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1 Paradoxical acclimation responses in the thermal performance of insect immunity

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20 *This work is part of Ferguson’s PhD, integrating ecophysiology, thermal biology, and*
21 *ecoimmunology. We measured insect immune performance across temperatures, finding that*
22 *thermal performance does not consistently respond to acclimation among, or even within,*
23 *physiological systems. Paradoxically, cold acclimation decreases low temperature immune*
24 *performance, revealing that cold tolerance trades off with immunity in the cold. Thus,*
25 *physiological systems differ in their responses to temperature, and conclusions about the impacts*
26 *of climate change cannot be based on a single performance measure.*

27

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29 Author Contributions: LVF and BJS conceived of the study and wrote the manuscript; LVF
30 carried out the design, lab work and statistical analysis; DEH participated in the design of the
31 study and drafting of the manuscript. All authors gave final approval for publication.

32 Abstract

33

34 Winter is accompanied by multiple stressors, and the interactions between cold and pathogen
35 stress potentially determine the overwintering success of insects. Thus, it is necessary to explore
36 the thermal performance of the insect immune system. We cold-acclimated spring field crickets,
37 *Gryllus veletis*, to 6°C for 7d and measured the thermal performance of potential (lysozyme and
38 phenoloxidase activity) and realised (bacterial clearance and melanisation) immune responses.
39 Cold acclimation decreased the critical thermal minimum from $-0.5 \pm 0.25^\circ\text{C}$ to $-2.1 \pm 0.18^\circ\text{C}$,
40 and chill coma recovery time after 72 h at -2°C from 16.8 ± 4.9 min to 5.2 ± 2.0 min. Measures
41 of both potential and realised immunity followed a typical thermal performance curve,
42 decreasing with decreasing temperature. However, cold acclimation further decreased realised
43 immunity at low, but not high, temperatures; effectively, activity became paradoxically
44 specialised to higher temperatures. Thus, cold acclimation induced mismatched thermal
45 responses between locomotor and immune systems, as well as within the immune system itself.
46 We conclude that cold acclimation in insects appears to preferentially improve cold tolerance
47 over whole-animal immune performance at low temperatures, and that the differential thermal
48 performance of physiological responses to multiple pressures must be considered when
49 predicting ectotherms' response to climate change.

50

51 Key words: cold, pathogen, thermal performance curve, biotic stressor, plasticity

52 Introduction

53 Ectotherms can respond to seasonal changes by maintaining homeostasis via phenotypic
54 or developmental plasticity. In insects, the cues that govern acclimation (in the laboratory) or
55 acclimatisation (in the field), such as temperature or photoperiod, often coordinate diverse
56 physiological adjustments to suit a new set of environmental conditions (Harrison et al. 2012).
57 For example, cold-acclimation of the beetle *Dendroides canadensis* elicits antifreeze protein
58 synthesis, removal of ice nucleators (Olsen and Duman 1997), and modification of epicuticular
59 waxes (Olsen et al. 1998) - all of which contribute to improved cold tolerance. In addition to
60 cold, there are multiple abiotic and biotic stressors associated with winter (Williams et al. 2015),
61 and these can select for thermal plasticity in multiple physiological systems. For instance, cold-
62 acclimation often increases desiccation resistance in insects, likely because of the high water
63 vapour deficits experienced during overwintering (Sinclair et al. 2013; Terblanche et al. 2005).
64 Biotic stressors, such as pathogens, are also likely to affect the success of insects at low
65 temperatures (Hokkanen 1992; Riedel and Steenberg 1998; Webberley and Hurst 2002; Williams
66 et al. 2015), yet the ability of insects to manage cold-related pathogen stress, and the role of
67 phenotypic plasticity in this response, is unclear.

68 Some insect pathogens are cold-active [e.g. fungi in the genera *Beauveria* and
69 *Metarhizium* (Fernandes et al. 2008)], or have increased virulence at low temperatures [e.g. the
70 bacterium *Yersinia enterocolitica* (Bresolin et al. 2006)]; thus there is capacity for these cold-
71 adapted pathogens to contribute to mortality of insects at low temperatures (Hokkanen 1992;
72 Steenberg et al. 1995). However, cold exposure appears to upregulate the insect immune system,
73 which may allow insects to respond to cold-associated pathogen stress (Sinclair et al. 2013). For
74 example, cold exposure increases fungal resistance in *Pyrrharctia isabella* caterpillars (Marshall

75 and Sinclair 2011) and adult *Drosophila melanogaster* (Le Bourg et al. 2009), and upregulates
76 genes encoding antimicrobial peptides in both *D. melanogaster* (Zhang et al. 2011) and the
77 solitary bee *Megachile rotundata* (Xu and James 2012). Further, this increased immunity appears
78 to translate into fitness: water striders (*Aquarius najas*) with stronger immune responses have
79 higher overwinter survival (Krams et al. 2011). Although cold-induced upregulation of immunity
80 may be a non-adaptive by-product of responses to cold (Fedorka et al. 2013; Sinclair et al. 2013),
81 the potential for conflicts between the energetic costs of immune responses (Ardia et al. 2012;
82 Freitak et al. 2003; Schmid-Hempel 2003) and energy conservation in the cold (Sinclair, in press)
83 instead suggest that the immune system is upregulated as an adaptive response to low-
84 temperature pathogen pressure (Irwin and Lee 2003; Sinclair, in press ; Williams et al. 2012).
85 However, most studies have been performed upon re-warming from cold exposure, and do not
86 necessarily reflect immune activity at low temperatures. Thus, to determine the ability of insects
87 to combat low-temperature pathogen stress, we must first explore the low-temperature
88 performance of the immune system, as well as the role of acclimation in shaping this
89 performance.

90 We expect the insect immune system to have reduced performance in the cold because it
91 relies on cellular and enzymatic processes that are likely temperature-sensitive (Collazos et al.
92 1994; Le Morvan et al. 1998; Marnila et al. 1995; Somero 1995). Indeed, phagocytosis and
93 encapsulation decrease at 4 °C in diapausing pupae of the giant silk moth, *Samia cynthia pryeri*,
94 although some immune function is maintained (Nakamura et al. 2011). However, this loss of
95 performance has the potential to be mitigated through phenotypic plasticity, including the
96 expression of cold-active isoforms of immune-related enzymes, or an increase in the
97 concentration of cells and molecules necessary for an immune response (Angilletta 2009;

98 Somero 1995). In either case, if there is an adaptive advantage to improved immunity at low
99 temperatures, then cold-acclimation would be expected to modify the thermal performance of the
100 immune system to increase activity at low temperatures (Angilletta 2009).

101 Thus, to understand the potential interactions of cold and immune stress in insects, and to
102 understand the role of biotic interactions in shaping ectotherm performance in a changing
103 climate, it is necessary to explore the thermal biology of the insect immune system. We explored
104 the thermal sensitivity and plasticity of the insect immune system by measuring the thermal
105 performance of immune-related enzymes *in vitro*, and immune responses *in vivo* [i.e. potential
106 immunity and realised immunity, respectively (Fedorka et al. 2007; Gershman 2008)] in warm-
107 and cold-acclimated spring field crickets (*Gryllus veletis*). We used a short-term acclimation to
108 explore the possibility of thermal plasticity in the immune system, as a first step in understanding
109 how the thermal biology of the immune system might impact the ecology of overwintering
110 insects. Cold acclimation differentially affected realised and potential immunity, in a direction
111 that is not predicted by the whole-organism response to cold acclimation. We suggest that
112 pathogen stress may be most prevalent upon re-warming from cold, and conclude that divergent
113 thermal performance of distinct stress responses must be considered when predicting ectotherms'
114 responses to climate change.

115

116 Material and Methods

117 We studied the thermal biology of the immune system in the spring field cricket, *Gryllus*
118 *veletis*. *Gryllus veletis* overwinters as a late-instar nymph in temperate North America
119 (Alexander 1968), and has a cold acclimation response (Coello Alvarado et al. 2015). Our
120 cricket colony was derived from a population collected in Lethbridge, Alberta, in 2010, and were

121 reared from egg to nymph at 25 °C (14 L:10 D) as described by Coello Alvarado et al. (2015).
122 We haphazardly assigned female 6th instar nymphs (the overwintering stage) into individual 180
123 mL clear plastic cups and provided *ad libitum* rabbit chow (Little Friends Rabbit Food, Martin
124 Mills, Elmira, ON, Canada) and water, with cardboard shelters. We cold-acclimated (CA)
125 individuals at 6 °C on a short light cycle (10 L: 14 D) or maintained them at rearing conditions
126 (warm-acclimated, WA) for 7 d.

127

128 **Thermal limits of locomotor activity**

129 We measured the critical thermal minimum (CT_{min} , the temperature at which an insect
130 enters chill coma) following MacMillan and Sinclair (2011), and chill-coma recovery (CCR) time
131 following MacMillan et al. (2012). Briefly, we cooled crickets at 0.25 °C/min from 22 °C to the
132 temperature at which movement ceased. Similarly, we measured the critical thermal maximum
133 (CT_{max}) by increasing temperature at 0.25 °C/min from 22 °C until we visually observed the
134 onset of spasms (Lutterschmidt and Hutchison 1997). For CCR, we cooled crickets at 0.25
135 °C/min from 22 °C to -2 °C and held them at -2 °C for 72 h. Crickets were returned to room
136 temperature and we recorded the time taken to achieve a coordinated righting response.

137

138 **Potential Immune Response**

139 We collected haemolymph for measuring potential humoral immunity following Adamo
140 (2004), and all measurements of potential immunity at different temperatures were tested on
141 extracted hemolymph. We pierced the membrane under the pronotum and collected 2 µL of
142 haemolymph with a micropipette. We mixed 2 µL of haemolymph with either 2 µL of
143 anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 6.8;

144 for lysozyme activity) or 50 μ L of phosphate-buffered saline [PBS; for phenoloxidase (PO)
145 activity] and snap-froze it in liquid nitrogen, followed by storage at -80 $^{\circ}$ C.

146 To estimate the bactericidal activity of lysozyme, we followed Vilcinskas and Matha
147 (1997), with some modifications. We added 4 μ L of the haemolymph-anticoagulant-buffer
148 suspension to 2 mm diameter wells on a petri plate containing *Micrococcus lysodeikticus* (*luteus*)
149 agar (1 % agar; 67 mM potassium phosphate, pH 6.4, 0.1 mg/mL streptomycin sulfate; 5 mg/ml
150 *M. lysodeikticus*). Plates were sealed with Parafilm[®] and incubated at one of 0, 6, 12, 18, 25 or 30
151 $^{\circ}$ C (MIR-153 incubators, Sanyo Scientific, Bensenville, IL, US). We then measured the area of
152 the cleared region around each well 24 and 48 h later using NIS Elements Imaging Software
153 (Nikon Instruments Inc, Melville, NY, USA).

154 Total PO activity, which indicates a potential broad-spectrum immune response, was
155 measured spectrophotometrically (Adamo 2004). Briefly, we mixed 25 μ L of thawed
156 hemolymph/PBS mixture with 70 μ L alpha-chymotrypsin (1.3 mg/ mL in PBS) and incubated it
157 for 20 min at room temperature (22 $^{\circ}$ C) before adding it to 0.9 mL of L-DOPA (4 mg/mL in
158 PBS). The rate of increase in absorbance was measured over 60 min at one of 6, 12, 18, 25, or 30
159 $^{\circ}$ C (Carey 100 Spectrophotometer with Peltier-effect Temperature Controller, Agilent, Santa
160 Clara, CA, USA).

161 Higher haemolymph protein concentration is linked to stronger immune responses in
162 insects, especially the melanisation response (Adamo 2004). To measure haemolymph protein
163 concentration, we followed methods as described by Adamo (2004) with some modifications.
164 Briefly, we used 10 μ L of the haemolymph/PBS mixture in a Bicinchoninic Acid kit (BCA; Life
165 Technologies, Carlsbad, CA, USA) and measured absorbance at 562 nm in a microplate
166 spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). We then converted

167 absorbance to concentration values using a standard curve created from bovine serum albumin.

168

169 **Realised Immune Response**

170 As an estimate of a broad-spectrum, realised immune response, we measured
171 melanisation by inserting a 2 mm piece of nylon filament (Krams et al. 2011) under the
172 pronotum and placing crickets at 0, 6, 12, 18, 25 or 30 °C for 12 h. We removed the filament
173 after 12 h, photographed it from two different angles at 30× magnification using a Nikon DSFI1
174 camera (Nikon Instruments Inc. Melville, NY, USA) attached to a stereomicroscope, and
175 determined the darkness of each filament using the average grey value calculated in ImageJ
176 (Rasband 1997-2014). We calculated relative melanisation as 255 - the grey value, such that a
177 higher number indicates more melanisation.

178 We measured the *in vivo* ability of crickets to clear *Staphylococcus aureus* (strain
179 Newman with chromosomally-encoded tetracycline resistance) from haemolymph following
180 (Haine et al. 2008). Briefly, we diluted *S. aureus* (grown overnight at 37 °C in tryptic soy broth)
181 to 1×10^9 CFU/mL in PBS and injected 2 µL of suspension into the thorax under the pronotum
182 (Adamo 2004) via a 30 G needle. Following 24 h post-challenge at 0.5, 4, 12, 18, 25, or 30 °C,
183 we homogenised whole crickets in 900 µL of PBS to ensure that we captured all remaining
184 bacteria (including those associated with tissue). We diluted and spotted of the homogenate on
185 tryptic soy agar (TSA) containing 2 µg/mL tetracycline and averaged the number of CFU over
186 four replicate spots, following 24 h at 37 °C. We homogenised a subset of crickets 1 min
187 following injection to obtain the true number of CFU injected and calculate percent clearance
188 (Haine et al. 2008). Control crickets injected with sterile PBS did not demonstrate any bacterial
189 growth on TSA containing tetracycline.

190

191 **Statistical analyses**

192 All analyses were performed in R v3.1.2 (Team 2010) and preliminary data exploration was
193 conducted according to (Zuur et al. 2010). We compared CT_{min} , CT_{max} , CCR, and protein
194 concentration between CA and WA crickets using Welch's two-sample t-test. We compared the
195 performance curves of immune activity between WA and CA crickets using ANOVA (Angilletta
196 2006). Where necessary, response variables were square-root- (lysozyme, phenoloxidase),
197 arcsine- (bacterial clearance), or log-transformed (melanisation) to satisfy the assumptions of the
198 ANOVA. We assessed the assumptions of ANOVA by plotting residuals against fitted values to
199 confirm homogeneity of variance, and standardised residuals against theoretical quantiles to
200 assess normality (Crawley 2007). We used polynomial contrasts (Lenth 2013) to compare means
201 between warm and cold-acclimated crickets at each temperature.

202

203 **3. Results**

204

205 **Thermal limits of locomotor activity**

206 Cold-acclimation enhanced low temperature locomotor activity of *G. veletis* and
207 shortened recovery time after cold exposure. The CT_{min} of WA crickets was significantly higher
208 than that of CA crickets ($t_{14,46} = 5.53$, $p < 0.001$; Fig. 1A); however, there was no difference
209 between the CT_{max} of WA and CA crickets (Fig. 1B; $t_5 = 0.11$, $p = 0.45$). Chill comarecovery
210 time was lower in CA crickets than WA crickets (Fig. 1C; $t_{5,34} = 2.19$, $p = 0.04$).

211

212 **Potential Immune Response**

213 Potential humoral immunity was sensitive to temperature but remained unaffected by acclimation

214 (Table 1). Specifically, both lysozyme and phenoloxidase activities decreased with decreasing
215 temperature in both WA and CA crickets (Fig. 2); however, there was no overall difference in the
216 activity of either enzyme in WA compared to CA crickets (Table 1). Haemolymph protein
217 concentration of CA and WA crickets did not differ significantly ($t_{22} = 0.59$, $p = 0.28$).

218

219 **Realised Immune Response**

220 Temperature also significantly affected realised immunity, including both bacterial
221 clearance and melanisation (Table 1). In addition, and in contrast to potential immunity,
222 acclimation had a significant effect on realised immunity (Table 1). Specifically, melanisation
223 and bacterial clearance were decreased in CA crickets at low temperatures, but largely
224 unchanged at warm temperatures (Fig. 3).

225

226 **Discussion**

227 We explored the capacity for cold-acclimation of the immune system in *G. veletis* using
228 an acclimation regime that improved locomotor activity at low temperatures [decreased CT_{min}
229 and CCR, recognised proxies for cold tolerance in insects (Andersen et al. 2015)], but had no
230 effect on heat tolerance (CT_{max}). Cold acclimation did not affect potential immunity nor realised
231 immunity at higher temperatures; however, realised immunity decreased in the cold in CA
232 crickets. We suggest that decreased activity in the cold may result from trade-off between some
233 components of immune activity and other physiological responses initiated by cold acclimation.

234 Theory suggests three ways by which the thermal performance of immunity could shift in
235 response to acclimation, if increased low temperature performance were important in the cold: 1)
236 a shift in the thermal performance curve (TPC) where T_{opt} decreases (i.e. beneficial acclimation;

237 Fig. 4A); 2) a shift in the TPC where T_{opt} is unchanged but maximal activity is higher across all
238 temperatures (i.e. colder is better; Fig. 4B); or 3) a reduction in thermal sensitivity, whereby the
239 TPC encompasses a larger range of activity, but maximal activity at the T_{opt} is lower [i.e.
240 generalist vs. specialist; Fig. 4C (Angilletta 2009)]. However, we found that cold-acclimation
241 resulted in a paradoxical narrowing of the TPC of realised immune responses in crickets,
242 whereby activity was specialised to higher instead of lower temperatures, and the T_{opt} and
243 maximal activity at the T_{opt} were unaffected (Fig. 3, 4D). Decreased performance in the cold may
244 result from trade-offs between the increased energy demands of improving cold tolerance (e.g.
245 production of cryoprotectants) and the immune system (Sinclair, in press). For example,
246 infection decreases CCR time in *Drosophila melanogaster* (Linderman et al. 2012), suggesting
247 that immune activity conflicts with cold tolerance. Thus, cold acclimation may preferentially
248 improve cold tolerance over whole-animal immune performance at low temperatures.

249 In addition to a decrease in realised immunity, cold acclimation produced mismatches
250 between potential and realised immunity. First, acclimation appeared only to decrease realised
251 immune responses in the cold, while potential immunity remained unchanged. Realised immune
252 responses, such as bacterial clearance, are generally mediated by the combined activity of
253 haemocytes, enzymes, and antimicrobial peptides (Gillespie and Kanost 1997), while the
254 potential immunity that we measured focused on the activity enzymes in isolation. Decreased
255 realised immune responses that are not paralleled in potential responses suggest that cold
256 acclimation has a stronger effect on cellular activity than it does on the activity of enzymes or
257 antimicrobial peptides, although we caution that we did not measure all components of the
258 immune system. Differences in potential and realised immune activity can create a disparate
259 estimate of overall immunocompetence (Fedorka et al. 2007), yet also hint at the relative

260 contributions of different immune components to protection against pathogens. In this case,
261 overall immunocompetence may decrease in the cold (realised immunity), yet a basal level of
262 protection may persist through the activity of enzymes and antimicrobial peptides (potential
263 immunity). We suggest that measuring multiple components of the immune system provides a
264 more comprehensive picture of the effects of thermal acclimation on immune performance, and
265 that both potential and realised responses should be considered when assessing the impact of the
266 abiotic environment on immunity.

267 Second, although PO activity and melanisation are linked as an immune response
268 (González-Santoyo and Córdoba-Aguilar 2012) their thermal optima were disparate; PO activity
269 peaked at 25 °C, whereas melanisation peaked at 18 °C, in both CA and WA crickets [similar to
270 phagocytic capacity in mosquitoes (Murdock et al. 2012)]. The lower T_{opt} of melanisation
271 compared to that of PO activity appears to reflect a disconnect between the T_{opt} , or thermal
272 sensitivity, of different components of the overall melanisation response. Temperature is likely to
273 drive the local adaptation of hosts and pathogens (Sternberg and Thomas 2014) and may have
274 driven the selection of *G. veletis* immune performance to a thermal optimum lower than enzyme
275 activity would predict. Thus, using thermal performance curves, we may gain insight into the
276 evolution of thermal sensitivity and plasticity of immune activity, and can begin to predict the
277 capacity for hosts to respond to pathogens under climate change scenarios.

278 Pathogen growth generally increases as temperatures increase (Harvell et al. 2002); thus,
279 re-warming from cold exposure is likely to lead to an increase in pathogen pressure, and require
280 an increase in immune activity. Despite decreased immune activity in the cold, realised immune
281 activity in CA crickets was maintained at optimal temperatures, which suggests that immune
282 activity may be required following, but not during, cold exposure. Seasonal immune activity in

283 other ectotherms, including fish [e.g. *Sparus aurata* (Tort et al. 1998)] and frogs [e.g. *Rana*
284 *pipiens* (Maniero and Carey 1997)], follows a pattern that reflects the effects of a short-term
285 acclimation on immune activity in crickets; specifically, immune activity decreases during the
286 winter but rapidly recovers, and even increases beyond control levels, upon re-warming. In
287 hibernators, such as the golden-mantled ground squirrel (*Spermophilus lateralis*), interbout
288 euthermia is accompanied by an increase in immune activity, thereby allowing the animal to
289 combat pathogens that have established in the cold (Prendergast et al. 2002). The thermal
290 performance of immune activity in *G. veletis* following a short acclimation to low temperatures
291 may thus reflect a seasonal pattern of immune activity in insects that fluctuates with seasonal
292 shifts in pathogen pressure.

293 The contrast between decreased immune activity in the cold and maintained immune
294 activity at high temperatures suggests that fluctuating temperatures will affect the ability of cold-
295 acclimated insects to fight cold-active pathogens and survive at low temperatures. Transient
296 increases in environmental temperature may facilitate a response to cold-active pathogens by
297 allowing for increased immune activity. For example, the expression of genes encoding immune
298 peptides in *M. rotundata* increases under warming provided by fluctuating thermal regimes
299 (Torson et al. 2015). Conversely, increased immune activity under periods of re-warming is
300 likely to decrease the energy available for responses to other stressors, such as cold. Further,
301 immune activity can trade-off with components of fitness, such as growth (Rantala and Roff
302 2005) and reproduction (Adamo et al. 2001; Ahmed et al. 2002); thus fluctuations in temperature
303 may create conflict between the response to pathogens and fitness- or stress-related physiological
304 processes. If climate change-related warming leads to an increase in both pathogen pressure and
305 immune activity, both transient and seasonal periods of re-warming will affect the interactions

306 between energy expenditure and pathogen response, thereby contributing to the impacts of cold
307 and winter on insects. We do caution, however, that the acclimation used in our study does not
308 reflect the type of long-term, seasonal acclimation that an insect would experience in preparation
309 for overwintering (Tauber et al. 1986), and thus we are limited in using our results to predict the
310 outcome of insect-pathogen responses in the wild.

311 As global temperatures shift with climate change, it is increasingly important to
312 understand the physiological capacity of organisms to respond to changes in their environment
313 (Araújo and Luoto 2007; Chown et al. 2010). Ecological physiology often quantifies this
314 capacity of ectotherms to respond to environmental change by measuring the thermal sensitivity
315 and plasticity of one trait or system; for example, thermal limits to activity (Terblanche et al.
316 2008) or reproduction (Cudmore et al. 2010). However, multiple abiotic and biotic pressures co-
317 occur, and we must instead consider what phenotypes are produced by the simultaneous activity
318 of multiple physiological systems in response to these pressures. Increased cold-tolerance,
319 coupled with decreased immune activity at low temperatures in cold-acclimated *G. veletis*,
320 demonstrates that thermal plasticity was disconnected among and within physiological systems;
321 this suggests that plasticity in one trait does not necessarily reflect the response of the whole
322 organism to connected shifts in its abiotic and biotic environment. Thus, to predict the phenotype
323 of an organism that will succeed under climate change scenarios, we must begin to measure
324 multiple physiological traits that correspond to multiple, integrated pressures in a changing
325 environment.

326

327 **Conclusions**

328 We show that cold acclimation improves cold tolerance in *G. veletis*, does not affect the
329 activity of immune enzymes *in vitro* (potential immunity), and decreases realised immune
330 activity at low temperatures. Thus, measures of whole-animal immune performance appear to
331 trade-off with cold tolerance, and we suggest that pathogen stress may be more prevalent upon
332 re-warming. Climate change will result in alterations to the interactions among multiple
333 stressors, such as between temperature and pathogens (Todgham and Stillman 2013), and the
334 thermal performance of the responses to these stressors will contribute to success under new
335 environmental conditions. However, we show that thermal performance does not consistently
336 respond to acclimation among – or even within – physiological systems. Therefore we caution
337 against predicting responses to climate change based on thermal performance of a single
338 physiological system.

339

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525

526 **Figures and Tables**

527 **Fig. 1** Locomotor activity related to cold tolerance in warm- and cold-acclimated *Gryllus veletis*.

528 (A) The critical thermal minima, or the temperatures at which crickets entered chill coma (n = 9
529 WA, 8 CA). (B) The critical thermal maxima, or temperatures indicating the onset of heat spasms
530 (n = 6 WA, 5 CA). (C) Time taken to recover from 72 h in chill coma at -2 °C (n = 5 per
531 acclimation)

532

533 **Fig. 2** Potential immune activity in warm- and cold-acclimated *Gryllus veletis*. (A) Lysozyme

534 activity *in vitro*, measured as the zone of inhibition of *Micrococcus luteus* from 24 h - 48 h (n =

535 6-8 per acclimation, per temperature) (B) Total phenoloxidase activity measured *in vitro* as an

536 increase in absorbance at 495 nm (n = 4-5 per acclimation, per temperature). Points represent

537 mean \pm SEM

538

539 **Fig. 3** Realised immune activity in warm- and cold-acclimated *Gryllus veletis*. (A) Melanisation,

540 represented as a reverse grey value, of an implanted nylon filament (n = 4-5 per acclimation, per

541 temperature) (B) The proportion of *Staphylococcus aureus* cleared from the haemolymph *in vivo*,

542 24 h following inoculation (n = 4-8 per acclimation, per temperature). Points represent mean \pm

543 SEM. Asterisks indicate significant differences between warm- and cold-acclimated crickets, $p <$

544 0.05

545

546 **Fig. 4** Thermal performance curves of the potential outcomes of cold-acclimation on immune

547 activity. A) The Beneficial Acclimation Hypothesis B) The Colder is Better Hypothesis C) The

548 Generalist-Specialist hypothesis D) Paradoxical narrowing of the TPC, representing

549 specialisation of activity to temperatures not predicted by acclimation temperature

- 1 **Table 1.** ANOVA results of the thermal performance of immune activity in warm- and cold-
 2 acclimated *Gryllus veletis*. Bolded p-values represent significant effects of each term
 3 (acclimation or temperature) on the response variable.

Immune activity		Term	df	F	P
Potential	Lysozyme	Temperature	5,73	36.22	<0.01
		Acclimation	1,73	0.45	0.50
		Temperature × Acclimation	5,73	1.24	0.30
	Phenoloxidase	Temperature	4,66	27.1	<0.01
		Acclimation	1,66	0.50	0.48
		Temperature × Acclimation	4,66	0.34	0.85
Realised	Bacterial clearance	Temperature	5,55	15.49	<0.01
		Acclimation	1,55	10.45	<0.01
		Temperature × Acclimation	5,55	1.31	0.27
	Melanisation	Temperature	5,34	9.98	<0.01
		Acclimation	1,34	30.6	<0.01
		Temperature × Acclimation	5,34	2.62	0.04

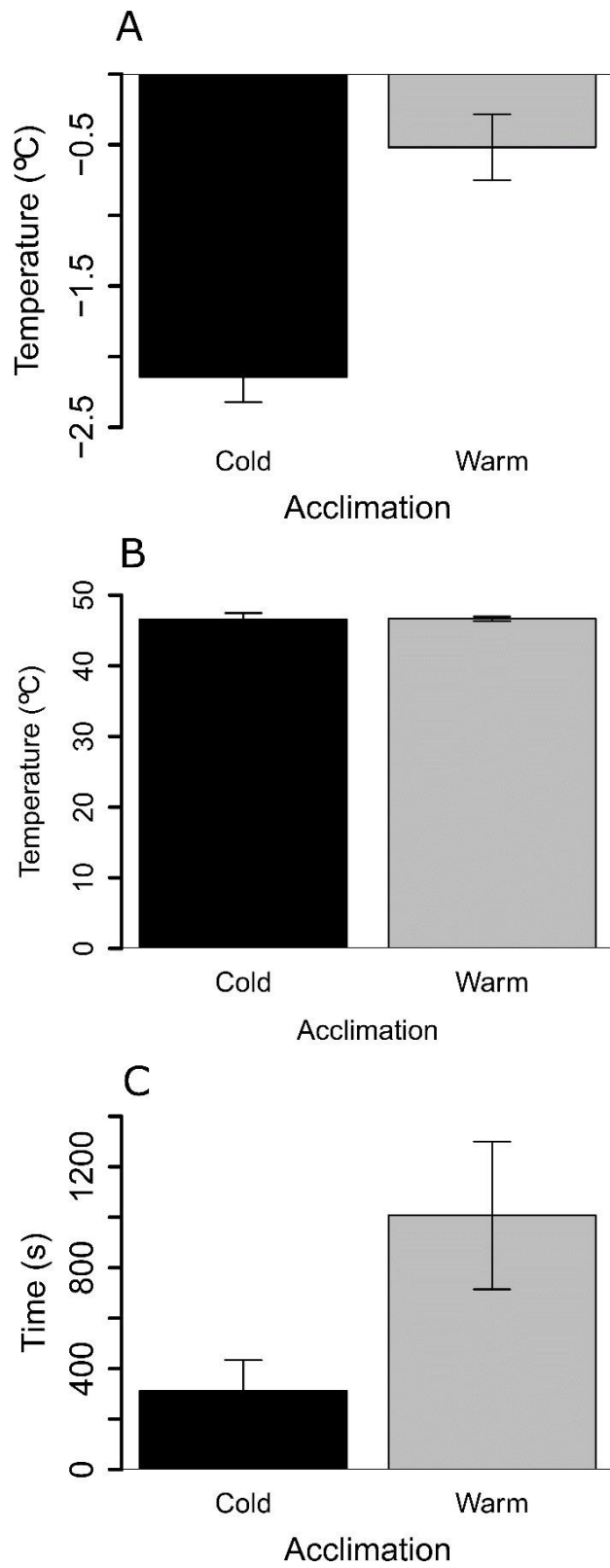


Figure 1.

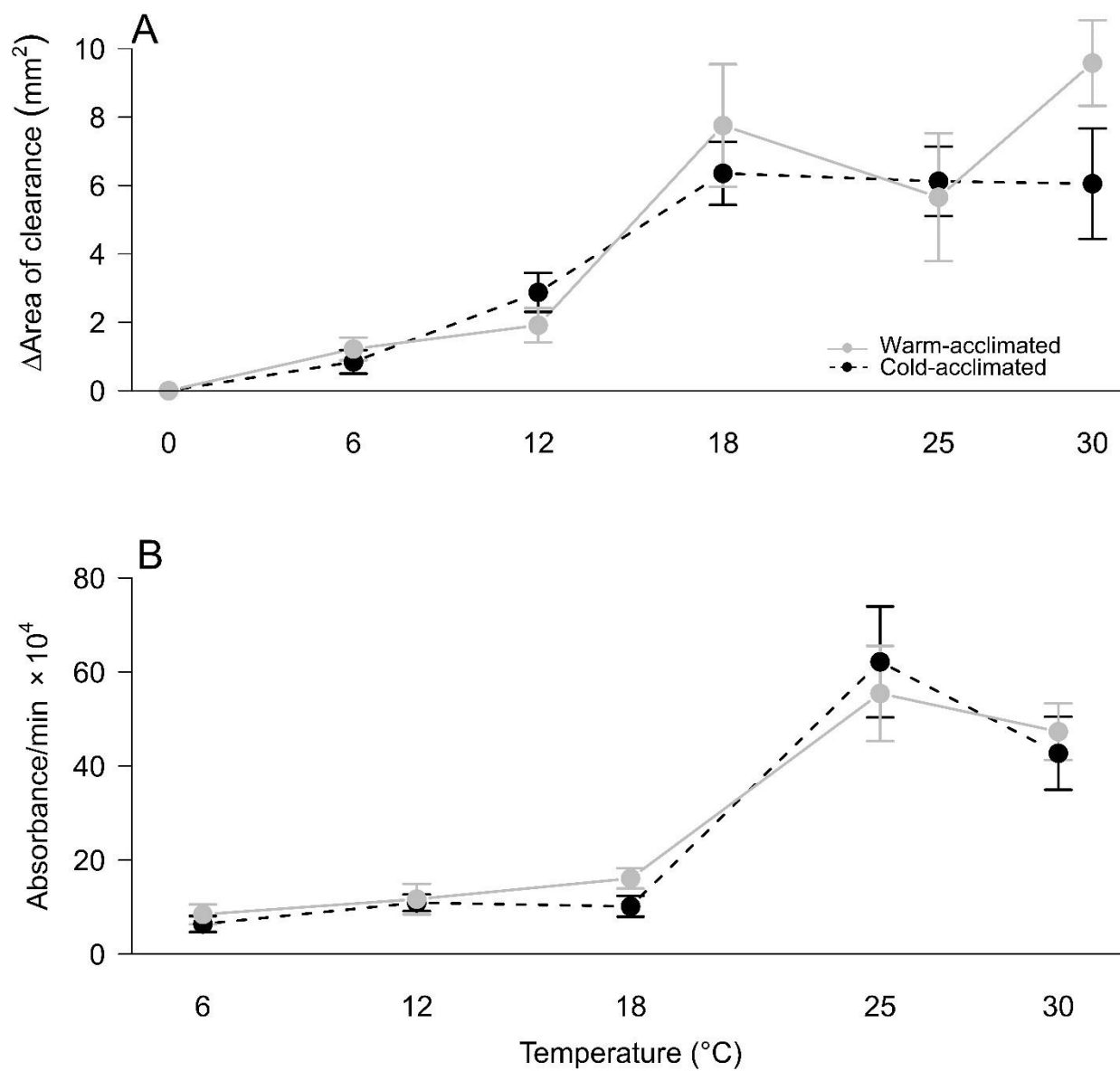


Figure 2.

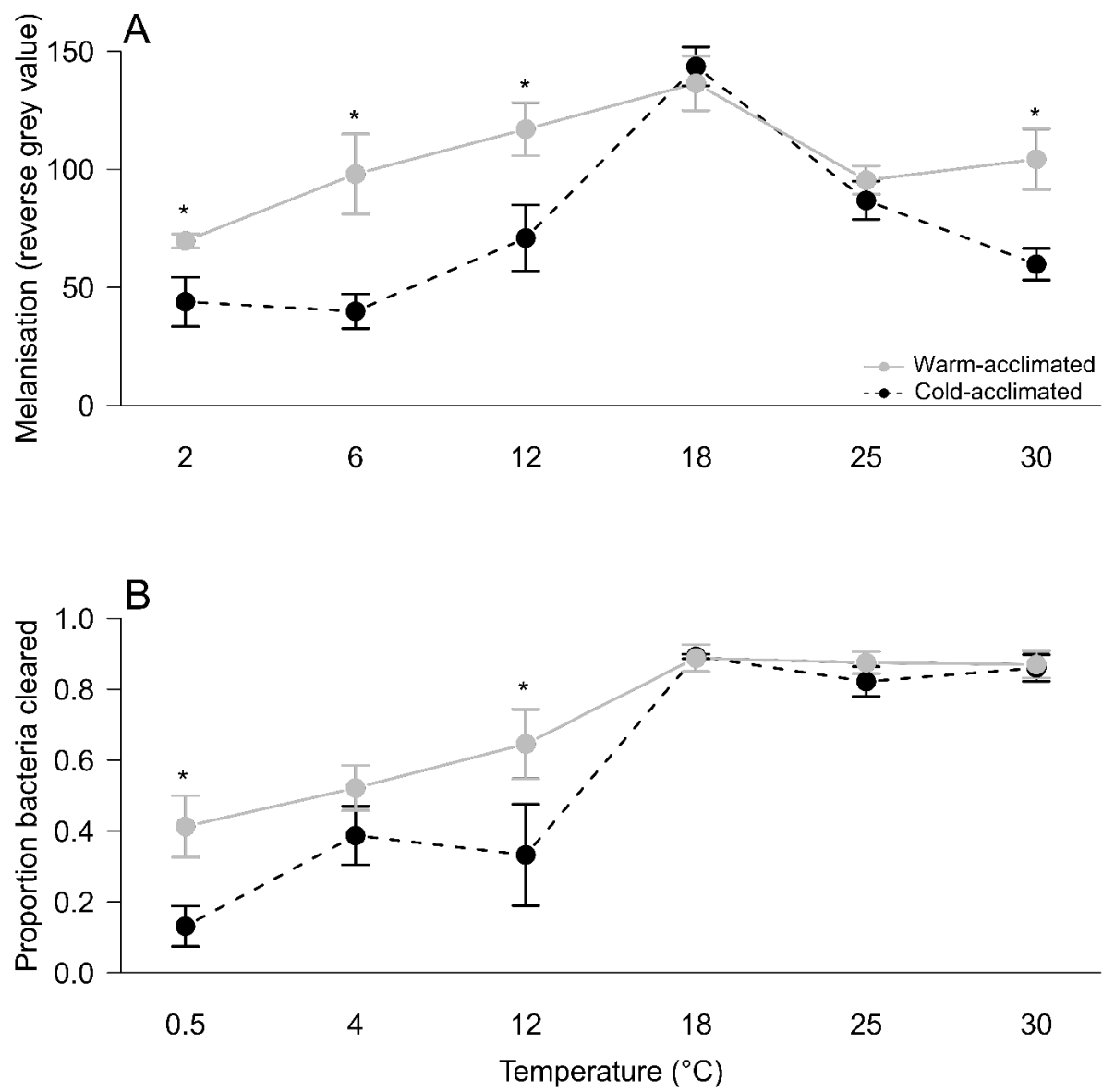


Figure 3.

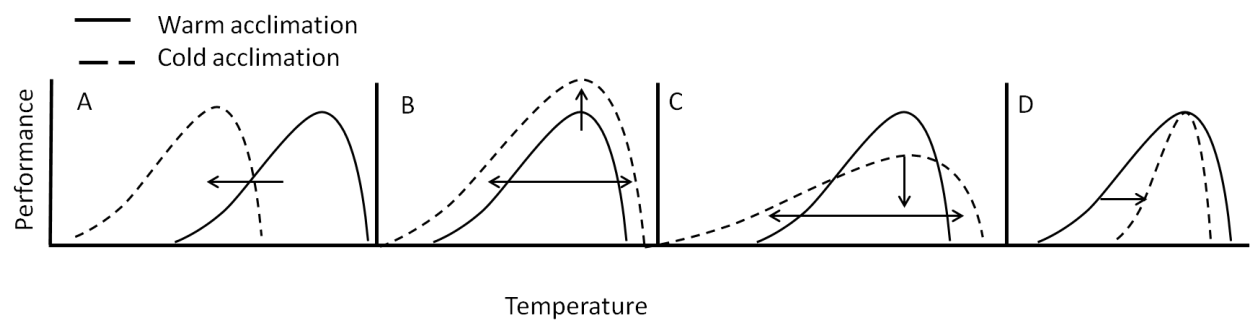


Figure 4.