Research

Cite this article: Bozinovic F, Cavieres G, Martel SI, Alruiz JM, Molina AN, Roschttardt H, Rezende EL. 2020 Thermal effects vary predictably across levels of organization: empirical results and theoretical basis. *Proc. R. Soc. B* **287**: 20202508. <http://dx.doi.org/10.1098/rspb.2020.2508>

Received: 8 October 2020
Accepted: 15 October 2020

Subject Category:
Ecology

Subject Areas:
biochemistry, ecology, physiology

Keywords:
temperature adaptation, integrative biology, coadaptation, thermal performance curves, climate change

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.5182592>.

Thermal effects vary predictably across levels of organization: empirical results and theoretical basis

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Thermal performance curves have provided a common framework to study the impact of temperature in biological systems. However, few generalities have emerged to date. Here, we combine an experimental approach with theoretical analyses to demonstrate that performance curves are expected to vary predictably with the levels of biological organization. We measured rates of enzymatic reactions, organismal performance and population viability in *Drosophila* acclimated to different thermal conditions and show that performance curves become narrower with thermal optima shifting towards lower temperatures at higher levels of organization. We then explain these results on theoretical grounds, showing that this pattern reflects the cumulative impact of asymmetric thermal effects that piles up with complexity. These results and the proposed framework are important to understand how organisms, populations and ecological communities might respond to changing thermal conditions.

1. Introduction

The world's climate is changing rapidly. Virtually all climate projections predict a dramatic increase in mean temperatures, ranging between 1.0 and 4.0°C, and thermal variability across the planet during this century [1]. Not surprisingly, the temperature has been the focus of a large body of research due to its impact on energy flow and limits to survival and reproduction. These effects are grounded on thermodynamics, which presumably constrains the potential responses that organisms might display to changing temperatures [2–4], and indeed most biological rate processes from biochemical reactions up to population growth seem to be affected in a consistent manner by temperature [1,5]. Indeed, this common unimodal and asymmetric shape currently described by thermal performance curves (TPCs) has been adopted as a baseline to compare thermal sensitivity across genotypes, populations or species [6,7] and to examine the response of organisms under scenarios of global warming [8–10]. Interestingly, while thermal effects on enzyme kinetics and denaturation rates can readily explain the shape of TPCs in biochemical reaction rates, it is currently unclear how these effects scale up to higher levels of the organization and translate into variation in organismal performance or population growth [11]. In other words, when we detect acclimation responses in TPCs at the whole-organismal level or differences in fitness between populations or species, what exactly is happening at lower levels? How are responses integrated from cellular processes up to entire physiological systems, and then how does the interplay between biological functions such as digestion or respiration translate into individual survival and reproduction? Ultimately, are there general rules that determine how TPCs vary between different levels of organization?

Here we try to provide some answers to these questions, by studying if TPCs are invariant across levels of organization and if they respond in an integrated fashion to different environmental thermal conditions. First, we report the results of an empirical experiment testing if traits at higher levels of organization exhibit narrower TPCs than lower level traits, as we recently predicted [11], and if these traits respond in a similar fashion to thermal acclimation. Then, we demonstrate how the outcome of this experiment and patterns reported in large-scale comparative analyses [11–13] can be explained on conceptual grounds. Finally, we discuss the repercussions of these findings for physiologists, ecologists and evolutionary biologists working in thermal biology and climate change.

To test experimentally if TPCs become narrower at higher levels of biological organization, we subjected *Drosophila melanogaster* to four thermal acclimation conditions: cold versus warm (15 and 27°C), constant versus variable temperatures (± 0 and 4°C) in a crossed design (see Methods). Then, we characterized the TPCs in the following traits at contrasting levels of organization: hexokinase (HK) specific enzymatic activity, whole-organismal metabolic rate, maximum running speed and viability (see Methods). We selected these variables primarily for logistic reasons, since they were relatively straightforward to measure across multiple temperatures and often required large sample sizes. We then performed a nonlinear fit employing the following function that describes how performance (pf) is affected by temperature (T):

$$pf = \begin{cases} (Ce^{T \ln Q_{10}/10}) & \text{if } T < T_{th} \\ (Ce^{T \ln Q_{10}/10}) \times (1 - d(T - T_{th})^2) & \text{if } T > T_{th} \end{cases} \quad (1.1)$$

As detailed in Rezende & Bozinovic [11], this function combines an exponential increase in reaction rates due to thermodynamics with a quadratic decrease in activity due to denaturation and contains four parameters: a constant C that shifts the curve up and down everything else being equal, Q_{10} describing the fold-increase in performance expected for a 10°C rise in temperature, a threshold temperature T_{th} in which denaturation starts taking place and the thermal sensitivity of denaturation d to temperatures above this value. From these parameters, we also calculated the optimal temperature in which performance is maximized (T_{opt}), the critical thermal maximum (CT_{max}) and the thermal breadth in which performance can be maintained at a 50% of its maximum ($T_{breadth}$). Importantly, with the exception of C that is expressed in units of performance, all parameters and descriptors of the TPC can be compared across traits, which allows us to study how TPCs vary across traits and treatments and also the mechanistic nature of these responses [11].

2. Methods

(a) Animal capture, maintenance and design

We measured traits at different levels of organization in the fruit fly *D. melanogaster*. Near 250 inseminated flies were collected in central Chile (33°26'S; 70°39'W at 500 m above sea level) during summer. After capture, flies were identified based on morphological characters [14]. Six groups were established with approximately 40 flies each. Groups were reared in controlled conditions at a constant ambient temperature $T_a = 24^\circ\text{C}$ and a 16 L:8 D photoperiod in Plexiglas cages ($27 \times 21 \times 16 \text{ cm}^3$) with Burdick culture medium plates [15] for five generations. To maintain a discrete generation regime, flies were allowed to

oviposit for 36 h in new food plates placed in each cage. The eggs were then placed in 130 ml bottles with medium (approx. 100 eggs per bottle to avoid overcrowding effects) and adult flies were transferred to a new cage as they emerged. Population size within each cage varied between roughly 1000 and 1500 breeding adult individuals, and we maintained six replicate cages that were pooled every generation to minimize the loss of genetic variation due to drift. Fifth-generation adult males and virgin females from this stock were randomly assigned to four thermal treatments in separate climatic chambers (PITEC, Model BIOREF) as described elsewhere [16]: $15 \pm 0^\circ\text{C}$ (15 C where 'C' stands for constant), $15 \pm 4^\circ\text{C}$ (15 V with 'V' for variable), $27 \pm 0^\circ\text{C}$ (27 C) and $27 \pm 4^\circ\text{C}$ (27 V). The photoperiod was 16 L:8 D. For variable temperatures, the temperature started to increase linearly at 07.00 up to the maximum at 09.00 and dropped from 19.00 to 21.00 h. The heating/cooling rate between the minimum and maximum temperatures was $0.08^\circ\text{C min}^{-1}$. The offspring were maintained from eggs to adults in each thermal treatment, and then we conducted the TPC experimental assays.

(b) Biochemistry

To characterize the effects of the thermal acclimation treatment on the TPC at the molecular level, we measured hexokinase specific enzymatic activity (U/total protein), using the Hexokinase Colorimetric Assay Kit from Sigma-Aldrich (MAK091). The protein extracts from multiple flies for each temperature treatment were homogenized in a grinding buffer provided by the kit. Total protein concentration for each sample was determined using Bradford Reagent from Sigma-Aldrich (B6916). All activity assays were conducted at a controlled temperature at 10, 20, 30, 40 or 50°C. Incubation at different temperatures was performed using a Biosystem 2720 thermal cycler. For all records, three replicates were assayed. Enzyme activity assays were carried out on a spectrophotometer UV mini 1240 Shimadzu.

(c) Metabolism and locomotor performance

We also estimated standard metabolic rate (SMR) in adult flies with a flow-through respirometry system. Individual flies were measured in 1 ml glass chambers; flow rate was 25 ml min^{-1} [16], and measurements were carried out at 12, 15, 20, 24, 27, 30, 32, 35 and 37°C (861 flies in total, $n \geq 20$ individuals at each temperature per treatment). Carbon dioxide production ($V\text{CO}_2$) was measured using the LI-7000 infrared CO_2 gas analyser (LICOR, Lincoln, NB, USA) attached to the MAVEN system (Sable Systems International SSI; Las Vegas, Nevada, USA) that allowed for the serial measurements of 16 metabolic chambers (one was empty as a baseline). Inflow CO_2 was scrubbed with Baralyme® and chambers were flushed with humidified air (above 90% relative humidity) to minimize desiccation. CO_2 production in each chamber was monitored twice for 5 min, resulting in trials lasting <3 h (=16 chambers $\times$ 5 min $\times$ two measurements). Data were extracted using LabAnalyst software (<https://warthog.ucr.edu>) and SMR was estimated for each 5 min period as the 30 s average when $V\text{CO}_2$ was stable and individuals presumably inactive.

As a proxy of locomotor performance, we quantified climbing speed (cm s^{-1}) by knocking down a fly in a narrow glass test tube ($12 \times 100 \text{ mm}$) and measuring the time required to walk up the tube to a height of 7.0 cm [17]. Individual flies from each treatment were kept for 10 min at the test temperature and then measured in the following the sequence: 12, 18, 24, 28, 32, 36, 38, 39 and 40°C (1404 measurements in total, $n = 10$ individuals per treatment). Animals remained at room temperature of $24 \pm 2^\circ\text{C}$ for around 25 min between batches. Therefore, each assay lasted approximately 45 s per fly, which then had 35 min between consecutive tests to recover. For both $V\text{CO}_2$ and locomotor performance, sexes were nearly balanced in each assay and were pooled in subsequent analyses.

(d) Viability

To estimate eggs-to-adult viability, new food plates were placed in maintenance cages for flies oviposition for 24 h. Then eggs were collected using a microscope Leica S6 [18]. After 24 h, 15 eggs were collected from each vial using a microscope. These eggs were transferred in batch to fresh vials, assigned and maintained in the climatic chambers at 12, 15, 20, 24, 27, 30 or 32°C until hatching occurred (552 vials in total, $n \approx 20$ replicates per temperature per acclimation treatment). Hatching was assessed every 24 h during a total period of 45 days, and viability for each replicate was calculated as the number of adults that successfully developed divided by the developmental time (we only focused on egg-to-adult developmental time and did not monitor other developmental stages). While viability is assessed primarily as the fraction of eggs that eclose and reach maturity, here we standardize by developmental time to obtain a more realistic and informative proxy of population growth rates at different temperatures.

(e) Statistical analyses

We fitted the TPCs described for biochemical reactions, organismal performance and viability employing the method developed by Rezende & Bozinovic [11]. Because the original method relies on mean estimates of performance for the nonlinear fit and may not always converge, to improve the probability of convergence and ensure the appropriate estimation of parameters we developed a function *ad hoc* in R (<https://cran.r-project.org>) that also takes into account the variation around performance means (the R functions employed to fit TPCs and estimate different parameters, worked examples and detailed explanations are provided in the electronic supplementary material). Briefly, the method samples a random performance estimate from a normal distribution with the mean $\pm$ s.d. obtained empirically for each temperature and fits the nonlinear model. This is done iteratively to obtain multiple estimates of C , Q_{10} , T_{thr} and d (equation (1.1)), whose median values provide TPCs that are highly consistent with results from the original nonlinear fit when there is convergence ($n = 100$ iterations). With these parameter estimates, we calculated descriptors T_{opt} , CT_{max} and $T_{breadth}$ for all 16 TPCs (=4 traits $\times$ 4 thermal treatments) (electronic supplementary material, table S1).

To determine how TPCs vary across traits and acclimation temperatures, and account for uncertainty in parameter estimation within each of the 16 treatments [19], we performed a mixed model with these variables as fixed effects, tested over treatment as a random factor (with a 100 fitted TPCs for each treatment). Preliminary analyses showed that there were virtually no differences between constant and variable regimes; therefore, this factor was not included for simplicity. Analyses were performed separately for parameters Q_{10} , T_{thr} , log-transformed d and descriptors T_{opt} , CT_{max} and $T_{breadth}$ (electronic supplementary material, table S2) and then for the first, second and third principal components obtained from a PCA analysis including all these variables (see [20] for similar analyses, results in electronic supplementary material, table S3). Parameter C was not included because it is expressed in different units for different traits and describes vertical shifts that have no effects in the overall shape of the TPC [11]. Details regarding the nonlinear fit and the functions available to perform these analyses are provided in the electronic supplementary material.

3. Results

The nonlinear regression provided a good fit to the empirical data, with $r^2 = 0.94 \pm 0.06$ ($\pm$ s.d.) between fitted versus observed mean values (figure 1). Diagnostic tests suggest that TPCs differ primarily between traits and acclimation temperatures, hence for simplicity we pooled constant and

Figure 1. Thermal performance curves across levels of organization. (a) Thermal effects on biochemical reaction rates, (b) whole-organism metabolic rates, (c) locomotor performance and (d) population viability under different acclimation regimes. Data points represent empirical measurements (mean $\pm$ s.d.) from flies subjected to different acclimation regimes (15°C and 27°C constant or variable, see Methods) and lines the nonlinear fitted curves (equation (1.1)). (Online version in colour.)

variable data and report results from a two-way mixed model (tables S2 and S3). According to this analysis, T_{opt} , CT_{max} and $T_{breadth}$ differ significantly between traits ($F_{3,11} > 38.15$, $p < 4.2 \times 10^{-6}$ in all cases) and T_{opt} and $T_{breadth}$ are also affected by acclimation temperature: cold-acclimated flies exhibit significantly lower T_{opt} ($F_{1,11} = 13.11$, $p = 0.0040$) and higher $T_{breadth}$ ($F_{1,11} = 11.43$, $p = 0.006$) than their warm-acclimated counterparts (figure 2). With regards to the parameters of the curve that should explain these results (equation (1.1)), we only detected significant differences between traits and acclimation temperatures primarily in Q_{10} ($F_{3,11} = 24.93$, $p = 3.3 \times 10^{-5}$ and $F_{1,11} = 7.18$, $p = 0.021$, respectively).

Figure 2. Disentangling the effects of acclimation and levels of organization. Comparisons between (a) temperature optima, (b) maximum critical temperatures and (c) thermal breadth for TPCs obtained for different traits and acclimation regimes. (d) A principal component analysis shows that these descriptors and fitted parameters (equation (1.1)) vary along two dimensions that account for nearly 85% of the variation, with the first dimension clearly discriminating traits at different levels of organization. Only mean estimates are shown for clarity, the analysis included 100 fitted curves per treatment ($n = 1600$ curves in total). Symbols as in figure 1. (Online version in colour.)

To determine how the overall shape of TPCs varies between traits and across acclimation treatments and to quantify and control for the covariation between different TPC parameters and descriptors, we performed a principal component analysis (PCA) including Q_{10} , T_{thr} , d log-transformed to improve normality, T_{opt} , CT_{max} and $T_{breadth}$. The first principal component accounted for 50.4% of the total variance across TPCs and encapsulated the most of the variation described by highly colinear T_{opt} , CT_{max} and $T_{breadth}$. Different traits fell along this component in accordance with their level of organization, with lower level HK activity exhibiting broad TPCs displaced towards high temperatures, population-level viability narrow TPCs displaced towards lower temperatures and whole-organismal metabolism and running speed lying in between these extremes (figure 2). According to the mixed model, differences between traits accounted for 98.0% of the total sums of squares in this component ($F_{3,11} = 302.5$, $p = 7.6 \times 10^{-11}$), whereas thermal acclimation accounted for an additional 0.8% of the variation ($F_{1,11} = 7.54$, $p = 0.019$). Variation in $T_{breadth}$ was mirrored by changes in Q_{10} and d , with low and high parameter values resulting, respectively, in

broad and narrow curves as described in other analyses [11]. The second principal component, which accounted for 28.5% of the variance, described the overall symmetry of the curves varying from left-skewed TPCs characterized by a low Q_{10} and high d to more symmetrical bell-shaped TPCs with a high Q_{10} and low d .

4. Discussion

We contend that the overall pattern described by the first principal component in our PCA—i.e. narrower TPCs displaced towards lower temperatures as complexity increases—constitutes a general biological rule that can be explained by first principles because (i) biochemical reactions exhibit TPCs that are inherently asymmetric and left skewed, (ii) reactions vary in their thermal sensitivities and therefore exhibit different TPCs and (iii) biological rates at higher levels emerge from the interaction of lower level processes (figure 3). To demonstrate how these principles give rise to this general behaviour, we adopt the net rate constant approach that

Figure 3. Thermal performance and complexity. Conceptual framework proposed to explain our empirical results. (a) As complexity and the number of limiting steps that vary with temperature increases, as shown for reactions k_1 , k_2 and k_3 , narrower TPCs with lower thermal optima emerge for the resulting reaction or process k_r . (b) This effect increases with the mismatch between TPCs at lower levels, as calculated for different k_1 , k_2 and k_3 (equation (4.1)). (c) Even though isozymes and parallel reactions can partly circumvent this limitation, we argue that this pattern constitutes a general rule in thermal biology, where cumulative energy costs are expected to increase with organization as more processes are involved (energy costs are schematically shown here for clarity and were not modelled in our study). (Online version in colour.)

designates, by analogy with electrical circuitry, the net rate constant k as the estimated conductance of a specific step within a set of reactions under steady-state [21,22]. This approach employs net rates instead of true reaction rates of opposite directions, reducing a sequence of reversible cycles into irreversible steps characterized by a net rate constant k_i . For a series of unbranched reactions of n steps, the resulting net rate k_r can be calculated from the rate of its components as [22]:

$$\frac{1}{k_r} = \sum_{i=1}^n \frac{1}{k_i}, \quad (4.1)$$

and therefore, when all steps present exactly the same net reaction rate ($k_i = k$), the right side of the equation can be reduced to $n k^{n-1} / k^n$ and we ultimately obtain $k_r = k/n$. That is, the net rate is inversely proportional to the number of steps in the reaction. If this pattern holds across different temperatures, the overall shape of the TPC for k_r will be identical to the TPC of each reaction step.

However, a more realistic scenario is that different steps will vary in thermal sensitivity and exhibit different TPCs, given not only the enormous array of functions and structural properties of different enzymes, but also the contrasting activation energies and dynamics of different reactions. Thus, k_i at each specific temperature will often differ. Expressing k_i as deviations from the average, we obtain $k_i = \bar{k} + e_i$ and this approximation based on a polynomial expansion for a large n :

$$\frac{1}{k_r} \approx \frac{\bar{k}^{n-1}}{\bar{k}^n} \sum_{i=1}^n \frac{1}{(1 + e_i/\bar{k})}, \quad (4.2)$$

The sum in the right side converges to n as e_i become increasingly smaller and, conversely, increases with the dispersion in e_i to values above n . In these circumstances, the net rate constant $k_r < \bar{k}/n$ and the magnitude of this

inequality is proportional to the variation in k_i . Because the mismatch between k_i is expected to be higher in the regions of the TPCs where reaction rates are increasing or decreasing sharply, the drop in k_r should be disproportionately larger in these regions resulting in a narrower TPC with higher Q_{10} and d (figure 3). Additionally, because TPCs are generally left skewed, this response is expected to be asymmetric and result in a displacement of T_{opt} and CT_{max} towards lower temperatures, with the ultimate constraint that the TPC for k_r must display the same CT_{max} as the lowest CT_{max} among its reaction steps (figure 3). This is because at this temperature one k_i tends to zero and this will drive k_r of the higher level reaction to zero as well (equation (4.1)), which implies that a reaction chain is only as resilient to temperature as its weakest link.

Overall, this exercise shows that we can explain all observed responses embedded in the first principal component of the *Drosophila* experiment, as well as a higher sensitivity to increasing temperatures (i.e. elevated Q_{10} , activation energy and curvature) and narrower thermal breadths at higher levels of organization [11–13], from first principles. And granted, this is an oversimplification. Metabolic networks are substantially more complex than sequential reactions, and isozymes specialized to work at different temperatures, reactions with branched pathways and the myriad of motifs detected in metabolic networks might partly compensate the inherent constraints described above. For parallel pathways, for instance, the net constant rate approach predicts $k_r = \sum k_i$, which may give rise, if two isozymes or sets of enzymes with contrasting T_{opt} are involved, to bimodal TPCs with a higher $T_{breadth}$ than each specific component [23]. Also, scaling up TPC responses above the individual level to populations or even communities involve processes that are not directly analogous to biochemical reactions. Nonetheless, we argue that this trend should be general and hold at higher levels of organization because it reflects an inherent constraint associated with increasing complexity, broadly defined here as the number of conditions that

must be fulfilled for a given task to be accomplished [24]. For instance, an individual can only reproduce in an environment where it can survive, remain active and digest its food, which is bound by the temperature range in which the TPCs for locomotion and digestion overlap. Similarly, the TPC of population fitness is bound by the range in which its individuals can reproduce, the TPC of species interactions at the community level [25] is bound by the thermal range in which their population sizes are high and sustainable, and so forth. Having said that, it is clear that these processes involve different temporal windows and both spatial and temporal variation in temperatures adds another layer of intricacy regarding how thermal effects at lower organization levels will ultimately affect populations' and species' responses to changing environmental conditions. Temporal effects are particularly important, as different models studying growth or survival rates predict narrower TPCs at larger temporal windows [26,27]. However, these effects are not mutually exclusive with the scenario proposed here, because increasingly complex processes often involve more time to be accomplished (e.g. the whole reaction described in figure 3a must take longer than each of its steps).

Our analyses suggest that thermal effects on performance are expected to vary predictably across different levels of biological organization (figure 3), which opens the venue to understand the mechanistic basis of thermal adaptation. While the general framework is supported by our empirical results and large-scale comparative analyses [11–13], future experiments including multiple traits within each category (e.g. TPCs obtained for many enzymes) or studies where the functional relationship between different processes is well-established (such as linear reaction systems) remain necessary to validate underlying assumptions, assess their generality and to study predictions in more detail. In spite of these limitations and the preliminary nature of our results, the proposed framework explains from first principles why variation in heat tolerance seems to be substantially lower than the estimated variability in cold tolerance, suggesting that the former is more conserved across lineages [28–30]. While it might be argued that other factors apart from biochemical reactions might account for observed thermal limits, we emphasize

that the fundamental assumption underlying our framework is that these factors also operate within a restricted thermal range (e.g. membrane properties such as fluidity or permeability also exhibit thermal optima and fall to zero at low and high thermal extremes). Also, this framework gives rise to several predictions that can be readily tested theoretically or empirically, such as the thermal coadaptation hypothesis, which predicts that the temperature dependence of different biological processes should coevolve and exhibit some degree of integration [31]. Varying degrees of coadaptation and physiological integration should in principle result in different levels of mismatch between traits at different levels of organization. More specifically, a high degree of coadaptation should give rise to TPCs with similar T_{opt} and $T_{breadth}$ across different levels of organization, whereas a low degree of coadaptation should result in narrower TPCs shifted towards lower temperatures at higher levels (figure 3b). This prediction can be put to the test empirically, for instance, with breeding experiments involving distant populations presumably adapted to different thermal environments and their hybrids, combined with the PCA analysis employed here (figure 2). Similarly, how symmetry and skewness in thermal effects differ between levels of organization can also be readily assessed by comparing extremes in this continuum, namely TPCs for biochemical reaction rates against those for intrinsic rates of population increase [11].

Data accessibility. The R script used to conduct the data analysis and the datasets are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.5hqbzkh49> [32].

Authors' contributions. F.B. and E.L.R. designed the research; H.R. performed the enzymatic assays, S.I.M., J.M.A., A.N.M. and G.C. the metabolism, locomotion and viability assays. F.B. and E.L.R. analysed the data and wrote a first draft that was edited by all authors.

Competing interests. The authors declare no competing interest.

Funding. This work was supported by FONDECYT 11190637 grant to G.C., and by ANID PIA/BASAL FB0002 and FONDECYT 1190007 grants to F.B.

Acknowledgements. We thank Mauro Santos for many discussions on the subject, and two anonymous reviewers for their detailed feedback on a first draft. In memoriam of A. Veloso.

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