Multigenerational exposure to increased temperature reduces metabolic rate but increases boldness

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February 22, 2024

Abstract

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Running head: Thermal history reduces metabolic temperature sensitivity.

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metabolic rate, reducing the temperature sensitivity of metabolism, with concomitant reductions in boldness and activity. We compared the temperature sensitivity of metabolic rate (acclimation at 20 versus 30°C) and allometric slopes of routine, standard, and maximum metabolic rates, in addition to boldness and activity behaviours, across eight recently divergent populations of a widespread fish species (*Gambusia affinis*). Our data reveal that warm-source populations express a reduced temperature sensitivity of metabolics, with relatively high metabolic rates at cool acclimation temperatures and relatively low metabolic rates at warm acclimation temperatures. Allometric scaling of metabolism did not differ with thermal history. Across individuals from all populations combined, higher metabolic rates were associated with higher boldness and activity. However, warm-source populations displayed relatively more bold behaviour at both acclimation temperatures, despite their relatively low metabolic rates at warm acclimation temperatures. Overall, our data suggest that in response to warming, multigenerational processes may not direct trait change along a simple "pace-of-life syndrome" axis, instead causing relative decreases in metabolism and increases in boldness. Ultimately, our data suggest that multigenerational warming may produce a novel combination of physiological and behavioural traits, with consequences for animal performance in a warming world.

Keywords: Mosquitofish, Gambusia, metabolism, temperature, pace-of-life, thermal history.

Introduction

Warming is expected to increase minimum energetic requirements and thus metabolic rate (Gillooly, Brown, West, Savage, & Charnov, 2001; Brown, Gillooly, Allen, Savage, & West, 2004), potentially influencing ecologically important behaviours and the strength of top-down effects (Angilletta & Dunham, 2003; Gardner, Peters, Kearney, Joseph, & Heinsohn, 2011; Sibly, Brown, & Kodric-Brown, 2012; Holt & Jorgensen, 2015; Norin, Malte, & Clark, 2016). The effects of thermal change may be particularly pronounced in ectothermic species, where environmental temperature regulates body temperature. Within species, populations may respond differently to warming depending on their history of temperature exposure. For example, populations chronically exposed to elevated temperatures may exhibit altered metabolic and behavioural traits (Crozier & Hutchings, 2014). When challenged with warming environmental temperatures, these trait differences can arise quickly due to plasticity (i.e., within generation plasticity, developmental plasticity) and evolutionary adaptation, potentially mediating overall trait responses to warming (Fryxell et al., 2020; Pilakouta et al., 2020).

Population differences in the temperature dependence of metabolic rates are often studied using species distributed over altitudinal or latitudinal gradients, where adaptive change may occur over long periods (White, Alton, & Frappell, 2012; McKenzie, Estivales, Svendsen, Steffensen, & Agnese, 2013; Gaitan-Espitia & Nespolo, 2014). However, it is less clear that these differences in metabolism can arise over short timescales, as could be the case under current warming. Laboratory experiments demonstrate that metabolic traits can respond quickly to temperature (Alton, Condon, White, & Angilletta, 2017; Mallard, Nolte, Tobler, Kapun, & Schlotterer, 2018; Morgan, Finnoen, Jensen, Pelabon, & Jutfelt, 2020). Alternatively, geothermally heated habitats can offer valuable natural experiments that overcome the limitations of other natural thermal gradients and experimental approaches. For example, the use of geothermal or artificially heated waterways has recently demonstrated that long-term exposure (e.g., 1000's of years) to increased temperatures may reduce the temperature sensitivity of metabolism in freshwater fishes (Bruneaux et al., 2014; Pilakouta et al., 2020). Similar reductions in metabolic temperature sensitivity may occur over shorter time scales (e.g., 10's to 100's of years) congruent with current environmental warming (Sandblom et al., 2016; Moffett. Fryxell, Palkovacs, Kinnison, & Simon, 2018; White & Wahl, 2020). Such moderations in metabolic rate may be associated with changes to other ecologically significant traits, such as animal behaviour, but these connections are largely unknown.

Individual differences in baseline metabolic requirements may lead to consistent behavioural differences (Biro & Stamps, 2010). For example, individuals with high standard metabolic rates may also express high boldness, exploration, and activity (Biro & Stamps, 2010; Biro, O'Connor, Pedini, & Gribben, 2013; Bartolini, Butail, & Porfiri, 2015). As such, increased metabolic demand with rising temperature may be associated with an increased frequency of risk-taking behaviours to maximise energy intake (Mathot

& Dingemanse, 2015). However, selective environments may modify these plastic responses to warming. For example, if warming selects for a "fast" pace-of-life syndrome, individuals may evolve faster metabolic rates, faster maturation, and bolder behaviours. Alternatively, if warming selects for a "slow" pace-of-life syndrome (countergradient selection), then metabolism and boldness may be reduced, counteracting effects of thermal plasticity alone (Sih, Bell, & Johnson, 2004; Reale et al., 2010). Individual traits may also respond in different ways to increased temperature. For example, metabolic rate may decrease while boldness increases, indicating no pace-of-life syndrome trait change (Royaute, Berdal, Garrison, & Dochtermann, 2018; Morgan et al., 2020). Ultimately, our ability to predict the ecological consequences of warming hinges on understanding responses of a suite of ecologically relevant traits, including metabolism and behaviour.

Here, we use populations of a globally distributed freshwater fish, Gambusia affinis (hereafter Gambusia), to test how metabolism and behaviour are affected by multiple generations of recent (100 years) exposure to elevated temperature in natural ecosystems (Fig. 1). *Gambusiashow inter-individual and inter-population* variation in behavioural traits (Cote, Fogarty, Weinersmith, Brodin, & Sih, 2010; Polverino, Santostefano, Diaz-Gil, & Mehner, 2018) and make an ideal model organism as they have recently invaded geothermal habitats of various temperatures (Table 1) (Fryxell & Palkovacs, 2017; Moffett et al., 2018). Previous work examining in situ metabolic rates of Gambusia affinis populations across a geothermal gradient showed that the temperature sensitivity of metabolism was about seven times less than the expectation of metabolic theory (Moffett et al., 2018). This pattern suggests that (1) Gambusia has a low inherent temperature sensitivity of metabolism or (2) that multigenerational exposure to increased temperatures in warmer-source populations has favoured reduced metabolic rates. Here, we use laboratory acclimation of geothermal and nongeothermal populations of *Gambusia* to test the hypothesis that geothermal populations exhibit a relatively low metabolic rate at high temperatures, with a concomitant reduction in boldness and activity. This result would suggest that multigenerational processes cause countergradient trait change along the pace-of-life syndrome axis (Conover, Duffy, & Hice, 2009). Alternatively, if fish from geothermal populations show relatively low metabolism but high behavioural rates, this result would suggest that multigenerational processes (i.e., selection on metabolic rate) act to modify trait relationships, giving rise to novel trait combinations in response to warming.

Materials and methods

Fish populations and collection

Gambusia were introduced to New Zealand in the 1930s and have spread throughout its North Island, including into geothermal streams (McDowall, 1978). Assuming two generations per year (Pyke, 2008), there have been approximately 170 generations since Gambusiaintroduction to New Zealand. We collected Gambusia in January 2016 from eight populations in the North Island of New Zealand that differ in thermal histories (Table 1). Four sites had geothermal influence and therefore had temperatures exceeding air temperature ('warm-source'), and four sites followed changes in air temperature ('ambient-source'). Both geothermal and ambient sites experienced daily and seasonal temperature variation. We did not have long-term continuous temperature profiles of all sites (Fig. 1 & S1), but at the time of fish collection, site temperature was, on average, 11degC higher for warm-source populations compared to ambient-source populations. Geothermal sites reached warmer temperatures and had warmer minimum temperatures (measured bi-monthly) than ambient sites (Table 1). Fish were collected by hand netting and transported to the laboratory in 20 L insulated buckets with water collected on-site and a portable aerator. At the time of fish collection, we measured dissolved oxygen, pH, conductivity, and temperature using hand-held meters (YSI Professional Plus; YSI ProODO).

Temperature Acclimation

Fish were acclimated in 20L tanks in the laboratory, with each tank containing fish from a single population. The eight populations were randomly assigned to tanks, and two tanks were established for each population (16 tanks total). We randomly allocated ~12 fish from a population to a tank (n = 198 fish total; details in S1). In each tank, we separated males and females using dividers to minimise sexually antagonistic interactions

that can affect survival; however, mosquitofish females store sperm, so most females were pregnant during the time of trait measurements, as they would be in nature. Each population was acclimated to two experimental temperatures (20 + 0.5 and 30 + 0.5 degC) over four months. Tank temperatures were initially set to the collection temperature for a given population and then adjusted by increasing or decreasing the set temperature of aquarium heaters by a maximum of 1degC every two days until the target temperature was reached. We started with water from the appropriate field site in each aquarium combined with treated tap water to remove chlorine (API Stress Coat) and progressively replaced it with treated water over two weeks. We fed fish twice daily by hand to satiation with freeze-dried *Daphnia* and Nutrafin MAX small tropical fish micro-granules and maintained a light cycle of 12:12 throughout the experiment. Each aquarium had artificial macrophytes and stones to provide refuge. Water was continuously filtered using sponge air filters, which we cleaned every second day. Fish mortality was low in most of the populations (see Table S1). We fasted individuals for 24 hours before measuring behavioural and metabolic traits to control for food digestion.

Metabolic Rate

We measured metabolism as maximum metabolic rate (MMR), standard metabolic rate (SMR), and routine metabolic rate (RMR). MMR is the maximum metabolic rate of an individual and sets the upper limit on organismal metabolic performance (Fry, 1971). In contrast, SMR is the minimum metabolic rate, measured after rest, with no digestion cost, on non-stressed fish and sets the lower requirement of an animal to sustain life. RMR was measured under similar conditions as SMR but allowed for some activity and sits between SMR and MMR. As RMR incorporates variation in activity between individuals, it may closely relate to behavioural traits (Mathot & Dingemanse, 2015).

We measured RMR and MMR using static respirometry and SMR using intermittent flow-through respirometry at each fish's acclimation temperature (Steffensen, 1989; Clark, Sandblom, & Jutfelt, 2013). We used respirometers comprising 40 mL acrylic chambers with magnetic stir bars in the chamber base to ensure water mixing throughout our oxygen measures in all assays. We measured metabolic rate as oxygen consumption (MO_2) using a FireSting four-channel oxygen logger with optical oxygen sensors (PyroScience, Germany). Respirometers were placed into 80 L aquaria, filled with treated tap water, fitted with a UV filtration system, an aerator, and a 100W aquarium heater.

Immediately following behavioural trials (see below), we measured RMR by placing individuals into chambers and measuring oxygen consumption over 15 minutes. Chambers were then connected to a recirculating pump and slowly flushed with oxygenated water for five minutes before beginning SMR measurements. Oxygen consumption measurements for SMR were taken overnight over an approximately 18-hour period. A computer-controlled aquarium pump intermittently flushed chambers for five minutes to ensure a complete turnover of water inside the chambers, then an oxygen measurement period of 15 minutes began after a 30 second wait period. We controlled oxygen flow and data logging through a PC using the software 'AquaResp' (Svendsen, 2017). Following SMR measurements, we measured MMR using an exhaustive chase protocol to induce maximum oxygen consumption (Clark et al., 2013; Norin & Clark, 2016). Fish were removed from chambers one by one and placed into a circular tank; in this tank, we used an aquarium net to chase the fish until exhaustion (defined as the lack of ability for burst swimming) (Norin & Clark, 2016). Fish were then immediately placed into a static respirometer, and oxygen consumption was measured for 5 minutes. We chose to measure MMR after SMR measurement to ensure our SMR measurement accuracy as metabolic rates may remain elevated for long periods after exhaustive exercise. We immediately euthanised the fish following the measurement of MMR using clove oil. Fish were then measured for mass, length, sex, and volume, then dried at 60degC for 48 hours and re-weighed for dry mass.

We controlled for microbial oxygen consumption in our metabolism assay water by subtracting the oxygen consumption in blanks (respirometers with water only), which were run before and after every trial. We assumed a linear increase in microbial oxygen consumption between measurements in blanks.

We calculated each SMR, MMR, and RMR as;

 $MO_2 = (V_r - V_f) \times \frac{\Delta C_{wO2}}{\Delta \tau}$

Where: MO_2 is oxygen consumption rate, V_r is respirometer volume, V_f is fish volume, ΔC_{wO2} is the change in oxygen concentration, Δt is the change in time.

We calculated SMR using the mean of the lowest 10 % of all measurements, excluding any outliers (\pm two standard deviations [SD] from the mean), aerobic scope as MMR-SMR, and factorial aerobic scope as MMR/SMR (Clark et al., 2013; Chabot, Steffensen, & Farrell, 2016).

Behaviour

Immediately before measuring metabolism, we conducted behavioural assays on individuals in a 60 L aquarium with a water depth of 20 cm and temperature set to the acclimation temperature. We fit the aquarium with an air pump and a UV filtration system to maintain high oxygen saturation and control microbial respiration. We measured individual' boldness' as latency to exit a refuge and individual 'activity' as time spent exploring a novel environment (Cote et al., 2010; Wilson, Godin, & Ward, 2010). For these behavioural measures, we placed individuals into a small enclosed and darkened area ('refuge,' 10cm \times 30cm) at one end of the 60 L aquarium. The aquarium was covered on all but one side to allow for observation. In the refuge, we provided artificial macrophytes and river stones. Fish were left in the refuge for 10 minutes before a 4 \times 4 cm door was opened remotely, allowing fish to exit and explore the remainder of the tank ('open area'). In the open area, we placed macrophytes opposite the refuge opening as a visual cue for exploration. We measured boldness using a stopwatch as the time it took the fish to leave the refuge. Fish that did not leave were assigned a maximum latency time of 600 seconds and were not measured for activity as forced tests may measure anxiety or fear traits (Brown, Burgess, & Braithwaite, 2007). Once the fish began exploring, we video-recorded their movement and later measured activity as time spent moving (versus remaining stationary) over five minutes following their emergence from the refuge.

Statistical analysis

Overview

We first constructed a full model incorporating all predictors (mass, acclimation temperature, and thermal history as geothermal or ambient) and their interactions to analyse the influence of our predictors on metabolism and behaviour. We included a random effect for population identity. We used the Akaike Information Criterion (AIC) to reduce these models (Säfken, Rügamer, Kneib, & Greven, 2018; Mazerolle, 2019). We ranked models by conditional Akaike information criterion (AICc) values and averaged candidate models with Δ AICc <4 using the R package 'MuMin' v.1.43.17 and removed models with interaction terms that were not significant (Burnham & Anderson, 2001; Barton, 2020) (Table S2 & S3). Second, for each metric of metabolism, we used linear regression on subsets of the data to calculate metabolic parameters b and Ea (described below), as is standard practice for analysing metabolism and behaviour across individuals from all populations combined. To do so, we used censored regression models to relate mass corrected metabolic rate to activity. We expressed metabolic rate per unit mass for models comparing metabolic rate to behaviour to avoid collinearity between metabolic rate and mass in the full model.

We constructed models using 'lme4' v.1.1.23 and calculated p values using 'LmerTest' v.3.1.2 package in R with Satterthwaite's degrees of freedom method (Bates, Mächler, Bolker, & Walker, 2015; Kuznetsova, Brockhoff, & Christensen, 2017). We performed all analyses using R version 4.0.0 and determined results to be statistically significant at the cut-off value $\alpha = 0.05$ (R Development Core Team, 2020).

Metabolism

To understand the relationship between metabolic traits (SMR, RMR, MMR, AS) and acclimation temperature or thermal history, we used a linear mixed effect model (LMM) with mass, thermal history, and acclimation temperature included as predictor variables. We chose to exclude the factor sex in preliminary analyses because sex was not significant in determining four of the five models used for metabolic traits. Further, sex is confounded by body size in this species (females are larger, Pyke, 2008), and by excluding sex, we increased our models' statistical power to detect other effects (Table S6).

We calculated allometric scaling coefficients (slope, b) using least-squares linear regression models of \log_{10} metabolic rate (µg O₂ min⁻¹) data against \log_{10} mass (mg) data, separately for each thermal history × acclimation temperature combination (n=4). The activation energy (Ea) of metabolism was calculated from Arrhenius plots of mass-normalised metabolic rates (MO₂ × M^{-b}), against acclimation temperature as an inverse function (1/kT) where T is the respirometry temperature (same as acclimation temperature) in degrees Kelvin, k is the Boltzmann constant (8.62 × 10⁻⁵ eV K⁻¹), and M is mass as in Gillooly *et al.* (2001).

Behaviour

Because our data are censored (fish had a maximum latency time of 600 seconds), we used a mixed-effects binomial logistic model to understand if thermal history, mass, and acclimation temperature influenced boldness. Then, taking individuals that left the refuge, we used a Poisson-lognormal generalised linear mixedeffects model to understand if thermal history and acclimation temperature influenced activity. Like in our metabolism models, we chose to exclude sex to avoid confounding trends between sex and body size and increase statistical power.

To understand if boldness was associated with mass corrected SMR, MMR, or RMR, we used censored regression models using 'censReg' v. 0.5.30 (Henningsen 2019). Similarly, to understand if behaviour, as activity, was related to SMR, MMR, or RMR, we used a Poisson-lognormal generalised linear mixed-effects model with source population as a random effect. We constructed separate models for each acclimation temperature to avoid confounding between metabolic rate and acclimation temperature, though this limited our ability to detect any interactions between metabolic rate and acclimation temperature.

Results

Based on model selection criteria, our best models for SMR (model 5), RMR (model 5), and MMR (models 4, 1, and 5) included history \times acclimation temperature and mass \times acclimation temperature interactions. For AS, the best model (1) included only main effects (see Table 2 for candidate models).

Allometric scaling of metabolism

The relationship between metabolic rate and mass depended on acclimation temperature (mass × acclimation temperature, p < 0.05, Fig. 2, Table S4). In particular, metabolism rose less with increasing mass at 30°C than at 20°C (Fig 1). The metabolic rates of smaller individuals were most sensitive to increasing acclimation temperature, as metabolic rates converged between acclimation temperatures for larger fish (Fig 1). Allometric slopes were similar between ambient- and warm-source fish at each of the acclimation treatment temperatures. Across all metabolic rate measurements, scaling exponents varied from 0.178 to 0.556 and were lowest for MMR (Table S7). Aerobic scope increased with mass and was higher when fish were acclimated at 30°C compared to 20°C (Table S4). The three measures of metabolic rate were related (linear regression, $r^2 = 0.596 - 0.829$; Figure S2). Overall, MMR was 1.6 and 1.8 times greater than SMR for fish at 30°C, respectively. RMR was 1.3-and 1.4 times greater than SMR for fish at 30 and 20°C, respectively (Fig. S2). Across all individual fish, factorial aerobic scope values ranged from 1.4 to 9.1.

Temperature sensitivity of metabolism

The effect of thermal history on all metabolic rate measures (SMR, RMR, and MMR) depended on acclimation temperature (i.e., significant thermal history × acclimation temperature interactions, p < 0.001, Table S4). Individuals from warm-source populations had lower metabolic rates than individuals from ambient-source populations at 30°C, but the reverse was true at 20°C (Fig 1A, B, C). We found no relationship between aerobic scope and thermal history (p = 0.871).

Similarly, temperature sensitivity (as activation energy, Ea) varied with population and acclimation temperature, where metabolic rates of individuals from warm-source populations had lower Ea compared to https://doi.org/10.22541/au.163383539.95423525/v1 — This is a preprint and has not been peer-reviewed. on 10 Oct $2021 - \mathrm{The}$ copyright holder is the author/funder. individuals from ambient populations (Fig. 3). Activation energies ranged from -1.655 to -0.998 eV for ambient-source fish and from -1.351 to -0.775 eV for warm-source fish.

Behaviour

Our best-supported model for boldness was model 1, which included only main effects (Table 2). Fewer individuals from ambient-source populations left the refuge compared to warm-source populations (n = 29 and 76 respectively) (Fig. 4). Individuals were bolder when they were smaller (z = -0.972, p = 0.044), originated from a warm-source population (z = 0.718, p = 0.044), and were acclimated to 30°C (z = 0.061, p = 0.046) (Table S5).

The best-supported models for activity were models 2, 5, and 4, which included interactions among all factors (Table 2). We found that the effect of thermal history on activity depended on acclimation temperature (z = 2.144, p = 0.032), where fish from warm-source populations were less active when acclimated at 20°C compared to fish from ambient-source populations. We also found an interaction between mass, thermal history, and acclimated at 30°C were more activity (z = 2.321, p = 0.020). As such, we show that smaller individuals acclimated at 30°C were more active than larger individuals acclimated at 20°C and that activity was higher for cool-source fish at 20°C than warm-source fish at 20°C.

Boldness and activity were both related to mass-corrected metabolic rate, but this effect was stronger when fish were acclimated at 20°C (Table S8 & S9). When acclimated at 20°C, individuals with higher SMRs (z = -2.216, p = 0.027) and RMRs (z = -2.149, p = 0.032) were bolder, but this behaviour was not related to MMR (z = -1.464, p = 0.143). Similarly, after acclimation at 20°C, individuals with higher SMRs (z = 6.187, p <0.0001) and RMRs (z = 2.436, p = 0.015) were more active, but there was no effect of MMR (z = 0.474, p = 0.635). When acclimated at 30°C no measure of metabolic rate was related to boldness (p > 0.05). In contrast, when acclimated to 30°C increased activity was related to higher RMRs (z =2.027, p = 0.043) and MMRs (z =2.249, p = 0.025), but SMR was not related to activity (z = 1.857, p = 0.063).

Discussion

Multigenerational exposure to increased temperatures may alter the temperature dependence of physiological and behavioural traits; however, the temperature dependence of physiological traits is not often examined over multiple generations (West, Brown, & Enquist, 1997; Persson, Leonardsson, de Roos, Gyllenberg, & Christensen, 1998; Cheung et al., 2012; Holt & Jorgensen, 2015). Here, our results demonstrate that populations with a recent history of multigenerational exposure to warmer temperatures (i.e., geothermal source populations) display a significantly reduced temperature sensitivity of metabolism. Moreover, at warmer acclimation temperatures, populations with a warmer thermal history show lower metabolic rates than populations from ambient conditions, suggesting that multigenerational processes (e.g., plasticity, adaptation) may counteract the metabolic consequences of temperature rise (Sandblom et al., 2016; Jutfelt, 2020). Further, we show that boldness and activity were positively related to metabolic rates at the individual level. However, when comparing populations, fish from warmer source populations showed relatively high boldness at both acclimation temperatures despite relatively low metabolic rates at the warmer acclimation temperatures despite relatively low metabolic rates at the warmer acclimation temperatures despite relatively low metabolic rates at the warmer acclimation temperatures despite relatively low metabolic rates at the warmer acclimation temperature. Together, these results suggest that multigenerational warming will cause a reduction in metabolic rate and an increase in boldness and activity, but that multigenerational processes may not act to direct these trait changes neatly along a "pace-of-life syndrome" axis.

Allometric scaling and temperature sensitivity of metabolism

In *Gambusia*, allometric slopes changed with acclimation temperature, and this change was similar between thermal histories (Fig. 2). Allometric slopes were shallower at the warm acclimation temperature, with small fish showing the largest difference in metabolic rates between acclimation temperatures. This difference in allometric slopes indicates that increased temperature may influence smaller individuals to a greater extent than larger individuals. The temperature-size rule demonstrates a reduction in body size with warming in ectotherms, which *Gambusia* show (Gardner *et al.* 2011; Moffett *et al.* 2018; Fryxell *et al.* 2020). As such, our data suggest that the effect of warming on *Gambusia* metabolism will be the most pronounced at smaller

body sizes. This increased temperature sensitivity of metabolism of small individuals with warming may have significant consequences for population size-structure (e.g., mortality/ reproduction) and the strength of top-down effects (e.g., consumption) as energetic demand increases parallel to declines in body size (Biro, Post, & Booth, 2007; Fryxell et al., 2020).

Our data suggest that multigenerational exposure to warm temperatures reduced the minimum energetic requirements of metabolism (SMR) at the warm acclimation temperature (Fig. 2). Similarly, sticklebacks (*Gasterosteus aculeatus*) and European perch (*Perca fluviatilis*, L.) show a reduction in SMR with a warm thermal history, indicating that such changes may be a common consequence of elevated thermal histories (Pilakouta et al., 2020; Sandblom et al., 2016). While metabolic change in sticklebacks occurred over a long period, change in the European perch was rapid, occurring over three decades. Here, change in metabolic rate in *Gambusia* occurred rapidly over ~100 years or ~170 generations (NLNZ, 1928; Pyke, 2008). Previous work with *Gambusia* in the wild demonstrated that metabolic temperature sensitivity was about seven times less than predicted by metabolic theory (Moffett et al., 2018). Here, our data suggest this discrepancy may be explained by multigenerational processes acting to reduce the metabolic rate of warm-exposed populations over multiple generations. Similarly, the metabolic rates of coral reef fishes originating from high latitudes were less sensitive to warming than those from low latitudes, suggesting such patterns may be widespread (Munday, McCormick, & Nilsson, 2012).

Further, we found that metabolic rates were relatively low for warm-source individuals when measured at 30°C but were relatively high at 20°C, demonstrating that thermal history can modify plastic responses to temperature itself. In contrast, metabolic rates in warm-source Stickleback were consistently lower than cool-source regardless of assay temperature (Pilakouta et al., 2020). In this study, the differences in metabolic rates with acclimation temperature may indicate a trade-off associated with warm adaptation. For example, if warm-source fish evolved down-regulation of enzymatic and mitochondrial density to save energy at warmer temperatures, this downregulation in mitochondrial density may lead to a lower ability to cold acclimate and to function well at cooler temperatures (Salin, Auer, Rey, Selman, & Metcalfe, 2015).

The moderating effects of thermal history on the temperature sensitivity of metabolism were consistent across all three measurements of metabolic rate. However, measurements of individual maximum metabolic rate showed the least variation between acclimation temperatures. Accordingly, our data suggest that minimum energy requirements may be more plastic than maximum energy requirements. Thermal tolerance may often evolve asymmetrically; for example, a species may show greater variation in their ability to adapt to cold temperatures than warm temperatures *via* greater thermal compensation in SMR compared to MMR (e.g., Addo-Bediako, Chown, & Gaston, 2000; Araújo et al., 2013; Sandblom et al., 2016; Bennett et al., 2021). Here, factorial aerobic scope (MMR / SMR) values in our study were somewhat low, ranging from 1.4 to 9.1, whereas in teleost fishes, values ranged from 1.8 to 12.4 (Killen et al., 2016). Lower factorial aerobic scope values may suggest that the exhaustive chase protocol used to measure MMR underestimated actual MMR (Andersson, Sundberg, & Eklöv, 2020). Nevertheless, any asymmetry in metabolic measures (SMR, RMR, MMR) could limit performance under warming if adaptive change is slow (Sterner & George, 2000).

4.2. Behaviour, metabolism, and temperature

Fish with higher metabolic rates were bolder and more active. Studies that have assessed the link between metabolism and behaviour have found mixed support for a relationship between these factors (Biro & Stamps, 2010; Niemela & Dingemanse, 2018; Royauté et al., 2018). Here, our data indicate that rising metabolic rate may play a significant role in regulating behaviour, where as metabolic rate increases, boldness and activity time also increase. For example, individuals with higher metabolic rates were more active and displayed less variability in activity (Fig. 4). Across all metabolic rate measurements, individual behaviour was most consistently related to RMR, suggesting that physiological measures may be best related to behaviour when they allow for, rather than control against, variation in behaviour (Careau, Thomas, Humphries, & Réale, 2008). Here, RMR measurement likely included some recovery from handling stress and any effect of being in a new environment. As such, the relatively strong correlation between RMR and behaviour may have resulted from muscle oxygen consumption, as activity was likely a significant component of RMR measurements.

Overall, our data suggest that increasing metabolic demand increases risk-taking and minimal behavioural activity as temperatures warm.

Despite the positive relationships among acclimation temperature, metabolic rate, and behaviour across all fish combined, we found these relationships to differ based on thermal history. If multigenerational processes had caused trait change along a pace-of-life syndrome axis, populations with relatively low metabolic rates should have shown low boldness and activity (Réale et al., 2010). However, warm-source populations displayed relatively bold behaviour at both acclimation temperatures, despite their relatively low metabolic rates at warm acclimation temperatures (Fig. 4). Patterns in activity were less clear, as warm-source individuals were less active than ambient-source individuals when measured at 20°C, while at 30°C, patterns were similar. Such patterns in activity with different thermal histories may be further evidence of a significant trade-off with warm adaptation, which may not only affect metabolic performance at lower temperatures (see above) but also behaviour. Thus, warmer environments seem to favour risk-taking behaviour while reducing routine activity, perhaps to conserve energy when resources are not perceived to be readily available. Other studies typically show an increase in boldness and activity with increased environmental or acclimation temperature (Careau et al., 2008; Biro, Beckmann, & Stamps, 2010; Forsatkar, Nematollahi, Biro, & Beckmann, 2016), but they rarely account for population differences. From our study, it appears that multigenerational processes may not direct trait change along a simple pace-of-life syndrome axis, instead producing a novel combination of physiological and behavioural traits.

Our results suggest that multiple generations of exposure to warmed temperatures result in significant changes to animal physiology and behaviour. Several processes may have generated the trait changes discovered here, including developmental plasticity, transgenerational plasticity, and evolution. However, ultimately, it is the net effect of these processes that will determine the outcomes of warming for individuals and populations. Importantly, our results show that the combination of these processes over several generations tends to counteract the increase in metabolism caused by acclimation to warm temperatures while intensifying acclimatory changes in some behavioural traits. This result calls to question the notion that warming will cause trait changes along a neat pace-of-life syndrome axis. Clearly, we must understand the novel trait combinations arising over multiple generations of exposure to warming to better predict outcomes for the ecology of individuals, populations, and ecosystems.

Data accessibility

All relevant data will be archived at Dryad Digital Repository.

Funding

Our research was supported by the Royal Society of New Zealand Marsden Fund (16-UOA-23). The Kate Edger Educational Charitable Trust provided partial support for ERM.

Competing interests

We declare we have no competing interests.

Author contributions

ERM and KSS conceived the idea and designed the methodology. ERM collected the data and processed the samples. ERM performed the statistical analyses with guidance from KSS and DCF. ERM wrote the initial draft, and KSS and DCF contributed to the editing of the manuscript.

Ethics statement

The use of fish was approved by the University of Auckland Animal ethics committee (Ref: 001089).

Tables:

Table 1. Characteristics from fish collection sites. We recorded temperature, conductivity, pH, and dissolved oxygen (DO) measurements at the time of fish collection. Annual temperature range measurements were

taken from bi-monthly measurements over one year. Warm-source fish are from geothermal systems, whereas ambient-source populations are from systems with no geothermal influence.

Source	Site	Temperature at fish collection (°C)	Annual temperature range (°C)	$\begin{array}{c} Conductivity \\ (\mu S/cm) \end{array}$	рН	DO (%)
Ambient	Twilight	19.6	13 -20	140	7.03	66
	Stream					
	Tahnua	21.3	12 -23	275	7.55	82.2
	Torea					
	Tourist	21.8	13 -23	134	6.99	51
	Stream					
	Auckland	24.8	12-25	307	7.64	55.5
	Domain					
Warm	Lake	30.0	26-30	337	7.51	104
	Ohakuri					
	Miranda	32.1	29-35	632	7.88	141.7
	Hot Spring					
	Awakeri	35.1	35-37	889	7.52	63.3
	Spring					
	Akatarewa	36.0	24-37	483	7.43	75.5
	Stream					

Table 2. Candidate models used in model selection for factors that influence fish metabolic and behavioural traits.

Model number	Model factors
1	Trait \sim mass + thermal history + acclimation temperature + (1 population)
2	Trait \sim mass \times thermal history \times acclimation temperature + (1 population)
3	Trait \sim mass \times thermal history + acclimation temperature + (1 population)
4	Trait \sim mass + thermal history × acclimation temperature + (1 population)
5	Trait ~ mass + thermal history + acclimation temperature + thermal history: acclimation temperature +
6	Trait \sim mass + thermal history + acclimation temperature + mass: acclimation temperature + thermal h

Figure legends

Figure 1. Summary of study, from left to right, *Gambusia affinis* were collected from warm- (pink) and ambient-source (blue) populations and taken into the laboratory where they were acclimated at 20 or 30°C for four months. After acclimation, fish behavioural (boldness, activity) and metabolic traits (standard, routine, maximum metabolic rates) were measured and compared.

Figure 2. Relationship between fish mass and A) standard metabolic rate (SMR), B) routine metabolic rate (RMR), and C) maximum metabolic rate (MMR) in *Gambusia affinis*. Dashed lines and open circles represent acclimation at 20°C, and solid lines and filled circles represent acclimation at 30°C. Warm and ambient refer to the population's thermal history (see Table 1). We fit data with simple linear regression models and denoted significance of these models as: <0.0001 '***', <0.001 '**', n = 198/ trait.

Figure 3. Temperature sensitivity of metabolism across laboratory acclimated ambient- and warm-source populations of Gambusia affinis. We show temperature sensitives for each measured metabolic trait separa-

tely. Data are averages ± 1 SE; n=8.

Figure 4. Relationship between thermal history and behaviour as A) boldness (i.e., emergence latency) and B) activity (i.e., time spent exploring) as population differences. Plots C and D show individual differences across all populations between mass corrected routine metabolic rate (RMR), expressed per unit mass, and behaviour as boldness and activity, respectively. We do not show individuals who did not leave the refuge, n = 76.

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Fig 1.

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Fig 2.

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Fig 3.

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Fig 4.

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