PREDATOR-DRIVEN PHENOTYPIC DIVERSIFICATION IN *GAMBUSIA AFFINIS*

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Abstract.—Predation is heterogeneously distributed across space and time, and is presumed to represent a major source of evolutionary diversification. In fishes, fast-starts—sudden, high-energy swimming bursts—are often important in avoiding capture during a predator strike. Thus, in the presence of predators, we might expect evolution of morphological features that facilitate increased fast-start speed. We tested this hypothesis using populations of western mosquitofish (*Gambusia affinis*) that differed in level of predation by piscivorous fish. Body morphology of *G. affinis* males, females, and juveniles diverged in a consistent manner between predatory environments. Fish collected from predator populations exhibited a larger caudal region, smaller head, more elongate body, and a posterior, ventral position of the eye relative to fish from predator-free populations. Divergence in body shape largely matched a priori predictions based on biomechanical principles, and was evident across space (multiple populations) and time (multiple years). We measured maximum burst-swimming speed for male mosquitofish and found that individuals from predator populations produced faster bursts than fish from predator-free populations (about 20% faster). Biomechanical models of fish swimming and intrapopulation morphology-speed correlations suggested that body shape differences were largely responsible for enhanced locomotor performance in fish from predator populations. Morphological differences also persisted in offspring raised in a common laboratory environment, suggesting a heritable component to the observed morphological divergence. Taken together, these results strongly support the hypothesis that divergent selection between predator regimes has produced the observed phenotypic differences among populations of *G. affinis*. Based on biomechanical principles and recent findings in other species, it appears that the general ecomorphological model described in this paper will apply for many aquatic taxa, and provide insight into the role of predators in shaping the body form of prey organisms.

Key words.—Common garden experiment, divergent natural selection, ecomorphology, fitness trade-offs, geometric morphometrics, population differentiation, predator-prey interactions.

Divergent natural selection between alternative environments often generates and maintains phenotypic diversity (e.g. Ehrlich and Raven 1969; Endler 1977; Rice and Hostert 1993; Orr and Smith 1998; Schluter 2000a; Albertson et al. 2003). Population differentiation (e.g. polymorphism, geographic variation) is typically believed to arise from such divergent selective regimes (reviewed in Robinson and Wilson 1994; Smith and Skúlason 1996; Magurran and May 1999; Schluter 2000a). Phenotypic diversification can stem from either genetic differentiation or phenotypic plasticity; either source of divergence can represent adaptive responses to selection and result in microevolutionary change within a species (e.g. Levins 1968; West-Eberhard 1989; Rice and Hostert 1993; Robinson and Wilson 1994; Orr and Smith 1998; Losos et al. 2000; Schluter 2000a; Agrawal 2001; Pigliucci and Murren 2003).

Empirical studies of diversification, particularly in vertebrates, have focused on resource competition as a primary agent driving divergence (e.g. Brown and Wilson 1956; Grant 1986; Schluter 1988, 1994, 2000b; Wainwright 1988; Losos 1990a, 2000; Robinson and Wilson 1994; Smith and Skúlason 1996; Rainey and Travisano 1998; Adams and Rohlf 2000; Streelman and Danley 2003). However, predation is among the most important factors structuring natural communities (e.g. Jeffries and Lawton 1984; Kerfoot and Sih 1987; Jack-

son et al. 2001; Silliman and Zieman 2001). Predators should not only affect the distribution and abundance of species, but also the distribution and abundance of phenotypes within species (e.g. Vermeij 1987; Brönmark and Miner 1992; Endler 1995; McPeek et al. 1996; Tollrian and Harvell 1999; DeWitt and Langerhans 2003; Reimchen and Nosil 2004).

For most prey, escape ability (i.e., avoidance of predatory encounters or escape during an encounter) is essential to survival. Prey organisms show a diverse array of predator escape mechanisms (reviewed in Edmunds 1974; Kerfoot and Sih 1987; Greene 1988), and predators often generate selection favoring enhanced escape performance (Brodie 1992; Nilsson et al. 1995; Watkins 1996; McPeek 1997; Van Buskirk and Relyea 1998; Van Buskirk and McCollum 1999; DeWitt and Langerhans 2003). These selection pressures may result in phenotypic differences among populations that vary in predation threat (Greene 1988; Reimchen 1994; Walker 1997; Storfer et al. 1999; DeWitt et al. 2000; Trussell 2000; O'Steen et al. 2002; Relyea 2002). This study tests the hypothesis that prey phenotypes associated with escape ability diverge between populations that differ in predator regime.

Livebearing fishes of the family Poeciliidae provide ideal models for studies of predator-driven evolution (e.g. Meffe and Snelson 1989a; Endler 1995; Magurran et al. 1995; Reznick 1996; Reznick et al. 1997; Langerhans and DeWitt 2004). These fish are small-bodied, mature rapidly, and inhabit environments that vary substantially in predator community. Most livebearing species are common prey items for a variety of predators, particularly piscivorous fish (Meffe and Snelson 1989b). Many studies have examined variation in life-history characteristics, behavior, and body color as-

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sociated with piscine predation in poeciliid fishes (e.g. Krumholz 1963; Seghers 1973; Sohn 1977; Endler 1980, 1983, 1995; Reznick and Endler 1982; Reznick 1989; Magurran et al. 1995; Houde 1997; Downhower et al. 2000; Johnson and Belk 2001; Jennions and Telford 2002). It is generally established that these phenotypic differences reflect fitness trade-offs between high- and low-predation environments. In this study, we compared body morphology and burst-swimming speed among populations of western mosquitofish (*Gambusia affinis*) that differed in predation threat.

Most fish, including poeciliids, exhibit a highly stereotyped escape response called a ''c-start'' when evading a predator strike (e.g. Weihs 1973; Howland 1974; Eaton et al. 1977; Webb 1978, 1986a; Harper and Blake 1990; Domenici and Blake 1997). During a c-start, the fish body bends into a ''C'' shape (stage 1) and then produces a propulsive stroke of the caudal region in the opposite direction (stage 2). This results in a sudden, high-energy burst of unsteady swimming activity. Burst-swimming speed is maximized by a long, deep caudal region and a relatively shallow anterior body/head region (Webb 1982a, 1986b; Webb and Blake 1985; Law and Blake 1996; Walker 1997). This derives from the fact that thrust is primarily generated by the caudal portion of fishes (highlighted in Fig. 1), whereas the anterior portion of the body contributes minimally to thrust, but substantially to drag (see Walker 1997, and references therein). Additionally, a body morphology optimized for burst speed might come at the cost of reduced performance at alternative locomotor activities, such as prolonged swimming (e.g. Dohm et al. 1996; Reidy et al. 2000). In fact, the body morphology necessary to maximize prolonged swimming efficiency (i.e. greatest depth in anterior body/head, shallow caudal region) is the opposite of that required to maximize burst speed (Keast and Webb 1966; Blake 1983; Webb 1984; Videler 1993; Vogel 1994). Thus, a fish cannot simultaneously optimize all types of swimming activities (i.e., functional trade-off).

These biomechanical principles allow us to generate hypotheses concerning expected ecomorphological relationships that can be evaluated with comparative data (Webb 1984; Endler 1986; Wainwright 1988, 1996; Losos 1990b; Emerson 1991; Williams 1992; Wainwright and Richard 1995; Walker 1997; Domenici 2003). We examined differences in body shape and c-start escape speed among populations of *G. affinis* in Texas, USA: three populations coexisting with piscivorous fish and three populations lacking predatory fish. Since piscivores might create selection favoring increased escape ability in *G. affinis*, we hypothesized that mosquitofish from populations sympatric with predatory fish would exhibit (1) a larger caudal region, (2) a shallower anterior body/head region, and (3) faster burst-swimming speeds relative to individuals from populations lacking predatory fish.

Materials and Methods

In this study, we asked three general questions: (1) Does body morphology differ between predator regimes? (2) Does divergence in body shape result in divergence of burst-swimming speed? (3) Is there a heritable basis to morphological divergence? We addressed the first question by comparing body shape of wild-caught fish from populations with and without piscivorous fish. To address the second question, we determined maximum burst-swimming speed for wild-caught adult males collected from predator and predator-free populations, and examined the relationship between body shape and burst speed. Finally, we examined whether morphological differences observed in wild-caught fish were retained in offspring raised in a common laboratory environment.

Populations

Gambusia affinis is a common livebearing fish that occupies a wide range of habitats varying in predator regime across the southern United States. We collected *G. affinis* from six populations in Brazos County, Texas. Although *G. affinis* serves as a prey item for a diverse array of predators, one of their greatest threats is from piscivorous fish (Meffe and Snelson 1989b). To examine phenotypic variation associated with predation from piscivorous fish, sites were chosen a priori so that they differed primarily in the presence of predatory fish. All collection sites were freshwater ponds (i.e. small, nonflowing collections of surface water at relatively low elevations) similar in many environmental parameters other than predator community (e.g. aquatic vegetation, surface area, perimeter, depth, turbidity, temperature, dissolved oxygen). We sampled three populations of *G. affinis* that did not coexist with any piscivorous fishes (Autumn, 30°38.4′N, 96°19.7′W; Hensel, 30°37.5′N, 96°20.8′W; Riverside Campus A, 30°38.0′N, 96°28.4′W) and three that did coexist with piscivorous fishes (Krenek Tap, 30°36.6′N, 96°17.6′W; Riverside Campus B, 30°38.1′N, 96°27.9′W; University Oaks, 30°37.2′N, 96°18.8′W). Throughout the paper, populations are termed ''predator'' and ''predator-free'' in relation to piscivorous fish presence, although other predators may have been present (e.g., invertebrates, turtles, birds). Although the phylogenetic relationships of the populations are unknown, we selected sites to minimize the likelihood of predator populations being more closely related to each other than to any predator-free population, and vice versa. This was accomplished by collecting from isolated ponds across the landscape, where populations of alternative predator regimes were geographically as close as or closer than populations of the same predator regime. For instance, Riverside Campus A and B populations represented alternative predator regimes and were only 0.75 km apart, the closest pair of populations, whereas some populations of the same predator regime were more than 14 km apart.

We surveyed populations for predatory fishes using seines, cast nets, dip nets, and hook-and-line angling. Common piscivorous fishes found in predator populations were native sunfishes (family Centrarchidae), including largemouth bass (*Micropterus salmoides*), green sunfish (*Lepomis cyanellus*), warmouth (*L. gulosus*), white crappie (*Pomoxis annularis*), longear sunfish (*L. megalotis*), and bluegill (*L. macrochirus*). *Gambusia affinis* were collected using seines and dip nets, and were immediately transferred to a laboratory at Texas A&M University.

Morphometrics

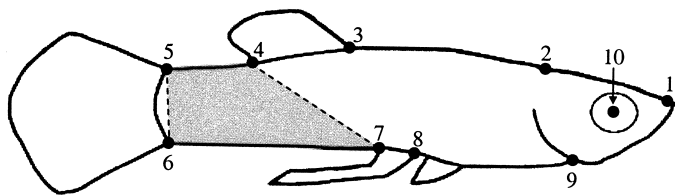To acquire morphometric information, we captured a lateral image of each specimen with a video camera equipped

FIG. 1. Landmarks used for morphometric analyses (male depicted). The shaded region highlights the caudal peduncle region.

with a telecentric lens. Images were obtained by placing live fish into a glass cage (30 mm high × 40 mm long × 5 mm wide) filled two-thirds with water, mounted in front of the video camera. The narrow cage prevented turning and restricted maneuverability, thus maintaining a constant angle of view. Video was captured at a resolution of 0.04 mm/ pixel. We obtained morphometric data from a total of 677 individuals. One author (R. B. Langerhans) digitized ten landmarks on each image using MorphoSys software version 1.29 (Meacham 1993) (Fig. 1). Since we hypothesized that natural selection might favor a body morphology that increases fast-start speed in the presence of piscivorous fishes, we highlight in our figures the body region most responsible for generating accelerated bursts (i.e., the caudal peduncle region, see Fig. 1).

We conducted geometric morphometric analyses using our digitized landmarks. Geometric morphometrics is a powerful tool for analyzing shape variation (Rohlf and Marcus 1993). A key feature of geometric morphometrics that sets it apart from more traditional approaches is that it retains information on spatial covariation among landmarks; linear-distance approaches do not take into consideration where a given measurement is taken relative to others. We used tpsRegr software (Rohlf 2000a) to obtain shape variables for analyses. TpsRegr rotates, translates, and scales landmark coordinates into alignment via generalized least squares superimposition (Bookstein 1991; Marcus et al. 1996). Superimposed landmark configurations were used to calculate affine and non-affine shape components (i.e., uniform components and partial warps) using tpsRegr. These shape variables served as dependent variables describing body morphology in our statistical analyses described below. We visualized variation in landmark positions using the thin-plate spline approach, which maps deformations in shape from one object to another (Bookstein 1991).

Wild-Caught Fish

For comparison with wild-caught fish, we examined fish collected from each of the six populations in the summers of 2001 (males, females, and juveniles) and 2003 (males only). Since *G. affinis* has approximately two generations per year, fish collected in 2001 and 2003 likely represented cohorts separated by several generations. A median of 23 individuals (range 18–27) from each age/gender class was collected from each population. Each age/gender class was analyzed separately. The 2003 collection of males was used to examine burst-swimming performance, as well as to evaluate temporal persistence of morphological differences between predator regimes.

To investigate body shape variation, independent of allometry, we included a measure of body size as a covariate in our statistical analyses. We used centroid size as our estimate of overall body size, as is typical in geometric morphometrics (Bookstein 1991). Centroid size is the square root of the summed, squared distances of all landmarks from their centroid. In this study, centroid size was highly correlated with standard length ($r \geq 0.99$, $P < 0.0001$ for each age/ gender class). All statistical analyses were conducted using JMP software (Ver. 4.04, SAS Institute Inc., Cary, NC) unless otherwise noted.

To test for morphological divergence between predatory environments, we performed a nested MANCOVA for each age/gender class. Morphological data (i.e., 14 partial warps, 2 uniform components) were tested for effects attributable to centroid size (covariate), predator regime, and population nested within predator regime. We first analyzed males collected in 2001 and 2003 separately. Since we found similar results regardless of year of collection (e.g., similar thin-plate spline transformations), we also present results from analyses conducted with data for males pooled across years.

A canonical axis was derived from the predator regime effect of each MANCOVA. Canonical axes represent sets of linear combinations of the response variables and serve as multivariate descriptions of morphological differences between predator regimes—''predator-free-shaped'' fish on one end, ''predator-shaped'' fish on the other. The nature of morphological divergence between predatory environments was assessed by examination of correlations between superimposed landmark coordinates and these axes, as well as production of thin-plate spline transformation grids. Thin-plate spline transformations were generated with tpsRegr using canonical scores and landmark coordinates to visualize shape variation along each canonical axis. We also used tpsSuper (Rohlf 2000b) to produce photographic representations of these transformations on the body shape of an actual specimen (see Langerhans et al. 2003). This procedure applied the thin-plate spline transformation to an image of an individual from the study with an intermediate canonical score.

A discriminant function analysis (DFA) was conducted for each age/gender class to provide an intuitive metric regarding the magnitude of morphological divergence (i.e., percent of fish correctly classified into predator regime). Each DFA used the geometric shape parameters as the dependent variables and predator regime as the independent variable. Thus, we examined the degree to which we could predict an individual fish's predator regime of origin based on its morphology. We used jackknife sampling as a cross-validation technique in our DFAs to avoid a classification bias resulting from the assignment of a case to a group using a discriminant function that was derived, in part, from that particular case (see Lance et al. 2000; Johnson and Wichern 2002; Rencher 2002). This procedure removed one individual from the dataset, classified that individual based on a DFA of the remaining data, returned the individual to the dataset, and then repeated this process for each individual. DFAs were performed using Systat (Ver. 10, SPSS Inc., Chicago, IL).

Burst-Swimming Performance

We examined burst-swimming performance of wild-caught adult males from each of the six populations in 2003. Burst

speed was obtained from 116 fish with a median of 19 fish per population (range 18–22). We only measured burst speed for males since swimming performance is affected by state of pregnancy in females (Plaut 2002) and since all age/gender classes exhibited similar morphological patterns between predator regimes (see Results). Fish were collected from the field, immediately transferred to a laboratory at Texas A&M University, housed in 56-L aquaria, and entered into a performance trial within 24 h.

A performance trial was initiated by transferring an individual to a 12×12 cm staging arena with a 5-mm square grid on the bottom. The staging arena was evenly illuminated and all sides were opaque. The arena was inside a 75-L aquarium that served as a water bath to help stabilize water temperature. A video camera was mounted above the arena and recorded performance trials directly to a computer (.avi file) at 30 frames/sec. We maintained a 1.5-cm water depth in the arena to limit vertical displacement of fish during escape responses. Fish were allowed to acclimate for 10 min before stimulated to perform an escape response. Water temperature (°C) was recorded at the time of escape response with a YSI-550 dissolved oxygen and temperature meter (YSI Inc., Yellow Springs, OH). We elicited a fast-start response by startling the fish with a sudden, downward thrust of a cylindrical wooden probe (5 mm diameter, 100 mm length), hitting the bottom of the stage within 3 cm of the fish. Three fast-starts were elicited for each fish, and the fastest response was retained for analyses.

We calculated burst-swimming speed by digitizing the center of mass for each frame of the fast-start response using tpsDig software (Rohlf 2003). The center of mass in the anterior-posterior direction was determined for 10 male *G. affinis* following Law and Blake (1996). This estimate was used to digitize the center of mass in video images. The fast-start response comprised three video frames in our data: frame 1: fish at rest; frame 2: fish in stage 1 (body in C-shaped bend); frame 3: fish in stage 2 (body moving rapidly away from stimulus just subsequent to propulsive tail stroke). The linear distance traveled between frames 2 and 3 was used to estimate burst speed in cm/sec.

Measurement precision might have been compromised and maximum burst speeds underestimated by recording movement at only 30 frames/sec. We were not, however, interested in obtaining the absolute maximum burst speed of these fish, but rather relative differences in burst speeds between predator regimes. To determine whether 30 frames/sec video provided adequate precision for calculating burst speed in *G. affinis*, we conducted a pilot study by measuring three escape responses for each of 18 fish, and calculating repeatability of the measurements following Lessells and Boag (1987). We found high repeatability of burst speed ($r = 0.89$, $P < 0.0001$), indicating an acceptable level of precision for the purposes of this study.

To assess whether burst speed differed between predatory environments, we conducted a nested ANCOVA with maximum burst speed as the dependent variable and centroid size (covariate), water temperature (covariate), predator regime, and population nested within predator regime as the independent variables. We also wished to investigate the relationship between body morphology and burst speed. To test

whether morphological differences between predator regimes were related to burst-swimming performance, we conducted a multiple regression that examined the effects of centroid size, water temperature, and the morphological axis of divergence on maximum burst speed. The predator regime canonical axis from the MANCOVA conducted with these particular fish (i.e., wild-caught males from 2003) served as the ''morphological axis of divergence.'' Since the axis is a linear combination of response variables, each fish received a score on the axis. We conducted a goodness-of-fit test and compared the total variance in maximum burst speed explained by the two models—the ANCOVA and the multiple regression—to evaluate whether body shape was as explanatory as the categorical variable for predator regime.

If the observed morphological divergence is causally related to burst speed, we would expect relatively predator-shaped fish to exhibit faster burst speeds *within* each population. To test this hypothesis, we conducted separate multiple regressions identical to that described above for each population. We extracted the standardized partial regression coefficient (i.e., slope, β') for the effect of morphology on speed from each model, and performed a sign test to examine whether we observed more positive slopes than expected by chance. If no relationship between body shape and speed existed, we should find as many negative as positive slopes between morphology and burst speed within populations. On the other hand, if relatively predator-shaped fish exhibited faster burst speeds than predator-free-shaped fish, regardless of population of origin, then we should find a positive slope within all six populations. Further, we determined the probability of observing the magnitude of positive slopes seen in the data by chance by calculating the product of one-tailed *P*-values from the multiple regressions.

Common-Garden Experiment

To examine whether morphological differences between predator regimes observed in wild-caught fish had a heritable basis, we acquired and reared offspring from each population in a common laboratory environment. We obtained offspring from six wild-caught females from each population. To reduce maternal effects, females were held in the laboratory for approximately one month before delivering offspring. Postfertilization maternal provisioning of embryos (matrotrophy) is known for several *Gambusia* species, including *G. affinis* (Marsh-Mathews et al. 2001; E. Marsh-Mathews, M. Brooks, and P. R. Deaton, unpubl. ms.). As a result, effects on offspring phenotype derived from the maternal environment, at least in part, likely arose while housed in the common laboratory environment. In addition, maternal effects on body *shape* appear rare in vertebrates (see Mousseau and Fox 1998), as opposed to body size or life-history traits. However, maternal effects cannot be ruled out using this design; thus persistent phenotypic differences should be cautiously regarded as evidence for heritable differences in body shape.

Offspring were raised in 15-L aquaria (two tanks per population) at 22–25°C for a 14L:10D photoperiod. Fish were fed newly hatched brine shrimp nauplii daily. We used reverse osmosis water for all aquaria, maintained constant water level, and exchanged water among tanks every other week

TABLE 1. Results of MANCOVAs examining body shape variation among populations of *Gambusia affinis*. *F*-ratios were approximated using Wilks's lambda values for the population nested within predator regime effect. Discriminant function analysis (DFA) results are the percent of fish correctly classified to predator regime using jackknife sampling. CG refers to common-garden juveniles.

		Centroid size			Predator regime			Pop (predator regime)			
	n	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	DFA results
2001 males	140	4.55	16, 118	<0.0001	12.66	16, 118	<0.0001	3.22	64, 464.23	<0.0001	82.9%
2003 males	127	5.45	16, 105	<0.0001	6.56	16, 105	<0.0001	4.12	64, 413.33	<0.0001	71.7%
Pooled males	267	12.08	16, 245	<0.0001	14.81	16, 245	<0.0001	3.88	64, 961.41	<0.0001	83.1%
2001 females	134	7.99	16, 112	<0.0001	12.07	16, 112	<0.0001	5.28	64, 440.74	<0.0001	85.1%
2001 juveniles	136	5.44	16, 114	<0.0001	6.37	16, 114	<0.0001	4.17	64, 448.57	<0.0001	72.8%
CG juveniles	140	7.17	16, 118	<0.0001	5.47	16, 118	<0.0001	3.53	64, 464.23	<0.0001	79.3%
Pooled juveniles	276	11.71	16, 254	<0.0001	10.24	16, 254	<0.0001	3.9	64, 996.64	<0.0001	77.5%

to ensure each tank experienced similar environmental conditions. Aquaria were arranged side-by-side in the laboratory, alternating between predator and predator-free populations, to reduce possible effects of microenvironmental factors. After 12 weeks, we captured images of fish for morphometric analyses. We examined juveniles exclusively (95% of fish were immature), rather than waiting until fish achieved sexual maturity, to ensure a large sample size (*n* = 140), and because analyses of wild-caught fish revealed similar morphological patterns for all age/gender classes (see Results). Final fish densities (number of fish per aquaria) did not differ between predator regimes or populations nested therein (ANOVA, both *P* > 0.25).

We tested for morphological differences between offspring of alternative parental predator regimes using a nested MANCOVA and DFA as described above. We used two approaches to evaluate whether common-garden fish retained divergent morphologies observed in wild-caught fish. First, we compared thin-plate spline transformation grids and correlations between landmark coordinates and canonical axes among common-garden and wild-caught juveniles. If the morphological divergence observed in wild-caught fish was maintained in laboratory-reared offspring, then these transformation grids and landmark-canonical variate correlations should be similar. Second, we pooled common-garden and wild-caught juvenile data and conducted the nested MANCOVA and DFA with the pooled dataset. If morphological differences between predator regimes were retained in common-garden fish, then a predator regime effect on body shape should be significant in the MANCOVA, and DFA should correctly classify a significant percentage of fish to the proper predator regime of origin based on morphology, irrespective of whether the fish was reared in the laboratory or collected from the field. To provide a quantitative metric describing similarity/dissimilarity in body shape among populations, we calculated Euclidean distances between population canonical means for wild-caught and common-garden fish from alternative predator regimes (i.e., nine pairwise population distances within six sets of comparisons, see Fig. 4). This distance metric offered a means of evaluating the degree to which wild-caught and common-garden fish from the same predator regime were more similar in body shape than fish from alternative predator regimes.

As in previous studies of this nature (e.g. Reznick and Endler 1982; Reznick 1989; Leips and Travis 1999; Kelly et al. 2000; Jennions and Telford 2002), populations were treated as fixed effects in all analyses since they were specifically chosen due to their known predator communities, and differences in population means were of interest (Bennington and Thayne 1994; Sokal and Rohlf 1995, pp. 196–203). In regard to the significance test for the predator regime effect, our statistical models are equivalent to conducting planned contrasts between predatory environments when population is the main effect in the analysis; however, the nested analysis is the more appropriate model for the present study. *F*-ratios used type-III sums of squares. Heterogeneity of slopes was tested (and never observed) in all models including a covariate.

RESULTS

Wild-Caught Fish

We found significant differences in body morphology of wild-caught fish (males, females, and juveniles) between predator and predator-free populations (Table 1). Body shape also varied with centroid size (i.e. multivariate allometry) and among populations nested within predator regime for each age/gender class. The predator regime effect of the MANCOVAs explained 63.2% of partial morphological variance (i.e., $SS_{effect}/SS_{effect} + SS_{error}$) for 2001 males, 50.0% for 2003 males, 63.3% for 2001 females, and 48.3% for 2001 juveniles. Since the nature of morphological divergence between predator regimes was very similar for males collected in 2001 and 2003 (i.e., similar thin-plate spline transformations and landmark correlations with canonical axes), we pooled data across years. The predator regime effect for males pooled across years explained 49.2% of partial shape variance. Examination of correlations between superimposed landmark coordinates and predator regime canonical axes revealed a consistent shift in morphology between predator regimes for males, females, and juveniles (Table 2). Canonical centroids and 95% confidence ellipses for populations of *G. affinis* along the respective canonical variate for each age/gender class, as well as thin-plate spline visualizations, are presented in Figure 2. Based on thin-plate spline transformations and correlations between landmark coordinates and canonical axes, fish found in predator populations exhibited four major morphological shifts: (1) larger caudal peduncle, (2) smaller head, (3) relatively posterior, ventral placement of the eye, and (4) more elongate body.

Discriminant function analyses for each age/gender class revealed highly significant differences in body shape between

TABLE 2. Pearson correlation between superimposed landmark coordinates and the predator regime canonical axis from MANCOVAs conducted for wild-caught fish. Correlations presented for males are from the pooled dataset. The direction of landmark differences observed in predator populations, relative to predator-free populations, is presented in the final column (e.g., landmark 2 is relatively anterior and ventral in predator populations). Variables that exhibited an average correlation across all age/gender classes ≥ ‖0.2‖ are bold.

Landmark	Males	Females	Juveniles	Direction
1X	**−0.6713**	−0.4912	−0.4627	posterior
1Y	−0.0248	−0.0508	−0.2354	—
2X	**0.1806**	**0.1663**	**0.3837**	anterior
2Y	**−0.5306**	**−0.3199**	**−0.3144**	ventral
3X	0.2407	0.0821	−0.1214	—
3Y	−0.0736	0.1447	−0.2089	—
4X	−0.0338	0.1537	−0.1090	—
4Y	0.0335	0.3161	−0.2486	—
5X	**−0.4060**	**−0.6106**	**−0.3357**	posterior
5Y	0.1711	0.0226	0.0013	—
6X	**−0.3353**	**−0.3646**	**−0.3551**	posterior
6Y	0.1051	−0.0981	0.0138	—
7X	**0.4767**	**0.6725**	**0.5438**	anterior
7Y	−0.3092	−0.1546	0.0595	—
8X	**0.4122**	**0.5089**	0.1788	anterior
8Y	**0.2207**	−0.0182	**0.4058**	dorsal
9X	0.1065	−0.0409	0.1628	—
9Y	**0.5907**	**0.6290**	**0.8081**	dorsal
10X	**−0.4323**	**−0.4205**	−0.1222	posterior
10Y	**−0.1193**	**−0.3659**	**−0.3409**	ventral

predator regimes (all $P < 0.0001$) and exhibited high predictability (Table 1). That is, based on morphology, a large majority of individual fish could be correctly classified to their predator regime of origin. Of particular importance is that predictability remained high when morphological data were pooled across years for wild-caught males—83.1% correctly classified to predator regime, irrespective of year of collection. This suggested that the nature of morphological divergence between predator regimes was consistent across years. To illustrate the temporal persistence of morphological divergence between predator regimes, we present thin-plate spline transformations for wild-caught males collected in 2001 and 2003 (Fig. 3A,B). We also present a photographic representation of the observed morphological divergence using data pooled across years (Fig. 3C).

Burst-Swimming Performance

ANCOVA revealed that maximum burst speed was strongly associated with predator regime of origin, and marginally associated with the covariate centroid size (larger fish tended to be faster; Table 3). On average, fish from predator populations were 19.9% faster than fish from predator-free populations (Fig. 4A). Note that centroid size did not differ between predator regimes ($F_{1,114} = 0.47$, $P = 0.49$). Using multiple regression, we found maximum burst speed increased with centroid size ($F_{1,112} = 3.97$, $P = 0.049$), water temperature ($F_{1,112} = 13.22$, $P = 0.0004$), and the morphological axis of divergence ($F_{1,112} = 17.79$, $P < 0.0001$). Comparing the two models—the ANCOVA and the multiple regression—we found that the ANCOVA was only slightly
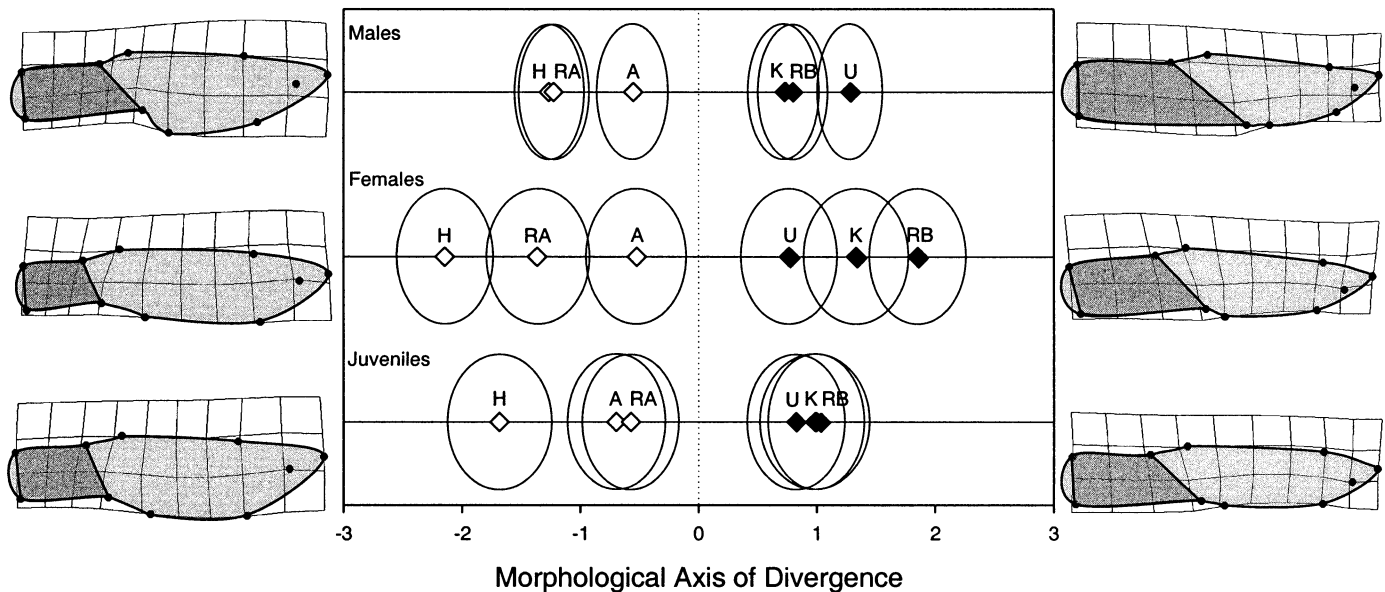

FIG. 2. Morphological divergence between predator-free (open symbols) and predator (filled symbols) populations for wild-caught males, females, and juveniles. Ellipses represent 95% confidence regions for each population along the respective morphological axis. Axes depict the canonical variates derived from the predator regime factor of the MANCOVA conducted separately for each age/gender class (i.e., three separate axes presented in one panel). Visualizations (i.e., thin-plate spline transformation grids) of the canonical shape axes are provided at the ends of each axis. Grids were generated using landmark coordinates and canonical scores in tpsRegr (Rohlf 2000a), and were magnified 2× to better illustrate morphological differences. Data are pooled across years for males. Population abbreviations are as follows: Autumn (A), Hensel (H), Riverside Campus A (RA), Krenek Tap (K), Riverside Campus B (RB), and University Oaks (U).

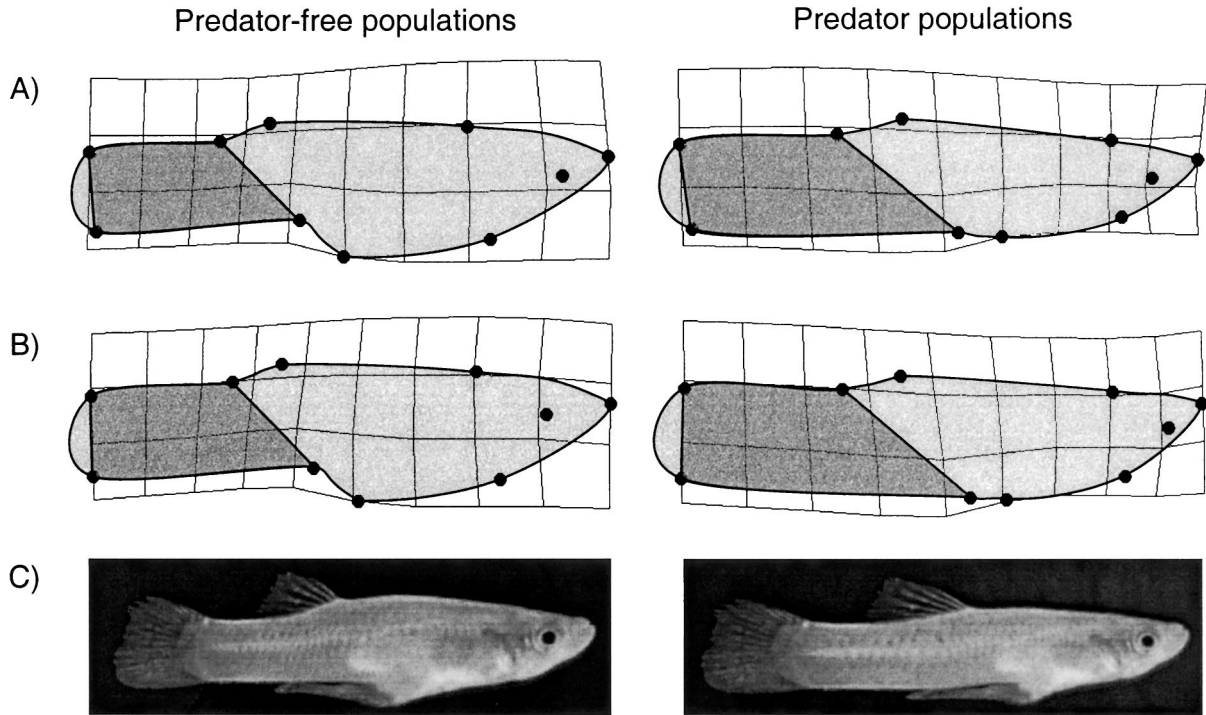## Predator-free populations   Predator populations

FIG. 3. Visualization of morphological divergence between predator regimes for *G. affinis* males. Thin-plate spline transformations (all magnified 2×) depict morphological differences in (A) 2001, (B) 2003, and (C) both years combined as described by canonical axes derived from the predator regime effect of each MANCOVA. Photographs in (C) represent deviations in landmark configurations between predatory environments applied to a single photograph of an individual with an intermediate canonical score.

more explanatory ($R^2 = 0.29$) than the multiple regression including the morphological axis of divergence ($R^2 = 0.26$), and did not provide a significantly better fit to the data ($F_{4,108} = 0.97$, $P = 0.42$). Thus, the morphological axis of divergence apparently captured much of the explanatory aspects of the categorical variable ''predator regime of origin.'' This suggested that body shape was a primary speed-determining factor among populations.

We found a consistent trend in the relationship between morphology and burst speed within populations: all six populations exhibited a positive standardized partial regression coefficient for the relationship between burst speed and the morphological axis of divergence (sign test of slopes, $P = 0.016$; Fig. 4B). Further, the probability of observing the magnitude of this relationship within each population by chance was extremely low (average $\beta' = +0.21$, $P < 0.0001$). Thus, within each population, relatively predator-shaped fish tended to produce faster burst speeds than relatively predator-free-shaped fish.

Common-Garden Experiment

As with wild-caught fish, body shape of common-garden fish varied with centroid size (i.e., multivariate allometry), between predator regimes, and among populations nested within predator regime (Table 1). The predator regime effect in the MANCOVA explained 42.6% of partial shape variance. Discriminant function analysis also indicated significant differences in morphology between predatory environments ($F_{16,123} = 9.26$, $P < 0.0001$) and exhibited high predictability (Table 1). Based on examination of thin-plate spline transformations, as well as landmark correlations with the predator regime canonical axes, morphological divergence between predator regimes observed in common-garden fish was very similar to that observed in wild-caught fish (Fig. 5A,B). Furthermore, when data were pooled for wild-caught and common-garden juveniles, MANCOVA revealed a highly significant effect of predator regime on body shape (Table 1). The predator regime effect in the pooled data explained

TABLE 3. Results of nested ANCOVA ($R^2 = 0.29$) examining variation in maximum burst speed (cm/sec) for *Gambusia affinis* males.

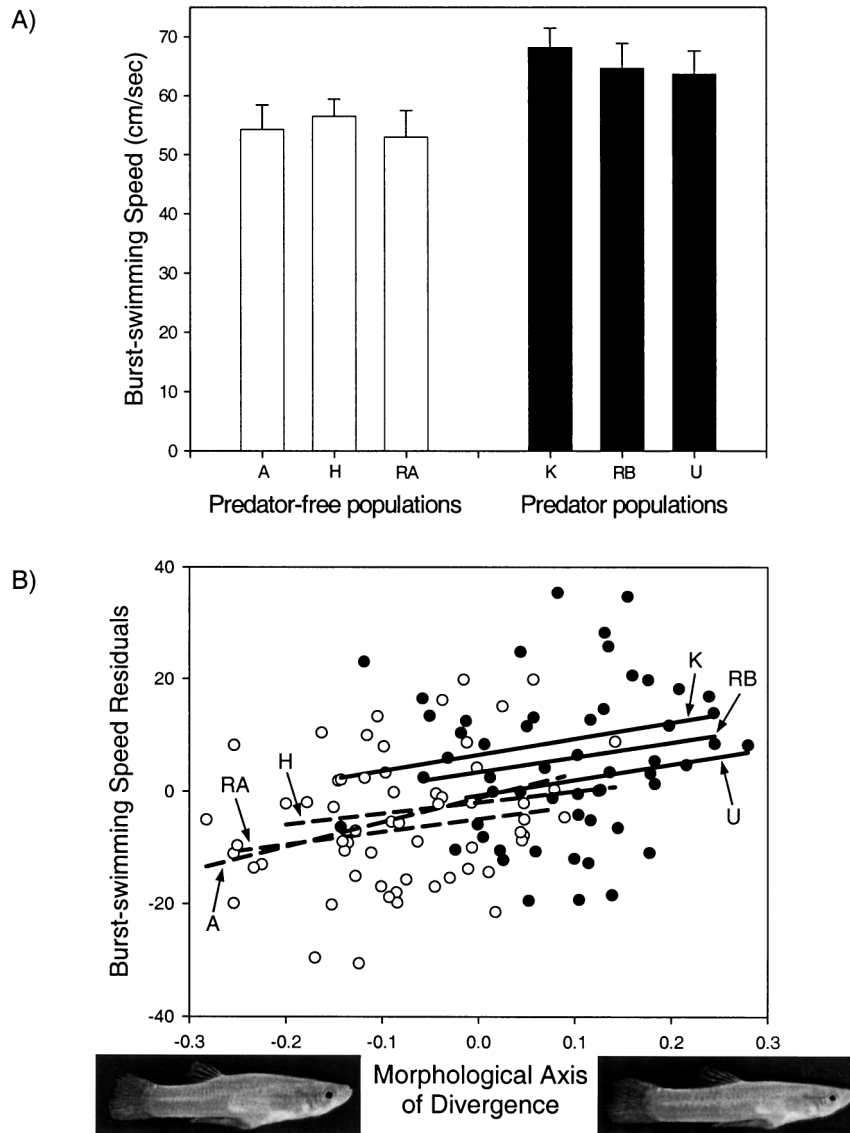| Source	MS	df	F	P
Predator regime	3376.03	1	20.10	<0.0001
Population (predator regime)	76.56	4	0.46	0.7679
Centroid size	627.85	1	3.74	0.0558
Water temperature	377.172	1	2.25	0.1369
Error	167.93	108		

FIG. 4.   Variation in burst-swimming speed of males (A) among populations (least squares means $\pm$ standard error), and (B) its relationship with body shape (predator regime canonical axis), controlling for effects of body size (centroid size) and water temperature. Open symbols and dashed lines, predator-free populations; filled symbols and solid lines, predator populations. Population abbreviations as in Figure 2.

39.2% of partial morphological variance. Evaluation of Euclidean distances between populations on this canonical axis clearly indicated that wild-caught and common-garden fish from the same predator regime were more similar in body shape than fish from alternative predator regimes (Fig. 5). Discriminant function analysis also found strong differences in body shape between predator regimes, regardless of whether fish were collected from the wild or reared in a common environment in the laboratory ($F_{16,259} = 10.24$, $P < 0.0001$; Table 1).

DISCUSSION

This study revealed clear phenotypic differences between predator and predator-free populations of *G. affinis*. Males, females, and juveniles exhibited a consistent pattern of mor-

phological divergence between predator regimes; a trend that persisted across years in wild-caught fish, and was retained in offspring raised in a common laboratory environment. Furthermore, differences in body shape and burst-swimming speed largely met a priori predictions, suggesting natural selection pressures associated with piscivorous fish were largely responsible for observed patterns.

Biomechanical Hypotheses and Observations

A powerful approach to the investigation of adaptive diversification is to generate hypotheses based on first principles and test them using comparative data (e.g. Wainwright 1988, 1996; Losos 1990b; Williams 1992; Walker 1997; Domenici 2003). We employed biomechanical models of fish swimming and ecological theory of predator-prey interactions
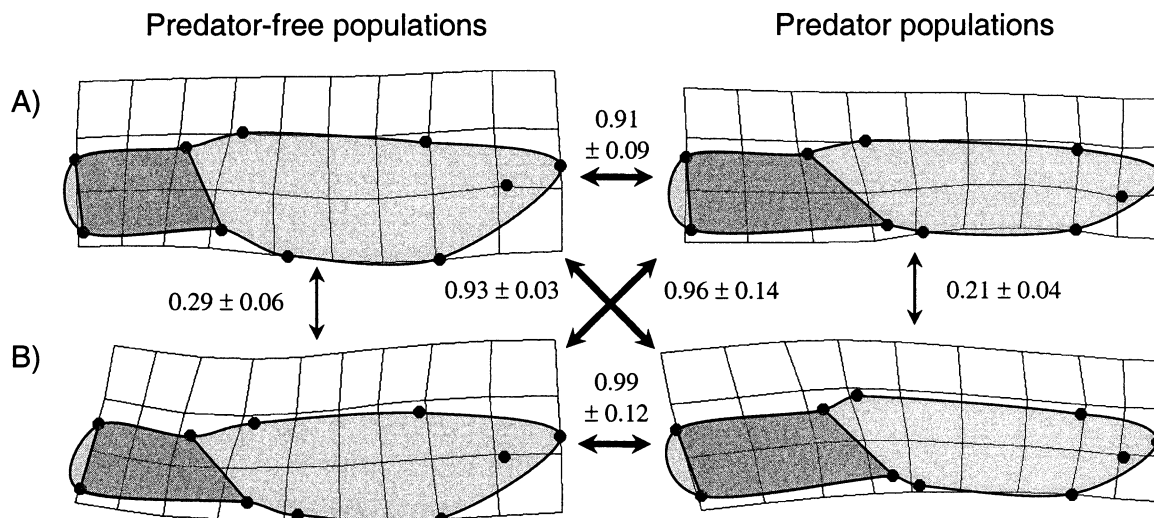
FIG. 5. Visualization of morphological divergence between predator regimes for (A) wild-caught and (B) common-garden *Gambusia affinis* juveniles. Thin-plate spline transformations (magnified 2×) depict morphological variation described by the predator regime factor of each MANCOVA. Arrows indicate the average Euclidean distance (± standard error) between population mean shapes along the predator regime canonical axis from a MANCOVA using pooled juvenile data.

to formulate simple predictions involving body form and locomotor performance in a prey species inhabiting environments that varied in level of predation threat. We hypothesized that predation from piscivorous fish would generate selection favoring phenotypes associated with enhanced escape ability in *G. affinis*. All three of our primary predictions were supported by the results: fish from predator populations exhibited (1) a larger caudal region, (2) a shallower anterior body/head region, and (3) faster burst-swimming speeds compared to fish from predator-free populations. Further, results supported the biomechanical prediction of a causal link between morphology and fast-start speed: divergence in body shape was deterministically related to burst-swimming speed in the manner predicted. This strong correspondence between predictions and observations suggests that observed phenotypic differences represent adaptive responses to divergent selection between populations with and without piscivorous fish.

Certain aspects of morphological differences observed between predator regimes were not predicted. These unpredicted characteristics might still reflect adaptive responses to alternative selective regimes, although they more likely involve performance features other than burst speed. For instance, fish from predator populations consistently exhibited a relatively posterior, ventral position of the eye compared to fish from predator-free populations. Location of the eye is unlikely to influence burst-swimming performance, however it might affect predator detection (e.g., fish predators typically attack *G. affinis* from below). Eye position might also influence foraging capabilities (e.g. prey detection/perception) or other visually coordinated activities. Surprisingly, this unpredicted pattern of divergence has recently been discovered in other fishes experiencing divergent predator regimes (i.e., the poeciliids *Brachyrhaphis rhabdophora* in Costa Rica and *Poecilia reticulata* in Trinidad [Langerhans and DeWitt 2004], and the cyprinid *Rastrineobola argentea* in East Africa [R. B. Langerhans, T. J. DeWitt, and L. E. Chap-

man, unpubl. data]). Divergence in eye position may reflect divergent selection associated with visual performance between predatory environments (e.g., a trade-off between predator detection and prey detection), and represents an interesting area for future research.

Burst-Swimming Performance

Although we focused on body shape as the proximate mechanism underlying locomotor performance, enhanced burst speeds in fish from predator populations might have resulted from a combination of factors, in addition to body morphology (i.e., the ''design box'' sensu Domenici 2003). For example, *G. affinis* individuals coexisting with piscivorous fish might perform c-start escape responses more often than fish in predator-free populations. This difference in frequency of muscle use might partially explain differences in burst-swimming speed. Additionally, ratio of muscle types might differ between predator regimes (due to either genetic differences or phenotypic plasticity), contributing to locomotor performance differences. This is because white muscle primarily powers burst swimming, whereas red muscle primarily powers sustained swimming (Mosse and Hudson 1977; Jayne and Lauder 1994).

Despite the potential role of muscle use and type in burst-swimming performance of *G. affinis*, we found that body shape was deterministically related to locomotor performance. Within populations, relatively predator-shaped individuals tended to exhibit faster burst-swimming speeds than relatively predator-free-shaped individuals. This suggested that body shape was largely responsible for differences in burst speeds between predator regimes. This was further supported by the finding that body shape was similar in explanatory ability to the categorical variable ''predator regime of origin.'' Thus, differences in burst speed between predator regimes can largely be explained—at least in the statistical sense—by differences in body shape.

Genetic Basis of Morphological Divergence

Morphological differences observed in wild-caught fish from different predatory environments could have been caused by genetic differences between populations, phenotypic plasticity, or a combination of both. We found that divergence in body shape observed in wild-caught fish was retained in offspring raised in a common-garden experiment. These results suggest that either genetic differentiation between populations or maternal special environmental effects that differ between predator regimes underlie phenotypic differences observed in common-garden fish (e.g. Falconer and Mackay 1996; Rossiter 1996; Mousseau and Fox 1998; Wolf et al. 1998; Agrawal et al. 1999). We specifically attempted to reduce maternal special environmental effects by holding females under common conditions for approximately one month before they delivered offspring; however, it is possible for such effects to have persisted. A second generation of fish reared in multiple environments (with and without predatory fish) might help resolve the relative magnitudes of genetic differentiation and phenotypic plasticity (including maternal special environmental effects) in producing phenotypic divergence between predator regimes in this system. Any of these sources of phenotypic variation can produce adaptation to divergent selective regimes between predator and predator-free populations in *G. affinis* (e.g., Rice and Hostert 1993; Robinson and Wilson 1994; Mousseau and Fox 1998; Orr and Smith 1998; Losos et al. 2000; Schluter 2000a; Agrawal 2001; Pigliucci and Murren 2003).

A Potential Trade-Off

The relationship between locomotor performance and fitness in the presence of piscivorous fish seems straightforward: increased burst speed should increase survival of predatory encounters (e.g. Howland 1974; Webb 1982b, 1986a; Weihs and Webb 1983). However, the nature of the burst speed-fitness relationship in the absence of predators is unclear. If a body shape maximizing burst-swimming performance is cost-free, then we might expect to observe this morphology in all populations. But since we observed a consistent pattern of morphological divergence between predator regimes, a trade-off likely exists involving body shape and fitness across environments. That is, a morphology that increases fitness in the presence of predators might necessarily decrease fitness in the absence of predators, and vice versa.

This trade-off might result from a particular morphology-performance trade-off predicted from biomechanical models of fish swimming: the optimal morphology for burst swimming is the opposite of that for prolonged swimming (e.g. Webb 1982a, 1984, 1986b; Videler 1993; Vogel 1994; Walker 1997). Empirical support also exists for this burst speed-endurance trade-off (Dohm et al. 1996; Reidy et al. 2000; Vanhooydonck et al. 2001; Wilson et al. 2002; Domenici 2003). Burst swimming is important for surviving predator strikes, whereas prolonged swimming is important for other activities, such as foraging and mate acquisition (Plaut 2001; Domenici 2003). Thus, a trade-off between burst-swimming speed and prolonged swimming might result in morphologies reflecting a balance between escape ability and competitive

ability, explaining the observed morphological divergence between predator regimes in *G. affinis*.

The fitness trade-off between predatory environments might also involve indirect effects of predators. Piscivorous fish largely restrict mosquitofish to shallow, vegetated areas, whereas the open-water habitat is used more frequently in predator-free environments (Winkelman and Aho 1993; R. B. Langerhans, C. A. Layman, A. M. Shokrollahi, and T. J. DeWitt, pers. obs.). Fish foraging in open-water environments are predicted to maximize prolonged-swimming ability relative to fish foraging in more complex habitats (Domenici 2003). Intraspecific morphological divergence between littoral and open-water habitats (e.g., benthic vs. limnetic) is known for many fish species (reviewed in Bell and Foster 1994; Robinson and Wilson 1994; Schluter 1996; McKinnon and Rundle 2002). Thus, both lethal and nonlethal (i.e., behavioral) effects of predators might contribute to a trade-off in *G. affinis*, resulting in different phenotypic optima between alternative predator regimes. The realization that body form is likely shaped by a suite of factors (e.g. escape ability, foraging efficiency) underscores the complexity of selection in nature. Further research explicitly examining the link between morphology, performance, and fitness in alternative environments will be critical in evaluating the importance of predators in shaping the morphology of *G. affinis*.

A New Ecomorphological Paradigm?

Many studies have demonstrated intraspecific differences in morphology of fishes across alternative habitats (reviewed in Echelle and Kornfield 1984; Robinson and Wilson 1994; Wimberger 1994; Schluter 1996; Smith and Skúlason 1996; Taylor 1999; Jonsson and Jonsson 2001). These studies have focused on variation in food resources and abiotic factors (e.g., water velocity, dissolved oxygen) among habitats. However, predation varies across space and time, and is presumably a key source of phenotypic diversity in many taxa (e.g. Havel 1987; Vermeij 1987; Endler 1995; Reznick 1996; Tollrian and Harvell 1999). This study supports recent work suggesting that predation plays a major role in the morphological diversification of fish (Brönmark and Miner 1992; Reimchen 1994; Walker 1997; Bergstrom 2002; Milano et al. 2002; Langerhans and DeWitt 2004).

In this paper, we outlined a general ecomorphological prediction based on biomechanical principles: fish coexisting with piscivorous fish should evolve a larger caudal region and a shallower anterior body/head region in order to increase burst-swimming speed. We found strong empirical support for these predictions. To our knowledge, this is the first study to describe and test this ecomorphological model.

This general model should apply not only to *G. affinis*, but to many fish species that employ burst swimming as an antipredator behavior. Strong support for the proposed model comes from recent work on two livebearing fish species, *B. rhabdophora* and *P. reticulata*. Both species exhibit the predicted pattern of morphological divergence between predator regimes (Langerhans and DeWitt 2004). Further, the proposed model is upheld by recent investigations of body shape variation in threespine stickleback (*Gasterosteus aculeatus*) of the Cook Inlet region of Alaska (Walker 1997; Walker

and Bell 2000). Additionally, temporal comparisons of a minnow, *R. argentea*, collected before and after the introduction of Nile perch (*Lates niloticus*, a predatory fish) into Lake Victoria revealed the predicted shift in morphology (R. B. Langerhans, T. J. DeWitt, and L. E. Chapman, unpubl. data). In each of these species, a posterior shift in body allocation is apparent in the presence of predatory fish. This convergent pattern of morphological divergence between predatory environments in distantly related species (i.e., three different orders) is remarkable, especially considering these species inhabit different geographic regions (e.g. Texas, Caribbean, Alaska, East Africa), different habitat types (e.g. ponds, streams, lakes), and environments with different predator species (e.g. sunfish, cichlids, trout, snook). This convergence strongly argues that natural selection pressures associated with piscivorous fish are responsible for observed patterns of morphological divergence.

The proposed ecomorphological model may also represent a broader paradigm useful for directing future research of morphological diversification in aquatic prey species. This paradigm should apply to many diverse aquatic organisms that use rapid locomotor responses when avoiding predation. For example, this ecomorphological model (i.e., larger caudal region, shallower anterior body/head in presence of predators) seemingly holds for many larval frogs (e.g. Lardner 2000; Relyea 2001; Van Buskirk 2002). Previous experiments have demonstrated that tadpoles exhibiting the predator-associated morphology experience enhanced survival of predatory encounters (e.g. McCollum and Van Buskirk 1996; Van Buskirk et al. 1997; Van Buskirk and Relyea 1998; Van Buskirk and McCollum 1999), and recent work has confirmed the predicted relationship between morphology and burst-swimming performance in two tadpole species (G. H. Dayton, D. Saenz, K. A. Baum, R. B. Langerhans, and T. J. DeWitt, unpubl. ms.). The utility of this paradigm in revealing convergent patterns among distantly related organisms (e.g. live-bearing fish, stickleback, tadpoles) suggests it is of broad applicability, and may provide substantial insight into the role of predation in the evolution of body form.

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