1  **A lack of repeatability creates the illusion of a trade-off between basal**

2  **and plastic cold tolerance**

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13  **Acronyms:**
14  CCO: chill-coma onset
15  CCRT: chill-coma recovery time
16  RTM: regression to the mean
17  $CT_{min}$: critical thermal minimum
18  LLT: lower lethal temperature
19  LTL: lower thermal limit
20  LMM: linear mixed-effects model

## Abstract

21

22   The thermotolerance-plasticity trade-off hypothesis predicts that ectotherms with greater basal

23   thermal tolerance have a lower acclimation capacity. This hypothesis has been tested at both high

24   and low temperatures but the results often conflict. If basal tolerance constrains plasticity (e.g.

25   through shared mechanisms that create physiological constraints), it should be evident at the

26   level of the individual, provided the trait measured is repeatable. Here, we used chill-coma onset

27   temperature and chill-coma recovery time (CCO and CCRT; non-lethal thermal limits) to

28   quantify cold tolerance of *Drosophila melanogaster* across two trials (pre- and post-acclimation).

29   Cold acclimation improved cold tolerance, as expected, but individual measurements of CCO

30   and CCRT in non-acclimated flies were not (or only slightly) repeatable. Surprisingly, however,

31   there was still a strong correlation between basal tolerance and plasticity in cold-acclimated flies.

32   We argue that this relationship is a statistical artefact (specifically, a manifestation of regression

33   to the mean; RTM) and does not reflect a true trade-off or physiological constraint. Thermal

34   tolerance trade-off patterns in previous studies that used similar methodology are thus likely to

35   be impacted by RTM. Moving forward, controlling and/or correcting for RTM effects is critical

36   to determining whether such a trade-off or physiological constraint truly exists.

## Introduction

37

38 Thermotolerance is widely considered a major determinant of ectotherm geographic distribution,

39 as thermal limits— particularly lower thermal limits— can strongly predict ectotherm range

40 limits [1–3]. In an era of global climate change [4], the ongoing persistence of ectotherms

41 already residing near their thermal limits will depend on the ability of these organisms to adapt

42 or respond plastically to changing thermal conditions [5,6]. Consequently, understanding

43 evolutionary and physiological constraints on ectotherm cold tolerance adaptation and plasticity

44 may be vital for predicting how ectotherms will be affected by global climate change [5].

45

46 Cold tolerance is the ability of an organism to survive exposure to low temperature extremes [7].

47 In many ectotherms, exposure to sublethal low temperatures physiologically primes the organism

48 to better respond to subsequent cold stresses (reviewed for insects in: [8,9]). Consequently, cold

49 tolerance can be conceptually divided into two categories: basal tolerance (an organism's

50 baseline level of cold tolerance; e.g. [10,11]), and induced tolerance (an organism's level of cold

51 tolerance following some adjustment period; e.g. [10]).  Physiological priming that results in an

52 improvement in thermotolerance is a form of phenotypic plasticity and is typically named

53 according to the length of the sublethal temperature exposure, from short-term (minutes-hours)

54 "hardening" to long-term (days-weeks) "acclimation" [12,13].

55

56 Basal and plastic cold tolerance can be quantified using a variety of metrics in chill-susceptible

57 insects (reviewed in [8]). For instance, critical thermal minimum ($CT_{min}$) is the temperature,

58 upon cold exposure, at which neuromuscular coordination of the organism becomes impaired

59 [14]. If cold exposure persists, $CT_{min}$ is followed by chill-coma onset (CCO), the temperature at

60    which neuromuscular function is entirely lost, resulting in complete paralysis [14]. Following

61    CCO and a period of time in the cold, chill-coma recovery time (CCRT) is the time taken for an

62    organism to regain neuromuscular function after being removed from coma-inducing conditions

63    [15]. For all three metrics— $CT_{min}$, CCO, and CCRT— low values indicate high cold tolerance.

64    In contrast to the above non-lethal metrics, lethal measures of cold tolerance include survivorship

65    (the proportion of organisms surviving some cold exposure; e.g. [11]), lower lethal temperature

66    (LLT; the temperature at which a certain proportion of individuals die under a fixed duration of

67    cold exposure (this is calculated using survivorship); e.g. [16]), and lower thermal limit (LTL; an

68    average temperature of mortality; e.g. [17]).

69

70    One critical uncertainty and point of contention among thermal biologists is whether basal

71    thermotolerance constrains plastic thermotolerance, such that animals with greater basal

72    tolerance— either cold tolerance (as described above) or heat tolerance— have a lower capacity

73    for acclimation [11,16–22]. To address this question, several researchers have used

74    thermotolerance metrics to test for a trade-off between basal and induced cold tolerance– called a

75    tolerance-plasticity trade-off (summarized in Table 1). While some studies (spanning a variety of

76    taxa) have documented evidence for such a trade-off at low temperatures (e.g. [11,20,22]), others

77    have found partial or no such evidence [16,17,21]. Similarly, in a recent review of heat

78    tolerance-plasticity trade-off studies, approximately half of the studies gathered— 17 out of 30—

79    did not support a trade-off [23].

80

81    Importantly, the studies described above and in Table 1 tested for a trade-off between some

82    measure of basal cold tolerance and plasticity predominantly at the species, lineage, or

83     population level. But if basal tolerance constrains plasticity through shared physiological

84     mechanisms of tolerance, such a pattern should arise at the level of the individual. The $CT_{min}$, for

85     example, is thought to occur as a result of sudden ionoregulatory failure (spreading

86     depolarization) in the nervous system [24,25], so, for example, constitutive expression of

87     ionoregulatory proteins (e.g. ion channels) that help to lower the basal $CT_{min}$ may approach the

88     "ceiling" to expression that can occur during thermal acclimation. Basal and induced cold

89     tolerance have similarly been suggested to share renal mechanisms that help prevent

90     ionoregulatory collapse (at least in *Drosophila*; [26–28]). We hypothesized that if such

91     constraints exist, they should be evident at the individual level and may underlie trade-off

92     patterns previously observed at higher levels of organization. To our knowledge, however, only

93     three studies have ever investigated the relationship between basal thermotolerance and plasticity

94     at the individual level in ectotherms, each time at high temperatures [29–31]. Thus, turning our

95     attention away from species/lineage/population level comparisons and towards individual

96     variation— which has historically been underutilized in physiology [32,33]— may prove

97     informative.

98

99     Here, we tested whether basal cold tolerance constrains plasticity at the individual level in

100     *Drosophila melanogaster.* We did so by quantifying cold tolerance in individual

101     *D. melanogaster* across two trials (pre- and post-cold-acclimation; Fig. 1). Pre-acclimation

102     measurements give an estimate of basal cold tolerance, while post-acclimation measurements

103     give an estimate of induced cold tolerance. If basal cold tolerance constrains plasticity, we

104     expected that individuals with higher basal cold tolerance (lower trial 1 measurements) would

105     tend to have diminished acclimation capacity (a reduced difference in thermotolerance between

106     trial 1 and trial 2) relative to individuals with lower basal cold tolerance. Such methodology,

107     where acclimation capacity is correlated with basal tolerance and is measured as the difference

108     between basal and induced tolerance, is a common method of testing the tolerance-plasticity

109     trade-off hypothesis (e.g. [11,16,17,22]; Table 1). Because different cold tolerance traits have

110     different underlying mechanisms and vary in different ways among and within populations and

111     lineages of *Drosophila* [3,8,34,35], we carried out our experiment twice using different metrics

112     of thermotolerance: chill-coma onset (CCO) and chill-coma recovery time (CCRT; Fig. 1).

113

**Table 1. Examples of studies that have tested for a trade-off between basal cold tolerance and plasticity.** "Support for a trade-off" refers only to results for a trade-off tested at low temperatures (some of the following studies also test for a trade-off at high temperatures).

| Reference | Taxon | Trait | Level | Methods | Support for a trade-off? |
|---|---|---|---|---|---|
| Stillman et al., 2013 [20] | *Petrolisthes* spp. (porcelain crabs) | Thermal limits of cardiac function | Species | Linear regression of acclimatory ability (difference between acclimated and unacclimated thermal limits of cardiac function) on maximal habitat temperature | Yes |
| Calosi et al., 2008 [17] | *Deronectes* spp. (diving beetles) | LTL | Species | Regression of acclimation capacity ($\Delta$LTL) on basal tolerance (LTL) | No |
| Nyamukondiwa et al., 2011 [16] | *Drosophila* spp. (fruit flies) | $LLT_{90}$, Survival | Species | Regression of RCH magnitude (difference in % survival between unhardened (control) and hardened groups) on basal tolerance ($LLT_{90}$) | Yes |
| Overgaard et al., 2011 [21] | *Drosophila* spp. (fruit flies) | $CT_{min}$ | Species | Correlation between southern latitude and plasticity (difference between unacclimated/unhardened and acclimated/hardened $CT_{min}$) | No |
| Gerken et al., 2015 [11] | *Drosophila melanogaster* | Survival | Lineage | Regression of RCH or DACC capacity (difference between unhardened/unacclimated and hardened/acclimated survivorship) on acute or chronic basal tolerance (survivorship) | Yes |
| Noh et al., 2017 [22] | *Drosophila melanogaster* | Survival, CCRT | Population, Lineage | Correlation between basal tolerance (survivorship or CCRT) and plasticity (short-term or developmental, respectively; difference between unhardened/unacclimated and hardened/acclimated survivorship or CCRT) | Yes |

LTL = lower thermal limit; $LLT_{90}$ = lower lethal temperature (at which mortality is 90%); $CT_{min}$ = critical thermal minimum; RCH = rapid cold hardening; DACC = developmental acclimation (acclimation occurring during an organism's development); CCRT = chill-coma recovery time

**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 non-adjusted repeatability does not. Search methods for finding the gathered studies are outlined in the Table S1.

| Reference | Trait | Taxon/Taxa | Repeatable? | Repeatability | Method of estimating repeatability |
|---|---|---|---|---|---|
| Andrew and Kemp, 2016 [36] | CCRT | *Eurema smilax* (small grass yellow butterfly) | Yes | 0.4; 0.405 | Adjusted (Pearson correlation coefficient); non-adjusted (Intraclass correlation coefficient) |
| Scharf et al., 2016 [37] | CCRT | *Myrmeleon hyalinus* (antlion); *Vermileo* sp. (wormlion) | Yes; No | 0.306; 0.198 | Adjusted (Pearson correlation coefficient) |
| Hawes, 2006 [38] | SCP | *Cryptopygus antarcticus* (Antarctic springtail) | Yes | 0.910 | Adjusted (Spearman's rank correlation) |
| Worland, 2005 [39] | SCP | *Tullbergia antarctica* (sub-Antarctic springtail) | Yes | 0.98 | Adjusted (methods unspecified) |
| Ditrich, 2018 [40] | SCP | *Pyrrhocoris apterus* (linden bug) | Yes | 0.61-0.84 | Adjusted (Pearson correlation coefficient) |
| Morgan et al., 2018 [29] | $CT_{max}$ | *Danio rerio* (zebrafish) | Yes | 0.447* | Adjusted (GLMM-based repeatability) |
| Bard and Kieffer, 2019 [41] | $CT_{max}$ | *Acipenser brevirostrum* (shortnose sturgeon) | Yes | N/A | Linear regression between first and second measurements |
| O'Donnell et al., 2020 [42] | $CT_{max}$ | *Salvelinus fontinalis* (brook trout) | Yes | 0.48 | Adjusted (LMM-based repeatability) |
| Grinder et al., 2020 [43] | $CT_{max}$ | *Poecilia reticulata* (Trinidadian guppy) | Yes | 0.43 | Adjusted; non-adjusted (GLMM-based repeatability) |
| Claireaux et al., 2013 [44] | UILT | *Dicentrarchus labrax* (European sea bass) | Yes | 0.35-0.68 | Adjusted (Pearson correlation coefficient) |

*when first trial was omitted. CCRT = chill-coma recovery time; SCP = supercooling point; $CT_{max}$ = critical thermal maximum; UILT = upper incipient lethal temperature; LMM = linear mixed-effects model; GLMM = generalized linear mixed-effects model
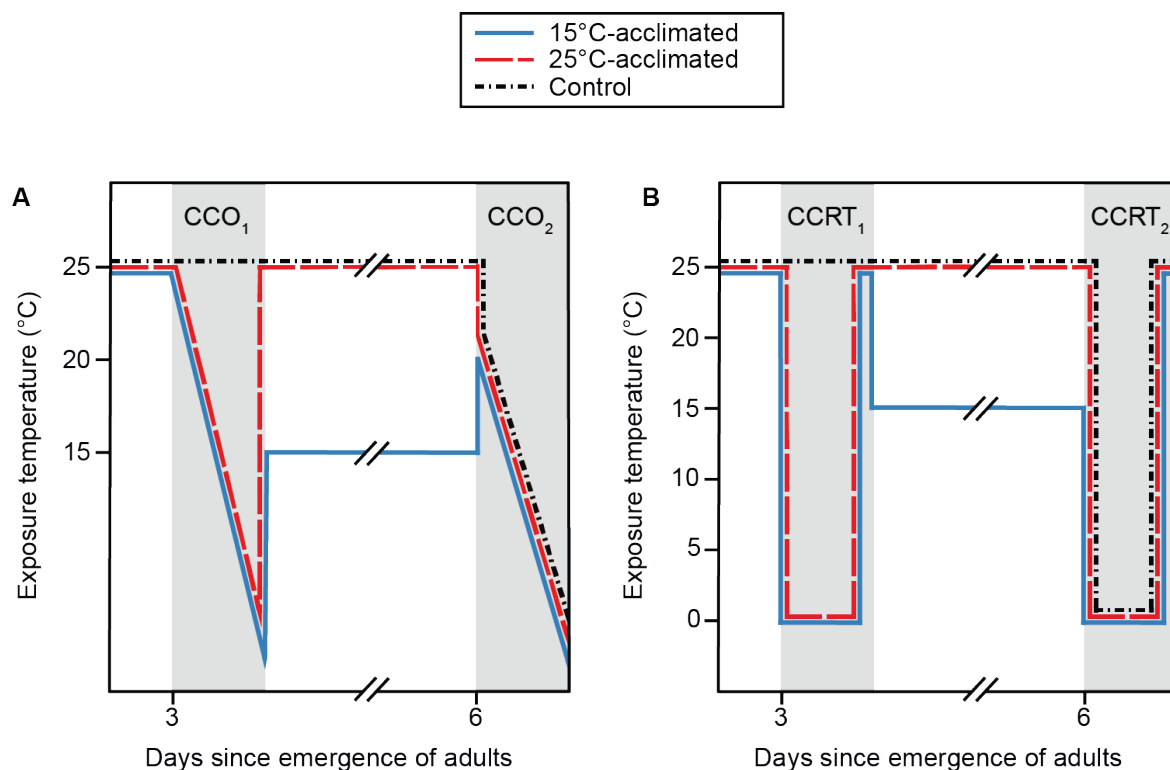
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139 **Figure 1. Outline of experimental design for CCO (A) and CCRT (B).** All flies (female
140 *D. melanogaster*) were reared at 25ºC. The 15ºC- and 25ºC-acclimated groups underwent an
141 initial thermotolerance measurement (CCO$_1$ or CCRT$_1$; pre-acclimation), and were subsequently
142 kept at 15ºC or 25ºC, respectively, for the next three days; the control group was kept at 25ºC
143 throughout this time. All three groups underwent the second thermotolerance measurement
144 (CCO$_2$ or CCRT$_2$; post-acclimation).

145

146 **Materials and Methods**

147 *Experimental overview*

148 Chill-coma onset and chill-coma recovery time (CCO and CCRT; non-lethal metrics of

149 thermotolerance) were used to quantify cold tolerance of individual flies across two trials (pre-

150 and post-acclimation; Fig. 1) to determine the relationship between basal tolerance and

151 acclimation capacity in cold-acclimated flies ("15°C-acclimated"; Fig. 1). Two other groups of

152 flies were included in the experiment: 1) flies that were not subject to cold acclimation between

153 trials ("25°C-acclimated"); 2) flies that were not subject to cold acclimation and that underwent

154 trial 2 but not trial 1 ("Control"; Fig. 1). The 25°C-acclimated flies were used to assess the

155 within-individual repeatability (hereafter, "repeatability") of CCO and CCRT in the absence of

156 cold acclimation, thereby estimating the repeatability of basal tolerance as measured by both

157 metrics. Repeatability is a descriptor of the within-individual consistency, or predictability, of a

158 trait over repeat measurements [45,46]. Repeatability of CCO and CCRT in non-cold-acclimated

159 flies is essential to addressing the trade-off hypothesis because otherwise measurements of CCO

160 and CCRT are unreliable indices of basal thermotolerance. Unhelpfully, the repeatability of

161 ectotherm thermotolerance traits is infrequently studied— repeatability has in fact never been

162 estimated for CCO and has only been estimated twice for CCRT (with conflicting results, and

163 neither time in *Drosophila;* [36,37]) — providing little hint of what to expect. Though, of the

164 ectotherm thermotolerance traits studied, most show at least moderate repeatability (Table 2).

165 Importantly, if trial 1 had some effect on trial 2 measurements, estimates of the repeatability of

166 CCO and CCRT in 25°C-acclimated flies (who by necessity underwent both trials) would not

167 necessarily be accurate representations of the repeatability of basal tolerance. Thus, control flies

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168    ("Control"; Fig. 1) were included to check whether trial 1 itself influenced thermotolerance in

169    trial 2.

170

171    *Animal husbandry*

172    *Drosophila melanogaster* used in this study originate from 35 isofemale lines originally captured

173    in London and Niagara on the Lake, Ontario, Canada [47]. Flies were reared in a 25°C incubator

174    (12 h:12 h light:dark cycle) in 250 mL bottles containing ~50 mL of a banana, corn syrup, and

175    yeast-based medium. To gather new eggs, adult flies were transferred to bottles containing fresh

176    media (~100 per bottle) and allowed to lay eggs for approximately 2 h before being removed.

177    The eggs laid in this time (~150 per bottle) were reared at 25°C until adult emergence (~10

178    days). On their day of emergence, adults were transferred to new bottles (such that all flies were

179    within 25 h of age), where they remained for ~24 h at 25°C to ensure all females were mated

180    before flies were anaesthetized (<15 min exposure to $CO_2$) and sorted by sex. It was important to

181    ensure females were mated because mating status can affect cold tolerance in insects (e.g.[48]).

182

183    Approximately 30 females (per run of the experiment) were then placed individually into 1.7 mL

184    microcentrifuge tubes. Prior to this, the microcentrifuge tubes were prepared by puncturing one

185    small hole in each tube lid and filling the tube with ~0.5 mL of the rearing diet. Early trials

186    confirmed that the food inside these tubes stayed well hydrated for at least 72 h and thus would

187    not cause desiccation stress. The microcentrifuge tubes were placed in a 25°C incubator where

188    they remained for typically 48-50 h (in one CCO run, flies remained for 53 h) before the first

189    thermotolerance measurement (trial 1). This delay was to prevent $CO_2$ exposure from interfering

190    with cold tolerance [49]. After trial 1 (pre-acclimation), flies were placed into new

191    microcentrifuge tubes (as described above) and held for three days at either 15°C or 25°C until

192   trial 2 (post-acclimation). Control flies did not undergo trial 1 measurements but were moved

193   into fresh microcentrifuge tubes while, or directly after, the trial was carried out (Fig. 1).

194

195   *Measurement of chill-coma onset*

196   Chill-coma onset (CCO) was measured by placing flies individually into 3.7 mL screw-top glass

197   vials, attaching these vials to an aluminum rack, and submerging the rack in a custom built

198   cooling bath (1:1 v/v water:ethylene glycol; as in [50]). The temperature of the cooling bath

199   began at 25°C (for trial 1) or 20°C (for trial 2) and decreased at a rate of 0.1°C/min. The

200   temperature was monitored by three type-K thermocouples placed at different locations in the

201   bath. After the temperature of the bath reached ~10°C, the glass vials were tapped frequently

202   with a metal rod to stimulate movement in the flies [27]. CCO was recorded as the temperature at

203   which a fly stopped moving and was unresponsive to tapping on the vial. The temperature

204   decreased until all flies had entered chill-coma, at which point the rack with vials was removed

205   from the bath. During trial 1, control flies were transferred into 3.7 mL screw-top glass vials and

206   kept in a 25°C incubator for the duration of the trial (~4 h) before being transferred into fresh

207   microcentrifuge tubes (to maintain similar levels of handling for the control and non-control

208   flies).

209

210   *Measurement of chill-coma recovery time*

211   Chill-coma recovery time (CCRT) was measured by placing flies individually into 3.7 mL

212   screw-top glass vials and placing these vials in a water-ice mixture within a Styrofoam box

213   inside a fridge (such that flies were exposed to ~0°C). The vials remained in this setup for 6 h,

214   after which they were removed from the water-ice mixture and set on a counter at room

215   temperature to record CCRT. CCRT was recorded as the time taken– from the moment the vials

216    were removed from the water-ice mixture– for flies to stand upright on all six limbs [27]. To

217    control for handling, control flies were transferred into fresh microcentrifuge tubes while trial 1

218    was being carried out. We also recorded room temperature during our CCRT recordings. Room

219    temperature varied among experimental runs (mean ± sd: 24.5 ± 1.3°C), and one run was

220    discarded and then replaced because CCRT tended to be slower (although not significantly) and

221    room temperature on that day was lower (21.9°C) than all other runs. Ultimately, room

222    temperature had no significant effect on CCRT, and was shared among all flies in each run (and

223    we included flies from all three treatment groups in each run), so we excluded it as a factor in

224    downstream analyses.

225

226    *Data analysis*

227    All data analyses were carried out in R version 3.6.2 [51]. Individuals that were lost or

228    accidentally crushed between trials were removed from the analysis. To test whether treatment

229    (15ºC vs. 25ºC acclimation) affected CCO or CCRT (that is, induced plasticity), linear mixed-

230    effects models were performed (fixed effects: trial (1 vs. 2), treatment group; random effects: run

231    date, individual ID (nested within run date)). Repeatability of CCO or CCRT in 25ºC-acclimated

232    flies was estimated using the rpt() function in the R package rptR (unadjusted repeatability with a

233    normal distribution of data assumed; number of parametric bootstraps = 1000 [52]). To test

234    whether disturbances to the flies associated with carrying out trial 1 affected trial 2

235    measurements, trial 2 measurements ($CCO_2$ or $CCRT_2$) of the control and 25ºC-acclimated

236    groups were compared using linear mixed-effects models (fixed effect: treatment group; random

237    effect: run date). Finally, to assess the relationship between basal tolerance ($CCO_1$ or $CCRT_1$)

238    and acclimation capacity ($CCO_1 - CCO_2$ or $CCRT_1 - CCRT_2$), two more linear mixed-effects

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239   models were carried out (fixed effects: $CCO_1$ or $CCRT_1$, treatment group; random effect: run

240   date); slopes of the 15°C- and 25°C-acclimated groups were compared by checking whether

241   there was a significant interaction between the effects of $CCO_1$ or $CCRT_1$ and treatment group

242   (15°C- vs. 25°C-acclimated) on acclimation capacity. All linear mixed-effects models were

243   carried out using the lme() function in the R package nlme [53].


244   **Results and Discussion**

245   *1. Cold acclimation induced plasticity*

246   As anticipated, 15°C-acclimation induced plasticity in CCO and CCRT. There was a significant

247   effect of trial (LMM: $F_{1,126} = 97.9$, $p < 0.0001$) and treatment (15°C- vs. 25°C-acclimation;

248   LMM: $F_{1,120} = 107.0$, $p < 0.0001$) on CCO, and a significant interaction between these effects

249   (LMM: $F_{1,126} = 106.4$, $p < 0.0001$; Fig. 2A). Likewise, there was a significant effect of trial

250   (LMM: $F_{1,191} = 18.0$, $p < 0.0001$) and treatment (LMM: $F_{1,185} = 34.0$, $p < 0.0001$) on CCRT, and

251   a significant interaction between these effects (LMM: $F_{1,191} = 30.7$, $p < 0.0001$; Fig. 2B). This

252   improvement in cold tolerance with cold acclimation is consistent with current literature [8].

253   Having verified that 15°C-acclimation did indeed induce plasticity in this study system, we

254   moved on to characterizing the relationship between basal tolerance and plasticity.
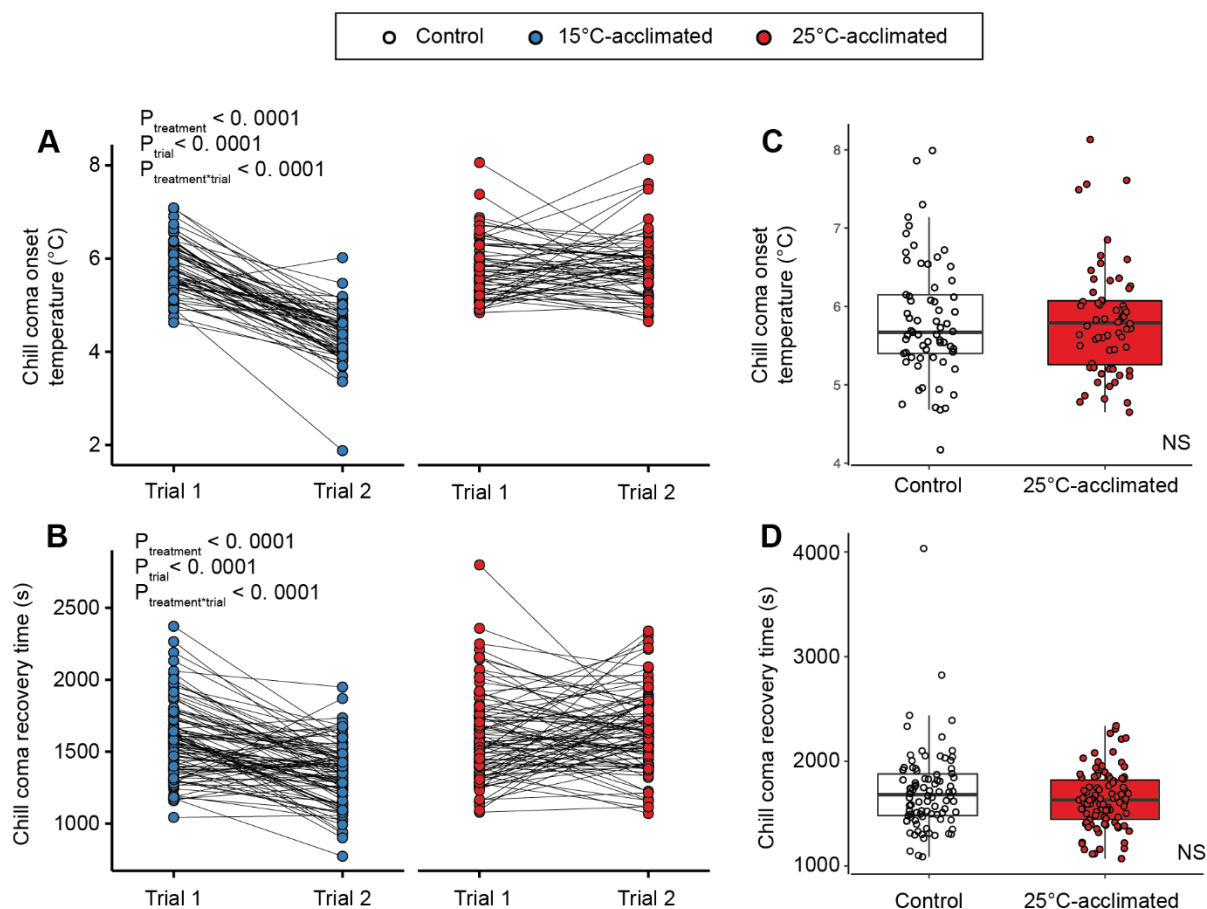
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**Figure 2. 15°C-acclimation induced plasticity in CCO (A) and CCRT (B) in female *D. melanogaster*, and conditions in trial 1 did not affect subsequent measurements of CCO (C) or CCRT (D).** Solid black lines in panels A and B connect an individual fly's trial 1 measurement ($CCO_1$ or $CCRT_1$) to the same individual's trial 2 measurement ($CCO_2$ or $CCRT_2$). The main effects of treatment and trial on CCO or CCRT were significant ($p < 0.0001$); there was a significant interaction effect between the two predictors ($p < 0.0001$). There was not a significant effect of treatment— control (did not undergo trial 1) vs. 25°C acclimation (did undergo trial 1)— on trial 2 measurements ($CCO_2$ (C) or $CCRT_2$ (D)).

*2. Repeatability of CCO and CCRT in 25°C-acclimated flies was low*

Earlier, within-individual repeatability was loosely defined as the consistency or predictability of

a trait within individuals over repeated measurements [45,46]. 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 [45,46]. "Subjects" may

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275    be any unit of measurement, from groups to— in this case— individuals. The non-repeatable

276    portion of the total variance (1–R) is composed of measurement error and fluctuations of the trait

277    within individuals [46,54]. We assumed that the lower the repeatability of CCO and CCRT in the

278    25°C-acclimated flies, the lower the likelihood of detecting a trade-off, if present, in the 15°C-

279    acclimated flies. This is because low repeatability indicates that measurements of CCO and

280    CCRT are unreliable indices of basal tolerance. This unreliability and consequential decreased

281    likelihood of detecting a trade-off has two-fold reasoning. First, low repeatability of CCO and

282    CCRT in the 25°C-acclimated flies — whether due to measurement error or "real" within-

283    individual fluctuations in CCO or CCRT— indicates that single estimates of basal tolerance are

284    not necessarily representative of a fly's "true" (mean) basal tolerance and therefore may not

285    predict a fly's acclimation capacity. Second, and perhaps more importantly, since repeatability

286    indicates the proportion of total variance attributable to differences among individuals [45,46],

287    low repeatability means that we lack meaningful variation in basal tolerance that is theoretically

288    necessary to detect a relationship between basal tolerance and plasticity. For both metrics,

289    repeatability was, in fact, low: CCO in the 25°C-acclimated flies was not significantly repeatable

290    ($R \pm SE = 0.14 \pm 0.11$, 95% CI = [0, 0.37], $p = 0.1$), while repeatability of CCRT in the 25°C-

291    acclimated flies was significant, but low ($R \pm SE = 0.23 \pm 0.09$, 95% CI = [0.033, 0.41],

292    $p = 0.01$).

293

294    Low repeatability in the 25°C-acclimated group may result if performing trial 1 had some effect

295    (damage or acclimation) on the thermotolerance of flies in trial 2. If this is the case, repeatability

296    in the 25°C-acclimated group does not give an accurate estimate of the repeatability of basal

297    tolerance, as acclimated or damaged tolerance in trial 2 would not be considered representative

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298    of basal tolerance. However, there was no significant effect of treatment group (Control vs.

299    25°C-acclimated) on $CCO_2$ (LMM: $F_{1,121} = 0.2$, $p = 0.7$; Fig. 2C). Similarly, there was no

300    significant effect of treatment group on $CCRT_2$ (LMM: $F_{1,177} = 2.9$, $p = 0.09$; Fig. 2D). This

301    suggests that conditions experienced by flies during trial 1 did not affect subsequent

302    measurements of thermotolerance. Thus, trial 2 measurements should be representative of basal

303    tolerance (in the 25°C-acclimated flies) and repeatability of CCO and CCRT in the 25°C-

304    acclimated flies should be accurate estimates of the repeatability of basal tolerance.

305

306    To some degree, low repeatability of CCO and CCRT is very likely due at least in part to

307    measurement error, but because we are familiar with these techniques and the amount of

308    measurement error that can be expected, we argue that true biological fluctuations and/or natural

309    stochasticity in CCO or CCRT within individuals are primary contributing factors to the low

310    repeatability we observed. Insect chill-coma has historically been associated with a reduction of

311    neuromuscular excitability and depolarization of neuromuscular membrane potential [8]. More

312    recent evidence has clarified the differing roles of the nervous and muscular systems in chill

313    coma entry and recovery: entry into chill coma (CCO) appears to be caused primarily by

314    depolarization of the central nervous system (CNS), while recovery from chill coma (CCRT)

315    after hours in the cold involves rapid recovery of CNS function, followed by slower

316    repolarization of muscle membrane potential as systemic ion balance is restored [55–57].

317    Within-individual stochasticity in mechanisms hypothesized to govern the maintenance of

318    neuromuscular excitability and membrane potential at low temperatures— e.g. modulation of

319    neuromuscular membrane composition (e.g.[58,59]), modulation of the thermal sensitivity of

320    voltage-gated ion channels responsible for action potentials (e.g. [60,61]), or changes in ion

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321    transporters in neuromuscular cells [55,62] may contribute to low repeatability. CCRT is

322    additionally influenced by an individual's motivation to stand [3], so within-individual

323    variability in motivation may lower repeatability of CCRT as well.

324

325    *3. The illusion of a trade-off between basal and plastic cold tolerance*

326    Given that estimates of the repeatability of basal tolerance were low for both CCO and CCRT,

327    we did not expect to find a significant relationship between basal tolerance and acclimation

328    capacity in the 15°C-acclimated flies. Surprisingly, however, we found a strong significant

329    relationship between both $CCO_1$ and acclimation capacity ($CCO_1 - CCO_2$) ($F_{1,118} = 106.0$,

330    $p < 0.0001$) and $CCRT_1$ and acclimation capacity ($CCRT_1 - CCRT_2$) ($F_{1,183} = 239.4$, $p < 0.0001$).
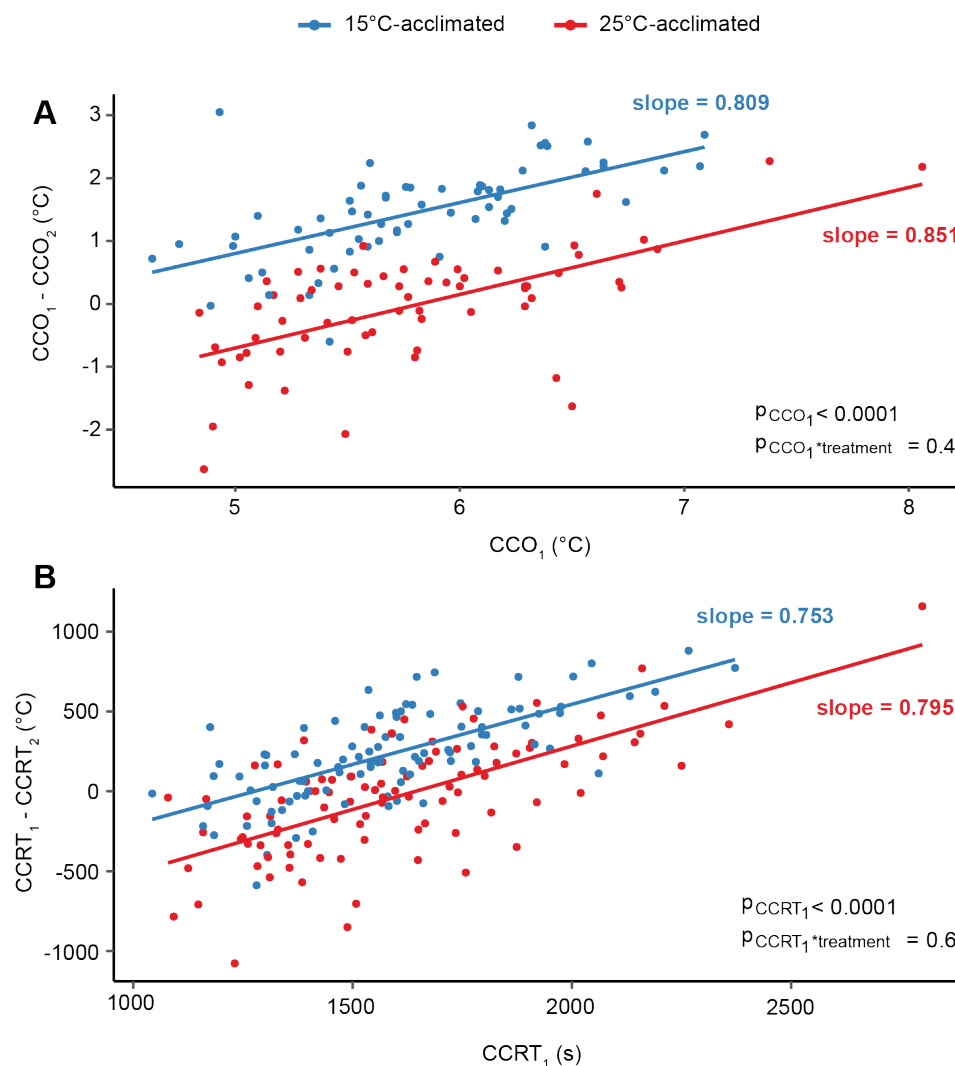
18

331

**Figure 3**. **Relationship between $CCO_1$ (A) or $CCRT_1$ (B) and acclimation capacity ($CCO_1$ –**
**$CCO_2$ or $CCRT_1$–$CCRT_2$) 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) $CCO_1$ 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) $CCRT_1$ 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 ($p = 0.6$).

339

340

341    A more thorough consideration of the relationship between trial 1 and trial 2 measurements

342    (particularly in the 25°C-acclimated flies) reveals how this significant trade off pattern arises in

343    our data despite low— and in the case of CCO, no— repeatability of basal tolerance. In the

344    25°C-acclimated flies, there was a striking— and unexpected— relationship between trial 1 and

19

345     trial 2 measurements within individuals: individuals that were more thermotolerant (lower CCO

346     or CCRT) in trial 1 tended to be less tolerant in trial 2, and individuals that were less tolerant in

347     trial 1 tended to be more tolerant in trial 2 (Fig. S1). One potential explanation for this pattern of

348     data is that the least thermotolerant flies in trial 1 were cold-hardened by trial 1 conditions, while

349     the most thermotolerant flies were ultimately damaged by trial 1 conditions. It is not illogical to

350     imagine that an individual's thermotolerance could subsequently improve because of exposure to

351     low temperatures during trial 1. After all, hardening responses can be induced by only minutes-

352     hours of sublethal cold exposure [13,63]. However, why the most tolerant flies in trial 1 would

353     not only fail to cold-harden but ultimately be damaged by trial 1 conditions when seemingly less

354     tolerant flies cold-hardened under the same conditions is unclear. Moreover, and more

355     importantly, there is a better explanation for the pattern observed.

356

357     When repeated measurements are taken from the same subject, these measurements are not likely

358     to be identical: some degree of random error— due to measurement error or fluctuation of the

359     given trait— is almost inevitable [64]. Because of this random error, such repeated

360     measurements are susceptible to a statistical phenomenon known as regression to the mean

361     (RTM; [64,65]). Put simply, subjects that have more-extreme measurements in one trial will tend

362     to have less-extreme measurements— measurements that are closer to their true mean— in the

363     next trial. The pattern of within-individual variation we are attempting to explain in the 25°C-

364     acclimated flies aligns with that expected from RTM (Fig. 4, S2). Due to this, and due to the

365     near-inevitability of RTM when taking repeated measurements, we attribute the relationship

366     between trial 1 and trial 2 measurements in the 25°C-acclimated flies to the effects of RTM, and

367     not to some biological phenomenon. Rank plots (Fig. S2) further emphasize this unexpected

368    positive correlation between basal thermotolerance and "acclimation capacity" in the 25°C-

369    acclimated flies, as "acclimation capacity" ($CCO_1 - CCO_2$ or $CCRT_1 - CCRT_2$) tends to be

370    negative in flies with lower trial 1 measurements and positive in flies with higher trial 1

371    measurements, indicative of RTM.

372

373    Falsely ascribing significance (e.g. biological, economic, psychological) to manifestations of

374    RTM is a common fallacy [65,66]. To avoid this error, one must separate effects expected from

375    RTM, and effects (if any) beyond those expected from RTM [64]. In the 25°C-acclimated flies,

376    differentiating such effects was straight-forward: there is no intervention applied or relevant

377    biological phenomena expected to be occurring across the experimental period, so any apparent

378    RTM effects can be reasonably attributed to RTM. In the 15°C-acclimated flies, on the other

379    hand, both RTM and a thermotolerance-plasticity trade-off are expected to manifest as a positive

380    correlation between basal tolerance and plasticity [64,65]. Thus, to determine whether the

381    positive correlation previously noted in the 15°C-acclimated flies (Fig. 3) is truly evidence for a

382    trade-off, we must determine how much of this correlation is attributable to RTM. In

383    experimental studies, like ours, differentiation between the effects of RTM and other phenomena

384    can be achieved quite easily with an adequate control group (i.e. a group for which repeat

385    measurements are taken but no treatment/intervention is applied between trials) [65]. As RTM is

386    expected to equally affect control and experimental groups, changes in the experimental group

387    different from those observed in the control group suggest phenomena at play besides RTM.

388    Happily, our 25°C-acclimated group acts as an ideal control for the 15°C-acclimated group in

389    this regard.

390

21

391    *4. The relationship between basal tolerance and acclimation capacity in the 15°C-acclimated*

392    *flies is underlain by regression to the mean*

393

394    If the slope between basal tolerance and "acclimation capacity" ($CCO_1 - CCO_2$ or $CCRT_1-$

395    $CCRT_2$) in 25°C-acclimated flies is the same as the equivalent slope in the 15°C-acclimated flies,

396    the apparent differential effect of cold acclimation on flies of high and low initial

397    thermotolerance is not greater than the differential change expected from RTM. There was a

398    significant relationship between $CCO_1$ and acclimation capacity ($CCO_1 - CCO_2$) in both the

399    15°C- and 25°C-acclimated groups ($F_{1,118} = 106.0$, $p < 0.0001$; Fig. 3). The slopes of the

400    relationships between $CCO_1$ and acclimation capacity did not significantly differ between

401    acclimation groups ($F_{1,118} = 0.6$, $p = 0.4$; Fig. 3A). Likewise, there was a significant relationship

402    between $CCRT_1$ and acclimation capacity ($CCRT_1-CCRT_2$) in both the 15°C- and 25°C-

403    acclimated groups ($F_{1,183} = 239.4$, $p < 0.0001$), and the slopes of the relationships between

404    $CCRT_1$ and acclimation capacity similarly did not significantly differ between 15°C- and 25°C-

405    acclimated flies ($F = 0.2$, $p = 0.6$; Fig. 3B). As the slopes of the 15°C- and 25°C-acclimated flies

406    (Fig. 3) did not significantly differ for either thermotolerance metric, we cannot attribute the

407    apparent differential effect of cold acclimation on flies of high or low initial thermotolerance to

408    anything other than RTM. There is therefore no evidence for a tolerance-plasticity trade-off in

409    the 15°C-acclimated flies.

410

411    *5. How big of a problem is regression to the mean in studies of thermal tolerance?*

412    When an independent variable (Y) contains the dependent variable (X) as a constituent (e.g. Y =

413    X – Z; as when plasticity = basal tolerance – induced tolerance), these variables are not

22

414    statistically independent [23,67]. Issues of interpretation resulting from this implicit statistical

415    association have been articulated before (e.g. when estimating trade-offs between constitutive

416    and induced defenses in plants [68]; when estimating costs of plasticity [67,69,70]; when

417    assessing mass-dependent mass loss in birds [71,72]). Yet, this methodology has only recently

418    been highlighted as a potential concern for thermotolerance-plasticity trade-off studies, and

419    remains widespread [23]. Conclusions made on the basis of a relationship between baseline

420    measurements and the difference between baseline and follow-up measurements may not

421    necessarily be problematic if the null hypothesis for such an investigation is properly identified

422    [71,72]. A proper null hypothesis should account for statistical bias resulting from phenomena

423    like RTM [65]. However, there is very little consideration of RTM (or repeatability, for that

424    matter) in previous thermotolerance-plasticity trade-off studies, precluding such identification

425    (but see Deery et al. [30], who do not mention RTM but do address statistical bias).

426

427    It is unclear to what extent the results of past thermotolerance-plasticity trade-off studies are

428    biased by RTM. We expect RTM to occur whenever repeated measurements of the same subjects

429    are taken (as random error is unavoidable in experimental biology), whether the study is at the

430    individual level or, as is the case for the vast majority of previous thermotolerance-plasticity

431    trade-off studies, above the individual level [64,65] (but see: [29–31]). However, it is not clear

432    under what specific methodological circumstances RTM occurs. For instance, if basal and

433    induced tolerance are measured for the same group but different individuals within the group are

434    used for each measurement, is RTM still expected to occur? This must be determined (e.g.

435    through modeling hypothetical data) before effects of RTM can be corrected for (per Kelly and

436    Price [65]).

437

438     In any case, the following certainly seems to be true: it is not yet known whether a

439     thermotolerance-plasticity trade-off exists [23]. This lack of clarity stems from more than the

440     inherent statistical association between basal tolerance and plasticity and issues of interpretation

441     arising from it. For instance, many tolerance-plasticity trade-off studies are carried out on field

442     organisms, meaning that environmental and genetic effects on thermotolerance cannot easily be

443     unwoven [23,73]. As well, misleading trade-off patterns may arise if more basally tolerant

444     organisms require more extreme temperatures/longer acclimation to induce plasticity (a

445     "threshold shift" as described in: [23]). Further, some metrics of thermotolerance— like

446     survivorship and CCRT— are bounded, such that there is an upper and/or lower limit to said

447     metric (e.g. 100% survival or a CCRT of 0 s). When testing for a trade-off using bounded

448     metrics, trade-off patterns may arise due to organisms with high basal tolerance hitting the upper

449     and/or lower limit of the metric after acclimation, rather than due to a trade-off [23,74]. The

450     above issues, as well as others, are discussed in detail by van Heerwaarden and Kellermann in

451     their recent review of heat tolerance-plasticity trade-off studies [23]. Considering the multitude

452     of issues with current approaches at testing for a thermotolerance-plasticity trade-off, a

453     reconsideration of methodology– statistical and otherwise– is vital if there is to be any hope of

454     determining whether such a trade-off truly exists.

455     *Conclusions*

456     Here, we found a strong positive correlation between basal cold tolerance and plasticity,

457     demonstrating apparent support for a cold tolerance-plasticity trade-off at the individual level in

458     *Drosophila melanogaster*. However, repeatability of CCO and CCRT in non-cold-acclimated

459     flies was low, indicating that estimates of basal tolerance were unreliable, and we should

24

460     therefore not be able to detect such a relationship. We argue that this pattern is likely a

461     manifestation of regression to the mean and is not reflective of a true trade-off. Concerningly,

462     many previous thermotolerance-plasticity trade-off studies, despite employing similar

463     methodology to that carried out herein— regressing plasticity on basal tolerance, where plasticity

464     is measured as the difference between basal and induced tolerance—, do not consider either

465     repeatability or RTM in their experimental design/analyses. Moving forward, we recommend

466     two courses of action: first, we must determine whether previous thermotolerance-plasticity

467     trade-off studies are indeed biased by RTM (and if, in fact, there is no or little existing evidence

468     for this hypothesis); second, we must carry out future thermotolerance-plasticity trade-off studies

469     with appropriate controls such that this bias is accounted for in the future.

470

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473
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## Author Contributions

481     All authors together conceived the study and designed the experiments. E.O. carried out the

482     experiments. E.O. ran the systematic literature search and developed Tables 1 and 2. E.O.

483    curated and analyzed the data and created visualizations. H.A.M. provided resources and

484    supervision. E.O. drafted the manuscript and all authors edited the manuscript.

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