Experimental evolution under combined thermal-nutritional stress in *Drosophila melanogaster* results in evolved shifts in larval thermotolerance and thermal plasticity

Authors: Yeuk Man Movis Choy, Teresa Kutz, Fiona E. Cockerell, Emily Lombardi, Sandra Hangartner, Christen K. Mirth*, Carla M. Sgrò*

*Equal supervision of the research. Authorship decided by coin toss.

School of Biological Sciences, Monash University, Wellington Rd, Clayton, 3800 Melbourne, Victoria Australia

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Abstract

Animals are facing increasing thermal and nutritional stress under climate change. Genetic adaptation is necessary to buffer long-term stress. However, it's unclear whether adaptation to combined stress can occur without compromising viability and thermal plasticity. We tested thermotolerance and thermal plasticity in larval *Drosophila melanogaster* selected under different temperatures (18°C, 25°C, 28°C) and diets (standard, diluted, low protein:carbohydrate). Basal cold tolerance was affected by both low protein concentration and temperature; larvae evolved higher basal cold tolerance on diluted and low protein:carbohydrate diets at 18°C and 28°C. Hardening increased cold tolerance for most lines, except those selected at 18°C and 28°C on low protein:carbohydrate diets and 25°C on standard diets. Basal heat tolerance was affected by selection temperature, with higher tolerance in 25°C selection lines. An

interaction between selection temperature, selection diet and hardening treatment affected plasticity in heat tolerance, with most selection lines showing reduced heat tolerance upon hardening, except for lines selected at 25°C on low protein diets and at 28°C on standard diets. Our results indicate that adaptation to combined stress allows basal cold tolerance and plasticity in cold tolerance to evolve independently, but not heat tolerance, highlighting the increased vulnerability of ectotherms to long-term climate change.

Introduction

Anthropogenic climate change is increasing the frequency of climatic extremes (Thomas *et al.* 2004; Diffenbaugh and Field 2013; Wiens 2016). Combined shifts in atmospheric CO_2, precipitation, and temperature (Van Der Putten *et al.* 2010; Pecl *et al.* 2017; Masson-Delmotte *et al.* 2018) will also alter plant growth, nutritional quality (Kreuzwieser and Gessler 2010; Lukac *et al.* 2010; Jin *et al.* 2019), and abundance (Van Der Putten *et al.* 2010; Pecl *et al.* 2017). These changes will likely impose increasing thermal and nutritional stress on organisms, both directly and indirectly through changes in microbial communities associated with plant decomposition (Bowes 1993; Richardson *et al.* 2002; Dwyer *et al.* 2007; DaMatta *et al.* 2010; Rosenblatt and Schmitz 2016). Ectothermic herbivores and frugivores, which rely on ambient temperature to maintain physiological performance and function (Hawlena and Schmitz 2010; Walters *et al.* 2012), will be particularly vulnerable to increased thermal and nutritional stress.

Organisms primarily respond to environmental stress by two means, phenotypic plasticity and genetic adaptation (Stillwell *et al.* 2007; Urban *et al.* 2014). Phenotypic plasticity, where a single genotype produces different phenotypes depending on the environment, is often the initial response to environmental change (Huey *et al.* 2002; Urban *et al.* 2014; Seebacher *et al.* 2015; Sgrò *et al.* 2016). Plasticity allows for immediate (within-generation) responses to environmental change (Padilla and Savedo 2013; Sgrò *et al.* 2016) and may contribute to population persistence (Chevin *et al.* 2010; Frankino *et al.* 2019; Noble *et al.* 2019), although it may not always be adaptive (Ghalambor *et al.* 2007; Leonard and Lancaster 2020). Nonetheless, an increasingly large body of work is focused on understanding plastic responses to a range of

stressors, particularly temperature and nutrition, to better predict population persistence under climate change. Many of these studies have focussed on plastic shifts in thermotolerance (van Heerwaarden *et al.* 2016; Rodrigues and Beldade 2020; van Heerwaarden and Kellermann 2020) since the ability to withstand thermal extremes is a significant predictor of climate sensitivity (Hoffmann *et al.* 2013; Noh *et al.* 2017; Kellermann and van Heerwaarden 2019; Ma *et al.* 2021).

Plastic shifts in thermotolerance in response to temperature and nutrition individually have been widely reported, particularly in insects (Lee, 1989; Hoffmann and Harshman 1999; Andersen *et al.* 2010; Sgrò *et al.* 2016; Mitchell *et al.* 2017; Ma *et al.* 2021). For example, diets with higher protein increased adult heat tolerance in insects (Andersen *et al.* 2010), while higher dietary carbohydrates facilitate synthesis of cold-resistant compounds, which in turn, enhance cold tolerance (Hazel 1995; Kostal and Simek 1998; Andersen *et al.* 2010; Colinet *et al.* 2013).

Thermal plasticity can arise through acclimation (Rako and Hoffmann 2006; Andersen *et al.* 2010; Kingsolver *et al.* 2016), which involves long-term exposure of days or weeks to temperatures above or below optimal temperatures but still within the normal viable range of an organism. Acclimation to higher developmental temperatures often increases basal heat tolerance (Kingsolver *et al.* 2016; Kellermann *et al.* 2017; Pumhan *et al.* 2020; Jerbi-Elayed *et al.* 2021; Willot *et al.* 2021), while acclimation to lower developmental temperatures can increase basal cold tolerance (Rako and Hoffmann 2006; Scharf, Galkin, *et al.* 2015; Noh *et al.* 2017).

Hardening, the short-term exposure of minutes or hours to sublethal temperatures, can also induce plastic shifts in thermotolerance (Hoffmann *et al.* 2003; Bowler 2005;

Loeschcke and Sørensen 2005; Teets *et al.* 2020; Ma *et al.* 2021). In insects, high and low temperature hardening treatments can improve subsequent heat (Chen *et al.* 1991; Sejerkilde *et al.* 2003; Bubliy *et al.* 2012; Manenti *et al.* 2015; Kalra *et al.* 2017) and cold tolerance (Czajka and Lee 1990; Chen *et al.* 1991; Kelty and Lee 2001; Sejerkilde *et al.* 2003; Jensen *et al.* 2007; Jakobs *et al.* 2015; Teets *et al.* 2020), respectively.

Plastic shifts in response to combinations of thermal and nutritional stress have been documented, but to date, such studies have primarily focussed on life history traits rather than thermal tolerance (Polak *et al.* 2004; Bubliy *et al.* 2012; Kutz *et al.* 2019; Chakraborty *et al.* 2020). Interestingly, these studies suggest that plastic responses to combinations of thermal and nutritional stress may actually be non-adaptive. For instance, larvae of *Tenebrio molitor* and *Drosophila melanogaster* developed at higher temperatures in combination with suboptimal diets had decreased survival (Rho and Lee 2017; Kutz *et al.* 2019).

Despite widespread evidence of plastic changes in thermotolerance, studies suggest that plasticity is unlikely to fully mitigate the negative consequences of ongoing temperature increases. For example, plasticity was found to have a limited effect on increasing the upper thermal limit of *D. melanogaster* (van Heerwaarden *et al.* 2016). There is also some evidence suggesting that insects with higher basal thermotolerance may be less plastic (Nyamukondiwa *et al.* 2011; Gerken *et al.* 2015; van Heerwaarden *et al.* 2016; Kellermann *et al.* 2017; Noh *et al.* 2017; Kellermann and Sgrò 2018), implying a potential trade-off between plasticity and basal thermotolerance (but see van Heerwaarden *et al.* (2024)). Thus, in the context of ongoing climate change, genetic adaptation, while occurring over longer timescales, will be key to the long-term

persistence of populations (Potvin and Tousignant 1996; Gienapp *et al.* 2008; Lande 2009).

Reliable predictions concerning whether and how populations might respond to selection imposed by climate change require answers to key questions (Cavicchi *et al.* 1995): do populations harbour the genetic variation necessary for rapid evolutionary responses to combinations of stressors? Does adaptation to specific thermal and nutritional conditions involve genetic changes in many traits? To what extent does adaptation affect the ability of populations to mount plastic responses to conditions other than those selected?

Experimental studies of evolution in the laboratory (Hoffmann and Harshman 1999; Rose *et al.* 2005) have addressed these questions with respect to genetic adaptation to thermal (Huey *et al.* 1991; Cavicchi *et al.* 1995; Bubliy and Loeschcke 2005) and nutritional (Kolss *et al.* 2009; Kristensen *et al.* 2011; Leftwich *et al.* 2017) stress individually. Using laboratory natural selection, where large populations are maintained for many generations at different non-extreme temperatures, Cavicchi *et al.* (1995) and Huey *et al.* (1991) observed rapid evolution of adult thermotolerance in *D. melanogaster*. Lines adapted to higher temperatures evolved higher heat tolerance (Huey *et al.* 1991; Cavicchi *et al.* 1995), while those adapted to cooler temperatures had faster development at lower temperature, but their cold tolerance was not assessed (Huey *et al.* 1991). Interestingly, Cavicchi *et al.* (1995) found that thermal adaptation resulted in correlated shifts in thermal plasticity. Lines evolved at 18°C showed a positive acclimation response (heat tolerance increased) to development at 25°C and 28°C, while 25°C evolved lines showed a positive acclimation response to 25°C, but a

122 negative acclimation response (heat tolerance decreased) to 28°C. Further, the evolved
123 lines also showed correlated changes in their hardening responses, with the 25°C
124 evolved lines having a higher hardening threshold (the temperature that induced a
125 hardening response) compared to the 18°C evolved lines. Notably, the 28°C evolved
126 lines lost the ability to mount a hardening response, and exposure to the highest
127 hardening temperature reduced thermal tolerance. These results suggest that adapting
128 to elevated temperatures may come at the cost of reduced plasticity. However, the
129 effect of thermal adaptation on larval thermotolerance traits was not examined.

130 Experimental evolution has also been used to assess adaptation to changes in diet.
131 Kolss *et al.* (2009) examined adaptation to nutritional stress in larvae of *D.*
132 *melanogaster* by exposing them to poor (25% diluted) or control diets throughout
133 development; adults were placed on the control diet every generation. Evolved lines
134 were tested on both poor and control diets to assess whether adaptation was
135 associated with any trade-offs in life-history traits and whether nutritional plasticity had
136 evolved. When tested on the poor diet, the selected lines had higher larval viability than
137 the control lines, but viability remained the same when tested on the control diet.
138 However, adults of lines selected on poor diets were smaller and had reduced
139 fecundity and lower starvation resistance than the controls. Interestingly, there was no
140 effect of selection or test diet on larval heat or cold tolerance. Similarly, Kristensen *et*
141 *al.* (2011) used experimental evolution to expose *D. melanogaster* larvae to standard or
142 protein-enriched diets. They found that adaptation to the high-protein diet resulted in
143 reduced larval-adult survival, suggesting that adaptation to changes in nutrition may be
144 limited by trade-offs between life-history traits. However, they did not examine larval or
145 adult thermotolerance. Finally, Leftwich *et al.* (2017) imposed nutritional selection on

146 larvae of the Mediterranean fruitfly and found that lines selected on the low calorie diet
147 evolved higher egg-adult survival compared to those on the standard diet after 30
148 generations of selection, when tested on both diets. Once again, larval
149 thermotolerance was not assessed.

150 Only one study has examined adaptation to both nutritional stress and temperature.
151 Bochdanovits and De Jong (2003) exposed larvae to two temperatures (17.5°C and
152 27.5°C) and poor (50% diluted) and control diets. Overall, adaptation to the higher
153 temperature resulted in reduced larval survival on both diets. The 17.5°C selected lines
154 evolved larger body size when also adapted to a poor diet, while the 27.5°C evolved
155 lines were smaller on both diets. This suggests that adaptation to combinations of
156 temperature and nutrition can result in trade-offs between adult size and larval survival,
157 and potentially other traits that were not measured. However, the consequences of
158 adaptation to both temperature and nutrition on thermal tolerance and its plasticity
159 were not assessed.

160 In this study, we aimed to understand how long-term adaptation to combined thermal-
161 nutritional stress affects larval thermotolerance and thermal plasticity. We assessed
162 basal and induced thermotolerance of larvae from nine *D. melanogaster* experimental
163 evolution lines that were forced adapted to combinations of three temperatures (18°C,
164 25°C and 28°C) and three diets (standard, diluted and low protein:high carbohydrate
165 (low P:C) diets) using laboratory natural selection (Alton *et al.* 2024). These conditions
166 were chosen to reflect variation in the nutritional context that developing larvae
167 experience in rotting fruit, with the diluted diet representing reduced food availability
168 and the low P:C diet representing reduced food quality (Markow *et al.* 1999; Kristensen

169 *et al.* 2011; Almeida de Carvalho and Mirth 2017; Silva-Soares *et al.* 2017) and to
170 simulate future thermal and nutritional conditions in the context of long-term climate
171 change (DaMatta *et al.* 2010; Gowik and Westhoff 2011; IPCC 2018). The diluted and
172 low P:C diets contained the same concentration of protein, and the standard and low
173 P:C diets contained the same caloric concentration by replacing the calories from
174 protein with carbohydrates. This allowed us to examine the effect of protein and
175 carbohydrate concentrations of the selection diets independently of each other.

176 Only larvae were exposed to selection to reflect their limited capacity to avoid stress by
177 behaviourally choosing or moving to more favourable microhabitats in the wild (Austin
178 and Moehring 2019). Based on previous studies examining thermal and nutritional
179 adaptation, we first predicted that adaptation to 28°C would increase larval heat
180 tolerance, and this effect would be strongest for lines adapted to the standard diet high
181 in protein. Second, we predicted that adaptation to 18°C would result in increased cold
182 tolerance, and that this effect would be greater for lines adapted to the low P:C diet.
183 Finally, we expected thermal plasticity to evolve in all lines. If basal thermotolerance
184 trade off with plasticity, we would expect plasticity to be reduced in the evolved lines
185 with higher basal thermotolerance.

186 Our results provide insight into the complexity of evolutionary responses to
187 combinations of thermal and nutritional stress, and what this means for our ability to
188 predict responses to ongoing climate change. They reveal that adaptation to
189 combinations of thermal and nutritional stress results in both genetic and plastic shifts
190 in pre-adult traits that are not easily predicted by adaptation to single stressors. Finally,
191 they highlight that studies that address the complexity of adaptation to multiple

192 stressors are needed to better understand responses to on-going environmental
193 change.

194

195 **Methods**

196 *Fly stock – Collection and initiation of experimental evolution lines*

197 Two hundred female *D. melanogaster* were collected in Duranbah, Queensland (28.3°
198 S, 153° E) in 2018 to initiate isofemale lines (Alton *et al.* 2024). Flies were returned to the
199 laboratory and were treated with tetracycline to remove *Wolbachia* (Min and Benzer
200 1997). Two generations after tetracycline treatment, five males and five virgin females
201 from each line were used to create a mass-bred base population. The base population
202 was maintained at 25°C under 12:12 hour light:dark cycles on standard diet (see below)
203 across 60 bottles each containing approximately 750-1000 flies for two generations
204 before the initiation of selection.

205

206 *Experimental evolution regimes and selection protocol*

207 Eggs were collected from the base population and were divided among nine combined
208 thermal-nutritional selection treatments with five replicate lines per treatment (Alton *et*
209 *al.* 2024). These treatments consisted of three temperatures (18°C, 25°C and 28°C) and
210 three diets (standard, diluted and low P:C) (Table S1) under a 12:12 light:dark cycle.
211 Only larvae were exposed to selection. All diets contained varying amounts of potato
212 flake, inactive yeast, agar, and dextrose, with the addition of nipagin and propionic acid
213 to prevent bacterial and fungal growth (Holleley *et al.* 2008) (Table S1). The standard

214 diet had a 1:3 protein-to-carbohydrate (P:C) ratio (yeast 36.36 g/L) and contained 320
215 kcal/L; the diluted diet had a 1:3 P:C ratio (yeast 9.09 g/L) and contained 80kcal/L; and
216 the low P:C diet had a 1:12 P:C ratio (yeast 8.18 g/L) and contained 320kcal/L (Table
217 S1).

218 These selection conditions reflect combinations of thermal and nutritional changes
219 that may be expected in the future under climate change; 18°C and 25°C represent the
220 current average Australian winter and summer temperatures respectively (Alton *et al.*
221 2024), while 28°C represents a 3°C increase of the average summer temperature
222 projected in twenty years (Australian Academy of Science 2021). The diluted diet and
223 the low P:C diet simulate a reduction of protein and varying carbohydrates in plant
224 composition projected under climate change (DaMatta *et al.* 2010), and span the range
225 of nutritional contexts experienced by developing larvae in rotting fruit (Matavelli *et al.*
226 2015; Silva-Soares *et al.* 2017).

227 At the start of each generation of selection, each selection replicate line was
228 established with three bottles containing 200-250 eggs on 70 mL of selection diets to
229 equalize density across all selection regimes. Animals were allowed to develop on their
230 respective diet and temperatures until adult eclosion. Upon eclosion, adults from each
231 bottle were collected daily until no more adults emerged to prevent selection for fast
232 development. Adults from the same replicate line were mixed and separated into two
233 bottles containing the standard diet at 25°C for three days to allow mating. On the
234 fourth day, adults were transferred to 250 mL egg-laying chambers containing 11 mL of
235 egg laying medium, the standard diet with double the concentration of agar of the
236 standard fly diet and a layer of autoclaved yeast for acclimation. We doubled the

concentration of agar to prevent females from embedding their eggs into the food, making egg collection easier. After the 24-hour acclimation, fresh medium was provided for egg-laying overnight. The eggs were collected the next morning to establish the next generation as described above.

At the time of experiment described below, generations of selection were as follows: 61 for the 18°C standard diet, 51 for the 18°C diluted and low P:C diets, 89 for the 25°C standard diet, 75 for the 25°C diluted and low P:C diets, 100 for the 28°C standard diet, 81 for the 28°C diluted diet, and 84 for the 28°C low P:C diet. Due to logistical limitations, three replicate lines were randomly selected from the five set up for each selection regime to perform the experiment described below. Prior to the experiment, all lines were placed in common garden conditions at 25°C on a standard diet for at least two generations to minimize parental effects (De Villemereuil *et al.* 2016).

Plastic and evolved shifts in larval thermotolerance

Experimental set up

After two generations of common garden rearing, adults were transferred to laying plates containing egg laying medium and a surface layer of autoclaved yeast to induce oviposition. Laying plates were placed at 25°C for 14 hours to allow egg laying. Eggs were collected from laying plates and transferred to vials containing 7.2mL of standard diet, at a density of twenty eggs per vial (Agnew *et al.* 2002). Five replicate vials were set up for each replicate line per selection regime. First instar larvae (hatched 24 hours after egg transfer) were exposed to the hardening and acute stress treatments. Vials

were maintained at 25°C under 12:12 hour light:dark cycles throughout the experiment, except when exposing larvae to the hardening and acute stress treatments described below. This common garden experimental design enabled us to investigate the effects of genetic adaptation to combined thermal-nutritional stress on larval thermotolerance and thermal plasticity.

Assessing induced (plastic) and basal thermotolerance

Larval cold plasticity was assessed by subjecting 24-hour-old first instar larvae, reared from embryos at 25°C on a standard diet, to a two-hour hardening treatment in a 0°C water bath, followed by a 24-hour recovery period at 25°C. After recovery, these 48-hour old larvae were exposed to a four-hour acute cold shock at 0°C in a water bath and then returned to 25°C under 12:12 hour light: dark cycles to complete development. Basal cold tolerance was assessed by exposing 48-hour-old larvae, reared from embryos at 25°C on a standard diet, to a four-hour acute cold shock at 0°C in a water bath, without prior hardening treatment. Then they were allowed to complete development at 25°C under 12:12 hour light: dark cycles. 48-hours old larvae were exposed to the acute cold shock treatment to equalise the stage at which larvae were exposed to the acute stress across the hardened and basal treatments.

Larval heat plasticity was assessed by subjecting 24-hour old first instar larvae, again reared from embryo at 25°C on a standard diet, to a one-hour hardening treatment in a 35°C water bath, followed by a 24-hour recovery period at 25°C. After recovery, these 48-hour old larvae were exposed to an acute heat shock of 39°C for half an hour. Those larvae were then allowed to develop at 25°C under 12:12 hour light: dark cycles until

adult eclosion. Basal heat tolerance was assessed by exposing 48-hour old larvae, reared from embryos on standard diet at 25°C, to an acute heat shock treatment of 39°C for 30 mins without any hardening treatment, then they were allowed to complete development at 25°C under 12:12 hour light: dark cycles.

A control treatment was also included, where vials contained larvae that were not exposed to any heat or cold stress treatments and were maintained on control food at 25°C under 12:12 hour light: dark cycles until adult eclosion.

The temperatures and durations for both heat and cold shock hardening and acute treatments were determined by pilot experiments (details in supplementary materials). Throughout these treatments, all vials were temporarily sealed with plastic caps and parafilm to prevent water leakage. After adult eclosion, larval-adult viability was subsequently measured as the ratio of the number of emerged adult flies to the initial egg number deposited in the vials.

Statistical analyses

Larval-adult viability was fitted using generalised linear mixed-effect models with a binomial distribution. We first tested for a response to selection on larval viability under control, non-stress conditions using analyses of variance (ANOVA) with selection temperature, selection diet, and their interaction, as fixed effects using the lme4 package (Bates *et al.* 2015) in R (R Core Team 2024). Block and replicate line were treated as random effects. Prior weights were set to the number of eggs deposited in the vial (i.e., 20). We performed multiple comparisons across treatments using the

304 ‘emmeans’ packages (Lenth 2023) to test for significant differences in larval-adult
305 viability across selection temperature and selection diet.

306 We used the same analyses to test for the effects of long-term adaptation on basal
307 thermotolerance and thermal plasticity of larvae, but with selection temperature,
308 selection diet, and treatment (hardened or basal) and their interactions included as
309 fixed effects, while block and replicate line were treated as random effects. We again
310 used the ‘emmeans’ packages (Lenth 2023) to test for significant differences in larval-
311 adult viability across factors.

312

313 **Results**

314 Our aim was to understand the effects of long-term adaptation to combined thermal-
315 nutritional stress on larval-adult viability, thermotolerance, and thermal plasticity. To
316 address this, we first asked whether adaptation to combinations of three temperatures
317 (18°C, 25°C and 28°C) and three diets (standard, diluted and low P:C) resulted in
318 evolved shifts in viability under control (25°C, standard diet) conditions. We then
319 assessed basal and induced (plasticity in) cold and heat tolerance of *D. melanogaster*
320 larvae from the evolved lines.

321

322 *Larval-adult viability under control conditions*

323 We first explored whether there was evidence for any response to selection in larval-
324 adult viability under control (non-stress) conditions. We found a significant interaction
325 between selection temperature and selection diet (Table 1, Figure 1), meaning that the

326 effect of adaptation to diet depended on the selection temperature. For instance,
327 adaptation to the standard diet resulted in higher viability when lines were also adapted
328 to 28°C, but not 18°C and 25°C (Figure 1, Table S2). On the other hand, adaptation to
329 the diluted diet selected for increased viability when lines had adapted to 25°C, but not
330 when lines had adapted to 28°C. Interestingly, adaptation to 28°C resulted in lower
331 viability in larvae also adapted to the diluted and low P:C diets (right panel, Figure 1),
332 whereas this pattern was largely reversed when lines had adapted to 25°C (middle
333 panel, Figure 1). Nonetheless, these effects of adaptation were small – mean viability
334 across all selection lines was 0.74, ranging between 0.698 - 0.778 across lines –
335 indicating that adaptation to combinations of temperature and diet did not incur large
336 costs in the form of large reductions in viability under control conditions.

337

338 *Basal cold tolerance*

339 We then explored whether adaptation to combined thermal-nutritional stress altered
340 basal cold tolerance. We found a significant two-way interaction between selection
341 temperature and selection diet on basal cold tolerance (Table 2, Figure 2), meaning that
342 any response to selection diet was modified by the selection temperature. This
343 interaction is illustrated by the fact that lines selected on both diluted and low P:C diets
344 had higher basal cold tolerance than those selected on standard diets but only when
345 they had evolved at 18°C and 28°C, and not 25°C (Figure 2, Table S4). Because the
346 diluted and low P:C diets had the same concentration of protein, this suggests that the
347 interaction between protein concentration and temperature is driving these effects
348 irrespective of carbohydrate levels.

Plasticity in cold tolerance (cold hardening capacity)

Plasticity in cold tolerance was significantly affected by selection diet, cold hardening treatment, and a three-way interaction between selection temperature, selection diet, and cold hardening treatment (Table 3, Figure 2). This means that the hardening response was dependent on the selection diet and selection temperature. Hardening induced an increase in cold tolerance in larvae from most selection treatments, but not for those selected on the low P:C diet at 18°C and 28°C and those selected on the standard diet at 25°C (Figure 2, Table S5). This means that diluted diet selected for increased plasticity in cold tolerance in general, low P:C diet selected for increased plasticity only at the optimal temperature, and standard diet (higher protein) selects for increased plasticity in cold tolerance only at non-optimal temperatures.

Basal heat tolerance

We then explored the effects of long-term adaptation to combined thermal-nutritional stress on basal heat tolerance. Basal heat tolerance was significantly affected by the selection temperature, but not selection diet (Table 4, Figure 3). The 25°C selection lines had higher basal heat tolerance than the 28°C, but not the 18°C, selection lines (Table S6). This suggests that long-term selection at higher temperatures does not necessarily lead to an increase in basal heat tolerance, contrary to previous studies (Cavicchi *et al.* 1995).

371 *Plasticity in heat tolerance (heat hardening capacity)*

372 Lastly, we explored the effects of long-term adaptation to combined thermal-nutritional
373 stress on plasticity in heat tolerance. We found a three-way interaction between
374 selection temperature, selection diet, and hardening treatment (Table 5, Table S7). This
375 means that plastic shifts in heat tolerance depended on both the selected diet and
376 selected temperature.

377 In contrast to the positive cold hardening response described above, where hardening
378 increased cold resistance in most selection lines (Figure 2), heat hardening resulted in
379 no change or a reduction in heat tolerance (Figure 3), and these effects varied across
380 selection diet and selection temperature. Lines selected at 25°C on low P:C diets and
381 at 28°C on standard diets who showed no significant response to hardening (Figure 3,
382 Table S7), however for all other lines heat hardening resulted in a significant reduction
383 in heat tolerance (Figure 3).

384

385 **Discussion**

386 Temperature and nutrition are amongst the most common stressors experienced by
387 animals in nature, and this will become increasingly so with ongoing climate change
388 (Rosenblatt and Schmitz 2016). However, assessments of climate risk have largely
389 focused on the ability to tolerate thermal extremes (Hoffmann *et al.* 2003; Bowler 2005;
390 Loeschcke and Sørensen 2005; Teets *et al.* 2020; Ma *et al.* 2021), rather than sensitivity
391 to combinations of stressors that organisms will increasingly experience in nature.
392 Genetic adaptation will play a key role in the long-term population persistence (Potvin

393 and Tousignant 1996; Gienapp *et al.* 2008; Lande 2009). However, we know very little
394 about the extent to which populations harbour the genetic variation needed to adapt to
395 combined thermal-nutritional stress, and whether such adaptation may be constrained
396 by trade-offs. We also have little insight into the consequences of adaptation to both
397 temperature and nutrition on the ability to shift phenotypes via plasticity, and whether
398 shifts in responses can be predicted by adaptation to single stressor alone. We
399 addressed these outstanding questions using lines of *D. melanogaster* whose larvae
400 evolved under combinations of three temperatures (18°C, 25°C, 28°C) and three diets
401 (standard, diluted, and low P:C) (Alton *et al.* 2024), and assessed the extent to which
402 adaptation to these stressors resulted in reduced viability or shifts in larval
403 thermotolerance and thermal plasticity.

404

405 *Temperature and nutrition interact to affect evolutionary shifts in pre-adult viability of D.*
406 *melanogaster under control conditions*

407 Previous studies have shown that populations harbour the genetic variation needed to
408 adapt to diet (Kolss *et al.* 2009; Kristensen *et al.* 2011) and temperature (Sørensen *et al.*
409 2001; Kimura 2004; Sgrò *et al.* 2010; Freda *et al.* 2019) individually and in combination
410 (Bochdanovits and De Jong 2003; Scharf, Braf, *et al.* 2015), which is consistent with the
411 current study. However, these previous studies provide mixed and context-dependent
412 evidence for trade-offs associated with adaptation. For example, adaptation to changes
413 in nutrition alone can result in reduced (Kristensen *et al.* 2011) or increased (Leftwich *et*
414 *al.* 2017) pre-adult viability regardless of the test diet, or an increase in viability only
415 when tested on the evolved diet (Kolss *et al.* 2009). Similarly, thermal evolution can

result in increased pre-adult survival only when tested at the temperature at which populations evolved (Partridge *et al.* 1994).

Such complexity is also evident when larvae are forced to adapt to both temperature and diet simultaneously. Specifically, Bochdanovits and De Jong (2003) found that adaptation to poor larval food in *D. melanogaster* resulted in increased viability when lines were selected at 27.5°C but decreased viability when lines were selected at 17.5°C. In contrast, we found that adaptation to low-protein diets (the diluted and low P:C diets) reduced larval viability compared to larvae evolved on standard food when selected at 28°C, whereas this pattern was largely reversed in lines evolving at 25°C. Our results suggest that simultaneous adaptation to nutritional stress and elevated temperature may negatively impact population growth, at least when reared under control (non-stress) conditions. Studies testing evolved shifts in viability and trade-offs under a range of conditions are needed to better understand the consequences for population growth.

Adaptation to temperature and nutrition in combination affects larval thermotolerance and thermal plasticity

Previous studies on adult thermal adaptation (Huey *et al.* 1991; Cavicchi *et al.* 1995; Bubliy and Loeschcke 2005) and dietary plasticity (Hazel 1995; Kostal and Simek 1998; Andersen *et al.* 2010; Colinet *et al.* 2013) suggest that adaptation to lower temperatures or a high-carbohydrate diet increases basal cold tolerance. Therefore, we predicted that lines exposed to 18°C on the low P:C diet (high carbohydrate) would evolve higher basal cold tolerance compared to lines selected at either 25°C or 28°C. Surprisingly,

lines selected on poor diets, both diluted and low P:C, evolved higher basal cold tolerance compared to lines selected on a standard diet, but only when they were selected at the non-optimal temperatures of 18°C and 28°C. This contradicts previous single nutritional stressor studies (Hazel 1995; Kostal and Simek 1998; Andersen *et al.* 2010; Colinet *et al.* 2013) that suggest a high-carbohydrate diet increases cold tolerance, except for Kolss et al. (2009), who, consistent with our findings, suggests that adaptation to poor (25% diluted) food at 25°C had no effect on basal cold tolerance. We found that cold tolerance may be enhanced when populations are forced to adapt to combinations of non-optimal thermal and low-protein nutritional conditions, as the diluted and low P:C diets were protein-matched. This further emphasises that evolutionary responses to combined stressors cannot be predicted by single-stressor studies alone. More studies are needed to better understand the effects of a broader range of temperature and nutritional contexts on basal cold tolerance and the underlying mechanisms.

While some studies suggest trade-offs between basal cold tolerance and cold plasticity (Bubliy and Loeschcke 2005; Nyamukondiwa *et al.* 2011; Noh *et al.* 2017), we found that most experimental evolution lines showed a positive cold hardening response—cold tolerance increased with hardening. This suggests that basal cold tolerance and plasticity in cold tolerance may evolve independently, depending on the environmental conditions to which populations are exposed.

Adaptation to elevated temperatures (Cavicchi *et al.* 1995; Bettencourt *et al.* 1999) and plastic responses to high-protein diets (Andersen *et al.* 2010) have been shown to increase adult heat tolerance. We therefore predicted that selection at 28°C on the

standard (high-protein) diet would increase larval heat tolerance. However, we found that larvae selected at 25°C had the highest average basal heat tolerance. This discrepancy between our results and previous studies (Andersen *et al.* 2010) may be due to the fact that previous studies focused on adult, not larval, thermotolerance, and insect life stages can differ in their thermal sensitivity (Kingsolver and Buckley 2020). For instance, thermal stress tolerance in *D. buzzatii* was either independent or weakly correlated across life stages (Sarup *et al.* 2006). Similarly, different life stages of *D. melanogaster* also vary in their basal heat tolerance (Freda *et al.* 2019; Moghadam *et al.* 2019), although the precise mechanisms controlling this variation remain unclear (Bowler and Terblanche 2008).

We also found that plasticity in heat tolerance became maladaptive after adaptation to most of the temperature-diet selection treatments; that is, the hardening treatment seemed to impose further heat stress on larvae and reduced, rather than increased, their survival and heat tolerance. Our results are partly consistent with previous work by Cavicchi *et al.* (1995), who found increased heat tolerance after hardening in lines adapted to 18°C and 25°C, while lines adapted to 28°C lost the ability to mount a hardening response. Additionally, the 28°C lines showed increased sensitivity to higher hardening temperatures (43°C), leading to a decrease in their hardened heat tolerance (Cavicchi *et al.* 1995). In contrast, we found that none of the evolved lines showed a significant positive hardening response; all significant hardening responses reduced larval heat tolerance.

Our results could be explained by plasticity in heat tolerance reaching a physiological limit. We found very weak evidence for evolved shifts in basal heat tolerance,

suggesting that the starting population may have had limited genetic variation for larval heat tolerance (Loeschcke and Krebs 1996; Lansing *et al.* 2000) and its ability to evolve plasticity. Limited responses to selection on larval heat tolerance also suggest that a trade-off between basal and hardened plasticity (Gunderson and Stillman 2015; van Heerwaarden *et al.* 2016; van Heerwaarden and Kellermann 2020) is unlikely to explain our findings.

Another explanation could be that the threshold for inducing a hardening response might have shifted down as a consequence of long-term adaptation to combined thermal-nutritional stress in the present study (Cavicchi *et al.* 1995; van Heerwaarden *et al.* 2024). Cavicchi *et al.* (1995) found that lines adapted to 18°C had a lower threshold for inducing a hardening response compared to those adapted to 25°C. In contrast, lines adapted to 28°C appeared to lose the capacity to mount a hardening response at lower hardening temperatures and exhibited a negative hardening response at the highest temperature. This is in part consistent with our results.

Plasticity of larval heat tolerance has been shown to vary across assay temperature and duration of exposure, and is species-specific (Chen *et al.* 1991; Moghadam *et al.* 2019; van Heerwaarden *et al.* 2024). Chen *et al.* (1991) demonstrates that the duration of hardening treatment significantly affected adult emergence of *Sarcophaga crassipalpis* after a heat shock; adult emergence was maximised with 2 hours of hardening at 40°C, but only achieved 50% with a 1-hour hardening treatment. Moghadam et al., (2019) further shows that heat hardening capacity depends on the temperature of acute heat stress. Hardening treatments had a negative effect on subsequent heat tolerance in 3rd instar larvae of *D. melanogaster* when the acute stress temperature exceeded 40°C,

but not when it was below 40°C. We only used a single acute heat stress temperature (39°C) to assess heat tolerance with and without hardening, but it is possible that a positive hardening response may have been observed if we had used a lower acute stress temperature. Our choice of temperature and duration for hardening and acute heat stress treatments might have been too stressful for the selected larvae. The pilot study (see supplementary materials) showed positive hardening responses for cold (Figure S2) and heat tolerance (Figure S3, albeit the effect was small). However, we only used larvae selected on the standard food in the pilot study.

In conclusion, we found that long-term adaptation to low protein diets and elevated temperature simultaneously came at the cost of reduced larval viability, at least under control conditions. Further, we observed unpredictable shifts in larval basal thermotolerance and its plasticity as a result of adaptation to combined thermal-nutritional stress, which could not be predicted from studies of thermal or nutritional adaptation individually. We found that basal cold tolerance and plasticity in cold tolerance may evolve independently. However, the evolved lines seemed to lose the ability to increase heat tolerance via positive heat hardening responses, and largely displayed reductions in heat tolerance following hardening. Taken together, our results suggest that being forced to adapt to shifts in both the thermal and nutritional environments simultaneously may increase the vulnerability of ectotherms to ongoing climate change. Future studies assessing evolutionary responses of thermotolerance and its plasticity to multiple stressors should explore a broader range of temperatures, nutritional contexts and hardening treatments/durations to better understand the complexity of adaptation to environmental change.

Results – table and graphs

Table 1. Results of analysis of variance testing for effects of selection temperature, selection diet, and their interaction on larval-adult viability under control (no stress) conditions. Significance is defined as a p-value less than 0.05.

Response: Larval-adult viability	**Chi-square**	**Df**	**P-value**
selection temperature	1.540	2	0.463
selection diet	0.335	2	0.846
selection temperature: selection diet	15.989	4	0.003

Table 2. Results of analysis of variance testing for effects of selection temperature, selection diet, and their interaction on larval basal (non-hardened) cold tolerance. Significance is defined as a p-value less than 0.05.

Response: Larval-adult viability	**Chi-square**	**Df**	**P-value**
selection temperature	1.125	2	0.121
selection diet	12.367	2	0.002
selection temperature: selection diet	11.922	4	0.018

Table 3. Results of analysis of variance testing for effects of selection temperature, selection diet, cold hardening treatment, and their interaction on larval cold tolerance. Significance is defined as a p-value less than 0.05.

Response: Larval-adult viability	Chi-square	Df	P-value
selection temperature	4.677	2	0.096
selection diet	15.226	2	<0.001
hardening treatment	70.606	1	<0.001
selection temperature: selection diet	6.969	4	0.138
selection temperature: hardening treatment	1.948	2	0.378
selection diet: hardening treatment	1.498	2	0.473
selection temperature: selection diet: hardening treatment	16.389	4	0.003

Table 4. Results of analysis of variance testing for effects of selection temperature, selection diet, and their interaction on larval basal (non-hardened) larval heat tolerance. Significance is defined as a p-value less than 0.05.

Response: Larval-adult viability	Chi-square	Df	P-value
selection temperature	6.142	2	0.046
selection diet	2.038	2	0.361
selection temperature: selection diet	1.079	4	0.898

Table 5. Results of analysis of variance testing for effects of selection temperature, selection diet, heat hardening treatment, and their interaction on larval heat tolerance. Significance is defined as a p-value less than 0.05.

Response: Larval-adult viability	Chi-square	Df	P-value
selection temperature	7.072	2	0.029
selection diet	2.557	2	0.278

hardening treatment	25.277	1	<0.001
selection temperature: selection diet	31.516	4	<0.001
selection temperature: hardening treatment	1.824	2	0.402
selection diet: hardening treatment	9.289	2	0.010
selection temperature: selection diet: hardening treatment	21.281	4	<0.001

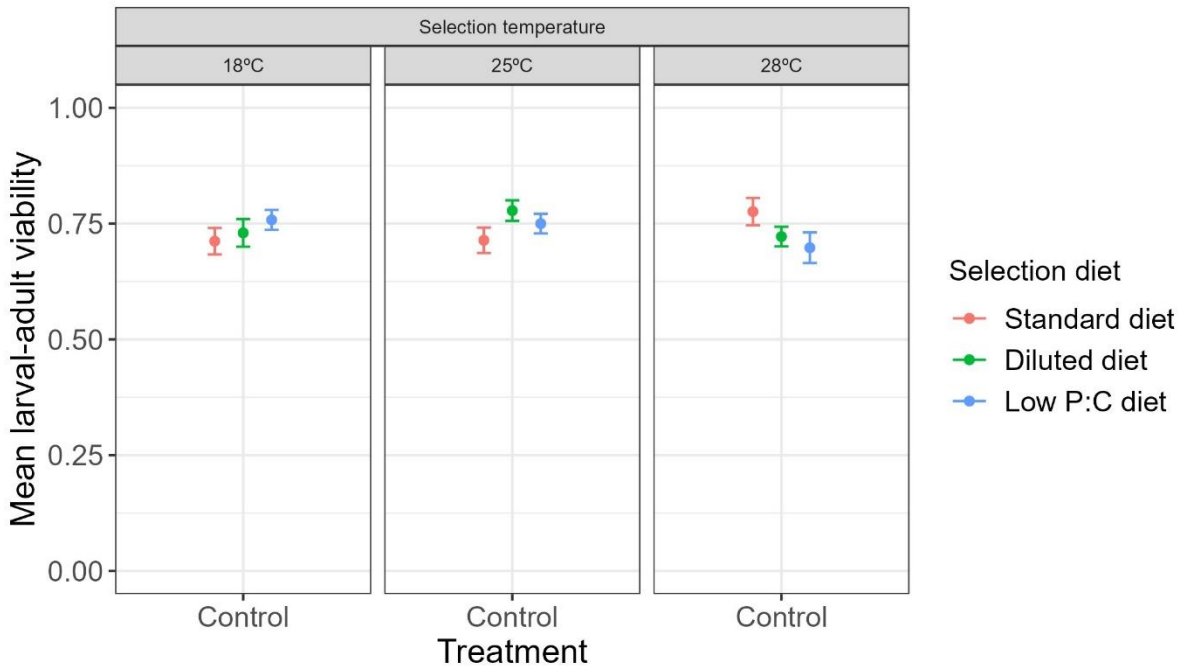

Figure 1. Mean larval-adult viability of experimental evolution lines tested under control treatments. Control treatment involved larvae developing on standard (control) food at 25°C under 12:12 light: dark cycles until adult eclosion. Error bars indicate ± 1 standard error.

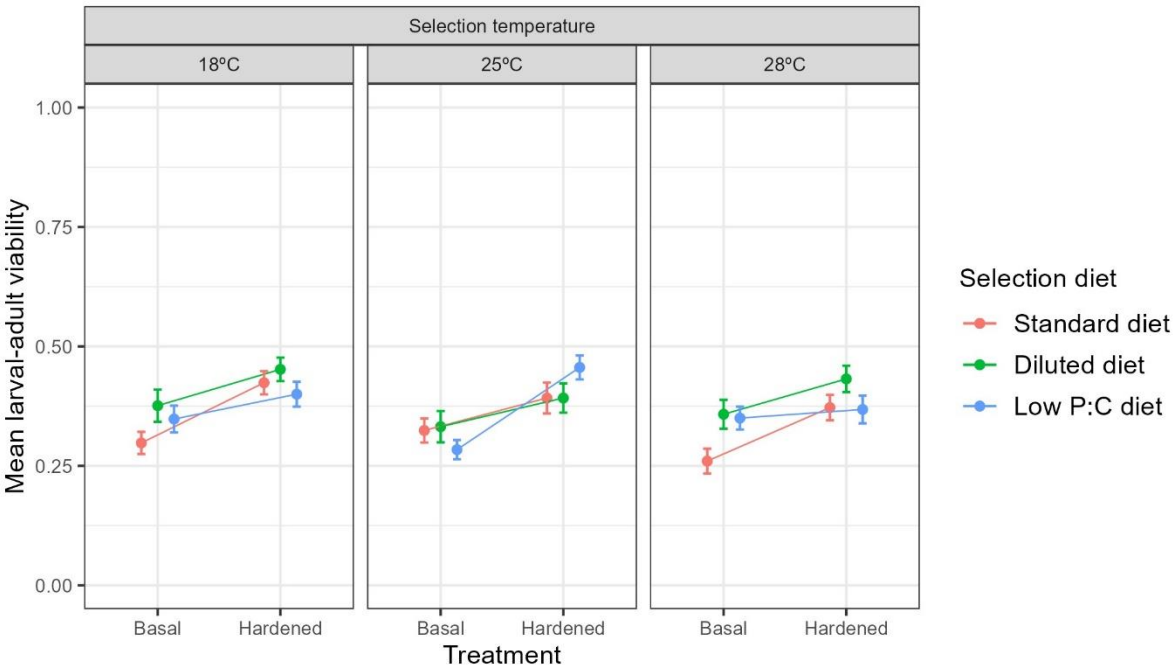

Figure 2. Mean larval-adult viability of basal (non-hardened) and hardened (2 h at 0°C) experimental evolution lines after exposure to an acute cold shock treatment of 4 h at 0°C. Error bars indicate ± 1 standard error.

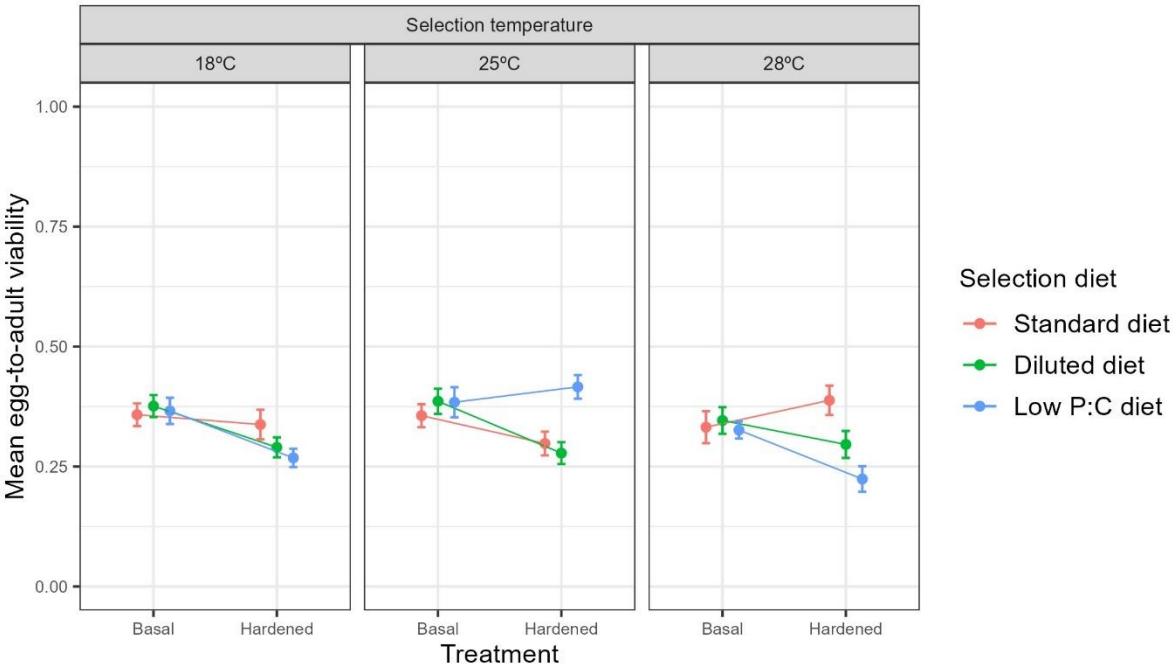

Figure 3. Mean larval-adult viability of basal (non-hardened) and hardened (1 h at 35°C) experimental evolution lines after exposure to an acute heat shock treatment of 0.5 h at 39°C. Error bars indicate ± 1 standard error.

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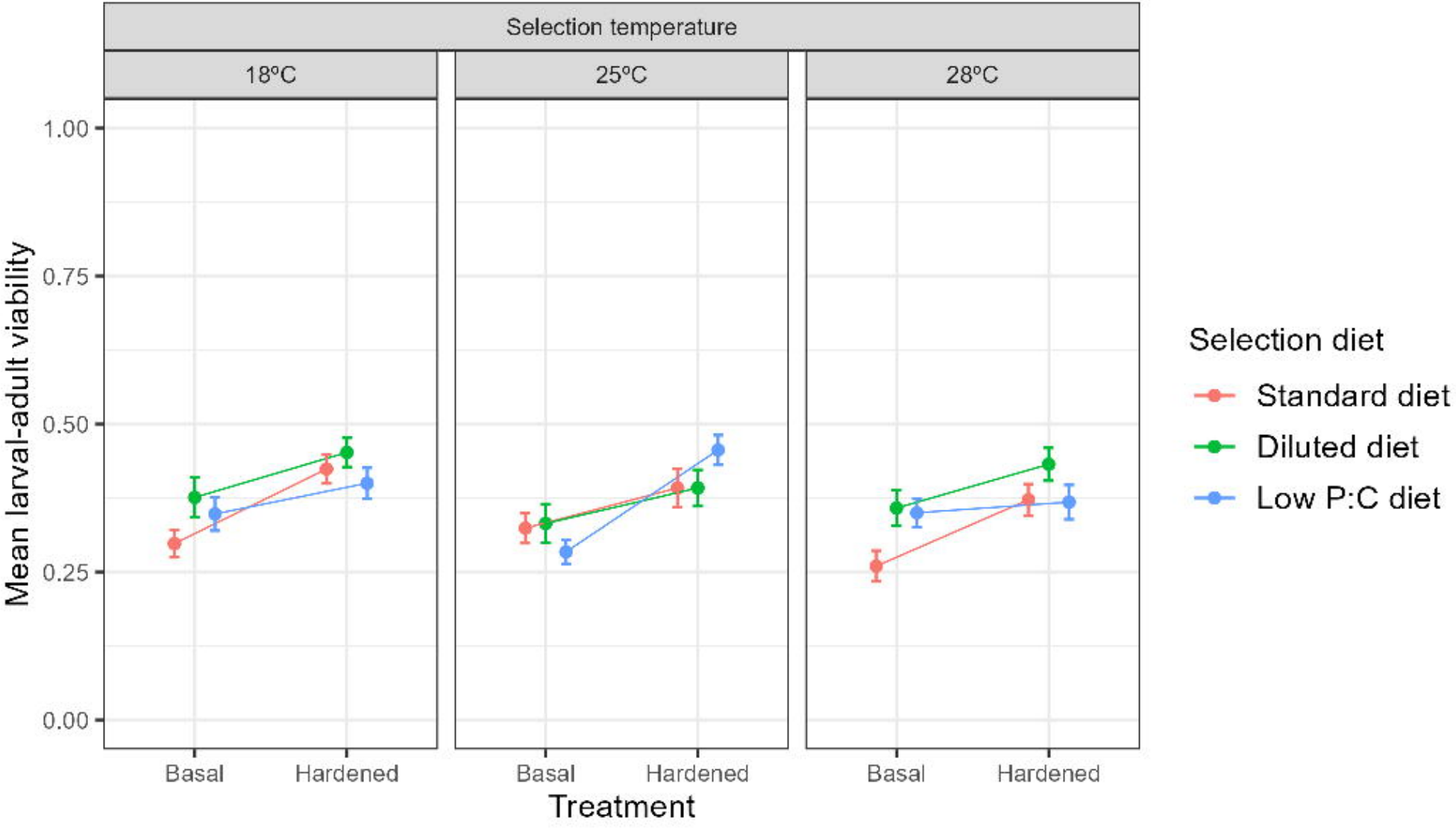
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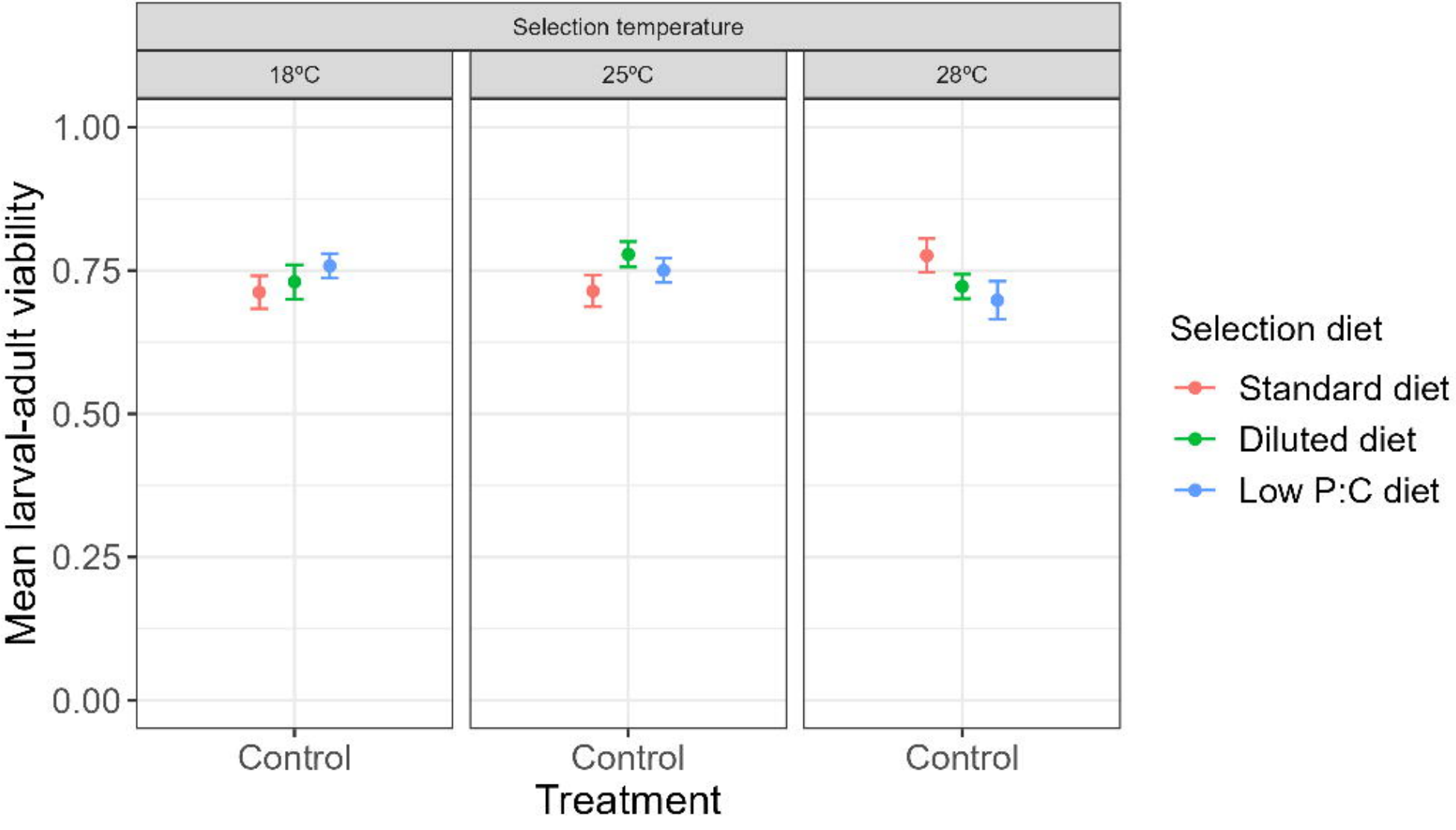
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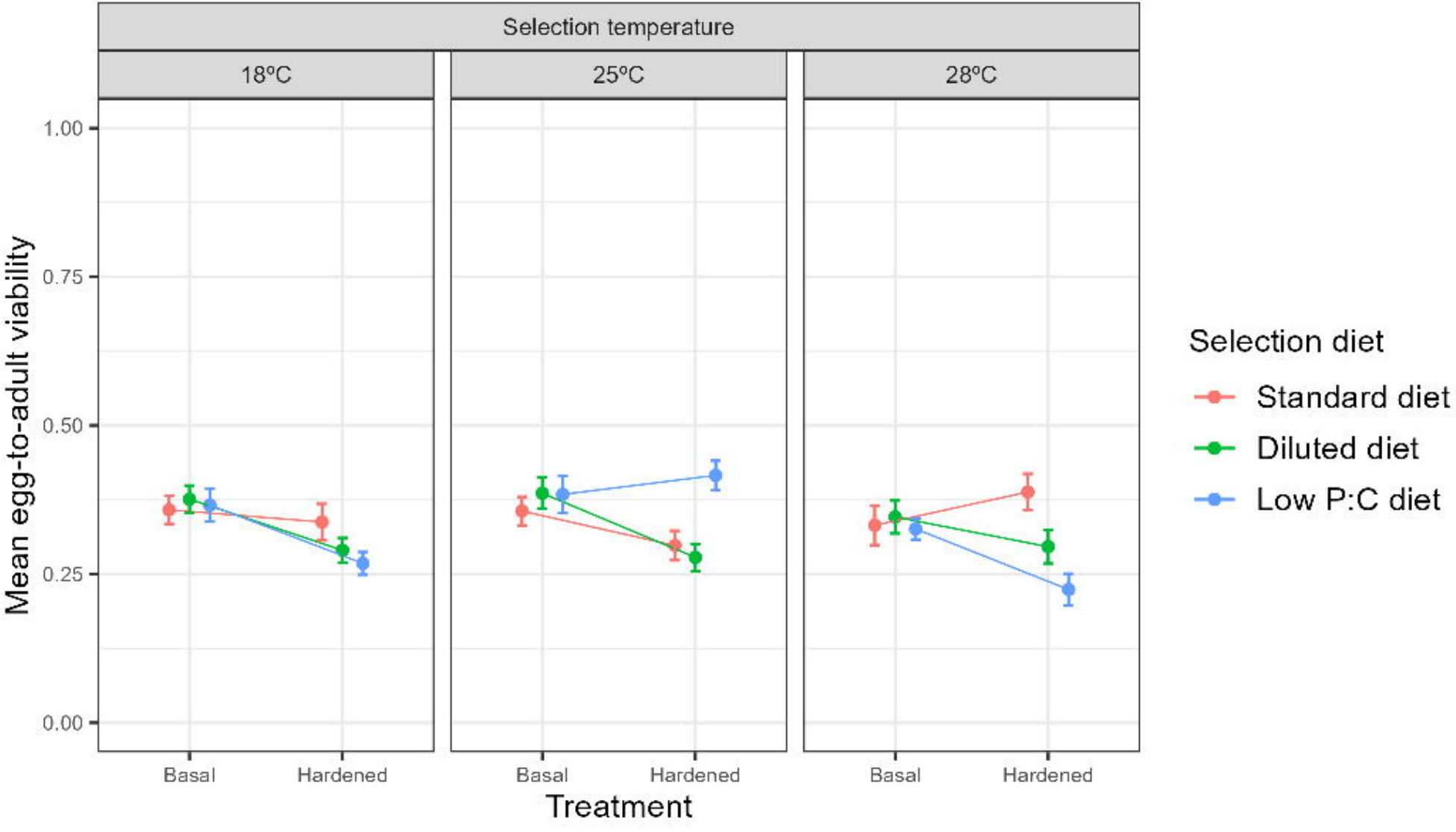
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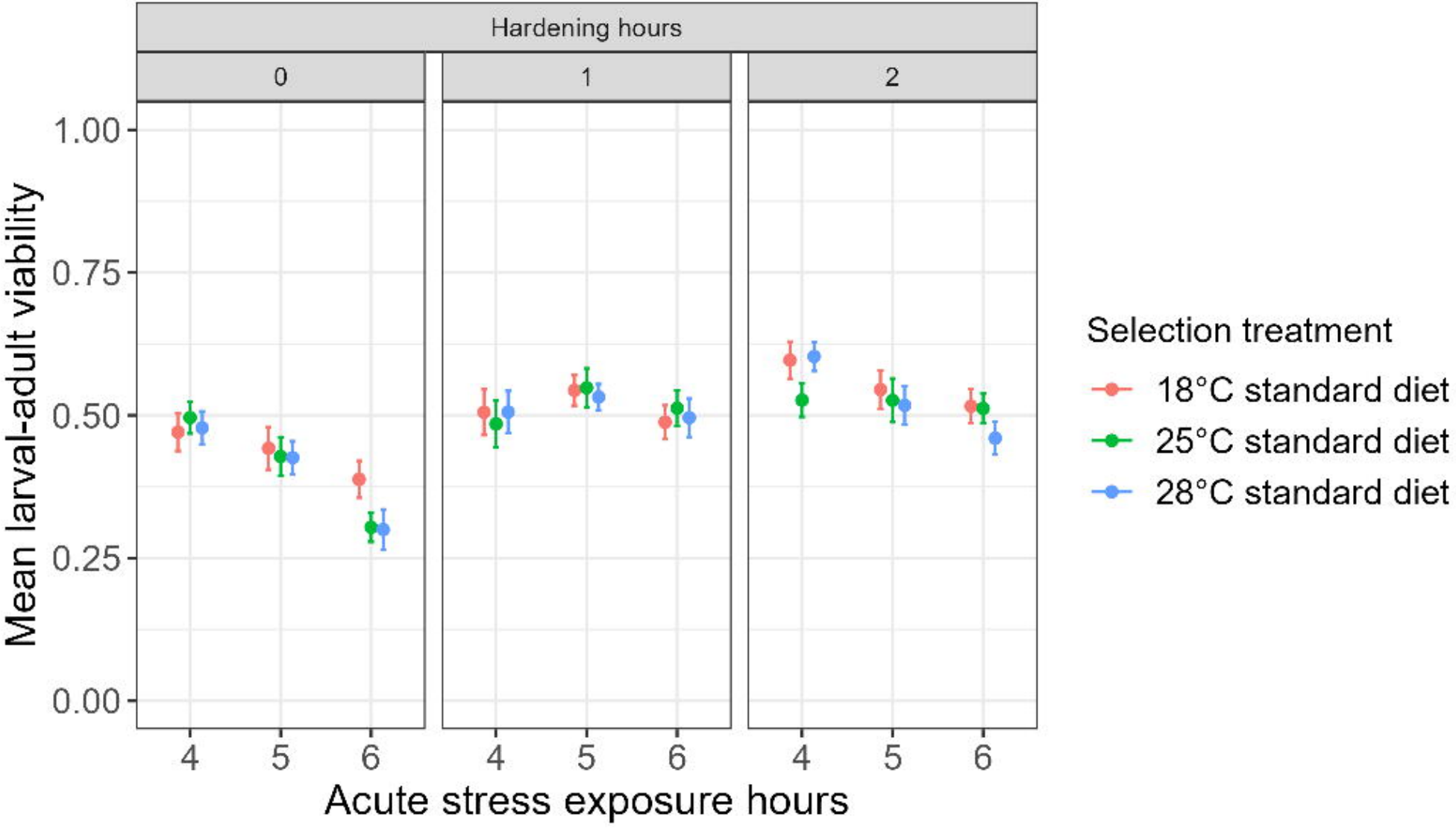
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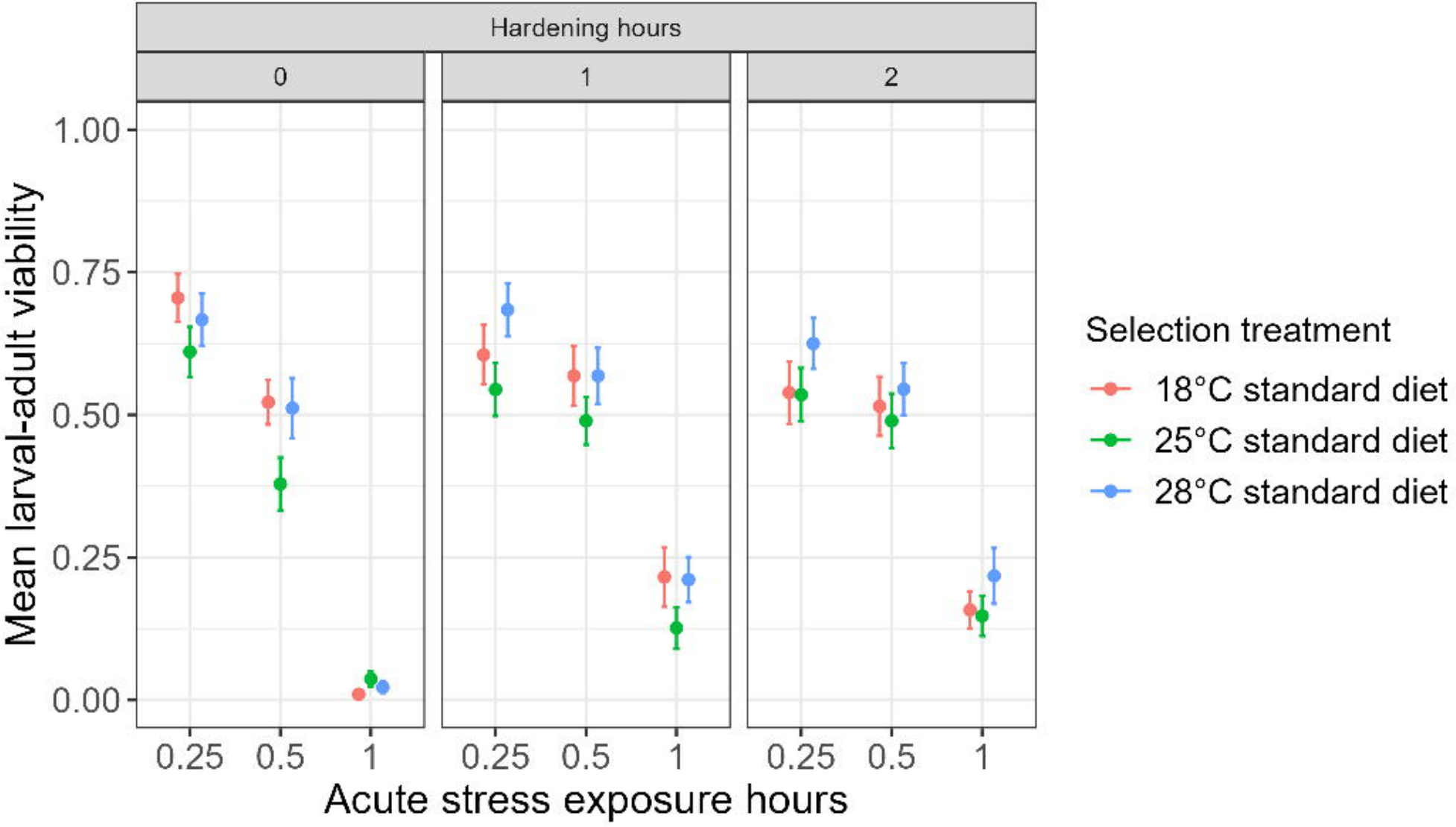
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Acute stress exposure hours
Selection treatment
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