5-1-2016

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

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

Abstract

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

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

Introduction

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

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

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

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

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

Thermal limits of locomotor activity

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

Potential Immune Response

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

for lysozyme activity) or 50 µL of phosphate-buffered saline [PBS; for phenoloxidase (PO) activity] and snap-froze it in liquid nitrogen, followed by storage at -80 °C.

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

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

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

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

Realised Immune Response

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

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

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

Realised Immune Response

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

Discussion

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

Theory suggests three ways by which the thermal performance of immunity could shift in response to acclimation, if increased low temperature performance were important in the cold: 1) 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

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

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

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

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

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

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

Acknowledgements

Thanks to Dr. Chris Guglielmo, for equipment access, Benjamin Arsic and Ron Flannagan for microbiological assistance, and Amanda Hu, Steven Villani, Steven Xia, and Joshua Zyss for cricket rearing. We are grateful to Caroline Williams and anonymous reviewers for comments that improved the manuscript. Supported by the Natural Sciences and Engineering Research Council of Canada via a Discovery Grant to BJS and a PGS-D scholarship to LVF; by a grant from the Canadian Foundation for Innovation to BJS; and by a grant from The Canadian Institutes of Health Research to DEH.

References

Adamo SA (2004) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket Gryllus texensis. J Insect Physiol 50:209-216. doi: 10.1016/j.jinsphys.2003.11.011

Adamo SA, Jensen M, Younger M (2001) Changes in lifetime immunocompetence in male and female Gryllus texensis (formerly G. integer): trade-offs between immunity and reproduction. Animal Behav 62:417-425. doi: 10.1006/anbe.2001.1786

Ahmed AM, Baggott SL, Maingon R, Hurd H (2002) The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. Oikos 97:371-377

Alexander RD (1968) Life cycle origins, speciation, and related phenomena in crickets. Q Rev Biol 43:1-41

Andersen JL et al. (2015) How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Func Ecol 29:55-65. doi: 10.1111/1365-2435.12310

Angilletta MJ (2006) Estimating and comparing thermal performance curves. J Therm Biol 31:541-545. doi: 10.1016/j.jtherbio.2006.06.002

Angilletta MJ, Jr. (2009) Thermal Adaptation: A Theoretical and Empirical Synthesis. Oxford University Press, New York, United States of America

Araújo MB, Luoto M (2007) The importance of biotic interactions for modelling species distributions under climate change. Global Ecol Biogeograph 16:743-753. doi: 10.1111/j.1466-8238.2007.00359.x

Ardia DR, Gantz JE, BC, Schneider, Strebel S (2012) Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. Func Ecol 26:732-739. doi: 10.1111/j.1365-2435.2012.01989.x

Bresolin G, Morgan JA, Ilgen D, Scherer S, Fuchs TM (2006) Low temperature-induced insecticidal activity of *Yersinia enterocolitica*. Molec Microbiol 59:503-512. doi: 10.1111/j.1365-2958.2005.04916.x

Chown SL, Hoffmann AA, Kristensen TN, Angilletta MJ, Stenseth NC, Pertoldi C (2010) Adapting to climate change: a perspective from evolutionary physiology. Climate Res 43:3-15. doi: 10.3354/cr00879

Coello Alvarado LE, MacMillan HA, Sinclair BJ (2015) Chill-tolerant *Gryllus* crickets maintain ion balance at low temperatures. J Insect Physiol 77:15-25. doi: 10.1016/j.jinsphys.2015.03.015

Collazos ME, Ortega E, Barriga C (1994) Effect of temperature on the immune system of a cyprinid fish (*Tinca tinca,* L). Blood phagocyte function at low temperatures. Fish Shellfish Immunol 4:231-238

Crawley MJ (2007) The R Book. John Wiley & Sons, Ltd, New York

Cudmore TJ, Björklund N, Carroll AL, Staffan Lindgren B (2010) Climate change and range expansion of an aggressive bark beetle: evidence of higher beetle reproduction in naïve host tree populations. J Appl Ecol 47:1036-1043. doi: 10.1111/j.1365-2664.2010.01848.x

Fedorka KM, Copeland EK, Winterhalter WE (2013) Seasonality influences cuticle melanization and immune defense in a cricket: support for a temperature-dependent immune investment hypothesis in insects. J Exp Biol 216:4005-4010. doi: 10.1242/jeb.091538

Fedorka KM, Linder JE, Winterhalter W, Promislow D (2007) Post-mating disparity between potential and realized immune response in *Drosophila melanogaster*. Proc R Soc B 274:1211-1217. doi: 10.1098/rspb.2006.0394

Fernandes EK, Rangel DE, Moraes AM, Bittencourt VR, Roberts DW (2008) Cold activity of *Beauveria* and *Metarhizium*, and thermotolerance of *Beauveria*. J Invert Pathol 98:69-78.

401 doi: 10.1016/j.jip.2007.10.011
402 Freitak D, Ots I, Vanatoa A, Horak P (2003) Immune response is energetically costly in white
403 cabbage butterfly pupae. Proc R Soc B 270:S220-222. doi: 10.1098/rsbl.2003.0069
404 Gershman SN (2008) Sex-specific differences in immunological costs of multiple mating in
405 *Gryllus vocalis* field crickets. Behav Ecol 19:810-815. doi: 10.1093/beheco/arn040
406 Gillespie JP, Kanost MR (1997) Biological mediators of insect immunity. Ann Rev Entomol
407 42:611-643
408 González-Santoyo I, Córdoba-Aguilar A (2012) Phenoloxidase: a key component of the insect
409 immune system. Entomol Exp Appl 142:1-16. doi: 10.1111/j.1570-7458.2011.01187.x
410 Haine ER, Moret Y, Siva-Jothy MT, Rolff J (2008) Antimicrobial defense and persistent infection
411 in insects. Science 322:1257-1259. doi: 10.1126/science.1165265
412 Harrison JF, Woods HA, Roberts SP (2012) Ecological and Environmental Physiology of
413 Insects. Oxford University Press, Oxford, New York
414 Harvell DH et al. (2002) Climate warming and disease risks for terrestrial and marine biota.
415 Science 296:2158-2162
416 Hokkanen HMT (1992) Overwintering survival and spring emergence in *Meligethes aeneus*:
417 effects of body weight, crowding, and soil treatment with *Beauveria bassiana*. Entomol
418 Exp Appl 67:241-246
419 Irwin JT, Lee RE, Jr. (2003) Cold winter microenvironments conserve energy and improve
420 overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta*
421 *solidaginis*. Oikos 100:71-78
422 Krams I, Daukšte J, Kivleniece I, Krama T, Rantala MJ (2011) Overwinter survival depends on
423 immune defence and body length in male *Aquarius najas* water striders. Entomol Exp
424 Appl 140:45-51. doi: 10.1111/j.1570-7458.2011.01132.x
425 Le Bourg E, Massou I, Gobert V (2009) Cold stress increases resistance to fungal infection
426 throughout life in *Drosophila melanogaster*. Biogerontol 10:613-625. doi:
427 10.1007/s10522-008-9206-y
428 Le Morvan C, Troutaud D, Deschaux P (1998) Differential effects of temperature on specific and
429 nonspecific immune defences in fish. J Exp Biol 201:165-168
430 Lenth RV (2013) lsmeans: Least-Squares means, R package version 1.06-05 edn
431 Linderman JA, Chambers MC, Gupta AS, Schneider DS (2012) Infection-related declines in chill
432 coma recovery and negative geotaxis in *Drosophila melanogaster*. PloS one 7:e41907.
433 doi: 10.1371/journal.pone.0041907
434 Lutterschmidt WI, Hutchison VH (1997) The critical thermal maximum: data to support the onset
435 of spasms as the definitive end point. Can J Zool 75:1530-1556
436 MacMillan HA, Sinclair BJ (2011) The role of the gut in insect chilling injury: cold-induced
437 disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. J Exp Biol
438 214:726-734. doi: 10.1242/jeb.051540
439 MacMillan HA, Williams CM, Staples JF, Sinclair BJ (2012) Reestablishment of ion homeostasis
440 during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. PNAS 109:20750–
441 20755
442 Maniero GD, Carey C (1997) Changes in selected aspects of immune function in the leopard
443 frog, *Rana pipiens,* associated with exposure to cold. J Comp Physiol B167:256-263
444 Marnila P, Tiiska A, Lagerspetz K, Lilius E-M (1995) Phagocyte activity in the frog *Rana*
445 *temporaria*: whole blood chemiluminescence method and the effects of temperature and
446 thermal acclimation. Comp Biochem Physiol 111A:609-614
447 Marshall KE, Sinclair BJ (2011) The sub-lethal effects of repeated freezing in the woolly bear
448 caterpillar *Pyrrharctia isabella*. J Exp Biol 214:1205-1212. doi: 10.1242/jeb.054569
449 Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, Thomas MB (2012) Complex
450 effects of temperature on mosquito immune function. Proc R Soc B 279:3357-3366. doi:
451 10.1098/rspb.2012.0638

452 Nakamura A et al. (2011) Innate immune system still works at diapause, a physiological state of
453 dormancy in insects. Biochem Biophys Res Comm 410:351-357. doi:
454 10.1016/j.bbrc.2011.06.015
455 Olsen TM, Duman JG (1997) Maintenance of the superooled state in overwintering pyrochroid
456 beetle larvae, *Dendroides canadensis*: role of hemolymph ice nucleators and antifreeze
457 proteins. J Comp Physiol B 167:105-113
458 Olsen TM, Sass SJ, Li N, Duman JG (1998) Factors contributing to seasonal increases in
459 inoculative freezing resistance in overwintering fire-colored beetle larvae *Dendroides*
460 *canadensis* (Pyrochroidae). J Exp Biol 201:1585-1594
461 Prendergast BJ, Freeman DA, Zucker I, Nelson RJ (2002) Periodic arousal from hibernation is
462 necessary for initiation of immune responses in ground squirrels. Am J Physiol
463 282:R1054–R1062
464 Rantala MJ, Roff DA (2005) An analysis of trade-offs in immune function, body size and
465 development time in the Mediterranean Field Cricket, *Gryllus bimaculatus*. Func Ecol
466 19:323-330. doi: 10.1111/j.1365-2435.2005.00979.x
467 Rasband WS (1997-2014) Image J, 1.45S edn. National Institutes of Health, Bethesda,
468 Maryland, USA
469 Riedel W, Steenberg T (1998) Adult polyphagous coleopterans overwintering in cereal
470 boundaries: winter mortality and susceptibility to the entomopathogenic fungus
471 *Beauveria bassiana*. BioControl 43:175-188
472 Schmid-Hempel P (2003) Variation in immune defence as a question of evolutionary ecology.
473 Proc R Soc B 270:357-366. doi: 10.1098/rspb.2002.2265
474 Sinclair BJ (in press) Linking energetics and overwintering in temperate insects. J Therm Biol
475 doi: 10.1016/j.jtherbio.2014.07.007
476 Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA (2013) Cross-tolerance and
477 cross-talk in the cold: relating low temperatures to desiccation and immune stress in
478 insects. Int Comp Biol 53:545-556. doi: 10.1093/icb/ict004
479 Somero GN (1995) Proteins and temperature. Ann Rev Physiol 57:43-68
480 Steenberg T, Langer V, Esbjerg P (1995) Entomopathogenic fungi in predatory beetles (col.:
481 *Carabidae* and *Staphylinidae*) from agricultural fields. Entomophaga 40:77-85
482 Sternberg ED, Thomas MB (2014) Local adaptation to temperature and the implications for
483 vector-borne diseases. Trends Parasitol 30:115-122. doi: 10.1016/j.pt.2013.12.010
484 Tauber MJ, Tauber CA, Masaki S (1986) Seasonal Adaptations of Insects. Oxford University
485 Press, New York
486 Team RDC (2010) R: A language and environment for statistical computing. R Foundation for
487 Statistical Computing, Vienna, Austria
488 Terblanche JS, Clusella-Trullas S, Deere JA, Chown SL (2008) Thermal tolerance in a south-
489 east African population of the tsetse fly *Glossina pallidipes* (Diptera, Glossinidae):
490 implications for forecasting climate change impacts. J Insect Physiol 54:114-127. doi:
491 10.1016/j.jinsphys.2007.08.007
492 Terblanche JS, Sinclair BJ, Jaco Klok C, McFarlane ML, Chown SL (2005) The effects of
493 acclimation on thermal tolerance, desiccation resistance and metabolic rate in *Chirodica*
494 *chalcoptera* (Coleoptera: Chrysomelidae). J Insect Physiol 51:1013-1023. doi:
495 10.1016/j.jinsphys.2005.04.016
496 Todgham AE, Stillman JH (2013) Physiological responses to shifts in multiple environmental
497 stressors: relevance in a changing world. Int Comp Biol 53:539-544. doi:
498 10.1093/icb/ict086
499 Torson AS, Yocum GD, Rinehart JP, Kemp WP, Bowsher JH (2015) Transcriptional responses to
500 fluctuating thermal regimes underpinning differences in survival in the solitary bee
501 *Megachile rotundata*.J Exp Biol. doi: 10.1242/jeb.113829
502 Tort L, Padros F, Rotillant J, Crespo S (1998) Winter syndrome in the gilthead sea bream

Sparus aurata. Fish Shellfish Immunol 8:37-47
Vilcinskas A, Matha V (1997) Effect of the entomopathogenic fungus *Beauveria bassiana* on the humoral immune response of *Galleria mellonella* larvae (Lepidoptera: Pyralidae). Eur J Entomol 94:461-472
Webberley KM, Hurst GDD (2002) The effect of aggregative overwintering on an insect sexually transmitted parasite system. J Parasitol 88:707-712
Williams CM, Henry HA, Sinclair BJ (2015) Cold truths: how winter drives responses of terrestrial organisms to climate change. Biol Rev Camb Philos Soc. doi: 10.1111/brv.12105
Williams CM, Marshall KE, MacMillan HA, Dzurisin JD, Hellmann JJ, Sinclair BJ (2012) Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. PloS one 7:e34470. doi: 10.1371/journal.pone.0034470
Xu J, James RR (2012) Temperature stress affects the expression of immune response genes in the alfalfa leafcutting bee, *Megachile rotundata*. Insect Molec Biol 21:269-280. doi: 10.1111/j.1365-2583.2012.01133.x
Zhang J, Marshall KE, Westwood JT, Clark MS, Sinclair BJ (2011) Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster.* J Exp Biol 214:4021-4029. doi: 10.1242/jeb.059535
Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common statistical problems. Methods Ecol Evol 1:3-14. doi: 10.1111/j.2041-210X.2009.00001.x

Figures and Tables

Fig. 1 Locomotor activity related to cold tolerance in warm- and cold-acclimated *Gryllus veletis*. (A) The critical thermal minima, or the temperatures at which crickets entered chill coma (n = 9 WA, 8 CA). (B) The critical thermal maxima, or temperatures indicating the onset of heat spasms (n = 6 WA, 5 CA). (C) Time taken to recover from 72 h in chill coma at -2 °C (n = 5 per acclimation)

Fig. 2 Potential immune activity in warm- and cold-acclimated *Gryllus veletis*. (A) Lysozyme activity *in vitro*, measured as the zone of inhibition of *Micrococcus luteus* from 24 h - 48 h (n = 6-8 per acclimation, per temperature) (B) Total phenoloxidase activity measured *in vitro* as an increase in absorbance at 495 nm (n = 4-5 per acclimation, per temperature). Points represent mean ± SEM

Fig. 3 Realised immune activity in warm- and cold-acclimated *Gryllus veletis*. (A) Melanisation, represented as a reverse grey value, of an implanted nylon filament (n = 4-5 per acclimation, per temperature) (B) The proportion of *Staphylococcus aureus* cleared from the haemolymph *in vivo,* 24 h following inoculation (n = 4-8 per acclimation, per temperature). Points represent mean ± SEM. Asterisks indicate significant differences between warm- and cold-acclimated crickets, $p < 0.05$

Fig. 4 Thermal performance curves of the potential outcomes of cold-acclimation on immune activity. A) The Beneficial Acclimation Hypothesis B) The Colder is Better Hypothesis C) The Generalist-Specialist hypothesis D) Paradoxical narrowing of the TPC, representing specialisation of activity to temperatures not predicted by acclimation temperature

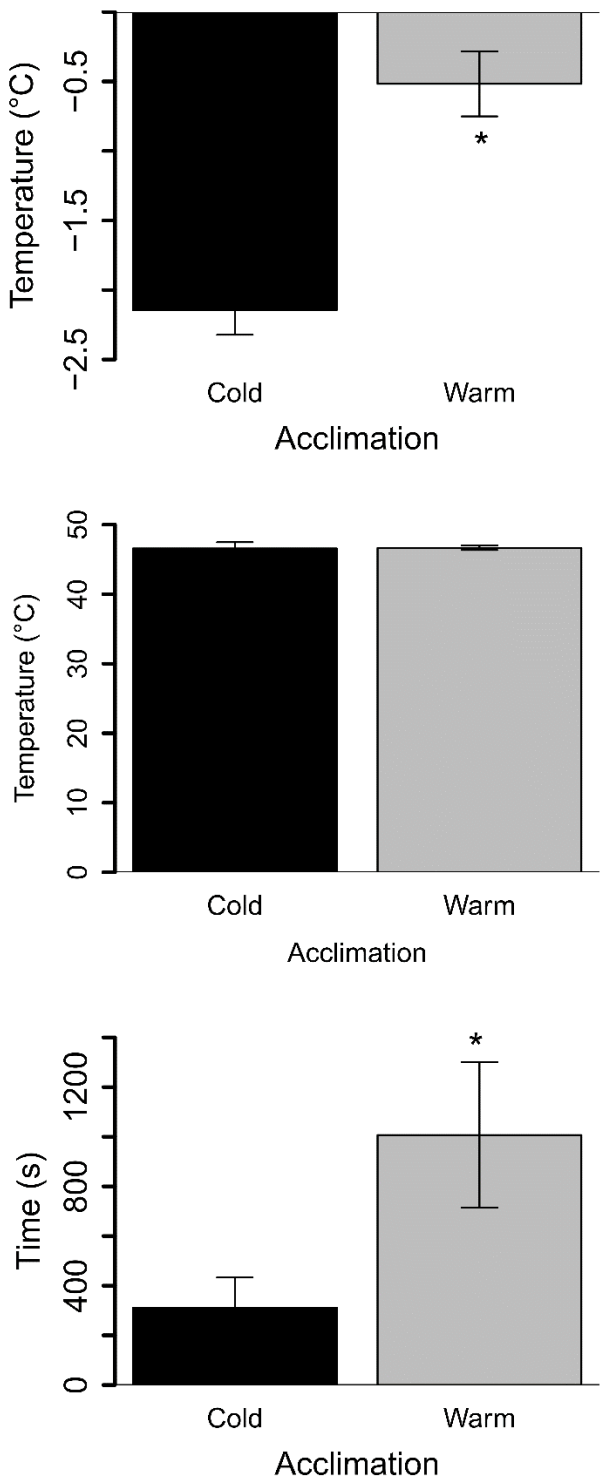
Temperature (°C)
−2.5
−1.5
−0.5
*
Cold
Warm
Acclimation
Temperature (°C)
0
10
20
30
40
50
Cold
Warm
Acclimation
Time (s)
0
400
800
1200
*
Cold
Warm
Acclimation

Figure 1.

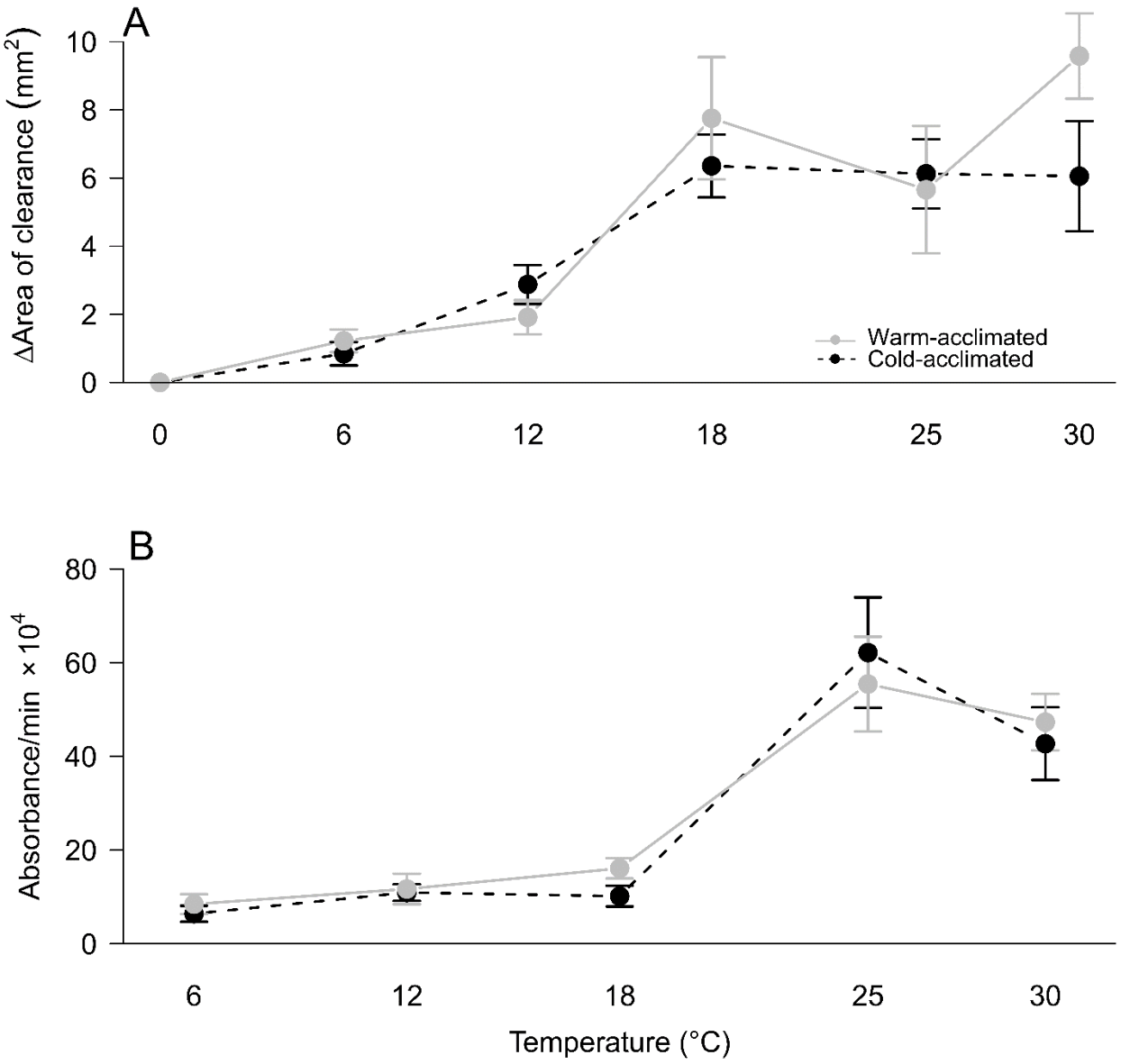
A
ΔArea of clearance (mm²)
10
8
6
4
2
0
0
6
12
18
25
30
Warm-acclimated
Cold-acclimated
B
Absorbance/min × 10⁴
80
60
40
20
0
6
12
18
25
30
Temperature (°C)

Figure 2.

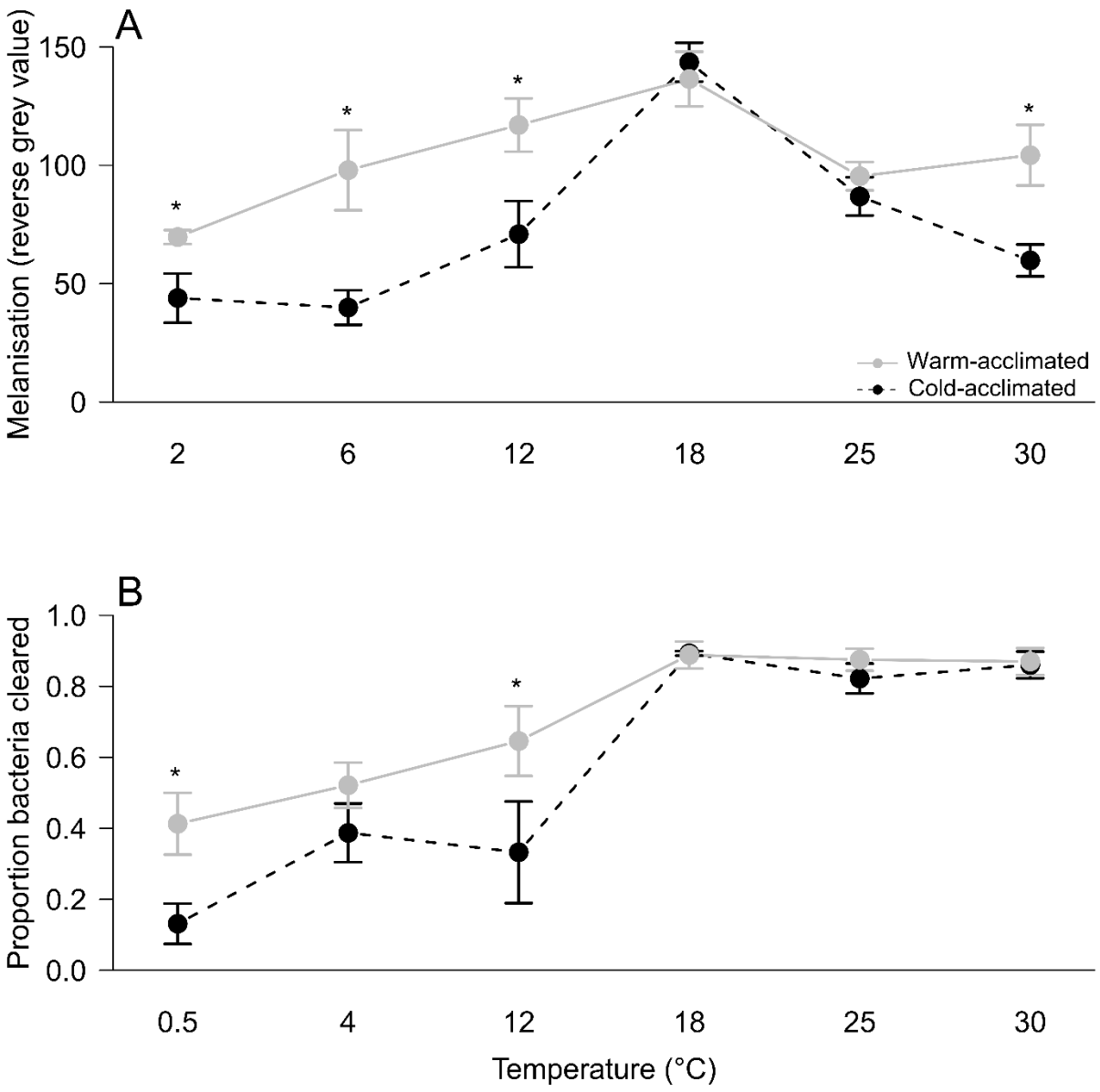
A
Melanisation (reverse grey value)
150
100
50
0
*
*
*
*
2
6
12
18
25
30
Warm-acclimated
Cold-acclimated
B
Proportion bacteria cleared
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0.5
4
12
18
25
30
Temperature (°C)

Figure 3.

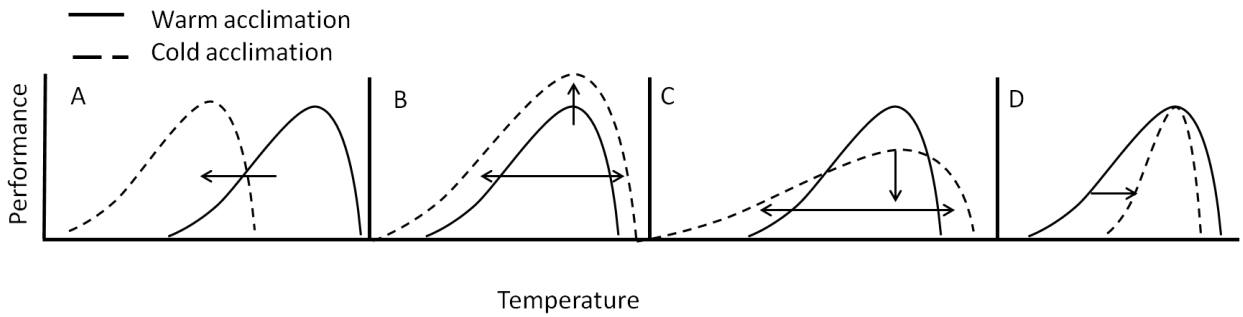
Warm acclimation
Cold acclimation
Performance
A
B
C
D
Temperature

Figure 4.

551