The evolution of Thermal Performance Curves and life-history traits in responses to thermal selection

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Author contributions

E.A.F. and S.P.M. conceived the study. S.P.M., I.R.M., M.-A.D., J.G. and C.G.-B. gathered the data. G.Z. and I.R.M. performed the statistical analyses. G. Z., I.R.M. and E.A.F. wrote the manuscript and all authors commented on the draft.

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Code and Data availability

Data and R-code is available at Dryad: https://doi.org/10.5061/dryad.q83bk3jxx

Conflict of interest statement

The authors declare no conflict of interest.

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Abstract

Thermal performance curves (TPCs) capture how population growth depends on temperature. When temperatures increase, such as during global change, TPCs may evolve to match new environmental temperatures. While previous studies mostly focus on population growth rate TPCs, evolution can also be strongly trait-dependent and require a multi-trait analysis. Here, we empirically tested how TPCs and multiple demographic, life-history and movement traits evolve by selecting four freshwater protist species at increased temperatures starting from clonal populations. After ten months of selection, populations showed a signature of evolutionary responses to the highest selection temperatures in different traits depending on the species. Particularly, we found consistent evolutionary reductions in body size in the three species having the largest cells and evolved changes in movement behaviour in all species. In contrast, we observed few modifications in population growth rate TPCs. These results suggest that adaptation, via evolution of TPCs, might involve the concurrent evolution of several traits. However, this may be species-specific and difficult from de-novo mutation alone, suggesting that natural populations that do not have sufficient standing genetic variation might have to rely on other means of mitigating the effects of climate change, such as dispersal.

Introduction

In ecology and evolutionary biology we strive to understand how populations respond to novel environments (Merilä and Hendry, 2014; Mouquet et al., 2015; Urban et al., 2016). Populations that experience substantial environmental stress, such as global climate change, must either adapt to the stress or disperse away from the stress, otherwise they will face population decline and an increased risk of extinction (Thomas et al., 2004; Parmesan, 2006; Gienapp et al., 2008; Urban et al., 2024).

One central aspect of global climate change is increasing temperatures and global warming (Hansen et al., 2006). Temperature is an environmental variable that affects nearly all biological processes, from the molecular and biochemical dynamics of enzymes (Gillooly et al., 2001) to population growth rates, spatial patterns and diversity of populations and communities (Angilletta, 2004; Wiens et al., 2006; Kingsolver, 2009).

To be able to predict the effects of global climate change on populations, it is imperative to understand if and how adaptation to increased temperatures can occur, how quickly evolution can act, and if these adaptations are associated with trade-offs or correlated responses more generally that would impact other traits which may limit their effectiveness (Bennett and Lenski, 2007; Hoffmann et al., 2023). For instance, advancements in the field of metabolic ecology allow us to predict how ecological rates (e.g., population growth rates, developmental rates) scale with temperature and related factors (e.g., body size) through the use of semi-mechanistic models (Brown et al., 2004).

Thermal performance curves (TPCs), which describe the thermal plasticity (or temperature niche) of a trait across a range of temperatures, are a powerful tool to investigate the impact of warming under controlled conditions (Schulte et al., 2011; Deutsch et al., 2008). Here, we specifically define the temperature niche, or thermal performance curve (TPC), of an organism as the range of body temperatures at which a non-density limited population will show positive population growth (Gvoždík, 2018). The TPC, and the reaction norms of other traits (e.g., developmental rates, fecundity, body size), is typically unimodal in ectotherms, and is often described by the temperature value at which the trait is maximised by (the thermal optimum, T_{opt}), and the range of temperatures at which an organism can respond (the width) (Kingsolver et al., 2011; Amarasekare and Savage, 2012; Grimaud et al., 2017). Further, the same shape (approx. left skewed unimodal distribution) of the temperature niche appears across a wide-range of ectotherm taxa (Deutsch et al., 2008; Kingsolver, 2009; Dell et al., 2011), likely due to the highly conserved nature of the physiological and biochemical processes that underlie these reaction norms (Gillooly et al., 2001; Have, 2002; Brown et al., 2004).

Multiple empirical studies have investigated TPC evolution: For instance, using outdoor mesocosms, Wang et al. (2023) found in the crustacean *Daphnia magna* rapid evolution of TPCs, but not for per-

formance traits related to energy gain such as ingestion rate and metabolic rate. Schaum et al. (2017) carried out a decade long warming experiment with the phytoplankton *Chlamydomonas reinhardtii* and found that isolates from warmed mesocosms had higher optimal growth temperatures (T_{opt}) than control isolates, and consequently had higher competitive ability at warmed temperatures than control isolates, a strong indication of adaptation to increased temperature. In a step-wise temperature selection experiment designed to generate thermally adapted lines of the coral photosymbionts *Symbiodinium*, Chakravarti and van Oppen (2018) observed stable adaptive change at the end of their exposure period (41–69 generations), resulting in populations that showed faster growth rates under acute heat stress, and higher photosynthetic efficiencies when compared with control populations, although these results were not found across all replicate lines. Bennett and Lenski (1993) evolved replicate lines of *Escherichia coli* for 2000 generations at three fixed selection temperature (32, 37, 42°C), or at a temperature regime that routinely fluctuated between the two extremes. They then measured the range of temperatures (12–44°C) that a population could maintain itself while undergoing serial dilutions. They found that the width of the temperature niche and extinction risk at high temperature was unaffected by the selection regime, but each selection line showed a pattern of local adaptation, an increase fitness at their selected temperature. In a similar experiment in *E. coli*, but specifically looking for trade-offs to high temperature (40°C) after adaptation to 20°C, Bennett and Lenski (2007) did find evidence of a general trade-off (a decline in mean fitness across all replicates), although they were not universal across replicates. A meta-analysis of the field of thermal selection experiments (Malusare et al., 2023) could recently show that TPCs have adaptive potential across ectothermic species (see references and studies used for the statistical analysis therein). This synthesis clarifies that trade-offs between adaptation to higher temperatures and fitness at lower temperatures can be generally observed across the tree of life. At the same time this study highlights the relative paucity of well-resolved TPCs and the limit of using only two test temperatures, as done by many studies, which hinders interpretation related to shape changes and responses at thermal limits. Overall, these studies provide empirical evidence and represent some proof of principle for how the TPC of different species can rapidly evolve in response to selection, in particular to increased temperatures.

Despite these recent advances and broad interest, more work remains to be done to increase our understanding of how thermal stress impacts the evolution of TPCs. Furthermore, many temperature selection studies focus on a single species, which makes comparisons of how species respond to identical selection regimes difficult. Phylogenetic comparative approaches can fill this gap and provide key insights. For instance, Kontopoulos et al. (2020) found a phylogenetic heritable signal across phytoplankton and prokaryotes for higher population growth rates at increased temperatures (i.e., thermal sensitivity of TPCs), which was further consistent for plants when using two physiological traits as proxy for growth.

The results suggest evolutionary convergence in such responses. Nonetheless, more experimental work remains important. In particular, a better understanding of the differences between species' ability to adapt their plastic response to increased temperature could help in predicting the evolution of community composition under climate change, as well as the relative rates of adaptation and extinctions in different environments (Hoffmann and Sgrò, 2011; Sinclair et al., 2016).

In order to address these challenges, we performed such a multi-species temperature selection experiment. After ten months of thermal selection at six different temperatures, we empirically tested how TPCs and multiple traits respond to selection across four life-history diverse protist species. All species showed responses to thermal selection compared to the evolutionary control treatment. Detailed responses differed across species, and mainly involved changes in body size and movement rather than population growth rate.

Materials and Methods

Study organisms

In this experiment we used four freshwater protist species, spanning a broad range of body sizes and trophic diversity: two genotypes of *Tetrahymena thermophila* (*Tet*; MT I: SB3539 and MT VII: CU428.2), a small (approx. 50 µm) bacterivorous ciliate commonly used in evolutionary and ecological microcosm experiments (Collins, 2012; Altermatt et al., 2015; de Melo et al., 2020; Moerman et al., 2020a,b), *Paramecium caudatum* (*Para*), a large (approx. 330 µm) widely studied bacterivorous ciliate (Magalon et al., 2010; Nørgaard et al., 2021; Zilio et al., 2023a), *Euglena gracilis* (*Eug*), a small (20-100 µm) mixotroph capable of both photosynthesis and phagocytosis (Altermatt et al., 2015; Harvey et al., 2017), and *Blepharisma* sp. (*Ble*), a large (approx. 200 µm) omnivore that feeds on bacteria and smaller ciliates (Giese, 1938; Saade et al., 2022; Tan et al., 2021).

These species were chosen due to their wide diversity in life-histories, their potential for studying biotic interactions among species in mixed species microcosms (Carrara et al., 2015), and their fast generation time. Our laboratory conditions (described below) preclude sexual reproduction, and all species reproduce asexually: *Tetrahymena thermophila* in 2–4 h (Frankel, 2000), *Paramecium caudatum* in 8–12 h (Zilio et al., 2023a), *Euglena gracilis* in 10–30 h (Buetow, 1962), and *Blepharisma* sp. in 14–16 h (Giese and McCaw, 1963). Populations were cultured in monoxenic medium. Medium was made from dehydrated organically grown salad and Volvic mineral water (1 g of salad in 1.6 L of water), autoclaved and then inoculated with the bacterium *Serratia fonticola* at 10% maximum density (tenfold dilution of one week old culture at 20°C is a food source for the protist species).

Selection protocol

Temperature selection lines (20, 25, 30, 33, 36 and 39°C) were generated for each species/strain using a ratchet protocol (modified from Huertas et al. 2011). Each selection temperature included three replicates that evolved independently, for a total of 90 selection lines (5 species × 6 temperatures × 3 replicates). This method strikes a balance between strong selection, through the step-wise increase in temperature, and maintaining high population sizes, to maximise the number of spontaneous mutations conferring adaptation to the increase in temperature.

In brief, three biological replicate populations of each organism were established in August 2020 from clonal lines derived from laboratory stock cultures that had been maintained in the lab at 20°C for several years. After six weeks, 10 mL samples were taken and established in 90 mL of 10% bacterized salad medium at the next higher selection temperature. If a population showed positive population growth after six weeks at a selection temperature, a sample was ratcheted to the next selection temperature (Fig. 1). If not, it was evaluated again after another six weeks. If an extinction occurred, a new population was established from the previous selection temperature. Selection lines were stored in temperature controlled incubators (randomised twice per week to avoid incubator effects) with a 12:12 light:dark photoperiod. To maintain excess food and prevent population growth rates declining, 10 mL from each selection line was regularly transferred into 90 mL of 10% bacterized salad medium (Tet I and Tet VII: 7 days, Para and Ble: 14 days, Eug: 21 days; the different timing reflects overall population growth rate differences).

As a reference, we used the 20°C selection line. We did this, as opposed to using ancestral measurements, to minimise time artefacts in the data and to ensure that the only difference between the reference selection line and other selection lines was the selection temperature. The selection regime may have introduced other unintended selection pressures (e.g., during weekly transfers), and ensuring that these were identical across all lines, including the reference, allowed for direct comparisons regarding the effects of temperature within and between selection lines.

Final Assay Protocol

Following the selection phase, the goal of the final assay was to measure population growth, as well as other traits (see below), of each selection line at ten different temperatures (10, 15, 20, 25, 30, 33, 36, 39 and 40°C) over a period of three weeks. We performed these assays after 10 months as all species had reached the highest selection temperature. The three replicates were measured in three temporal blocks (see Fig. 1, dashed line). Prior to beginning the temperature assay, we kept the selection lines in a 20°C common garden phase to minimise parental effects, adjusting approximately the time proportional to each species' generation times (*Tet* I and *Tet* VII: three days; *Para* and *Ble*: five days; *Eug:* seven

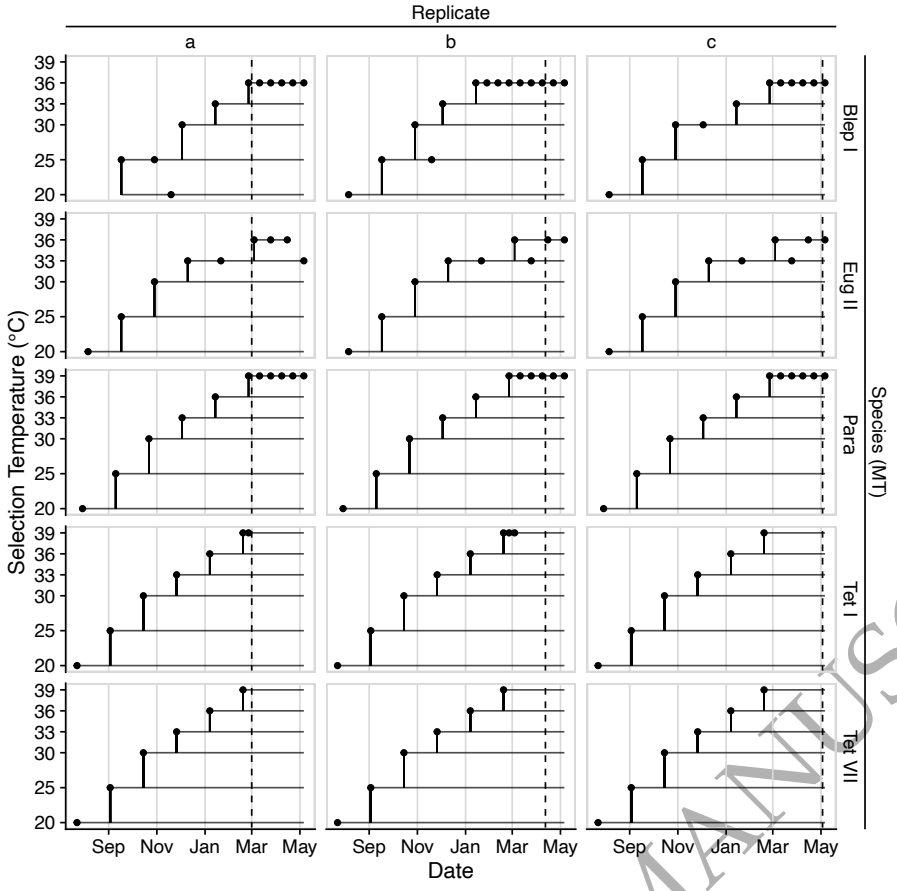

Figure 1: Chronogram showing the selection experiment history over 10 months, from August 2020 to May 2021. Vertical black lines between selection temperatures (step) represent an initial transfer, which occurred in case of positive growth after 6 weeks at a certain selection temperature. For example, in the case of the replicate "a" of Tet VII (bottom left panel), the starting line was transferred at 25°C (September 2020) after spending 6 weeks at 20°C and showing positive growth. The starting line was also maintained and propagated at 20°C. Dots on the line after the first establishment of a line at higher temperature represent extinctions where the population had to be re-established from the previous selection temperature. Dashed lines show the starting date for the final assay for each population.

days). To establish the assay lines, we added 200 µL (20 µL for *Tet* I and *Tet* VII) of selection line protist culture to sterile plastic vials containing 20 mL of 10% bacterized salad medium and stored them in temperature controlled incubators (randomised twice per week) with a 12:12 light:dark photoperiod. Three times per week, we adjusted the volumes of each assay line vial to 18 mL and added 2 mL of 10% bacterialised salad medium to ensure sufficient food availability.

From the established 20mL assay line vials, we placed 350 μL samples into 24-well clear bottom microplates (Porvair Sciences, UK). We imaged each sample using a microscope plate reader (Cytation 5, BioTek) for 10 seconds (150 frames). The video files were then analysed using the 'BeMoVi' R package (Pennekamp et al., 2015) to extract density, cell size data and swimming movement data (for exact settings and modifications see https://github.com/efronhofer/analysis_script_bemovi/tree/ffmpeg_overlays). The

whole procedure was repeated twice a day for the first two days, once a day for the third and fourth day, and then once a day every two days. We first used this protocol to determine the ancestral TPC and traits of each species, and it was then repeated after 10 months and two years of selection to determine evolutionary changes. During the 2 years, 18 lines across different species and selection temperature went extinct, leaving us with 83 lines for the first 10 months, and 72 thereafter (Table S1). In addition, during the final assays in year 2 several lines did not grow for unknown reasons, even in the evolutionary control treatment and at lower (permissive) temperature conditions for which positive growth always occurred. Given these issues for which we do not have a satisfactory explanation, and due to the fact that data from year 1 and 2 show no overall different pattern (Fig. S1), we decided to focus on data from year 1 (after 10 months).

Statistical analyses

All statistical analyses were performed using R v4.2.0 (R Core Team, 2022) and the packages '*rstan*' v2.21.5 (Stan Development Team, 2022) and '*brms*' v2.17.0 (Bürkner, 2017).

Data check and cleaning

In order to facilitate the statistical analysis, we first proceeded to visually inspect each population time series collected during the assays and clean data to exclude potential outliers and cases in which populations did not grow. Upon inspection of the video files and 'BeMoVi' overlays, we found that some outliers in population densities were caused by the plate reader having failed to auto-focus correctly. In order to remove such outliers systematically, we first fitted LOESS splines using the `loess()` function in R to the data and removed any point that deviated too far (3 times greater, respectively lesser) from the spline fit. We then excluded any populations with less than 7 data points in their time series, reducing the initial 830 populations to 766.

Population growth rates and other traits

To extract estimates of population growth rates (r_0; unit: h^{-1}), we fitted Bayesian linear models to the population density estimates (log-transformed) during the exponential phase. The exponential phase was identified using visual inspection of all growth curves. We opted for this approach because testing and comparing methods that automatically screen growth curves and search for the steepest slope (exponential growth phase) were more prone to spurious results and overestimation. We used an MCMC chain length of 30,000 iterations (15,000 warmup iterations) and vaguely informative priors, with the intercepts and slope parameters following a normal distribution and half-normal distribution respectively, with mean 0 and standard deviation 1. The adapt_delta parameter was set to 0.999 to eliminate post-warmup divergent

transitions. The estimated slope parameters corresponded to the population growth rates. Measures of other traits included the major and minor cell axes (proxies for body size), bioarea (proxy for biomass), as well as swimming speed and tortuosity (standard deviation of the turning angle distribution). These were averaged over the exponential growth phase selected. We additionally considered the time point at which populations attained their maximum density as a proxy for equilibrium density.

TPC fitting

To explore how the TPC varies after selection at increased temperatures, we fitted thermal performance curves to the r_0 estimates obtained as described above. We use a high temperature inactivation version of the Sharpe-Schoolfield equation (Schoolfield et al., 1981), a commonly used semi-mechanistic model that extends the Arrhenius equation to incorporate a decline in population growth rates beyond an optimum value. The model is given by the equation

$$r_0 = \frac{r_{0_{T_{ref}}} \cdot exp^{\frac{-E}{k}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)}}{1 + exp^{\frac{E_h}{k}\left(\frac{1}{T_h} - \frac{1}{T}\right)}} \quad (1)$$

where T is temperature in Kelvin (K), k is the Boltzmann constant ($8.617 \times 10^{-5} eVK^{-1}$), E is the average activation energy (eV), E_h is the high temperature deactivation energy, T_h is the temperature at which growth rates are 50% decreased due to high temperature and r_{0ref} is the r_0 value at a reference temperature (here, 20℃). The optimum value (T_{opt}) of Eq. 1 is given by

$$T_{opt} = \frac{E_h T_h}{E_h + kT_h ln\left(\frac{E_h}{E} - 1\right)} \quad (2)$$

and the maximal growth rate (r_{max}) can be calculated by substituting T_{opt} into Eq. 1.

We again made use of a Bayesian statistical model to fit the TPC function to the r_0 estimates, which allowed for the propagation of the error associated with r_0 estimates throughout the analysis. We estimated parameter values for $r_{0_{T_{ref}}}$, E, E_h and T_h using the '*brms*' and '*rstan*' R packages (for R code, see Supplementary Methods). As before, we used priors that were informative, making use of published values to inform mean and variance estimates of E and E_h, and using realistic, but broad priors for T_h. The assumption of a universal temperature dependence (i.e., that values of E are highly constrained by thermodynamics), is often called into question (Clarke, 2004; Clarke and Fraser, 2004; Clarke, 2006; Gillooly et al., 2006), hence we used wider priors for E than those directly predicted from metabolic theory of ecology. The only prior that differed between species was $r_{0_{T_{ref}}}$ (for full information on the priors used, see Table S2). We included a measure of the error on each r_0 estimate by including each estimate as a normal distribution with a mean and standard deviation taken from the posterior distribution of the

previous analysis (normal distribution on the log scale). For each selection temperature of each species of each replicate, we ran chains with 10000 iterations (warmup=5000 iterations, adapt_delta=0.99).

TPC parameters

The TPC parameter posteriors $r_{0_{T_{ref}}}$, E, E_h and T_h directly estimated from the TPC fitting were scaled and centred around their means. These were analysed using multi-variate multilevel models (warmup = 10,000 iterations, chain = 20,000 iterations) with a normal error structure. We conducted two main analyses for each species. Firstly, to assess potential TPC responses to laboratory conditions we compared the intercept model to the one including the ancestral populations and the evolutionary control lines at 20°C as explanatory factors (i.e., evolutionary conditions). For model comparison and weight we used the Watanabe-Akaike Information Criterion (WAIC; Watanabe and Opper, 2010), a generalized version of the Akaike Information Criterion (Gelman et al., 2014). Secondly, we applied the same statistical procedure to test for TPC differences across the selection temperatures, from the evolutionary control at 20°C to the highest-temperature evolutionary treatment at 39°C. In this second analysis we considered selection temperature as a continuous response variable, and we further included replicates as a random factor. In all models we adopted the default vaguely informative priors of the 'brms' package. Additionally, we used the obtained 4-parameter matrix for complementary graphical inspection by means of principal component analysis (PCA).

For the calculated parameters T_{opt} and r_{max} we proceeded similarly. We conducted 2 separate main analyses for each of the 5 species using univariate multilevel models (warmup = 10,000 iterations, chain = 20,000 iterations) with a normal error structure. In one analysis we compared WAIC of the intercept to the evolutionary conditions model, and in the other the intercept to the selection temperature model. We also propagated the error associated with T_{opt} and r_{max} estimate calculation. In some cases, when analysing r_{max}, the inclusion of the selection line random effect in the selection temperature models produced few divergent transitions ($< 0.01\%$) even after applying guidelines for corrections. Following Stan Development Team diagnostics, careful inspections revealed no pattern in their locations, and the models had ideal convergence (Rhat = 1.00) and effective sample size (ESS) values. Further, the results were qualitatively comparable to models without random terms (and divergent transitions). We therefore decided to maintain the models with the random effect structure.

Multi-trait evolution

We applied the same statistical approach as for the TPC parameters described above. For each species, we run Bayesian multi-variate models (warmup = 10,000 iterations, chain = 20,000 iterations) on 7 traits, using normal error structure and model comparison. The phenotypic traits were r_0, swimming speed and

tortuosity, major and minor cell axis, bioarea and maximum density, all of which were scaled and centred prior to the four main analyses. In the first two analyses, we compared ancestral and evolutionary control lines (two factors variable) assayed at their own selection regime (20°C). We also compared ancestral and evolutionary control lines assayed at the other temperatures. Specifically, we compared WAIC of the intercept-only to the evolutionary conditions model, and in the other the intercept-only to the selection temperature model. In the other two analyses, we similarly compared evolved lines assayed at their own selection treatment or in the other temperatures and compared WAIC for the intercept-only and the full model. For the latter analysis (other temperatures), given a non-linear trend after visual inspection (Fig. 4), we fitted an additional model with a quadratic term, applying the same procedure of WAIC comparison, but could show that the non-linear model was not supported statistically ($\Delta AIC = 0.44$). We considered selection temperature as a continuous variable and we also included a selection line random effect. The few cases of divergent transitions were carefully treated as previously described.

Results

TPC parameters

For all species, we observed TPC changes in response to laboratory conditions and handling (evolutionary conditions: WAIC weight > 0.96; Table S3 and S4). The evolutionary control lines maintained and propagated during a year at standard 20°C showed indeed a different combination of niche parameters compared to the ancestral ones (Fig. S3). Thus, the TPC of the evolutionary control at 20°C is the most reliable baseline information to compare the evolutionary impact of the selection temperatures.

We found some signals of TPC evolution after selection to increased temperatures in both *Tetrahymena thermophila* strains (selection temperature: Tet I WAIC = 192.87; SE = 21.45; WAIC weights = 0.95; Tet VII WAIC = 172.7; SE = 19.89; WAIC weights = 0.98), but not across all species (Fig. 2, see also Fig. S6 showing the average predictions only). For *Tetrahymena thermophila* strain VII, selection at the highest temperatures of 36°C and 39°C decreased $r_{0_{T_{ref}}}$ by 136% and 464% respectively and thus led to a reduction in population growth at the reference 20°C control temperature (slope= -0.096; 95% CI= -0.166, -0.027, Fig. 3). For *Tetrahymena thermophila* strain I, the signal was less clear. Even though no parameter correlation was formally different from zero (Fig. 3; Table S6), we observed an overall shift in the parameters space for lines selected at the highest temperature of 39°C (Fig. S2 A) similarly to what was found for strain VII (Fig. S2 B). In contrast, the TPCs of *Paramecium caudatum*, *Blepharisma* sp. and *Euglena gracilis* were unaffected by the selection temperature (Fig. 2; evolutionary conditions: WAIC weight < 0.2). Nonetheless, lines of *Euglena gracilis* selected at 30°C showed a visual tendency for an overall upwards shift of the whole TPC.

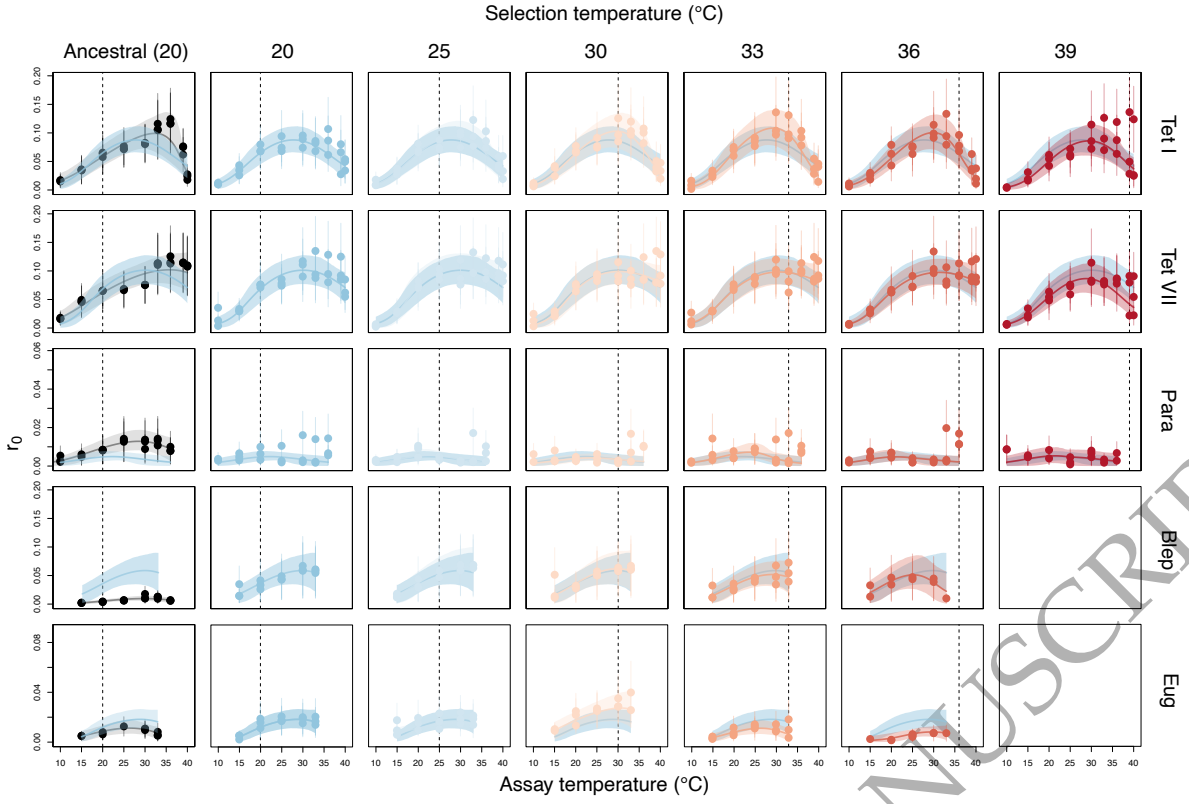

Figure 2: Thermal Performance Curve (TPC) for r_0 (population growth rate) estimates across different assayed temperatures ranging from 10 to 40°C for four protist species subjected to six temperature selection regimes for 10 months (colour gradient from left to right). Averaged fitted curves and 95% credible intervals, lines and polygons respectively, are derived from a Bayesian implementation of the modified Sharpe-Schoolfield equation (Eq. 1) fitted to r_0 and SE values (solid points and bars). The latter were obtained from population density recorded over a period of three weeks (growth curves). r_0 and SE represent the raw data used for the statistical analysis and TPC fitting (3 biological replicates). The ancestral conditions are represented in grey. The evolutionary control at 20°C is shown in the second column from the left, and it was considered as reference. The averaged model predictions and 95% credible intervals of the evolutionary control are repeated in each panel for visual comparison. Changes in the TPC would correspond, for example, to complete or partial shift from the evolutionary control towards higher temperatures with (Hotter is Better) or without changes in shape (see Knies et al., 2009; Malusare et al., 2023). Dashed vertical lines indicate matching selection and assay temperatures. Tet I and VII are two mating types of *Tetrahymena thermophila*, Para is *Paramecium caudatum*, Blep is *Blepharisma* sp., and Eug is *Euglena gracilis*.

Complementary analyses evaluating TPC evolution using the ancestor rather than the evolutionary control as reference (see Fig. S5) suggested slightly stronger signals for evolutionary change. In particular, additionally to the *Tetrahymena thermophila* strains, this analysis also indicates TPC evolution in *Blepharisma* sp. (Table S5; Blep WAIC = 128.03; SE = 15.63; WAIC weights = 0.99). Nonetheless, the more detailed parameter correlation analysis showed no quantitative changes in comparison to the analysis reported above (Fig. 3). We found indeed only two formally significant effects for *Tetrahymena thermophila* (Table S7, red regression lines in Fig. S7).

There were no evolutionary changes in the optimal growth temperature T_{opt} and maximal growth

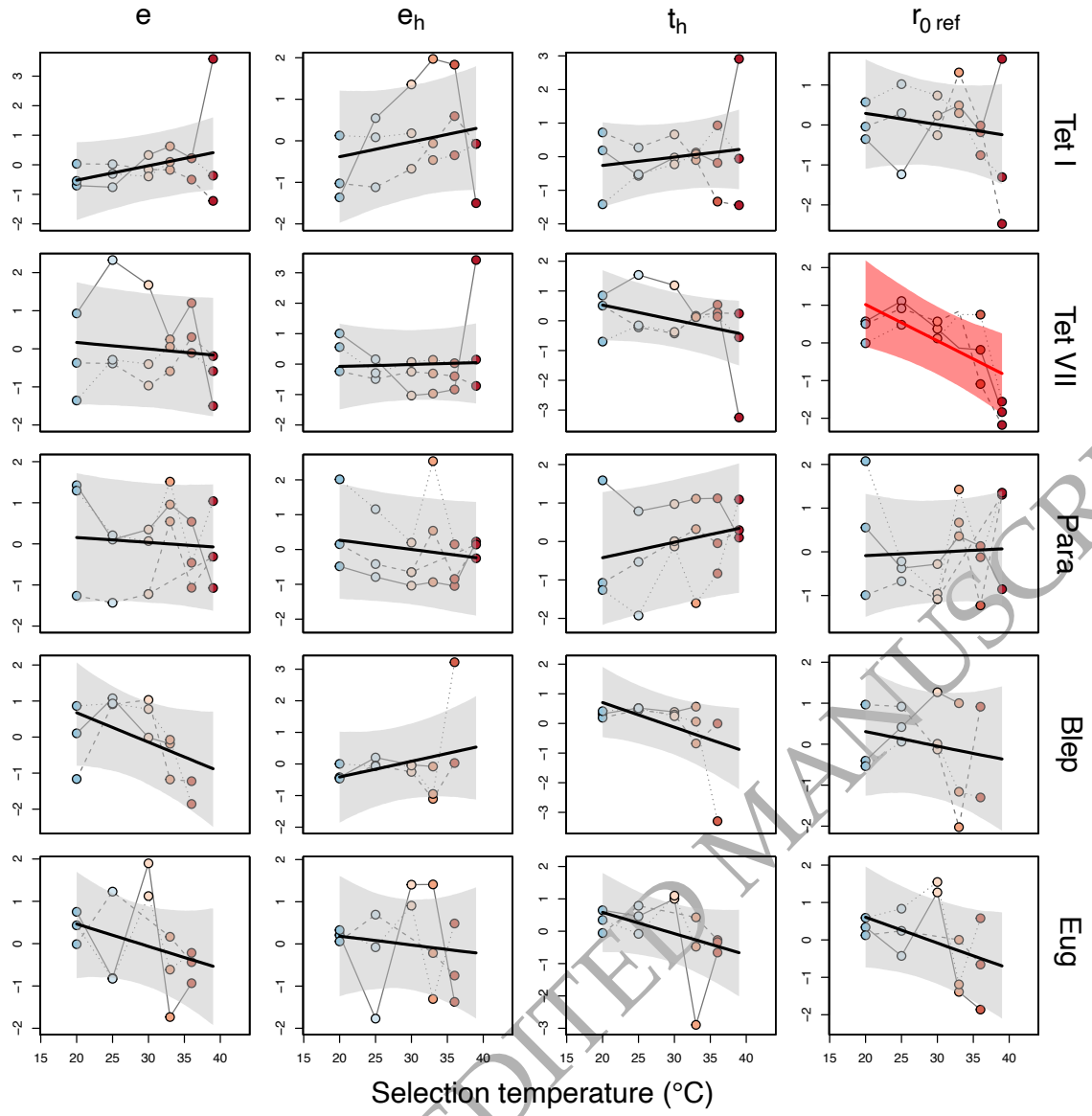

Figure 3: Median model predictions (solid lines) and 95% compatibility interval (shaded areas) obtained from the multivariate analysis of the TPC parameters E, E_h, T_h, $r_{0_{T_{ref}}}$ (niche fitting from Eq. 1). Each point is a standardized niche parameter value (raw data for the corresponding analysis) corresponding to a biological replicate. Each biological replicate is connected by a different line type. The colours are the different selection temperatures experienced during evolution, as also indicated on the x-axis. Model predictions with slope parameter different from 0 are shown in red. Tet I and VII are two mating types of *Tetrahymena thermophila*, Para is *Paramecium caudatum*, Blep is *Blepharisma* sp., and Eug is *Euglena gracilis*.

r_{max} of all five species compared to the ancestral conditions (T_{opt}: WAIC weight < 0.47, Table S8; r_{max} : WAIC weight < 0.43, Table S11). The absence of responses to increased selection temperature was also found across temperatures (T_{opt}: WAIC weight < 0.33, Table S9; r_{max} : WAIC weight < 0.47, Table S12). Additional analysis using the ancestral lines as references found the same absence of responses for both T_{opt} and r_{max} (Tables S10 and S13). The r_{max} of *Paramecium caudatum* evolutionary control lines

tended to have the opposite trend and be higher than the ancestral conditions, although this remained a weak signal (WAIC = -20.09; SE = 1.18; WAIC weights = 0.57), which was not found when comparing lines across temperatures (WAIC = -78.79; SE = 3.48; WAIC weights = 0.39).

Multi-trait evolution

Ancestral and evolution control evolved differently, due to laboratory conditions. This was observed when lines were assayed at 20°C matching their evolutionary conditions (Fig. S3, left column; WAIC weight > 0.83), and also when tested across the range of temperatures, from 10°C to 40°C (Fig. S3 right column; WAIC weight = 1; see Table S14 and S15). The evolutionary control lines of all species evolved smaller sizes (reduced minor and major cell axes), but increased their intrinsic growth rate (r_0) and reached higher maximum density, independently of the assayed temperatures (left vs. right column in Fig. S3). *Paramecium caudatum* was somewhat different: In response to laboratory conditions it evolved reduced cell size as the other species, whilst decreasing maximum density compared to its ancestral lines (Fig. S3 E, F). When these evolved lines were tested across temperatures they further exhibited reduced growth rate (Fig. S3 F).

As for the TPC above, we considered the evolutionary control lines as a baseline for additional comparisons. Analyses comparing to the ancestral lines are provided in Fig. S8 and Tables S19 and S21, and show no qualitative difference to the main results reported here. After selection at increased temperatures, populations rarely showed adaptation to their selection temperature, with notable exceptions (Table S16, see also Fig. S4 for a visual representation of the multi-trait evolutionary trajectories). We found direct responses to selection (own temperature) only in Tet I (WAIC = 310.78; SE = 14.72; WAIC weight = 1) and *Blepharisma* sp. (WAIC = 239.69; SE = 7.42; WAIC weight = 0.99).

Note that for *Blepharisma* sp., lines selected at 36°C did not grow at their own selection temperature, and lines at 39°C were not available overall (Table S1). Despite the signal remaining weaker, we also found a trend for direct responses to selection in Tet VII (ΔWAIC = 0.57), which was confirmed by inspecting the model output (Table S17). Tet I and Tet VII reached lower maximum density after selection at higher temperatures (Fig. 4; Tet I slope= -0.121, 95% CI=-0.169; -0.072; Tet VII slope= -0.074, 95% CI=-0.146; -0.001). In addition, Tet VII decreased its bioarea (slope= -0.072, 95% CI=-0.143; -0.001) and became a slower swimmer (slope= -0.082, 95% CI=-0.157; -0.006). We observed the same trend for trait changes in bioarea and swimming speed in Tet I (Table S17). *Blepharisma* sp. evolved at 33°C and assayed at the same selection temperature increased its intrinsic growth rate (slope= 0.126, 95% CI=-0.001; 0.249). We detected no clear direct responses to selection in *Paramecium caudatum* and *Euglena gracilis* (Intercept: WAIC weight > 0.89).

However, when considering phenotypic traits measured across temperature after selection, all species

exhibited evolutionary responses (Table S18; Selection temperature: WAIC weight > 0.99). We found stronger and consistent patterns for *Paramecium caudatum*, *Blepharisma* sp. and *Euglena gracilis* (Fig. 4, Table S20). Namely, selection at the highest temperature led to smaller body size (decrease in major axis (length) by 56%, 62% and 17% in Para, Blep and Eug, respectively) and impacted mobility traits in all 3 species (Fig. 4; increase in tortuosity by 33%, 49% and 224% in Para, Blep and Eug, respectively, with Eug also showing an increase in speed of 133%). The swimming behaviour of the Tet VII strain was also affected by selection at higher temperatures (speed decreased by 9%).

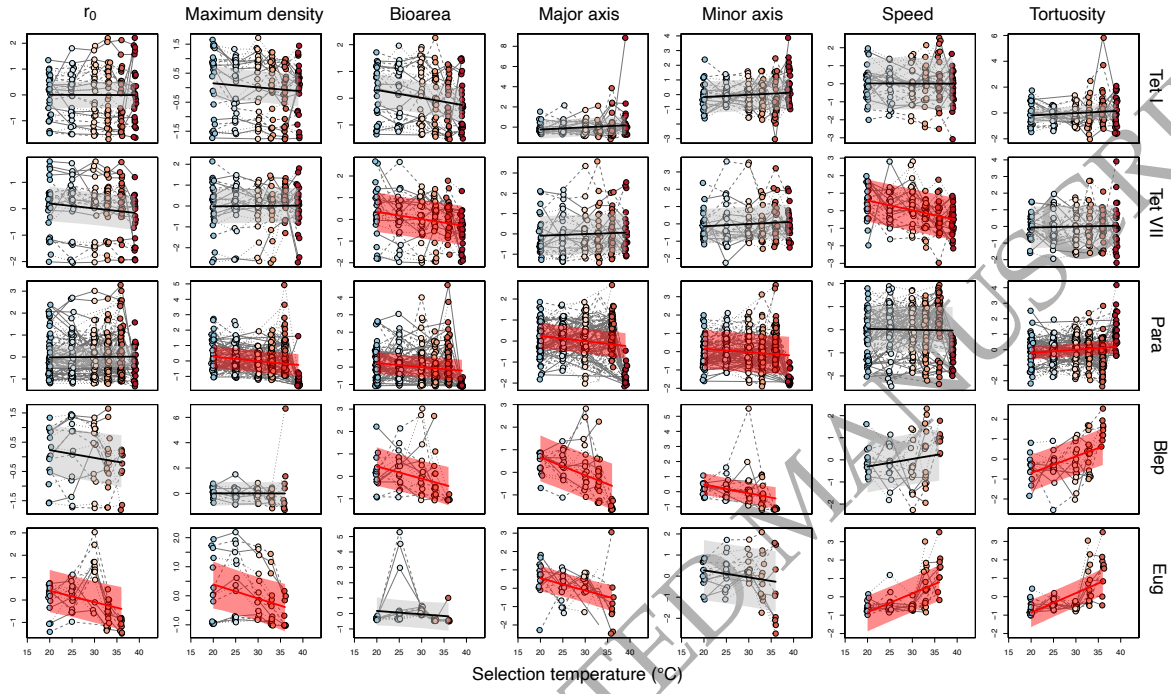

Figure 4: Median model predictions (solid lines) and 95% compatibility interval (shaded areas) obtained from the multivariate analysis of the 7 standardized phenotypic traits (r_0, maximum density, bioarea, major axis, minor axis, swimming speed and tortuosity) assayed at different temperatures ranging from 10 to 40°C. Each point is a standardized trait value (raw data), and corresponds to a biological replicate. Each biological replicate is connected by a different line type. The colours are the different selection temperature (evolutionary treatment of origin) as also indicated on the x-axis. Model prediction with parameter slopes different from 0 are highlighted in red. Tet I and VII are two mating types of *Tetrahymena thermophila*, Para is *Paramecium caudatum*, Blep is *Blepharisma* sp., and Eug is *Euglena gracilis*. Given some non-linear trends after visual inspection, we fitted an additional model with a quadratic term, but could show that the non-linear model was not supported statistically ($DeltaAIC = 0.44$).

Discussion

Climate change has had wide-ranging impacts on species, communities and ecosystems (Walther, 2010) and remains one of the biggest threats to biodiversity worldwide (Urban et al., 2016). Globally warming temperatures are well documented (Masson-Delmotte et al., 2021), making it important to understand

if and how species can adapt to increased temperatures. This understanding is central for predicting the impacts of climate change on species persistence, extinction rates and changes to community compositions, for example.

In order to increase our understanding of evolutionary responses to temperature increases, we here experimentally evolved four different protist species at multiple increased temperatures and compared their thermal performance curves and multiple traits after ten months of selection. In general, we found only weak signals for the evolution of population growth (Fig. 3, red regression line), but stronger responses for body size and movement (Fig. 4, red regression lines).

Evolution of population growth rate: weak signatures of adaptation

Using a Sharpe-Schoolfield model to fit TPCs on measured population growth rate data (Fig. 2) we only found weak signals of TPC evolution (Fig. 3). This lack of a clear evolutionary response was unexpected, since all the protist species underwent selection for ten months, in a selection regime far more intense than would be experienced in wild populations under the worst case scenarios for global warming (Masson-Delmotte et al., 2021). We did find signals of TPC evolution for growth rate in *T. thermophila*, more specifically $r_{0_{T_{ref}}}$ decreased with increasing selection temperature (Fig. 3, red regression line). We also report a general shift in niche parameter space and niche shape at the most extreme selection temperature (39°C; Fig. 2, Fig. S2). Lastly, we observed an overall up-shift of the whole TPC for *Euglena gracilis* selected at 30°C (Fig. 2), a pattern that corresponds to all replicates selected at 30°C having a tendency to higher r_0, no matter the assayed temperatures (Fig. 4 bottom left panel; note that the non-linearity is not supported statistically since the linear and the non-linear models have similar WAICs). We speculate that in this species, selection at 30°C, a temperature close to the ancestral optimum (Fig. 2), may have led to direct and correlated responses in r_0 which may be in line with predictions of partial shifts of the TPC without changes in shape (see Knies et al., 2009).

We had particularly expected responses in *T. thermophila* because the (effective) population sizes in the selection lines were rather large (at equilibrium density approx 3.2×10^6 cells at 20°C and 1.7×10^6 at 39°C) and were selected for at least 857 generations (approximate estimate assuming demographic replacement and following standard calculations: time · dilution rate / ln(2); see Gresham and Hong 2015). Furthermore, *T. thermophila* has an estimated macro-nuclear mutation rate ($U = 0.0333$ per haploid macronucleus genome per generation) that is orders of magnitudes higher than estimates for other protists, and eukaryotes in general (Brito et al., 2010). Of course, micro-nuclear mutation rates are a lot lower (e.g. Sung et al., 2012; Long et al., 2016), but since recombination does not occur in our experimental setup (see e.g., Moerman et al., 2020b) we do not consider these as relevant here. Therefore, we assumed that there should have been ample opportunity for evolution to occur. Finally,

T. thermophila has been shown to successfully adapt to other selection pressures (e.g., pH Moerman et al. 2020a; and dispersal: Fronhofer et al. 2017) and has shown evolutionary change in response to temperature in previous studies (de Melo et al., 2020).

Most strikingly, T_{opt} and r_{max} were completely unaffected by our temperature treatments in all five species (Tables S8 – S13). Our results also do not support the "hotter-is-better" hypothesis, similarly to what was found in a meta-analysis on TPC evolution (Malusare et al., 2023). Several experimental studies in ectotherms have illustrated the rapid evolution of T_{opt} (Santos et al., 2006; Carbonell and Stoks, 2020; Mesas et al., 2021; Wang et al., 2023), but the associated evolution of r_{max} is usually inconsistent, and an overall clear consensus for evolutionary trajectories of TPCs is currently lacking. A recent evolutionary experiment in which eight species (51 genotypes) of the *Saccharomyces* genus were exposed to increasing temperatures for 600 generations found strong between- and within-species differences in TPC evolution. Interestingly, the authors identified two different evolutionary trajectories: genotypes either exhibited a "hotter is wider" or a "jack of all temperatures is a master of none" pattern (Molinet and Stelkens, 2025). This study highlights the complexity of adaptive responses to thermal selection with the presence of genotype-by-environmental interactions.

The absence of responses in our study and others may be explained by the biochemical adaptation (Montagnes et al., 2022) or the perfect compensation hypothesis (Frazier et al., 2006). These postulate that a fixed r_{max} may be the evolutionary result of compensating for thermodynamic constraints at a given temperature. The apparent absence of clearer responses could be also due to our current limitations in mechanistic theory, and therefore nonsensical estimates in TPC parameters. For instance, metabolic theory of ecology (Brown et al., 2004) has not yet integrated how the biochemical kinetics of the metabolism contribute to a single activation energy parameter E (O'Connor et al., 2007).

The lack of adaptation in growth rate to increased temperature in this study may suggest that adaptation to thermal stress from de novo mutations is more difficult than adaptation to other stressors, such as to pH (Moerman et al., 2020a), for example. While mechanistic targets of selection are less well defined for temperature, often observed strategies of adaptation to temperature include the evolution of 'flexible' enzymes (Marx et al., 2006), paralogous isozymes through duplication and mutation of genes (Somero, 1995), membrane composition (Hazel, 1995) and mitochondrial density/ capacity (Pörtner, 2001), for example. Importantly, as temperature affects the folding of proteins and the kinetics of biochemical reactions within a cells, it seems likely that a wide range of adaptations would be needed to confer thermal adaptation. Indeed, for example in *E. coli*, adaptation to extreme temperature was characterised by many epistatic interactions across the genome (Tenaillon et al., 2012). Further, a study of the thermal limits of 2000 taxa found that ancestral adaptation to cold temperatures happens far quicker than adaptation to warm temperatures (Bennett et al., 2021).

Evolution of body size and swimming behaviour

The three largest protist species, *P. caudatum*, *Blepharisma* sp. and *E. gracilis*, exhibited consistent evolutionary changes in traits other than growth rate: Selection at the highest temperatures led to a reduction in their body size and modifications in swimming behaviour (swimming speed and tortuosity) across the assayed temperatures (Fig. 4). Changes in swimming speed were also found in one strain of *T. thermophila*.

We observed an overall reduction in body size of the evolved lines compared to ancestral conditions in all species (Fig. S3). Body size reduction might be adaptive and in line with expectations from Bergmann's rule (Watt et al., 2010). This considers ecological and evolutionary responses of body size to temperatures, and hypothesizes increase size in colder climates as an evolutionary strategy to conserve heat due to lower surface-to-volume ratio in larger organisms, although this might not be universal (Angilletta and Dunham, 2003; Kingsolver and Huey, 2008). At warmer temperatures instead, smaller body sizes might be favoured because of their lower metabolic demands and efficiency in facing oxygen limitations (Gardner et al., 2011; Verberk et al., 2020). Thus, the reduction in body size in life-history diverse protist species might be a general response to the warmest temperatures experienced during the 10 month selection experiment. *T. thermophila* was the exception here (Fig 4), and this was in contrast to a previous study showing evolutionary decreases in body size in response to thermal selection (de Melo et al., 2020)). *T. thermophila* is the smallest of the four species in the study, and it may have been harder or even impossible to respond towards such evolutionary trajectory. It is possible that *T. thermophila* has already attained its biophysical limit due to laboratory handling, and could therefore not additionally decrease its body size. Several thermal experimental evolution studies have shown a direct relationship between increased temperature and the evolution of reduced sizes (Anderson, 1973; Cavicchi et al., 1989; Partridge et al., 1994), and this is also generally expected in aquatic ecosystems due to global warming (Daufresne et al., 2009). However, the general trend is still debated, and several additional factors should be considered such as intra- and inter-specific variation or the association with other traits, making predictions on body size evolution not straightforward. For instance, in their meta-analysis (Siepielski et al., 2019) found little support for adaptive evolutionary shift to shrinking body size in function of increased global temperatures.

The concurrent evolution of the swimming movement (Fig. 4) is a result that should be interpreted carefully, especially considering how the experimental design had no explicitly spatial component. Previous evolutionary experiments with *P. caudatum* (Zilio et al., 2023b,a) and *T. thermophila* (Fronhofer and Altermatt, 2015) have provided evidence for the rapid evolution of swimming behaviour. Similarly to our findings showing for *P. caudatum* the evolution of higher tortuosity with warming temperatures,

selection at the front of laboratory-simulated range expansions led to the evolution of higher dispersal, which was associated with the evolution of increased tortuosity (Zilio et al., 2023b,a). Tortuosity is the propensity to change directions while swimming, and can be interpreted as a more exploratory swimming behaviour, which could indeed facilitate dispersal or allow to closely track micro-variation in the environmental conditions. It is tempting to (i) speculate how selection at the highest temperature, likely representing the most stressful conditions, might have selected *P. caudatum* for increased tortuosity and potentially dispersal to more benign conditions, and (ii) to generalize this to *Blepharisma* sp. and *E. gracilis* which also showed the same pattern for reduced body size. However, in the studies mentioned above the trait under selection was dispersal, and evolutionary changes in swimming behaviour might be simply correlative responses or part of an emerging dispersal syndromes (Cote et al., 2016). Such speculative arguments do not necessarily hold for Tet VII. In *T. thermophila*, it is higher swimming speed rather than tortuosity to be associated with dispersal (Fronhofer and Altermatt, 2015), but our results suggest the evolution of reduced swimming speed at increased selection temperature. Nonetheless, *T. thermophila* already responded to laboratory conditions by increasing its swimming speed (Fig. S3). Thus, similarly to what was proposed before for body size, *T. thermophila* might have already reached some intrinsic limits and could not further increase swimming speed.

Experimental design and limitations

Although we found evolutionary responses to thermal selection, it is important to put these results into context with some limits placed upon evolution in our experiment. Each replicate line was established from a clonal line without any standing genetic variation (Fig. 1), a scenario that is unlikely to be the norm during adaptation to increased temperatures in wild populations. Standing genetic variation is expected to increase the speed of adaptation as i) the pace of evolution is a function of the amount of variation present, ii) the variation already present may be 'pre-tested' (i.e., it was not so deleterious as to be purged from the gene pool) and iii) there is a higher probability of fixation of weakly beneficial alleles as fixation probability is not only a function of the magnitude of the beneficial effect, but also of the effective population size (Barrett and Schluter, 2008). Further, as only a single mating type was present in each selection line, no recombination could occur, which many ciliates do under stressful conditions (Collins, 2012), and could be expected to increase the speed of adaptation (Cooper, 2007; McDonald et al., 2016). Our selection regime also imposed (comparatively mild) regular bottlenecks (1:10) during transfers to new bacterized medium, an often unavoidable feature of experimental evolution using serial transfer batch cultures (Wahl et al., 2002), which would act to limit the effective population size of the populations and therefore increase the effect of drift, reducing the probability that a beneficial mutation will reach fixation (Frankham et al., 1999).

It is also worth noting how the ratchet design will have introduced some variability among selection lines in how long they were kept at each selection temperature (Fig. 1). For instance, lines that evolved at the highest temperatures (e.g., 36–39°C) spent overall less absolute time in their selection regime compared to lines exposed to 20°C or 25°C. However, these organisms, as many other ectotherms, can have faster reproduction at higher temperatures(Fig. 2) therefore experiencing more divisions per absolute amount of time. As a consequence, it is hard if not impossible to standardise time in our experiment.

Finally, wild populations are unlikely to experience such strong, persistent selection pressures and constant temperatures, but rather fluctuations (e.g., due to diurnal and seasonal temperature cycles) or heatwaves (Mazdiyasni and AghaKouchak, 2015), which impact how we might expect populations to respond to climate change. Temperature fluctuations can have important consequences for biological processes (Slein et al., 2023). They can interact with other stressors (Verheyen et al., 2019; Chang et al., 2022) and modify biotic interactions (Raffel et al., 2012), leading to different outcomes than constant thermal regimes (for a discussion for adaptation to fluctuating temperatures see also Malusare et al., 2023). In a recent meta-analysis and synthesis, Stocker et al. (2024) found that biological responses to thermal change were mostly driven by mean temperatures and not fluctuations, but this was context-dependent. For instance, in freshwater environments (habitat of the protist species used in this study), the general pattern was indeed reversed, and the fluctuating temperatures had a stronger impact on biological responses than stable temperatures, suggesting how fluctuations might be the most relevant temperature regime to be investigated using protists.

Implications and conclusions

While both local adaptation and dispersal (movement) in response to a warming world will undoubtedly play a role in species' persistence (see e.g., Norberg et al., 2012; Thompson and Fronhofer, 2019; Usui and Angert, 2024), there are many cases in which dispersal may be too limited, for instance due to anthropogenic fragmentation and loss of connecting habitat (Harrison and Bruna, 1999; Fahrig, 2017; Fletcher et al., 2018; Kamal et al., 2025). In such cases, understanding how a population can be rescued (Gonzalez et al., 2013; Chevin and Bridle, 2025) from environmentally induced declines via adaptation is vital to truly evaluate the potential effects of climate change. Reduction in body size, for example, could be adaptive against extreme temperature, but it may have detrimental consequences, affecting eco-evolutionary dynamics and ecosystem functioning (Dossena et al., 2012). Populations with reduced body size may be more exposed to other global change stressors (e.g., pollutants) or suffer more from antagonistic biotic interactions (e.g., parasites or predators), potentially increasing their extinction probability. In fact, change towards smaller body sizes is even used as a proxy and predictor to detect and manage populations at risk of collapse (Cardillo, 2021; Williams et al., 2021).

In this study, we have assayed the population growth of multiple protist species that have undergone selection at increased temperatures. Our results highlight the importance of using multiple-traits approaches to investigate a species response to increasing temperatures and TPC evolution, and have implications for how species may respond to climate change and impact whole ecosystems.

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