Complex phenotype–environment associations revealed in an East African cyprinid

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Abstract

Environmental factors influence phenotypes directly, as well as indirectly via trait correlations and interactions with other environmental variables. Using nine populations of the African cyprinid *Barbus neumayeri*, we employed path analysis to examine direct, indirect and total effects of two environmental variables, water flow (WF) and dissolved oxygen (DO), on several morphological traits. WF and DO directly influenced relative gill size, body shape and caudal fin shape in manners consistent with *a priori* predictions. Indirect effects also played an important role in the system: (1) strong, oppositely signed direct and indirect effects of WF on body shape resulted in a nonsignificant total effect; (2) DO had no direct effect on body shape, but a strong total effect via indirect effects on gill size; (3) WF indirectly influenced gill size via effects on DO. Only through examination of multiple environmental parameters and multiple traits can we hope to understand complex relationships between environment and phenotype.

Introduction

In natural systems, organisms face a multitude of ecological challenges and often respond with phenotypic shifts. Because organisms experience multiple selective and/or inducing agents, observed phenotypes generally reflect the influence of multiple environmental variables, in addition to other evolutionary factors (e.g. gene flow, genetic drift, genetic/developmental constraints) (e.g. Felsenstein, 1976; Lowell, 1987; Slatkin, 1987; Robinson & Wilson, 1994; Schluter, 2000; DeWitt & Langerhans, 2003; Ackermann & Cheverud, 2004). Environmental influences on traits can arise via genetically based responses to selection – including both environment-contingent phenotype production (i.e. phenotypic plasticity) and environment-independent phenotype production – as well as potentially nonadaptive effects of environment on phenotype (e.g. Levins, 1968; Schluter, 2000; Pigliucci, 2001; Pigliucci & Murren, 2003; West-Eberhard, 2003; DeWitt & Scheiner, 2004).

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Regardless of the adaptive nature or the form of genetic basis of phenotypic variation, environmental effects on phenotypes can be complex because of the interplay of direct and indirect effects on traits. That is, environmental factors can directly influence phenotypic values, and also indirectly influence phenotypes through trait correlations and interactions with other environmental factors (e.g. Gould & Lewontin, 1979; Lande & Arnold, 1983; Koehl, 1996; DeWitt & Langerhans, 2003; Pigliucci & Preston, 2004; Marks *et al.*, 2005). For example, one environmental factor might directly increase the value of a particular trait, but indirectly decrease the value of that trait via effects on other environmental factors or correlated traits. This scenario might produce a situation where no relationship is observed between phenotype and environment (if examined in isolation of other phenotypes and environmental factors), when the true relationship is quite strong, albeit complex.

Ecologists and evolutionary biologists seek to understand relationships between environment and phenotype; but we are challenged by the complex networks of direct and indirect effects on traits. One useful approach to address this complexity is to apply a methodology that assesses both direct and indirect effects of multiple environmental factors on multiple phenotypes (DeWitt

& Langerhans, 2003; Schaack & Chapman, 2003; Caumul & Polly, 2005; Hoverman *et al*., 2005). This assessment will be stronger if made in relation to an existing *a priori* understanding of how environmental factors and phenotypes are predicted to affect one another. Such an analysis should reveal how environment and phenotype are associated, even if this relationship is complex.

We examined effects of two abiotic environmental factors, dissolved oxygen (DO) concentration and water flow (WF), on several morphological traits of a cyprinid fish. We selected DO because it is a strong predictor of morphological variation in fishes, as well as other aquatic organisms, such as amphibians (Bond, 1960; Burggren & Mwalukoma, 1983) and arthropods (Jacobsen, 2000; Roast & Jones, 2003). In earlier studies, we have demonstrated population differentiation and/or phenotypic plasticity in respiratory traits for a suite of fish species that occur across DO gradients (e.g. Chapman & Liem, 1995; Chapman *et al*., 1999, 2000, 2002; Timmerman & Chapman, 2004). WF is generally believed to be a critically important factor in the evolution of morphology (including evolution of morphological plasticity) in fishes (e.g. Hubbs, 1941; McLaughlin & Grant, 1994; Hendry *et al*., 2000; Pakkasmaa & Piironen, 2000; Brinsmead & Fox, 2002; Imre *et al*., 2002; Langerhans *et al*., 2003; McGuigan *et al*., 2003; Collyer *et al*., 2005; Hendry *et al*., 2006; Sidlauskas *et al*., 2006), as well as other aquatic taxa (e.g. Denny, 1988, 1994; Johnson & Koehl, 1994; Gaylord *et al*., 2001; Carrington, 2002). Thus, these two abiotic variables may serve as major selective or inducing agents in many aquatic organisms.

Both environmental gradients (DO and WF) are predicted to influence morphological traits in particular manners. Using a comparative analysis of nine populations of the African cyprinid *Barbus neumayeri*, we tested *a priori* hypotheses regarding direct effects of each factor on particular phenotypes and direct effects of phenotypes on other phenotypes (Table 1). We developed a set of hypothesized direct effects (see details below), and used

these predictions to build a path model. The path model was employed to understand the direct, indirect and total effects of environmental factors on organismal traits. We did not offer predictions regarding indirect or total effects, but rather these effects are designed to be revealed by a path analysis.

We proposed specific predictions regarding the nature of direct effects for five of seven hypothesized direct effects (Table 1). These predictions were made based on physical, physiological, biomechanical and architectonical knowledge of the system (see references in Table 1). For instance, physical interaction of atmospheric oxygen with turbulent surface waters causes rapid dissolution of oxygen into water (where oxygen concentration is much lower than air), and thus generally leads to a positive association between WF and DO. Each prediction concerning direct effects of environment on phenotype reflects an adaptive hypothesis (via either plasticity and/or fixed responses). For example, larger gills can extract oxygen more efficiently (but may be costly to produce), and thus selection should favour larger gills in hypoxic waters and relatively small gills in well-oxygenated waters. Further, to reduce the energetic expenditure necessary to maintain position in high-flowing water, fish morphologies should minimize drag and maximize thrust for steady swimming; this is accomplished with a large anterior body depth and narrow caudal peduncle (i.e. fusiform shape), and a large, high aspect ratio caudal fin (morphologies that compromise other aspects of swimming that employ the same propulsors, such as fast starts). In addition, we hypothesized that DO might directly affect overall body shape in an unknown manner, that larger gills would result in fish with larger head regions, and that body shape might influence caudal fin form in an unknown manner. This latter prediction describes the hypothesis that caudal fin form may partially derive from changes in body morphology (e.g. caudal peduncle depth could necessarily increase caudal fin height). However, alternative hypotheses exist

Table 1 Defined *a priori* direct effects, predictions and references supporting predictions.

Direct effect	Predicted nature of effect	Reference
Water flow → dissolved oxygen	Higher WF → higher DO	1–3
Water flow → body shape	Higher WF → more fusiform body shape	4–11
Water flow → caudal fin form	Higher WF → larger, higher aspect ratio caudal fin	4–11
Dissolved oxygen → gill size	Lower DO → larger gills	12–19
Dissolved oxygen → body shape	Unknown	
Gill size → body shape	Larger gills → larger head region	15,17,20–21
Body shape ← ? → caudal fin form	Unknown	

These hypothesized direct effects were used to construct a path model (see Fig. 5).
References: 1 = Lampert *et al*., 1997, 2 = Cushing & Allan, 2001, 3 = Allan, 2004, 4 = Keast & Webb, 1966, 5 = Lighthill, 1975, 6 = Blake, 1983, 7 = Webb, 1984, 8 = Videler, 1993, 9 = Vogel, 1994, 10 = Boily & Magnan, 2002, 11 = Blake, 2004, 12 = Galis & Barel, 1980, 13 = Chapman & Liem, 1995, 14 = Chapman *et al*., 1999, 15 = Chapman *et al*., 2000, 16 = Chapman & Hulen, 2001, 17 = Schaack & Chapman, 2003, 18 = Timmerman & Chapman, 2004, 19 = Brauner & Val, 2005, 20 = Smits *et al*., 1996, 21 = Bouton *et al*., 2002.

regarding the relationship between body shape and caudal fin form, and thus we evaluate alternative hypotheses using a model selection approach.

Methods

Study system

Barbus neumayeri is widely distributed in East Africa (Greenwood, 1962, 1966), and is found in a variety of habitats within Kibale National Park, Uganda (Olowo & Chapman, 1996). This cyprinid reaches a maximum total length of approximately 11 cm within the park and feeds principally on small insect larvae, aquatic plants and detritus (Corbet, 1961; Schaack & Chapman, 2004). We sampled *B. neumayeri* populations across a gradient of DO and WF within or near Kibale National Park (0°13′– 0°41′N, 30°19′–30°32′E). Approximately 60% of the 795-km^2 park is characterized by moist evergreen forest, and the remainder is comprised of a mosaic of wetland, grassland, pine plantation, thicket and colonizing forest (Butynski, 1990). The forest is drained by two major everflowing rivers, Dura and Mpanga; both are tributaries of Lake George and are fed by numerous small forest streams. Extensive valley swamps dominated by papyrus (*Cyperus papyrus*) characterize both systems. *Barbus neumayeri* inhabits all of these types of aquatic environments (i.e. rivers, streams and swamps; Chapman *et al.*, 1999).

Collection sites

Nine collection sites were selected for the survey of *B. neumayeri* to maximize the range of variation in DO and WF, while simultaneously: (1) reducing the strong positive correlation between these two variables to as great an extent as possible (i.e. selecting sites with relatively high WF and low DO and vice versa) and (2) minimizing any potentially confounding environmental parameters (e.g. selecting sites similar in abiotic parameters other than DO and WF). Selected sites included swamps, streams and rivers (Fig. 1), and spanned a broad range of DO and WF (Table 2; see description below). Although we could not eliminate the positive correlation between DO and WF, we were able to minimize the correlation through careful selection of study sites, thus keeping multicollinearity relatively low in our analyses (see Results). We were quite effective in avoiding major confounding variables, as no measured environmental parameter (water temperature, depth and pH measured for all nine sites; conductivity for eight sites; transparency for six sites) was significantly correlated with DO, and only one parameter (transparency) exhibited a significant relationship with WF ($P = 0.037$).

Our two swamp sites were ≈500 m apart within the Rwembaita Swamp, one of the largest swamps in Kibale National Park (6.5 km long). In the swamp interior, minimal exposure to incident light (caused by dense papyrus averaging 5 m in height), reduced mixing and high rates of organic decomposition produce hypoxic conditions (Chapman *et al.*, 1998, 1999, 2000). At the eastern edge of the swamp, water exits the wetland through an open-water stream (Rwembaita/Njuguta outflow stream), picking up oxygen as it flows through the forest, until it meets the well-oxygenated waters of the Njuguta River. Fish were collected in both the outflow stream and the river. We collected from three stream sites (1–2 m in width) situated in secondary forest: Mikana Stream, Inlet Stream East and Inlet Stream West. We also sampled both major everflowing rivers in this region: a fast-flowing well-oxygenated section of the Dura River running through primary forest, and a section of the Mpanga River running through a landscape of *Acacia* woodland and agricultural plots downstream of a large papyrus swamp producing moderate levels of hypoxia.

Prior molecular analyses have demonstrated significant genetic differentiation among five *B. neumayeri* populations within Mpanga River drainage, and genetic distances among sites are not associated with geographic distances (Chapman *et al.*, 1999). Thus, we treated populations as statistically independent in analyses described below.

Environmental sampling

Dissolved oxygen and WF was measured bi-monthly over a 2-year period (April 2001 to May 2003) at a series of representative habitats within all sites with the exception of Dura River and Mpanga River. We measured DO and WF monthly for 2 years at Dura River (April 1996 to May 1998) and monthly for 1 year at Mpanga River (July 1995 to June 1996). DO was measured using a YSI meter (Model 51B or Model 95) in the upper 20 cm of the water column. WF of each site was ranked on a scale of 0–3 (0 = no flow, 1 = low flow, 2 = fast flow and 3 = very fast flow; ranked using increments of 0.5 units). In the closed cover of the forest and papyrus swamp habitats, diel variation in DO and water temperature tends to be very low (Chapman & Liem, 1995). Therefore, measurements were taken once per sampling day in the mid-morning to mid-afternoon period.

Morphometrics

In May–June 2003, fish were collected with minnow traps, killed in buffered MS222 (tricane methane sulphonate), weighed to the nearest 0.1 g, and placed on a laminated grid mounted on a level structure. We captured a lateral image of the right-hand side of each fish (resolution of 0.05 mm/pixel) using a digital camera (Nikon Coolpix 4500) mounted on a tripod. Fish were then preserved in paraformaldehyde (35 g l^{-1}). We measured body morphology and caudal fin form using
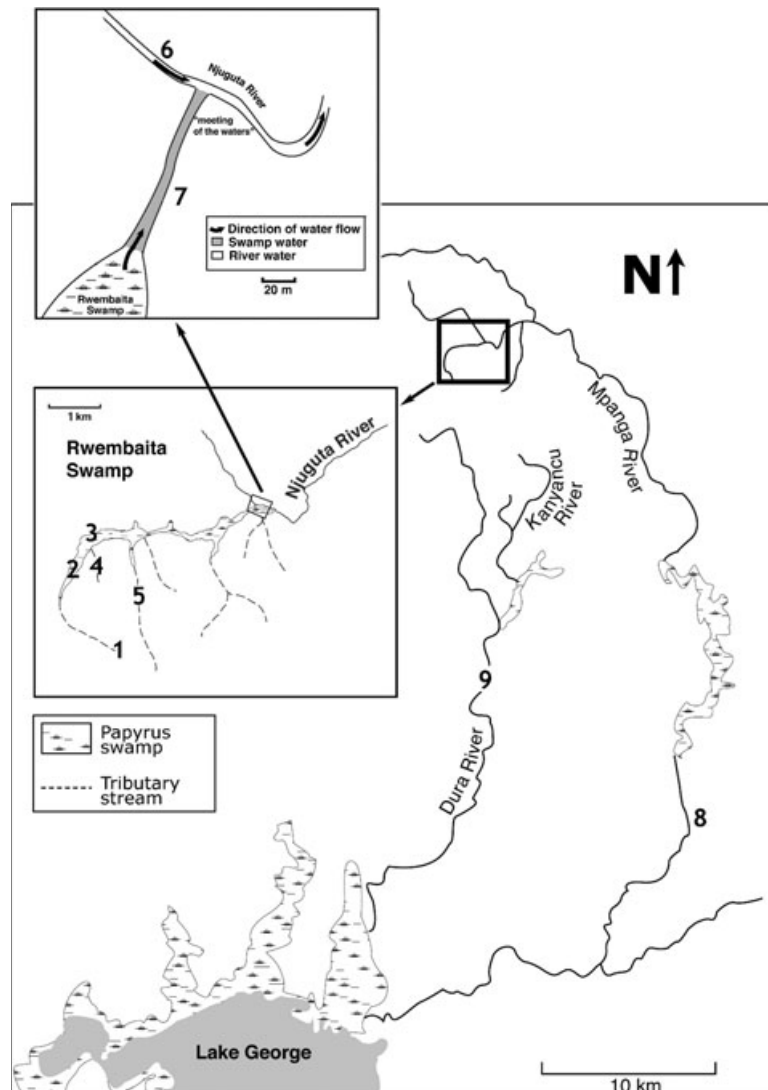
Fig. 1 Map of the Kibale National Park region of Uganda, indicating the two major drainage systems (Dura River, Mpanga River), the Rwembaita Swamp system, and the nine sites where *Barbus neumayeri* were sampled. 1 = Mikana Stream, 2 = Rwembaita Swamp-up, 3 = Rwembaita Swamp-mid, 4 = Inlet Stream West, 5 = Inlet Stream East, 6 = Njuguta River, 7 = Rwembaita/Njuguta outflow stream, 8 = Mpanga River, 9 = Dura River.

the digital images, and measured gill structure using preserved specimens. As sexual dimorphism is not reported in this species (Greenwood, 1962, 1966), we pooled sexes for analyses.

For the analysis of body morphology, we digitized 11 landmarks (Fig. 2) on each image using the software program tpsDig (Rohlf, 2004a). We conducted geometric morphometric analyses (Rohlf & Marcus, 1993; Adams *et al.*, 2004; Zelditch *et al.*, 2004) using our digitized landmarks. We used tpsRegr software (Rohlf, 2004b) to obtain shape variables (uniform components and partial warps) for analyses described below. TpsRegr rotates, translates and scales landmark coordinates into align-

ment via generalized Procrustes analysis (Bookstein, 1991; Marcus *et al.*, 1996). Superimposed landmark configurations were used to calculate affine and nonaffine shape components using tpsRegr. We visualized variation in landmark positions using the thin-plate spline approach, which maps deformations in shape from one object to another (Bookstein, 1991).

We measured caudal fin form using two calculations: caudal fin length (CFL) and caudal fin aspect ratio (CFAR). CFL was measured as the distance between the midpoint of a line connecting landmarks 5 and 6 and the midpoint of a line connecting the dorsoposterior and ventroposterior tips of the caudal fin (Fig. 2). Caudal fin

Table 2 Dissolved oxygen concentration, water flow (ranked between 0 and 3; see text), sample sizes and standard length (SL) of *Barbus neumayeri* used in analyses for each of the nine collection sites (two swamp, four stream and three river sites).

Site	Dissolved oxygen (mg l⁻¹)	Water flow (rank)	Sample size		SL, x̄ (min–max)
			Body/tail	Gills	
Rwembaita Swamp-mid (3)	1.35 ± 0.18	0.32 ± 0.03	30	10	56.1 (33.2–76.9)
Rwembaita Swamp-up (2)	2.78 ± 0.20	0.46 ± 0.06	30	6	44.0 (33.0–66.0)
Mpanga River (8)	4.26 ± 0.42	0.83 ± 0.08	9	6	49.2 (37.9–62.4)
Rwembaita/Njuguta outflow stream (7)	4.31 ± 0.14	0.93 ± 0.07	30	7	52.3 (35.3–80.5)
Inlet Stream East (5)	5.15 ± 0.18	1.14 ± 0.05	30	6	48.7 (32.1–75.8)
Inlet Stream West (4)	5.58 ± 0.16	0.43 ± 0.03	30	6	49.0 (34.0–73.1)
Njuguta River (6)	6.55 ± 0.14	0.90 ± 0.04	30	6	55.6 (36.5–88.7)
Mikana Stream (1)	6.74 ± 0.12	0.97 ± 0.04	30	10	52.9 (31.1–77.5)
Dura River (9)	7.32 ± 0.12	1.28 ± 0.06	30	6	59.7 (38.8–93.4)

Numbers in parentheses beside each site name refer to site labels in Fig. 1. Environmental values represent averages of monthly (Dura River and Mpanga River) or bimonthly (all other sites) samples (mean ± 1 SE).
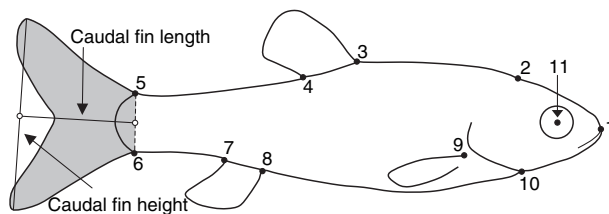

Fig. 2 Landmarks and measurements used for analysis of body morphology and caudal fin form of *Barbus neumayeri*. Open symbols represent midpoints used in the measurement of caudal fin length. The shaded region depicts the caudal fin surface area used in the calculation of caudal fin aspect ratio.

height was measured as the distance between the dorsoposterior and ventroposterior tips (Fig. 2). Caudal fin surface area was measured as the lateral area enclosed within the caudal fin margin and a line connecting landmarks 5 and 6 (shaded region in Fig. 2). CFAR was calculated as height²/surface area. All measurements were conducted in tpsDig.

We measured a series of gill features on the left branchial basket for a subset of preserved specimens, selected to span the range of observed body size within each population. We selected five gill measurements that represent the size of gills, as well as potentially reflect spatial constraints in the head region: total gill filament length (TGFL), total gill filament number (TGFN), total area of the hemibranchs (THA), total perimeter of the hemibranchs (THP) and average length of the hemibranch (AHL) (Fig. 3). We selected TGFL, because it is more likely to reflect spatial constraints of large gills on neighbouring structures than another commonly measured parameter, gill surface area, and because it has been well documented that *B. neumayeri* from hypoxic waters have a larger TGFL than fish from well-oxygenated sites (Chapman & Liem, 1995; Chapman *et al.*, 1999). Further, previous studies have found that for two fish species, populations with larger TGFL are characterized by a
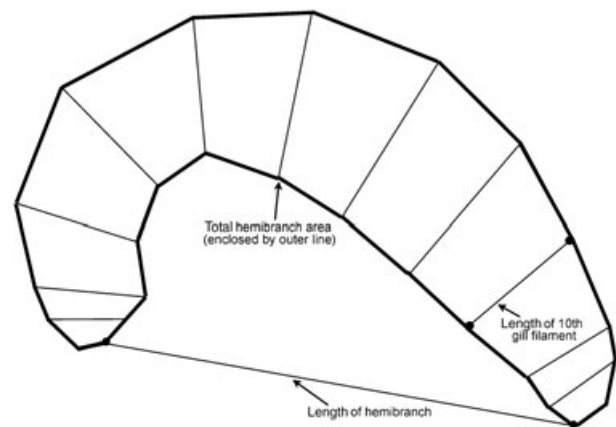

Fig. 3 Illustration of measurements used for analyses of gill size and shape of *Barbus neumayeri*. The length of filaments was used to estimate total gill filament length (TGFL); the length of the hemibranch was used to quantify average hemibranch length (AHL), and the total hemibranch area (THA) and perimeter (THP) were estimated from the outer line connecting the filament base and tips (bold line).

larger gill surface area (*Pseudocrenilabrus multicolor victoriae*, Chapman *et al.*, 2000; *Poecilia latipinna*, Timmerman & Chapman, 2004).

To quantify TGFL, each of the four gill arches from the left side of the branchial basket was separated and laid flat on a microscope slide. For each hemibranch, the length of every fifth gill filament was measured across the arch, with additional measurements at each end to increase resolution. Two successive measurements were averaged to estimate the length of intermediate filaments. Filament lengths were summed for the eight hemibranchs and multiplied by two to produce an estimate of TGFL. The total number of filaments was counted for each hemibranch on one side of the fish and multiplied by two to produce an estimate of the total

number of filaments on the branchial basket (TGFN). To estimate hemibranch areas, we digitized the area of the gill filaments on the eight hemibranchs on one side of the fish. This was multiplied by two to produce an estimate of the total hemibranch area (THA, not including the bony arches). Total hemibranch perimeter (THP) was estimated as the sum of the perimeters of the eight hemibranchs on one side of the fish (not including the bony arches) multiplied by two. Hemibranch length was estimated as the AHL from the bottom of the first filament to the last. This value was averaged over the eight hemibranchs on one side of the fish to produce an estimate of average hemibranch length (AHL).

Statistical analyses

As we wished to examine the influence of environmental factors on size-independent morphology, we first size-adjusted all morphometric data. For each type of morphometric data, we conducted a MANCOVA using population as the main effect and an estimate of body size as the covariate, and saved least-squares means of size-adjusted values for each population (the population term was highly significant in all cases, $P < 0.0001$). Caudal fin and gill measurements were log-transformed prior to analysis. The logarithm of centroid size (square root of the summed, squared distances of all landmarks from their centroid) was used as the covariate for body shape, log-standard length for caudal fin form and log body mass for relative gill size. In all MANCOVAS, we tested for heterogeneity of slopes and included the interaction term when significant. Slopes were largely homogeneous, having minimal influence on results even when significantly heterogeneous (for body shape and caudal fin form) as revealed by the high correlation among size-adjusted values derived with and without inclusion of the interaction term (mean $r = 0.97$, all $P < 0.0001$). To reduce dimensionality, we conducted a principal components analysis (PCA) of size-adjusted values for each type of morphometric data. For these analyses, we used the covariance matrix for variables measured on the same scale (body shape) and the correlation matrix for variables that differed in scale (caudal fin form and relative gill size).

We used path analysis (e.g. Wright, 1934; Mitchell, 1992; Shipley, 1997; Mitchell, 2001; Kline, 2005) to investigate direct and indirect effects of WF and DO on our three morphometric parameters (i.e. body shape, caudal fin form and relative gill size). We formulated a path model representing the *a priori* defined direct effects (Table 1), substituting principal component (PC) scores for each morphometric parameter. Because we had alternative hypotheses regarding the form of the relationship between body shape and caudal fin form, we evaluated separate path models using the Akaike information criterion (AIC; Akaike, 1992) to select the most appropriate model for the data. The best model had the lowest AIC_C value and differed from the next best model by at least two units (Burnham & Anderson, 2002). We modelled four alternative hypotheses: (1) body shape directly influences caudal fin form; (2) caudal fin form directly influences body shape; (3) body shape and caudal fin form reciprocally influence each other; and (4) residuals for body shape and caudal fin form are correlated.

The overall fit of our selected model was evaluated using the Bollen–Stine bootstrap test (Bollen & Stine, 1992), which tests the null hypothesis that the *a priori* designation of paths is correct. All path coefficients (i.e. partial regression coefficients) were estimated using 10 000 bootstraps of the data set. We assessed significance of direct effects (path coefficients), indirect effects (multiplication of path coefficients) and total effects (sum of direct and indirect effects) using a bootstrap approximation obtained by constructing two-sided bootstrapped confidence intervals. As results were only significant using the first PC for each morphometric parameter (compared with models substituting subsequent PCs), we hereafter refer only to this path analysis. Path analysis was conducted with Amos version 5.0.1 (Arbuckle & Wothke, 1999; Arbuckle, 2003).

Results

We retained the first PC for each set of morphometric variables (Table 3). Body shape variation described by the first PC is visualized in Fig. 4. AIC unambiguously selected the path model where body shape directly influences caudal fin form (ΔAIC = 4.6 for second best model). Using Akaike weights, the selected model was approximately 10 times more likely to be the best model for the observed data than the next best alternative model (Akaike weights of 0.81 vs. 0.08). There was no indication that the selected path model was inadequately structured (Bollen–Stine bootstrap $P = 0.4383$) and multicollinearity was reasonably low for those components of the analysis simultaneously considering multiple related factors (all variance inflation factors < 5.02). Results of the path analysis largely matched *a priori* predictions (Fig. 5), and most direct, indirect and total effects in the path analysis were significant (Table 4). We summarize the major findings of the path analysis below.

We found that higher WF resulted in an increased DO and was directly associated with deep-bodied fish having relatively deep heads and large, high aspect ratio caudal fins. Interestingly, indirect effects of WF on body shape, via indirect effects on relative gill size, opposed direct effects, resulting in a nonsignificant total effect of WF on body shape for the populations studied. DO was negatively associated with relative gill size. Although DO had no direct relationship with body shape, it indirectly influenced body shape via effects on gill size, resulting in a strong total effect. Other findings

Table 3 Pearson correlation coefficients between size-adjusted morphological variables and principal components.

Variable	Principal component 1
Relative gill size	56.90% of variance
log TGFN	0.36
log TGFL	0.94
log THA	0.96
log THP	0.94
log AHL	0.10
Caudal fin form	63.50% of variance
log CFL	0.80
log CFAR	0.80
Body shape	41.70% of variance
1x	0.63
1y	0.17
2x	−0.93
2y	0.84
3x	0.25
3y	0.70
4x	−0.02
4y	0.71
5x	0.29
5y	0.60
6x	0.80
6y	−0.41
7x	0.75
7y	−0.73
8x	−0.44
8y	−0.71
9x	−0.45
9y	−0.67
10x	−0.44
10y	−0.82
11x	0.87
11y	−0.10

Abbreviations follow the text and Fig. 3. Body shape variables reflect the x and y dimensions of each landmark depicted in Fig. 2 (positive values represent shifts right [x] or up [y] associated with positive principal component scores).

Fig. 5 Path analysis results. Numerical values indicate path coefficients, and line thickness reflects the strength of the path. Solid lines represent positive effects, and dashed lines represent negative effects.

included a direct effect of relative gill size on body shape (larger gills resulted in fish with relatively deeper bodies and particularly large heads) and a direct effect of body shape on caudal fin form (fish with relatively deeper bodies and particularly large heads also had large, high aspect ratio caudal fins). Because body shape directly influenced caudal fin form, there were also significant total effects of DO and relative gill size on caudal fin form via their effects on body shape.

Discussion

Environmental impacts on phenotypes can be complex. Our path analysis uncovered a complex set of direct and indirect effects of WF and DO on morphological characters in *B. neumayeri*. We found an array of significant direct, indirect and total effects of environment on phenotype, as well as effects of phenotypes on other phenotypes.

Results of the path analysis largely matched *a priori* predictions for all five direct effects for which we proposed specific hypotheses: (1) higher WF resulted in
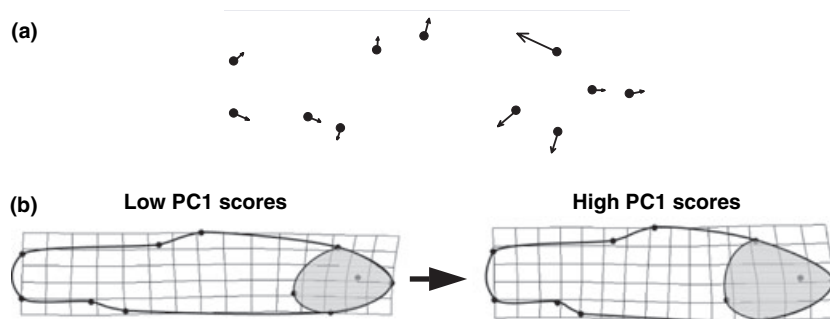

Fig. 4 Visualization of the first principal component for body shape. Populations varied significantly in morphology (MANCOVA, $P < 0.0001$; Procrustes distances ranged from 0.01 to 0.03). (A) Vectors describing the direction and relative magnitude of change in the location of each landmark (landmarks follow Fig. 2; arrows point toward positive PC1 values). (B) Thin-plate spline visualization illustrating the nature of body shape variation described by the first principal component for body shape (magnified 3× to more clearly illustrate variation; grid transformations are relative to the mean landmark configuration). The head region is shaded to emphasize differences in relative head size.
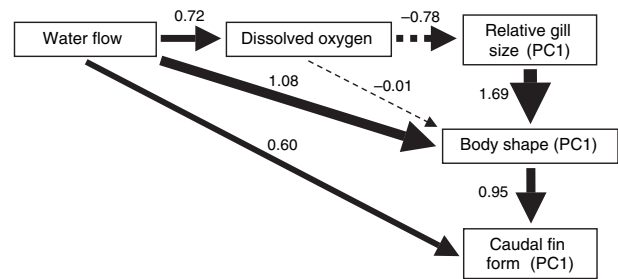
Table 4 Direct, indirect and total effects for the path analysis.

| Variable | Effect on | | | | | | | | | | | |
| | Dissolved oxygen | | | Relative gill size (PC1) | | | Body shape (PC1) | | | Caudal fin form (PC1) | | |
	DE	IE	TE	DE	IE	TE	DE	IE	TE	DE	IE	TE
Water flow	**0.72**	–	**0.72**	–	**−0.56**	**−0.56**	**1.08**	**−0.96**	0.12	**0.60**	0.11	**0.71**
Dissolved oxygen	–	–	–	**−0.78**	–	**−0.78**	−0.01	**−1.32**	**−1.33**	–	**−1.27**	**−1.27**
Relative Gill Size (PC1)	–	–	–	–	–	–	**1.69**	–	**1.69**	–	**1.61**	**1.61**
Body Shape (PC1)	–	–	–	–	–	–	–	–	–	**0.95**	–	**0.95**
r^2		0.54			0.69			0.73			0.86	

Direct effects are path coefficients as depicted in Fig. 5. DE = direct effect, IE = indirect effect, TE = total effect. Significant effects ($P < 0.05$) are in bold.

higher DO; (2) higher WF resulted in fish with a relatively more fusiform body shape (e.g. greater anterior depth); (3) higher WF was directly associated with larger, higher aspect ratio caudal fins; (4) populations experiencing lower DO exhibited relatively larger gills; and (5) populations with relatively large gills had relatively large heads. Such correspondence between observations and predictions suggests that our *a priori* understanding of the system's direct effects is well founded, and that phenotypic shifts likely reflect adaptive responses through either phenotypic plasticity and/or divergent genetically based traits. We also found that body shape was associated with caudal fin form. This association could reflect phenotypic integration of functionally related traits (e.g. body morphology might influence caudal fin form architectonically, or through effects on developmental pathways) (Olson & Miller, 1958; Pigliucci, 2003; Pigliucci & Preston, 2004). Further, we found no evidence for a direct effect of DO on body shape, although we suspected that such an effect might exist because of effects of oxygen on growth, development and gene expression (e.g. Stewart, 1967; Cech, 1984; Rombough, 1988; Secor & Gunderson, 1998; Gracey *et al.*, 2001; Kajimura *et al.*, 2006).

The use of path analysis provided important insights into the indirect and total effects observed among factors in the system. For example, had we simply examined the bivariate relationship between WF and body shape (i.e. ignored variation in DO and relative gill size), we would have detected a nonsignificant association in the opposite direction, leading to the false conclusion that WF was not strongly associated with body shape. This suggests that prior studies reporting a nonsignificant association, or an association in the opposite direction of that observed here, between WF and body shape in fishes might have actually obtained a spurious result because of indirect effects of WF on body shape. That is, WF can influence DO, and thus gill size, which can cause indirect effects on body shape that mask the direct effects. This is a genuine possibility in some cases; however, in the present study we specifically chose populations exhibiting marked variation in both WF and DO; many previous studies likely did not examine populations exhibiting such variation in DO – although, DO is rarely measured and reported in such studies. We suggest that future studies examining the relationship between WF and body shape take DO into consideration in light of the results observed in this study.

Another interesting finding was that caudal fin form reflected the influence of multiple environmental factors and multiple phenotypes, despite having only two direct effects connecting it to the rest of the system. WF, DO, relative gill size and body shape all had significant total effects on caudal fin form, illustrating how some phenotypes can be influenced by more factors (environmental and phenotypic) than others. Effects of DO and gill size on caudal fin form resulted via indirect effects mediated through body shape. If this apparent phenotypic integration between body shape and tail form is a general phenomenon in fishes, it suggests that caudal fins can be influenced by a large number of factors as body shape is known to be influenced by numerous selective/inducing agents and other phenotypic attributes (e.g. Wimberger, 1992; Schluter, 1993; Robinson & Wilson, 1994; Walker, 1997; Robinson *et al.*, 2000; Ruber & Adams, 2001; Langerhans *et al.*, 2003; Ghalambor *et al.*, 2004; Langerhans & DeWitt, 2004; Sidlauskas *et al.*, 2006). Such findings are only possible through an examination of multiple environmental factors and multiple phenotypes, coupled with path analysis to distinguish direct and indirect connections among factors in the system.

Integrative studies examining multiple phenotypes and multiple selective/inducing agents are required to gain a more complete understanding of how phenotype and environment are associated. We employed such an approach in this study, and revealed how two abiotic environmental variables directly and indirectly influence three types of morphological parameters in *B. neumayeri*. Through the examination of multiple environmental factors and multiple traits, we should acquire a better understanding of phenotype–environment relationships, even if complex.

Acknowledgments

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