A lack of repeatability creates the illusion of a trade-off between basal and plastic cold tolerance

Erica O’Neill, Hannah E. Davis and Heath A. MacMillan

Department of Biology, Carleton University, ON K1S 5B6, Canada

The thermotolerance–plasticity trade-off hypothesis predicts that ectotherms with greater basal thermal tolerance have a lower acclimation capacity. This hypothesis has been tested at both high and low temperatures but the results often conflict. If basal tolerance constrains plasticity (e.g. through shared mechanisms that create physiological constraints), it should be evident at the level of the individual, provided the trait measured is repeatable. Here, we used chill-coma onset temperature and chill-coma recovery time (CCO and CCRT; non-lethal thermal limits) to quantify cold tolerance of Drosophila melanogaster across two trials (pre- and post-acclimation). Cold acclimation improved cold tolerance, as expected, but individual measurements of CCO and CCRT in non-acclimated flies were not (or only slightly) repeatable. Surprisingly, however, there was still a strong correlation between basal tolerance and plasticity in cold-acclimated flies. We argue that this relationship is a statistical artefact (specifically, a manifestation of regression to the mean; RTM) and does not reflect a true trade-off or physiological constraint. Thermal tolerance trade-off patterns in previous studies that used similar methodology are thus likely to be impacted by RTM. Moving forward, controlling and/or correcting for RTM effects is critical to determining whether such a trade-off or physiological constraint exists.

1. Introduction

Thermotolerance is widely considered a major determinant of ectotherm geographic distribution, as thermal limits—particularly lower thermal limits (LTLs)—can strongly predict ectotherm range limits [1–3]. In an era of global climate change [4], the ongoing persistence of ectotherms already residing near their thermal limits will depend on the ability of these organisms to adapt or respond plastically to changing thermal conditions [5,6]. Consequently, understanding evolutionary and physiological constraints on ectotherm cold tolerance adaptation and plasticity may be vital for predicting how ectotherms will be affected by global climate change [5].

In many ectotherms, exposure to sublethal low temperatures physiologically primes the organism to better respond to subsequent cold stresses (reviewed for insects in: [7,8]). Consequently, cold tolerance can be conceptually divided into two categories: basal tolerance (an organism’s baseline level of cold tolerance; e.g. [9,10]) and induced tolerance (an organism’s level of cold tolerance following some adjustment period; e.g. [9]). Physiological priming that results in an improvement in thermotolerance is a form of phenotypic plasticity and is typically named according to the length of the sublethal temperature exposure, from short-term (minutes-hours) ‘hardening’ to long-term (days-weeks) ‘acclimation’ [11,12].

Basal and plastic cold tolerance can be quantified using a variety of metrics in chill-susceptible insects (reviewed in [7]). For instance, critical thermal minimum (\(CT_{\text{min}}\)) is the temperature, upon cold exposure, at which neuromuscular coordination of the organism becomes impaired [13]. If cold exposure persists, \(CT_{\text{min}}\) is followed by chill-coma onset (CCO), the temperature at which...
neuromuscular function is entirely lost, resulting in complete paralysis [13]. Following CCO and a period of time in the cold, chill-coma recovery time (CCRT) is the time taken for an organism to regain neuromuscular function after being removed from coma-inducing conditions [14]. For all three metrics—CT\text{min}, CCO and CCRT—low values indicate high cold tolerance. In contrast with the above non-lethal metrics, lethal measures of cold tolerance include survivorship (the proportion of organisms surviving some cold exposure; e.g. [10]), lower lethal temperature (LLT; the temperature at which a certain proportion of individuals die under a fixed duration of cold exposure (this is calculated using survivorship); e.g. [15]) and LTL (an average temperature of mortality; e.g. [16]).

One critical uncertainty and point of contention among thermal biologists is whether basal thermotolerance constrains plastic thermotolerance, such that animals with greater basal tolerance—either cold tolerance (as described above) or heat tolerance—have a lower capacity for acclimation or hardening [10,15–21]. To address this question, several researchers have used thermotolerance metrics to test for a trade-off between basal and induced cold tolerance (a ‘tolerance-plasticity trade-off’; summarized in table 1). While some studies (spanning a variety of taxa) have documented evidence for such a trade-off at low temperatures (e.g. [10,19,21]), others have found partial or no such evidence [15,16,20]. Similarly, in a recent review of heat tolerance plasticity trade-off studies, approximately half of the studies gathered—17 out of 30—did not support a trade-off [22].

Importantly, the studies described above and in table 1 tested for a trade-off between some measure of basal cold tolerance and plasticity predominantly at the species, lineage, or population level. But if basal tolerance constrains plasticity through shared physiological mechanisms of tolerance, such a pattern should arise at the level of the individual. The CT\text{min} for example, is thought to occur as a result of sudden ionoregulatory failure (spreading depolarization) in the nervous system [23,24], so, for example, constitutive expression of ionoregulatory proteins (e.g. ion channels) that help to lower the basal CT\text{min}, may approach the ‘ceiling’ to expression that can occur during thermal acclimation. Basal and induced cold tolerance have similarly been suggested to share renal mechanisms that help prevent ionoregulatory collapse (at least in Drosophila; [25–27]). We hypothesized that if such constraints exist, they should be evident at the individual level and may underlie trade-off patterns previously observed at higher levels of organization. To our knowledge, however, only three studies have ever investigated the relationship between basal thermotolerance and plasticity at the individual level in ectotherms, each time at high temperatures [28–30]. Thus, turning our attention away from species/lineage/population-level comparisons and towards individual variation—which has historically been underused in physiology [31,32]—may prove informative.

Here, we tested whether basal cold tolerance constrains plasticity at the individual level in Drosophila melanogaster. We did so by quantifying cold tolerance in individual D. melanogaster across two trials (pre- and post-cold acclimation; figure 1). Pre-acclimation measurements give an estimate of basal cold tolerance, while post-acclimation measurements give an estimate of induced cold tolerance. If basal cold tolerance constrains plasticity, we expected that individuals with higher basal cold tolerance (lower trial 1 measurements) would tend to have diminished acclimation capacity (a reduced difference in thermotolerance between trial 1 and trial 2) relative to individuals with lower basal cold tolerance. Such methodology, where acclimation capacity is correlated with basal tolerance and is measured as the difference between basal and induced tolerance, is a common method of testing the tolerance-plasticity trade-off hypothesis (e.g. [10,15,16,21]; table 1). Because different cold tolerance traits have different underlying mechanisms and vary in different ways among and within populations and lineages of Drosophila [3,7,33,34], we carried out our experiment twice using different metrics of thermostolerance: CCO and CCRT (figure 1).

2. Material and methods

(a) Experimental overview

CCO and CCRT (non-lethal metrics of thermostolerance) were used to quantify cold tolerance of individual flies across two trials (pre- and post-acclimation; figure 1) to determine the relationship between basal tolerance and acclimation capacity in cold-acclimated flies (15°C-acclimated; figure 1). Two other groups of flies were included in the experiment: (i) flies that were not subject to cold acclimation between trials (25°C-acclimated) and (ii) flies that were not subject to cold acclimation and that underwent trial 2 but not trial 1 (‘Control’; figure 1). The 25°C-acclimated flies were used to assess the within-individual repeatability (hereafter, ‘repeatability’) of CCO and CCRT in the absence of cold acclimation, thereby estimating the repeatability of basal tolerance as measured by both metrics. Repeatability is a descriptor of the within-individual consistency, or predictability, of a trait over repeat measurements [35,36]. Repeatability of CCO and CCRT in non-cold-acclimated flies is essential to address the trade-off hypothesis because otherwise measurements of CCO and CCRT are unreliable indices of basal thermotolerance. Unhelpfully, the repeatability of ectotherm thermotolerance traits is infrequently studied—repeatability has in fact never been estimated for CCO and has only been estimated twice for CCRT (with conflicting results, and neither time in Drosophila; [37,38])—providing little hint of what to expect. Though, of the ectotherm thermotolerance traits studied, most show at least moderate repeatability (table 2). Importantly, if trial 1 had some effect on trial 2 measurements, estimates of the repeatability of CCO and CCRT in 25°C-acclimated flies (who by necessity undergone both trials) would not necessarily be accurate representations of the repeatability of basal tolerance. Thus, control flies (‘Control’; figure 1) were included to check whether trial 1 itself influenced thermostolerance in trial 2.

(b) Animal husbandry

Drosophila melanogaster used in this study originate from 35 iso-female lines originally captured in London and Niagara on the Lake, Ontario, Canada [46]. Flies were reared in a 25°C incubator (12 h : 12 h light : dark cycle) in 250 ml bottles containing approximately 50 ml of a banana, corn syrup and yeast-based medium. To gather new eggs, adult flies were transferred to bottles containing fresh media (approx. 100 per bottle) and allowed to lay eggs for approximately 2 h before being removed. The eggs laid in this time (approx. 150 per bottle) were reared at 25°C until adult emergence (approx. 10 days). On their day of emergence, adults were transferred to new bottles (such that all flies were within 25 h of age), where they remained for approximately 24 h at 25°C to ensure all females were mated before flies were...
Anaesthetized (less than 15 min exposure to CO₂) and sorted by sex. It was important to ensure females were mated because mating status can affect cold tolerance in insects (e.g. [47]).

Approximately 30 females (per run of the experiment) were then placed individually into 1.7 ml microcentrifuge tubes. Prior to this, the microcentrifuge tubes were prepared by puncturing one small hole in each tube lid and filling the tube with approximately 0.5 ml of the rearing diet. Early trials confirmed that the food inside these tubes stayed well hydrated for at least 72 h and thus would not cause desiccation stress. The microcentrifuge tubes were placed in a 25°C incubator where the food temperature decreased until all flies had entered chill-coma, at which point the rack with vials was removed from the bath. During trial 1, control flies were transferred into 3.7 ml screw-top glass vials and placed in a water–ice mixture (1:1 v/v water: ethylene glycol; as in [49]). The temperature of the cooling bath began at 25°C (for trial 1) or 20°C (for trial 2) and decreased at a rate of 0.1°C min⁻¹. The temperature was monitored by three type-K thermocouples placed at different locations in the bath. After the temperature of the bath reached approximately 10°C, the glass vials were tapped frequently with a metal rod to stimulate movement in the flies [26]. CCO was recorded as the temperature at which a fly stopped moving and was unresponsive to tapping on the vial. The temperature decreased until all flies had entered chill-coma, at which point the rack with vials was removed from the bath. During trial 1, control flies were transferred into 3.7 ml screw-top glass vials and kept in a 25°C incubator for the duration of the trial (approx. 4 h) before being transferred into fresh microcentrifuge tubes (to maintain similar levels of handling for the control and non-control flies).

(c) Measurement of chill-coma onset

CCO was measured by placing flies individually into 3.7 ml screw-top glass vials and placing these vials in a water–ice mixture within a Styrofoam box inside a fridge (such that flies were exposed to approximately 0°C). The vials remained in this set-up for 6 h, after which they were removed from the water–ice mixture and set on a counter at room temperature to record CCRT. CCRT was recorded as the time taken—for the moment the vials were removed from the water–ice mixture—for flies to stand upright on all six limbs [26]. The position of flies was

(d) Measurement of chill-coma recovery time

CCRT was measured by placing flies individually into 3.7 ml screw-top glass vials and placing these vials in a water–ice mixture within a Styrofoam box inside a fridge (such that flies were exposed to approximately 0°C). The vials remained in this set-up for 6 h, after which they were removed from the water–ice mixture and set on a counter at room temperature to record CCRT. CCRT was recorded as the time taken—for the moment the vials were removed from the water–ice mixture—for flies to stand upright on all six limbs [26]. The position of flies was
not altered after the vials were set upright. To control for handling, control flies were transferred into fresh microcentrifuge tubes while trial 1 was being carried out. We also recorded room temperature during our CCRT recordings. Room temperature varied among experimental runs (mean ± s.d.: 24.5 ± 1.3°C), and one run was discarded and then replaced because CCRT tended to be slower (although not significantly) and room temperature on that day was lower (21.9°C) than all other runs. Ultimately, room temperature had no significant effect on CCRT and was shared among all flies in each run (and we included flies from all three treatment groups in each run), so we excluded it as a factor in downstream analyses.

(e) Data analysis
All data analyses were carried out in R v. 3.6.2 [50]. Individuals that were lost or accidentally crushed between trials were removed from the analysis. To test whether treatment (15°C versus 25°C acclimation) affected CCO or CCRT (that is, induced plasticity), linear mixed-effects models (LMMs) were performed (fixed effects: trial (1 versus 2), treatment group; random effects: run date, individual ID (nested within run date)). Repeatability of CCO or CCRT in 25°C-acclimated flies was estimated using the rpt() function in the R package rptR (unadjusted repeatability = 97.9, number of parametric bootstraps = 1000 [51]). Because cold tolerance is plastic and can respond rapidly to changes in the environment, we wanted to make sure our measurements themselves did not induce substantial plasticity or cause injury that might alter subsequent measurements. To test whether disturbances to the flies associated with carrying out trial 1 affected trial 2 measurements, trial 2 measurements (CCO2 or CCRT2) of the control and 25°C-acclimated groups were compared using LMMs (fixed effect: treatment group; random effect: run date). Finally, to assess the relationship between basal tolerance (CCO1 or CCRT1) and acclimation capacity (CCO2–CCO1 or CCRT2–CCRT1), two more LMMs were carried out (fixed effects: CCO1 or CCRT1, treatment group; random effect: run date); slopes of the 15°C- and 25°C-acclimated groups were compared by checking whether there was a significant interaction between the effects of CCO1 or CCRT1 and treatment group (15°C- versus 25°C-acclimated) on acclimation capacity. All LMMs were carried out using the lme() function in the R package nlme [52].

3. Results and discussion
(a) Cold acclimation induced plasticity
As anticipated, 15°C-acclimation induced plasticity in CCO and CCRT. There was a significant effect of trial (LMM: \(F_{1,126} = 97.9, p < 0.0001\)) and treatment (15°C- versus 25°C-acclimation; LMM: \(F_{1,120} = 107.0, p < 0.0001\)) on CCO and a significant interaction between these effects (LMM: \(F_{1,126} = 106.4, p < 0.0001\); figure 2a). Likewise, there was a significant effect of trial (LMM: \(F_{1,191} = 18.0, p < 0.0001\)) and treatment (LMM: \(F_{1,185} = 34.0, p < 0.0001\)) on CCRT, and a significant interaction between these effects (LMM: \(F_{1,191} = 30.7, p < 0.0001\); figure 2b). This improvement in cold tolerance with cold acclimation is consistent with current literature [7]. Having verified that 15°C-acclimation did indeed induce plasticity in this study system, we moved on to characterizing the relationship between basal tolerance and plasticity.

(b) Low repeatability in 25°C-acclimated flies
Earlier, within-individual repeatability was loosely defined as the consistency or predictability of a trait within individuals over repeated measurements [35,36]. More formally, repeatability (typically represented as ‘R’) is the proportion of total variance attributable to differences among (rather than within) subjects over repeat measurements of a trait [35,36]. ‘Subjects’ may be any unit of measurement, from groups to—in this case—individuals. The non-repeatable portion of

---

**Figure 1.** Outline of experimental design for CCO (a) and CCRT (b). All flies (female *D. melanogaster*) were reared at 25°C. The 15°C- and 25°C-acclimated groups underwent an initial thermotolerance measurement (CCO or CCRT; pre-acclimation) and were subsequently kept at 15°C or 25°C, respectively, for the next 3 days; the control group was kept at 25°C throughout this time. All three groups underwent the second thermotolerance measurement (CCO2 or CCRT2; post-acclimation). (Online version in colour.)
Table 2. Summary of studies that have estimated within-individual repeatability of thermotolerance traits in ectotherms. ‘Repeatable?’ refers to whether the repeatability of the trait in question is statistically significant (that is, significantly different from 0); repeatability ranges from 0 (no repeatability) to 1 (high repeatability). Adjusted repeatability controls for group-wide shifts in trait values from trial to trial while unadjusted repeatability does not. Search methods for finding the gathered studies are outlined in the electronic supplementary material, table S1.

| reference | trait | taxon/taxa | repeatable? | repeatability | method of estimating repeatability |
|-----------|-------|------------|-------------|---------------|-----------------------------------|
| O’Neill et al. (present study) | C\textsubscript{Tmax}; CCRT | Drosophila melanogaster | no; yes | 0.14; 0.23 | unadjusted (LMM-based repeatability) |
| Andrew & Kemp [37] | CCRT | Eurema smilax (small grass yellow butterfly) | yes | 0.4; 0.405 | adjusted (Pearson correlation coefficient); unadjusted (intraclass correlation coefficient) |
| Scharf et al. [38] | CCRT | Myrmeleon hyalinus (antlion); Vermileo sp. (wormlion) | yes; no | 0.306; 0.198 | adjusted (Pearson correlation coefficient) |
| Hawes [39] | SCP | Cryptopygus antarcticus (Antarctic springtail) | yes | 0.910 | adjusted (Spearman’s rank correlation) |
| Worland [40] | SCP | Tullbergia antarctica (subantarctic springtail) | yes | 0.98 | adjusted (methods unspecified) |
| Ditrich [41] | SCP | Pyrrhocoris apterus (linden bug) | yes | 0.61–0.84 | adjusted (Pearson correlation coefficient) |
| Morgan et al. [28] | C\textsubscript{Tmax} | Danio rerio (zebrafish) | yes | 0.447\textsuperscript{a} | adjusted (GLMM-based repeatability) |
| Bard & Kieffer [42] | C\textsubscript{Tmax} | Aciropenser brevirostrum (shortnose sturgeon) | yes | n.a. | linear regression between first and second measurements |
| O’Donnell et al. [43] | C\textsubscript{Tmax} | Salvelinus fontinalis (brook trout) | yes | 0.48 | adjusted (LMM-based repeatability) |
| Grinder et al. [44] | C\textsubscript{Tmax} | Poecilia reticulata (Trinidadian guppy) | yes | 0.43 | adjusted; unadjusted (GLMM-based repeatability) |
| Claireaux et al. [45] | UILT | Dicentrarchus labrax (European sea bass) | yes | 0.35–0.68 | adjusted (Pearson correlation coefficient) |

\textsuperscript{a}When first trial was omitted. CCRT = chill-coma recovery time; SCP = supercooling point; C\textsubscript{Tmax} = critical thermal minimum; C\textsubscript{Tmax} = critical thermal maximum; UILT = upper incipient lethal temperature; LMM = linear mixed-effects model; GLMM = generalized linear mixed-effects model.

The total variance (1–R) is composed of measurement error and fluctuations of the trait within individuals [36,53]. We assumed that the lower the repeatability of CCO and CCRT in the 25°C-acclimated flies, the lower the likelihood of detecting a trade-off, if present, in the 15°C-acclimated flies. This is because low repeatability indicates that measurements of CCO and CCRT are unreliable indices of basal tolerance within an individual. This unreliability and consequential decreased likelihood of detecting a trade-off has twofold reasoning. First, low repeatability of CCO and CCRT in the 25°C-acclimated flies—whether due to measurement error or ‘real’ within-individual fluctuations in CCO or CCRT—indicates that single estimates of basal tolerance are not necessarily representative of a fly’s ‘true’ (mean) basal tolerance and therefore may not predict a fly’s acclimation capacity. Second, and perhaps more importantly, since repeatability indicates the proportion of total variance attributable to differences among individuals [35,36], low repeatability means that we lack meaningful variation in basal tolerance that is theoretically necessary to detect a relationship between basal tolerance and plasticity. For both metrics, repeatability was, in fact, low: CCO in the 25°C-acclimated flies was not significantly repeatable (R ± s.e. = 0.14 ± 0.11, 95% CI = [0, 0.37], p = 0.1), while repeatability of CCRT in the 25°C-acclimated flies was significant, but low (R ± s.e. = 0.23 ± 0.09, 95% CI = [0.033, 0.41], p = 0.01).

Low repeatability in the 25°C-acclimated group may result if performing trial 1 had some effect (damage or acclimation) on the thermotolerance of flies in trial 2. If this is the case, repeatability in the 25°C-acclimated group does not give an accurate estimate of the repeatability of basal tolerance, as acclimated or damaged tolerance in trial 2 would not be considered representative of basal tolerance. However, there was no significant effect of treatment group (Control versus 25°C-acclimated) on CCO\textsubscript{2} (LMM: F\textsubscript{1,121} = 0.2, p = 0.7; figure 2c). Similarly, there was no significant effect of treatment group on CCRT\textsubscript{2} (LMM: F\textsubscript{1,177} = 2.9, p = 0.09; figure 2f). This suggests that conditions experienced by flies during trial 1 did not affect subsequent measurements of thermotolerance (which differs from what was found by Morgan et al. for the zebrafish C\textsubscript{Tmax} [28]). Thus, trial 2 measurements should be representative of basal tolerance (in the 25°C-acclimated flies) and repeatability of CCO and
CCRT in the 25°C-acclimated flies should be accurate estimates of the repeatability of basal tolerance.

To some degree, low repeatability of CCO and CCRT is very likely due at least in part to measurement error, but because we are familiar with these techniques and the amount of measurement error that can be expected, we argue that true biological fluctuations and/or natural stochasticity in CCO or CCRT within individuals are primary contributing factors to the low repeatability we observed. Insect chill-coma has historically been associated with a reduction of neuromuscular excitability and depolarization of neuromuscular membrane potential [7]. More recent evidence has clarified the differing roles of the nervous and muscular systems in chill-coma entry and recovery: entry into chill-coma (CCO) appears to be caused primarily by depolarization of the central nervous system (CNS), while recovery from chill-coma (CCRT) after hours in the cold involves the rapid recovery of CNS function, followed by slower repolarization of muscle membrane potential as systemic ion balance is restored [54–56]. Within-individual stochasticity in mechanisms hypothesized to govern the maintenance of neuromuscular excitability and membrane potential at low temperatures—e.g. modulation of neuromuscular membrane composition (e.g. [57,58]), modulation of the thermal sensitivity of voltage-gated ion channels responsible for action potentials (e.g. [59,60]) or changes in ion transporters in neuromuscular cells [54,61]—may contribute to low repeatability. CCRT is additionally influenced by an individual’s motivation to stand [3], so within-individual variability in motivation may lower the repeatability of CCRT as well.

(c) The illusion of a trade-off between basal and plastic cold tolerance

Given that estimates of the repeatability of basal tolerance were low for both CCO and CCRT, we did not expect to find a significant relationship between basal tolerance and acclimation capacity in the 15ºC-acclimated flies. Surprisingly, however, we found a strong significant relationship between both CCO and acclimation capacity (CCO1–CCO2) (F1,118 = 106.0, p < 0.0001) and CCRT1 and acclimation capacity (CCRT1–CCRT2) (F1,183 = 239.4, p < 0.0001).

A more thorough consideration of the relationship between trial 1 and trial 2 measurements (particularly in the 25ºC-acclimated flies) reveals how this significant trade-off pattern arises in our data despite low—and in the case of CCO, no—repeatability of basal tolerance. In the 25ºC-acclimated flies, there was a striking—and unexpected—relationship between trial 1 and trial 2 measurements within individuals: individuals that were more thermotolerant (lower CCO or CCRT) in trial 1 tended to be less tolerant in trial 2, and individuals that were less tolerant in trial 1 tended to be more tolerant in trial 2 (electronic supplementary material, figure S1). This pattern could relate to individuals experiencing thermal stress differently. For example, one potential explanation for this pattern of data...
is that the least thermotolerant flies in trial 1 were cold-hardened by trial 1 conditions, while the most thermotolerant flies were ultimately damaged by trial 1 conditions. It is not illogical to imagine that an individual’s thermotolerance could subsequently improve because of exposure to low temperatures during trial 1. After all, hardening responses can be induced by only minutes–hours of sublethal cold exposure [12,62]. However, why the most tolerant flies in trial 1 would not only fail to cold-harden but ultimately be damaged by trial 1 conditions when seemingly less tolerant flies cold-hardened under the same conditions is unclear. Moreover, and more importantly, there is a better explanation for the pattern observed.

When repeated measurements are taken from the same subject, these measurements are not likely to be identical: some degree of random error—due to measurement error or fluctuation of the given trait—is almost inevitable [63]. Because of this random error, such repeated measurements are susceptible to a statistical phenomenon known as regression to the mean (RTM; [63,64]). Put simply, subjects that have more-extreme measurements in one trial will tend to have less-extreme measurements—measurements that are closer to their true mean—in the next trial. The pattern of within-individual variation we are attempting to explain in the 25°C-acclimated flies aligns with that expected from RTM (figure 3; electronic supplementary material, figure S2). Due to this, and due to the near-inevitability of RTM when taking repeated measurements, we attribute the relationship between trial 1 and trial 2 measurements in the 25°C-acclimated flies to the effects of RTM, and not to some biological phenomenon. Rank plots (electronic supplementary material, figure S2) further emphasize this unexpected positive correlation between basal thermotolerance and ‘acclimation capacity’ in the 25°C-acclimated flies, as ‘acclimation capacity’ (CCO1–CCO2 or CCRT1–CCRT2) tends to be negative in flies with lower trial 1 measurements and positive in flies with higher trial 1 measurements, indicative of RTM.

Falsely ascribing significance (e.g. biological, economic and psychological) to manifestations of RTM is a common fallacy [64,65]. To avoid this error, one must separate effects expected from RTM and effects (if any) beyond those expected from RTM [63]. In the 25°C-acclimated flies, differentiating such effects was straightforward: there is no intervention applied or relevant biological phenomena expected to be occurring across the experimental period, so any apparent RTM effects can be reasonably attributed to RTM. In the 15°C-acclimated flies, on the other hand, both RTM and a thermotolerance–plasticity trade-off are expected to manifest as a positive correlation between basal tolerance and plasticity [63,64]. Thus, to determine whether the positive correlation previously noted in the 15°C-acclimated flies (figure 3) is truly evidence for a trade-off, we must determine how much of this correlation is attributable to RTM. In experimental studies, like ours, differentiation between the effects of RTM and other phenomena can be achieved quite easily with an adequate control group (i.e. a group for which repeat measurements are taken but no treatment/intervention is applied between trials) [64]. As RTM is expected to equally affect control and experimental groups, changes in the experimental group different from those observed in the control group suggest phenomena at play besides RTM. Happily, our 25°C-acclimated group acts as an ideal control for the 15°C-acclimated group in this regard.

![Figure 3](royalsocietypublishing.org/journal/rspb)  
**Figure 3.** Relationship between CCO1 (a) or CCRT1 (b) and acclimation capacity (CCO1 − CCO2 or CCRT1 − CCRT2) in female *D. melanogaster*. Fitted lines and slopes are based on a simple linear regression for each group for illustrative purposes (p-values derived from mixed effects models). (a) CCO1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.4). (b) CCRT1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.6). The slopes being parallel means that these relationships are a statistical artefact driven by regression to the mean, as no such variation in acclimation capacity should otherwise appear in the 25°C-acclimated flies. (Online version in colour.)

**Figure 3.** Relationship between CCO1 (a) or CCRT1 (b) and acclimation capacity (CCO1 − CCO2 or CCRT1 − CCRT2) in female *D. melanogaster*. Fitted lines and slopes are based on a simple linear regression for each group for illustrative purposes (p-values derived from mixed effects models). (a) CCO1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.4). (b) CCRT1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.6). The slopes being parallel means that these relationships are a statistical artefact driven by regression to the mean, as no such variation in acclimation capacity should otherwise appear in the 25°C-acclimated flies. (Online version in colour.)

**Figure 3.** Relationship between CCO1 (a) or CCRT1 (b) and acclimation capacity (CCO1 − CCO2 or CCRT1 − CCRT2) in female *D. melanogaster*. Fitted lines and slopes are based on a simple linear regression for each group for illustrative purposes (p-values derived from mixed effects models). (a) CCO1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.4). (b) CCRT1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.6). The slopes being parallel means that these relationships are a statistical artefact driven by regression to the mean, as no such variation in acclimation capacity should otherwise appear in the 25°C-acclimated flies. (Online version in colour.)

**Figure 3.** Relationship between CCO1 (a) or CCRT1 (b) and acclimation capacity (CCO1 − CCO2 or CCRT1 − CCRT2) in female *D. melanogaster*. Fitted lines and slopes are based on a simple linear regression for each group for illustrative purposes (p-values derived from mixed effects models). (a) CCO1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.4). (b) CCRT1 strongly predicted acclimation capacity in both groups (p < 0.0001). The slopes of the 15°C- and 25°C-acclimated groups were not significantly different (p = 0.6). The slopes being parallel means that these relationships are a statistical artefact driven by regression to the mean, as no such variation in acclimation capacity should otherwise appear in the 25°C-acclimated flies. (Online version in colour.)
How big of a problem is RTM?

When an independent variable (Y) contains the dependent variable (X) as a constituent (e.g., Y = X – Z; as when plasticity = basal tolerance – induced tolerance), these variables are not statistically independent [22,66]. Issues of interpretation resulting from this implicit statistical association have been articulated before (e.g., when estimating trade-offs between constitutive and induced defenses in plants [67]; when estimating costs of plasticity [66,68,69]; when assessing mass-dependent mass loss in birds [70,71]). Yet, this methodology has only recently been highlighted as a potential concern for thermotolerance–plasticity trade-off studies and remains widespread [22]. Conclusions made on the basis of a relationship between baseline measurements and the difference between baseline and follow-up measurements may not necessarily be problematic if the null hypothesis for such an investigation is properly identified [70,71]. A proper null hypothesis should account for statistical bias resulting from phenomena like RTM [64]. However, there is very little consideration of RTM (or repeatability, for that matter) in previous thermotolerance–plasticity trade-off studies, precluding such identification (but see Deery et al. [29], who do not mention RTM but do address statistical bias).

It is unclear to what extent the results of past thermotolerance–plasticity trade-off studies are biased by RTM. We expect RTM to occur whenever repeated measurements of the same subjects are taken (as random error is unavoidable in experimental biology), whether the study is at the individual level or, as is the case for the vast majority of previous thermotolerance–plasticity trade-off studies, above the individual level [63,64] (but see: [28–30]). However, it is not clear under what specific methodological circumstances RTM occurs. For instance, if basal and induced tolerance are measured for the same group but different individuals within the group are used for each measurement, is RTM still expected to occur? This must be determined (e.g., through modelling hypothetical data) before effects of RTM can be corrected for [per Kelly & Price (64)].

In any case, the following certainly seems to be true: it is not yet known whether a thermotolerance–plasticity trade-off exists [22]. This lack of clarity stems from more than the inherent statistical association between basal tolerance and plasticity and issues of interpretation arising from it. For instance, many tolerance–plasticity trade-off studies are carried out on field organisms, meaning that environmental and genetic effects on thermotolerance cannot easily be unwoven [22,72]. As well, misleading trade-off patterns may arise if more basally tolerant organisms require more-unwoven [22,72]. As well, misleading trade-off patterns and genetic effects on thermotolerance cannot easily be performed on field organisms, meaning that environmental and genetic effects on thermotolerance cannot easily be unwoven [22,72].

relationships in European diving beetles (Coleoptera: Dytiscidae), J. Anim. Ecol. 79, 194–204. (doi:10. 1111/j.1365-2656.2009.01611.x)

4. Conclusion

Here, we found a strong positive correlation between basal cold tolerance and plasticity, demonstrating apparent support for a cold tolerance–plasticity trade-off at the individual level in Drosophila melanogaster. However, repeatability of CCO and CCRT in non-cold-acclimated flies was low, indicating that estimates of basal tolerance were unreliable, and we should therefore not be able to detect such a relationship. We argue that this pattern is a manifestation of RTM and is not reflective of a true trade-off. Concerningly, many previous thermotolerance–plasticity trade-off studies, despite employing similar methodology to that carried out herein—regressing plasticity on basal tolerance, where plasticity is measured as the difference between basal and induced tolerance—do not consider either repeatability or RTM in their experimental design/analyses. Moving forward, we recommend two courses of action: first, we must determine whether previous thermotolerance–plasticity trade-off studies are indeed biased by RTM (and if, in fact, there is no or little existing evidence for this hypothesis) and second, we must carry out future thermotolerance–plasticity trade-off studies with appropriate controls such that this bias is accounted for in the future.

Data accessibility. The data are provided in the electronic supplementary material [74].

Authors’ contributions. E.O.: conceptualization, data curation, formal analysis, visualization, writing — original draft, writing the review and editing; H.E.D.: conceptualization, methodology, writing the review and editing; H.M.: conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, visualization, writing the review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

Funding. This research was supported by Natural Sciences and Engineering Research Council of Canada Discovery Grant (grant no. RGPIN-2018-05322) to H.A.M., an Undergraduate Student Research Award to E.O. and a Canada Graduate Scholarship to H.E.D. Equipment used in this study was acquired through support from the Canadian Foundation for Innovation and Ontario Research Fund (to H.A.M.).

Acknowledgements. The authors wish to thank the other MacMillan lab members for their unfailing support.
functional traits behind species’ climate niches: patterns of desiccation and cold resistance across 95 Drosophila species. Evolution (NY) 66, 3377–3389. (doi:10.1111/j.1558-5646.2012.01685.x)

3. Andersen JL, Manenti T, Sørensen JG, MacMillan HA, Loeschke V, Overgaard J. 2015 How to assess Drosophila cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Funct. Ecol. 29, 55–65. (doi:10.1111/1365-2435.12310)

4. IPCC. 2014 Climate change 2014: synthesis report. Contribution of working groups i, ii and iii to the fifth assessment report of the intergovernmental panel on climate change. Geneva, Switzerland: IPCC.

5. Pörtner HO, Bennett AF, Bozinovic F, Clarke A, Lardies MA, Lucassen M, Pelster B, Schiemer F, Stillman JH. 2006 Trade-offs in thermal adaptation: the need for a molecular to ecological integration. Physiol. Biochem. Zool. 79, 295–313. (doi:10.1086/499986)

6. Hoffmann GE, Todgham AE. 2010 Living in the now: physiological mechanisms to tolerate a rapidly changing environment. Annu. Rev. Physiol. 72, 127–145. (doi:10.1146/annurev-physiol-021909-135900)

7. Overgaard J, MacMillan HA. 2017 The integrative physiology of insect chill tolerance. Annu. Rev. Physiol. 79, 187–208. (doi:10.1146/annurev-physiol-022516-031442)

8. Teets NM, Denlinger DL. 2013 Physiological mechanisms of seasonal and rapid cold-hardening in insects. Physiol. Entomol. 38, 105–116. (doi:10.1111/j.1479-8203.2012.01209)

9. Esper K, Kjaergaard A, Walters RJ, Berger D, Blankenhorn WU. 2016 Plastic and evolutionary responses to heat stress in a temperate dung fly: negative correlation between basal and induced heat tolerance? J. Evol. Biol. 29, 900–915. (doi:10.1111/jeb.12832)

10. Gerken AR, Eller OC, Hahn DA, Morgan TJ. 2015 Constraints, independence, and evolution of thermal plasticity: probing genetic architecture of long- and short-term thermal acclimation. Proc. Natl Acad. Sci. USA 112, 4399–4404. (doi:10.1073/pnas.1306356112)

11. Colinet H, Hoffmann AA. 2012 Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in Drosophila melanogaster. Funct. Ecol. 26, 84–93. (doi:10.1111/j.1365-2435.2011.01898.x)

12. Hoffmann AA, Sørensen JG, Loeschke V. 2003 Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches. J. Therm. Biol. 28, 175–216. (doi:10.1016/S0306-4565(02)00057-8)

13. Hazell SP, Bale JS. 2011 Low temperature thresholds: are chill coma and CT_max synonymous? J. Insect Physiol. 57, 1085–1089. (doi:10.1016/j.jip.2011.04.004)

14. Géza T, Moré Beau B, Pétavy G, Karan D, David JR. 2001 Chill-coma tolerance, a major climatic adaptation among Drosophila species. Evolution (NY) 55, 1063–1068. (doi:10.1111/j.0014-3820.2001.tb00623.x)

15. Nyamukondiwa C, Terblanche JS, Marshall KE, Sinclair BJ. 2011 Basal cold but not heat tolerance constrains plasticity among Drosophila species (Diptera: Drosophilidae). J. Evol. Biol. 24, 1927–1938. (doi:10.1111/j.1420-9101.2010.01934.x)

16. Calosi P, Bilton DT, Spicer JL. 2008 Thermal tolerance, acclimation capacity and vulnerability to global climate change. Biol. Lett. 4, 99–102. (doi:10.1098/rsbl.2007.0408)

17. Angilletta MJ. 2009 Thermal adaptation: a theoretical and empirical synthesis. (doi:10.1093/acprof:oso/978190578085.1)
44. Grinder RM, Bassar RD, Auer SK. 2020 Upper thermal limits are repeatable in Trinidadian guppies. *J. Therm. Biol.* 90, 102597. (doi:10.1016/j.jtherbio.2020.102597)

45. Claineaux G, Theron M, Primeau M, Dussauze M, Merlin FX, Le Floch S. 2013 Effects of oil exposure and dispersant use upon environmental adaptation performance and fitness in the European sea bass, *Dicentrarchus labrax*. *Aquat. Toxicol.* 130–131, 160–170. (doi:10.1016/j.aquatox.2013.01.004)

46. Marshall KE, Sinclair BJ. 2010 Repeated stress exposure results in a survival—production trade-off in *Drosophila melanogaster*. *Proc. R. Soc. B* 277, 963–969. (doi:10.1098/rspb.2009.1807)

47. Facón B, Estoup A, Huffbauer RA, Foucaud J, Taye A. 2017 Maternal stress influences cold tolerance and subsequent reproduction in the invasive ladybird *Harmonia axyridis*. *Front. Ecol. Evol.* 5, 108. (doi:10.3389/fevo.2017.00108)

48. Nilson TL, Sinclair BJ, Roberts SP. 2006 The effects of carbon dioxide anesthesia and anaoxia on rapid cold-hardening and chill coma recovery in *Drosophila melanogaster*. *J. Insect Physiol.* 52, 1027–1033. (doi:10.1016/j.jinsphys.2006.07.001)

49. MacMillan HA, Yerushalmi GY, Jonusaite S, Kelly SP, Donini A. 2017 Thermal acclimation mitigates cold-induced paracellular leak from the *Drosophila* gut. *Sci. Rep.* 7, 8807.

50. R Development Core Team. 2019 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See http://www.R-project.org/.

51. Stoffel MA, Nakagawa S, Schielzeth H. 2017 rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods Ecol. Evol.* 8, 1639–1644. (doi:10.1111/2041-210X.12797)

52. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2020 *lme4: linear and nonlinear mixed effects models*. R package version 3.1-147.

53. Wolak ME, Fairbairn DJ, Paulsen YR. 2012 Guidelines for estimating repeatability. *Methods Ecol. Evol.* 3, 129–137. (doi:10.1111/j.2041-210X.2011.00125.x)

54. Andersen MK, Overgaard J. 2019 The central nervous system and muscular system play different roles for chill coma onset and recovery in insects. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 233, 10–16. (doi:10.1016/j.cbpa.2019.03.015)

55. Bayley JS, Sørensen JG, Moes M, Køstal V, Overgaard J. 2020 Cold-acclimation increases depolarization resistance and tolerance in muscle fibers from a chill-susceptible insect: *Locusta migratoria*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 319, R439–R447. (doi:10.1152/ajpregu.00068.2020)

56. Bayley JS, Overgaard J, Pedersen TH. 2021 Quantitative model analysis of the resting membrane potential in insect skeletal muscle: implications for low temperature tolerance. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 257, 110970. (doi:10.1016/j.cbpa.2021.110970)

57. Slotsbo S, Sørensen JG, Holmstrup M, Kostal V, Kellersmann V, Overgaard J. 2016 Tropical to subpolar gradient in phospholipid composition suggests adaptive tuning of biological membrane function in *drosophilids*. *Func. Ecol.* 30, 759–768. (doi:10.1111/1365-2435.12568)

58. Tomcala A, Tollarová M, Overgaard J, Simek P, Kostal V. 2006 Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression. *J. Exp. Biol.* 209, 4102–4114. (doi:10.1242/jeb.02484)

59. Finder A, Overgaard J, Pedersen TH. 2016 Reduced L-type Ca2+ current and compromised excitability induce loss of skeletal muscle function during acute cooling in locust. *J. Exp. Biol.* 219, 2340–2348. (doi:10.1242/jeb.137604)

60. Frolow RV, Singh S. 2013 Temperature and functional plasticity of L-type Ca2+ channels in *Drosophila*. *Cell Calcium* 54, 287–294. (doi:10.1016/j.ccal.2013.07.005)

61. Cheshock A, Andersen MK, MacMillan HA. 2021 Thermal acclimation alters Na+/K+-ATPase activity and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression. *J. Exp. Biol.* 209, 4102–4114. (doi:10.1242/jeb.02484)

62. Lee RE, Chen CP, Denlinger DL. 1987 A rapid cold-hardening process in insects. *Science* 238, 1415–1417.

63. Barnett AG, van der Pols JC, Dobson AJ. 2005 Regression to the mean: what it is and how to deal with it. *Int. J. Epidemiol.* 34, 215–220. (doi:10.1093/ije/dyh299)

64. Kelly C, Price TD. 2005 Correcting for regression to the mean in behavior and ecology. *Am. Nat.* 166, 700–707. (doi:10.1086/497402)

65. Stigler SM. 1997 Regression towards the mean, historically considered. *Stat. Methods Med. Res.* 6, 103–114. (doi:10.1177/096228029700600202)

66. Roff DA. 2011 Measuring the cost of plasticity: a problem of statistical non-independence. *Proc. R. Soc. B* 278, 2724–2725. (doi:10.1098/rspb.2011.0595)

67. Norris WF, Tsaw MB, Bergelson J. 2006 On testing for a tradeoff between constitutive and induced resistance. *Oikos* 112, 102–110.

68. Auld JR, Agrawal AA, Relyea RA. 2011 Measuring the cost of plasticity: avoid multi-collinearity. *Proc. R. Soc. B* 278, 2726–2727. (doi:10.2307/2408842)

69. Auld JR, Agrawal AA, Relyea RA. 2010 Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proc. R. Soc. B* 277, 503–511. (doi:10.1098/rspb.2009.1355)

70. Cichon M, Menilia J, Hillström L, Wiggins D. 1999 Mass-dependent mass loss in breeding birds: getting the null hypothesis right. *Oikos* 87, 191–194.

71. Gebhardt-Henrich SG. 2000 When heavier birds lose more mass during breeding: statistical artefact or biologically meaningful? *J. Avian Biol.* 31, 245–246. (doi:10.1093/oxfordjournals.10.105/488200.2000.2102546)

72. Armbruster WS, Schweagerle KE. 1996 Causes of covariation of phenotypic traits among populations. *J. Evol. Biol.* 9, 261–276. (doi:10.1046/j.1420-9101.1996.030261.x)

73. Sørensen JG, Kristen J, Overgaard J. 2016 Evolutionary and ecological patterns of thermal acclimation capacity in *Drosophila*: is it important for keeping up with climate change? *Curr. Opin. Insect Sci.* 17, 98–104. (doi:10.1016/j.cois.2016.08.003)

74. O'Neill E, Davis HE, MacMillan HA. 2021 A lack of repeatability creates the illusion of a trade-off between basal and plastic cold tolerance. *Figshare.*