included stark differences in predator community. Both localities were characterized by a pool-riffle-pool stream structure, with *Brachyrhaphis* predominantly occurring in pools. However, as in *B. rhabdophora* [14], these riffles did not represent a barrier to fish movement, even under low-water conditions during the dry season. To account for possible movement in or out of our target pools, we employed a serial mark-recapture design (see below), thus constraining our focus on two populations for this study. However, the populations we surveyed have been previously shown to differ dramatically in life-history, morphology [15], and behavior [16, 17], falling within the range of variation found in other populations of *B. roseni* and *B. terrabensis.*

Mark–recapture experiment

We estimated mortality rates and transitional growth rates in *Brachyrhaphis roseni* and *B. terrabensis* by using a serial mark-recapture design with a sampling protocol that covered a 5-wk period in the dry season of 2013 (March and April). At each location, we selected a single focal pool and, with the aid of two field assistants, attempted to collect all fish in the pool by repeated seining (typically 40–50 seine hauls over the course of 1-2 hours). Individuals were collected early in the morning and held in a stream-side tank. Each fish was then anesthetized in a solution of MS-222, measured, sexed, and marked with sub-cutaneous injections of latex paint (suspended in fish Ringer’s solution) in the caudal peduncle. By using combinations of six different paint colors, we were able to individually identify each marked fish in order to generate personalized capture histories and characterize stage specific mortality rates. Following marking, we allowed individuals to recover in a holding tank before being released back to their natural pool at the end of the same day. Marking mortalities were extremely rare (<1%) and marked fish held under controlled conditions through the duration of the experiment did not lose their marks for any of the colors used.

We used a serial-mark recapture design [18] to account for the potential impact of migration (in or out of the pool) or incomplete sampling on recapture rates. This required returning to the sampling pools every 7 d over a 5-wk time span. During each visit, we collected all possible fish from each pool as we did during the initial visit. We re-measured previously marked individuals and held them in the recovery pool while unmarked fish were sexed, measured, and given a new identification mark before being placed in the recovery pool. At the end of the day, all fish were released back to the pool where they were collected. This protocol was repeated each week until the end of the experiment, resulting in an individualized recapture history for each fish in the study. In total, we marked 223 *B. roseni* and 266 *B. terrabensis*. The serial mark-recapture design has several advantages over a single mark and single recapture protocol. First, recapture histories can be used to generate estimates of survival rates (by sex or size class) that account for the potential impact of migration or inefficient seining [18]. This gives more confidence in our survival estimates than if we marked, released, and recaptured just once. Additionally, this protocol allowed us to track individual growth rates of fish over the duration of the five week sampling period, allowing us to estimate the proportion of fish growing into new size classes each generation, a statistic key for assessing various demographic processes (see below).

In the current study we focus on females, given the assumption that female gestation and interbrood interval ultimately constrain recruitment [19]. We divided females of each species into five size classes that correspond to five stages of the *B. roseni* and *B. terrabensis* life cycle (based on life-history data from Belk et al., in review). For *B. roseni,* the first stage is composed of newborn fish <18 mm in standard length (SL); the juvenile 1 stage is composed of small individuals (18 mm to <24 mm); the juvenile 2 stage consists of individuals of intermediate size (24 mm to <30 mm) that are approaching sexual maturity; adult 1 stage contains small adult females (30 mm to <36 mm); and adult 2 contains large adult females (> 36mm) with the highest fecundity. For *B. terrabensis,* the first stage is composed of newborn fish <20 mm in standard length (SL); the juvenile 1 stage is composed of small individuals (20 mm to <28 mm); the juvenile 2 stage consists of individuals of intermediate size (28 mm to <36 mm) that are approaching sexual maturity; adult 1 stage contains small adult females (36 mm to <44 mm); and adult 2 contains large adult females (> 44mm) with the highest fecundity. We used these size classes to estimate mortality rates, and as the basis for calculating growth rates between stages and stage-specific fecundity estimates.

We estimated mortality rates using a model selection framework implemented in the software program MARK [20]. For each fish in our study, we created a recapture record with five recapture events, equally spaced at 1-wk intervals (see above). We assigned each captured fish to one of the five life-cycle stages defined above, allowing us to estimate size-specific mortality rates for each of the two focal populations, as well as assign different recapture probabilities to different stages. We derived mortality rates from model-averaged estimates taken from 12 distinct variations of 4 primary mark–recapture models (as per MARK; [20]). Our four primary competing mark-recapture models were as follows (See Table S1-S2 for models and ranking): 1) mortality rates constrained to be identical across all five stages; 2) mortality rates allowed to vary across all five stages; 3) mortality rates for the three non-reproducing stages constrained to be identical and mortality rates for the two adult stages constrained to be identical; and 4) mortality rates for the three non-reproducing stages and small-adult stage constrained to be identical but allowed to vary from the large-adult stage. The latter two models test the idea that newborn/juvenile and adult mortality rates differ from one another or that mortality in the largest adult size class differs from the other four ontogenetic stages. These models can be compared to the two competing models of 'no difference among stages' and 'all stages differ from one another,' models 1 and 2, respectively. We predicted that model 3 or 4 would show the greatest support, as this would indicate a difference between adult and newborn/ juvenile mortality rates, consistent with the age specific mortality hypothesis. From these four general models we created a total of twelve models that accounted for potential differences in recapture probabilities. These twelve models were as follows: 1) mortality rates and recapture rates constrained to be identical across all five stages; 2A) mortality rates allowed to vary across all five stages and recapture probabilities held constant; 2B) mortality rates allowed to vary across all five stages and recapture probabilities held constant in all non-newborn stages; 3A) mortality rates for the three non-reproducing stages constrained to be identical and mortality rates for the two adult stages constrained to be identical, recapture probabilities held constant; 3B) mortality rates for the three non-reproducing stages constrained to be identical and mortality rates for the two adult stages constrained to be identical, recapture probabilities held constant; 3C) mortality rates for the three non-reproducing stages constrained to be identical and mortality rates for the two adult stages constrained to be identical, recapture probabilities held constant in all but newborns; 3D) mortality rates for the three non-reproducing stages constrained to be identical and mortality rates for the two adult stages constrained to be identical, recapture probabilities allowed to vary between newborns, juvenile 1- juvenile 2, and adult 1-adult 2; 3E) mortality rates for the three non-reproducing stages constrained to be identical and mortality rates for the two adult stages constrained to be identical, recapture probabilities allowed to vary in large adults, newborns, and juvenile 1-juvenile 2-adult 1; 4A) mortality rates for the three non-reproducing stages and small-adult stage constrained to be identical but allowed to vary from the large-adult stage, recapture probabilities held constant in all but newborns; 4B) mortality rates for the three non-reproducing stages and small-adult stage constrained to be identical but allowed to vary from the large-adult stage, recapture probabilities held constant; 4C) mortality rates for the three non-reproducing stages and small-adult stage constrained to be identical but allowed to vary from the large-adult stage, recapture probability allowed to vary between newborns, juvenile 1- juvenile 2- adult 1, and adult 2; 4D) mortality rates for the three non-reproducing stages and small-adult stage constrained to be identical but allowed to vary from the large-adult stage, recapture probability allowed to vary between Newborns, , juvenile 1- juvenile 2, and adult 1-adult 2. We generated and used model-weighted estimates for all subsequent analyses (see Table S2.1-S2.2 for model weights). Taking model-weighted survival estimates allowed us to consider the support from each competing model, thereby providing a more robust estimate than considering any single model alone [21].

Population projection matrix and life cycle graph

We used a population projection matrix approach to model population dynamics in *B. roseni* and *B. terrabensis.* The first step of this approach required identifying a set of biologically meaningful life stages in *B. roseni* and *B. terrabensis*. We delineated five ontogenetic stages based on life-history data for these species (see *Mark-recapture experiment* above for size classes). To complete the matrix model, we used previously documented estimates of fecundity for each adult size class (from Belk et al., in review), and used model-averaged estimates of mortality from MARK for each stage. We then calculated the proportion of individuals in each size class that grew into the next size class and used this as a rate of transition due to growth. Finally, it was necessary to establish an appropriate time interval for a single iteration of the life cycle model. The interbrood interval for *Brachyrhaphis* species is approximately 29 days [14]. We thus calculated our fecundity, mortality, and transition rates for both *B. roseni* and *B. terrabensis* over this time period. We used these data to construct a 5 3 5 projection matrix that can be visually depicted as a life cycle graph (Figure S2.1). The matrix elements (a_{ij}) (detailed in Caswell 2001) are identified in the matrix as follows: (1) stage-specific fecundity occurs in the first row of the matrix; (2) stage-specific survival for individuals that remain in the same stage (stasis) is found along the main diagonal of the matrix; and (3) survival with progression to larger size classes (growth) is found along the sub-diagonals. Mortality and transition rates were implemented as described above. Stage-specific fecundity was measured as the mean number of embryos per female in each ontogenetic stage in each species. We partitioned stage-specific survival estimates into ‘stasis’ and ‘growth’ components using the observed proportion of individuals that remained in the same size class over a four-week period vs. those that grew to a larger size class.

Estimating population demography

We used population projection matrices to examine several demographic patterns (Table S2.3). Specifically, we examine three population traits: the finite rate of population increase (λ), which is the dominant eigenvalue of the matrix; the stable stage structure (*w*) taken from the right eigenvector of the matrix; and the distribution of stage-specific reproductive values (*v*) taken from the left eigenvector of the matrix [19]. In order to identify which life history traits, which stages, and which demographic pathways (i.e., growth, fecundity, and stasis) have the greatest overall impact on population growth rates, we used elasticity analysis [22-24]. This required that we first calculate the sensitivity of population growth rate to perturbation of each element in the matrix (Table S2.4). Sensitivities (s_{ij}) measure the absolute change in λ that would result from absolute changes in each matrix entry ($s_{ij} = \partial\lambda / \partial a_{ij}$). Matrix elements are measured in different units (e.g., fecundity as number or offspring vs. survival rate as a proportion of survivors), and thus sensitivities are difficult to compare without standardizing values. To standardize sensitivities we used elasticity analysis. Elasticities are standardized sensitivities ($e_{ij} = s_{ij} \times [a_{ij}/\lambda]$), which by definition sum to one [25]. They are thus a valuable tool for comparing the relative influence of changes among vital rates, stage classes, etc., on population growth rates [22]. Elasticity values also serve as a measure of selective forces acting on a given vital rate [19, 24]. Higher elasticity values for a given trait indicate a greater contribution of that trait to overall population growth rate, and, in terms of selective forces, indicate stronger selection acting on this trait. Similarly, we can decompose the overall life cycle into distinct demographic sub-cycles, also known as 'loops,' and can evaluate their respective elasticities ([26]).

To generate confidence intervals (95%) for λ and elasticities we conducted a resampling procedure. We first resampled 1000 new random values for survival (φ) and transition rates based on a normal distribution with a mean equal to the point estimate of each parameter and a standard deviation equal to the standard error of each parameter. We also generated 1000 new average fecundity values per stage and population using a bootstrapping method. From these resampled values of φ, transition rates, and fecundity values, we calculated 1000 new values for the matrix entries and 1000 new values of λ and matrix elasticities. We then sorted values of the resulting distribution and used the 25th and 975th values as the lower and upper limits, respectively. This procedure allowed us to compare values of λ and elasticities among species. To assess selection on a given life-history stage we summed the matrix elasticity values for each stage column. To assess selection on a given life-history process (i.e., growth, fecundity, and stasis), we summed the elasticities for the matrix cells that corresponded to the input values for each life-history process (Figure S2.2; Table S2.5 for 95% CIs)

Supplemental results

Mortality rate comparisons

Estimates of mortality rates from our top models (Tables S2.1 and S2.2) revealed that overall *B. roseni* suffered higher mortality rates than *B. terrabensis* (Figure S2.2). Newborn and juvenile survival did not differ between species, but adult *B. roseni* suffered higher mortality rates than *B. terrabensis* (Figure S2.2). Mortality rates were relatively constant at all stages of *B. terrabensis,* while larger *B. roseni* (Adult 1 and Adult 2 classes) suffered increased mortality risk.

Demographic results

Population projection matrices and other demographic estimates (e.g., stage-specific reproductive value) are found in Table S2.3. In *B. rhabdophora,* differences in mortality rates between habitat types resulted in a much higher reproductive value for large adults in the non-predator environment relative to those in the predator environment [14]. This pattern is similar, although more exaggerated, in sister species *B. roseni* and *B. terrabensis.* In low-predation *B. terrabensis,* the reproductive value of large adults is more than double that of small adults of *B.*

terrabensis, and more than double that of both small and large adults of *B. roseni*. Furthermore, the small and large adult size classes in *B. roseni* have nearly an identical reproductive value.

Elasticity values for fecundity, growth, and stasis in *B. roseni* and *B. terrabensis* are remarkably similar to *B. rhabdophora* and *Poecilia reticulata* from divergent predation regimes (Figure S2.3; [14]). In all cases, fecundity had the lowest elasticity, growth had an intermediate elasticity, and stasis had the highest elasticity. This consistency across a diversity of taxa and levels of divergence suggests a clear phenomenon: survival (stasis) is more important than fecundity or growth in terms of impact on population fitness. These results also confirm that predation is more important in terms of its direct impact on population dynamics through predator-induced mortality (represented by stasis) than its indirect impact through the evolution of life-histories (represented by the fecundity measure). This pattern seems to be even more exaggerated in this system than in populations of both *B. rhabdophora* and *P. reticulata* from divergent predation regimes [14]. Indeed, the CIs for stasis were higher in *B. roseni* and *B. terrabensis* than they were in Javilla and Grande (Table S2.5). Confidence intervals for estimates of λ for *B. roseni* and *B. terrabensis* overlapped each other and 1 (*B. roseni:* 0.994 ± 0.056; *B. terrabensis:* 0.965 ± 0.059). This result suggests that, as in *B. rhabdophora,* stable and similar population growth rates can be maintained even in the face of differential mortality rates [14].

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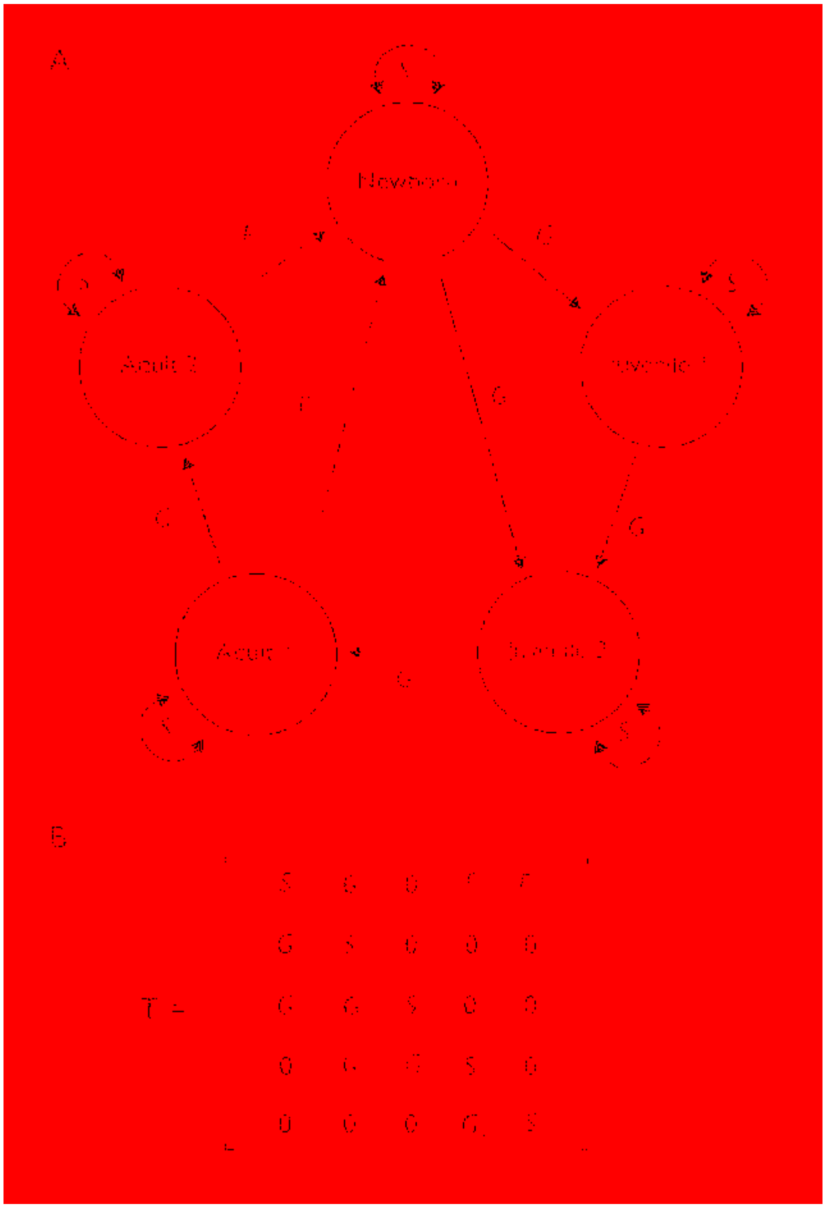

Figure S2.1: Life cycle graph.

(A) Life cycle graph of the live-bearing fish *Brachyrhaphis roseni* and *B. terrabensis*. Nodes represent five ontogenetic stages that individuals progress through from birth to the end of life. Solid arrows represent transitions from one stage to another or stasis in a particular stage (*G*, survival with progression to a larger size class; *S*, survival staying in the same size class). Dashed arrows represent reproduction (*F*, mean fecundity of females in that stage). Subscripts identify size ontogenetic stages as follows: 1, newborn; 2, juvenile 1; 3, large juvenile 2; 4, adult 1; and

5, adult 2. The decimal subscripts on $G_{1.1}$ and $G_{1.2}$ represent the growth of newborn individuals into the small-juvenile (juvenile 1) and large-juvenile (juvenile 2) stages, respectively. (B) Projection matrix for *B. roseni* and *B. terrabensis*. Terms in the matrix coincide with those in the life cycle graph. The matrix can be interpreted as a numeric depiction of the life cycle graph. Zero values represent cases not observed in our study.

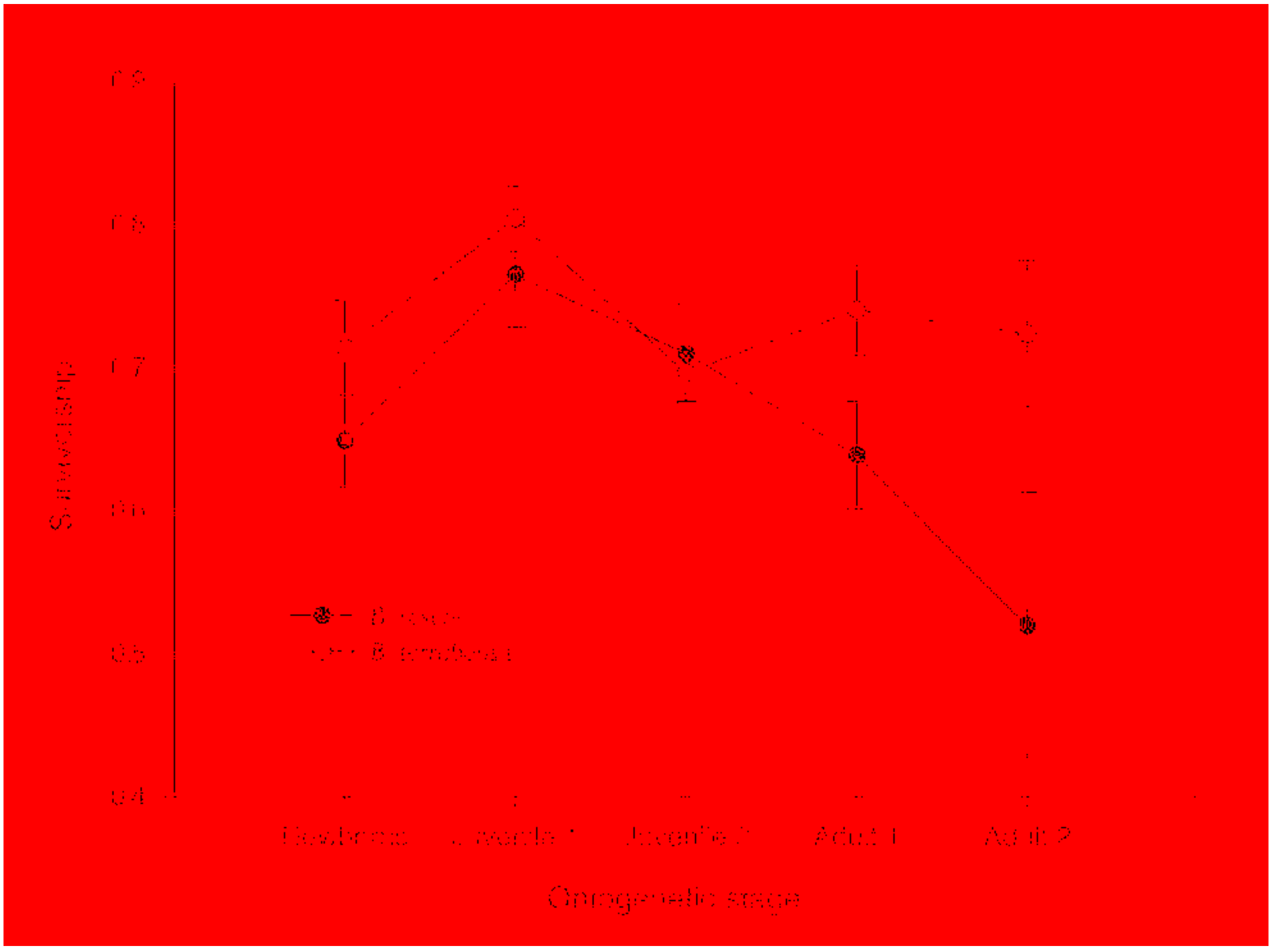

Figure S2.2: Survival rates for *B. roseni* and *B. terrabensis.*

Survival rates (mean ± SE) across the five ontogenetic stages used in this study. Values are presented for both *B. roseni* (predators present) and *B. terrabensis* (predators absent).

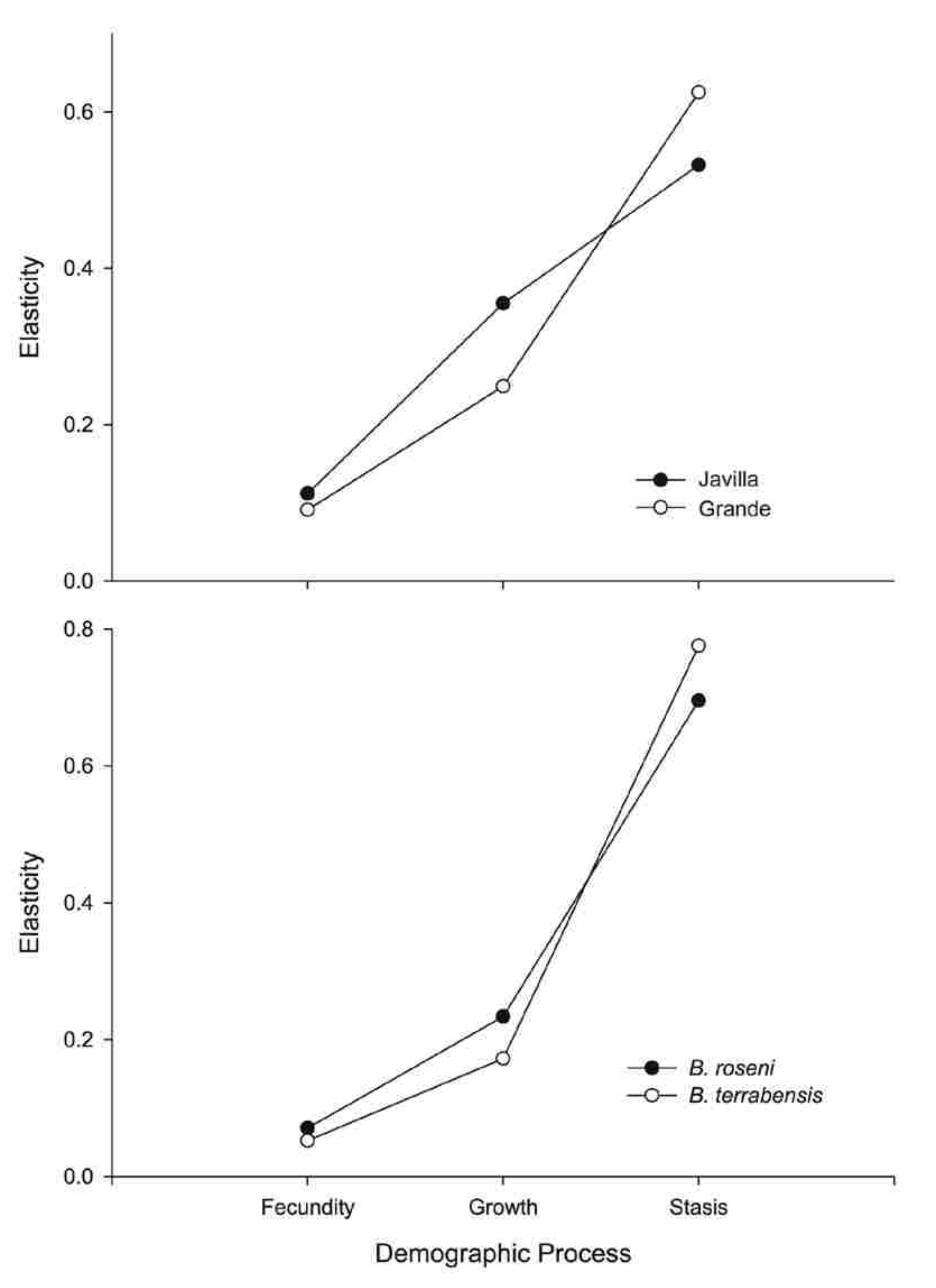

Figure S2.3: Summed elasticities for each life-history process.

A) B. rhabdophora Javilla and Grande; and B) B. roseni and B. terrabensis.

Table S2.1: Competing models of survival in *Brachyrhaphis roseni.*

Model	AICc	Delta AICc	AICc Weights	Model Likelihood	Num. Par	Deviance
4C	581.79	0.00	0.40	1.00	5	129.58
4D	583.71	1.92	0.15	0.38	5	131.50
4A	584.30	2.51	0.11	0.29	4	134.12
3C	584.83	3.04	0.09	0.22	4	134.66
3D	585.10	3.31	0.08	0.19	5	132.89
3E	585.37	3.58	0.07	0.17	5	133.16
1	586.83	5.04	0.03	0.08	2	140.71
3B	587.42	5.63	0.02	0.06	4	137.24
3A	588.19	6.40	0.02	0.04	3	140.05
4B	588.40	6.62	0.01	0.04	3	140.26
2B	589.40	7.61	0.01	0.02	7	133.08
2A	590.61	8.82	0.00	0.01	6	136.35

Table S2.2: Competing models of survival in *Brachyrhaphis terrabensis*.

Model	AICc	Delta AICc	AICc Weights	Model Likelihood	Num. Par	Deviance
4A	922.62	0.00	0.21	1.00	4	140.30
2B	922.82	0.20	0.19	0.90	7	134.36
4C	923.39	0.77	0.15	0.68	5	139.03
3C	923.39	0.77	0.15	0.68	4	141.07
3E	923.63	1.01	0.13	0.60	5	139.27
4D	924.31	1.69	0.09	0.43	5	139.95
2D	925.06	2.44	0.06	0.30	5	140.70
2A	927.46	4.84	0.02	0.09	6	141.05
1	942.41	19.79	0.00	0.00	2	164.15
4B	943.93	21.31	0.00	0.00	3	163.65
3A	944.43	21.81	0.00	0.00	3	164.14
3B	946.40	23.78	0.00	0.00	4	164.08

Table S2.3: Population projection matrices and demographic results.

Population projection matrices and demographic results for populations of *Brachyrhaphis terrabensis* (A) and *B. roseni* (B). Abbreviations are: q_x, estimates of mortality rates in each life stage; *w*, the projected stable stage distribution; and *v*, stage-specific reproductive values.

Life-history stage	Newborn	Juvenile 1	Juvenile 2	Adult 1	Adult 2	*w*	*v*
A) *B. terrabensis*							
Newborn	0.71	0.00	0.00	6.15	11.94	0.68	1.00
Juvenile 1	0.05	0.80	0.00	0.00	0.00	0.20	5.30
Juvenile 2	0.00	0.12	0.70	0.00	0.00	0.09	7.20
Adult 1	0.00	0.00	0.06	0.74	0.00	0.02	34.09
Adult 2	0.00	0.00	0.00	0.03	0.72	0.003	49.28
q_x	0.24	0.20	0.18	0.20	0.25		
B) *B. roseni*							
Newborn	0.65	0.00	0.00	6.61	13.83	0.61	1.00
Juvenile 1	0.12	0.76	0.00	0.00	0.00	0.31	2.99
Juvenile 2	0.00	0.06	0.71	0.00	0.00	0.06	12.33
Adult 1	0.00	0.00	0.13	0.64	0.00	0.02	27.53
Adult 2	0.00	0.00	0.00	0.11	0.52	0.005	29.13
q_x	0.24	0.24	0.24	0.23	0.37		

Table S2.4: Population sensitivity estimates.

Population sensitivity estimates for *B. terrabensis* and *B. roseni,* and populations of *B. rhabdophora* from Grande and Javilla. Sensitivity estimates for *B. rhabdophora* were taken from Johnson and Zuniga-Vega [14].

Stage	*B. terrabensis* (Predators absent)					*B. roseni* (Predators present)				
Newborn	0.208	0	0	0.007	0.001	0.203	0	0	0.007	0.002
Juvenile 1	1.100	0.323	0	0	0	0.606	0.306	0	0	0
Juvenile 2	0	0.439	0.194	0	0	0	1.262	0.246	0	0
Adult 1	0	0	0.920	0.232	0	0	0	0.550	0.197	0
Adult 2	0	0	0	0.335	0.043	0	0	0	0.209	0.048
Stage	**Grande (Predators absent)**					**Javilla (Predators present)**				
Newborn	0.15	0	0	0.02	0.01	0.2	0	0	0.02	0.0
Juvenile 1	0.24	0.11	0	0	0	0.45	0.11	0	0	0
Juvenile 2	0.65	0.31	0.22	0	0	0	0.31	0.2	0	0
Adult 1	0	0	0.63	0.27	0	0	0	0.49	0.34	0
Adult 2	0	0	0	0.84	0.25	0	0	0	0.51	0.05

Table S2.5: Results of permutation analyses.

Confidence intervals (95%) resulting from permutation analysis for summed elasticities of each demographic process (fecundity, growth, and stasis).

Population	Fecundity	Growth	Stasis
Javilla	0.095 – 0.119	0.313 – 0.375	0.506 – 0.593
Grande	0.079 – 0.104	0.226 – 0.275	0.586 – 0.665
B. roseni	0.059 – 0.077	0.203 – 0.260	0.664 – 0.733
B. terrabensis	0.055 – 0.077	0.194 – 0.256	0.669 – 0.747

Chapter 3

Morphological Divergence Driven by Predation Environment Within and Between Species of *Brachyrhaphis* Fishes

Spencer J. Ingley[1, 2], Eric J. Billman[1], Mark C. Belk[1], and Jerald B. Johnson[1, 3]

[1]Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT 84602

[2] Corresponding Author: E-mail: sjingley@byu.edu

[3] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602

Abstract

Natural selection often results in profound differences in body shape among populations from divergent selective environments. Predation is a well-studied driver of divergence, with predators having a strong effect on the evolution of prey body shape, especially for traits related to escape behavior. Comparative studies, both at the population level and between species, show that the presence or absence of predators can alter prey morphology. Although this pattern is well documented in various species or population pairs, few studies have tested for similar patterns of body shape evolution at multiple stages of divergence within a taxonomic group. Here, we examine morphological divergence associated with predation environment in the livebearing fish genus *Brachyrhaphis*. We compare differences in body shape between populations of *B. rhabdophora* from different predation environments to differences in body shape between *B. roseni* and *B. terrabensis* (sister species) from predator and predator free habitats, respectively. We found that in each lineage, shape differed between predation environments, consistent with the hypothesis that locomotor function is optimized for either steady swimming (predator free) or escape behavior (predator). Although differences in body shape were greatest between *B. roseni* and *B. terrabensis*, we found that much of the total morphological diversification between these species had already been achieved within *B. rhabdophora* (29% in females and 47% in males). Interestingly, at both levels of divergence we found that early in ontogenetic development, females differed in shape between predation environments; however, as females matured, their body shapes converged on a similar phenotype, likely due to the constraints of pregnancy. Finally, we found that body shape varies with body size in a similar way, regardless of predation environment, in each lineage. Our findings are important because they provide evidence that the

same source of selection can drive similar phenotypic divergence independently at multiple divergence levels.

Keywords

Brachyrhaphis, geometric morphometrics, predation environment, pregnancy, mating strategies, trade-offs

Introduction

Numerous studies have documented adaptation to divergent natural selection regimes [1-8]. However, most studies examining fine-scale evolutionary diversification are limited to either between species or within species differences, and as a result, fail to adequately address how the same source of selection drives phenotypic divergence at varying taxonomic levels (a broad but general exception being studies of convergent and parallel evolution). Indeed, few studies have looked at the evolution of adaptive strategies across a speciation continuum (i.e., both within and between species) with the intent of determining how much diversification takes place across different stages of speciation [9-11]. The paucity of such studies may be due to the difficulty of identifying systems where similarly divergent selection regimes have driven or are driving divergence at multiple taxonomic levels. These studies are valuable to our understanding of evolutionary diversification, and can help explain how predictable phenotypic divergence is when populations or species are subject to similar selective environments.

Predation has been a focal mechanism of divergent selection since Darwin outlined his theory of evolution by natural selection [12]; indeed, Darwin saw predation-prey interactions as some of the clearest cases of natural selection, and cited numerous examples of adaptation in both predator and prey [12]. Predation is known to affect numerous traits in both predator and prey, including behavior, life history, and morphology [7,8,13-25]. Morphological adaptations resulting from different predation environments are of particular importance because they reflect both behavioral and life-history adaptations, and such adaptations have been observed in numerous and diverse taxa [8,20,26-36]. Predators can have a profound effect on the evolution of prey body shape, especially for traits related to escape behavior [37]. Comparative studies of taxa from different 'predation environments,' both between populations within species and between species pairs, show a strong link between the presence of predators and overall prey morphology [13,20,31-36].

Livebearing fishes (Poeciliidae) have been used as model systems in a diversity of ecological and evolutionary studies [6,23,38-45]. Many of these studies have focused on adaptation to divergent predation environments, specifically examining life-history evolution and morphological divergence driven in large part by the presence or absence of predators [6,21,46-52]. The live-bearing fish genus *Brachyrhaphis* has become an important model for studying the evolution of predator-mediated adaptations [6,13,23,46]. *Brachyrhaphis* occur primarily in lower Central America (LCA), with many species endemic to Costa Rica and Panama. Several species of *Brachyrhaphis* exhibit adaptation to divergent predation environments, including changes to life-history [46] and morphology [6,13]. *Brachyrhaphis rhabdophora,* for example, has evolved divergent life-history strategies associated with predation environment that are similar to those observed in numerous other poeciliid species [46,53]. Studies of adaptation in *Brachyrhaphis* have so far focused exclusively on intra-specific variation, where populations of a given species occur in either 'predator free' or 'predator' environments. Interestingly, similar patterns of morphological divergence may be present at deeper phylogenetic levels within *Brachyrhaphis* (i.e., between sister species rather than populations within a species; see below).

If this is the case, then *Brachyrhaphis* would provide an ideal model system for studying morphological variation both among populations and between species from divergent predation environments, and testing for similar patterns of divergence among different phylogenetic levels to determine how similar selective regimes drive phenotypic divergence.

Brachyrhaphis roseni and *B. terrabensis* are sister species [54] that have similar distributions, occurring from southeastern Costa Rica to central Panama along the Pacific versant [55]. Although these species frequently occur within the same drainages, *B. terrabensis* typically occupies higher elevation headwater streams, while *B. roseni* occupies lower elevation coastal streams [55]. Consequently, *B. terrabensis* occurs in streams that are primarily void of piscivorous predators, while *B. roseni* co-occurs with numerous and abundant predators (e.g., *Hoplias microlepis*). This pattern is similar to that observed among populations *within* other poeciliid species [13,21,23,27,47,50,51], including the well-studied sister species to this species pair, *B. rhabdophora* [24,25,43,46,56]. However, *B. roseni* and *B. terrabensis* are unique because they themselves do not span both predator and predator free environments, but rather are segregated into predator and predator free environments, respectively (Belk et al. in review; unpublished data). Furthermore, *Brachyrhaphis roseni* and *B. terrabensis* have evolved similarly divergent life histories (Belk et al. in review) to those observed between populations of *B. rhabdophora* [46], *B. episcopi* [23], and other poeciliids [21], namely smaller size at maturity with more and smaller offspring in predator environments than in predator free environments. The hypothesis that these species are sister taxa, and the fact that they occur in divergent predation environments and display predictable patterns of life-history divergence, suggests that the selective forces driving divergence between populations of *B. rhabdophora* (i.e., predator vs. predator free environments) might also have driven divergence between *B. roseni* and *B.*

terrabensis. This provides an opportunity to compare morphological variation both within (recently diverged) and between species of *Brachyrhaphis* from opposing predation environments in two closely related evolutionary lineages. In addition to testing for gross differences in prey morphology associated with predation environment, our data set allows us to test for similar patterns of morphological divergence both between sexes and among size classes.

In this study, we use geometric morphometric analyses to test four hypotheses related to morphological divergence driven by predation environment in three species of *Brachyrhaphis* fishes. We focus on contrasts between *B. roseni* and *B. terrabensis* and between populations of *B. rhabdophora* from divergent predation environments. Our hypotheses are as follows.

First, we predict that body shape differs between *B. roseni* and *B. terrabensis*, and between populations of *B. rhabdophora* from different predation environments. We predict that populations from predator environments (*B. roseni* and predator *B. rhabdophora*) will be more streamlined and have a more robust caudal peduncle region than populations from predator free environments (*B. terrabensis* and predator free *B. rhabdophora*) due to morphological optimization for different swimming modes [8,49,57-62]. Co-occurrence with predators should favor the evolution of a body form optimized for fast-start swimming (i.e., greater burst speed ability), needed to evade predator strikes [8]. In contrast, increased resource competition often associated with predator free environments should favor the evolution of a body form optimized for more efficient prolonged swimming, important for finding and consuming food, acquiring mates, and conserving energy for reproduction [8,49]. Given that these two swimming types are optimized by different propulsor arrangements (i.e., fin size and shape, muscle size and shape), optimizing body shape for one swimming mode necessarily compromises the other. Prolonged swimming performance is optimized with a relatively shallow caudal peduncle, and a deep anterior body/head region. Fast-start swimming is optimized by the opposite trait values, including deep caudal peduncle and a shallow anterior body/head [8,49,57-62].

Second, we expect to find similar, but more pronounced (i.e., greater magnitude), morphological divergence occurs between sister taxa *Brachyrhaphis roseni* and *B. terrabensis* than occurs between populations of *B. rhabdophora* from different predation environments. This hypothesis focuses on determining how much divergence occurs between populations of *B. rhabdophora* from different predation environments versus between sister species *B. roseni* and *B. terrabensis* from different predation environments. We predict that divergence in body shape between *B. roseni* and *B. terrabensis* will be associated with predation environments as predicted by theory, and that these differences will be similar but more exaggerated than those observed between populations of *B. rhabdophora.* This difference in magnitude could be attributed to several factors, including for example a greater time since divergence or differences in the balance between strength of divergent selection and homogenizing gene flow.

Third, we predict that body shape will vary between sexes, both for the among-species and among-population comparisons. Although the pattern of variation described above is predicted to occur between populations from different predation environments due to divergent natural selection, it is also likely that, within populations, these morphological traits are affected by differences in reproductive roles between sexes, mating strategies among size classes, and ontogenetic changes. Given that *Brachyrhaphis* are live-bearing, females of all three species may be constrained morphologically by pregnancy in the same way [37]. Therefore, we test if patterns of sexual dimorphism show equal magnitude and direction of divergence between contrasting selective environments, essentially addressing the question, do differences in male and female reproductive roles constrain or magnify shape responses to variation in predation environment?

We predict that female body shape will converge between predation environments relative to males due to the constraint of pregnancy.

Finally, we test the hypothesis that body shape differs among size classes across predation environments. This hypothesis tests for an interaction between size and species, and addresses potential differences in reproductive roles, alternative-mating strategies among size classes, and ontogenetic effects. We predict that shape will not vary consistently across sizes (i.e., as individuals mature and grow) because of the potential for variation in male reproductive strategy across size classes in *Brachyrhaphis* (i.e., coercive mating versus coaxing), and differences in female reproductive allocation at different sizes.

Materials and methods

Molecular Laboratory Methods and Analysis of Genetic Distance

A primary purpose of this study is to determine how body shape evolves at different phylogenetic levels of divergence (i.e., within and between species) when populations are subject to similarly divergent selective regimes. Although a previous study of *Brachyrhaphis rhabdophora* indicated little molecular divergence among populations from different predation environments [43], the amount of molecular divergence among populations of *B. rhabdophora* compared to the amount of divergence between sister species *B. roseni* and *B. terrabensis* remains relatively unexplored (but see Mojica et al. 1997). Thus, we generated mitochondrial DNA sequences from the cytochrome *b* (cyt*b*) gene for four representative populations of *B. rhabdophora* from different predation environments and for six populations of *B. roseni* and *B. terrabensis.* We isolated DNA using the Qiagen DNeasy96 tissue protocol (QIAGEN Sciences, Maryland, USA) and amplified cyt*b* fragments for each sample by PCR, using forward primer

GLU31 [63] and reverse primer HD15680 [64]. We followed [65] for amplification and sequencing reactions, clean up, and sequence visualization. We assembled contigs and checked amino acid coding for errors (stop codons) while viewing electropherograms in Geneious [66], and manually aligned sequences in Mesquite v. 2.75 [67]. We obtained a total of 26 *B. rhabdophora,* 16 *B. roseni,* and 18 *B. terrabensis* sequences of a cyt*b* fragment 1140 bp in length (plus ~65 bp of the downstream gene) representing four, three, and three populations, respectively. All sequences were deposited on Genbank under accession numbers KJ081551 - KJ081609.

In order to test for varying levels of molecular divergence within and among species of *Brachyrhaphis,* we computed pairwise genetic distances using MEGA5 [68]. We first computed raw pairwise genetic distance. Next, we used a model selection framework (AIC, [69]) within jModelTest 2 [70] to determine the best-fit model of molecular evolution for our data set. We then calculated model-corrected pairwise genetic distances using the best-fit model, TrN+G [71], with the Tamura-Nei model and gamma distributed rates among sites in MEGA5 [68]. Our results show that *B. roseni* and *B. terrabensis* show a greater level of genetic divergence than populations of *B. rhabdophora* from different predation environments. Pairwise population comparisons of cyt*b* among populations of *B. rhabdophora* from different predation environments revealed remarkably little variation (mean model corrected pairwise genetic distance = 0.004). On the contrary, pairwise population comparisons between *B. roseni* and *B. terrabensis* showed genetic distance an order of magnitude greater (mean model corrected pairwise genetic distance = 0.04). Thus, with an expanded sampling both in terms of numbers of base pairs and sequences, we find strong evidence that supports the findings of Johnson (2001) and refute the findings of Mojica et al. (1997). Collectively, these data validate our comparison as one consisting of two levels of phylogenetic divergence.

Study Sites and Characterizing Predation Environment

We collected *Brachyrhaphis roseni* and *B. terrabensis* with a handheld seine from eight streams in the Chiriquí province of Panama between 20 and 29 August 2011, and one population of each species from eastern Costa Rica during 2007 (Figure 3.1). We collected *Brachyrhaphis rhabdophora* from two predator free and three predator environments in Guanacaste region of Costa Rica between 5 and 12 May 2006. All animal collecting was conducted under Brigham Young University IACUC committee approval. All necessary permits were obtained for the described field studies, and no collecting took place on private or protected lands. Collecting and export permits were provided by the Autoridad Nacional del Ambiente in Panama and under the Costa Rican Ministerio del Ambiente y Energía Sistema Nacional de Areas de Conservasión in Costa Rica.

The streams are characterized by a pool-riffle-pool structure, similar to that observed in other *Brachyrhaphis* species [25]. A primary environmental indicator of *B. roseni, B. terrabensis,* and *B. rhabdophora* life history divergence is the presence or absence of piscivorous predators (e.g., *Parachromis dovii* and *Hoplias microlepis* [24,25,46], unpublished data). Although predation pressure may be the selective force of most importance in this system, ‘predation environment’ is characterized by the presence (‘predator’) or absence (‘predator free’) of predators and a suite of other confounded environmental factors. For example, resource availability, stream gradient, and stream width may play an important role in determining life-history evolution and resulting morphology and are known to co-vary with presence or absence of predators in *B. rhabdophora* [56]. In this study, we consider 'predation environment' to be this suite of ecological features, which included either the presence or absence of piscivorous predators. *Brachyrhaphis roseni*, *B. terrabensis,* and *B. rhabdophora* typically occur in low velocity stream habitats (i.e., side-channels and pools found in small tributaries), although higher elevation sites (typical of *B. terrabensis* populations) tend to have steeper gradients and slightly faster stream velocities. *Brachyrhaphis terrabensis* primarily occurs in the same river drainages as *B. roseni,* although at higher elevations. *Brachyrhaphis roseni* habitat is characterized by low-elevation streams that are predator environments, while *B. terrabensis* occurs in predator free environments. *Brachyrhaphis rhabdophora* is found in both habitat types, predator free (typically high-elevation) and predator (typically low-elevation).

Geometric Morphometric Analyses

We used a total of 802 fish in the geometric morphometric analysis: 211 *B. terrabensis* (predator free), 289 *B. roseni* (predator), and 302 *B. rhabdophora* (201 from predator, and 101 from predator free sites). For all sites, there were roughly equal numbers of males and females, and a representative sample of the range of size variation observed within each population. For each fish, we measured standard length (mm), and digitized thirteen biologically homologous landmarks (or semi-landmarks) on a lateral image of each fish (tpsDig; [72]). Landmarks were defined as: (1) anterior tip of the snout; (2), anterior extent of the eye; (3) semi-landmark midway between landmarks 1 and 4; (4) anterior insertion of the dorsal fin; (5) posterior insertion of the dorsal fin; (6) semi-landmark midway between landmarks 5 and 7; (7) dorsal origin of the caudal fin; (8) ventral origin of the caudal fin; (9) semi-landmark midway between landmarks 8 and 10; (10) posterior insertion of anal fin or gonopodium in males; (11) anterior insertion of the anal fin or gonopodium in males; and (12) semi-landmark midway between landmarks 11 and 13; (13) intersection of the operculum with the ventral outline of the body.

We summarized shape variation from digital landmarks into relative warps (i.e., principal components) using tpsRelw [73]. We used generalized Procrustes analysis [74] to remove all non-shape variation due to position, orientation, and scale of the specimens for each image. For sliding semi-landmarks we used the minimize d^2 option in tpsRelw. Relative warps are defined as linear combinations of affine and non-affine shape components that describe some portion of the variation observed in the specimens [73]. We used the first 10 relative warps, which combined explained more than 96% of the shape variation, in subsequent analyses. By using only the top ten relative warps we effectively reduce the number of variables and account for the reduced dimensionality from use of sliding semi-landmarks. We analyzed the data using mixed model multivariate analysis of variance (MANOVA) in ASREML-R version 3.00[75] within R (R Core Development Team 2010). Within each model, we included sampling site as a random factor to ensure that outlier sites did not drive the patterns we observed. Given that relative warps are orthogonal and ordered according to the amount of variation they explain, they can be treated as repeated measures with the use of an 'index variable' analogous to time in traditional repeated measures models. This method has been successfully employed in similar studies of shape variation in *B. rhabdophora* [6] and other livebearing fishes [76]. Thus, the order number of the relative warps (i.e. 1–10; reflecting the order of the warps but not the value) was treated as an index variable and included in the repeated statement for mixed model analyses. The use of the index variable arises out of mathematical necessity, and is crucial for this method to work and to interpret the results. It is the interaction of the main effect with the index variable that allows us to test the hypothesis that shape differs between groups on any one or any linear combination of relative warps. This is the same hypothesis tested in a standard MANOVA, but the index variable allows us to test this hypothesis in a mixed model framework. We tested each of our four hypotheses (detailed above) using these data.

To test for overall shape differences between predation environments (hypothesis 1), and for shape differences between predation environment and across sexes (hypothesis 3), we first tested for main effects and interactions of predation environment, sex, centroid size (a covariate; hereafter size), and index variable for the whole dataset ($N = 802$). Within each model, we included sampling site as a random factor to ensure that outlier sites did not drive the patterns we observed. Our initial global model estimated shape as ~ index variable + species + sex + size + (index variable: species) + (index variable: sex) + (index variable: size) + (index variable: species: sex) + (index variable: species: size) + (index variable: sex: size) + (index variable: species: sex: size). We used model selection techniques (i.e., AIC) to determine if a reduced model (all possible models maintaining the fixed effects) resulted in a better model fit (i.e., lowest AIC score; [69,77]). In our analysis, interactions between main effects and the index variable served as the most direct test of our hypotheses. Simple interactions of main effects are less informative because the interaction with the index variable tests for differences in shape on each of the relative warps independently, while simple interactions do not. If we do not consider the interaction with the index variable we are simply testing for differences among treatments when averaged across all relative warps. Relative warps are independent from each other (i.e., they explain different axes of variation); therefore the magnitude and direction of differences between levels of the main effects may vary differently and randomly across relative warps. Interactions with the index variable allow relative warps to vary independently (i.e., not to be considered as a whole) and thus allow the interaction to be significant even if the main effects alone, or their interactions, are not [6].

Given that in both of our taxonomic contrasts we found a significant interaction between predation environment, sex, and the index variable in the MANOVA, we applied a phenotypic change vector analysis (PCVA; [78-80]) to determine the specific nature of the interaction to test for differences in shape changes between sexes. This analysis has been used previously and effectively in another *Brachyrhaphis* species [6]. The PCVA tests whether the significant interaction between main effects and the index variable resulted from differences in magnitude (*MD*) or direction (Θ) of morphological change. The PCVA tests magnitude and direction across all relative warps. Specifically, we used the PCVA to compare the amount and direction of sexual dimorphism between *B. roseni* and *B. terrabensis,* and between populations of *B. rhabdophora* from different predation environments. Here, we compared both size and direction of the phenotypic trajectories to test for differences in magnitude of sexual dimorphism and for different effects of predation on males and females (i.e., to determine if predation affects sexes differently), respectively. We conducted the PCVA using ASREML-R version 3.00 [75] within R (R Core Development Team 2010). We plotted LS means on the first two relative warp axes, which accounted for 64.36% of the shape variation, to visualize differences in magnitude and direction of shape change (Figure 3.2).

To test for a difference in magnitude of variation between predation environment (hypothesis 2), and for differences between predation environment across sizes (hypothesis 4), we tested for main effects and interactions of species group (*B. roseni* /*B. terrabensis* and *B. rhabdophora* from divergent predation environments), predation environment, size, and index variable for each sex (males $N = 278$; females $N = 523$) using a mixed model MANOVA. We included location as a random variable in the model. Our full model estimated shape as = index variable + group + environment + size + (index variable: group) + (index variable: environment) + (index variable: size) + (index variable: group: environment) + (index variable: group: size) + (index variable: environment: size) + (index variable: group: environment: size). We used model selection techniques to determine if a reduced model resulted in a better model fit [69,77]. Where the interaction of group, environment, and index variable was significant in the MANOVA, we applied the PCVA to determine whether the significant interaction between main effects and the index variable resulted from differences in *MD* or Θ of morphological change. Following significant interaction between size and the index variable, we generated thin-plate splines in tpsRegr [81] using centroid size and superimposed landmark coordinates to visualize shape variation along the centroid size axis.

Results

Effects of Predation Environment on Body Shape

Consistent with the predictions in our first hypothesis, we found that body shape differed between predation environments both within *Brachyrhaphis rhabdophora* and between *B. roseni* and *B. terrabensis*. The best-fit model estimated shape as ~ index variable + species + sex + size + (index variable: species) + (index variable: sex) + (index variable: size) + (index variable: species: sex) + (index variable: species: size) + (index variable: sex: size) + (index variable: species: sex: size). Morphology differed significantly for the interaction of species group, predation environment, and index variable for both females and males (Table 3.1). Thus, we conducted a PCVA analysis to determine if the significant differences were caused by the magnitude of change, the direction/angle of change, or both for each sex (hypothesis 2). For females, the PCVA revealed that the magnitude of shape variation was greater in the *B. roseni/B. terrabensis* species group (*MD* = 0.0348; P = 0.001); the trajectories also differed in orientation (θ = 80.14°; P = 0.001). Similarly, the PCVA revealed that the magnitude of shape variation in males was greater in the *B. roseni/B. terrabensis* species group (*MD* = 0.0247; P = 0.001) and that the trajectories differed in orientation (θ = 81.80°; P = 0.002). Consistent with the predictions for our second hypothesis, greater morphological differentiation occurred between *B. roseni/B. terrabensis* than between populations of *B. rhabdophora* from different predation environments. Specifically, *B. rhabdophora* achieved 29 % (females) and 47% (males) of the divergence present between *B. roseni/B. terrabensis.*

Morphology differed significantly for the interaction of predation environment, sex, and index variable (Table 3.2). Thus, we conducted a PCVA analysis to determine if the significant difference was caused by the magnitude of change, the direction/angle of change, or both. Summary statistics revealed that there was significant variation in the magnitude of sexually dimorphic shape change among the four taxa ($\mathrm{Var}_{\mathrm{size}}$ = 0.0000977; P = 0.003) and significant variation in the direction of shape change ($\mathrm{Var}_{\mathrm{orient}}$ = 257.57; P = 0.001). Within species groups, the magnitude of shape change was not significantly different; however, the magnitude of sexually dimorphic shape change was significantly greater in the *B. roseni/B. terrabensis* species group in all pairwise comparisons with the *B. rhabdophora* group (Table 3.3). The direction of shape change was significant in all pairwise comparisons (Table 3.3). For within species comparisons, the direction of shape change represented a convergence of shape in females, which was consistent with the predictions of our third hypothesis.

To determine how shape varies across size classes (hypothesis 4) in females (due to changes associated with pregnancy) and males (due to potential differences in mating strategies and ontogenetic effects), we generated thin-plate splines in tpsRegr [81] using centroid size and superimposed landmark coordinates to visualize shape variation along the centroid size axis in females (Figure 3.3) and males (Figure 3.4) of both species. We found that females showed a shift in morphology from small to large that was characterized by an increase in abdomen size and a decrease in caudal peduncle area. Adult males showed a shift in morphology from small to large that was characterized by a shortening and deepening of the head region and a reduction in the caudle peduncle region.

Discussion

The principal objective of our study was to test for divergent morphologies driven by predation environment in *Brachyrhaphis* fishes at two taxonomic levels in two phylogenetically sister lineages, and determine how much variation occurs within populations and species that have evolved in similarly divergent selective regimes. We predicted that the divergent morphology observed between these species and populations would reflect body shape optimized for their native predation environment, although the magnitude of morphological divergence would be greater between *B. roseni* and *B. terrabensis* than between populations of *B. rhabdophora* from different predation environments. We also tested for differences in shape between sexes and across size classes, and predicted that shape optimization would differ across sex and size class according to potential differences in mating strategies or reproductive constraints.

Parallel Morphological Evolution at Two Levels of Divergence

Our results strongly support divergent morphologies between *Brachyrhaphis roseni* and *B. terrabensis,* and between populations of *B. rhabdophora* from different predation environments as predicted by theory (Table 3.2; Figure 3.2) [8,51,57-62,82]. As predicted, individuals from predator environments showed a deeper caudal peduncle and a shallower anterior body/head than individuals from predator free environments. This pattern is strikingly similar to that observed in other poeciliids [8,13], and strongly suggests that 'predation environment' is the principal driver of parallel patterns of shape variation between both sister species (*B. roseni* and *B. terrabensis*) and populations within a species (*B. rhabdophora*). Importantly, although our results suggest that both male and female body shape was significantly more divergent (i.e., more pronounced) between *B. roseni* and *B. terrabensis* than between *B. rhabdophora* populations from different predation environments (Figure 3.2), 47% (males) and 29% (females) of the variation in body shape was already present between populations of *B. rhabdophora*. Therefore, although sister species *B. roseni* and *B. terrabensis* are clearly at a point of greater divergence (i.e., phylogenetically but also potentially ecologically), both taxon pairs are on a similar evolutionary trajectory and *B. rhabdophora* has already reached a substantial level o cf evolutionary diversification. Intraspecific evolutionary divergence of this type has been noted in a variety of poeciliid fishes for several different traits [13,39,40,46-49]. Interestingly, we found that in *B. rhabdophora* divergence in male morphology was greater than divergence in female morphology, at least relative to variation noted between *B. roseni and B. terrabensis*. This pattern of males evolving more rapidly than females has previously been noted in guppies in work that focused on life history traits [83]. Following an introduction experiment, which involved transplanting populations from high-predation to low-predation sites, evolution of male life-history traits was significantly more rapid than female life-history traits [83]. This finding was largely attributed to a difference in heritability, possibly associated with Y chromosome-linked traits [83]. The pattern observed in *Brachyrhaphis* suggests that female body shape is less variable, perhaps due to constraints associated with pregnancy (see below). The fact that male *B. rhabdophora* have achieved a greater amount of divergence relative to females may be due to greater existing variation in male body shape. One possible explanation is that males that employ alternative mating strategies have evolved different morphologies to accommodate these strategies (see below). If males of different sizes do in fact tend to adopt alternative mating strategies, it would be likely that greater genetic variance would occur in males relative to females, possibly contributing to the greater differentiation achieved in male *B. rhabdophora* relative to female *B. rhabdophora.* Overall, we see four possible explanations for why greater divergence occurs between *B. roseni* and *B. terrabensis* than occurs within *B. rhabdophora,* although we did not explicitly test any of these hypotheses, and only briefly state them here. First, the time since *B. roseni* and *B. terrabensis* diverged could be greater than the time since populations of *B. rhabdophora* from predator and predator free environments. Second, *B. roseni* and *B. terrabensis* could be experiencing stronger divergent selection than *B. rhabdophora.* Third, populations of *B. rhabdophora* and sister species *B. roseni-B. terrabensis* could be experiencing differences in the balance between selection and gene flow. And finally, greater heritable variation could be present between *B. roseni* and *B. terrabensis* relative to *B. rhabdophora.* These hypotheses should be tested further to determine the exact nature of this difference in relative morphological divergence.

The idea that *Brachyrhaphis roseni* and *B. terrabensis* are sister taxa that occur in the same drainages but in different predation regimes suggests the possibility that divergent natural selection has driven and maintains reproductive isolation between these two species. Numerous lines of evidence suggest that the most recent common ancestor of this species pair likely occurred across a range of predation habitats within the drainages where *B. roseni* and *B.*

terrabensis are currently found, a pattern strikingly similar to that found in congenerics *B. rhabdophora* [24,25,43,46,56] and *B. episcopi* [23,42,84]. For example, multiple recently diverged populations of *B. rhabdophora* have evolved life-history phenotypes that are adaptive for their specific predation environments [24,25,43,46,56]. *Brachyrhaphis roseni* and *B. terrabensis* have evolved nearly identical, although more pronounced, life-history phenotypes as a result of divergent selection regimes (Belk et al., in review). Likewise, our results suggest that body shape evolution is also occurring in parallel, with similar but more pronounced divergence in *B. roseni* and *B. terrabensis* than is found in *B. rhabdophora.* This begs the question: have similarly divergent selection regimes also driven the evolution of reproductive isolation in parallel? Previous studies suggest that body shape plays a key role in mate choice in other livebearing fish, and that individuals prefer as mates those who have a body shape optimized for selection regimes similar to their own [7]. If this holds true in *Brachyrhaphis,* it is likely that reproductive isolation due to assortative mating for body shape may already occur between populations of *B. rhabdophora,* and is even stronger between *B. roseni* and *B. terrabensis*. Studies in our lab are currently underway to test these predictions.

Reproductive Constraints on Morphological Evolution

Although shape varied between *B. roseni* and *B. terrabensis,* and between populations of *B. rhabdophora* from different predation environments as predicted (hypothesis 1), the degree of variation was not equal across sexes (hypothesis 3). As predicted, both male and female diverged as a function of predation environment; however, divergence in female shape was less than divergence in male shape (Figure 3.2). One explanation for this is that *Brachyrhaphis* are livebearing fishes with a female body shape constrained by pregnancy [6], regardless of predation environment. Hence, immature females from different predation environments might initially differ in body shape, but these differences go away once females become pregnant. This difference is predicted by a tradeoff that occurs between reproduction and fast-start swimming performance (i.e., pregnant females have reduced fast-start speeds), as observed in another poeciliid species [6,37]. This observation of female shape convergence also illuminates previous patterns observed regarding mortality rates in the closely related *B. rhabdophora* [25]. Johnson and Zuniga-Vega (2009) showed that differential mortality rates drive life-history evolution in *B. rhabdophora* (i.e., higher survivorship in predator free environments than in predator environments), and that in predator environments mortality rates were relatively constant across size classes until individuals reached the largest size class where mortality increases. This pattern is reversed in predator free environments (i.e., survivorship increases in the largest size class). If convergence in body shape coincides with divergent mortality rates as size increases, then our data suggest that *B. roseni* and *B. terrabensis* should also be experiencing differences in size-specific mortality rates. A possible explanation is the negative impact that pregnancy may have on fast start swimming performance (useful in predator environments) as seen in related poeciliid fish [37].

Morphological Evolution across Size Classes: Role of Sexual Selection and Alternative Mating Strategies?

In addition to finding gross differences in morphology between predation environments, we found evidence that shape did not vary consistently among size classes of adult females (Figure 3.3) and males (Figure 3.4) of all *Brachyrhaphis* species studied. In other words, we found allometric differences in shape among size classes in each taxon. We predicted that shape would not vary consistently across sizes (i.e., as individuals mature and grow) because of the potential variation in male reproductive strategy across size classes in *Brachyrhaphis,* and differences in female reproductive allocation at different sizes. As adult females increase in size, the predominant shape change that occurs is a relative increase in abdomen size and a resulting relative decrease in the caudal peduncle region. This finding complements Wesner et al. (2011), who found that late in pregnancy, female body shape converges due to constraints of pregnancy on body shape. The patterns observed between female *B. roseni* and *B. terrabensis,* and *B. rhabdophora* from different predation environments, is remarkable similar.

The pattern of shape change with size in mature males follows a different pattern, potentially consistent with different reproductive strategies between small and large males (i.e., sneaker males vs. displaying males) in each species. Patterns of shape variation with size observed in males of *B. roseni, B. terrabensis,* and *B. rhabdophora* are consistent with shapes that are optimized for behaviors associated with reproductive mode; within taxonomic units, small males had a body shape that facilitated burst swimming more than large males (e.g., more streamlined with a more robust caudal peduncle), who demonstrated a body shape that was more conducive to endurance swimming necessary for displaying behaviors (i.e., deeper anterior body/head region with a relatively shallow peduncle) [12-14,51,55]. The size at which a male reaches maturity has a large effect on mode of reproduction in numerous livebearing fish [85-87] because males typically do not grow after maturing. Relatively smaller males ("sneakers") often rely on forced copulations (i.e., coercion) rather than courting females to win mates, although the degree to which this pattern holds is highly species specific; mating strategy is context dependent [82,86-90] in some species (i.e., relative size determines mating strategy), while in others mating strategy is genetically based and not plastic [86,87,91]. Preliminary observations suggest that small *Brachyrhaphis* males tend to sneak (especially in the presence of larger males), while larger males devote more of their reproductive efforts to displaying to win mates (personal observation). Although species-specific variation in mating strategies exists, some patterns can be generalized. Forced copulation generally relies on short swimming bursts [86,87] that allow the male to copulate with a female before she can defend herself and potentially injure the male. Alternatively, relatively large males adopt larger, showier features and often rely on a courting strategy of reproduction (i.e., coaxing) [86,87]. Displaying males are often required to swim alongside a female until she concedes copulation (personal observation). We hypothesize that this mode of reproduction is likely optimized by a more fusiform body shape that allows the male to have greater swimming endurance during courtship. Just as livebearing reproduction interacts antagonistically with predation environment in generating female morphology (i.e., pregnancy constraints and resulting swimming performance trade-offs), reproductive mode and predation environment may exert opposing selective pressures on body shape in males. We propose that the nearly identical patterns we observed at both taxonomic levels we tested here suggests that selection could favor different body forms that may be associated with reproductive roles and mating strategies, and that the potential adaptive nature of different behaviors is paralleled by morphological divergence. Our findings, although they do not provide conclusive evidence in support of this hypothesis, highlight a gap in our knowledge related to the role of morphology in alternative mating strategies. Future work should focus on determining how body shape and size interplay with mating strategies, whether genetically determined or plastic.

Summary

In conclusion, sister taxa *Brachyrhaphis roseni* and *B. terrabensis* differed dramatically in body shape and the differences observed correspond to divergent predation regimes that favor different body shapes. *Brachyrhaphis rhabdophora* from different predation environments also differ as predicted by predation environment, and these differences are parallel, although less exaggerated, to those observed between *B. roseni* and *B. terrabensis.* Our study provides evidence that evolution acts in a predictable manner when similar selection pressures are at work by showing that body shape evolution follows dramatically similar trajectories at multiple levels of divergence (i.e., both between and within species). We also conclude that shape appears to be optimized differently in males and females, and across a range of sizes, and that these differences may correspond to reproductive roles and mating strategies, respectively. The fact that closely related species in geographic proximity and similar selective environments have evolved nearly identical morphological characteristics is strong evidence that evolution acts in a predictable manner, and provides a framework for future studies on speciation in this unique system.

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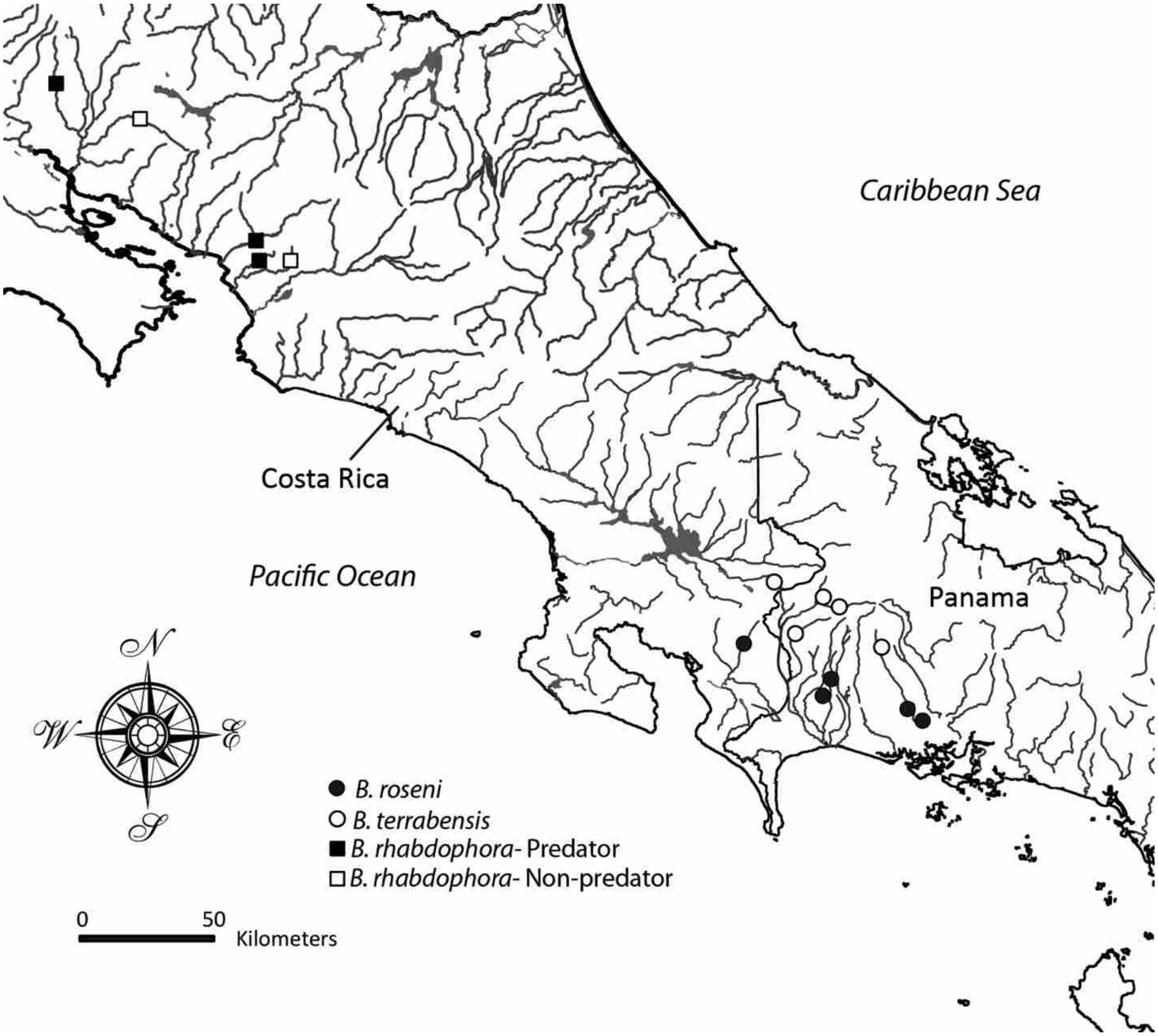

Figure 3.1: Map of collection sites geometric morphometric analyses.

Brachyrhaphis terrabensis (open circles) occur at higher elevations in streams that are void of fish predators. *Brachyrhaphis roseni* (closed circles) occur at lower elevations in streams that have abundant predators. *Brachyrhaphis rhabdophora* occur at sites that are both predator (closed squares) and predation free (open squares).

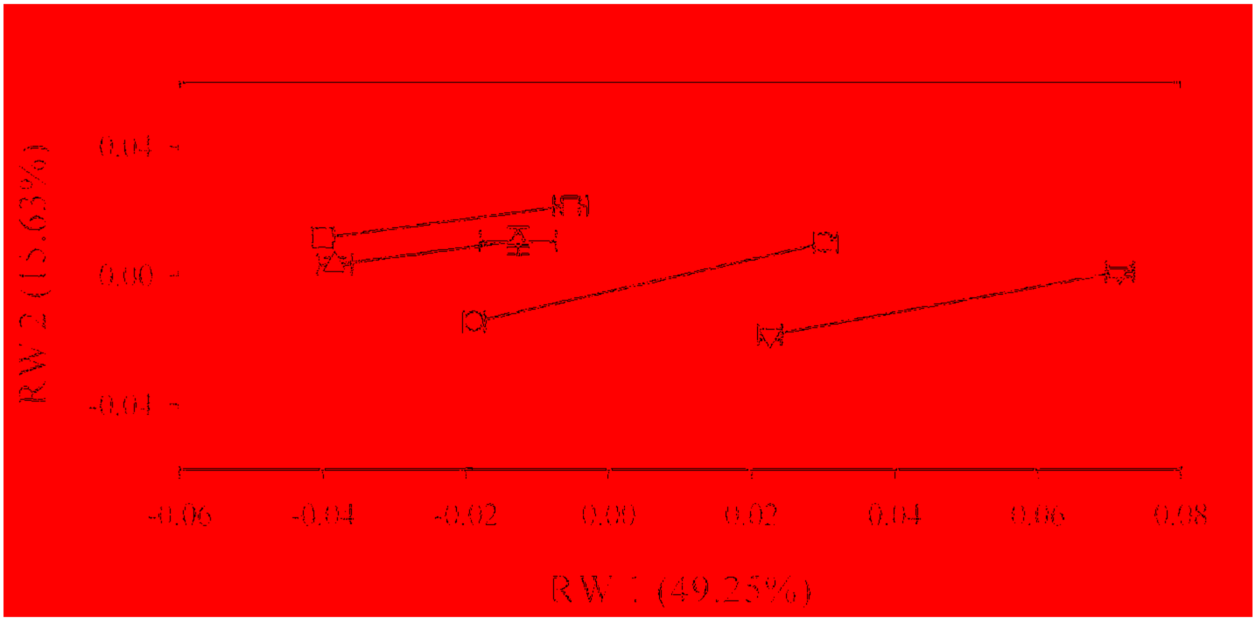

Figure 3.2: Least Square Means of Relative Warps.

Graph of least square means of relative warp (RW) scores (±SE) for *Brachyrhaphis roseni* (●), *B. terrabensis* (▼), *B. rhabdophora* from predator environments (■), and *B. rhabdophora* from predator free environments (▲). Filled symbols represent males, and open symbols represent females. Female body shape converges relative to male body shape in *B. roseni, B. terrabensis* and populations of *B. rhabdophora* from divergent predation environments.

Figure 3.3: Morphological Divergence in Female *Brachyrhaphis.*

Visualization of morphological divergence with centroid size in female *Brachyrhaphis roseni* (a), *B. terrabensis* (b), and *B. rhabdophora* from predator (c) and predator free (d) environments. Thin-plate spline transformations depict the end points of the centroid size axis (i.e. the smallest and largest individuals). Shaded regions are drawn to aid in interpretation. Note the increase in abdomen distension and decrease in caudle peduncle region in large females. Deformations are scaled to 3X to assist interpretation of the shape differences.

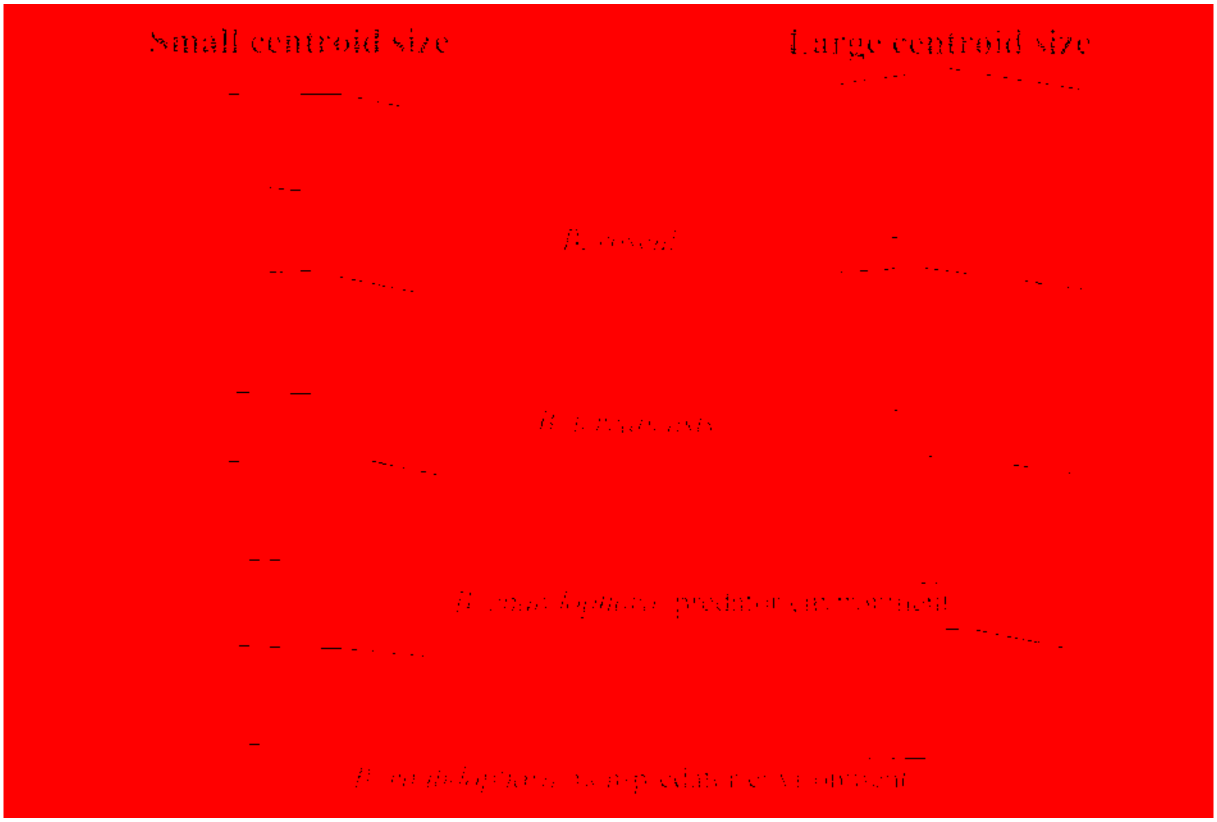

Figure 3.4: Morphological Divergence in Male *Brachyrhaphis*.

Visualization of morphological divergence with centroid size in male *Brachyrhaphis roseni* (a), *B. terrabensis* (b), and *B. rhabdophora* from predator (c) and predator free (d) environments. Thin-plate spline transformations depict the end points of the centroid size axis (i.e. the smallest and largest individuals). Shaded regions are drawn to aid in interpretation. Note the shortening and deepening of the head region and the reduction in the caudle peduncle region in large males. Deformations are scaled to 3X to assist interpretation of the shape differences.

Table 3.1: Results for MANOVA tests comparing body shape.

Results of mixed-repeated-measures MANOVA testing for interactions between combinations of species-group, predation-environment, size and index-variable.

Effect	DF (fm)	F (f)	P (f)	F (m)	P (m)
Index variable	10	869.1	< 0.001	1464.9	< 0.001
Species group	1	78.4	< 0.001	9.8	0.002
Predation	1	22.8	< 0.001	0.2	0.649
Centroid size	1	16.2	< 0.001	1.8	0.177
Species group × index variable	9	1756.8	< 0.001	904.8	< 0.001
Predation × index variable	9	697.5	< 0.001	565.5	< 0.001
Centroid size × index variable	9	517.0	< 0.001	197.8	< 0.001
Species group × predation × index variable	10	664.0	< 0.001	118.6	< 0.001

DF = degrees of freedom, f = females, m = males.

Table 3.2: MANOVA results evaluating sexual dimorphism.

Results of mixed-repeated-measures MANOVA examining shape variation and sexual dimorphism in *Brachyrhaphis*.

Effect	DF	F	P
Index variable	10	0.1	1
Species	3	50.8	< 0.001
Sex	1	762.5	< 0.001
Centroid size	1	3.4	0.06455
Species × index variable	27	4491.1	< 0.001
Sex × index variable	9	1892.3	< 0.001
Centroid size × index variable	9	663.2	< 0.001
Species × sex × index variable	30	440.8	< 0.001

DF = degrees of freedom.

Table 3.3: Phenotypic trajectory analysis results.

Statistical assessment of differences in trajectory size/ direction among trajectories characterizing sexual dimorphism in *Brachyrhaphis.*

Comparison	$MD_{1,2}$	P_{size}	$\theta_{1,2}$	P_θ
1, 2	0.0019	0.583	**14.32**	**0.007**
1, 3	**0.0190**	**0.001**	**26.41**	**0.004**
1, 4	**0.0206**	**0.003**	**50.31**	**0.002**
2, 3	**0.0209**	**0.001**	**33.41**	**0.002**
2, 4	**0.0225**	**0.001**	**56.90**	**0.002**
3, 4	0.0016	0.808	**26.60**	**0.005**

$MD_{1,2}$ = trajectory size, $\theta_{1,2}$ = trajectory direction, Taxa codes: 1 = *Brachyrhaphis roseni*, 2 = *B. terrabensis*, 3 = *B. rhabdophora* from predator environments, and 4 = *B. rhabdophora* from predator free environments. Significant differences generated empirically from 1,000 permutations are indicated in bold.

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Supplemental material

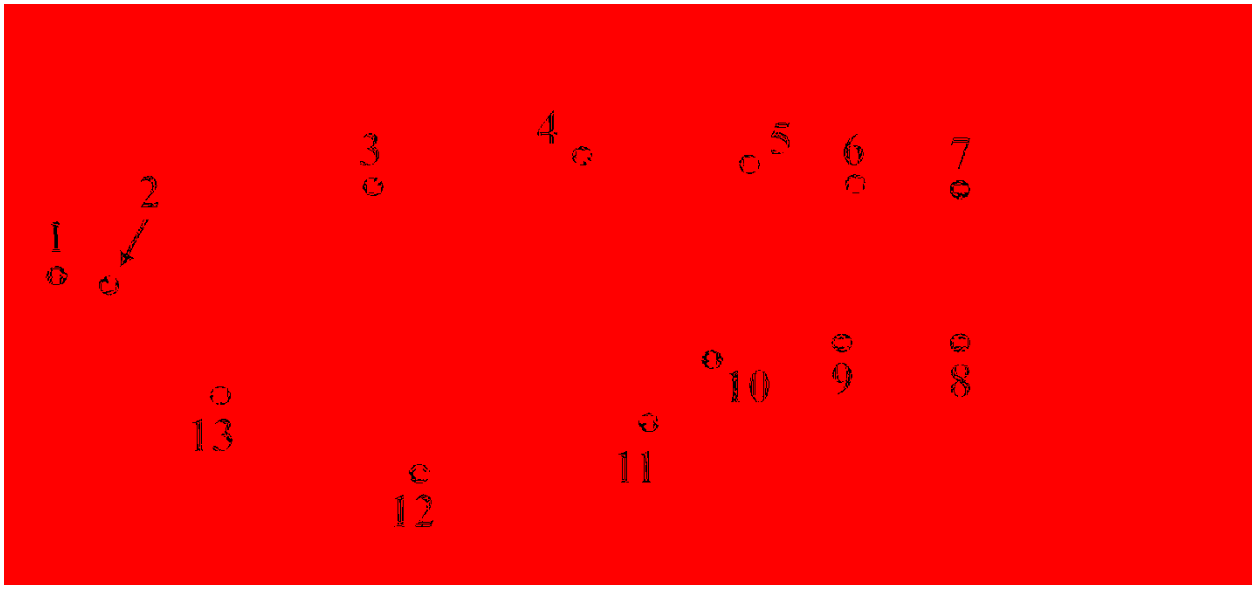

Figure S3.1: Geometric morphometric landmark configuration.

Landmark locations used for geometric morphometric analyses on *Brachyrhaphis roseni*, *B. terrabensis,* and *B. rhabdophora.*

Table S3.1: Geometric morphometric sampling localities.

Population data for samples used in the geometric morphometrics portion of this study, including total N, drainage and country of origin, and coordinates.

Species	**Total (N)**	**Drainage**	**Country**	**Coordinates**
B. roseni	41	Rio Chiriquí Viejo	Panama	N 8.5184, W 82.7115
B. roseni	38	Rio Chiriquí Viejo	Panama	N 8.53371, W 82.6734
B. roseni	132	Rio Chiriquí	Panama	N 8.4251, W 82.4176
B. roseni	40	Rio Chiriquí	Panama	N 8.4304, W 82.4209
B. roseni	38	Rio Coto	Costa Rica	N 8.6551, W 82.9463
B. terrabensis	40	Rio Chiriquí Viejo	Costa Rica	N 8.8802, W 82.8571
B. terrabensis	69	Rio Chiriquí Viejo	Panama	N 8.7924, W 82.6566
B. terrabensis	21	Rio Chiriquí Viejo	Panama	N 8.8294, W 82.7154
B. terrabensis	33	Rio Chiriquí Viejo	Panama	N 8.7183, W 82.8118
B. terrabensis	49	Rio Chiriquí	Panama	N 8.6609, W 82.5206
B. rhabdophora high-predation	114	Rio Jesus Maria	Costa Rica	N 9.9604, W 84.6066
B. rhabdophora high-predation	43	Rio San Rafael	Costa Rica	N 9.9844, W84.6252
B. rhabdophora high-predation	44	Rio Piedras	Costa Rica	N 10.5297, W 85.2809
B. rhabdophora no-predation	65	Quebrada Grande	Costa Rica	N 10.4415, W 84.9877
B. rhabdophora no-predation	35	Rio Machuca	Costa Rica	N 9.9632, W 84.4911

Table S3.2: Population genetic sampling localities.

Population data for samples used in the pairwise analyses of genetic distance, including total sample size (N), drainage and country of origin, and coordinates. All sequences are deposited on Genbank under accession numbers KJ081551 - KJ081609.

Species	Total (N)	Drainage	Country	Coordinates
B. roseni	4	Rio Chiriquí	Panama	N 8.4251, W 82.4176
B. roseni	8	Rio Chiriquí Viejo	Panama	N 8.5184, W 82.7115
B. roseni	3	Rio Coto	Costa Rica	N 8.6551, W 82.9463
B. terrabensis	8	Rio Chiriquí	Panama	N 8.6609, W 82.5206
B. terrabensis	7	Rio Chiriquí Viejo	Panama	N 8.7183, W 82.8118
B. terrabensis	2	Rio Chiriquí Viejo	Costa Rica	N 8.8802, W 82.8571
B. rhabdophora high-predation	6	Rio Javilla	Costa Rica	N 10.4024, W 85.0755
B. rhabdophora high-predation	5	Rio Ciruelas (lower)	Costa Rica	N 10.0603, W 84.7586
B. rhabdophora no-predation	7	Quebrada Grande	Costa Rica	N 10.4415, W 84.9877
B. rhabdophora no-predation	6	Rio Ciruelas (upper)	Costa Rica	N 10.1008, W 84.7250

Table S3.3: Pairwise genetic distances for *B. rhabdophora*.

Pairwise genetic distances based on 1140 base pairs of cytochrome *b* (plus ~65 bp of the downstream gene) for *Brachyrhaphis rhabdophora* from high- (HP) and low-predation (LP) environments. Raw pairwise differences are presented above the diagonal, and adjusted pairwise differences using TrN+G model of evolution are presented below the diagonal.

	Grande (LP)	**Javilla (HP)**	**Lower Ciruelas (HP)**	**Upper Ciruelas (LP)**
Grande (LP)	-	0.003	0.008	0.003
Javilla (HP)	0.003	-	0.007	0.003
Lower Ciruelas (HP)	0.009	0.008	-	0.004
Upper Ciruelas (LP)	0.003	0.003	0.005	-

Table S3.4: Pairwise genetic distances for *B. roseni* – *B. terrabensis.*

Pairwise genetic distances based on 1140 base pairs of cytochrome *b* (plus ~65 bp of the downstream gene) for *Brachyrhaphis roseni* and *B. terrabensis*. Raw pairwise differences are presented above the diagonal, and adjusted pairwise differences using TrN+G model of evolution are presented below the diagonal. Population abbreviations for drainage of origin are as follows: Rio Chiriquí (Ch.); Rio Chiriquí Viejo (CV); and Rio Coto (C). Two populations of *B. terrabensis* were taken from the Rio Chiriquí Viejo drainage, and are designated with subscripts representing their country of origin (CV_{CR} and CV_P for Costa Rica and Panama, respectively).

	***B. roseni* – Ch.**	***B. roseni* - CV**	***B. roseni* - C**	***B. terrabensis* – Ch.**	***B. terrabensis* – CV_P**	***B. terrabensis* – CV_{CR}**
***B. roseni* – Ch.**	-	0.006	0.014	0.036	0.043	0.042
***B. roseni* -CV**	0.006	-	0.010	0.034	0.043	0.042
***B. roseni* - C**	0.014	0.010	-	0.037	0.045	0.046
***B. terrabensis* – Ch.**	0.039	0.036	0.040	-	0.026	0.025
***B. terrabensis* – CV_P**	0.047	0.047	0.049	0.028	-	0.001
***B. terrabensis* – CV_{CR}**	0.045	0.045	0.050	0.027	0.001	-

Chapter 4

Evolving beyond paths of least resistance: mismatching trade-offs in response to predation within and among populations

Spencer J. Ingley*[1], Henry Camarillo[1], Hannah Willis[1] and Jerald B. Johnson[1,2]

[1] Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT 84602, USA

[2] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602, USA

* Corresponding author: sjingley@gmail.com

Abstract

Specialization is fundamentally important in biology because specialized traits allow species to expand into new environments, in turn promoting population differentiation and speciation. Specialization often results in trade-offs between traits that maximize fitness in one environment but not others. Despite the ubiquity of trade-offs, we know relatively little about how often between population trade-offs evolve from trade-offs within populations. Here, we present a case study on *Brachyrhaphis* fishes from different predation environments. We evaluate within/between population trade-offs in burst-speed and endurance at two levels of evolutionary diversification: high- and low-predation populations of *B. rhabdophora*, and sister species *B. roseni* and *B. terrabensis,* which occur in high- and low-predation environments, respectively. We show that at both levels of diversification strong trade-offs between burst and endurance swimming occur, suggesting that they have evolved rather rapidly and persisted post-speciation. However, we did not find corresponding trade-offs within populations. This suggests that populations from divergent environments have solved performance challenges in ways that are distinct from the way these challenges are solved within populations, apparently by decoupling the morphological features that underlie different swimming modes. Our study shows that populations have the ability to evolve along evolutionary trajectories other than those of least resistance.

Keywords

Swimming performance; trade-offs; natural selection; Poeciliidae; *Brachyrhaphis*

Introduction

Local adaptation has been a central topic in ecology and evolution because adaptive, specialized traits can allow species to expand into new environments, which in turn can help promote reproductive isolation and speciation (Coyne and Orr 2004; Funk 1998; Rundle and Nosil 2005; Sandoval and Nosil 2005; Schluter 2000). Populations within a species that occur in different selective environments often evolve phenotypic trade-offs (i.e., negative correlations among beneficial traits) in ecologically relevant, fitness determining traits (Joshi and Thompson 1995; Schluter 2000; Via et al. 2000). Such trade-offs often result in the occurrence of closely related populations that differ substantially in one or more traits. Where divergent traits are locally adaptive, but maladaptive in non-native selective regimes, movement of individuals among populations, and consequently gene flow, can be restricted by several reproductive isolating mechanisms. Although trade-offs *between* populations that occur in divergent selective regimes have been identified for numerous traits in several taxonomic groups (Agrawal et al. 2010; Franssen et al. 2013; Joshi and Thompson 1995; Langerhans 2009b; Martin et al. 2015; Pfennig and Pfennig 2005; Schluter 2000), how often these between population trade-offs reflect *within* population trade-offs is less well known.

Micro-evolutionary processes can influence macro-evolutionary processes (and trade-offs) if correlations within ancestral populations bias the divergence of correlated traits among descendent populations. In other words, descending populations could be restricted to genetic ‘lines of least resistance’ that stem from ancestral trait correlations (Schluter 1996). For example, if an ancestral population shows substantial variation in a morphological trait that corresponds to an efficiency trade-off in the acquisition of different food sources available in their local environment [e.g., beak size and seed use in finches (Grant 1986; Grant and Grant 2006)], individuals at either extreme of the trait distribution are phenotypically ‘primed’ for the invasion of more specialized foraging niches. Matching intra- and inter-population trade-off patterns are indicative of trade-offs or adaptive correlations among traits, and suggest that trade-offs within the ancestral population provided material upon which selection could act once populations moved into divergent environments. In contrast, when intra-population trade-offs do not match inter-population trade-offs, it is likely that populations have arrived at different adaptive solutions than those found within the ancestral populations (Agrawal et al. 2010). Although evidence of intra- and inter-population trade-offs is abundant (Schluter 2000), the extent to which populations are constrained by ancestral trade-offs remains relatively unknown (Agrawal et al. 2010). Furthermore, we know little about how predictable these patterns are among lineages within the same clade, which has potentially important implications for variation in rates of diversification within and among lineages (Hendry and Kinnison 1999; Kinnison and Hendry 2001).

Members of the Neotropical livebearing fish genus *Brachyrhaphis* (Poeciliidae) have emerged as a model system in ecology and evolution in recent years (Archard and Braithwaite 2011a; Archard and Braithwaite 2011b; Ingley et al. 2014a; Ingley et al. 2014b; Ingley et al. 2014c; Ingley et al. 2015; Johnson 2001a; Johnson 2001b; Johnson and Zuniga-Vega 2009), and are ideal for examining correspondence between intra- and inter-population trade-offs. Several species within *Brachyrhaphis* contain populations that occur in divergent predation environments, and have repeatedly and independently evolved life-history (Jennions and Telford 2002; Johnson 2001a), morphological (Ingley et al. 2014a; Wesner et al. 2011) and behavioral (Archard and Braithwaite 2011a; Ingley et al. 2014b; Ingley et al. 2014c) adaptations to their respective environments. Similar patterns have recently been documented at the *between* species level (Ingley et al. 2014a; Ingley et al. 2014b), with sister species occurring primarily in divergent predation environments and showing similar behavioral and life-history adaptations to those seen *within* several species of *Brachyrhaphis* (e.g., *B. rhabdophora*). This pattern suggests that divergent predation environments might be a primary driver of evolution in this group, and make it ideal for studying potential within and between population trade-offs at different stages of divergence. Here, we focus on evaluating trade-offs between and within populations of *B. rhabdophora* that occur in either high- ('Javilla') or low-predation ('Grande') environments, and between and within sister species of *Brachyrhaphis* that occur in high- (*B. roseni,* hereafter '*BR*') and low-predation (*B. terrabensis,* hereafter '*BT*') environments. We focus specifically on trade-offs between burst-speed and endurance swimming performance because these traits are likely under strong divergent natural selection, are ecological relevant (Domenici 2010; Langerhans and Reznick 2010), and are predicted to be tied to previously documented morphological differences observed in these species (Ingley et al. 2014a). High-predation environments have been shown to select for increased burst-speed ability because burst-speed is a strong predictor of predator escape ability (Domenici 2010; Langerhans 2009a). In contrast, low-predation environments often select for increased endurance because these environments tend to have higher population densities, resulting in increased intraspecific competition for resources and mates (Abrams 1993; Langerhans 2009b). Theory predicts that a locomotor trade-off should occur between these two swimming modes because a morphological arrangement that optimized one swimming mode necessarily compromises the other, and vice a versa (Langerhans 2009b; Langerhans et al. 2004). Although many studies have evaluated burst-speed and endurance swimming performance in fish and other taxa (Fu et al. 2015; Langerhans 2009b; Vanhooydonck et al. 2001; Wilson et al. 2002; Yan et al. 2012; Yan et al. 2013), few have evaluated both traits in the same individuals, and are thus limited in their ability to identify individual level trade-offs and determine if within and between population trade-off patterns parallel each other. Moreover, to our knowledge these traits have never been evaluated in pairs of populations that occur in similarly divergent environments but at different stages of evolutionary divergence, thus limiting our understanding of how predictable these trade-offs are within lineages, and how often among population trade-offs evolve from within population ancestral variation.

The objectives of our study are three fold. First, we test for overall differences in burst-speed and endurance in populations that occur in different predation environments, both at early (Javilla vs. Grande) and late (*BR* vs. *BT*) stages of divergence, and attempt to identify what traits (e.g., body shape and size) drive these patterns. We predict that, at both stages of divergence, populations from high-predation environments will have higher burst-speeds than low-predation populations, and that low-predation populations will have higher endurance than high-predation populations. We also predict that, although the magnitude might differ, the same pattern will be present both at early and late stages of divergence. Finally, we expect that variation in body shape will be the primary driver of variation in swimming ability, as has been observed in other poeciliids (Langerhans 2009a; Langerhans 2009b).

Second, as an extension of our first objective, we test for an among population trade-off between burst and endurance. We predict that, overall, populations will exhibit patterns indicative of a trade-off between burst and endurance swimming ability, namely a strong negative correlation between these swimming modes. We expect this trade-off to be more pronounced between sister species *BR* and *BT* than between Javilla and Grande given that the former show deeper levels of genetic and morphological divergence than the latter (Ingley et al. 2014a).

Finally, we test whether between population trade-offs run parallel to within population, among individual trade-offs. Do individuals within a population show a negative correlation between burst and endurance swimming ability? Matching intra- and inter-population trade-off patterns would suggest that trade-offs or adaptive correlations drive differences among traits, and that trade-offs within the ancestral population provided material upon which selection could act once populations moved into different selective environments. If these intra-population trade-offs in swimming ability are lacking it would suggest that populations have arrived at different adaptive solutions to a trade-off than those found within the ancestral populations.

Methods

Study System and Sample Populations

Brachyrhaphis roseni and *B. terrabensis* have recently emerged as a model system for studying patterns of trait divergence in recently diverged species that occur in different selective environments (Ingley 2014; Ingley 2015; Ingley et al. 2014a; Ingley et al. 2014b; Ingley et al. 2014c; Ingley et al. 2015). Previous work has shown that these species diverge in numerous traits, such as behavior (Ingley et al. 2014b; Ingley et al. 2014c) and morphology (Ingley et al. 2014a), which correspond to different predation environments. These sister species are co-distributed in Pacific slope streams throughout western Panama and southeastern Costa Rica, although the majority of populations occur in allopatry and in divergent environments with respect to predation (Ingley et al. 2014a; Ingley et al. 2014b; Ingley et al. 2014c). For this study, we collected live fish from two streams in the Rio Caño Seco drainage in Puntarenas, Costa Rica. *Brachyrhaphis roseni* were collected from a low-elevation tributary (N 8.65427, W 82.93489; elevation 70 m) and *BT* was collected from a high-elevation tributary (N 8.81299 W 82.97408;

elevation 962 m). Both streams were characteristic of the respective species (Ingley et al. 2014a), with the primary difference being the presence or absence of piscine predators and differences in population densities. Collection and transportation to Brigham Young University (BYU) took place in April 2014, and trials were conducted between April and July 2015. Fish were held in small group tanks (~10 fish per 38-L tank) until immediately before our trials began. Prior to beginning our trials, we removed a subset of males (~30 per species) and placed them in tanks where individuals could be easily identified by standard length (SL). Males do not grow after reaching sexual maturity, so individuals were easily identified when placed in tanks containing 3 or 4 individuals of different SL. Fish were allowed to acclimate to their new groupings for at least one week prior to testing. We held all tanks in an environmentally controlled laboratory with natural lighting (12h day, 12h night) and temperature conditions. We provided natural cover and aeration in each tank, and fed fish twice daily with TetraMin flakes supplemented with brine shrimp and fruit flies.

We collected live *B. rhabdophora* from two streams in Guanacaste, Costa Rica in April 2014 and immediately transported them to BYU. High-predation fish were collected from the low-elevation Rio Javilla (N 10.40245, W 85.07610; elevation 99 m; hereafter referred to as 'Javilla') and low-predation fish were collected from high-elevation Quebrada Grande (N 10.44194, W 84.98804; elevation 363 m; hereafter referred to as 'Grande'). These populations have been the subject of extensive study in the context of morphological (Ingley et al. 2014a; Wesner et al. 2011) and life-history (Johnson 2001a; Johnson 2001b; Johnson 2002; Johnson and Belk 2001; Johnson and Zuniga-Vega 2009) divergence in response to different predation environments. Both streams were characteristic of high- and low-predation populations, with the primary differences being the presence or absence of piscine predators and population density.

We followed the same grouping protocol to that we followed for *BR* and *BT*, and held fish under the same laboratory conditions.

Geometric Morphometrics

Following burst-speed performance trials, but prior to the endurance swimming trials (see below), we removed each fish from the test arena and anesthetized it with MS-222. We then took a lateral photograph of each fish for geometric morphometric analyses following the methods of Ingley et al. (2014). In short, we digitized 13 morphological landmarks (or semi-landmarks) on lateral images of fish using tpsDig (Rohlf 2005). Landmarks were defined as: (1) anterior tip of the snout; (2), anterior extent of the eye; (3) semi-landmark midway between landmarks 1 and 4; (4) anterior insertion of the dorsal fin; (5) posterior insertion of the dorsal fin; (6) semi-landmark midway between landmarks 5 and 7; (7) dorsal origin of the caudal fin; (8) ventral origin of the caudal fin; (9) semi-landmark midway between landmarks 8 and 10; (10) posterior insertion of the gonopodium; (11) anterior insertion of the gonopodium; (12) semi-landmark midway between landmarks 11 and 13; and (13) intersection of the operculum with the ventral outline of the body. We then summarized shape variation for all individuals tested in each comparison (i.e., Javilla – Grande, and *BR* – *BT*) into relative warps (i.e., principal components) using tpsRelw (Rohlf 2003). We used generalized Procrustes analysis (Rohlf and Slice 1990) to remove all non-shape variation due to position, orientation, and scale of the specimens for each image. Relative warps are defined as linear combinations of affine and non-affine shape components that describe some portion of the variation observed in the landmarked individuals (Rohlf 2003). In each comparison we used one of the relative warps as a morphological axis of divergence that captured a large amount of between species differences in body shape that are predicted to correspond to locomotor trade-offs (described in Ingley et al., 2014). Thus, at one extreme of the axis are fish with ‘high-predation’ body shapes (i.e., streamlined head, enlarged caudal peduncle), and at the other extreme are fish with ‘low-predation’ body shapes. Each fish therefore fell somewhere along this continuous morphological axis of divergence. This allowed us to test for a relationship between swimming performance and body shape, with the prediction that more ‘high-predation like’ fish will have higher burst-speeds but lower endurance than more ‘low-predation like’ fish.

Burst-speed Swimming Performance

We removed fish from grouping tanks and placed them in 11-L individual tanks 24 hours prior to testing. Fish were fasted during this time to ensure that they were in a post-absorptive state (Niimi and Beamish 1974). For each trial, we gently removed the test fish from their individual tank and placed them in a clear cylinder (13 cm in diameter) that was found within the burst-speed test arena. The test arena was a 60 cm wide octagonal tank that was positioned within a 244-L (125 cm x 65 cm x 30cm) buffer tank. The fish were allowed to acclimate for five minutes before lowering the acclimation cylinder remotely and eliciting an escape response. To elicit an escape response we struck the arena with an acrylic hammer mechanism within ~ 1 body length of the fish. We filmed each trial with a high-speed Phantom v4.2 camera (Vision Research) at 400 fps, and analyzed videos using Phantom v630 software. Prior to each trial we measured water temperature with a Eutech PCSTestr 35 probe, as temperature has been found previously to influence burst-speed performance (Langerhans et al. 2004). To quantify burst-speed from each trial video we approximately followed the methods of Langerhans et al. (2004). In short, we calculated burst-speed by digitizing the center of mass for each frame of the fast-start response. We used measurement functions within Phantom v630 software to calculate the linear distance traveled and speed of the fish from the time it initiated the C-start response to the time when the fish was moving rapidly away from the probe just subsequent to the propulsive tail stroke. We repeated this protocol three times for each fish, allowing us to account for potential variability in response within each fish. For subsequent analyses we used an average of the three burst-speed responses.

Endurance swimming trials and kinematics

We investigated endurance swimming performance in the same fish described above. Our intent was to be able to directly compare performance in two swimming modes, burst-speed and endurance swimming, in individual fish so as to test for individual level trade-offs in locomotor performance. Individual level trade-offs are hypothesized to occur when propulsive mechanisms for different swimming types are linked (Langerhans 2009b). Such trade-offs are often assumed, though they go untested. As with the burst-speed trials, all fish were starved for 24 hours prior to testing to ensure that they were in a post-absorptive state (Niimi and Beamish 1974). We conducted all swimming trials in a 5-L Loligo Systems swim tunnel system (Loligo Systems ApS, Denmark). This system consists of a 5-L swim chamber with flow straighteners on the upstream end and a steel mesh on the downstream end. Water cycles through the chamber continuously and is flushed with a submerged pump that generates flow from a large buffer tank into the swim chamber. The propeller motor is not housed within the flow chamber and does not therefore affect water temperature, allowing water temperature to remain relatively constant. The swim chamber and holding tanks were kept in the same laboratory and therefore were subject to the same temperature and lighting conditions. A biological aeration filter was placed in the buffer tank of the flow system in order to ensure that the water was sufficiently oxygenated.

For each trial, we placed the test fish in the flow chamber and allowed it to acclimate in still water for one minute. Following this acclimation period, we increased the flow rate gradually (over approximately 5 seconds) to 0.1 m s^{-1}. This speed was sufficient to force the subject to begin swimming with the flow (or be pushed to the downstream screen if unresponsive), but not fast enough to present an aerobically challenging effort. We allowed fish to acclimate at this low flow rate for one minute before increasing the flow rate to 0.2 m s^{-1}. After one minute of swimming at this flow rate we captured a 5 second video at 400 frames per second using a Phantom v4.2 camera positioned directly above the chamber. We mounted a small mirror on the side of the chamber at a 45-degree angle so that we could capture both lateral and dorsal views of the fish simultaneously. If the fish had not fatigued after 15 minutes at 0.2 m s^{-1}, we increased the flow rate to 0.32 m s^{-1}, and at 30 minutes we further increased the flow to 0.38 m s^{-1} where it remained until the fish fatigued. For fish that swam beyond 15 minutes at 0.2 m s^{-1}, we captured high-speed videos at 0.32 and 0.38 m s^{-1}, although we do not present these results here.

The protocol we followed allowed us to accomplish several goals. First, we obtained an estimate of swimming endurance for each fish by measuring time to fatigue in seconds (F_t; hereafter ‘endurance’), defined as the time from initiation of high flow (i.e., 0.2 m s^{-1}) until the fish was unable to continue swimming and fell back against the downstream screen. This measurement is intended to provide a metric of organism-level fitness, particularly in high-competition environments, assuming that individuals that can swim at sustained speeds for longer can spend more time foraging and pursuing potential mates (Blake 2004; Domenici 2003; Langerhans 2009b; Plaut 2001; Vogel 1994). Low-predation environments correspond to high population densities and increased intraspecific competition. Therefore, we predict that fish from low-predation environments will have higher endurance than high-predation fish.

Second, high-speed video of sustained swimming allowed us to conduct frame-by-frame analyses of swimming behavior to extract a series of kinematic variables that were likely to have a direct relationship to overall endurance (all variables described below were extracted from videos using Phantom v630 software). Assuming a fairly simplistic model of undulatory swimming [i.e., where the fish is modeled as an actuator-driven, flexible body; (Langerhans 2009b; McHenry et al. 1995)], swimming speed can be controlled by modifying body stiffness, driving frequency, and driving amplitude. More efficient swimmers are predicted to have more stiff bodies, lower tail-beat frequencies, and decreased driving amplitude. Here, we estimated the following over three complete tail beats: body stiffness by measuring propulsive wavelength, λ (double the posterior half-wavelength); driving frequency as tail-beat frequency, f (inverse of the average period of ten complete tail-beat cycles); and driving amplitude as rostral amplitude, R (half the distance between right and left excursions of the anterior tip of the rostrum). These three parameters determine propulsive wave speed (calculated as $c = \lambda f$) and tail-beat amplitude, H (also measured from video sequences here), which consequently determines swimming speed, U (held constant at the rates described above). Thus, if fish from populations that occur in divergent predation environments differ in their endurance swimming abilities, then at least one of these parameters should differ (Langerhans 2009b). If fish from different predation environments do indeed differ in one of these traits, they will have to compensate by modifying one or more of the other kinematic variables in order to maintain a constant speed. These modifications to kinematics are predicted to lead to greater hydromechanical work produced by high-predation fish compared to low-predation fish swimming at the same speed. We therefore followed Langerhans (2009) by calculating total hydromechanical power (*P*) as an overall summary of the magnitude of thrust production. To do so, we used Lighthill's elongated-body theory (Lighthill 1975; Videler 1993) which indicates that the mean thrust generated during swimming can be calculated from conditions at the trailing edge of the fin. In addition to the above kinematic variables, we measured the caudal fin trailing-edge depth (*B,* mm) as the vertical distance between the dorsal-most and ventral-most points on the caudal fin. This allowed us to estimate power, or the mechanical rate of working, as $P \propto f^2H^2B^2(1\text{-}U/c)$. Given a constant swimming speed, a lower value of *P* would indicate greater overall locomotor efficiency (i.e., less power used to overcome drag forces). We predict that fish from low-predation environments will exhibit more efficient kinematics, which will in turn lower the power needed to maintain a constant speed and thus increase endurance.

Body shape comparisons

To test for overall differences in body shape between populations from different predation environments we conducted a multivariate analysis of covariance (MANCOVA). We conducted one MANCOVA for each population pair. For each MANCOVA, geometric shape variables (relative warps) were used as response variables, with centroid size as a covariate (controlling for multivariate allometry), and population as a main effect. Our sampling only included males, so sex was not included as an effect. We also conducted a discriminant function analysis (DFA) for each comparison to provide an intuitive metric of the magnitude of morphological divergence that occurs between each population pair. This method builds a linear model based on the input data that will maximize the explanatory power of the categorical grouping variables assigned. We used all 22 relative warps as predictor variables in the DFA in order to maximize the potential explanatory power of the model. Once the original model had been created, we conducted a leave-one-out cross validation procedure to test the predictive power of the DFA model.

Burst-speed comparisons

To test for overall differences in burst-speed among populations, we conducted an analysis of covariance (ANCOVA). Each population pair was tested separately. For each model we used average burst-speed as the response variable, species (or population) as a main effect, and SL as a covariate. We also conducted a multiple regression analysis to test for a relationship between burst-speed and body shape, both within and between populations. In each case we used body shape and SL as predictor variables of burst-speed to test for the impact of each on burst-speed ability.

Endurance and kinematic variables

We tested for overall differences in kinematics by conducting a MANCOVA with the five kinematic variables as dependent variables, body shape and SL as covariates, and population as a main effect. We then conducted separate univariate ANCOVAs for caudal fin depth, each of the five measured kinematic variables, power, and endurance time. This allowed us to test which specific traits differed among populations. For each ANCOVA we included species as a main effect and SL as a covariate to test for overall differences between populations while controlling for differences in SL. We tested for differences in SL among population pairs using ANOVA. We used the following data transformations for all analyses to meet assumptions for parametric tests: for *BR – BT,* we used a natural log transformation for tail-beat frequency, power, and propulsive wave speed; log10 for rostral amplitude; and square root for endurance time; for Javilla – Grande we used natural log transformation for tail-beat frequency, power, caudal fin depth, and propulsive wave speed; and log10 for rostral amplitude. Prior to these analyses and data transformations we standardized caudal fin depth, rostral amplitude, tail-beat amplitude, propulsive wavelength, and propulsive wave speed by dividing raw values by SL.

Path analysis and pairwise comparisons for endurance and kinematic variables

We conducted a series of path analyses to test for a relationship among morphology, kinematics, and endurance. Path analysis is a method that is frequently used to quantify how natural selection acts on traits (Alcala and Dominguez 2005; Arnold 1983; Conner 1996; Crespi and Bookstein 1989; Johnson et al. 2008; Kaplan and Phillips 2006; Kingsolver and Schemske ; Mitchell 1992; Scheiner et al. 2000; Shipley 1997; Sinervo and DeNardo 1996). Path analysis is particularly useful for incorporating intermediate variables – kinematics in this case – between traits (e.g., morphology) and fitness (e.g., endurance), allowing researchers to clarify functional relationships between traits and fitness (Kingsolver and Schemske ; Scheiner et al. 2000). In a classic paper on this subject, Arnold (1983) provides a methodological approach that highlights performance as an intermediary between traits in the traditional sense (e.g., physiology or morphology) and measures of fitness (e.g., survival), suggesting that these traits do not directly determine fitness, but that fitness is instead determined by various aspects of performance to which such traits contribute (Brodie and Ridenhour 2003). Garland and Losos (1994) expanded the Arnold model by including direct pathways between traits and fitness, thus allowing path models to test both direct and indirect pathways between traits, performance, and fitness. Expanding this approach further by testing relationships between traits, performance, and fitness in a model selection framework (Johnson and Omland 2004) provides a powerful tool for determining which traits are under the strongest selection.

To assess the effects of morphology on performance (i.e., kinematic variables) and fitness, and the effects of performance on fitness, we conducted path analyses using AMOS 19 (Arbuckle 2010). We conducted path analyses that included either all individuals from both populations, or a population singly, conducting analyses for each population pair separately. This approach allowed us to test for significant relationships among morphology, performance (kinematics), and fitness (endurance) both within and among populations. We employed a bootstrapping method (5000 replicates) within AMOS to assess path significance. We used maximum likelihood (ML) methods to estimate path relationships. In addition to generating estimates of direct effects of each path, we also generated estimates of indirect effects. Indirect effects can be interpreted as the effect of a phenotypic trait (e.g., SL or body shape) on endurance mediated by kinematic variables. These indirect effects are in addition to any direct effects that body shape or SL have on endurance (Kline 2005). To reduce dimensionality and multicolinearity, we conducted a principal components analysis (PCA) using correlation matrices for the five kinematic variables. We retained all PC axes that explained more variation than expected under a broken-stick model (Frontier 1976; Jackson 1993) and used them to construct our path models (first two PC axes for both comparisons, explaining a total of 85.8% and 92.1% of variation for Javilla – Grande and *BR – BT,* respectively). We did not include caudal fin depth in our analyses because it was highly correlated with SL ($R^2 > 0.5$, $P < 0.001$). For each path analysis we generated and tested all biologically plausible competing models (Table S4.1) that excluded one or more direct and/or indirect paths between morphology, performance, and fitness, and used the top model (based on AIC) to generate path estimates.

Finally, to compare differences between paths for different populations, we conducted a critical-ratio-differences test (Byrne 2010; Hopwood 2007). This method allows for the comparison of the strength and direction (sign) of a path between models of different groups (e.g., the strength and direction of the relationship between SL and endurance in *BR* vs. *BT*). Using this method we conducted two pairwise comparisons: *BR* vs. *BT* and Javilla vs. Grande.

Between and within population performance trade-offs

We assessed between and within population trade-offs in endurance and burst-speed ability by conducting multiple regression analyses. In each case, we used endurance as the response variable and burst-speed and SL as predictor variables. This allowed us to control for the effect of SL when evaluating the relationship between endurance and burst-speed. To test for between population trade-offs, we included all individuals from both high- and low-predation populations. We conducted one analysis for each comparison (i.e., Javilla – Grande, *BR* – *BT*). For within population comparisons, we built regression models that included only individuals from a single population. These analyses allowed us to test whether population level trade-offs reflected within population trade-offs. With the exception of the path models, all statistical analyses were conducted in R (R Core Development Team 2013).

Results

Divergent body shape

Body shape was significantly different between predation environments for both levels of comparison (Table 4.1). Patterns of morphological divergence matched those previously documented in these species (Ingley et al. 2014a). The DFA provided additional evidence for morphological divergence between population pairs that occur in different predation environments. The DFA correctly assigned 22/22 *BR* (100%), 33/33 *BT* (100%), 25/26 Javilla (96.2%), and 29/29 Grande (100%). The cross validation procedure we conducted indicated that the model was robust to data removal, correctly assigning 22/22 *BR,* 33/33 *BT,* 21/26 Javilla and 26/29 Grande. Thus, variation in body shape alone is sufficient for correctly assigning the vast majority of fish to their population of origin.

Burst-speed comparisons

Our combined Javilla – Grande multiple regression analysis ($R^2 = 0.184$, $P = 0.005$) found that burst-speed increased with morphological axis of divergence ($F_{1,52} = 9.81$, $P = 0.004$) but not with centroid size ($F_{1,52} = 2.50$, $P = 0.119$). Our ANCOVA model found evidence that burst-speed was strongly associated with predation environment, but not associated with centroid size (Table 4.2; Figure 4.1). However, our analysis of Javilla ($R^2 = 0.169$, $P = 0.119$) and Grande ($R^2 = 0.042$, $P = 0.572$) separately revealed that, within populations, burst-speed did not increase with morphological axis of divergence (Javilla: $F_{1,23} = 3.73$, $P = 0.066$; Grande: $F_{1,26} = 1.00$, $P = 0.33$), although in Javilla the relationship was only marginally non-significant. Centroid size did not have an effect on burst-speed when populations were analyzed separately (Javilla: $F_{1,23} = 0.93$, $P = 0.344$; Grande: $F_{1,26} = 0.14$, $P = 0.71$).

Our combined *BR – BT* multiple regression analysis ($R^2 = 0.494$, $P < 0.001$) found that burst-speed increased with morphological axis of divergence ($F_{1,52} = 49.71$, $P < 0.001$) but not with centroid size ($F_{1,52} = 0.95$, $P = 0.34$). Our ANCOVA model found evidence that burst-speed was strongly associated with predation environment, but not associated with centroid size (Table 4.2). However, our multiple regression analysis of *BR* ($R^2 = 0.106$, $P = 0.344$) and *BT* ($R^2 = 0.051$, $P = 0.457$) separately revealed that, within populations, burst-speed did not increase with morphological axis of divergence (*B.roseni*: $F_{1,19} = 1.33$, $P = 0.26$; *BT*: $F_{1,30} = 1.31$, $P = 0.26$), nor did it increase with centroid size (*B.roseni*: $F_{1,19} = 0.93$, $P = 0.35$; *BT*: $F_{1,30} = 0.30$, $P = 0.59$)

Divergent kinematics and endurance performance

Overall, populations from different predation environments showed significant differences in swimming kinematics (Javilla – Grande: $F_{5,48} = 9.199$, $P < 0.001$; *BR – BT* $F_{5,48} = 19.816$, $P < 0.001$). Standard length also had a significant effect on kinematics (Javilla – Grande: $F_{5,48} = 14.032$, $P < 0.001$; *BR – BT* $F_{5,48} = 9.744$, $P < 0.001$). Based on univariate comparisons we found significant differences in both SL and caudal fin depth (Table 4.3). Furthermore, univariate tests revealed that tail-beat frequency, rostral amplitude, and tail-beat amplitude were higher in high-predation populations compared to low-predation populations, but that there was no difference in propulsive wave speed (Table 4.3; Figure 4.2). Propulsive wavelength was higher in *BT* than in *BR,* but did not differ between Javilla and Grande. Hydromechanical power, which we used as a measure of energy efficiency (with higher power indicating lower efficiency), was significantly higher in high-predation than in low-predation populations (Figure 4.3). Finally, endurance was significantly higher in low-predation than in high-predation populations (Table 4.3; Figure 4.1). These results demonstrate that fish from divergent predation environments engage in distinctly different swimming styles, with low-predation fish producing thrust in a more energetically efficient way with low-amplitude (rostral and tail-beat), long-wavelength undulations than high-predation fish. These kinematic differences result in dramatically higher endurance in low-predation populations relative to high-predation populations.

Path analysis and pairwise comparisons for endurance swimming

Path analysis of the morphology-performance-fitness pathway allowed us to determine how morphology influenced kinematics, and how kinematics in turn influenced endurance. The results for the top models for each path analysis are found in Figure 4, and the results for each pairwise path comparison are found in Table S4.2. Indirect path estimates and their significance are found in Table S4.3. Overall, propulsive wave speed (*c*), tail-beat frequency (*f*), and rostral amplitude (*R*) loaded heavily on the first two PC axes and had the greatest effect on endurance. Body shape had a significant effect on rostral amplitude in the combined *BR* – *BT* model and in the *BR* model, with more 'high-predation like' individuals suffering from larger rostral amplitudes. As a result, body shape had a significant indirect effect on endurance, with more high-predation like individuals having lower endurance (Figure 4; Table S4.3). Body shape did not affect kinematics or endurance in the *BT* model or any of the *B. rhabdophora* models, with the exception of the Javilla model. In the Javilla model, body shape had a significant indirect effect on endurance, with more high-predation like individuals having lower endurance. Body size did have a strong effect on endurance in all models, which was manifested both directly and indirectly. Larger individuals consistently had lower values for propulsive wave speed, tail-beat frequency, and rostral amplitude, which resulted in higher endurance in all models except for *BT*. Our pairwise path comparisons revealed that the strength and direction (sign) of paths did not differ between Javilla and Grande. However, the paths connecting SL to endurance, and PC1 (on which propulsive wave speed, tail-beat frequency, and rostral amplitude loaded heavily) to endurance, differed significantly between *BR* and *BT* (Table S4.2). This difference appeared to be due to a strong positive effect of SL on endurance in *BT* but no effect in *BR,* and reversed path signs in the path between PC1 and endurance.

Between and within species trade-offs

Our multiple regression analyses indicated that between populations, there was a significant trade-off between burst-speed and endurance (Javilla – Grande: $R^2 = 0.149$, $F_{2,52} = 4.535$, $P =$

0.015; *BR* – *BT*: $R^2 = 0.352$, $F_{2,52} = 14.11$, $P < 0.001$). Overall, endurance increased as burst-speed decreased both between Javilla and Grande, and between *BR* and *BT.* However, we found no evidence for *within* species trade-offs in any population we tested (Javilla: $R^2 = 0.079$, $F_{2,23} = 2.081$, $P = 0.148$; Grande: $R^2 = 0.023$, $F_{2,26} = 0.292$, $P = 0.749$; *BR*: $R^2 = 0.089$, $F_{2,19} = 0.926$, $P = 0.413$; *BT*: $R^2 = 0.003$, $F_{1,31} = 0.109$, $P = 0.743$; Figure S4.1). These results suggest that whatever is driving this trade-off at the between population level is absent within populations.

Discussion

Our results provide strong evidence that different predation environments have driven the evolution of divergent swimming behaviors in *Brachyrhaphis* fishes, with low-predation environments favoring high endurance, and high-predation environments favoring high burst-speeds. Although between population differences in swimming performance were strong and consistent across our comparisons, the traits that affected differences in swimming performance were less consistent. Furthermore, we found that the magnitude of change at different stages of divergence was similar for burst-speed, but less exaggerated for endurance swimming between Javilla and Grande compared to *BR* and *BT.* Our results also suggest that, within environments, individuals do not suffer from trade-offs between burst and endurance swimming. This indicates that descendent populations solved between population trade-offs in a way that is unique to within population trait correlations likely found in ancestral populations.

Body shape, burst-speed and endurance differ among predation environments

As in previous studies of *Brachyrhaphis,* we found that body shape differed consistently among predation environments. Patterns of body shape divergence in our current samples were similar to those that we have documented previously (Ingley et al. 2014a). Although these patterns were similar to those observed in other poeciliids – where they had a significant effect on swimming ability (Langerhans 2009a; Langerhans 2009b) – body shape differences within populations of *Brachyrhaphis* did not have a strong effect on swimming ability. However, these body shape differences did correlate strongly with burst-speed swimming *between* populations. This lack of within population effects could be due to limited body shape variation within populations, or it could results from some unmeasured trait that better accounts for individual differences in burst-speed. For example, differences in red-white muscle ratios could account for inter-individual variation in burst-speed, as this ratio often corresponds to differences in aerobic vs. anaerobic swimming performance (Goolish 1989) and is not necessarily expected to vary with body shape.

Although our measured morphological traits did not correlate with burst-speed performance, among population divergence in burst-speed was strong both at early and late stages of divergence (Figure 4.1). In fact, burst-speed values for populations from the same predation environments, regardless of their stage of divergence, were indistinguishable. Burst-speed is under strong selection by predators in high-predation environments (unpublished data), with faster individuals consistently out-surviving slower individuals in the presence of a predator. This pattern is present both within and among species of *Brachyrhaphis* from different predation environments, and is consistent with previous work, which has found that faster burst-speeds increase predator escape ability (Domenici 2010). Although we have not evaluated how selection acts on burst-speed in low-predation environments, our results show that populations quickly lost their burst-speed swimming ability (i.e., fish from Grande), and that this loss has persisted in late stages of divergence (i.e., *BT*). Given that we find no evidence for within population trade-offs in burst and endurance swimming ability (see below), the loss of burst-speed swimming ability in low-predation environments could be associated with strong selection on some other trait that is negatively correlated with burst-speed. Alternatively, this difference could be partly environmental, with low-predation fish simply falling 'out of practice' when found in habitats lacking predators. Environmental conditioning does have the potential to influence swimming performance (Goolish 1989), and this could result in among individual or population differences in traits that could underlie swimming trade-offs (Young and Cech 1993). However, all fish used in this study were held under common conditions (no-flow) for one year prior to testing, suggesting that their current level of conditioning was equivalent. In either case, burst-speed performance appears to diverge predictably with predation environment in *Brachyrhaphis* at early and late stages of divergence.

Endurance differed significantly among populations of *Brachyrhaphis* from divergent predation environments, with low-predation fish having higher endurance than high-predation fish (Figure 4.1, Table 4.3). Endurance is considered to be a good indicator of organism level fitness in areas with high population-densities, as fish with higher endurance can spend more time foraging and pursuing potential mates (Langerhans 2009b). Given that low-predation environments tend to have higher intraspecific competition due to higher population densities, individuals that have higher endurance can spend more time engaging in these competitive interactions, and thus increase their overall fitness (Blake 2004; Domenici 2003; Langerhans 2009b; Plaut 2001; Vogel 1994). Although the difference was less exaggerated between Javilla – Grande, we found consistent differences in endurance between populations at both stages of divergence that we examined. Furthermore, we found consistent differences in kinematic variables, with measured traits varying among predation environments in line with our predictions. Tail-beat frequency, rostral amplitude, and tail-beat amplitude were higher in high-predation populations compared to low-predation populations (Figure 4.2, Table 4.3). Therefore, on average low-predation fish had less flexible bodies and used longer, lower amplitude undulations during swimming. As predicted, these differences resulted in between population variation in the amount of power used to maintain a constant speed, with both high-predation populations using significantly more power than their low-predation counterparts (Figure 4.3, Table 4.3). These patterns were remarkably consistent at different stages of divergence within *Brachyrhaphis* (Figures 2 and 3), and they are also parallel to patterns observed in distantly related poeciliids [e.g., (Langerhans 2009b)].

Our path analysis allowed us to determine which traits directly and/or indirectly affected overall endurance. Propulsive wave speed, tail-beat frequency, and rostral amplitude had the greatest and most consistent effects on endurance in all our comparisons, with higher values of these variables generally leading to lower endurance (Figure 4.4). Body shape only affected kinematics and endurance in the model that included both *BR* – *BT* and *BR* singly. More 'high-predation like' individuals had larger rostral amplitudes, although this did not manifest itself as a significant indirect effect on endurance. Body shape did have a significant indirect effect on endurance in Javilla only, which was mediated by rostral amplitude. In contrast, body size was a strong predictor of endurance, either directly or indirectly, in all models. Overall, larger fish had higher endurance. The one anomaly in our study was *BT,* in which size had a direct positive effect on endurance, but an indirect negative effect. The relationship between kinematics variables and endurance was also reversed in *BT* relative to our predictions and the patterns observed in the other populations. *BT* had the highest endurance and exerted the lowest power of all of the populations we tested (Figures 1 and 3). It is possible that *BT* has evolved other adaptations for endurance swimming that we did not measure here (e.g., red-white muscle ratio) and that are under opposing selection to measured kinematics variables, such that even individuals with seemingly less efficient kinematics have higher endurance. Despite this contrasting pattern, size did have a strong positive effect on endurance in all populations we examined, suggesting that selection for higher endurance could be an additional factor leading to divergence in body size among populations that occur in different predation environments.

Between and within population trade-offs

Brachyrhaphis fishes appear to have evolved divergent swimming strategies in response to different predation environments, both within *B. rhabdophora* and between *BR* and *BT.* Our combined regression analyses indicated that, at the among population level, increased performance in one swimming mode compromises performance in the other. Our comparative approach further revealed that this trade-off could evolve rather quickly. Javilla and Grande show low levels of genetic divergence (Ingley et al. 2014a; Johnson 2001b), yet they have achieved the same magnitude of divergence in burst-speed performance as observed between *BR* and *BT,* and nearly that observed in endurance swimming (Figure 4.1). Trade-offs at the population level are common in nature (Schluter 2000), and ecological divergence and local adaptation appear to be significant drivers of speciation (Funk et al. 2006; Nosil 2012). Indeed, population level trade-offs in swimming ability appear to be common, with different predation environments favoring different locomotor adaptations in a diversity of taxa (Arendt 2009; Fu et al. 2015; Johnson et al. 2008; Katzir 1993; Webb 1986). Our results suggest that divergent predation environments select for divergent swimming modes, and that this results in a population level trade-off between endurance and burst-speed swimming.

Although we found strong evidence for a trade-off between burst and endurance swimming at the between population level, we did not find corresponding trade-offs at the *within* population level. This lack of matching suggests that populations of *Brachyrhaphis* that occur in divergent predation environments have arrived at between population trade-offs in a way that is distinct from within population trade-offs. Genetic correlations of traits within ancestral species have the potential to bias phenotypic divergence of correlated traits in descendent species (Schluter 1996). Such a bias could cause descendent species to occupy a relatively restricted area of bivariate trait space, unless selection or drift is strong enough to break this bias (Agrawal et al. 2010). Many studies have tested whether divergence tends to occur primarily along trajectories found within species, and most have found that this is the case. Although *Brachyrhaphis* is not alone in this regard, relatively few species have been found to diverge substantially from within species correlations (Agrawal et al. 2010; Hansen and Houle 2008; Hunt 2007; Marroig and Cheverud 2005; Schluter 1996).

Several processes could provide an explanation for the mismatch between within and between population trade-offs. For example, body shape is a poor predictor of within population differences in both burst and endurance swimming in *Brachyrhaphis*. Previous work has found that trade-offs in swimming performance are tightly linked to body shape differences that optimize one form of swimming but not the other (Blake 1983; Langerhans 2009b; Langerhans et al. 2004; Langerhans and Reznick 2010; Lighthill 1975; Videler 1993; Vogel 1994; Webb 1984). The absence of this relationship within *Brachyrhaphis* suggests that some other trait is mediating among individual differences in swimming performance; one that does not necessarily result in among individual trade-offs in swimming performance. Furthermore, in *Brachyrhaphis,* different swimming modes appear to be influenced by different morphological traits. Size has a strong influence on endurance, with larger individuals having better endurance, but has no effect on burst-speed. Within population trade-offs are expected when values of a phenotypic trait corresponds to high values of one performance trait, but low values of another. Here, it appears that populations have solved different performance challenges in ways that do not compromise within species performance, in a way decoupling different swimming modes such that individuals do not suffer from within population trade-offs.

The lack of within population trade-offs could also be attributed to weaker directional selection on traits within populations. Antagonistic pleiotropy, which refers to genes that have positive effects on one fitness component but negative effects on another (Williams 1957), is generally regarded as the most common mechanism underlying negative genetic correlations. An implicit assumption of this model is that trade-offs involve traits that are under strong directional selection. Thus, we would expect that when strong directional selection favors the highest values of both burst-speed and endurance, these characters would become negatively correlated (Sorci et al. 1995). Although selection appears to be strong and positive on swimming traits in *Brachyrhaphis*, it is possible that populations have reached a performance level that is sufficient, and that increasing performance traits beyond observed levels could be accompanied by unfavorable trade-offs.

Conclusions

Our results provide further evidence that divergent predation environments favor the evolution of between environment trade-offs in swimming performance. However, we show that the traits underlying these trade-offs are less predictable than often assumed. Furthermore, we show that populations of *Brachyrhaphis* that occur in divergent predation environments have solved different performance challenges in ways that do not compromise within species performance. Hence, these populations appear to have solved between population trade-offs in a way that is unique to within population trait correlations likely found in ancestral populations. Given that similar patterns were observed both at early (Javilla – Grande) and late (*BR* – *BT*) stages of divergence, these between population trade-offs appear to have evolved rather quickly. In short, our study shows that populations have the ability to quickly and persistently evolve along evolutionary trajectories other than those of least resistance.

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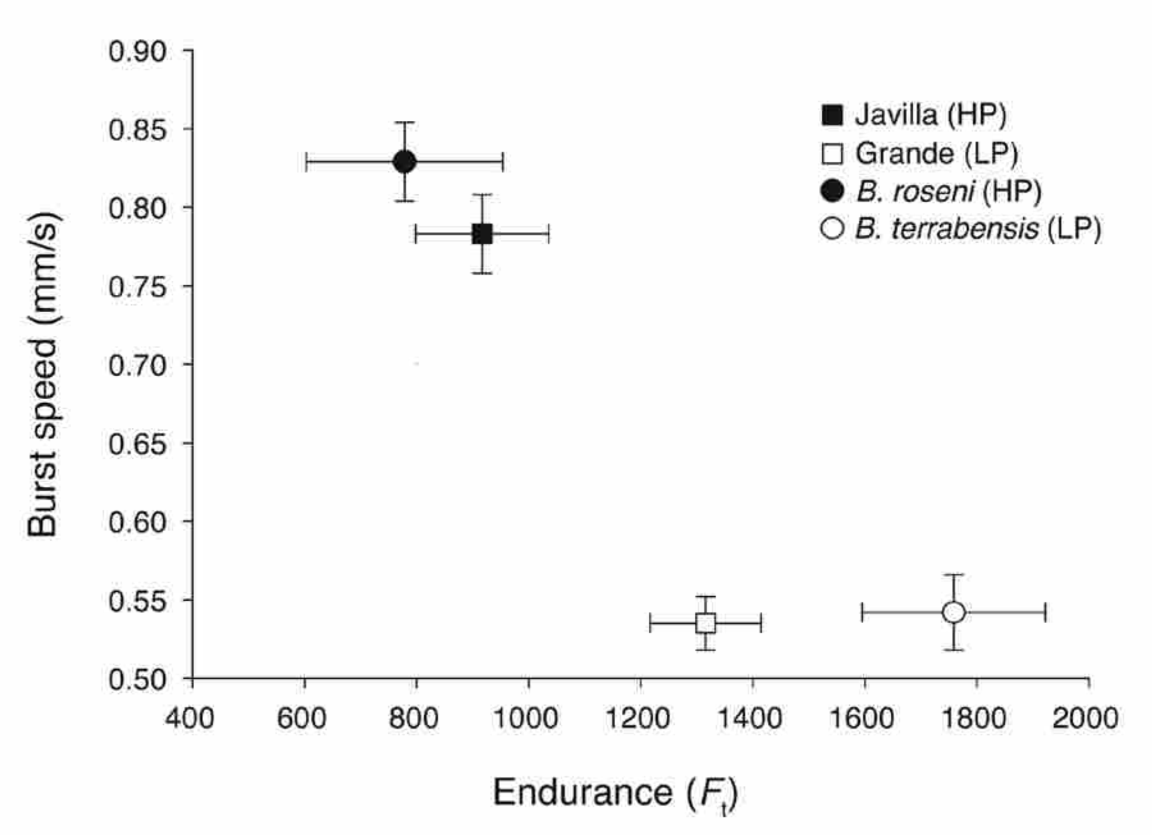

Figure 4.1: Burst-speed and endurance trade-offs.

Means and standard errors for endurance (F_t) as a function of burst-speed (mm/s) for each population tested.

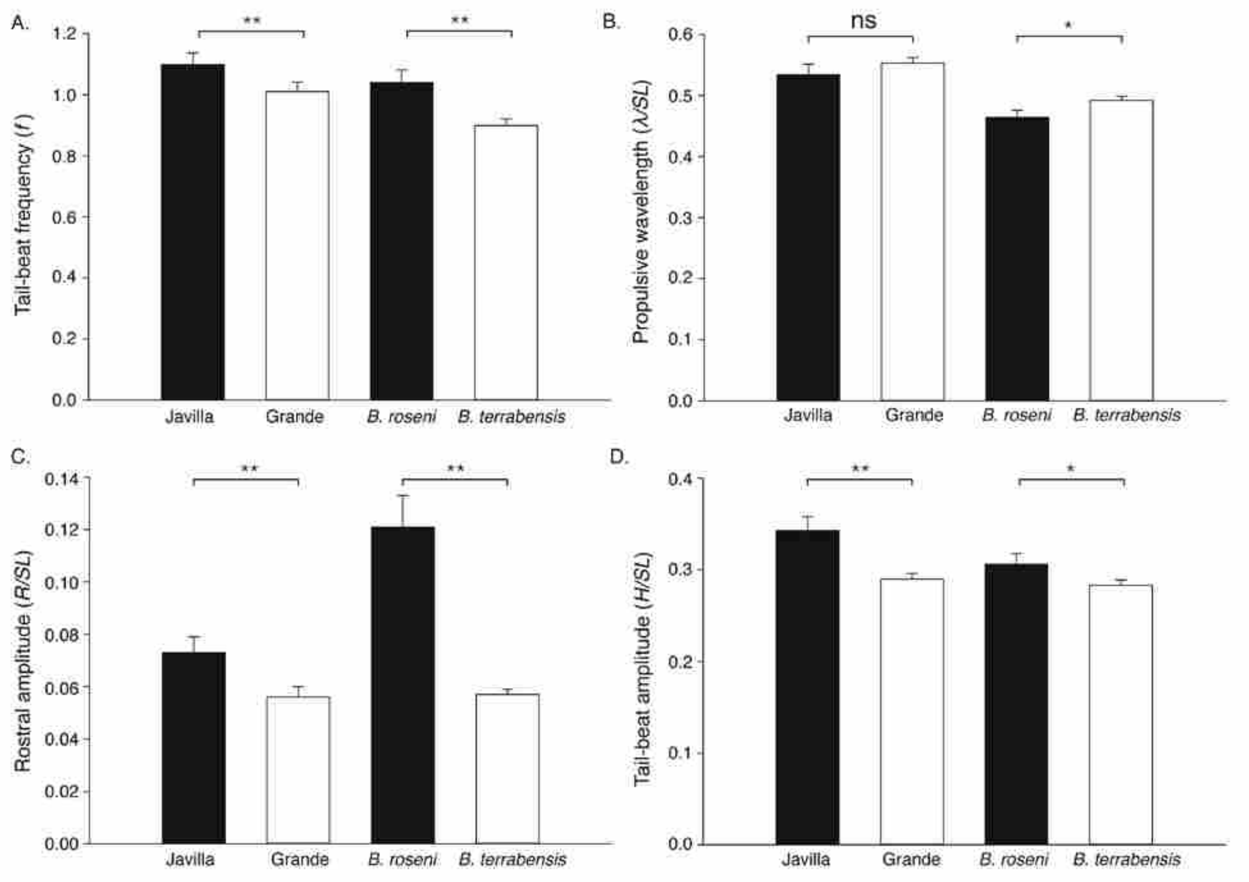

Figure 4.2: Population means for kinematic variables.

Univariate comparisons of kinematic variables representing population means and standard errors. Kinematics variables shown are: A) tail-beat frequency (*f*); B) standardized propulsive wavelength (*λ/SL*); C) standardized rostral amplitude (*R/SL*); and D) standardized tail-beat amplitude (*H/SL*). $P > 0.05$, ns; *$P < 0.05$; **$P < 0.01$.

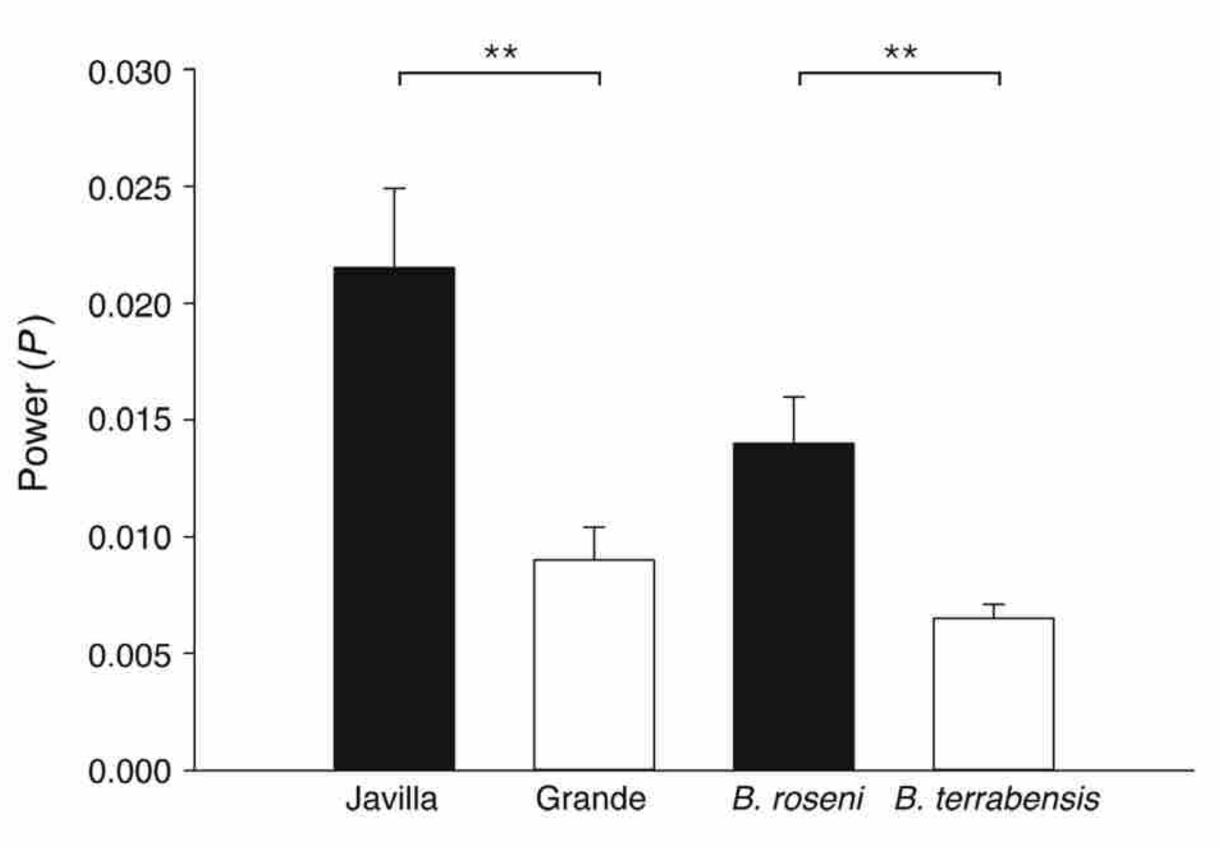

Figure 4.3: Population means for hydromechanical power.

Population means and standard errors for power (*P*), calculated as $P \propto f^2H^2B^2(1\text{-}U/c)$. High values for power indicate less efficient swimming kinematics. $^{**}P < 0.01$.

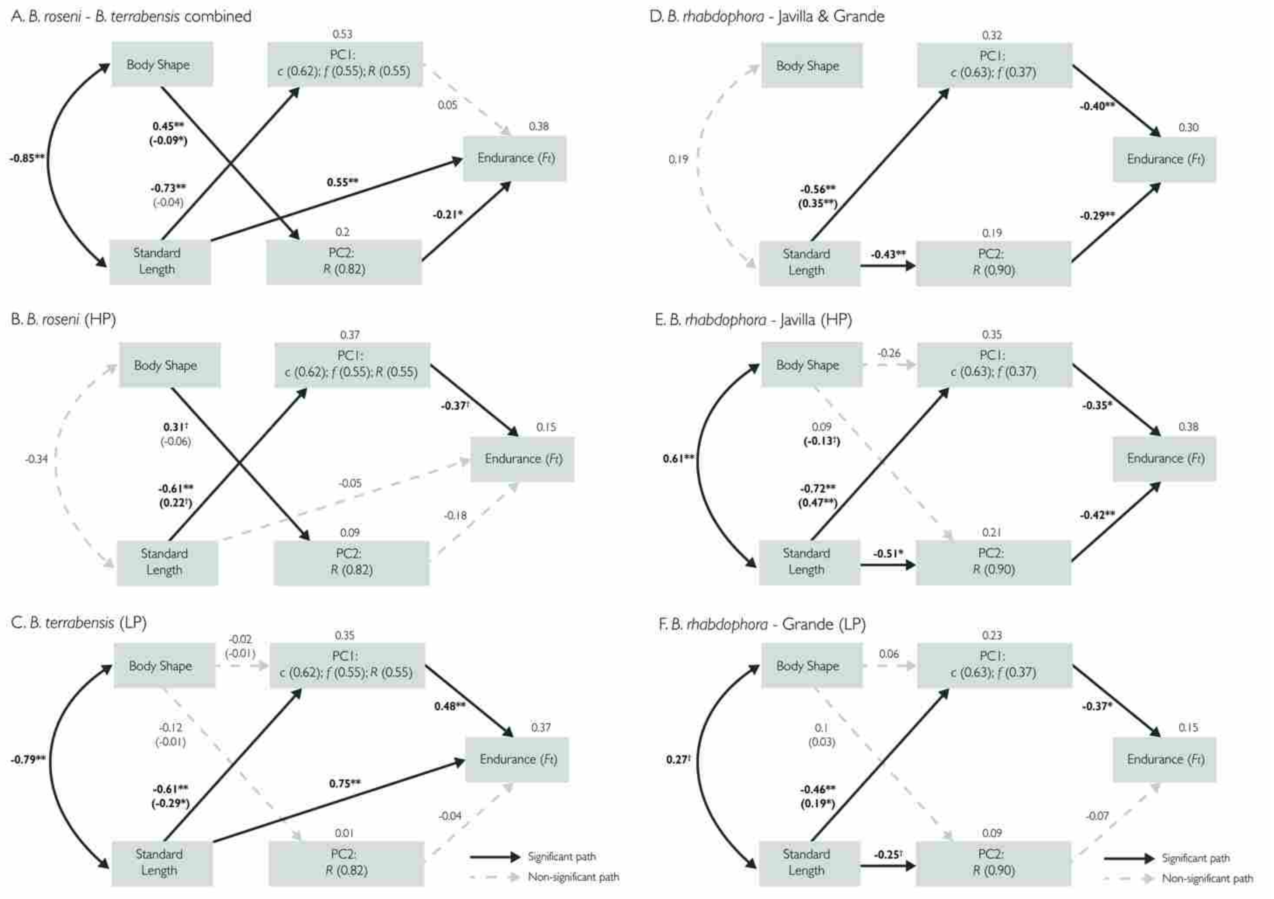

Figure 4.4: Path analysis results for endurance swimming.

Path analysis results for all top models with populations analyzed together (A and D) and individually (B, C, E, and F). Significant paths, assessed through bootstrapping (5000 replicates) are shown in solid dark-grey arrows, while non-significant paths are shown in broken light-grey arrows. Double-headed arrows represent correlations between body-shape and SL. Values near each arrow represent regression coefficients, and values above each ellipsis represent squared correlation coefficients. Indirect effects of SL and body-shape on survival are shown in parentheses. Indirect effects can be interpreted as mediated effects that occur in addition to any direct effect that these kinematics variables have on survival. †$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

Table 4.1: Results for body shape analyses.

Results for multivariate analysis of covariance (MANCOVA) comparing body shape between: A) Javilla and Grande; and between B) *B. roseni* – *B. terrabensis*.

Comparison	Effect	DF	*F*	*P*
A. Javilla – Grande				
	Population	22, 31	6.43	<0.001
	Centroid size	22, 31	1.03	0.46
B. *B. roseni* – *B. terrabensis*				
	Species	22, 31	47.8	<0.001
	Centroid size	22, 31	7.1	<0.001

Table 4.2: Results for burst-speed comparisons.

Results for analysis of covariance (ANCOVA) comparing burst-speed between: A) Javilla and Grande; and between B) *B. roseni* – *B. terrabensis*.

Comparison	Effect	DF	*F*	*P*
A. Javilla – Grande				
	Population	1	63.17	<0.001
	Centroid	1	0.04	0.84
	Residuals	52		
B. *B. roseni* – *B. terrabensis*				
	Species	1	63.13	<0.001
	Centroid	1	0.01	0.93
	Residuals	52		

Table 4.3: Results for comparisons in body size, kinematics, and endurance.

Results for analysis of covariance (ANVOCA) and analysis of variance (ANOVA) testing for variation in body size, kinematics, and endurance between: A) Javilla and Grande; and B) *Brachyrhaphis roseni* and *B. terrabensis.* Cohen's *d* is the standardized effect size for the population (i.e., predation environment) term.

			Predation regime		Standard Length	
A.	Dependent variable	Cohen's d (95% CI)	*F*	*P*	*F*	*P*
	Standard length (*SL,* mm)	1.243 (0.639, 1.848)	21.18	<0.001		
	Caudal fin depth (log *B/SL*)	-0.700 (-1.269, -0.131)	7.831	0.007	9.704	0.003
	Tail-beat frequency (log *f*)	-0.503 (-1.063, 0.058)	3.652	0.062	3.876	0.054
	Rostral amplitude (log10 *R/SL*)	-0.683 (-1.251, -0.115)	9.345	0.003	25.427	<0.001
	Tail-beat amplitude (*H/SL*)	-0.977 (-1.562, -0.392)	22.86	<0.001	40.55	<0.001
	Propulsive wavelength (*λ/SL*)	0.283 (-0.271, 0.838)	1.228	0.273	7.184	0.009
	Propulsive wave speed (log *c/SL*)	-0.236 (-0.789, 0.318)	0.963	0.331	14.846	<0.001
	Power (log *P*)	-1.104 (-1.698, -0.511)	30.53	<0.001	44.76	<0.001

Fatigue time (Ft)	0.698 (0.129, 1.267)	7.027	0.012	3.736	0.059

B. Dependent variable	Cohen's d (95% CI)	F	P	F	P
Standard length (SL)	-2.946 (-2.946, -1.514)	65.64	<0.001		
Caudal fin depth (B/SL)	1.233 (0.619, 1.847)	22.686	<0.001	7.892	0.007
Tail-beat frequency (log f)	0.943 (0.350, 1.536)	13.73	<0.001	10	0.003
Rostral amplitude (log10 R/SL)	2.014 (1.324, 2.703)	74.7	<0.001	21.98	<0.001
Tail-beat amplitude (H/SL)	0.521 (-0.051, 1.093)	5.022	0.029	22.302	<0.001
Propulsive wavelength (λ/SL)	-0.574 (-1.148, 0.001)	4.403	0.041	1.712	0.197
Propulsive wave speed (log c/SL)	0.415 (-0.153, 0.983)	2.693	0.107	10.77	0.002
Power (log P)	1.286 (0.669, 1.904)	38.45	<0.001	41.32	<0.001
Fatigue time ($\sqrt{Ft}$)	-1.316 (-1.936, -0.696)	25.364	<0.001	6.806	0.012

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Supplemental material

Figure S4.1: Within population relationships between burst-speed and endurance.

Population level relationships between mean burst-speed and endurance swimming performance. A) Javilla (high-predation B. rhabdophora); B) Grande (low-predation B. rhabdophora); C) B. roseni (high-predation); C) B. terrabensis (low-predation).

Table S4.1: Model comparisons for endurance path analyses.

Model comparison results for competing models for all path analyses performed for endurance data. Top models are shown in bold, the estimates for which are included in the results (Figure 4.4).

Comparison	Model	AIC
B. roseni – B. terrabensis combined		
	Direct + indirect	160.140
	Body shape ➔ PC1, SL ➔ PC2, and Body shape ➔ Endurance removed	144.819
	SL ➔ PC2 and Body shape ➔ Endurance removed	150.734
B. roseni		
	Direct + indirect	40.144
	Body shape ➔ PC1, SL ➔ PC2, and Body shape ➔ Endurance removed	38.047
	SL ➔ PC2 and Body shape ➔ Endurance removed	36.119
B. terrabensis		
	Direct + indirect	68.006
	Body shape ➔ PC1, SL ➔ PC2, and Body shape ➔ Endurance removed	62.418
	SL ➔ PC2 and Body shape ➔ Endurance removed	64.407
B. rhabdophora – Javilla		

and Grande		
	Direct + indirect	127.341
	Body shape ➔ PC1, Body shape ➔ PC2, Body shape ➔ Endurance, and SL ➔ Endurance removed	106.755
	Body shape ➔ PC2, Body shape ➔ Endurance, and SL ➔ Endurance removed	110.886
	Body shape ➔ Endurance and SL ➔ Endurance removed	116.412
B. rhabdophora - Grande		
	Direct + indirect	42.819
	Body shape ➔ PC1, Body shape ➔ PC2, Body shape ➔ Endurance, and SL ➔ Endurance removed	36.767
	Body shape ➔ PC2, Body shape ➔ Endurance, and SL ➔ Endurance removed	37.170
	Body shape ➔ Endurance and SL ➔ Endurance removed	38.995
B. rhabdophora - Javilla		
	Direct + indirect	40.389
	Body shape ➔ PC1, Body shape ➔ PC2, Body shape ➔ Endurance, and SL ➔ Endurance removed	33.239
	Body shape ➔ PC2, Body shape ➔ Endurance, and SL ➔ Endurance removed	35.123
	Body shape ➔ Endurance and SL ➔ Endurance removed	36.837

Table S4.2: Pairwise comparisons of path analyses.

Results for pairwise comparisons of paths across endurance models. Significance was assessed using a critical ratio difference test.

Comparison	Path	Z	*P*
B. roseni - B. terrabensis			
	Body shape ➔ PC2	1.581	0.114
	SL ➔ PC1	-1.333	0.182
	PC2 ➔ Endurance	-0.532	0.595
	SL ➔ Endurance	-2.405	0.016
	PC1 ➔ Endurance	-2.980	0.003
Javilla - Grande			
	SL ➔ PC1	0.678	0.498
	SL ➔ PC2	-0.725	0.468
	Body shape ➔ PC1	-1.084	0.278
	Body shape ➔ PC2	0.680	0.497
	PC1 ➔ Endurance	0.058	0.954
	PC2 ➔ Endurance	-1.544	0.122

Table S4.3: Indirect path estimates for endurance swimming path analyses.

Indirect path estimates and bootstrap standard errors (SE) for all path models generated to test relationships among body shape, swimming kinematics, and endurance swimming. One-tailed significance was estimated using a bootstrapping method (5000 replicates).

Model	Path	Indirect estimate	SE	*P* (1-tailed)
A. Javilla - Grande				
	SL ➔ Endurance	0.35	0.092	<0.001
B. Javilla				
	SL ➔ Endurance	0.466	0.154	0.009
	Body shape ➔ Endurance	-0.132	0.107	0.10
C. Grande				
	SL ➔ Endurance	0.187	0.108	0.038
	Body shape ➔ Endurance	0.028	0.08	0.298
D. *B. roseni - B. terrabensis*				
	SL ➔ Endurance	-0.04	0.118	0.365
	Body shape ➔ Endurance	-0.093	0.056	0.016
E. *B. roseni*				
	SL ➔ Endurance	0.222	0.188	0.067

F. *B. terrabensis*

	SL ➔ Endurance	-0.284	0.162	0.039
	Body shape ➔ Endurance	0.005	0.034	0.302

Chapter 5

Divergent natural selection promotes immigrant inviability at early and late stages of evolutionary divergence

Spencer J. Ingley[*1] and Jerald B. Johnson[1,2]

[1] Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT 84602, USA

[2] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602, USA

[*] Corresponding author: sjingley@gmail.com

Abstract

Natural selection's role in speciation has been of fundamental importance since Darwin first outlined his theory. Recently, work has focused on understanding how selection drives trait divergence, and subsequently reproductive isolation. 'Immigrant inviability' (II), a barrier that arises from selection against immigrants in their non-native environment, appears to be of particular importance. Although II is likely ubiquitous, we know relatively little about how selection acts on traits to drive II, and how important II is at early-versus-late stages of divergence. We present a study evaluating the role of predation in the evolution of II in recently-diverged population pairs and a well-established species pair of *Brachyrhaphis* fishes. We evaluate performance in a high-predation environment by assessing survival in the presence of a predator, and swimming endurance in a low-predation environment. We find strong signatures of local adaptation and II of roughly the same magnitude both early and late in divergence. We find remarkably conserved selection for burst-speed swimming (important in predator evasion), and selection for increased size in low-predation environments. Our results highlight the consistency with which selection acts during speciation, and suggest that similar factors might promote initial population differentiation and maintain differentiation at late stages of divergence.

Keywords

Path analysis; predation; *Brachyrhaphis*; Poeciliidae; mesocosm; swimming performance

Introduction

Speciation is of fundamental importance in the biological sciences (Coyne and Orr 2004; Butlin et al. 2012). The means by which new species arise, and the relationship among living species, has been a topic that has captivated both lay and scientific observers for centuries. In recent years, the study of speciation has enjoyed increased attention (Schluter 2001; Coyne and Orr 2004; Nosil et al. 2009; Schluter 2009; Nosil 2012; Ingley and Johnson 2014), resulting in significant advances in our understanding of how species form, with impressive progress made toward understanding both the traits and genes involved in the evolution of reproductive isolating mechanisms (Schluter and Conte 2009; Presgraves 2010; Barrett and Hoekstra 2011; Nosil and Feder 2012; Gompert et al. 2013). A major theme in this resurgence of speciation research has focused on identifying mechanisms of reproductive isolation (RI) between evolutionary units, and subsequently teasing apart the genetic mechanisms underlying such barriers (Feder et al. 2012; Nosil and Feder 2012; Nosil and Feder 2013; Egan et al. 2015). These efforts stem largely from the early classification of RI mechanisms laid out by Dobzhansky (1937) and Mayr (1942), which fundamentally influenced thinking about the process of speciation (Coyne and Orr 2004). Although our understanding of the processes that contribute to speciation has increased dramatically in recent years, our knowledge of how reproductive barriers accumulate as speciation proceeds is still limited (Nosil 2012). Thus, studies that evaluate trait divergence and its consequences at early verses late stages of divergence can provide valuable insight into the speciation process.

The RI mechanisms described by Dobzhansky (1937) and Mayr (1942) and extended by several others (Schluter 2000; Naisbit et al. 2001; Rundle and Whitlock 2001) can be generally classified as premating-prezygotic barriers (e.g., sexual/behavioral isolation), postmating-prezygotic barriers (e.g., gametic incompatibility), and postmating-postzygotic barriers (Coyne and Orr 2004). More recently, Nosil et al. (2005) synthesized prior work and proposed an additional, then largely unrecognized, reproductive barrier: immigrant inviability (II). The concept of II implies that RI (i.e., the reduction of gene flow) between two populations can be driven by decreased survival of maladapted immigrants in their non-native environments [e.g., different host-plants (Dickey and Medina 2011), differences in predator susceptibility (Nosil and Crespi 2006), or differences in toxin levels (Tobler 2009)], which results in a reduction of encounter rates and mating opportunities among individuals from different environments (Nosil 2012). In its most extreme form, II can completely exclude populations from occurring in certain geographic and ecological areas, a process that has been referred to as 'eco-geographic isolation' (Ramsey et al. 2003; Lowry et al. 2008; Sobel et al. 2010).

Immigrant inviability can result from a variety of selective agents acting on numerous targets of selection. Nosil (2012) presented an analysis of published data sets of systems where multiple reproductive barriers have been evaluated, and found that II was either the strongest reproductive barrier measured, or at least competed in strength with the strongest measures. In short, the impact of II on speciation is likely profound. Yet, despite the importance and apparent ubiquity of II, we still lack a general understanding of how important this process, including how divergent selection acts on traits to drive II (Nosil 2012).

The livebearing fish genus *Brachyrhaphis* has emerged in recent years as a model for understanding trait evolution at different stages of divergence (Johnson and Zuniga-Vega 2009; Ingley et al. 2014a; Ingley et al. 2015). Within *Brachyrhaphis,* several species contain populations that occur in different predation environments, where they have independently and repeatedly evolved divergent adaptations in traits such as life-history (Johnson 2001a; Johnson and Belk 2001; Jennions and Telford 2002; Johnson and Zuniga-Vega 2009) and morphology (Ingley et al. 2014a). For example, populations of *B. rhabdophora* from high-predation environments have evolved life-history strategies that include younger age and smaller size at maturity relative to low-predation populations (Johnson 2001b, a; Johnson and Belk 2001;

Johnson 2002; Johnson and Zuniga-Vega 2009). Furthermore, recent work has found similar patterns of trait divergence at later stages of diversification, such as between sister species *Brachyrhaphis roseni* (*BR*) and *B. terrabensis* [*BT;* (Ingley et al. 2014a; Ingley et al. 2014b; Ingley et al. 2014c)]. These species, as in populations of *B. rhabdophora* from different predation environments, have evolved life-history strategies and morphologies that appear to be adaptive for their native environments, such that *BR* (high-predation) matures earlier and has more, smaller offspring than *BT* (low-predation). This unique system thus provides an excellent model to test the importance of trait divergence for processes such as II at early and late stages of speciation, where similar agents of selection appear to be driving repeated trait divergence.

Here, we test for similar patterns of selection and resulting effects on fitness at two levels of divergence that can be viewed as falling on extreme ends of the speciation continuum. First, we test for predator driven II by conducting a series of mesocosm experiments. We use a path analysis framework to evaluate patterns of selection on morphology-performance-fitness pathways by evaluating the relationship between body shape/size, predator escape behavior, and survival in the presence of a predator, between populations of *B. rhabdophora* from high- (Javilla) and low-predation (Grande) environments and between sister species *BR* (high-predation) and *BT* (low-predation). We predict that, in both comparisons, previously documented differences in body shape (Ingley et al. 2014a) will drive high-predation fish to have faster burst-speeds than low-predation fish, resulting in lower mortality risk in fishes that co-occur with predators relative to those that do not.

Second, we test for divergence in endurance, a common indicator of fitness for fish living in low-predation environments (Vogel 1994; Plaut 2001; Domenici 2003; Blake 2004; Langerhans 2009b). Low-predation environments often correspond with higher population densities and increased competition for food and mates. Thus, individuals with higher endurance have the potential to out-compete poor swimmers. We conduct path analyses to determine how morphology affects swimming kinematics, and in turn how kinematics affect endurance. We predict that, in both comparisons, high-predation populations will have lower endurance than low-predation populations, reflecting a morphological and locomotor trade-off between endurance and burst-speed swimming (Langerhans 2009b). We also predict that populations from different predation environments will differ significantly in swimming kinematics, and that these variables will serve as strong predictors of endurance.

Methods

Study System and Sample Populations

Brachyrhaphis roseni and *B. terrabensis* have become a model system for studying patterns of trait divergence in recently diverged species that occur in different selective environments (Ingley 2014; Ingley et al. 2014a; Ingley et al. 2014b; Ingley et al. 2014c; Ingley 2015; Ingley et al. 2015). These sister species are found in Pacific slope streams throughout western Panama and southeastern Costa Rica. For this study, we collected fish from two streams in the Rio David drainage in Chiriquí, Panama. *Brachyrhaphis roseni* were collected from a low-elevation tributary to Rio David (N 8.50497, W 82.41128; elevation 124 m) and *BT* was collected from a high-elevation tributary to Rio David (N 8.62653, W 82.49213; elevation 604 m). Both streams were characteristic of the respective species (Ingley et al. 2014a), with the primary difference being the presence or absence of piscine predators. Furthermore, these populations have been the subjects of previous studies on behavioral (Ingley et al. 2014b; Ingley et al. 2014c) and morphological (Ingley et al. 2014a) divergence associated with predation environments.

All trials and collecting for the mesocosm experiments took place between April and June 2013. Upon collection, we transported fish to large (300 l) holding pools near the sites where they were fed Tetramin flakes twice daily until tested. Pools were held under natural lighting (12h day, 12h night) and temperature conditions, and we provided fish with natural cover and aeration. We kept each species in separate pools, but each species pool had members of each sex, approximating the natural population conditions.

For endurance trials, collection and transportation to Brigham Young University (BYU) took place in April 2014, and trials were conducted between April and July 2015. Fish were held in small group tanks (~10 fish per 38-L tank) until immediately before our trials began. Prior to testing, we removed ~30 males per species and placed them in tanks where individuals could be identified by standard length (SL). Male poeciliids do not grow once mature, so individuals could be identified when placed in tanks containing 3 or 4 individuals of different SL. Fish were allowed to acclimate to their new groupings for at least one week prior to testing. All tanks were held in environmentally controlled laboratories with natural lighting (12h day, 12h night) and temperature conditions. Each tank contained natural cover and aeration, and fish were fed twice daily with TetraMin flakes supplemented with brine shrimp and fruit flies.

We collected live *B. rhabdophora* from two streams in Guanacaste, Costa Rica. High-predation fish were collected from the low-elevation Rio Javilla (N 10.40245, W 85.07610; elevation 99 m; hereafter referred to as ‘Javilla’) and low-predation fish were collected from high-elevation Quebrada Grande (N 10.44194, W 84.98804; elevation 363 m; hereafter referred to as ‘Grande’). These populations have been studied extensively in the context of morphological (Wesner et al. 2011; Ingley et al. 2014a), and life-history (Johnson 2001a; Johnson and Belk 2001; Johnson 2002; Johnson and Zuniga-Vega 2009) evolution. For predator mesocosm experiments, collecting took place in March 2015. Following collection, we transported fish to BYU, where they were held in large (200 l) holding tanks and fed Tetramin flakes twice daily until tested. We kept each population in separate tanks, but each species pool had members of each sex. For endurance trials, collection and transportation to BYU took place in April 2014. Trials were conducted between April and July 2015. We followed the same grouping, housing, and feeding protocol as outlined above for *BR* and *BT* specimens.

Predator Mesocosm Experiment

Burst-speed swimming

On the morning of a trial, twenty adult fish were selected haphazardly from the holding pools (equal number of males and females for each population). No juveniles were used in the study, as we were primarily interested in the performance of potentially reproductive adults. Subjects were moved to a 75 l aquarium before testing burst-speed. The testing order of the fish was random. Burst-speed trials for *BR* and *BT* differed slightly from those for Javilla and Grande as they were conducted in different laboratory settings. For each trial of *BR* and *BT*, the focal fish was placed in a circular arena (23 cm in diameter), with 1.5 cm water depth to limit vertical displacement of the subject during burst-speed responses. The arena had opaque sides and a 1 cm grid printed on the bottom, and was evenly illuminated. We allowed fish to settle for 5 minutes prior to eliciting a burst-speed response, and tested each fish three times. We elicited a burst-speed response by quickly striking within one body length of the fish with a cylindrical wooden probe (5 mm diameter, 200 mm length). We used a Eutech PCSTestr 35 to measure water temperature for every trial, as temperature could influence burst-speed swimming performance (Langerhans et al. 2004). We conducted trials in a room that was kept at the same temperature of the water used in the trials, which helped limit variation in temperature among/within trials. We recorded trials with a video camera at 30 frames per second (fps). Although measurement precision might have been compromised by recording at only 30 fps, we were interested in calculating and comparing relative differences in burst-speed between species, not absolute maximum burst-speed. We calculated burst-speed (following Langerhans et al. 2004) by digitizing the center of mass for each frame of the fast-start response. We used tpsDig (Rohlf 2005) to calculate the linear distance traveled from the time the fish initiated the C-start response to the time when the fish was moving rapidly away from the probe just subsequent to the propulsive tail stroke. Our procedure for populations of *B. rhabdophora* was the same with the exception of the tank size and camera used. We conducted trials for *B. rhabdophora* in a 60 cm wide octagonal tank that was positioned within a 244 l (125 cm x 65 cm x 30cm) buffer tank. Fish were acclimated in a small, clear cylinder (13 cm diameter) that could be remotely removed from beneath the arena. Trials were filmed using a high-speed Phantom v4.2 camera (Vision Research) at 400 fps, and videos were analyzed using Phantom v630 software following the methods outlined above.

Geometric morphometrics

Following burst-speed trials, we removed subjects from the test arena and anesthetized them with MS-222. We took a lateral photograph of each fish for geometric morphometric analyses, following the methods of Ingley et al. (2014). In short, we digitized 13 morphological landmarks (or semi-landmarks) on lateral images of fish using tpsDig (Rohlf 2005). We summarized shape variation for each comparison (i.e., Javilla – Grande, and *BR* – *BT*) into relative warps (i.e., principal components) using tpsRelw (Rohlf 2003). We used generalized Procrustes analysis (Rohlf and Slice 1990) to remove all non-shape variation due to position, orientation, and scale of the specimens for each image. In each comparison one of the relative warps can be considered a morphological axis of divergence that represented between species differences in body shape that are predicted to correspond to locomotor trade-offs (described in Ingley et al., 2014). Thus, at one extreme are fish with ‘high-predation’ body shapes (i.e., streamlined head, enlarged caudal peduncle), and at the other extreme are fish with ‘low-predation’ body shapes (i.e., deeper body and more narrow caudal peduncle). Each fish therefore fell somewhere along this morphological axis of divergence. This allowed us to test for a relationship between swimming performance and body shape, with the prediction that more ‘high-predation’ like fish will have better burst-speed performance than more ‘low-predation’ like fish, and that the reverse should be true for endurance.

Predator mesocosm

Once subjects had been tested for burst-speed and photographed, we marked them with a single subcutaneous mark (latex paint suspended in Ringer’s solution) that allowed us to identify each individual and track their fitness (i.e., survival). Each individual received a single mark near the tail region of the body to minimize any adverse effects. We tested burst-speed for a group of control individuals before and after marking, and found no difference in their performance. Fish recovered for at least 6-hours prior to being introduced to the predator mesocosm. In all cases, individuals returned to normal activity within a few minutes of marking.

Following the recovery period, we introduced 20 marked fish (5 males and 5 females for both species) to a 300 l pool for each trial. The pool had a sand and gravel bottom with abundant refugia (e.g., rocks and roots of emergent vegetation) on one side of the pool and a sloping shore on the other side. We made every possible effort to create an environment that mimicked a natural 'high-predation' environment, thus providing fish the opportunity to behave naturally during their encounter with a predator. Following the release of the fish, we released one predator (*Hoplias microlepis* for *BR – BT* trials, and *Parachromis dovii* for *B. rhabdophora* trials) into the pool. We starved the predator for 48 hours before each trial. Each trial started at dusk and lasted for at least 48 hours. This time period allowed the predator to eat some, but not all of the test fish (5.18 ± 1.6 fish for *BR – BT* trials; 6.44 ± 1.5 fish for *B. rhabdophora* trials). We allowed some *B. rhabdophora* trials to run beyond 48 hours (82.667 ± 32 hours) in order to allow sufficient mortality (we visually inspected pools at the end of each day following the initial 48 hours, and continued for another 24 hours if fewer than 3 fish had been eaten). At the end of each trial, we removed the predator and drained the pool to detect and remove all survivors. We used individual subcutaneous marks to identify survivors. We conducted 11 trials with 220 test fish for *BR – BT,* and 9 trials with 180 fish for Javilla – Grande.

Testing for Divergent Endurance Swimming Performance

Endurance trials

We investigated endurance in males for each population. Fish were starved for 24 hours prior to testing to ensure that they were in a post-absorptive state (Niimi and Beamish 1974). We conducted swimming trials in 5 l Loligo Systems swim tunnel (Loligo Systems ApS, Denmark), which consists of a 5 l swim chamber with flow straighteners on the upstream end and a steel mesh downstream. The propeller motor is not housed within the flow chamber and does not therefore affect water temperature. The swim chamber and holding tanks were kept in the same laboratory and in similar temperature and lighting conditions. A biological aeration filter was placed in the buffer tank of the flow system in order to ensure that the water was sufficiently oxygenated.

For each trial, we placed the test fish in the flow chamber for a one-minute acclimation period in still water. We then gradually (over approximately 5 seconds) increased flow to 0.1 m s^{-1}, a speed sufficient to force the subject to begin swimming, but not fast enough to present an aerobically challenging effort. Fish acclimated to the chamber at this low flow rate for one minute before increasing the flow rate to 0.2 m s^{-1}. After one minute at this rate we captured a 5 second video at 400 frames per second using a Phantom v4.2 camera positioned directly above the chamber. A small mirror was mounted to the side of the chamber at a 45-degree angle to capture both lateral and ventral views of the fish simultaneously. If the fish had not fatigued after 15 minutes at 0.2 m s^{1}, we increased flow to 0.32 m s^{1}, and at 30 minutes we increased flow to 0.38 m s^{1} where it remained until the fish fatigued. When applicable, we captured additional high-speed videos of the fish at 0.32 and 0.38 m s^{-1}, although these data are not presented here.

Extracting kinematic variables

Our endurance protocol allowed us to accomplish several goals. First, we obtained an estimate of endurance for each fish by measuring endurance (F_t), defined as the time from initiation of high flow (i.e., 0.2 m s^{1}) until the fish was unable to continue swimming and fell back against the downstream screen for > 5 seconds. This provides a metric of organism-level fitness in a low-predation environment, and assumes that individuals who are able to swim at sustained speeds can spend more time foraging and pursuing mates. Given that low-predation environments tend to have higher population densities due to a lack of predation, competition for mates and resources are exaggerated relative to high-predation streams. We therefore use endurance as an indicator of fitness in low-predation environments, allowing us to assess the viability of hypothetical immigrants (i.e., fish from high-predation environments).

Second, high-speed video of sustained swimming allowed us to conduct frame-by-frame analyses to extract kinematic variables hypothesized to have a direct relationship with endurance (variables were extracted from videos using Phantom v630 software). Assuming a fairly simplistic model of undulatory swimming [i.e., where the fish is modeled as an actuator-driven, flexible body (McHenry et al. 1995)], swimming speed can be controlled by modifying body stiffness, driving frequency, and driving amplitude. Here, we roughly followed Langerhans (2009b) to estimate the following over three complete tail beats: body stiffness by measuring propulsive wavelength, λ (double the posterior half-wavelength); driving frequency as tail-beat frequency, f (inverse of the average period of ten complete tail-beat cycles); and driving amplitude as rostral amplitude, R (half the distance between right and left excursions of the anterior tip of the rostrum). These three parameters determine propulsive wave speed (calculated as $c = \lambda f$) and tail beat amplitude (H, also measured from video sequences), which consequently determines swimming speed (U, held constant at the rates described above). Thus, if individuals from divergent predation environments differ in endurance, at least one of these parameters should differ among populations (Langerhans 2009b). Where fish differ, they have to compensate by modifying one or more of the other kinematic variables to maintain a constant speed. These modifications to swimming kinematics are predicted to lead to greater hydromechanical work while swimming at the same speed. We therefore followed Langerhans (2009b) by calculating total hydromechanical power (P) as an overall summary of the magnitude of thrust production. To do so, we used Lighthill's elongated-body theory (Wu 1971; Lighthill

1975; Videler 1993), which indicates that the mean thrust generated during swimming can be calculated from conditions at the trailing edge of the fin. In addition to the above kinematic variables, we measured the caudal fin trailing-edge depth (*B,* mm) as the vertical distance between the dorsal-most and ventral-most points on the caudal fin. This allowed us to estimate power, or the mechanical rate of working, as $P \propto f^2H^2B^2(1\text{-}U/c)$. Given a constant swimming speed, a lower value of P would indicate greater overall locomotor efficiency (i.e., less force produced to overcome drag).

Geometric morphometrics

Following each endurance trial, we removed the test fish from the chamber and anesthetized it with MS-222. We then took a lateral photograph of each fish for geometric morphometric analyses and followed the same methods outlined above for digitizing landmarks and summarizing shape variation (see *Predator Mesocosm Experiment: Geometric morphometrics*).

Statistical Analysis

Geometric morphometric comparisons

To test for overall differences in body shape between populations from different predation environments we conducted a multivariate analysis of covariance (MANCOVA). We conducted MANCOVA for each population pair, and analyzed data for the predator mesocosm and endurance trials separately, although the analyses were identical with the exception that ‘sex’ was excluded as a factor in the endurance data analyses (only males were tested). For each MANCOVA, geometric shape variables (relative warps) were used as response variables, with centroid size as a covariate (controlling for multivariate allometry), and population and sex (for predator mesocosm only) as main effects. We included an interaction term for population and sex in each model for the predator mesocosm data. We further conducted a discriminant function analysis (DFA) for each comparison to provide a metric of the magnitude of morphological divergence that occurs between predation environments. This method builds a linear model based on the input data that will maximize the explanatory power of the categorical grouping variables assigned. We used all 22 relative warps as predictor variables in the DFA. Once the original model had been created, we conducted a leave-one-out cross validation procedure to test the predictive power of the DFA model.

Comparing mortality rates in predator mesocosms

To test for overall differences in mortality rates among populations, and to test for effects of sex, SL, and burst-speed, we conducted an analysis of covariance (ANCOVA). Each population pair was tested separately. For each model we used relative fitness (calculated for each trial replicate) as the response variable, sex and population as main effects, and burst-speed and SL as covariates. ANOVA was used to compare SL among population and sexes, and to test for differences in burst-speed among population, sexes, and survivors vs. non-survivors. All statistical analyses were conducted in R (R Core Team, 2013).

Path analysis and pairwise comparisons for predator mesocosms

We conducted a series of path analyses to test for a relationship among measured traits (i.e., SL, body shape, and burst-speed) and survival. Path analysis is a method that is frequently employed to quantify the manner in which natural selection acts on traits (Kingsolver and Schemske ; Arnold 1983; Crespi and Bookstein 1989; Sinervo and DeNardo 1996; Shipley 1997; Scheiner et al. 2000; Alcala and Dominguez 2005; Kaplan and Phillips 2006). Path analysis is particularly useful for incorporating intermediate variables, such as metrics of performance, between traits (e.g., morphology) and fitness, allowing researchers to clarify functional relationships between traits and fitness (Kingsolver and Schemske ; Scheiner et al. 2000). We used this approach in a model selection framework (Johnson and Omland 2004), allowing us to determine the model that best represents selection on these traits.

For each comparison (i.e., *BR* vs. *BT,* and Javilla vs. Grande), we used AMOS 19 (Arbuckle 2010) to conduct path analyses that included either all individuals from both populations, or populations singly. This allowed us to test for significant relationships both within and among populations. We employed a bootstrapping method (5000 replicates) within AMOS to assess path significance. We used both maximum likelihood (ML) and Bayesian methods. Both methods produced nearly identical results, and we therefore report only ML estimates. In addition to generating estimates of direct effects of each path, we generated estimates of indirect effects and their significance. Indirect effects can be interpreted as the effect of a phenotypic trait (e.g., SL or body shape) on survival mediated by burst-speed. These effects are in addition to any direct effect that body shape or SL has on survival (Kline 2005). In each case we generated and tested 7 competing models (Table S5.1) that excluded one or more paths between morphology, performance, and survival. We report estimates for the top model from each analysis. Temperature, which has been found to influence burst-speed performance in other fishes (Langerhans 2009a), did not have a significant relationship with burst-speed for any populations included in our data set. Therefore, none of our models include temperature as a direct effect on burst-speed.

Finally, to compare differences between paths for different populations and between sexes, we conducted a critical-ratio differences test (Hopwood 2007; Byrne 2010). This method allows for the comparison of the strength and direction of a path between models of different groups, either among populations (e.g., the strength and direction of the relationship between SL and burst-speed in *BR* vs. *BT*) or between sexes within populations. Using this method we conducted eight pairwise comparisons: *BR* vs. *BT* and Javilla vs. Grande (to test for differences in selection on populations from different predation environments); *BR* vs. Javilla and *BT* vs. Grande (to test for differences in selection between populations from different species and at different stages of divergence that occupy similar predation environments); and between sexes within each population (to test for differences in selection between sexes but within species).

Endurance swimming and kinematic variable comparisons

To test for overall differences in kinematics, we conducted a MANCOVA with the five kinematic variables as dependent variables, SL as a covariate, and population as a main effect. We followed this by conducting univariate ANCOVAs for caudal fin depth, each kinematic variable, power, and endurance. For each ANCOVA we included population as a main effect and SL as a covariate. We tested for differences in SL among population pairs using ANOVA. For all analyses we used the following data transformations to meet assumptions for parametric tests: for *BR – BT,* we used natural log of tail-beat frequency, power, and propulsive wave speed, log10 of rostral amplitude, and square root of endurance time; for Javilla – Grande, we used natural log of tail-beat frequency, power, caudal fin depth, and propulsive wave speed, and log10 of rostral amplitude. Prior to these analyses and data transformations we standardized caudal fin depth, rostral amplitude, tail-beat amplitude, propulsive wavelength, and propulsive wave speed by SL.

Path analysis and pairwise comparisons for endurance and kinematics

To assess the effects of morphology on performance (i.e., kinematic variables) and fitness, and the effects of performance on fitness, we conducted path analyses. For each taxonomic comparison we used AMOS 19 (Arbuckle 2010) to conduct path analyses that included either all individuals from both populations, or populations singly. This approach allowed us to test for significant relationships among morphology, performance (kinematics), and fitness (endurance) both within and among populations. We employed the same methods described above to test path significance and generate indirect estimates in addition to direct path relationships. To reduce dimensionality and multicolinearity, we conducted a principal components analysis (PCA) using correlation matrices for the five kinematic variables. We retained all principal component (PC) axes that explained more variation than expected under a broken-stick model (Frontier 1976; Jackson 1993) and used them to construct our path models (first two PC axes for both comparisons, explaining a total of 85.8% and 92.1% of variation for Javilla – Grande and *BR* – *BT,* respectively). We did not include caudal fin depth from our analyses because it was highly correlated with body length ($R^2 > 0.5$, $P < 0.001$) and was uninformative. For each path analysis we generated and tested competing models (Table S5.2) that excluded one or more paths and present results from the top model (based on AIC). We conducted pairwise comparisons using a critical-ratios differences test to determine if selection acted differently on morphology and kinematics in different populations. We followed the same protocol outlined above, however, due to our use of PC axes as response variables (which were constructed using separate PCA and loaded differently on kinematics variables for each population pair), we only compared *BR* to *BT,* and Javilla to Grande.

Results

Geometric Morphometrics

Body shape differed significantly between predation environments for both levels of comparison for both mesocosm and endurance groups (Table 5.1). Body shape differed significantly between sexes in the predator mesocosm data (Table 5.1). Patterns of morphological divergence uncovered matched patterns previously documented in these species (Ingley et al. 2014a). The DFA provided additional evidence for morphological divergence between population pairs. For the predator mesocosm data, the DFA correctly assigned 108/109 *BR* (99.1%)*,* 109/110 *BT* (99.1%)*,* 70/90 Javilla (77.8%), and 70/90 Grande (77.8%). The cross validation procedure indicated that the model was robust to data removal, correctly assigning 103/109 *BR,* 107/110 *BT,* 61/90 Javilla and 63/90 Grande. Results were similar with the endurance data, with the DFA correctly assigning 22/22 *BR* (100%)*,* 33/33 *BT* (100%)*,* 25/26 Javilla (96.2%), and 29/29 Grande (100%). The cross validation procedure model correctly assigned 22/22 *BR,* 33/33 *BT,* 21/26 Javilla and 26/29 Grande. Thus, the vast majority of fish can be correctly assigned to their population of origin based on body shape alone, although the degree to which body shape distinguishes populations was less in the Javilla – Grande comparison than in *BR* – *BT*. This result follows Ingley et al. (2014a), who found that these population pairs are diverging along parallel evolutionary trajectories, although at different magnitudes of divergence.

Predator Mesocosm – Differential Mortality Rates

Predation regime had a significant effect on mortality rates in both comparisons (Table 5.2). Low-predation populations suffered higher mortality than their high-predation counterparts (Figure 5.1). Surprisingly, sex did not have an effect on survival (Table 5.2; Figure 5.1). In both comparisons, burst-speed had a significant effect on survival (Table 5.2), with survivors having faster burst-speeds than non-survivors both within and between populations.

Path Analysis and Pairwise Comparisons for Predator Mesocosms

Results for top models for each of the six path analyses are found in Figure 5.3, and results for all pairwise path comparisons are found in Table S5.3. Indirect path estimates and their significance are found in Table 5.3. Briefly, burst-speed had a strong positive effect on survival in all groups. Pairwise comparisons indicated that the strength and direction of selection on burst-speed did not differ between any species, population, or sex comparisons (all $P > 0.15$l; Table S5.3).

Body shape had a strong effect (direct and indirect) on burst-speed in all path models except for Javilla (Figure 5.3; Table 5.3). These patterns revealed that individuals with more 'high-predation like' body shapes had higher burst-speeds. Despite this pattern, pairwise comparisons between Javilla and Grande provided no evidence for a difference in the strength/direction of selection on the body shape to burst-speed path (Z = -1.306, $P = 0.192$; Table S5.3). However, pairwise comparisons did indicate significantly stronger selection on the body shape to burst-speed path in *BR* compared to Javilla (Z = -2.837, $P = 0.005$; Table S5.3). Furthermore, body shape appeared to have a strong consistent indirect effect on survival, mediated by burst-speed (Figure 5.3; Table 5.3). This was the case in all models except for Javilla, where the indirect effect of body shape on survival was very weak (Figure 5.3; Table 5.3).

Standard length had a significant direct effect on survival only in Javilla and in the Javilla – Grande model. Although this was the case, selection on the SL – survival path did not differ across any of our pairwise comparisons (all $P > 0.05$; Table S5.3). Although the direct effect of SL on survival was minimal, SL had a significant indirect effect in the combined *BR* – *BT* model, the *BR* model, and the Grande model (Figure 5.3; Table 5.3). In the *BR* and Grande model, increasing SL had a positive indirect effect on survival, while the relationship was reversed in the combined *BR* – *BT* model. This pattern was likely driven by size differences between species, as *BR* (28.624 ± 0.535 mm) was significantly smaller than *BT* (42.805 ± 0.650 mm; $F_{1,217} = 283.1$, $P < 0.001$).

Kinematics and Endurance Swimming Performance

Populations from different predation environments showed significant differences in kinematics (Javilla – Grande: $F_{5,48} = 9.199$, $P < 0.001$; *BR* – *BT* $F_{5,48} = 19.816$, $P < 0.001$). Body length also had a significant effect on kinematics (Javilla – Grande: $F_{5,48} = 14.032$, $P < 0.001$; *BR* – *BT* $F_{5,48} = 9.744$, $P < 0.001$). Univariate comparisons found significant differences in both SL and caudal fin depth (Table 5.4). Univariate tests also revealed that tail-beat frequency, rostral amplitude, and tail-beat amplitude were higher in high-predation populations compared to low-predation populations, but that there was no difference in propulsive wave speed (Table 5.4). Propulsive wavelength was higher in *BT* than in *BR,* but did not differ between Javilla and Grande (Table 5.4). Hydromechanical power, which we used as a measure of energy efficiency (with higher power indicating lower efficiency), was significantly higher in high-predation than in low-predation populations. Finally, endurance was significantly higher in low-predation than in high-predation populations (Figure 5.4; Table 5.4). These results demonstrate that fish from different predation environments have distinctly different swimming styles, with low-predation fish producing thrust in a more energetically efficient way with low-amplitude (rostral and tail-beat), long-wavelength undulations. These kinematic differences result in dramatically higher endurance in low-predation populations relative to high-predation populations.

Path Analysis and Pairwise Comparisons for Endurance Swimming

Results for top models for path analyses are found in Figure 5, and results for all pairwise path comparisons are found in Table S5.4. Indirect path estimates and their significance are found in Table 5. Overall, propulsive wave speed (*c*), tail-beat frequency (*f*), and rostral amplitude (*R*) loaded heavily on the first two PC axes and had the greatest effect on endurance. Body shape had a significant effect on rostral amplitude in the combined *BR* – *BT* model and in the *BR* model, with more 'high-predation like' individuals having larger rostral amplitudes. Consequently, body shape had a significant indirect effect on endurance, with more high-predation like individuals having lower endurance (Figure 5; Table 5). Body shape did not affect kinematics or endurance in the *BT* model or any of the *B. rhabdophora* models, with the exception of the Javilla model. In the Javilla model, body shape had a significant indirect effect on endurance, with more high-predation like individuals having lower endurance. Body size had a strong effect on endurance in all models, either directly, indirectly, or both. Larger individuals consistently had lower values for propulsive wave speed, tail-beat frequency, and rostral amplitude, which resulted in higher endurance in all models except for *BT*. Our pairwise comparisons indicated that the strength and directions of paths did not differ between Javilla and Grande. However, the paths connecting SL to endurance and PC1 (on which propulsive wave speed, tail-beat frequency, and rostral amplitude loaded heavily) to endurance differed significantly between *BR* and *BT* (Table S5.4). This difference resulted from a strong positive effect of SL on endurance in *BT* but no effect in *BR,* and reversed path signs in the path between PC1 and endurance.

Discussion

Our results provide strong evidence that divergent natural selection promotes population differentiation in *Brachyrhaphis* fishes, both at early and late stages of divergence. High-predation fish consistently had higher survival in the presence of a predator relative to low-predation fish. Conversely, low-predation fish consistently outperformed fish from high-predation environments in endurance, a common indicator of fitness for species living in more densely populated, resource-limited, low-predation environments. In both cases, fitness appeared to be tightly linked to performance traits (i.e., burst-speed and kinematics), which was in large part determined by morphological traits. Overall, our study provides a valuable glimpse into the repeatability of evolution, with remarkably similar patterns of selection acting on populations at early and late stages of divergence.

Predator Driven Immigrant Inviability

Immigrant inviability is a strong driver of RI because it often acts quickly to reduce or eliminate potential future mating interactions between locally adapted and maladapted individuals (Nosil 2012). This form of RI appears to be ubiquitous, and is often as strong or stronger than other forms (Nosil et al. 2005; Lowry et al. 2008; Nosil 2012). Nevertheless, our understanding of the way by which divergent selection generates II remains limited, due in part to the rarity of experimental studies addressing both the agents and targets of selection (Schluter 2000; Nosil 2012). Our study provides evidence that predation acts on numerous traits and results in strong II in *Brachyrhaphis* by driving differential mortality rates in populations adapted to divergent predation environments.

Predation is a significant driver of trait divergence and II in several taxa (Kruuk and Gilchrist 1997; Hatfield and Schluter 1999; Riechert and Hall 2000; Vamosi 2002; Nosil 2004;

Langerhans 2009a; Tobler 2009). This pattern holds in *Brachyrhaphis,* where predation appears to be a strong agent of selection, quickly eliminating ~40% of immigrants over a short (48 hour) period. Although we did not directly test for a relationship between mortality and encounter rates among heterospecifics, our results are strongly suggestive that predator driven selection within high-predation environments is sufficient to dramatically reduce mating opportunities between predator-adapted and non predator-adapted populations. These results were consistent both between sexes and across species (Figures 1 and 3). Furthermore, differential mortality rates between recently diverged populations (Javilla and Grande) were similar to the more divergent species pair (*BR* and *BT*). Although the general pattern between the two comparisons was similar (Figure 5.1), Javilla – Grande trials tended to last longer (82.67 ± 32 hours) than *BR* – *BT* trials (limited to 48 hours), suggesting that predator driven selection against immigrants is slightly weaker in *B. rhabdophora*. This result corresponds to previously observed differences in body shape (Ingley et al. 2014a), and differences in burst-speed performance presented here.

Conserved Selection on Burst-speed Performance

The conserved nature of selection on burst-speed performance that we observed is remarkable (Figure 5.3). We consistently observed that burst-speed was the best predictor of survival, overshadowing any direct effects of body size or shape. Our pairwise comparisons found no differences in this path between any of our populations; in every case faster burst-speeds increased survival rates (Table S5.3). This suggests that, regardless of the underlying causal mechanisms, burst-speed is a trait that ubiquitously impacts survival in the presence of predators.

Although burst-speed had the greatest direct effect on survival, body shape consistently had a significant indirect effect, mediated by burst-speed. In other words, body shape only impacted survival in so much as it affected burst-speed, providing no evidence that predators cue-in on body shape, as they do in other organisms (Johnson et al. 2008). The relationship between body shape and burst-speed swimming was also largely consistent across species and sexes. In fact, the only comparison that showed significant differences in this path was *BR* – Javilla (Table S5.3). This difference appeared to be driven by the lack of a relationship between body shape and burst-speed in Javilla. The reason for this is unclear, but could stem from limited trait variation within our sample population. Fish from Javilla suffer high predator-induced mortality rates in the wild (Johnson and Zuniga-Vega 2009), which could act to erode additive genetic variance in traits related to body shape, thus reducing the material upon which selection can act. Alternatively, this result could be an artifact of sampling bias. Regardless, our results show that, in general, fish with more 'high-predation like' body shapes have higher burst-speeds, which in turn increases survival. The impact of SL on survival is less clear. SL had a positive effect on burst-speed in *BR* and Grande, but not in *BT* or Javilla (Figure 5.3). However, SL did have a direct effect on survival in Javilla, with larger individuals benefiting from higher survival rates. This result seems to contradict mark-recapture data for Javilla, which found that mortality was highest in large adults (Johnson and Zuniga-Vega 2009).

Several other traits could also contribute to differential survival rates. For example, behavior in the presence of a predator could affect predator encounter rates and predator avoidance. *Brachyrhaphis* fishes show divergence in several behaviors that could be related to survival. Fish from high-predation environments are more bold, active, and prone to explore than fish from low-predation environments (Ingley et al. 2014b; Ingley et al. 2014c). Ingley et al. (2014b) posited that these patterns could be driven by differences in predation environment, with high-predation level favoring bold and active individuals who can better identify predators and avoid their attacks (Godin and Davis 1995), as well as increase encounter rates with potential mates.

Divergent Endurance

Our work provides additional evidence that low-predation environments, which often correspond to higher population densities and increased intraspecific competition, favor the evolution of increased endurance (Hassell 1975; Holt 1985; Chesson and Huntly 1997; Gurevitch et al. 2000; Reznick et al. 2001; Langerhans 2009b). Fish that are able to sustain active swimming for longer are hypothesized to benefit from an increased ability to search for food and engage in energetically demanding courtship activities (Domenici 2003; Blake 2004; Langerhans 2009b). Given that endurance is of critical importance, competition is predicted to favor the evolution of more energetically efficient means of swimming (Langerhans 2009b). Consistent with this hypothesis, we found that low-predation populations had higher endurance than high-predation populations at early and late stages of divergence, although this pattern was more pronounced between *BR* and *BT.* Kinematics also differed significantly among predation environments at both levels of divergence (Table 5.4). Our path analysis revealed that these differences had a causal relationship with endurance. In all cases but *BT,* individuals with lower tail beat frequency, rostral amplitude, and propulsive wave speed had better endurance (Figure 5.5), suggesting that the population level differences in these traits reflect a pattern of local adaptation, where low-predation environments favor the evolution of more efficient swimming form.

Body Size, not Shape, Affects Endurance

Previous work has hypothesized that individual level trade-offs between burst and endurance swimming should occur based on the prediction that certain body shapes favor one form of swimming but compromise the other (Blake 1983; Webb 1984; Langerhans 2009b; Langerhans and Reznick 2010). Although we found evidence for a relationship between body shape and burst-speed, we found limited evidence for a relationship with endurance (direct or indirect). Body shape affected rostral amplitude in the *BT* – *BR* comparison and in *BR* when analyzed independently, but in no other populations. However, we did find evidence for selection on body size, with larger individuals exhibiting higher endurance in all populations. Larger individuals had lower tail-beat frequencies, rostral amplitude, and propulsive wave speed (Figure 5.5). Consequently, larger fish were more efficient (less power exerted) and had higher endurance in all populations but *BT*. *Brachyrhaphis terrabensis* had extremely high endurance. Path analysis revealed that, although SL had a positive direct effect on endurance, it had a negative indirect effect notwithstanding that larger individuals had lower tail-beat frequencies, rostral amplitude, and propulsive wave speed. This result is paradoxical, and could stem from unmeasured traits (e.g., red-white muscle ratio) that could be associated with larger body size and have the ability to overpower the effects of less efficient kinematics. Despite this surprising result, our findings suggest that increased body size is under strong selection in environments with high-intraspecific competition due to its relationship with endurance.

Conclusions and Implications for Speciation

Our results provide strong evidence that divergent predation environments, where individuals are subject to either increased predation or intraspecific competition, drive the evolution of performance related traits. By comparing population pairs at different points of divergence (i.e., early and late stages), we have shown that selection acts uniformly on traits related to predator escape and intraspecific competition. The repeated and parallel patterns of trait evolution in different levels of divergence in *Brachyrhaphis* suggest that natural selection is acting in both systems to drive trait evolution that could contribute to RI. Given that locally maladapted immigrants had dramatically poorer performance in their non-native environments, heterospecifics encounters are likely rare in nature. These selective forces could thus play an important role in driving population differentiation and RI at early stages of divergence (e.g., Javilla – Grande), and in maintaining population differentiation in late stages of divergence (e.g., *BR* – *BT*). Future work should evaluate other RI mechanisms at work in this system, and test for a role of natural selection in these barriers (e.g., sexual isolation based on body-size/shape).

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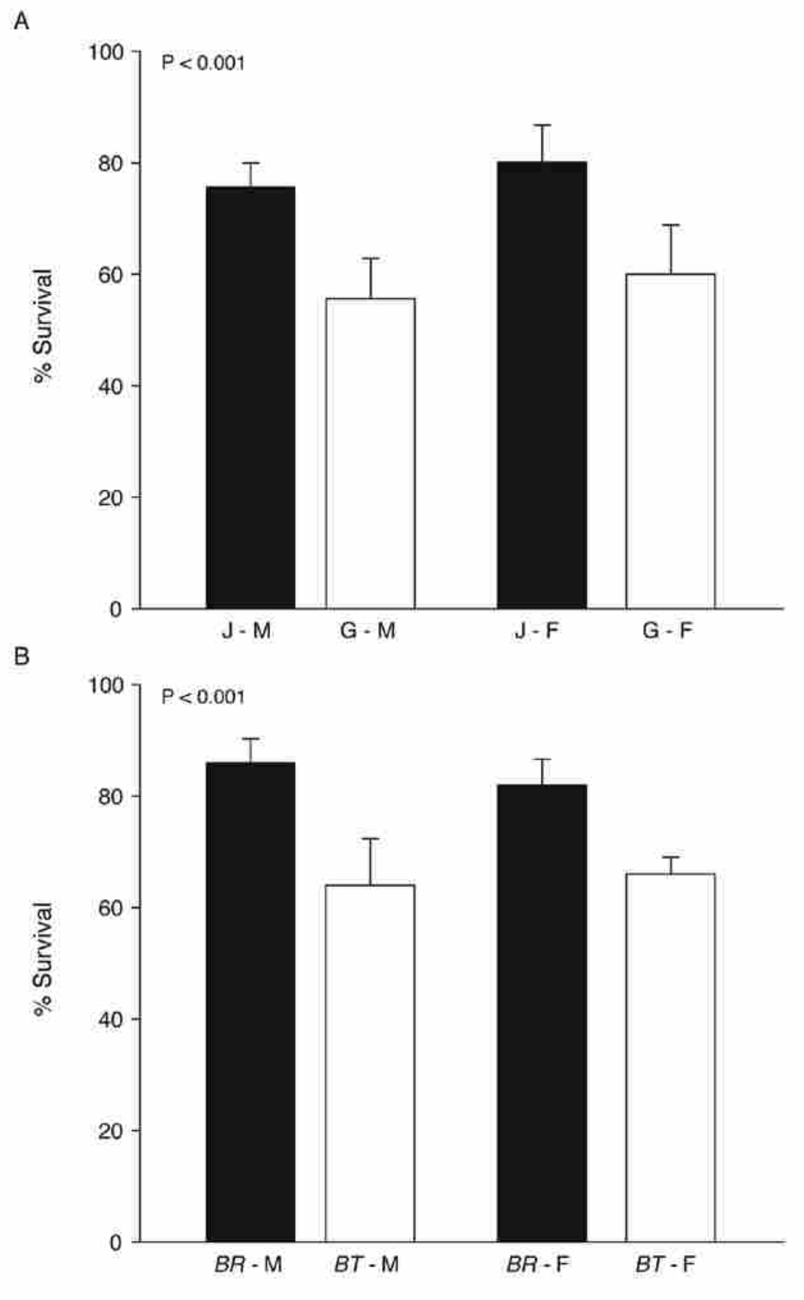

Figure 5.1: Survival rates for mesocosm experiments.

Survival rates (as % survival during a period of exposure to a predator) of males (M) and females (F) during the mesocosm experiment for: A) Javilla (J) and Grande (G); and B) *Brachyrhaphis roseni* (*BR*) and *B. terrabensis* (*BT*). P-values indicate significance of the ‘Species’ or ‘Population’ effect. Survival rates within each species did not differ between males and females.

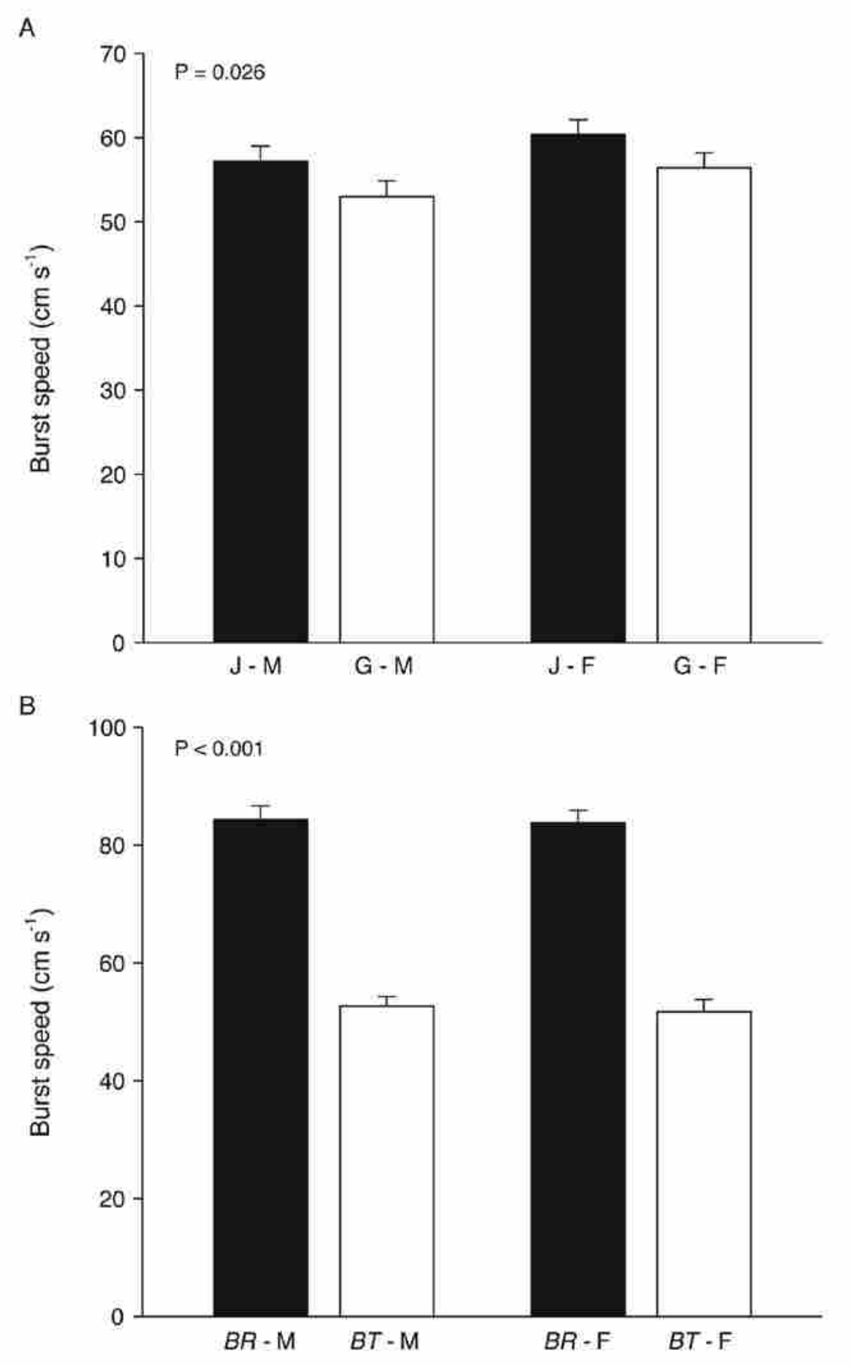

Figure 5.2: Population means for burst-speed swimming.

Burst-speed swimming performance (cm/s) for males (M) and females (F): A) Javilla (J) and Grande (G); and B) *Brachyrhaphis roseni* (*BR*) and *B. terrabensis* (*BT*). P-values indicate significance of the 'Species' or 'Population' effect. Burst-speed within each species did not differ between males and females.

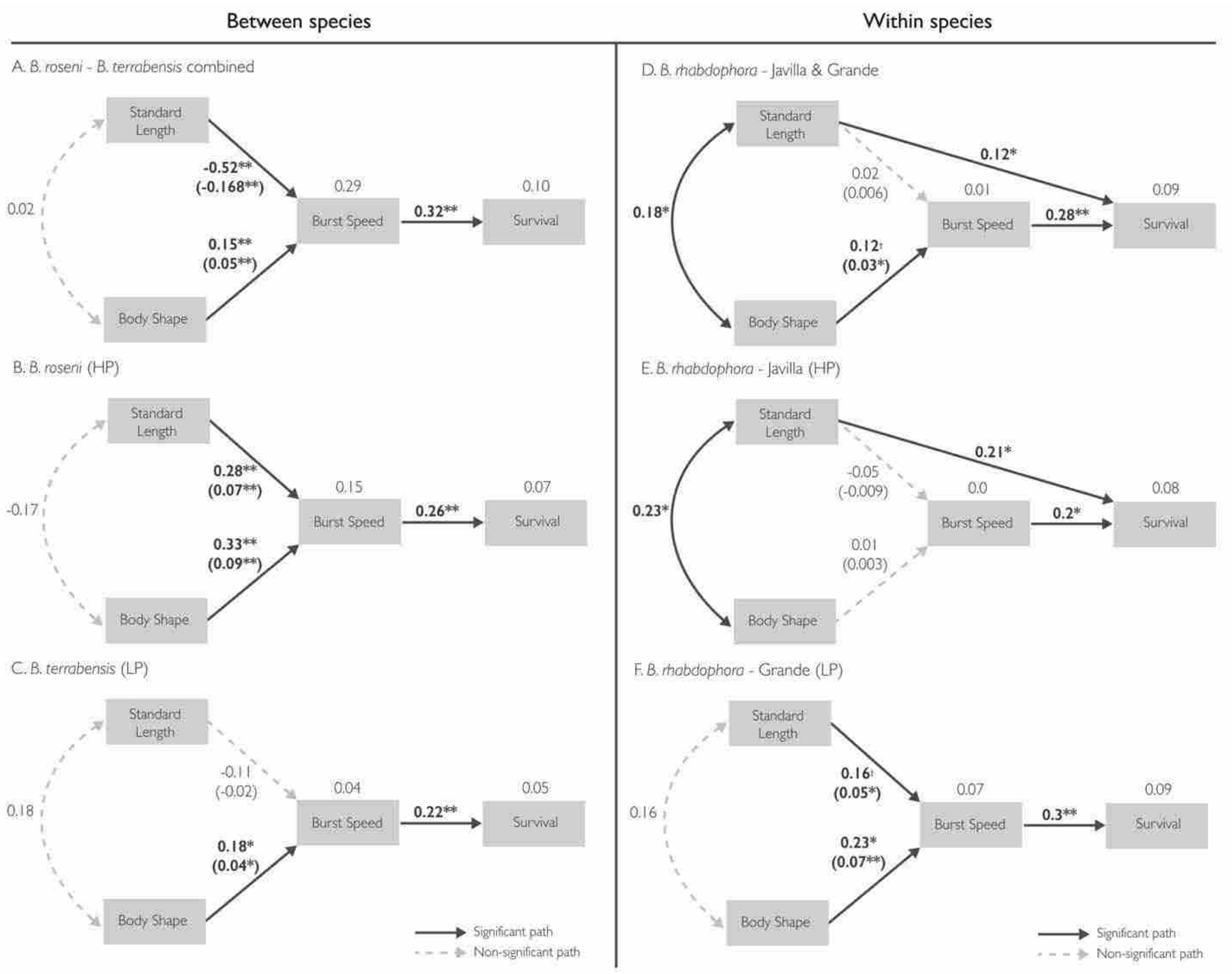

Figure 5.3: Path analysis results for mesocosm experiments.

Path analysis results for mesocosm experiments for all top models with populations analyzed together (A and D) and individually (B, C, E, and F). Significant paths are shown in solid dark-grey arrows, while non-significant paths are shown in broken light-grey arrows. Double-headed arrows represent correlations between body shape and SL. Values near each arrow represent regression coefficients, and values above each ellipsis represent squared correlation coefficients. Indirect effects of SL and body shape on survival are shown in parentheses. Indirect effects can be interpreted as mediated effects that occur in addition to any direct effect that these variables have on survival. †$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

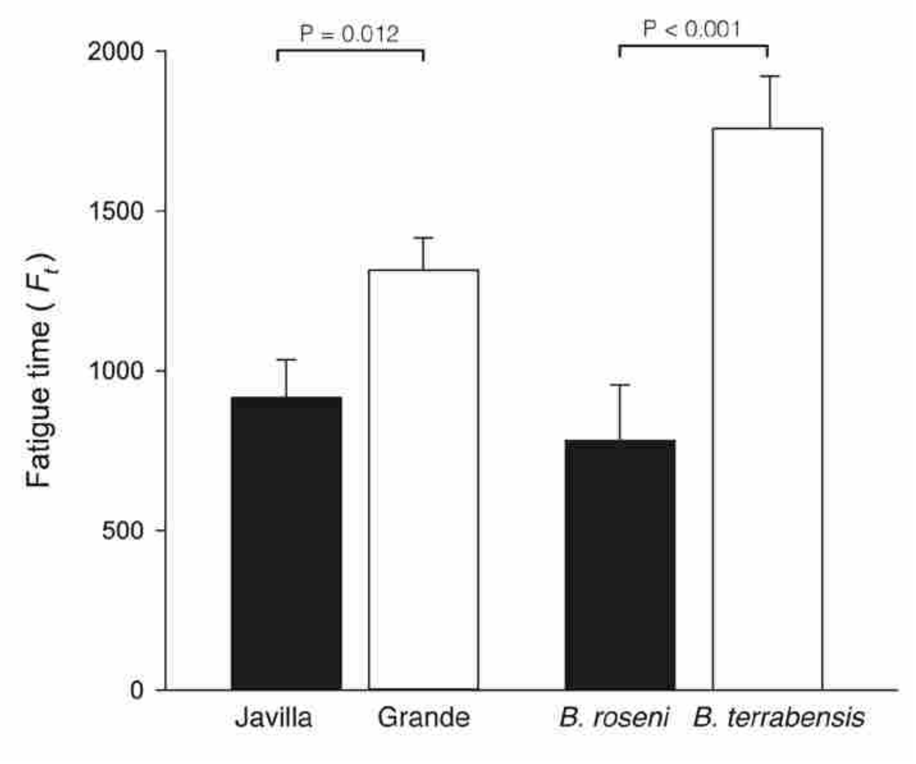

Figure 5.4: Population means for endurance swimming.

Endurance swimming performance (fatigue time, F_t; seconds) for Javilla and Grande, and *B. roseni* and *B. terrabensis.* P-values indicate significance of the 'Species' or 'Population' effect.

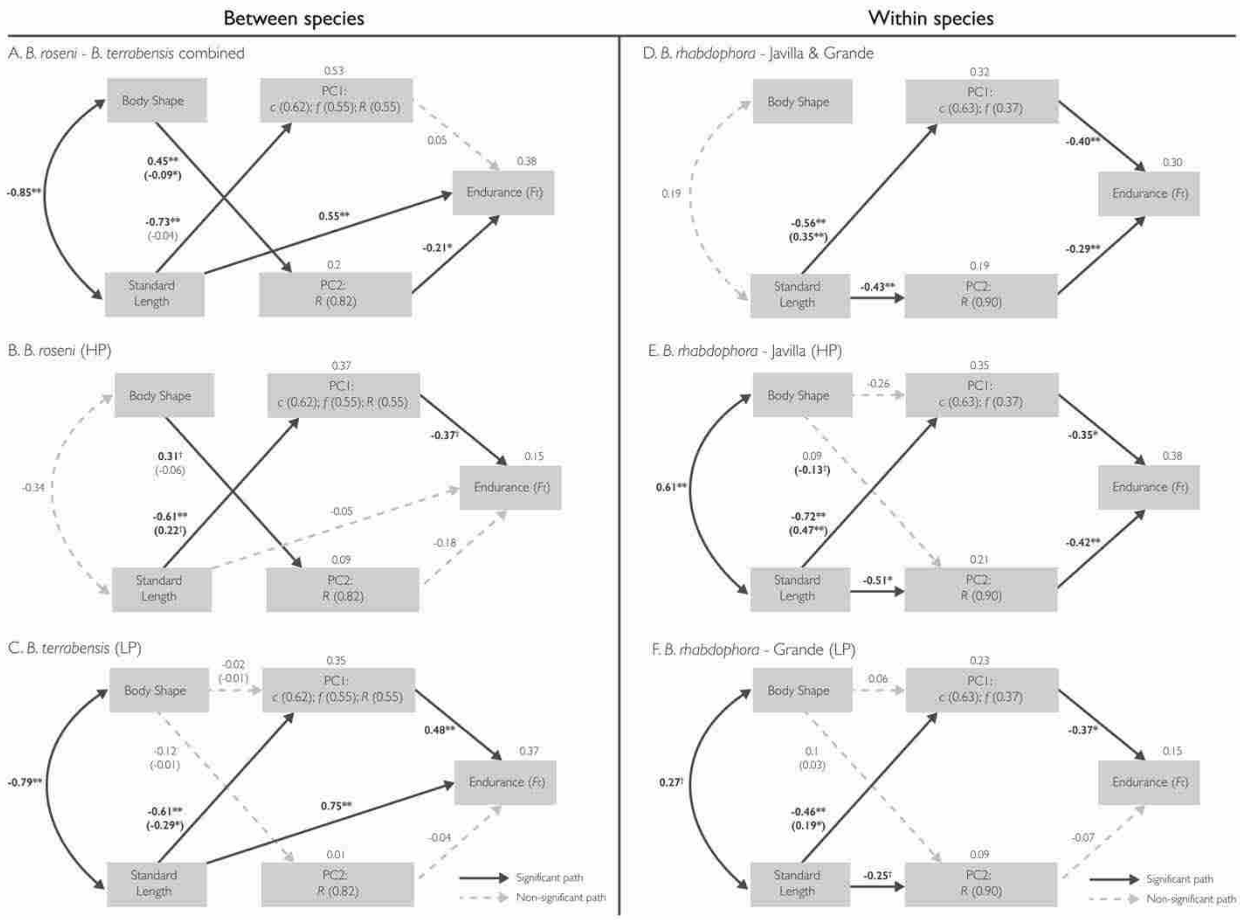

Figure 5.5: Path analysis results for endurance swimming.

Path analysis results for endurance data for all top models with populations analyzed together (A and D) and individually (B, C, E, and F). Significant paths are shown in solid dark-grey arrows, while non-significant paths are shown in broken light-grey arrows. Double-headed arrows represent correlations between body shape and SL. Values near each arrow represent regression coefficients, and values above each ellipsis represent squared correlation coefficients. Indirect effects of SL and body shape on survival are shown in parentheses. Indirect effects can be interpreted as mediated effects that occur in addition to any direct effect that these kinematics variables have on survival. †*P* < 0.10; **P* < 0.05; ***P* < 0.01.

Table 5.1: Results for body shape comparisons.
Results for multivariate analysis of covariance (MANCOVA) comparing body shape between: A) Javilla and Grande; and between B) *B. roseni – B. terrabensis*.

		Predator Mesocosm			Endurance Swimming		
Comparison	Effect	DF	F	P	DF	F	P
A. Javilla – Grande							
	Population	22, 154	4.032	<0.001	22, 31	6.43	<0.001
	Sex	22, 154	18.516	<0.001	-	-	-
	Centroid size	22, 154	1.032	0.429	22, 31	1.03	0.46
	Population: Sex	22, 154	0.2.778	<0.001	-	-	-
B. B. roseni – B. terrabensis							
	Species	22, 193	46.444	<0.001	22, 31	47.8	<0.001
	Sex	22, 193	51.645	<0.001	-	-	-
	Centroid size	22, 193	1.531	0.067	22, 31	7.1	<0.001
	Species: Sex	22, 193	7.359	<0.001	-	-	-

Table 5.2: Mortality rate comparisons for mesocosm experiments.

Results for analysis of covariance (ANCOVA) comparing mortality rates between: A) Javilla and Grande; and between B) *B. roseni – B. terrabensis*.

Comparison	Effect	DF	*F*	*P*
A. Javilla – Grande				
	Population	5, 174	11.228	<0.001
	Sex	5, 174	0	1
	Burst-speed	5, 174	11.086	0.001
	SL	5, 174	8.386	0.004
	Population: Sex	5, 174	0.854	0.357
B. *B. roseni – B. terrabensis*				
	Species	5, 213	13.1618	<0.001
	Sex	5, 213	0.0003	0.987
	Burst-speed	5, 213	13.2508	<0.001
	SL	5, 213	0.1692	0.681
	Species: Sex	5, 213	0.1244	0.725

Table 5.3: Indirect path estimates for mesocosm experiments.

Indirect path estimates and bootstrap standard errors (SE) for all path models generated to test relationships among body shape, size, burst-speed, and survival. Significance (1-tailed) of paths was estimated through bootstrapping (5000 replicates).

Model	Path	Indirect estimate	SE	*P* (1-tailed)
Javilla - Grande				
	SL -> Survival	0.006	0.019	0.349
	Body shape -> Survival	-0.033	0.023	0.039
Javilla				
	SL -> Survival	-0.009	0.023	0.233
	Body shape -> Survival	-0.003	0.023	0.397
Grande				
	SL -> Survival	0.047	0.03	0.023
	Body shape -> Survival	-0.065	0.039	0.008
B. roseni - B. terrabensis				
	SL -> Survival	-0.168	0.033	<0.001
	Body shape -> Survival	0.05	0.02	0.003
B. roseni				
	SL -> Survival	0.074	0.036	0.004

	Body shape -> Survival	0.086	0.038	0.003
B. terrabensis				
	SL -> Survival	-0.023	0.026	0.112
	Body shape -> Survival	0.04	0.028	0.028

Table 5.4: Results for variation in body size, kinematics, and endurance.

Results for analysis of covariance (ANVOCA) and analysis of variance (ANOVA) testing for variation in body size, kinematics, and endurance between: A) Javilla and Grande; and B) *Brachyrhaphis roseni* and *B. terrabensis.* Cohen's *d* is the standardized effect size for the population (i.e., predation environment) term.

			Predation regime		Standard Length	
A.	Dependent variable	Cohen's d (95% CI)	F	P	F	P
	Standard length (SL, mm)	1.243 (0.639, 1.848)	21.18	<0.001		
	Caudal fin depth (log B/SL)	-0.700 (-1.269, -0.131)	7.831	0.007	9.704	0.003
	Tail-beat frequency (log f)	-0.503 (-1.063, 0.058)	3.652	0.062	3.876	0.054
	Rostral amplitude (log10 R/SL)	-0.683 (-1.251, -0.115)	9.345	0.003	25.427	<0.001
	Tail-beat amplitude (H/SL)	-0.977 (-1.562, -0.392)	22.86	<0.001	40.55	<0.001
	Propulsive wavelength (λ/SL)	0.283 (-0.271, 0.838)	1.228	0.273	7.184	0.009
	Propulsive wave speed (log c/SL)	-0.236 (-0.789, 0.318)	0.963	0.331	14.846	<0.001
	Power (log P)	-1.104 (-1.698, -0.511)	30.53	<0.001	44.76	<0.001

Fatigue time (Ft)	0.698 (0.129, 1.267)	7.027	0.012	3.736	0.059

B. Dependent variable	Cohen's d (95% CI)	F	P	F	P
Standard length (SL)	-2.946 (-2.946, -1.514)	65.64	<0.001		
Caudal fin depth (B/SL)	1.233 (0.619, 1.847)	22.686	<0.001	7.892	0.007
Tail-beat frequency (log f)	0.943 (0.350, 1.536)	13.73	<0.001	10	0.003
Rostral amplitude (log10 R/SL)	2.014 (1.324, 2.703)	74.7	<0.001	21.98	<0.001
Tail-beat amplitude (H/SL)	0.521 (-0.051, 1.093)	5.022	0.029	22.302	<0.001
Propulsive wavelength (λ/SL)	-0.574 (-1.148, 0.001)	4.403	0.041	1.712	0.197
Propulsive wave speed (log c/SL)	0.415 (-0.153, 0.983)	2.693	0.107	10.77	0.002
Power (log P)	1.286 (0.669, 1.904)	38.45	<0.001	41.32	<0.001
Fatigue time ($\sqrt{Ft}$)	-1.316 (-1.936, -0.696)	25.364	<0.001	6.806	0.012

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Supplemental material

Table S5.1: Predator mesocosm model comparisons.

Model comparison results for 7 competing models for all path analyses performed for predator mesocosm data. Top models are shown in bold.

Comparison	Model	AIC
B. roseni – B. terrabensis combined		
	Direct only	169.286
	Direct + Body shape ➔ Burst-speed	159.140
	Direct + SL ➔ Burst	101.399
	Direct + indirect	84.00
	Indirect + Body shape ➔ Burst-speed	78.482
	Indirect + SL ➔ Burst-speed	79.027
	Indirect only	**73.412**
B. roseni		
	Direct only	42.174
	Direct + Body shape ➔ Burst-speed	35.387
	Direct + SL ➔ Burst	38.595
	Direct + indirect	28.000
	Indirect + Body shape ➔ Burst-speed	26.064
	Indirect + SL ➔ Burst-speed	26.225
	Indirect only	**24.373**

B. terrabensis		
	Direct only	28.176
	Direct + Body shape ➔ Burst-speed	27.258
	Direct + SL ➔ Burst	29.557
	Direct + indirect	28.00
	Indirect + Body shape ➔ Burst-speed	26.397
	Indirect + SL ➔ Burst-speed	26.721
	Indirect only	**24.945**
B. rhabdophora – Javilla and Grande		
	Direct only	80.820
	Direct + Body shape ➔ Burst-speed	80.728
	Direct + SL ➔ Burst	85.142
	Direct + indirect	84.00
	Indirect + Body shape ➔ Burst-speed	85.370
	Indirect + SL ➔ Burst-speed	**78.239**
	Indirect only	80.443
B. rhabdophora - Grande		
	Direct only	30.176
	Direct + Body shape ➔ Burst-speed	28.461
	Direct + SL ➔ Burst	30.717
	Direct + indirect	28.000

	Indirect + Body shape ➔ Burst-speed	27.107
	Indirect + SL ➔ Burst-speed	26.001
	Indirect only	**25.160**
B. rhabdophora - Javilla		
	Direct only	24.226
	Direct + Body shape ➔ Burst-speed	26.180
	Direct + SL ➔ Burst	26.013
	Direct + indirect	28.000
	Indirect + Body shape ➔ Burst-speed	29.742
	Indirect + SL ➔ Burst-speed	**26.088**
	Indirect only	28.300

Table S5.2: Model comparisons for endurance swimming.

Model comparison results for competing models for all path analyses performed for endurance data. Top models are shown in bold.

Comparison	Model	AIC
B. roseni – B. terrabensis combined		
	Direct + indirect	160.140
	Body shape ➔ PC1, SL ➔ PC2, and Body shape ➔ Fatigue removed	**144.819**
	SL ➔ PC2 and Body shape ➔ Fatigue removed	150.734
B. roseni		
	Direct + indirect	40.144
	Body shape ➔ PC1, SL ➔ PC2, and Body shape ➔ Fatigue removed	**38.047**
	SL ➔ PC2 and Body shape ➔ Fatigue removed	36.119
B. terrabensis		
	Direct + indirect	68.006
	Body shape ➔ PC1, SL ➔ PC2, and Body shape ➔ Fatigue removed	62.418
	SL ➔ PC2 and Body shape ➔ Fatigue removed	**64.407**
B. rhabdophora – Javilla and Grande		
	Direct + indirect	127.341

	Body shape ➔ PC1, Body shape ➔ PC2, Body shape ➔ Fatigue, and SL ➔ Fatigue removed	**106.755**
	Body shape ➔ PC2, Body shape ➔ Fatigue, and SL ➔ Fatigue removed	110.886
	Body shape ➔ Fatigue and SL ➔ Fatigue removed	116.412
B. rhabdophora - Grande		
	Direct + indirect	42.819
	Body shape ➔ PC1, Body shape ➔ PC2, Body shape ➔ Fatigue, and SL ➔ Fatigue removed	36.767
	Body shape ➔ PC2, Body shape ➔ Fatigue, and SL ➔ Fatigue removed	37.170
	Body shape ➔ Fatigue and SL ➔ Fatigue removed	**38.995**
B. rhabdophora - Javilla		
	Direct + indirect	40.389
	Body shape ➔ PC1, Body shape ➔ PC2, Body shape ➔ Fatigue, and SL ➔ Fatigue removed	33.239
	Body shape ➔ PC2, Body shape ➔ Fatigue, and SL ➔ Fatigue removed	35.123
	Body shape ➔ Fatigue and SL ➔ Fatigue removed	**36.837**

Table S5.3: Pairwise comparisons for predator mesocosm path models.

Results for pairwise comparisons of paths across predator mesocosm models. Significance was assessed using a critical ratio difference test. Significantly different paths are indicated in bold.

Comparison	Path	Z	P
B. roseni – *B. terrabensis*			
	Burst-speed (cm/s) 🡐 SL	-3.173	**0.002**
	Burst-speed (cm/s) 🡐 Body shape	-1.337	0.181
	Survival🡐 SL	-0.63	0.529
	Survival 🡐 Burst-speed (cm/s)	0.179	0.858
	Survival 🡐 Body shape	0.964	0.335
Javilla – Grande			
	Burst-speed (cm/s) 🡐 SL	-1.465	0.143
	Burst-speed (cm/s) 🡐 Body shape	-1.306	0.192
	Survival🡐 SL	0.324	0.746
	Survival 🡐 Burst-speed (cm/s)	-0.768	0.442
	Survival 🡐 Body shape	-0.193	0.847
B. roseni – Javilla			
	Burst-speed (cm/s) 🡐 SL	-2.671	**0.008**
	Burst-speed (cm/s) 🡐 Body shape	-2.837	**0.005**

	Survival← SL	1.307	0.191
	Survival ← Burst-speed (cm/s)	0.26	0.795
	Survival ← Body shape	0.12	0.904
B. terrabensis – Grande			
	Burst-speed (cm/s) ← SL	-1.936	0.053
	Burst-speed (cm/s) ← Body shape	0.384	0.701
	Survival← SL	-1.227	0.219
	Survival ← Burst-speed (cm/s)	-0.874	0.382
	Survival ← Body shape	0.689	0.491
B. roseni M – F			
	Burst-speed (cm/s) ← SL	0.262	0.793
	Burst-speed (cm/s) ← Body shape	-0.702	0.483
	Survival← SL	1.772	0.076
	Survival ← Burst-speed (cm/s)	-0.803	0.422
	Survival ← Body shape	0.031	0.975
B. terrabensis M – F			
	Burst-speed (cm/s) ← SL	-0.904	0.366
	Burst-speed (cm/s) ← Body shape	0.248	0.804
	Survival← SL	-0.787	0.431

	Survival ← Burst-speed (cm/s)	0.469	0.639
	Survival ← Body shape	0.241	0.809
Javilla M – F			
	Burst-speed (cm/s) ← SL	0.79	0.429
	Burst-speed (cm/s) ← Body shape	-1.036	0.300
	Survival← SL	-0.684	0.494
	Survival ← Burst-speed (cm/s)	-1.188	0.235
	Survival ← Body shape	0.285	0.776
Grande M – F			
	Burst-speed (cm/s) ← SL	-0.728	0.467
	Burst-speed (cm/s) ← Body shape	0.281	0.779
	Survival← SL	0.804	0.422
	Survival ← Burst-speed (cm/s)	-1.407	0.159
	Survival ← Body shape	-0.522	0.602

Table S5.4: Pairwise comparisons for endurance swimming.

Results for pairwise comparisons of paths across endurance models. Significance was assessed using a critical ratio difference test. Significantly different paths are indicated in bold.

Comparison	Path	Z	P
B. roseni - B. terrabensis			
	Body shape ➔ PC2	1.581	0.114
	SL ➔ PC1	-1.333	0.182
	PC2 ➔ Endurance	-0.532	0.595
	SL ➔ Endurance	-2.405	**0.016**
	PC1 ➔ Endurance	-2.980	**0.003**
Javilla - Grande			
	SL ➔ PC1	0.678	0.498
	SL ➔ PC2	-0.725	0.468
	Body shape ➔ PC1	-1.084	0.278
	Body shape ➔ PC2	0.680	0.497
	PC1 ➔ Endurance	0.058	0.954
	PC2 ➔ Endurance	-1.544	0.122