Thermal regime drives a latitudinal gradient in morphology and life history in a livebearing fish

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Within-species diversity is often driven by changing selective regimes along environmental gradients. Here, we provide a direct test of the environmental factors underlying phenotypic diversity across the wide native distribution of eastern mosquitofish (*Gambusia holbrooki*). We investigated life-history and body-shape divergence (including multiple measures of body size) across more than 14 degrees of latitude in North America, and used Akaike's information criterion-based model selection to determine the relative contributions of thermal regime, population densities and habitat productivity as potential drivers of latitudinal phenotypic variation. We found thermal regime to be the most important driver of large-scale latitudinal phenotypic patterns: populations in colder climates with greater seasonality and range in temperature exhibited larger body size, larger reproductive investment coupled with smaller offspring size, and shallower bodies with a smaller head and more anterodorsally positioned pectoral fins. Nonetheless, population density and habitat productivity also influenced trait divergence, but independent of latitudinal patterns, and some variation in body shape was due to apparent covariation with life histories. Our study confirms thermal regime as an important driver of latitudinal phenotypic differentiation even in ectotherms, but also uncovers multiple additional factors that shape phenotypic diversity, emphasizing the importance of the multivariate approach we employed here.

ADDITIONAL KEYWORDS: body size – Bergmann's rule – *Gambusia holbrooki* – population density – productivity – temperature gradient.

INTRODUCTION

Studying biotic responses along environmental gradients has been a staple of evolutionary ecology for decades, and provides a unique opportunity to investigate patterns of natural selection (Curtis & McIntosh, 1951; Whittaker, 1967; Endler, 1986; Schweiger *et al.*, 2016; Riesch *et al.*, 2018b). The variation in environmental conditions along these gradients affects the survival and reproduction of organisms, and thus is fundamental in driving patterns of biodiversity by facilitating both phenotypic and genetic variation within and between species (McArthur *et al.*, 1988; Culumber *et al.*, 2012). Hence, large-scale geographical patterns of trait variation can provide insights into micro- and macroevolutionary patterns, pointing to putative selective agents responsible for major trends of phenotypic evolution (Endler, 1986; Culumber *et al.*, 2012). Evidence for adaptive diversification along environmental gradients stems from many different taxa (e.g. plants: Abbott & Brennan, 2014; invertebrates: Diamond & Chick, 2018; Gordon *et al.*, 2018; reptiles: Álvarez-Ruiz *et al.*, 2018; birds and mammals: Weir & Schluter, 2007), but is particularly varied for fishes,

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which have, for example, been studied along elevational gradients (Rahel & Hubert, 1991; Olinger *et al.*, 2016), salinity gradients (Mück & Heubel, 2018), temperature gradients (Ohlberger *et al.*, 2008) and predation gradients (Walsh & Reznick, 2009). However, these studies are often constrained by focusing on a single environmental variable, when it is more likely that across the studied populations additional gradients exist that might select for similar or different responses compared to the focal gradient (Reznick *et al.*, 2001; Riesch *et al.*, 2015). This exacerbates the difficulty of properly interpreting results when data do not conform to a priori predictions.

Amongst ecological gradients, latitudinal gradients are particularly well studied (e.g. Schemske *et al.*, 2009). For example, many birds lay larger clutches in higher latitudes (Jetz *et al.*, 2008), while eastern fence lizards (*Sceloporus undulatus*) from southern populations are smaller, and differ in reproductive output from their northern counterparts by producing smaller and fewer eggs as part of their first clutch. However, lizards from southern populations are also more likely to produce a second clutch than lizards from northern populations (Du *et al.*, 2014). For fishes, Braaten & Guy (2002) reported that longevity of four of their five focal species increased with increasing latitude, while growth rates also tended to increase from low to high latitudes. Similarly, recent studies on invasive eastern mosquitofish (*Gambusia holbrooki*) in Europe found increased investment into reproduction and lower body condition in southern compared to northern latitudes (Benejam *et al.*, 2009). Many of these studies pointed to thermal regime as being one of the, if not the, most important drivers of these phenotypic patterns (Benejam *et al.*, 2009; Parra *et al.*, 2014). However, most previous studies on latitudinal gradients used latitude as a surrogate for putative, causal environmental factors (although air or water temperatures were sometimes measured as well), and so competing hypotheses about the underlying causes of broad biogeographical patterns across taxa (e.g. competition, predation or primary productivity) were difficult to address specifically. Thus, the actual environmental selective agents often remained unclear and untested (Ashton *et al.*, 2000; Belk & Houston, 2002; Angilletta *et al.*, 2004; Olalla-Tárraga, 2011; Rypel, 2014).

Here, we address this problem by exploring specific environmental drivers of broad, geographical patterns in life histories and morphology. We measured eight life-history traits (including two estimates of body size) and used geometric morphometrics to measure body shape across a bioclimatic gradient in the native range of a livebearing ectotherm, the eastern mosquitofish (Poeciliidae). To explicitly investigate some of the potential underlying mechanisms responsible for latitudinal patterns of trait divergence, we quantified a range of environmental variables hypothesized to underlie latitudinal associations, of which thermal regime is just one. We examined ten populations along the eastern coast of the USA, spanning more than 14 degrees latitude and asked two specific questions tied to a set of a priori hypotheses that are outlined in Table 1: (1) Do life histories and body shape exhibit hypothesized clines across the latitudinal gradient? (2) Do thermal regime, habitat productivity and population density explain variation in these traits?

MATERIAL AND METHODS

STUDY POPULATIONS AND HABITAT CHARACTERISTICS

During 19–28 September 2011, we collected sexually mature adult fish from ten different natural *G. holbrooki* populations across their native range along the Atlantic Coast of the USA, spanning > 14 degrees of latitude from New Jersey to southern Florida [Fig. 1A, B; see Online Supplementary Material (OSM), Table A1; mean sample size per sex and population = 19, range = 2–30]. We conducted all collections during a short period to avoid confounding factors of time of year, to include adults that could vary in age from ~2 months to > 1.5 years for all sites, and to capture a time after summer, but preceding winter (which varies dramatically across latitude). Furthermore, in an attempt to standardize our sampling approach across this large latitudinal range, we decided a priori to only sample water bodies with still water or very weak current located within 4 km of the nearest ocean waters. In this way, we minimized confounding environmental factors known to influence phenotypic variation in some livebearing fishes (e.g. Alcaraz & Garcia-Berthou, 2007; Carmona-Catot *et al.*, 2011; Jourdan *et al.*, 2016), permitting us to focus more directly on environmental factors of interest in this study. We collected *G. holbrooki* using a combination of seine (4 m long, 3-mm mesh-width) and dip nets (1.6-mm mesh-width). Immediately after collection we killed the fish in an aqueous solution of tricaine methanesulfonate (MS 222) and preserved them in 95% ethanol. Life histories and morphologies were subsequently measured using preserved specimens (see details below).

At the time of collection, we measured conductivity using a YSI 85 meter (Yellow Springs, OH, USA), measured the photosynthetic pigment chlorophyll *a* using a fluorometer (AquaFluor model, Turner Designs, Sunnyvale, CA, USA), and estimated population density (see Table A1). Conductivity is often reported to exhibit positive associations with nutrient availability, primary productivity, and fish growth rate and body size (e.g. McFadden & Cooper, 1962; Scarnecchia & Bergerson, 1987; Trippel &

Table 1. Potential life-history and body-shape shifts in response to environmental variation across the East Coast of the USA in eastern mosquitofish (*G. holbrooki*)

Selective agent(s)	Potential pattern	Rationale	References
Thermal regime	↑Body size with ↑latitude	Greater overwintering potential for larger individuals at high latitudes and/or harsher conditions at higher latitudes facilitate stronger male–male competition, favouring larger individuals.	Thompson *et al.*, 1991; Daniels & Felley, 1992; Felley & Daniels, 1992; Dadda *et al.*, 2005; Shoup & Wahl, 2011; Booksmythe *et al.*, 2013
	More rounded and compact body shape at ↑latitude	Higher fat reserves (i.e. more rotund body shapes) should increase overwintering survival at higher latitudes.	Thompson *et al.*, 1991; Griffiths & Kirkwood, 1995
Thermal regime + productivity	↓Body size with ↑latitude	Higher temperatures and decreased seasonality in lower latitudes coupled with increased productivity result in increased growth rates, while fish in higher latitudes could be *r*-selected.	Pianka, 1970; Gross *et al.*, 1988; Reznick *et al.*, 2002
	↓Offspring size, ↑fecundity and ↑RA with ↑latitude	*r*-selected phenotypes due to shorter reproductive periods, higher winter mortality, and more unpredictable mortality across the year as a result of resource- and climatic fluctuations at higher latitudes.	Pianka, 1970; Winemiller & Rose, 1992; Reznick *et al.*, 2002
Productivity	↑Body size, ↑fat content, ↑lean weight, ↑RA, ↑fecundity and deeper bodies with ↑productivity	More available resources result in greater body condition and a large investment into each reproductive bout.	Reznick & Yang, 1993; Spoljaric & Reimchen, 2007; Riesch *et al.*, 2016
Density	↑Body size, ↑offspring size coupled with ↓fecundity, ↓RA and shallower bodies at higher densities	*K*-selected life-history strategies at high population densities due to high competition, resulting in fewer resources per individual being available to invest into body condition and reproduction.	Pianka, 1970; Reznick *et al.*, 2002; Landy & Travis, 2015
Life history – morphology covariation	Larger abdominal regions, smaller heads and more anteriorly positioned pectoral fins with ↑reproductive effort in females, and ventral deepening of abdominal regions with ↑reproductive effort in males.	Covariation with life histories could result in correlated responses in body shape across populations.	Wesner *et al.*, 2011; Banet *et al.*, 2016

Predictions were derived from life-history theory and previous studies on the effects of environmental gradients on life histories and body shape in poeciliid and non-poeciliid fishes. RA, reproductive allocation.

Beamish, 1989; Chételat *et al.*, 1999), while chlorophyll *a* serves as an estimate of algal biomass and a proxy of overall primary productivity. Conspecific density was estimated as the total number of *G. holbrooki* caught during five standardized seine hauls or during 20 min of dip-netting (depending on the accessibility of the habitat), and scored as 1 = fewer than 100 individuals, 2 = greater than 100 but fewer than 500, and 3 = greater than 500. As additional important climatic variables, we downloaded temperature seasonality, annual mean temperature, temperature isothermality, temperature diurnal range and annual precipitation for each site from WorldClim (Version 1, Hijmans *et al.*, 2005) at 2.5-min (of a longitude/latitude degree) spatial resolution to capture regional differences between our collection sites.

We then conducted a principal component analysis (PCA) using a correlation matrix on the geographical/
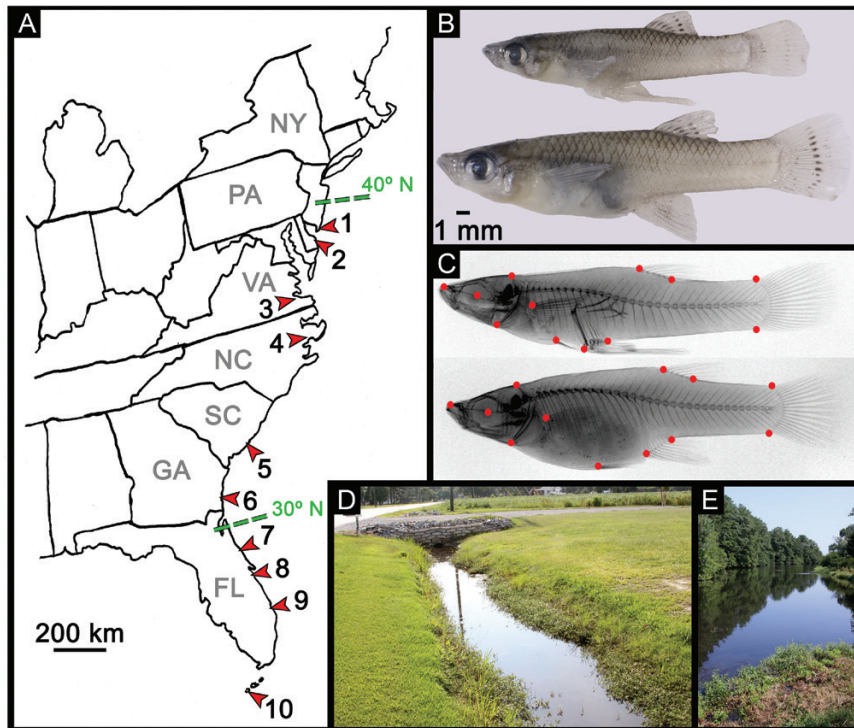
Figure 1. A, map of sampling localities along the East Coast of the USA with arrows and numbers indicating sample sites (see OSM Table A1 for details). B, male (top) and female (bottom) eastern mosquitofish, *Gambusia holbrooki*. C, landmarks used for morphometric analysis shown on a representative X-ray of a male (top) and female (bottom). Habitat photos of site 4 in Hyde County, NC (D) and site 2 in Rehoboth Beach, DE (E).

environmental data to reduce dimensionality of environmental variation (hereafter environmental PC, and comprising conductivity, chlorophyll *a*, density and the five bioclimatic variables from WorldClim data). We retained the first four axes, which explained over 96% of environmental variation (OSM Table B1). Based on the axis loadings, PC1 was associated with more northern (latitude) and eastern (longitude) populations, greater seasonality, and lower, more variable temperatures. PC2 was associated with higher precipitation and diurnal temperature range, as well as lower conductivity and lower *G. holbrooki* density. PC3 was associated with chlorophyll *a*, and PC4 with greater *G. holbrooki* density.

POPULATION GENETIC ANALYSES

Because *G. holbrooki* have been, and continue to be, purposefully transported by humans across large distances in the USA for mosquito control (e.g. Pyke, 2008), we conducted a population genetic analysis based on 15 previously published nuclear microsatellite markers to evaluate whether any evidence supports a history of human-mediated translocations in our focal populations (see OSM, Section C for details on methods). Because the genetic data did not reveal any obvious patterns of non-natural genetic structuring (see OSM, Section C for details on results), we treated all sampled populations as natural populations and included them in our subsequent analyses.

LIFE-HISTORY MEASUREMENTS

We conducted life-history dissections on all pregnant females and mature males. Both sexes exhibit indeterminate growth, but males strongly reduce their growth rates after reaching sexual maturity (Snelson, 1989). Following the protocol of Reznick & Endler (1982), all preserved fish were weighed and measured for standard length. For females, we removed the reproductive tissue and all developing offspring. Offspring were counted and their stage of development was determined (scored after Riesch *et al.*, 2011). Somatic tissues (including the gut), reproductive tissues and embryos were then dried for 24 h at 55 °C and weighed again. To assess female and embryo condition, somatic tissues and embryos were rinsed up to six times for at least 6 h in petroleum ether to extract soluble non-structural fats (Heulett *et al.*, 1995) and were then redried and reweighed. Furthermore, we calculated reproductive effort for both sexes by calculating reproductive allocation (RA) for females and the

gonadomosatic index (GSI) for males. RA was calculated by dividing offspring weight by the sum of offspring weight plus somatic dry weight (Reznick & Endler, 1982; Riesch *et al.*, 2013), while GSI was calculated by dividing a male's reproductive tissue dry weight by the sum of reproductive tissue dry weight and somatic dry weight (Riesch *et al.*, 2013). We classified males as mature based on the complete development of the terminal structures of their modified anal fin (i.e. gonopodium), following methods described by Turner (1941).

In total, we measured eight life-history traits. For both males and females, we measured standard length [SL (mm)], wet weight (g), lean weight [(g), i.e. dry weight after fat extraction; a proxy for muscle mass], fat content (%), and reproductive investment [females: RA (%), males: GSI (%)]. For females, we also measured the offspring-related traits fecundity (i.e. no. developing offspring), offspring lean weight [(mg); our measure of offspring size] and offspring fat content (%).

We log$_{10}$-transformed (adult SL, adult wet and lean weight, and embryo dry and lean weight), square-root transformed (fecundity), or arcsine (square root)-transformed (adult fat content, embryo fat content, RA and GSI) all life-history variables to meet assumptions of statistical analyses (i.e. these transformations greatly facilitated normality of model residuals). We tested for differences in SL between populations by means of two sex-specific univariate analyses of variance (ANOVAs). We tested for population differences in all other traits by employing two sex-specific multivariate analyses of covariance (MANCOVAs). We included SL and – in the case of female life-history data – embryonic stage of development (Riesch *et al.*, 2011) as covariates. All analyses were conducted in JMP software (SAS Institute, Cary, NC, USA).

ANALYSIS OF BODY SHAPE

For geometric morphometrics, a lateral X-ray image was taken of each individual using a custom-built digital X-ray unit comprising a micro-focus X-ray source (Hamamatsu L6731-01, Hamamatsu Corporation, Bridgewater, NJ, USA) and a digital X-ray detector (PaxScan 2520E, Kodex Inc., Nutley, NJ, USA) housed in a lead-shielded cabinet (see Beckmann *et al.*, 2015). One of us (RM) digitized the following landmarks on digital images using tpsDig2 (Rohlf, 2010a): (1) most anterodorsal point of premaxilla (tip of snout), (2) most posterodorsal point of skull, (3) anterior insertion of dorsal fin, (4) posterior insertion of dorsal fin, (5) dorsal insertion of caudal fin, (6) ventral insertion of caudal fin, (7) posterior insertion of anal fin, (8) anterior insertion of anal fin, (9) insertion of pelvic fin, (10) most posteroventral point of skull and (11) centre of the eye orbit (Fig. 1C). We used geometric morphometric methods to examine morphological variation (Marcus *et al.*, 1996; Mitteroecker & Gunz, 2009). Using tpsRelw (Rohlf, 2010b), we performed generalized Procrustes analyses separately for each sex (i.e. align landmark coordinates by rotating, translating and scaling coordinates to remove positioning effects and isometric size effects; Bookstein, 1991; Marcus *et al.*, 1996) and obtained shape variables (relative warps) for statistical analysis.

We first confirmed population differences in body shape with MANCOVA (using relative warps as dependent variables and centroid size as the covariate) and then derived divergence vectors (**d**) describing multivariate axes of morphological differences among populations, controlling for allometry (Langerhans, 2009). For males, we retained the first four axes, explaining 95.1% of among-population shape variation. For females, we retained the first five axes, explaining 94.3% of among-population shape variation. We used population means for each **d** axis as described for life-history traits, while examining sexes separately.

MODEL SELECTION

To examine the competing hypotheses for each trait, we used a model selection approach (Burnham & Anderson, 2002) that used the 17 traits (see Table 2) as dependent variables and the four environmental PCs as potential independent variables describing both broad-scale and local-scale environmental variation. For body size, we examined both SL and wet weight. For life-history traits other than body size, we first calculated marginal means for each population to control for effects of body size on these traits. To accomplish this, we conducted general linear models including SL as a covariate, and saved least-squares means for either the population term (for traits only measured in females) or the interaction between population and sex (for traits measured in both sexes). In this way, we examined 'size-free' life-history variables, comparing average values for each population for an average-sized fish. Results were qualitatively similar whether using mean values, means of residual values or marginal means for all analyses with one exception: examination of residuals of fat content additionally suggested the importance of environmental PC1 in explaining fat variation, but we only present results using marginal means, which suggested that environmental PC1 was suggestive but not significant.

We used Akaike's information criterion corrected for small sample sizes (AIC$_c$; Burnham *et al.*, 2011), examined ΔAIC$_c$ values, and conducted model averaging with the subset of models that fulfilled a cumulative AIC$_c$ weight of 0.95 (*sensu* Burnham & Anderson, 2002; Burnham *et al.*, 2011). We assessed models by both inspecting the significance of model terms in the top selected models for each trait (ΔAIC$_c$ ≤ 2.0), as well as inspecting model-averaged coefficients and standard errors.

Table 2. Summary of model selection results

Trait	PC1	PC2	PC3	PC4
Body size (length)	**positive**		**positive**	
Body size (weight)	**positive**		**positive**	**negative (F)**
Reproductive effort	**positive**			**negative (F)**
Fecundity	positive			negative
Offspring size	**negative**			
Offspring fat		**negative**		
Relative lean weight			positive	negative
Fat content			**positive**	
Male $\mathbf{d}_1$		positive		
Male $\mathbf{d}_2$	**positive**			
Male $\mathbf{d}_3$	(negative)			
Male $\mathbf{d}_4$			positive	
Female $\mathbf{d}_1$	**(positive)**	**(positive)**		
Female $\mathbf{d}_2$				
Female $\mathbf{d}_3$			**negative**	**positive**
Female $\mathbf{d}_4$	**positive**			
Female $\mathbf{d}_5$		negative		
	6 (1)	1 (2)	4 (2)	3 (2)

Note: Bold indicates strongest evidence (supported by both model averaging and significant *P* value within selected models); non-bold text indicates suggestive evidence (supported by either model averaging or significance within selected models). 'F' in parentheses indicates the effect was most apparent in females. Trends in parentheses disappeared when controlling for covariation with life-history traits. Numbers at the bottom of the table reflect the sum of significant effects (and suggestive trends in parentheses) for each environmental PC; PC1 was associated with more northern latitudes and eastern longitudes, greater seasonality, and lower, more variable temperatures. PC2 was associated with higher precipitation and diurnal temperature range, as well as lower conductivity and *G. holbrooki* density. PC3 was associated with chlorophyll *a*, and PC4 with greater *G. holbrooki* density.

For body shape, we additionally performed a second model selection procedure to evaluate whether apparent associations between morphology and the four environmental PCs might be explained by covariation with life-history traits. In this manner, we could uncover whether observed morphology–environment associations actually reflected spurious correlations due to underlying life-history–morphology correlations. To accomplish this, we created a model set separately for each morphological axis that included all measured life-history traits (separately for each sex) in addition to the environmental PCs identified as important in the first model selection procedure based on either model averaging or a significant *P*-value in selected models. If the top model included a life-history trait, but not an environmental PC, then we concluded that covariation between morphology and life history had produced a spurious association between morphology and the relevant PC(s).

RESULTS

ESTABLISHING LIFE-HISTORY AND MORPHOLOGICAL VARIATION ALONG THE BIOCLIMATIC GRADIENT

We found broad support for life-history and morphological variation along the latitudinal gradient in bioclimatic parameters. Descriptive statistics for life histories of all sampled fish can be found in OSM Tables D1 and D2. Both estimates of body size differed significantly among populations (ANOVA, males, SL: $F_{9,149} = 6.87$, $P < 0.001$, wet weight: $F_{9,149} = 7.26$, $P < 0.001$; females, SL: $F_{9,213} = 24.75$, $P < 0.001$, wet weight: $F_{9,213} = 29.01$, $P < 0.001$). Our sex-specific MANCOVAs further uncovered significant differences in all other life-history traits between populations (males, SL: $F_{3,146} = 659.50$, $P < 0.001$, population: $F_{27,427} = 7.64$, $P < 0.001$; females, SL: $F_{6,206} = 713.46$, $P < 0.001$, embryonic stage of development: $F_{6,206} = 29.54$, $P < 0.001$, population: $F_{54,1055} = 27.53$, $P < 0.001$). The same was true for body shape variation (males, centroid size: $F_{20,132} = 6.07$, $P < 0.001$, population: $F_{180,1107.2} = 6.17$, $P < 0.001$; females, centroid size: $F_{20,197} = 17.21$, $P < 0.001$, population: $F_{180,1643.5} = 9.06$, $P < 0.001$). We therefore proceeded to test our specific predictions as to why these life-history and morphological traits differed between populations. Model selection results are presented in OSM Tables E1 and E2.

BODY SIZE AS A FUNCTION OF LATITUDE

We found larger adult body sizes at higher latitudes with lower temperatures and greater seasonality and temperature range (Fig. 2A, B; Table 2, OSM Table E1).
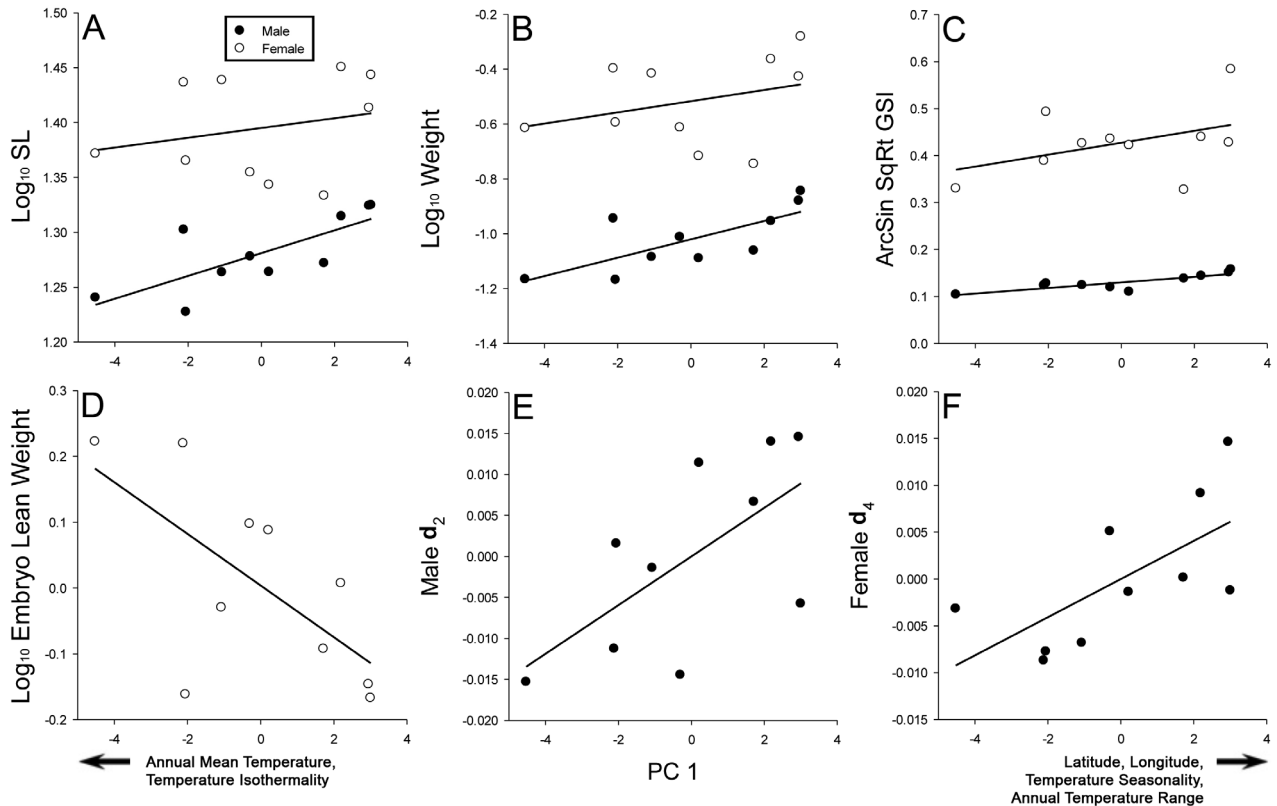
Figure 2. Significant associations between latitude/thermal regime (PC1) and (A) standard length (SL), (B) wet body weight, (C) reproductive investment, gonadomosatic index (GSI) (female GSI is usually referred to as reproductive allocation, RA), (D) offspring size, (E) male body shape (d_2) and (F) female body shape (d_4).

REPRODUCTIVE STRATEGIES AS A FUNCTION OF LATITUDE

Latitude had the predicted association with reproductive strategies, and reproductive investment (GSI and RA) was greater, while offspring size was smaller at higher latitudes and lower/more variable temperatures. The evidence for an influence of latitude on fecundity (greater at higher latitudes and lower/ more variable temperatures) was only suggestive (Fig. 2C, D; Table 2, OSM Table E1).

BODY SHAPE AS A FUNCTION OF LATITUDE

Both males and females exhibited shallower bodies with a smaller head and more anterodorsally positioned pectoral fins at higher latitudes (male d_2 and female d_4, respectively; Figs 2E, F, 3; Table 2), which was contrary to our a priori predictions based on overwintering potential. Neither of these patterns was affected by possible covariation with life-history traits. On the other hand, other observed associations between body shape and latitude appeared to reflect underlying body shape – life-history correlations. We found a suggestive association between male d_3 and

PC1 that was apparently due to covariation with GSI (only GSI was included in the top model; males with deeper abdominal regions exhibited larger GSI; see OSM Fig. F1). Female d_1 exhibited significant associations with PC1 and PC2, but this was apparently due to covariation with fecundity (only fecundity was included in the top model; females with longer and deeper mid-body/abdominal regions exhibited greater fecundity; see OSM Fig. F1).

BODY SHAPE AND LIFE HISTORIES AS A FUNCTION OF PRODUCTIVITY AND DENSITY

With respect to productivity (PC3), we found support for our predictions regarding body size (both SL and wet weight) and fat content [all greater in habitats with higher productivity; Fig. 4A, B (SL not shown)], the trend for relative lean weight was only suggestive, but we found no support for increased fecundity or reproductive investment (Table 2). We further found support for our prediction of deeper bodies in habitats with greater productivity, but only in females (Fig. 4C; Table 2).

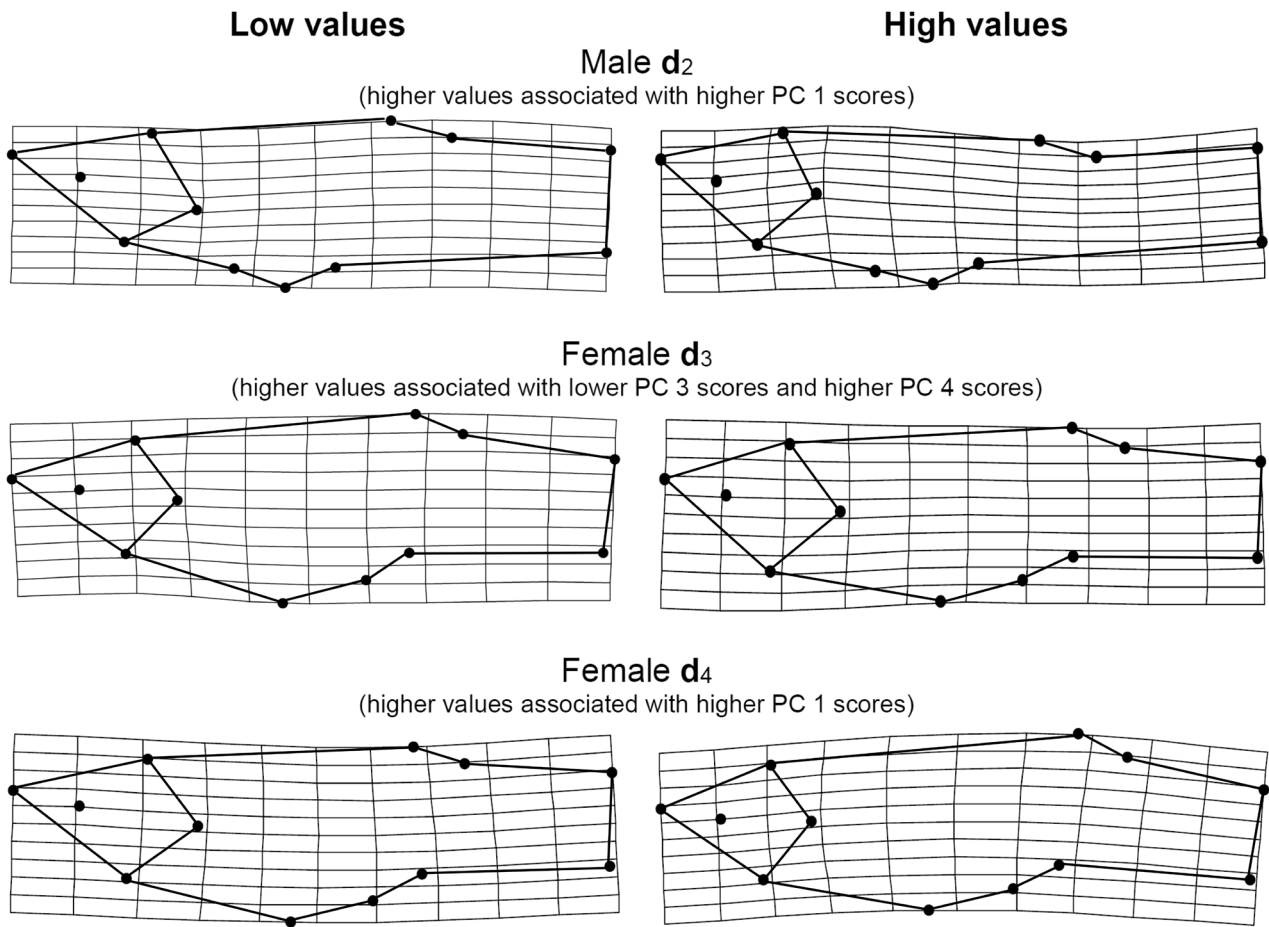With respect to population density (PC4), we only found significant support for one of our predictions

Low values   High values

Male d_2
(higher values associated with higher PC 1 scores)

Female d_3
(higher values associated with lower PC 3 scores and higher PC 4 scores)

Female d_4
(higher values associated with higher PC 1 scores)

Figure 3. Visualization of body shape variation (based on thin-plate spline transformations for each sex) along divergence vectors (**d**) that were significantly associated with environmental factors (see main results). Landmarks reflect those depicted in Figure 1C.

in that females had a lower reproductive investment at sites with higher population densities (the same trend was very weak for males; Fig. 4E; Table 2). We further found some suggestive evidence for fecundity and relative lean weight, but no support for offspring size (Table 2). Several other traits exhibited patterns opposite to our predictions as females (and very weakly also males) actually exhibited smaller body size with higher population densities (Fig. 4D). Females also exhibited shallower bodies in sites with lower population densities (Fig. 4F; Table 2).

DISCUSSION

In line with our a priori predictions, we found that populations at higher latitudes had larger body size, greater relative investment in reproduction in both sexes, and smaller offspring size. In contrast to these clear latitudinal patterns consistent with a priori predictions, other predictions generally fared poorly in comparison when faced with our empirical tests. For instance, patterns for body shape across the latitudinal gradient were contrary to our a priori predictions in that at higher latitudes both sexes had shallower bodies with smaller heads and more anterodorsally positioned pectoral fins. With respect to productivity and population density, we only found some support for our a priori predictions, with greater body size and fat content, and deeper female bodies at sites with higher productivity, and lower female reproductive investment at higher densities, while other traits exhibited patterns contrary to our predictions. There might be some limitations to our study, such as the number of populations and at least one case of a small sample size. Nonetheless, many strong patterns were uncovered, some matching a priori predictions, and if we exclude the one case of small sample size, results were qualitatively unchanged.
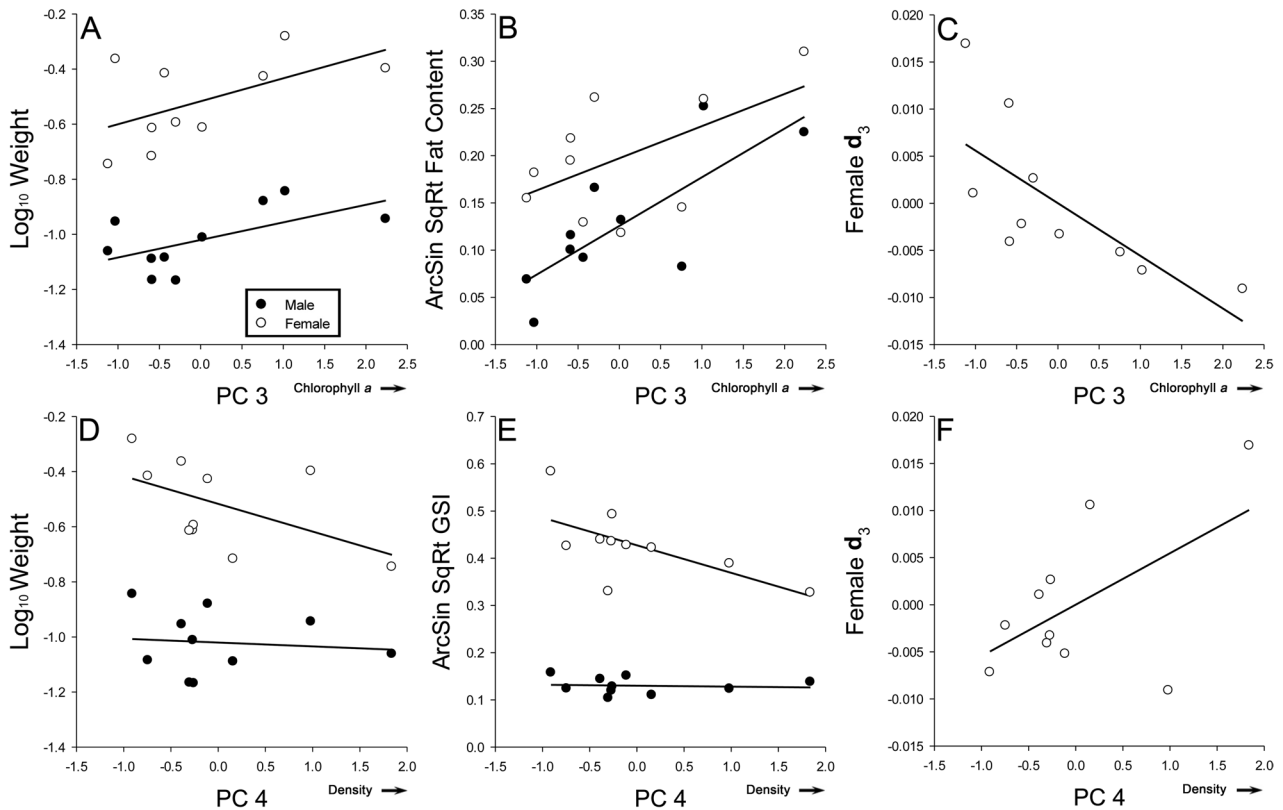
Figure 4. Significant associations between productivity (PC3) and (A) wet body weight, (B) fat content and (C) female body shape (d_3), as well as between population density (PC4) and (D) wet weight, (E) reproductive investment, gonadomosatic index (GSI) (female GSI is usually referred to as reproductive allocation, RA) and (F) female body shape (d_3).

BODY SIZE AS A FUNCTION OF LATITUDE

We uncovered that body size increased with increasing latitude, which mirrors patterns predicted for endotherms by Bergmann's rule (Bergmann, 1847). However, because the standard explanation for body size patterns following Bergmann's rule (i.e. an explanation based on thermoregulation) is not applicable to ectotherms (Bergmann, 1847; Salewski & Watt, 2017), several recent studies have tried to evaluate alternative explanations (Vinarski, 2014; Osorio-Canadas *et al.*, 2016). Body size–latitude patterns (or lack thereof) in ectotherms are likely to be system-specific, with a combination of factors that covary with latitude underlying the observed patterns (Vinarski, 2014; Rollinson & Rowe, 2018). Here, we hypothesized that *G. holbrooki* may exhibit larger body size at higher latitudes because larger individuals in many fish species have a greater overwintering survival probability (Thompson *et al.*, 1991; Daniels & Felley, 1992; Shoup & Wahl, 2011). In support of this prediction, we found that thermal regime, and no other ecological factors, explains larger body sizes at higher latitudes in *G. holbrooki*. Future work should directly test the association between body size and

overwintering survival in *G. holbrooki* to investigate this putative mechanism. Here we focused exclusively on adult individuals collected late in the growing season to minimize examination of individuals at different ontogenetic growth stages and centre on adults that could potentially overwinter. If *G. holbrooki* have evolved a similar latitudinal gradient in growth capacity as has been reported for other fish from the same region – where high-latitude individuals grow comparatively fast during their shorter growing season (e.g. Conover & Present, 1990; Chavarie *et al.*, 2010; Snover *et al.*, 2015) – then timing of measurements during growth trajectories of fish could prove critically important in uncovering latitudinal patterns in adult body size. Future research in *G. holbrooki* should investigate temporal patterns of body size across seasons as well as temperature-specific growth rates (and population differences therein) to investigate the role of growth trajectories in explaining this latitudinal trend.

Previous evidence investigating patterns of body size across latitudes in fishes had been rather conflicting. One study, which examined body size patterns across 18 species of North American freshwater

fishes (including *Gambusia affinis*, a close relative of *G. holbrooki*), found that body size decreased with latitude (Belk & Houston, 2002), while another study comprising 29 different North American freshwater fishes found that body size increased with latitude in cool-/cold-water species but decreased in warm-water species (Rypel, 2014). An obvious difference between these previous studies and our present study is that we specifically sampled fish at approximately the same time for patterns of body size and other phenotypic variables (i.e. our study was specifically designed to test this hypothesis), while the two previous, multi-species studies used publicly available angling records (Rypel, 2014), museum specimens and other published datasets (Belk & Houston, 2002) that stem from different years and different seasons. This means that some of the variation in previous studies could have resulted from time-of-year effects or exploitation of certain sizes by fisheries (Kuparinen & Merilä, 2007), which might have stronger effects in certain latitudes than in others.

REPRODUCTIVE STRATEGIES AS A FUNCTION OF LATITUDE

We predicted that *G. holbrooki* from more northern latitudes would exhibit reproductive traits characteristic of an *r*-selected life-history strategy (Pianka, 1970; Reznick *et al.*, 2002), and our results were consistent with that prediction. Specifically, males and females invested more in reproduction, and females also produced smaller offspring at higher latitudes. Moreover, females tended to produce larger clutches at higher latitudes, even though this effect was not statistically significant. These patterns suggest that mosquitofish at higher latitudes experience higher mortality rates than mosquitofish from lower latitudes, which could arise from a higher overall mortality rate across age/size classes (Mattingly & Butler, 1994; Bronikowski *et al.*, 2002), higher mortality of larger individuals (Gadgil & Bossert, 1970; Michod, 1979) or a combination of both. We hypothesize that higher overall mortality across age/size classes in eastern mosquitofish may occur in higher latitudes due to greater variance of climate and resources throughout the year, combined with harsher winter conditions. Our analyses partially supported this interpretation, as environmental PC1 – which correlated with these life-history variables – was characterized by high loadings of not only latitude (positive), but also temperature seasonality (positive), temperature range (positive) and temperature isothermality (negative). This suggests that the variable and unpredictable climate could have driven the observed patterns of life-history divergence. Thus, high mortality under northern climate regimes appears to have selected for *G. holbrooki* that

invest greatly into every reproduction event given the uncertainty regarding survival to subsequent reproductive bouts (Pianka, 1970; Roff, 2002).

Our present sampling scheme provided only a single measure and snapshot estimate of habitat productivity, so our data did not allow us to investigate if fluctuations in resource availability might play a role as well. However, given what is known about the influence of latitude on resource availability in aquatic freshwater habitats (Brylinsky & Mann, 1973; Gross *et al.*, 1988), and how this can affect life histories (Boyce, 1979), we consider it likely to play an important role in shaping the observed patterns as well.

BODY SHAPE AS A FUNCTION OF LATITUDE

Based only on considerations regarding adaptive morphological divergence, we predicted a priori that fish from northern latitudes would have more rounded and compact body shapes relative to mosquitofish from southern latitudes, because we expected higher body condition (i.e. fat reserves) in late September prior to the onset of winter. Surprisingly, our results were contrary to our predictions with both males and females exhibiting shallower bodies with a smaller head and more anterodorsally positioned pectoral fins at higher latitudes. Further research should investigate the mechanisms underlying this pattern, and we offer hypotheses of interest here. First, populations in northern latitudes might exhibit elevated growth of muscle at the expense of metabolically expensive brain tissue (Isler & van Schaik, 2006) to enhance growth and reproduction during shorter growing seasons, even though brain morphology was not directly examined here. Second, fish in northern climates may also exhibit shallower bodies that partially reflect lower overall condition. Consistent with this notion, male d_2 (the male shape axis associated with latitude) was negatively associated with lean weight, our proxy for muscle mass ($P = 0.05$), and showed a suggestive negative trend with fat content ($P = 0.06$); female d_4 (the female shape axis associated with latitude) also had a suggestive negative association with fat content ($P = 0.09$). However, these body shape–latitude trends persisted when statistically controlling for measured life-history traits. Third, changes in head size and position of the pectoral fin may reflect trophic shifts related to changes in thermal regime. This awaits further study, for example using gut content or stable isotope analyses.

We observed patterns partially congruent with our predictions based on co-variation of body shape with life-history traits. In essence, fish from higher latitudes had body morphologies that reflected/facilitated increased investment into reproduction and a tendency to produce larger clutch sizes. In environments where

selection favours *r*-selected life-history strategies, indirect selection probably favours a body shape that increases body cavity space to house the larger testes (for males) and larger clutches (for females; for a similar pattern in *G. sexradiata* and *G. yucatana* see Jourdan *et al.*, 2016). Our observed patterns are consistent with this: first, males in northern latitudes tended to exhibit ventral deepening of the body ($\mathbf{d}_3$; OSM Fig. F1), which was statistically explained by larger relative testis size in these populations; second, females exhibited longer and deeper abdominal regions in northern latitudes ($\mathbf{d}_1$; OSM Fig. F1), which was statistically explained by increased fecundity. Thus, some, but not all, of the morphological clines observed involved accommodations of life-history shifts.

LIFE HISTORIES AND BODY SHAPE AS A FUNCTION OF PRODUCTIVITY AND DENSITY

Both habitat productivity and population density had a strong influence on aspects of body shape and life-history divergence. In agreement with our a priori predictions, body size and fat content were greater in habitats with higher productivity, and females, but not males, also had deeper bodies at sites with higher productivity and lower population densities. These patterns are congruent with studies on the influence of resource availability in other fishes (e.g. Trinidadian guppies, *Poecilia reticulata*: Reznick & Yang, 1993; three-spined sticklebacks, *Gasterosteus aculeatus*: Spoljaric & Reimchen, 2007; Atlantic mollies, *Poecilia mexicana*: Riesch *et al.*, 2016). However, we found no support for our prediction regarding increased investment into reproduction and fecundity in habitats with higher productivity, and the pattern for lean weight was only suggestive.

We further predicted that high densities should result in larger body size, lower fecundity, larger offspring size at birth and reduced investment in reproduction (Pianka, 1970; Weeks, 1993; Reznick *et al.*, 2002), while high-density fish should also have more shallow body shapes (Landy & Travis, 2015). However, our data only strongly supported the prediction for lower reproductive investment and shallower bodies at higher densities in females, while support for fecundity was merely suggestive. We found no evidence for increasing offspring size with increasing population density, and the pattern for body size in females (but not males) was opposite to our prediction, with larger size at lower population densities, potentially reflecting larger size at sites with reduced intraspecific competition for resources.

Regarding our predictions for responses to productivity and population density that were not supported, we propose several, not mutually exclusive explanations. First, some population-specific patterns in fecundity and offspring size, as well as male body shape, might have largely been driven by population-specific habitat characteristics that were not quantified in our present study. In particular, reproductive life histories and body shape in livebearing fishes are known to be strongly shaped by differential predation regimes (life histories, guppies: Reznick & Endler, 1982; *Brachyrhaphis rhabdophora*: Johnson & Belk, 2001; *Gambusia hubbsi*: Riesch *et al.*, 2013; body shape, *G. affinis*: Langerhans *et al.*, 2004; *G. hubbsi*: Langerhans *et al.*, 2007), so some of the noise in our dataset is probably due to differences in predation pressures and other (not quantified) habitat characteristics (e.g. more precise estimates of productivity or population density measured over multiple time points). Second, different selective agents were predicted to select for different (often opposite) life histories and body shapes (Table 1). Hence, some of the different selective agents might have cancelled each other out, or, potentially synergistic effects of certain combinations of selective agents might have resulted in somewhat different patterns of divergence from population to population (e.g. Langerhans & Riesch, 2013). For example, based on latitude/climate, population 3 (Suffolk, Virginia) was predicted to exhibit a relatively high fecundity, but based on productivity and population density, a relatively low fecundity was predicted. What we found was relatively low fecundity at that site compared to other populations, suggesting that selection by the combination of productivity and population density might have outweighed selection due to latitude/climate (non-additive interaction of selective agents; see OSM Tables A1, D2). On the other hand, all quantified selective agents were expected to select for high fecundity in population 2 (Rehoboth Beach, Delaware), and indeed that population exhibited by far the highest fecundity of all sampled populations (OSM Tables A1, D2). Moreover, note that this population also exhibited extremely low allelic richness for the microsatellites we analysed (OSM Table C1), suggesting that the population might have undergone a recent population bottleneck. This would further facilitate an *r*-selected life-history strategy (Pianka, 1970; Reznick *et al.*, 2002).

Finally, we cannot fully discount the fact that our point-estimate for population density was relatively crude and might not have fully represented the actual population density at each site, because it relied largely on indirect density estimates at the exact point of sampling rather than a direct density-sampling approach of the whole habitat. A similar point can be made for our point-estimate of productivity. This could have resulted in some non-representative density estimates, for example, in generally low-density habitats in which we by chance happened to sample a high-density patch, or vice versa.

CONCLUSIONS

We were able to identify thermal regime as the primary driver of organismal (in our case fish) phenotypic responses along latitudinal gradients, particularly patterns of adult and offspring size, investment in reproduction, and some aspects of body morphology. With respect to North American freshwater fishes, this is one of the first studies to describe body size patterns following Bergmann's rule, as well as other large-scale phenotypic patterns in responses to different environmental variables. However, our study also highlights how multivariate phenotypes are often the result of multifarious selective regimes, which does not always make it easy to fully identify the respective selective forces shaping patterns of differentiation in individual traits. Furthermore, different traits and trait suites may covary so that a response to selection in one trait could lead to a correlated response in another trait, potentially suggesting a direct response to selection when in fact there is none (e.g. Losos, 2011), or even resulting in phenotypes contrary to what would be expected based on certain selective pressures (e.g. Langerhans & Riesch, 2013). The picture becomes even more complex in light of a recent study by Fischer *et al.* (2016), who demonstrated that trait correlations are not necessarily stable in space or time, so that certain responses to a given set of environmental variables might not be replicated in other, similar, environments. In fact, certain population-specific phenotypic patterns might be driven by unquantified selective forces, which could mask or even override phenotypic responses to measured environmental characteristics. Any combination of these factors could lead to over- or underestimation of phenotypic patterns along large-scale environmental gradients, which reinforces the need to evaluate habitats in more multivariate terms when trying to match phenotypes to certain habitat characteristics.

Even though we observed some strong patterns consistent with a priori predictions, our ability to accurately predict phenotypic differentiation across environmental gradients was relatively low overall in this study, with 33% correct predictions for males and 47% for females (42% and 65%, respectively, if we include the suggestive trends). This is a lower predictive ability than some recent studies have reported based on analyses of dozens of evolutionary studies (e.g. Langerhans, 2017; Oke *et al.*, 2017). This suggests that at this broad geographical scale, traits may be influenced by such a range of factors – which can have conflicting effects on traits – that identification of the most important selective agents for each trait becomes increasingly difficult (Moore *et al.*, 2016). Also, contrary to those prior studies, some traits in *G. holbrooki* even exhibited significant patterns opposite to predictions, indicating that we have a much better understanding

of adaptive differentiation for some traits (e.g. size, RA and offspring size) than for others.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table A1. Summary of collection localities, water chemistry, population density estimates and sample sizes for life-history (numerator) and morphology (denominator) datasets.

Table B1. Principal components analysis of environmental variables associated with populations of *Gambusia holbrooki* sampled across a latitudinal gradient along the Atlantic Coast of the USA.

Table C1. Genetic diversity in Eastern mosquitofish (*Gambusia holbrooki*). For each population and locus, observed (H_O) and expected (H_E) heterozygosities and allelic richness (A) are given. Zero values indicate that the locus is monomorphic in this population; for population information, please refer to Table 1.

Table C2. Pairwise genetic divergence among ten populations of *Gambusia holbrooki*. Above the diagonal are F_{ST} distances calculates using FSTAT (Goudet, 2001), while below the diagonal are F_{ST} distances calculated in FreeNA (Chapuis and Estoup, 2007) using the ENA method, which considers potential null alleles. For population information, please refer to Table 1.

Table C3. Pairwise Nei's D_A distances among ten populations of *Gambusia holbrooki* (Nei *et al.*, 1983). For population information, please refer to Table 1.

Figure C1. Population assignment using STRUCTURE version 2.3.4104. $K = 2$ was recovered as the most likely number of genetic clusters; for population information, please refer to Table 1.

Figure C2. Neighbour-joining tree based on Nei's D_A distances among ten populations of *Gambusia holbrooki* (Nei *et al.*, 1983). For population information, please refer to Table 1.

Figure C3. Relationship between allelic richness (A) and latitude (Pearson's $r = -0.66$, $P = 0.038$).

Table D1. Descriptive life-history traits (mean ± SEM) for 159 male eastern mosquitofish (*Gambusia holbrooki*) from ten populations along the US Atlantic Coast, spanning > 14 degrees of latitude.

Table D2. Descriptive life-history traits (mean ± SEM) for 223 pregnant female eastern mosquitofish (*Gambusia holbrooki*) from ten populations along the US Atlantic Coast, spanning > 14 degrees of latitude.

Table E1. Summary of best-fitting models and model-averaged coefficients (β) and standard errors (SE) for our tests of environmental drivers (PC1–4) of life-history trait variation in *Gambusia holbrooki*.

Table E2. Summary of best-fitting models and model-averaged coefficients (β) and standard errors (SE) for our tests of environmental drivers (PC1–4) of body shape variation in *Gambusia holbrooki*.

Figure F1. Visualization of body shape variation (based on thin-plate spline transformations for each sex) along divergence vectors (**d**) that exhibited associations with latitude that were explained by covariation with life-history traits (see main results).

SHARED DATA

The data associated with this article can be found on Dryad (Riesch *et al.*, 2018a).