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RESEARCH ARTICLE

Plasticity and evolution shape the scaling of metabolism and excretion along a geothermal temperature gradient

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Abstract

1. Physiological rates are heavily dependent on temperature and body size. Most current predictions of organisms' response to environmental warming are based on the assumption that key physiological rates such as metabolism and excretion scale independently with body size and temperature and will not evolve. However, temperature is a significant driver for phenotypic variability in the allometric scaling and thermal sensitivity of physiological rates within ectotherm species, suggesting that evolution may play a role in shaping these parameters.
2. We common-reared six populations of western mosquitofish that have recently established (~100 years ago) in geothermal springs along a broad thermal gradient (19–33°C) to determine whether these scaling parameters are affected by evolutionary and/or plastic responses to warming over ecological timescales. Each population was reared at four different temperatures (23, 26, 30 and 32°C). We measured routine metabolic and nitrogen excretion rates on mosquitofish across a wide body size range.
3. We found evidence for plasticity, but not evolution, increasing the allometric scaling of metabolic rate with temperature. Plasticity in metabolism allometry reflected a decrease in thermal sensitivity at smaller body sizes.
4. We found evidence for evolution of phenotypic plasticity on the allometry of excretion rate, reflecting evolutionary differences in how thermal sensitivity varies with body size across different populations.
5. Evolutionary differences in excretion rate scaling did not influence the relationship between excretion and metabolism across rearing temperatures, suggesting that warming does not affect the balance between mosquitofish energy demands and nutrient recycling rates.

KEYWORDS
allometric scaling, common rearing, evolution, excretion rate, *Gambusia*, metabolic rate, phenotypic plasticity, thermal sensitivity

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1 | INTRODUCTION

Ecological responses to environmental warming depend on organisms' physiological characteristics and the extent to which these can change in response to increasing temperature (Chown et al., 2010). Beneficial physiological responses can be achieved via two interacting mechanisms: adaptive phenotypic plasticity and evolutionary adaptation (Ghalambor et al., 2007; Stamp & Hadfield, 2020). Plasticity can allow organisms to cope with warming (Crozier & Hutchings, 2014; Merilä & Hendry, 2014), but if temperature rises beyond the range of existing adaptive plastic responses, population persistence may depend on the potential for rapid evolutionary change (Visser, 2008). Moreover, even within the thermal window for persistence, ongoing evolutionary adaptation may tune physiology in a manner that alters population ecology and species interactions (Hendry, 2016; Nadeau & Urban, 2019). In this way, the ecological outcomes of warming rest on a combination of plastic and evolved changes of key physiological traits (Levis & Pfennig, 2020; Price et al., 2003).

Physiological processes are heavily influenced by body size and temperature. Of the physiological processes that shape organism performance and function, metabolism is one of the strongest predictors of changes in population dynamics, community composition and ecosystem processes as climates warm (Bruno et al., 2015). Metabolic rate dictates energy demand, influencing food intake and top-down processes (Carr & Bruno, 2013; Jochum et al., 2012). Thus, the body size and temperature dependence of metabolism and associated physiological processes are fundamental parameters in many models attempting to predict the ecological outcome of environmental warming (Bruno et al., 2015; Schramski et al., 2015; Sentis et al., 2017). Most models are based on the assumptions of the metabolic theory of ecology (MTE), which predicts that metabolic rate B scales exponentially with temperature and hypo-allometrically with body size, according to $B = b_0 M^{\alpha} e^{-E/kT}$, where b_0 is a normalisation constant, M is mass, α is the allometric scaling exponent (i.e. body size dependence), E is the activation energy (i.e. thermal sensitivity or temperature dependence), k is Boltzmann's constant and T is temperature (Brown et al., 2004; Gillooly et al., 2001). The MTE assumes that metabolism scales independently with body size and temperature, which can be problematic, as it disregards the interactive influence of body size and temperature on metabolic scaling, as well as the potential for plasticity and evolution shaping metabolic scaling parameters (Lindmark et al., 2018; Moffett et al., 2018). Counter to MTE predictions, the effects of size and temperature on metabolism may not be independent (Glazier, 2005; Killen et al., 2010; Lindmark et al., 2018). For example, positive relationships between α and temperature have been observed in mosquitofish (Moffett et al., 2018), black porgy (Li et al., 1999) and some intertidal gastropod species (Huebner, 1973; Newell, 1973), among others (Glazier, 2005). In these cases, warmer temperatures are associated with α closer to one, meaning that as temperature rises, metabolic rate per unit of mass becomes more similar across the body size range (Moffett et al., 2018). The opposite pattern has also been found in several aquatic invertebrate and fish species (Glazier, 2005; Killen et al., 2010; Ohlberger et al., 2012).

Most studies on the size dependence and temperature dependence of metabolism and associated rates have been examined at the interspecific level. The allometric slope of metabolism (α) can evolve across species, commonly decreasing with the temperature at which different species were assayed (Glazier, 2005; Rubalcaba et al., 2020; White et al., 2019). Although intraspecific variability in the size scaling and thermal sensitivity of metabolic rate has been examined within several species, it is difficult to conclusively determine whether it results from phenotypic plasticity and/or evolutionary change at the intraspecific level (Fossen et al., 2019; Glazier, 2005; Moffett et al., 2018; Nespolo, 2003; Réveillon et al., 2019). Populations from warmer environments commonly display metabolic rates below expectations, suggesting that evolutionary adaptation can reduce E ('countergradient variation', 'metabolic cold adaptation hypothesis'; Pilakouta et al., 2020; White et al., 2012; although see Alton et al., 2017). Studies of fish species have recently demonstrated that metabolic reduction at warmer temperatures can arise over few generations (<100 years), but these studies have not assessed the relative contribution of evolutionary change versus plasticity in shaping these patterns (Moffett et al., 2018; Pilakouta et al., 2020).

Along with metabolism, the excretion of nutrients in physiological waste is a key process by which consumers can influence ecological processes. In particular, excretion can change the availability of nutrients at the base of aquatic food webs, influencing ecosystems from the bottom-up via nutrient subsidy (Vanni, 2002). Metabolic demands drive food acquisition and processing; thus, it is reasonable to expect nutrient excretion rates to scale similarly to metabolism with body size and temperature. However, α of excretion rate tends to be more variable than α of metabolism, as factors such as body stoichiometry, food composition and starvation period can also influence excretion rate's scaling (Hall et al., 2007; Hirst et al., 2017; Vanni & McIntyre, 2016). Given that the biomass and abundance of organisms in ecosystems can depend on the balance between energy demands and recycling rates (O'Gorman et al., 2012), a key question is how the relationship between excretion and metabolism is affected by temperature, and how plastic and evolutionary responses in either rate can exacerbate or mitigate the effect of warming.

We disentangled the plastic and evolutionary responses of metabolic and nutrient excretion rates to warming in a model organism, the western mosquitofish *Gambusia affinis*. We examined six recently established (~100 years) mosquitofish populations in geothermal springs that span a broad temperature gradient (19–33°C). Prior field surveys of these populations have shown that body sizes decline and allometric slopes (α) of metabolism rise in the populations from low to high temperature (Moffett et al., 2018). Furthermore, common-rearing experiments revealed that somatic growth rates evolved in a countergradient fashion such that populations in warmer springs have evolved reduced adult body size (Fryxell et al., 2020). Although evolutionary influences on metabolic scaling have not been tested, growth rates and metabolic rates are interdependent (Kerkhoff, 2012), so the countergradient evolution of growth may reflect a similar pattern in metabolism. On the other hand, excretion rate may show the opposite pattern to growth, as slower growing fish tend to excrete more nutrients than fish with faster growth rates because they retain less nitrogen for protein synthesis and growth (Bucking, 2017).

We common reared fish from each population at four different rearing temperatures (23, 26, 30 and 32°C) to test for the relative contribution of evolutionary change and plasticity to shaping metabolic and excretion scaling parameters and the relationship between the physiological rates. If patterns found in the wild result primarily from phenotypic plasticity alone, we expected to see a similar effect of rearing temperature on metabolic and excretion scaling parameters across all populations. However, if evolution is involved, we expected some degree of differing allometric responses across populations, consistent with the previously observed patterning with temperature in the wild as described above (Moffett et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Study organism and focal populations

The western mosquitofish *Gambusia affinis* is a small (<6 cm), livebearing fish native to the southern United States and northern Mexico. It was introduced to diverse environments worldwide for mosquito biocontrol throughout the 20th century, creating a natural experiment in phenotypic and genotypic responses to environmental changes (Fryxell et al., 2021). Mosquitofish are thermal generalists, capable of growth and reproduction at water temperatures ranging from 16 to 35°C, but also tolerant of short-term exposures to temperatures between 0 and 45°C (Pyke, 2005, 2008; Wood et al., 2020).

Here, we studied mosquitofish populations that have recently invaded geothermal springs in California. In 1922, mosquitofish were taken from 1–2 localities in Texas, stocked into a single pond in California, and systematically spread across the state through a series of transplantations (Dill & Cordone, 1997). Our focal populations occur in six thermally stable geothermal springs in Inyo and Mono Counties, with temperatures ranging from 19 to 33°C. These populations experience relatively constant temperatures around the year (Figure 1) and have natural or human-made barriers to dispersal among habitats with different temperatures (Fryxell et al., 2020). In addition, the springs display similar water chemistry characteristics, are geographically close to each other (maximum distance between sites ~70 km, Figure 1) and do not contain other piscivorous fishes, making them an ideal system to study evolutionary responses to temperature without typical confounding factors along other thermal gradients (Fryxell et al., 2020; Table S1).

2.2 | Field collection and common rearing

We collected fish from the wild, transported them to controlled environment rearing facilities, and then performed common rearing for two generations. Fish collections were approved by our institutional animal ethics committee (UCSC protocols PALKE-1311 and PALKE-1801) and the local wildlife agency (CADFW permit SC-12752). Collection and rearing protocols are described in detail in Fryxell et al. (2020). Briefly, we collected adult mosquitofish from the six populations using hand nets in February 2018. We transported these F0 (wild-caught generation) fish to an environment-controlled greenhouse at UC Santa Cruz, California. F0 fish from different populations were reared separately in 568 L tanks heated to the intermediate temperature of 26°C (i.e. populations were in separate tanks). After 1 month, we began collecting offspring (i.e. F1 generation fish). Groups of newborn F1 fish

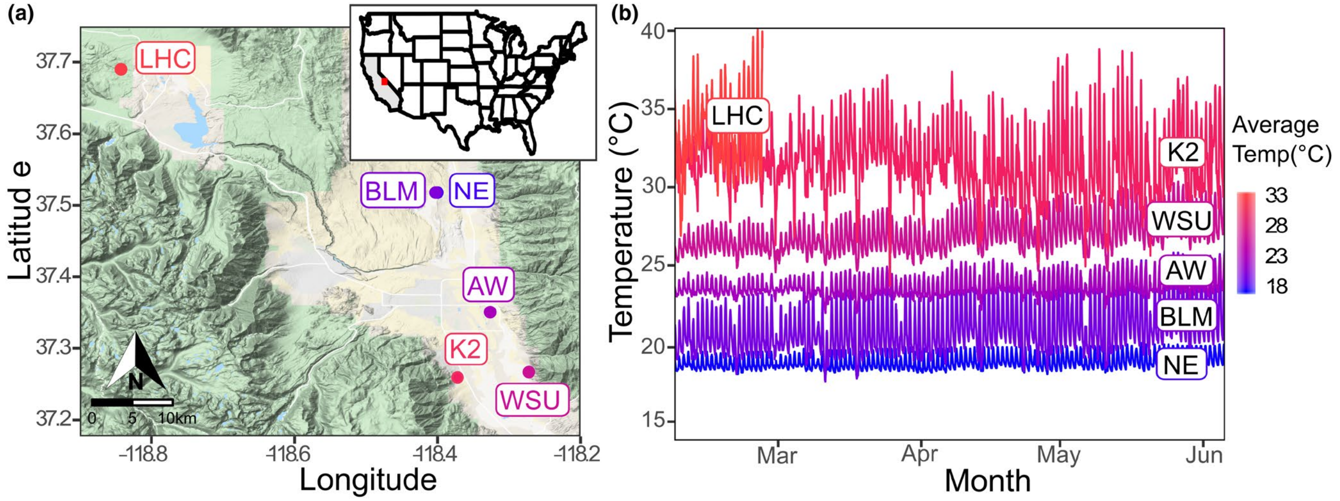

FIGURE 1 Study sites. (a) Map of the geothermal spring sites (eastern California); colours correspond to the temperature gradient. (b) Temperature profile of each geothermal spring logged at 15-min intervals over springtime 2014. The warmest site temperature logger ('LHC', 33.3°C) failed on February 25

from the same population and day of birth were reared together in mesh baskets hung in 57 L tanks in the same room at 26°C. When we had collected at least 90 F1 fish per population, F0 fish were euthanised, and F1 fish were introduced to the 568 L tanks after they had been drained, cleaned and refilled, again with populations in separate tanks. F1 fish were grown to maturity, and their offspring (F2 generation) were collected over several months. F0 and F1 fish were fed a controlled combination of flake food and pellets 2× daily throughout rearing.

We transferred F2 fish on their day of birth into controlled environment rooms (R.W. Smith & Co) for rearing at one of the four treatment rearing temperatures (23, 26, 30 and 32°C), which spanned most of the natural temperature range experienced by these populations. F2 fish were assigned a unique ID number and reared individually inside mesh cylinders with a petri dish bottom and an open-top (250μm mesh, 7cm diameter, 20cm height) such that F2 had no contact with each other during the common rearing. Cylinders were sunk upright into 100 L plastic tubs (91 × 61 × 20 cm) filled with off-gassed city water. Tubs were allocated between two controlled environment rooms. In one room, we set air temperature to 23°C and heated tubs to 23, 30 and 32°C. In the other room, we set air temperature to 19°C and heated tubs to 26°C. Tubs in each room were randomly assigned rearing temperatures, and each temperature treatment had five replicate tubs. F2 fish from a given population were assigned treatment temperatures sequentially as they were born to equalise sample sizes across rearing temperatures for each population. Fish were assigned to replicate tubs to minimise tub differences in fish density through time. Thus, each tub had similar densities of fish from various populations and birth dates. F2 fish were fed an excess of Frystartr food (Skretting, Stavanger, Norway) three times daily. Metabolism and nutrient excretion assays were performed on F2 fish after being reared between 12 and 118 days, as we needed to measure fish at a range of body sizes to determine allometric scaling coefficients. A different number of fish were assayed per population, depending on fish availability for each population (Table S2).

2.3 | Metabolism and nutrient excretion assays

We measured routine metabolic rate (RMR) as oxygen consumption (MO_2) of individual fish (Sinclair et al., 2006), using the same experimental protocol used to measure the field metabolic rates of these populations in the wild (Moffett et al., 2018). Routine and field metabolic rates are comparable and are more ecologically relevant than basal metabolic rates because they are measured under normal activity, including spontaneous movements (Clarke, 2017; Hudson et al., 2013). We used four closed-system respirometers that consisted of 40 ml rectangular acrylic chambers fitted with an optical sensor to measure dissolved oxygen concentration (DO) and temperature. Sensors were monitored with a FireSting four-channel oxygen logger (PyroScience, Aachen, Germany), allowing us to run the four respirometers simultaneously. Nitrogen excretion rate was estimated as change in ammonia (NH_4^+–N) concentration in the closed respirometers over the metabolism assays, as described in Moffett et al. (2018).

Metabolism and nutrient excretion assays were performed over 5 weeks between September 11 and October 20 2018. Individual fish were assayed at the temperature at which they were reared. Each week, we conducted assays on fish from the four temperatures with one temperature assayed per day. The order in which fish from different temperatures were assayed was randomised each week. Before each measuring day, we selected 20–30 mosquitofish of different populations and body sizes reared at a given rearing temperature. We attempted to balance the number of fish assayed per origin population on each day, but sample size differences arose due to population differences in reproductive rates. Food digestion can increase fish MO_2 (Chabot et al., 2016). Mosquitofish digestion time can vary with temperature (Pyke, 2005); thus, selected fish were individually starved for 16 hr before assays. To do so, selected fish were held individually in 475 ml plastic cups placed inside a 40 L plastic tub (69 × 47 × 22 cm) filled with water at the selected rearing temperature to ensure fish guts were empty during the assays. Measurements were performed inside a 95 L 'assay' bin of dechlorinated city water heated to the appropriate temperature and aerated overnight with an aquarium bubbler. Assay water was changed daily.

To start assays, respirometers were filled with water from the assay bin, a single fish was added to each respirometer, and chambers were sealed and placed inside the assay bin to keep temperature constant. We allowed fish to settle inside the respirometers for a few minutes before monitoring DO consumption for 10–15 min, with duration adjusted to result in a similar magnitude of DO change across assays. When fish were large enough to mix water inside chambers with their fin movements, we used the slope of the linear declines in oxygen over time ($r^2 > 0.9$; Clark et al., 2013; Moffett et al., 2018). In the case of some small juveniles, it was necessary to mix the water in the chambers at the end of the run, and the difference between initial and final DO concentration divided by time in the chamber was used instead of linear relationships (59% of measurements). Each day we ran two blanks at the beginning and the end of the assays. The MO_2 of blanks was similar at the start and the end of assays, so we averaged all blanks and subtracted the value from fish MO_2. Routine metabolic rate was calculated as μmol O_2/min.

For excretion rate measurement, we took a 5 ml water sample from each chamber at the end of each metabolism run, filtered it through a 0.7 μm glass microfiber filter (GF/F) and stored it on ice inside a 15 ml tube. We then euthanised fish to obtain blotted wet weight measurements at the end of each measuring day. Each week after the four metabolism measuring days, NH_4^+ concentration was measured by fluorimetry (Holmes et al., 1999) using a Trilogy Laboratory Fluorimeter (Turner Designs). To calculate NH_4^+ excretion rates, we subtracted the NH_4^+ concentration of blanks from NH_4^+ concentration in respirometers with fish and divided it by the total time fish spent in the chamber. The NH_4^+ concentrations in blanks increased linearly across runs in a day, so we estimated the

NH_4^+ concentration of a blank for each run using a linear regression between the NH_4^+ concentration of the blanks in the first and the last runs of each measuring day (Moffett et al., 2018). Fish with highly fluctuating MO_2 traces were excluded from analyses; thus, a total of 415 fish were analysed for RMR, and 452 fish were analysed for excretion rate.

2.4 | Statistical analyses

First, we examined the allometric relationships of RMR and excretion rate with body mass across all individuals, not accounting for origin temperature (evolved differences) or rearing temperature (phenotypic plasticity), by calculating simple linear regression models on ln-transformed data. Second, we assessed the effects of origin and rearing temperatures on allometric scaling (α) of physiological rates by fitting OLS regression models. We started with the full model specification: *ln(rate) ~ ln(mass) + RearingTemp + OriginTemp + ln(mass) × RearingTemp + ln(mass) × OriginTemp + RearingTemp × OriginTemp + ln(mass) × RearingTemp × OriginTemp*. The most appropriate set of predictors was selected by sequentially removing individual terms from full models and comparing the resulting reduced models using log-Likelihood ratios tests (χ^2). A significant interaction term between mass and rearing temperature would indicate broadly shared plastic effects of rearing temperature on α (i.e. a common pattern of change in allometric scaling with temperature across common-reared populations). An interaction between mass and origin would suggest an independent effect of evolution on α. Finally, a significant interaction between mass, rearing temperature and origin temperature would support that the plastic effects of rearing temperature on α are themselves mediated by heritable evolution (evolution of plasticity; Table 1).

Prior work in the field (Moffett et al., 2018) found that thermal sensitivity (as activation energy, *E*) of metabolism increased with body size in mosquitofish. We explored this for RMR and excretion rate by estimating Arrhenius relationships between ln-transformed rates and rearing temperature (expressed as $1/kT$; Brown et al., 2004) at three different body sizes (minimum, mean and maximum mass values in the datasets). The ln-transformed rates were predicted for each body size at each rearing temperature using the coefficients from the previously estimated reduced models. We calculated these Arrhenius relationships at each origin temperature in the case of evolutionary differences in the allometry of the rates across origin temperatures.

We estimated the slopes of the relationship between ln-transformed excretion rate and RMR on all assayed fish pooled together and on fish divided by origin and rearing temperatures to test how temperature affects the relationship between nutrient excretion and metabolic rate. We assessed the effect of origin and rearing temperatures on the slopes of the excretion versus metabolism relationships by fitting an OLS regression model with the full model specification: *Slope ~ RearingTemp + OriginTemp + RearingTemp × OriginTemp*.

All statistical analyses were performed in R v3.6.2 (R Core Team, 2020). The OLS regression models were fitted using R's lm() function. For the model selection process, log-Likelihood ratios tests (χ^2) were calculated with the lrtest() function of the LMTEST v0.9-37 package (Zeileis & Hothorn, 2002). Final model assumptions were verified by plotting residuals versus fitted values and versus each covariate in the model, whereas we calculated the variance inflation factor (VIF) to assess collinearity among covariates using the vif() function of the CAR v3.0-8 package (Fox & Weisberg, 2019). The VIF values for ln(body mass), rearing temperature and origin temperature were lower than the cut-off value of 5 recommended for removing collinear variables in a model (Gareth et al., 2013). We used the summary() function to extract the coefficients of the reduced models, and the function predict() to predict log-transformed rates and ±95% confidence intervals at given body masses, rearing temperatures and origin temperatures. α and *E* estimated at specific rearing temperatures and body sizes were extracted from the allometric and Arrhenius relationship using the emtrends() function of the EMMEANS v1.4.7 package (Lenth, 2020).

TABLE 1 Terms included in the full OLS models used to assess the effects of origin and rearing temperatures on the scaling of metabolic and excretion rates. Interpretations and expectations of what each would be evidence for if significant

Model term	Interpretation	Pattern/expectation
ln(mass)	Mass-scaling of the physiological rate	0.75 power function
RearingTemp	Effect of temperature on the mean physiological rate	Rate intercept increases with rearing temperature
OriginTemp	Effect of evolution on mean physiological rate	Rate intercept differs between with origin temperature
ln(mass) × RearingTemp	Plasticity of mass-scaling of the physiological rate	α scales with rearing temperature as in Moffett et al. (2018)
ln(mass) × OriginTemp	Evolution of mass-scaling of the physiological rate	α scales with origin temperature as in Moffett et al. (2018)
RearingTemp × OriginTemp	Effect of temperature on mean physiological rate shaped by evolution	Warmer populations show less temperature sensitivity
ln(mass) × RearingTemp × OriginTemp	Evolution of plasticity of mass-scaling of the physiological rate	Rearing and origin temperatures interaction results in differences in α similar to Moffett et al. (2018)

3 | RESULTS

Across all fish combined, RMR increased with mass ($\alpha = 0.76 \pm 0.06$) consistent with MTE predictions ($R^2 = 0.58$, $p < 0.001$). The model selection process showed no effect of origin temperature or its interaction terms with body mass and rearing temperature on RMR, suggesting a common pattern in metabolic scaling across populations (Figure 2). Instead, the best model included body mass, rearing temperature and their interaction. The significant interaction between mass and rearing temperature ($p = 0.049$), and lack of a population effect on metabolic rate, indicated that variation in α was a plastic response to rearing temperature (Tables S3 and S4). Thus, fish from different origin temperatures were pooled to estimate α and thermal sensitivity (E) of RMR. We found α rose from 0.60 to 0.76 across the rearing temperature gradient (Table S5; Figure 3a). Consequently, the predicted E of RMR differed with body size, rising from 0.17 to 0.68 eV from the smallest to the largest body size value in the dataset (Table S6; Figure 3b).

The α of excretion rate across all fish was steeper ($\alpha = 0.82 \pm 0.06$; $R^2 = 0.60$, $p < 0.001$) than that observed for RMR ($\alpha = 0.76 \pm 0.06$). In contrast to RMR, the best model for excretion rate included the three-way interaction between mass, rearing temperature and origin temperature, indicating plastic and evolutionary differences in the allometry of excretion rate (log-likelihood = −323.80; $\chi^2 = 6.58$; $p = 0.010$). Allometry of excretion rate was examined separately at each rearing and origin temperature, as all terms in the full model affected excretion rate (Table S7), reflecting differences in the response of α to rearing temperature across origin temperatures (Figure 4). Notably, the pattern of change in α of excretion with rearing temperature reversed across the gradient of population origin temperature (Table S8; Figure 4a). For the coldest population, α decreased with rising rearing temperature, but the pattern reversed across the gradient of origin temperature, with the warmest population exhibiting an

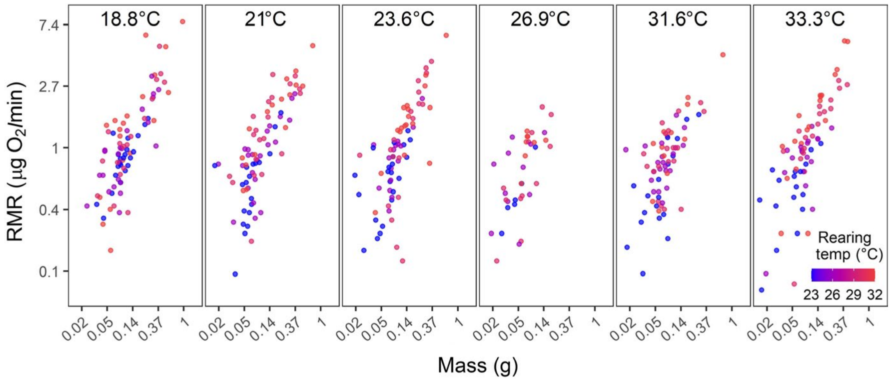

FIGURE 2 Allometry of RMR of each population (panels, origin temperature shown) at each rearing temperature (colours). Each point represents one fish measurement. No prediction lines were estimated across populations, as the model selection process showed no effect of origin temperature or its interaction terms with body mass and rearing temperature on RMR. Mass and RMR are plotted on the ln scale

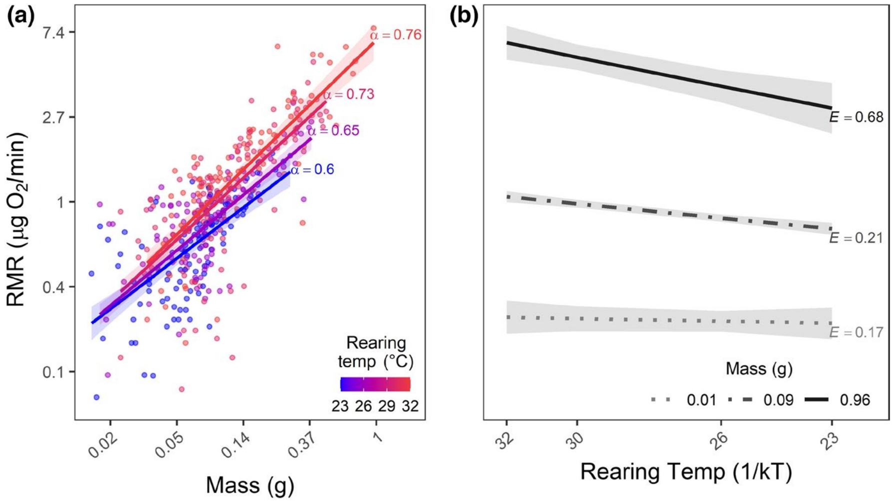

FIGURE 3 Allometry of RMR at each rearing temperature and temperature dependence (E) by body size, based on data pooled across populations. (a) RMR allometric relationships, prediction lines were estimated at each rearing temperature (colours). Each point represents one fish measurement. Light shading shows 95% confidence intervals of regressions. (b) Predicted Arrhenius relationships, 95% confidence intervals and E for different size classes across rearing temperatures. Mass and RMR are plotted on the ln scale. Temperature is reversed to display data in an Arrhenius format

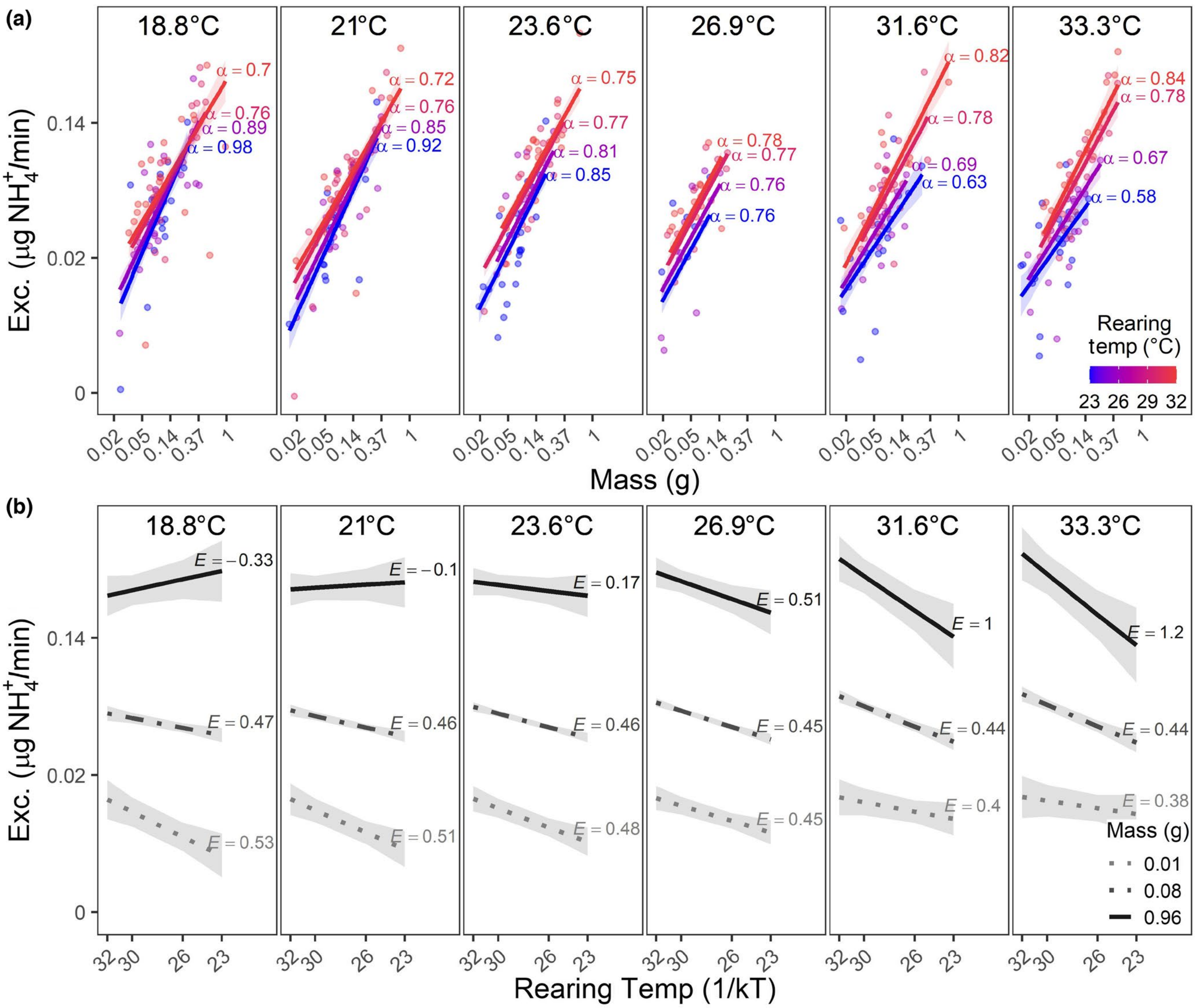

FIGURE 4 Allometric relationship and temperature dependence of excretion rate calculated at different origin temperatures. (a) Excretion rate allometric relationship (panels, origin temperature shown), prediction lines were estimated at each rearing temperature (colours) and origin temperature (panels). Each point represents one fish measurement. Light shading shows 95% confidence intervals of regressions. (b) Predicted Arrhenius relationships, 95% confidence intervals and *E* for different size classes across rearing and origin temperatures. Mass and excretion rate are plotted on the ln scale. Temperature is reversed to display data in an Arrhenius format

increase in α with increasing rearing temperature. Thus, the pattern of the predicted *E* at different body sizes changed across origin temperatures. In particular, *E* of large fish increased and *E* of small fish declined as origin temperature rose (Table S9; Figure 4b).

Excretion rate scaled hypo-allometrically with RMR across all fish (slope = 0.74 ± 0.08, $R^2 = 0.47$, $p < 0.001$; Figure 5). The model selection process indicated no effect of rearing or origin temperature in the slope of the relationship between excretion rate and RMR, despite differences in α and *E* between rates (Tables S10; Figure S1).

4 | DISCUSSION

We common-reared six recently established geothermal populations of mosquitofish across four temperatures to disentangle plastic and evolutionary change in the allometry and temperature dependence of metabolic and nitrogen excretion rates. Previously, common rearing demonstrated that mosquitofish from these populations evolved slower juvenile growth rates and greater reproductive investment at small sizes in response to increasing temperature, contributing to reduced body sizes with warming (Fryxell et al., 2020). At the same time, metabolic rate measurements on these populations in the wild showed a positive linear relationship between the allometric scaling of metabolic rate and environmental temperature, reflecting an increasing thermal sensitivity of metabolism with increasing body size (Moffett et al., 2018). Our results indicate that the differences in metabolic scaling parameters are primarily due to a shared pattern of phenotypic plasticity, not genetic evolution. In contrast, we found evidence for the evolution of phenotypic plasticity in the scaling of excretion rate. Despite the different responses of both rates to temperature, the relationship between excretion and metabolism was not affected by rearing or origin temperature.

4.1 | Body size scaling and thermal sensitivity of routine metabolic rate

Across all individuals and rearing treatments, the allometry of metabolic rate followed MTE expectations of $\alpha \sim 0.75$ ($\alpha = 0.76 \pm 0.06$). However, when rearing and origin temperatures were taken into account, we found an increase in the allometric scaling of metabolism with rearing temperature, similar to what was found in the wild (Moffett et al., 2018). In other words, as temperature increased, fish of different body sizes had more similar metabolic rates per unit of mass. This increase in allometric scaling reflects a plastic increase in thermal sensitivity of metabolism with body size such that body size and temperature affected mosquitofish metabolism interdependently, contrary to MTE expectations (Glazier, 2005; Lindmark et al., 2018; Moffett et al., 2018). Although negative relationships between the allometric scaling of basal (or standard) metabolic rate and temperature have been found within and across multiple fish species, activity level can change these relationships (Glazier, 2020; Killen et al., 2010; Ohlberger et al., 2012; Rubalcaba et al., 2020). Routine metabolic rate measurements used in this study allowed for normal activity and spontaneous movement; thus, it may be more ecologically and evolutionary relevant for fish and other non-sessile organisms, better reflecting energetic demands experienced in nature (Hudson et al., 2013).

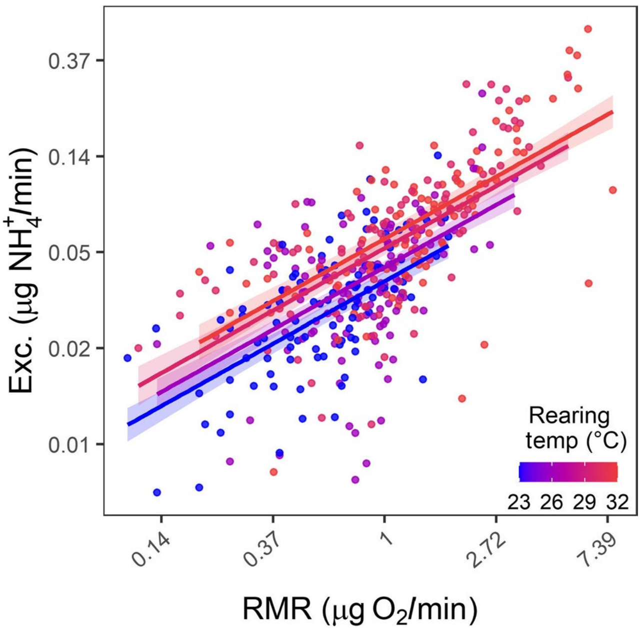

FIGURE 5 Relationship between excretion rate and RMR. Prediction lines were estimated at each rearing temperature. Each point represents one fish measurement. Light shading shows 95% confidence intervals of regressions. RMR and excretion rate are plotted the ln scale

Plasticity in metabolism allometry is common within species, and differences in allometric relationships are even found across species' ontogeny (Gaitán-Espitia et al., 2013; Glazier, 2006; Norin & Gamperl, 2018). High plasticity in metabolism's size dependence and temperature dependence may slow evolutionary change by shielding genes from natural selection (Price et al., 2003). Phylogenetic comparative studies suggest that body size and metabolic rate evolve in a correlated fashion across species (White et al., 2019). Therefore, both plasticity in the scaling of body mass and metabolic rate, and the genetic correlation between these traits, are likely to constrain the capacity of body size and metabolic rate traits to evolve independently, constraining the evolutionary potential of intraspecific allometric relationships at ecologically relevant timescales (Futuyma, 2010).

Although evolution did not directly affect metabolism allometric scaling, natural selection may operate indirectly on mosquitofish metabolism via its connection to body size. Per Fryxell et al. (2020), there is good evidence for a heritable component of early growth rates. Hence, metabolic phenotype differences among mosquitofish populations in the wild are likely shaped by a combination of the indirect evolutionary effects of growth rates interacting with local environmental (plastic) effects on the size dependence and temperature dependence of metabolic rate (Figure 6). Evolution towards smaller body sizes can help mosquitofish cope with the energetic constraints

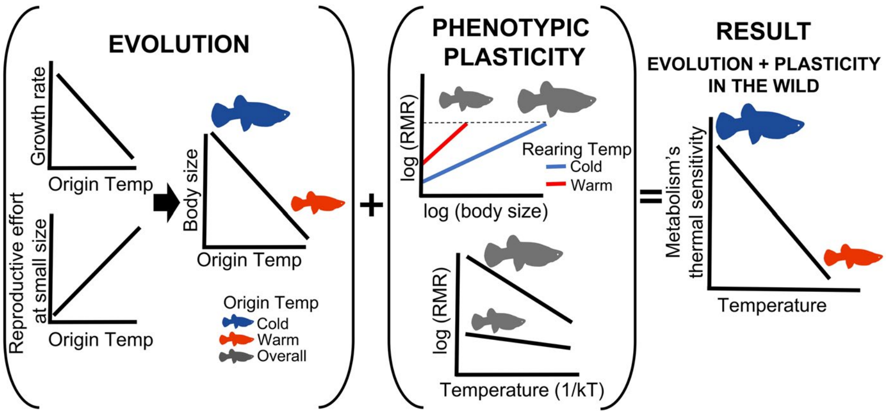

FIGURE 6 Conceptual diagram showing how evolution in body size (results from Fryxell et al., 2020) and plasticity in metabolic rate scaling parameters (results from this study) can interact to reduce metabolism's thermal sensitivity in the wild

imposed by higher temperatures by reducing whole-organism energetic demands. Additionally, our results suggest that reduced body sizes probably reduce metabolism thermal sensitivity for warmer populations (Brown et al., 2004; Fryxell et al., 2020; Réveillon et al., 2019; Riemer et al., 2018). The increase in the allometric scaling of metabolism could also facilitate the evolution of smaller body sizes even further, as it equalises size-specific metabolic rates of small and large individuals, potentially eliminating the advantage of higher energetic efficiency at larger body sizes (Lindmark et al., 2018; Moffett et al., 2018). We note that age likely plays into this process, as smaller fish tended to be younger. It is possible that the metabolism of young fish had a lower thermal sensitivity independently of body size because fish age can affect metabolic allometry. For example, Norin and Gamperl (2018) found that the size dependence of fish metabolism decreases with age. Ultimately, size and age are inextricably linked and difficult to disentangle.

4.2 | Body size scaling and thermal sensitivity of excretion rate

Contrary to metabolic rate, we found evidence for the evolution of phenotypic plasticity in the allometry of nitrogen excretion rate. Across all fish, the allometric scaling of excretion increased more than expected by MTE (α = 0.82 ± 0.06), but there was great variation in allometry across origin and rearing temperatures (α = 0.58–0.98). Field-tested mosquitofish also exhibited differences in the allometric scaling of excretion rate across springs, but these differences had no relationship with spring temperature and were probably driven by differences in diet composition across springs (Moffett et al., 2018, 2021; Vanni & McIntyre, 2016). Our results suggest a nonlinear response of excretion's allometric scaling to temperature, reflecting an increase in the thermal sensitivity of large individuals and a decrease in the thermal sensitivity of small individuals as origin temperature increases. Thus, the evolutionary decline of mosquitofish body size also results in a decline in the thermal sensitivity of excretion rate. Although our results suggest that plasticity and evolutionary adaptation can influence excretion rate's response to warming, most studies exploring excretion's thermal sensitivity are normally based on assays performed on fish from a single origin acclimated to different temperatures for short periods, ignoring the potential for local adaptation (Morgan et al., 2001; Wright et al., 2001).

Nutrient excretion rates can reflect how fish allocate resources between structural growth and other vital activities (Hirst et al., 2017; Wright et al., 2001). Nitrogen excretion rate reflects the portion of fishes' metabolic activity fuelled by proteins (Wright et al., 2001). As temperature decreases towards fishes' lower thermal tolerance limit, the proportion of metabolism supported by protein tends to decrease (Alsop et al., 1999; Hirst et al., 2017). Consistently, mosquitofish reared at colder temperatures tended to have lower nitrogen excretion rates than fish reared at warmer temperatures, except for large fish from colder origin temperatures, suggesting an enhanced cold tolerance in these fish. Large body sizes are associated with enhanced cold tolerance in mosquitofish (Wood et al., 2020). However, in large fish from the warmer populations, protein-fuelled metabolic activity decreased abruptly with decreasing rearing temperatures, potentially reflecting lower cold tolerances and suggesting that cold tolerance in mosquitofish involves other mechanisms besides increasing body size. On the other hand, fishes' nitrogen excretion rates and growth rates tend to exhibit a negative relationship, as slow-growing fish retain a lower proportion of consumed amino acids for protein synthesis and growth than faster growing fish (Bonaldo et al., 2011; Bucking, 2017). Consistently, fish from warmer populations had higher nitrogen excretion rates across all rearing temperatures at small body sizes, which might be driven by the evolutionary decrease in growth rates with origin temperature found in juvenile fish from these populations (Fryxell et al., 2020).

4.3 | Relationship between metabolism and excretion

Evolutionary differences in excretion rate scaling did not alter the balance between mosquitofish's energy demands and nutrient recycling rates. If consumers' energy demands and recycling rates scale in a coupled fashion, ecosystems may be able to maintain their current organism abundance and biomass despite increasing temperatures, as higher nutrient recycling rates can facilitate a faster turnover of primary producers to support consumers communities (O'Gorman et al., 2012). Across all fish, nitrogen excretion rate increased less than proportionally with metabolic rate (slope = 0.74 ± 0.08), similar to what was previously found in field-tested fish (slope = 0.85; Moffett et al., 2018). The similarity between results from field surveys and common-reared fish suggests that differences in the relationship between metabolism and excretion in the wild are driven by factors other than body size, temperature, food composition and starvation period (Carter & Brafield, 1992; Uliano et al., 2010; Vanni & McIntyre, 2016; Wright et al., 2001). The relationship between nitrogen excretion and metabolism may be influenced by physiological processes, like fish metabolising their glycogen and lipids reserves instead of protein after being subjected to short periods of starvation (Ferreira et al., 2019; Wright et al., 2001); and methodological limitations, as we did not measure nitrogen excretion in the form of urea, which can account for 10%–30% of teleost fish nitrogenous waste (Uliano et al., 2010).

5 | CONCLUSIONS

Plastic and evolved responses in physiological rates will influence the ecological effects of climate change. Results from this study and previous studies in this system indicate that a combination of body size evolution and phenotypic plasticity in metabolic scaling parameters can reduce populations' total energetic demands in the face of warming (Figure 6; Fryxell et al., 2020; Moffett et al., 2018). Furthermore, we found evidence for the evolution of phenotypic plasticity in the body size and temperature dependence of nitrogen excretion rate, potentially reflecting differences in the thermal tolerances of fish of different body sizes across origin temperatures. Evolutionary differences across populations highlight the importance of intraspecific variation in the scaling of physiological rates, as different populations may respond differently to changes in temperature. Finally, we found that the balance between nitrogen recycling rates and energy demand is not affected by temperature, suggesting that ecosystems may be able to maintain their current organism abundance and biomass in the face of warming. Overall, our findings suggest that ecological change caused by warming will probably depend on the interaction of contemporary evolution and plastic responses across multiple traits. Combining natural experiments with common-rearing experiments can help us parse the contribution of phenotypic plasticity and evolutionary change on each of these traits, improving our understanding of how species cope with rapid ecological change.

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CONFLICT OF INTEREST

We declare we have no conflict of interest associated with this publication.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study design; D.C.F. led the common rearing (with help from J.N.B.); J.N.B. conducted the metabolism and excretion assays (with help from D.C.F. and K.S.S.), performed the statistical analyses, and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

ANIMAL ETHICS

Fish collections were approved by our institutional animal ethics committee (UCSC protocols PALKE-1311 and PALKE-1801) and the local wildlife agency (CADFW permit SC-12752).

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.2280gb5ts (Benavente et al., 2022).

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