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Laura V Ferguson

David E Heinrichs

Brent J Sinclair

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Laura V. Ferguson[1*], David E. Heinrichs[2], Brent J. Sinclair[1]

[1]Department of Biology, University of Western Ontario, London, ON, Canada N6A 5B7

[2]Department of Microbiology and Immunology, University of Western Ontario, London, ON, Canada N6A 5B7

*Author for correspondence: lfergus9@uwo.ca

Department of Biology, University of Western Ontario, 1151 Richmond Street N, London, ON, Canada N6A 5B7. Phone: 1-519-661-2111 ext. 89158. Fax: 1-519-661-3935

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.

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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;

144 for lysozyme activity) or 50 µL of phosphate-buffered saline [PBS; for phenoloxidase (PO)
145 activity] and snap-froze it in liquid nitrogen, followed by storage at -80 °C.
146 To estimate the bactericidal activity of lysozyme, we followed Vilcinskas and Matha
147 (1997), with some modifications. We added 4 µL 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 °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 µL of thawed
156 hemoymph/PBS mixture with 70 µL alpha-chymotrypsin (1.3 mg/ mL in PBS) and incubated it
157 for 20 min at room temperature (22 °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 °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 µL 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

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.

Statistical analyses

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

3. Results

Thermal limits of locomotor activity

Cold-acclimation enhanced low temperature locomotor activity of *G. veletis* and shortened recovery time after cold exposure. The CT_{min} of WA crickets was significantly higher than that of CA crickets ($t_{14.46} = 5.53$, $p < 0.001$; Fig. 1A); however, there was no difference between the CT_{max} of WA and CA crickets (Fig. 1B; $t_5 = 0.11$, $p = 0.45$). Chill coma recovery time was lower in CA crickets than WA crickets (Fig. 1C; $t_{5.34} = 2.19$, $p = 0.04$).

Potential Immune Response

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

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 lateralus*), 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

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

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

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.

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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

Table 1
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Table 1. ANOVA results of the thermal performance of immune activity in warm- and cold-acclimated *Gryllus veletis*. Bolded p-values represent significant effects of each term (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
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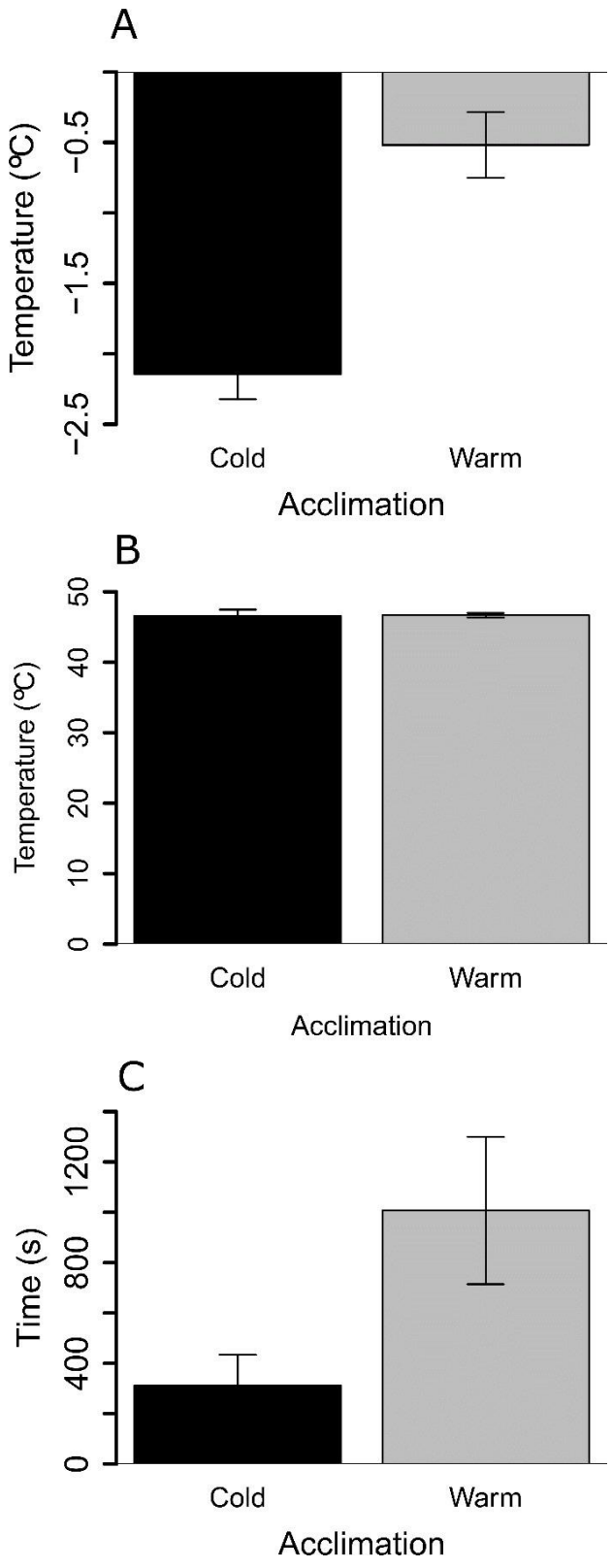

Figure 1.

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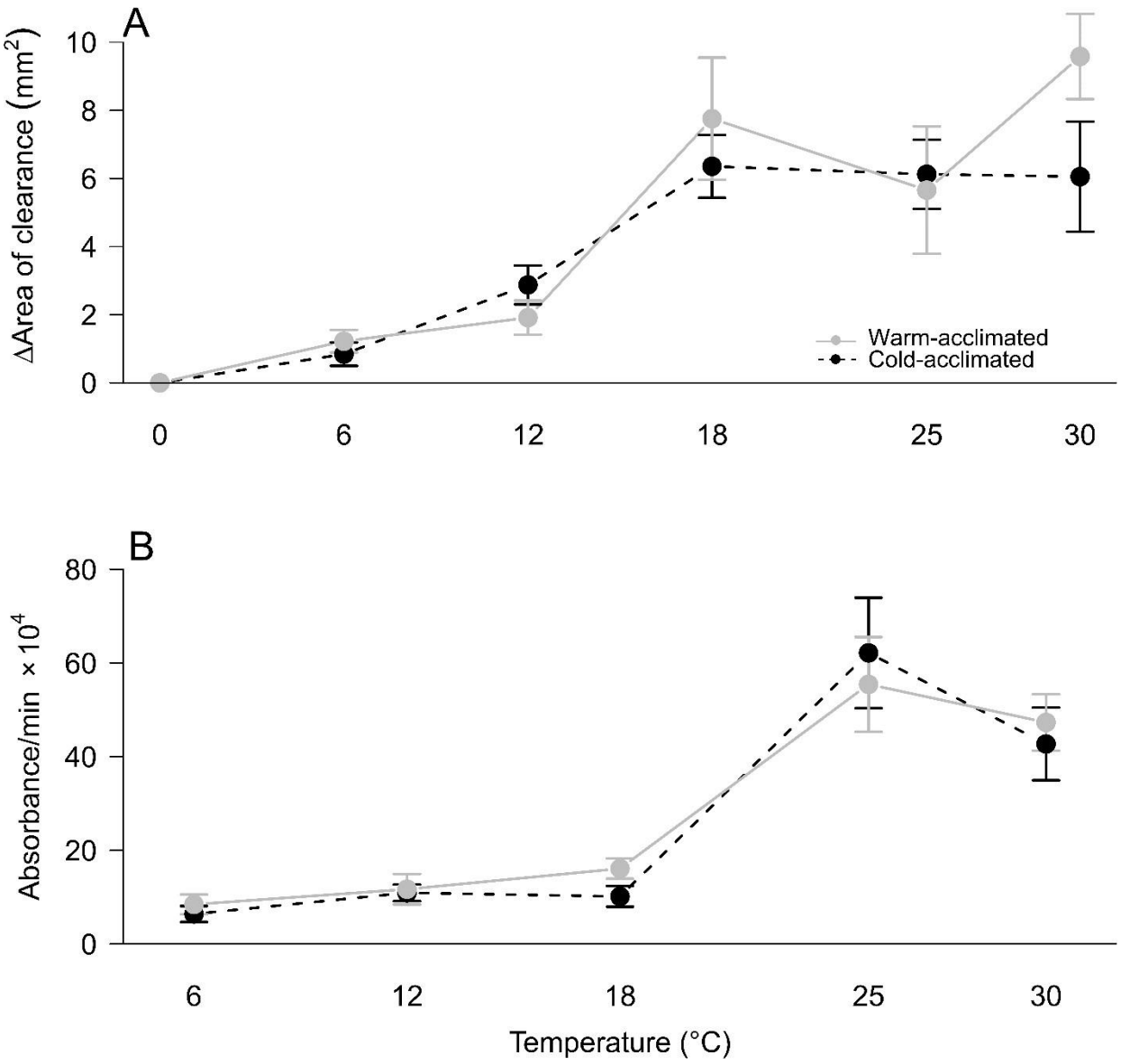

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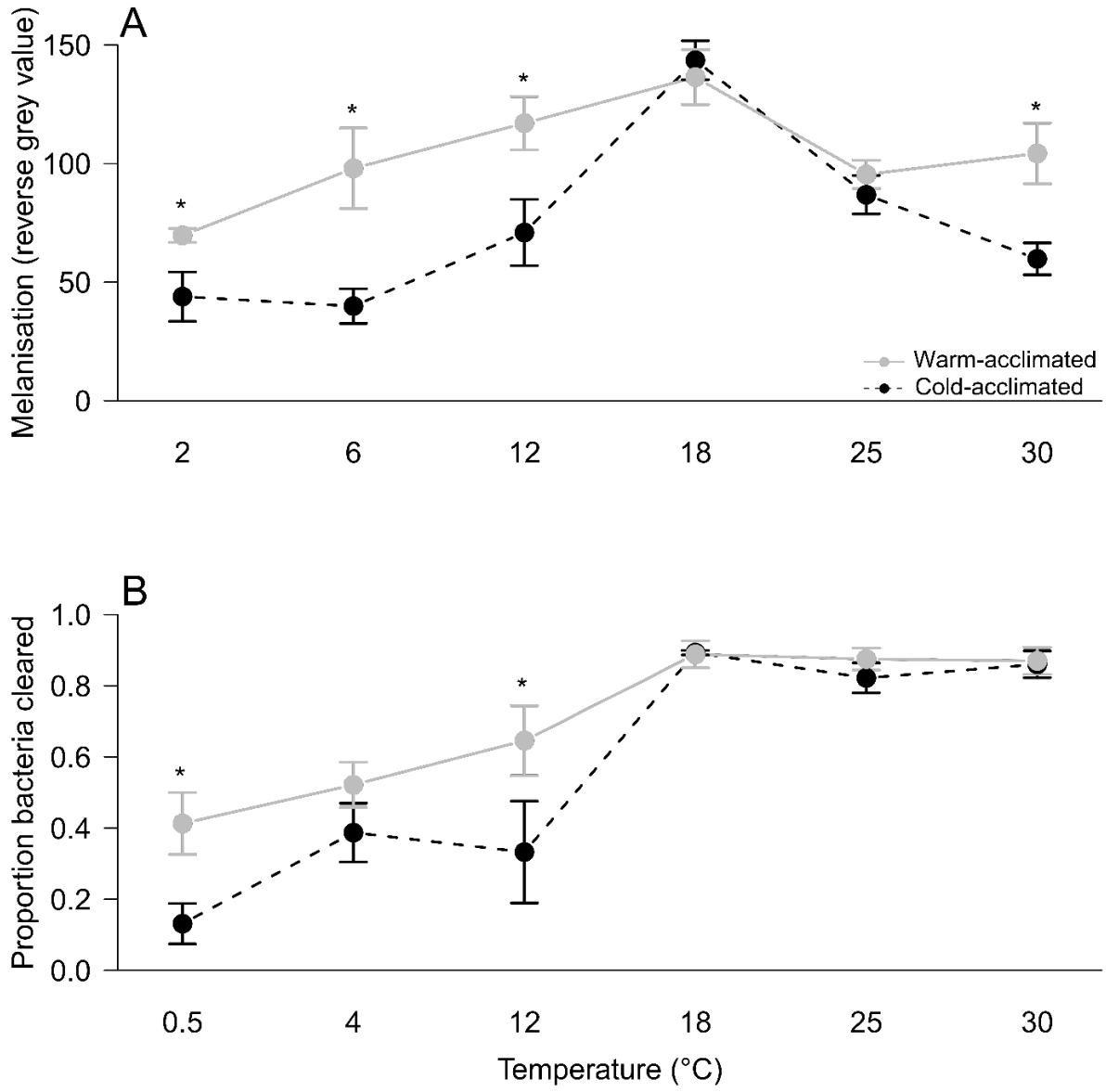

Figure 3.

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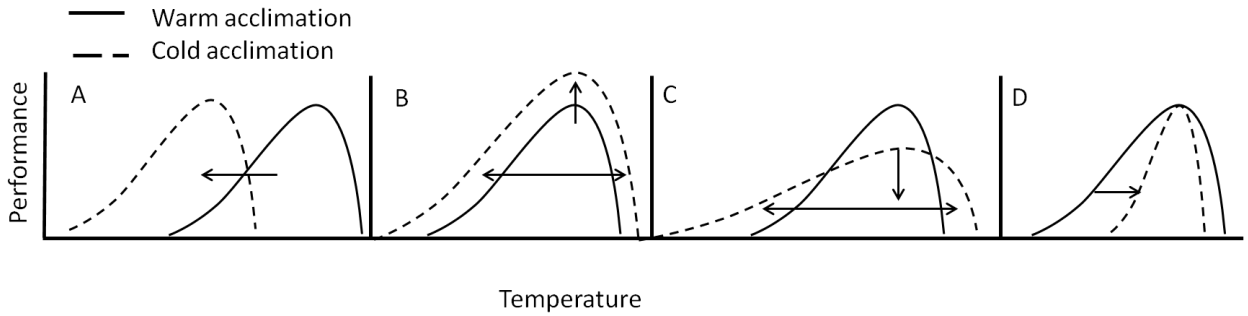

Figure 4.