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Phenotypic complexity: integrated responses of life-history characters to multiple environmental factors

Santiago Salinas[1] and Stephan B. Munch[2]

[1]*Center for Stock Assessment Research, University of California Santa Cruz, Santa Cruz, California, USA and* [2]*Southwest Fisheries Science Center, National Oceanic and Atmospheric Administration, Santa Cruz, California, USA*

ABSTRACT

Question: Most of our theoretical and empirical knowledge of phenotypic plasticity is limited to changes in single traits under variation of a single environmental variable. Are insights drawn from this 'univariate' world-view different than if we were to study individuals as the integration of many traits in response to many environmental variables?

Organism: Sheepshead minnows, *Cyprinodon variegatus*, from Gulf Islands National Seashore, Florida.

Methods: We reared individuals at different combinations of temperature and food availability (3×3 factorial design) over approximately 6 months. We measured growth, age and size at maturation, gonadosomatic index, hepatosomatic index, and body shape. We also estimated levels of phenotypic integration and relative fitness for males and females in each of the nine treatments.

Results/conclusions: Most traits responded to temperature and food directly and some exhibited interactions in their response. Phenotypic integration and fitness changed substantially under different environments, and differently for males versus females. Studying responses from this integrated perspective led to insights that could not have been obtained studying single traits or single environmental variables.

Keywords: phenotypic plasticity, phenotypic integration, reaction norm, growth rate, age at maturation, size at maturation.

INTRODUCTION

Phenotypic plasticity plays a key role in coping with environmental changes. Its relevance to ecology and evolutionary biology, therefore, cannot be underestimated (DeWitt and Scheiner, 2004). Theoretical and empirical work highlights the role of plasticity in population dynamics (Reed *et al.*, 2010), ecological processes (Miner *et al.*, 2005), and evolutionary trajectories (West-Eberhard,

Correspondence: S. Salinas, Department of Biological Sciences, University of the Pacific, 3601 Pacific Ave., Stockton, CA 95211, USA. e-mail: ssalinas@pacific.edu

2003; Schlichting, 2004). Although novel sets of tools for understanding multivariate plasticity are being developed (e.g. Robinson and Beckerman, 2013), most studies of plasticity focus on the change of *one* trait over various levels of *one* environmental variable (Pigliucci, 2001). Although convenient from an experimental and theoretical point of view, univariate reaction norms are likely to be incomplete descriptions of trait change.

An individual is the integration of a collection of traits. Much in the same way multivariate trait evolution changed how we conceptualize evolutionary theory (Lande and Arnold, 1983), thinking about plasticity at a multivariate, whole-organism scale could also lead to conceptual and practical advances. The phenotype responds to the environment as a conglomerate (Cheverud, 1982), and studying it thus has led to some interesting observations. Some plants, for example, exhibit different phenotypic correlations among traits ('phenotypic integration') depending on the environment (Lechowicz and Blais, 1988; Schlichting, 1989). This plasticity in phenotypic integration can significantly alter a population's response to selection and subsequent evolutionary trajectory (Schlichting, 1986; Price *et al.*, 2003). Phenotypic integration may also constrain phenotypic plasticity, as a given trait will be less variable the more integrated it is with other traits (in the same way that a person held by one arm can move around to some degree while another held by both arms has very limited motion) (Gianoli and Palacio-López, 2009).

An individual is the integration of a collection of traits in response to a collection of environmental variables. Environmental variables interact in non-linear ways in shaping phenotypes, making univariate reaction norms of limited utility (Kingsolver *et al.*, 2006). Studies of fishes that analysed thermal reaction norms of growth or metabolism, for example, have repeatedly shown that food mediates the response to temperature [growth-specific examples include: Brett *et al.* (1969), Wurtsbaugh and Cech (1983), Fonds *et al.* (1992), Reznick (1993), and Hutchings *et al.* (2007)]. Maturation decisions also depend on combinations of temperature and food availability. For instance, reducing either temperature or food tends to reduce growth, but a decrease in temperature results in delayed maturation at a *larger* size, while a decrease in food causes delayed maturation at a *smaller* size (Atkinson, 1994; Berrigan and Charnov, 1994; Thorpe, 2004). Thus, important life-history traits such as growth and maturation are determined by the interaction of environmental variables. There is, however, a paucity of studies on the responses of integrated suites of traits to simultaneous changes in multiple environmental variables. Furthermore, how the level of integration relates to fitness estimates has received relatively little empirical attention, despite its potential importance in dictating the direction of evolution (Pigliucci and Preston, 2004).

In this paper, we evaluate empirically how growth, age and size at maturation, gonadosomatic index, hepatosomatic index, and body shape were affected by temperature and food availability over a period of about 6 months in the sheepshead minnow, *Cyprinodon variegatus*. We then use these data to evaluate likely fitness differences among the treatments. Studying responses from this integrated perspective leads to insights that could not have been obtained studying single traits or single environmental variables.

METHODS

Study system

The sheepshead minnow, *Cyprinodon variegatus*, is a widely distributed estuarine fish found along the Atlantic and Gulf coasts of the United States, the Caribbean, and northern

Venezuela, inhabiting shallow water habitats that feature little current or wave action (Environmental Protection Agency, 2002). These environments experience rapid changes in salinity and temperature, and sheepshead minnows are adapted to tolerate these changes. They can withstand temperatures from −1.5 to 41.6°C (Bennett and Beitinger, 1997) and salinity fluctuations between 0.1 and 125 psu (Nordlie, 2006).

Experimental set-up

We collected wild adult sheepshead minnows from Gulf Islands National Seashore, Florida in August 2009, and transported them to our facility at Stony Brook University. These fish were bred in the lab and their offspring (F1) maintained at 21–22°C, 20 psu, and food *ad libitum*. To produce the experimental fish (F2), we acclimated size-matched F1 adults to one of three temperatures: 24°C, 29°C, or 34°C. After 7 days, we introduced spawning mats and collected eggs, which were hatched and reared at the temperature in which they were spawned. Larvae were fed freshly hatched brine shrimp nauplii twice a day until the experiment began. Due to low hatching success in the 24°C eggs, and to begin the experiment with similarly aged larvae, the 24°C parents were spawned again 2 weeks later under identical conditions to create a second batch of experimental fish.

We started the experiment with 23-day-old larvae (29°C and 34°C) and 39- and 25-day-old larvae (individuals in the first and second batch of 24°C larvae exhibited very similar growth trajectories; see Results). Two sea tables were used per temperature treatment, each controlled by digital thermostats. Daily care followed standard protocols (Cripe *et al.*, 2009; Salinas and Munch, 2012).

Offspring from each of the temperatures were transferred to individual containers and randomly assigned to one of the high, medium, or low food availability treatments, corresponding to 100%, 80%, and 60% of average maximum consumption (sample sizes: $n = 27$ for all temperatures at mid and high food rations, $n = 28$ for all temperatures at low rations). Containers consisted of cylindrical 140-mm diameter dishes, 2-mm mesh walls, and a mesh subdivision along the centre of the dish (each container housed two individuals separated by a mesh wall). Over the range of sizes in this experiment, average maximum consumption rate increased linearly with length as follows (Salinas, 2012):

$$\text{Max Consumption (mg/day)} = 0.6 \times \text{Total Length (mm)} + 7.6.$$

Based on this relationship, daily rations were determined using the observed length for each individual, updated weekly. All treatments were fed flake food four times a day throughout the experiment.

Since the goal of this study was to evaluate the combined influence of temperature and food availability on a suite of traits, we measured growth, body shape, age and size at maturation, gonadosomatic index (GSI, a proxy for reproductive investment), and hepatosomatic index (HSI, a proxy for fish condition) for each individual. Each week we digitally photographed all fish dorsally with a tripod-mounted 10.1-megapixel EOS 40D Canon camera with macro lens (fish remained in their containers, in ~10 mm of water, during the photographing so that there was no need for anaesthesia). The following day, we measured the photographs of the fish using ImagePro Plus (Media Cybernetics, Bethesda, MD). In addition to weekly length measurements, we conducted daily maturation checks, beginning at 45 days post-hatch. For males, we checked for signs of secondary sexual coloration (male sheepshead minnows turn iridescent blue on their dorsal side and orange on their ventral

side). Females, on the other hand, are more cryptic after becoming mature. Thus, we visually inspected females for signs of enlarged gonad cavity on a daily basis. If we suspected a female had matured, we gently compressed her abdomen and only recorded her as mature if eggs were released. To ensure that no females were missed using this procedure, we also checked for eggs on three different days (five females per day) when we did not suspect females had matured (but close to the expected maturation period). All these checks resulted in no eggs.

At the conclusion of the experiment (~5.5 months post-hatch), we sacrificed each individual with a lethal dose of tricaine methanesulfonate (MS-222) and immediately photographed the fish for geometric morphometric analysis. All 216 fish that survived to the end of the experiment were laterally photographed under identical lighting and exposure conditions with the same digital camera used for length measurements. After the fish were photographed, we weighed them and then removed and weighed both gonad and liver to calculate GSI and HSI. The GSI is a measure of energy devoted to reproduction (calculated as gonad mass/total body mass), while HSI is typically used as a condition index (liver mass/total body mass) (Helfman *et al.*, 1997). We obtained measurements of all variables of interest in 220 individuals (variable-specific sample sizes: $N_{\mathbf{maturation}} = 234$, $N_{\mathbf{GSI}} = 224$, $N_{\mathbf{HSI}} = 235$, $N_{\mathbf{morphometrics}} = 235$).

For the geometric morphometric analysis, we first digitized 12 landmarks for each individual (Fig. S6 inset, see evolutionary-ecology.com/data/2888Appendix.pdf) using tpsDIG2 (Rohlf, 2006). These landmarks represent standard characters used to compare shape variation in fishes (Cadrin, 2000). Then, we performed a full Procrustes fit and projection onto tangent space to obtain shape variables independent of size, translation, and rotation (Zelditch *et al.*, 2004). This and all subsequent morphometric analyses were performed in MorphoJ (Klingenberg, 2011). Outlier analysis revealed that two fish strongly deviated from the mean shape (one fish from mid temp/mid food, the other from low temp/high food), and were removed from all analyses.

Statistical analysis: univariate responses

Growth

We tested for variation in growth trajectories among treatment groups with MANOVA and visualized these differences with canonical variate analysis. We also tested for differences in juvenile growth rate (growth between days 23–59 post-hatch for the 29°C and 34°C treatments, and days 25–60 for the 24°C treatments) via a three-way ANOVA with temperature, food, sex, and all interaction terms (and 'table' as a random effect in this and subsequent analyses). In multi-trait analyses, we summarized each individual's growth trajectory using principal component analysis (instead of including 20 highly correlated weekly length measurements). The first two principal axes explained 91.4% of the variation in the collection of growth trajectories from the entire experiment and we used individual projections on these axes as indices of overall growth. Additionally, for fish of each sex, we estimated final length–final weight allometries ($\text{Weight} = b\ \text{Length}^c$) and compared the treatment-specific (temperature/food/sex) exponents via a two-way ANOVA with temperature, food, and interaction terms.

Age and size at maturation

We assessed the effects of food and temperature on age and size at maturation using two-way generalized linear mixed models [GLMM (Bolker *et al.*, 2009)] for each life-history character. To evaluate the combined effects of food and temperature on age at maturation, we used a GLMM with a negative binomial likelihood and log link function. This likelihood is appropriate if the age at maturation is the result of a sum of discrete waiting times (McCullagh and Nelder, 1989). Because of its robustness (Bolker *et al.*, 2009), we chose the Laplace method to approximate the likelihood. The model included temperature, food, sex, and all possible two- and three-way interactions as predictors. Because maturation is often treated as a state-dependent decision process (Van Dooren *et al.*, 2005), we also included growth (first and second growth trajectory principal components) as a random effect.

We also analysed maturation decisions under different treatments by comparing observed and predicted sizes at maturation. We obtained predicted maturation sizes by estimating the average growth trajectory for each treatment, and then using observed ages at maturation to get predicted maturation sizes (for a schematic of the approach, see 2888Appendix.pdf, Fig. S1). We then compared the ratio of observed variance to predicted variance to determine whether size or age at maturation is more variable (i.e. if the ratio < 1, size matters more in the maturation decision, and vice versa).

GSI and HSI

Similarly, the gonadosomatic and hepatosomatic indices were analysed via GLMMs (with arcsine-transformed data and a normal distribution and identity link). In addition, to evaluate whether variation in size at maturation is simply driven by differences in time to maturation among groups with different growth rates, we plotted juvenile growth rate (calculated linearly between the last measurement before maturation and the length at first measurement) against the slope of age versus size at maturation for each of the nine treatments.

Morphometrics

For shape variation, we analysed samples (Procrustes coordinates) via canonical variate analysis with food and temperature treatments as the grouping variables. In addition, we computed pairwise comparisons among all treatments based on Mahalanobis distances, a measure of the distance between centroids on a scale adjusted to the within-group variance in the direction of the group difference (Strauss, 2010). To summarize shape variation for inclusion in integration and multivariate analyses, we used the first two principal components of shape (a PCA of Procrustes coordinates is similar to relative warp analysis excluding bending energy weighting), which explained 52.5% of the variation.

Statistical analysis: multivariate responses

Since we are ultimately interested in how food and temperature interact to shape all of the life-history traits simultaneously, we tested for treatment effects and their interaction with MANOVA. To do this, we arcsine-transformed the GSI and HSI data. However, as the results of the phenotypic integration analyses make clear below, the fundamental assumption of constant covariance matrices within treatment groups is strongly suspect, so significance levels are only nominal.

Phenotypic integration

To assess the effects of food and temperature on phenotypic integration, we calculated correlation coefficients among traits for individuals within each of the nine food and temperature treatments. Specifically, we used age at maturation, size at maturation, final weight, arcsine-transformed GSI, arcsine-transformed HSI, the first and second principal components of growth, and the first and second principal components of shape. We added final weight to this analysis to reflect potential size differences not described in the growth principal components. We only included individuals with data for all nine traits ($n = 220$).

We used two indices to evaluate differences in phenotypic integration among treatments, based on the eigenvalues of the complete phenotype covariance matrix (i.e. the matrix composed of length at each time step, age at maturation, size at maturation, final weight, GSI, HSI, and the first and second principal components of shape). Variance in the eigenvalues

$$(\mathrm{Var} = \frac{\Sigma_i^N(\lambda_i - 1)^2}{N})$$

is typically used as an index of integration, with low variance corresponding to low integration (Pavlicev *et al.*, 2009). However, it is difficult to interpret *a priori* what constitutes low or high variance for a given set of traits. We therefore introduce the 'effective dimension' of the correlation matrix as an intuitive measure of integration with clearly interpretable limits. The effective dimension also uses the eigenvalues of the correlation matrix and is calculated as:

$$\mathrm{EffDim} = \frac{(\Sigma\lambda)^2}{\Sigma\lambda^2}.$$

This dimensionless index ranges between 1 (perfect integration) and the number of traits measured (complete lack of integration), providing an intuitive comparison of the degree of integration across treatments. Note that when based on correlation matrices, these two measures of integration are related by the identity $\mathrm{EffDim} = N/(\mathrm{Var} + 1)$.

Fitness

We constructed a simple model to estimate lifetime fitness for each individual aiming to assess the importance of trait integration and multivariate plasticity. We stress that our goal here was not to develop a comprehensive fitness model but to compare the importance of different traits and environments against a common currency. As our measure of fitness, we used lifetime reproductive output, $R_0 = \int_0^\infty \varphi(a) e^{-\int_0^a \mu(s)ds} da$, where $\varphi(a)$ and $m(a)$ are age-specific fecundity and mortality, respectively. In fishes, fecundity, $\varphi(a)$, is typically proportional to gonad mass (Eros, 2003). We used the observed length–weight allometry in each treatment (keeping the sexes separate) and individual estimates of GSI to convert individual length measurements into age-specific fecundity. Specifically, we used

$$\varphi_i(a) = GSI_i \cdot b\, l_i(a)^c, \tag{1}$$

where $l_i(a)$ is the length of individual i at age a, and b and c are the treatment-specific intercept and exponent of the final weight–final length relationship.

Based on reviews of size-dependent mortality in fishes (Pepin, 1991; Sogard, 1997), we modelled age-specific mortality, $\mu(a)$, as a function of length-at-age given by

$$\mu(a) = \mu_0 + \frac{\mu_1}{l(a)}. \quad (2)$$

Meredith and Lotrich (1979) report juvenile and adult mortality rates for cyprinodontids of 0.995 and 0.54 per year, respectively. We chose mortality parameters to match these observed mortality rates, by integrating (2) over the average growth trajectory of fish in the experiment before and after maturation. Doing so yields estimates of $\mu_0 = 0.001155$ per day and $\mu_1 = 0.014781$ mm/day. Over the duration of the experiment, observed sizes were linearly interpolated to obtain continuous estimates of length at age. To carry our fitness calculation out over the expected lifespan of each individual, length-at-age after the experimental period was estimated from individual-specific von Bertalanffy growth curves. We report the results of these calculations in terms of sex-specific relative fitness, which is obtained by dividing each individual's fitness estimate by the overall average fitness for their sex. To determine whether any single trait is an adequate proxy for fitness, we calculated the percent variance in fitness explained by each single trait. Two individuals (both females, one from 24°C-low food and one from 34°C-mid food) were identified as outliers and removed from these analyses.

RESULTS

We begin by summarizing the responses to food and temperature for each life-history character separately. We then describe the results of the multivariate analysis, the treatment effects on phenotypic integration, and fitness.

Univariate responses

Growth

Of the 246 fish at the start of the experiment, 235 (96%) survived to the last day. Growth trajectories were generally more variable at lower temperatures, with coefficients of variation of final length being approximately twice as large for fish at 24°C than those at 34°C (2888Appendix.pdf, Fig. S2, Table S1). Growth was fastest at 29°C, regardless of food treatment, and at high food, regardless of temperature (Fig. S3). Juvenile growth rate, defined as growth between days 23–59 post-hatch for the 29°C and 34°C treatments and 25–60 days for the 24°C treatments (a period during which growth was linear for all fish; Fig. S1), was sensitive to temperature, food availability, and their interaction (Fig. 1a, Table S2). The multivariate analysis of growth revealed significant temperature (Wilks' $\lambda = 0.203$, $P < 0.001$) and food (Wilks' $\lambda = 0.513$, $P < 0.001$) effects, but the interaction was not significant (Wilks' $\lambda = 0.649$, $P = 0.310$).

In addition, growth before and after maturation (comparing slopes of linear regressions before and after maturation) was uncorrelated (2888Appendix.pdf, Fig. S4). However, 24°C fish grew faster after maturation compared with individuals at 29°C and 34°C (Fig. S4, inset). The comparison of length–weight exponents showed no differences based on temperature, food, or their interaction for either sex (all $P > 0.05$).

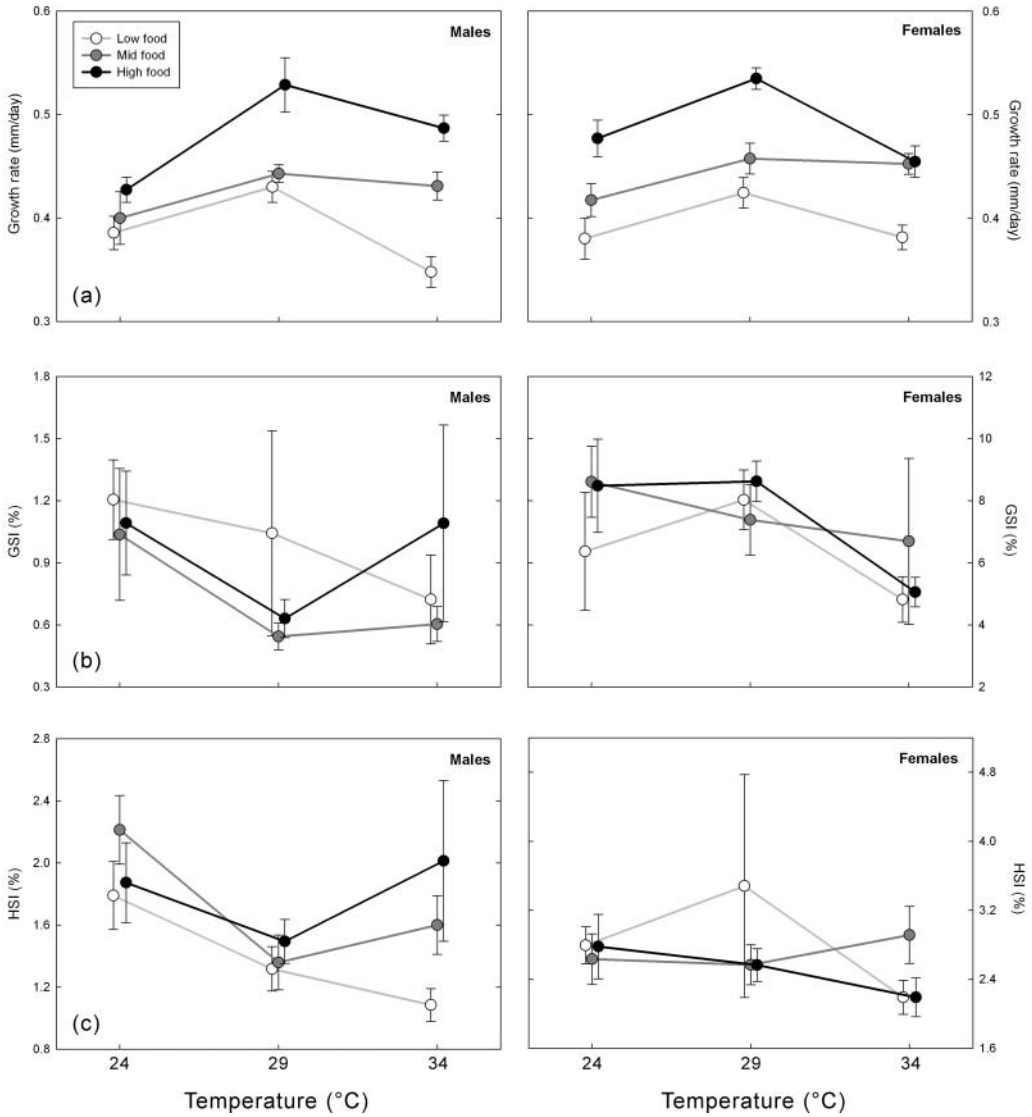

Fig. 1. Reaction norms for (a) juvenile growth rate, (b) GSI, and (c) HSI (±1 S.E.). Shading represents food treatment (white = low food, 60% of maximum consumption; grey = mid food, 80% of maximum consumption; black = high food, 100% of maximum consumption.

Age and size at maturation

Age at maturation was significantly affected by both temperature and food availability after conditioning on growth (2888Appendix.pdf, Fig. S5, Table S3). There was, however, no interaction between the two variables (Table S3). Fish matured earliest in the mid temperature/high food (mean = 68.3 days, S.D. = 10.0) and latest in the low temperature/low food treatment (mean = 89.4 days, S.D. = 23.0) respectively. Age at maturation, like juvenile growth rate, was much more variable at lower temperatures (Fig. S5).

Sheepshead minnows appear to change maturation decisions based on the temperature they experience. The ratio of observed to predicted variance in size at maturity, based on observed age at maturity and mean treatment-specific growth trajectory, was larger than 1 for all high temperature treatments (high temperature/low food: 2.41; high temperature/mid food: 1.82; high temperature/high food: 1.53). Conversely, all low temperature treatments showed ratios less than 1 (low temperature/low food: 0.67; low temperature/mid food: 0.88; low temperature/high food: 0.54), while the intermediate temperature treatments had ratios close to 1 (mid temperature/low food: 1.00; mid temperature/mid food: 1.88; mid temperature/high food: 0.73). Therefore, size at maturation is more flexible at high temperatures and strongly constrained at low temperatures, after accounting for the variation in age at maturation.

GSI and HSI

The gonadosomatic index (GSI) differed between the sexes, as females tended to allocate more to reproduction (Fig. 1b; 2888Appendix.pdf, Table S3). The hepatosomatic index (HSI) also differed between males and females, the latter being in relatively better condition (Fig. 1c, Table S3). HSI was also significantly affected by temperature, but not food availability, with higher HSI values generally observed in the low temperature treatment (Fig. 1c, Table S3).

Shape

Fish shape was closely linked to temperature (MANOVA $P < 0.001$; 2888Appendix.pdf, Fig. S6). Food environment, on the other hand, was of little relevance in shaping individuals (MANOVA $P = 0.246$; Fig. S6). The first canonical variate axis, which explains 62.6% of the variation, appears to separate fish from each of the three temperatures evenly, with the 24°C fish on the 'stockier' side (particularly on the caudal peduncle). The second canonical variate axis, explaining 17.1% of variation, divides the 29°C from the 24°C and 34°C fish, with the 29°C fish showcasing a less upturned mouth and slightly less prominent hump (Fig. S6).

Multivariate response

All traits responded to temperature (Wilks' $\lambda = 0.343$, $P < 0.001$; Fig. 2) and food (Wilks' $\lambda = 0.652$, $P < 0.001$; Fig. 2) in multivariate space. There was not, however, a significant interaction term (Wilks' $\lambda = 0.861$, $P = 0.694$; Fig. 2).

Phenotypic integration

Phenotypic correlations differed substantially depending on the environment. Only the first principal component of growth and final weight were significantly correlated across all nine treatments (Fig. 3). Age and size at maturation showed positive correlations at low temperature (across food treatments) and low food (across temperature treatments). The two indices of integration used here, variance and effective dimension of eigenvalues were, not surprisingly, highly correlated ($r = -0.880$, $P = 0.002$). The treatments with the highest degree of phenotypic integration were mid temperature/high food and high temperature/low food. Conversely, high temperature/high food showed the least integration (Table 1).

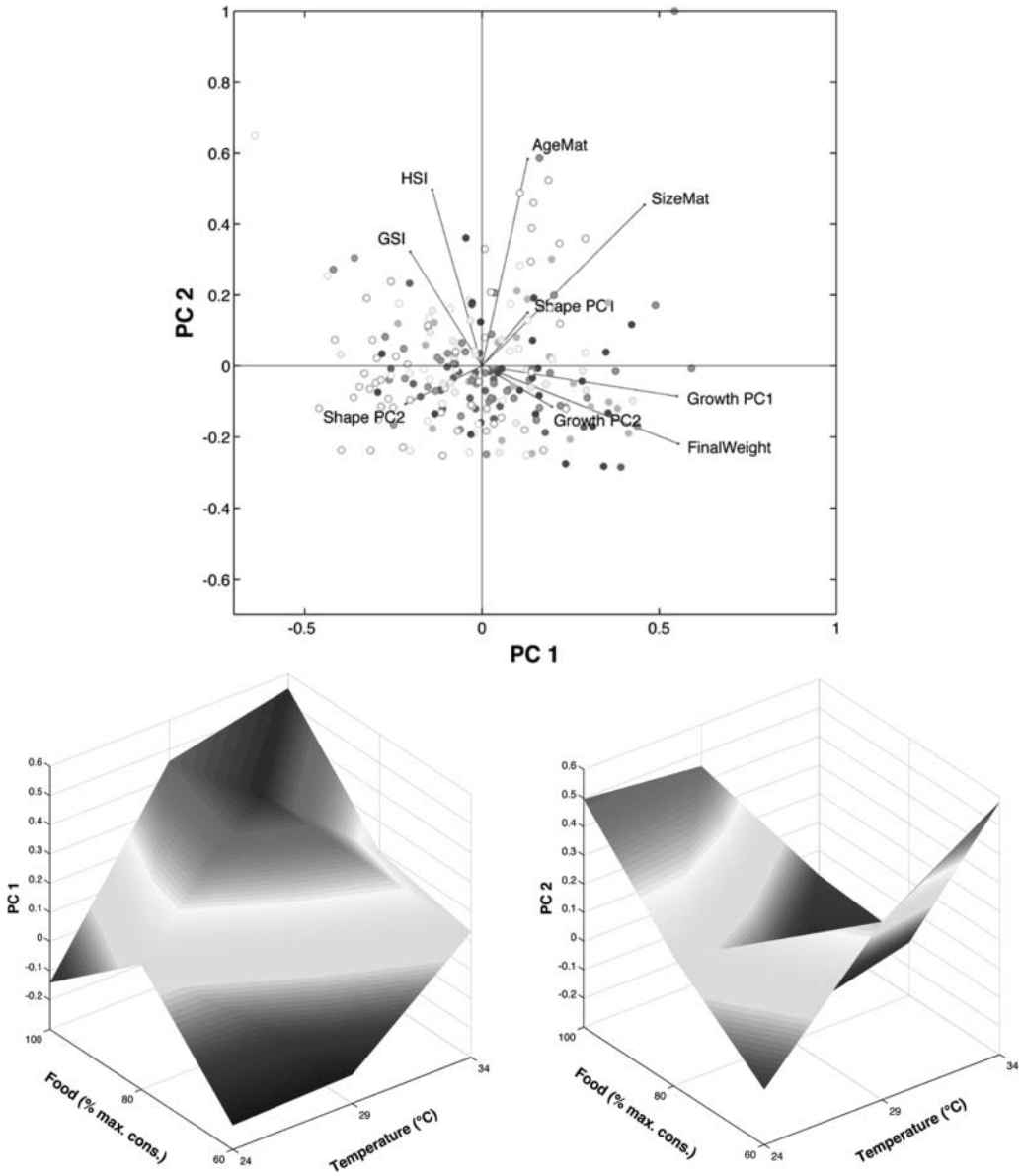

Fig. 2. Multivariate response of growth (PC1 and PC2), age and size at maturation, GSI, HSI, and shape (PC1 and PC2) to temperature and food variation.

Fitness

Relative fitness was highest at the low temperature/high food combination for both males and females ($\bar{W}_M = 1.668$ and $\bar{W}_F = 1.914$) and lowest at the high temperature/low food combination ($\bar{W}_M = 0.444$ and $\bar{W}_F = 0.506$). The fitness surface (Fig. 4) exhibited signs of interaction between temperature and food, although the interactions were not similar between males and females. Relative fitness among females at high and intermediate food

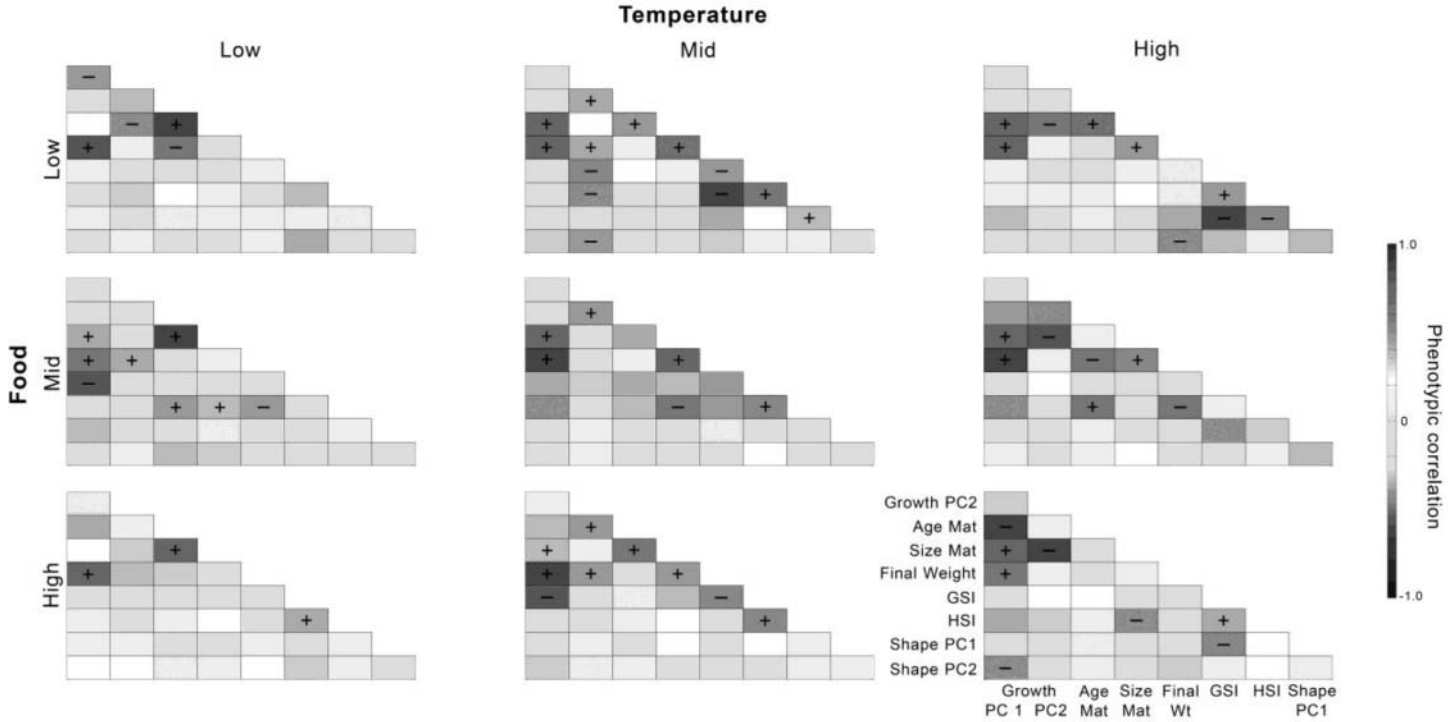

Fig. 3. Phenotypic correlations for all traits measured at the nine temperature × food combinations. Significant correlations are highlighted with ' + ' if positive, ' – ' if negative.

Table 1. Phenotypic integration based on the eigenvalues of the phenotype covariance matrix

Temperature (°C)	Food	Variance	EffDim
24	60	22.323	7.901
24	80	13.909	9.067
24	100	9.220	8.167
29	60	16.943	9.590
29	80	13.470	8.526
29	100	26.709	7.034
34	60	24.616	6.457
34	80	10.271	9.116
34	100	9.196	10.186

Note: Indices evaluated are: (1) variance of eigenvalues, a commonly used index (low variance = low integration); and (2) effective dimension, defined as $\text{EffDim} = \frac{(\Sigma\lambda)^2}{\Sigma\lambda^2}$, where 1 = perfect integration, n traits measured = complete lack of integration.

decreased evenly as temperature increased, but was low at all temperatures under low food (Fig. 4b). Males, on the other hand, showed a steep drop in relative fitness going from low temperature to intermediate and high temperatures, regardless of food treatment (Fig. 4a). There was no relationship between relative fitness and level of phenotypic integration, regardless of index used ($P > 0.8$). In males, three traits exhibited significant correlations between trait value and fitness: the second principal component of growth ($r = 0.301$, $P = 0.004$), GSI ($r = 0.842$, $P < 0.001$), and HSI ($r = 0.322$, $P = 0.004$). In females, different traits were significantly correlated with fitness: the first principal component of growth ($r = 0.385$, $P < 0.001$), GSI ($r = 0.720$, $P < 0.001$), final weight ($r = 0.390$, $P < 0.001$), and both principal components of shape ($r_{\text{PC1}} = 0.333$, $P_{\text{PC1}} < 0.001$; $r_{\text{PC2}} = -0.308$, $P_{\text{PC2}} = 0.001$).

DISCUSSION

Organisms live in complex worlds, facing combinations of various environmental factors, and it is the coordinated response of the entire suite of life-history traits that ultimately determines fitness. Here, we studied the response of life-history traits of the sheepshead minnow to different combinations of temperature (24°C, 29°C, or 34°C) and food availability (60%, 80%, or 100% of maximum consumption). We found that most traits responded to temperature and food directly and that juvenile growth rate exhibited significant food × temperature interactions in their response. Furthermore, phenotypic integration and fitness changed substantially under different environments, and differently for males and females.

Mechanistic understanding of plasticity is likely to yield the best predictions for populations facing climatic changes (Chown *et al.*, 2010). For instance, in coastal and marine food webs, both temperature and the food available to individuals are likely to change (Harley *et al.*, 2006; Wiklund *et al.*, 2009) and a broader view of integrated responses, as opposed to inferences made from univariate, single-trait reaction norms, seems warranted. In this study,

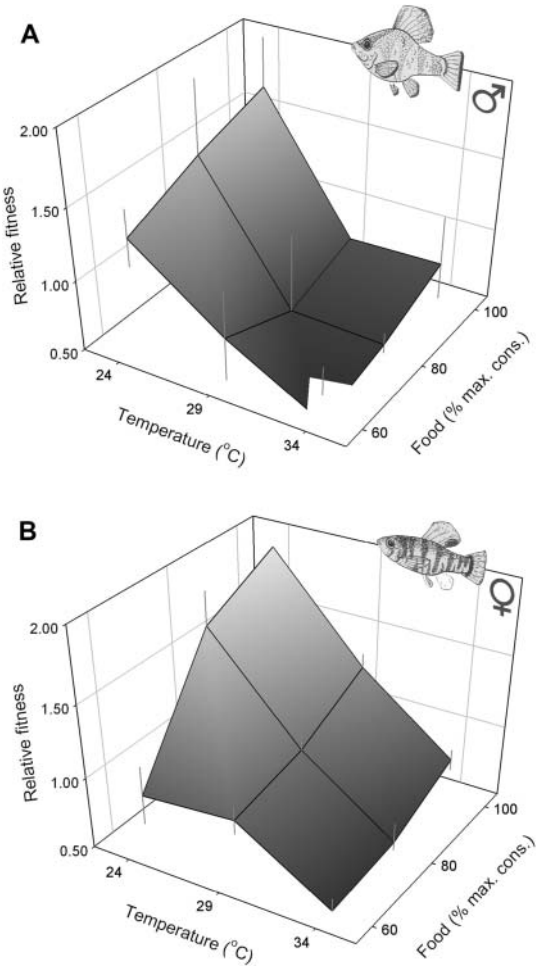

Fig. 4. Relative fitness (± S.E.) of males (A) and females (B) at each of the nine treatments.

we found that the thermal response of juvenile growth rate in sheepshead minnows was mediated by food availability (i.e. there was a statistical interaction between the two), as was shown for many other ectotherms (e.g. Petersen *et al.*, 2000; Giebelhausen and Lampert, 2001; Kingsolver *et al.*, 2006; Stillwell *et al.*, 2007). Inferences obtained from univariate thermal reaction norms would have led us to believe that juvenile males grow 14% faster when going from 24°C to 34°C (at a high food ration, the most common experimental protocol). However, in a low food environment, fish at 34°C actually grew 10% slower than those at 24°C.

The level of phenotypic integration was not consistent across treatments, and, importantly, could not have been predicted *a priori*. The low correlation between integration and fitness is at odds with our expectations from previous analyses; we suspect this is because the estimated fitness function is based directly on lifetime reproductive output rather than on an approximation using the quadratic form. Relative fitness, which we modelled using most of the traits measured, was also not uniform in response to the interaction of environments and, furthermore, showed different interactions among males and

females. If true for most species, as it appears to be (Schlichting, 1986), predicting phenotypic changes, both plastic and evolutionary, will be a daunting task. Integration could lead to slowed and constrained responses (Pigliucci, 2004), and these responses would be erroneously forecast if based on univariate reaction norms. In populations experiencing rapid environmental changes (e.g. invasive species, populations subjected to climate change), multivariate plastic responses will determine evolutionary trajectories (Price *et al.*, 2003; Parsons and Robinson, 2006).

Importantly, the fitness consequences of different food and temperature levels could not be approximated by any of the traits measured alone. This is particularly relevant since single traits are typically used as fitness proxies. Here, growth, possibly the most commonly used proxy for fitness, explained roughly 10% of the variation in fitness. The best single-trait proxy for fitness is GSI, accounting for 51% and 71% of the variation in females and males, respectively.

Trait-specific analyses of interacting environmental variables also revealed some unexpected results. Age at maturation was a function of temperature and food availability, even after conditioning on observed growth. This has important consequences for the probabilistic maturation reaction norm [PMRN (Heino *et al.*, 2002)]. The PMRN technique was designed to disentangle plastic from evolutionary changes in age at maturation by assuming that the relevant environmental effects are integrated in an individual's growth trajectory and beyond that maturation decisions are independent of environment (Dieckmann and Heino, 2007). As shown here, and in recent studies on white-spotted charr (Morita *et al.*, 2009), ninespine stickleback (Kuparinen *et al.*, 2011), zebrafish (Uusi-Heikkila *et al.*, 2011), and guppies (Pauli and Heino, 2013), this assumption is incorrect. Mollet *et al.* (2007) have suggested that the PMRN approach can be salvaged by the incorporation of other environmental variables, but without experimental validation (Uusi-Heikkila *et al.*, 2011) or explicit changes in gene frequencies (Therkildsen *et al.*, 2013), separating plasticity from evolution using observed phenotypic changes in natural populations will remain ambiguous.

In addition to growth and maturation, geometric morphometrics revealed significant changes in fish shape due to temperature. In general, the most pronounced feature was the depth of the body, as fish grown at lower temperatures tended to be deeper. This finding appears to contradict the common observation that cold water leads to more slender bodies in fishes [e.g. Atlantic cod (Marcil *et al.*, 2006); European sea bass (Georgakopoulou *et al.*, 2007); zebrafish (Georga and Koumoundouros, 2010; Sfakianakis *et al.*, 2011)]. These other species, however, are much better swimmers than sheepshead minnows, which are lazy swimmers that live in warm, shallow habitats at high densities. Food availability had little impact on determining shape, even though it was found to be important in closely related [Amargosa river pupfish (Lema and Nevitt, 2006)] and other species [e.g. Chinook salmon and rainbow trout (Currens *et al.*, 1989); pearl cichlid and redhump eartheater (Wimberger, 1992); Atlantic cod (Marcil *et al.*, 2006)]. Warmer temperatures may lead to shallower, more slender bodies [which are less energetically costly during swimming (Herbing, 2002)] in response to elevated physiological rates, or it could be a response to different habitat use at different temperatures [deeper bodies are associated with hovering and manoeuvrability (Peres-Neto and Magnan, 2004)]. Increased body depth may also allow slow-growing (low temperature) fish to escape predation by outgrowing predators' gapes.

Understanding the nature of interacting reaction norms across populations could shed light on the evolution of and constraints on phenotypic plasticity (David *et al.*, 2004; Stillwell *et al.*, 2007). Sheepshead minnow growth appears to be phenotypically plastic with respect to

temperature along the US east coast (Berry, 1987), while growth in the mummichog, *Fundulus heteroclitus*, exhibits local adaptation (Schultz *et al.*, 1996) despite its physiological, ecological, and geographical similarities (Collette and Klein-MacPhee, 2002; Nordlie, 2006; Haney *et al.*, 2007). A comparative study of plasticity in these two species could illuminate conditions favouring the evolution of phenotypic plasticity versus those leading to local adaptation, an area that has enjoyed much theoretical but little empirical attention.

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