Stress Responses and Candidate Gene Expression Investigated Across Species and Ecosystems in the Field

Noer, Natasja Krog

DOI (link to publication from Publisher):
10.54337/aau510736737

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

STRESS RESPONSES AND CANDIDATE GENE EXPRESSION INVESTIGATED ACROSS SPECIES AND ECOSYSTEMS IN THE FIELD

BY
NATASJA KROG NOER

DISSERTATION SUBMITTED 2022

AALBORG UNIVERSITY
DENMARK

STRESS RESPONSES AND CANDIDATE GENE EXPRESSION INVESTIGATED ACROSS SPECIES AND ECOSYSTEMS IN THE FIELD

PHD THESIS

BY

NATASJA KROG NOER

DEPARTMENT OF CHEMISTRY AND BIOSCIENCE

FACULTY OF ENGINEERING AND SCIENCE

AALBORG UNIVERSITY

Dissertation submitted 2022

AALBORG UNIVERSITY
DENMARK

Dissertation submitted: 31-08-2022

PhD supervisor: Professor Torsten Nygaard Kristensen
Aalborg University

Assistant PhD supervisor: Associate Professor Simon Bahrndorff
Aalborg University

PhD committee: Associate Professor Simone Daniela Langhans (chair)
Aalborg University, Denmark

Professor, Dr. Klaus Fischer,
University of Koblenz-Landau, Germany

Associate Professor Nicholas M. Teets,
University of Kentucky, USA

PhD Series: Faculty of Engineering and Science, Aalborg University

Department: Department of Chemistry and Bioscience

ISSN (online): 2446-1636
ISBN (online): 978-87-7573-837-3

Published by:
Aalborg University Press
Kroghstræde 3
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Credits: In-text photographs or figures by Natasja Krog Noer unless stated otherwise
Front/back cover photo: Nysius groenlandicus by Simon Bahrndorff

Printed in Denmark by Stibo Complete, 2022

PREFACE

This thesis represents the final product (**PART B**) of four years of PhD study at the Department of Chemistry and Bioscience, Aalborg University, Denmark. It consists of the second part of an integrated PhD (4+4) based on work from September 2020 to August 2022. The final product is a revised and elaborated version of the first part (**PART A**) of the integrated PhD from the period September 2018 to August 2020. The thesis, **PART B,** consists of two segments: The first is an introduction to the background, state-of-the-art, discussion of current work, and emphasis on the future work perspectives. The second part will include three accepted papers, a submitted paper under editorial assessment, and the preliminary results from a planned paper. Finally, several co-authored papers have been listed in the appendix. These papers and projects are the principal products of my thesis.
It is important for me to stress that I consequently use the 'I' form when describing our results in the introduction. However, all work in this thesis is the results of contributions and fruitful collaboration between all involved collaborators.

Natasja Krog Noer
Aalborg, August 2022

ACKNOWLEDGEMENT

I owe several people my gratitude for their contribution to the work in this thesis. First, a special thanks to my supervisor Torsten Nygaard Kristensen for supporting me in my choices and enabling my pursuit of an academic career. I am grateful for your optimistic and sympathetic mindset, the network and opportunities you have provided for me, and your crazy take on life. On equal terms, I would like to thank my co-supervisor Simon Bahrndorff for supporting me in my work and trusting me with great responsibilities. I have valued our scientific discussions and you have been a great source of inspiration for me throughout the years. You have both helped shaping me as a researcher through your committed participation and collaboration in the field and behind the desk. Above all, we have shared incredible adventures together which has given me fun and happy memories that will last for a lifetime.

I am grateful to all of my collaborators who have educated me in scientific disciplines. To my Australian collaborators Ary A. Hoffmann and Michele Schiffer, thank you for welcoming me at Bio21 and Daintree Rainforest Observatory. You made my cumbersome and logistically challenging experiments possible, and I appreciate the educational and fruitful discussions. 'Merci' to my French collaborators David Renault and Hervé Colinet for going through the trouble of hosting me during a pandemic and for giving me the tools and knowhow to analyze complex omics data. Thanks you Anders Malmendal for welcoming me at your office in Roskilde for private lessons on NMR metabolomics. I would also like to thank Kåre Lehmann Nielsen for private tutoring on RNA sequencing and analysis, and for being the optimistic, kind, and educational person you are. Finally, but not least, especially thanks to my talented collaborators Michael Ørsted and Palle Duun Rohde from whom I have learned much. Your work ethics and dedication to science has been an inspiration. Also, for my collaborator and travel companion Jesper Givskov Sørensen. I admire your ability keep science and tedious work fun, you amazing organizational and leadership abilities, and your plethora of research ideas and advice.

Thank you to all the people at Biochemistry and Science. I enjoyed coming to work owing to my wonderful colleagues and office mates with whom I shared many 'coffee talks' with when needed. Moreover, I would never have made it without our lab technicians Helle, Susan, Sofie, and Gitte. Thank you for always jumping in last minute when needed and for always bringing joy to the lab.

A huge thanks to my friends and colleagues Laura Skrubbeltrang Hansen, Andreas Havbro Faber, and Alex Jørgensen for the invaluable scientific discussions and the emotional support during the hard and the good times. You were always supporting my scientific quests and celebrating the successes (and failures). Thank you to Mathias Hamann Sørensen for the fun and wonderful adventures in the Arctic and in Denmark and for bearing with me (and feeding me) when my blood sugar was low. You have all kept me sane during this journey and reminded me to enjoy life and nurse my hobbies. Finally, thank you to my family and friends for your patience and support in this odd choice of career.

ENGLISH SUMMARY

The recurring theme of this thesis is the ability of insects to cope with variable environmental conditions by phenotypic plasticity. Species are continuously exposed to changes in abiotic factors in nature. The ability to respond to variable environmental conditions depends on phenotypic plasticity and evolutionary adaptation to the local environment. With global change, the intensity and frequency of extreme and unpredictable temperature fluctuations will increase. How insects cope with these changes and the interaction with other abiotic and biotic factors in nature is largely unknown. Currently, most work on thermal adaptation has been done using laboratory studies on model-organisms under strictly controlled conditions potentially with little ecological relevance. The common denominator for the work during my PhD was the use of ecologically relevant environmental conditions to examine the capacity for phenotypic plasticity to buffer the detrimental impacts of variable and occasionally stressful temperatures on insects adapted to habitats characterized by diverse temporal temperature heterogeneity. First, I provide an overview of the research field in the **Introduction** followed by four papers and additional results.

In **PAPER I**, I examined the extent to which thermal plasticity to daily field temperature variation occurs in 12 insect species from temperate and tropical regions. High levels of plasticity in heat tolerance were found across all the tested species, but the magnitude and direction of the plastic response was species- and sex-specific. Generally, the temperature at the collection time was a strong driver of plastic adjustments in HKDT in temperate insects, but the environmental factors driving plastic change in tropical species were less obvious. This highlighted the importance of considering species thermoregulatory behavior, microhabitat scale climate variables, and local adaptation in order to understand future responses of species to increasing variable environments. These findings have implications for the classification of species vulnerabilities based on predictive models and call for a better understanding of heat tolerance and plasticity in species in nature, especially species inhabiting regions most impacted by climate change.

In **PAPER II**, I synthesized existing knowledge on terrestrial polar and subpolar arthropods' sensitivity to high and increasing temperatures and extended this knowledge with own data. The review of published studies and our own data revealed a large gap in knowledge on plasticity and the importance of evolutionary adaptation to periodically high and increasing temperatures in polar regions. This lack of information obstructs predictions on the consequences of warming temperatures on polar ecosystems. Analysis of the available estimates on upper critical temperature (CT_{max}) showed extensive variation in CT_{max} and the underlying mechanisms of heat tolerance which suggests that fine-scale spatial adaptation to microhabitat drive differences in heat tolerance. I argue that the highly variable thermal conditions in arctic/subarctic environments provide a unique opportunity to disentangle effects of thermal regime on heat tolerance, plasticity and evolutionary adaptation to temperature changes.

In **PART A** (the midterm exam product for the 4+4 PhD study), I presented results showing that a subarctic arthropod, *Nysius groenlandicus*, was able to induce an extremely fast and strong plastic response to high temperature exposure. These results led me to use this species to examine underlying mechanisms of thermal tolerance plasticity in **PAPER III** and **PAPER VI**. First, I showed that *N. groenlandicus* females responded to immediate changes in environmental temperature and tracks their ambient environment by plasticity. Second, in **PAPER III** I showed that physiological responses to heat, but less so cold, are visible at the transcriptomic level when gradually exposed to high and low temperature in the laboratory. High temperature exposures led to induction of the heat shock response and increased transcription of vitellogenins (essential for oocyte growth). The induction of these (and other) transcript in the field occurred at much lower temperatures compared to what was seen in the laboratory. This stressed the importance of validating the ecologically relevance of gene expression patterns found in laboratory studies. In addition, I showed that individuals collected at the most extreme time points (highest/lowest field temperatures, though relatively benign) were enriched for transcripts related to translation, cellular respiration, and metabolism, suggesting that energetic demands are challenged at extreme temperature variations. In **PAPER IV,** I showed that environmental temperatures had a big impact on metabolite composition of field collected individuals. Especially exposure to cold field temperatures resonated in the metabolite profiles showing increased levels of multiple sugars across all timepoints subsequent to low night temperatures. Sugars have been associated with increased cold tolerance through stabilizing and colligative features, and the observed daily effects of temperatures cold tolerance could be explained by this mechanism. Finally, hourly differences in metabolic intermediates and amino acids complemented RNAseq profiles from **PAPER III** suggesting that the species face energetic challenges when temperatures fluctuate over large temperature spans.

Last, I present data from an ongoing *Drosophila melanogaster* study investigating the quantitative genetic basis of diurnal variation in heat tolerance and locomotor activity. Preliminary results suggest time and temperature specific genetic architectures of the investigated traits suggesting potentially strong impact of increasing future temperature variability on the evolutionary trajectories of these traits.

In summary, this thesis investigated the extent and rate of phenotypic plastic adjustments in various thermal tolerance measures and the physiological modifications and genetic architecture that facilitates such responses in insect species in nature.

DANSK RESUMÉ

Det gennemgående tema i denne afhandling er arters evne til at overkomme varierende mikroklimatiske miljøforhold via fænotypisk plasticitet. Arter udsættes kontinuert for ændringer i abiotiske faktorer i naturen. Evnen til at tilpasse sig til variable miljøfaktorer afhænger af fænotypisk plasticitet og evolutionær tilpasning til det lokale miljø. Som følge af klimaforandringerne forventes hyppigere og mere intense og uforudsigelige temperaturekstremer. Hvordan arter håndterer disse ændringer i temperaturregimer og interaktioner med andre miljøfaktorer i stadig uvist. Hidtil har de fleste studier som beskæftiger sig med organismers tilpasning til temperaturændringer været udført i laboratoriet ved brug såkaldte "model-arter" under stærkt kontrollerede forhold som ikke er særligt naturtro. Denne afhandling undersøger de fysiologiske mekanismer som ligger bag tilpasning til variable og, til tider, stressfulde temperaturer, samt plastiske responser til variable temperaturer på tværs af arter tilhørende forskellige taxa og en "ikke-modelart" i sit naturlige miljø. Dette inkluderer en undersøgelse af omfanget og hastigheden af fænotypisk tilpasning i adskillige mål for temperaturtolerance og de fysiologiske modifikationer som faciliterer disse responser. I denne afhandling giver jeg først et overblik over forskningsfeltet i introduktionen efterfulgt af fire artikler samt supplerende resultater fra et igangværende projekt.

I **ARTIKEL I** undersøges omfanget af plastisk tilpasning i temperaturtolerance til daglig temperaturvariation i 12 insektarter fra tempererede og tropiske egne. De plastiske responser var kraftige på tværs af alle arterne, men størrelse og retning på responserne var arts- og kønsspecifikke. Generelt var temperaturen ved indsamlingstidspunktet vigtig for kortids-justeringer i varmetolerance i temperere arter, men miljøfaktorerne som drev ændringer i varmetolerance for tropiske arter var mindre tydelige. Det markerede vigtigheden af at overveje adfærdsmedieret temperaturregulering, lokal tilpasning til mikrohabitater og evolution af plasticitet for at forstå arters responser til øget temperaturvariabilitet i fremtiden. Disse resultater har implikationer for hvordan vi klassificerer arters sårbarhed baseret på prædiktive modeller og kræver at vi øger vores forståelse for varmetolerance af plasticitet i arter i naturen, især for arter som lever i de regioner der er mest påvirket af klimaforandringer.

I **ARTIKEL II** syntetiserede jeg eksisterende viden på terrestriske polare og subpolare leddyrs sensitivitet til høje og stigende temperaturer, og udvidede denne viden med empiri fra egne studier. Syntesen af de publicerede studier og vores eget data afslørede en stor mangel på viden omkring plasticitet og vigtigheden af evolutionær tilpasning til periodisk høje og stigende temperaturer. Denne mangel på information betyder, at vores evne til at forudsige konsekvenserne af stigende temperaturer på polare økosystemer er utilstrækkelig. Analyse af de tilgængelige estimater på øvre kritiske temperaturer (CT_{max}) viste at der er massive forskelle i varmetolerance og de underliggende mekanismer bag disse, hvilket antyder at finskala rummelig tilpasning til mikrohabitater driver disse forskelle. Jeg argumenterede for at

de kraftigt varierende temperaturforhold i arktiske/subarktiske miljøer byder på unikke muligheder for at skelne mellem effekter af temperaturgennemsnit, -fluktuationer og -ekstremer på varmetolerance, plasticitet og evolutionær tilpasning til temperaturændringer.

I **DEL A** (produktet af midtvejseksamen for 4+4 PhD ordningen) præsenterede jeg resultater som viste at det subarktiske insekt, *Nysius groenlandicus*, var i stand til at inducere en ekstremt hurtigt og stærk plastisk respons ved præ-eksponering til en stressende varm temperatur. Disse resultater ledte mig til at bruge denne art til at undersøge de underliggende mekanismer bag plastisk regulering af temperaturtolerancer i **ARTIKEL III** og **ARTIKEL IV**. Først viste jeg at *N. groenlandicus* hunner øjeblikkeligt ændrede deres tolerance til temperaturændringer og følger disse via kontinuer plasticitet. Derefter viste jeg i **ARTIKEL III** at fysiologiske responser til varme, men i mindre grad kulde, er synlige på transkriptom-niveau, når individerne udsættes for gradvis stigende eller faldende temperaturer under kontrollerede laboratorieforhold. Høje temperaturer medførte induktion af den traditionelle varmechok-respons og øget mængde vitellogenin-transkripter (som er essentielle for oocyt-vækst). Temperaturen som niveauet af disse gen-transkripter (samt andre varme-inducerede transkripter) blev øget ved var betydeligt lavere i felten sammenlignet med laboratoriet. Dette understreger vigtigheden af at validere resultater fra laboratorieeksperimenter i felten i den naturlige økologiske kontekst. Yderligere fandt jeg at individer indsamlet fra felten under de mest ekstreme temperaturforhold (højeste/laveste temperaturer, trods disse var relativt milde) var beriget for gen-transkripter som var relateret til translation, cellulær respiration og metabolisme, hvilket påpeger at energikravet var udfordret under disse miljøforhold.

I **ARTIKEL IV** viste jeg, at temperaturen i mikromiljøet havde en stor indflydelse på metabolitindholdet i individerne, og især kolde nattemperaturer resonerede i metabolitprofilerne i form af øgede niveauer af sukkerstoffer på tværs af alle testtidspunkter på den efterfølgende dag. Sukkerstoffer er blevet associeret med øget kuldetolerance gennem stabiliserende og kolligative egenskaber, og de observerede daglige forskelle i kuldetolerance kan muligvis tilskrives disse funktioner. Forskelle i niveauet af mellemprodukter fra metabolisme og aminosyrer på timelig basis understøttede transkriptom-profilerne fra **ARTIKEL III**, hvilken antydede at arter er udfordret på energikrav når de udsættes for store temperatureudsving.

Sidst præsenterer jeg resultater fra et igangværende *Drosophila melanogaster* studie som undersøger den kvantitativt genetiske basis bag daglig variation i varmetolerance og aktivitetsniveauer. De foreløbige resultater indikerer tids- og temperaturspecifik genetisk arkitektur af de undersøgte egenskaber, hvilket kan betyde potentielt stærke påvirkninger af den stigende temperaturvariabilitet på evolutionære udfald af disse egenskaber.

Opsummeret undersøger denne afhandling omfanget og hastigheden af fænotypisk plasticitet i forskelle mål for temperaturtolerance, og de fysiologiske modifikationer samt den genetiske arkitektur som faciliterer disse responser i insektarter i naturen.

TABLE OF FIGURES

TABLE OF CONTENTS

LIST OF PAPERS

PAPER I **Noer, N. K.**, Ørsted, M., Schiffer, M., Hoffmann, A. A., Bahrndorff, S.*, and Kristensen, T. N.* (2021). Into the wild – a field study on the evolutionary and ecological importance of thermal plasticity in ectotherms across temperate and tropical regions. *Philosophical Transactions of the Royal Society B*. 377, 20210004. doi: 10.1098/rstb.2021.0004

PAPER II Bahrndorff, S., Lauritzen, J. M. S., Sørensen, M. H., **Noer, N. K.***, and Kristensen, T. N.* (2021). Responses of terrestrial polar arthropods to high and increasing temperatures. *Journal of Experimental Biology*. 224, jeb230797. doi:10.1242/jeb.230797.

PAPER III **Noer, N. K.**, Nielsen, K. L., Sverrisdóttir, E., Kristensen, T. N.* and Bahrndorff, S.* (2022). Temporal regulation of temperature tolerances and gene expression in an arctic insect. (Submitted to *OIKOS*)

PAPER IV **Noer, N. K.**, Sørensen, M. H., Colinet, H., Renault, D., Bahrndorff, S., and Kristensen, T. N. (2022). Rapid adjustments in thermal tolerance and the metabolome to daily environmental changes – A field study on the arctic seed bug *Nysius groenlandicus*. *Frontiers in Physiology.* 13, 818485. doi:10.3389/fphys.2022.818485/bibtex.

* Shared last authorship

In addition, I will present and briefly discuss results from ongoing work entitled "Strong experimental evidence for diurnal variation in genetic architecture of behavior and heat tolerance traits revealed in *Drosophila melanogaster* under natural temperature conditions".

Papers presented in **PART A** of the thesis (2018-2020) and additional co-authored papers will listed in Appendix A.

INTRODUCTION

INTERPLAY BETWEEN ARTHROPODS AND THE THERMAL ENVIRONMENT

Terrestrial organisms, including invertebrates and plants, are particularly sensitive to changes in their thermal environment because of the direct link between their physiology and the ambient temperature (Huey and Kingsolver, 1989; Angilletta, 2009; Rodrigues et al., 2021). Temperature partly determines metabolic rates and cellular activity, which affect fitness and life history of the organism with consequences at the community and ecosystem levels (Helmuth et al., 2005; Sinclair et al., 2016). Hence, as the thermal environment varies on multiple spatial and temporal scales, the success of populations depends on the ability of individuals to anticipate and compensate for these changes within and / or across generations. In the absence of a proper phenotypic response to an abiotic stressor, or in the presence of a maladaptive response, a reduction in fitness can be measured which is referred to as "environmental stress" (Hoffmann and Parsons, 1991; Chevin and Hoffmann, 2017). Adjustment of the phenotype to meet environmental stressors can occur across generations by rapid transgenerational effects (1-10 generations) and by slower evolutionary adaption changing the genetic constitution of the population, or within the lifetime of the organism by phenotypic plasticity (Hoffmann and Sgró, 2011; Kristensen et al., 2020).

Species are continually exposed to fast changes in the environment on a short-term scale. Changes can occur on the scale of minutes to hours, such as the daily cyclic temperatures, or in a matter of days to months, including day-to-day temperature variation and strong seasonal temperature gradients. When encountered with severe short-term temperature changes, populations must respond through adaptive physiological, behavioral, or morphological adjustments (Hoffmann and Sgró, 2011). Such responses are termed *phenotypic plasticity* and defined as the ability of a genotype to produce different phenotypes when encountering distinctive environmental conditions (Scheiner, 1993; West-Eberhard, 2003). The phenotypic change can be induced by exposure to short-term, sub-lethal conditions referred to as "hardening" or "conditioning", or a longer-term exposure to non-lethal conditions experienced in the environment referred to as "acclimatization" or "acclimation" (Loeschcke and Hoffmann, 2002; Colinet and Hoffmann, 2012; Teets and Denlinger, 2013). These short-term responses can modulate traits strongly and thus affect survival to environmental stress or allow individuals to track and optimize their phenotypic performance with the diel and seasonal changes in the environment (Angilletta, 2009). On the long-term scale, anthropogenic climate change has caused temperatures to rise by ~1°C globally since pre-industrial times and this has been linked to observed shifts in species distribution and declines (Parmesan and Yohe, 2003; Lister and Garcia, 2018; Halsch et al., 2021). The rise in mean temperatures is projected to continue accompanied by more frequent, severe and prolonged events of extreme high temperatures (Easterling et al., 2000; Schär et al., 2004; Perkins et al.,

2012; Diffenbaugh and Field, 2013). In isolated populations that are unable to track the most suitable habitat in space, evolutionary adaptation is thus necessary to maintain high fitness or performance in the changing environment (Chevin and Hoffmann, 2017). Even though there are examples of rapid evolutionary adaptation occurring both in the field and in controlled environments at ecological timescales (reviewed by Hairston et al., 2005; Carroll et al., 2007), there is also evidence for slow and constrained evolution of populations to high temperatures and concomitant environmental changes, and thus limits to genetic responses to global change (e.g. Kellermann et al., 2009, 2012b, 2012a; Kelly et al., 2012; Araújo et al., 2013; Schou et al., 2014; Kristensen et al., 2015). Evolutionary responses depend, among others, on the amount of additive genetic variation present in populations, genetic correlations and linkage between traits, and interplay with other evolutionary forces (drift, gene flow, mutation etc.) (Frankham et al., 2004; Davis et al., 2005). In addition, because evolution occurs across generations it also depends on the development/generation time of the species. This can be problematic for some taxa and populations as generation time can last for several years as discussed in **PAPER II**, often in regions affected by high rates of warming compared to the global mean such as the Arctic. Phenotypic plasticity of temperature tolerance is in this context often highlighted as crucial for coping with increasing mean and variability in temperatures in future climates (Sørensen et al., 2016a). Hence, phenotypic plasticity will serve as the major focal point of this thesis.

STUDYING ENVIRONMENTAL STRESS IN THE FACE OF CLIMATE CHALLENGES

A central goal for ecological physiologists has been to understand the evolutionary and plastic adaptation of organisms to harsh and changing climates. With human-induced climate change, this knowledge on temperature-trait interactions has proven valuable for generating evolutionary and predictive models that can forecast organismal responses to global change (Kearney and Porter, 2009). The standard methodology for evaluating ecological consequences of temperature change has been to measure the performance of biological rates or fitness (enzyme kinetics, metabolic rate, locomotion, reproductive output etc.) of individuals as a function of body temperature (T_B). This relationship is described by Thermal Performance Curves (TPC, Figure 1) and is normally based on performance measured across a range of constant acute or chronic temperatures (Kingsolver and Buckley, 2017; Ma et al., 2021). Further, the measurements are generally limited to few taxa, often *Drosophila* spp., with short reproductive lifecycles and minimal requirements for maintenance (Chown et al., 2010; Huey et al., 2012). The TPC for traits generally take a universal shape where the performance rate rises slowly with temperature until reaching an optimum (T_{opt}) followed by a rapid drop. This non-linear and asymmetrical curve is only optimized at a short range of temperatures and performance delimited by the critical thermal minima (CT_{min}) and critical thermal maxima (CT_{max}).

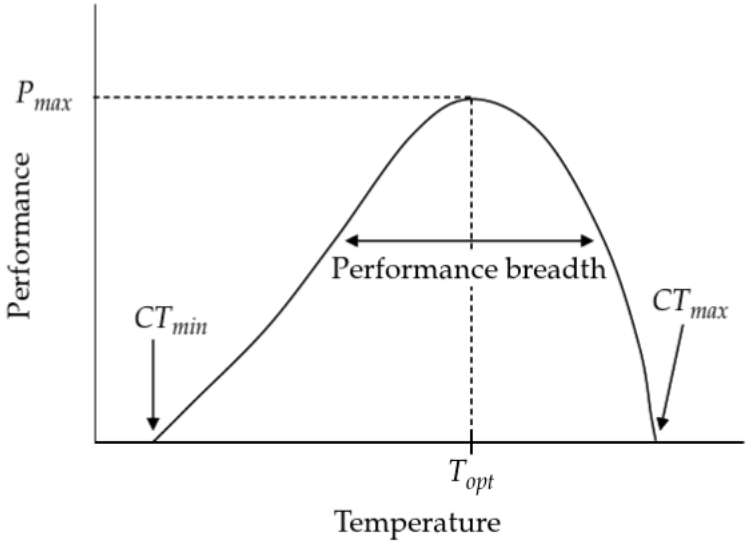


Figure 1: Performance curve of an ectotherm as a function of body temperature. The temperature at which performance is maximized (P_{max}) is termed the optimum body temperature (T_{opt}). The critical temperatures are the points at which performance is zero and defines the limits of the tolerance zone. Performance breadth is the temperature range where performance is above an arbitrary threshold, typically 80–90% (Angilletta, 2009).

Both natural selection and phenotypic plasticity can shape performance curves in a variety of ways: shifting the curve along the x-axis thereby adjusting T_{opt} to the new conditions; changing the performance breadth allowing performance over wider or narrower range of environments; or changing the height of the curve thereby maximizing performance (Schulte et al., 2011; Sørensen et al., 2016a). This knowledge on plastic and evolutionary impact on thermal performance is crucial for understanding species ability to cope with current and future climatic scenarios. However, there are several problems with this way of measuring and interpreting thermal performances. First, temperatures in nature are not static and an increasing number of studies show that thermal variability interacts with mean temperatures in their impact on thermal performance (Fischer et al., 2011; Colinet et al., 2015; Vázquez et al., 2017). In **PART A**, I used three taxonomically distinct species (*Arabidopsis thaliana*, *Drosophila melanogaster*, and *Orchesella cincta*) to demonstrate that fluctuating temperatures within a non-stressful thermal range will benefit thermal performance compared to constant temperature acclimation due to Jensen's inequality (**Noer et al., 2020; APPENDIX A**). Briefly, this theory describes how the temperature mean interact with fluctuations because the TPC is left-skewed

and T_{opt} is closer to the upper threshold for performance. This means that at low mean temperature, the gain from fluctuation towards T_{opt} will be disproportionately high because of the steep increase of the performance curve. Contrary, if the mean temperature is around T_{opt} or higher, then the net gain of fluctuations will be negative, because performance quickly declines when temperatures surpass T_{opt} (Ruel and Ayres, 1999; Vasseur et al., 2014; Colinet et al., 2015). In our study, the theoretical consideration held true for two species, *D. melanogaster* and *A thaliana*. These experienced a significant increase in the survival proportion after exposure to lethal temperature stress compared to individuals acclimated to constant temperatures with the same mean temperature. Several empirical studies have examined this relationship between temperature mean and variability for thermal adaptation in different traits including growth rate, development time, reproduction (Siddiqui and Barlow, 1972; Brakefield and Kesbeke, 1997; Pétavy et al., 2004; Kingsolver et al., 2009, 2015; Fischer et al., 2011; Cavieres et al., 2018), and stress resistance (Bozinovic et al., 2011; Manenti et al., 2014, 2017; Boher et al., 2016; Sørensen et al., 2016b; Salachan and Sørensen, 2017). These have shown that the effect of thermal fluctuations will depend on the properties of the thermal regime (mean, amplitude, duration, etc.), the interaction with other environmental factors, and the thermal sensitivity of the traits measured. Therefore, it is important to study the combined effects of variability and averages to increase our understanding of adaptation to variable environments (Kingsolver et al., 2015). However, the impact of thermal fluctuations can be even more complex as also shown by other studies (Terblanche et al., 2010; Bozinovic et al., 2011; Vasseur et al., 2014). This was substantiated by our own results, that found no effect of temperature fluctuations for the last species, *O. cincta* (Noer et al., 2020, Appendix A). I hypothesized that the local adaptation to their microhabitat and the behavioral thermoregulatory ability of the three species determined the outcome of acclimation. Plants and fly pupal stages have low capacity to buffer short-term changes in temperature using behavior and they may therefore be subject to a higher selection pressure for plasticity (Stevenson, 1985; Huey et al., 2002; Huey and Tewksbury, 2009). Contrary to these findings, the springtail, *O. cincta*, which occupy a buffered and invariable environment showed little phenotypic adjustments to fluctuating temperatures. It is therefore important to test theoretical considerations in non-model species systems.

Another complication that arises from constant temperature acclimation is that mismatches in responses can be a consequence of either lowered performance at constant temperature due to thermal stress from the chronic exposure or due to beneficial acclimation during the chronic exposure. For instance, Niehaus et al. 2012 showed that empirical models based on constant rearing temperature of the frog *Limnodynastes peronii* poorly predicted performance of growth and development at fluctuating temperatures and that this could be explained by chronic stress from rearing at constant temperatures. This underpins that methodological procedures affect the outcome of temperature treatments on performance. There is an infinite number of possible combinations of treatments to examine plastic capacity including different acclimation/hardening temperatures, exposure times, recovery times, assays, traits etc. The outcome of these treatments can differ substantially, especially when

considered in concert with other abiotic or biotic stressors. Unfortunately, logistics often limit the number of feasible treatments to include in experiments. Estimates of plastic scope are therefore often based on few acclimation/hardening temperatures or exposure times in a single stressor experimental setup (Chown et al., 2009; Rezende et al., 2014; Pintor et al., 2016). Consequently, the choice of treatments must be considered carefully, and results be interpreted with caution to avoid misleading conclusions on ecological and evolutionary outcomes (Sørensen et al., 2016a; Salachan et al., 2019). Likewise should the choice of assay reflect the research question investigated. For instance, using 10 *Drosophila* species we showed that dynamic heat ramping assays better separate species differences in heat tolerance, whereas static heat knockdown assays better disclose subtle differences in heat tolerances due to acclimation (**Bak et al. 2020, Appendix A**). All of the abovementioned complications suggest that results obtained under constant laboratory conditions may not reflect the situation in natural systems and limit the usage of data from laboratory studies to make prediction to the field. This, is supported by an increasing amount of evidence from field studies implying that laboratory results on thermal plastic adaptation can be misleading to findings under natural conditions (e.g. Brakefield and Kesbeke, 1997; Koveos, 2001; Kelty, 2007; Loeschcke and Hoffmann, 2007; Kristensen et al., 2008; Overgaard and Sørensen, 2008; Schou et al., 2015; Gleason et al., 2017; Jensen et al., 2019). In this thesis, I focus on addressing the latter concerns, hence I wanted to understand the extent of and the temporal scale at which acclimatization and hardening occurs in species in their natural environments, if we can generalize these thermal plasticity patterns across taxa, and provide an ecological context for these observations. Further, I aimed at improving our understanding of the underlying basis behind field-based plastic responses.

PLASTICITY IN REAL TIME

In **PAPER I,** I examined the extent to which thermal plasticity to daily temperature variation occurs in non-model insect populations in temperate and tropical regions. This was examined by collecting individuals from the field belonging to a range species with either temperate or tropical origin followed by immediate test for heat knockdown time. The collection and thermal assessment proceeded over multiple days and time points within days for each species. Common for this paper, and all papers presented as part of my thesis, is the emphasis on the ecological relevance of the experimental designs. The field experiments are designed to test the effects of temperature variation on performance at multiple timescales in the ecological context that the species are realizing. Hence, the plastic responses to temperature variation were measured as they are shaped by the interaction with multiple abiotic (soil moisture, humidity, light etc.) and biological (food availability, competition, predation, behavior etc.) factors (Sinclair et al. 2016). Some of these factors are known to impact thermal performance either in isolation or in concert with each other (e.g. Coulson and Bale, 1991; Holmstrup et al., 2002; Benoit et al., 2009; Fischer et al., 2010, 2014; Bubliy et al., 2012, 2013; Sinclair et al., 2013; Chidawanyika et al., 2017). Other factors, which currently do not represent stressors, could become so when

experienced in combination with other factors, because the cost of mounting the response to one stressor might compromise the ability to cope with additional stressors (Schulte et al. 2014). These considerations should stress the importance of supplementing laboratory investigations on thermal performance with examinations in the natural environments of the species.

In **PAPER I**, the original idea was threefold. First, I wanted to examine the extent of plastic thermal adaptation in the field as a response to natural environmental variation. This was partly to investigate the plastic ability of a range of non-model species to confirm the importance of plasticity in nature, and partly to examine how the impact of natural acclimatization and hardening affects thermal tolerances to provide an ecological context of plastic responses. Second, I investigated what climate variables that are important for inducing plastic responses in heat tolerance and the temporal scale of induction. This should enable a better understanding of time needed to induce acclimatization and hardening responses in laboratory studies. Third, I wanted to examine differences in plastic scope related to species origin to get an idea as to whether adaptation to variable environments results in more plastic phenotypes (discussed in section on adaptation to variable environments).

The results from **PAPER I** suggested that high levels of plasticity in heat tolerance occurred within and across days for all species, no matter the region of origin. All species showed some sort of plastic environmental tracking and continually adjusted their heat tolerance, most often in a quadratic shape throughout the days similar to the course of daily temperature variation. From laboratory experiments we know that short-term exposures to environmental stress can induce such rapid adaptive hardening responses that can alter thermal tolerances to reduce injury from potentially lethal stress (Bowler, 2005; Terblanche et al., 2007; Sgrò et al., 2016). However, our results suggest that plastic adjustment is a continuous process in nature, which might help insect optimizing performance at the various temperatures encountered throughout the day. Sublethal temperature stress is seemingly not required to induce rapid responses, instead temperature likely interact with other environmental or biological factors (mentioned above) to contribute to these daily patterns of heat tolerance adjustments.

This continuous process was however not only affected by both short-term (test time) changes in abiotic/biotic conditions, but in interaction with long-term (test day) changes for many of the tested species. The use of single or few laboratory treatments to examine the scope for plasticity in heat tolerance could therefore be misleading to findings in nature. Using the same field sampling approach, I also showed a significant effect of sampling time and day on both heat and cold tolerances in the arctic bug *Nysius groenlandicus* in **PAPER IV**. The temperature immediately prior to sampling individuals from the field was correlated with heat and cold tolerances, but this relationship was not evident for male bugs when the data was corrected for daily temperature in **PAPER III**. Thus, acclimatization and hardening effects should be considered together in order to understand the full scope for plasticity. It is however important to note that the full potential for plasticity might not have been uncovered for the individual species in these studies because only a small range of the

environmental conditions that the species face in their habitat was investigated. Likewise, the studies only subjected the individuals to temperatures within the current climate and limits our ability to make predictions of responses to future climates.

In an attempt to pinpoint the climatic variables of importance and temporal scale at which they impact on heat tolerance in **PAPER I**, I showed that association between heat tolerance and climatic variables is very complex. The magnitude and direction of correlations between temperature or humidity with heat tolerances were species- and sex-specific. Generally, the mean temperature at the collection time was a strong driver of plasticity in temperate insects, but the environmental factors driving plastic change in tropical species were less obvious. However, it seemed that the impact of humidity was especially important for tropical species, which has been shown in other studies as well (e.g. Kellermann et al., 2009; Fischer et al., 2014; Fischer and Kirste, 2018; Gigante et al., 2021). As for temperate species, I found a strong impact of mean temperature at the collection time for the arctic *N. groenlandicus* in **PAPER I, III and IV.** There has generally been a lack of strong association between the abiotic environment and heat tolerances in literature. Several studies have argued that this is because insects utilize fine-scale habitat temperature variation for optimizing performance, which disconnect their thermal biology from the coarse resolution of the climatic variables typically measured (Potter et al., 2013; Gutiérrez-Pesquera et al., 2016; Pincebourde et al., 2016). Though I endeavored to measure the microhabitat temperature in **PAPER I, III and IV** with dataloggers placed just above the soil surfaces, some studies are pointing to extreme microscale temperature heterogeneity, such as 12°C temperature gradient on apple leaves, which is exploited by some species (Pincebourde and Suppo, 2016; Pincebourde et al., 2016; Pincebourde and Casas, 2019; Poitou et al., 2021). Exploiting extreme microclimatic variation could be of high importance for tropical species living in highly biodiverse environments where niches are readily occupied by competing species. Thus, it is plausible that thermoregulatory behavior at extreme microspatial scale account for the large daily plastic responses in tropical species but disconnect these from ambient air temperatures.

The results from **PAPER I** have implications for the way that we model and predict species range shifts and vulnerabilities with climate change. Hence, in order to improve the accuracy of predictive models, we need to consider fine-scale environmental variation and phenotypic plasticity. While such data on heat tolerance and plasticity is available for a large number of temperate species in Europe and North America, almost no data exist on species from higher latitudes (Seebacher et al., 2015a). This is problematic as species at tropical and polar regions are deemed most vulnerable to changes in temperature due to already high average temperatures in the Tropics and accelerated rate of climate change in polar regions (Deutsch et al., 2008; Sunday et al., 2011). In **PAPER** II, I synthesized existing knowledge on terrestrial polar and subpolar arthropods' sensitivity to high and increasing temperatures and extended this knowledge with empirical results. I found 10 studies reporting CT_{max} for different polar species. Few other studies documented heat tolerances based on various experimental protocols and in concert with different climatic conditions, but

comparison of such data are difficult. In addition, only few of these studies report on plasticity in CT_{max}. Hence our understanding on polar species' responses to climate change is limited so far. Overall, these deficits in data confuses the conclusion on the outcome of warming temperatures on polar ecosystems. Thus, increasing temperatures could either benefit species at high latitudes due to alleviation of cold stress and extension of the short growing season, or contrary result in costs from increased metabolic rate, heat/desiccation stress and phenological mismatches. It is therefore difficult to assess who will be most threatened to climate change with current knowledge.

ADAPTATION TO VARIABLE ENVIRONMENTS

In the previous chapters I focused on the methodological aspects of measuring thermal plasticity and the climatic variables of importance for precise estimation of species vulnerabilities. In this section I will focus on evolutionary aspects of heat tolerance and plasticity of heat tolerances.

Evolution, i.e. change in the genetic constitution of a population, is limited by the available additive genetic variation expressed for the traits that selection act on and by the selection intensity (Falconer and Mackay, 1996). There are numerous examples of evolutionary constraints in heat resistance and central life-history traits in arthropods (e.g. Kellermann et al., 2009; Mitchell and Hoffmann, 2010; Kelly et al., 2012, 2013; Hoffmann et al., 2013; Kristensen et al., 2015). Conclusions on evolutionary constraints are often based on laboratory selection experiments imposing artificial selective pressures on traits hypothesized to be of importance to cope with climate change, or based on measures of evolutionary potential, often inferred by estimates of trait heritability derived from one or few environments. Research has tended to focus on heat and cold stress survival with the notion that these will be decisive of the faith of populations with climate change (Saxon et al., 2018). However, temperature variation within existing temperature ranges may have sublethal impact on survival and many of these sublethal traits seem to be more paramount to fitness.

In addition, the ability to withstand heat stress varies in a diurnal fashion over a range of environments in insect species including *D. melanogaster* according to **PAPER I**, **III**, and **IV**. We have little knowledge about the genetic background for this variation and to what extent it can evolve. In the **Additional Results** I present preliminary results from an ongoing experiment using the *Drosophila melanogaster* Genetic Reference Panel (DGRP) to examine the genetic architecture of behavioral traits under variable temperature conditions, how daily temperature fluctuations affect the ability to perform under stressfully high temperatures, and the heritability of locomotor performance and heat resistance under high temperatures. The DGRP consists of 205 genome-wide homozygous lines derived by 20 generations of consecutive full-sib mating of wild-caught flies (Mackay et al., 2012). Because the genome sequence data of the DGRP lines are publicly available, the genetic basis for any quantitative trait phenotype can be investigated using this panel (Mackay et al., 2012), which I aim to do in future analyses.

The preliminary results show that the heritability of locomotor activity under semi-natural field conditions varies greatly across the day, and with a non-linear relationship with field temperatures. These results indicate that the genetic architecture of this behavioral trait is time and / or temperature specific and highlight the need to test performance at multiple environments in order to determine the potential for evolutionary responses to temperature variation and extremes (Hoffmann and Sgró, 2011). Further, the heritabilities of heat tolerance and different locomotor traits (total activity, peak size) under heat stress varies with the environmental conditions experienced prior to the thermal stress. These traits showed relatively strong positive genetic correlations suggesting that selection on heat tolerance will affect locomotor activity in the same direction, thus the traits seem not to constrain each other.

With the remaining dataset from this experiment, I will be looking further into the evolutionary potential of plasticity in the examined traits. Trait plasticity is often defined as the slope of the phenotype of specific genotype as a function of the environment (termed 'reaction norm'), whereas the trait mean is defined by the y-intercept of the reaction norm. Both the trait mean and trait plasticity is controlled by different genes and consequently capable of responding to selection (Scheiner and Lyman, 1989; Stearns et al., 1991; Ketola et al., 2014; Manenti et al., 2015; Sørensen et al., 2016b; Ørsted et al., 2018). The evolution of plasticity is regarded as extremely important for species persistence in the context of environmental change because plasticity can allow species to respond to sudden stressful conditions, but also utilize novel conditions and facilitate range shifts and adaptation (Matesanz et al., 2010; Valladares et al., 2014; Murren et al., 2015).

Theory specify that temperature variability should select for increased thermal plasticity (Schlichting, 1986; Pigliucci, 2005; Ghalambor et al., 2006; Matesanz et al., 2010; Lande, 2014). These theoretical considerations suggest that high levels of temporal heterogeneity will lead to selection for increased capacity for phenotypic plasticity if 1) the environmental cues for change are reliable (Reed et al., 2010; Scheiner, 1993; but see Manenti et al., 2017), 2) the heterogeneity occurs at a timescale relevant for the lifespan of the organism, and 3) genetic variation for plasticity in the given trait is present (Matesanz et al., 2010; Oostra et al., 2018; Scheiner, 1993; Sgrò et al., 2016). Empirical support for this hypothesis is however few, e.g. studies have found that artificial selection for plasticity in thermally fluctuating environments does not increase levels of plasticity, and contrarily, selection in constant environments does not result in lost ability to induce plastic responses in several life history- and stress resistance traits (Kingsolver et al., 2009; Van Heerwaarden and Sgrò, 2011; Manenti et al., 2015, 2017).

Other studies have, based on this hypothesis, suggested that increasing seasonality with increasing latitude from equator should select for plastic phenotypes better able to cope with thermal fluctuations (Gaston and Chown, 1999; Addo-bediako et al., 2000; Ghalambor et al., 2006). Organisms living at low latitudes, e.g. tropical regions, are then expected to be sensitive to even small increases in temperatures because they have evolved within narrow and relatively stable thermal ranges and are consequently

less plastic, but also because they live at temperatures closer to their upper thermal limits (thermal safety margin) (Deutsch et al., 2008; Tewksbury et al., 2008; Sunday et al., 2014). However, the results from such studies are conflicting and support for this hypothesis has generally only been consistent for plants and marine organisms and not terrestrial ectotherms (Sgrò et al., 2010; Overgaard et al., 2011; Gunderson and Stillman, 2015; Seebacher et al., 2015b; Schou et al., 2017; Kellermann and Sgrò, 2018; MacLean et al., 2019). In **PAPER I**, I found no relationship between the levels of plasticity and origin of the tested species (temperate/tropical), but the interspecific levels of HKDT and CT_{max} differed across all species. No hard conclusions can be based on the comparison of the species in this paper, because the species were not tested under common garden conditions, I did not account for developmental or adult acclimation, nor controlled for phylogeny. However, it provides a great insight into the daily plastic scope to temperatures across a range of species to supplement typical examinations of *Drosophila* species. These results, and results from our previous work (**Noer et al. 2020; Appendix A**) indicate that species differences in heat tolerance and plasticity in terrestrial environments might be owing to the occupied microhabitats and thermoregulatory capability. This aspect was also considered briefly in **PAPER II**.

Arctic and subarctic regions provide some interesting environments for studying local adaptations to temperatures and the effects of temperature extremes on physiology. These regions are characterized by high temperature variability within and across days and habitats, and temperatures can span from sub-zero at night to daily peaks of 30–40°C in the summer. In **PAPER II**, the compilation of CT_{max} values from studies on polar species and our own data provided CT_{max} on 39 species. The tolerances ranged extensively with 20.7°C difference in the lowest and highest CT_{max} value. To examine local adaptation, the species were grouped into two categories: 1) day/night active, and 2) habitat type, i.e. airborne, surface- and soil-dwelling species. The CT_{max} values were lowest for soil-dwelling species and highest for surface-dwelling, and higher for day active arthropods compared to night-active. These findings suggest that species exposed to the diurnal temperature fluctuations of the surface environment have adapted their intrinsic heat tolerance and / or plasticity of heat tolerance. This is substantiated by other studies showing great fine-scale local differences in physiology (Spacht et al., 2021). However, there was almost no work done on plasticity of heat tolerances in the reviewed literature. In **PART A**, I presented results on the plastic scope for heat tolerance in the subarctic arthropod, *Nysius groenlandicus* (**Sørensen et al. 2019; Appendix A**). The species belongs to the group of surface-dwelling day active arthropods, and I hypothesized that *N. groenlandicus* is adapted to the highly variable micro-environment in polar regions where coping with both high and low temperature extremes on a daily scale is necessary for survival. The species had extremely fast and strong plastic response to high temperature hardening / conditioning and the response was only detectable using acute temperature exposure assays because it induced its hardening response during dynamic assays. Because of these remarkably rapid and large phenotypic responses to temperature changes, I

decided to use this species as a model to understand some of the underlying mechanisms behind plastic responses in **PAPER III** and **IV**.

MECHANISMS OF PLASTICITY – FROM GENES TO PHENOTYPE

I have now discussed physiological and evolutionary impacts of natural temperature variation on species. In the following section, I will focus on the functional understanding of species plasticity to temperature changes and temperature stress in organisms. As I have discussed in the previous sections, plastic responses and thermal tolerances are often idiosyncratic and our understanding of their impact on ecological systems elusive. Exposing the mechanisms underlying plasticity and stress responses is an important aspect of understanding how species will cope with climate change. Moreover, it can be a useful tool to pinpoint molecular markers that reveals the state of the individual or population and perhaps be used to make generalizations across species, or groups of species, inhabiting specific environments.

The mechanisms behind plastic responses to temperature acclimation and stress have been studied widely for species acclimated to constant laboratory conditions. Some core cellular stress responses have been identified to function across most taxa and environments, including molecular chaperone activity, changes in membrane lipid composition, altered energy metabolism, and build-up of cryoprotectants / osmoprotectants (reviewed by Hazel, 1995; Feder and Hofmann, 1999; Kültz, 2005). Hence, it is evident that features of thermal acclimation and stress are manifested at various levels of biological organization, from molecules and membranes to whole organisms and ecosystems. Studies should therefore aim at integrating multi-omic levels investigations to gain holistic information on organism's cellular biology (Pörtner et al., 2007).

In **PAPER III** and **PAPER IV**, the initial idea was to use *N. groenlandicus*, which inhabits an environment that frequently reaches cold and warm extremes, as a model-system for dissecting the effects of environmental variation on physiology through investigation of the transcriptome and metabolomic fingerprint. Transcriptomics is the characterization of the momentarily presence and quantity of RNA transcripts in a biological sample (Alvarez et al. 2015). Using high-throughput technology such as RNA sequencing, mRNA transcripts, i.e. protein coding transcripts (~1% of RNA), can be enriched for the purpose of comparing differential regulation in response to e.g. environmental stimuli across samples. The mRNA levels ultimately contribute to the formation of proteins, cellular phenotype, and organismal phenotype, however, the interpretation is complex because of feedback loops, posttranslational modifications, and mRNA instability and translation rates (Hayward, 2014; Alvarez et al., 2015). Metabolomics constitutes the profiling of the complete set of substrates and products of metabolism in a biological sample that drive essential cellular functions. These include energy productions and storage, signal transduction and apoptosis. Hence, the metabolome provides a much closer link to the phenotype compared to transcriptomics or proteomics (Johnson et al., 2016). However, due to the high variability of the elemental composition of metabolites, the requirements for comprehensive and quantitative analysis are complex. Therefore, several methods are

needed to gain insight into the whole metabolome and studies examining metabolites often only include a fingerprint or a subset of metabolites (Hayward, 2014).

In **PAPER III**, I used RNA sequencing to examine the transcriptome response of *N. groenlandicus* to changes in the environment. This was done by investigating the effects of gradual cooling/heating of laboratory acclimated individuals in order to understand their stress physiology, and second to link these transcriptomic responses to individuals caught under natural field conditions over different timepoints and days. I found that responses to gradual heating, but not freezing, are visible at the transcriptomic level. This is in agreement with other studies that have argued that RCH is rapid and therefore relies on existing gene products rather than synthesis of new. Instead, responses are mediated by post translational modification of proteins and cell signaling (Sinclair et al., 2007; Teets et al., 2008, 2012, 2013). This calls for investigation of cold responses at other cellular levels (Hayward, 2014). In our study, the gradual heating activated a plethora of chaperones, many of which have been interpreted as causative of increased heat tolerance in insects (Sørensen et al., 2003; King and Macrae, 2015). Further, two vitellogenin transcripts were up-regulated at high temperatures, which is in contrast with findings showing that vitellogenins are not temperature sensitive in *Bicyclus anynana* (Geister et al., 2008). Vitellogenins are imperative for oocyte growth and completion of the reproductive cycle but has also been associated with antioxidant properties (Seehuus et al., 2006; Havukainen et al., 2013). These transcripts, and chaperones, might explain the extremely rapid and large plastic heat hardening response that was previously observed for this species under controlled laboratory conditions (**Sørensen et al., 2019; APPENDIX A**), though not validated here. In order to infer the function of genes present in an organism, an important step is to reveal context-specific expression in the species' natural habitat (Pavey et al., 2012). Comparison between laboratory treated and field collected individuals showed that the induction temperature of abovementioned responses in the laboratory was much lower in the field collected individuals, showing the importance of ecological context on short-term transcriptional responses. Besides a large overlap in the number of heat induced genes in the laboratory and field generated transcriptomes, I found some unique differences in gene expression within individuals sampled across the largest temperature gradient in the field; i.e. from morning to noon of the second field day. This shift in temperature was associated with increased levels of polyubiquitin-associated gene transcripts and cytoskeletal components, maybe related to repair of cold damage (Teets et al., 2012). Finally, the largest differences observed between days were related to cellular respiration, thus energy expenditure is highly affected by differences in environmental temperature.

In **PAPER IV**, I applied Gas Chromatography Mass Spectrometry (GC-MS) to a set of field samples collected simultaneously with the set of field samples from **PAPER III.** GC coupled with MS is a method for identifying and quantifying volatile compounds. GC vaporizes and separates substances into individual components under heat, whereafter MS detect the substances by their mass (Fiehn, 2002). With this method, 33 metabolites in the form of non-structural carbohydrates, polyols, amino and organic acids was detected. I found that in terms of metabolites, the metabolic fingerprints were more distinct (non-overlapping) on the test day characterized by variable field temperatures and the greatest separation of metabolite profiles were

found at the sampling times with the greatest difference in environmental temperature (as for the transcriptomic profile). The separation of metabolic profiles on an hourly basis (between sampling times) was largely driven by differences in some amino acids and metabolic intermediates. These findings complement the mRNA profiles showing increased translational activity, cellular respiration and metabolism between time points. Finally, the sugars galactose and glucose differed by a factor two across experimental days. Based on the experimental procedure in this study, it cannot be concluded if this is a consequence of temperature or other abiotic/biotic factors, e.g. increased sap feeding to compensate for metabolic demands under more stressful conditions. But as discussed in the **PAPER IV**, rapid cold hardening and cold shock responses are associated with sugar accumulation. In our study the sugars accumulated at the coldest timepoint in the night and remained high throughout the day as found by others (Overgaard et al., 2007), which might explain the interaction between 'day' and 'time' on thermal tolerance. This indicated that cold hardening is of ecological importance for this species.

CONCLUSIONS AND PERSPECTIVES

Insect populations are threatened by multiple anthropogenic stresses and in the light of rapid climate change and species declines there is a need to improve the assessment of effects of environmental stress on species. Studies have often focused on effect of constant rearing regimes and single stressor impact on species, largely ignoring natural thermal regimes, multiple stressors, and ecological context. Thus information on species responses to temperature fluctuations **(Noer et al., 2020)**, the climatic variables and temporal resolution of responses across tropical, temperate (**PAPER I**) and polar (**PAPER II**, **III**, and **IV**) environments is important for our understanding and projection of the impact of environmental stress on species and ecosystem with environmental change.

In **PAPER I,** I concluded that high levels of plasticity in heat tolerance occurred across all the tested species. Both short- and long-term variability of temperature and humidity affected plastic adjustments of heat tolerance within and across days, but with species differences. Our results revealed that plastic changes in heat tolerance occur rapidly at a daily scale and that environmental factors on a relatively short timescale are important drivers of the observed variation in thermal tolerance. These results have several implications. First, they highlight that the current use of single constant laboratory treatments for examination of plasticity scope could result in misleading conclusions about species vulnerability to temperature changes. Second, climatic variables and the temporal scale of variability that determine plastic responses are species- and sex-specific and generalizations based on *Drosophila* studies cannot be applied.

The consequences of rapidly increasing temperatures for terrestrial insects in arctic and Antarctic regions are uncertain due to paucity in baseline inventories and systematic monitoring as highlighted in **PAPER II**. However, some studies indicate significant decline in several arthropod species groups, and increase in other groups, since the beginning of monitoring efforts, likely due to indirect climate-driven habitat

changes e.g. (Schmidt et al., 2016; Bowden et al., 2018; Koltz et al., 2018; Gillespie et al., 2020; Høye et al., 2021) or direct effects of warming and increasingly variable and less predictable temperatures (Vasseur et al., 2014; Dillon et al., 2016). We are thus in urgent need to increase our understanding of the capacity of terrestrial arthropods from polar regions to respond to future temperature warming and understand the costs of increasing temperatures on sublethal traits. As specified in **PAPER II**, however, this can be challenging as data on high temperature performance is missing and costs of high temperatures on sublethal trait can be challenging to measure because we do not understand the fundamental biology of most species in these regions. It has proven laborious to maintain and encourage reproduction in arctic non-model insects in captivity which limits our ability to conduct controlled laboratory experiments. Despite of this, this rapidly changing environment provides unique opportunities to disentangle effects of climate change on ecosystems due to the low biodiversity, simple trophic complexity, and highly variable thermal conditions.

In **PAPER III** and **IV**, I used molecular techniques to unravel the 'status' of the arctic insect *N. groenlandicus* through a time series sampling of individuals from the field. From **PAPER III**, I concluded that daily large temperature fluctuations in the field was associated with many heat stress induced gene transcripts even at relatively benign daily peak temperature in the field. These results indicated that increasing temperature variability and more frequent occurrence of warm peaks can have costs to *N. groenlandicus* and likely other arctic species as well. In **PAPER IV,** I concluded that the phenotypic responses to temperature fluctuations were visible at the metabolic level, with large modifications in sugar content across time of day and days, but more subtle changes in amino acids and metabolic intermediates within days. These findings supported that within-day environmental fluctuations were associated with increased translation activity, breakdown of proteins, cellular respiration and lipid metabolism, suggesting high costs of maintaining normal functioning of the organism. Though challenging, the results from these studies suggest that future studies should aim at examining if there are costs associated with temperature fluctuations and extreme high temperatures, and the consequence of repeated exposure to high temperature variability on fitness should be examined.

In addition to these findings, I also found several correlative patterns of gene and metabolite regulations that can be crucial for responding to heat and cold stress in the field environment. For example, sugars seem to respond to cold temperatures and have previously been associated with increased cold resistance through colligative and stabilization of macromolecules and membranes. Further, in **PAPER III** the heat stress response was not only associated with excessive expression of HSPs, but also apolipoproteins, which might be crucial for heat tolerance in arctic insects.

In order to validate the causative effect of these products we should however move beyond the correlative patterns. With current technology it is becoming feasible to design experiments that manipulates e.g. transcription in non-model species, including transgenics, RNAi, and CRISPER/Cas. An imperative step will thus be to validate the importance of e.g. HSPs or vitellogenin for heat tolerance for instance by

gene knockdown or knockout experiments. At the metabolomics level, validation can be obtained from e.g. dietary manipulation or direct metabolite injections on environmental stress resistance (Sømme, 1968; Wolfe et al., 1998; Benoit et al., 2009; Koštál et al., 2016; Bujan and Kaspari, 2017; Toxopeus et al., 2019). This has for instance proven efficient in documenting the effect of trehalose as a cryoprotectant that increase cold shock survival the Antarctic midge *Belgica antarctica* (Benoit et al., 2009). However, I argue that an important step on the way is to identify the impact of these manipulated gene or metabolite products in concert with ecologically relevant traits in a natural setting to refine our understanding of well-known and novel genetic pathways of interest in model and non-model species.

REFERENCES

Addo-bediako, A. A., Chown, S. L., and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. R. Soc. B Biol. Sci.* 267, 739–745. doi: 10.1098/rspb.2000.1065

Alvarez, M., Schrey, A. W., and Richards, C. L. (2015). Ten years of transcriptomics in wild populations: What have we learned about their ecology and evolution? *Mol. Ecol.* 24, 710–725. doi:10.1111/mec.13055.

Angilletta, M. J. (2009). "Thermal acclimation," in *Thermal Adaptation: A Theoretical and Empirical Synthesis* (Oxford University Press: New York), 126–156.

Araújo, M. B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P. A., Valladares, F., and Chown, S. L. (2013). Heat freezes niche evolution. *Ecol. Lett.* 16, 1206–1219. doi:10.1111/ele.12155.

Benoit, J. B., Lopez-martinez, G., Elnitsky, M. A., Lee, R. E., and Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comp. Biochem. Physiol. Part A.* 152, 518–523. doi:10.1016/j.cbpa.2008.12.009.

Boher, F., Trefault, N., Estay, S. A., and Bozinovic, F. (2016). Ectotherms in variable thermal landscapes: A physiological evaluation of the invasive potential of fruit flies species. *Front. Physiol.* 7, 302. doi:10.3389/fphys.2016.00302.

Bowden, J. J., Hansen, O. L. P., Olsen, K., Schmidt, N. M., and Høye, T. T. (2018). Drivers of inter-annual variation and long-term change in High-Arctic spider species abundances. *Polar Biol.* 41, 1635–1649. doi:10.1007/s00300-018-2351-0.

Bowler, K. (2005). Acclimation, heat shock and hardening. *J. Therm. Biol.* 30, 125–130. doi:10.1016/j.jtherbio.2004.09.001.

Bozinovic, F., Bastias, D. A., Boher, F., Clavijo-Baquet, S., Estay, S. A., Angilletta Jr., M. J., et al. (2011). The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiol. Biochem. Zool.* 84, 543–552. doi:10.1086/662551.

Brakefield, P. M., and Kesbeke, F. (1997). Genotype-environment interactions for insect growth in constant and fluctuating temperature regimes. *Proc. R. Soc. B Biol. Sci.* 264, 717–723. doi:10.1098/RSPB.1997.0102.

Bubliy, O. A., Kristensen, T. N., Kellermann, V., and Loeschcke, V. (2012). Plastic responses to four environmental stresses and cross-resistance in a laboratory population of *Drosophila melanogaster*. *Funct. Ecol.* 26, 245–253. doi:10.1111/j.1365-2435.2011.01928.x.

Bubliy, O. A., Kristensen, T. N., and Loeschcke, V. (2013). Stress-induced plastic responses in *Drosophila simulans* following exposure to combinations of temperature and humidity levels. *J. Exp. Biol.* 216, 4601–4607. doi:10.1242/jeb.092502.

Bujan, J., and Kaspari, M. (2017). Nutrition modifies critical thermal maximum of a dominant canopy ant. *J. Insect Physiol.* 102, 1–6.

doi:10.1016/J.JINSPHYS.2017.08.007.
Carroll, S. P., Hendry, A. P., Reznick, D. N., and Fox, C. W. (2007). Evolution on ecological time-scales. *Funct. Ecol.* 21, 387–393. doi:10.1111/J.1365-2435.2007.01289.X.
Cavieres, G., Bogdanovich, J. M., Toledo, P., and Bozinovic, F. (2018). Fluctuating thermal environments and time-dependent effects on fruit fly egg-hatching performance. *Ecol. Evol.* 8, 7014–7021. doi:10.1002/ECE3.4220.
Chevin, L. M., and Hoffmann, A. A. (2017). Evolution of phenotypic plasticity in extreme environments. *Philos. Trans. R. Soc. B Biol. Sci.* 372, 20160138. doi:10.1098/RSTB.2016.0138.
Chidawanyika, F., Nyamukondiwa, C., Strathie, L., and Fischer, K. (2017). Effects of thermal regimes, starvation and age on heat tolerance of the parthenium beetle *Zygogramma bicolorata* (Coleoptera: Chrysomelidae) following dynamic and static protocols. *PLoS One.* 12, e0169371. doi:10.1371/JOURNAL.PONE.0169371.
Chown, S. L., Hoffmann, A. A., Kristensen, T. N., Angilletta, M. J., Stenseth, N. C., and Pertoldi, C. (2010). Adapting to climate change: A perspective from evolutionary physiology. *Clim. Res.* 43, 3–15. doi:10.3354/cr00879.
Chown, S. L., Jumbam, K. R., Sørensen, J. G., and Terblanche, J. S. (2009). Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. *Funct. Ecol.* 23, 133–140. doi:10.1111/J.1365-2435.2008.01481.X.
Colinet, H., and Hoffmann, A. A. (2012). Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. *Funct. Ecol.* 26, 84–93. doi:10.1111/j.1365-2435.2011.01898.x.
Colinet, H., Sinclair, B. J., Vernon, P., and Renault, D. (2015). Insects in fluctuating thermal environments. *Annu. Rev. Entomol.* 60, 123–140. doi:10.1146/annurev-ento-010814-021017.
Coulson, S. J., and Bale, J. S. (1991). Anoxia induces rapid cold hardening in the housefly *Musca domestica* (Diptera: Muscidae). *J. Insect Physiol.* 37, 497–501. doi:10.1016/0022-1910(91)90026-V.
Davis, M. B., Shaw, R. G., and Etterson, J. R. (2005). Evolutionary responses to changing climate. *Ecology.* 86, 1704–1714. doi:10.1890/03-0788.
Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., et al. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. U. S. A.* 105, 6668–6672. doi:10.1073/pnas.0709472105.
Diffenbaugh, N. S., and Field, C. B. (2013). Changes in ecologically critical terrestrial climate conditions. *Science.* 341, 486–492. doi:10.1126/SCIENCE.1237123.
Dillon, M. E., Woods, H. A., Wang, G., Fey, S. B., Vasseur, D. A., Telemeco, R. S., et al. (2016). Life in the frequency domain: The biological impacts of changes in climate variability at multiple time scales. *Integr. Comp. Biol.* 56, 14–30. doi:10.1093/icb/icw024.
Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., and

Mearns, L. O. (2000). Climate extremes: Observations, modeling, and impacts. *Science.* 289, 2068–2075. doi: 10.1126/science.289.5487.2068
Falconer, D. S., and Mackay, T. F. C. (1996). *Introduction to quantitative genetics*. 4th ed. Longmans Green, Harlow: Essex, UK.
Feder, M. E., and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response. *Annu. Rev. Physiol.* 61, 243-282. doi: 10.1146/annurev.physiol.61.1.243
Fiehn, O. (2002). Metabolomics-the link between genotypes and phenotypes. *Plant Mol. Biol.* 48, 155–171.
Fischer, K., Dierks, A., Franke, K., Geister, T. L., Liszka, M., Winter, S., et al. (2010). Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. *PLoS One.* 5, e15284. doi:10.1371/journal.pone.0015284.
Fischer, K., and Kirste, M. (2018). Temperature and humidity acclimation increase desiccation resistance in the butterfly *Bicyclus anynana*. *Entomol. Exp. Appl.* 166, 289–297. doi:10.1111/eea.12662.
Fischer, K., Klockmann, M., and Reim, E. (2014). Strong negative effects of simulated heat waves in a tropical butterfly. *J. Exp. Biol.* 217, 2892–2898. doi:10.1242/jeb.106245/257785.
Fischer, K., Kölzow, N., Höltje, H., and Karl, I. (2011). Assay conditions in laboratory experiments: Is the use of constant rather than fluctuating temperatures justified when investigating temperature-induced plasticity? *Oecologia.* 166, 23–33. doi:10.1007/s00442-011-1917-0.
Frankham, R., Ballou, J. D., and Briscoe, D. A. (2004). *A primer of conservation genetics*. Cambridge University Press: Cambridge, UK.
Gaston, K. J., and Chown, S. L. (1999). Elevation and climatic tolerance: A test using dung beetles. *Oikos.* 86, 584–590. doi: 10.2307/3546663
Geister, T. L., Lorenz, M. W., Meyering-Vos, M., Hoffmann, K. H., and Fischer, K. (2008). Effects of temperature on reproductive output, egg provisioning, juvenile hormone and vitellogenin titres in the butterfly *Bicyclus anynana*. *J. Insect Physiol.* 54, 1253–1260. doi:10.1016/j.jinsphys.2008.06.002.
Ghalambor, C. K., Huey, R. B., Martin, P. R., Tewksbury, J. J., and Wang, G. (2006). Are mountain passes higher in the tropics? Janzen's hypothesis revisited. *Integr. Comp. Biol.* 46, 5–17. doi:10.1093/icb/icj003.
Gigante, E. T., Lim, E. J., Crisostomo, K. G., Cornejo, P., and Rodriguez, L. J. (2021). Increase in humidity widens heat tolerance range of tropical Ceratosolen fig wasps. *Ecol. Entomol.* 46, 573–581. doi:10.1111/een.13003.
Gillespie, M. A. K., Alfredsson, M., Barrio, I. C., Bowden, J. J., Convey, P., Culler, L. E., et al. (2020). Status and trends of terrestrial arthropod abundance and diversity in the North Atlantic region of the Arctic. *Ambio.* 49, 718–731. doi:10.1007/S13280-019-01162-5.
Gleason, L. U., Miller, L. P., Winnikoff, J. R., Somero, G. N., Yancey, P. H., Bratz, D., et al. (2017). Thermal history and gape of individual *Mytilus californianus* correlate with oxidative damage and thermoprotective osmolytes. *J. Exp. Biol.* 220, 4292–4304. doi:10.1242/JEB.168450.
Gunderson, A. R., and Stillman, J. H. (2015). Plasticity in thermal tolerance has

limited potential to buffer ectotherms from global warming. *Proc. R. Soc. B Biol. Sci.* 282, 20150401. doi:10.1098/rspb.2015.0401.
Gutiérrez-Pesquera, L. M., Tejedo, M., Olalla-Tárraga, M. A., Duarte, H., Nicieza, A., and Solé, M. (2016). Testing the climate variability hypothesis in thermal tolerance limits of tropical and temperate tadpoles. *J. Biogeogr.* 43, 1166–1178. doi:10.1111/JBI.12700.
Hairston, N. G., Ellner, S. P., Geber, M. A., Yoshida, T., and Fox, J. A. (2005). Rapid evolution and the convergence of ecological and evolutionary time. *Ecol. Lett.* 8, 1114–1127. doi:10.1111/J.1461-0248.2005.00812.X.
Halsch, C. A., Shapiro, A. M., Fordyce, J. A., Nice, C. C., Thorne, J. H., Waetjen, D. P., et al. (2021). Insects and recent climate change. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2002543117. doi:10.1073/PNAS.2002543117.
Havukainen, H., Münch, D., Baumann, A., Zhong, S., Halskau, Ø., Krogsgaard, M., et al. (2013). Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *J. Biol. Chem.* 288, 28369–28381. doi:10.1074/JBC.M113.465021.
Hayward, S. A. L. (2014). Application of functional "Omics" in environmental stress physiology: Insights, limitations, and future challenges. *Curr. Opin. Insect Sci.* 4, 35–41. doi:10.1016/J.COIS.2014.08.005.
Hazel, J. R. (1995). Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* 57, 19–42. doi:10.1146/annurev.ph.57.030195.000315.
Helmuth, B., Kingsolver, J. G., and Carrington, E. (2005). Biophysics, physiological ecology, and climate change: Does mechanism matter? *Annu. Rev. Physiol.* 67, 177–201. doi:10.1146/annurev.physiol.67.040403.105027.
Hoffmann, A. A., Chown, S. L., and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: How constrained are they? *Funct. Ecol.* 27, 934–949. doi:10.1111/j.1365-2435.2012.02036.x.
Hoffmann, A. A., and Parsons, P. A. (1991). *Evolutionary genetics and environmental stress*. Oxford University Press: New York.
Hoffmann, A. A., and Sgró, C. M. (2011). Climate change and evolutionary adaptation. *Nature.* 470, 479–485. doi:10.1038/nature09670.
Holmstrup, M., Hedlund, K., and Boriss, H. (2002). Drought acclimation and lipid composition in *Folsomia candida*: Implications for cold shock, heat shock and acute desiccation stress. *J. Insect Physiol.* 48, 961–970. doi:10.1016/S0022-1910(02)00175-0.
Høye, T. T., Loboda, S., Koltz, A. M., Gillespie, M. A. K., Bowden, J. J., and Schmidt, N. M. (2021). Nonlinear trends in abundance and diversity and complex responses to climate change in Arctic arthropods. *Proc. Natl. Acad. Sci. U. S. A.* 118, 1–8. doi:10.1073/PNAS.2002557117.
Huey, R. B., Carlson, M., Crozier, L., Frazier, M., Hamilton, H., Harley, C., et al. (2002). Plants versus animals: Do they deal with stress in different ways? *Integr. Comp. Biol.* 42, 415–423. doi:10.1093/icb/42.3.415.
Huey, R. B., Kearney, M. R., Krockenberger, A., Holtum, J. A. M., Jess, M., and Williams, S. E. (2012). Predicting organismal vulnerability to climate warming: Roles of behaviour, physiology and adaptation. *Philos. Trans. R.*

Soc. B Biol. Sci. 367, 1665–1679. doi:10.1098/rstb.2012.0005.
Huey, R. B., and Kingsolver, J. G. (1989). Evolution of thermal sensitivity of ectotherm performance. *Trends Ecol. Evol.* 4, 131–135. doi:10.1016/0169-5347(89)90211-5.
Huey, R. B., and Tewksbury, J. J. (2009). Can behavior douse the fire of climate warming? *Proc. Natl. Acad. Sci. U. S. A.* 106, 3647–3648. doi:10.1073/pnas.0900934106.
Jensen, A., Alemu, T., Alemneh, T., Pertoldi, C., and Bahrndorff, S. (2019). Thermal acclimation and adaptation across populations in a broadly distributed soil arthropod. *Funct. Ecol.* 33, 833–845. doi:10.1111/1365-2435.13291.
Johnson, C. H., Ivanisevic, J., and Siuzdak, G. (2016). Metabolomics: beyond biomarkers and towards mechanisms. *Nat. Rev. Mol. Cell Biol.* 17, 451-459. doi:10.1038/nrm.2016.25.
Kearney, M., and Porter, W. (2009). Mechanistic niche modelling: Combining physiological and spatial data to predict species' ranges. *Ecol. Lett.* 12, 334–350. doi:10.1111/j.1461-0248.2008.01277.x.
Kellermann, V., Loeschcke, V., Hoffmann, A. A., Kristensen, T. N., Fløjgaard, C., David, J. R., et al. (2012a). Phylogenetic constraints in key functional traits behind species' climate niches: Patterns of desiccation and cold resistance across 95 *Drosophila species*. *Evolution.* 66, 3377–3389. doi:10.1111/j.1558-5646.2012.01685.x.
Kellermann, V., Overgaard, J., Hoffmann, A. A., Fljøgaard, C., Svenning, J. C., and Loeschcke, V. (2012b). Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci. U. S. A.* 109, 16228–16233. doi:10.1073/pnas.1207553109.
Kellermann, V., and Sgrò, C. M. (2018). Evidence for lower plasticity in CTmax at warmer developmental temperatures. *J. Evol. Biol.* 31, 1300–1312. doi:10.1111/jeb.13303.
Kellermann, V., van Heerwaarden, B., Sgrò, C. M., and Hoffmann, A. A. (2009). Fundamental evolutionary limits in ecological traits drive *Drosophila* species distribution. *Science.* 325, 3–5. doi:10.1126/science.1175443.
Kelly, M. W., Grosberg, R. K., and Sanford, E. (2013). Trade-offs, geography, and limits to thermal adaptation in a tide pool copepod. *Am. Nat.* 181, 846–854. doi:10.1086/670336.
Kelly, M. W., Sanford, E., and Grosberg, R. K. (2012). Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. B Biol. Sci.* 279, 349–356. doi:10.1098/rspb.2011.0542.
Kelty, J. (2007). Rapid cold-hardening of *Drosophila melanogaster* in a field setting. *Physiol. Entomol.* 32, 343–350. doi:10.1111/j.1365-3032.2007.00584.x.
Ketola, T., Kellermann, V. M., Loeschcke, V., López-Sepulcre, A., and Kristensen, T. N. (2014). Does environmental robustness play a role in fluctuating environments? *Evolution.* 68, 587–594. doi:10.1111/EVO.12285.
King, A. M., and Macrae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annu. Rev. Entomol.* 60, 59–75. doi:10.1146/annurev-ento-011613-

162107.
Kingsolver, J. G., and Buckley, L. B. (2017). Evolution of plasticity and adaptive responses to climate change along climate gradients. *Proc. R. Soc. B Biol. Sci.* 284, 20170386. doi:10.1098/rspb.2017.0386.
Kingsolver, J. G., Higgins, J. K., and Augustine, K. E. (2015). Fluctuating temperatures and ectotherm growth: Distinguishing non-linear and time-dependent effects. *J. Exp. Biol.* 218, 2218–2225. doi:10.1242/jeb.120733.
Kingsolver, J. G., Ragland, G. J., and Diamond, S. E. (2009). Evolution in a constant environment: Thermal fluctuations and thermal sensitivity of laboratory and field populations of *Manduca Sexta*. *Evolution.* 63, 537–541. doi:10.1111/j.1558-5646.2008.00568.x.
Koltz, A. M., Schmidt, N. M., and Høye, T. T. (2018). Differential arthropod responses to warming are altering the structure of Arctic communities. *R. Soc. Open Sci.* 5. doi:10.1098/RSOS.171503.
Koštál, V., Korbelová, J., Poupardin, R., Moos, M., and Šimek, P. (2016). Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*. *J. Exp. Biol.* 219, 2358–2367. doi:10.1242/jeb.142158.
Koveos, D. S. (2001). Rapid cold hardening in the olive fruit fly *Bactrocera oleae* under laboratory and field conditions. *Entomol. Exp. Appl.* 101, 257–263. doi:10.1046/j.1570-7458.2001.00910.x.
Kristensen, T. N., Hoffmann, A., Overgaard, J., Sørensen, J. G., Hallas, R., and Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 105, 216–221. doi:10.1073/pnas.0708074105.
Kristensen, T. N., Ketola, T., and Kronholm, I. (2020). Adaptation to environmental stress at different timescales. *Ann. N. Y. Acad. Sci.* 1476, 5–12. doi:10.1111/nyas.13974.
Kristensen, T. N., Overgaard, J., Lassen, J., Hoffmann, A. A., and Sgrò, C. (2015). Low evolutionary potential for egg-to-adult viability in *Drosophila melanogaster* at high temperatures. *Evolution.* 69, 803–814. doi:10.1111/evo.12617.
Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol.* 67, 225–257. doi:10.1146/annurev.physiol.67.040403.103635.
Lande, R. (2014). Evolution of phenotypic plasticity and environmental tolerance of a labile quantitative character in a fluctuating environment. *J. Evol. Biol.* 27, 866–875. doi:10.1111/jeb.12360.
Lister, B. C., and Garcia, A. (2018). Climate-driven declines in arthropod abundance restructure a rainforest food web. *Proc. Natl. Acad. Sci. U. S. A.* 115, E10397–E10406. doi:10.1073/PNAS.1722477115.
Loeschcke, V., and Hoffmann, A. A. (2002). The detrimental acclimation hypothesis. *Trends Ecol. Evol.* 17, 407–408. doi:10.1016/S0169-5347(02)02558-2.
Loeschcke, V., and Hoffmann, A. A. (2007). Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature.

Am. Nat. 169, 175–183. doi:10.1086/510632.
Ma, C.-S., Ma, G., and Pincebourde, S. (2021). Survive a warming climate: Insect responses to extreme high temperatures. *Annu. Rev.* 66, 163–84. doi:https://doi.org/10.1146/annurev-ento-041520-074454.
Mackay, T. F. C., Richards, S., Stone, E. A., Barbadilla, A., Ayroles, J. F., Zhu, D., et al. (2012). The *Drosophila melanogaster* Genetic Reference Panel. *Nature.* 482, 173–178. doi:10.1038/nature10811.
MacLean, H. J., Sørensen, J. G., Kristensen, T. N., Loeschcke, V., Beedholm, K., Kellermann, V., et al. (2019). Evolution and plasticity of thermal performance: An analysis of variation in thermal tolerance and fitness in 22 *Drosophila* species. *Philos. Trans. R. Soc. B Biol. Sci.* 374, 20180548. doi:10.1098/rstb.2018.0548.
Manenti, T., Loeschcke, V., Moghadam, N. N., and Sørensen, J. G. (2015). Phenotypic plasticity is not affected by experimental evolution in constant, predictable or unpredictable fluctuating thermal environments. *J. Evol. Biol.* 28, 2078–2087. doi:10.1111/jeb.12735.
Manenti, T., Sørensen, J. G., and Loeschcke, V. (2017). Environmental heterogeneity does not affect levels of phenotypic plasticity in natural populations of three *Drosophila* species. *Ecol. Evol.* 7, 2716–2724. doi:10.1002/ece3.2904.
Manenti, T., Sørensen, J. G., Moghadam, N. N., and Loeschcke, V. (2014). Predictability rather than amplitude of temperature fluctuations determines stress resistance in a natural population of *Drosophila simulans*. *J. Evol. Biol.* 27, 2113–2122. doi:10.1111/jeb.12463.
Matesanz, S., Gianoli, E., and Valladares, F. (2010). Global change and the evolution of phenotypic plasticity in plants. *Ann. N. Y. Acad. Sci.* 1206, 35–55. doi:10.1111/J.1749-6632.2010.05704.X.
Mitchell, K. A., and Hoffmann, A. A. (2010). Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. *Funct. Ecol.* 24, 694–700. doi:10.1111/j.1365-2435.2009.01666.x.
Murren, C. J., Auld, J. R., Callahan, H., Ghalambor, C. K., Handelsman, C. A., Heskel, M. A., et al. (2015). Constraints on the evolution of phenotypic plasticity: Limits and costs of phenotype and plasticity. *Heredity.* 115, 293–301. doi:10.1038/hdy.2015.8.
Ørsted, M., Rohde, P. D., Hoffmann, A. A., Sørensen, P., and Kristensen, T. N. (2018). Environmental variation partitioned into separate heritable components. *Evolution.* 72, 136–152. doi:10.1111/evo.13391.
Overgaard, J., Kristensen, T. N., Mitchell, K. A., and Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical *Drosophila* species: Does phenotypic plasticity increase with latitude? *Am. Nat.* 178, S80–S96. doi:10.1086/661780.
Overgaard, J., Malmendal, A., Sørensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C., et al. (2007). Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *J. Insect Physiol.* 53, 1218–1232. doi:10.1016/j.jinsphys.2007.06.012.

Overgaard, J., and Sørensen, J. G. (2008). Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. *Cryobiology* 56, 159–162. doi:10.1016/j.cryobiol.2008.01.001.
Parmesan, C., and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature.* 421, 37–42. doi:10.1038/nature01286.
Pavey, S. A., Bernatchez, L., Aubin-Horth, N., and Landry, C. R. (2012). What is needed for next-generation ecological and evolutionary genomics? *Trends Ecol. Evol.* 27, 673–678. doi:10.1016/j.tree.2012.07.014.
Perkins, S. E., Alexander, L. V., and Nairn, J. R. (2012). Increasing frequency, intensity and duration of observed global heatwaves and warm spells. *Geophys. Res. Lett.* 39, 20714. doi:10.1029/2012GL053361.
Pétavy, G., David, J. R., Debat, V., Gibert, P., and Moreteau, B. (2004). Specific effects of cycling stressful temperatures upon phenotypic and genetic variability of size traits in *Drosophila melanogaster*. *Evol. Ecol. Res.* 6, 873–890.
Pigliucci, M. (2005). Evolution of phenotypic plasticity: where are we going now? *Trends Ecol. Evol.* 20, 481–486. doi:https://doi.org/10.1016/j.tree.2005.06.001.
Pincebourde, S., and Casas, J. (2019). Narrow safety margin in the phyllosphere during thermal extremes. *Proc. Natl. Acad. Sci.* 116, 5588–5596. doi:10.1073/PNAS.1815828116.
Pincebourde, S., Murdock, C. C., Vickers, M., and Sears, M. W. (2016). Fine-scale microclimatic variation can shape the responses of organisms to global change in both natural and urban environments. *Integr. Comp. Biol.* 56, 45–61. doi:10.1093/icb/icw016.
Pincebourde, S., and Suppo, C. (2016). The vulnerability of tropical ectotherms to warming is modulated by the microclimatic heterogeneity. *Integr. Comp. Biol.* 56, 85–97. doi:10.1093/ICB/ICW014.
Pintor, A. F. V, Schwarzkopf, L., and Krockenberger, A. K. (2016). Extensive acclimation in ectotherms conceals interspecific variation in thermal tolerance limits. *PLoS One.* 11, e0150408. doi:10.1371/journal.pone.0150408.
Poitou, L., Robinet, C., Suppo, C., Rousselet, J., Laparie, M., and Pincebourde, S. (2021). When insect pests build their own thermal niche: The hot nest of the pine processionary moth. *J. Therm. Biol.* 98, 102947. doi:10.1016/j.jtherbio.2021.102947.
Pörtner, H. O., Peck, L., and Somero, G. (2007). Thermal limits and adaptation in marine antarctic ectotherms: an integrative view. *Philos. Trans. R. Soc. B.* 362, 2233–2258. doi:10.1098/rstb.2006.1947.
Potter, K. A., Arthur Woods, H., and Pincebourde, S. (2013). Microclimatic challenges in global change biology. *Glob. Chang. Biol.* 19, 2932–2939. doi:10.1111/gcb.12257.
Rezende, E. L., Castañeda, L. E., and Santos, M. (2014). Tolerance landscapes in thermal ecology. *Funct. Ecol.* 28, 799–809. doi:10.1111/1365-2435.12268.
Rodrigues, L. R., McDermott, H. A., Villanueva, I., Djukarić, J., Ruf, L. C., Amcoff, M., et al. (2021). Fluctuating heat stress during development exposes

reproductive costs and putative benefits. *J. Anim. Ecol.* 91, 391–403. doi:10.1111/1365-2656.13636.
Ruel, J. J., and Ayres, M. P. (1999). Jensen's inequality predicts effects of environmental variation. *Trends Ecol. Evol.* 14, 361–366. doi:https://doi.org/10.1016/S0169-5347(99)01664-X.
Salachan, P. V., Burgaud, H., and Sørensen, J. G. (2019). Testing the thermal limits: Non-linear reaction norms drive disparate thermal acclimation responses in *Drosophila melanogaster*. *J. Insect Physiol.* 118, 103946. doi:10.1016/j.jinsphys.2019.103946.
Salachan, P. V., and Sørensen, J. G. (2017). Critical thermal limits affected differently by developmental and adult thermal fluctuations. *J. Exp. Biol.* 220, 4471–4478. doi:10.1242/JEB.165308/262745.
Saxon, A. D., O'Brien, E. K., and Bridle, J. R. (2018). Temperature fluctuations during development reduce male fitness and may limit adaptive potential in tropical rainforest *Drosophila*. *J. Evol. Biol.* 31, 405–415. doi:10.1111/jeb.13231.
Schär, C., Vidale, P. L., Lüthi, D., Frei, C., Häberli, C., Liniger, M. A., et al. (2004). The role of increasing temperature variability in European summer heatwaves. *Nature.* 427, 332–336. doi:10.1038/nature02300.
Scheiner, S. M. (1993). Genetics and evolution of phenotypic plasticity. *Annu. Rev. Ecol. Evol. Syst* 24, 35–68. doi:10.1146/annurev.es.24.110193.000343.
Scheiner, S. M., and Lyman, R. F. (1989). The genetics of phenotypic plasticity I. Heritability. *J. Evol. Biol.* 2, 95–107. doi:10.1046/J.1420-9101.1989.2020095.X.
Schlichting, C. D. (1986). The evolution of phenotypic plasticity in plants. *Annu. Rev. Ecol. Syst.* 17, 667–693. doi:10.1146/annurev.es.17.110186.003315.
Schmidt, N. M., Mosbacher, J. B., Nielsen, P. S., Rasmussen, C., Høye, T. T., and Roslin, T. (2016). An ecological function in crisis? The temporal overlap between plant flowering and pollinator function shrinks as the Arctic warms. *Ecography.* 39, 1250–1252. doi:10.1111/ecog.02261.
Schou, M. F., Kristensen, T. N., Kellermann, V., Schlötterer, C., and Loeschcke, V. (2014). A *Drosophila* laboratory evolution experiment points to low evolutionary potential under increased temperatures likely to be experienced in the future. *J. Evol. Biol.* 27, 1859–1868. doi:10.1111/JEB.12436.
Schou, M. F., Loeschcke, V., and Kristensen, T. N. (2015). Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. *PLoS One.* 10, e0130307. doi:10.1371/journal.pone.0130307.
Schou, M. F., Mouridsen, M. B., Sørensen, J. G., and Loeschcke, V. (2017). Linear reaction norms of thermal limits in *Drosophila*: predictable plasticity in cold but not in heat tolerance. *Funct. Ecol.* 31, 934–945. doi:10.1111/1365-2435.12782.
Schulte, P. M., Healy, T. M., and Fangue, N. A. (2011). Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr. Comp. Biol.* 51, 691–702. doi:10.1093/icb/icr097.
Seebacher, F., White, C. R., and Franklin, C. E. (2015a). Physiological plasticity increases resilience of ectothermic animals to climate change.

doi:10.1038/nclimate2457.
Seebacher, F., White, C. R., and Franklin, C. E. (2015b). Physiological plasticity increases resilience of ectothermic animals to climate change. doi:10.1038/nclimate2457.
Seehuus, S. C., Norberg, K., Gimsa, U., Krekling, T., and Amdam, G. V. (2006). Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* 103, 962–967. doi:10.1073/PNAS.0502681103.
Sgrò, C. M., Overgaard, J., Kristensen, T. N., Mitchell, K. A., Cockerell, F. E., and Hoffmann, A. A. (2010). A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from eastern Australia. *J. Evol. Biol.* 23, 2484–2493. doi:10.1111/J.1420-9101.2010.02110.X.
Sgrò, C. M., Terblanche, J. S., and Hoffmann, A. A. (2016). What can plasticity contribute to insect responses to climate change ? *Annu Rev Entomol.* 61, 433–451. doi:10.1146/annurev-ento-010715-023859.
Siddiqui, W. H., and Barlow, C. A. (1972). Population growth of *Drosophila melanogaster* (Diptera: Drosophilidae) at constant and alternating temperatures. *Ann. Entomol. Soc. Am.* 65, 993–1001. doi:10.1093/AESA/65.5.993.
Sinclair, B. J., Ferguson, L. V., Salehipour-Shirazi, G., and Macmillan, H. A. (2013). Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. *Integr. Comp. Biol.* 53, 545–556. doi:10.1093/ICB/ICT004.
Sinclair, B. J., Gibbs, A. G., and Roberts, S. P. (2007). Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Mol. Biol.* 16, 435–443. doi:10.1111/J.1365-2583.2007.00739.X.
Sinclair, B. J., Marshall, K. E., Sewell, M. A., Levesque, D. L., Willett, C. S., Slotsbo, S., et al. (2016). Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? *Ecol. Lett.* 19, 1372–1385. doi:10.1111/ele.12686.
Sømme, L. (1968). The effect of acclimation and glycerol injection on mortality and pupation in larvae of *Ephestia kuehniella* after exposures at low temperatures. *Entomol. Exp. Appl.* 11, 143–148. doi:10.1111/J.1570-7458.1968.TB02040.X.
Sørensen, J. G., Kristensen, T. N., and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6, 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x.
Sørensen, J. G., Kristensen, T. N., and Overgaard, J. (2016a). Evolutionary and ecological patterns of thermal acclimation capacity in *Drosophila*: is it important for keeping up with climate change? *Curr. Opin. Insect Sci.* 17, 98–104. doi:10.1016/J.COIS.2016.08.003.
Sørensen, J. G., Schou, M. F., Kristensen, T. N., and Loeschcke, V. (2016b). Thermal fluctuations affect the transcriptome through mechanisms independent of average temperature. *Sci. Reports.* 6, 1–11. doi:10.1038/srep30975.

Spacht, D. E., Gantz, J. D., Devlin, J. J., McCabe, E. A., Lee, R. E., Denlinger, D. L., et al. (2021). Fine-scale variation in microhabitat conditions influences physiology and metabolism in an Antarctic insect. *Oecologia.* 197, 373–385. doi:10.1007/S00442-021-05035-1.

Stearns, S., de Jong, G., and Newman, B. (1991). The effects of phenotypic plasticity on genetic correlations. *Trends Ecol. Evol.* 6, 122–126. doi:10.1016/0169-5347(91)90090-K.

Stevenson, R. D. (1985). The relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms. *Am. Nat.* 126, 362–386. doi:10.1086/284423.

Sunday, J. M., Bates, A. E., and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proc. R. Soc. B Biol. Sci.* 278, 1823–1830. doi:10.1098/rspb.2010.1295.

Sunday, J. M., Bates, A. E., Kearney, M. R., Colwell, R. K., Dulvy, N. K., Longino, J. T., et al. (2014). Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proc. Natl. Acad. Sci. U. S. A.* 111, 5610–5615. doi:10.1073/pnas.1316145111.

Teets, N. M., and Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiol. Entomol.* 38, 105–116. doi:10.1111/phen.12019.

Teets, N. M., Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., Denlinger, D. L., and Lee, R. E. (2008). Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica*: Cellular cold-sensing and a role for calcium. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 294, R1938-46. doi:10.1152/ajpregu.00459.2007.

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A., et al. (2012). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiol. Genomics.* 44, 764–777. doi:10.1152/physiolgenomics.00042.2012.

Teets, N. M., Yi, S. X., Lee, R. E., and Denlinger, D. L. (2013). Calcium signaling mediates cold sensing in insect tissues. *Proc. Natl. Acad. Sci. U. S. A.* 110, 9154–9159. doi:10.1073/PNAS.1306705110.

Terblanche, J. S., Deere, J. A., Clusella-Trullas, S., Janion, C., and Chown, S. L. (2007). Critical thermal limits depend on methodological context. *Proc. R. Soc. B Biol. Sci.* 274, 2935–2942. doi:10.1098/rspb.2007.0985.

Terblanche, J. S., Nyamukondiwa, C., and Kleynhans, E. (2010). Thermal variability alters climatic stress resistance and plastic responses in a globally invasive pest, the Mediterranean fruit fly (*Ceratitis capitata*). *Entomol. Exp. Appl.* 137, 304–315. doi:10.1111/j.1570-7458.2010.01067.x.

Tewksbury, J. J., Huey, R. B., and Deutsch, C. A. (2008). Putting the heat on tropical animals. *Science.* 320, 1296–1297. doi:10.1126/science.1159328.

Toxopeus, J., Koštál, V., and Sinclair, B. J. (2019). Evidence for non-colligative function of small cryoprotectants in a freeze-tolerant insect. *Proc. R. Soc. B* 286, 20190050. doi:10.1098/RSPB.2019.0050.

Valladares, F., Matesanz, S., Guilhaumon, F., Araujo, M., Balaguer, L., Benito-Garzon, M., et al. (2014). The effects of phenotypic plasticity and local

adaptation on forecasts of species range shifts under climate change. *Ecol. Lett.* 17, 1351–1364. doi:10.1111/ele.12348.

Van Heerwaarden, B., and Sgrò, C. M. (2011). The effect of developmental temperature on the genetic architecture underlying size and thermal clines in *Drosophila melanogaster* and *D. simulans* from the east coast of Australia. *Evolution* 65, 1048–1067. doi:10.1111/J.1558-5646.2010.01196.X.

Vasseur, D. A., Delong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., Mccann, K. S., et al. (2014). Increased temperature variation poses a greater risk to species than climate warming. *Proc. R. Soc. B Biol. Sci.* 281, 1–8. doi:10.1098/rspb.2013.2612.

Vázquez, D. P., Gianoli, E., Morris, W. F., and Bozinovic, F. (2017). Ecological and evolutionary impacts of changing climatic variability. *Biol. Rev.* 92, 22–42. doi:10.1111/brv.12216.

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. New York: Oxford University Press.

Wolfe, G. R., Hendrix, D. L., and Salvucci, M. E. (1998). A thermoprotective role for sorbitol in the silverleaf whitefly, *Bemisia argentifolii*. J. Insect Physiol. 44, 597-603. doi:10.1016/s0022-1910(98)00035-3.

PAPER I

INTO THE WILD—A FIELD STUDY ON THE EVOLUTIONARY AND ECOLOGICAL IMPORTANCE OF THERMAL PLASTICITY IN ECTOTHERMS ACROSS TEMPERATE AND TROPICAL REGIONS

Published in Philosophical Transactions of the Royal Society B

Natasja K. Noer, Michael Ørsted, Michele Schiffer, Ary A. Hoffmann, Simon Bahrndorff, and Torsten N. Kristensen

PHILOSOPHICAL TRANSACTIONS B

royalsocietypublishing.org/journal/rstb

Research

Cite this article: Noer NK, Ørsted M, Schiffer M, Hoffmann AA, Bahrndorff S, Kristensen TN. 2022 Into the wild—a field study on the evolutionary and ecological importance of thermal plasticity in ectotherms across temperate and tropical regions. *Phil. Trans. R. Soc. B* **377**: 20210004. https://doi.org/10.1098/rstb.2021.0004

Received: 16 July 2021
Accepted: 18 October 2021

One contribution of 11 to a theme issue 'Species' ranges in the face of changing environments (part 1)'.

Subject Areas:
evolution, physiology

Keywords:
insects, climate predictors, microhabitat environment, phenotypic plasticity, species distribution, thermal tolerance

Author for correspondence:
Natasja K. Noer
e-mail: nkn@bio.aau.dk

Into the wild—a field study on the evolutionary and ecological importance of thermal plasticity in ectotherms across temperate and tropical regions

Natasja K. Noer[1], Michael Ørsted[2], Michele Schiffer[3], Ary A. Hoffmann[1,4], Simon Bahrndorff[1,†] and Torsten N. Kristensen[1,†]

[1]Department of Chemistry and Bioscience, Aalborg University, Aalborg E 9220, Denmark
[2]Zoophysiology, Department of Biology, Aarhus University, Aarhus C 8000, Denmark
[3]Daintree Rainforest Observatory, James Cook University, Cape Tribulation, Douglas, Queensland 4873, Australia
[4]School of BioSciences, Bio21 Institute, the University of Melbourne, Parkville, Victoria 3010, Australia

NKN, 0000-0002-4430-0342; MØ, 0000-0001-8222-8399; MS, 0000-0002-5203-2480; AAH, 0000-0001-9497-7645; SB, 0000-0002-0838-4008; TNK, 0000-0001-6204-8753

Understanding how environmental factors affect the thermal tolerance of species is crucial for predicting the impact of thermal stress on species abundance and distribution. To date, species' responses to thermal stress are typically assessed on laboratory-reared individuals and using coarse, low-resolution, climate data that may not reflect microhabitat dynamics at a relevant scale. Here, we examine the daily temporal variation in heat tolerance in a range of species in their natural environments across temperate and tropical Australia. Individuals were collected in their habitats throughout the day and tested for heat tolerance immediately thereafter, while local microclimates were recorded at the collection sites. We found high levels of plasticity in heat tolerance across all the tested species. Both short- and long-term variability of temperature and humidity affected plastic adjustments of heat tolerance within and across days, but with species differences. Our results reveal that plastic changes in heat tolerance occur rapidly at a daily scale and that environmental factors on a relatively short timescale are important drivers of the observed variation in thermal tolerance. Ignoring such fine-scale physiological processes in distribution models might obscure conclusions about species' range shifts with global climate change.

This article is part of the theme issue 'Species' ranges in the face of changing environments (part 1)'.

1. Introduction

Temperature is an important abiotic environmental variable that can constrain the abundance and geographical distribution of species [1–4]. This can be either directly by temperatures exceeding physiological tolerance limits [5], or indirectly through interactions with other abiotic or biotic stressors [5,6]. Understanding how changing environmental conditions, such as increasing and less predictable temperatures, affect thermal performance and tolerances is thus crucial for predicting future species' range limits (e.g. [7,8]). This is highlighted by the current focus on modelling how future global warming scenarios will affect species abundance and distribution. This can be through the use of mechanistic species distribution models that incorporate physiological information on range-limiting processes, which has been suggested to provide robust predictions of future species distributions, and allows extrapolation beyond current climates [9,10].

However, fine-tuning such process-based models presents many challenges. A key challenge, which trait-based studies have attempted to answer for decades, is what physiological metrics serve as the best predictor of species' vulnerability to environmental change across species [10]. Commonly, species'

[†]Shared last-authorship.

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5762692.

THE ROYAL SOCIETY PUBLISHING

critical thermal limits have been used as proxies for species' vulnerability, as they define the space for the performance of vital physiological functions such as locomotion, growth and reproduction [4,11]. Studies have been successful in linking lower thermal limits to large-scale climate patterns, showing that cold tolerance increases with latitudinal change in climate [11–16], including the mean temperature of the coldest quarter, absolute monthly minimum and mean annual temperature for reptiles [8,14,15]. However, upper thermal limits vary less, or at negligible levels, across latitude [11–14,16,17], which makes upper thermal limits a weak predictor of species' vulnerabilities. This is despite the obvious role of heat tolerance in coping with increasing temperatures with climate change [4,18,19]. Likewise, empirical evidence suggests that these tolerance metrics may result in misleading conclusions on species' vulnerabilities to climate change (e.g. [20,21]). Efforts to explain this perplexity have suggested that species' thermoregulatory behaviour [22–25], low genetic variability for heat tolerance [15,17,26,27], physiological adjustments [28–31] and methodology [32–36] obscure the effectiveness of using heat tolerance as a predictor of range limits [35,37,38].

Studies examining how physiological adjustments impact on heat tolerance and the evolutionary and plastic capacity to alter heat tolerance have typically been executed in the laboratory, and on populations adapted to laboratory conditions (e.g. [37,39,40]). Further, common garden laboratory studies are limited to species that can be reared successfully in the laboratory, constraining the number of species studied, which have typically been model species of temperate and tropical origin (e.g. [10,38]). This can be problematic if those studies aim to predict future responses to climate change in the field, because laboratory conditions do not reflect natural variable and unpredictable temperatures, and also because responses shown by model species may not be representative [41,42]. In addition, very few studies have addressed the realized thermal niche across different temporal scales, which can lead to both underestimates and overestimates of acclimatization, e.g. upper thermal limits (but see [43,44]). Finally, thermoregulatory behaviour is unaccounted for when organisms are restricted under laboratory conditions. Thus, species have no opportunity to behaviourally shape their thermal environment, which will often alter responses to environmental stress [3,9].

When fine-tuning mechanistic models with the goal to provide robust predictions of future distributions, an issue is defining the scale of bioclimatic variables that best explain physiological information [45–47]. To date, species' responses to environmental change are typically assessed using coarse, interpolated and low-resolution climate data that is measured in the air, high above the ground level, and at distances spanning several km^2 [45,48,49]. Such data ignore the climate-forcing processes that operate near the ground, and thermal heterogeneity across the environment [46,50], and studies report that the microclimate can deviate by up to 35°C [51] from air temperature. Thus, the conditions met by small organisms in the field bear little resemblance to the macroclimate [52]. Further, currently used climate data usually consist of long-term measures (monthly averages) and do not account for fine-scale spatio-temporal variability [25], thereby disregarding exposures to e.g. extreme conditions on the short time-scale (minutes, hours, days). Ignoring the frequency that organisms are exposed to stressful conditions may cause inaccurate predictions of species' ranges.

In order to provide field data on the ability to respond to daily fluctuations in temperature, and obtain climatic predictors of heat tolerance, we here examine the daily temporal variation in heat tolerance in seven insects collected in temperate Melbourne, Australia (*Nysius caledoniae*, *Stenophyella macreta*, *Uroleucon sonchi*, *Hyperomyzus lactucae*, *Aphis nerii*, *Drosophila melanogaster*, Psyllidae sp.) and in five species from tropical Cape Tribulation, Australia (*Pseudopachybrachius guttus*, *Oecophylla smaragdina*, *Drosophila rubida*, *Scaptodrosophila novoguineensis*, Cicadelliadae sp.). In order to do this, we collected individuals at four different time points on multiple days, and immediately tested for heat tolerance using an acute heat knockdown assay. Further, data on local microclimate (temperature and humidity) were recorded at the collection sites up to two weeks prior to testing to pinpoint microclimatic parameters on a temporal scale that could affect changes in thermal tolerance and plasticity for individual species across time. We observed marked and highly species-specific plasticity in heat tolerance, providing evidence that some species can change their heat knockdown time (HKDT) by up to 90% in a day relative to the lowest recorded daily knockdown time whereas others are much more constrained. Our data also showed that both means of temperature and humidity, as well as their variability experienced prior to organisms being tested, were useful for predicting heat tolerance. These results suggest that the input data typically used in mechanistic models will often not provide accurate measures of thermal robustness, because they fail to take into account local thermal conditions and the ability of many species to respond strongly to temperature variability on a daily scale.

2. Methods

(a) Study regions and microhabitat climates

Insects were collected and tested for heat tolerance in temperate (Melbourne, Australia, latitude 37.8° S) and tropical (Cape Tribulation, Australia, latitude 16.1° S) locations, characterized by highly variable temperatures and humidity at the temperate location and more constant climatic conditions at the tropical location (figure 1). The specific sites, dates and times of collection of insects for thermal assessment are presented in electronic supplementary material, table S1. At each field site, the temperature and humidity were recorded every 5 min using Easylog USB data loggers (LASCAR Electronics, EL-USB-2^+). The data loggers were placed in the shade at 20 cm above the soil surface. The microclimatic variables recorded at each study site were associated with the heat tolerance of each insect species collected at the specific sites at a given time point.

(b) Field experiment

We used sweep nets to catch adult individuals of each species at four time points throughout the day; morning (*ca* 8.00), noon (*ca*. 12.00), afternoon (*ca* 16.00) and night (*ca* 20.00). This was done for 4–8 days for each species. The specific collection times depended on weather conditions and the abundance of the species, given that sufficient numbers were required for tolerance tests. Individuals were caught within a radius of 25 m from the microhabitat data loggers and near laboratory facilities. In the field, individuals were placed into 4 ml screw-cap glass vials (45 × 14.7 mm) held in the shade until a sufficient number of individuals had been collected. We used 15–20 individuals of each sex for every assay (for those species where we could differentiate between sexes; table 1). The individuals were sexed by eye

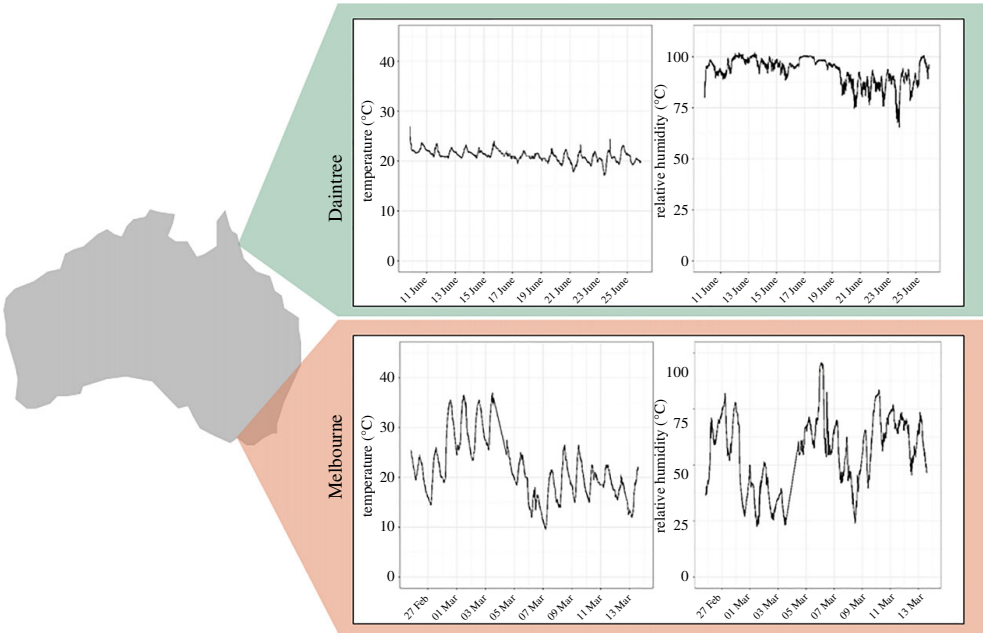


Figure 1. Study sites used for insect collection and local temperature and humidity data. The insects were collected at the Daintree Research Observatory (latitude 16.1° S) in tropical Australia (green) from 3 June to 28 June 2019, and in Melbourne (latitude 37.8° S) in temperate Australia (orange) from 1 February to 2 June 2019. A period of the microhabitat temperature (°C) and air humidity (%RH) recorded 20 cm above the soil surface are displayed for each sampling site from each region. (Online version in colour.)

in the field prior to the assays and the sexes were verified under a stereo microscope after the thermal assay had been completed. After field collection the vials were quickly transported to the laboratory facilities, and heat tolerance of individuals was determined (see next section), 30–90 min following collection in the field. Subsequently, individuals were stored in 90% ethanol and identified using identification keys [53,54], or by comparison against insect collections at La Trobe University in Melbourne and at the Daintree Rainforest Observatory, with guidance from experienced entomologists.

(c) Heat tolerance assessment

We used a static knockdown assay to assess heat tolerance across days and sampling times for each species. The temperature used for the heat knockdown assays depended on the species, and was initially determined by finding critical upper lethal limits (CT_{max}) on haphazardly collected individuals of each species using a dynamic ramping assay. The heat knockdown temperature used was set 1.4°C below the species-specific CT_{max}, and 1.9°C below CT_{max} for *S. novoguineensis* owing to immediate knockdown when the temperature was set only 1.4°C below CT_{max} (table 1 for CT_{max} and knockdown temperatures). For this initial work, field-collected individuals were placed into 4 ml screw-cap glass vials (45 × 14.7 mm) and submerged in a water bath at 25°C. The temperature was then increased gradually at a rate of 0.2°C min^{-1}, and CT_{max} was scored as the temperature at which all movement ceased, i.e. individuals went into heat coma [55].

In the acute knockdown assays, the vials were submerged into a water bath heated to the species-specific test temperature. HKDT was then scored as the time it took for individuals to go into a heat-induced coma [55]. The knockdown temperatures used resulted in individuals going into a coma within 20–40 min, and thus there was a limited opportunity for hardening effects to develop during the tests.

(d) Statistical analysis

The recorded climate data at each study location were summarized by daily mean temperature and daily coefficient of variation (CV).

Differences in CT_{max} between species were tested using a Kruskal–Wallis test followed by Dunn's *post hoc* test to clarify the observed differences. The *p*-values from Dunn's test were corrected using Holm adjustments. For each species, we used *N*-way ANOVAs to examine differences in HKDT according to the independent categorical variables 'day', 'time of day' and 'sex' (when relevant). In most cases, residuals of the models adhered to normal distributions; however, the HKDT data were transformed using the rank inverse transformation to ensure normal distribution for all species before ANOVAs were carried out. The *p*-values generated across the two-way or three-way ANOVAs were then corrected for multiple testing using Benjamini–Hochberg false discovery rate (FDR) adjustments. In addition, the changes in HKDT within days were visualized for each day, species and sex using polynomial regressions on raw HKDT data. Thus, polynomial curves were fitted when the regressions had a significant quadratic term, but not when only linear terms were significant. Regressions with no significant terms were not considered further.

Daily adjustments in HKDT were quantified for each species in two ways: (i) using %CV as an unbiased measure of variability, i.e. s.d./mean × 100. Within each day, %CV was calculated based on raw HKDTs for each sex (when relevant). The daily %CVs were then averaged across days. (ii) Calculating percentage change in 'mean HKDT' relative to the lowest mean within each day. Specifically, this was calculated as the difference between the maximal and the minimal mean HKDT recorded within a day, relative to the minimal knockdown time:

$$\Delta\mathrm{HKDT} = \frac{\overline{\mathrm{HKDT}}_{\max} - \overline{\mathrm{HKDT}}_{\min}}{\overline{\mathrm{HKDT}}_{\min}} \times 100\,.$$

Finally, short-term (hardening) and long-term (acclimatization) effects of climate on adjustments in HKDT were examined

Table 1. Overview of the species used for heat knockdown time assessment and their thermal performance. The species-specific knockdown temperatures, T_{HKDT}, used were based on the species' critical thermal maximum, CT_{max} (s.e.m., *n*). Superscript letters a–f denote significant differences in CT_{max} between species, based on Dunn's *post hoc* tests. The average knockdown time, $HKDT_{avr}$ (s.e.m. in seconds, *n*), measured on the total number of individuals tested across days and times are displayed. Plasticity is expressed as the mean of the daily coefficients of variation (CVs) recorded across test days, and the minimum and maximum CVs are provided in parentheses. The daily change in mean HKDT relative to the minimum (ΔHKDT) is displayed as an average across days and the lowest and highest recorded daily changes are provided in parentheses. Plasticity and ΔHKDT were only calculated when more than 3 days of measurements were available, and otherwise are indicated by 'n.a.'. Sex is indicated as male (m), female (f) or not available (n.a.).

region	order	species	CT_{max} (°C)	T_{HKDT} (°C)	$HKDT_{avr}$ (min : s)	plasticity (%CV)	ΔHKDT (%)	sex
temperate	Hemiptera	*Stenophyella macreta*	51.1 (0.1, 30)^{f}	49.7	16 : 20 (22, 475)	22.9 (14.2–29.1)	23.1 (9.2–46.6)	m + f
temperate	Hemiptera	*Nysius caledoniae*	49.9 (0.2, 20)^{ef}	48.5	11 : 49 (13, 1069)	21.2 (14.1–35.5)	17.6 (5.8–48.4)	m + f
tropical	Hymenoptera	*Oecophylla smaragdina*	48.0 (0.1, 28)^{ef}	46.6	24 : 47 (31, 592)	22.9 (18.3–25.2)	14.6 (5.6–26.8)	n.a.
tropical	Hemiptera	*Pseudopachybrachius guttus*	46.2 (0.1, 38)^{ce}	44.8	23 : 55 (43, 939)	24.6 (21.8–30.8)	14.5 (8.0–25.7)	m + f
tropical	Hemiptera	Cicadellidae sp.	44.8 (0.2, 40)^{ac}	43.4	30 : 51 (101, 799)	28.1 (18.9–41.0)	32.9 (9.5–63.7)	m + f
temperate	Hemiptera	Psyllidae sp.	44.2 (0.2, 15)^{abc}	42.8	11 : 49 (28, 349)	31.6 (22.7–38.7)	21.0 (11.8–39.9)	n.a.
temperate	Hemiptera	*Aphis nerii*	43.9 (0.0, 28)^{ab}	42.5	12 : 13 (47, 137)	n.a.	n.a.	f
temperate	Hemiptera	*Uroleucon sonchi*	43.8 (0.0, 24)^{ab}	42.4	10 : 23 (21, 382)	22.9 (19.1–27.6)	21.9 (8.7–37.4)	f
temperate	Hemiptera	*Hyperomyzus lactucae*	43.3 (0.1, 19)^{ab}	41.9	14 : 01 (35, 253)	27.2 (21.4–34.7)	31.6 (15.4–47.7)	f
tropical	Diptera	*Scaptodrosophila novoguineensis*	42.6 (0.1, 39)^{bd}	40.7	26 : 59 (78, 946)	35.9 (32.2–43.2)	33.4 (19.6–87.6)	m + f
temperate	Diptera	*Drosophila melanogaster*	41.4 (0.1, 13)^{bd}	40.0	15 : 28 (49, 920)	32.8 (22.5–46.2)	35.7 (16.9–59.8)	m + f
tropical	Diptera	*Drosophila rubida*	40.0 (0.1, 45)^{d}	38.6	26 : 22 (42, 954)	41.9 (28.1–51.7)	31.7 (6.4–90.3)	m + f

by Pearson's correlations. First, the mean and variability (CV) of field temperature and humidity recordings were extracted at a range of time intervals. Short-term intervals consisted of climate measures extracted in rolling 1 h bins within the first 24 h preceding thermal assessment, while longer-term intervals consisted of 24 h bins in the period 14 days prior to the assessment of heat tolerance (as illustrated in electronic supplementary material, figure S1). Additionally, the climate variables were extracted in 'windows' by moving the past time boundary back in time, in either 1 h (short-term window) or 24 h (long-term window) intervals, thereby accumulating the time windows across which climate measures were extracted (electronic supplementary material, figure S1). Both 'bins' and 'windows' were used for the analyses to explore whether accumulated impacts, or short bursts of temperature change were more important for HKDT of species in their natural environments. Before correlating 'mean HKDT' with the short-term responses, potentially confounding effects of long-term acclimation responses on short-term correlations were controlled. We first regressed 'mean HKDT' on 'test day' for each species and extracted the residuals from the regressions. The residuals were then correlated with all short-term climate variables (less than 24 h prior to tests). The long-term responses were examined by correlating 'mean HKDT' with the extracted long-term bins or windows (1–14 days preceding tests). All analyses were carried out using the software R [56], and raw files for the analyses can be accessed in the electronic supplementary material.

3. Results

(a) Contrasting climatic conditions at study locations

The temperate study sites were characterized by high-temperature variability both within days and between months (figure 1 and electronic supplementary material, table S2). During the field experiments, the daily mean temperature averaged across month dropped from 21.30°C in February to 13.10°C in May. The daily variability in temperature (CV) ranged from 0.18 in March to 0.27 in April. As expected, temperatures were more stable at the tropical sites (figure 1 and electronic supplementary material, table S2). The daily average temperature in June was 21.04–21.53°C, and the daily temperature variability was extremely low, with CVs of 0.04–0.05 across the study sites.

Relative humidity (%RH) was highly correlated with the temperature at the temperate sites (electronic supplementary material, figure S2), but not at the tropical sites. The daily mean RH ranged from 60.9 to 75.4% at the temperate sites, and daily CVs were in the range of 0.14–0.23. In the tropical study sites, RH was high at all times, with daily means of 86.9–90.5% and CVs of 0.03–0.05 across study sites.

(b) Both tolerance and plasticity for tolerance varied within and between days for many species

CT_{max} scores differed between the collected insect groups (Kruskal–Wallis $H(11) = 314.71$, $p < 0.001$, table 1), with the temperate species *S. macreta* being the most heat-tolerant, and the tropical species *D. rubida* being the least heat-tolerant. For all species, HKDT varied markedly within and across days (figure 2; electronic supplementary material, tables S3 and S4). Females had higher HKDTs than males in six of the seven species for which we had information on the sex (figure 2; electronic supplementary material, table S3). In addition, HKDT varied differently for the sexes between days, but not within days except for *D. melanogaster* and the Cicadellidae sp. The relationship between HKDT and the time of the day that individuals were tested was examined further by regression. In the majority of cases (71.2%), we observed a significant quadratic relation between 'time of day' and HKDT (74 out of 104 combinations of species and test days with more than one sampling time), and few cases (4.8%) had a linear shape. Overall, HKDT varied within days in 76% of the tested sex–species combinations; however, the direction in the change of HKDT varied across days.

In order to quantify variation in heat tolerance, we calculated the variability in HKDT compared with the mean HKDT value (%CV) for each day as a measure of plasticity, and the daily change in mean HKDT relative to the lowest mean HKDT (table 1). The mean CV ranged between 21.2 and 41.9%, with *N. caledoniae* and *D. rubida* representing the extremes. Plasticity levels varied notably across days for all species originating from both regions. The highest daily CVs were 51.7 and 46.2% for the tropical and temperate flies *D. rubida* and *D. melanogaster*, respectively, and the lowest daily CVs were 14.1 and 14.2% for the temperate *N. caledoniae* and *S. macreta*, followed by 18.3 and 18.9% for the tropical ant *O. smaragdina* and Cicadellidae sp., respectively.

The daily change in mean HKDT relative to the lowest mean for these species reflected the CVs. For instance, *N. caledoniae* had a relatively low average change in mean HKDT of 17.6% compared with that of *D. rubida* of 31.7% or *D. melanogaster* of 35.7%. Despite this, the daily change in HKDT for *N. caledoniae* was high on some days and reached a change of 48.4% in HKDT relative to the daily minimum. The maximum daily increase in HKDT was recorded for *D. rubida*, and reached 90.3% on one of the test days, followed by *S. novoguineensis*, for which the highest recorded daily change in HKDT was 87.6%.

(c) Species-specific associations between microclimate variables and heat tolerance

The correlations between residuals of HKDT and short-term mean temperature bins were highly species- and time-specific; thus both the strength and direction of correlations varied in time for the different species (figure 3*a*). For most of the temperate species, the mean temperature experienced in the time prior to testing thermal tolerance was positively associated with HKDT for up to 10–12 h (figure 3). For example, the correlations of HKDT with mean temperature for *D. melanogaster* were strongest in the first 11 h, thereafter decreasing slightly. These short-term association patterns were less evident when using 'time windows' for extraction of climate variables (electronic supplementary material, figure S3A).

Long-term correlations between HKDT and mean temperature and humidity bins were more variable than short-term correlations, especially for temperate species. However, for several species of both temperate and tropical origin (e.g. *D. melanogaster*, *U. sonchi*, *H. lactucae*, Cicadellidae sp. and *P. guttus*), we found relatively strong positive correlations with long-term mean temperatures. Of these cases, *H. lactucae*, Cicadellidae sp. and *P. guttus* had no or very weak associations of HKDT with short-term temperature bins (figure 3*a*). Notably, long-term correlations for species of tropical origin were stronger and more directional compared with the short-term responses. An example is *D. novoguineensis*, a species for which long-term correlations between mean

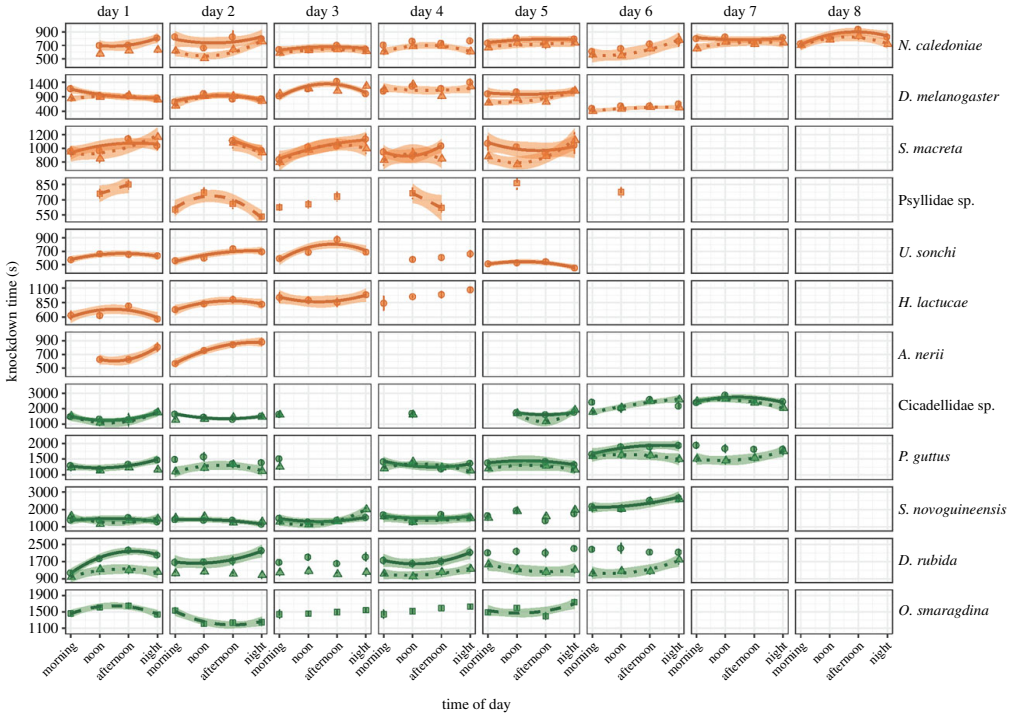


Figure 2. Mean knockdown times (HKDT) measured multiple times (1–4) daily and across days for each species from temperate locations (orange) and tropical locations (green). The knockdown temperatures are displayed for males (triangles) and females (circles) separately when sex was identified, and squares display species for which sex could not be determined. Bars are standard errors of the means. Significant relations between the HKDT and 'time of day' were visualized by polynomial or linear regressions with 95% confidence bands. Solid regression lines represent females, dotted lines are for males, and dashed lines are provided for species of unknown sex. (Online version in colour.)

temperature and HKDT were mostly negative, but short-term associations were weaker and positive.

Comparison of temperature and humidity means revealed opposing directions of correlations for the temperate species (figure 3*a*,*c*). For example, HKDT of *D. melanogaster* was positively correlated with mean temperature but negatively correlated with mean humidity for both the short- and long-term bins. This pattern was not evident for most tropical species; for instance, long-term temperature and humidity means were both positively correlated with heat tolerance in *P. guttus*, or negatively correlated with heat tolerance in *S. novoguineensis*. The same correlations are apparent based on 'time windows' (electronic supplementary material, figure S3A,C).

Variability in temperature and humidity were only sporadically associated with HKDT in both the short and long term (less than 24 h) when assessing CV across 1 or 24 h bins, where the temperature does not vary much (figure 3*b*,*d*). Using accumulated CVs (time windows) for correlations with HKDT, in six of the species (50%) HKDT correlated with long-term mean temperature and humidity windows (electronic supplementary material, figure S3B,D). Temperature and humidity CVs were generally positively associated with HKDT in the late short-term windows, but the associations with variability in humidity were stronger for most species, e.g. the bugs *N. caledoniae* and *S. macreta* (10–16 h) (electronic supplementary material, figure S3D). Contrary to the climatic means, the CVs had the same direction of correlations for both temperature and humidity, in both the short and long term (electronic supplementary material, figure S3B,D).

4. Discussion

(a) Extensive and species-specific plasticity

In the present study, we evaluated the temporal variation in heat tolerance of field-sampled individuals from different arthropod species from temperate and tropical Australia. We observed high levels of variability in heat tolerance for both tropical and temperate species (%CV, table 1). Further, some species, such as the tropical flies *S. novoguineensis* and *D. rubida*, occasionally increased their HKDT substantially relative to the lowest recorded HKDT within a day. Several studies have deemed tropical species especially vulnerable to increases in temperature as they are considered to be currently living at the edge of their thermal safety margins [3,4], and have limited capacity for adaptive and plastic responses to warm temperatures [11–14,16,17] and concomitant desiccation stress [57,58]. Our results suggest that many arthropods in nature have a high capacity to adjust their upper thermal tolerance to small alterations in the environment. Thus, the realized thermal acclimation likely plays an important role for species' ability to cope with fluctuating temperatures at a daily or monthly scale and the ability to induce plasticity is likely under strong selection.

The variability in thermal tolerance for the individual species observed within and across days may, however, not solely be related to climatic variation. Perhaps different genotypes are caught at different time points during the days and across sampling days, affecting thermal tolerances [59], although a recent field study on *Orchesella cincta* attributed

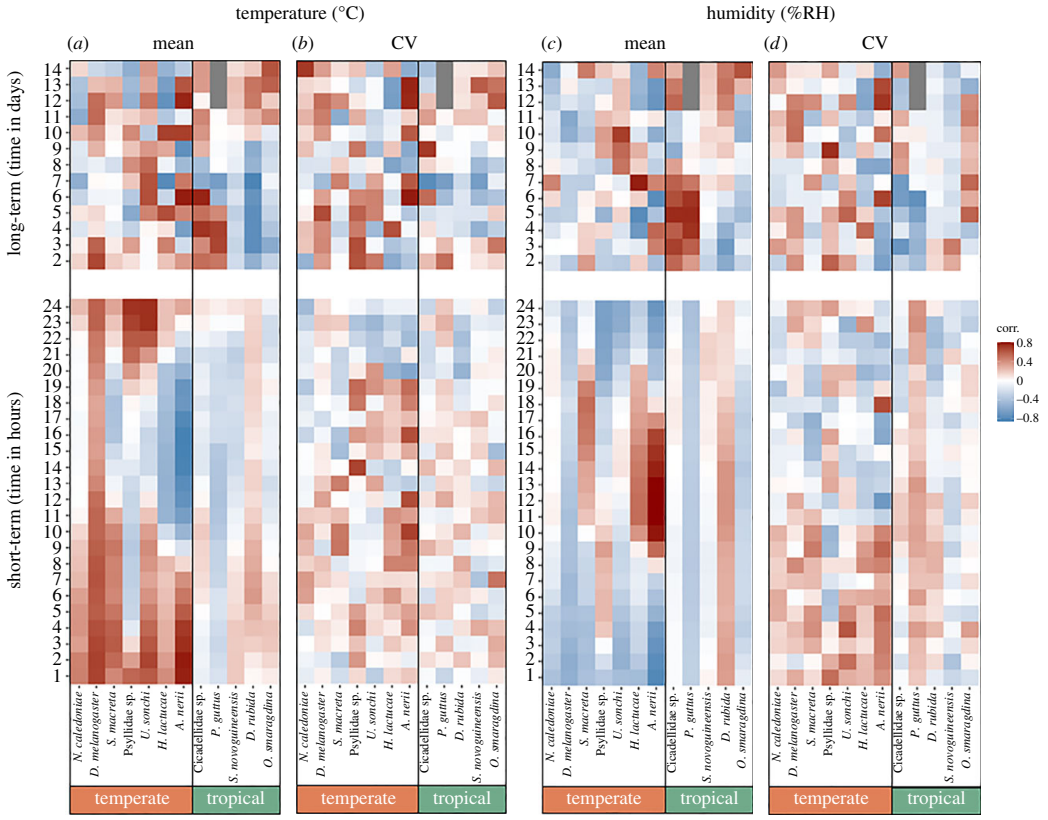


Figure 3. Heatmap showing correlations between HKDT for each species and the mean and CV of microclimate temperature (*a*) and humidity (*b*) extracted in short-term (less than 24 h) and long-term (2–14 days) 'time bins'. The species are grouped into origin from either temperate or tropical Australia. Grey boxes indicate that climate data were not available.

monthly variation in heat tolerance to acclimation rather than genetic differences between animals [44]. Age [60–64], nutritional status [60,65–67] and mating status [68] are other factors known to affect heat tolerance in a range of insect species. These factors may differ in the individuals tested at different time points, which means that any differences in heat tolerance within and across days need to be interpreted cautiously.

Despite these issues, our data suggest that species have the potential for adjusting to new temperature and humidity regimes within a species' current range, and perhaps also to conditions outside their current distribution range. This could allow some species to exhibit larger range sizes in the future [69,70]. However, while the flexibility in heat tolerance detected within and across days in our study may be adaptive, some caution is required given that the fitness consequences of trait changes will be context-dependent [71], and that the same environmental changes might trigger adaptive plastic responses in some traits but trigger costly changes for others [72]. Moreover, recent studies have shown that non-lethal endpoints such as behavioural and reproductive traits might be impacted differently by temperature changes [20,21]. Male fertility thermal limits may often be lower than heat tolerance limits, and many species may, therefore, be exposed to temperatures closer to their upper thermal limits than currently presumed. Our assays also do not capture the effects of temperature plasticity on traits such as the ability to find food resources [73] or the ability to respond to more variable temperature conditions [44,74].

Finally, the acclimation potential of species may be much greater than observed in wild populations [75]. This may be particularly true for temperate species which are exposed to larger seasonal variation and unpredictable changes in temperatures when compared with tropical species. This could lead to underestimates of the actual acclimatization capacity of both tropical and temperate species in the present study as they have not been examined under the full range of temperatures that they potentially could endure. On the other hand, laboratory acclimation treatments are often inconsistent or insufficient to produce the maximal plastic responses for different species and exclude the option of species to behaviourally modify their thermal tolerance [75]. A combination of field and laboratory studies is needed to further investigate such issues on the same species.

(b) Climatic conditions explaining heat tolerance

Optimizing process-based distribution models requires accurate understanding of the physiological metrics that serve as the best predictor of species' vulnerability to environmental change across phylogenetically diverse species and geographical regions. In this study, we found large variation

in heat tolerance across days and hours for all species, suggesting that the fine-scale spatio-temporal environment has a great impact on small invertebrates, and this might not be reflected in the macroclimate, which is typically used for modelling species' vulnerabilities. Defining the climate variables that best explain observed differences, and the spatio-temporal scale that these vary at, are thus central for understanding species' responses to increasingly warm and variable temperatures.

We found that short-term variation in the microhabitat mean temperature and humidity (variation 24 h prior to the assessment of heat tolerance) had a strong influence on some tropical and temperate species (figure 3*a*,*c* and electronic supplementary material, figure S3A,C). For most species, the short-term correlations between HKDT and mean habitat temperatures were positive, implying that heat hardening responses to shifts in microhabitat environmental temperatures take place in most organisms. This relationship was not evident for the bug species *P. guttus*, which had a negative correlation with temperature, thus being less heat-resistant at warm habitat temperatures. It is unclear whether this indicates a cost of warm temperatures and possibly other environmental factors on fitness components, or if resources are allocated for other vital physiological functions at high temperatures. In addition, the temporal scale at which the positive associations were observed differed markedly between species. Thus, for some, e.g. the Psyllidae sp., the mean temperature in the 2–3 h prior to testing was critical for heat tolerance, whereas for *D. melanogaster*, temperatures experienced up to 14 days prior to testing were typically positively associated with heat tolerance. For *D. melanogaster*, the life cycle is short (one to a few weeks depending on temperatures) [76], and it is well-known from laboratory studies that developmental temperature has a high impact on adult heat tolerance in insects (e.g. [77–79]). Thus, the strong correlations observed between long-term mean environmental temperatures and HKDT for *D. melanogaster* and several other species, might be explained by developmental or adult acclimation.

The impact of temperature on HKDT had opposing effects compared with the impact of humidity for some temperate species (figure 3*a*,*c*; electronic supplementary material, figure S3A,C). Thus, while recent exposure to high temperatures was associated with high HKDT, exposure to high humidity was associated with low HKDT. This pattern was likely caused by strong negative correlations between environmental temperature and humidity in species from the temperate locations (electronic supplementary material, figure S2), which complicates our ability to separate the effects of temperature and humidity. These variables were not correlated in the tropical locations, and the direction of correlations of temperature and humidity on HKDT did not oppose each other in tropical species (figure 3*a*,*c*). This suggests that both temperature and humidity are important climate variables for predicting tropical species' tolerances [57,80,81].

Finally, variability of temperature and humidity did not seem to affect thermal tolerances on a day to day basis (figure 3*b*,*d*), but CV measured over multiple days using 'time windows' for extraction of climate variables showed that climatic variability had an increasing significant association with HKDT on the long-term scale for several species. In accordance with this, a study that examined monthly differences in thermal tolerances in natural populations of the collembolan *O. cincta* found that diurnal range was the best predictor for HKDT and CT_{max} [44].

In conclusion, we found strong evidence for the importance of plasticity in insects for coping with variable thermal conditions in the field, and that climatic variables affecting heat tolerance were species-specific. While further investigations are needed, our results suggest that the evolution of plasticity is important to understand future responses of species to increasingly variable thermal environments. Obviously, studies like ours cannot be performed easily and on a large number of species throughout their range. However, our results suggest that microhabitat temperatures need to be considered in correlative and mechanistic species distribution modelling. Methods to obtain these climate data on fine temporal and spatial scales do exist [49,82–84]. Our results also indicate that trait information incorporated in mechanistic models should take into account the plasticity of these traits. For species of particular interest from a conservation or agricultural pest perspective, relevant data from the laboratory and ideally the field should be generated, including a consideration of populations from the edges of a species' distribution. Incorporating such information into models would improve the prediction of expected future species distributions. Standardized ways to measure the plasticity of relevant traits and to store data in an open-access database would facilitate this process.

Data accessibility. All data are presented in the main manuscript and electronic supplementary material. Data and raw files are provided in the electronic supplementary material [85].

Authors' contributions. S.B., T.N.K., A.A.H. and N.K.N. conceived the ideas and designed the methodology. M.S., S.B., T.N.K. and N.K.N. collected the data. M.Ø. and N.K.N. analysed the data. T.N.K. and N.K.N. led the writing of the manuscript, and all authors contributed to the drafts and gave final approval for publication. All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Competing interests. The authors declare no conflicts of interest.

Funding. This work was supported by a grant from the Danish Council for Independent Research (grant no. DFF-8021-00014B) to T.N.K and an Australian Research Council Discovery grant no. 120100916 to A.A.H. Travel grants were provided to N.K.N. from Otto Mønsted Fond, Knud Højgaards Fond and the Royal Danish Academy of Sciences and Letters.

Acknowledgements. We are grateful to Dr Peter Ridland at Bio21, Melbourne University, and Dr Mallik B. Malipatil at AgriBio, La Trobe University, for guidance on insect identification and searching for suitable sampling locations, and to Palle Duun Rohde, Aalborg University, for advice on the statistical analyses. We further thank the Staff at Melbourne Zoo, especially Thomas Meek for providing sampling permissions and access to the zoo backstage for collection of *D. melanogaster*.

References

1. Sinclair BJ *et al.* 2016 Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? *Ecol. Lett.* **19**, 1372–1385. (doi:10.1111/ele.12686)

2. Bale JS *et al.* 2002 Herbivory in global climate change research: direct effects of rising

temperature on insect herbivores. *Glob. Change Biol.* **8**, 1–16. (doi:10.1046/j.1365-2486.2002.00451.x)

3. Sunday JM, Bates AE, Kearney MR, Colwell RK, Dulvy NK, Longino JT, Huey RB. 2014 Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proc. Natl Acad. Sci. USA* **111**, 5610–5615. (doi:10.1073/pnas.1316145111)
4. Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC, Martin PR. 2008 Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl Acad. Sci. USA* **105**, 6668–6672. (doi:10.1073/pnas.0709472105)
5. Bale JS. 2002 Insects and low temperatures: from molecular biology to distributions and abundance. *Phil. Trans. R. Soc. Lond. B* **357**, 849–862. (doi:10.1098/rstb.2002.1074)
6. Araújo MB, Luoto M. 2007 The importance of biotic interactions for modelling species distributions under climate change. *Glob. Ecol. Biogeogr.* **16**, 743–753. (doi:10.1111/j.1466-8238.2007.00359.x)
7. Parmesan C, Yohe G. 2003 A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**, 37–42. (doi:10.1038/nature01286)
8. Clusella-Trullas S, Blackburn TM, Chown SL. 2011 Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *Am. Nat.* **177**, 738–751. (doi:10.1086/660021)
9. Kearney M, Porter W. 2009 Mechanistic niche modelling: combining physiological and spatial data to predict species' ranges. *Ecol. Lett.* **12**, 334–350. (doi:10.1111/j.1461-0248.2008.01277.x)
10. Evans TG, Diamond SE, Kelly MW. 2015 Mechanistic species distribution modelling as a link between physiology and conservation. *Conserv. Physiol.* **3**, cov056. (doi:10.1093/conphys/cov056)
11. Overgaard J, Kearney MR, Hoffmann AA. 2014 Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. *Glob. Change Biol.* **20**, 1738–1750. (doi:10.1111/gcb.12521)
12. Addo-bediako AA, Chown SL, Gaston KJ. 2000 Thermal tolerance, climatic variability and latitude. *Proc. R. Soc. Lond. B* **267**, 739–745. (10.1098/rspb.2000.1065)
13. Sunday JM, Bates AE, Dulvy NK. 2011 Global analysis of thermal tolerance and latitude in ectotherms. *Proc. R. Soc. Lond. B* **278**, 1823–1830. (doi:10.1098/rspb.2010.1295)
14. MacLean HJ, Sørensen JG, Kristensen TN, Loeschcke V, Beedholm K, Kellermann V, Overgaard J. 2019 Evolution and plasticity of thermal performance: an analysis of variation in thermal tolerance and fitness in 22 *Drosophila* species. *Phil. Trans. R. Soc. B* **374**, 20180548. (doi:10.1098/rstb.2018.0548)
15. Kellermann V, Loeschcke V, Hoffmann AA, Kristensen TN, Fløjgaard C, David JR, Svenning JC, Overgaard J. 2012 Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution* **66**, 3377–3389. (doi:10.1111/j.1558-5646.2012.01685.x)
16. Overgaard J, Kristensen TN, Mitchell KA, Hoffmann AA. 2011 Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *Am. Nat.* **178**, S80-S96. (doi:10.1086/661780)
17. Kellermann V, Overgaard J, Hoffmann AA, Fljøgaard C, Svenning JC, Loeschcke V. 2012 Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl Acad. Sci. USA* **109**, 16 228–16 233. (doi:10.1073/pnas.1207553109)
18. Kingsolver JG, Diamond SE, Buckley LB. 2013 Heat stress and the fitness consequences of climate change for terrestrial ectotherms. *Funct. Ecol.* **27**, 1415–1423. (doi:10.1111/1365-2435.12145)
19. Kingsolver JG, Buckley LB. 2017 Quantifying thermal extremes and biological variation to predict evolutionary responses to changing climate. *Phil. Trans. R. Soc. B* **372**, 20160147. (doi:10.1098/rstb.2016.0147)
20. Parratt SR, Walsh BS, Metelmann S, White N, Manser A, Bretman AJ, Hoffmann AA, Snook RR, Price TAR. 2021 Temperatures that sterilize males better match global species distributions than lethal temperatures. *Nat. Clim. Change* **11**, 481–484. (doi:10.1038/s41558-021-01047-0)
21. van Heerwaarden B, Sgrò CM. 2021 Male fertility thermal limits predict vulnerability to climate warming. *Nat. Commun.* **12**, 2214. (doi:10.1038/s41467-021-22546-w)
22. Bogert CM. 1949 Thermoregulation in reptiles; a factor in evolution. *Evolution* **3**, 195–211. (doi:10.1111/j.1558-5646.1949.tb00021.x)
23. Kearney M, Shine R, Porter WP. 2009 The potential for behavioral thermoregulation to buffer 'cold-blooded' animals against climate warming. *Proc. Natl Acad. Sci. USA* **106**, 3835–3840. (doi:10.1073/pnas.0808913106)
24. Huey RB, Tewksbury JJ. 2009 Can behavior douse the fire of climate warming? *Proc. Natl Acad. Sci. USA* **106**, 3647–3648. (doi:10.1073/pnas.0900934106)
25. Huey RB, Kearney MR, Krockenberger A, Holtum JAM, Jess M, Williams SE. 2012 Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Phil. Trans. R. Soc. B* **367**, 1665–1679. (doi:10.1098/rstb.2012.0005)
26. Araújo MB, Ferri-Yáñez F, Bozinovic F, Marquet PA, Valladares F, Chown SL. 2013 Heat freezes niche evolution. *Ecol. Lett.* **16**, 1206–1219. (doi:10.1111/ele.12155)
27. Kelly MW, Sanford E, Grosberg RK. 2012 Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. B* **279**, 349–356. (doi:10.1098/rspb.2011.0542)
28. Hoffmann AA, Chown SL, Clusella-Trullas S. 2013 Upper thermal limits in terrestrial ectotherms: how constrained are they? *Funct. Ecol.* **27**, 934–949. (doi:10.1111/j.1365-2435.2012.02036.x)
29. Valladares F *et al*. 2014 The effects of phenotypic plasticity and local adaptation on forecasts of species range shifts under climate change. *Ecol. Lett.* **17**, 1351–1364. (doi:10.1111/ele.12348)
30. Fox RJ, Donelson JM, Schunter C, Ravasi T, Gaitán-Espitia JD. 2019 Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Phil. Trans. R. Soc. B* **374**, 20180174. (doi:10.1098/rstb.2018.0174)
31. Davis MB, Shaw RG. 2001 Range shifts and adaptive responses to quaternary climate change. *Science* **292**, 673–679. (doi:10.1126/science.292.5517.673)
32. Hoffmann AA, Sørensen JG, Loeschcke V. 2003 Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175–216. (doi:10.1016/S0306-4565(02)00057-8)
33. Mitchell KA, Hoffmann AA. 2010 Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. *Funct. Ecol.* **24**, 694–700. (doi:10.1111/j.1365-2435.2009.01666.x)
34. Hoffmann AA, Dagher H, Hercus M, Berrigan D. 1997 Comparing different measures of heat resistance in selected lines of *Drosophila melanogaster*. *J. Insect Physiol.* **43**, 393–405. (doi:10.1016/S0022-1910(96)00108-4)
35. Chown SL, Jumbam KR, Sørensen JG, Terblanche JS. 2009 Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. *Funct. Ecol.* **23**, 133–140. (doi:10.1111/j.1365-2435.2008.01481.x)
36. Jørgensen LB, Malte H, Ørsted M, Klahn NA, Overgaard J. 2021 A unifying model to estimate thermal tolerance limits in ectotherms across static, dynamic and fluctuating exposures to thermal stress. *Scient. Rep.* **11**, 12840. (doi:10.1038/s41598-021-92004-6)
37. Ribeiro PL, Camacho A, Navas CA. 2012 Considerations for assessing maximum critical temperatures in small ectothermic animals: insights from leaf-cutting ants. *PLoS ONE* **7**, 32083. (doi:10.1371/journal.pone.0032083)
38. Rezende EL, Tejedo M, Santos M. 2011 Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. *Funct. Ecol.* **25**, 111–121. (doi:10.1111/j.1365-2435.2010.01778.x)
39. Dahlgaard J, Loeschcke V, Michalak P, Justesen J. 1998 Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Funct. Ecol.* **12**, 786–793. (doi:10.1046/j.1365-2435.1998.00246.x)
40. Bahrndorff S, Mariën J, Loeschcke V, Ellers J. 2009 Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. *Funct. Ecol.* **23**, 233–239. (doi:10.1111/j.1365-2435.2009.01541.x)

41. Manenti T, Sørensen JG, Moghadam NN, Loeschcke V. 2014 Predictability rather than amplitude of temperature fluctuations determines stress resistance in a natural population of *Drosophila simulans*. *J. Evol. Biol.* **27**, 2113–2122. (doi:10.1111/jeb.12463)
42. Manenti T, Loeschcke V, Moghadam NN, Sørensen JG. 2015 Phenotypic plasticity is not affected by experimental evolution in constant, predictable or unpredictable fluctuating thermal environments. *J. Evol. Biol.* **28**, 2078–2087. (doi:10.1111/jeb.12735)
43. Schou MF, Loeschcke V, Kristensen TN. 2015 Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. *PLoS ONE* **10**, e0130307. (doi:10.1371/journal.pone.0130307)
44. Jensen A, Alemu T, Alemneh T, Pertoldi C, Bahrndorff S. 2019 Thermal acclimation and adaptation across populations in a broadly distributed soil arthropod. *Funct. Ecol.* **33**, 833–845. (doi:10.1111/1365-2435.13291)
45. Lenoir J, Hattab T, Pierre G. 2017 Climatic microrefugia under anthropogenic climate change: implications for species redistribution. *Ecography* **40**, 253–266. (doi:10.1111/ecog.02788)
46. Lembrechts JJ, Lenoir J. 2020 Microclimatic conditions anywhere at any time! *Glob. Change Biol.* **26**, 337–339. (doi:10.1111/gcb.14942)
47. Pincebourde S, Salle A. 2020 On the importance of getting fine-scale temperature records near any surface. *Glob. Change Biol.* **26**, 6025–6027. (doi:10.1111/gcb.15210)
48. Potter KA, Woods HA, Pincebourde S. 2013 Microclimatic challenges in global change biology. *Glob. Change Biol.* **19**, 2932–2939. (doi:10.1111/gcb.12257)
49. Kearney MR, Shamakhy A, Tingley R, Karoly DJ, Hoffmann AA, Briggs PR, Porter WP. 2014 Microclimate modelling at macro scales: a test of a general microclimate model integrated with gridded continental-scale soil and weather data. *Methods Ecol. Evol.* **5**, 273–286. (doi:10.1111/2041-210X.12148)
50. Sears MW, Riddell EA, Rusch TW, Angilletta MJ. 2019 The world still is not flat: lessons learned from organismal interactions with environmental heterogeneity in terrestrial environments. *Integr. Comp. Biol.* **59**, 1049–1058. (doi:10.1093/icb/icz130)
51. Kinzner MT, Kinzner MC, Kaufmann R, Hoffmann AA, Arthofer W, Schlick-Steiner BC, Steiner FM. 2019 Is temperature preference in the laboratory ecologically relevant for the field? The case of *Drosophila nigrosparsa*. *Glob. Ecol. Conserv.* **18**, e00638. (doi:10.1016/j.gecco.2019.e00638)
52. Bahrndorff S, Loeschcke V, Pertoldi C, Beier C, Holmstrup M. 2009 The rapid cold hardening response of Collembola is influenced by thermal variability of the habitat. *Funct. Ecol.* **23**, 340–347. (doi:10.1111/j.1365-2435.2008.01503.x)
53. Malipatil MB. 2010 Review and revision of *Nysius* Dallas of Australia and South West Pacific (Hemiptera: Heteroptera: Orsillidae). *Zootaxa* **44**, 29–44. (doi:10.11646/zootaxa.2410.1.2)
54. Eyles AC, Malipatil MB. 2010 *Nysius caledoniae* Distant, 1920 (Hemiptera: Heteroptera: Orsillidae) a recent introduction into New Zealand, and keys to the species of *Nysius*, and genera of Orsillidae, in New Zealand. *Zootaxa* **1920**, 45–52. (doi:10.11646/zootaxa.2484.1.4)
55. Overgaard J, Kristensen TN, Sørensen JG. 2012 Validity of thermal ramping assays used to assess thermal tolerance in arthropods. *PLoS ONE* **7**, e32758. (doi:10.1371/journal.pone.0032758)
56. R Core Team. 2021 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See http://www.R-project.org/.
57. Hoffmann AA, Hallas RJ, Dean JA, Schiffer M. 2003 Low potential for climatic stress adaptation in a rainforest *Drosophila* species. *Science* **301**, 100–102. (doi:10.1126/science.1084296)
58. Kellermann V, van Heerwaarden B, Sgrò CM, Hoffmann AA. 2009 Fundamental evolutionary limits in ecological traits drive *Drosophila* species distribution. *Science* **325**, 3–5. (doi:10.1126/science.1175443)
59. Fournier-Level A, Perry EO, Wang JA, Braun PT, Migneault A, Cooper MD, Metcalf CJE, Schmitt J. 2016 Predicting the evolutionary dynamics of seasonal adaptation to novel climates in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **113**, E2812–E2821. (doi:10.1073/PNAS.1517456113)
60. Chidawanyika F, Nyamukondiwa C, Strathie L, Fischer K. 2017 Effects of thermal regimes, starvation and age on heat tolerance of the parthenium beetle *Zygogramma bicolorata* (Coleoptera: Chrysomelidae) following dynamic and static protocols. *PLoS ONE* **12**, e0169371. (doi:10.1371/JOURNAL.PONE.0169371)
61. Bowler K, Terblanche JS. 2008 Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? *Biol. Rev.* **83**, 339–355. (doi:10.1111/j.1469-185X.2008.00046.x)
62. Slotsbo S, Schou MF, Kristensen TN, Loeschcke V, Sørensen JG. 2016 Reversibility of developmental heat and cold plasticity is asymmetric and has long-lasting consequences for adult thermal tolerance. *J. Exp. Biol.* **219**, 2726–2732. (doi:10.1242/JEB.143750)
63. Kristensen TN, Loeschcke V, Tan Q, Pertoldi C, Mengel-From J. 2019 Sex and age specific reduction in stress resistance and mitochondrial DNA copy number in *Drosophila melanogaster*. *Scient. Rep.* **9**, 12305. (doi:10.1038/s41598-019-48752-7)
64. Alemu T, Alemneh T, Pertoldi C, Ambelu A, Bahrndorff S. 2017 Costs and benefits of heat and cold hardening in a soil arthropod. *Biol. J. Linn. Soc.* **122**, 765–773. (doi:10.1093/biolinnean/blx092)
65. Bujan J, Kaspari M. 2017 Nutrition modifies critical thermal maximum of a dominant canopy ant. *J. Insect Physiol.* **102**, 1–6. (doi:10.1016/J.JINSPHYS.2017.08.007)
66. Manenti T, Cunha TR, Sørensen JG, Loeschcke V. 2018 How much starvation, desiccation and oxygen depletion can *Drosophila melanogaster* tolerate before its upper thermal limits are affected? *J. Insect Physiol.* **111**, 1–7. (doi:10.1016/J.JINSPHYS.2018.09.002)
67. Andersen LH, Kristensen TN, Loeschcke V, Toft S, Mayntz D. 2010 Protein and carbohydrate composition of larval food affects tolerance to thermal stress and desiccation in adult *Drosophila melanogaster*. *J. Insect Physiol.* **56**, 336–340. (doi:10.1016/J.JINSPHYS.2009.11.006)
68. Xue Q, Ma C Sen. 2020 Aged virgin adults respond to extreme heat events with phenotypic plasticity in an invasive species, *Drosophila suzukii*. *J. Insect Physiol.* **121**, 104016. (doi:10.1016/J.JINSPHYS.2020.104016)
69. Hampe A, Petit RJ. 2005 Conserving biodiversity under climate change: the rear edge matters. *Ecol. Lett.* **8**, 461–467. (doi:10.1111/j.1461-0248.2005.00739.x)
70. Hoffmann AA, Sgró CM. 2011 Climate change and evolutionary adaptation. *Nature* **470**, 479–485. (doi:10.1038/nature09670)
71. Bahrndorff S, Gertsen S, Pertoldi C, Kristensen TN. 2016 Investigating thermal acclimation effects before and after a cold shock in *Drosophila melanogaster* using behavioural assays. *Biol. J. Linn. Soc.* **117**, 241–251. (doi:10.1111/BIJ.12659)
72. Hendry AP. 2016 Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *J. Hered.* **107**, 25–41. (doi:10.1093/JHERED/ESV060)
73. Kristensen TN, Hoffmann A, Overgaard J, Sørensen JG, Hallas R, Loeschcke V. 2008 Costs and benefits of cold acclimation in field-released *Drosophila*. *Proc. Natl Acad. Sci. USA* **105**, 216–221. (doi:10.1073/pnas.0708074105)
74. Overgaard J, Sørensen JG. 2008 Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. *Cryobiology* **56**, 159–162. (doi:10.1016/j.cryobiol.2008.01.001)
75. Pintor AF, Schwarzkopf L, Krockenberger AK. 2016 Extensive acclimation in ectotherms conceals interspecific variation in thermal tolerance limits. *PLoS ONE* **11**, e0150408. (doi:10.1371/journal.pone.0150408)
76. Fernández-Moreno MA, Farr CL, Kaguni LS, Garesse R. 2007 *Drosophila melanogaster* as a model system to study mitochondrial biology. *Methods Mol. Biol.* **372**, 33–49. (doi:10.1007/978-1-59745-365-3_3)
77. Schou MF, Kristensen TN, Pedersen A, Karlsson G, Loeschcke V, Malmendal A. 2017 Metabolic and functional characterization of effects of developmental temperature in *Drosophila melanogaster*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **312**, R211–R222. (doi:10.1152/ajpregu.00268.2016)

78. Shah AA, Funk WC, Ghalambor CK. 2017 Thermal acclimation ability varies in temperate and tropical aquatic insects from different elevations. *Integr. Comp. Biol.* **57**, 977–987. (doi:10.1093/icb/icx101)
79. Terblanche JS, Chown SL. 2006 The relative contributions of developmental plasticity and adult acclimation to physiological variation in the tsetse fly, *Glossina pallidipes* (Diptera, Glossinidae). *J. Exp. Biol.* **209**, 1064–1073. (doi:10.1242/jeb.02129)
80. Angilletta MJ. 2009 Thermal acclimation. In *Thermal adaptation: a theoretical and empirical synthesis*, pp. 126–156. Oxford, UK: Oxford University Press.
81. Chown SL, Hoffmann AA, Kristensen TN, Angilletta MJ, Stenseth NC, Pertoldi C. 2010 Adapting to climate change: a perspective from evolutionary physiology. *Clim. Res.* **43**, 3–15. (doi:10.3354/cr00879)
82. Kearney MR, Gillingham PK, Bramer I, Duffy JP, Maclean IMD. 2019 A method for computing hourly, historical, terrain-corrected microclimate anywhere on earth. *Methods Ecol. Evol.* **11**, 38–43. (doi:10.1111/2041-210X.13330)
83. Maclean IMD. 2019 Predicting future climate at high spatial and temporal resolution. *Glob. Change Biol.* **26**, 1003–1011. (doi:10.1111/gcb.14876)
84. Maclean IMD *et al*. 2021 On the measurement of microclimate. *Methods Ecol. Evol.* **12**, 1397–1410 (doi:10.1111/2041-210x.13627)
85. Noer NK, Ørsted M, Schiffer M, Hoffmann AA, Bahrndorff S, Kristensen TN. 2022 Into the wild—a field study on the evolutionary and ecological importance of thermal plasticity in ectotherms across temperate and tropical regions. Figshare.

SUPPLEMENTAL MATERIALS FOR PAPER I

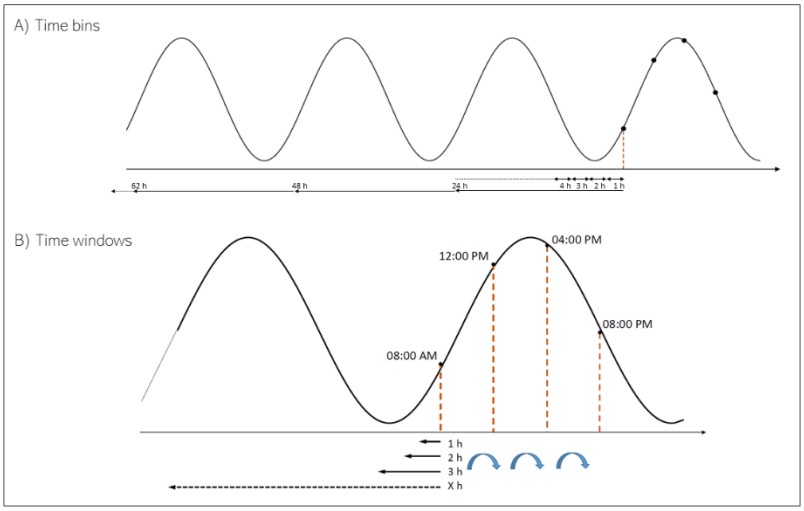


Figure S1: Extraction of the microclimate variables 'mean' and 'coefficient of variation' from recorded temperature and humidity data. The variables were extracted as (A) 'time bins', where variables were extracting in rolling, non-overlapping, 1 or 24 h bins, and (B) 'time windows', where variables were extracted in expanding windows that increased by 1 h intervals in the first 24 hours and in intervals of 24 hours and up to 14 days.

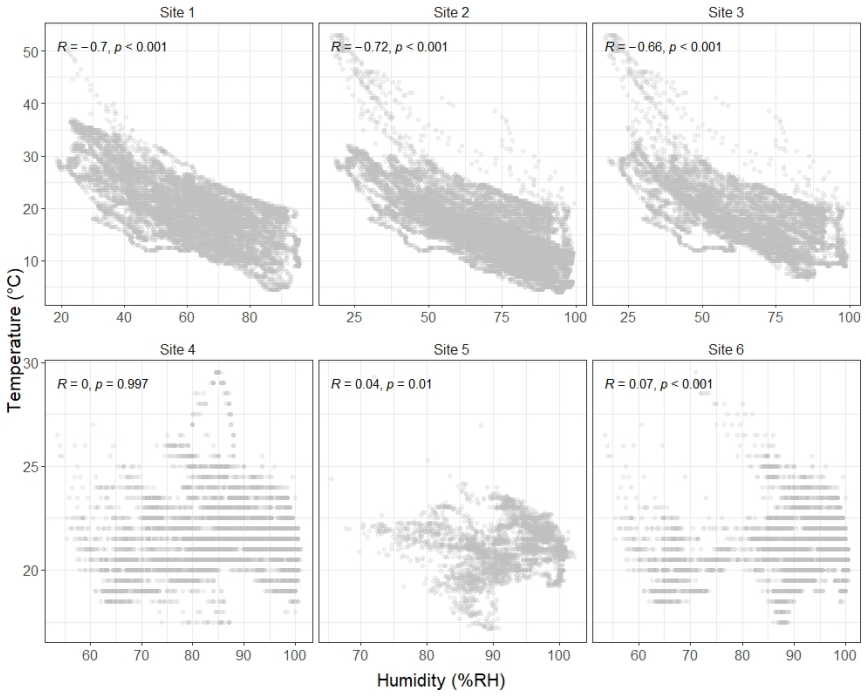


Figure S2: Relationship between temperature and humidity in the study sites in temperate (top panel) and tropical (bottom panel) sites. Pearson's correlations were used to examine the relationship between temperature and humidity at each study site (upper panels Temperate and lower panels Tropical sites). Correlation coefficients and significance of correlations are displayed in the plots. All climate recordings from each location are presented, thus, the temperate sites had more data points due to the longer period of field work which appears as finer resolution of the data. However, the resolution and recording interval was the same for each site, except for Site 5. At site 5, an iButton (DS1923-F5#, Hygrochron, iButtonLink) was used to record data (back-up) because the Easylog malfunctioned. The iButton had a resolution of 0.1°C, whereas Easylogs record with 0.5°C resolution.

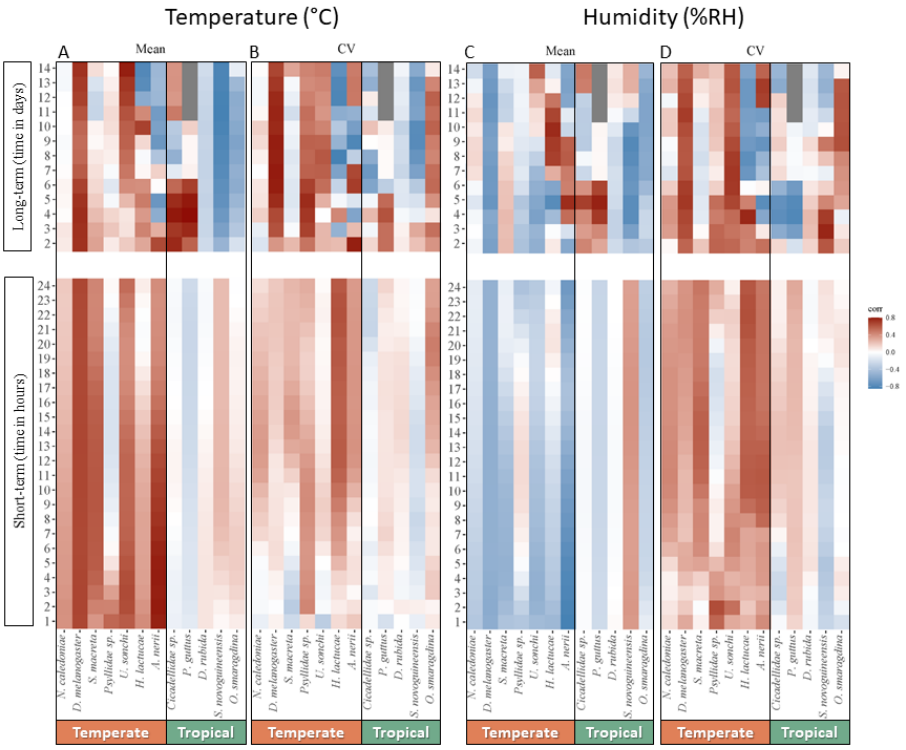


Figure S3: Heatmap showing correlations between knockdown time for each species and the mean and CV of microclimate temperature (A) and humidity (B) extracted in short-term (< 24 h) and long-term (2-14 days) windows. The species are grouped into origin from either temperate or tropical Australia.

Table S1 – Overview of insect collection dates, HKDT test times, collection sites, and number of individuals tested for HKDT

Species	Date (dd-mm-yyyy)	Test time	Site	# of ind.
Nysius caledoniae	15-02-2019	11:59	Site 1	38
Nysius caledoniae	15-02-2019	15:55	Site 1	38
Nysius caledoniae	15-02-2019	20:00	Site 1	39
Nysius caledoniae	17-02-2019	08:07	Site 1	33
Nysius caledoniae	17-02-2019	11:57	Site 1	39
Nysius caledoniae	17-02-2019	16:00	Site 1	37
Nysius caledoniae	17-02-2019	20:05	Site 1	38
Nysius caledoniae	20-02-2019	08:39	Site 1	36
Nysius caledoniae	20-02-2019	12:56	Site 1	38
Nysius caledoniae	20-02-2019	16:41	Site 1	40
Nysius caledoniae	20-02-2019	20:28	Site 1	38
Nysius caledoniae	25-02-2019	08:30	Site 1	38
Nysius caledoniae	25-02-2019	12:22	Site 1	40
Nysius caledoniae	25-02-2019	16:25	Site 1	40
Nysius caledoniae	25-02-2019	20:34	Site 1	36
Nysius caledoniae	02-03-2019	08:14	Site 1	40
Nysius caledoniae	02-03-2019	12:05	Site 1	34
Nysius caledoniae	02-03-2019	16:06	Site 1	34
Nysius caledoniae	02-03-2019	20:18	Site 1	37
Nysius caledoniae	04-03-2019	08:39	Site 1	30
Nysius caledoniae	04-03-2019	12:05	Site 1	30
Nysius caledoniae	04-03-2019	16:09	Site 1	28
Nysius caledoniae	04-03-2019	19:45	Site 1	30
Nysius caledoniae	05-03-2019	08:21	Site 1	30
Nysius caledoniae	05-03-2019	12:17	Site 1	30
Nysius caledoniae	05-03-2019	16:16	Site 1	29
Nysius caledoniae	05-03-2019	20:19	Site 1	30
Nysius caledoniae	07-03-2019	09:48	Site 1	30
Nysius caledoniae	07-03-2019	12:06	Site 1	30
Nysius caledoniae	07-03-2019	16:03	Site 1	30
Nysius caledoniae	07-03-2019	20:01	Site 1	29
Stenophyella macreta	22-03-2019	10:05	Site 2	30
Stenophyella macreta	22-03-2019	13:48	Site 2	29
Stenophyella macreta	22-03-2019	16:48	Site 2	30
Stenophyella macreta	22-03-2019	20:49	Site 2	30
Stenophyella macreta	23-03-2019	16:47	Site 2	29
Stenophyella macreta	23-03-2019	20:21	Site 2	30
Stenophyella macreta	24-03-2019	09:44	Site 2	29
Stenophyella macreta	24-03-2019	12:46	Site 2	29
Stenophyella macreta	24-03-2019	16:16	Site 2	30
Stenophyella macreta	24-03-2019	20:25	Site 2	30
Stenophyella macreta	27-03-2019	10:39	Site 2	30
Stenophyella macreta	27-03-2019	14:00	Site 2	29

Table S1 continued

Species	Date (dd-mm-yyyy)	Test time	Site	# of ind.
Stenophyella macreta	27-03-2019	17:24	Site 2	29
Stenophyella macreta	16-04-2019	08:47	Site 2	29
Stenophyella macreta	16-04-2019	12:45	Site 2	22
Stenophyella macreta	16-04-2019	16:36	Site 2	25
Stenophyella macreta	16-04-2019	19:49	Site 2	15
Uroleucon sonchi	26-03-2019	17:16	Site 3	20
Uroleucon sonchi	26-03-2019	12:14	Site 3	20
Uroleucon sonchi	26-03-2019	08:42	Site 3	19
Uroleucon sonchi	26-03-2019	20:10	Site 3	20
Uroleucon sonchi	28-03-2019	08:24	Site 3	20
Uroleucon sonchi	28-03-2019	12:18	Site 3	20
Uroleucon sonchi	28-03-2019	15:52	Site 3	20
Uroleucon sonchi	28-03-2019	20:10	Site 3	19
Uroleucon sonchi	29-03-2019	08:33	Site 3	20
Uroleucon sonchi	29-03-2019	12:07	Site 3	20
Uroleucon sonchi	29-03-2019	16:15	Site 3	20
Uroleucon sonchi	29-03-2019	20:17	Site 3	20
Uroleucon sonchi	01-04-2019	13:38	Site 3	20
Uroleucon sonchi	01-04-2019	16:37	Site 3	19
Uroleucon sonchi	01-04-2019	20:34	Site 3	19
Uroleucon sonchi	15-04-2019	08:42	Site 3	25
Uroleucon sonchi	15-04-2019	12:27	Site 3	20
Uroleucon sonchi	15-04-2019	16:08	Site 3	21
Uroleucon sonchi	15-04-2019	20:15	Site 3	20
Aphis nerii	01-04-2019	13:21	Site 3	19
Aphis nerii	01-04-2019	16:20	Site 3	20
Aphis nerii	01-04-2019	20:06	Site 3	19
Aphis nerii	02-04-2019	08:38	Site 3	20
Aphis nerii	02-04-2019	12:36	Site 3	20
Aphis nerii	02-04-2019	15:55	Site 3	20
Aphis nerii	02-04-2019	19:51	Site 3	19
Hyperomyzus lactucae	04-04-2019	08:07	Site 3	13
Hyperomyzus lactucae	04-04-2019	12:32	Site 3	12
Hyperomyzus lactucae	04-04-2019	16:34	Site 3	15
Hyperomyzus lactucae	04-04-2019	20:08	Site 3	15
Hyperomyzus lactucae	05-04-2019	08:49	Site 3	14
Hyperomyzus lactucae	05-04-2019	12:43	Site 3	19
Hyperomyzus lactucae	05-04-2019	16:40	Site 3	14
Hyperomyzus lactucae	05-04-2019	20:49	Site 3	15
Hyperomyzus lactucae	07-04-2019	10:11	Site 3	14
Hyperomyzus lactucae	07-04-2019	12:35	Site 3	15
Hyperomyzus lactucae	07-04-2019	16:32	Site 3	11
Hyperomyzus lactucae	07-04-2019	20:29	Site 3	15
Hyperomyzus lactucae	15-04-2019	09:00	Site 3	22
Hyperomyzus lactucae	15-04-2019	12:48	Site 3	22
Hyperomyzus lactucae	15-04-2019	16:22	Site 3	19

Table S1 continued

Species	Date (dd-mm-yyyy)	Test time	Site	# of ind.
Drosophila melanogaster	09-04-2019	09:35	Site 2	30
Drosophila melanogaster	09-04-2019	12:32	Site 2	39
Drosophila melanogaster	09-04-2019	16:14	Site 2	30
Drosophila melanogaster	09-04-2019	20:32	Site 2	29
Drosophila melanogaster	16-04-2019	09:30	Site 2	40
Drosophila melanogaster	16-04-2019	13:32	Site 2	41
Drosophila melanogaster	16-04-2019	17:22	Site 2	39
Drosophila melanogaster	16-04-2019	21:01	Site 2	38
Drosophila melanogaster	17-04-2019	08:44	Site 2	40
Drosophila melanogaster	17-04-2019	12:04	Site 2	40
Drosophila melanogaster	17-04-2019	17:47	Site 2	39
Drosophila melanogaster	17-04-2019	21:34	Site 2	39
Drosophila melanogaster	25-04-2019	08:53	Site 2	41
Drosophila melanogaster	25-04-2019	12:40	Site 2	40
Drosophila melanogaster	25-04-2019	16:52	Site 2	38
Drosophila melanogaster	25-04-2019	20:38	Site 2	38
Drosophila melanogaster	15-05-2019	08:52	Site 2	41
Drosophila melanogaster	15-05-2019	11:55	Site 2	40
Drosophila melanogaster	15-05-2019	16:17	Site 2	38
Drosophila melanogaster	15-05-2019	20:32	Site 2	41
Drosophila melanogaster	11-04-2019	08:52	Site 2	39
Drosophila melanogaster	11-04-2019	12:24	Site 2	40
Drosophila melanogaster	11-04-2019	16:25	Site 2	40
Drosophila melanogaster	11-04-2019	20:43	Site 2	40
Psyllidae sp.	23-04-2019	13:24	Site 2	20
Psyllidae sp.	23-04-2019	17:41	Site 2	19
Psyllidae sp.	06-05-2019	09:03	Site 2	26
Psyllidae sp.	06-05-2019	12:31	Site 2	32
Psyllidae sp.	06-05-2019	16:23	Site 2	31
Psyllidae sp.	06-05-2019	20:56	Site 2	29
Psyllidae sp.	07-05-2019	09:12	Site 2	30
Psyllidae sp.	07-05-2019	12:36	Site 2	29
Psyllidae sp.	07-05-2019	17:54	Site 2	28
Psyllidae sp.	17-05-2019	12:33	Site 2	33
Psyllidae sp.	17-05-2019	17:07	Site 2	19
Psyllidae sp.	18-05-2019	13:11	Site 2	23
Psyllidae sp.	23-05-2019	13:10	Site 2	30
Pseudopachybrachius guttus	05-06-2019	08:47	Site 4	38
Pseudopachybrachius guttus	05-06-2019	12:25	Site 4	38
Pseudopachybrachius guttus	05-06-2019	16:26	Site 4	38
Pseudopachybrachius guttus	05-06-2019	20:32	Site 4	38
Pseudopachybrachius guttus	06-06-2019	08:38	Site 4	38
Pseudopachybrachius guttus	06-06-2019	12:37	Site 4	36
Pseudopachybrachius guttus	06-06-2019	17:00	Site 4	40
Pseudopachybrachius guttus	06-06-2019	20:05	Site 4	39
Pseudopachybrachius guttus	07-06-2019	08:12	Site 4	40

Table S1 continued

Species	Date (dd-mm-yyyy)	Test time	Site	# of ind.
Pseudopachybrachius guttus	08-06-2019	13:46	Site 4	40
Pseudopachybrachius guttus	08-06-2019	17:33	Site 4	40
Pseudopachybrachius guttus	08-06-2019	20:38	Site 4	40
Pseudopachybrachius guttus	09-06-2019	10:26	Site 4	39
Pseudopachybrachius guttus	09-06-2019	15:14	Site 4	38
Pseudopachybrachius guttus	09-06-2019	19:07	Site 4	40
Pseudopachybrachius guttus	13-06-2019	08:39	Site 4	40
Pseudopachybrachius guttus	13-06-2019	12:22	Site 4	40
Pseudopachybrachius guttus	13-06-2019	16:11	Site 4	40
Pseudopachybrachius guttus	13-06-2019	20:08	Site 4	39
Pseudopachybrachius guttus	14-06-2019	08:44	Site 4	40
Pseudopachybrachius guttus	14-06-2019	12:35	Site 4	42
Pseudopachybrachius guttus	14-06-2019	16:41	Site 4	36
Pseudopachybrachius guttus	14-06-2019	20:19	Site 4	40
Cicadellidae sp.	05-06-2019	09:25	Site 4	39
Cicadellidae sp.	05-06-2019	13:00	Site 4	40
Cicadellidae sp.	05-06-2019	17:12	Site 4	39
Cicadellidae sp.	05-06-2019	21:13	Site 4	39
Cicadellidae sp.	06-06-2019	09:25	Site 4	35
Cicadellidae sp.	06-06-2019	13:15	Site 4	36
Cicadellidae sp.	06-06-2019	17:36	Site 4	38
Cicadellidae sp.	06-06-2019	20:45	Site 4	38
Cicadellidae sp.	07-06-2019	08:56	Site 4	36
Cicadellidae sp.	08-06-2019	14:23	Site 4	37
Cicadellidae sp.	09-06-2019	11:08	Site 4	39
Cicadellidae sp.	09-06-2019	16:00	Site 4	37
Cicadellidae sp.	09-06-2019	19:51	Site 4	34
Cicadellidae sp.	19-06-2019	08:30	Site 4	40
Cicadellidae sp.	19-06-2019	11:59	Site 4	38
Cicadellidae sp.	19-06-2019	16:28	Site 4	40
Cicadellidae sp.	19-06-2019	19:59	Site 4	39
Cicadellidae sp.	20-06-2019	08:00	Site 4	40
Cicadellidae sp.	20-06-2019	12:00	Site 4	40
Cicadellidae sp.	20-06-2019	15:54	Site 4	37
Cicadellidae sp.	20-06-2019	19:50	Site 4	38
Oecophylla smaragdina	11-06-2019	10:11	Site 4	30
Oecophylla smaragdina	11-06-2019	14:36	Site 4	30
Oecophylla smaragdina	11-06-2019	18:39	Site 4	30
Oecophylla smaragdina	11-06-2019	21:34	Site 4	28
Oecophylla smaragdina	19-06-2019	09:49	Site 4	29
Oecophylla smaragdina	19-06-2019	13:14	Site 4	30
Oecophylla smaragdina	19-06-2019	17:42	Site 4	30
Oecophylla smaragdina	19-06-2019	21:13	Site 4	26
Oecophylla smaragdina	20-06-2019	09:20	Site 4	29
Oecophylla smaragdina	20-06-2019	13:30	Site 4	30
Oecophylla smaragdina	20-06-2019	17:07	Site 4	30

Table S1 continued

Species	Date (dd-mm-yyyy)	Test time	Site	# of ind.
Oecophylla smaragdina	24-06-2019	09:20	Site 4	29
Oecophylla smaragdina	24-06-2019	11:44	Site 4	30
Oecophylla smaragdina	24-06-2019	15:56	Site 4	30
Oecophylla smaragdina	24-06-2019	19:53	Site 4	31
Oecophylla smaragdina	25-06-2019	07:56	Site 4	30
Oecophylla smaragdina	25-06-2019	11:50	Site 4	30
Oecophylla smaragdina	25-06-2019	15:52	Site 4	30
Oecophylla smaragdina	25-06-2019	19:41	Site 4	31
Scaptodrosophila novoguineensis	16-06-2019	08:58	Site 5/6	39
Scaptodrosophila novoguineensis	16-06-2019	13:11	Site 5/6	38
Scaptodrosophila novoguineensis	16-06-2019	17:11	Site 5/6	40
Scaptodrosophila novoguineensis	16-06-2019	21:21	Site 5/6	37
Scaptodrosophila novoguineensis	17-06-2019	09:00	Site 5/6	40
Scaptodrosophila novoguineensis	17-06-2019	13:06	Site 5/6	39
Scaptodrosophila novoguineensis	17-06-2019	17:07	Site 5/6	39
Scaptodrosophila novoguineensis	17-06-2019	20:58	Site 5/6	40
Scaptodrosophila novoguineensis	18-06-2019	09:16	Site 5/6	39
Scaptodrosophila novoguineensis	18-06-2019	12:44	Site 5/6	40
Scaptodrosophila novoguineensis	18-06-2019	16:58	Site 5/6	40
Scaptodrosophila novoguineensis	18-06-2019	21:04	Site 5/6	40
Scaptodrosophila novoguineensis	21-06-2019	09:05	Site 5/6	39
Scaptodrosophila novoguineensis	21-06-2019	12:56	Site 5/6	39
Scaptodrosophila novoguineensis	21-06-2019	17:18	Site 5/6	40
Scaptodrosophila novoguineensis	21-06-2019	21:07	Site 5/6	40
Scaptodrosophila novoguineensis	22-06-2019	09:00	Site 5/6	39
Scaptodrosophila novoguineensis	22-06-2019	12:58	Site 5/6	39
Scaptodrosophila novoguineensis	22-06-2019	17:05	Site 5/6	39
Scaptodrosophila novoguineensis	22-06-2019	21:11	Site 5/6	40
Scaptodrosophila novoguineensis	26-06-2019	09:05	Site 5/6	40
Scaptodrosophila novoguineensis	26-06-2019	13:13	Site 5/6	40
Scaptodrosophila novoguineensis	26-06-2019	17:05	Site 5/6	40
Scaptodrosophila novoguineensis	26-06-2019	20:34	Site 5/6	40
Drosophila rubida	16-06-2019	08:02	Site 5/6	40
Drosophila rubida	16-06-2019	12:04	Site 5/6	40
Drosophila rubida	16-06-2019	15:59	Site 5/6	40
Drosophila rubida	16-06-2019	20:28	Site 5/6	40
Drosophila rubida	17-06-2019	08:00	Site 5/6	39
Drosophila rubida	17-06-2019	12:05	Site 5/6	40
Drosophila rubida	17-06-2019	15:58	Site 5/6	39
Drosophila rubida	17-06-2019	20:05	Site 5/6	40
Drosophila rubida	18-06-2019	08:07	Site 5/6	40
Drosophila rubida	18-06-2019	11:42	Site 5/6	40
Drosophila rubida	18-06-2019	16:02	Site 5/6	39
Drosophila rubida	18-06-2019	19:54	Site 5/6	40
Drosophila rubida	21-06-2019	08:00	Site 5/6	40
Drosophila rubida	21-06-2019	11:57	Site 5/6	39

Table S1 continued

Species	Date (dd-mm-yyyy)	Test time	Site	# of ind.
Drosophila rubida	21-06-2019	20:00	Site 5/6	40
Drosophila rubida	22-06-2019	08:00	Site 5/6	40
Drosophila rubida	22-06-2019	11:52	Site 5/6	40
Drosophila rubida	22-06-2019	15:58	Site 5/6	40
Drosophila rubida	22-06-2019	20:00	Site 5/6	39
Drosophila rubida	26-06-2019	07:59	Site 5/6	40
Drosophila rubida	26-06-2019	11:55	Site 5/6	39
Drosophila rubida	26-06-2019	15:59	Site 5/6	40
Drosophila rubida	26-06-2019	19:27	Site 5/6	40

Table S2 Summary of monthly temperature and humidity measures during the field experiment. The mean, coefficient of variation (CV), absolute minimum, and maximum values recorded are given over varying number of days (n_{obs}) for each month/sampling site

2019			Temperature (°C)					Humidity (%RH)				
Month	Region	Site	Mean	CV	Min_{abs}	Max_{abs}	n_{obs}	Mean	CV	Min_{abs}	Max_{abs}	n_{obs}
Feb	Temperate	Site 1	21.30	0.19	10.50	50.50	15	60.90	0.19	22.00	87.50	15
Mar	Temperate	Site 1	19.93	0.18	7.00	37.00	31	61.94	0.18	18.50	95.50	31
Mar	Temperate	Site 2	19.36	0.24	7.00	53.00	18	67.23	0.23	17.00	99.50	18
Apr	Temperate	Site 1	15.90	0.27	4.50	30.00	12	64.61	0.22	26.50	96.00	12
Apr	Temperate	Site 2	16.27	0.25	4.50	38.50	30	63.84	0.21	22.50	96.50	30
May	Temperate	Site 2	13.10	0.21	4.00	23.50	31	75.42	0.14	36.50	99.50	31
Jun	Tropical	Site 3	21.53	0.05	17.50	29.50	26	86.86	0.05	53.50	101.00	26
Jun	Tropical	Site 4	21.14	0.04	17.14	29.33	17	93.04	0.03	65.45	102.25	17
Jun	Tropical	Site 5	21.04	0.04	17.50	29.50	25	90.48	0.04	53.50	100.50	25

Table S3: Summary of 3-way ANOVAs. The analyses were run on rank inverse transformed HKDT values as dependent variable and 'Day', 'Time of day', and 'Sex' as independent variables. The *p*-values were corrected for multiple testing using Benjamini-Hochberg False Discovery Rate adjustments (FDR).

Spp.	Variables	Df	Sum Sq	Mean Sq	F-value	*p*-value	FDR
D. melanogaster	Day	5	340.283	68.057	123.806	< 0.001	**< 0.001**
	Time of day	3	22.918	7.639	13.897	< 0.001	**< 0.001**
	Sex	1	10.508	10.508	19.116	< 0.001	**< 0.001**
	Day : Time of day	15	36.979	2.465	4.485	< 0.001	**< 0.001**
	Day : Sex	5	4.699	0.940	1.710	0.130	0.193
	Time of day : Sex	3	2.754	0.918	1.670	0.172	0.234
	Day : Time of day : Sex	15	21.207	1.414	2.572	0.001	**< 0.01**
	Residuals	872	479.340	0.549			
N. caledoniae	Sex	7	109.740	15.677	20.791	< 0.001	**< 0.001**
	Day	3	30.889	10.296	13.655	< 0.001	**< 0.001**
	Time of day	1	75.856	75.856	100.597	< 0.001	**< 0.001**
	Sex : day	20	66.018	3.301	4.378	< 0.001	**< 0.001**
	Sex : Time of day	7	10.172	1.453	1.927	0.062	0.109
	Day : Time of day	3	1.2548	0.418	0.555	0.645	0.687
	Sex : day : Time of day	20	14.413	0.721	0.956	0.515	0.587
	Residuals	1007	759.333	0.754			
S. macreta	Sex	4	16.100	4.025	4.731	0.001	**< 0.01**
	Day	3	25.943	8.648	10.163	<0.001	**< 0.001**
	Time of day	1	9.205	9.205	10.819	0.001	**< 0.01**
	Sex : day	9	27.828	3.092	3.634	< 0.001	**< 0.001**
	Sex : Time of day	4	4.411	1.103	1.296	0.271	0.340
	Day : Time of day	3	1.532	0.511	0.600	0.615	0.674
	Sex : day : Time of day	9	13.444	1.494	1.756	0.075	0.118
	Residuals	441	375.234	0.851			
Cicadellidae sp.	Sex	6	313.596	52.266	97.490	< 0.001	**< 0.001**
	Day	3	3.728	1.243	2.318	0.074	0.118
	Time of day	1	4.510	4.510	8.413	0.004	**< 0.01**
	Sex : day	11	47.505	4.319	8.055	< 0.001	**< 0.001**
	Sex : Time of day	6	0.875	0.146	0.272	0.950	0.950
	Day : Time of day	3	2.915	0.972	1.813	0.143	0.207
	Sex : day : Time of day	11	18.728	1.704	3.176	< 0.001	**< 0.001**
	Residuals	757	405.842	0.536			
P. guttus	Sex	6	191.230	31.872	43.594	< 0.001	**< 0.001**
	Day	3	0.615	0.205	0.280	0.840	0.857
	Time of day	1	51.754	51.754	70.789	< 0.001	**< 0.001**
	Sex : day	14	17.227	1.230	1.683	0.054	0.098
	Sex : Time of day	6	5.932	0.989	1.352	0.231	0.306
	Day : Time of day	3	2.995	0.998	1.365	0.252	0.325
	Sex : day : Time of day	14	16.529	1.181	1.615	0.069	0.117
	Residuals	891	651.414	0.731			
S. novoguineensis	Sex	5	200.464	40.093	54.377	< 0.001	**< 0.001**
	Day	3	10.032	3.344	4.535	0.004	**< 0.01**
	Time of day	1	0.095	0.095	0.129	0.719	0.750
	Sex : day	15	54.195	3.613	4.900	< 0.001	**< 0.001**
	Sex : Time of day	5	2.602	0.520	0.706	0.619	0.674
	Day : Time of day	3	3.894	1.298	1.760	0.153	0.214
	Sex : day : Time of day	15	11.308	0.754	1.022	0.429	0.500
	Residuals	898	662.107	0.737			

Table S3 continued

Spp.	Variables	Df	Sum Sq	Mean Sq	F-value	*p*-value	FDR
D. rubida	Sex	5	28.778	5.756	7.901	< 0.001	**< 0.001**
	Day	3	12.186	4.062	5.576	0.001	**< 0.01**
	Time of day	1	206.750	206.750	283.824	< 0.001	**< 0.001**
	Sex : day	15	22.435	1.496	2.053	0.010	**< 0.05**
	Sex : Time of day	5	4.003	0.800	1.099	0.359	0.440
	Day : Time of day	3	2.101	0.700	0.961	0.410	0.490
	Sex : day : Time of day	15	16.471	1.098	1.507	0.096	0.146
	Residuals	906	659.972	0.728			

Table S4: Summary of 2-way ANOVAs. The analyses were run on rank inverse transformed HKDT values as dependent variable and 'Day' and 'Time of day' as independent variables. The *p*-values were corrected for multiple testing using Benjamini-Hochberg False Discovery Rate adjustments (FDR).

Spp.	Variables	Df	Sum Sq	Mean Sq	F-value	*p*-value	FDR
Psyllidae sp.	Day	5	28.012	5.602	6.379	< 0.001	**< 0.001**
	Time of day	3	14.836	4.945	5.631	0.001	**< 0.01**
	Day : Time of day	4	9.754	2.439	2.777	0.027	**< 0.05**
	Residuals	336	295.089	0.878			
U. sonchi	Day	4	83.146	20.786	30.785	< 0.001	**< 0.001**
	Time of day	3	25.110	8.370	12.396	< 0.001	**< 0.001**
	Day : Time of day	11	27.337	2.485	3.681	< 0.001	**< 0.001**
	Residuals	363	245.103	0.675			
H. lactucae	Day	3	53.101	17.701	25.620	< 0.001	**< 0.001**
	Time of day	3	13.551	4.517	6.538	< 0.001	**< 0.001**
	Day : Time of day	9	21.312	2.368	3.427	0.001	**< 0.001**
	Residuals	237	163.738	0.691			
A. nerii	Day	1	3.367	3.3674	5.093	0.026	**< 0.05**
	Time of day	3	43.788	14.600	22.073	< 0.001	**< 0.001**
	Day : Time of day	2	2.599	1.299	1.965	0.144	0.1546
	Residuals	130	85.961	0.661			
O. smaragdina	Day	4	46.545	11.636	13.416	< 0.001	**< 0.001**
	Time of day	3	2.267	0.756	0.8712	0.456	0.4557
	Day : Time of day	12	45.164	3.764	4.339	< 0.001	**< 0.001**
	Residuals	572	496.113	0.867			

PAPER II

RESPONSES OF TERRESTRIAL POLAR ARTHROPODS TO HIGH AND INCREASING TEMPERATURES

Published in Journal of Experimental Biology

Simon Bahrndorff, Jannik M. S. Lauritzen, Mathias H. Sørensen, Natasja K. Noer* and Torsten N. Kristensen* (shared last-author)

© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb230797. doi:10.1242/jeb.230797

REVIEW

Responses of terrestrial polar arthropods to high and increasing temperatures

Simon Bahrndorff[1,‡], Jannik M. S. Lauritzen[1], Mathias H. Sørensen[1], Natasja K. Noer[1,*] and Torsten N. Kristensen[1,2,*]

ABSTRACT

Terrestrial arthropods in the Arctic and Antarctic are exposed to extreme and variable temperatures, and climate change is predicted to be especially pronounced in these regions. Available ecophysiological studies on terrestrial ectotherms from the Arctic and Antarctic typically focus on the ability of species to tolerate the extreme low temperatures that can occur in these regions, whereas studies investigating species plasticity and the importance of evolutionary adaptation to periodically high and increasing temperatures are limited. Here, we provide an overview of current knowledge on thermal adaptation to high temperatures of terrestrial arthropods in Arctic and Antarctic regions. Firstly, we summarize the literature on heat tolerance for terrestrial arthropods in these regions, and discuss variation in heat tolerance across species, habitats and polar regions. Secondly, we discuss the potential for species to cope with increasing and more variable temperatures through thermal plasticity and evolutionary adaptation. Thirdly, we summarize our current knowledge of the underlying physiological adjustments to heat stress in arthropods from polar regions. It is clear that very little data are available on the heat tolerance of arthropods in polar regions, but that large variation in arthropod thermal tolerance exists across polar regions, habitats and species. Further, the species investigated show unique physiological adjustments to heat stress, such as their ability to respond quickly to increasing or extreme temperatures. To understand the consequences of climate change on terrestrial arthropods in polar regions, we suggest that more studies on the ability of species to cope with stressful high and variable temperatures are needed.

KEY WORDS: Climate change, Insects, Arctic, Antarctic, Heat stress, Adaptation

Introduction

Temperatures in polar regions are in many ways harsh and extreme, with long, cold winters, and short summers with periodically high temperatures (Convey, 1996; Danks, 2004). These conditions strongly influence the fitness of individual organisms, and the extreme and variable temperatures experienced in polar regions, together with the fast changes in climate currently taking place, are thus likely to be important drivers of evolutionary changes in polar species. Terrestrial arthropods living in Arctic and Antarctic regions are exposed to and have adapted to these extreme thermal conditions on different spatial and temporal scales (Danks, 2004; Denlinger and Lee, 2010). For example, development required to complete the life cycle of many arthropods is not possible within one season. Thus, different life stages can be exposed to very different thermal conditions at a temporal scale, suggesting selection for highly thermally plastic genotypes. Further, variation in microhabitat temperatures, as discussed below, suggests that different species may be exposed locally to very different thermal conditions.

Most studies on the thermal biology of terrestrial ectotherms from high latitudes, which we focus on here, have investigated how species cope with cold temperatures during winter (e.g. Block, 2003; Danks et al., 1994; Holmstrup, 2014; Sinclair et al., 2015). Currently, it is unclear how terrestrial arthropods from polar regions respond physiologically to stressful high temperatures, whether they are exposed to temperatures close to their upper thermal limit, and whether they show similar upper thermal limits to those of species from temperate and tropical regions. Because air temperatures in polar regions are typically low, it has been assumed that the temperatures that terrestrial polar species can tolerate are well above the temperatures experienced in their habitat (Addo-Bediako et al., 2000; Deutsch et al., 2008), and that episodes of extremely high temperatures will be countered behaviorally (Everatt et al., 2014; Hayward et al., 2003). Some polar species may even benefit from climate change; for example, warming of the polar regions may alleviate the stress of living in a low-temperature environment (Peck et al., 2006). However, as discussed below, microhabitat temperature recordings suggest that temperatures can easily reach 30–40°C in polar regions, and that development of polar species will take place under such conditions during the short polar summers. Such temperatures can be stressful for some species, but it is unclear whether the thermal tolerance limits of specific species are close to the microhabitat temperatures that they experience, and to what degree thermoregulatory behavior may help species to avoid stressful temperatures (Sunday et al., 2014).

In this Review, we provide an overview of current knowledge on thermal adaptation of terrestrial polar arthropods to high temperatures, and we discuss variation in heat tolerance across species, habitats and geographical regions. Furthermore, we discuss the potential for species to cope with increasing temperatures both within generations (thermal plasticity; see Glossary) and across them (evolutionary adaptation; see Glossary), and we investigate whether there is evidence that terrestrial arthropods in polar regions show unique physiological adjustments to heat stress. We argue that the ability to cope with high temperatures is increasingly important for the survival of terrestrial arthropods in polar regions, and that it is essential to generate additional data on this if we are to predict future species distributions and abundance of terrestrial invertebrates in Arctic and Antarctic regions.

Temperature regimes in polar regions and the impact of climate change

Although northern and southern polar regions share many features, they equally differ in many ways, and it is difficult to compare these

[1]Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220 Aalborg, Denmark. [2]Department of Animal Science, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark.
*Shared last authorship

‡Author for correspondence (sba@bio.aau.dk)

S.B., 0000-0002-0838-4008; N.K.N., 0000-0002-4430-0342; T.N.K., 0000-0001-6204-8753

Glossary

Acclimation response ratio (ARR)
The change in the upper thermal tolerance relative to the change in mean temperature. Quantified in experiments as the slope of the relationship between the upper thermal tolerance and acclimation temperature.

Critical thermal maximum (CT_{max})
Broadly defined as the high temperature at which individuals lose motor control or the ability to move body parts.

Critical thermal minimum (CT_{min})
Broadly defined as the low temperature at which individuals lose motor control or the ability to move body parts.

Evolutionary adaptation
A process of genetic change of a population owing to natural selection.

Heat hardening
A process by which an organism's thermal sensitivity can be increased by a brief exposure to an intermediately high temperature which, in turn, provides protection from injury at a more severe high temperature.

Heat knockdown time (HKT)
The time at which an individual is unable to locomote effectively or remain upright in a static assay.

Rapid cold hardening
A process by which ectotherms rapidly enhance their cold tolerance in response to brief (minutes to hours) chilling or another acclimation cue.

Thermal acclimation
A physiological, morphological or behavioral phenotypic change of an individual in response to a change in temperature.

Thermal plasticity
Ability of an individual to produce more than one phenotype when exposed to different thermal environments.

Univoltine
Referring to organisms having one brood per year.

regions directly. For example, Antarctic latitudes are generally colder than their Arctic counterparts (Convey, 1996). Seasonally, temperatures may vary by as much as 80°C in the Antarctic (Peck et al., 2006). The continental or frigid Antarctic has mean monthly temperatures that rarely and only locally exceed 0°C in summer. However, temperatures in sub-Arctic continental Canada and Siberia are colder than in the equivalent sub-Antarctic zone (Convey, 1996; Pienitz et al., 2004). Further, surface temperatures vary as much as 35°C over the ice sheets, but only about 11°C over sea ice in the Southern Hemisphere, whereas in the Northern Hemisphere the temperatures over sea ice and the ice sheet vary by about 28°C (Comiso, 1994). Also, within Antarctic and Arctic regions, we see large differences in climate patterns. For example, the sub-Antarctic will experience positive mean monthly sea level air temperatures for at least 6 months of the year, whereas the maritime Antarctic will experience such temperatures for 2–4 months out of every 12, and the continental Antarctic will only rarely and locally experience temperatures above 0°C (Convey, 1996). Further, extreme temperature variation is reduced by the maritime climate in the cold Antarctic regions. Thus, in conclusion, large differences in thermal environments are observed between northern and southern polar regions and also within Arctic and Antarctic zones.

Even though polar regions are generally characterized by extremely low temperatures during winter, terrestrial microhabitats, such as south-facing slopes in the Arctic, can occasionally reach high and potentially stressful temperatures. Extreme temperature variation can thus be observed not only across seasons but also on a daily basis across microhabitats (Peck et al., 2006). Temperatures at the microhabitat scale may differ substantially from air temperatures, which are typically measured in the shade 2 m above the ground. For example, solar energy can result in short-term temperature maxima of 30–40°C at both High Arctic and Antarctic continental locations (Hodkinson, 2005; Sinclair et al., 2006; Smith, 1988; Sørensen et al., 2019), and ground surface and soil temperatures can differ substantially from air temperature (Convey et al., 2018; Peck et al., 2006). Temperature recordings from 20 High Arctic and maritime Antarctic sites show that summer ground and sub-surface temperatures vary more than air temperatures, and that accumulated thermal sum (cumulative degree days – using 0°C as a baseline, the sum of mean daily temperature above zero multiplied by the number of days with that mean temperature) in the ground exceeds the sum in the air (Convey et al., 2018). Further, in the maritime Antarctic, maximum temperature recorded during spring/summer on Signy Island shows a high level of inter-day variation, whereas daily minimum temperatures are relatively constant and close to 0°C (Davey et al., 1992). This is similar to observations in southern Greenland (Sørensen et al., 2019). Together, this highlights the complex and highly heterogeneous terrestrial thermal environment in polar regions, where species are dependent both on maximizing development during a short summer, with variable and periodically high and stressful temperatures, and on survival over long, cold winters during which limited resources are available. Another important point is that we are currently lacking data that enable us to link air temperature warming trends with ground surface or microhabitat temperature trends (Convey et al., 2018). This may be further complicated by changes in plant communities caused by climate change. For example, researchers have found shifts in Arctic vegetation under climate change that will affect temperatures at both the macro- and micro-scale (Asmus et al., 2018; Pearson et al., 2013).

The Arctic and Antarctic regions are also vastly impacted by climate change, as demonstrated by some of the fastest temperature changes observed on Earth (Overland et al., 2017; Turner et al., 2014). For example, Arctic temperatures have exceeded previous records every year from 2014 to 2018, and – even more worrying – Arctic air temperature continues to increase at double the rate of the global mean air temperature increase (Overland et al., 2017), which will have major impacts on terrestrial ecosystems. Patterns of climate change in the Antarctic are more complex. Temperature records from the last 50 years collected at 19 stations show warming trends at 11 of these, whereas seven have cooling trends in their annual data, indicating the spatial complexity of change that has occurred across the Antarctic in recent decades (Turner et al., 2005, 2014). Thus, surface temperature trends show significant warming across the Antarctic Peninsula and to a lesser extent in the rest of West Antarctica since the early 1950s, with little change across the rest of the continent. Further, since the late 1990s, warming has paused on the Antarctic Peninsula, which reflects the extreme natural internal variability of the regional atmospheric circulation (Turner et al., 2016).

Current knowledge on heat tolerance in terrestrial arthropods from Arctic and Antarctic regions

Comparing upper thermal limits for polar arthropods

Here, we have compiled published data on upper thermal limits (measured as critical thermal maxima, CT_{max}; see Glossary) for terrestrial arthropods in polar regions (see Table 1). It can for many reasons be difficult to compare species' thermal responses across polar regions directly. For example, the terms Arctic, Antarctic, sub-Arctic and sub-Antarctic are commonly used to describe different regions, although they are not always used in the same way. For the purpose of this Review, we used a climatological aspect (the 10°C summer isotherms) to define polar regions, enabling us to compare the thermal tolerance of polar terrestrial arthropods in the most straightforward way. This criterion can be used for both polar regions and provides a solid basis for comparison of thermal

Table 1. Published studies on upper thermal limits of arthropods in polar regions

Species	Collection date	Time of day	Acclimation status	Ramping rate (°C min^{-1})	Life stage	Collection latitude (°)	Collection longitude (°)	Ecotype	Habitat	CT_{max}	*N*	Reference
Eurois occulta	Aug. 2017	Night	Field collected	0.2	Adults	61	−45	Air	–	40.8±0.3	33	Unpublished*
Rhyacia quadrangula	Aug. 2017	Night	Field collected	0.2	Adults	61	−45	Air	–	41.5±0.3	27	Unpublished*
Spaelotis clandestina	Aug. 2017	Night	Field collected	0.2	Adults	61	−45	Air	Under stones	43.6±0.3	24	Unpublished*
Delia fabricii	Aug. 2017	Day	Field collected	0.2	Adults	61	−45	Air	Grassland	40.0±0.5	33	Unpublished*
Dolichopus groenlandicus	Aug. 2017	Day	Field collected	0.2	Adults	61	−45	Air	Banks of fresh waters	41.4±0.2	35	Unpublished*
Otiorhynchus arcticus	Aug. 2017	Day/ night	Field collected	0.2	Adults	61	−45	Surface	Grassland	43.4±0.1	44	Unpublished*
Nabis flavomarginatus	Aug. 2017	Day	Field collected	0.2	Adults	61	−45	Surface	Grassland	45.4±0.3	34	Unpublished*
Psammotettix lividellus	Aug. 2017	Day	Field collected	0.2	Adults	61	−45	Surface	Grassy south facing slopes	47.2±0.2	58	Unpublished*
Nysius groenlandicus	Aug. 2017	Day	Field collected	0.2	Adults	61	−45	Surface	Grassy south facing slopes	49.4±0.0	60	Unpublished*
Wyochernes asiaticus	–	Day	Field collected	0.25	Adults	66	−136	Soil	Stones	37.8±1.1	10	Anthony et al., 2016
Pardosa groenlandica	Jun.–Aug. 2016	Day	Field collected	0.25	Juvenile	61	−45	Surface	Rocky cobble	45.1±0.2	11	Anthony et al., 2019
Pardosa groenlandica	Jun.–Aug. 2016	Day	Field collected	0.25	Adult female	61	−45	Surface	Rocky cobble	45.3±0.1	10	Anthony et al., 2019
Paradosa furcifera	Jun.–Aug. 2016	Day	Field collected	0.25	Juvenile	61	−45	Surface	Moss- and lichen-dominated fen	43.4±0.4	10	Anthony et al., 2019
Paradosa furcifera	Jun.–Aug. 2016	Day	Field collected	0.25	Adult female	61	−45	Surface	Moss- and lichen-dominated fen	46.6±0.5	11	Anthony et al., 2019
Paradosa hyperborea	Jun.–Aug. 2016	Day	Field collected	0.25	Juvenile	61	−45	Surface	Moss- and lichen-dominated fen	46.0±0.2	11	Anthony et al., 2019
Paradosa hyperborea	Jun.–Aug. 2016	Day	Field collected	0.25	Adult female	61	−45	Surface	Moss- and lichen-dominated fen	43.6±0.2	16	Anthony et al., 2019
Paradosa glacialis	Jun.–Aug. 2016	Day	Field collected	0.25	Adult female	69	−53	Surface	Moss (hot springs)	43.2±0.3	14	Anthony et al., 2019
Paradosa glacialis	Jun.–Aug. 2016	Day	Field collected	0.25	Adult female	67	−136	Surface	Scree field	46.6±0.4	9	Anthony et al., 2019
Pardosa lapponica	Jul. 2015	Day	Field collected	0.25	Adult female	65	−137	Surface	Tundra	42.9±3.1	5	Anthony et al., 2019
Pardosa lapponica	Jul. 2015	Day	Field collected	0.25	Adult female	67	−136	Surface	Scree field	45.5±1.0	6	Anthony et al., 2019
Pardosa moesta	Jul. 2015	Day	Field collected	0.25	Adult female	67	−136	Surface	Scree field	44.7±0.5	7	Anthony et al., 2019
Pardosa sodalis	Jul. 2015	Day	Field collected	0.25	Adult female	65	−137	Surface	Tundra	46.4±0.5	4	Anthony et al., 2019
Pardosa sodalis	Jul. 2015	Day	Field collected	0.25	Adult female	67	−136	Surface	Scree field	46.8±0.3	3	Anthony et al., 2019
Megaphorura arctica	Aug. 2011	Day	Acclimation (4°C)	0.2	Adults	78	11	Soil	Moss covered slopes	31.7	30	Everatt et al., 2013a

(Continued)

Journal of Experimental Biology (2021) 224, jeb230797. doi:10.1242/jeb.230797

Table 1. (Continued)

Species	Collection date	Time of day	Acclimation status	Ramping rate (°C min^{-1})	Life stage	Collection latitude (°)	Collection longitude (°)	Ecotype	Habitat	CT_{max}	*N*	Reference
Cryptopygus antarcticus	Jan.–Mar. 2012	Day	Acclimation (4°C)	0.2	Adults	−67	−68	Soil	Moss and algae	30.1	30	Everatt et al., 2013a
Alaskozetes antarcticus	Jan.–Mar. 2012	Day	Acclimation (4°C)	0.2	Adults	−67	−68	Soil	Moss and algae	34.1	30	Everatt et al., 2013a
Friesea grisea	Dec. 2002	Day	Field collected	0.25	Adults	−72	170	Soil	Moss banks	34.9±0.2	15	Sinclair et al., 2006
Cryptopygus cisantarcticus	Nov. 2002	Day	Field collected	0.25	Adults	−72	170	Soil	Algal flats	28.7±0.2	15	Sinclair et al., 2006
Isotoma klovstadi	Nov. 2002	Day	Field collected	0.25	Adults	−72	170	Soil	Scree field	33.2±0.3	13	Sinclair et al., 2006
Bothrometopus elongatus	Dec.–Feb. 1998/1999	Day	Field collected	0.25	Adults	−46	37	Surface	Supralittoral rock faces and fellfields	37.6±0.6	10	Klok and Chown, 2003
Bothrometopus parvulus	Dec.–Feb. 1998/1999	Day	Field collected	0.25	Adults	−46	37	Surface	Rock faces and fellfield	38.1±0.4	10	Klok and Chown, 2003
Bothrometopus randi	Dec.–Feb. 1998/1999	Day	Field collected	0.25	Adults	−46	37	Surface	Rock faces and fellfield	36.6±0.5	10	Klok and Chown, 2003
Ectemnorhinus marioni	Dec.–Feb. 1998/1999	Day	Field collected	0.25	Adults	−46	37	Surface	Rock faces and fellfield	38.0±0.2	10	Klok and Chown, 2003
Ectemnorhinus similis	Dec.–Feb. 1998/1999	Day	Field collected	0.25	Adults	−46	37	Surface	Moss field/lowland vegetation	38.7±0.2	10	Klok and Chown, 2003
Palirhoeus eatoni	Dec.–Feb. 1998/1999	Day	Field collected	0.25	Adults	−46	37	Surface	Moss field/lowland vegetation	39.2±0.2	10	Klok and Chown, 2003
Canonopsis sericeus	Dec.–Feb. 2000/2001	Day	Field collected	0.25	Adults	−53	73	Surface	Moss field/lowland vegetation	38.7±0.5	10	Klok and Chown, 2003
Bothrometopus brevis	Dec.–Feb. 2000/2001	Day	Field collected	0.25	Adults	−53	73	Surface	–	34.4±0.7	10	Klok and Chown, 2003
Ectemnorhinus viridis	Dec.–Feb. 2000/2001	Day	Field collected	0.25	Adults	−53	73	Surface	Rocky cobble/lowland vegetation	34.2±0.7	10	Klok and Chown, 2003
Bothrometopus gracilipes	Dec.–Feb. 2000/2001	Day	Field collected	0.25	Adults	−53	73	Surface	Moss field and fellfield	36.8±0.5	10	Klok and Chown, 2003
Embryonopsis halticella	May 1997	Day	Field collected	0.5	Larvae	−46	37	Surface	Tussock grassland	39.7±0.2	10	Klok and Chown, 1998
Halmaeusa atriceps	Apr. 2001/May 2002	Day	Field collected	0.5	Adults	−46	37	Surface	Coastal plain/decomposing plant material	33.2±0.3	10	Slabber and Chown, 2005
Halmaeusa atriceps	Apr. 2001/May 2002	Day	Field collected	0.5	Larvae	−46	37	Surface	Coastal plain/decomposing plant material	32.0±0.3	10	Slabber and Chown, 2005
Myro kerguelensis	2005	Day	Field collected	0.25	Adults	−46	37	Surface	Dry fellfield	35.4±0.2	10	Jumbam et al., 2008
Prinerigone vagans	2005	Day	Field collected	0.25	Adult female	−46	37	Surface	Mire vegetation	35.8±0.1	10	Jumbam et al., 2008
Mucrosomia caeca	–	Day	Acclimation (10°C)	0.05	Adults	−54	158	Soil	–	33.5	–	Kuyucu and Chown, 2021
Parochlus steinenii	Dec.–Feb. 2015–2019	Day	Field collected	0.1	Adults	−62	−58	Air	Herb–moss cover and fellfield	31.4±0.2	10	Contador et al., 2020

CT_{max}, upper thermal limit (means±s.e.m.); *N*, number of individuals. Dashes indicate that no data were available. If multiple populations or registrations were present, the highest value was chosen. *For more details, see Appendix (data are available from Dryad).

Journal of Experimental Biology (2021) 224, jeb230797. doi:10.1242/jeb.230797

Journal of Experimental Biology (2021) 224, jeb230797. doi:10.1242/jeb.230797

tolerance (Pienitz et al., 2004). The number of polar species that have been investigated is limited, and we have only been able to locate 10 published studies, as well as unpublished results from southern Greenland from our laboratory. This paucity is especially pronounced for studies on species obtained south of 60°S or north of 60°N (see Table 1). We found that the average values of CT_{max} across all investigated terrestrial arthropod species from the Antarctic and Arctic regions were 35.2 and 43.7°C, respectively. CT_{max} ranged from 28.7 to 49.4°C in species across polar regions. The variation in heat tolerance across species on a local scale was extensive. For example, CT_{max} values of species found in the same area of southern Greenland ranged from 40.0 to 49.4°C (Table 1; see Appendix).

Generally, CT_{max} seems to be lower for soil-dwelling species, whereas some surface-dwelling species, such as spiders and seed bugs, show high upper thermal limits (Table 1, Fig. 1). As discussed above, microhabitat temperatures can vary substantially across spatial scales, and this may partly explain the differences in thermal tolerance observed across habitats. For example, more variable and extreme surface and air temperatures may have led to selection for genotypes with higher thermal tolerance, whereas species in the soil are inhabiting a more buffered thermal environment (Bahrndorff et al., 2009a).

Assessing thermal tolerance – methods and limitations

Different ways of assessing heat tolerance in insects are extensively discussed in the literature (Kristensen et al., 2008; Rezende et al., 2011; Santos et al., 2011; Sinclair et al., 2015; Terblanche et al., 2011) and will not be elaborated on here. Studies addressing thermal tolerance typically use static or dynamic ramping assays with predetermined endpoints, where the temperature at which individuals succumb to heat (or cold) stress is registered, e.g. CT_{max} (Box 1). In this analysis, we chose to focus on results from dynamic temperature-ramping assays, where individuals are exposed to gradually increasing temperatures, and the temperature where activity ceases is registered as the CT_{max}. Results from this assay provide measures of heat tolerance that enable comparison across studies (but see the potential pitfalls discussed in Box 1). Other studies have examined heat tolerance using other assays on a range of species from polar regions, including midges (Rinehart et al., 2006), collembolans (Aunaas et al., 1983; Block et al., 1994; Everatt et al., 2013b; Hodkinson et al., 1996; Slabber et al., 2007), mites (Deere et al., 2006; Everatt et al., 2013b; Hodkinson et al., 1996), bumblebees (Martinet et al., 2015), spiders and beetles (Aunaas et al., 1983; Bale et al., 2000; van der Merwe et al., 1997), and other arthropods (Slabber and Chown, 2004). However, experimental protocols, cross-tolerance examination and life stages differ between studies, which makes comparisons difficult. Some studies suggest that heat tolerance of polar species is lower than that observed for their temperate counterparts or compared with that of invasive species (Martinet et al., 2015; Slabber et al., 2007).

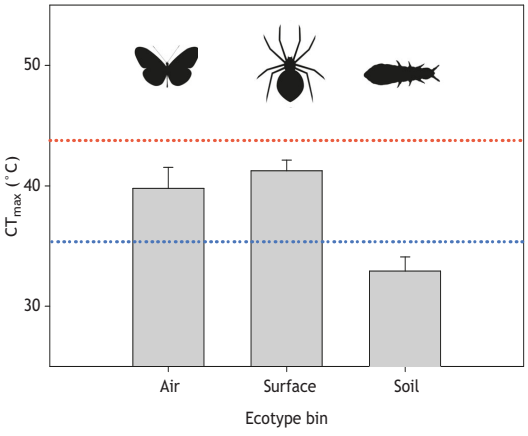


Fig. 1. Upper critical thermal limits (CT_{max}) of terrestrial invertebrates across habitats (air, surface and soil) found in polar regions. Data (means±s.e.m.) are based on published studies and unpublished results (Table 1). The dotted red line indicates mean CT_{max} of data from terrestrial invertebrates from Arctic regions, and the dotted blue line indicates mean CT_{max} of data from terrestrial invertebrates from Antarctic regions. Illustrations above columns indicate species representatives of each habitat.

Studies on ectotherms have shown that adult tolerance to thermal extremes correlates well with their current distribution (Kellermann et al., 2012; Overgaard et al., 2014), but polar arthropods are not included in such studies. Of course, the ecological relevance of the results obtained from both static and dynamic ramping assays can be questioned. Individuals in nature might avoid extreme temperatures by migrating to more benign microhabitats. Thus, they might never be exposed to extreme high or low temperatures in their natural environments. Further, sub-lethal impacts of temperature on many fitness components – such as predation capability, behavior or reproduction traits – might be affected negatively by temperatures much lower (or higher) than those established for critical thermal thresholds in laboratory tests (Walsh et al., 2019; Yao et al., 2019). Less is known about the impacts of sub-lethal stress on surviving individuals, although it has been argued that these traits may be of greater ecological importance than the ability to survive temperature extremes per se (Everatt et al., 2013a). Many polar arthropods need more than a year to complete their life cycle, which can make it difficult to include reproduction output as an endpoint. Thus, some sub-lethal endpoints may be more suited than others addressing the effects of thermal extremes on polar arthropods. For example, locomotion may be a suitable sub-lethal endpoint to consider. Everatt et al. (2013a) showed that locomotion in a species of Antarctic mite, *Alaskozetes antarcticus*, was affected by thermal exposure, increasing with increasing temperature until reaching 25°C. Thus, at temperatures above 25°C, locomotion will decrease; this is well below the CT_{max} of this species (approximately 31°C for summer-acclimated individuals). We argue that future studies examining the ecological and evolutionary impacts of climate change in polar regions should investigate field-relevant measures of thermal robustness, which is vital for the assessment of biodiversity impacts of climate change in these vastly understudied parts of the world. In the context of new assays allowing more ecologically relevant traits to be assessed, it is important that frameworks that allow for comparison of trait values across species and studies are developed, as suggested for thermal fertility limits by Walsh et al. (2019).

The effect of humidity

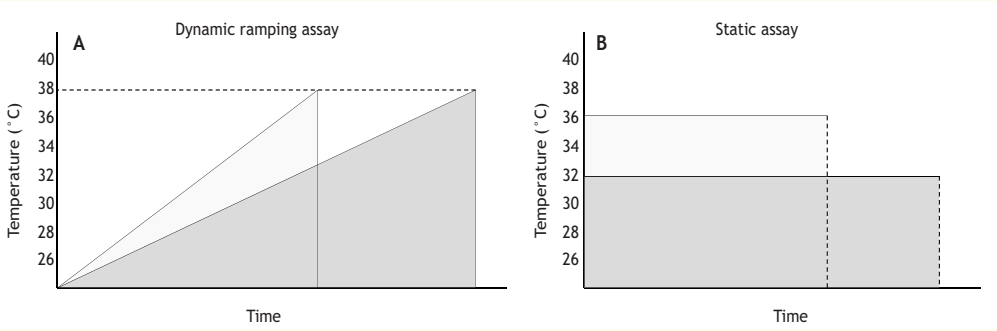
Species responses of polar terrestrial arthropods to high temperatures are also dependent on humidity, although the nature of the relationship between heat stress and humidity varies. For example, for some polar species, survival following heat stress increases with increasing humidity or shows no dependence (Block et al., 1994; Hodkinson et al., 1996), whereas other species tolerate heat stress better at low humidity levels (Benoit et al., 2009). Increasing temperatures in polar regions will also increase the likelihood of summer drought. It will therefore be relevant in the future to look at

Journal of Experimental Biology (2021) 224, jeb230797. doi:10.1242/jeb.230797

Box 1. Quantifying heat tolerance and plasticity

Two different approaches are typically used to quantify arthropod heat tolerance. The first is a dynamic ramping assay (left panel), where the organism is exposed to gradually increasing temperatures and the temperature at which a predetermined endpoint (e.g. heat coma, death, loss of motor function) is reached is recorded. The second (right panel) is a static assay, where an organism is exposed to a constant, stressful temperature, and the time it takes to reach the predetermined endpoint is recorded (Hoffmann et al., 2003; Lutterschmidt and Hutchison, 1997; Overgaard et al., 2012). Which assay is the most ecologically relevant has been widely discussed. The dynamic ramping assay reflects the temperature changes that species might encounter in nature, and thus provides a relevant measure of the accumulation of deleterious effects of heat stress (Somero, 2005; Terblanche et al., 2007). However, the dynamic assay is long lasting (typically >3 h), creating an unnatural environment with interacting stressors such as starvation and desiccation that may confound results. The static assay is shorter (typically lasting <1 h); however, species are rarely exposed to such acute temperature changes in nature (Rezende et al., 2011). Several studies have thus also focused on the intensity of heat stress and the exposure duration (Jørgensen et al., 2019; Rezende et al., 2014, 2020). For example, static and dynamic assays give comparable information on heat tolerance across *Drosophila* species (Jørgensen et al., 2019), but static assays may prove superior to measure small differences in thermal tolerance (Bak et al., 2020).

The static assay may better reflect plasticity in heat tolerance for species with a very fast hardening response because such species will undergo hardening during temperature ramping, thus concealing the effects of pre-hardening treatments (Sørensen et al., 2019). However, this effect will depend on the thermal sensitivity of the species and the assay conditions applied, which affect time–temperature interactions on heat tolerance. For the dynamic assay, high starting temperature and fast ramping rates (left panel; steeper line) will probably result in higher critical thermal maximum (CT_{max}) estimates for most species compared with low starting temperatures and slow ramping rates, and thus are also more likely to estimate thermal plasticity. Likewise, the time it will take to reach an endpoint using the static assay depends on the chosen knockdown temperature (right panel; indicated by the two lines; Sørensen et al., 2013). These time–temperature interactions can be problematic, as comparison of CT_{max} and knockdown times across species and treatments is dependent on assay conditions as well as the species' thermal tolerance and level of plasticity (Bak et al., 2020; Overgaard et al., 2011; Sørensen et al., 2019).



not only the effect of high temperatures alone but also heat tolerance at different humidity levels. As pointed out by Everatt et al. (2015), studies addressing the effect of cross-tolerance between desiccation and high temperatures in polar organisms are few, and the outcome may be dependent on the species' strategy to cope with water stress.

More data are needed

Given the limited number of published studies on thermal biology of terrestrial arthropods in response to high temperatures in polar regions, there is a need for additional data on the ability of such species to thrive under increasing future temperatures if we are to expand our understanding of thermal adaptation across species, habitats and polar regions. It is also important that we include species from different classes, orders, etc., as this will allow for an evaluation of the importance of phylogenetic relatedness in determining thermal tolerance. For example, results suggest a strong phylogenetic signal in heat resistance for some species groups – reflecting phylogenetic inertia rather than common selection pressures (Kellermann et al., 2012) – but not in other groups (García-Robledo et al., 2016). Such information may help us better understand thermal responses across species and the extent to which changes in upper thermal limits, through physiological changes within the lifetime of an individual or through evolutionary responses, might be constrained (Hoffmann et al., 2013). In general, such data will be instrumental in forecasting the impact of climate change on arthropods in polar regions and for our basic understanding of possible differences across regions. It is clear that there will be some limitations in comparing the thermal tolerance of polar organisms directly with that of temperate species; for example, as biodiversity is generally lower in polar regions. Similarly, differences in species richness exist across the Arctic and Antarctic regions. Considering the Antarctic region, the sub-Antarctic has the most species-rich animal community, but still shows a low biodiversity when compared with habitats at corresponding Arctic latitudes (Peck et al., 2006). These differences reflect the evolutionary history of the polar regions, e.g. results suggest post-glacial colonization and the presence of glacial refugia for the Arctic region (Coulson et al., 2014), whereas the Antarctic is extremely isolated (Convey, 2007). The bio-geographical biodiversity patterns may thus partly explain the dearth of ecophysiological information available within and across polar regions (Convey, 1996).

Evolutionary adaptation to high and increasing temperatures

Adaptation to high temperatures through evolutionary changes is typically slow, and in some species heat tolerance has been shown to be constrained by genetic trade-offs and a lack of adaptive genetic variation (Araújo et al., 2013; Hoffmann et al., 2013). In polar regions, where climate change is most extreme, the developmental rate of arthropods is typically slow – as discussed above, many species require several years to complete their life cycle (Convey,

1996; Denlinger and Lee, 2010). Thus, it is likely that evolutionary adaptation of polar arthropods will proceed at an even slower pace compared with that of tropical or temperate species that are exposed to higher average temperatures and typically have faster life cycles (Bleiweiss, 1998; Dillon, 2006; but see Berteaux et al., 2004). Thus, the relative contribution of evolutionary adaptation to increasing temperatures in insects from polar regions might be limited; evolution may not proceed with sufficient speed to enable adaptation to rapidly changing temperatures (Chown and Nicholson, 2004; Sørensen et al., 2016). Studies suggest that some polar species display genetic variation in thermal tolerance across populations (e.g. Bahrndorff et al., 2007), though there is a lack of studies addressing variation in upper thermal limits across populations. It is clear from the literature that large differences in upper thermal limits exist across polar arthropod species (Table 1). This demonstrates the importance of past evolutionary processes on current ecological dynamics. However, as emphasized, we have little information on the levels of genetic variation present within and across populations for different species of arthropods and thus also their evolutionary potential to respond to future environmental changes. Thus, evidence for past natural selection on heat tolerance in polar arthropods needs to be investigated. Further, we need to pinpoint whether adaptation through evolutionary processes is likely to occur fast enough to keep up with climate change and whether genetic constraints exist, for example, as a result of a lack of genetic variation in upper thermal tolerance limits or genetic trade-offs limiting the potential for evolutionary changes. Together, this will increase our fundamental understanding of the ability of polar arthropods to cope with warmer and more variable temperatures.

Physiological acclimation to high temperature

Individuals can show thermal acclimation (see Glossary) to changing and stressful environments by responding plastically, i.e. by altering their physiology, morphology or behavior in response to environmental changes (DeWitt and Langerhans, 2004; West-Eberhard, 2003). Plastic responses can be adaptive or maladaptive, but may be important for coping with diurnal and seasonal changes in temperature (Gunderson and Stillman, 2015; Jensen et al., 2019). There are several published examples of cold acclimation and rapid cold hardening (see Glossary) in polar arthropods (e.g. Bahrndorff et al., 2007; Everatt et al., 2013a; Lee et al., 2006; Teets and Denlinger, 2014; Worland and Convey, 2001), but few studies have investigated physiological acclimation of polar terrestrial arthropods to high temperatures. Further, thermal acclimation and heat hardening (see Glossary) typically increase heat tolerance by only a small fraction of the inducing temperature (e.g. a 10°C increase in temperature increases heat tolerance by 1°C; Chown and Nicholson, 2004). Morley et al. (2019) showed that polar terrestrial arthropods (>55° latitude) had a high acclimation response ratio (ARR; see Glossary) for CT_{max} (*Alaskozetes antarcticus*, ARR=0.3; *Cryptopygos antarcticus*, ARR=0.6); however, their study only included data for two species, both from the Southern Hemisphere. We have found that field-collected individuals of the seed bug *Nysius groenlandicus* from southern Greenland show a high basal heat tolerance (Box 2, Table 1), but a low acclimation response when using CT_{max} as the endpoint (Sørensen et al., 2019). Everatt et al. (2013a,b) found that rapid heat hardening had little effect on heat tolerance for two Antarctic species, and long-term acclimation (1 week at 10°C) did not enhance the heat tolerance of either species. Thus, there seems to be little or no acclimation ability allowing an increase in their upper thermal limits (CT_{max}), supporting the contention that thermal tolerance shows less phenotypic plasticity at higher temperatures than at lower temperatures in invertebrates (Hoffmann et al., 2013). However, recent studies also suggest that the choice of assay may strongly affect conclusions drawn on the ecological role of thermal plasticity (Bak et al., 2020; MacLean et al., 2017; Sørensen et al., 2019). For example, for *N. groenlandicus*, thermal plasticity for heat tolerance was marked when using a static assay, but not when using a dynamic ramping assay (Box 2). Further, studies on temperate arthropods have shown that heat hardening can have both positive and negative effects, and it can affect other life-history traits, such as the ability to locate resources and reproductive traits (e.g. Alemu et al., 2017; Loeschcke and Hoffmann, 2007; Zizzari and Ellers, 2011). In addition, findings from lab studies on costs and benefits of heat and cold acclimation responses have led to different conclusions compared with findings under natural conditions (Kristensen et al., 2008). These studies highlight that, in order to fully understand the costs and benefits of heat hardening in polar arthropods, further studies are needed; such studies should look at sub-lethal endpoints and use different assays when scoring heat tolerance.

It is important that we increase our understanding of whether the basal thermal tolerance of polar arthropod species gives them sufficient capacity to cope with future climate scenarios or whether plasticity in heat tolerance will be necessary to allow them to cope with more variable and unpredictable temperatures in the future. We suggest that future studies should address the plasticity of the upper thermal limits of polar arthropods and should measure species-specific upper thermal limits using not only lethal but also sub-lethal endpoints. It is also crucial for future studies to obtain information on the microhabitat temperatures that reflect temperatures experienced by each species. We know from existing time series of Arctic and Antarctic microclimates that large temperature differences at soil surfaces and in the vegetation exist both within and across short temporal and spatial scales (e.g. Convey et al., 2018; Davey et al., 1992). For example, some Arctic species, such as seed bugs, may experience extremely high temperatures during daytime in the summer (Box 2). By contrast, nocturnal species, such as moths, may be more buffered from thermal fluctuations. In accordance with this, we found 9°C differences in CT_{max} when comparing day-active and night-active species from southern Greenland (Table 1). Further, we also lack information on thermal acclimation of single species under natural conditions and the importance of warming as a factor driving future extinction rates, particularly in polar regions (Seebacher et al., 2015).

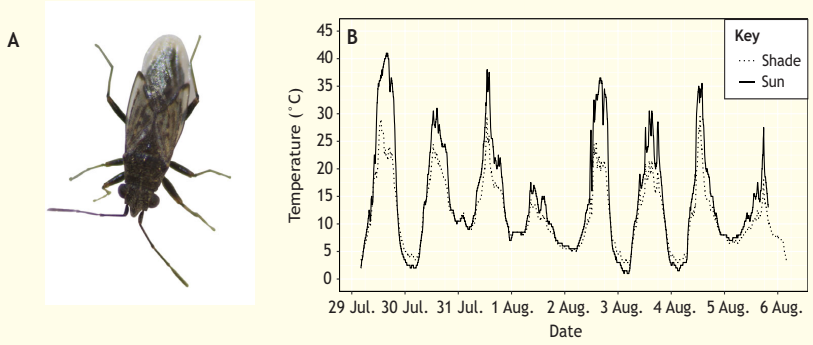
Underlying physiological mechanisms of heat response

Polar terrestrial arthropods can be exposed to high microhabitat temperatures and water stress during their lifetime as discussed above. Even though some polar species show upper thermal limits of up to ∼50°C, many species show substantially lower thermal limits, and other life-history traits are likely to be affected at lower temperatures. Thus, polar organisms are highly dependent on physiological adjustments that allow them to cope with high temperatures. However, the physiological mechanisms underlying the plasticity and evolutionary adaptation of upper thermal limits in polar arthropods have not received as much attention as those underlying cold tolerance. Some reviews on the physiological mechanisms that allow insects to cope with high temperatures exist, and in recent years different -omics approaches have additionally increased our knowledge of the underlying physiological mechanisms (Denlinger and Yocum, 1998; González-Tokman

Box 2. An Arctic insect exposed to highly variable temperatures

One of the most widespread and abundant arthropod species inhabiting the Arctic is the seed bug *Nysius groenlandicus* (Zetterstedt), a true bug (Heteroptera) in the family Lygaeidae. The species is widely distributed across all of Greenland and often appears in dense communities in warm and dry sites dominated by herbs and grasses. It feeds on a wide variety of grass and flower seeds (Böcher, 1972). The species is univoltine and utilizes high local temperatures to complete its life cycle within the short summer season. In July–August, adults emerge, mate and lay eggs. The eggs overwinter in a diapause state, and the first of five nymphal stages appears after snowmelt (Böcher, 1975; Böcher and Nachman, 2001).

The Artic and sub-Arctic summer is characterized by cold average air temperatures, but extremely variable conditions close to the soil surface (Böcher and Nachman, 2011). We measured the temperature at ∼20 cm above ground in the sun (solid line) and shade (dotted line) in a grass-covered site in Narsarsuaq, southern Greenland from 29 July to 6 August, 2018. The highest measured temperatures were 29.5°C in the shade and 41°C in the sun, whereas the coldest temperatures were 2.5°C in the shade and 1.0°C in the sun. The largest daily temperature span reached 39 and 26.5°C in the sun and shade, respectively. The seed bug is well adapted to these changes in temperature; the adult life stage of the species has a critical thermal minimum (CT_{min}; see Glossary) of −3.2 to 3.4°C and a critical thermal maximum (CT_{max}) of 49.4 to 52°C (Bahrndorff et al., 2021a; Böcher and Nachman, 2001; Sørensen et al., 2019). Adult *N. groenlandicus* have a strong preference for high temperatures (above 30°C), which is thought to enable rapid growth, development and reproduction in the short and warm summers (Böcher and Nachman, 2001). Further, the heat tolerance is adjusted rapidly by means of phenotypic plasticity to cues in the microenvironment (Sørensen et al., 2019). For instance, the species shows a remarkable ability to quickly induce thermal tolerance to high temperatures [measured as the time spent at high temperatures (48°C) before going into heat coma (heat knockdown time, HKT; see Glossary)]. Individuals almost double their HKT within 45 min of exposure to a hardening temperature of 42°C. The gain in heat tolerance is quickly reversible: 2 h after the hardening treatment, HKT is back to pre-hardening level, indicating efficient regulatory mechanisms. The rapid reversal of the response may allow allocation of energy to processes that are important for completing a fast life cycle. Such rapid phenotypic adjustments seem to be an adaptation to the variable Arctic environment, and suggest that this species might even be able to cope with temperature rises and increased temperature fluctuations predicted in the near future.



et al., 2020; Neven, 2000). However, as pointed out by González-Tokman et al. (2020), the stress response mediated by heat shock proteins (Hsps) dominates investigations of the physiological mechanisms of heat tolerance in arthropods. Therefore, in this section, we begin by briefly discussing the role of Hsps and go on to consider what we know about other mechanisms by which polar arthropods may cope with high temperatures. There is a need for studies addressing not only the stress response mediated by Hsps but also neuronal mechanisms important for detecting and responding to heat, transcriptomic, proteomic and metabolic responses to heat, thermoregulation and the involvement of hormones that coordinate developmental and behavioral responses at warm temperatures (see review by González-Tokman et al., 2020). This is particularly true for polar organisms, as the nature of the thermal environment in which these species dwell is, in many ways, extreme (Box 2).

It is clear that Hsps play an important role in the heat shock response, but this group of proteins can also be induced by many different environmental stressors (e.g. low temperature, radiation and desiccation) (Sørensen et al., 2003). The heat shock response in invertebrates has received substantial attention, and results suggest that the underlying mechanisms differ across not only species but also habitats. Thus, soil-dwelling species inhabiting more temperature-buffered habitats show a distinct heat shock response compared with, for example, species inhabiting areas with more variable temperatures (Bahrndorff et al., 2009b; Dahlgaard et al., 1998). This suggests that unique physiological responses may also be found for polar species exposed to extreme and highly variable temperatures. In accordance with this, results from our laboratory show that *N. groenlandicus* can quickly increase heat tolerance following heat hardening, but that the hardening response is reversible within hours of hardening, which is much faster than observed in other model arthropod species (Bahrndorff et al., 2009b; Dahlgaard et al., 1998; Sørensen et al., 2019). Several studies have investigated the underlying physiological mechanisms of the heat response in the Antarctic midge, *Belgica antarctica*. Rinehart et al. (2006) found unique thermal adaptations in the heat acclimation response of this insect: there is a dichotomy in survival strategies exploited at different stages of the life cycle. *Belgica antarctica* larvae constitutively up-regulate their Hsps (small hsp, hsp70 and hsp90): these proteins are not further up-regulated by high or low temperature exposure, and the larvae maintain a high inherent tolerance to temperature stress. In contrast, adults show no constitutive up-regulation of their Hsps, have a lower intrinsic tolerance to high temperatures, but are able to upregulate their Hsps when exposed to thermal stress, resulting in enhanced thermotolerance relative to that of adults not exposed to stress. The larval strategy of expressing Hsps continuously while still sustaining growth in *B. antarctica* is unusual and apparently costly. However, this strategy may facilitate proper protein folding in a continually cold habitat that is more thermally stable than that of the adults. Lopez-Martinez et al. (2008) looked at different stressors (all of which would normally be expected to increase the expression of

Hsps) and found that neither heat shock nor freezing and anoxia are able to induce Hsps in larvae of *B. antarctica*, whereas more recent studies have indicated that dehydration stress can induce Hsps (Lopez-Martinez et al., 2009; Teets et al., 2012).

It is not only Hsps that are constitutively up-regulated in the larvae of *B. antarctica*. Lopez-Martinez et al. (2008) also found that two enzymes, catalase and superoxide dismutase, are expressed continuously in larvae, but also in heat-exposed larvae. These enzymes prevent oxidative stress by inactivating reactive oxygen species, thereby limiting damage to lipids, proteins and DNA under stressful conditions. Additionally, Michaud et al. (2008) compared the abundance of metabolites in larvae of *B. antarctica* exposed to a short-term heat-hardening treatment and a control group. Alpha-ketoglutarate and putrescine levels were higher in the hardened group, and glycerol, glucose and serine levels were suppressed. By contrast, Benoit et al. (2009) found the sugar trehalose to be important for heat tolerance in larvae of *B. antarctica*. Larvae injected with trehalose show significantly increased survival following heat shock for 3 h at 30°C compared with control groups. Trehalose accumulates during slow dehydration of *B. antarctica* larvae at 98% and 75% relative humidity. Slow dehydration further increases heat tolerance 3.5-fold compared with that of fully hydrated controls. Together, these results suggest that trehalose is important for mitigating the effects of heat stress. The studies discussed above highlight unique physiological adjustments in polar arthropods to an extreme thermal environment that have not been found in temperate or tropical species.

Altogether, we have a limited knowledge of the physiological responses to heat stress in polar arthropods, and more studies are needed across species in order to better understand the physiological adaptations (and maladaptations) to high and variable temperatures in polar regions. Such knowledge will provide basic information on physiological responses in an extreme environment and allow researchers to better understand what drives evolutionary responses to heat stress.

Responses to climate change in polar regions

As discussed above, we have limited knowledge of thermal tolerance, plasticity of upper thermal limits and the evolutionary adaptive potential for increased heat tolerance of polar terrestrial arthropods. The limited knowledge on species from polar regions means that we have little understanding of how species distribution and abundance in these regions, covering more than 20% of the Earth's area, will respond to climate change.

Several studies have linked shifts in species distribution, including polar arthropods, to changes in climate (e.g. Jepsen et al., 2011; Parmesan and Yohe, 2003; Pearson et al., 2013). There is currently an emphasis on understanding and modeling how future global warming scenarios will affect species abundance and distribution. This can be through the use of trait-based approaches for assessing the relative susceptibility of species to changing temperatures and/or through incorporation of acclimation and genetic adaptation into mechanistic species distribution models (Chown, 2012; Clusella-Trullas et al., 2011; Deutsch et al., 2008; Overgaard et al., 2014). Developments in modeling approaches now allow us to consider processes such as physiology, dispersal, demography and biotic interactions, which permits more robust predictions of future species distribution (Briscoe et al., 2019). However, for the most part, these models have not yet been applied to species in polar regions, partly because an understanding of the fundamental biology of most species is still not available. The modeling of species distribution under future climate scenarios for terrestrial ectotherms in polar regions is based on a very limited number of studies; we have a much better understanding for species living under temperate conditions. However, a recent study employed an ecological niche model using ecophysiological data to predict the future distribution of the Antarctic winged midge *Parochlus steinenii*; it was suggested that this species could be used as an indicator species of the impacts of climate change in the Antarctic (Contador et al., 2020). In addition, a combination of correlative and mechanistic niche models have been used to better understand, predict and manage biological invasions for an invasive insect in the sub-Antarctic; the mechanistic model indicated a slightly larger invasive potential based on larval performance at different temperatures (Bartlett et al., 2020; see also Pertierra et al., 2020). Thus, physiological data will help to provide input to species distribution models, enabling more accurate predictions of the effect of global climate change on terrestrial arthropods in Arctic and Antarctic regions.

The low biodiversity and simple trophic complexity of polar regions (Peck et al., 2006) can provide a unique opportunity to disentangle the effects of climate change on ecosystems, including both direct and indirect effects of factors such as changes in temperature (Høye, 2020). Currently, predictions on how terrestrial arthropods will respond to climate change in cold environments are conflicting. Increasing temperatures could alleviate cold stress and/or lengthen the growing season (Bale and Hayward, 2010), but may also lead to population declines as a result of heat stress (Block et al., 1994), desiccation (Hodkinson et al., 1998) and phenological mismatches (Høye et al., 2013). A number of approaches have been used to link environmental conditions with terrestrial invertebrate numbers (Coulson et al., 1996; Høye et al., 2018; Turney et al., 2018). For example, analysis of long-term datasets on terrestrial invertebrate numbers at a High Arctic site suggests that responses to warming differ for above-ground and soil-dwelling arthropods, and that herbivores, but not detritivores, may benefit from climate change (Koltz et al., 2018). However, most often, direct and indirect effects are not separated, and a deeper understanding of polar terrestrial arthropod responses to high temperatures is needed. We suggest that more species – inhabiting different microhabitats – should be studied, and that thermal tolerance should be more directly linked to habitat temperatures. Further, different thermal assays and a broader choice of traits should be included in future studies, and this should be done in a systematic way, allowing comparisons across studies. Some species may prove more suitable for this than others; for example, a species such as *N. groenlandicus* has a wide geographical distribution and a life cycle that is univoltine (see Glossary), it occurs at very high population sizes and may thus constitute an Arctic and sub-Arctic model species that can provide information on both evolutionary and plastic thermal responses. Likewise, the molecular work conducted on *B. antarctica* can provide a framework for a physiological understanding of thermal responses in polar arthropods in an extreme environment.

Conclusions

Terrestrial arthropods in polar regions have adapted to extreme and harsh environments, with low temperatures during winter, but where microhabitat temperatures can occasionally reach high and potentially stressful levels. Further, the Arctic and Antarctic regions are vastly impacted by climate change, as demonstrated by some of the fastest temperature changes observed on Earth. Generally, we see large variation in upper thermal limits across polar regions, habitats and species. Further, the polar species investigated thus far

show unique physiological adjustments to heat stress in, for example, being able to respond quickly to increasing temperatures. However, there is little information on the evolutionary potential of upper thermal limits and sub-lethal endpoints within and between species of arthropods in polar regions. Some studies have addressed thermal plasticity of terrestrial polar arthropods, but results seem to be dependent on the assay used. The polar regions could provide a unique opportunity to disentangle both the direct and indirect effects of climate change on ecosystems in general. By increasing our fundamental knowledge of key species, thermal tolerance of polar arthropods, how different life-history traits are affected by high temperatures, and the underlying physiological and molecular basis, we will better be able to predict the future abundance and distribution of arthropods in polar regions. This will be invaluable in the light of unprecedented anthropogenic changes affecting these habitats.

APPENDIX

Upper thermal tolerance limits (CT_{max}) data

Upper thermal tolerance limits (CT_{max}) were measured on nine species collected at two locations in Narsarsuaq, Greenland (Table S1). The individuals used for thermal assays were collected in the field using species-specific catch methods (Table S1). Adults of unknown age and sex were tested. To measure CT_{max}, a dynamic ramping assay was used. Field-caught individuals were placed in 15 ml plastic vials with screw caps with a droplet of 2% agar to prevent desiccation during exposure. The vials were mounted to a rack and lowered into a water bath with a temperature of 25°C. Subsequently, the temperature was increased by 0.2±0.01°C min^{-1} using an immersion circulator (Polyscience MX Immersion Circulator model: MX-CA12E). Individuals in each vial were continuously stimulated with a flashlight and tapping on the screw cap with a rod until reaching a temperature at which movement ceased (heat coma). The temperature of heat coma was recorded for each individual. Individuals were stored in 70% ethanol after the thermal assay for later identification. Species were identified based on morphological features using a species identification key (see Böcher et al., 2015).

Acknowledgements

We thank Pete Convey, two anonymous reviewers and the Editor for invaluable suggestions and help in improving this Review and Toke Thomas Høye for help with access to field sites in Narsarsuaq, Greenland.

Competing interests

The authors declare no competing or financial interests.

Funding

We thank the Danish Council for Independent Research (Natur og Univers, Det Frie Forskningsråd grant no. DFF-8021-00014B), the Carlsberg Foundation (Carlsbergfondet, CF17-0415), North to North and the Aalborg Zoo Conservation Foundation (Aalborg Zoos Naturbevaringsfond, AZCF) for supporting this work.

Data availability

Data from our study are available from the Dryad digital repository (Bahrndorff et al., 2021b): dryad.cc2fqz65q

Supplementary information

Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.230797.supplemental

References

Addo-Bediako, A., Chown, S. L. and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. R. Soc. London B Biol. Sci.* **267**, 739-745. doi:10.1098/rspb.2000.1065

Alemu, T., Alemneh, T., Pertoldi, C., Ambelu, A. and Bahrndorff, S. (2017). Costs and benefits of heat and cold hardening in a soil arthropod. *Biol. J. Linn. Soc.* **122**, 765-773. doi:10.1093/biolinnean/blx092

Anthony, S. E., Buddle, C. M. and Sinclair, B. J. (2016). Thermal biology and immersion tolerance of the Beringian pseudoscorpion *Wyochernes asiaticus*. *Polar Biol.* **39**, 1351-1355. doi:10.1007/s00300-015-1849-y

Anthony, S. E., Buddle, C. M., Høye, T. T. and Sinclair, B. J. (2019). Thermal limits of summer-collected *Pardosa* wolf spiders (Araneae: Lycosidae) from the Yukon Territory (Canada) and Greenland. *Polar Biol.* **42**, 2055-2064. doi:10.1007/s00300-019-02580-7

Araújo, M. B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P. A., Valladares, F. and Chown, S. L. (2013). Heat freezes niche evolution. *Ecol. Lett.* **16**, 1206-1219. doi:10.1111/ele.12155

Asmus, A. L., Chmura, H. E., Høye, T. T., Krause, J. S., Sweet, S. K., Perez, J. H., Boelman, N. T., Wingfield, J. C. and Gough, L. (2018). Shrub shading moderates the effects of weather on arthropod activity in arctic tundra. *Ecol. Entomol.* **43**, 647-655. doi:10.1111/een.12644

Aunaas, T., Baust, J. G. and Zachariassen, K. E. (1983). Ecophysiological studies on arthropods from Spitsbergen. *Polar Res.* **1**, 235-240. doi:10.3402/polar.v1i3.6990

Bahrndorff, S., Petersen, S. O., Loeschcke, V., Overgaard, J. and Holmstrup, M. (2007). Differences in cold and drought tolerance of high arctic and sub-arctic populations of *Megaphorura arctica* Tullberg 1876 (Onychiuridae: Collembola). *Cryobiology* **55**, 315-323. doi:10.1016/j.cryobiol.2007.09.001

Bahrndorff, S., Loeschcke, V., Pertoldi, C., Beier, C. and Holmstrup, M. (2009a). The rapid cold hardening response of Collembola is influenced by thermal variability of the habitat. *Funct. Ecol.* **23**, 340-347. doi:10.1111/j.1365-2435.2008.01503.x

Bahrndorff, S., Mariën, J., Loeschcke, V. and Ellers, J. (2009b). Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. *Funct. Ecol.* **23**, 233-239. doi:10.1111/j.1365-2435.2009.01541.x

Bahrndorff, S., Alemu, T., Kristensen, T. N., Sørensen, M. H., Høye, T. T. and Holmstrup, M. (2021a). Thermal adaptations of adults and eggs in the Arctic seed bug *Nysius groenlandicus* (Insecta: Hemiptera) from South Greenland. *Polar Biol.* doi:10.1007/s00300-021-02807-6

Bahrndorff, S., Lauritzen, J., Sørensen, M., Noer, N. and Kristensen, T. (2021b). Responses of terrestrial polar arthropods to high and increasing temperatures. *Dryad Dataset*. doi:10.5061/dryad.cc2fqz65q

Bak, C. W., Bahrndorff, S., Krog Noer, N., Jørgensen, L. B., Overgaard, J. and Kristensen, T. N. (2020). Comparison of static and dynamic assays when quantifying thermal plasticity of *Drosophilids*. *Insects* **11**, 537. doi:10.3390/insects11080537

Bale, J. S. and Hayward, S. A. L. (2010). Insect overwintering in a changing climate. *J. Exp. Biol.* **213**, 980-994. doi:10.1242/jeb.037911

Bale, J. S., Block, W. and Worland, M. R. (2000). Thermal tolerance and acclimation response of larvae of the sub-Antarctic beetle *Hydromedion sparsutum* (Coleoptera: Perimylopidae). *Polar Biol.* **23**, 77-84. doi:10.1007/s003000050011

Bartlett, J. C., Convey, P., Pertierra, L. R. and Hayward, S. A. L. (2020). An insect invasion of Antarctica: the past, present and future distribution of *Eretmoptera murphyi* (Diptera, Chironomidae) on Signy Island. *Insect Conserv. Divers.* **13**, 77-90. doi:10.1111/icad.12389

Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **152**, 518-523. doi:10.1016/j.cbpa.2008.12.009

Berteaux, D., Réale, D., McAdam, A. G. and Boutin, S. (2004). Keeping pace with fast climate change: Can Arctic life count on evolution? *Integr. Comp. Biol.* **44**, 140-151. doi:10.1093/icb/44.2.140

Bleiweiss, R. (1998). Slow rate of molecular evolution in high-elevation hummingbirds. *Proc. Natl. Acad. Sci.* **95**, 612-616. doi:10.1073/pnas.95.2.612

Block, W. (2003). Water or ice? - the challenge for invertebrate cold survival. *Sci. Prog.* **86**, 77-101. doi:10.3184/003685003783238680

Block, W., Webb, N. R., Coulson, S., Hodkinson, I. D. and Worland, M. R. (1994). Thermal adaptation in the Arctic collembolan *Onychiurus arcticus* (Tullberg). *J. Insect Physiol.* **40**, 715-722. doi:10.1016/0022-1910(94)90099-X

Böcher, J. (1972). Feeding biology of *Nysius groenlandicus* (Zett.) (Heteroptera: Lygaeidae) in Greenland, with a note on oviposition in relation to food source and dispersal of the species. *Meddelelser om Grønl.* **191**, 1-41.

Böcher, J. (1975). Notes on the reproductive biology and egg-diapause in *Nysius groenlandicus* (Heteroptera: Lygaeidae). *Vidensk. Meddelelser fra Dansk Naturhistorisk Foren.* **138**, 21-38.

Böcher, J. and Nachman, G. (2001). Temperature and humidity responses of the arctic-alpine seed bug *Nysius groenlandicus*. *Entomol. Exp. Appl.* **99**, 319-330. doi:10.1046/j.1570-7458.2001.00831.x

Böcher, J. and Nachman, G. (2011). Coexistence of bisexual and unisexual populations of *Nysius groenlandicus* in the Zackenberg Valley, Northeast Greenland. *Entomol. Exp. Appl.* **140**, 196-206. doi:10.1111/j.1570-7458.2011.01153.x

Böcher, J., Kristensen, N. P., Pape, T. and Vilhelmsen, L. (2015). The Greenland Entomofauna. An Identification Manual of Insects, Spiders and their Allies. Vol. 44. Lieden, The Netherlands: Brill.

Briscoe, N. J., Elith, J., Salguero-Gómez, R., Lahoz-Monfort, J. J., Camac, J. S., Giljohann, K. M., Holden, M. H., Hradsky, B. A., Kearney, M. R., McMahon, S. M. et al. (2019). Forecasting species range dynamics with process-explicit models: matching methods to applications. *Ecol. Lett.* **22**, 1940-1956. doi:10.1111/ele.13348

Chown, S. L. (2012). Trait-based approaches to conservation physiology: Forecasting environmental change risks from the bottom up. *Philos. Trans. R. Soc. B Biol. Sci.* **367**, 1615-1627. doi:10.1098/rstb.2011.0422

Chown, S. L. and Nicholson, S. (2004). *Insect Physiological Ecology: Mechanisms and Patterns*. New York: Oxford University Press, Inc.

Clusella-Trullas, S., Blackburn, T. M. and Chown, S. L. (2011). Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *Am. Nat.* **177**, 738-751. doi:10.1086/660021

Comiso, J. C. (1994). Surface temperatures in the polar regions from Nimbus 7 temperature humidity infrared radiometer. *J. Geophys. Res.* **99**, 5181-5200. doi:10.1029/93JC03450

Contador, T., Gañan, M., Bizama, G., Fuentes-Jaque, G., Morales, L., Rendoll, J., Simoes, F., Kennedy, J., Rozzi, R. and Convey, P. (2020). Assessing distribution shifts and ecophysiological characteristics of the only Antarctic winged midge under climate change scenarios. *Sci. Rep.* **10**, 9087. doi:10.1038/s41598-020-65571-3

Convey, P. (1996). The influence of environmental characteristics on life history attributes of antarctic terrestrial biota. *Biol. Rev.* **71**, 191-225. doi:10.1111/j.1469-185X.1996.tb00747.x

Convey, P. (2007). Influences on and origins of terrestrial biodiversity of the sub-Antarctic islands. *Pap. Proc. R. Soc. Tasmania* **141**, 83-93. doi:10.26749/rstpp.141.1.83

Convey, P., Coulson, S. J., Worland, M. R. and Sjöblom, A. (2018). The importance of understanding annual and shorter-term temperature patterns and variation in the surface levels of polar soils for terrestrial biota. *Polar Biol.* **41**, 1587-1605. doi:10.1007/s00300-018-2299-0

Coulson, S. J., Hodkinson, I. D., Webb, N. R., Block, W., Bale, J. S., Strathdee, A. T., Worland, M. R. and Wooley, C. (1996). Effects of experimental temperature elevation on high-arctic soil microarthropod populations. *Polar Biol.* **16**, 147-153. doi:10.1007/BF02390435

Coulson, S. J., Convey, P., Aakra, K., Aarvik, L., Ávila-Jiménez, M. L., Babenko, A., Biersma, E. M., Boström, S., Brittain, J. E., Carlsson, A. M. et al. (2014). The terrestrial and freshwater invertebrate biodiversity of the archipelagoes of the Barents Sea; Svalbard, Franz Josef Land and Novaya Zemlya. *Soil Biol. Biochem.* **68**, 440-470. doi:10.1016/j.soilbio.2013.10.006

Dahlgaard, J., Loeschcke, V., Michalak, P. and Justesen, J. (1998). Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Funct. Ecol.* **12**, 786-793. doi:10.1046/j.1365-2435.1998.00246.x

Danks, H. V. (2004). Seasonal adaptations in Arctic insects. *Integr. Comp. Biol.* **44**, 85-94. doi:10.1093/icb/44.2.85

Danks, H. V., Kukal, O. and Ring, R. A. (1994). Insect cold-hardiness: insights from the Arctic. *Arctic* **47**, 391-404. doi:10.14430/arctic1312

Davey, M. C., Pickup, J. and Block, W. (1992). Temperature variation and its biological significance in fellfield habitats on a maritime Antarctic island. *Antarct. Sci.* **4**, 383-388. doi:10.1017/S0954102092000567

Deere, J. A., Sinclair, B. J., Marshall, D. J. and Chown, S. L. (2006). Phenotypic plasticity of thermal tolerances in five oribatid mite species from sub-Antarctic Marion Island. *J. Insect Physiol.* **52**, 693-700. doi:10.1016/j.jinsphys.2006.03.009

Denlinger, D. L. and Lee, R. E. (eds.) (2010). *Low Temperature Biology of Insects*. Cambridge University Press.

Denlinger, D. L. and Yocum, G. D. (1998). Physiology of heat sensitivity. Chapter 2. In *Temperature Sensitivity in Insects and Application in Integrated Pest Management* (ed. G. J. Hallman and D. L. Denlinger), pp. 7-57. Boulder, CO: Westview Press.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. USA* **105**, 6668-6672. doi:10.1073/pnas.0709472105

DeWitt, T. J. and Langerhans, R. B. (2004). Integrated solutions to environmental heterogeneity: theory of multimoment reaction norms. In *Phenotypic Plasticity. Functional and Conceptual Approaches* (ed. T. J. DeWitt and S. M. Scheiner), pp. 98-101. New York: Oxford University Press.

Dillon, M. E. (2006). Into thin air: Physiology and evolution of alpine insects. *Integr. Comp. Biol.* **46**, 49-61. doi:10.1093/icb/icj007

Everatt, M. J., Bale, J. S., Convey, P., Worland, M. R. and Hayward, S. A. L. (2013a). The effect of acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates. *J. Insect Physiol.* **59**, 1057-1064. doi:10.1016/j.jinsphys.2013.08.003

Everatt, M. J., Convey, P., Worland, M. R., Bale, J. S. and Hayward, S. A. L. (2013b). Heat tolerance and physiological plasticity in the Antarctic collembolan, *Cryptopygus antarcticus*, and mite, *Alaskozetes antarcticus*. *J. Therm. Biol.* **38**, 264-271. doi:10.1016/j.jtherbio.2013.02.006

Everatt, M. J., Convey, P., Worland, M. R., Bale, J. S. and Hayward, S. A. L. (2014). Are the Antarctic dipteran, *Eretmoptera murphyi*, and Arctic collembolan, Megaphorura arctica, vulnerable to rising temperatures? *Bull. Entomol. Res.* **104**, 494-503. doi:10.1017/S0007485314000261

Everatt, M. J., Convey, P., Bale, J. S., Worland, M. R. and Hayward, S. A. L. (2015). Responses of invertebrates to temperature and water stress: A polar perspective. *J. Therm. Biol.* **54**, 118-132. doi:10.1016/j.jtherbio.2014.05.004

García-Robledo, C., Kuprewicz, E. K., Staines, C. L., Erwin, T. L. and Kress, W. J. (2016). Limited tolerance by insects to high temperatures across tropical elevational gradients and the implications of global warming for extinction. *Proc. Natl. Acad. Sci. USA* **113**, 680-685. doi:10.1073/pnas.1507681113

González-Tokman, D., Córdoba-Aguilar, A., Dáttilo, W., Lira-Noriega, A., Sánchez-Guillén, R. A. and Villalobos, F. (2020). Insect responses to heat: physiological mechanisms, evolution and ecological implications in a warming world. *Biol. Rev.* **95**, 802-821. doi:10.1111/brv.12588

Gunderson, A. R. and Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proc. R. Soc. B Biol. Sci.* **282**, 20150401. doi:10.1098/rspb.2015.0401

Hayward, S. A. L., Worland, M. R., Convey, P. and Bale, J. S. (2003). Temperature preferences of the mite, *Alaskozetes anrarcticus*, and the collembolan, *Cryptopygus antarcticus* from the maritime Antarctic. *Physiol. Entomol.* **28**, 114-121. doi:10.1046/j.1365-3032.2003.00327.x

Hodkinson, I. D. (2005). Adaptations of invertebrates to terrestrial Arctic. *Transactions of the Royal Norwegian Society of Sciences and Letters* 2005, 1-45.

Hodkinson, I. D., Coulson, S. J., Webb, N. R. and Block, W. (1996). Can high Arctic soil microarthropods survive eleveated summer temperatures? *Funct. Ecol.* **10**, 314-321. doi:10.2307/2390278

Hodkinson, I. D., Webb, N. R., Bale, J. S., Block, W., Coulson, S. J. and Strathdee, A. T. (1998). Global change and Arctic ecosystems: Conclusions and predictions from experiments with terrestrial invertebrates on Spitsbergen. *Arct. Alp. Res.* **30**, 306-313. doi:10.2307/1551978

Hoffmann, A. A., Sørensen, J. G. and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175-216. doi:10.1016/S0306-4565(02)00057-8

Hoffmann, A. A., Chown, S. L. and Clusella-trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Funct. Ecol.* **27**, 934-949. doi:10.1111/j.1365-2435.2012.02036.x

Holmstrup, M. (2014). The ins and outs of water dynamics in cold tolerant soil invertebrates. *J. Therm. Biol.* **45**, 117-123. doi:10.1016/j.jtherbio.2014.09.001

Høye, T. T. (2020). Arthropods and climate change – arctic challenges and opportunities. *Curr. Opin. Insect Sci.* **41**, 40-45. doi:10.1016/j.cois.2020.06.002

Høye, T. T., Post, E., Schmidt, N. M., Trøjelsgaard, K. and Forchhammer, M. C. (2013). Shorter flowering seasons and declining abundance of flower visitors in a warmer Arctic. *Nat. Clim. Chang.* **3**, 759-763. doi:10.1038/nclimate1909

Høye, T. T., Bowden, J. J., Hansen, O. L. P., Hansen, R. R., Henriksen, T. N., Niebuhr, A. and Skytte, M. G. (2018). Elevation modulates how Arctic arthropod communities are structured along local environmental gradients. *Polar Biol.* **41**, 1555-1565. doi:10.1007/s00300-017-2204-2

Jensen, A., Alemu, T., Alemneh, T., Pertoldi, C. and Bahrndorff, S. (2019). Thermal acclimation and adaptation across populations in a broadly distributed soil arthropod. *Funct. Ecol.* **33**, 833-845. doi:10.1111/1365-2435.13291

Jepsen, J. U., Kapari, L., Hagen, S. B., Schott, T., Vindstad, O. P. L., Nilssen, A. C. and Ims, R. A. (2011). Rapid northwards expansion of a forest insect pest attributed to spring phenology matching with sub-Arctic birch. *Glob. Chang. Biol.* **17**, 2071-2083. doi:10.1111/j.1365-2486.2010.02370.x

Jørgensen, L. B., Malte, H. and Overgaard, J. (2019). How to assess *Drosophila* heat tolerance: unifying static and dynamic tolerance assays to predict heat distribution limits. *Funct. Ecol.* **33**, 629-642. doi:10.1111/1365-2435.13279

Jumbam, K. R., Terblanche, J. S., Deere, J. A., Somers, M. J. and Chown, S. L. (2008). Critical thermal limits and their responses to acclimation in two sub-Antarctic spiders: *Myro kerguelenensis* and *Prinerigone vagans*. *Polar Biol.* **31**, 215-220. doi:10.1007/s00300-007-0349-0

Kellermann, V., Overgaard, J., Hoffmann, A. A., Flojgaard, C., Svenning, J.-C. and Loeschcke, V. (2012). Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci. USA* **109**, 16228-16233. doi:10.1073/pnas.1207553109

Klok, C. J. and Chown, S. L. (1998). Interactions between desiccation resistance, host-plant contact and the thermal biology of a leaf-dwelling sub-antarctic caterpillar, *Embryonopsis halticella* (Lepidoptera: Yponomeutidae). *J. Insect Physiol.* **44**, 615-628. doi:10.1016/S0022-1910(98)00052-3

Klok, C. J. and Chown, S. L. (2003). Resistance to temperature extremes in sub-Antarctic weevils: Interspecific variation, population differentiation and acclimation. *Biol. J. Linn. Soc.* **78**, 401-414. doi:10.1046/j.1095-8312.2003.00154.x

Koltz, A. M., Schmidt, N. M. and Høye, T. T. (2018). Differential arthropod responses to warming are altering the structure of arctic communities. *R. Soc. Open Sci.* **5**. doi:10.1098/rsos.171503

Kristensen, T. N., Hoffmann, A. A., Overgaard, J., Sørensen, J. G., Hallas, R. and Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 216-221. doi:10.1073/pnas.0708074105

Kuyucu, A. C. and Chown, S. L. (2021). Time course of acclimation of critical thermal limits in two springtail species (Collembola). *J. Insect Physiol.* **130**, 104209. doi:10.1016/j.jinsphys.2021.104209

Lee, R. E., Jr., Elnitsky, M. A., Rinehart, J. P., Hayward, S. A. L., Sandro, L. H. and Denlinger, D. L. (2006). Rapid cold-hardening increases the freezing tolerance of the Antarctic midge *Belgica antarctica*. *J. Exp. Biol.* **209**, 399-406. doi:10.1242/jeb.02001

Loeschcke, V. and Hoffmann, A. A. (2007). Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature. *Am. Nat.* **169**, 175-183. doi:10.1086/510632

Lopez-Martinez, G., Elnitsky, M. A., Benoit, J. B., Lee, R. E. and Denlinger, D. L. (2008). High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins. *Insect Biochem. Mol. Biol.* **38**, 796-804. doi:10.1016/j.ibmb.2008.05.006

Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **179**, 481-491. doi:10.1007/s00360-008-0334-0

Lutterschmidt, W. I. and Hutchison, V. H. (1997). The critical thermal maximum: data to support the onset of spasms as the definitive end point. *Can. J. Zool.* **75**, 1553-1560. doi:10.1139/z97-782

MacLean, H. J., Kristensen, T. N., Overgaard, J., Sørensen, J. G. and Bahrndorff, S. (2017). Acclimation responses to short-term temperature treatments during early life stages causes long lasting changes in spontaneous activity of adult *Drosophila melanogaster*. *Physiol. Entomol.* **42**, 404-411. doi:10.1111/phen.12212

Martinet, B., Lecocq, T., Smet, J. and Rasmont, P. (2015). A protocol to assess insect resistance to heat waves, applied to bumblebees (*Bombus latreille*, 1802). *PLoS ONE* **10**, 1-10. doi:10.1371/journal.pone.0118591

Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica*. *J. Insect Physiol.* **54**, 645-655. doi:10.1016/j.jinsphys.2008.01.003

Morley, S. A., Peck, L. S., Sunday, J. M., Heiser, S. and Bates, A. E. (2019). Physiological acclimation and persistence of ectothermic species under extreme heat events. *Glob. Ecol. Biogeogr.* **28**, 1018-1037. doi:10.1111/geb.12911

Neven, L. G. (2000). Physiological responses of insects to heat. *Postharvest Biol. Technol.* **21**, 103-111. doi:10.1016/S0925-5214(00)00169-1

Overgaard, J., Kristensen, T. N., Mitchell, K. A. and Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *Source Am. Nat.* **178**, 80-96. doi:10.1086/661780

Overgaard, J., Kristensen, T. N. and Sørensen, J. G. (2012). Validity of thermal ramping assays used to assess thermal tolerance in arthropods. *PLoS ONE* **7**, e32758. doi:10.1371/journal.pone.0032758

Overgaard, J., Kearney, M. R. and Hoffmann, A. A. (2014). Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. *Glob. Chang. Biol.* **20**, 1738-1750. doi:10.1111/gcb.12521

Overland, J. E., Hanna, E., Hanssen-Bauer, I., Kim, S.-J., Walsh, J. E., Wang, M., Bhatt, U. S. and Thoman, R. L. (2017). *Surface air temperature*.

Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**, 37-42. doi:10.1038/nature01286

Pearson, R. G., Phillips, S. J., Loranty, M. M., Beck, P. S. A., Damoulas, T., Knight, S. J. and Goetz, S. J. (2013). Shifts in Arctic vegetation and associated feedbacks under climate change. *Nat. Clim. Chang.* **3**, 673-677. doi:10.1038/nclimate1858

Peck, L. S., Convey, P. and Barnes, D. K. A. (2006). Environmental constraints on life histories in Antarctic ecosystems: Tempos, timings and predictability. *Biol. Rev. Camb. Philos. Soc.* **81**, 75-109. doi:10.1017/S1464793105006871

Pertierra, L. R., Bartlett, J. C., Duffy, G. A., Vega, G. C., Hughes, K. A., Hayward, S. A. L., Convey, P., Olalla-Tarraga, M. A. and Aragón, P. (2020). Combining correlative and mechanistic niche models with human activity data to elucidate the invasive potential of a sub-Antarctic insect. *J. Biogeogr.* **47**, 658-673. doi:10.1111/jbi.13780

Pienitz, R., Douglas, M. S. V. and Smol, J. P. (2004). Paleolimnological research in polar regions: an introduction. In *Long-term Environmental Change in Arctic and Antarctic Lakes* (ed. J. P. Smol, R. Pienitz and M. S. V. Douglas), pp. 1-17. Dordrecht: Springer. doi:10.1007/978-1-4020-2126-8_1

Rezende, E. L., Tejedo, M. and Santos, M. (2011). Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. *Funct. Ecol.* **25**, 111-121. doi:10.1111/j.1365-2435.2010.01778.x

Rezende, E. L., Castañeda, L. E. and Santos, M. (2014). Tolerance landscapes in thermal ecology. *Funct. Ecol.* **28**, 799-809. doi:10.1111/1365-2435.12268

Rezende, E. L., Bozinovic, F., Szilágyi, A. and Santos, M. (2020). Predicting temperature mortality and selection in natural *Drosophila* populations. *Science (80-.).* **369**, 1242-1245. doi:10.1126/science.aba9287

Rinehart, J. P., Hayward, S. A. L., Elnitsky, M. A., Sandro, L. H., Lee, R. E. and Denlinger, D. L. (2006). Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proc. Natl. Acad. Sci. USA* **103**, 14223-14227. doi:10.1073/pnas.0606840103

Santos, M., Castañeda, L. E. and Rezende, E. L. (2011). Making sense of heat tolerance estimates in ectotherms: lessons from *Drosophila*. *Funct. Ecol.* **25**, 1169-1180. doi:10.1111/j.1365-2435.2011.01908.x

Seebacher, F., White, C. R. and Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nat. Clim. Chang.* **5**, 61-66. doi:10.1038/nclimate2457

Sinclair, B. J., Terblanche, J. S., Scott, M. B., Blatch, G. L., Jaco Klok, C. and Chown, S. L. (2006). Environmental physiology of three species of Collembola at Cape Hallett, North Victoria Land, Antarctica. *J. Insect Physiol.* **52**, 29-50. doi:10.1016/j.jinsphys.2005.09.002

Sinclair, B. J., Coello Alvarado, L. E. and Ferguson, L. V. (2015). An invitation to measure insect cold tolerance: Methods, approaches, and workflow. *J. Therm. Biol.* **53**, 180-197. doi:10.1016/j.jtherbio.2015.11.003

Slabber, S. and Chown, S. L. (2004). Thermal tolerance and cold hardiness strategy of the sub-Antarctic psocid *Antarctopsocus jeanneli* Badonnel. *Polar Biol.* **28**, 56-61. doi:10.1007/s00300-004-0649-6

Slabber, S. and Chown, S. L. (2005). Differential responses of thermal tolerance to acclimation in the sub-Antarctic rove beetle *Halmaeusa atriceps*. *Physiol. Entomol.* **30**, 195-204. doi:10.1111/j.1365-3032.2005.00448.x

Slabber, S., Roger Worland, M., Petter Leinaas, H. and Chown, S. L. (2007). Acclimation effects on thermal tolerances of springtails from sub-Antarctic Marion Island: Indigenous and invasive species. *J. Insect Physiol.* **53**, 113-125. doi:10.1016/j.jinsphys.2006.10.010

Smith, R. I. L. (1988). Recording bryophyte microclimate in remote and severe environments. In *Methods in Bryology* (ed. J. M. Glime), pp. 275-284. Hattori Botanical Laboratory.

Somero, G. N. (2005). Linking biogeography to physiology: Evolutionary and acclimatory adjustments of thermal limits. *Front. Zool.* **2**, 1-9. doi:10.1186/1742-9994-2-1

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037. doi:10.1046/j.1461-0248.2003.00528.x

Sørensen, J. G., Loeschcke, V. and Kristensen, T. N. (2013). Cellular damage as induced by high temperature is dependent on rate of temperature change - investigating consequences of ramping rates on molecular and organismal phenotypes in *Drosophila melanogaster*. *J. Exp. Biol.* **216**, 809-814. doi:10.1242/jeb.076356

Sørensen, J. G., Kristensen, T. N. and Overgaard, J. (2016). Evolutionary and ecological patterns of thermal acclimation capacity in Drosophila: Is it important for keeping up with climate change? *Curr. Opin. Insect Sci.* **17**, 98-104. doi:10.1016/j.cois.2016.08.003

Sørensen, M. H., Kristensen, T. N., Lauritzen, J. M. S., Noer, N. K., Høye, T. T. and Bahrndorff, S. (2019). Rapid induction of the heat hardening response in an Arctic insect. *Biol. Lett.* **15**. doi:10.1098/rsbl.2019.0613

Sunday, J. M., Bates, A. E., Kearney, M. R., Colwell, R. K., Dulvy, N. K., Longino, J. T. and Huey, R. B. (2014). Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proc. Natl. Acad. Sci. USA* **111**, 5610-5615. doi:10.1073/pnas.1316145111

Teets, N. M. and Denlinger, D. L. (2014). Surviving in a frozen desert: Environmental stress physiology of terrestrial Antarctic arthropods. *J. Exp. Biol.* **217**, 84-93. doi:10.1242/jeb.089490

Teets, N. M., Peyton, J. T., Colinet, H., Renault, D., Kelley, J. L., Kawarasaki, Y., Lee, R. E. and Denlinger, D. L. (2012). Gene expression changes governing extreme dehydration tolerance in an Antarctic insect. *Proc. Natl. Acad. Sci. USA* **109**, 20744-20749. doi:10.1073/pnas.1218661109

Terblanche, J. S., Deere, J. A., Clusella-Trullas, S., Janion, C. and Chown, S. L. (2007). Critical thermal limits depend on methodological context. *Proc. R. Soc. B Biol. Sci.* **274**, 2935-2943. doi:10.1098/rspb.2007.0985

Terblanche, J. S., Hoffmann, A. A., Mitchell, K. A., Rako, L., le Roux, P. C. and Chown, S. L. (2011). Ecologically relevant measures of tolerance to potentially lethal temperatures. *J. Exp. Biol.* **214**, 3713-3725. doi:10.1242/jeb.061283

Turner, J., Colwell, S. R., Marshall, G. J., Lachlan-Cope, T. A., Carleton, A. M., Jones, P. D., Lagun, V., Reid, P. A. and Iagovkina, S. (2005). Antarctic climate change during the last 50 years. *Int. J. Climatol.* **25**, 279-294. doi:10.1002/joc.1130

Turner, J., Barrand, N. E., Bracegirdle, T. J., Convey, P., Hodgson, D. A., Jarvis, M., Jenkins, A., Marshall, G., Meredith, M. P., Roscoe, H. et al. (2014). Antarctic climate change and the environment: an update. *Polar Rec. (Gr. Brit).* **50**, 237-259. doi:10.1017/S0032247413000296

Turner, J., Lu, H., White, I., King, J. C., Phillips, T., Hosking, J. S., Bracegirdle, T. J., Marshall, G. J., Mulvaney, R. and Deb, P. (2016). Absence of 21st century

warming on Antarctic Peninsula consistent with natural variability. *Nature* **535**, 411-415. doi:10.1038/nature18645

Turney, S., Altshuler, I., Whyte, L. G. and Buddle, C. M. (2018). Macroinvertebrate and soil prokaryote communities in the forest–tundra ecotone of the Subarctic Yukon. *Polar Biol.* **41**, 1619-1633. doi:10.1007/s00300-018-2330-5

van der Merwe, M., Chown, S. L. and Smith, V. R. (1997). Thermal tolerance limits in six weevil species (Coleoptera, Curculionidae) from sub-Antarctic Marion Island. *Polar Biol.* **18**, 331-336. doi:10.1007/s003000050196

Walsh, B. S., Parratt, S. R., Hoffmann, A. A., Atkinson, D., Snook, R. R., Bretman, A. and Price, T. A. R. (2019). The impact of climate change on fertility. *Trends Ecol. Evol.* **34**, 249-259. doi:10.1016/j.tree.2018.12.002

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. New York: Oxford University Press.

Worland, M. R. and Convey, P. (2001). Rapid cold hardening in Antarctic microarthropods. *Funct. Ecol.* **15**, 515-524. doi:10.1046/j.0269-8463.2001.00547.x

Yao, F.-L., Zheng, Y., Ding, X.-L., Zhao, J.-W., Lu, X.-S., Desneux, N., He, Y.-X. and Weng, Q.-Y. (2019). Effects of heat shock on survival and predation of an important whitefly predator, *Serangium japonicum*. *Entomol. Exp. Appl.* **167**, 476-489. doi:10.1111/eea.12794

Zizzari, Z. V. and Ellers, J. (2011). Effects of exposure to short-term heat stress on male reproductive fitness in a soil arthropod. *J. Insect Physiol.* **57**, 421-426. doi:10.1016/j.jinsphys.2011.01.002

SUPPLEMENTAL MATERIALS FOR PAPER II

Table S1: List of species collected in Nasarsuaq, Greenland, during August (2017) in the field and used for measuring upper critical temperatures (CT_{max})

Table S1: List of species collected in Nasarsuaq, Greenland, during August (2017) in the field and used for measuring upper thermal tolerance limits (CT_{max}). Collection time, method and location are provided for each species.

Species	Catch method	Time	Location GPS WGS 84
Eurois occulta	Light trap	Night	61°09'17.3''N 45°25'37.0''W
Rhyacia quadrangula	Light trap	Night	61°09'17.3''N 45°25'37.0''W
Spaelotis clandestina	Light trap	Night	61°09'17.3''N 45°25'37.0''W
Delia fabricii	Net	Day	61°10'58.0''N 45°22'15.3''W
Dolichopus groenlandicus	Net	Day	61°10'58.0''N 45°22'15.3''W
Otiorhynchus arcticus	Hand	Night	61°10'58.0''N 45°22'15.3''W
			61°09'17.3''N 45°25'37.0''W
Nabis flavomarginatus	Net	Day	61°10'58.0''N 45°22'15.3''W
Psammotettix lividellus	Net	Day	61°10'58.0''N 45°22'15.3''W
			61°09'17.3''N 45°25'37.0''W
Nysius groenlandicus	Net	Day	61°10'58.0''N 45°22'15.3''W
			61°09'17.3''N 45°25'37.0''W

PAPER III

TEMPORAL REGULATION OF TEMPERATURE TOLERANCES AND GENE EXPRESSION IN AN ARCTIC INSECT

Submitted to OIKOS

Natasja Krog Noer, Kåre Lehmann Nielsen, Elsa Sverrisdóttir, Torsten Nygaard Kristensen, Simon Bahrndorff

Temporal regulation of temperature tolerances and gene expression in an arctic insect

Natasja Krog Noer[1], Kåre Lehmann Nielsen[1], Elsa Sverrisdóttir[1], Torsten Nygaard Kristensen[1,*] and Simon Bahrndorff[1,*]

[1]Department of Chemistry and Bioscience, Aalborg University, Aalborg 9220, Denmark

*Shared last authorship

Abstract

Terrestrial arthropods in the Arctic are exposed to highly variable temperatures that frequently reach cold and warm extremes. Yet, ecophysiological studies on arctic insects typically focus on the ability of species to tolerate low temperatures, whereas studies investigating species' physiological adaptations to periodically warm and variable temperatures are few. In this study, we investigate temporal changes in thermal tolerances and the transcriptome in the Greenlandic seed bug *Nysius groenlandicus*, collected in the field across different times and temperatures in Southern Greenland. We find that plastic changes in heat and cold tolerances occur rapidly and at a daily scale in the field and that these changes are correlated with diurnal temperature variation. Using RNA sequencing we provide molecular underpinnings of the rapid adjustments in thermal tolerance across ambient field temperatures and in the laboratory. We show that transcriptional responses are sensitive to daily temperature changes, and days characterized by high temperature variation induce markedly different expression patterns than thermally stable days. Further, genes associated with laboratory induced heat and cold stress responses, including expression of heat shock proteins and vitellogenins, are shared across laboratory and the field, but they are induced at much lower temperatures in the field.

Keywords: Arctic, phenotypic plasticity, thermal tolerance, climate change, RNA sequencing, heat shock proteins

1. Introduction

The environmental conditions in the polar regions pose significant challenges to terrestrial arthropods, which are exposed to highly variable temperatures that frequently reach cold and warm extremes (Danks, 2004; Bahrndorff et al., 2021b). Coping with such extremes is critical for survival and reproduction of insects living in these regions. Polar species have developed a suite of adaptations that allow coping with temporal changes in thermal conditions. Especially adaptations to cold temperatures that enable survival during long and cold winters have been the center of attention for species in these regions (Young and Block, 1980; Sømme, 1981; Block and Sømme, 1982; Sømme and Block, 1982; Cannon and Schenker, 1985; Worland et al., 1998; Block and Convey, 2001; Convey et al., 2003; Danks, 2004; Purać et al., 2008; Teets et al., 2012a). However, with increasing mean temperatures and temperature variability (IPCC, 2014), changes in both cold and heat tolerance over short timescales might also be critical for behavior, survival and reproduction during the short summer period (Rinehart et al., 2000; Worland and Convey, 2001; Shreve et al., 2004). Accordingly, several studies have examined the potential for plasticity through rapid cold hardening (RCH) in polar terrestrial arthropods and they have shown how RCH can improve survival and reproduction after exposure to cold extremes (Lee et al., 1987; Worland and Convey, 2001; Sinclair et al., 2003a, 2003b). Less is known about rapid physiological adjustments that can alter heat tolerance at short temporal scales, such as heat hardening (HH), despite arctic and subarctic species being frequently exposed to high daily temperature peaks at a microhabitat scale (Mølgaard, 1982; Bahrndorff et al., 2021b; Noer et al., 2022b). Moreover, most knowledge on RCH and HH in arthropods, including species from Arctic and Subarctic regions, is derived from studies conducted under highly controlled laboratory conditions (but see Koveos, 2001; Kelty, 2007; Loeschcke and Hoffmann, 2007; Overgaard and Sørensen, 2008; Schou et al., 2015; Jensen et al., 2019; Teets et al., 2020). However, acclimation and hardening responses to diurnal and seasonal variation in temperature and other environmental variables have been shown to differ between laboratory and field conditions and more field studies are needed to fully understand thermal plasticity under field conditions (Kristensen et al., 2008; Vasseur et al., 2014; Colinet et al., 2015; Sørensen et al., 2016; Jensen et al., 2019; Noer et al., 2022b).

An arctic species that has adapted to the highly variable thermal conditions in Arctic and Subarctic regions is the Greenlandic seed bug *Nysius groenlandicus* (Zetterstedt) (Bahrndorff et al., 2021a). The species is univoltine and utilizes high local temperatures to complete its life cycle within the short summer season where daily temperatures can vary by >30°C and reach subzero temperatures at night. Recent work has revealed that this species has a broad thermal tolerance range (Böcher and Nachman, 2001; Sørensen et al., 2019; Bahrndorff et al., 2021b; Noer et al., 2022b) and that it can rapidly increase acute heat tolerance when exposed to high and stressful temperatures under laboratory (Sørensen et al., 2019) and field conditions (Noer et al., 2022b). However, we lack a comprehensive understanding of temporal changes in thermal tolerances of this species in the field and the molecular responses associated with diurnal environmental variation.

Here we investigated temporal changes in thermal tolerances of *N. groenlandicus* collected in the field at different timepoints during days differing markedly in mean temperatures and temperature variation. We further used an RNA-seq approach to 1) identify genes that, under field conditions, differed in expression levels across days and timepoints within days, and 2) identified genes that were affected by gradual heating and cooling under controlled laboratory conditions to obtain gene profiles of laboratory hardening responses. With these data we investigated the association between field temperatures, heat and cold tolerance, and gene expression patterns. Further we contrasted gene expression patterns in the field and the laboratory to investigate whether similar or contrasting responses were observed.

2. Materials and Methods

2.1 Experimental design

To investigate how the environment affects thermal tolerances and potentially induces alterations in gene expression levels in *N. groenlandicus*, we conducted two experiments. First, a field experiment (section 2.1.1) was carried out to examine how natural temperature dynamics in concert with omnipresent environmental variables affect heat tolerance, measured as 'heat knockdown time' (HKDT), and cold tolerance, measured as 'temperature at chill coma recovery' ($T_{recovery}$; procedure described in Noer et al. (2022b)). In addition, the underlying gene expression levels were investigated in individuals collected simultaneously in the field using an RNA-seq approach. Second, a laboratory experiment (section 2.1.2) was conducted to examine gene expression levels in individuals that were exposed to controlled heat and cold temperature treatments.

2.1.1 Field collection

The field work was conducted in July-August 2018 in Narsarsuaq, Greenland (61.160°N, 45.424°W). Insect collections and thermal tolerance assays followed the procedures described in Noer et al. (2022b) with small modifications. Briefly, adult individuals of *N. groenlandicus* were collected at four time points during the day for five days. The time of collection were dependent on weather conditions and the abundance of the species; for exact collection dates and times, see Table S1. Specimens were placed individually in 4 mL screw-cap glass vials (45 × 14.7 mm), the sex of individuals was then assessed by eye, and the vials transferred to the laboratory within 30-45 minutes of collection. Immediately after returning, the HKDT and $T_{recovery}$ was scored using 20 females and 20 males for each assay. The HKDT was scored by submerging the vials containing the bugs into a temperature-controlled water bath (PolyScience MX Immersion Circulator: MX-CA12E) pre-set at 48°C. The individuals were observed and stimulated with flashes of light and gentle taps on the vial caps with a metal rod. The endpoint noted was the time at which all movement had stopped. $T_{recovery}$ was scored by submerging the vials into another temperature-controlled water bath (LAUDA Proline Edition X RP 1845-C) with a glycol-water solution maintained at -3°C at which the bugs quickly went into a chill coma. The glycol-water was then gradually heated at a rate of

0.2°C min^{-1}. The temperature at which the flies recovered from the chill induced coma, i.e. first movement observed, was recorded as $T_{recovery}$.

For RNA-seq, female individuals were collected at the same four sampling times at two days (i.e. 22/08/18 and 27/08/18, see Table S1), representing a day with high and low temperature variation, respectively. At each collection time, six samples of 10 females were collected and transferred directly into ice cold RNAlater-ICE Frozen Tissue Transition Solution (Invitrogen, Cat. No. AM7030) to prevent degradation of RNA during subsequent storage at -20°C in Narsarsuaq for approximately one week. The samples were then transferred to our laboratory where they were stored at -80°C and later used for RNA sequencing.

The air temperature at the collection site was continuously recorded with 5-minute intervals in the shade using Easylog USB data loggers (LASCAR Electronics, EL-USB-2^{+}). The loggers were placed 15 cm above the soil surface to reflect the thermal environment at the top of the grasses where *N. groenlandicus* was most abundant and was caught with a sweeping net.

2.1.2 Laboratory experiment

The laboratory experiment was conducted using *N. groenlandicus* collected in 2019 from the same field site as used for the field experiment (section 2.1.1). The specimens were collected from the field and transported to the laboratory in Aalborg, Denmark, within two days from collection. The bugs were placed randomly in large petri dishes (145 × 20 mm) with a density of approximately 40 individuals per petri dish and fed with sunflower seeds, grasses from the field site, and 10% sucrose solution in Eppendorf tubes plugged with small cotton balls. The petri dishes with animals were kept under constant rearing temperature at 23°C and a 12/12 light dark cycle for three days before initiating the experiment.

Three temperature treatments were initiated simultaneously: gradual cold exposure, gradual heat exposure, and a control group kept at the constant rearing temperature of 23°C. The heat- and cold treatments were initiated simultaneously with the sampling of the control group for RNA-seq for this group to serve as a control for the stress treatments. Females were placed in screw cap glass vials and submerged into two different water baths at 25°C. The temperatures in the baths were then slowly increased or decreased with a rate of 0.2˚C min^{-1}. Four replicates of five females were then sampled at two different temperatures during heat ramping (33 and 43˚C) and two temperatures during cold ramping (13 and 3˚C) and snap frozen in liquid nitrogen. All samples from the laboratory experiment were stored at -80˚C until subsequent RNA-seq analysis.

2.2 RNA sequencing

2.2.1 RNA extraction and purification

Both field and laboratory collected samples were prepared for RNA-seq using the following protocol. Total RNA was extracted from pools of female *N. groenlandicus* using TRIzol™ (Invitrogen, Cat. No. 15596018) and PureLink™ RNA mini kit (Invitrogen, Cat. No.

12183018A) following the manufacturer's protocol with few modifications. Pools of bugs (15-20 mg tissue from field collected samples and 8-12 mg tissue from laboratory collected samples) were homogenized in 1 mL of TRIzol using a FP-120 FastPrep bead beater for 2x30 sec at 6500 rpm and 5 sec rest in between. The samples were incubated for 5 minutes on ice. Subsequently, 0.2 mL of chloroform was added, the samples shaken, and incubated for 2–3 minutes. The samples were centrifuged for 15 minutes at 12,000 × g at 4°C. The upper aqueous phase (~400-600 µL) was transferred to new tubes and an equal volume (400-600 µl) of 70% ethanol was added and mixed. Total RNA was obtained following the protocol from this step but discarding the use of on-column purelink DNase treatment. RNA concentrations were examined with a NanoDrop 2000 (Thermo Scientific) and RNA quality spot checked on random samples with an Agilent 2200 TapeStation system.

2.2.2 Library prep and sequencing

From the RNA extract, mRNA was poly-A selected and reverse transcribed to cDNA libraries using a TruSeq Stranded mRNA Library Prep kit (Illumina, Cat. No. RS-122-2101). Each library was given an individual 8-base barcode adapter to allow multiplexing and subsequently all libraries were pooled. The library pool was checked for the correct insert size (~150 bp) on an Agilent 2200 TapeStation system and paired-end sequenced on an Illumina HiSeq 3000/4000 platform for 150 cycles.

De novo contig assembly of N. groenlandicus transcriptome

Pre-processing and assembly were performed with CLC Genomics Workbench v. 20.0 (QIAGEN, RRID:SCR_011853). RNA-seq reads were trimmed for quality (Phred score limit of 0.05) and sequences with ambiguous nucleotides (N) larger than 2 were removed. Homopolymers (T's) were removed from the 3' ends and poly-A's allowed to remain at the 5' ends. Sequences shorter than 40 bp were removed to avoid sequences generated from technical artifacts and improve the de novo assembly.

Initially, a naïve draft de novo transcript model was created using 373,541,899 preprocessed RNA-seq reads with automatic bubble and word size and a minimum contig length of 200 bp. This resulted in 215,802 transcript models. However, many of these contigs were supported by only a few reads and unlikely to represent true transcripts. Therefore, all RNA-seq reads (373,541,899) were mapped to the draft transcript model using a similarity parameter of 0.8 and a length parameter of 0.5. 346,731,148 (92.8%) mapped to the draft model overall. Transcript models with an average read coverage of 1000 was included in the final transcript set that contained 7005 transcript models (FASTA files provided in supporting information Appendix 1). Despite that the final set contained only 3,2% of the naïve transcripts, 77,7% of the reads mapping to the draft transcript model mapped to the final transcript model supporting that the final set is an inclusive representation of the true transcriptome. The final transcript model set was searched against the NCBI protein database specified for all organisms using BLASTx. Significant positive matches (e-value $< 10^{-4}$) were identified and gene ontology (GO) terms assessed with the CLC workbench plugin Blast2GO (Conesa et al., 2005).

2.3 Statistical analysis

The temporal scale at which adjustments of thermal tolerances are affected by field temperature, was examined by the following procedure. First, the mean temperature and temperature variability (Coefficient of Variation, CV) were extracted from the field-site microhabitat temperature recordings. The metrics were extracted in a range of time intervals, spanning 1 to 24 hours, preceding the timepoints at which the bugs were collected from the field (exact timepoints in Table S1) and tested for thermal tolerances. In details, each metric was extracted in time 'windows' by moving the past time boundary back in time in 1-h intervals, thereby accumulating the data input for each measure. Next, we corrected the phenotypic data for long-term acclimation effects to separate the short-term (minutes to hours) effects of temperature on physiological adjustments from long-term acclimation effects (days-months). This was done by regressing HKDT and $T_{recovery}$ on 'test day', whereafter the residuals from the regression were extracted and used for further analysis. Thus, only hourly adjustments in thermal tolerances were considered further. Last, the residuals were correlated with the different temperature metrics at each of the extracted time intervals using Pearson's correlations (for further description of the method, see Noer et al., (2022a). All microclimate data and phenotype data were analyzed using the software 'R' (R Core Team, 2020).

Differentially expressed genes (DEG) were determined using the CLC Genomics Workbench. Pairwise comparisons on TMM normalized read counts were performed on laboratory groups with ´temperature treatment´ as the independent variable. In addition, pairwise comparisons were performed to test the effect of 'sampling time' within each sampling day, and finally pairwise comparison of the effect of 'day' within each sampling time. An FDR corrected *p*-value of < 0.05 and absolute $\log_2$ fold change of > 1 were used as significance criteria of DEGs. Enriched GO terms were determined using Fisher's exact test in the R-package TopGO (Alexa and Rahnenführer, 2020) with restricted output to GO terms with "Biological Process" ontology only.

Finally, we compiled a list of specific genes associated with temperature stress responses in insects based on literature (Table S2) and used this to search the lists of DEG for indications of stress responses.

3. Results

3.1 Thermal tolerances associated with short-term temperature changes in the field

The correlations between residuals of both HKDT and $T_{recovery}$ and the mean temperature in the field varied over time for both sexes but differed in the strength and temporal scale of adjustments (Fig. 1). Generally, females had stronger correlations with field temperatures than males, i.e. stronger adjustments in tolerances with changes in environmental temperatures. The association between HKDT, $T_{recovery}$, and the mean temperature in the field ahead of collecting was strongest for females when using the field temperature in the time windows closest to the collection time. Thus, females were more heat tolerant (higher HKDT) when the field temperatures experienced just prior to testing were high, and less heat tolerant

when field temperatures experienced before testing were low (Fig. 1A). The association decreased gradually with time and was strongest within the first hour after collecting the animals (Fig. 1A). The pattern of association between $T_{recovery}$ and the field temperature was similarly highest at the time point leading up to insect collection for females with a gradual decline with time (Fig. 1B). Hence chill coma recovery temperature was lowest (fast recovery) at cold environmental temperatures for females and *vice versa* at warm temperatures (Fig. 1B). The association between HKDT and temperature variation (CV) peaked at 10-12 hours ahead of testing females (Fig. 1A), but for $T_{recovery}$ this association was weaker and peaked at 22-23 hours before testing (Fig 1B).

Heat tolerance of males showed no association with short-term mean field temperature, though there was a weak positive relationship with variation in temperature (CV; Fig. 1A). Adversely, $T_{recovery}$ for males was very sensitive to changes in field temperature (Fig. 1B), thus a high mean field temperature up to 8-10 hours prior to sampling resulted in slow recovery times from chill coma. As for females, temperature CV was associated with $T_{recovery}$ peaking at 22-23 hours ahead of testing (Fig 1B).

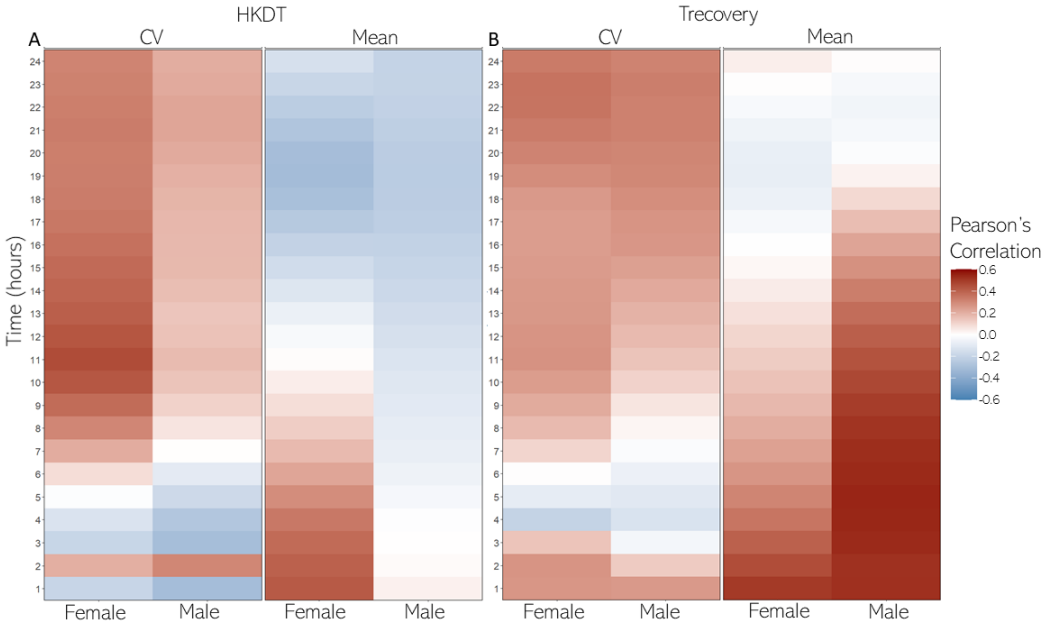


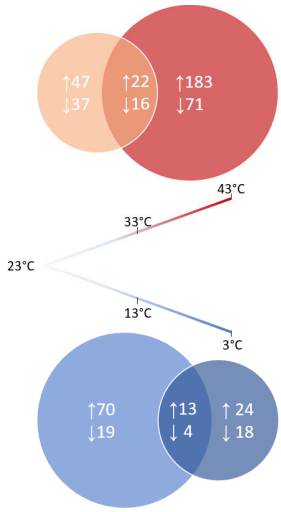
Figure 1: Heatmap displaying correlations between temperature variables (CV and mean) and A) HKDT and B) $T_{recovery}$ of male and female *N. groenlandicus*. The temperature variables were extracted in time-windows of 1-24 hours prior to testing thermal tolerances. Red colors are positive correlations and blue are negative.

3.2 Distinct transcriptomic responses to gradual heating and cooling

To pinpoint transcriptomic responses to gradual changes in temperature, we used RNA-seq to first construct de novo transcript models (see methods for details) and second, quantify gene expression levels in female individuals that had been exposed to increasingly warm and cold temperatures in the laboratory within the range of temperatures occurring in the natural habitat of *N. groenlandicus* during summer.

The treatment causing the most substantial changes in gene expression was warm temperature ramping from 23 to 43°C, which resulted in 292 differentially expressed (DE) transcripts of the 7005 transcript models relative to the 23°C control group (Fig. 2). A temperature increase from 23 to 33°C and decrease from 23 to 13°C caused 122 and 106 DE transcripts, respectively, and the lowest response of 59 DE transcripts was observed at the low temperature ramping from 23 to 3°C. In the groups exposed to the most extreme temperature treatments, i.e. temperature ramping from 23 to 3°C and from 23 to 43°C, 24 DE transcripts were shared, of which six obtained a significant match during the BLASTx search of the protein database; dolichyl-phosphate beta-glucosyltransferase, hypothetical protein acetyl-L-carnitine (ALC)60_11283, cytochrome oxidase subunit I, probable ATP-dependent rRNA helicase RRP3. The first three of these were found in all treatments together with nine other shared transcripts, but with no annotation (Fig. S1). This observation supports that response to heat and cold treatments are distinct and does not involve a major common stress response. These transcripts appear to be entrants in basal metabolism and likely random hyper-variables.

The GO enrichment analysis found 28 enriched terms with a p-value < 0.01 for the heat treatments (Table S3) and 10 enriched terms for the cold treatments. The top 10 GO enrichment terms for individuals exposed to heat ramping (combined 33 and 43°C treatments) was dominated by processes concerned with protein function (protein folding, translation, peptide metabolic process, organonitrogen compound biosynthetic process, cellular amide metabolic process, amide biosynthetic process) as well as glycolytic energy processes (glycolytic process, pyruvate metabolic process and ATP generation from ADP). Together this suggests that the main challenge for the individuals is exposed to heat stress is related to maintaining protein function and stability, at least in part by synthesizing novel proteins and providing energy to this effort. In contrast, the combined cold response (13 and 3°C) showed an abundance of GO terms related to DNA structure and conformational change (DNA conformation change, DNA geometric change, DNA duplex unwinding, chromosome organization), as well as providing energy to these processes during cellular respiration and energy production (cellular respiration, energy derivation by oxidation of organic compounds, aerobic respiration, ATP metabolic process and oxidative phosphorylation). This suggests that a challenge to be met during cold treatment is maintaining DNA dynamics, such as the ability to transcribe.



GO.ID	Term	Annotated	Significant	Expected	classicFisher
GO:0043604	amide biosynthetic process	171	23	7.81	7.7e-07
GO:0006412	translation	161	22	7.35	1.1e-06
GO:0043043	peptide biosynthetic process	163	22	7.45	1.3e-06
GO:0043603	cellular amide metabolic process	186	23	8.50	3.6e-06
GO:0006518	peptide metabolic process	173	22	7.90	3.8e-06
GO:1901566	organonitrogen compound biosynthetic pro...	260	26	11.88	4.0e-05
GO:0006457	protein folding	49	10	2.24	4.4e-05
GO:0006090	pyruvate metabolic process	26	7	1.19	0.00010
GO:0006096	glycolytic process	20	6	0.91	0.00018
GO:0006757	ATP generation from ADP	20	6	0.91	0.00018

GO.ID	Term	Annotated	Significant	Expected	classicFisher
GO:0071103	DNA conformation change	15	3	0.15	0.00039
GO:0006091	generation of precursor metabolites and ...	298	9	3.08	0.00140
GO:0032392	DNA geometric change	7	2	0.07	0.00206
GO:0032508	DNA duplex unwinding	7	2	0.07	0.00206
GO:0045333	cellular respiration	257	8	2.66	0.00245
GO:0015980	energy derivation by oxidation of organi...	258	8	2.67	0.00251
GO:0009060	aerobic respiration	201	7	2.08	0.00268
GO:0046034	ATP metabolic process	213	7	2.20	0.00376
GO:0051276	chromosome organization	33	3	0.34	0.00420
GO:0006119	oxidative phosphorylation	182	6	1.88	0.00783

Figure 2: Differential expression between benign control individuals (23°C) and individuals exposed to heat and cold ramping in the laboratory. The temperature was increased or decreased at a rate of 0.2°C min^{-1} from a start temperature of 23°C. Individuals for RNA sequencing were sampled at five different temperatures illustrated on the figure. The coral and red diagram show the number up and down regulated contigs in the individuals sampled at 33 and 43°C, respectively, and blue and dark blue diagrams represent differentially expressed genes in individuals sampled at 13 and 3°C. Top 10 GO enrichment terms are presented for the DE transcripts expressed at both heat treatments combined and cold treatments combined.

Targeted search for genes involved in the temperature stress responses (heat and cold stress, oxidative stress, desiccation, immune response, etc.) was performed using the list of genes compiled from literature in supporting information Table S2. None of these candidate genes were found or significantly differentially expressed at 3 and 13°C compared to 23°C controls whereas expression of 16 candidate stress-related genes was significantly increased at 43°C compared to 23°C, and one candidate gene was significantly increased at 33°C (Table 1). The majority of these were molecular chaperones and *vitellogenin (vg) 1* and *2*. The expression level of few of these transcripts was notable, including 37-fold change in the inducible *Hsp70*, followed by 24-fold increase in *vitellogenin 2*, 15-fold increase in *Hsp83*, and 9-fold increase in one *Hsp90* variant. This supports the observation based on GO term enrichment, that a major challenge of increased heat is maintaining protein functioning. In addition, vg is crucial for ovarian development, and it may not be surprising that maintaining proper egg development, by maintaining a suitable amount of functional vg, even under increased temperature promoting protein denaturation, is a biologically fundamental important process, for which a protective response has evolved.

Despite not finding stress-related genes in the other temperature contrasts, some transcripts changed substantially, including the reverse transcriptase rna-directed dna polymerase from mobile element jockey-like protein or the membrane component hypothetical protein ALC60_11283 that increased expression 256-fold and 393-fold in the individuals ramped to 13°C, respectively. Likewise, three transcripts in the oxidative phosphorylation in mitochondria, *cytochrome b* and two *cytochrome oxidase subunit 1,* transcripts responded strongly to ramping to 3°C with 11-, 182-, and 380-fold decreases compared to the 23°C exposed individuals, suggesting decreased ATP production at lower temperature.

Table 1: Genes up- or downregulated significantly in response to warm and cold treatments in the laboratory. The fold changes are relative to the control group 23°C, accordingly positive values indicate upregulated transcripts and negative values downregulated transcripts in the ramped group compared to the control.

Contig ID	Transcript	GO Names	Log2 Change	Fold Change	FDR *p*-value
23°C vs. Heat 33°C					
Gene 13178	Glutathione peroxidase 6	F:glutathione peroxidase activity; P:response to oxidative stress; F:hydrolase activity; P:obsolete oxidation-reduction process; P:cellular oxidant detoxification	1.09	2.13	0.012
23°C vs. Heat 43°C					
Gene 57	heat shock cognate protein	F: ATP binding	1.37	2.58	0.009
Gene 58	heat shock 70 kDa cognate protein	F: ATP binding	1.83	3.56	0.040
Gene 37163	heat shock 70 kDa protein cognate 5	F: ATP binding; C: mitochondrion; F: ATP hydrolysis activity; F: heat shock protein binding; P: cellular response to unfolded protein; P: protein refolding; F: protein folding chaperone; F: unfolded protein binding; P: chaperone cofactor-dependent protein refolding; F: misfolded protein binding	1.25	2.38	0.049
Gene 5701	major heat shock 70 kDa protein Ba-like	F: ATP binding	5.22	37.20	< 0.001
Gene 83	vitellogenin 2	F: lipid transporter activity; P: lipid transport	4.57	23.83	< 0.001
Gene 1582	dnaJ homolog subfamily A member 1	F: ATP binding; C: cytosol; P: protein folding; P: response to heat; F: heat shock protein binding; F: metal ion binding; F: unfolded protein binding	1.21	2.32	< 0.001
Gene 49033	dnaJ protein homolog 1	P:protein folding; F:unfolded protein binding	3.52	11.50	< 0.001
Gene 1703	heat shock protein 83	F: ATP binding; P: protein folding; F: unfolded protein binding	3.92	15.12	< 0.001
Gene 3471	vitellogenin 1	F: lipid transporter activity; P: lipid transport	1.49	2.81	0.045
Gene 679	apolipoprotein D-like	P:response to reactive oxygen species; F:binding; C:cytoplasm; P:lipid metabolic process	1.43	2.70	< 0.001
Gene 7239	heat shock protein 90	F: ATP binding; P: protein folding; F: unfolded protein binding	1.97	3.93	< 0.001
Gene 13722	heat-shock protein 90	F: ATP binding; P: protein folding; F: unfolded protein binding	2.92	7.58	< 0.001
Gene 13723	heat-shock protein 90	F: ATP binding; P: protein folding; F: unfolded protein binding	1.69	3.22	< 0.001
Gene 17311	heat-shock protein 90	F: ATP binding; P: protein folding; F: unfolded protein binding	3.21	9.24	< 0.001
Gene 19974	60 kDa heat shock protein, mitochondrial-like	F: ATP binding; P: protein refolding	1.14	2.21	0.005
Gene 23751	BAG domain-containing protein Samui isoform X2	F: chaperone binding	1.55	2.92	< 0.001

3.3 Gene expression highly affected by field temperatures

In the field, samples for RNA-seq were collected four times daily on days with low (day 1) or high (day 2) temperature amplitude (Fig. 3). To understand the effect of natural field temperatures on the transcriptional response we 1) compared the transcripts and expression levels at each collection timepoint between the two days (Fig. 3), 2) quantified the number of genes expressed differentially between the collection timepoints within each day (Fig. 4), and 3) examined if any of the transcripts expressed differentially under the laboratory ramped groups were expressed during the field sampling days (Fig. 5).

3.3.1 Expression across days in the field

Differential expression examined across days at each timepoint showed that at all collection timepoints, except for the morning group (08:00), more transcripts were upregulated on day 2 (high amplitude) relative to day 1. Peculiarly, the number of genes upregulated in the evening group (20:00) was much higher on day 2 relative to day 1 despite the temperature being almost identical for both days at this timepoint. In the morning, more genes were downregulated on day 2 relative to day 1, though not many genes were expressed differentially at this time. Downstream GO enrichment showed that genes that were differentially expressed between the two days in the morning and evening (08:00 and 20:00) were associated with cellular respiration and proton transmembrane transport related to the electron transport chain (Table S4). These findings indicate a high energy demand at cold field temperatures. At the warmest timepoint (12:00), lipid storage/localization and ATP-synthesis dominated the GO terms, whereas gene expression/translation and cellular respiration transcripts were overrepresented in the afternoon (16:00).

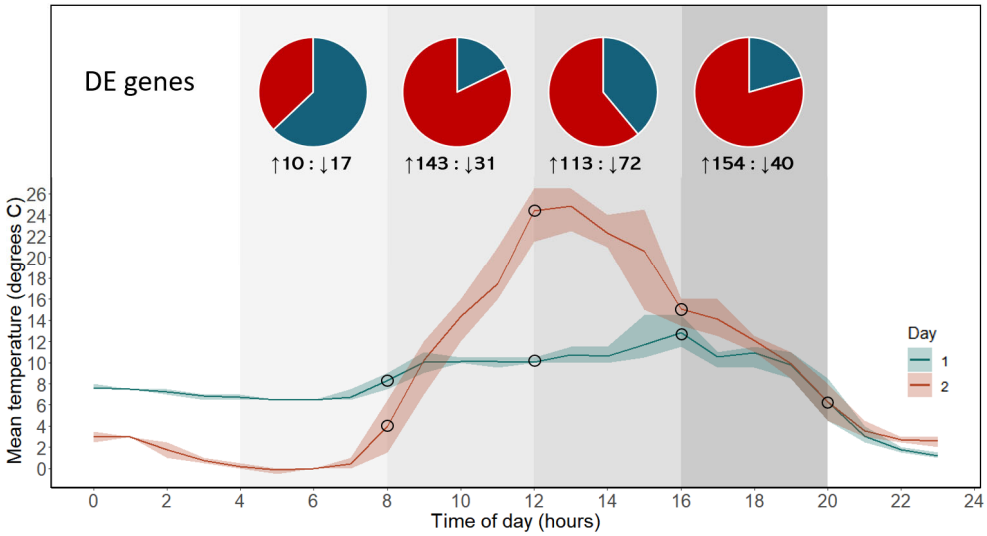


Figure 3: Field temperatures recorded at the sampling sites and comparison of genes expressed differentially at day 2 relative to day 1 for each timepoint. The temperature curves show the mean temperature within each hour and shaded areas are the minimum and maximum temperature within each hour. Individuals were sampled for RNA-seq at 8, 12, 16, and 20 hours (marked by open circles). The percent and number of genes significantly up (red) and down (blue) regulated on day 2 relative to day 1 are shown for each sampling time.

3.4.1 Expression within days and overlap with genes impacted by temperature ramping in the laboratory

The number of transcripts expressed differentially between timepoint in field collected individuals varied little on the thermally stable day, ranging between 34 and 39 transcripts (Fig. 4, day 1). Contrary to this, on the high amplitude day the number of transcripts upregulated changed substantially by 198 DE transcript in individuals collected at noon relative to the morning (Fig. 4, day 2) where the temperatures changed the most (Fig. 3). A large proportion of these upregulated transcripts overlapped with the transcripts expressed in the laboratory groups exposed to gradual cooling and heating, namely individuals ramped to 43, 33, and 13°C (Fig. 5). Notably, between the morning and noon 50 DE transcripts overlapped with the DE transcripts in the females that were heat-ramped from 23 to 43°C in the laboratory, and 10 transcripts overlapped with DE transcripts in females exposed to 33°C heat ramping. The great overlap between field induced transcription responses and transcriptional response to the 43°C heat-ramped vanished between noon and afternoon (Fig. 5). This indicates that many of the pathways regulated at high temperatures in the laboratory are shared with responses to temperature fluctuations in the field, but at lower induction temperatures.

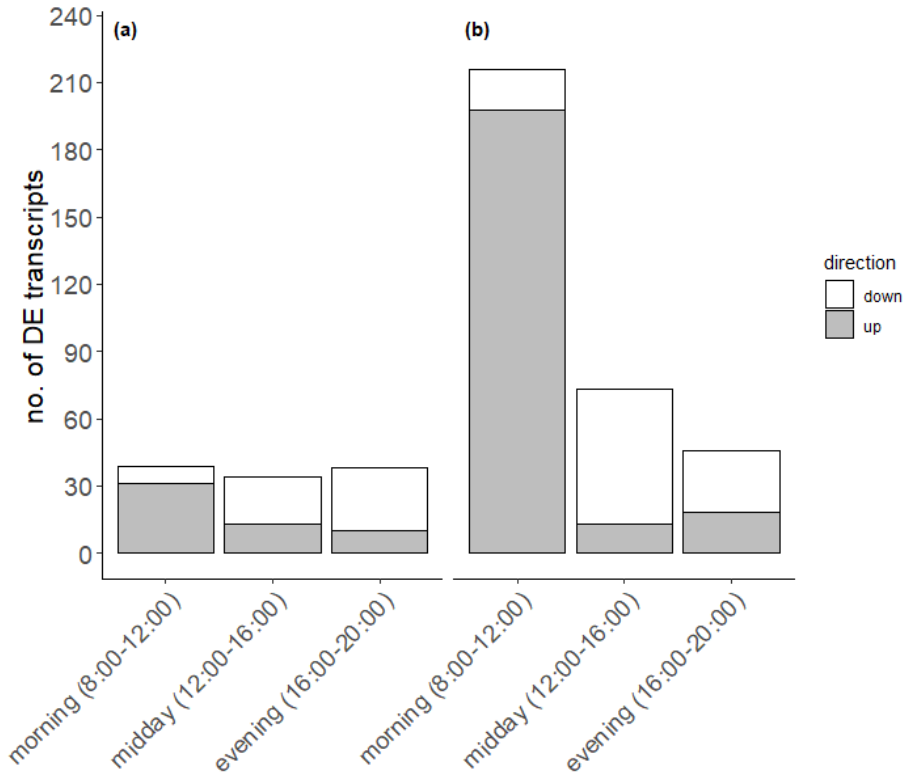


Figure 4: Number of differentially expressed genes between field sampling timepoints for a) day 1 and b) day 2. Grey and white indicate up- or down-regulation of genes relative to the past sampling timepoint.

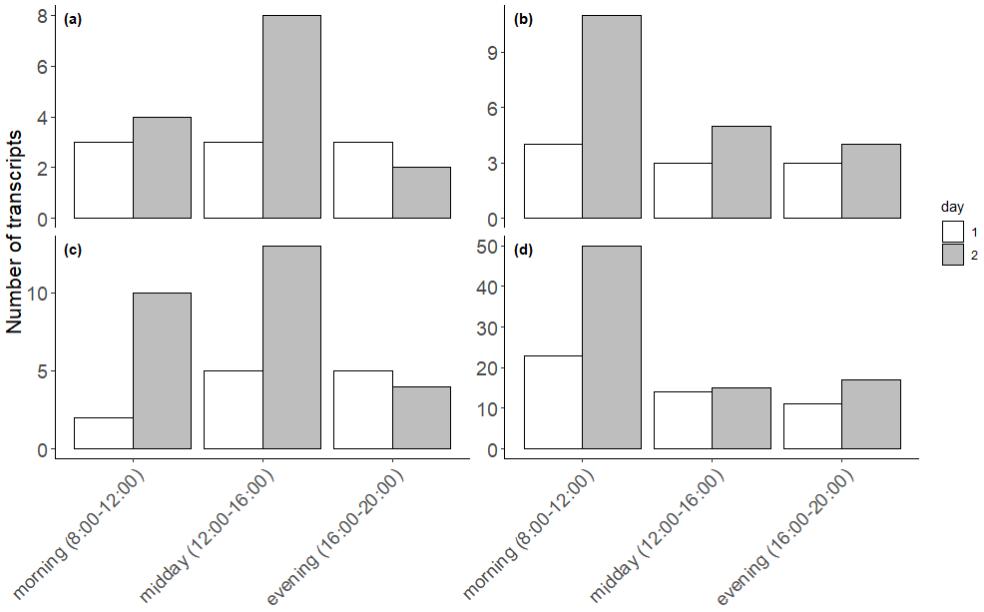


Figure 5: Overlap between transcripts expressed differentially between collection timepoints in the field collected individuals (x-axis) and the DE transcripts induced in the laboratory temperature ramping from 23°C to a) 3°C, a) 13°C, c) 33°C and d) 43°C. Colors indicate field collection days.

4. Discussion:

The ability of animals to cope with environmental stress partly depends on past experiences, yet studies that examine the effect of environmental factors influencing thermal tolerance and underlying physiological responses under natural conditions remains sparse (Koveos, 2001; Kelty, 2007; Loeschcke and Hoffmann, 2007; Kristensen et al., 2008; Overgaard and Sørensen, 2008; Schou et al., 2015; Gleason et al., 2017; Jensen et al., 2019). Here we show that recent thermal exposures drive changes in the transcriptome and thermal tolerances of *N. groenlandicus*. That is, past thermal experience, leading to hardening or acclimation responses affect thermal tolerances in a trait and sex specific manner (Fig. 1). These results add to earlier work on this species showing that HKDT and $T_{recovery}$ of field collected individuals varied markedly across days and time of day (Noer et al., 2022b, suppl. Figure S2 and Table S2). Together these results suggest that females can change their cold and heat tolerance rapidly through plastic responses, and that thermal performance is highly linked to temperatures experienced in the time period prior to testing. On the contrary, male heat tolerance did not respond strongly to short-term changes in mean field temperature (Fig. 1). Sexual dimorphism in thermal tolerances have been described by several studies, accordingly with male arthropod species having lower tolerances and plastic capacity than their female

counterparts (Bradley, 1978; Willett, 2010; Schou et al., 2017; Ørsted et al., 2018; Noer et al., 2022a). Such dimorphism can lead to, and cause, sex-specific evolution of life-history traits including age of sexual maturity, body size, longevity etc. (De Block and Stoks, 2003; Stillwell and Fox, 2007; Stillwell et al., 2009; Rogell et al., 2014). Thus, increasing temperatures can pose a risk of disparate evolutionary trajectories for male and female bugs. Alternatively, this discrepancy between plasticity in heat tolerance for male and female *N. groenlandicus* might reflect behavioral differences in the natural environment, for instance females basking in the sun to complete their reproductive life during the short arctic summer.

On a longer time-scale, the correlation between mean field temperature and HKDT was negative for both males and females, thus colder temperatures >12 hours prior to test resulted in higher HKDTs. Studies using temperature fluctuations show that interruption from high temperature stress can have positive effects on heat tolerance (Renault et al., 2004; Colinet et al., 2006; Koštál et al., 2007, Ørsted et al. in press). This is because alleviation from high temperatures allows repair of accumulated cell injuries and could explain the observed patterns in our study, suggesting a low thermal limit for injury accumulation for the species.

The associations between $T_{recovery}$ and mean field temperature suggest that males, like females, are highly plastic for this trait. This is in accordance with hypotheses suggesting that cold tolerance is under stronger selective pressures than heat tolerance (Huey et al., 2003; Bahrndorff et al., 2007; Sunday et al., 2011, 2014). Especially, species living in cold and highly seasonal environments are expected to be exposed to more variation in lower critical temperatures compared to warm extremes and have evolved stronger phenotypic responses to cold conditions (Gaston and Chown, 1999; Sinclair et al., 2003a; Ghalambor et al., 2006; Sunday et al., 2011; Bahrndorff et al., 2021b).

The gradual laboratory heat and cold ramping treatments caused distinct transcriptomic profiles with few shared DE transcripts (Fig. 2). This indicates that the mechanisms operating during cold and warm temperature stress are different, which is also reflected in the tradeoff between heat and cold tolerances found for female individuals. With climate change, temperatures are expected to become more variable and the frequency of which small organism are exposed to both cold and warm extremes within short timeframes are likely to increase (IPCC, 2014). This will potentially have negative impacts on species, like *N. groenlandicus*, living in temporally heterogenous environments because frequent induction of heat and cold responses are energetically costly and reduce energy allocated to thermal defense, reproduction and longevity (Feder and Hofmann, 1999; Sørensen et al., 2003).

The most substantial increase in DE transcripts occurred in females heat ramped to 43°C which induced 3-6x more DE transcripts than the other treatments, and the treatment initiated the heat shock response (Figure 2 and Table 1). The heat shock response is the cellular protective response induced by exposure to stressful high temperatures (and other environmental variables) and is found to be a conserved mechanism across animal and plant kingdoms (Feder et al., 1992; Feder and Hofmann, 1999; Sørensen et al., 2001; Bahrndorff et al., 2009; González-Tokman et al., 2020). During heat stress, levels of molecular chaperones increase in the cells where they stabilize and refold unstable or denatured proteins

and macromolecules. We found an increase in 12 different molecular chaperones, including four different transcripts of *Hsp90*, with heat ramping ending at 43°C. Expectedly, the largest fold change occurred in inducible *Hsp70* which is one of the major heat shock proteins that has been studied most extensively in relation to heat stress in arthropods (e.g. Feder et al., 1992; Dahlgaard et al., 1998; Krebs and Feder, 1998; Sørensen et al., 2001; Bettencourt et al., 2008; Bahrndorff et al., 2009; King and Macrae, 2015), followed by *Hsp90* and *Hsp83*. No chaperones were found when ramping the temperature to 33°C, so apparently short-term exposure to this temperature in the laboratory is not within the thermal range inducing this stress response. Further, at 43°C the expression of *vg 1 and 2* was upregulated quite markedly. Vg's are apolipoproteins and egg-yolk precursors which transport lipids and proteins to the oocyte and is thus crucial for ovarian development (Wu et al. 2021). The almost 27-fold increase in *vg* support our pondering of females utilizing high temperatures to complete their life cycle rapidly. Additionally, vg has antioxidant properties and can neutralize free radicals, which are byproducts of increased metabolism at higher temperature (Seehuus et al., 2006; Corona et al., 2007; Havukainen et al., 2013; Salmela et al., 2016). We cannot deduce whether vg has an impact on the lifecycle or heat tolerance from our results, but the different proposed functions of vg are not mutually exclusive, and vg might have several critical functions in this species under high temperature exposures.

We did not detect DE genes previously associated with cold hardening/acclimation in our study where we ramped temperatures from 23 to 3°C. The critical lethal temperature (CTmin) of *N. groenlandicus* ranges from −3.2 to 3.4°C (Bahrndorff et al., 2021b), thus we would expect that the cold ramping would induce a cold shock response as the temperature approaches CTmin. It is not uncommon that the transcriptional cold response is relatively weak despite a marked functional phenotypic response to cold hardening/acclimation (Sinclair et al., 2007; Sørensen et al., 2007; Von Heckel et al., 2016; Königer and Grath, 2018). For instance, Teets et al. (2012b) found no upregulated transcripts during RCH in the flesh fly (*Sarcophaga bullata*). Despite this, the study found that metabolic pathways increasing gluconeogenesis (glucose synthesis), and synthesis of amino acids and polyols were affected by the cold shock treatment. Thus, our results support the notion that RCH does not require synthesis of new gene products (Sinclair et al., 2007; Teets et al., 2012b). Instead, posttranslational processes and intracellular signaling are sufficient to induce RCH, hence the response should be detectable at higher levels of biological organization such as metabolite level such as shown in Noer et al., 2022b and Teets et al., 2012b. In this context, our results indicate that DNA dynamics (conformational changes) rather than gene expression might be critical for adjustments of cold tolerance. Chromatin structure, histones and other DNA modifications participate actively in regulation of gene activity, and these might be crucial for maintaining gene regulation at low temperatures, e.g. chromatin and histone modifications help access of regulatory proteins necessary for regulation of cold responsive genes (Zeng et al., 2019).

In the field, expression patterns changed in individuals collected across time and days. We found a strong effect of environmental temperature on gene expression patterns (Fig. 3 and

4) with more genes expressed differentially across all time points, except morning, on the day with large temperature fluctuations compared to the day with less temperature fluctuations (Fig. 4). This is in accordance with more upregulated genes at warm temperatures in the laboratory ramped individuals. Though the increase in transcription occurs across many transcripts and it might not have a physiological impact. mRNA transcripts are labile and break down faster at higher body temperature. Hence increased/constant transcription is required to maintain the same levels of transcripts as at lower temperature. Likewise, higher transcription levels might be a product of greater turnover of proteins. Thus, the protein levels might not change if the transcription and protein turnover is tightly coupled (Podrabsky and Somero, 2004). These observations are supported by the GO-terms enriched during the laboratory heat ramping from 23 to 33 and 43°C combined, which suggests that protein function and stability was challenged and dominated by synthesis of novel proteins and energy. Between days, there was also a significant increase in genes related to lipid storage and localization, including increased expression of *vg1* and *vg2* on the warm day. Thus, vg seems to have an ecological relevance in natural environments as well, but at much lower induction temperatures than in the laboratory.

Within days, the far greatest response was observed when comparing morning with noon of the temperature variable day which is also the timepoint with the largest change in temperature. Between these two time points, several transcripts found to be differentially expressed in the laboratory were also expressed differentially in the field. For instance, between the earliest sampling point and the noon, we found a small increase of the chaperones *Hsp83* and *Hsp90*. Though, the expression levels were magnitudes smaller than observed in the laboratory, it is noticeable that the induction temperature of Hsp expression is much lower than in the laboratory. There could be several reasons for this. First, the temperature of induction of the heat shock response varies according to evolutionary adaptation (Gehring and Wehner, 1995; Boher et al., 2016) / or acclimatization to the environment (Buckley et al., 2001; Karl et al., 2009). The individuals used for the laboratory experiment had been acclimated to 23°C for three days prior to the heat ramping. Based on the sensitive plastic acclimation responses of *N. groenlandicus*, this acclimation period might have been sufficient to raise the Hsp induction temperature. Second, several studies have shown that during recovery from cold shock, the level of Hsp's, including *Hsp22*, *Hsp23*, and *Hsp70*, in the cells increase as a consequence of cold damages to the cells (Colinet et al., 2010; Teets et al., 2012b). This might be the case here as the nighttime/early morning temperature is around the freezing point (Fig. 3). Third, multiple stressors act in concert in the natural environment, and a range of abiotic (temperature, desiccation) and biotic (predation, competition, starvation) stressors can affect the expression of Hsp's in the field.

In summary our results show marked thermal plasticity in *N. groenlandicus*, a widespread arctic arthropod, adapted to extreme thermal conditions. We show that the investigated population of this species continuously tracks the thermal environment and adjust its thermal tolerance and gene expression to match the environmental temperature. At the transcriptional level, individuals collected in the field on a day with high temperature variation showed a distinct expression profile compared to individuals collected on a day

with low temperature variation, thus increasing temperature variability might have energetic cost at a transcriptional level for species in heterogenous environments. Finally, we show that transcriptional pathways activated by high temperatures in the laboratory are shared but induced at much lower temperatures in the field reminding of the importance of performing ecologically relevant field studies to understand how species cope with thermal variation in their natural habitat.

Acknowledgement:

We thank Toke Thomas Høye for collecting *Nysius groenlandicus* from the field site in Narsarsuaq, 2019, for use for controlled laboratory experiments. We thank Finn Thomsen and Storch Lund for access to field sites and laboratory facilities in Narsarsuaq, Greenland.

Funding:

This work was supported by a grant from the Danish Council for Independent Research (DFF8021-00014B) and Carlsberg (Carlsbergfondet, CF17-0415). Travel grants was provided by North2North and SAFT (Selskab for Arktisk Forskning og Teknologi).

References:

Alexa, A., and Rahnenführer, J. (2020). Gene set enrichment analysis with topGO. Available at: http://www.mpi-sb.mpg.de/~alexa [Accessed April 25, 2022].

Arrese, E. L., and Soulages, J. L. (2010). Insect fat body: Energy, metabolism, and regulation. *Annu. Rev. Entomol.* 55, 207–225. doi:10.1146/annurev-ento-112408-085356.

Bahrndorff, S., Alemu, T., Kristensen, T. N., Sørensen, M. H., Høye, T., and Holmstrup, M. (2021a). Thermal adaptations of adults and eggs in the arctic insect, *Nysius groenlandicus*. *Polar Biol.* 44, 491–498. doi:10.1007/s00300-021-02807-6.

Bahrndorff, S., Lauritzen, J. M. S., Sørensen, M. H., Noer, N. K., and Kristensen, T. N. (2021b). Responses of terrestrial polar arthropods to high and increasing temperatures. *J. Exp. Biol.* 224, jeb230797. doi:10.1242/jeb.230797.

Bahrndorff, S., Mariën, J., Loeschcke, V., and Ellers, J. (2009). Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. *Funct. Ecol.* 23, 233–239. doi:10.1111/j.1365-2435.2009.01541.x.

Bahrndorff, S., Petersen, S. O., Loeschcke, V., Overgaard, J., and Holmstrup, M. (2007). Differences in cold and drought tolerance of high arctic and sub-arctic populations of *Megaphorura arctica* Tullberg 1876 (Onychiuridae: Collembola). *Cryobiology.* 55, 315–323. doi:10.1016/J.CRYOBIOL.2007.09.001.

Bettencourt, B. R., Hogan, C. C., Nimali, M., and Drohan, B. W. (2008). Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in Hsp70 null flies. *BMC Biol.* 6, 5. doi:10.1186/1741-7007-6-5.

Block, W., and Convey, P. (2001). Seasonal and long-term variation in body-water content of an Antarctic springtail - A response to climate change? *Polar Biol.* 24, 764–770. doi:10.1007/s003000100282.

Block, W., and Sømme, L. (1982). Cold hardiness of terrestrial mites at Signy Island, maritime Antarctic. *Oikos.* 38, 157–167. doi: 10.2307/3544015

Böcher, J., and Nachman, G. (2001). Temperature and humidity responses of the arctic-alpine seed bug *Nysius groenlandicus*. *Entomol. Exp. Appl.* 99, 319–330. doi:10.1046/j.1570-7458.2001.00831.x.

Boher, F., Trefault, N., Estay, S. A., and Bozinovic, F. (2016). Ectotherms in variable thermal landscapes: A physiological evaluation of the invasive potential of fruit flies species. *Front. Physiol.* 7, 302. doi:10.3389/fphys.2016.00302.

Bradley, B. P. (1978). Increase in range of temperature tolerance by acclimation in the copepod *Eurytemora affinis*. *Bulletin.* 154, 177–187. doi:10.2307/1541120

Buckley, B. A., Owen, M. E., and Hofmann, G. E. (2001). Adjusting the thermostat: The threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *J. Exp. Biol.* 204, 3571–3579. doi:10.1242/jeb.204.20.3571

Cannon, R. J. C., and Schenker, R. (1985). Cold tolerance of a cryptostigmatid mite at Signy Island, maritime Antarctic. *Br. Antarct. Surv. Bull.* 67, 1–5.

Colinet, H., Lee, S. F., and Hoffmann, A. (2010). Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. *FEBS J.* 277, 174–185. doi:10.1111/J.1742-4658.2009.07470.X.

Colinet, H., Renault, D., Hance, T., and Vernon, P. (2006). The impact of fluctuating thermal regimes on the survival of a cold-exposed parasitic wasp, *Aphidius colemani*. *Physiol. Entomol.* 31, 234–240. doi:10.1111/J.1365-3032.2006.00511.X.

Colinet, H., Sinclair, B. J., Vernon, P., and Renault, D. (2015). Insects in fluctuating thermal environments. *Annu. Rev. Entomol.* 60, 123–140. doi:10.1146/annurev-ento-010814-021017.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674–3676. doi:10.1093/BIOINFORMATICS/BTI610.

Convey, P., Block, W., and Peat, H. J. (2003). Soil arthropods as indicators of water stress in Antarctic terrestrial habitats? *Glob. Chang. Biol.* 9, 1718–1730. doi:10.1046/J.1365-2486.2003.00691.X/FULL.

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A., et al. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. U. S. A.* 104, 7128–7133. doi:10.1073/PNAS.0701909104.

Dahlgaard, J., Loeschcke, V., Michalak, P., and Justesen, J. (1998). Induced

thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Funct. Ecol.* 12, 786–793. doi:10.1046/j.1365-2435.1998.00246.x

Danks, H. V. (2004). Seasonal adaptations in arctic insects. *Integr. Comp. Biol.* 44, 85–94. doi:10.1093/ICB/44.2.85.

De Block, M., and Stoks, R. (2003). Adaptive sex-specific life history plasticity to temperature and photoperiod in a damselfly. *J. Evol. Biol.* 16, 986–995. doi:10.1046/J.1420-9101.2003.00581.X.

Feder, J. H., Rossi, J. M., Solomon, J., Solomon, N., and Lindquist, S. (1992). The consequences of expressing hsp70 in *Drosophila* cells at normal temperatures. *Genes Dev.* 6, 1402–1413. doi:10.1101/GAD.6.8.1402.

Feder, M. E., and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response. *Annu. Rev. Physiol.*, 243. doi:10.1146/annurev.physiol.61.1.243

Gaston, K. J., and Chown, S. L. (1999). Elevation and climatic tolerance: A test using dung beetles. *Oikos.* 86, 584–590. doi:10.2307/3546663

Gehring, W. J., and Wehner, R. (1995). Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proc. Natl. Acad. Sci. U. S. A.* 92, 2994. doi:10.1073/PNAS.92.7.2994.

Ghalambor, C. K., Huey, R. B., Martin, P. R., Tewksbury, J. J., and Wang, G. (2006). Are mountain passes higher in the tropics? Janzen's hypothesis revisited. *Integr. Comp. Biol.* 46, 5–17. doi:10.1093/icb/icj003.

Gleason, L. U., Miller, L. P., Winnikoff, J. R., Somero, G. N., Yancey, P. H., Bratz, D., et al. (2017). Thermal history and gape of individual *Mytilus californianus* correlate with oxidative damage and thermoprotective osmolytes. *J. Exp. Biol.* 220, 4292–4304. doi:10.1242/JEB.168450.

González-Tokman, D., Córdoba-Aguilar, A., Dáttilo, W., Lira-Noriega, A., Sánchez-Guillén, R. A., and Villalobos, F. (2020). Insect responses to heat: physiological mechanisms, evolution and ecological implications in a warming world. *Biol. Rev.* 95, 802–821. doi:10.1111/BRV.12588.

Havukainen, H., Münch, D., Baumann, A., Zhong, S., Halskau, Ø., Krogsgaard, M., et al. (2013). Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *J. Biol. Chem.* 288, 28369–28381. doi:10.1074/JBC.M113.465021.

Huey, R. B., Hertz, P. E., and Sinervo, B. (2003). Behavioral drive versus behavioral inertia in evolution: A null model approach. *Am. Nat.* 161, 357–366. doi:10.1086/346135.

IPCC (2014). Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 p.

Jensen, A., Alemu, T., Alemneh, T., Pertoldi, C., and Bahrndorff, S. (2019). Thermal acclimation and adaptation across populations in a broadly distributed soil arthropod. *Funct. Ecol.* 33, 833–845. doi:10.1111/1365-2435.13291.

Karl, I., Sørensen, J. G., Loeschcke, V., and Fischer, K. (2009). HSP70 expression in the copper butterfly *Lycaena tityrus* across altitudes and temperatures. *J. Evol. Biol.* 22,

172–178. doi:10.1111/J.1420-9101.2008.01630.X.
Kelty, J. (2007). Rapid cold-hardening of *Drosophila melanogaster* in a field setting. *Physiol. Entomol.* 32, 343–350. doi:10.1111/j.1365-3032.2007.00584.x.
King, A. M., and Macrae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annu. Rev. Entomol.* 60, 59–75. doi:10.1146/ANNUREV-ENTO-011613-162107.
Königer, A., and Grath, S. (2018). Transcriptome analysis reveals candidate genes for cold tolerance in *Drosophila ananassae*. *Genes.* 9, 624. doi:10.3390/GENES9120624.
Koštál, V., Renault, D., Mehrabianová, A., and Bastl, J. (2007). Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: Role of ion homeostasis. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 147, 231–238. doi:10.1016/J.CBPA.2006.12.033.
Koveos, D. S. (2001). Rapid cold hardening in the olive fruit fly *Bactrocera oleae* under laboratory and field conditions. *Entomol. Exp. Appl.* 101, 257–263. doi:10.1046/j.1570-7458.2001.00910.x.
Krebs, R. A., and Feder, M. E. (1998). Hsp70 and larval thermotolerance in *Drosophila melanogaster*: How much is enough and when is more too much? *J. Insect Physiol.* 44, 1091–1101. doi:10.1016/S0022-1910(98)00059-6.
Kristensen, T. N., Hoffmann, A., Overgaard, J., Sørensen, J. G., Hallas, R., and Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 105, 216–221. doi:10.1073/pnas.0708074105.
Lee, R. E., Chen, C., and Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science.* 238, 1415–1417. doi:10.1126/science.238.4832.1415
Loeschcke, V., and Hoffmann, A. A. (2007). Consequences of heat hardening on a field fitness component in Drosophila depend on environmental temperature. *Am. Nat.* 169, 175–183. doi:10.1086/510632.
Mølgaard, P. (1982). Temperature observations in high arctic plants in relation to microclimate in the vegetation of Peary Land. *Arct. Alp. Res.* 14, 105–115. doi:10.1080/00040851.1982.12004287.
Noer, N. K., Ørsted, M., Schiffer, M., Hoffmann, A. A., Bahrndorff, S., and Kristensen, T. N. (2022a). Into the wild—a field study on the evolutionary and ecological importance of thermal plasticity in ectotherms across temperate and tropical regions. *Philos. Trans. R. Soc. B.* 377, 20210004. doi:10.1098/RSTB.2021.0004.
Noer, N. K., Sørensen, M. H., Colinet, H., Renault, D., Bahrndorff, S., and Kristensen, T. N. (2022b). Rapid adjustments in thermal tolerance and the metabolome to daily environmental changes – a field study on the arctic seed bug Nysius groenlandicus. *Front. Physiol.* 13, 6. doi:10.3389/FPHYS.2022.818485/BIBTEX.
Ørsted, M., Malmendal, A., Muñoz, J., and Kristensen, T. N. (2018). Metabolic and functional phenotypic profiling of Drosophila melanogaster reveals reduced sex differentiation under stressful environmental conditions. *Biol. J. Linn. Soc.* 123, 155–162. doi:10.1093/biolinnean/blx120.
Overgaard, J., and Sørensen, J. G. (2008). Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. *Cryobiology.* 56, 159–162. doi:10.1016/j.cryobiol.2008.01.001.

Podrabsky, J. E., and Somero, G. N. (2004). Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. *J. Exp. Biol.* 207, 2237. doi:10.1242/jeb.01016.

Purać, J., Burns, G., Thorne, M. A. S., Grubor-Lajšić, G., Worland, M. R., and Clark, M. S. (2008). Cold hardening processes in the Antarctic springtail, *Cryptopygus antarcticus*: Clues from a microarray. *J. Insect Physiol.* 54, 1356–1362. doi:10.1016/J.JINSPHYS.2008.07.012.

R Core Team (2020). R: A Language and Environment for Statistical Computing. Available at: https://www.r-project.org.

Renault, D., Nedved, O., Hervant, F., and Vernon, P. (2004). The importance of fluctuating thermal regimes for repairing chill injuries in the tropical beetle Alphitobius diaperinus (Coleoptera: *Tenebrionidae*) during exposure to low temperature. *Physiol. Entomol.* 29, 139–145. doi:10.1111/J.0307-6962.2004.00377.X.

Rinehart, J. P., Yocum, G. D., and Denlinger, D. L. (2000). Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis*. *Physiol. Entomol.* 25, 330–336. doi:10.1111/J.1365-3032.2000.00201.X.

Rogell, B., Widegren, W., Hallsson, L. R., Berger, D., Björklund, M., and Maklakov, A. A. (2014). Sex-dependent evolution of life-history traits following adaptation to climate warming. *Funct. Ecol.* 28, 469–478. doi:10.1111/1365-2435.12179.

Salmela, H., Stark, T., Stucki, D., Fuchs, S., Freitak, D., Dey, A., et al. (2016). Ancient duplications have led to functional divergence of vitellogenin-like genes potentially involved in inflammation and oxidative stress in honey bees. *Genome Biol. Evol.* 8, 495–506. doi:10.1093/GBE/EVW014.

Schou, M. F., Kristensen, T. N., Pedersen, A., Karlsson, G., Loeschcke, V., and Malmendal, A. (2017). Metabolic and functional characterization of effects of developmental temperature in *Drosophila melanogaster*. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 312, R211–R222. doi:10.1152/ajpregu.00268.2016.

Schou, M. F., Loeschcke, V., and Kristensen, T. N. (2015). Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. *PLoS One* 10, e0130307. doi:10.1371/journal.pone.0130307.

Seehuus, S. C., Norberg, K., Gimsa, U., Krekling, T., and Amdam, G. V. (2006). Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* 103, 962–967. doi:10.1073/PNAS.0502681103/ASSET/00F6C22C-FC06-4E85-871F-474BD2BCC4E6/ASSETS/GRAPHIC/ZPQ0040608950005.JPEG.

Shreve, S. M., Kelty, J. D., and Lee, R. E. (2004). Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. *J. Exp. Biol.* 207, 1797–1802. doi:10.1242/JEB.00951.

Sinclair, B. J., Addo-Bediako, A., and Chown, S. L. (2003a). Climatic variability and the evolution of insect freeze tolerance. *Biol. Rev. Camb. Philos. Soc.* 78, 181–195. doi:10.1017/S1464793102006024.

Sinclair, B. J., Gibbs, A. G., and Roberts, S. P. (2007). Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect*

Mol. Biol. 16, 435–443. doi:10.1111/J.1365-2583.2007.00739.X.
Sinclair, B. J., Vernon, P., Klok, C. J., and Chown, S. L. (2003b). Insects at low temperatures: An ecological perspective. *Trends Ecol. Evol.* 18, 257–262. doi:10.1016/S0169-5347(03)00014-4.
Sømme, L. (1981). Cold tolerance of alpine, Arctic, and Antarctic Collembola and mites. *Cryobiology* 18, 212–220. doi:10.1016/0011-2240(81)90091-2.
Sømme, L., and Block, W. (1982). Cold hardiness of Collembola at Signy Island , maritime Antarctic. *Oikos.* 38, 168–176. doi:10.2307/3544016
Sørensen, J. G., Dahlgaard, J., and Loeschcke, V. (2001). Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. *Funct. Ecol.* 15, 289–296. doi:10.1046/J.1365-2435.2001.00525.X.
Sørensen, J. G., Kristensen, T. N., and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6, 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x.
Sørensen, J. G., Nielsen, M. M., and Loeschcke, V. (2007). Gene expression profile analysis of *Drosophila melanogaster* selected for resistance to environmental stressors. *J. Evol. Biol.* 20, 1624–1636. doi:10.1111/j.1420-9101.2007.01326.x.
Sørensen, J. G., Schou, M. F., Kristensen, T. N., and Loeschcke, V. (2016). Thermal fluctuations affect the transcriptome through mechanisms independent of average temperature. *Sci. Reports.* 6, 1–11. doi:10.1038/srep30975.
Sørensen, M. H., Kristensen, T. N., Lauritzen, J. M. S., Noer, N. K., Høye, T. T., and Bahrndorff, S. (2019). Rapid induction of the heat hardening response in an arctic insect. *Biol. Lett.* 15, 20190613. doi:10.1098/rsbl.2019.0613.
Stillwell, R. C., Blanckenhorn, W. U., Teder, T., Davidowitz, G., and Fox, C. W. (2009). Sex differences in phenotypic plasticity affect variation in sexual size dimorphism in insects: from physiology to evolution. *Annu. Rev. Entomol.* 55, 227–245. doi:10.1146/ANNUREV-ENTO-112408-085500.
Stillwell, R. C., and Fox, C. W. (2007). Environmental effects on sexual size dimorphism of a seed-feeding beetle. *Oecologia.* 153, 273–280. doi:10.1007/S00442-007-0724-0.
Storey, K. B. (1997). Organic solutes in freezing tolerance. *Comp. Biochem. Physiol. - A Physiol.* 117, 319–326. doi:10.1016/S0300-9629(96)00270-8.
Sunday, J. M., Bates, A. E., and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proc. R. Soc. B Biol. Sci.* 278, 1823–1830. doi:10.1098/rspb.2010.1295.
Sunday, J. M., Bates, A. E., Kearney, M. R., Colwell, R. K., Dulvy, N. K., Longino, J. T., et al. (2014). Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proc. Natl. Acad. Sci. U. S. A.* 111, 5610–5615. doi:10.1073/pnas.1316145111.
Teets, N. M., Gantz, J. D., and Kawarasaki, Y. (2020). Rapid cold hardening: ecological relevance, physiological mechanisms and new perspectives. *J. Exp. Biol.* 223. doi:10.1242/JEB.203448/223657.
Teets, N. M., Peyton, J. T., Colinet, H., Renault, D., Kelley, J. L., Kawarasaki, Y., et al. (2012a). Gene expression changes governing extreme dehydration tolerance in an

Antarctic insect. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20744–20749. doi:10.1073/pnas.1218661109.

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A., et al. (2012b). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiol. Genomics.* 44, 764–777. doi:10.1152/PHYSIOLGENOMICS.00042.2012/SUPPL_FILE/TABLES8.XLSX.

Vasseur, D. A., Delong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., Mccann, K. S., et al. (2014). Increased temperature variation poses a greater risk to species than climate warming. *Proc. R. Soc. B Biol. Sci.* 281, 1–8. doi:10.1098/rspb.2013.2612

Von Heckel, K., Stephan, W., and Hutter, S. (2016). Canalization of gene expression is a major signature of regulatory cold adaptation in temperate *Drosophila melanogaster*. *BMC Genomics* 17, 574. doi:10.1186/s12864-016-2866-0.

Willett, C. S. (2010). Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution.* 64, 2521–2534. doi:10.1111/J.1558-5646.2010.01008.X.

Worland, A. M. R., and Convey, P. (2001). Rapid cold hardening in antarctic microarthropods. *Funct. Ecol.* 15, 515–524. doi:10.1046/j.0269-8463.2001.00547.x

Worland, M. R., Grubor-Lajsic, G., and Montiel, P. O. (1998). Partial desiccation induced by sub-zero temperatures as a component of the survival strategy of the Arctic collembolan *Onychiurus arcticus* (Tullberg). *J. Insect Physiol.* 44, 211–219. doi:10.1016/S0022-1910(97)00166-2.

Young, S. R., and Block, W. (1980). Experimental studies on the cold tolerance of *Alaskozetes antarcticus*. *J. Insect Physiol.* 26, 189–200. doi:10.1016/0022-1910(80)90080-3.

Zeng, Z., Zhang, W., Marand, A. P., Zhu, B., Buell, C. R., and Jiang, J. (2019). Cold stress induces enhanced chromatin accessibility and bivalent histone modifications H3K4me3 and H3K27me3 of active genes in potato. *Genome Biol.* 20, 1–17. doi:10.1186/S13059-019-1731-2/FIGURES/9.

Ørsted, M., Jørgensen, L. B., and Overgaard, J. (in press). Finding the right thermal limit: a framework to reconcile ecological, physiological, and methodological aspects of CTmax in ectotherms. *J. Exp. Biol.*

SUPPLEMENTAL MATERIALS FOR PAPER III

Table S1: Date and time of field collection and thermal assays for testing thermal tolerance of Nysius groenlandicus

Table S2: Gene transcripts related to cellular stress used for targeted search of stress responses induced by gradual cold and heat ramping in the laboratory acclimated individuals

Figure S1: Venn diagram showing the number of overlapping transcripts of gradual heat and cold ramping

Table S3: Gene Ontology Enrichment Analysis on transcripts that were differentially expressed for heat ramped treatments.

Table S4: Gene Ontology Enrichment Analysis on transcripts that were differentially expressed across days

Table S1: Overview of dates and times for field collection of *N. groenlandicus*, start times of thermal assays (heat knockdown time (HKDT) and chill coma recovery temperature ($T_{recovery}$)). The insects were kept in glass containers in the shadow in the field site until assay start. Stars (*) indicate time points when samples for RNA sequencing were collected simultaneously with individuals for thermal tolerance tests (modified from Noer et al. 2022).

		Assay start	
	Sampling date	**HKDT**	**$T_{recovery}$**
Day 1	07-08-2018	08:30	08:28
	07-08-2018	12:10	12:09
	07-08-2018	16:08	16:06
	07-08-2018	19:58	19:58
Day 2	10-08-2018	08:22	08:14
	10-08-2018	12:20	12:15
	10-08-2018	17:08	17:09
	10-08-2018	20:35	20:32
Day 3	11-08-2018	08:14	08:08
	11-08-2018	12:15	12:09
	11-08-2018	16:16	16:09
	11-08-2018	20:20	20:11
Day 4	22-08-2018*	08:51	08:44
	22-08-2018*	12:22	12:15
	22-08-2018*	16:22	16:15
	22-08-2018*	20:20	20:15
Day 5	27-08-2018*	08:20	08:13
	27-08-2018*	12:39	12:33
	27-08-2018*	16:29	15:25
	27-08-2018*	20:25	16:21

Table S2: Transcripts related to cellular stress used for targeted search of stress responses induced by gradual cold and heat ramping in the laboratory acclimated individuals.

Keyword	Stressor	reference
heat shock	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
cold shock	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
oxidative stress	Oxidative	Gonzáles-Tokman et al. 2020 - Biol. Rev
antioxidant	Oxidative	Gonzáles-Tokman et al. 2020 - Biol. Rev
immune	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
salinity	Osmotic	Gonzáles-Tokman et al. 2020 - Biol. Rev
chaperone	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
co-chaperone	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
dnaJ	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
hsp	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
protein folding	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
refolding	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
apoptosis	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
disulfid	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
calreticulin	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
desaturase	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
fatty acid	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
cholesterol	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
HMG	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
CoA	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
hydroxymethylglutaryl	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
ADRP	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
GPI	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
glycine	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
elongation	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
HMGB1	Temperature	Podrabsky and Somero, 2004 - J. Exp. Biol.
crystallin	Temperature	Gracey et al. 2008 - Curr. Biol.
HSPBP1	Temperature	Gracey et al. 2008 - Curr. Biol.
peroxidase	Oxidative	Torson et al. 2015 - J. Exp. Biol.; Franke et al. 2019 - BMC
glutathione	Oxidative	Torson et al. 2015 - J. Exp. Biol.

Table S2 continued

Keyword	Stressor	reference
S-transferase	Oxidative	Torson et al. 2015 - J. Exp. Biol.
P450	Oxidative	Torson et al. 2015 - J. Exp. Biol.; Franke et al. 2019 - BMC
toll	Temperature	Torson et al. 2015 - J. Exp. Biol.
superoxide dismutase	Oxidative	Torson et al. 2015 - J. Exp. Biol.
ecdysone	Oxidative	Gonzáles-Tokman et al. 2020 - Biol. Rev
juvenile hormone	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
gustatory	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
transient receptor	Temperature	Gonzáles-Tokman et al. 2020 - Biol. Rev
thiol	Oxidative	Gonzáles-Tokman et al. 2020 - Biol. Rev
ascorbic	Oxidative	Gonzáles-Tokman et al. 2020 - Biol. Rev
catalase	Oxidative	Gonzáles-Tokman et al. 2020 - Biol. Rev
vitellogenin	Oxidative	Torson et al. 2019 - J. Insect Phys.
apolipoprotein	Oxidative	Torson et al. 2019 - J. Insect Phys.
cuticular protein	Temperature	Teets et al. 2014 - J. Exp. Biol.
CHK1	Temperature	Teets et al. 2014 - J. Exp. Biol.
aquaporin	Temperature	Teets et al. 2014 - J. Exp. Biol.
calcium	Temperature	Teets et al. 2008 - Am J Physiol Regul Integr Comp Physiol; Teets et al. 2020 - J. Exp. Biol.
p38	Temperature	Teets et al. 2008 - Am J Physiol Regul Integr Comp Physiol; Teets et al. 2020 - J. Exp. Biol.
LEA	Osmotic	Bahrndorff et al- 2009 - J. Insect Phys.
ATMP synthase	Temperature	Michaud and Denlinger, 2010
HIF-1	Temperature	Michaud and Denlinger, 2010

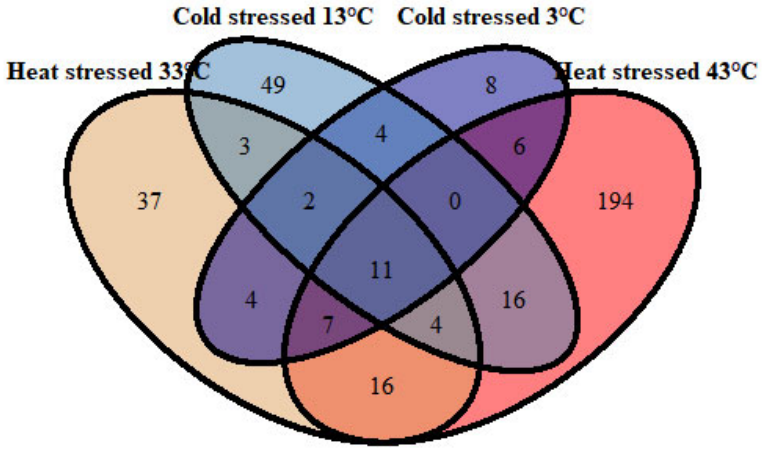


Figure S1: Venn diagram showing the number of overlapping transcripts that was differentially expressed in individuals exposed to either of two heat ramping and cold ramping treatments compared to a control groups acclimated to 23 °C.

Table S3: Gene Ontology Enrichment Analysis on transcripts that were differentially expressed for heat ramped treatments (pdf). Significant GO-terms for cold ramped individuals are presented in figure 2.

GO enrichment - Heat treatments

GO.ID	Term	Annotated	Significant	Expected	classicFisher
GO:0043604	amide biosynthetic process	171	23	7.81	7.7e-07
GO:0006412	translation	161	22	7.35	1.1e-06
GO:0043043	peptide biosynthetic process	163	22	7.45	1.3e-06
GO:0043603	cellular amide metabolic process	186	23	8.50	3.6e-06
GO:0006518	peptide metabolic process	173	22	7.90	3.8e-06
GO:1901566	organonitrogen compound biosynthetic pro...	260	26	11.88	4.0e-05
GO:0006457	protein folding	49	10	2.24	4.4e-05
GO:0006090	pyruvate metabolic process	26	7	1.19	0.00010
GO:0006096	glycolytic process	20	6	0.91	0.00018
GO:0006757	ATP generation from ADP	20	6	0.91	0.00018
GO:0006165	nucleoside diphosphate phosphorylation	21	6	0.96	0.00024
GO:0046939	nucleotide phosphorylation	21	6	0.96	0.00024
GO:0009135	purine nucleoside diphosphate metabolic ...	22	6	1.00	0.00031
GO:0009179	purine ribonucleoside diphosphate metabo...	22	6	1.00	0.00031
GO:0009185	ribonucleoside diphosphate metabolic pro...	22	6	1.00	0.00031
GO:0046031	ADP metabolic process	22	6	1.00	0.00031
GO:0030388	fructose 1,6-bisphosphate metabolic proc...	4	3	0.18	0.00036
GO:0009132	nucleoside diphosphate metabolic process	23	6	1.05	0.00041
GO:0016052	carbohydrate catabolic process	23	6	1.05	0.00041
GO:0044271	cellular nitrogen compound biosynthetic ...	321	27	14.66	0.00058
GO:0034645	cellular macromolecule biosynthetic proc...	289	24	13.20	0.00159
GO:0009059	macromolecule biosynthetic process	296	24	13.52	0.00224
GO:1901576	organic substance biosynthetic process	408	29	18.64	0.00547
GO:0031398	positive regulation of protein ubiquitin...	3	2	0.14	0.00600
GO:1903322	positive regulation of protein modificat...	3	2	0.14	0.00600
GO:1901564	organonitrogen compound metabolic proces...	627	40	28.64	0.00601
GO:0009058	biosynthetic process	414	29	18.91	0.00681
GO:0044249	cellular biosynthetic process	400	28	18.27	0.00813
GO:0006869	lipid transport	20	4	0.91	0.01121
GO:0009150	purine ribonucleotide metabolic process	56	7	2.56	0.01212

Table S4: Gene Ontology Enrichment Analysis on transcripts that were differentially expressed across days. (pdf).

GO.ID	Term	Annotated	Significant	Expected	p-value (Fisher's exact)
08:00					
GO:0022900	**electron transport chain**	256	9	1.53	7.4e-07
GO:0045333	cellular **respiration**	257	9	1.54	7.6e-07
GO:0015980	**energy derivation by oxidation of organi...**	258	9	1.54	7.9e-07
GO:0006091	generation of precursor metabolites and ...	298	9	1.78	2.8e-06
GO:0006119	oxidative phosphorylation	182	6	1.09	0.00026
GO:1902600	**proton transmembrane transport**	200	6	1.20	0.00044
GO:0009060	aerobic respiration	201	6	1.20	0.00046
GO:0098662	inorganic cation transmembrane transport	211	6	1.26	0.00060
GO:0046034	ATP metabolic process	213	6	1.27	0.00063
GO:0098655	cation transmembrane transport	215	6	1.29	0.00066
12:00					
GO:0010876	**lipid localization**	24	5	0.52	0.00012
GO:1902769	regulation of choline O-acetyltransferas...	2	2	0.04	0.00046
GO:1902600	**proton transmembrane transport**	200	12	4.35	0.00070
GO:0098662	inorganic cation transmembrane transport	211	12	4.59	0.00114
GO:0046034	ATP metabolic process	213	12	4.63	0.00124
GO:0098655	cation transmembrane transport	215	12	4.68	0.00135
GO:0098660	inorganic ion transmembrane transport	217	12	4.72	0.00147
GO:0006754	ATP biosynthetic process	12	3	0.26	0.00183
GO:0015985	**energy coupled proton transport, down el...**	12	3	0.26	0.00183
GO:0015986	ATP synthesis coupled proton transport	12	3	0.26	0.00183
16:00					
GO:0006412	**translation**	161	24	5.87	2.9e-10
GO:0043043	peptide biosynthetic process	163	24	5.94	3.8e-10
GO:0043604	amide biosynthetic process	171	24	6.23	1.1e-09
GO:0006518	peptide metabolic process	173	24	6.30	1.4e-09

Table S4 continued

GO:00 43603	cellular amide metabolic process	186	24	6.78	6.5e-09
GO:19 01566	organonitrogen compound biosynthetic pro...	260	25	9.47	1.2e-06
GO:00 22904	respiratory electron transport chain	93	13	3.39	1.6e-05
GO:00 34645	cellular macromolecule biosynthetic proc...	289	24	10.53	3.2e-05
GO:00 09059	macromolecule biosynthetic process	296	24	10.78	4.8e-05
GO:00 06091	generation of precursor metabolites and ...	298	24	10.86	5.4e-05
20:00					
GO:00 22900	electron transport chain	256	15	5.57	0.00015
GO:00 45333	cellular respiration	257	15	5.59	0.00015
GO:00 15980	energy derivation by oxidation of organi...	258	15	5.61	0.00016
GO:00 06091	generation of precursor metabolites and ...	298	15	6.48	0.00083
GO:00 08152	metabolic process	1349	37	29.34	0.00226
GO:19 02600	proton transmembrane transport	200	11	4.35	0.00256
GO:00 98662	inorganic cation transmembrane transport	211	11	4.59	0.00394
GO:00 06119	oxidative phosphorylation	182	10	3.96	0.00424
GO:00 46034	ATP metabolic process	213	11	4.63	0.00425
GO:00 98655	cation transmembrane transport	215	11	4.68	0.00457

PAPER IV

RAPID ADJUSTMENTS IN THERMAL TOLERANCE AND THE METABOLOME TO DAILY ENVIRONMENTAL CHANGES – A FIELD STUDY ON THE ARCTIC SEED BUG *NYSIUS GROENLANDICUS*

Published in Frontiers of Physiology

Natasja Krog Noer, Mathias Hamann Sørensen, Hervé Colinet, David Renault, Simon Bahrndorff and Torsten Nygaard Kristensen

frontiers in Physiology

ORIGINAL RESEARCH
published: 16 February 2022
doi: 10.3389/fphys.2022.818485

Rapid Adjustments in Thermal Tolerance and the Metabolome to Daily Environmental Changes – A Field Study on the Arctic Seed Bug *Nysius groenlandicus*

Natasja Krog Noer[1], Mathias Hamann Sørensen[1], Hervé Colinet[2], David Renault[2,3], Simon Bahrndorff[1†] and Torsten Nygaard Kristensen[1†]*

[1] Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark, [2] UMR 6553, CNRS, Ecosystèmes, Biodiversité, Évolution, University of Rennes 1, Rennes, France, [3] Institut Universitaire de France, Paris, France

OPEN ACCESS

Edited by:
Frank Seebacher, The University of Sydney, Australia

Reviewed by:
Vladimír Košťál, Centre for Biology, Academy of Sciences of the Czech Republic (ASCR), Czechia
Folco Giomi, Independent Researcher, Padua, Italy

***Correspondence:**
Natasja Krog Noer
nkn@bio.aau.dk

[†] *These authors share last authorship*

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *22 November 2021*
Accepted: *03 January 2022*
Published: *16 February 2022*

Citation:
Noer NK, Sørensen MH, Colinet H, Renault D, Bahrndorff S and Kristensen TN (2022) Rapid Adjustments in Thermal Tolerance and the Metabolome to Daily Environmental Changes – A Field Study on the Arctic Seed Bug Nysius groenlandicus. Front. Physiol. 13:818485. doi: 10.3389/fphys.2022.818485

Laboratory investigations on terrestrial model-species, typically of temperate origin, have demonstrated that terrestrial ectotherms can cope with daily temperature variations through rapid hardening responses. However, few studies have investigated this ability and its physiological basis in the field. Especially in polar regions, where the temporal and spatial temperature variations can be extreme, are hardening responses expected to be important. Here, we examined diurnal adjustments in heat and cold tolerance in the Greenlandic seed bug *Nysius groenlandicus* by collecting individuals for thermal assessment at different time points within and across days. We found a significant correlation between observed heat or cold tolerance and the ambient microhabitat temperatures at the time of capture, indicating that *N. groenlandicus* continuously and within short time-windows respond physiologically to thermal changes and/or other environmental variables in their microhabitats. Secondly, we assessed underlying metabolomic fingerprints using GC-MS metabolomics in a subset of individuals collected during days with either low or high temperature variation. Concentrations of metabolites, including sugars, polyols, and free amino acids varied significantly with time of collection. For instance, we detected elevated sugar levels in animals caught at the lowest daily field temperatures. Polyol concentrations were lower in individuals collected in the morning and evening and higher at midday and afternoon, possibly reflecting changes in temperature. Additionally, changes in concentrations of metabolites associated with energetic metabolism were observed across collection times. Our findings suggest that in these extreme polar environments hardening responses are marked and likely play a crucial role for coping with microhabitat temperature variation on a daily scale, and that metabolite levels are actively altered on a daily basis.

Keywords: arctic, climate change, diurnal environmental variation, GC-MS metabolomics, insects, phenotypic plasticity, temperature variation, thermal tolerance

INTRODUCTION

Terrestrial ectotherms are subject to large spatial and temporal variability in their thermal environment (Kearney and Porter, 2009). In terrestrial ecosystems, daily changes in temperature can be substantial, and vary greatly with microhabitat characteristics such as topography and orientation, vegetation cover, shading and more (Sears et al., 2019; Kearney et al., 2020; Lembrechts and Lenoir, 2020). Some of the most extreme environments are found in the polar regions where the winters are long and cold, and the summers short and periodically hot (Bahrndorff et al., 2021b). During the arctic summer, daily temperatures can vary by >30°C and reach subzero temperatures at night (Convey et al., 2018; Davey et al., 2021). Organisms, including insects, living in these environments must therefore be able to survive and reproduce over a wide range of temperatures (Deere et al., 2006; Bahrndorff et al., 2021a). This can be achieved by evolutionary adaptation to the local thermal conditions across generations, or by fast adjustments of the physiology within the lifetime of an organism *via* phenotypic plasticity (Scheiner, 1993; Fusco and Minelli, 2010; Kristensen et al., 2020). Evolutionary adaptation to changing and periodically stressful temperatures can be slow, and are sometimes constrained by genetic trade-offs or lack of adaptive genetic variation (Araújo et al., 2013; Hoffmann et al., 2013). Conversely, rapid plastic adaptive changes can rescue individuals exposed to biotic and abiotic challenges at a shorter timescale, including daily environmental fluctuations (Colinet and Hoffmann, 2012; Noer et al., 2022). Plastic changes might therefore be particularly relevant for arctic species exposed to unpredictable and rapid changes in the environment.

Organisms can respond plastically to short-term exposure to sub-optimal temperatures through hardening or by acclimation at longer term exposures (Colinet and Hoffmann, 2012; Schou et al., 2017). Hardening responses to extreme or acute temperatures are thought to counter rapid thermal stress, such as daily temperature extremes and stochastic events (Koveos, 2001; Hoffmann et al., 2003; Kelty, 2007; Overgaard and Sørensen, 2008; Jensen et al., 2019). The other form of more gradual acclimation includes seasonal acquisition of cold or heat tolerance that is induced by changes in temperature and photoperiod interacting with other abiotic factors (reviewed by Chown and Terblanche, 2006; Teets and Denlinger, 2013). There are several published examples of cold acclimation and rapid cold hardening in arctic arthropods (e.g., Bahrndorff et al., 2007; Everatt et al., 2013), but very few studies have investigated physiological acclimation of polar terrestrial arthropods to high temperatures (Sørensen et al., 2019; Bahrndorff et al., 2021b). Traditionally, thermal plasticity of insects has been investigated using model-organisms kept and hardened/acclimated to constant controlled temperatures in the laboratory (Angilletta, 2009; Colinet et al., 2015; Javal et al., 2016; Ketola and Kristensen, 2017). However, recent work on the impacts of temperature variability on thermal tolerance have emphasized that thermal performance based on constant temperatures do not always accurately predict performance under variable conditions in the laboratory (reviewed by Colinet et al., 2015; Vázquez et al., 2017), nor in the field (see e.g., Kingsolver and Nagle, 2007; Loeschcke and Hoffmann, 2007; Kristensen et al., 2008; Ketola and Kristensen, 2017). This potential mismatch in the conclusions arising from investigations based on constant *versus* fluctuating temperatures partly results from the non-linear impact of temperatures on thermal performance (Jensen's inequality) (Ruel and Ayres, 1999; Colinet et al., 2015), time-by-temperature interactions (Foray et al., 2013; Kingsolver et al., 2015), and methodology (Chown et al., 2009; Mitchell and Hoffmann, 2010; Bahrndorff et al., 2016). Based on such results, the potential for transferring the knowledge obtained from the laboratory to field conditions, and thus forecast reliable predictions of the effects of climate change on the responses and geographic distribution of insects, is being increasingly questioned (Fischer et al., 2011; Kingsolver et al., 2015; Kinzner et al., 2019; Taylor et al., 2021).

The physiological and molecular mechanisms enabling arthropods to tolerate temperature stress has previously focused on controlled laboratory studies (but see Tomanek and Somero, 1999, 2002; Buckley et al., 2001; Gracey et al., 2008; Kristensen et al., 2012; Vasquez et al., 2019). Studies on temperate and polar species suggest (causation is typically lacking in such studies) that metabolites such as sugars, free amino acids and polyols can be associated with changes in cold tolerance measures (Zachariassen, 1985; Fields et al., 1998; Sømme, 1999; Holmstrup et al., 2002; Michaud and Denlinger, 2007, 2010; Overgaard et al., 2007, 2014). Changes in polyols on the other hand have been associated with changes in heat tolerance (Hendrix and Salvucci, 1998; Wolfe et al., 1998; Salvucci et al., 2000; Benoit et al., 2009). However, few have attempted to describe how these metabolites are affected by dynamic and fluctuating temperatures as encountered in nature (but see Kristensen et al., 2012; Noer et al., 2020; Sheldon et al., 2020).

In this study, we examined the effects of daily variation in the microhabitat temperatures on plastic adjustments of heat and cold tolerance of the Greenlandic seed bug *Nysius groenlandicus* (Zetterstedt) during summer in Southern Greenland. *Nysius groenlandicus* is a univoltine species, and widespread and abundant in Arctic and sub-Arctic regions. Previous work on the species have revealed that it can rapidly increase heat tolerance when exposed to high and stressful temperatures under laboratory conditions (Sørensen et al., 2019), and thus *N. groenlandicus* represents a valuable polar insect model for field-based description of daily changes in individuals' thermal tolerance. Further, we examined the metabolic fingerprints within days with high and low temperature variation using a quantitative targeted gas chromatography-mass spectrometry (GC-MS) approach. We hypothesized that the ability to tolerate low and high temperatures is constantly fine-tuned to respond to temporally fluctuating temperatures in field-collected individuals of *N. groenlandicus* as an adaptation to the highly variable environmental conditions within and across days. Thus, we expected specimens collected in early morning and late evening to have the highest cold tolerance, while those collected at midday exhibiting the highest heat tolerance. Finally, we expected to see daily changes in metabolites known to improve cold tolerance (sugars and amino acids) in individuals collected in early morning and late evening, while metabolites enhancing

heat tolerance (polyols) would show higher concentrations in individuals sampled during the warm periods of the day.

MATERIALS AND METHODS

Field Collection and Experimental Design

The field work was conducted in July–August 2018 in Narsarsuaq (Southern Greenland, 61.160°N, 45.424°W). This region is characterized by cool temperatures, long winters and short and thermally variable summers (Böcher and Nachman, 2001; Bahrndorff et al., 2021b). The study site was a heath-like, grass-covered area, where adult *N. groenlandicus* were collected from the grasses using a sweep net (**Figure 1**). All individuals used in the experiment were caught in a 50 m × 50 m area less than 100 m from the laboratory facilities.

Adult individuals of *N. groenlandicus* were collected at four time points (08:00 am, 12:00 pm, 04:00 pm, and 08:00 pm) during each of 5 days (depending on weather conditions and the abundance of *N. groenlandicus*; see **Supplementary Table 1** for exact collection times and dates), thereafter referred to as day 1 to day 5. At the time of field-collection, each individual was placed in a 4 mL screw-cap glass vial (45 mm × 14.7 mm) and placed in the shade on the ground to prevent abrupt changes in the thermal conditions. The sex of individuals was then assessed by eye, and the vials transferred to the laboratory within 30–45 min of collection. Immediately after returning, the heat and cold tolerances were scored using 20 females and 20 males for each assay (see next section).

Additional individuals were collected for subsequent metabolomics analysis at the same four sampling times at two dates (22/08/18 and 27/08/18, representing day 4 and 5, respectively, see **Supplementary Table 1**), representing days with either high or low observed temperature variation. At each collection time, eight samples of five females (only females were used for the metabolomics studies) were collected, transferred directly into ice cold RNAlater, and stored at −20°C for approximately 1 week. Then the samples were transferred to our laboratory in Denmark where they were stored at −80°C and later used for metabolomic fingerprinting. Collection of

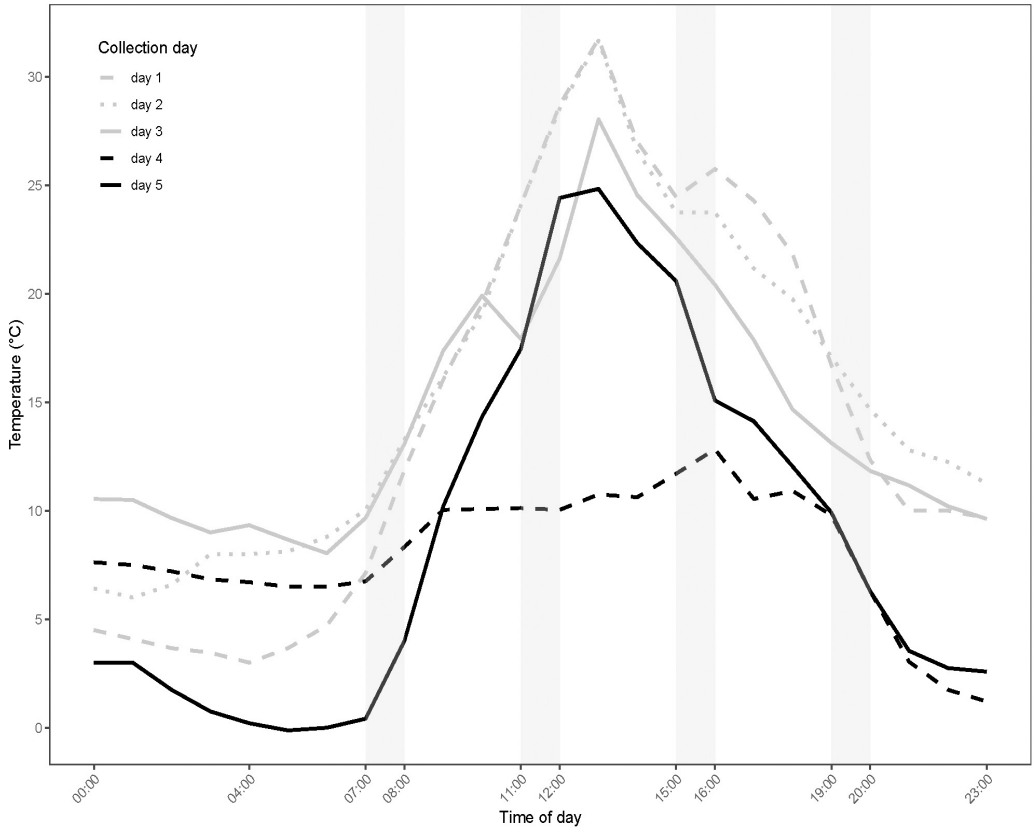


FIGURE 1 | Microhabitat temperature (°C) averaged in 1-h bins across the five experimental days. The temperatures were measured at 15 cm above the soil surface in the shade every 5 min. Samples for GC-MS analysis were collected on day 4–5 (black lines). Shaded columns indicate the timespans where specimen are collected.

samples in RNAlater is amenable for downstream metabolomics analysis if stored at subzero temperatures (van Eijsden et al., 2013; Harris, 2018).

The air temperature at the collection site was continuously recorded with 5-min intervals in the shade using Easylog USB data loggers (LASCAR Electronics, EL-USB-2^+). The temperature was measured in the shade to avoid warm temperature-spikes in the measurements caused by direct solar radiation (Maclean et al., 2021) and the loggers were placed 15 cm above the soil surface to reflect the thermal environment at the top of the grasses where *N. groenlandicus* was most abundant, and was caught with the sweeping net. Based on these recordings, the mean temperature was calculated for the 1-h timespan prior to testing thermal tolerances. The mean temperature immediately prior to thermal assessment has been shown to be highly correlated with the heat tolerance in a range of insect species collected in the field (Noer et al., 2022).

Heat and Cold Tolerance Assays

Heat Knockdown Time

Heat tolerance was measured as heat knockdown time (HKDT), i.e., a measure of the time before individuals go into a heat-induced coma/die at a high stressful temperature (Terblanche et al., 2007; Bak et al., 2020). For *N. groenlandicus* HKDT constitutes a direct measure of heat tolerance as we register the time individuals can withstand before they die. The vials containing field-collected individuals were mounted to a rack and submerged into a temperature-controlled water bath (PolyScience MX Immersion Circulator: MX-CA12E) maintained at 48°C. The individuals were then observed and stimulated with flashes of light and gentle taps on the vial caps with a metal rod. The time until movement ceased was noted for each individual. The chosen HKDT temperature was based on experiences from previous work on the species (Sørensen et al., 2019), and on unpublished preliminary results showing that *N. groenlandicus* individuals went into coma within 20–40 min at 48°C. Earlier results suggest a fast heat hardening response for this species and since we wanted a measure of their acute heat tolerance, and aimed for reducing hardening responses induced while testing, this HKDT was found relevant.

Chill Coma Recovery

Cold tolerance was measured as the temperature at which the bugs regained the ability to move after being knocked down by exposure to a low temperature ($T_{recovery}$) following a modified procedure of the method described in Overgaard et al. (2011). Thus, we used a proxy rather than direct measures of cold tolerance. The glass vials containing the individuals were mounted to a rack and submerged into a glycol-water solution that was kept at −3°C using a thermostat (LAUDA Proline Edition X RP 1845-C, LAUDA DR. R. WOBSER GMBH & CO., KG, Germany). This temperature was based on the lower critical temperature (CTmin) which induces a cold coma for the species (Bahrndorff et al., 2021b). Immediately after submersion, the temperature of the solution was increased at a rate of 0.2°C/min. Pilot studies showed that this temperature (−3°C) was sufficient to induce chill coma within a few minutes with full survival upon returning to room temperature (data not presented). Following the HKDT procedure, the individuals were observed and provoked using light flashes and gentle taps, and the temperature at which individuals first moved any body part was noted as their chill coma recovery temperature ($T_{recovery}$). A low $T_{recovery}$ is interpreted as high cold tolerance.

Metabolomic Fingerprinting

We adapted the methods detailed in Thiébaut et al. (2020) for detecting and quantifying the metabolite content from whole-body extracts of female *N. groenlandicus*. For each field sampling time, eight replicates were used, each consisting of five pooled females to obtain sufficient biomass (∼4 mg dry mass per sample). Each sample was vacuum dried (Speed Vac Concentrator, miVac; Genevac Ltd., Ipswich, England) and weighed (Mettler Toledo UMX2, accurate to 0.001 mg) before extractions so that metabolite concentrations could be reported according to dry mass. The samples were first homogenized for 90 s with two tungsten beads in 450 μL of a solution of ice cold methanol-chloroform (ratio 2:1, v:v) using a bead beater (Qiagen MM301; Retsch GmbH, Haan, Germany) set at 25 Hz. To separate the homogenate in two distinct phases (lipid-rich containing phase, and aqueous phase containing metabolites), 300 μL of cold ultrapure water was added to each tube before centrifugation (Sigma 2-16K, Sigma GmbH, Harz, Germany) for 10 min at 4000 *g* at 4°C. The supernatants containing metabolites were transferred to new tubes and stored at −80°C until analysis by GC-MS. Prior to the analysis, 120 μL of the metabolite extract was transferred to glass vials and vacuum dried.

The derivatization of the samples (dry residues) was automatized with a CTC CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Dried samples were re-suspended in 30 μL of 25 mg mL^{-1} methoxyamine-hydrochloride (CAS Number: 593-56-6, SIGMA-ALDRICH, St. Louis, MO, United States) in pyridine prior to incubation under orbital shaking at 40°C for 60 min. Following incubation, 30 μL of N-methyl-bis(trifluoroacetamine) (BSTFA, CAS Number: 685-27-8) was added, and derivatization was conducted at 40°C for 60 min under agitation. An Agilent 7890B gas chromatograph coupled to a 5977B mass spectrometer was used for the separation and detection of the metabolites. For each sample, 1 μL was injected (Injector temperature: 250°C; split mode with a split ratio of 25:1); the temperature of the oven increased from 70 to 170°C at a rate of 5°C/min, from 170 to 280°C at 7°C/min, and from 280 to 320°C at 15°C/min, and then remained at 320°C for 4 min. We used a 30-m fused silica column (HP5 MS 30 m, I.D. 0.25 mm, thickness 0.25 μm, 5% Diphenyl/95% Dimethylpolysiloxane, Agilent Technologies), and the gas carrier (Helium) had a flow of 1 mL per min. The temperatures of the transfer line and ion source were 280 and 230°C, respectively. Metabolite fragmentation and ionization were carried out by electronic impact (electron energy: 70 eV) and detected with the full scan mode. The detected peaks were identified and annotated with MassHunter (Agilent). Most detected metabolites were identified, and calibration curves were used with pure compounds for calculating the concentration of each metabolite.

Statistical Analysis

Differences in thermal tolerance with sampling time were examined using two-way ANOVAs for male and female *N. groenlandicus* separately with "time to knockdown" or "chill coma recovery temperature" as dependent variables, and "time of day" and "day" as independent variables. The tests were run on individuals. To ensure normal distribution, the data were transformed using rank inverse transformation and the models were run on both transformed and non-transformed data for validation. In addition, we used Pearson's correlations to examine if microhabitat temperature affected thermal tolerances rather than sampling time alone. The Pearson's correlations were calculated using the mean field temperatures observed in the 1 h preceding each collection round and the mean HKDT and $T_{recovery}$ for each assay and sex ($n = 20$).

We used one-way ANOVAs to examine if individual metabolite concentrations within each day differed according to collection time. The concentrations of all quantified metabolites were then scaled and mean centered. For each sampling day, between-class PCA (R-package "ade4"), were run to explore the daily temporal structure of the metabolomic profiles. Monte Carlo tests (1000 permutations) were used to examine the significance of differences in metabolite profiles among classes of individuals from the four sampling times. Further, the metabolites that contributed the most to separation of groups were identified and ranked by their correlation to the principal components that described most of the inertia in the data. All analyses were carried out using the software "R" (R Core Team, 2020). Raw temperature and GC-MS files used for the analyses are available in the **Supplementary Material**.

RESULTS

Microhabitat Temperatures

Observed microhabitat temperatures of the different sampling periods are shown in **Figure 1**. The largest daily amplitude recorded was 28.8°C on day 1, while the lowest of 11.6°C was on day 4. The warmest average temperature in the 1-h timespan prior to thermal tolerance tests was 26.3°C (see **Supplementary Table 1** for exact temperatures) and was recorded at midday on day 2; the coldest average temperature prior to tests was 1.0°C and was recorded in the morning of day 5.

The microhabitat temperatures varied markedly between the two experimental days where samples were collected for metabolomic profiling (day 4 and 5; **Figure 1**). Within day 4, the temperatures were relatively constant with only 4.9°C difference in the average temperatures at the four daily collection times. Day 4 was also characterized by the lowest recorded daily amplitude. Day 5 was characterized by a high temperature variation with an amplitude of 25°C; the average temperatures at the four collection times differed by 21°C (**Figure 1** and **Supplementary Table 1**).

Thermal Tolerance

Thermal tolerances varied significantly within and between days for both male and female individuals (**Supplementary Figure 2** and ANOVA **Supplementary Table 2**). The average HKDT at each collection time and day was correlated with the average field temperature observed 1 h prior to heat tolerance assessment. The correlation was significant for females but not for males (**Figure 2A**). The regression slope, representing the change in HKDT per °C change in field temperature, was 0.36 min/°C for females and 0.18 min/°C for males. In addition, HKDT was overall higher for females than for males at any given field temperature with a difference of 5 min at the intercept. The maximal difference in mean HKDT measured across all temperatures was 23 min for females and 12 min for males. The relation between mean $T_{recovery}$ and the average microhabitat temperature preceding cold assays revealed a significant positive relationship for both females and males (**Figure 2B**). The increase in recovery temperature per °C change in field temperature were 0.09 and 0.07°C for females and males, respectively. The largest difference in $T_{recovery}$ found across all days was 3.3°C for females and 3.4°C for males.

For the days where females were collected for metabolomic fingerprinting, the HKDT and $T_{recovery}$ varied markedly across the thermally variable day (day 5), and less so on the thermally stable day (day 4) (**Supplementary Figure 2**). Thus, the correlation between the field temperature and heat tolerance of the females collected only at the time points used for metabolomic profiling was strong and highly significant ($R = 0.94$, $p = <0.001$). The relationship between field temperature and $T_{recovery}$ of females collected on day 4 and 5 was also positive and directional, however, not significant ($R = 0.52$, $p = 0.19$).

Metabolomic Profiling

Description of the Quantified Metabolites in the Samples

The full scan monitoring allowed us to identify and quantify 33 metabolites with the quadrupole GC-MS platform (**Supplementary Table 3**; and see raw metabolite concentrations in **Supplementary Table 4**). We quantified 13 free amino acids, five sugars, six polyols, six metabolic intermediates, and three other metabolites. Xylitol and ethanolamine concentrations were below the quantification limit (i.e., below the lowest concentration of the calibration curve) in many samples and were therefore excluded from the quantitative profiling. The most abundant metabolites across all sampling times and days were phosphoric acid, proline, glutamate, and tyrosine.

Effects of Collection Time on Metabolic Fingerprints

Individual metabolite concentrations for each sampling time are displayed in **Figure 3**. We observed that a large number of metabolites varied in concentrations across collection times on day 5. There was a significant effect of "time of day" on the levels of eight metabolites (**Supplementary Table 5**). Conversely, the metabolite concentrations varied less on collection day 4, though there was a significant effect of "time of day" on concentration for seven of these (**Supplementary Table 5**). To examine whether the concentration levels varied similarly across collection times for the two collection days, we ran 2-way ANOVAs for each metabolite. Six metabolites varied distinctively across the four collection times for the 2 days, and these are depicted as

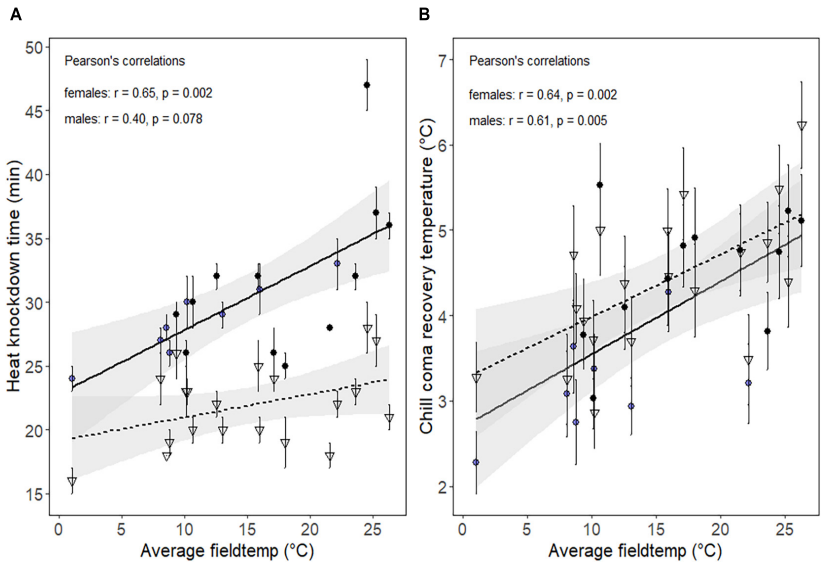


FIGURE 2 | Scatter plot showing the mean **(A)** heat knockdown time in minutes, and **(B)** chill coma recovery temperature ($T_{recovery}$) of females (circles) and males (triangles), as a function of the average microhabitat temperature 1 h prior to assay start. At eight sampling times, females were collected for metabolomic fingerprints simultaneously with individuals used for the thermal assays (blue circles). Black points are data points with no metabolomic fingerprints associated. Solid (female) and dotted (male) lines represent regressions between field temperature and thermal tolerances. Bars are standard errors of the mean.

the interaction between collection day and time on **Figure 3**. Finally, the sugars glucose, fructose, and galactose and the polyol glycerol occurred in larger concentrations in individuals collected on day 5.

Between-class PCAs were run separately on the metabolite concentrations from individuals sampled on day 4 (**Figure 4A**) and day 5 (**Figure 4B**). PC1 and PC2 cumulated 80 and 83% inertia on day 4 and 5, respectively; thus, on both days, the first two PCs explained most of the between-class variation. All classes showed a clear-cut separation meaning that metabolomic fingerprints differed among the different sampling times. The separation appeared stronger on day 5 (**Figure 4B**) than on day 4 (**Figure 4A**). Monte-Carlo randomization tests confirmed differences in metabolomic profiles among classes on day 5 ($p < 0.001$) and day 4 ($p = 0.026$) and the significance levels underpins that metabolomic profiles differed more markedly between sampling times on the thermally variable day 5 compared to on the less thermal variable day 4, as evidenced by much lower ellipses overlap.

Effects of Low Daily Thermal Fluctuations on Metabolic Fingerprints

On day 4, which was characterized by low temperature variation, the metabolomic profiles of individuals collected in the morning and evening differed from the profiles from midday and afternoon along PC1 (**Figure 4A**). Further, the metabolic fingerprints of individuals collected during midday and afternoon separated along PC2.

The metabolites most positively correlated to PC1 were the sugars galactose and glucose (**Figure 4A**). These two sugars were more abundant in individuals sampled in the morning and evening compared to those sampled at midday and afternoon. Lactic acid and citrulline were the metabolites that were most negatively correlated to PC1, and they were more abundant in individuals sampled at midday and afternoon. The polyol glycerol-3-phosphate and succinic acid had the highest positive associations with PC2 (**Supplementary Figure 3**) and were characterized by higher concentrations in individuals sampled in the morning and afternoon. Only one metabolite, glucose-6-phosphate, were negatively associated with PC2 and thus more abundant in the morning.

Effects of High Daily Thermal Fluctuations on Metabolic Fingerprints

On day 5, metabolomic profiles of individuals collected in the morning separated strongly from individuals sampled in the afternoon (**Figure 4B**). These two collection times were also the two thermal extremes of the day (i.e., the lowest and highest temperatures of the day, see **Figure 1** and **Supplementary Table 1**); this observation suggests that PC1 explained metabolic changes correlated to high diel thermal variation. Further, the metabolic fingerprints of the individuals collected at these time points separated from the fingerprints of individuals collected at midday and in the evening along PC2.

The metabolites that were positively associated with PC1 on day 5 were phenylalanine, glutamic acid, citric acid, and to a lesser extent other amino acids, including valine, leucine, serine, and alanine (**Figure 4B**). These were more abundant in individuals sampled in the morning compared to individuals from the other collection times. The metabolites that were most negatively

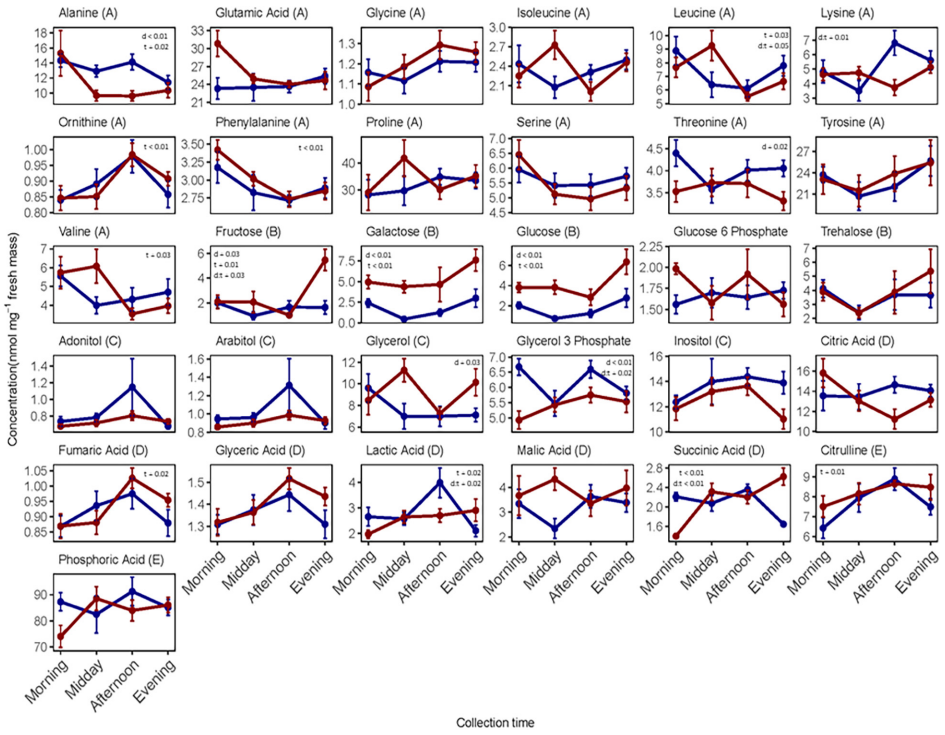


FIGURE 3 | Individual metabolite concentrations (nmol mg^{-1} dry mass) measured in whole-body extracts of female *N. groenlandicus* collected from the field at four consecutive time points (morning, midday, afternoon, and evening) during day 4 (blue) and day 5 (red). Each point represent mean concentration (n = 8) and bars are standard errors of the mean. Differences between concentrations (log-transformed) and collection days (d), collection times (t), and the interaction between day and time (d:t) was investigated using 2-way ANOVAs and significance p-values are shown on each plot if significant. The metabolites are classified from A to E in the header according to functional group; A, amino acids; B, sugars; C, polyols; D, metabolic intermediates; and E, other metabolites.

correlated to PC1 were succinic acid, fumaric acid, glyceric acid and ornithine. Further, negative correlations included several polyols (adonitol, glycerol-3-phosphate, arabitol) whose concentrations were higher in individuals collected in the afternoon than in the morning. The sugars fructose, glucose and galactose, and the amino acids isoleucine and lysine, as well as succinic acid and glycerol were positively associated with PC2 (**Supplementary Figure 3**), and were thus more abundant in individuals sampled in the evening. Negative associations were few, but the most negatively associated metabolite was the sugar glucose-6-phosphate which was more abundant in the morning and afternoon.

DISCUSSION

Diel Variations in the Thermal Tolerance of Field-Sampled Insects

In our study, we showed a linear relationship between ambient microhabitat temperature and measures of both cold and heat tolerance of field-collected specimens of *N. groenlandicus*. Given the temperatures observed in the field during summer in Narsarsuaq includes subzero night temperatures and peak day temperatures above 40°C (Sørensen et al., 2019; Bahrndorff et al., 2021b) we advocate that the ability to withstand and remain active at high temperatures and recover fast from low temperature coma is ecologically important, especially for a univoltine species such as *N. groenlandicus*. This allows the species to, e.g., forage and mate in a transient environment and short summer season. Thus, our results point to plasticity in thermal tolerances being of strong importance for survival of insects in this region. Recently, the heat hardening capacity of *N. groenlandicus* has been examined in the laboratory, showing that heat tolerance can be increased within 45 min when the insects are exposed to high temperatures (Sørensen et al., 2019). It was also found that the heat hardening effect was reverted within 2 h when the insects were returned to cooler temperatures. Our results are consistent with these former observations, and the rapid adjustments that we observed in both heat and cold tolerance from field-sampled *N. groenlandicus* additionally support the assumption that hardening responses are important for coping with rapid changes in ambient temperature in the field. This is in contrast to the slower hardening responses observed in some temperate

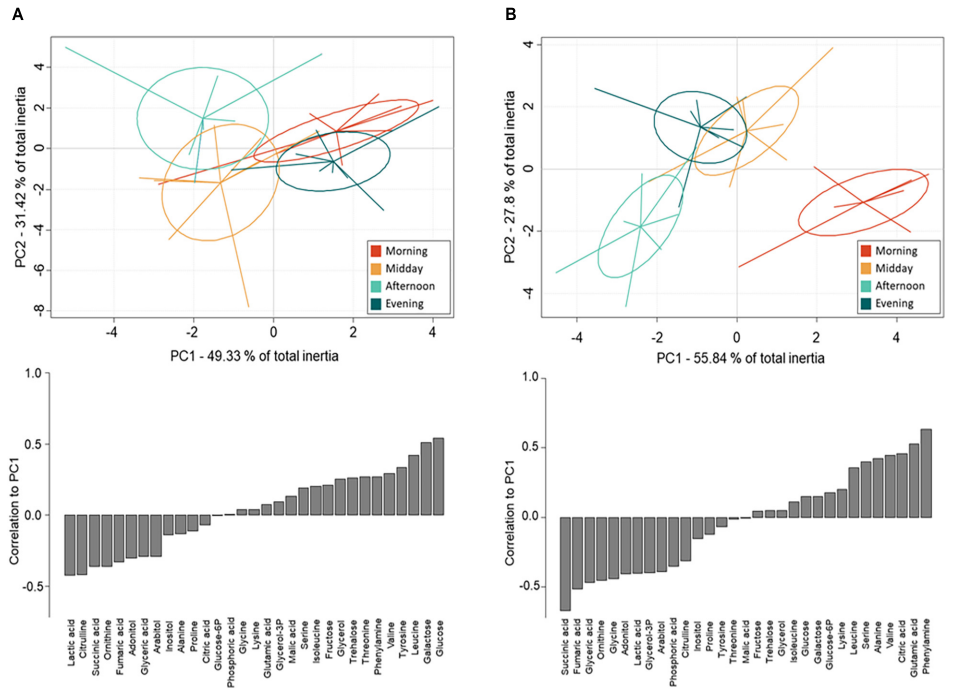


FIGURE 4 | Between-class Principal Component Analyses (top) and metabolite correlations to PC1 (bottom) based on GC-MS quantification of metabolites from whole-body extracts of female *N. groenlandicus* sampled in the field at four consecutive sampling time points (8:00 am, 12:00 pm, 4:00 pm, 8:00 pm) on **(A)** the thermally stable sampling day (day 4) and **(B)** the thermally variable sampling day (day 5). Lines represents individual sample positions respective to centroids (n = 8).

insect species and indicates that high thermal variability of the environment can be a selective agent for rapid plastic responses (Dahlgaard et al., 1998; Bahrndorff et al., 2009; Alemu et al., 2017).

Interestingly, the adjustments occurred not only at extreme temperatures, but also at temperatures that are not considered as stressful or sub-lethal to the species. The body temperatures experienced by *N. groenlandicus,* like other insect species, might differ from the temperatures measured in the shade due to behavioral thermoregulation (e.g., Stevenson, 1985; Kearney et al., 2009), such as seeking microhabitat temperatures that deviate from air temperatures (Stevenson, 1985; Böcher and Nachman, 2001; Danks, 2004; Kearney et al., 2009). However, as sampled individuals are collected in the same microhabitat as where air temperatures are measured, we do argue that the difference between air and body temperatures is likely to be minor. The adult life-stage of *N. groenlandicus* seemingly has a high preferred body temperature of approximately 30°C (Böcher and Nachman, 2001) and an extreme thermal tolerance breadth spanning from critical lower limits (CTmin) of −3.4°C to critical upper thermal limits (CTmax) of 48.5–52°C (Böcher and Nachman, 2001; Sørensen et al., 2019; Bahrndorff et al., 2021b). Thus, only the lowest temperatures recorded in the field approximated sub-lethal conditions for the species. Typically, hardening responses are described as being induced by stressful conditions (Angilletta, 2009). For example it has been described from laboratory and field studies performed on *Drosophila* spp. that hardening temperatures ca. 10–15°C above optimal rearing temperatures are needed to induce adaptive increases in heat tolerance, and cause upregulation of heat shock proteins (Sørensen et al., 2003; King and MacRae, 2015). Here, despite exposure to temperatures well within their thermal comfort zone, we show that heat and cold tolerance changed daily in *N. groenlandicus*. This finding might represent an evolutionary adaptation to the extreme climatic variations of arctic environments, but may also suggest that temperature variation act in concert with changes in air humidity and/or other climate variables to affect thermal tolerances as found for several other polar and sub-polar species (Block et al., 1994; Hodkinson et al., 1996; Benoit et al., 2009; Everatt et al., 2015).

Another important discovery was that the patterns of plastic changes in cold tolerance were similar for males and females, while distinct patterns were seen for heat tolerance for the two sexes (**Figure 2**). Females had a higher HKDT (+5 min HKDT) compared to males and tended to exhibit a stronger plasticity for that trait when field temperatures varied. Often, studies on thermal plasticity in insects find that the variation in upper thermal limits is constrained, and less plastic, compared to lower thermal limits (reviewed by Hoffmann et al., 2013; see also Chown, 2001; Overgaard et al., 2011; Alford et al., 2012). This

is likely resulting from the difficulty of insects to seek shelter from cold temperatures, thus resulting in a stronger selection pressure for plasticity of cold tolerance (Hoffmann et al., 2013). Our findings suggest that the selection pressure for cold tolerance may have been similar in male and female *N. groenlandicus* because no differences were observed in $T_{recovery}$. Conversely, heat stress is often countered by behavioral thermoregulation in ectotherms, for instance by seeking shadow or migrating below-ground (Huey and Tewksbury, 2009; Kearney et al., 2009). The higher heat tolerance and plasticity for this trait in females could be explained by the univoltine life history and the short arctic summers requiring females to seek out warm temperatures to rapidly complete their life cycle (Bahrndorff et al., 2021a). Further, our results indicate a trade-off between heat and cold tolerance. Thus, individuals sampled at middays and afternoons are overall more heat tolerant and less cold tolerant compared to individuals sampled during mornings and evenings. Similar results have been found for thermal tolerance of the fruit fly *Drosophila melanogaster* kept under natural and semi-natural conditions (Overgaard and Sørensen, 2008; Schou et al., 2015). A consequence might be maladaptive plastic responses to environmental cues, as climatic conditions are prospected to become more unpredictably variable in the future (Kingsolver and Huey, 1998; Huey et al., 1999; Manenti et al., 2014).

Daily Thermal Variations and Metabolic Fingerprints

The separation of the metabolic fingerprints from field-sampled *N. groenlandicus* was much stronger when individuals were collected during the thermally variable day (day 5), as compared with the less temperature variable day (day 4). This finding supports our hypothesis that microhabitat environmental conditions have a strong impact on diurnal changes of the physiology of adult *N. groenlandicus*. It also suggests that the observed diurnal variation in metabolic fingerprints cannot be explained by circadian clock regulations alone because similar patterns would be expected on the two collection days if that was the case.

On the less variable day, the average temperature prior to testing the insects was rather similar for the four collection periods (maximal temperature difference of 4.9°C; **Figure 1**). The measured changes in metabolite concentrations on this day were thus mostly independent of temperature, and rather reflected adjustments in energetic metabolism over the day or circadian regulated responses independent of temperature. This assumption is supported by the grouping pattern of the metabolomic profiles of individuals collected at the four different time points of day 4. Metabolomic profiles in the morning and evening were more similar and separated from those of individuals collected on the midday and afternoon. This pattern may reflect that the activity of the individuals was higher during midday and afternoon and in turn increased the energetic needs and metabolism in general. Consistently, sugars (glucose, fructose, galactose), some metabolic intermediates (citric and fumaric acid), and a range of free amino acids (phenylalanine, valine, serine, glutamine, and tyrosine), all being important substrates for glycolysis and Krebs cycle, varied in rhythmic patterns on both day 4 and 5 (**Figure 3**). These patterns might constitute circadian clock mechanisms that are regulated independently of temperature, humidity and other variable abiotic factors as seen, e.g., in *D. melanogaster* (Rhoades et al., 2018).

On the temperature variable day, the pattern of separation was markedly different than the one reported for day 4. The groups separating the strongest and explaining most of the variation in the data belonged to the individuals collected in the morning and afternoon, representing the time points with the lowest and the highest temperatures of day 5. Throughout this day, the sugars fructose, glucose and galactose, occurred in higher quantities compared to day 4 and especially fructose and glucose accumulated 2–3 fold in the evening in the individuals sampled on day 5, despite the temperature not being different from the temperature on day 4 at this time point (**Figures 1**, **3**). Typically, sugar accumulation is associated with cold shock responses (Jagdale et al., 2005; Lalouette et al., 2007; Michaud and Denlinger, 2007; Overgaard et al., 2007; Holmstrup et al., 2010; Teets et al., 2011) and seasonal preparation for diapause (Koštál et al., 2001; Watanabe, 2002; Vasquez et al., 2019). Sugars have osmoprotective properties that may play important protective roles in cold tolerance possibly through stabilization of cell membranes and macromolecular structures even at low concentration (Gekko and Timasheff, 1981; Yancey, 2005; Koštál et al., 2016) or by maintaining haemolymph osmolality despite low [Na+] and [K+] due to cold exposure (MacMillan et al., 2015). Thus, sugars might be accountable for the higher cold tolerance observed in individuals on days characterized by large temperature amplitude.

Polyols accumulated during the warmest periods of the days and especially inositol, fluctuated on day 5. Accumulation of the polyols mannitol and sorbitol in whiteflies and aphids with daily warm peaks has been associated with increased heat tolerance under natural and semi-natural conditions (Hendrix and Salvucci, 1998; Wolfe et al., 1998; Salvucci et al., 2000). This could indicate that polyols contribute to regulation of heat tolerance in *N. groenlandicus*.

It is possible that oscillations of sugars and polyols were affected by temperature-dependent activity patterns such as feeding, mating and general metabolism, and this might confound the effects of temperature and humidity alone on thermal tolerance. Foraging or feeding rates are partly governed by upper and lower activity thresholds of organisms (Everatt et al., 2013). Our own unpublished data on the activity of *N. groenlandicus* show that the species is virtually inactive at temperatures below 15°C and activity peaks at 35–40°C. This might suggest that feeding is constricted to the warmest periods of the day (typically between 20 and 30°C at the given study site). *Nysius* species feed on phloem sap and plant seeds (Böcher, 1972; Broadle et al., 1986; Böcher et al., 2015; Tiwari and Wratten, 2019; Maharjan et al., 2020), and therefore ingest large quantities of sucrose, which is produced by photosynthesis in plants. In other hemipteran phloem-feeders, sucrose is hydrolyzed to glucose and fructose when ingested and rapidly converted to trehalose or polyols, which are less toxic compounds to store

in the hemolymph at high concentrations (Becker et al., 1996; Hendrix and Salvucci, 1998). Thus, there might be a direct link between feeding behavior and sugar and polyol levels. For instance, trehalose concentrations increased more in the afternoon/evening on the warm day when feeding rates are expected to be higher (**Figure 3**).

Additionally, selective feeding on protein- and lipid rich diets impact on thermal tolerance in several insect species. For instance, the dung beetle *Thorectes lusitanicus* has been found to selectively supplement its diet with acorn, and experiments showed that beetles that were fed on acorn had a hemolymph supercooling point that was 5°C lower than individuals fed on cow-dung (Verdú et al., 2010). The shift was associated with alterations in hemolymph cryoprotectant content. Likewise, Rho and Lee (2017) showed that the beetle *Tenebrio molitor* selectively chose a carbohydrate rich diet at cold and warm temperatures, opposed to a more balanced protein-carbohydrate diet at intermediate temperatures. Switches in feeding preference with cold stress have also been found for *Drosophila* species (Brankatschk et al., 2018; Strassburger and Teleman, 2018). These aspects should be examined further in future studies.

Whether the oscillations of sugars and polyols constitute protective responses rather than consequences of altered energetic metabolism or temperature-dependent feeding is not evident from our results. However, the contrasting patterns of sugar and polyol oscillation may indicate that polyols are converted to sugars during the coldest times of the thermally variable day, maybe as a protective response. It might also explain the negative trade-off observed between heat and cold tolerance in this study. However, this is speculative and should be examined further in future studies along with common garden experiments revealing the importance of circadian rhythm regulation of *N. groenlandicus* behavior and physiology.

CONCLUSION

Here, we showed that thermal tolerance was correlated with ambient microhabitat temperature in the Greenlandic seed bug, *N. groenlandicus*. Thus, we show that hardening responses observed under constant laboratory conditions in a previous study (Sørensen et al., 2019) occur also in field settings for this species. Interestingly, we found that the plastic adjustments of thermal tolerance occurred at relative benign temperatures contrasting experimental evidence from laboratory studies on primarily model species, suggesting that these responses occur at sub- or supra lethal temperatures. Thus, plasticity of thermal tolerance is likely affected by multiple factors under natural conditions and constitute an example of evolutionary adaption to the extreme and variable Arctic and sub-Arctic habitats of *N. groenlandicus*. Further, we showed that field heat hardening causes increased heat tolerance and reduced cold tolerance and *vice versa* with cold acclimation, suggesting a trade-off. GC-MS investigation of field collected individuals revealed candidate metabolites that are regulated according to thermal and other abiotic and biotic conditions that vary on a diurnal basis. Distinct metabolic fingerprints associated with temperature on the thermally variable day (day 5) were not observed at the thermal stable day (day 4). This suggests that our results cannot be explained by circadian clock regulated mechanisms alone, but that these metabolites are partly regulated by temperature variability (or variation in correlated environmental or physiological variables) and constitute important physiological mechanisms controlling diurnal variability in thermal tolerances in the species. The strong plastic responses observed in heat and cold tolerance and in the metabolomic profiles in our study suggests ongoing strong selection for plasticity in this highly fluctuating polar environment. The genetic architecture of these traits are mainly investigated in model organisms where studies on *D. melanogaster* suggest significant heritable variation for plasticity of thermal stress tolerance traits and metabolite profiles (Gerken et al., 2015; Hangartner and Hoffmann, 2015; Ørsted et al., 2017; Rohde et al., 2021).

DATA AVAILABILITY STATEMENT

All data, including raw field temperature files and GC-MS data, are presented in this article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SB, TNK, and NKN conceived the ideas and designed the methodology. MHS, SB, TNK, and NKN collected the data. DR, HC, and NKN processed samples for metabolomics analysis and analyzed the metabolomics data. TNK and NKN led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

FUNDING

This work was supported by a grant from the Danish Council for Independent Research (DFF8021-00014B), the European co-funded Partnership BiodivClim-191 ASICS (0156-00024B), and Carlsberg (Carlsbergfondet, CF17-0415). Travel grants was provided by North2North and SAFT (Selskab for Arktisk Forskning og Teknologi). Metabolomics analyses were supported by IRP CNRS "PRICES" and IPEV 136 "Subanteco."

ACKNOWLEDGMENTS

We thank Finn Thomsen and Storch Lund for access to field sites and laboratory facilities in Narsarsuaq, Greenland. Further, we are grateful to EcoChimie Platform (EcoChim) from UMS OSUR 3343 for target quantitative metabolomics analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.818485/full#supplementary-material

REFERENCES

Alemu, T., Alemneh, T., Pertoldi, C., Ambelu, A., and Bahrndorff, S. (2017). Costs and benefits of heat and cold hardening in a soil arthropod. *Biol. J. Linn. Soc.* 122, 765–773. doi: 10.1093/biolinnean/blx092

Alford, L., Blackburn, T. M., and Bale, J. S. (2012). Effect of latitude and acclimation on the lethal temperatures of the peach-potato aphid *Myzus persicae*. *Agric. For. Entomol.* 14, 69–79. doi: 10.1111/j.1461-9563.2011.00553.x

Angilletta, M. J. (2009). "Thermal acclimation," in *Thermal Adaptation: A Theoretical and Empirical Synthesis*, ed. M. J. Angilletta (New York, NY: Oxford University Press), 126–156.

Araújo, M. B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P. A., Valladares, F., and Chown, S. L. (2013). Heat freezes niche evolution. *Ecol. Lett.* 16, 1206–1219. doi: 10.1111/ele.12155

Bahrndorff, S., Gertsen, S., Pertoldi, C., and Kristensen, T. N. (2016). Investigating thermal acclimation effects before and after a cold shock in *Drosophila melanogaster* using behavioural assays. *Biol. J. Linn. Soc.* 117, 241–251. doi: 10.1111/bij.12659

Bahrndorff, S., Lauritzen, J. M. S., Sørensen, M. H., Noer, N. K., and Kristensen, T. N. (2021b). Responses of terrestrial polar arthropods to high and increasing temperatures. *J. Exp. Biol.* 224:jeb230797. doi: 10.1242/jeb.230797

Bahrndorff, S., Alemu, T., Kristensen, T. N., Sørensen, M. H., Høye, T., and Holmstrup, M. (2021a). Thermal adaptations of adults and eggs in the arctic insect, *Nysius groenlandicus*. *Polar Biol.* 44, 491–498. doi: 10.1007/s00300-021-02807-6

Bahrndorff, S., Mariën, J., Loeschcke, V., and Ellers, J. (2009). Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. *Funct. Ecol.* 23, 233–239. doi: 10.1111/j.1365-2435.2009.01541.x

Bahrndorff, S., Petersen, S. O., Loeschcke, V., Overgaard, J., and Holmstrup, M. (2007). Differences in cold and drought tolerance of high arctic and sub-arctic populations of *Megaphorura arctica* Tullberg 1876 (Onychiuridae: Collembola). *Cryobiology* 55, 315–323. doi: 10.1016/j.cryobiol.2007.09.001

Bak, C. W., Bahrndorff, S., Noer, N. K., Jørgensen, L. B., Overgaard, J., and Kristensen, T. N. (2020). Comparison of static and dynamic assays when quantifying thermal plasticity of drosophilids. *Insects* 11:537. doi: 10.3390/insects11080537

Becker, A., Schlöder, P., Steele, J. E., and Wegener, G. (1996). The regulation of trehalose metabolism in insects. *Experientia* 52, 433–439.

Benoit, J. B., Lopez-martinez, G., Elnitsky, M. A., Lee, R. E., and Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 152, 518–523. doi: 10.1016/j.cbpa.2008.12.009

Block, W., Webb, N. R., Coulson, S., Hodkinson, I. D., and Worland, M. R. (1994). Thermal adaptation in the Arctic collembolan *Onychiurus arcticus* (Tullberg). *J. Insect Physiol.* 40, 715–722. doi: 10.1016/0022-1910(94)90099-X

Böcher, J. (1972). Feeding biology of *Nysius groenlandicus* (Heteroptera: Miridae) in Greenland. With a note on oviposition in relation to food-source and dispersal of the species. *Medd. Grønland* 191, 1–41.

Böcher, J., Kristensen, N. P., Pape, T., and Vilhelmsen, L. (2015). *The Greenland Entomofauna: An Identification Manual of Insects, Spiders and Their Allies (Fauna Entomologica Scandinavica)*. Leiden: Brill. doi: 10.1163/ej.9789004157705.i-265.29

Böcher, J., and Nachman, G. (2001). Temperature and humidity responses of the arctic-alpine seed bug *Nysius groenlandicus*. *Entomol. Exp. Appl.* 99, 319–330. doi: 10.1046/j.1570-7458.2001.00831.x

Brankatschk, M., Gutmann, T., Knittelfelder, O., Palladini, A., Prince, E., Grzybek, M., et al. (2018). A temperature-dependent switch in feeding preference improves *Drosophila* development and survival in the cold. *Dev. Cell* 46, 781–793.e4. doi: 10.1016/J.DEVCEL.2018.05.028

Broadle, R., Simpson, B., and Beavis, C. (1986). Damage by "*Nysius*" spp. (Hemiptera: Lygaeidae) in non-stressed sunflower ('*Helianthus annus*' L.) crops. *Gen. Appl. Entomol.* 18, 17–24.

Buckley, B. A., Owen, M. E., and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *J. Exp. Biol.* 204, 3571–3579.

Chown, S. L. (2001). Physiological variation in insects: hierarchical levels and implications. *J. Insect Physiol.* 47, 649–660. doi: 10.1016/S0022-1910(00)00163-3

Chown, S. L., Jumbam, K. R., Sørensen, J. G., and Terblanche, J. S. (2009). Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. *Funct. Ecol.* 23, 133–140. doi: 10.1111/J.1365-2435.2008.01481.X

Chown, S. L., and Terblanche, J. S. (2006). Physiological diversity in insects: ecological and evolutionary contexts. *Adv. Insect Phys.* 33, 50–152. doi: 10.1016/S0065-2806(06)33002-0

Colinet, H., and Hoffmann, A. A. (2012). Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. *Funct. Ecol.* 26, 84–93. doi: 10.1111/j.1365-2435.2011.01898.x

Colinet, H., Sinclair, B. J., Vernon, P., and Renault, D. (2015). Insects in fluctuating thermal environments. *Ann. Rev. Entomol.* 60, 123–140. doi: 10.1146/annurev-ento-010814-021017

Convey, P., Coulson, S. J., Worland, M. R., and Sjöblom, A. (2018). The importance of understanding annual and shorter-term temperature patterns and variation in the surface levels of polar soils for terrestrial biota. *Polar Biol.* 41, 1587–1605.

Dahlgaard, J., Loeschcke, V., Michalak, P., and Justesen, J. (1998). Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Funct. Ecol.* 12, 786–793. doi: 10.1046/j.1365-2435.1998.00246.x

Danks, H. V. (2004). Seasonal adaptations in Arctic insects. *Integr. Comp. Biol.* 44, 85–94. doi: 10.1093/ICB/44.2.85

Davey, M. C., Pickup, J., and Block, W. (2021). Temperature variation and its biological significance in fellfield habitats on a maritime Antarctic island. *Antarct. Sci.* 4, 383–388. doi: 10.1017/S0954102092000567

Deere, J. A., Sinclair, B. J., Marshall, D. J., and Chown, S. L. (2006). Phenotypic plasticity of thermal tolerances in five oribatid mite species from sub-Antarctic Marion Island. *J. Insect Physiol.* 52, 693–700. doi: 10.1016/j.jinsphys.2006.03.009

Everatt, M. J., Bale, J. S., Convey, P., Worland, M. R., and Hayward, S. A. L. (2013). The effect of acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates. *J. Insect Physiol.* 59, 1057–1064. doi: 10.1016/j.jinsphys.2013.08.003

Everatt, M. J., Convey, P., Bale, J. S., Worland, M. R., and Hayward, S. A. (2015). Responses of invertebrates to temperature and water stress: a polar perspective. *J. Therm. Biol.* 54, 118–132. doi: 10.1016/j.jtherbio.2014.05.004

Fields, P. G., Fleurat-Lessard, F., Lavenseau, L., Febvay, G., Peypelut, L., and Bonnot, G. (1998). The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in *Sitophilus granarius* and *Cryptolestes ferrugineus* (Coleoptera). *J. Insect Physiol.* 44, 955–965. doi: 10.1016/S0022-1910(98)00055-9

Fischer, K., Kölzow, N., Höltje, H., and Karl, I. (2011). Assay conditions in laboratory experiments: is the use of constant rather than fluctuating temperatures justified when investigating temperature-induced plasticity? *Oecologia* 166, 23–33. doi: 10.1007/s00442-011-1917-0

Foray, V., Desouhant, E., Voituron, Y., Larvor, V., Renault, D., Colinet, H., et al. (2013). Does cold tolerance plasticity correlate with the thermal environment and metabolic profiles of a parasitoid wasp? *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 164, 77–83. doi: 10.1016/j.cbpa.2012.10.018

Fusco, G., and Minelli, A. (2010). Phenotypic plasticity in development and evolution: facts and concepts. *Philos. Trans. R. Soc. B* 365, 547–556. doi: 10.1098/rstb.2009.0267

Gekko, K., and Timasheff, S. N. (1981). Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* 20, 4667–4676. doi: 10.1021/bi00519a023

Gerken, A. R., Eller, O. C., Hahn, D. A., and Morgan, T. J. (2015). Constraints, independence, and evolution of thermal plasticity: probing genetic architecture of long-and short-term thermal acclimation. *Proc. Natl. Acad. Sci. U.S.A.* 112, 4399–4404.

Gracey, A. Y., Chaney, M. L., Boomhower, J. P., Tyburczy, W. R., Connor, K., and Somero, G. N. (2008). Rhythms of gene expression in a fluctuating intertidal environment. *Curr. Biol.* 14, 1501–1507. doi: 10.1016/j.cub.2008.08.049

Hangartner, S., and Hoffmann, A. A. (2015). Evolutionary potential of multiple measures of upper thermal tolerance in *Drosophila melanogaster*. *Funct. Ecol.* 30, 442–452. doi: 10.1111/1365-2435.12499

Harris, D. T. (2018). Biobanking and omics. *Front. Biol.* 13:287–292. doi: 10.1007/s11515-018-1505-3

Hendrix, D. L., and Salvucci, M. E. (1998). Polyol metabolism in homopterans at high temperatures: accumulation of mannitol in aphids (Aphididae:

Homoptera) and sorbitol in whiteflies (Aleyrodidae: Homoptera). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 120, 487–494. doi: 10.1016/S1095-6433(98)10058-2

Hodkinson, I. D., Coulson, S. J., Webb, N. R., and Block, W. (1996). Can high Arctic soil microarthropods survive eleveated summer temperatures? *Funct. Ecol.* 10, 314–321. doi: 10.2307/2390278

Hoffmann, A. A., Chown, S. L., and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Funct. Ecol.* 27, 934–949. doi: 10.1111/j.1365-2435.2012.02036.x

Hoffmann, A. A., Sørensen, J. G., and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* 28, 175–216. doi: 10.1016/S0306-4565(02)00057-8

Holmstrup, M., Bayley, M., Pedersen, S. A., and Zachariassen, K. E. (2010). "Interactions between cold, desiccation and environmental toxin," in *Low Temperature Biology of Insects*, eds D. L. Denlinger and R. E. Lee (Cambridge: Cambridge University Press), 166–190.

Holmstrup, M., Hedlund, K., and Boriss, H. (2002). Drought acclimation and lipid composition in *Folsomia candida*: implications for cold shock, heat shock and acute desiccation stress. *J. Insect Physiol.* 48, 961–970. doi: 10.1016/S0022-1910(02)00175-0

Huey, R. B., Berrigan, D., Gilchrist, G. W., and Herron, J. C. (1999). Testing the adaptive significance of acclimation: a strong inference approach. *Am. Zool.* 39, 323–336. doi: 10.1093/icb/39.2.323

Huey, R. B., and Tewksbury, J. J. (2009). Can behavior douse the fire of climate warming? *Proc. Natl. Acad. Sci. U.S.A.* 106, 3647–3648. doi: 10.1073/pnas.0900934106

Jagdale, G. B., Grewal, P. S., and Salminen, S. O. (2005). Both heat-shock and cold-shock influence trehalose metabolism in an entomopathogenic nematode. *J. Parasitol.* 91, 988–994. doi: 10.1645/GE-504R.1

Javal, M., Renault, D., and Colinet, H. (2016). Impact of fluctuating thermal regimes on *Drosophila melanogaster* survival to cold stress. *Anim. Biol.* 66, 427–444. doi: 10.1163/15707563-00002510

Jensen, A., Alemu, T., Alemneh, T., Pertoldi, C., and Bahrndorff, S. (2019). Thermal acclimation and adaptation across populations in a broadly distributed soil arthropod. *Funct. Ecol.* 33, 833–845. doi: 10.1111/1365-2435.13291

Kearney, M., Gillingham, P. K., Bramer, I., Duffy, J. P., and MacLean, I. M. D. (2020). A method for computing hourly, historical, terrain-corrected microclimate anywhere on earth. *Methods Ecol. Evol.* 11, 38–43. doi: 10.1111/2041-210X.13330

Kearney, M., and Porter, W. (2009). Mechanistic niche modelling: combining physiological and spatial data to predict species' ranges. *Ecol. Lett.* 12, 334–350. doi: 10.1111/j.1461-0248.2008.01277.x

Kearney, M., Shine, R., and Porter, W. P. (2009). The potential for behavioral thermoregulation to buffer "cold-blooded" animals against climate warming. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3835–3840. doi: 10.1073/pnas.0808913106

Kelty, J. (2007). Rapid cold-hardening of *Drosophila melanogaster* in a field setting. *Physiol. Entomol.* 32, 343–350. doi: 10.1111/j.1365-3032.2007.00584.x

Ketola, T., and Kristensen, T. N. (2017). Experimental approaches for testing if tolerance curves are useful for predicting fitness in fluctuating environments. *Front. Ecol. Evol.* 5:129. doi: 10.3389/fevo.2017.00129

King, A. M., and MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Ann. Rev. Entomol.* 60, 59–75. doi: 10.1146/annurev-ento-011613-162107

Kingsolver, J. G., Higgins, J. K., and Augustine, K. E. (2015). Fluctuating temperatures and ectotherm growth: distinguishing non-linear and time-dependent effects. *J. Exp. Biol.* 218, 2218–2225. doi: 10.1242/jeb.120733

Kingsolver, J. G., and Huey, R. B. (1998). Evolutionary analyses of morphological and physiological plasticity in thermally variable environments. *Am. Zool.* 38, 545–560. doi: 10.1093/icb/38.3.545

Kingsolver, J. G., and Nagle, A. (2007). Evolutionary divergence in thermal sensitivity and diapause of field and laboratory populations of *Manduca sexta*. *Physiol. Biochem. Zool.* 80, 473–479. doi: 10.1086/519962

Kinzner, M. T., Kinzner, M. C., Kaufmann, R., Hoffmann, A. A., Arthofer, W., Schlick-Steiner, B. C., et al. (2019). Is temperature preference in the laboratory ecologically relevant for the field? The case of *Drosophila nigrosparsa*. *Glob. Ecol. Conserv.* 18:e00638. doi: 10.1016/j.gecco.2019.e00638

Koštál, V., Korbelová, J., Poupardin, R., Moos, M., and Šimek, P. (2016). Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*. *J. Exp. Biol.* 219, 2358–2367. doi: 10.1242/jeb.142158

Koštál, V., Šlachta, M., and Šimek, P. (2001). Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of *Pyrrhocoris apterus* (Heteroptera: Insecta). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 130, 365–374. doi: 10.1016/S1096-4959(01)00441-9

Koveos, D. S. (2001). Rapid cold hardening in the olive fruit fly *Bactrocera oleae* under laboratory and field conditions. *Entomol. Exp. Appl.* 101, 257–263. doi: 10.1046/j.1570-7458.2001.00910.x

Kristensen, T. N., Hoffmann, A., Overgaard, J., Sørensen, J. G., Hallas, R., and Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 216–221. doi: 10.1073/pnas.0708074105

Kristensen, T. N., Ketola, T., and Kronholm, I. (2020). Adaptation to environmental stress at different timescales. *Ann. N. Y. Acad. Sci.* 1476, 5–12. doi: 10.1111/nyas.13974

Kristensen, T. N., Overgaard, J., Hoffmann, A. A., Nielsen, N. C., and Malmendal, A. (2012). Inconsistent effects of developmental temperature acclimation on low-temperature performance and metabolism in *Drosophila melanogaster*. *Evol. Ecol. Res.* 14, 821–837.

Lalouette, L., Kostal, V., Colinet, H., Gagneul, D., and Renault, D. (2007). Cold exposure and associated metabolic changes in adult tropical beetles exposed to fluctuating thermal regimes. *FEBS J.* 274, 1759–1767. doi: 10.1111/j.1742-4658.2007.05723.x

Lembrechts, J. J., and Lenoir, J. (2020). Microclimatic conditions anywhere at any time! *Glob. Change Biol.* 26, 337–339. doi: 10.1111/gcb.14942

Loeschcke, V., and Hoffmann, A. A. (2007). Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature. *Am. Nat.* 169, 175–183. doi: 10.1086/510632

Maclean, I. M. D., Duffy, J. P., Haesen, S., Govaert, S., De Frenne, P., Vanneste, T., et al. (2021). On the measurement of microclimate. *Methods Ecol. Evol.* 12, 1397–1410. doi: 10.1111/2041-210x.13627

MacMillan, H. A., Ferguson, L. V., Nicolai, A., Donini, A., Staples, J. F., and Sinclair, B. J. (2015). Parallel ionoregulatory adjustments underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance. *J. Exp. Biol.* 218, 423–432. doi: 10.1242/jeb.115790

Maharjan, R., Yoon, Y., Jang, Y., Jeong, M., Jung, T. W., Ha, T. J., et al. (2020). Oviposition and development response of perilla seed bugs (*Nysius* sp.) (Heteroptera: Lygaeidae) to five crop seeds. *J. Appl. Entomol.* 144, 806–816. doi: 10.1111/JEN.12814

Manenti, T., Sørensen, J. G., Moghadam, N. N., and Loeschcke, V. (2014). Predictability rather than amplitude of temperature fluctuations determines stress resistance in a natural population of *Drosophila simulans*. *J. Evol. Biol.* 27, 2113–2122. doi: 10.1111/jeb.12463

Michaud, M. R., and Denlinger, D. L. (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 177, 753–763. doi: 10.1007/s00360-007-0172-5

Michaud, R., and Denlinger, D. L. (2010). "Genomics, proteomics and metabolomics: finding the other players in insect cold-tolerance," in *Low Temperature Biology of Insects*, eds D. L. Denlinger and R. E. Lee (New York, NY: Cambridge University Press), 91–115.

Mitchell, K. A., and Hoffmann, A. A. (2010). Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. *Funct. Ecol.* 24, 694–700. doi: 10.1111/j.1365-2435.2009.01666.x

Noer, N. K., Ørsted, M., Schiffer, M., Hoffmann, A. A., Bahrndorff, S., and Kristensen, T. N. (2022). Into the wild – a field study on the evolutionary and ecological importance of thermal plasticity in ectotherms across temperate and tropical regions. *Philos. Trans. R. Soc. B.* 376:20210004. doi: 10.1098/rstb.2021.0004

Noer, N. K., Pagter, M., Bahrndorff, S., Malmendal, A., and Kristensen, T. N. (2020). Impacts of thermal fluctuations on heat tolerance and its metabolomic basis in *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Orchesella cincta*. *PLoS One* 15:e0237201. doi: 10.1371/journal.pone.0237201

Ørsted, M., Rohde, P. D., Hoffmann, A. A., Sørensen, P., and Kristensen, T. N. (2017). Environmental variation partitioned into separate heritable components. *Evolution* 72, 136–152. doi: 10.1111/evo.13391

Overgaard, J., Kristensen, T. N., Mitchell, K. A., and Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *Am. Nat.* 178, S80–S96. doi: 10.1086/661780

Overgaard, J., Malmendal, A., Sørensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C., et al. (2007). Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *J. Insect Physiol.* 53, 1218–1232. doi: 10.1016/j.jinsphys.2007.06.012

Overgaard, J., and Sørensen, J. G. (2008). Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. *Cryobiology* 56, 159–162. doi: 10.1016/j.cryobiol.2008.01.001

Overgaard, J., Sørensen, J. G., Com, E., and Colinet, H. (2014). The rapid cold hardening response of *Drosophila melanogaster*: complex regulation across different levels of biological organization. *J. Insect Physiol.* 62, 46–53. doi: 10.1016/j.jinsphys.2014.01.009

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rho, M. S., and Lee, K. P. (2017). Temperature-driven plasticity in nutrient use and preference in an ectotherm. *Oecologia* 185, 401–413. doi: 10.1007/S00442-017-3959-4/FIGURES/4

Rhoades, S. D., Nayak, K., Zhang, S. L., Sehgal, A., and Weljie, A. M. (2018). Circadian- and light-driven metabolic rhythms in *Drosophila melanogaster*. *J. Biol. Rhythms* 33, 126–136. doi: 10.1177/0748730417753003

Rohde, P. D., Kristensen, T. N., Sarup, P., Muñoz, J., and Malmendal, A. (2021). Prediction of complex phenotypes using the *Drosophila melanogaster* metabolome. *Heredity* 126, 717–732. doi: 10.1038/s41437-021-00404-1

Ruel, J. J., and Ayres, M. P. (1999). Jensen's inequality predicts effects of environmental variation. *Trends Ecol. Evol.* 14, 361–366. doi: 10.1016/S0169-5347(99)01664-X

Salvucci, M. E., Stecher, D. S., and Henneberry, T. J. (2000). Heat shock proteins in whiteflies, an insect that accumulates sorbitol in response to heat stress. *J. Therm. Biol.* 25, 363–371. doi: 10.1016/S0306-4565(99)00108-4

Scheiner, S. M. (1993). Genetics and evolution of phenotypic plasticity. *Ann. Rev. Ecol. Evol. Syst.* 24, 35–68. doi: 10.1146/annurev.es.24.110193.000343

Schou, M. F., Kristensen, T. N., Pedersen, A., Karlsson, G., Loeschcke, V., and Malmendal, A. (2017). Metabolic and functional characterization of effects of developmental temperature in *Drosophila melanogaster*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 312, R211–R222. doi: 10.1152/ajpregu.00268.2016

Schou, M. F., Loeschcke, V., and Kristensen, T. N. (2015). Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. *PLoS One* 10:e0130307. doi: 10.1371/journal.pone.0130307

Sears, M. W., Riddell, E. A., Rusch, T. W., and Angilletta, M. J. (2019). The world still is not flat: lessons learned from organismal interactions with environmental heterogeneity in terrestrial environments. *Integr. Comp. Biol.* 59, 1049–1058. doi: 10.1093/icb/icz130

Sheldon, K. S., Padash, M., Carter, A. W., and Marshall, K. E. (2020). Different amplitudes of temperature fluctuation induce distinct transcriptomic and metabolomic responses in the dung beetle *Phanaeus vindex*. *J. Exp. Biol.* 223(Pt 23):jeb233239. doi: 10.1242/jeb.233239

Sømme, L. (1999). The physiology of cold hardiness in terrestrial arthropods. *Eur. J. Entomol.* 96, 1–10.

Sørensen, J. G., Kristensen, T. N., and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6, 1025–1037. doi: 10.1046/j.1461-0248.2003.00528.x

Sørensen, M. H., Kristensen, T. N., Lauritzen, J. M. S., Noer, N. K., Høye, T. T., and Bahrndorff, S. (2019). Rapid induction of the heat hardening response in an Arctic insect. *Biol. Lett.* 15:20190613. doi: 10.1098/rsbl.2019.0613

Stevenson, R. D. (1985). The relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms. *Am. Nat.* 126, 362–386. doi: 10.1086/284423

Strassburger, K., and Teleman, A. A. (2018). Flies eat their veggies to survive the cold. *Dev. Cell* 46, 671–672. doi: 10.1016/j.devcel.2018.05.030

Taylor, E. N., Diele-Viegas, L. M., Gangloff, E. J., Hall, J. M., Halpern, B., Massey, M. D., et al. (2021). The thermal ecology and physiology of reptiles and amphibians: a user's guide. *J. Exp. Zool. A Ecol. Integr. Physiol.* 335, 13–44. doi: 10.1002/jez.2396

Teets, N. M., and Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiol. Entomol.* 38, 105–116. doi: 10.1111/phen.12019

Teets, N. M., Kawarasaki, Y., Lee, R. E., and Denlinger, D. L. (2011). Survival and energetic costs of repeated cold exposure in the Antarctic midge, *Belgica antarctica*: a comparison between frozen and supercooled larvae. *J. Exp. Biol.* 214, 806–814. doi: 10.1242/jeb.051912

Terblanche, J. S., Deere, J. A., Clusella-Trullas, S., Janion, C., and Chown, S. L. (2007). Critical thermal limits depend on methodological context. *Proc. R. Soc. B Biol. Sci.* 274, 2935–2942. doi: 10.1098/rspb.2007.0985

Thiébaut, G., Tarayre, M., Jambon, O., Le Bris, N., Colinet, H., and Renault, D. (2020). Variation of thermal plasticity for functional traits between populations of an invasive aquatic plant from two climatic regions. *Hydrobiologia* 848, 2077–2091. doi: 10.1007/s10750-020-04452-2

Tiwari, S., and Wratten, S. D. (2019). Profile biology and management of the New Zealand endemic wheat bug, *Nysius huttoni* (Hemiptera: Lygaeidae). *J. Integr. Pest Manag.* 10, 34–35. doi: 10.1093/jipm/pmz032

Tomanek, L., and Somero, G. N. (1999). Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J. Exp. Biol.* 202, 2925–2936.

Tomanek, L., and Somero, G. N. (2002). Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of hsp gene expression. *J. Exp. Biol.* 205, 677–685. doi: 10.1242/jeb.205.5.677

van Eijsden, R. G. E., Stassen, C., Daenen, L., Van Mulders, S. E., Bapat, P. M., Siewers, V., et al. (2013). A universal fixation method based on quaternary ammonium salts (RNAlater) for omics-technologies: *Saccharomyces cerevisiae* as a case study. *Biotechnol. Lett.* 35, 891–900. doi: 10.1007/s10529-013-1163-0

Vasquez, M. C., Lippert, M. R., White, C., Walter, R. K., and Tomanek, L. (2019). Proteomic changes across a natural temperature gradient in a marine gastropod. *Mar. Environ. Res.* 149, 137–147. doi: 10.1016/j.marenvres.2019.06.002

Vázquez, D. P., Gianoli, E., Morris, W. F., and Bozinovic, F. (2017). Ecological and evolutionary impacts of changing climatic variability. *Biol. Rev.* 92, 22–42. doi: 10.1111/brv.12216

Verdú, J. R., Casas, J. L., Lobo, J. M., and Numa, C. (2010). Dung beetles eat acorns to increase their ovarian development and thermal tolerance. *PLoS One* 5:e10114. doi: 10.1371/journal.pone.0010114

Watanabe, M. (2002). Cold tolerance and myo-inositol accumulation in overwintering adults of a lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Eur. J. Entomol.* 99, 5–9. doi: 10.14411/eje.2002.002

Wolfe, G. R., Hendrix, D. L., and Salvucci, M. E. (1998). A thermoprotective role for sorbitol in the silverleaf whitefly, *Bemisia argentifolii*. *J. Insect Physiol.* 44, 597–603. doi: 10.1016/s0022-1910(98)00035-3

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* 208, 2819–2830. doi: 10.1242/jeb.01730

Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiol. Rev.* 65, 799–832. doi: 10.1152/physrev.1985.65.4.799

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

SUPPLEMENTAL MATERIALS FOR PAPER IV

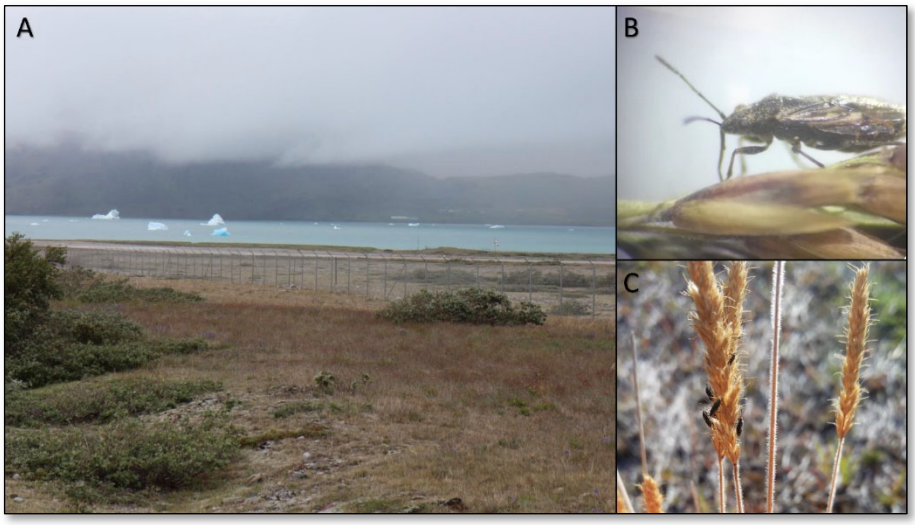


Figure 1: The fieldwork site (A) is a grass-covered and dry habitat located along the bank of Tunulliarfik Fjord. Adult *N. groenlandicus* was collected from the grasses (B and C) using a sweep net.

Table 1: Overview of dates and times for field collection of *N. groenlandicus*, start times of thermal assays (heat knockdown time (HKDT) and chill coma recovery temperature ($T_{recovery}$)), and microhabitat temperature summary. The insects were kept in glass containers in the shadow in the field site until assay start. Stars (*) indicate time points when samples for GC-MS analysis were collected simultaneously with individuals for thermal tolerance tests. Microhabitat temperatures were summarized by average, minimum, maximum, and daily range (max-min) in the 1 hour timespan prior to assay start.

	Sampling date	HKDT assay start	$T_{recovery}$ assay start	Avr	Min	Max	Range
Day 1	07-08-2018	08:30	08:28	9.4	7.0	11.5	4.5
	07-08-2018	12:10	12:09	25.3	22.0	28.0	6.0
	07-08-2018	16:08	16:06	24.5	23.5	26.0	2.5
	07-08-2018	19:58	19:58	17.1	15.0	19.5	4.5
Day 2	10-08-2018	08:22	08:14	10.7	10.0	12.0	2.0
	10-08-2018	12:20	12:15	26.3	23.0	28.5	5.5
	10-08-2018	17:08	17:09	23.6	22.0	24.5	2.5
	10-08-2018	20:35	20:32	15.9	14.5	17.0	2.5
Day 3	11-08-2018	08:14	08:08	10.1	8.5	11.0	2.5
	11-08-2018	12:15	12:09	18.0	17.5	19.0	1.5
	11-08-2018	16:16	16:09	21.5	20.0	24.0	4.0
	11-08-2018	20:20	20:11	12.5	12.0	13.5	1.5
Day 4	22-08-2018*	08:51	08:44	8.1	7.0	8.5	1.5
	22-08-2018*	12:22	12:15	10.2	10.0	10.5	0.5
	22-08-2018*	16:22	16:15	13.0	11.5	14.5	3.0
	22-08-2018*	20:20	20:15	8.8	7.0	10.0	3.0
Day 5	27-08-2018*	08:20	08:13	1.0	0.5	2.5	2.0
	27-08-2018*	12:39	12:33	22.2	18.0	25.0	7.0
	27-08-2018*	16:29	15:25	16.0	13.5	21.5	8.0
	27-08-2018*	20:25	16:21	8.6	7.0	10.0	3.0

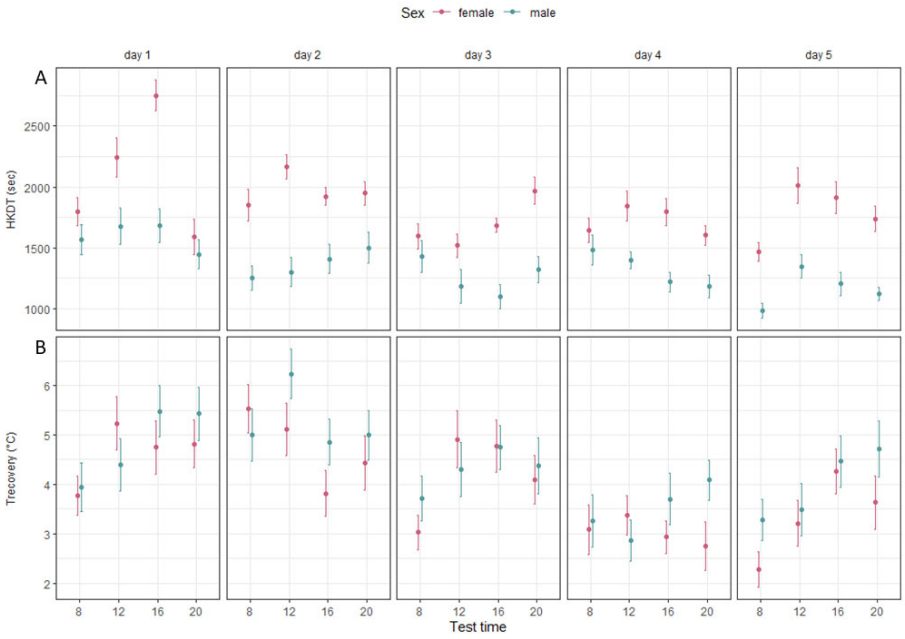


Figure 2: A) Mean heat knockdown time (seconds) and B) chill coma recovery temperature (°C) for female (red) and male (blue) *N. groenlandicus* collected across different times and days. Bars are standard errors of the mean.

Table 2: Summary of two-way ANOVAs on rank inverse transformed HKDT and $T_{recovery}$ of individuals (n=20 pr assay) as dependent variables and 'day' and 'Time of day' as independent variables.

ANOVA Heat knockdown time

sex	variable	Df	Sum sq	mean sq	F value	Pr(>F)	
females	day	4	27.25	6.81	8.62	1.14E-06	***
	time	3	23.37	7.79	9.86	2.83E-06	***
	day:time	12	47.61	3.97	5.02	9.15E-08	***
	Residuals	379	299.47	0.79			
males	day	4	28.30	7.08	7.70	5.65E-06	***
	time	3	0.70	0.24	0.26	0.855	
	day:time	12	20.00	1.67	1.82	0.044	*
	Residuals	379	348.60	0.92			

ANOVA chill coma recovery temperature

sex	variable	Df	Sum sq	mean sq	F value	Pr(>F)	
females	day	4	40.80	10.21	11.77	5.10E-09	***
	time	3	9.20	3.06	3.52	0.015	*
	day:time	12	18.90	1.57	1.81	0.045	*
	Residuals	378	327.80	0.87			
males	day	4	32.50	8.13	9.07	5.22E-07	***
	time	3	12.20	4.06	4.53	0.004	**
	day:time	12	13.60	1.13	1.26	0.238	
	Residuals	379	339.40	0.90			

Table 3: Metabolites detected by GC-MS on whole-body extract on female *N. groenlandicus*. A total of 33 metabolites were detected. Two metabolites (marked by *) occurred at concentrations below the quantification limit.

Free amino acids	***Sugars***	***Polyols***	***Metabolic intermediates***	***Other metabolites***
Alanine (Ala)	Fructose (Fru)	Adonitol	Citric acid	Citrulline
Isoleucine (Ile)	Galactose (Gal)	Arabitol	Fumaric acid	Ethanolamine*
Leucine (Leu)	Glucose (Glc)	Inositol	Glyceric acid	Phosphoric Acid
Lysine (Lys)	Glucose-6-phosphate (G6P)	Glycerol	Lactic acid	
Glutamic acid (Glu)	Trehalose (Tre)	Glycerol-3-Phosphate	Malic acid	
Glycine (Gly)		Xylitol*	Succinic acid	
Ornithine (Orn)				
Phenylalanine (Phe)				
Proline (Pro)				
Serine (Ser)				
Threonine (Thr)				
Tyrosine (Tyr)				
Valine (Val)				

Table 4: Raw data of detected metabolite concentrations from *N. groenlandicus* whole-body extract by GC-MS. Concentrations (nmol.mg-1) are listed for each metabolite (columns) and each replicate (rows) of the different sampling times and days (Excel).

The table can be accessed via Figshare: 10.6084/m9.figshare.17041397

Table 5: Results from one-way Analysis of Variance (ANOVA) performed on log-transformed metabolite data for day 4 and day 5 separately. The dependence of sampling time (morning, midday, afternoon, and evening) on metabolite concentration was examined for each metabolite (Excel).

ANOVA		**Day 4**					**Day 5**			
Lactic_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	1.5645	0.5215	4.8046	0.008008	3	0.5419	0.180633	2.2496	0.1063
Residuals	28	3.0392	0.10854			26	2.0877	0.080297		
Alanine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.27911	0.093037	2.1612	0.1149	3	0.73556	0.245188	2.6892	0.06708
Residuals	28	1.20538	0.043049			26	2.37059	0.091177		
Valine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.5321	0.17735	1.4098	0.2607	3	1.3478	0.44928	3.8538	0.02091
Residuals	28	3.5225	0.1258			26	3.0311	0.11658		
Leucine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.7468	0.24892	2.2001	0.1102	3	0.93243	0.310809	4.0458	0.01739
Residuals	28	3.1679	0.11314			26	1.9974	0.076823		
Phosphoric_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.06305	0.021017	0.6149	0.6111	3	0.14057	0.046857	2.6207	0.07202
Residuals	28	0.95709	0.034182			26	0.46487	0.01788		

Glycerol	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.5392	0.17973	1.3107	0.2905	3	0.7805	0.26018	2.1062	0.1238
Residuals	28	3.8395	0.13713			26	3.2118	0.12353		
								:		
Isoleucine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.15721	0.052404	0.9843	0.4143	3	0.37251	0.124169	2.7381	0.06377
Residuals	28	1.49072	0.05324			26	1.17906	0.045349		
Proline	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.6314	0.21048	1.1726	0.3378	3	0.6614	0.22048	1.0921	0.37
Residuals	28	5.026	0.1795			26	5.2489	0.20188		
Glycine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.04385	0.014615	0.7146	0.5515	3	0.13642	0.045475	2.1198	0.122
Residuals	28	0.57266	0.020452			26	0.55775	0.021452		
Succinic_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.57128	0.19043	7.7251	0.000653	3	1.54519	0.51506	18.253	1.40E-06
Residuals	28	0.69021	0.02465			26	0.73368	0.02822		
Glyceric_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.0502	0.016732	0.9926	0.4106	3	0.083568	0.027856	2.71	0.06565
Residuals	28	0.47199	0.016857			26	0.267255	0.010279		
Fumaric_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.06463	0.021544	1.2101	0.3243	3	0.12967	0.043224	4.4009	0.01244
Residuals	28	0.49852	0.017804			26	0.25537	0.009822		
Serine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.05183	0.017275	0.4033	0.7518	3	0.29029	0.096765	2.0692	0.1288
Residuals	28	1.19949	0.042839			26	1.21586	0.046764		
Threonine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.20233	0.067442	1.7257	0.1845	3	0.06158	0.020527	0.4735	0.7034
Residuals	28	1.09424	0.03908			26	1.12724	0.043355		

Malic_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	1.2187	0.40625	2.0671	0.1272	3	0.3804	0.12682	0.5726	0.6381
Residuals	28	5.5028	0.19653			26	5.7582	0.22147		

Citrulline	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.45316	0.151055	3.2427	0.03685	3	0.09637	0.032124	0.9123	0.4486
Residuals	28	1.30431	0.046583			26	0.91549	0.035211		

Glutamic_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.05749	0.019164	0.3806	0.7677	3	0.2559	0.085301	4.034	0.01759
Residuals	28	1.40993	0.050355			26	0.54978	0.021145		

Phenylalanine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.09595	0.031985	1.1015	0.365	3	0.20032	0.066774	5.9448	0.003152
Residuals	28	0.81304	0.029037			26	0.29204	0.011232		

Arabitol	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.34919	0.116395	1.5734	0.2179	3	0.069	0.023001	1.6591	0.2002
Residuals	28	2.07135	0.073977			26	0.36045	0.013864		

Adonitol	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.4789	0.15963	1.3368	0.2823	3	0.09984	0.03328	1.9033	0.1539
Residuals	28	3.3435	0.11941			26	0.45462	0.017485		

Glycerol_3P	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.25277	0.084257	3.8719	0.01959	3	0.09716	0.032388	1.636	0.2053
Residuals	28	0.60931	0.021761			26	0.51473	0.019797		

Ornithine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.10898	0.036327	1.9595	0.143	3	0.10859	0.036198	3.3069	0.03578
Residuals	28	0.51908	0.018538			26	0.2846	0.010946		

Citric_acid	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)

Time of day	3	0.06358	0.021194	0.4824	0.6972	3	0.39313	0.131042	2.6357	0.0709
Residuals	28	1.2301	0.043932			26	1.29267	0.049718		
Fructose	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	2.5304	0.84345	1.1962	0.3292	3	11.594	3.8647	6.5631	0.001884
Residuals	28	19.7431	0.70511			26	15.31	0.5889		
Glucose	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	9.9916	3.3305	4.0946	0.01575	3	3.3953	1.1318	2.6308	0.07126
Residuals	28	22.7752	0.8134			26	11.1851	0.4302		
Lysine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	3.2905	1.09684	3.4044	0.03126	3	0.54995	0.183318	2.0671	0.1291
Residuals	28	9.0211	0.32218			26	2.30577	0.088683		
Galactose	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	12.69	4.2299	4.2992	0.01292	3	4.51	1.50333	2.248	0.1065
Residuals	28	27.549	0.9839			26	17.387	0.66873		
Tyrosine	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.24024	0.080079	1.5882	0.2144	3	0.09758	0.032527	0.4059	0.75
Residuals	28	1.4118	0.050421			26	2.08352	0.080135		
Inositol	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.09638	0.032127	0.5829	0.6311	3	0.22211	0.074037	1.7025	0.191
Residuals	28	1.54312	0.055111			26	1.13064	0.043486		
Glucose_6P	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	0.05096	0.016986	0.2953	0.8285	3	0.38818	0.129393	1.8794	0.1579
Residuals	28	1.61067	0.057524			26	1.79007	0.068849		
Trehalose	Df	Sum sq	Mean sq	F value	Pr(>F)	Df	Sum sq	Mean sq	F value	Pr(>F)
Time of day	3	1.4615	0.48718	1.1037	0.3641	3	1.5651	0.52171	1.2512	0.3116
Residuals	28	12.359	0.44139			26	10.8409	0.41696		

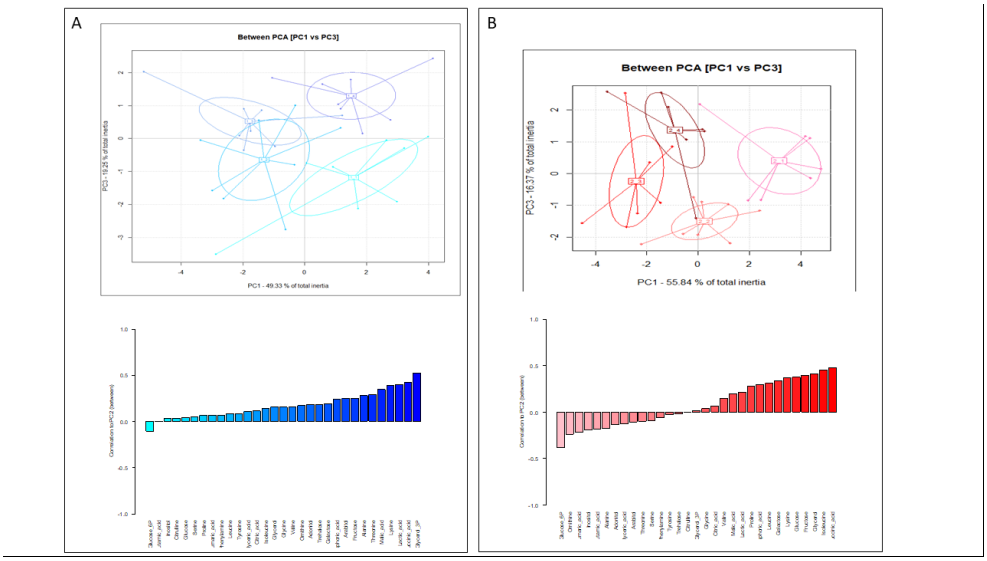


Figure S3: Between-class Principal Component Analyses (PCA) based on GC-MS on whole-body extracts of female *N. groenlandicus* sampled in the field at four consecutive sampling time points (8:00 am, 12:00 pm, 4:00 pm, 8:00 pm) on **A)** day 4 and **B)** day 5. Scores for PC1 and PC3 are depicted. Lines represents individual sample position respective to centroids (n=8). Correlations of metabolite concentrations (relative proportions) to PCs in the between-class PCA. A and B represent metabolite correlations to PC2 for day 4 and day 5, respectively.

ADDITIONAL RESULTS

STRONG EXPERIMENTAL EVIDENCE FOR DIURNAL VARIATION IN GENETIC ARCHITECTURE OF BEHAVIOR AND HEAT TOLERANCE TRAITS REVEALED IN *DROSOPHILA MELANOGASTER* UNDER NATURAL TEMPERATURE CONDITIONS

Natasja Krog Noer, Palle Duun Rhode, Simon Bahrndorff, and Torsten Nygaard Kristensen

Strong experimental evidence for diurnal variation in genetic architecture of behavior and heat tolerance traits revealed in *Drosophila melanogaster* under natural temperature conditions

Natasja Krog Noer[1], Palle Duun Rohde[2], Simon Bahrndorff[1], and Torsten Nygaard, Kristensen[1]
[1]Department of Chemistry and Bioscience, Aalborg University, 9220 Aalborg, Denmark
[2]Department of Health Science and Technology, Aalborg University, 9220 Aalborg, Denmark

Introduction

Species are continuously exposed to changes in abiotic factors in nature. The ability to cope with variable environmental conditions depends on phenotypic plasticity and evolutionary adaptation to the local environment (Hoffmann and Parsons, 1991; Kristensen et al., 2020). With global change, mean temperatures will increase with concomitant increase in frequency of intense and severe temperature events (IPCC, 2013). How ectotherms respond to altered temperature regimes will on a short-term scale depend on the existence of thermoregulatory behavior and plastic changes of physiological limits (Sunday et al., 2014; Sørensen et al., 2016; Kristensen et al., 2020). On a long-term scale, ectotherms with limited possibility of migration must cope with the steadily increasing mean temperatures by evolutionary adaptation (Chevin and Hoffmann, 2017). Such changes in the genetic constitution of a population depend on the amount of additive genetic variation expressed for the traits that selection act on and on the selection intensity (Falconer and Mackay, 1996). However, a number of studies imply that evolution in upper heat tolerance and central life-history traits are constrained in some species, and that this is due low genetic variation (Kellermann et al., 2009; Mitchell et al., 2011; Kelly et al., 2012; Hoffmann et al., 2013; Kristensen et al., 2015). These conclusions on population evolutionary trajectories are often based on estimates of genetic variances and heritabilities in single or few environments. This is despite variance components and heritabilities are thought to be sensitive to the environment, suggesting that the potential for evolutionary changes of phenotypes is environment specific (Hoffmann and Parsons, 1991). This raises questions about the validity of deducing genetic constraints from studies in one environment (Weigensberg and Roff, 1996; Sgrò and Hoffmann, 2004). One aspect that has received attention is that environmental stress or exposure to unfavorable conditions might impact on heritable variation (Hoffmann and Merilä, 1999; Charmantier and Garant, 2005). Environmental conditions vary spatially among habitats and at multiple temporal scales including minutes, days, seasons, and years. Organisms are therefore expected to encounter physiological stress or unfavorable conditions at one or more of these spatiotemporal scales (Weigensberg and Roff, 1996; Hoffmann and Merilä, 1999). The studies examining the consequences of unfavorable conditions have been conflicting, some suggesting increasing heritability of life-history traits under stressful conditions due to increased additive genetic variation (Hoffmann and Parsons, 1991; Hoffmann and Merilä,

1999; Charmantier and Garant, 2005). However, the trend is towards lower heritabilities at stressful environments. Generally, little is known about temperature dependent genetic architectures of thermal performance traits, but Ørsted et al. 2018 showed a reduced evolvability of cold stress resistance across a gradient of developmental temperatures. We argue that there is a need for studies examining the environmental dependence of genetic architecture to improve our knowledge on evolutionary trajectories with global change.

Lethal endpoints such as upper critical temperature have been studied profoundly under the notion that survival at high temperatures underpins the fitness or success of the species. However, there are a number of traits that are affected by stresses less extreme and more regularly encountered in nature than those causing mortality (Braschler et al., 2021). Evidence for the importance of some these sublethal traits on fitness and survival is escalating. For instance, several studies show that male *Drosophila* suffer from infertility at sub-lethal temperatures with implications for the reproductive output of the population (Jørgensen et al., 2006; Sales et al., 2018, 2021; Parratt et al., 2021; van Heerwaarden and Sgrò, 2021; Walsh et al., 2021). Other sub-lethal traits important fitness components are courtship, foraging, and predator avoidance and activity. These traits are all encompassed by locomotor activity which can therefore be hypothesized to be central for fitness of insects (Everatt et al., 2013). Locomotor activity is a complex trait, and the genetic component is governed by many loci with small effects that are sensitive to environmental conditions, and likely their interactions (Rohde et al., 2018). Several studies have revealed abundant genetic variation for different locomotor activity measures in *Drosophila* (Burnet et al., 1988; Jordan et al., 2006, 2007; Rohde et al., 2018). However, the genetic basis of this complex trait is largely uncharacterized and the impact of natural and increasing temperatures fluctuations on these estimates unknown.

Using the Drosophila Genetic Reference Panel (DGRP), we set out to investigate 1) the genetic architecture (h^2, V_A, r_g etc) of behavioral traits investigated under a range variable field temperature conditions during Danish summer, 2) How exposure temperatures in the field impact on behavior and upper thermal limits when subsequently assessed in the laboratory at high temperatures, 3) how the numerous traits investigated are connected phenotypically and genetically and 4) the genetic architecture of the heritability of these traits. This dataset gives us unforeseen insight into the complex genetic nature of ecologically relevant thermal performance traits and how these are likely to evolve in concert with future climate changes.

Note that these data are work in progress and the figures presented are based on data from one (of three) test day only. These preliminary results show strong temperature and time of day effects on heritabilities and variance components of a behavioral trait whereas the genetic architecture of heat tolerance. Curiosity driven research with strong importance for mechanistic SDMs suggesting that trait genetic architectures are much more complex than hitherto assumed.

Materials and Methods

Drosophila stocks and maintenance:

A total of 127 lines of the *Drosophila melanogaster* Genetic Refence Panel (DGRP) were obtained from Bloomington *Drosophila* Stock Center (NIH P40OD018537; supplemental Table S1). The flies were maintained on a standard *Drosophila* oatmeal-sugar-yeast-agar medium (7 ml) at 20 °C, and a 12:12 h light/dark photoperiod for two generations before experimental start. The flies were pre-fed with additional dry yeast on the surface of the *Drosophila* medium before oviposition for experimental flies.

Experimental setup:

Experimental flies were produced from three replicate vials per line of ~15 adult flies for the 100 DGRP lines that laid eggs for four 12 h periods (flies tipped to new vials every 12 hours). Within 48 hours of eclosion, flies were sexed under CO_2 anaesthesia and males were transferred to new vials with fresh food. When the male flies were five days old, 16 individuals from each line were transferred to 5 mm polycarbon tubes (TriKinetics, US) containing a droplet of *Drosophila* standard medium and sealed with parafilm to prevent the media from drying out (Photo 1). Not all lines produced enough flies, and the number of lines and replicates differs slightly for each run. The 16 replicates x 100 lines ≈ 1600 tubes were sealed with moist pipe cleaners, after which they were arranged randomly in 50 *Drosophila* Activity Monitors (DAM2, TriKinetics, US) in a block design. The monitors have four rows x eight columns (see Fig. 1A) and in each column we placed four replicate tubes of a line (referred to as block). Hence, the four blocks consisting of 16 replicate tubes were randomized across different monitors and columns in the monitors. The monitors quantify movement of animals over time by counting the number of times an animal crosses a laser centered at the middle of the polycarbon tube at a fixed time interval. The monitors were placed at an outdoor roofed field-site (57°00'52.6"N, 9°59'04.5"E) for 24 or 32 hours in two cohorts at each experimental day before testing heat performance phenotypes (next section; Fig. 1B); one cohort was tested in the morning (08:00 AM) and the other cohort at afternoon (16:00 PM). The idea with this set up was to ensure that the flies were exposed to a wide range of temperatures. To get activity measures of flies in the semi-field situation, the early cohort were monitored in the DAM systems for the entire duration of the field acclimation (24 hours). The experiment was repeated for 3 days in April-June 2022 to achieve as much variation in temperatures as possible. The experimental setup is illustrated in Figure 1.

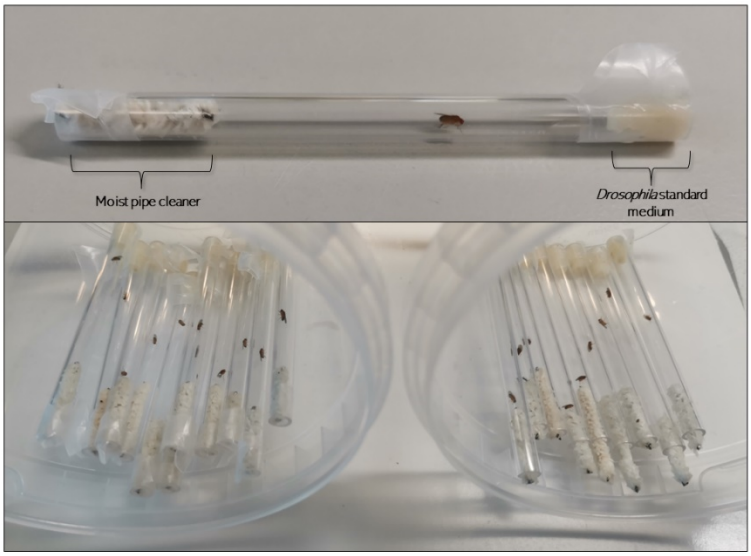


Photo 1 | Experimental setup. Flies used for the experiment were transferred to individual tubes containing *Drosophila* standard medium and a moist pipe cleaner to prevent desiccation. For each of the ~100 DGRP lines, 16 replicate tubes were established and placed by a randomized block design in DAM monitors (Fig. 1).

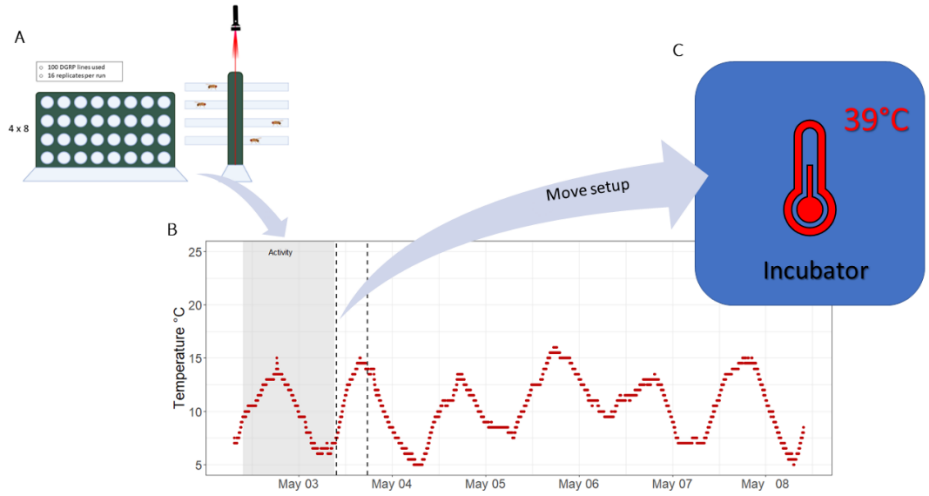


Figure 1 | Experimental setup. A) The 16 replicates of each ~100 *Drosophila* lines were randomized and placed in 50 DAM monitors counting the frequency of flies crossing a centered laser, B) the DAM monitors were placed in the field to monitor the activity of flies for 24 hours during the natural temperature variation (shaded grey area), C) after 24 and 32 hours (dashed black lines), the monitors were moved to a pre-heated incubator for assessment of heat tolerance and activity measures of the flies.

Heat performance traits:

After 24 hours acclimation in the field, the DAMs containing the 16 males from 100 lines were transferred to a preheated incubator (WT 450, Weiss Umwelttechnik GmbH, Germany) at 39 °C for 2 hours (Fig. 1C). The DAMs monitored the activity levels every 30 seconds during the heat exposure. From the activity data, a suite of traits was extracted: time to heat knockdown (laser crossings cease), time to peak activity, size of peak activity, and total activity (Fig. 4 for demonstration of phenotypes). The heat stress temperature was based on pilot test of eight DGRP lines and aimed to knockdown the flies within 20-30 minutes to avoid acclimation or heat hardening effects to occur during the heat exposure.

Data analysis:

Locomotor activity data

The DAM monitors return the individual counts of infra-red laser beam breaks per 30 seconds. We refer to the count of breaks per time unit as the activity. Individuals showing no activity during field monitoring and during heat exposure were considered dead and discarded from the analysis. The field activity was summarized for each individual by the total number of laser beam breaks per 20 minutes for the 24-hour monitoring period. The line activity was found by averaging the summarized activity across the 16 individuals per line.

During heat exposure at 39°C, the activity was monitored per 30 seconds for 2 hours and four different phenotypes were defined and assessed based on this data (see figure 4 for visual description). The phenotypes included 1) heat knock down time (HKDT) here defined as the time (seconds) until last beam-break for each replicate and averaged across the 16 individuals for each line, 2) Total activity, defined as the summarized number of laser beam breaks for the entire heat exposure period across individuals, 3) Peak size, defined as the maximum frequency of laser breaks recorded , and 4) Time to peak activity (TTP) defined as the time (seconds) for each fly to reach its maximum frequency of laser crossings. ‘peak size’ and ‘peak time’ for each line was calculated by averaging the measure for four individuals in a monitor (block) and finally averaging the four blocks. This was to reduce the effect of multiple activity peaks across different individuals.

Statistical genetic analyses

Consider the linear model that contains an observation vector of the trait(s) of interest (y), the fixed effects (d) that explain systematic differences in y, and the random genomic effects g, and random residual effects e:

$$\boldsymbol{y = Xd + Zg + e}, \quad \textbf{(1)}$$

where X and Z are known design matrices linking the fixed effects d and random genomic effects g to the observations y. The additive genomic effects are defined as the sum of the effects of all makers:

$$\boldsymbol{g = \sum_{i=1}^{m} w_{ij} b_i}, \quad \textbf{(2)}$$

where m is the total number of markers, w_{ij} is the *i*-th sequence variant for individual *j*, and b_i is the effect of sequence variant *i*. For a two-trait model, the joint distribution of marker effects is assumed to be:

$$\begin{bmatrix} \boldsymbol{b_1} \\ \boldsymbol{b_2} \end{bmatrix} \sim \boldsymbol{N}\left(\begin{bmatrix} \boldsymbol{0} \\ \boldsymbol{0} \end{bmatrix}, \begin{bmatrix} \boldsymbol{I\sigma^2_{b_1}} & \boldsymbol{I\sigma_{b_1 b_2}} \\ \boldsymbol{I\sigma_{b_2 b_1}} & \boldsymbol{I\sigma^2_{b_2}} \end{bmatrix}\right), \quad \textbf{(3)}$$

where the *I's* represents identity matrices and $\sigma^2_{b_1}$ (and $\sigma^2_{b_2}$) is the prior variance of marker effects for trait 1 (trait 2), and $\sigma_{b_1 b_2}$ is their prior covariance. The joint distribution of the genomic values for trait 1 and 2 follows a multinormal distribution:

$$\begin{bmatrix} \boldsymbol{g_1} \\ \boldsymbol{g_2} \end{bmatrix} \sim \boldsymbol{N}\left(\begin{bmatrix} \boldsymbol{0} \\ \boldsymbol{0} \end{bmatrix}, \begin{bmatrix} \boldsymbol{G\sigma^2_{g_1}} & \boldsymbol{G\sigma_{g_1 g_2}} \\ \boldsymbol{G\sigma_{g_2 g_1}} & \boldsymbol{G\sigma^2_{g_2}} \end{bmatrix}\right), \quad \textbf{(4)}$$

where $\sigma^2_{g_1}$ and $\sigma^2_{g_2}$ is the genomic variance for trait 1 and 2, $\sigma_{g_2 g_1}$ is the genomic covariance between the two traits, and G is the genomic relationship among the DGRP lines estimated as $G = \frac{1}{m} WW'$, where W is a centred and scaled genotype matrix. Each column vector of W is $w_i = \frac{a_i - 2p_i}{\sqrt{2p_i(1-p_i)}}$, with p_i being the allele frequency of the *i*-th variance, and a_i is the *i*-th

column vector of a allele count matrix contain the genotyped encoded as 0, 1 and 2 referring to the number of the minor allele.

Similarly, the joint distribution of the random residuals (Eq. 1) is assumed to be:

$$\begin{bmatrix} \boldsymbol{e_1} \\ \boldsymbol{e_2} \end{bmatrix} \sim \boldsymbol{N}\left(\begin{bmatrix} \boldsymbol{0} \\ \boldsymbol{0} \end{bmatrix}, \begin{bmatrix} \boldsymbol{I\sigma_{e_1}^2} & \boldsymbol{I\sigma_{e_1e_2}} \\ \boldsymbol{I\sigma_{e_2e_1}} & \boldsymbol{I\sigma_{e_2}^2} \end{bmatrix} \right). \quad \textbf{(5)}$$

The narrow-sense heritability (h^2) for trait 1 is estimated as:

$$\boldsymbol{h_{g_1}^2} = \frac{\boldsymbol{\sigma_{g_1}^2}}{\boldsymbol{\sigma_{g_1}^2 + \sigma_{e_1}^2}}, \quad \textbf{(6)}$$

and the genetic correlation among two traits is defined as:

$$\boldsymbol{r_g} = \frac{\boldsymbol{\sigma_{g_1g_2}}}{\sqrt{\boldsymbol{\sigma_{g_1}^2\sigma_{g_2}^2}}}. \quad \textbf{(7)}$$

The models above are implemented using empirical Bayesian methods using a Bayesian, Markov chain Monte Carlo approach where the hyperparameters was estimated with maximum likelihood, and conditional on these, the model was fitted using Markov chain Monte Carlo. The approach is implemented in the R package qgg (Rohde et al., 2020). The prior distribution of the marker effects was a Gaussian variance (Sørensen et al., 2015). Assigning a Gaussian prior to b implies that the posterior means are equivalent to the Best Linear Unbiased Predictor (BLUP) estimates.

$$\boldsymbol{p(b)} = \boldsymbol{N(0, \sigma_b^2)}. \quad \textbf{(8)}$$

To quantify the uncertainties of the estimated genetic parameters (i.e., heritability and genetic correlations) we performed non-parametric bootstrap (using 100 random samplings with replacement), where the bootstrap standard error is the standard deviation of the bootstrap sampling distribution.

Results and Discussion

Field activity

The field activity was summarized for each line within 20-minute bins throughout 24 hours monitoring-period. Among the lines we found substantial phenotypic variation in the beam-break frequency and time course of the activity throughout this period (not shown). The average field activity profile across the 100 DGRP lines showed a uniform distribution with a single activity peak in the afternoon and almost no activity during the night (Fig. 2). The locomotor activity tracked the field temperature peaking between 17:00-18:00. This affirm results from other studies showing that activity levels are shaped by temperature in part (Klepsatel et al., 2013; MacLean et al., 2017; Shaw et al., 2019). However, other factors such as circadian clock, light, and social housing have a strong impact on daily activity levels as well and these interact in complex ways (Menegazzi et al., 2012; Shaw et al., 2019). For instance, Shaw et al. (2019) showed that locomotor activity *D. suzukii* measured in the field is delayed relative to temperature peak in the spring, whereas activity more closely track the daily temperature course during summer months, likely due to these complex interactions. Finally, in our data the rapid decline in activity at the start of the monitoring likely reflect disturbance of the flies during the transition from laboratory to field conditions and also followed by a period of cold stress before acclimating to the cold field temperatures.

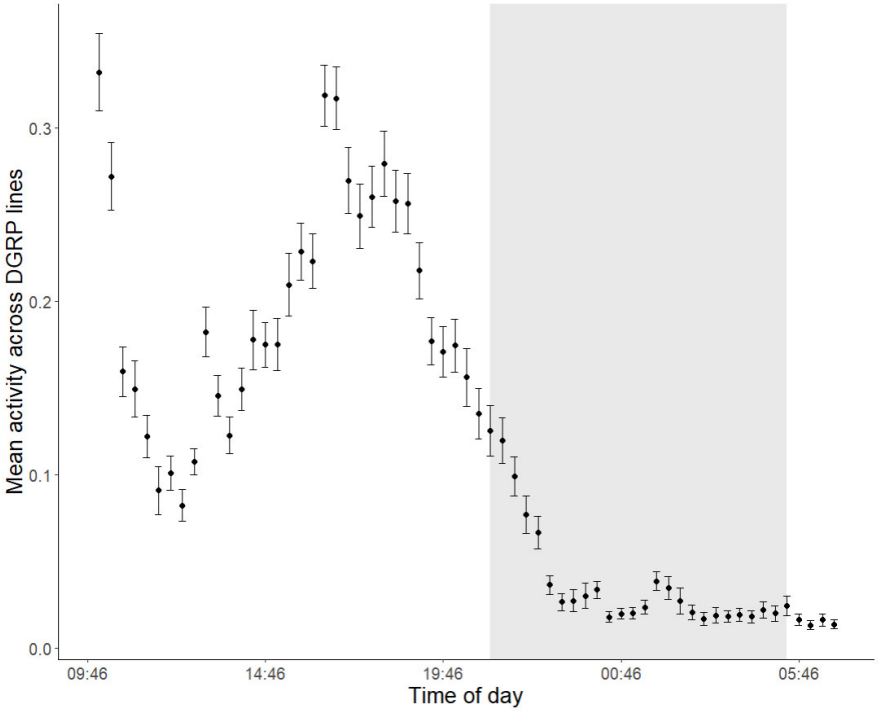


Figure 2 | Field activity levels. The mean activity of all 100 DGRP lines in the field site summarized by 20-minute time bins. Error bars are standard error of the mean**.** The natural light regime in the field is indicated by white (light) and grey (no light) background shading.

The narrow sense heritability for activity was calculated in each 20-minute bin (Fig. 1), starting time at 09:46, across the 24-hour field monitoring (Fig. 2A). There was large variability in the heritability estimates which changed at least four-fold during the day ranging between ~0 and 0.20. Ignoring the first hour of the day due to uncertainty in activity measurements, the heritability estimates peaked after 11 hours (20:46) and was lowest during the night and early morning. A previous study estimated a narrow sense heritability of 0.26 for locomotor activity at 25°C constant laboratory conditions in 204 DGRP lines (Rohde et al., 2018) and others found a narrow-sense heritability of 0.16 of *D. melanogaster* also kept at 25°C laboratory conditions (Jordan et al., 2007). The estimated narrow-sense heritability in our study is seemingly in the range of these previous estimates. However, our results show that there is a considerable effect of natural variation in environmental conditions on this estimate and the genetic background for activity seem to vary across the day and large part of the variation might be attributed to variation in temperature (Fig. 2B). Plotting the

heritability as a function of field temperature resulted in a non-linear relationship with the highest heritability estimates at 10°C and declining estimates towards higher and lower temperatures (Fig. 2B), suggesting that the quantitative genetic architecture is environment dependent even at a rapid short temporal scale. Other studies have found environmental dependence of stress resistance, life-history, and morphological traits (Van Heerwaarden and Sgrò, 2011; Kristensen et al., 2015; Ørsted et al., 2019) and these have proposed that this is caused by different sets of genes being important under different environmental conditions (Hoffmann and Merilä, 1999). Thus, it will be interesting to investigate this further and e.g. detect 'time of day specific' or temperature specific candidate genes of importance for explaining variation in activity patterns.

In the field occupied with forecasting species vulnerability and biogeographical shifts under future climate change it has long been argued that evolutionary adaptation should be integrated in prediction models and that current models ignoring adaptive genetic variation overestimate species vulnerabilities and future losses (Kearney et al., 2009; Bush et al., 2016). However, our results suggest that this will be complex as the genetic parameters included in models will vary not only with mean temperatures (e.g. Ørsted et al. 2018), but by thermal variability across days.

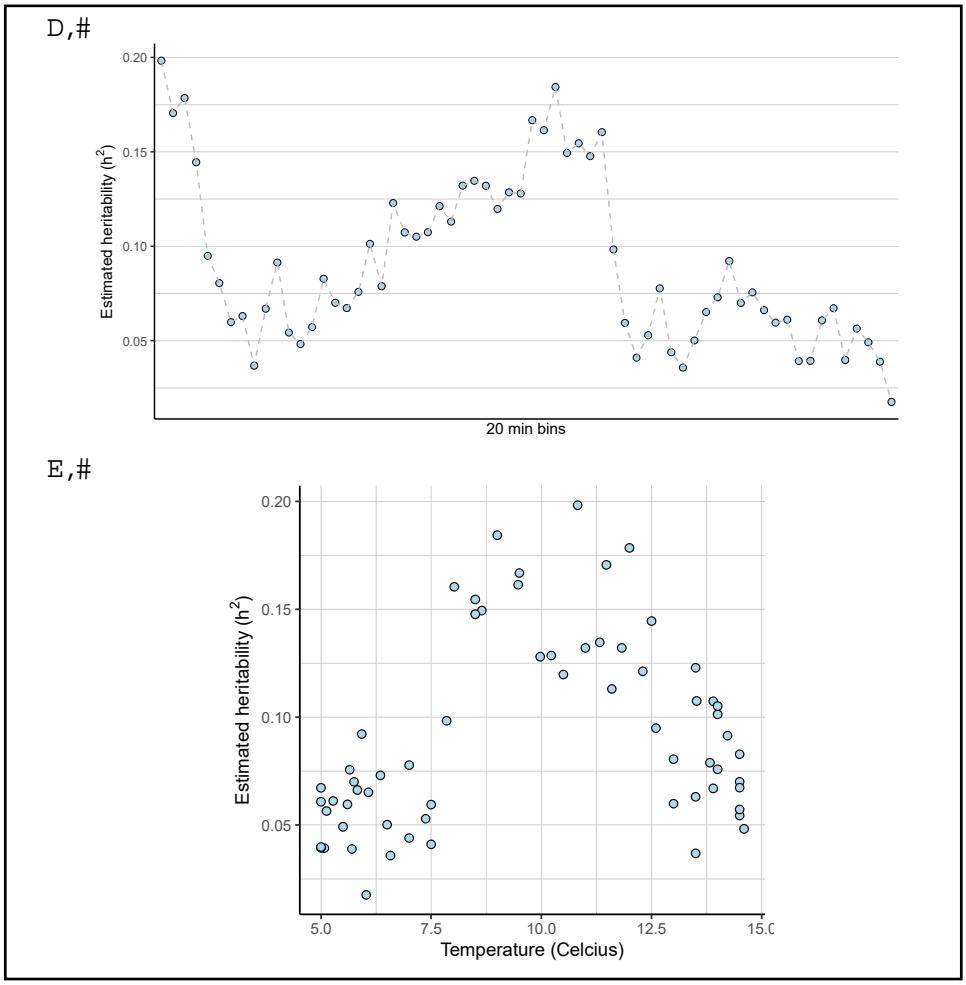


Figure 3 | Estimated narrow-sense heritability (h^2) for 20-min total-activity as function of A) time in the field across 1280 minutes, and B) mean temperature in the 20-min windows.

Heat performance phenotypes

After acclimation to field temperatures, the four heat performance phenotypes were assessed (illustrated on Fig. 4A). There was considerable phenotypic variation among the 100 DGRP lines in all four traits (Fig. 4 and S1). Notably, the ranking of genotypes differed between phenotypic assessment in the morning and the afternoon, i.e. there was considerable

genotype-by-environment interaction for all traits as not all genotypes responded equally to the field acclimation (see Supplemental Fig. 4). Two general trends were clear from visual inspection of the plots (Fig. 4 B). First, HKDT was seemingly higher in the morning relative to the afternoon in most genotypes, which was unexpected as *Drosophila* species usually show some beneficial acclimation response to temperatures (Schou et al., 2017). This might indicate a cost of exposure to the cold night temperatures since the transition from rearing at 20°C in the laboratory to the cold field temperature of 6°C during the night was quite abrupt. Cold injuries can accumulate slowly and might not be visible before 24 hours after cold exposure, hence a delayed negative effect on HKDT (Sinclair et al., 2015). Second, total activity seemed higher in the afternoon relative to morning. This can either be explained by the total activity to reflect a stress response as a consequence of lower heat resistance at this time point. Alternatively, higher activity levels can be an adaptive plastic response to higher temperatures yielding an increased capacity for e.g. foraging, mate-seeking, courtship, and escape from stressors etc. However, this needs to be evaluated by experiments designed to answer this question.

With the continued analysis of the other test days, we can evaluate whether higher environmental variation and higher mean temperatures changes the conclusions on genotype-by-environment interactions.

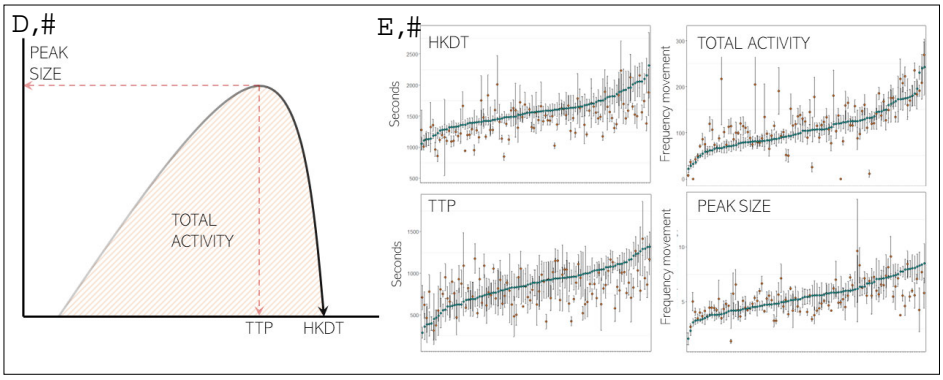


Figure 4 | Temperature stress phenotypes. The left-hand graph illustrates the four different measures extracted for each line from the monitoring data during the acute heat exposure at 39°C. These include 1) HKDT, time to ceased movement, 2) Total activity, the summarized number of laser crossings for the entire heat exposure, 3) Peak size, the maximum frequency of laser crossing, and 4) TTP, time to peak activity, i.e. the maximum frequency of laser crossing is reached. The right-hand plots show the variation in the four heat performance phenotypes for all lines ranked by the morning cohort. The morning cohort is illustrated by blue points and the afternoon cohort by orange points. Error bars are standard error of the mean.

The heritability estimates of the heat performance phenotypes did not differ across test times except for HKDT which was higher in the afternoon relative to morning (Fig. 5). The highest heritability estimates were found for total activity and peak size ranging between 0.20 and 0.25. The high phenotypic correlation between these phenotypes (Fig. 6) can partly be explained by methodological artefact from higher peak size being equal to more total activity, however it is not necessarily the case always. The heritability estimates for time to peak activity and time to knockdown were relatively low. The heritability for HKDT was similar to other estimates for *Drosophila* and higher than found for dynamic estimates of heat tolerance, i.e. upper critical temperature CT_{max} (e.g. Mitchell et al., 2011; Blackburn et al., 2014). These results support general findings suggesting that potential for evolutionary adaptation of heat tolerances is relatively low (Mitchell et al., 2011; Araújo et al., 2013; Hoffmann et al., 2013). This is often contributed to depletion of additive genetic variance as a consequence of strong directional selection over time and ultimately fixation of alleles. With the additional data that we have not yet analyzed we further wish to examine how more stressful / unfavorable / novel conditions affect heritability.

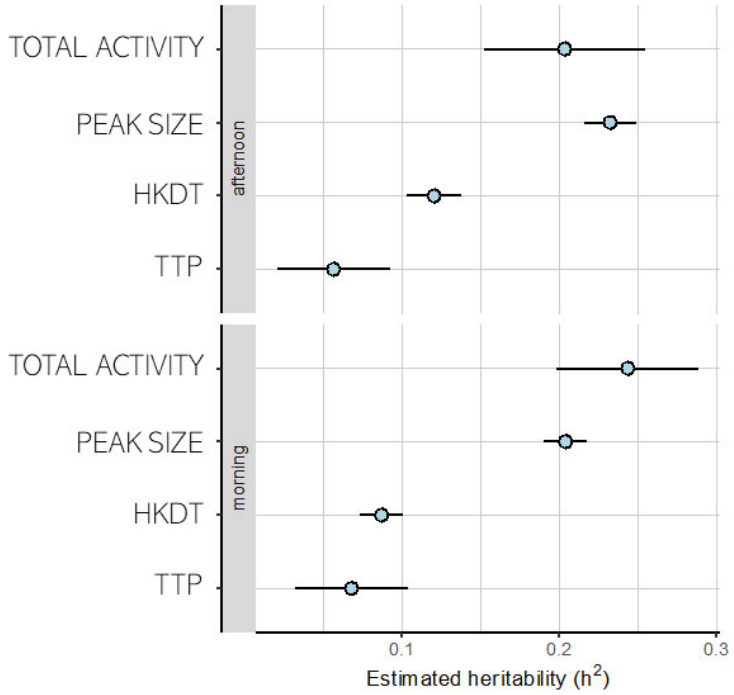


Figure 5 | Estimated heritability (h^2) for traits assessed in the laboratory after exposure to natural temperature variability in a natural habitat. Error bars represents the bootstrap standard error.

Genetic correlation across environments were high to moderate strong for all traits (>0.48) (Fig. 6). The phenotypic correlations showed the same tendency, but correlations were weaker. For now, we have limited power (two environments) to conclude whether this pattern is consistent across different environmental conditions. Studies have shown that the correlations will decrease as a consequence of increased environmental dissimilarity (Ørsted et al., 2018; Stinchcombe et al., 2010;). This will be considered with subsequent analyses using the data from higher and more variable field conditions.

We found relatively strong and positive correlations between the four different traits. The highest phenotypic and genetic correlations were found between HKDT and activity, and peak size and activity. These positive correlations indicate a high degree of shared genetic mechanisms between the investigated traits and that high activity levels are associated with high heat tolerance.

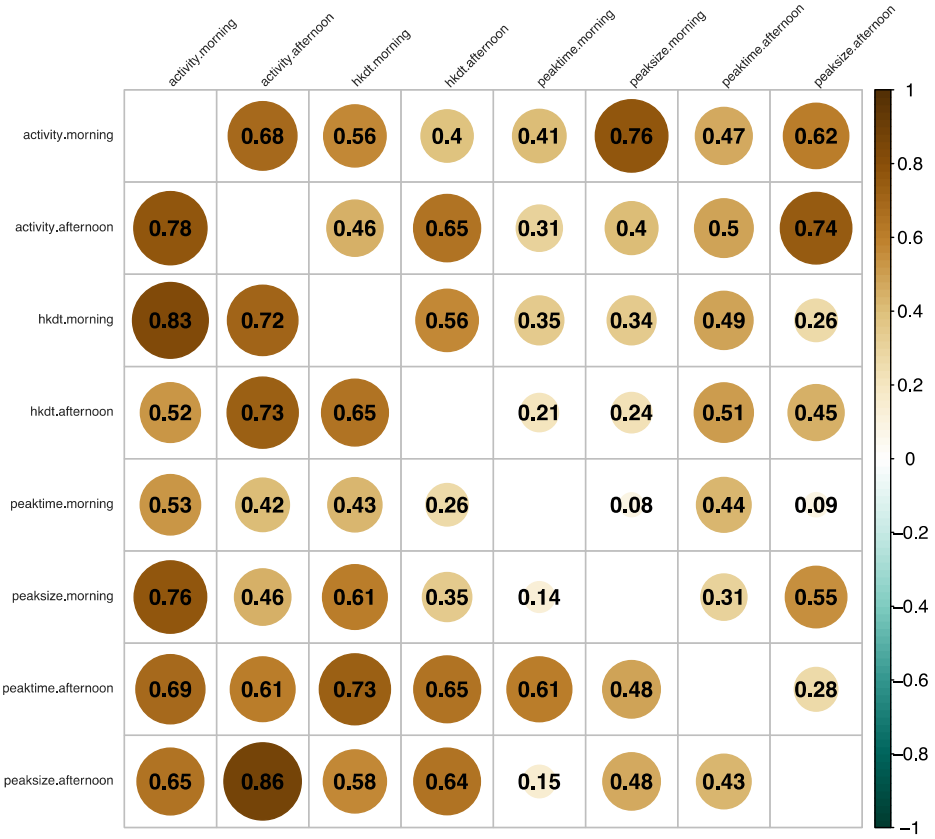


Figure 6 | Estimated genetic (lower triangular matrix) and phenotypic (upper triangular matrix) correlations between the morning and afternoon assessments of the four traits measured in the laboratory after exposure in nature. Size/color of the dots illustrate strength of correlations.

In summary the ongoing investigation of data from this experiment suggest some interesting patterns: 1) the genetic architecture of locomotor activity is specific to the time of day / temperature at which the individuals were tested, 2) there is high genetic correlations between heat tolerance and activity measures under heat exposure, 3) we found evidence for interactions between line and test time, i.e. genotype-by-environment interactions for all traits. Further analysis including data from the remaining test days where temperatures were higher will provide more information on abovementioned patterns.

References:

Araújo, M. B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P. A., Valladares, F., and Chown, S. L. (2013). Heat freezes niche evolution. *Ecol. Lett.* 16, 1206–1219. doi:10.1111/ele.12155.

Blackburn, S., Van Heerwaarden, B., Kellermann, V., and Sgro, C. M. (2014). Evolutionary capacity of upper thermal limits: Beyond single trait assessments. *J. Exp. Biol.* 217, 1918–1924. doi:10.1242/JEB.099184/257307.

Braschler, B., Chown, S. L., and Duffy, G. A. (2021). Sub-critical limits are viable alternatives to critical thermal limits. *J. Therm. Biol.* 101, 103106. doi:10.1016/J.JTHERBIO.2021.103106.

Burnet, B., Burnet, L., Connolly, K., and Williamson, N. (1988). A genetic analysis of locomotor activity in *Drosophila melanogaster*. *Heredity.* 61, 111–119. doi:10.1038/hdy.1988.96.

Bush, A., Mokany, K., Catullo, R., Hoffmann, A., Kellermann, V., Sgrò, C., et al. (2016). Incorporating evolutionary adaptation in species distribution modelling reduces projected vulnerability to climate change. *Ecol. Lett.* 19, 1468–1478. doi:10.1111/ELE.12696.

Charmantier, A., and Garant, D. (2005). Environmental quality and evolutionary potential: lessons from wild populations. *Proc. R. Soc. B Biol. Sci.* 272, 1415–1425. doi:10.1098/RSPB.2005.3117.

Chevin, L. M., and Hoffmann, A. A. (2017). Evolution of phenotypic plasticity in extreme environments. *Philos. Trans. R. Soc. B Biol. Sci.* 372, 20160138. doi:10.1098/RSTB.2016.0138.

Everatt, M. J., Bale, J. S., Convey, P., Worland, M. R., and Hayward, S. A. L. (2013). The effect of acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates. *J. Insect Physiol.* 59, 1057–1064. doi:10.1016/j.jinsphys.2013.08.003.

Falconer, D. S., and Mackay, T. F. C. (1996). *Introduction to quantitative genetics*. 4th ed. Longmans Green, Harlow: Essex, UK.

Hoffmann, A. A., Chown, S. L., and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: How constrained are they? *Funct. Ecol.* 27, 934–949. doi:10.1111/j.1365-2435.2012.02036.x.

Hoffmann, A. A., and Merilä, J. (1999). Heritable variation and evolution under favourable and unfavourable conditions. *Trends Ecol. Evol.* 14, 96–101. doi:10.1016/S0169-5347(99)01595-5.

Hoffmann, A. A., and Parsons, P. A. (1991). *Evolutionary genetics and environmental stress*. New York: Oxford University Press; Oxford Science Publications doi:10.1016/0169-5347(91)90017-r.

Huang, W., Massouras, A., Inoue, Y., Peiffer, J., Ràmia, M., Tarone, A. M., et al. (2014). Natural variation in genome architecture among 205 *Drosophila melanogaster* Genetic Reference Panel lines. *Genome Res.* 24, 1193–1208. doi:10.1101/GR.171546.113.

IPCC (2013). Climate Change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate

Change.
Jordan, K. W., Carbone, M. A., Yamamoto, A., Morgan, T. J., and Mackay, T. F. C. (2007). Quantitative genomics of locomotor behavior in *Drosophila melanogaster*. *Genome Biol.* 8, 1–17. doi:10.1186/GB-2007-8-8-R172/FIGURES/7.
Jordan, K. W., Morgan, T. J., and Mackay, T. F. C. (2006). Quantitative trait loci for locomotor behavior in *Drosophila melanogaster*. *Genetics* 174, 271–284. doi:10.1534/genetics.106.058099.
Jørgensen, K. T., Sørensen, G., and Bundgaard, J. (2006). Heat tolerance and the effect of mild heat stress on reproductive characters in *Drosophila buzzatii* males. *J. Therm. Biol.* 31, 280–286. doi:10.1016/j.jtherbio.2005.11.026.
Kearney, M., Porter, W. P., Williams, C., Ritchie, S., and Hoffmann, A. A. (2009). Integrating biophysical models and evolutionary theory to predict climatic impacts on species' ranges: the dengue mosquito *Aedes aegypti* in Australia. *Ecology* 23, 528–538.
Kellermann, V., van Heerwaarden, B., Sgrò, C. M., and Hoffmann, A. A. (2009). Fundamental evolutionary limits in ecological traits drive *Drosophila* species distribution. *Science.* 325, 3–5. doi:10.1126/science.1175443.
Kelly, M. W., Sanford, E., and Grosberg, R. K. (2012). Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. B Biol. Sci.* 279, 349–356. doi:10.1098/rspb.2011.0542.
Klepsatel, P., Gáliková, M., De Maio, N., Huber, C. D., Schlötterer, C., and Flatt, T. (2013). Variation in thermal performance and reaction norms among populations of *Drosophila melanogaster*. *Evolution.* 67, 3573–3587. doi:10.5061/dryad.vclql.
Kristensen, T. N., Ketola, T., and Kronholm, I. (2020). Adaptation to environmental stress at different timescales. *Ann. N. Y. Acad. Sci.* 1476, 5–12. doi:10.1111/nyas.13974.
Kristensen, T. N., Overgaard, J., Lassen, J., Hoffmann, A. A., and Sgrò, C. (2015). Low evolutionary potential for egg-to-adult viability in *Drosophila melanogaster* at high temperatures. *Evolution.* 69, 803–814. doi:10.1111/evo.12617.
MacKay, T. F. C., Richards, S., Stone, E. A., Barbadilla, A., Ayroles, J. F., Zhu, D., et al. (2012). The *Drosophila melanogaster* Genetic Reference Panel. *Nature.* 482, 173–178. doi:10.1038/nature10811.
MacLean, H. J., Kristensen, T. N., Overgaard, J., Sørensen, J. G., and Bahrndorff, S. (2017). Acclimation responses to short-term temperature treatments during early life stages causes long lasting changes in spontaneous activity of adult *Drosophila melanogaster*. *Physiol. Entomol.* 42, 404–411. doi:10.1111/PHEN.12212.
Menegazzi, P., Yoshii, T., and Helfrich-Förster, C. (2012). Laboratory versus nature: The two sides of the *Drosophila* circadian clock. *J. Biol. Rhythms* 27, 433–442. doi:10.1177/0748730412463181.
Mitchell, K. A., Sgrò, C. M., and Hoffmann, A. A. (2011). Phenotypic plasticity in upper thermal limits is weakly related to *Drosophila* species distributions. *Funct. Ecol.* 25, 661–670. doi:10.1111/j.1365-2435.2010.01821.x.
Ørsted, M., Hoffmann, A. A., Rohde, P. D., Sørensen, P., and Kristensen, T. N. (2019). Strong impact of thermal environment on the quantitative genetic basis of a key stress tolerance trait. *Heredity.* 122, 315–325. doi:10.1038/S41437-018-0117-7.

Parratt, S. R., Walsh, B. S., Metelmann, S., White, N., Manser, A., Bretman, A. J., et al. (2021). Temperatures that sterilize males better match global species distributions than lethal temperatures. *Nat. Clim. Chang.* 11, 481–484. doi:10.1038/s41558-021-01047-0.

Rohde, P. D., Østergaard, S., Kristensen, T. N., Sørensen, P., Loeschcke, V., Mackay, T. F. C., et al. (2018). Functional validation of candidate genes detected by genomic feature models. *G3 Genes, Genomes, Genet.* 8, 1659–1668. doi:10.1534/g3.118.200082.

Sales, K., Vasudeva, R., Dickinson, M. E., Godwin, J. L., Lumley, A. J., Michalczyk, Ł., et al. (2018). Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. *Nat. Commun.* 9, 4771. doi:10.1038/S41467-018-07273-Z.

Sales, K., Vasudeva, R., and Gage, M. J. G. (2021). Fertility and mortality impacts of thermal stress from experimental heatwaves on different life stages and their recovery in a model insect. *R. Soc. open Sci.* 8, 201717. doi:10.1098/RSOS.201717.

Schou, M. F., Kristensen, T. N., Pedersen, A., Karlsson, G., Loeschcke, V., and Malmendal, A. (2017). Metabolic and functional characterization of effects of developmental temperature in *Drosophila melanogaster*. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 312, R211–R222. doi:10.1152/ajpregu.00268.2016.

Sgrò, C. M., and Hoffmann, A. A. (2004). Genetic correlations, tradeoffs and environmental variation. *Heredity.* 93, 241–248. doi:10.1038/sj.hdy.6800532.

Shaw, B., Fountain, M., and Wijnen, H. (2019). Control of daily locomotor activity patterns in *Drosophila suzukii* by the circadian clock, light, temperature and social interactions. *J. Biol. Rhythms* 34, 463–481. doi:10.1177/0748730419869085.

Sinclair, B. J., Coello Alvarado, L. E., and Ferguson, L. V (2015). An invitation to measure insect cold tolerance: Methods, approaches, and workflow. *J. Therm. Biol.* 53, 180–197. doi:10.1016/j.jtherbio.2015.11.003.

Sørensen, J. G., Kristensen, T. N., and Overgaard, J. (2016). Evolutionary and ecological patterns of thermal acclimation capacity in *Drosophila*: is it important for keeping up with climate change? *Curr. Opin. Insect Sci.* 17, 98–104. doi:10.1016/J.COIS.2016.08.003.

Sunday, J. M., Bates, A. E., Kearney, M. R., Colwell, R. K., Dulvy, N. K., Longino, J. T., et al. (2014). Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proc. Natl. Acad. Sci. U. S. A.* 111, 5610–5615. doi:10.1073/pnas.1316145111.

van Heerwaarden, B., and Sgrò, C. M. (2021). Male fertility thermal limits predict vulnerability to climate warming. *Nat. Commun.* 12, 1–11. doi:10.1038/s41467-021-22546-w.

Van Heerwaarden, B., and Sgrò, C. M. (2011). The effect of developmental temperature on the genetic architecture underlying size and thermal clines in *Drosophila melanogaster* and *D. simulans* from the east coast of Australia. *Evolution* 65, 1048–1067. doi:10.1111/J.1558-5646.2010.01196.X.

Walsh, B. S., Parratt, S. R., Mannion, N. L. M., Snook, R. R., Bretman, A., and Price, T. A. R. (2021). Plastic responses of survival and fertility following heat stress in pupal and

adult *Drosophila* virilis. *Ecol. Evol.* 11, 18238–18247. doi:10.1002/ECE3.8418.
Weigensberg, I., and Roff, D. A. (1996). Natural heritabilities: can they be reliably estimated in the laboratory? *Evolution.* 50, 2149–2157. doi:10.1111/J.1558-5646.1996.TB03605.X.

SUPPLEMENTAL MATERIALS FOR ADDITIONAL RESULTS

Table S1: DGRP lines included in the study

Figure S1: Reaction norms for the four heat performance phenotypes across environments

Table S1 | DGRP line IDs. The table provides and overview of the DGRP lines included in the experiment original RAL-IDs.

Synonym (Bloomington)	stock	ID (homemade)	Genotype (Bloomington)
RAL-21	28122	1	21
RAL-26	28123	2	26
RAL-28	28124	3	28
RAL-38	28125	4	38
RAL-41	28126	5	41
RAL-45	28128	6	45
RAL-59	28129	7	59
RAL-69	28130	8	69
RAL-73	28131	9	73
RAL-75	28132	10	75
RAL-83	28134	11	83
RAL-88	28135	12	88
RAL-91	28136	13	91
RAL-93	28137	14	93
RAL-101	28138	15	101
RAL-105	28139	16	105
RAL-109	28140	17	109
RAL-129	28141	18	129
RAL-136	28142	19	136
RAL-138	28143	20	138
RAL-142	28144	21	142
RAL-149	28145	22	149
RAL-153	28146	23	153
RAL-161	28148	24	161
RAL-176	28149	25	176
RAL-177	28150	26	177
RAL-181	28151	27	181
RAL-189	28152	28	189

Table S1 continued

Synonym (Bloomington)	stock	ID (homemade)	Genotype (Bloomington)
RAL-195	28153	29	195
RAL-208	25174	30	208
RAL-217	28154	31	217
RAL-227	28156	32	227
RAL-228	28157	33	228
RAL-239	28161	34	239
RAL-280	28164	35	280
RAL-287	28165	36	287
RAL-301	25175	37	301
RAL-303	25176	38	303
RAL-304	25177	39	304
RAL-307	25179	40	307
RAL-309	28166	41	309
RAL-313	25180	42	313
RAL-315	25181	43	315
RAL-317	28167	44	317
RAL-318	28168	45	318
RAL-324	25182	46	324
RAL-332	28171	47	332
RAL-335	25183	48	335
RAL-336	28172	49	336
RAL-338	28173	50	338
RAL-340	28174	51	340
RAL-350	28176	52	350
RAL-356	28178	53	356
RAL-357	25184	54	357
RAL-358	25185	55	358
RAL-359	28179	56	359
RAL-360	25186	57	360
RAL-361	28180	58	361
RAL-362	25187	59	362

Table S1 continued

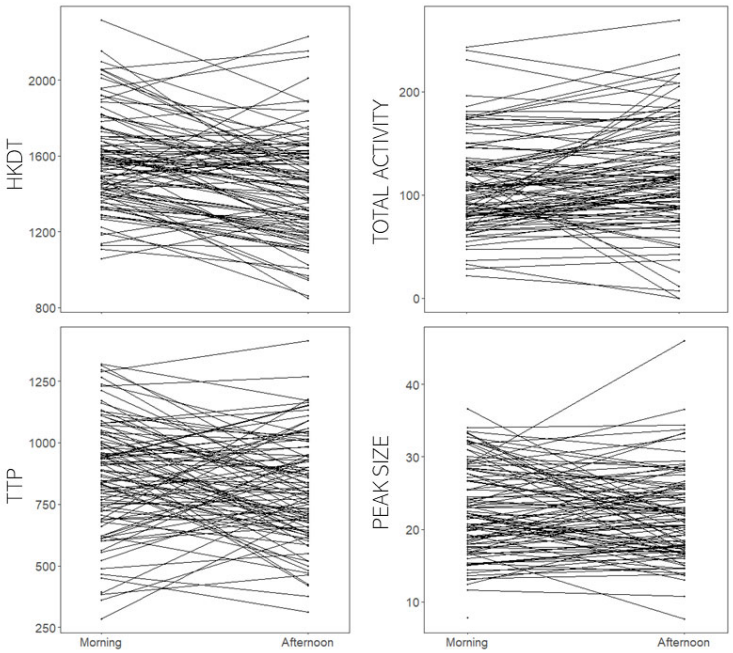
Synonym (Bloomington)	stock	ID (homemade)	Genotype (Bloomington)
RAL-365	25445	60	365
RAL-370	28182	61	370
RAL-371	28183	62	371
RAL-373	28184	63	373
RAL-374	28185	64	374
RAL-375	25188	65	375
RAL-377	28186	66	377
RAL-379	25189	67	379
RAL-380	25190	68	380
RAL-381	28188	69	381
RAL-382	28189	70	382
RAL-383	28190	71	383
RAL-385	28191	72	385
RAL-386	28192	73	386
RAL-391	25191	74	391
RAL-392	28194	75	392
RAL-399	25192	76	399
RAL-426	28196	77	426
RAL-427	25193	78	427
RAL-437	25194	79	437
RAL-440	28197	80	440
RAL-441	28198	81	441
RAL-443	28199	82	443
RAL-461	28200	83	461
RAL-486	25195	84	486
RAL-491	28202	85	491
RAL-492	28203	86	492
RAL-502	28204	87	502
RAL-508	28205	88	508
RAL-517	25197	89	517
RAL-531	28207	90	531

Table S1 continued

Synonym (Bloomington)	stock	ID (homemade)	Genotype (Bloomington)
RAL-535	28208	91	535
RAL-555	25198	92	555
RAL-563	28211	93	563
RAL-584	28212	94	584
RAL-589	28213	95	589
RAL-595	28215	96	595
RAL-639	25199	97	639
RAL-646	28217	98	646
RAL-703	28218	99	703
RAL-705	25744	100	705
RAL-707	25200	101	707
RAL-712	25201	102	712
RAL-714	25745	103	714
RAL-716	28219	104	716
RAL-721	28220	105	721
RAL-730	25202	106	730
RAL-732	25203	107	732
RAL-738	28223	108	738
RAL-748	28224	109	748
RAL-757	28226	110	757
RAL-761	28227	111	761
RAL-765	25204	112	765
RAL-774	25205	113	774
RAL-776	28229	114	776
RAL-783	28230	115	783
RAL-786	25206	116	786
RAL-787	28231	117	787
RAL-790	28232	118	790
RAL-796	28233	119	796
RAL-799	25207	120	799
RAL-801	28234	121	801

Table S1 continued

Synonym (Bloomington)	stock	ID (homemade)	Genotype (Bloomington)
RAL-802	28235	122	802
RAL-804	28236	123	804
RAL-805	28237	124	805
RAL-820	25208	125	820
RAL-852	25209	126	852
RAL-859	25210	127	859



Supplemental Figure S1 | Reaction norms for the four heat performance phenotypes. Mean trait value for each line tested in the morning and afternoon after acclimatization to different field temperatures. Each black line represents the reaction norm for a DGRP line.

APPENDIX A

Papers included in PART A:

Noer, N. K., Pagter, M., Bahrndorff, S., Malmendal, A., and Kristensen, T. N. (2020). Impacts of thermal fluctuations on heat tolerance and its metabolomic basis in *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Orchesella cincta*. *PLoS One* 15, e0237201. doi:10.1371/journal.pone.0237201.

Sørensen, M. H., Kristensen, T. N., Lauritzen, J. M. S., **Noer, N. K**., Høye, T. T., and Bahrndorff, S. (2019). Rapid induction of the heat hardening response in an Arctic insect. *Biol. Lett.* 15, 20190613. doi:10.1098/rsbl.2019.0613.

Additional co-authored papers:

Bak, C. W., Bahrndorff, S., **Noer, N. K**., Jørgensen, L. B., Overgaard, J., and Kristensen, T. N. (2020). Comparison of static and dynamic assays when quantifying thermal plasticity of drosophilids. *Insects* 11, 1–11. doi:10.3390/insects11080537.

Laursen, S. F., Hansen, L. S., Bahrndorff, S., Nielsen, H. M., **Noer, N. K**., Renault, D., et al. (2021). Contrasting manual and automated assessment of thermal stress responses and larval body size in black soldier flies and houseflies. *Insects* 12, 380. doi:10.3390/INSECTS12050380/S1.

Popular science publications:

Kristensen, T. N., **Noer, N. K.**, and Pertoldi, C. (2019). Genetikken giver os nye muligheder for at redde truede arter (Genetics provide novel tools to save threatened species). Videnskab.dk. https://videnskab.dk/naturvidenskab/genetikken-giver-os-nye-muligheder-for-at-redde-truede-arter

SUMMARY

Terrestrial arthropods are continuously exposed to temperature changes at multiple spatiotemporal scales. Because of the tight link between arthropod physiology and the environment, they must anticipate and respond to these changes within and across generations to maintain high fitness. Hence, survival and reproductive success to temperature variation and global warming depend on phenotypic plasticity in physiological, behavioral and morphological responses, and on evolutionary adaptation. Currently, knowledge on species ability to respond and adapt to environmental stress is based on studies conducted under laboratory condition using model organisms. These estimates have little external validity and often conclusions based on laboratory studies does not match fitness in nature.

This thesis investigates the extent to which phenotypic plasticity occurs in species in their natural environments with a focus on the temporal scale that the species respond to temperature fluctuations, the climatic predictors of plastic changes, and the evolutionary adaptation to environments characterized by different thermal variabilities. This is supplemented with a mechanistic understanding of the underlying molecular basis behind field-based plastic responses. With these endeavors, this thesis provides an ecological context for phenotypic plasticity and evolutionary adaptation.

ISSN (online): 2446-1636
ISBN (online): 978-87-7573-837-3

AALBORG UNIVERSITY PRESS