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Yuanjie Li. Impact of climate change on aphid communities : physiological and molecular responses to temperature. Ecology, environment. Université de Rennes, 2023. English. NNT : 2023URENB033 . tel-04707691

HAL Id: tel-04707691

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THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES

ECOLE DOCTORALE N° 600

Ecologie, Géosciences, Agronomie, Alimentation

Spécialité : *Ecologie Evolution*

Par

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Impact of climate change on aphid communities: physiological and molecular responses to temperature

Thèse présentée et soutenue à Rennes, le 21 Septembre 2023

Unité de recherche : UMR 6553 ECOBIO

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Résumé

Le changement climatique entraîne une augmentation de la température moyenne mais aussi des températures extrêmes plus fréquentes et plus intenses, et ces hausses devraient encore augmenter dans un avenir proche. Les insectes, qui sont des ectothermes, sont sensibles aux changements de température de leur environnement. Les écologistes sont donc confrontés au défi qui consiste à comprendre et anticiper comment le réchauffement climatique et les températures extrêmes influencent les réponses des individus et des populations d'insectes, ainsi que les conséquences écologiques sur la structure de la communauté et le fonctionnement de l'écosystème. Pour élucider les liens de causalité entre le changement climatique et les réponses des insectes, il est crucial de comprendre les liens mécanistiques qui existent entre les stress environnementaux causés par le changement climatique et les fonctions biologiques des organismes. Dans cette optique, l'étude physiologique des réponses des organismes au changement climatique, y compris leurs limites de tolérance thermique, est essentielle.

Cette thèse se concentre sur les guildes de pucerons des céréales, comprenant trois espèces : *Sitobion avenae*, *Rhopalosiphum padi* et *Metopolophium dirhodum*. Ces pucerons sont mondialement connus comme des ravageurs de cultures et des vecteurs de divers virus, dont le virus de la jaunisse nanisante de l'orge (BYDV) qui réduit considérablement les rendements céréaliers. Des observations de terrain menées dans l'est de la Chine et dans l'ouest de la France ont révélé l'influence notable du changement climatique sur l'abondance relative de ces trois espèces de pucerons : *S. avenae* était auparavant considéré comme l'espèce dominante de la guildes. Cependant, avec l'augmentation de la fréquence et de l'intensité des épisodes de températures extrêmes, *R. padi* a progressivement bénéficié de ces conditions, ce qui a entraîné un changement dans la dominance relative annuelle des populations de pucerons dans certaines régions méridionales de la Chine. Inversement, *M. dirhodum* semblait préférer les zones plus fraîches du nord-ouest de la Chine. Ces pucerons apparaissent en petites populations au début du printemps et disparaissent ensuite au cours des saisons.

L'hypothèse principale de cette thèse est que la permutation des espèces dominantes (*S. avenae* remplacé par *R. padi*) pourrait être étroitement liée au changement climatique en cours, qui pourrait affecter les espèces concernées de manière non uniforme et disproportionnée, en favorisant certaines espèces (« *the winners* ») et en défavorisant d'autres (« *the losers* »). En effet, si les espèces de la guildes possèdent des niveaux différents de tolérance thermique, cela conduirait à ces phénomènes de changement de communauté, comme cela a déjà été observé dans d'autres taxons. Les différents niveaux de tolérance thermique (c'est-à-dire les réponses individuelles) peuvent être la conséquence de réponses physiologiques distinctes au stress thermique (c'est-à-dire les réponses individuelles).

L'accumulation de cryoprotectants (molécules de faible poids moléculaire) et la synthèse de protéines de choc thermique sont des mécanismes physiologiques essentiels pour améliorer la capacité

à tolérer le stress thermique chez de nombreuses espèces, y compris les espèces d'insectes. Ainsi, des tolérances thermiques différentielles chez les différentes espèces de pucerons pourraient provenir de l'induction de réponses métaboliques et moléculaires distinctes au cours du stress thermique.

Pour tester les hypothèses mentionnées ci-dessus, nous avons mené une série d'expériences pour comparer les variations interspécifiques des tolérances au chaud et au froid chez les trois espèces de pucerons (*Sitobion avenae*, *Rhopalosiphum padi* et *Metopolophium dirhodum*) afin de relier ces variations aux changements dans l'abondance de la communauté de pucerons (chapitre 2). En outre, nous avons étudié les différences dans les niveaux d'expression des gènes des protéines de choc thermique (*hsps*) entre les trois espèces de pucerons en réponse au stress thermique chaud et froid (chapitre 3). Enfin, nous avons effectué une analyse comparative de la réponse métabolique au stress thermique chaud et froid chez les trois espèces de pucerons par des méthodes de métabolomique ciblée en GC-MS (chapitres 4 et 5).

Dans le chapitre 2, nous avons utilisé une approche de « *thermal tolerance landscape* » pour comparer la tolérance thermique des trois espèces de pucerons (*M. dirhodum*, *S. avenae*, et *R. padi*). Plus précisément, nous avons construit des modèles de temps de mort thermique (TDT) basés sur des jeux de données complexes de survie (à différentes températures et durées d'exposition) des trois espèces de pucerons, et ce, à trois âges différents. Nous avons pour cela testé une large gamme de températures chaudes (34~40 °C) et froides (-3~-11 °C). En utilisant les paramètres TDT, nous avons effectué une évaluation du risque de létalité thermique, en calculant l'accumulation potentielle de lésions thermiques quotidiennes associées aux variations régionales de température dans trois sites de culture du blé le long d'un gradient de latitude en Chine. Nos résultats ont révélé que *R. padi* était plus tolérant à la chaleur et plus vulnérable au froid que *S. avenae* et *M. dirhodum*. Pendant l'hiver, nous avons estimé que *R. padi* accumulait plus de lésions dues au froid que les deux autres espèces, tandis que *M. dirhodum* accumulait plus de lésions dues à la chaleur pendant l'été. Les individus se développant dans le site le plus chaud présentaient des risques plus élevés de lésions dues à la chaleur et ceux du site le plus frais présentaient des risques plus élevés de lésions dues au froid le long d'un gradient de latitude. De plus, les jeunes nymphes ont généralement montré une tolérance thermique plus faible que les nymphes plus âgées ou les adultes. Ces résultats corroborent les récentes observations de terrain selon lesquelles la proportion relative des populations de *R. padi* augmente avec la fréquence accrue des vagues de chaleur. En résumé, cette recherche a montré que des différences interspécifiques de tolérance thermique pourraient être à l'origine de changements dans l'abondance des communautés de pucerons.

Les protéines de choc thermique (HSP) sont des chaperons moléculaires bien connus pour leur rôle vital dans la protection des cellules et des protéines en particulier contre les effets néfastes du stress thermique. Néanmoins, peu d'études sur les chaperons moléculaires ont été menées chez les pucerons des céréales. Dans le chapitre 3, nous avons examiné les profils d'expression de sept gènes *hsp* (*hsp10*, *hsp60*, *hsc70*, *hsp70A*, *hsp70B*, *hsp75*, *hsp90*) après des expositions correspondant à des

niveaux équivalents de lésions thermiques (c'est-à-dire le temps létal médian, Lt_{50}) mais aussi après des durées identiques d'exposition à des températures élevées (34 et 37 °C) ou basses (-5 et -8 °C). Nous avons émis l'hypothèse selon laquelle une réponse moléculaire au stress différentielle entre les espèces pourrait expliquer en partie les variations interspécifiques de tolérance au chaud et au froid comme observé dans le chapitre 2. Nos résultats ont montré que : 1) Les gènes *Hsps* sont induits plus fortement par la chaleur que par le froid, *hsp70A* était le gène le plus exprimé en réponse aux deux facteurs de stress. 2). *R. padi* avait plus de gènes inductibles au chaud et des niveaux d'ARNm de *hsp70A*, *hsp10*, *hsp60* et *hsp90* significativement plus élevés que les deux autres espèces après une exposition au chaud, et ce, pour les deux niveaux de stress (i.e. lésion similaire ou durée similaire). 3) A 37 °C, les *Hsps* ont cessé d'être exprimées chez *M. dirhodum* et *S. avenae*, tandis que l'expression a été maintenue chez *R. padi*. 4) *M. dirhodum* avait plus de gènes inductibles au froid que les deux autres espèces de pucerons étudiées. Cet ensemble de données confirme des réponses moléculaires différentielles entre espèces, avec des patrons qui vont dans le sens de nos hypothèses.

Dans le chapitre 4, nous avons comparé les signatures métaboliques de ces espèces de pucerons lorsqu'elles sont exposées au stress chaud par un profilage par chromatographie en phase gazeuse et spectrométrie de masse (GC-MS). Nous avons comparé deux conditions expérimentales : 1) une exposition à 34 °C pendant des durées croissantes, correspondant au $1/2 Lt_{50}$ (temps létal médian) et au $2/2 Lt_{50}$ propres à chaque espèce, ainsi que 2) une même durée d'exposition pour les trois espèces. Nos résultats indiquent que l'homéostasie métabolique des pucerons est perturbée pendant le stress thermique chaud, les degrés de perturbation augmentant au fur et à mesure que la durée d'exposition s'allonge. Pour des pucerons exposés à un niveau de blessure modéré ($1/2 Lt_{50}$) une période de récupération a permis de rétablir complètement les profils métaboliques (homéostasie). En revanche, les profils métaboliques des pucerons exposés à un niveau de blessure plus élevé ($2/2 Lt_{50}$) sont restés distincts de ceux des témoins (perte d'homéostasie). Pendant le stress thermique, les pucerons ont présenté une diminution des niveaux de saccharose et une accumulation des intermédiaires du cycle Krebs, ainsi que des produits finaux du métabolisme anaérobie, à savoir l'alanine et l'acide lactique. Ces résultats suggèrent une dépendance significative au métabolisme anaérobie. Le mannitol est apparu comme le principal polyol impliqué dans la réponse au stress thermique chaud chez les trois espèces. Le glycérol ne s'est accumulé de manière significative que chez l'espèce la plus tolérante au chaud, *R. padi*. En outre, l'espèce de puceron la plus tolérante au chaud, *R. padi*, a montré moins de perturbations dans les profils métaboliques mais des niveaux d'accumulation de polyols nettement plus élevés que les autres espèces lorsqu'elle est exposée à des températures élevées pendant la même durée. Cet ensemble de données confirme encore une fois des réponses physiologiques et métaboliques différentielles entre espèces, avec des patrons qui vont à nouveau dans le sens des hypothèses.

Dans le chapitre 5, nous avons à nouveau effectué une analyse métabolomique comparative par GC-MS sur les trois espèces de pucerons afin d'obtenir des informations sur leur capacité à tolérer

cette fois un stress froid. Plus précisément, nous avons soumis les pucerons à une basse température (-5 °C) pendant des durées croissantes correspondant à 1/2 Lt₅₀ et 2/2 Lt₅₀ (i.e. intensité croissante des lésions), ainsi qu'à une durée d'exposition identique pour les trois espèces. Les résultats ont indiqué que: 1) Le stress du froid perturbait de manière significative l'homéostasie métabolique des pucerons, avec des perturbations plus sévères au fur et à mesure que la durée d'exposition et donc les lésions au froid augmentaient. 2) Les espèces les plus tolérantes au froid (*M. dirhodum* et *S. avenae*) présentent des profils métaboliques similaires pendant le stress, tandis que l'espèce sensible au froid (*R. padi*) présente des profils distincts. Lors de la récupération après le stress froid, les espèces tolérantes au froid présentent des niveaux accrus de cryoprotecteurs (par exemple, tréhalose, proline et asparagine). 3) Lorsqu'exposée à un stress froid de durée identique, l'espèce sensible au froid (*R. padi*) présente un plus grand nombre de métabolites dont les concentrations sont altérées que les deux autres espèces ; signe d'une perturbation plus intense. Cela suggère que cette espèce a une plus faible capacité à maintenir l'homéostasie métabolique, en particulier dans le métabolisme énergétique. En conclusion, ces résultats impliquent que la tolérance au froid est associée à une inertie/robustesse métabolique et que cette capacité physiologique diffère bien entre espèces comme nous l'avions supposé.

Cette recherche confirme les hypothèses de départ à savoir que les espèces de la guilda possèdent des niveaux différents de tolérance thermique et que ces différences se manifestent par des réponses physiologiques au stress distinctes (moléculaires et métaboliques). Cela pourrait conduire *in fine* à des phénomènes de changement dans la communauté. Ce travail comporte certaines limites, qui sont discutées dans la partie discussion (chapitre 6).

Pour dresser des pistes d'amélioration et perspectives, nous pouvons souligner que le modèle d'évaluation des risques basé sur les courbes TDT du chapitre 2 ne tenait pas compte des facteurs tels que la capacité de régulation thermique de l'insecte, des effets d'acclimatation, et de l'impact du stress thermique sur d'autres traits du cycle de vie (par exemple, la fécondité). Ces facteurs devront être considérés car ils peuvent entraver notre capacité à extrapoler les processus du niveau individuel au niveau populationnel. Ensuite, il est évident que les gènes *hsps* mesurés dans le chapitre 3 ne constituent qu'un sous-ensemble de tous les chaperons. De plus la manière dont l'expression différentielle de ces gènes confère, ou pas, une thermotolérance différentielle aux trois espèces de pucerons dans des environnements naturels devra encore être vérifiée expérimentalement. Avec la méthode analytique quantitative mais ciblée utilisée dans les chapitres 4 et 5, nous n'avons quantifié qu'un sous-ensemble des petites molécules (32 métabolites). Il était donc inopportun de tenter d'extrapoler sur les voies métaboliques potentiellement impliquées. En outre, les changements métaboliques observés pouvaient soit indiquer des ajustements biochimiques adaptatifs (par exemple, l'accumulation de cryoprotectants), soit représenter des conséquences de capacités physiologiques perturbées. Par conséquent, il reste à étudier si l'accumulation de certains métabolites tels qu'observés chez certaines espèces, peut effectivement conférer une plus grande tolérance thermique au chaud comme au froid.

Au cours de cette thèse, nous avons mis en évidence les variations interspécifiques substantielles de tolérance thermique entre les trois espèces de pucerons. Ces variations peuvent être sans doute partiellement attribuées à leurs réponses moléculaires et métaboliques distinctes au stress thermique. L'espèce tolérante à la chaleur, *R. padi*, possédait des mécanismes de thermoprotection plus robustes contre le stress thermique chaud que les deux autres espèces. Cette thermoprotection renforcée contribue possiblement à la meilleure survie des pucerons. Cela se traduirait *in fine* par des bénéfices pour les populations au sein des communautés confrontées à des événements extrêmes (chauds) de plus en plus fréquents et intenses liés au changement climatique. En examinant de manière exhaustive ces réponses aux niveaux individuel et sub-individuel (niveaux biochimique et moléculaire), notre recherche a contribué à une meilleure compréhension des liens mécanistiques entre les stress environnementaux causés par le changement climatique et les fonctions biologiques des organismes. Bien que l'identification des mécanismes biochimiques et moléculaires régissant la tolérance thermique chez ces espèces à compléter, nos travaux nous ont permis de proposer et d'affiner plusieurs hypothèses. Dans l'ensemble, cette étude met en lumière la relation complexe entre le stress thermique et la survie des espèces. Elle améliore notre compréhension de la dynamique écologique et de la résilience des populations dans le contexte de conditions climatiques changeantes. Ces connaissances ont des implications pour les stratégies de conservation et de gestion des ravageurs face au changement climatique.

Acknowledgements

I am sincerely grateful for the immense support and assistance I have received throughout my PhD journey.

First and foremost, I would like to express my heartfelt appreciation to my supervisors, Hervé Colinet, Chun-Sen Ma, and David Renault. Their invaluable expertise is crucial in shaping the research questions and methodologies of this PhD. I am grateful for their insightful feedback and unwavering support, which elevated the quality of my work. I would also like to thank Joan Van Baaren for providing valuable comments on my research.

I am grateful to Si-Yang Chen, Yi Yan, and Xiang-Rui Li from Institute of Plant Protection, Chinese Academy of Agricultural Sciences, for their assistance during the experiment.

I would also like to thank Nathalie Le Bris and Virginie Daburon from Ecobio for providing technical trainings.

I would like to acknowledge Lisa Bjerregaard Jørgensen and Johannes Overgaard from Aarhus University for their contributions, which greatly enriched my research experience. I am grateful for their wonderful cooperation.

I extend my profound gratitude to my family members, for their unwavering support.

Finally, I would like to express my thanks to my friends and colleagues in China and France for their delightful company.

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Chapter 1 General introduction

1 The impact and risks of climate change on insects

1.1 *Climate change and extreme temperatures*

Climate has warmed at an unprecedented rate. The global average temperature has increased by 1.09 °C (with a range of 0.95 to 1.2 °C) between 2011-2020 and 1850-1900, with larger increases observed over land (1.59 °C) than over oceans (0.88 °C) (Allan et al. 2021). Global climate impacts will increase significantly after 2100 without rapid mitigation (Lyon et al. 2022). Even with substantial greenhouse gas mitigation, there is still a possibility of failing to keep global warming below the 2 °C threshold that the United Nations Paris Agreement aims to achieve (Diffenbaugh and Barnes 2023). Climate change is also expected to increase temperature variability (Folland et al. 2002, Ummenhofer and Meehl 2017), which can result in more frequent and intense extreme temperature events (Figure 1). The projected changes in the variance of daily minimum and maximum temperatures in some north-temperate regions may experience an increase of up to 3 °C (Donat and Alexander 2012).

As a result of the increasing mean temperature and temperature variability (Figure 1), heat waves, such as those that occurred in Chicago in 1995 and Paris in 2003, are expected to become more intense, frequent, and longer lasting in the second half of the 21st century (Meehl and Tebaldi 2004, Ummenhofer and Meehl 2017). Despite a global increase in mean winter temperatures and a decrease in extremely low minimum temperature, predicting the temporal variation in winter climate at the regional level is difficult due to the complex interactions (e.g., the atmospheric circulation pattern) (Williams et al. 2015). Cold temperature anomalies can occur due to atmospheric circulation patterns, such as the negative phase of the North Atlantic Oscillation in Europe in 2010 (Cattiaux et al. 2010). Arctic sea ice loss may also lead to more cold extremes over mid-latitude continents (Palmer 2014, Sun et al. 2016). The combination of warming trends and temperature variability associated with climate change has caused previously rare spring frost damage events to become more common in recent decades, especially in Europe and Asia (Augspurger 2013, Zohner et al. 2020). Overall, from a biological perspective, some organisms may still experience recurring or more severe cold spells in the future, despite an overall trend of winter warming (Williams et al. 2015, Teets et al. 2022)

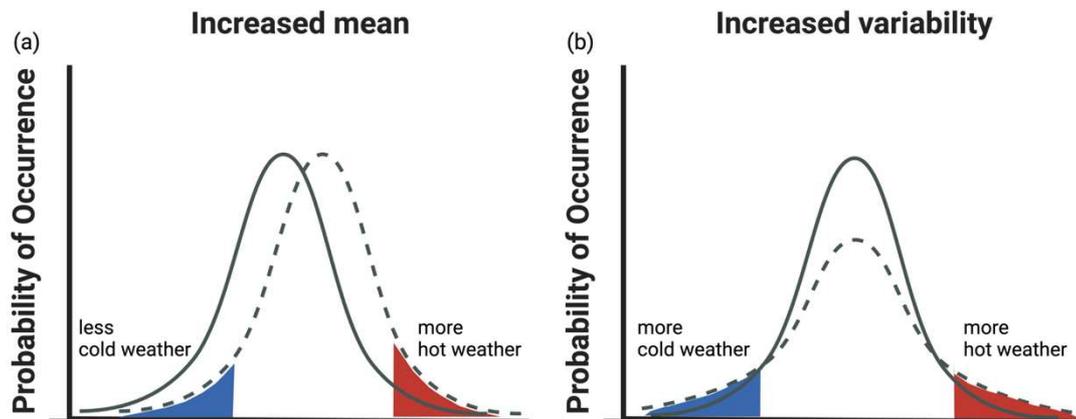


Figure 1. Schematic highlighting the effect of changes in the temperature distribution on extreme climate events occurrence between present and future climate conditions. (a) effects of a simple shift of the entire distribution toward a warmer climate; (b) effects of an increase in temperature variability with no shift in the mean. Adapted from Ummenhofer and Meehl (2017).

1.2 The impact of climate change on insects

Insects are a remarkably diverse group of animals and comprise 80% of animal life on Earth. They provide critical ecological services such as dung burial, pest control, pollination, and wildlife nutrition (Losey and Vaughan 2006). Being ectothermic, insects do not produce heat through metabolic processes and instead rely on the external environment to maintain their function (Björkman and Niemelä 2015). Therefore, insects are highly susceptible to temperature changes in the environment. Generally, how ambient temperature affects different attributes of ectotherms, from protein function to life-history traits, can be described as a thermal performance curve (TPC) (Figure 2) (Rezende and Bozinovic 2019). Typically, this curve is nonlinear and asymmetric with an optimum temperature (T_{opt}) and the operative temperature range between the critical minimum temperature (T_{min}) and the critical maximum temperature (T_{max}) (Huey and Kingsolver 1989).

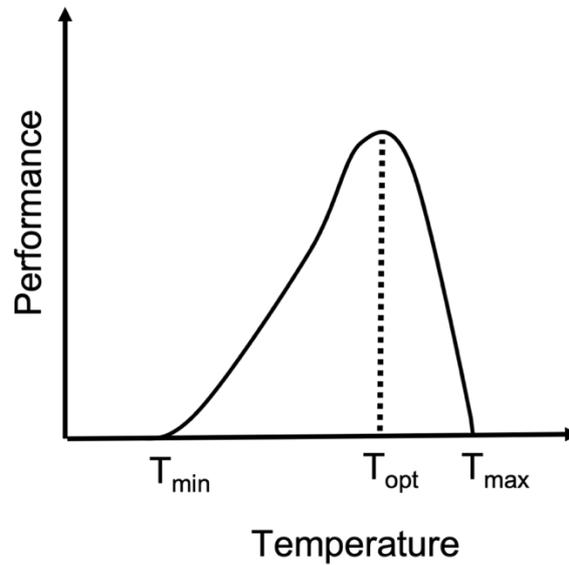


Figure 2. A hypothetical thermal performance curve (TPC). The temperature at which performance is maximized is termed the T_{opt} . The points at which performance is zero are termed the critical temperatures T_{max} and T_{min} .

Over the past two decades, the impacts of warming on insects have been widely recognized by ecologists and extensively reviewed (Parmesan and Yohe 2003, Musolin and Saulich 2012, Vasseur et al. 2014). Briefly, in response to rising mean temperatures, various insect species have begun to shift their ranges towards the north or to higher elevations as well as shrink their distribution in the southern edges of their ranges (Parmesan 2006, Chen et al. 2011, Kerr et al. 2015). Yet this 'climatic emigration' is not always possible for many species for a variety of reasons: lack of connectivity between habitats, low dispersal capacity, necessary interactions with other organisms, or simply lack of access to microhabitats (Ofori et al. 2017). Phenology in the onset of development advances in spring and shifts later in the autumn (Musolin and Saulich 2012). The altered phenology and distribution of individual species are likely to disrupt species interaction, like pollination (Memmott et al. 2007). The individual responses to climate change may affect the structure and composition of communities through cascading effects, such as altered species interaction and changes in ecosystem function (Filazzola et al. 2021). It has been reported that downstream thermophilic invertebrate taxa in France have replaced upstream cold-water taxa, during a period when the water temperature has increased by 1.5 °C (Daufresne et al. 2004).

Recent research has shifted its attention to climatic extremes and variability. Because temperature variations can affect species more significantly than changes in mean temperatures (Paaijmans et al. 2013, Colinet et al. 2015, Ma et al. 2015a). The widespread bumble bee species decline can be explained by the increasing frequency of high temperatures that exceed historically

observed tolerances (Soroye et al. 2020). Temperature extremes are likely to asymmetrically affect different insect species living within the same community for instance, three co-occurring aphid species differed in their demographic responses when exposed to high-temperature extremes, which cause shifts in insect community features (Ma et al. 2015b). Extremely warm mid-winter temperatures cause an irreversible loss of winter acclimatization and the resumption of development in the emerald ash borer *Agrilus planipennis* making them susceptible to subsequent cold snaps (Sobek-Swant et al. 2012). Species that enter dormancy early in autumn will experience increased energy drain due to delayed winter onset, and higher thermal variability in autumn may worsen this effect (Williams et al. 2015).

Few studies have focused on the effects of cold spells, despite their potential to severely impact ecosystem structure. A notable example is the severe cold spell that occurred in early 2010 in Subtropical Florida, which had devastating consequences for terrestrial and marine species, including coral reef communities, non-native crabs, and important gamefish (Lirman et al. 2011). Additionally, cold spells have been associated with reduced occurrences of outbreaks by some tree-killing pests. Large drops in temperature without previous cold hardening can be energetically demanding and result in rapid lethal effects, ultimately diminishing the tree-killing ability of the next season's progeny (Sambaraju et al. 2012). Furthermore, cold spells can alter the abundance of species within communities due to their species-specific response to low temperatures, as observed in a parasitoid system (Bonsignore et al. 2020).

Despite the extensive research, it is still hard to conclude how climate change will impact individuals, populations, and communities in the future. This is because responses to these changes are individualistic, multifaceted, and ecologically complex (Harvey et al. 2020, Lehmann et al. 2020). A meta-analysis of 31 major global pest species' response to ongoing climate warming suggests that a single species can both increase and decrease in severity, highlighting the need for individual species assessment to predict their response more accurately (Lehmann et al. 2020). Furthermore, even within the same broad taxonomic groups, species exhibit individualistic dynamics, which are likely driven by their responses to different short-term events associated with climatic variability (Palmer et al. 2017).

1.3 Assessing the vulnerability of insects to climate change

Assessing the vulnerability of insects to climate change is crucial due to their significant ecological and economic roles. The vulnerability of a species is determined by its exposure and sensitivity to climate change (Williams et al. 2008). Sensitivity is determined by intrinsic factors including thermal traits such as physiological tolerance limits (Williams et al. 2008). One commonly used thermal trait is the thermal safety margin (TSM). Various definitions are in use, but TSM is generally inversely proportional to the risk of an animal experiencing temperatures above T_{\max} (Sinclair et al. 2016). As illustrated in Figure 3a, we use the definition of TSM given by Deutsch et al. (2008), which is the difference between an organism's optimal temperature (T_{opt}) and the air

temperatures it typically encounters in its habitat (T_{hab}). TSM often increases considerably with latitude (Figure 3b) (Deutsch et al. 2008). This suggests that tropical insects would be more vulnerable than temperate species because they already live close to their optimal temperature and could easily reach their upper limits under global warming, while temperate species may benefit from global warming because current mean temperatures tend to be well below their optimal temperatures (Deutsch et al. 2008, Diamond et al. 2012). However, these results did not consider daily thermal fluctuations, which may increase the frequency of extreme temperatures, depress thermal safety margins, and exacerbate the potentially detrimental effects of climate change (Vasseur et al. 2014). Using fine-grained climate projections, it has been discovered that temperate species would be at the greatest risk of performance declines, and mid-latitude species would be particularly susceptible to heat stress (Kingsolver et al. 2013, Vasseur et al. 2014). Nevertheless, these studies typically rely on air temperature, which may not reflect an ectotherm's body temperature. A study using operative temperatures, which estimated an ectotherm's steady-state body temperature and provided more biophysically accurate indices of microclimates experienced by ectotherms, predicted that most ectotherms do not have a physiological TSM for heat, indicating they must rely on behavior to avoid overheating (Sunday et al. 2014). Likewise, species living at temperate latitudes and in alpine habitats must retreat to avoid lethal cold exposure (Sunday et al. 2014).

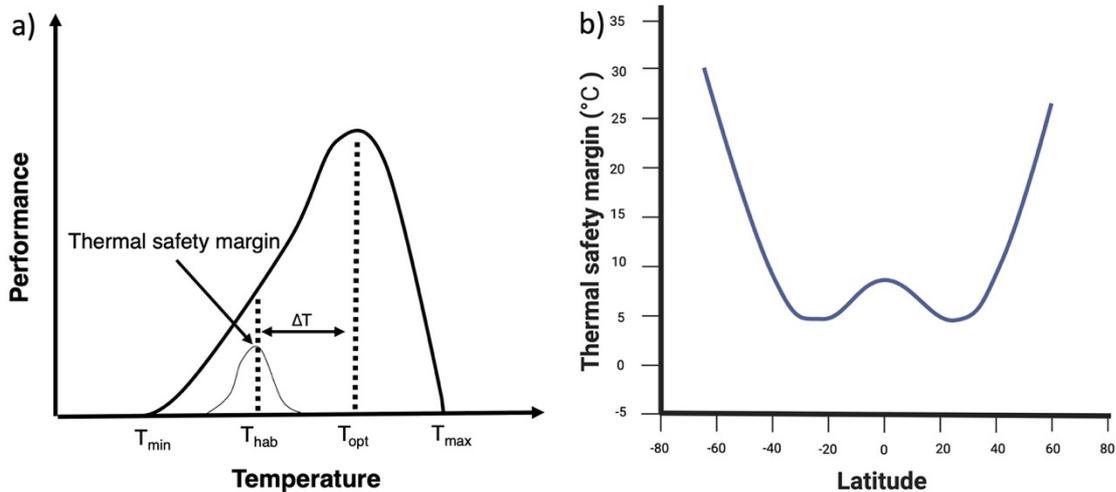


Figure 3. Illustration of thermal safety margin (following Deutsch et al. 2008) and its change against the latitudinal position. a) Thermal performance curve of ectotherms. T_{opt} is the optimal temperature for performance. T_{hab} is the average temperature to which the organism is exposed in its habitat during an average year. The thermal safety margin is calculated as the distance between T_{opt} and T_{hab} . b) Changes in thermal safety margin against latitude. This figure is adapted from Clusella-Trullas et al. 2011.

Significant differences in thermal safety margin (TSM) exist between interacting species. Generally higher trophic species have lower heat tolerance. For example, parasitoids have lower heat tolerance and narrower TSM than their herbivore hosts (Wenda et al. 2023). As a result, animals at higher trophic levels may become more vulnerable to climate change and temperature extremes, which might further disturb interactions between species (Diamond et al. 2017, Agosta et al. 2018, Chen et al. 2019, Wenda et al. 2023).

Overall, extreme temperatures due to climate change have the potential to surpass the thermal limits, pushing insects exposed to resulting unprecedented thermal stresses and disrupting species interactions (Harvey et al. 2020). The importance of thermal traits and the effect of thermal stress has been recognized as crucial for predicting the potential climate risks to ectotherms and species interactions (Williams et al. 2008).

2 Effects of thermal stress on cellular and individual levels

2.1 What is thermal stress?

Despite the word “thermal stress” being used frequently, a formal definition has not yet been provided. To provide a definition, we refer to the niche-based definition of “stress” proposed by Korsloot et al. 2004. Thermal stress is a condition induced in an organism by environmental temperatures that bring the organism near or over the edges of its ecological niche, as illustrated in Figure 3, which includes the optimal and suboptimal thermal ranges. Outside of this range, the organism may survive temporarily but cannot grow and reproduce normally (sublethal and lethal thermal ranges). (Korsloot et al. 2004).

As ecological niches vary between populations within one species or even different genotypes within a population, what is experienced as thermal stress for one population or genotype may be normal for another (Korsloot et al. 2004). Moreover, thermal stress is often divided into heat stress and cold stress as shown in Figure 4. Depending on whether the ambient temperature is above or below the freezing point of the organism’s body fluid, cold stress can be further divided into chilling stress and freezing stress (Bale 1996).

Initially, “stress” referred to environmental forces, while “strain” referred to the extent of deformation or alteration of a biological system resulting from stress (Kültz 2020). Over time, however, “stress” has become synonymous with the internal consequence of an external factor (Korsloot et al. 2004). As a result, the term "stressor" was introduced to refer to an external factor, while "stress" now refers to the internal state brought about by a stressor (Korsloot et al. 2004). For thermal stress in this thesis, the stressor is environmental temperature.

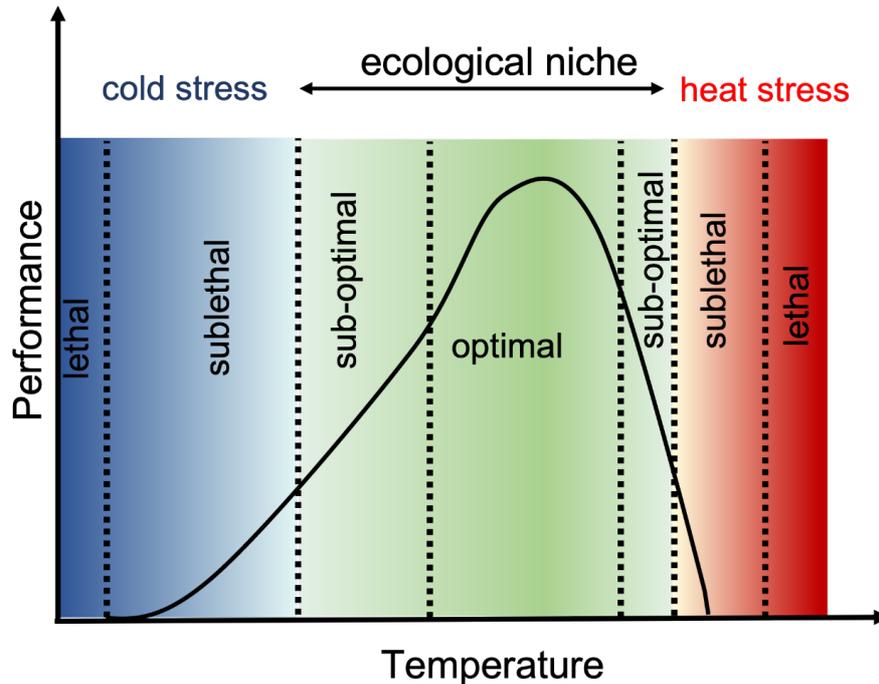


Figure 4. Thermal range based on the performance curve. Ecological niches include optimal and sub-optimal thermal ranges, where organisms can grow and reproduce. When temperatures fall outside the ecological niche, which includes lethal and sublethal thermal ranges, thermal stress occurs. This graph has been adapted from Henry (2018).

2.2 Manifestation of thermal stress on insects

- **On cellular level**

Environmental stressors can be deleterious to biological activity leading to perceptible disturbances at all levels of biological organization, from the most elementary such as the molecular or cellular level to more complex levels such as individuals or populations (Bijlsma and Loeschcke 1997). Whatever the level of the manifestation of thermal stress, the basic attack and subsequent physiological changes occur at the cellular level on macromolecules and macrostructures (Korsloot et al. 2004).

There is a startling degree of similarity in the stress responses of various species at the cellular level, even to greatly divergent environmental stresses (Kültz 2005). Both heat and cold stress deform and damage macromolecules, mainly membrane lipids, proteins, and DNA by destabilizing weak interactions such as van der Waals, and ionic and hydrogen bonds (Kültz 2005, Somero 2020). DNA damage is a significant consequence of both heat and cold stress, leading to double- and single-stranded breaks of DNA, with the extent of damage depending on temperature and duration of exposure (Yao and Somero 2012, Lubawy et al. 2019). Under normal cellular functioning, proteins are typically folded, but various processes such as transport, polypeptide synthesis, and assembly of multimeric proteins, can cause them to temporarily unfold. Stress can also induce unfolding. The

exposed amino acid side groups, especially hydrophobic residues, promote interactions between these 'non-native' proteins and folded proteins, inducing the latter to unfold (Chown and Terblanche 2006). As a result, irreversible aggregations of unfolded proteins occur, depleting the cellular pool of functional proteins and potentially causing cytotoxic (Feder and Hofmann, 1999; Korsloot et al., 2004). The most dramatic secondary effects of protein damage and enzyme inhibition are probably disturbance in the respiratory chain and uncoupling of oxidative phosphorylation in the mitochondrion (Korsloot et al. 2004). The uncoupling effect may result in a drop in ATP level, preventing NADH, the short-term buffer of energy in the cell, from being able to unload its electrons (Welch 1992). Therefore, aerobic energy metabolism ceases and shifts to anaerobic metabolism, resulting in a lower ATP yield. With the high energy demand under stressful conditions, this switch is enforced and can be considered an escape from further detrimental effects of stress (Korsloot et al. 2004).

Although heat and cold stress harm macromolecules and cellular metabolism in a similar way, some injuries are exclusive to heat or cold, or heat and cold produce different cascades of failure (MacMillan 2019). I'll go on to quickly summarize the heat and cold injuries individually next.

High temperatures disrupt the structure of cellular membranes. When exposed to high temperature, the phospholipid bilayer transit from a liquid crystalline phase to a non-bilayer, hexagonal phase, resulting in the loss of membrane integrity (Denlinger and Lee Jr 2010). Heat stress increases the bilayer's permeability to K^+ , which results in K^+ leakage into the hemolymph from tissues, causing hyperkalaemia, which impairs neurophysiological function (O'Sullivan et al. 2017). In addition, actin microfilament integrity can be disrupted by heat stress, damaging synaptic connections, and causing heat coma (Chown and Terblanche 2006). (Figure 5).

The direct mechanism initiating cell death in insects at high temperatures remains unclear (Macmillan 2019). One hypothesis proposed by Bowler is that the cascading deleterious effect of membrane damage plays a significant role in cell death (Bowler 2018). The rise in permeability of the membrane causes hyperkalemia, which may induce furthermore symptomatic of either apoptotic or necrotic cell death (Bowler 2018). However, a recent study suggests that while hyperkalemia may contribute to further injury in the heat, it is not the proximate mechanism of cell death (O'Sullivan et al. 2017). Instead, it seems to be a secondary outcome resulting from cell death occurring for other still unknown reasons (O'Sullivan et al. 2017). Another hypothesis is oxygen and capacity limitation. Higher oxygen consumption results from the exponential relationship between metabolic rate and temperature (Pörtner et al. 2017). However, due to reduced cardiac performance, impaired mitochondrial function, or limited oxygen-carrying capacity, there is a mismatch between oxygen supply and demand (Macmillan 2019). This leads to tissue and cellular hypoxia and a decline in aerobic metabolism, resulting in decreased ATP supply (Macmillan 2019). Prolonged periods of high metabolic rates cause mortality due to energy depletion (Gonzalez-Tokman et al. 2020). Additionally, the increased metabolic rate and the mismatch between oxygen supply and demand imply higher

production of free radicals resulting from respiration. This elevated oxidative stress can result in damage to lipids, proteins, and nucleic acids (Gonzalez-Tokman et al. 2020).

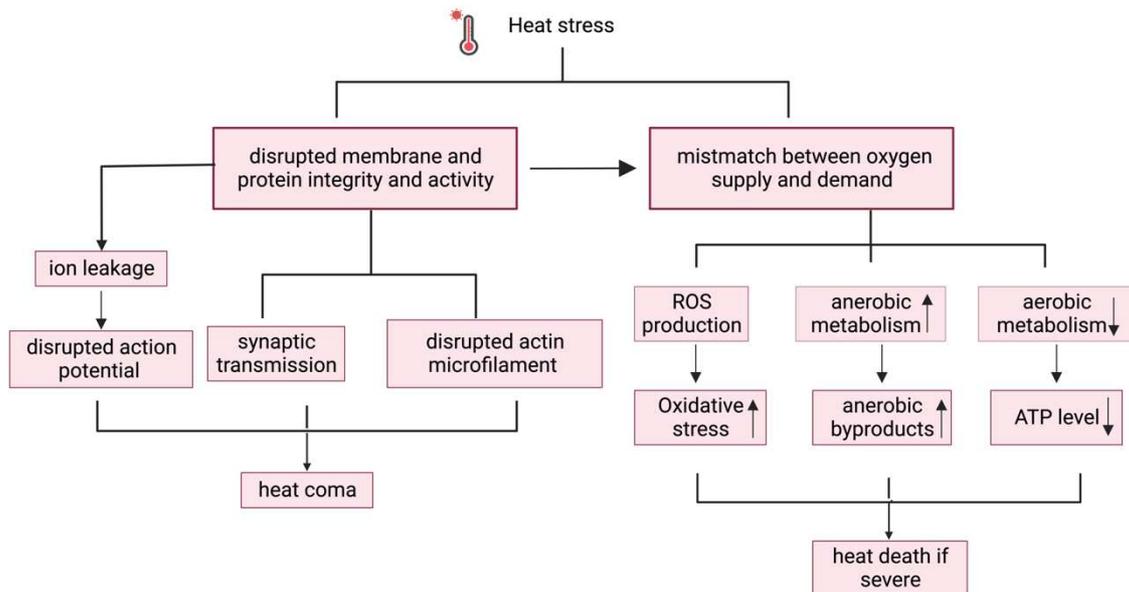


Figure 5. A hypothetical model for cell injury and death caused by heat stress inspired by the previous works (Bowler 2018, Macmillan 2019)

Freezing stress occurs when temperatures drop below the freezing point of an insect's body fluids. The primary risk associated with this stress is the formation of ice/ crystallization (Figure 6). Crystallization occurs in the extracellular space through the aggregation of water molecules into an ice nucleus or by gathering around a substance or irregularity (Chown and Terblanche 2006). As the ice lattice grows, it can cause damage to tissues and organs, particularly when it forms between adjacent cell layers. The growing mass of ice pulls apart the layers of tissue, resulting in mechanical injury that persists even after thawing (Denlinger and Lee Jr, 2010). During the freezing process, additional water is added to the nucleus or nuclei, causing the insect to start to desiccate. The removal of water from the solution leads to a progressive concentration of body fluids, resulting in pH changes, protein denaturation, and alterations in membrane properties (Chown and Terblanche 2006). These changes, in turn, affect electrochemical gradients and transport properties (Chown and Terblanche 2006). Furthermore, as water is removed from the cells, cellular shrinkage may occur, which can further damage the cell membrane (Chown and Terblanche 2006).

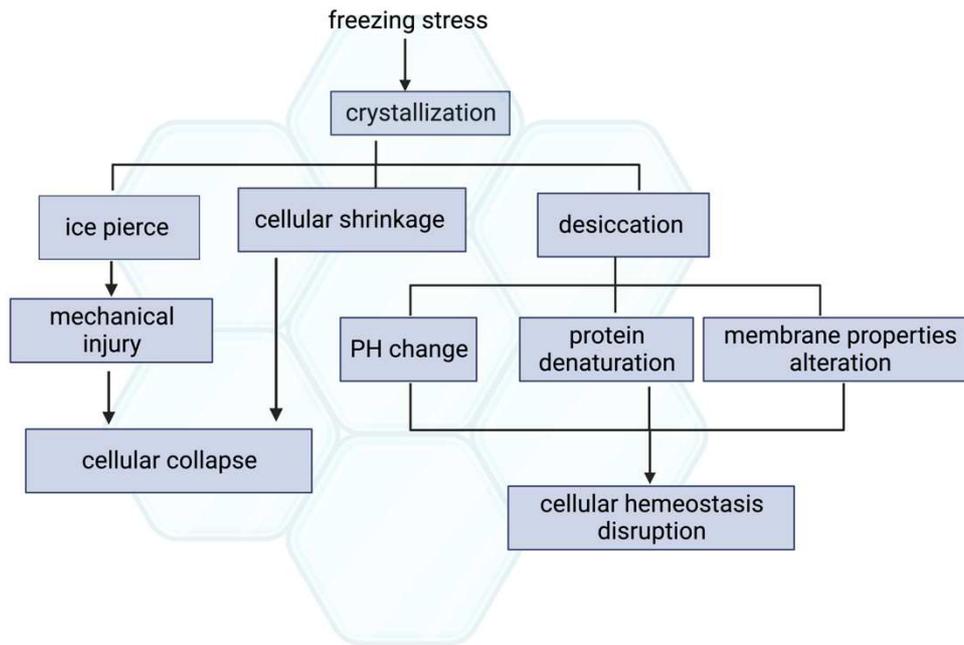


Figure 6. A hypothetical model for cell injury caused by freezing stress.

Chilling stress occurs when an organism is rapidly cooled without the formation of extracellular ice. When temperatures decrease, membrane lipids become closely packed in a gel phase, reducing fluidity (Denlinger and Lee Jr, 2010). This fluid-to-gel phase change leads to the separation of membrane proteins and lipids, altering membrane permeability and causing a decline in the activity of membrane-bound enzymes (Chown and Terblanche 2006). Meantime, with the decreasing temperature, the active transport process slows down to a point where they are insufficient to counterbalance the passive movement of solutes and water, leading to loss of homeostasis (Denlinger and Lee Jr 2010, Overgaard and MacMillan 2017). Reduced active transport leads to loss of hemolymph volume and disturbance of ion balance, notably an increase in extracellular $[K^+]$ (i.e. hyperkalemia) (Figure 7) (Overgaard et al. 2021). Hyperkalemia causes cellular depolarization, triggering voltage-dependent Ca^{2+} influx and initiating necrotic and/or cell death (Overgaard and MacMillan 2017). This probably drives a feed-forward loop of further $[K^+]$ release from cells, further cell depolarization, and cell death (MacMillan 2019).

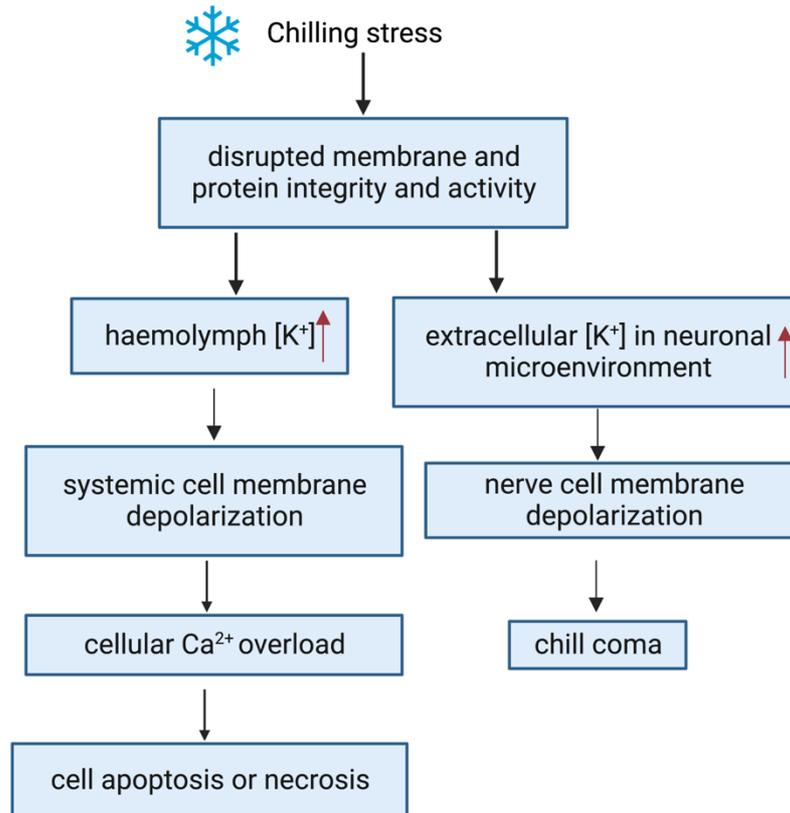


Figure 7. A hypothetical model for cell injury and death caused by chilling stress. Adapted from MacMillan (2019).

- **At the individual level**

As demonstrated by extensive empirical studies, with temperature shifting from optimal temperature to stressful temperature, the performance of individuals including various life-history traits generally decreases, as predicted by TPC (see Figure 2). Different life history traits differ in their thermal sensitivity (Ma et al. 2021). Among the main life history traits, reproduction has the narrowest thermal range, followed by development, while survival has the widest (Ma et al. 2021). For instance, in the moths *Plutella xylostella*, a single heat episode lasting a few hours had little effect on survival but did reduce reproduction (Zhang et al. 2013). In *Drosophila melanogaster*, egg-to-adult viability is limited to the range 10-32 °C whereas male fertility has a narrow range from 12 °C to 30 °C (Hoffmann 2010). A sublethal cold exposure can also delay mortality but decreased female fecundity, which has been found in flies (Coulson and Bale 1992, Košťál et al. 2019) and aphids (Hutchinson and Bale 1994). Abnormalities, defects, and delays in development in response to thermal stress are also commonly observed (Neven 2000, Košťál et al. 2019). For instance, heat exposure caused a supernumerary molt to a sixth instar in a codling moth (Neven 2000).

The effects of thermal stress can vary throughout an organism's ontogeny due to variations in environmental history and any "carry-over" effects thereof as well as morphological, physiological,

and behavioral adjustment (Mutamiswa et al. 2023). Generally, the tolerance to high temperatures decreases with age and into more advanced life stages although evidence to data suggests that the form of this negative relationship can be either non-linear or linear within life stages (Bowler and Terblanche 2008). These differences may be attributed to the variation in mobility of a particular life stage with less-mobile life stages expected to have greater thermal tolerance (Bowler and Terblanche 2008). However, there are exceptions to this pattern. For example, in some insects, eggs, despite being highly immobile, exhibit lower tolerance to high temperatures (Bowler and Terblanche 2008). Regarding low temperatures, eggs tend to be the most tolerant, while adult females are usually the least tolerant (Bowler and Terblanche 2008). Again, it is difficult to generalize the difference in thermal tolerances between life stages across species due to the existence of exceptions. Furthermore, because developmental life phases are interrelated, temperature stress at one stage can influence performance at later stages or across generations (Jeffs and Leather 2014, Mutamiswa et al. 2023).

3 Thermal tolerance and how to measure it?

3.1 Definition of thermal tolerance

Thermal tolerance refers to an organism's ability to survive short-term exposure to extreme temperatures (Maclean et al. 2019). Basal thermal tolerance refers to an organism's ability to withstand extreme temperatures without prior acclimation, while induced thermal tolerance refers to its ability to withstand extreme temperatures after prior exposure to moderate temperatures (Park and Yun 2013). In this thesis, I use thermal tolerance to refer to basal thermal tolerance.

3.2 Macrophysiology view of thermal tolerance in ectotherms

Thermal tolerance limits are reliable predictors of the geographical distribution of ectothermic animals (Sunday et al. 2011) and climate change exceeding tolerance limits can explain their current redistributions (Sunday et al. 2012). Lower thermal limits in terrestrial ectotherms decrease with latitude, but upper thermal limits vary little across geographic distribution. (Addo-Bediako et al. 2000, Sunday et al. 2011). Tolerance to heat is also largely conserved across lineages and appears unrelated to climate ancestry (Sunday et al. 2011, Kellermann et al. 2012, Bennett et al. 2021). Conversely, tolerance to cold varies between and within species according to the environmental temperature in the habitat from which they originated (Araujo et al. 2013, Bennett et al. 2021).

There are physiological boundaries that constrain the evolution of tolerance of terrestrial organisms to high temperatures, but the evolution of cold tolerance should be more frequent and has allowed species to persist in colder environments (Araujo et al. 2013). If the evolution rate for upper thermal limits continues as it has in the past, adaptive responses in thermal limits will have limited potential to rescue most species given the unprecedented rate of contemporary climate change (Bennett et al. 2021).

3.3 Common Indices of thermal tolerance

Thermal limits of animals have significant implications for our understanding of animal distribution and ecology. When animals are exposed to temperatures that restrict their behavior, activity, and survival, they may become ecologically nonviable or are heading in that direction (Macmillan 2019). Table 1 presents a list of commonly used indices that help measure an organism's thermal tolerance limits, such as knockdown temperature, knockdown time, lethal temperature/time, and recovery time (Angilletta 2009).

To determine these indices, diverse methods are employed but generally divided into static or dynamic methods. The static assays expose organisms to constant stressful temperatures and measure the time required to reach an endpoint, or score survival after a fixed duration (Jørgensen et al. 2019). For example, LT_{50} (median lethal temperature) is commonly estimated by exposing samples to a range of constant temperatures for a fixed duration and assessing insect survival following a recovery period. A logistic regression with a logit link and binomial error distribution (i.e., probit analysis) is used to linearize the sigmoidal survival curve and provide an accurate estimate of the LT_{50} value (Sinclair et al. 2015). Following a similar principle, Lt_{50} (median lethal time) is determined by exposing an insect sample to a pre-determined stressful temperature for various durations before recovery and probit analysis is performed. Survival is usually assessed within the first 24 h to a few days after exposure. Thus, LT_{50} or Lt_{50} estimates may ignore mortality that occurs in the longer term and also disregard sublethal effects on behavior or reproductive capacity (Sinclair et al. 2015). Furthermore, measurements of LT_{50} or Lt_{50} demand a considerably larger number of animals, particularly if there is no prior knowledge of mortality on which to base exposure temperatures or times (Andersen et al. 2015).

Table 1. Common indices of thermal tolerance (Angilletta 2009).

Indices	Definition
CT_{\min} / CT_{\max} (Critical thermal minimum or maximum)	The temperature at which neuromuscular coordination is impaired
LT_{50} (Median lethal temperature)	The temperature at which 50% of individuals survive a given exposure duration
Lt_{50} (Median lethal time)	The time at a given temperature that causes 50% mortality
Knockdown time	The time at a given temperature after which an insect is no longer able to stand upright
CCO (Chill coma onset temperature)	The temperature that induces complete paralysis
CCRT (Chill common recovery time)	The time required to recover the ability to stand following cold exposure

Dynamic assays expose organisms to a ramping rate of increasing or decreasing temperatures and report the temperature at which the organisms reach an endpoint (Rezende et al. 2014, Jørgensen et al. 2019). For example, CT_{min} or CT_{max} , estimate the temperature at which insects lose motor function and are more ecologically relevant than lethal temperature as an organism's ability to move is critical for finding food, a mate, or escaping predation. As the temperature continues to decrease or increase beyond the CT_{min} or CT_{max} , the insects will eventually become completely paralyzed, which is referred to as the chill or heat coma. However, this approach has been criticized for several reasons: 1) indices show invariance with the ramping rates, start temperatures, and the duration of experiments (Terblanche et al. 2007). 2) the ramping rate may not accurately reflect the reality that insects experienced in the field, for example, $1\text{ }^{\circ}\text{C min}^{-1}$ remains the experimental standard for determining CT_{min} (Sinclair et al. 2003). 3) the endpoint (loss of motor function) can be defined as a lack of spontaneous movement, a loss of coordination, or a fully comatose state in literature. From a physiological perspective, these measures of different endpoints that appear to be conceptually equivalent may be measuring entirely different physiological endpoints (Macmillan 2019).

Although it is not listed in Table 1, many studies examining the cold tolerance of insects typically begin with preliminary measurements of supercooling point (SCP). The SCP is the temperature at which freezing begins and ice formation typically spreads from the site of nucleation to other parts of the insect's body (Sinclair et al. 2015). Based on the ability to survive SCP, insects fall into the following categories: 1) Chill susceptible species; they are killed by cold in the absence of internal ice formation. 2) Freeze-avoidance species (sometimes referred to as freezing-intolerance); they can survive cold in the absence of internal ice formation but are killed by any internal ice. 3) Freeze-tolerant species, they can tolerate the formation of ice in their body tissues and fluids (Bale 1996).

The choice of indices to use depends on the goal of the study. For instance, when evaluating the potential for overwintering survival, it may be more suitable to consider LT_{50} after exposure to a longer time period (Sinclair et al. 2015). Andersen et al. assessed five indices of cold tolerance and determined that CT_{min} , LT_{50} , and Lt_{50} were the best predictor to describe the variation in latitude distribution among *Drosophila* species. These three indices also showed strong correlations suggesting that there are related physiological processes underlying the interspecific variation in these traits (Andersen et al. 2015).

3.4 Thermal tolerance landscape

Thermal death time curve

These indices in Table 1 use different endpoints, starting temperatures, and ramping rates, making it onerous to compare results among studies and subject to measurement errors based on methodology (Cooper et al. 2008). Moreover, it is questioned whether such a critical thermal limit is adequate or realistic (Rezende et al. 2014). As the impact of thermal stress depends on both its intensity and duration, a single parameter is insufficient to describe a response that is embedded in two dimensions. Different thermal tolerance estimates likely represent snapshots of a single underlying relationship between temperature, exposure time, and mortality (Rezende et al. 2014). To resolve the above problems, Rezende et al. (2014) reintroduced the framework of thermal tolerance landscapes (TTLs).

Thermal tolerance landscape (TTL) is based on thermal death time (TDT) curves, which describe that the tolerable duration at stressful temperatures exponentially decreases with increasing stress intensity using multiple static experiments across a temperature range (Figure 8). This log-linear dose–response relation between stress intensity has been used for almost a century in food science (Bigelow, 1921) and has also been found in many insects’ studies (Nedvĕd et al. 1998, Colinet et al. 2011, Rezende et al. 2014, Jørgensen et al. 2019, Tarapacki et al. 2021). Two parameters, z (thermal sensitivity coefficient) and sCT_{max} (critical thermal maximum of static assays) can be obtained from this linear TDT curve (Castañeda et al. 2015, Rezende et al. 2014). z is the negative inverse slope and describes the temperature difference required for a 10-fold change in the mortality rate. sCT_{max} is the extrapolated average temperature that would result in a knockdown time of $\log_{10} t = 0$ (i.e., thermal tolerance at 1 min) (Rezende et al. 2014, Castañeda et al. 2015, Truebano et al. 2018). Furthermore, Rezende et al (2014) suggested that z and sCT_{max} together describe thermal tolerance strategies of ectotherms, such that a high sCT_{max} describes high tolerance to acute heat stress while a low z (i.e., steep slope) indicates high tolerance to moderately lower (chronic) temperatures. In this respect, it was suggested that the positive correlation between sCT_{max} and z in ectotherms, in general, indicated a general trade-off between acute and chronic heat tolerance (Rezende et al. 2014). This has also been reported in *Drosophila subobscura* from a latitudinal cline (Castañeda et al. 2015), and in the ontogeny of an intertidal gastropod (Truebano et al. 2018). However, Jørgensen et al (2019) showed these surprising “trade-offs” could arise as an analytical artifact of the model caused by insufficient data collection and excessive data extrapolation since z and sCT_{max} are derived from the same linear regression.

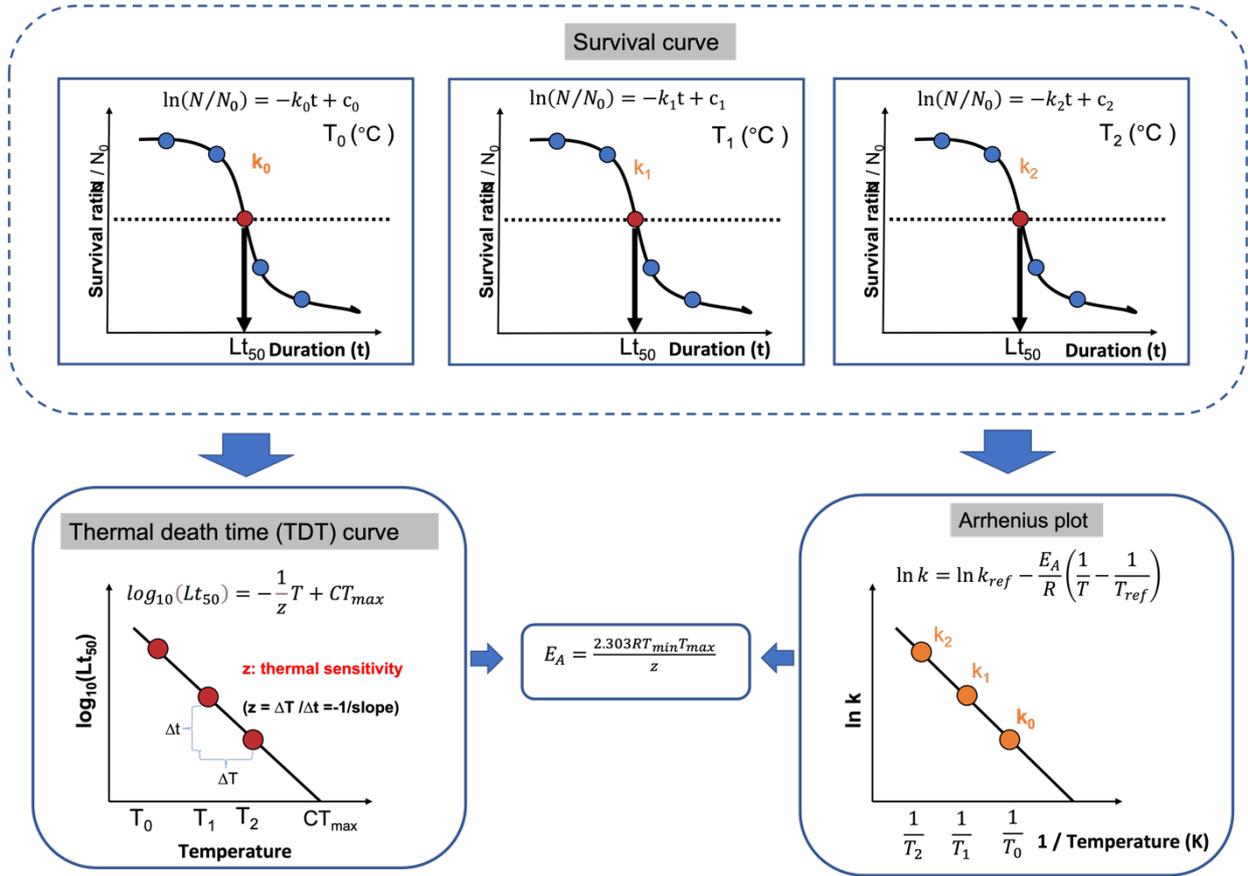


Figure 8. The illustration of the thermal death time curve, Arrhenius plot, and their correlation.

The TDT model offers several advantages. Firstly, once the parameters z and sCT_{max} are estimated from multiple static measurements, it is possible to calculate the knockdown time at any given other stressful temperatures (Jørgensen et al. 2019). Secondly, this approach can reconcile the discrepancies and unify findings obtained from static and dynamic assays. Thirdly, TDT models enable the calculation of the accumulated injury (mortality), as the temperature can be viewed as the determinant of an injury accumulation rate that increases exponentially with temperature. The injury sustained at different static temperatures can be completely additive (Jørgensen et al. 2021, Ørsted et al. 2022). Therefore, the TDT model provides a comprehensive understanding of how thermal tolerance duration varies with temperatures (Tarapacki et al. 2021, Willot et al. 2022) and links the thermal tolerance to the injury accrued under natural temperatures (Rezende et al. 2020, Alruiz et al. 2022).

The theoretical basis of TDT analysis and correlation with other models

- Survival curve and Lethal time (Lt)

At each temperature, the mean survival proportion as a function of exposure times is used to develop the thermal death kinetic models. The kinetic model is based on the following equation (Gazit et al. 2004):

$$\frac{d(N/N_0)}{dt} = -k(N/N_0)^n \quad (1)$$

Where k is the thermal death rate constant (min^{-1}), n is the kinetic order of the reaction, N and N_0 are the surviving and initial numbers of insects, and t is the exposure time (minutes). Mathematically, logarithmic TDT curves correspond to a first-order reaction, in which the mortality rate at any given temperature remains constant through time (Wang et al. 2007). The integration form of equation 1 can be obtained as follows for the first-order reaction ($n = 1$).

$$\ln(N/N_0) = -kt + c \quad (2)$$

For each temperature, survival (N / N_0) is regressed against exposure time (t) and then the values of k and c can be derived from the regression equation.

Lethal time (Lt) is defined as the time needed to kill $y\%$ (referred to as mortality rate) of the insects at a given temperature. Based the equation 2, the Lt value can be inversely derived:

$$Lt = \frac{\ln(N/N_0) - c}{k} \quad (3)$$

For example, Lt_{50} can be estimated by inputting 0.5 for N / N_0 into a developed kinetic model.

- TDT analysis

A thermal death time (TDT) curve can be developed by plotting each temperature against the log-transformed Lt_{50} .

$$\log_{10}(Lt_{50}) = -\frac{1}{z}T + CT_{max} \quad (4)$$

The z value represents thermal sensitivity. It is the temperature difference required for a 10-fold change in the mortality rate, which can be obtained from the negative inverse of the slope of the TDT curve (Celsius).

- Arrhenius analysis

Another way to model the effect of temperature T (K) on the change in the rate constant k (min^{-1}) in equations 1 and 2 that quantifies the decrease in the surviving number of individuals is to use the Arrhenius relationship, as follows (Wang et al. 2007):

$$k = k_{ref} e^{\frac{-E_A}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right)} \quad (5)$$

Where T is the absolute temperature (K), k_{ref} is the reaction rate constant at the reference temperature T_{ref} , E_A is the activation energy (J / mol) and R is the universal gas constant (8.314 J / mol K). Taking the natural logarithm on both sides of equation 5 yields the following equation:

$$\ln k = \ln k_{ref} - \frac{E_A}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \quad (6)$$

By integrating equation 3 and equation 6, Arrhenius plots were also created by regressing the inverse of the test temperature ($1/T$ in Kelvin) against the natural logarithm (\ln) to Lt_{50} :

$$\ln Lt_{50} = \ln \frac{\ln 0.5 - c}{k_{ref}} + \frac{E_A}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \quad (7)$$

The activation energy (E_A) for the thermal death of test organisms, which represents the thermal sensitivity, can be calculated from the slope times the value of R (gas constant, 8.314 J / mol K). The higher the activation energy, the more sensitive organisms are to temperature changes.

As the coefficients of determination of the Arrhenius fits were similar to the TDT curve, more studies preferred to use the “simpler” exponential relation rather than the Arrhenius fits for analysis (Jørgensen et al. 2019, Jørgensen et al. 2021, Tarapacki et al. 2021).

TDT and Arrhenius analysis both produce the estimate of thermal sensitivity: z and E_A , respectively. It is noted that these two indices are related. z could be used to calculate the activation energy (E_A , J / mol) needed for the thermal death of test insects according to the following relationship (Tang et al. 2000):

$$E_A = \frac{2.303RT_{min}T_{max}}{z} \quad (8)$$

Where R is the universal gas constant (8.314 J/mol K), and T_{min} and T_{max} are the minimum and maximum absolute temperatures (K) employed as stressful temperatures in the static assays.

- Q_{10}

Another commonly used index for thermal sensitivity is the Q_{10} factor (Smith 1957, Tarapacki et al. 2021, Jørgensen et al. 2022). This factor is the ratio of the metabolic rate at a given temperature divided by the metabolic rate at a temperature 10 °C lower. There is also a relationship between the z and the Q_{10} (Holdsworth and Simpson 2007):

$$Q_{10} = 10^{10/z} = 10^{10 E_A / 2.303 R T_{min} T_{max}} \quad (9)$$

That is, the Q_{10} for thermal death increases exponentially with the activation energy at elevated temperatures.

4. Biochemical and molecular protective mechanisms against thermal stress

For insects to successfully tolerate cold or heat, they must employ mechanisms to protect against or alleviate the adverse effects of stressful temperatures. Heat shock, for instance, has the potential to destabilize or even denature the structure of proteins (e.g., enzymes) (Somero 2020). Similarly, severe cold stress can lead to the inactivation or unfolding of some proteins (Szyperski et al. 2006). Once proteins become unfolded, even partially, they lose their functional competence (denaturation) and must be degraded and recycled. This process, known as proteolysis, is often cited as a potential cause of injury (Neven 2000, Bowler 2018). Maintaining a functional proteome (i.e., proteostasis) is considered crucial for the proper functioning of cellular processes in the organism (Kültz 2005). When proteostasis is disrupted by a stress such as heat or cold, the unfolded protein

response pathway is activated causing the induction of heat shock proteins (HSPs) in particular (Korsloot et al. 2004, Kültz 2005).

4.1 Heat shock proteins

The genes coding for heat shock proteins (HSPs, plain and uppercase names refer to heat shock proteins, as opposed to italicized and lowercase names which refer to the genes coding for heat shock protein) were first discovered as chromosome puffs in 1962 in *Drosophila* species after exposure to high temperatures, thus the term “heat shock” was coined (Ritossa 1962). However, it wasn't until 1973 that the protein product was discovered in the salivary glands of *Drosophila melanogaster* (Tissières et al. 1974). The function of HSPs as molecular chaperones was soon elucidated (Parsell and Lindquist 1993, Feder and Hofmann 1999). These molecular chaperones assist in refolding or degrading damaged proteins and/or prevent the aggregation of proteins damaged by thermal stress or other proteotoxic stress as shown in Figure 9 (Chen et al. 2018). Furthermore, the production of HSPs is one of the most important molecular responses to thermal stress, with anoxia, starvation, and desiccation also identified as inducers (Parsell and Lindquist 1993).

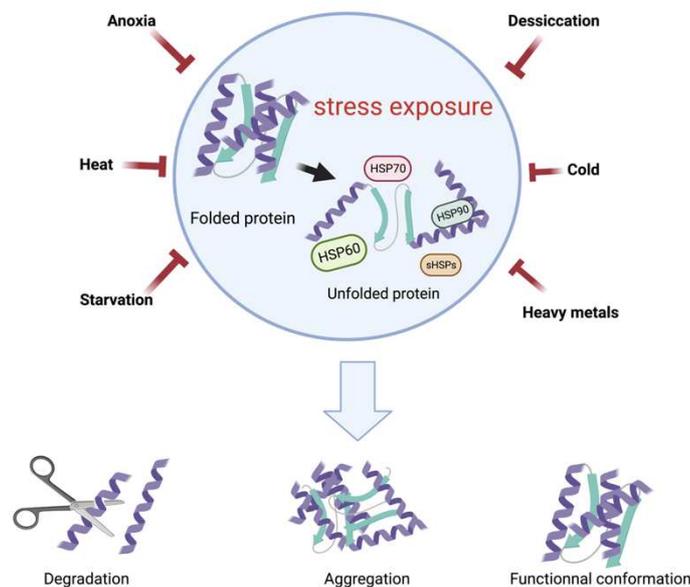


Figure 9. Cellular function of heat shock proteins (HSPs). The fate of proteins with non-functional conformations after stress exposure may be either to re-obtain the functional conformation, form aggregations with other misfolded proteins or become degraded. HSPs play a helper role in shifting the equilibrium in the direction of more functional proteins or degradation of damaged proteins (Sørensen et al. 2003). Created with BioRender.com.

In insects, the recognized HSPs are divided into four families according to their molecular weight: small heat shock proteins (sHSPs), HSP60, HSP70, and HSP90 (King and MacRae 2015). The genes coding these proteins are highly conserved at the molecular level, making them relatively easy to clone and evaluate in divergent species, even where genome data for that species is limited or unavailable. Therefore, *hsp* gene expression studies are popular (Clark and Worland 2008).

Among the various HSP families, HSP70 is the largest and most important family and plays a central role in defending the cell against stress. The primary function of HSP70 is to bind unfolded or partially unfolded proteins to prevent their aggregation and to release them for folding (Clare and Saibil 2013). It consists of two types of stress proteins: heat shock cognates 70 (HSC70) and heat shock proteins (HSP70). The former is constitutively present, whereas the HSPs are induced by stress (very low concentration in normal conditions) (Korsloot et al. 2004). Even though *hsc70* is constitutively expressed, it can also be significantly upregulated in response to heat shock in insects (Wang et al. 2008, Cheng et al. 2016, Li et al. 2017). The upregulation of *hsp70A* may also contribute to the repair of cold injury. After suppressing the *hsp70* up-regulation response to cold in *Pyrrhocoris apterus* adults using RNAi, the proportion of insects that were not able to fully repair cold injury (uncoordinated crawling) increased (Košťál and Tollarová-Borovanská 2009). In *Drosophila melanogaster*, *hsp70*-null mutants (lacking all copies of *hsp70* gene) showed higher mortality during the recovery from severe cold shock than the wild-type strain (Štětina et al. 2015).

HSP90 has been reported to be responsible for heat-shocked induced defenses in many insect species (Xu et al. 2010, Chen et al. 2014, Quan et al. 2022). HSP90 might cooperate with HSP70 chaperone systems in protein repair. the former acts to prevent the aggregation of thermally unfolded reporter enzymes (Bose et al. 1999); the latter is responsible for the refolding of these enzymes (Mayer and Bukau 1999).

HSP60 is primarily localized in the mitochondria (Cheng et al. 1989), where it is involved in the de novo folding and refolding of imported proteins (Martin et al. 1992). *Hsp60* was heat-inducible in some insects (Cui et al. 2010, Zhang et al. 2015, Cai et al. 2017) but not in others (Chen et al. 2015). HSP60 can enhance cold hardiness and promote membrane stability by stabilizing microfilaments in insects (Kayukawa and Ishikawa 2009). *Hsp60* was slightly upregulated in response to cold exposure in *Belgica antarctica* (Teets et al. 2019), *Monochamus alternatus* (Cai et al. 2017) and *Galeruca daurica* (Tan et al. 2018).

Compared to ATP-dependent HSP60, HSP70, and HSP90, small heat shock proteins (sHSPs) are ATP-independent proteins with molecular weights ranging from 12 to 43 kDa and generally exhibit a greater variation in size, sequence, and function (Li et al. 2009). Some sHSPs likely act as chaperones for maintaining cytoskeletal structural elements during stress, as well as preventing the aggregation of denatured proteins (Lockwood et al. 2010, Basha et al. 2012). Colinet et al. (2010a) showed that some *sHsp* genes were upregulated during the recovery from cold stress in *Drosophila Melanogaster* but were not modulated during cold stress itself. The removal of *hsp22* and *hsp23*

mRNA by RNAi in *Drosophila melanogaster* disrupted recovery from chill injury (Colinet et al. 2010). The knockdown of two *sHsps* in the leaf beetle *Gastrophysa atrocyanea* decreased viability and heat tolerance, which suggests sHSPs may protect against heat stress (Atungulu et al. 2006).

Different organisms initiate HSP production at a wide range of temperatures, but in each case, rather than at a single universal temperature threshold, organisms do so when their temperature rises above that which is suitable for them. For instance, HSPs are activated in arctic fish at about 5 °C and in thermophilic bacteria at about 100 °C (Parsell and Lindquist 1993). Continuous exposure to thermal stress can lead to a decline in HSPs (Wang et al., 2008), probably as a strategy to minimize the energetic demands of the heat shock response.

4.2 Cytoprotective molecules

Accumulation of various low-weight small molecules in response to various environmental stressors is commonly found in diverse organisms (Yancey 2005). These low molecular weight metabolic products are often called “organic compatible osmolytes”, as they were first described in organisms in high-salinity environments. But the terms “cytoprotective molecules” or “cytoprotectants” are better since these molecules have a variety of protective functions (Kostal et al. 2016). Chemically, cytoprotectants (CPs) fall into a few classes: polyols and sugars, free amino acids and their derivatives, methylamines, and urea (Yancey et al., 1982). According to Yancey (2005), the cytoprotective properties fall into two broad categories: (1) metabolic protection (antioxidation, redox balance maintenance, detoxification) (2) stabilization of macromolecules, which is based on specific and diverse interactions between the molecules of solvent (water), solute (cytoprotectant) and a protected structure (protein, nucleic acid, lipid bilayer). Next, I will discuss the roles of these molecules in cold and heat tolerance, respectively.

- **Response to cold stress**

Universal responses to cold include homeoviscous adaptation (remodeling of membrane phospholipids) and the accumulation of cryoprotectants (Haywards and Colinet 2023). Diapause, acclimation (rapid or gradual), and cold adaptation all induce the accumulation of a specific arsenal of cryoprotectants in insects (Colinet et al. 2012a, Colinet et al. 2012b, Durak et al. 2021, Huang et al. 2022). The primary function of cryoprotectants relates to their colligative effects at high concentrations in body fluid (i.e., lowering the freezing point) (Haywards and Colinet 2023). When in low concentration, cryoprotectants can have non-colligative effects, such as the stabilization and protection of macromolecules and macrostructures such as proteins and membranes (Haywards and Colinet 2023).

The accumulation of polyols involved in cold tolerance was first found in diapausing eggs of the Bombyx silk moth (Chino 1957). Later it was found that the elevation of polyols conferred cold tolerance, for instance, larvae of the flesh fly, *Sarcophaga bullata*, with high glycerol levels were 3-4 times more tolerant of a 2h exposure to -10 °C than control (Yoder et al. 2006). Injection of

exogenous glycerol into larvae protected against low-temperature injury (Yoder et al. 2006). The accumulation of massive concentrations of polyols is linked to freeze-protective functions such as the diminution of the supercooling point via a colligative effect (Crosthwaite et al., 2011). At low concentrations, polyols may play other protective functions, likely through a “preferential” exclusion of solutes from proteins, which help to stabilize their structures (Gekko and Timasheff 1981, Kim et al. 2018). Furthermore, the types and amounts of cryoprotective polyols vary significantly by species, even the closely related ones; For example, *Dermacentor silvarum* tick accumulated large amount of glycerol in response to cold (Wang et al. 2017), whereas the glycerol level did not change in the close-related species *D. variabilis* (Rosendale et al. 2022).

In addition to polyols, many overwintering insects use trehalose, the sugar found in insects, as a seasonal cryoprotectant (Thompson 2003). The development of cold hardiness and the drop in supercooling point were both connected with the high quantities of trehalose seen in overwintering insects (Khani et al. 2007). Trehalose also contributes to rapid cold hardening (Colinet et al. 2012a) and cold adaptation (Overgaard et al. 2007).

Some amino acids are also known to possess cryoprotective properties. For example, proline increased cold and freeze tolerance of *Drosophila melanogaster* (Košťál et al. 2012), and diet supplementation of proline permitted larvae from the fly *Chymomyza costata* to withstand exposure to liquid nitrogen (Košťál et al. 2011). Ticks that received exogenous betaine and valine also demonstrated enhanced cold tolerance (Rosendale et al. 2022). The cold-protective mechanisms of free amino acids (against freezing mainly) involve a range of different interactions, such as preferential exclusion, protection of native protein structure, binding of partially unfolded proteins, stabilization of membrane structure, and vitrification (Yancey 2005, Grgac et al. 2022).

- **Response to heat stress**

Under heat stress, the small organic molecules (i.e., cytoprotectants CPs) may act as stabilizers of proteins, RNA, and membranes (Somero 2020). The concentrations of CPs, including the strongest stabilizing solutes currently known, rise quickly when the culture temperature rises in the extremely thermophilic Archaea and Bacteria, which appears to play a crucial role in supporting the extreme thermal tolerance of these bacteria (Martins et al. 1997, Lamosa et al. 2013). Heat-stressed yeast rapidly produces high concentrations of the disaccharide trehalose, which is a strong protein stabilizer, by preventing the further unfolding of proteins and reducing the formation of cytotoxic protein aggregates. Trehalose thus is regarded as a “chemical chaperone.” Besides, trehalose is also a strong stabilizer of membrane and its accumulation under thermal stress might have widespread roles in protecting the cell membrane's integrity (Somero et al., 2017; Yancey et al., 1982).

The preliminary research on insects have revealed that aphids and whiteflies are two examples of species that accumulate mannitol and sorbitol molecules under hot temperatures, suggesting thermo- and osmo-protection (Hendrix and Salvucci 1998, Salvucci 2000). Six-carbon polyols, like mannitol, play a crucial role as osmotic pressure regulators, as they have limited ability

to cross cell membranes easily (Garcia- Perez and Burg, 1991, Hendrix and Salvucci, 1998). Mannitol also functions as a scavenger of increased free radicals, thus prolonging survival time during heat stress (Niu et al. 2003). Furthermore, polyols contribute to protein stabilization by strengthening the pairwise hydrophobic interaction between hydrophobic groups, thus protecting proteins from denaturation under heat stress (Back et al. 1979). Glycerol is known to preserve cells from hyperthermic cell death and enhance the stability of thermolabile enzymes, preventing their permanent inactivation under unfavorable conditions (Henle and Warters 1982, Zancan and Sola-Penna 2005). Increased glycerol concentrations also contribute to improved dehydration tolerance and a reduction in the rate of water loss (Yoder et al. 2006, Benoit et al. 2007).

4.3 Metabolomic as a technique

Metabolomics is defined as the comprehensive analysis of metabolites in a biological specimen (Clish 2015). Metabolomics has been traditionally viewed as a complement to genomics, transcriptomics, and proteomics (Lankadurai 2013). However, it has the potential to overcome limitations associated with other omics methods. When an organism encounters an external stressor, it triggers changes in gene expression and protein production, regulated by homeostatic controls and feedback mechanisms. As a result, the metabolite profile undergoes alterations, making metabolomics a more sensitive and integrated indicator of the external stressor compared to other omics technologies (Lankadurai 2013).

In insects research, metabolomics focuses on the study of small molecules found in an organism's tissues and fluids, particularly non-protein substrates like sugars, amino acids, metabolic intermediates, and polyols (Denlinger and Lee Jr 2010). Currently, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) are commonly used for metabolite detection and quantification (Hayward and Colinet 2023). Among them, GC-MS has been used most extensively, owing to its high resolution, high detection sensitivity, and numerous open-access spectral libraries (Hayward and Colinet 2023).

When studying metabolic responses to cold / heat in insects, we can expect that the observed metabolite changes reflect either i) an adaptive biochemical change (e.g., accumulation of CPs) that preserves vital functions, or ii) downstream consequences of a disrupted physiological capacity (Hayward and Colinet 2023). For example, an increase in amino acids might indicate adaptation as cytoprotectants or relate to protein breakdown. Alternatively, it might result from a decrease in the TCA (tricarboxylic acid) cycle turnover rate, since many amino acids are components of biosynthetic pathways linked to glycolysis and TCA. Since both responses can be found concurrently, it is important to consider, or even test, the adaptive potential of a biochemical change.

5 Aphids as model species for studying the impacts of thermal stress

Aphids are a highly economically important group of insects, that belong to the superfamily Aphidoidea, which comprises approximately ~4400 species (Heie 1994). Most of these species are found in temperate regions of the northern hemisphere (Heie 1994). Of these, around 250 species are notorious and cosmopolitan pests due to the rapid propagation capacity, which causes considerable damage and fitness cost to crops by directly feeding on the phloem and indirectly transmitting viral infections (Will and Vilcinskas 2015).

Aphids are prime models for studying the impact of climate change on insects due to their high sensitivity to temperature changes, short life cycles, and high fecundity (Hullé et al. 2010). More importantly, since aphids can reproduce parthenogenetically, it is easy to compare their responses to temperature while excluding the confounding effect of genetic variation (Enders et al. 2015).

5.1 Aphids' life cycle

Aphids have various life cycles that can be classified based on how they utilize host plants (host alternating and non-host alternating) and how they reproduce (asexual and sexual) (Van Emden and Harrington 2017). Host alternating aphids live on a single plant species (usually a woody plant) in winter and migrate to completely unrelated herbaceous plants in the summer months, where parthenogenetic generations continue exploiting stages of rapid plant growth (Figure 10). Aphid parthenogenesis is generally associated with viviparity and the telescoping of generations (where the granddaughters of a female aphid are already developing within the daughters inside her). This confers a high demographic potential (i.e., can produce massive numbers of individuals under the right conditions) during the asexual phase of aphids (Simon et al. 2002). In late summer or early autumn, they migrate back to the woody plant and produce frost-resistant eggs through sexual reproduction triggered by short photoperiods and low temperatures (Simon et al. 2010). Aphids that interrupt parthenogenesis with sexual reproduction in this way are termed “holocyclic”, which is the typical annual life cycle of aphids. Non-host alternating aphids stay on the same or closely related host species throughout the year. Some aphids continue parthenogenetic reproduction all year round and are known as “anholocyclic” aphids. Some aphid species show both holocyclic and anholocyclic life cycles in different clones. The life-cycle polymorphisms of aphids have been shown to correlate broadly with climate variables (Simon et al. 2002). Holocyclic aphid clones are confined mainly to regions with severe winters (Simon et al. 2002).

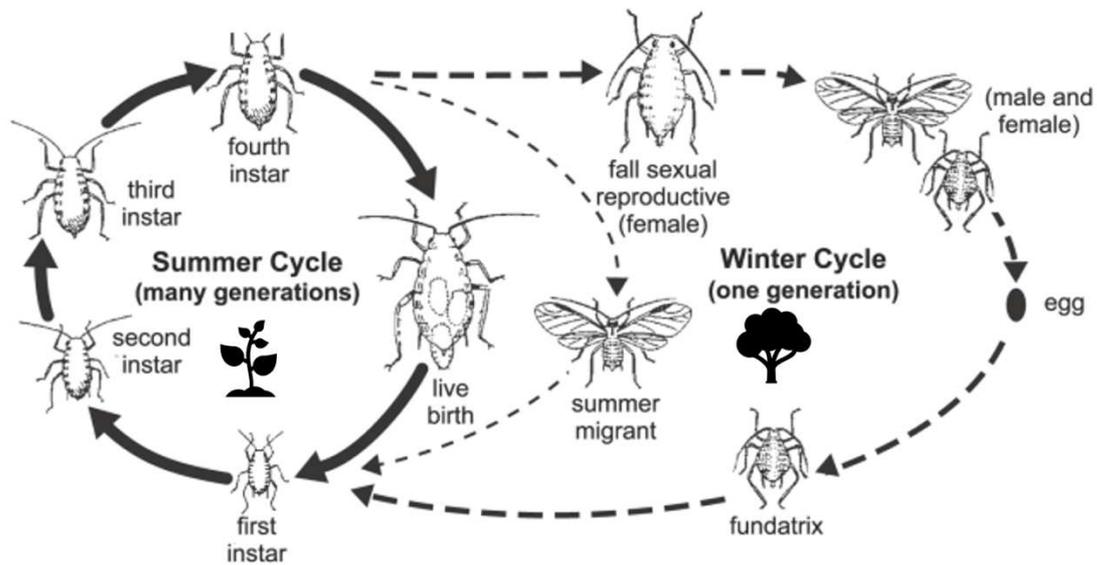


Figure 10. The general life cycle of aphids. Asexual reproduction occurs during summer cycles. Holocyclic aphid species produce a generation of sexual individuals that produce overwintering eggs in winter cycles (Dreistadt et al. 2004).

5.2 Aphids' response to climate change

Global warming has altered the phenology of aphids. Long-term data on the seasonal appearance of flying aphids have shown significant changes in aphid synchrony over time, with first flights occurring much earlier due to climate change in England (Bell et al. 2015). Warmer winters, for instance, cause aphid species to migrate 4–19 days earlier per 1 °C increase in normal winter temperature in England (Zhou et al. 1995, Bell et al. 2015). In China, aphids have also been observed to have earlier first occurrence and first migration dates, whereas their final occurrence and final migration dates were significantly later (Wu et al. 2020).

The predictive effects of climate change on aphid population dynamics are ambiguous as modeling studies have found aphid populations may decline, increase, or remain unaffected (Bezemer et al. 1998, Newman 2004, Newman 2005). This may be due to the different optimum temperatures for maximum growth among aphid species, which are often based on their habitat and climatic ranges.

Laboratory simulation studies have found that increasing mean temperature or increasing the amplitude of high temperatures can have negative or positive impacts on aphids depending on the species. Chen's experiment found that different levels of warming (from 1.1 °C to 6.4 °C) negatively impacted both life history traits (development rate, longevity, and fecundity) and demographic parameters (mean generation time, net reproduction rate and intrinsic rate of increase) of *Aphis craccivora* (Chen et al. 2013). However, with a 2.5–3.5 °C increase in mean temperatures, green peach aphids developed faster and had greater fecundity than the control (Davis et al. 2006). This suggests that the aphids were able to recover once temperatures returned to below the upper

developmental threshold, although fluctuating temperatures extended threshold limits (Davis et al. 2006).

The effects of extreme temperatures have received increased emphasis in recent years. Research has found that even if the average temperature remains within the optimal range, an increased magnitude of high-temperature fluctuations has a negative effect on aphid population growth (Ma et al. 2015a, Gao et al. 2016). The number and distribution of hot events between normal days also matter. Increasing the duration of hot days in heat waves has a negative effect on various demographic rates and the lifetime fitness of aphids, but the magnitude of this effect typically depends on the temporal clustering of hot periods (Ma et al. 2018a). Sporadic short hot events during a long mild period did not significantly affect aphid vital rates (Zhu et al. 2019). The interactive effects of normal and hot periods are likely driven by a shift in the balance of damage versus repair periods (Ma et al. 2018a).

Moreover, night warming associated with climate change may exacerbate the detrimental effects of hot days on aphids by reducing recovery opportunities (Ma et al. 2020). In the grain aphid *Sitobion avenae*, night warming was observed to decrease nymphal survival and depressed adult performance, such as lifetime fecundity and longevity, and ultimately results in a reduced population growth (Zhao et al. 2014).

5.3 Aphids' response to thermal stress

- **Cold stress**

Many aphids are chill-susceptible as they die at a higher temperature than their freezing point (Knight and Bale, 1986b, Worland et al. 2010). However, they can survive the cold stress through the following strategies.

Holocyclic aphids produce eggs that are considerably cold-hardy to survive the severely cold winter. *Pemphigus bursarius* overwinters as asexual hiemalis, a special winter morph that has more cold hardiness than the summer morph (Phillips et al. 2000).

Aphids exhibit a high level of phenotypic plasticity in cold tolerance through acclimation or hardening effect. Acclimation at low temperatures can decrease the lower lethal temperature of both first-instar and adult aphids (Knight and Bale 1986a, Bale et al. 1988, Alford et al. 2012a). Nymphs and adults of *Myzus persicae* maintained at 5 °C had LT_{50s} 8.3 °C and 4.7 °C, respectively lower than those for non-acclimated individuals (Clough et al. 1990). Low-temperature acclimation also enabled aphids to maintain mobile at lower temperatures (Alford et al. 2012b). It is worth noting that aphids can begin the process of acclimation before birth, thanks to their telescoping reproduction, which potentially facilitates progressive increases in levels of acclimation with each subsequent generation (Powell and Bale 2008). In a previous study of intergenerational acclimation using a single clone of the aphid *Sitobion avenae*, the LT₅₀ decreased from -10.6 °C at a constant 20 °C to -12.3 °C after one generation at 10 °C and then to -14.8 °C following three generations at 10 °C (Powell and Bale, 2008).

Insects are also capable of enhancing cold tolerance on a much shorter time scale, in a process called rapid cold-hardening (RCH) (Teets and Denlinger 2013). RCH allows insects to improve cold tolerance almost instantaneously (i.e., within minutes to hours) to cope with sudden cold snaps and regularly-occurring diurnal drops in temperature, for example, hardening aphid nymphs for 2 hours at 0 °C resulted in the survival rate increasing from 18% to 83% (Powell and Bale 2004). Moreover, RCH has no ecological costs of development, longevity, or fecundity for aphids (Powell and Bale 2004, Saeidi et al. 2017).

Research about metabolic responses to cold stress in aphids is relatively scarce. Saeidi et al (2012) found that the amount of glucose, mannitol, myo-inositol, and trehalose increased in the overwintering cabbage aphid *Brevicoryne brassicae*, which was concomitant with the development of the aphid cold hardiness in response to a decrease in air temperature from autumn to winter. Similar metabolites increased in another overwintering aphid *Cinara tujaefilina* (Durak and Durak 2021). Saeidi et al (2017) also found that trehalose and glucose increased considerably through cold acclimation and rapid cold hardening treatments. These findings strongly indicate that aphids possess the ability to respond to cold temperatures through biochemical adaptation.

Although there have been numerous studies investigating heat shock proteins (HSPs), limited research exists regarding the response of aphids' HSPs to cold temperatures. One notable study by Saeidi et al. (2021) discovered that HSP70 levels significantly increased in both the Russian wheat aphid (*Diuraphis noxia*) and melon aphid (*Aphis gossypii*) following one week of cold acclimation.

- **Heat stress**

Previous studies have consistently shown a negative correlation between elevated temperatures and key biological parameters in aphids, such as the nymphal period, reproductive period, adult longevity, and total longevity (Ma et al., 2004; Chiu et al., 2015; Ma et al., 2015a). However, aphids have developed various strategies to cope with heat stress. One such strategy is thermal regulation behavior, whereby aphids can actively avoid heat stress. For instance, *Rhopalosiphum padi* have been observed hiding their bodies beneath the soil to avoid detrimental high temperatures at the soil surface (Wikteliuss 1987). *Sitobion avenae* escapes heat stress by dropping off from the plant (Ma et al. 2018).

The potential of heat hardening and acclimation to enhance the upper lethal temperature (CT_{max}) in aphids is limited, as indicated by studies conducted by Alford et al. (2012a) and Zhao et al. (2017). Moreover, there is no evidence supporting intergenerational acclimation effects on either CT_{max} or heat coma, as reported by Hazell et al. (2010).

Aphids may use metabolic changes to protect themselves from heat stress. One such change involves the induction of polyols in response to high temperatures, although the specific type of polyol varies depending on the aphid species. For instance, elevated temperatures promoted the production of mannitol in the cotton aphid (*Aphis gossypii*) (Hendrix and Salvucci 1998). In contrast, *Acyrtosiphon pisum* exhibited a significantly stronger response in the production of sorbitol

compared to mannitol when exposed to high temperatures (Burke et al. 2010). Furthermore, repeated exposure to high temperatures led to the accumulation of glycerol in *Myzus persicae* compared to aphids subjected to prolonged single exposure (Ghaedi and Andrew 2016). Additionally, after experiencing heat exposure, pea aphids demonstrated an increase in β -Alanine, which may act as a chemical chaperone preserving enzyme function during heat shock (Burke et al., 2010).

The heat shock response in aphids involves the activation of HSPs. Previous studies on HSPs in aphids have consistently demonstrated that heat shock induces the expression of *hsp70* in various aphid species (Wilson et al., 2006; Li et al., 2017; Zhang et al., 2019; Saeidi et al., 2021; Chen et al., 2022). Furthermore, the expression levels of *hsp60* and *hsc70* were also found to be upregulated in *Rhopalosiphum padi* when exposed to heat (Li et al., 2017). It is worth noting that the regulation of cellular responses in aphids may involve the simultaneous upregulation of molecular chaperones in aphid symbionts (Wilson et al., 2006; Zhang et al., 2019). These findings highlight the coordinated efforts of both aphids and their symbionts in mounting a robust heat shock response.

6 The objectives and outline of the thesis

6.1 Research questions and hypothesis

Ecologists face the critical task of comprehending the effects of environmental stresses, particularly those associated with climate change, on individual organisms and the subsequent repercussions on populations, communities, and ecosystems (Sulmon et al., 2015). Organisms residing at the edges of their ecological niches encounter stress, as they confront environmental conditions that may surpass the ranges necessary for normal growth and development (Korsloot et al., 2004). The ability of organisms to cope with these stressors determines their ecological limits and significantly influences their life history traits, thereby impacting population dynamics and community composition.

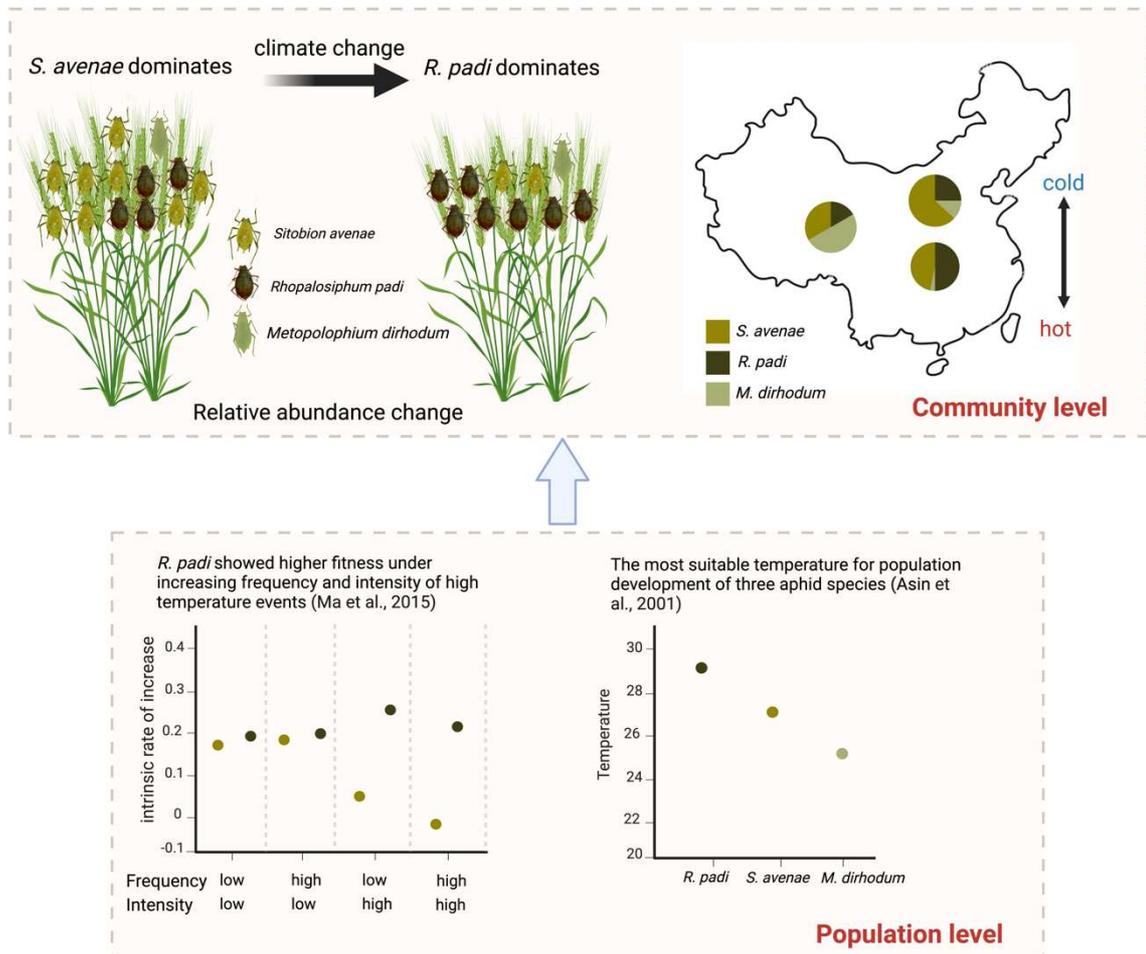
Temperature, as a prominent abiotic factor, can trigger a stress response, especially in ectothermic animals and plants that lack the capacity to regulate their internal temperature (Roelofs et al., 2007). Physiological plasticity, enabling organisms to adapt to diverse environmental conditions, assumes paramount importance in the context of climate change. Nevertheless, our understanding of the physiological responses of organisms to climate-induced environmental variations at the community level remains limited, as such data are scarce in the existing literature. This scarcity impedes our ability to develop consistent predictions regarding community structure and ecosystem functioning.

The focus of this research is on the cereal aphid guild, comprising three species: *Sitobion avenae*, *Rhopalosiphum padi*, and *Metopolophium dirhodum*. These aphids are globally recognized as pests and vectors of various viruses, including the Barley Yellow Dwarf Virus (BYDV), which significantly reduces cereal yields (Brault et al., 2010). Extensive field observations conducted in eastern China over several years have revealed the substantial influence of climate change on the

relative abundances of these three species (Ma et al., 2015b; Zhu et al., 2021). These changes appear to be primarily driven by variations in the thermotolerance of each species.

Sitobion avenae used to be considered the most dominant species (Zhang et al., 2009). However, with the increasing frequency and intensity of high-temperature events, *Rhopalosiphum padi* has exhibited relatively higher fitness, resulting in a shift in its annual relative dominance in certain southern regions of China (Ma et al., 2015b; Zhu et al., 2021). Conversely, *Metopolophium dirhodum* seems to prefer cooler areas in northwest China. These aphids appear in small populations during early spring and quickly disappear (Hu and Gui, 1985; Chen et al., 1994).

Regarding their population development, the most suitable constant temperature for *Rhopalosiphum padi* was found to be 28.5 °C, followed by 26.5 °C for *Sitobion avenae* and 24.5 °C for *Metopolophium dirhodum* (Figure 11)



The general hypothesis of this PhD thesis is that the shift in dominant species could be closely linked to current climate change that would act in a non-uniform way on the species concerned. Indeed, if the species within the guild have different levels of thermal tolerance, this will lead to these community shift phenomena under climate change, as has already been observed in other groups

(Daufresne et al. 2004, Hansen et al. 2017). Different levels of thermal tolerance (i.e., individual response) may be driven by distinct physiological tolerance to thermal stress (i.e., sub-individual response). The accumulation of low molecular weight cytoprotectants and the synthesis of heat shock proteins are critical physiological mechanism in increasing physiological tolerance to thermal stress (Teets and Denlinger 2013, Olsson et al. 2016, Gonzalez-Tokman et al. 2020). Thus, species with varying thermal tolerance could be attributed to their distinct response in metabolic and molecular responses to thermal stress.

The objectives of this thesis are:

- 1) To compare the interspecific variation in heat and cold tolerances among the three aphid species and relate this variation to the changes in aphid community abundance.
- 2) To compare the expression levels of heat shock protein genes (*hsps*) among the three aphid species and the differences in *hsps* expression in response to heat vs cold.
- 3) To compare the metabolic response to heat and cold stress among the three aphid species.

The thesis aims to test the following hypotheses:

- 1) The three aphid species will exhibit significant variations in heat and cold tolerance, as indicated by differences in the parameters of their thermal death time curves. We expect *R. padi* will have higher heat tolerance but be more vulnerable to cold, while *M. dirhodum* could be more susceptible to heat stress but exhibit greater cold tolerance. (Chapter 2)
- 2) Interspecific variations in thermal tolerance may manifest in differences in the potential thermal injuries accrued by aphids under natural temperature regimes. (Chapter 2)
- 3) In comparison to control conditions, aphids are expected to show high expression levels of *hsps* during/after heat stress. However, they may not express *hsps* during/after cold stress or only moderately express *hsps* during recover from cold stress. (Chapter 3)
- 4) Species suffering similar stress levels may show similar expression levels of *hsps*. (Chapter 3)
- 5) The most tolerant species are likely to employ a large network of active molecular chaperones following the same exposure duration to heat and cold stress. (Chapter 3)
- 6) Aphids exposed to heat or cold stress are expected to exhibit metabolic disorders compared to control conditions. The severity of these metabolic disorders is predicted to vary according to the stress levels, with longer durations of stress (i.e., higher injury level) resulting in increasingly challenging recovery. (Chapter 4 &5)
- 7) When exposed to the same duration of heat/cold stress, the tolerant species is expected to show fewer metabolic perturbations and more efficient restoration of homeostasis during recovery than the intolerant species. (Chapter 4 &5)
- 8) Aphids are expected to accumulate cytoprotectants in response to heat/cold stress. Specifically, levels of some polyols, such as mannitol and sorbitol, may increase in response

to heat stress, while trehalose and some amino acids (e.g., proline) may upregulate in response to cold stress. (Chapter 4 &5)

- 9) More direct and pronounced effects/perturbations are predicted for heat stress compared to cold stress due to the dissymmetric nature of thermal performance curves. (Chapter 4 &5)

6.2 Thesis outline

To test the above hypotheses, a series of experiments were designed and conducted across chapters 2 to 5.

- In Chapter 2, we compared the thermal tolerance of three aphid species (*Metopolophium dirhodum*, *Sitobion avenae*, and *Rhopalosiphum padi*) using a thermal tolerance landscape approach. Specifically, we built the thermal death time (TDT) models based on detailed survival datasets for each species at different ages across a broad range of stressful high (34~40 °C) and low (-3~-11 °C) temperatures. Using these TDT parameters, we performed a thermal risk assessment by calculating the potential daily thermal injury accumulation associated with the regional temperature variations in three wheat-growing sites along a latitude gradient.
- In Chapter 3, we investigated interspecific differences in the expression of *hsps* among the three aphid species. We exposed aphids to temperatures of 34 and 37 °C (typical summer temperatures) and -5 and -8 °C (typical winter temperatures in temperate regions). We cloned *hsp* sequences and compared the interspecific differences in mRNA levels of seven *hsps* (*hsp10*, *hsc70*, *hsp60*, *hsp70*, *hsp75*, and *hsp90*) after the same exposure durations, but also after the same injury levels (i.e., Lt_{50}).
- In Chapter 4, we explored the interspecific differences in the metabolic signatures during heat stress and recovery using targeted metabolomics. Based on our result obtained in Chapter 2, we designed two experiments to achieve this goal: subjecting the three aphid species to a high temperature (34 °C) for durations corresponding to increasing injury levels ($1/2 Lt_{50}$ and Lt_{50}) as well as the same exposure time. We compared the deviations in metabolites from homeostasis of the three aphid species during stress and recovery.
- In Chapter 5, we investigated the interspecific differences in the metabolic signatures during cold stress and recovery using targeted metabolomics. The study involved subjecting the three species to a low temperature (-5 °C) for the time corresponding to increasing injury levels ($1/2 Lt_{50}$ and Lt_{50}) and also the same exposure time. We compared the deviations in metabolites from homeostasis among the three aphid species during stress and recovery.

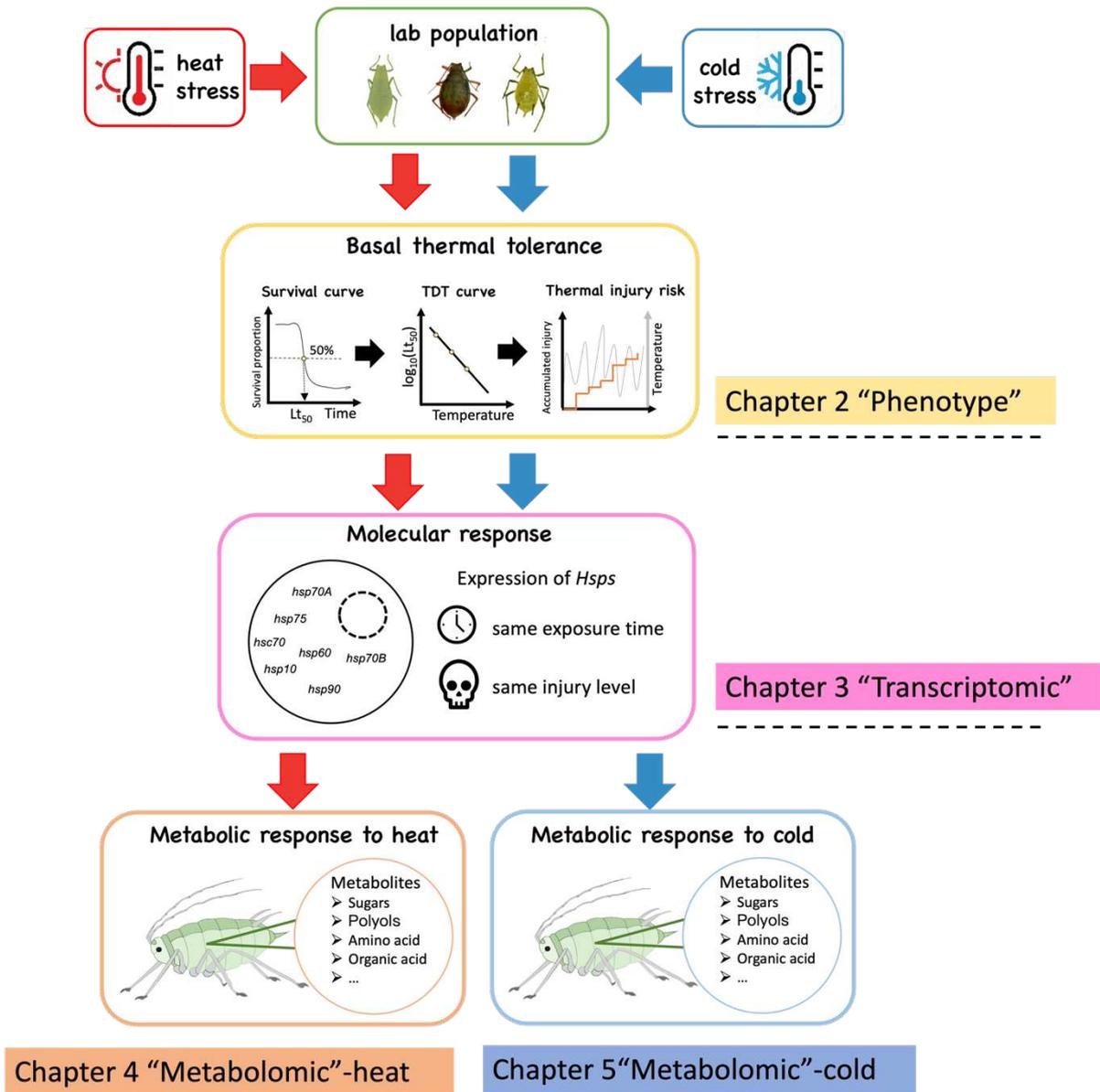


Figure 12. Illustration of the thesis structure aiming at comparing the interspecific variations in basal thermal tolerance and the molecular and metabolic responses to thermal stress.

Chapter 2 Comparative thermal tolerance

Article 1. Interspecific differences in thermal tolerance landscape explain aphid community abundance under climate change

Three co-occurring cosmopolitan species are found within the guild of cereal aphids: *Sitobion avenae*, *Ropalosiphum padi* and *Metopolophium dirhodum*. Earlier reports have shown that increasing frequency of temperature extremes causes a shift in dominant species within guilds of cereal aphids by differently altering the population's growth (Ma et al. 2015b). We hypothesize that this phenomenon is due to the uneven effect of ongoing climate change on the species concerned. Indeed, if the species present in the guild have different levels of thermal tolerance, then this would lead to these community shift phenomena under climate change, as has already been observed in other groups (Daufresne et al. 2004, Hansen et al. 2017). To test the hypothesis, we used a thermal tolerance landscape approach to compare the interspecific thermal tolerance among the three aphid species.

Main findings:

- 1) The thermal tolerance landscape reflected the survivals of cereal aphids at extreme temperatures.
- 2) *R. padi* was more heat-tolerant and cold-vulnerable than *S. avenae* and *M. dirhodum*.
- 3) Young nymphs generally had lower thermal tolerance than old nymphs or adults.
- 4) According to estimated thermal injury, *R. padi* was severely injured by cold while *M. dirhodum* was by heat.
- 5) Interspecific differences in thermal tolerance may drive the aphid community abundance change.



Interspecific differences in thermal tolerance landscape explain aphid community abundance under climate change

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ARTICLE INFO

Keywords:

Extreme temperature
Thermal death time curve
Cereal aphids
Sitobion avenae
Rhopalosiphum padi
Metopolophium dirhodum

ABSTRACT

A single critical thermal limit is often used to explain and infer the impact of climate change on geographic range and population abundance. However, it has limited application in describing the temporal dynamic and cumulative impacts of extreme temperatures. Here, we used a thermal tolerance landscape approach to address the impacts of extreme thermal events on the survival of co-existing aphid species (*Metopolophium dirhodum*, *Sitobion avenae* and *Rhopalosiphum padi*). Specifically, we built the thermal death time (TDT) models based on detailed survival datasets of three aphid species with three ages across a broad range of stressful high (34–40 °C) and low (–3~–11 °C) temperatures to compare the interspecific and developmental stage variations in thermal tolerance. Using these TDT parameters, we performed a thermal risk assessment by calculating the potential daily thermal injury accumulation associated with the regional temperature variations in three wheat-growing sites along a latitude gradient. Results showed that *M. dirhodum* was the most vulnerable to heat but more tolerant to low temperatures than *R. padi* and *S. avenae*. *R. padi* survived better at high temperatures than *Sitobion avenae* and *M. dirhodum* but was sensitive to cold. *R. padi* was estimated to accumulate higher cold injury than the other two species during winter, while *M. dirhodum* accrued more heat injury during summer. The warmer site had higher risks of heat injury and the cooler site had higher risks of cold injury along a latitude gradient. These results support recent field observations that the proportion of *R. padi* increases with the increased frequency of heat waves. We also found that young nymphs generally had a lower thermal tolerance than old nymphs or adults. Our results provide a useful dataset and method for modelling and predicting the consequence of climate change on the population dynamics and community structure of small insects.

1. Introduction

Climate change leads to more frequent extreme temperature events (Perkins-Kirkpatrick and Lewis 2020; Arias et al., 2021), which trigger consequences from individual physiology to ecosystem functions (Ma et al., 2021; Secretariat et al., 2021). Insects' body temperature is mainly determined by the microclimates of their habitats, making them highly sensitive to temperature changes (Pincebourde and Woods 2020). Exposure to a short-term extreme temperature that reaches or exceeds critical thermal limits can cause an immediate effect on fecundity, fertility and even mortality (Zhang et al., 2013; Liang et al., 2014; Sales

et al., 2021). Therefore, thermal tolerance is often correlated with population abundance, species distribution, and phenology, and thus plays an important role in predicting the effect of climate change (Sunday et al., 2012; Amundrud and Srivastava 2020; Alruiz et al., 2022).

A single temperature estimated from static or dynamic assays has been widely adopted as an upper or lower thermal tolerance limit (Winther Bak et al., 2020). However, the critical thermal limit depends on the methodological context including the exposure time (Rezende et al., 2014; Terblanche et al., 2007). Moreover, this limit, which represents a static snapshot of thermal tolerance, is not able to describe the

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temporal dynamic and cumulative impact of environmental extreme temperatures on survival (Alruiz et al., 2022).

Thermal Death Time (TDT) curve, which describes that the tolerable duration at stressful temperatures exponentially decreases with increasing stress intensity, can fully and accurately characterize species' thermal tolerance (Bigelow 1921; Fry et al., 1946; Nedv ed et al., 1998; Colinet et al., 2011; Rezende et al., 2014; J rgensen et al., 2019; Tarapacki et al., 2021). The TDT framework has recently been re-examined and parameterized to enable the analysis of the combined impact of extreme temperatures and exposure times on survival, which is now referred to as the "thermal tolerance landscape". (Rezende et al., 2014; J rgensen et al. 2019, 2021;  rsted et al., 2022). Furthermore, the injury accumulation rate increases exponentially with temperature and the injury sustained at different static temperatures can be completely additive (J rgensen et al., 2021;  rsted et al., 2022). Thus, through the TDT model, we can not only get a holistic view of how thermal tolerance duration varies with temperatures (Tarapacki et al., 2021; Willot et al., 2022), but also link the thermal tolerance to the injury accrued under natural temperatures. However, few studies employ this approach to extrapolate the thermal tolerance estimated in the laboratory to natural settings.

The same dose of exposure to thermal extremes might lead to different reactions among co-existing species (Ma et al. 2015a, 2015b) because some species can be more stress-resilient than others (Kaspari et al., 2015; Buckley and Huey 2016). The interspecific differences in thermal tolerance can shift the community structure through cascading effects (Harvey et al., 2020; Filazzola et al., 2021), altering the relative abundance of coexisting insect species (Ma et al., 2015b; Birkett et al., 2018; Zhu et al., 2021). This is particularly important for practice in agricultural pest management (Mohammed et al., 2019). In addition, the natural population of insects commonly consists of multiple life stages or age cohorts that have different thermal tolerance (Bowler and Terblanche 2008; Zhang et al., 2015; Zhao et al., 2019), even the stages living in the same microhabitat also show different heat tolerance (Zhao et al., 2017). When the mixed-stage population experiences extreme temperature events, the most sensitive stages are likely to be injured and die, whereas the most tolerant may survive and develop to the adult stage for reproduction during the following mild phases, thereby contributing to population recovery (Ma et al., 2021). Therefore, clarifying the thermal tolerance in different developmental stages is essential for understanding the ecological consequences of extreme temperature events.

Here we used three species of co-existing global pests (family Aphididae) in cereal crops, *Sitobion avenae* (Fabricius, 1775), *Rhopalosiphum padi* (Linnaeus, 1758), and *Metopolophium dirhodum* (Walker, 1849) as focus species. We aimed to answer the following questions: 1) can the change in aphid community abundance be related to interspecific differences in the basal thermotolerance of the three aphid species? 2) do older aphids suffer less than younger ones from heat/cold stress? To answer these questions, we conducted an elaborate study to characterize the interspecific variation in thermal tolerance among the three cereal aphid species using the TDT framework at both high and low temperatures. Then, we examined the effect of developmental stage/age on the intraspecific variations in thermal tolerance. Finally, we used the TDT model parameters to perform a risk assessment predicting the potential injuries under the natural environments for three aphid species in three sites along a latitude gradient in China.

2. Material and methods

2.1. Aphid stock rearing

Populations of three common aphid species developing on winter wheat, *S. avenae*, *M. dirhodum* and *R. padi* were originally collected in May 2010 from wheat fields near Beijing (39°48'N, 116°28'E) (Zhao et al., 2014). The colonies were then refreshed annually with

field-collected aphids. The aphids were reared in screened cages (60 × 60 × 60 cm) at 22 ± 0.5 °C, RH 70%, and a photoperiod of 16 L: 8 D. Aphids were fed on 10–20 cm tall winter wheat seedlings that were renewed weekly. Aphids used for experiments were prepared by placing newly molted adult aphids in a pot with wheat seedlings at 20 °C. After 24 h, the apterous adult aphids were removed so that only newly-born nymphs remained. Subsequently, these nymphs were allowed to develop for 2, 6, 12 days to generate three different stages: 2nd, 3rd or 4th instar (depending on species) and adult, respectively.

2.2. Tests of heat and cold survival

For heat tolerance tests, a multifactorial experiment involved the three aphid species × three ages (2, 6, 12 days) × seven temperature regimes (34, 35, 36, 37, 38, 39, 40 °C) × ≥ five exposure times (ranging from 0 to 2000 min depending on the temperature) was conducted. Similarly, a multifactorial experiment for cold tolerance included the three aphid species × three ages (2, 6, 12 days) × nine temperature regimes (−3, −4, −5, −6, −7, −8, −9, −10, −11 °C) × ≥ five exposure times (ranging from 0 to 10162 min depending on the temperature) was conducted. All the treatments were replicated at least 3 times, and for each replicate, 7–12 aphids were tested (Table S1). To obtain a reliable survival curve at each temperature, we tried to find and test the exposure times that result in survival rates close to 0%, 25%, 50%, 75%, and 100%, respectively. To reduce handling stress and provide food as well as a water source, we cut off the wheat leaves with aphids and then transferred them directly into glass vials (10 mL). Then, we closed each vial with a dry sponge and placed a rack with vials into a glycol bath (Ministat 230-cc-NR; Huber Ltd., Germany, accuracy ± 0.01 °C) pre-set at the experimental temperature. For each temperature and exposure time, the exposed aphids were removed from the bath and allowed to recover at 20 °C for 24h before checking the survival of each individual. Aphids were considered alive if the individual moved following a gentle tactile stimulation with a brush. In total, 14980 and 14633 individuals were tested, for heat and cold tolerance, respectively, corresponding to 1502 and 1536 replicates.

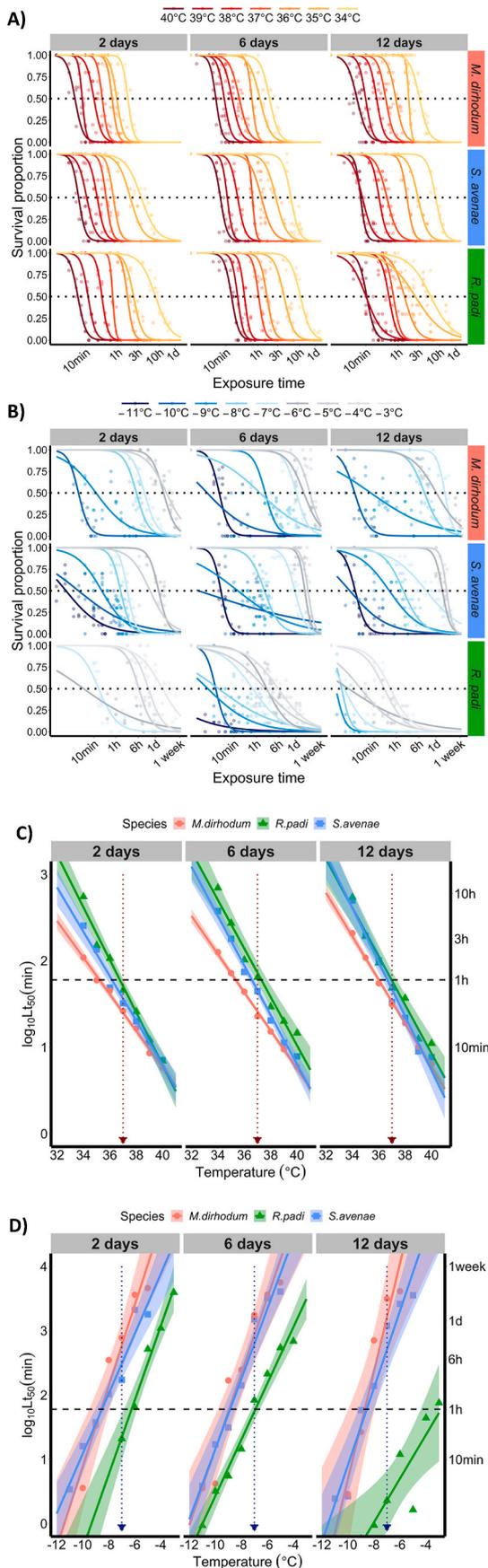
2.3. Survival curves, Lt_{50} , and thermal death time (TDT) curves

For each tested temperature, the survival proportion was fitted to a logistic regression model using the *glm* function and the Lt_{50} (i.e. the temperature-specific exposure duration resulting in 50% mortality) was estimated using the *dose.p* function in the R package "MASS" (Ripley et al., 2013).

TDT curves were generated by regressing $\log_{10}(Lt_{50})$ against test temperature for each species/age-group combination, at both high and low temperatures. The output of the TDT analysis is typically given by model estimates of temperature causing 50% mortality after a specific time (e.g., the temperature that causes 50% mortality after 1 h), as well as the thermal sensitivity, which is often expressed from z (−1/slope). The latter parameter reveals how many degrees are required to change tolerance duration 10-fold (Rezende et al., 2014). Note that for cold tolerance, thermal sensitivity is expressed by z' which corresponds to 1/slope.

2.4. Examining effects of species and age

The effect of test temperature, species, and age on Lt_{50} was examined in a three-way ANCOVA including all interaction terms, with test temperature as a continuous variable and species and age as categorical variables. Interspecific differences in heat or cold tolerance were examined independently for each age group by comparing differences in the parameters of the TDT curves. Specifically, slopes of the TDT curves (thermal sensitivity) were first compared pairwise using the functions *emtrends()* and *pairs()* in the R package "emmeans" (Lenth et al., 2019), which are based on least-squares means. Further, we tested whether the



(caption on next column)

Fig. 1. Survival curves (panel A for heat, B for cold) and thermal death time curves (panel C for heat, D for cold) for the three ages (2, 6, and 12 days) of *M. dirhodum*, *S. avenae*, and *R. padi*. The curves in different colors (A and B) were fitted by the logistic regression model at each temperature. Each point in panels A and B represented the survival proportion of aphids in a single replicate and each point in panels C and D represented the $\log_{10}(Lt_{50})$ value estimated from the survival curve at a tested temperature. Lines and shades in C and D illustrated the linear regression lines and the corresponding 95% confidence intervals. CT_{max} and CT_{min} were the intercepts with the black dashed line ($Lt_{50} = 1$ h). Thermal sensitivity for high temperature (z) or low temperature (z') was derived from the slope of the line ($z = -1/\text{slope}$, $z' = 1/\text{slope}$). The $\log_{10}(Lt_{50})$ at 37 °C and $\log_{10}(Lt_{50})$ at -7 °C where red and blue dashed arrows pointed were pairwise compared between species and ages. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

intercepts (Log-transformed Lt_{50} estimate) differed between species by comparing the intercept at 37 °C for heat tolerance and -7 °C for cold tolerance using the function *emmeans()* in “emmeans” package (Lenth et al., 2019). These temperatures were chosen as they represented intermediate values in the range of temperatures used to assess heat and cold tolerance, respectively. A similar approach, comparing slopes and intercepts, was used to examine the effects of aphid age on both heat and cold tolerance for each species. All data analyses were performed in R version 4.1.2.

2.5. Relating TDT curves to natural temperature regimes

Following the method described by Jørgensen et al., (2021), we used parameters of TDT curves (slope and intercept) to estimate the cumulative daily thermal injury experienced by aphids during natural thermal fluctuations. This basic risk analysis has of course some limits, for instance, it ignores the importance of microhabitat choice and behavioral thermoregulation by using the simple assumption that aphid body temperature is equal to air temperature. Further, this analysis does not consider if cold or heat tolerance changes seasonally due to acclimatization to winter or summer conditions. Despite these caveats, this simple approach can provide a raw proportional risk assessment to compare species or regions based on both the intensity and duration of thermal extremes. Specifically, it calculates, minute by minute, the accumulation of thermal stress over 24 h (1440 min) using the formula:

$$\text{Accumulated injury} = \sum_{i=1}^{i=1440} \frac{100 \cdot (t_{i+1} - t_i)}{10^{(\beta \cdot \max(T_i; T_{i+1}) + \alpha)}}$$

where α is the slope and β is the intercept of the TDT curve, t_i and t_{i+1} , are the i min and the $i+1$ min, respectively, $\max(T_i; T_{i+1})$ is the maximum temperature between the i and $i+1$ min; for winter, it is the $\min(T_i; T_{i+1})$, the minimum temperature between the i and $i+1$ min. Note that thermal injuries only accumulate in the stressful temperature range (above a critical temperature T_c for heat stress or below a critical temperature T_c' for cold stress) (Jørgensen et al., 2021, Ørsted et al., 2022). We defined T_c or T_c' as the temperature estimated to cause 50% mortality (Lt_{50}) in one week. Specifically, we calculated this T_c or T_c' from the TDT curves. For 6-day-old *M. dirhodum*, *R. padi* and *S. avenae*, T_c was estimated as 25.29 °C, 29.34 °C and 28.82 °C, respectively and cold T_c' was -5.15 °C, -1.81 °C and -4.79 °C, respectively.

To associate thermal tolerance measures to natural climate conditions, we downloaded hourly temperature data for winter (from November to February 2016/2017) and summer (from May to August 2016) months from three sites in China where grow winter wheat infested by cereal aphids: southern site (Wuhan, N 30.78°, E 114.21°), intermediate site (Xinxiang, N 35.30°, E 113.92°) and northern site (Beijing, N 40.07°, E 116.58°) (Fig. 4A). Data were downloaded from a global weather API (<https://www.visualcrossing.com/weather-data>) in May 2022. We calculated minute by minute temperature by linear

Table 1

Parameters of thermal death time curves for each age group of the three species at high temperatures. Superscript lowercase letters in thermal sensitivity (z) and $\log_{10}(Lt_{50})$ at 37 °C marked significant differences in slope and intercept between species with the same age ($p < 0.05$). Superscript uppercase letters marked significant differences between age groups for the same species ($p < 0.05$).

Species	R ²	P value	Thermal sensitivity (z)	CT _{max} (°C)	$\log_{10}(Lt_{50})$ at 37 °C
2 days					
<i>M. dirhodum</i>	0.99	<1*10 ⁻⁴	4.71 ^{B, b}	35.21	1.40 ^{A, a}
<i>S. avenae</i>	0.98	<1*10 ⁻⁴	3.89 ^{A, a}	36.16	1.56 ^{A, b}
<i>R. padi</i>	0.99	<1*10 ⁻⁴	3.29 ^{A, a}	36.76	1.71 ^{A, c}
6 days					
<i>M. dirhodum</i>	0.99	<1*10 ⁻⁴	4.52 ^{B, b}	35.35	1.41 ^{A, a}
<i>S. avenae</i>	0.99	<1*10 ⁻⁴	3.49 ^{A, a}	36.58	1.66 ^{A, b}
<i>R. padi</i>	0.97	<1*10 ⁻⁴	3.57 ^{A, a}	37.29	1.86 ^{B, c}
12 days					
<i>M. dirhodum</i>	0.99	<1*10 ⁻⁴	3.97 ^{A, a}	36.00	1.53 ^{B, a}
<i>S. avenae</i>	0.98	<1*10 ⁻⁴	3.19 ^{A, a}	36.65	1.67 ^{A, b}
<i>R. padi</i>	0.97	<1*10 ⁻⁴	3.56 ^{A, a}	36.97	1.77 ^{A, c}

interpolation from the hourly temperature using the na.approx function in the “zoo” package (Zeileis et al., 2014). The accumulated daily heat stress dose was calculated from midnight to midnight and the accumulated daily cold stress dose from noon to noon. In total, this analysis produced 122 estimates of daily heat stress doses and 120 estimates of daily cold stress doses for each species and region.

We used Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction to compare the differences in the calculated daily accumulated injury between species within sites and sites with the same species. Note that to avoid comparing a lot of days without any injury accumulation, only days with injury accumulation were compared.

3. Results

3.1. Survival curves, TDT curves: estimation of critical thermal limits

The survival curves generally fitted the logistic function well, with a median R² of 0.81 (Fig. 1A and B, 74% of assays had McFadden’s pseudo-R² > 0.70, See Table S2). For both heat and cold tolerance, the estimated Lt₅₀ decreased exponentially with increasing stress intensity (temperature) as predicted by the TDT curves (Fig. 1C and D). Linear regressions of $\log_{10}(Lt_{50})$ against temperature generated nine TDT curves (three species × three ages) for high and low temperatures respectively. All curves had a high determination coefficient (R²) ranging from 0.78 to 0.99 ($P < 0.01$), especially for those at high temperatures (see Table 1 and Table 2).

Table 2

Parameters of thermal death time curves for each age group of the three species at low temperatures. Superscript lowercase letters in thermal sensitivity (z') and $\log_{10}(Lt_{50})$ at -7 °C marked significant differences in slope and intercept between species with the same age ($p < 0.05$). Superscript uppercase letters marked significant differences between age groups for the same species ($p < 0.05$).

Species	R ²	P value	Thermal sensitivity (z')	CT _{min} (°C)	$\log_{10}(Lt_{50})$ at -7 °C
2 days					
<i>M. dirhodum</i>	0.94	<1*10 ⁻²	1.59 ^{A, a}	-8.58	2.77 ^{A, b}
<i>S. avenae</i>	0.96	<1*10 ⁻³	2.14 ^{A, a}	-8.52	2.49 ^{A, b}
<i>R. padi</i>	0.98	<1*10 ⁻²	1.73 ^{A, a}	-6.24	1.34 ^{B, a}
6 days					
<i>M. dirhodum</i>	0.93	<1*10 ⁻³	1.70 ^{A, a}	-8.94	2.92 ^{A, b}
<i>S. avenae</i>	0.96	<1*10 ⁻⁴	1.83 ^{A, a}	-8.86	2.80 ^{A, b}
<i>R. padi</i>	0.98	<1*10 ⁻⁴	2.29 ^{A, a}	-6.92	1.74 ^{B, a}
12 days					
<i>M. dirhodum</i>	0.93	<1*10 ⁻²	1.19 ^{A, a}	-8.70	3.21 ^{A, b}
<i>S. avenae</i>	0.98	<1*10 ⁻³	1.67 ^{A, ab}	-8.55	2.71 ^{A, b}
<i>R. padi</i>	0.78	<1*10 ⁻²	2.94 ^{A, b}	-2.84	0.36 ^{A, a}

Here, we used the temperature causing 50% mortality after 1 h (CT_{max(1h)}/CT_{min(1h)}) as the upper or lower critical thermal limit. *R. padi* ranked first in CT_{max(1h)} (36.76 °C–37.29 °C in different ages), 0.97–1.94 °C higher than *M. dirhodum* and *S. avenae* proved intermediate of the 2 species (Table 1). The thermal sensitivity at high temperatures (z) ranged from 3.19 to 4.17 (Table 1). CT_{min(1h)} fell between -8.24 ~ -2.84 °C (Table 2). Aphids had high cold sensitivity as the lower z' values indicated greater thermal sensitivity (i.e., a larger change in Lt₅₀ with a change in exposure temperature).

3.2. Interspecific variation in thermal tolerance

The effects of temperature, species, and age on Lt₅₀ were examined in a three-way ANCOVA including all interaction terms. This analysis found that species interacted with both age and temperature at both high and low temperatures (Tables S3 and S4). Accordingly, we split the dataset according to age so that TDT slopes and intercepts from different species could be compared. *R. padi* generally had the highest heat tolerance, as depicted by the significantly higher $\log_{10}(Lt_{50})$ value (1.71–1.86) at 37 °C for all age groups, followed by *S. avenae*, and then *M. dirhodum* (Table 1, see p-values in Table S5). The thermal sensitivity (z) showed few interspecific differences; *M. dirhodum* was characterized by a lower thermal sensitivity (higher z) than *R. padi* and *S. avenae* (Table 1, See p-values in Table S6).

For cold tolerance, *R. padi* showed a significantly lower $\log_{10}(Lt_{50})$ at -7 °C for all age groups than the other two species (Table 2, see p-values in Table S7). The difference between *M. dirhodum* and *S. avenae* was relatively small. The thermal sensitivity (z') did not differ significantly among species, except for 12-day adults of *R. padi* that had a statistically higher z' (lower thermal sensitivity) than *M. dirhodum*. (Table 2, See p-values in Table S8).

3.3. Developmental stage variation in thermal tolerance

Adults (12-d-old) or old nymphs (6-d-old) generally had higher thermal tolerance than young nymphs (2-d-old). For *M. dirhodum* and *S. avenae*, adults (12-d-old) had a lower z -value (higher thermal sensitivity) and longer $\log_{10}(Lt_{50})$ at 37 °C than younger life stages, although this was only statistically significant for *M. dirhodum* (Table 1). For *R. padi*, old nymphs (6-d-old) were the most tolerant among all life stages (Table 1).

For cold tolerance, none of the species displayed a significant difference in thermal sensitivity (z') between ages. Adult (12-d-old) *R. padi* showed a significantly lower $\log_{10}(Lt_{50})$ at -7 °C than nymphs (Table 2, see p-values in Table S7).

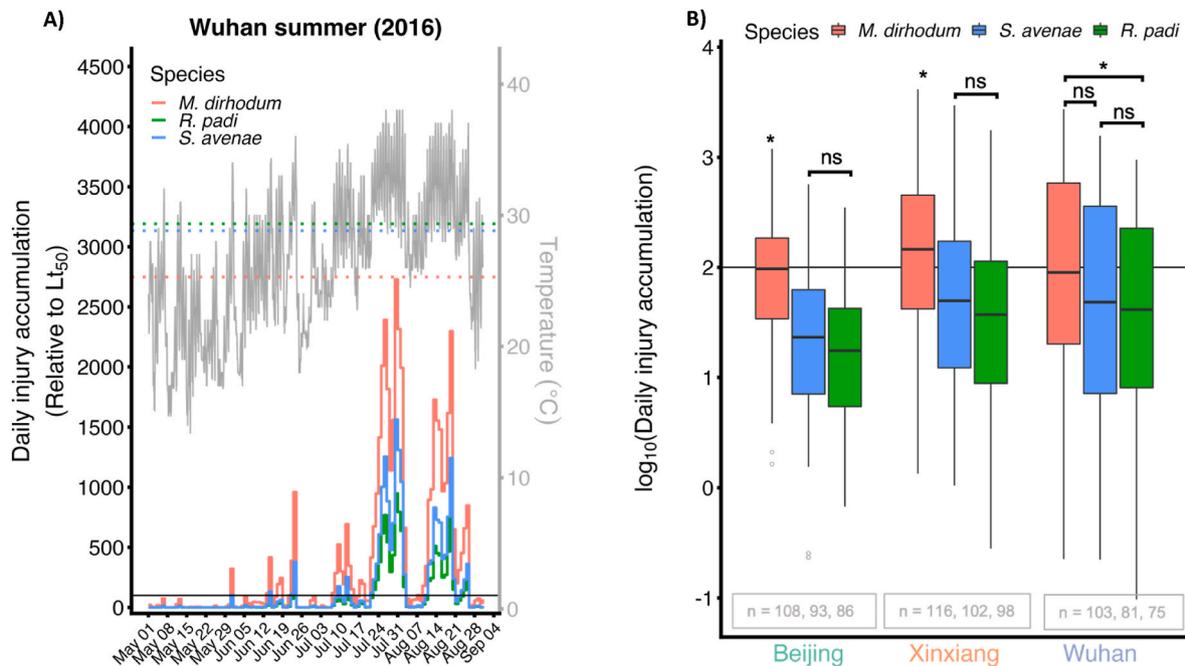


Fig. 2. Predicted daily injury accumulation based on the TDT parameters for the three species (age 6 days) under natural temperature fluctuations in the summer (May–August) of 2016. **A)** The temperature profile (grey, secondary y-axis) was used to project the daily accumulation of injury by use of the TDT parameters (see the formula in the method part). The species-colored lines/steps at the bottom of the graph indicated the amount of injury accumulated *per day* relative to the amount of injury that would result in half the aphid dying (here set to 100 with the black line). In the current run of this model, only temperatures above a T_c of 25.29 °C (red dotted line), 29.34 °C (green dotted line) and 28.82 °C (blue dotted line) were set to result in injury for *M. dirhodum*, *R. padi* and *S. avenae*, respectively. **B)** Boxplot summarizing the data from (A) and similar analyses for Beijing and Xinxiang in the summer (see Figs. S1A and B). Note that the y-axis was \log_{10} -transformed and the injury accumulation resulting in median lethal (100) was marked by the line at $\log_{10}(\text{Daily injury accumulation}) = 2$. To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as *n* in the graph) were summarized in this plot. Comparisons among species within sites were examined using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction – results were indicated on the plot. Asterisks marked significant differences ($p < 0.05$) between two groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Relating TDT curves to natural temperature regimes

To associate thermal tolerance measures with natural climate conditions, we calculated the accumulated thermal injury of three aphid species in three age groups using the TDT model and regional temperatures. Results were similar for all three age groups (2-d-old, 6-d-old, 12-d-old, see Figs. S3–6), thus here we only present the result of 6-day-old aphids (Figs. 2–4).

In each site, the estimated heat injury differed among species ($\chi^2 = 53.3$, $df = 2$; $p < 1 \times 10^{-11}$ in Beijing; $\chi^2 = 36.0$, $df = 2$; $p < 1 \times 10^{-7}$ in Xinxiang; $\chi^2 = 9.0$, $df = 2$; $p < 0.05$ in Wuhan, Fig. 2B). *M. dirhodum* always accumulated more heat injury than the two other species in Beijing and Xinxiang (Pairwise Wilcoxon Rank Sum Tests, $p < 0.05$).

According to the calculation, *R. padi* might suffer cold injury across the whole winter months while the two other species might be affected only during the coldest days (Fig. 3A). Estimated accumulated cold injury differed among species in Beijing ($\chi^2 = 8.5$, $df = 2$; $p < 0.05$; Fig. 3B). Specifically, *R. padi* had higher daily injury accumulation than *S. avenae* in Beijing (Pairwise Wilcoxon Rank Sum Tests, $p < 0.05$), while no significant interspecific difference was found in Xinxiang. *M. dirhodum* and *S. avenae* might not accumulate any injury in Wuhan during winter (Fig. 3B).

According to the calculation, heat injury of three species might differ with sites ($\chi^2 = 6.4$, $df = 2$; $p < 0.05$ for *M. dirhodum*; $\chi^2 = 9.9$, $df = 2$; $p < 0.01$ for *S. avenae*; $\chi^2 = 10.6$, $df = 2$; $p < 0.01$ for *R. padi*, Fig. 4C). In Beijing, the northernmost site, aphids accumulated less injury during summer (Pairwise Wilcoxon Rank Sum Tests, $p < 0.05$, Fig. 4C) but more injury during winter than the other two sites (Pairwise Wilcoxon Rank Sum Tests, $p < 0.05$, Fig. 4D).

4. Discussion

The previous studies often inferred the impact of climate change on the coexisting species based on a single estimate of thermal tolerance, such as CT_{max} and CT_{min} , or LT_{50} and Lt_{50} (Warren and Chick 2013; Birkett et al., 2018). High-tolerant species will increase their population or expand their range, while low-tolerant species will decrease their population or shrink their range under extreme climate scenarios. However, thermal tolerance is not constant but varies with the dose determined by temperature and exposure duration as shown in our study. In nature, extreme high and low temperatures occur regularly in a diurnal and seasonal cycle, and irregularly across time and space, thereby altering the mortality of insects both dynamically and cumulatively. It is difficult to use a single estimate of thermal tolerance to calculate the dynamic and cumulative thermal injury. Studies that assess demographic performances of co-existing species under limited temperature scenarios (e.g., Ma et al., 2015b) may not fully reflect the abundance responses to arbitrary fluctuating temperatures in nature. Here we measured the mortality under a broad range of extreme high/low temperatures and multiple exposure durations, thus providing a solid dataset to build the TDT model or to do other analyses for the impact of extreme climate on cereal aphid species. Through the group of survival curves and TDT model, we can have a holistic and comprehensive view of the interspecific and developmental stage variations in thermal tolerance. Moreover, using the values of TDT parameters, we made a thermal stress risk assessment of three aphid species in natural temperature regimes. Our data and approach provide a framework for calculating the cumulative thermal mortality of aphids at different ages at a given time (from one day to a year), thus could quantitatively estimate the relative abundance of co-existing species.

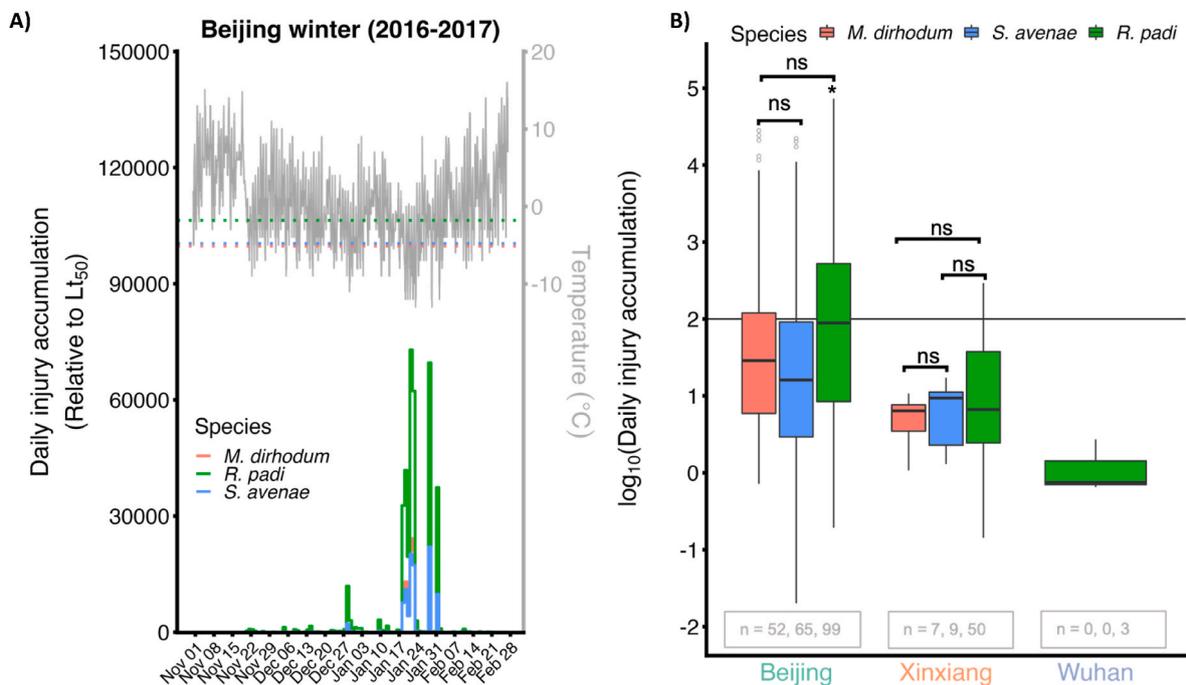


Fig. 3. Predicted daily injury accumulation based on the TDT parameters for the three species of cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) (age 6 days) under natural temperature fluctuations found in the winter (November–February) of 2016–2017. **A)** The temperature profile (grey, secondary y-axis) was used to project the daily accumulation of injury by use of the TDT parameters (see the formula in the method part). The species-colored lines/steps at the bottom of the graph indicated the calculated amount of injury accumulated *per day* relative to the amount of injury that would result in half the aphids dying (here set to 100 with the black line). Injuries only accumulated when temperature dropped below a T_c of $-5.15\text{ }^{\circ}\text{C}$ (red dotted line), $-1.81\text{ }^{\circ}\text{C}$ (green dotted line) and $-4.79\text{ }^{\circ}\text{C}$ (blue dotted line) for *M. dirhodum*, *R. padi* and *S. avenae*, respectively **B)** Boxplot summarizing the data from (A) and similar analyses for Wuhan and Xinxiang in the winter (see Fig. S2 A and B). Note that the y-axis was \log_{10} -transformed and the injury accumulation resulting in median lethal (100) was marked by the line at $\log_{10}(\text{Daily injury accumulation}) = 2$. To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as n in the graph) were summarized in this plot. Comparisons between species within sites were examined using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction. Asterisks marked significant differences ($p < 0.05$) between two groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.1. TDT curves, interspecific and developmental stage variations in thermal tolerance

Here we aimed to examine if/how both thermal stress intensity and duration affected the survival of three cereal aphid species. We found strong linear relationships between high/low temperatures and \log -transformed L_{t50} across different ages of three aphid species as in other ectotherms (Jørgensen et al., 2022). The steep slope (low z' value) of TDT curves from our data at low temperatures is similar to the typical freezing time-temperature curve (Salt 1966), which indicates that the cold tolerance we measured might reflect the susceptibility to freezing rather than chill injury (Tarapacki et al., 2021).

It is intuitive to compare interspecific tolerance using a single value, such as CT_{max} , CT_{min} , LT_{50} , or L_{t50} . However, we should realize that interspecific differences in thermal tolerance are not constant but change with the thermal dose (intensity and exposure duration). The TDT curves of three aphid species at high temperatures converge due to their different slopes (thermal sensitivity) (Fig. 1C), which means the L_{t50} values of the three species are getting closer with increasing intensity. Even, the interspecific differences in tolerate duration can reverse depending on the temperature, for example, at temperatures below $-9\text{ }^{\circ}\text{C}$, *S. avenae* could sustain a longer duration than *M. dirhodum* (Fig. 1D). However, our results generally showed that *R. padi* survived longer at high but shorter at low temperatures, indicating it was the most heat-tolerant but cold-vulnerable species. Whereas *M. dirhodum* performed as the most heat-vulnerable but cold-tolerant species. These results are supported by the previous studies under lifetime constant temperature exposures or the static thermal tolerance experiments (Asin and Pons 2001; Alford et al. 2014, 2016).

We found that the effect of the developmental stage was similar at both high and low temperatures. The old nymphs survived longer than young nymphs at either high or low temperatures in the three aphid species. However, the thermal tolerance of adults was species-specific. For *S. avenae* and *M. dirhodum*, adults (12-d-old) showed high tolerance, but the tested adults of *R. padi* were extremely sensitive to cold stress. This pattern was also found by Alford et al. (2014). Typically, thermal tolerance is highest at eclosion and then declines during the early days of adult life (Bowler and Terblanche 2008; Colinet et al., 2013). In our case, the development rate of *R. padi* was faster than the other two species, thus 12-d-old *R. padi* was at the 3rd or 4th day of adult life (but 1st or 2nd day for *S. avenae* and *M. dirhodum*). Therefore, the intolerant 12-d-old *R. padi* might be resulting from a rapid decline of thermal tolerance in adult life, as observed in other insects (Bowler 1967; Davison 1969; Colinet et al., 2013).

4.2. Relating TDT curves to natural temperature regimes

The injury risk analysis showed that *R. padi* was the least affected by high temperatures during summer, which seems to fit the field observation showing that the relative abundance of *R. padi* increased with extremely high-temperature frequency in south area (Ma et al., 2015b; Zhu et al., 2021). In Wuhan, *S. avenae* has the largest proportional abundance (78%) in 2008 when hot-day (daily mean temperature $>=30\text{ }^{\circ}\text{C}$) frequency was low (Ma et al., 2015b). However, *R. padi* became the most abundant species (79%) in the aphid community in 2011 when hot days occurred more frequently (Ma et al., 2015b). In the middle region (near Xinxiang), the proportional abundance of *R. padi* has increased from 13% in 1998 to 83% in 2012 (Zhu et al.,

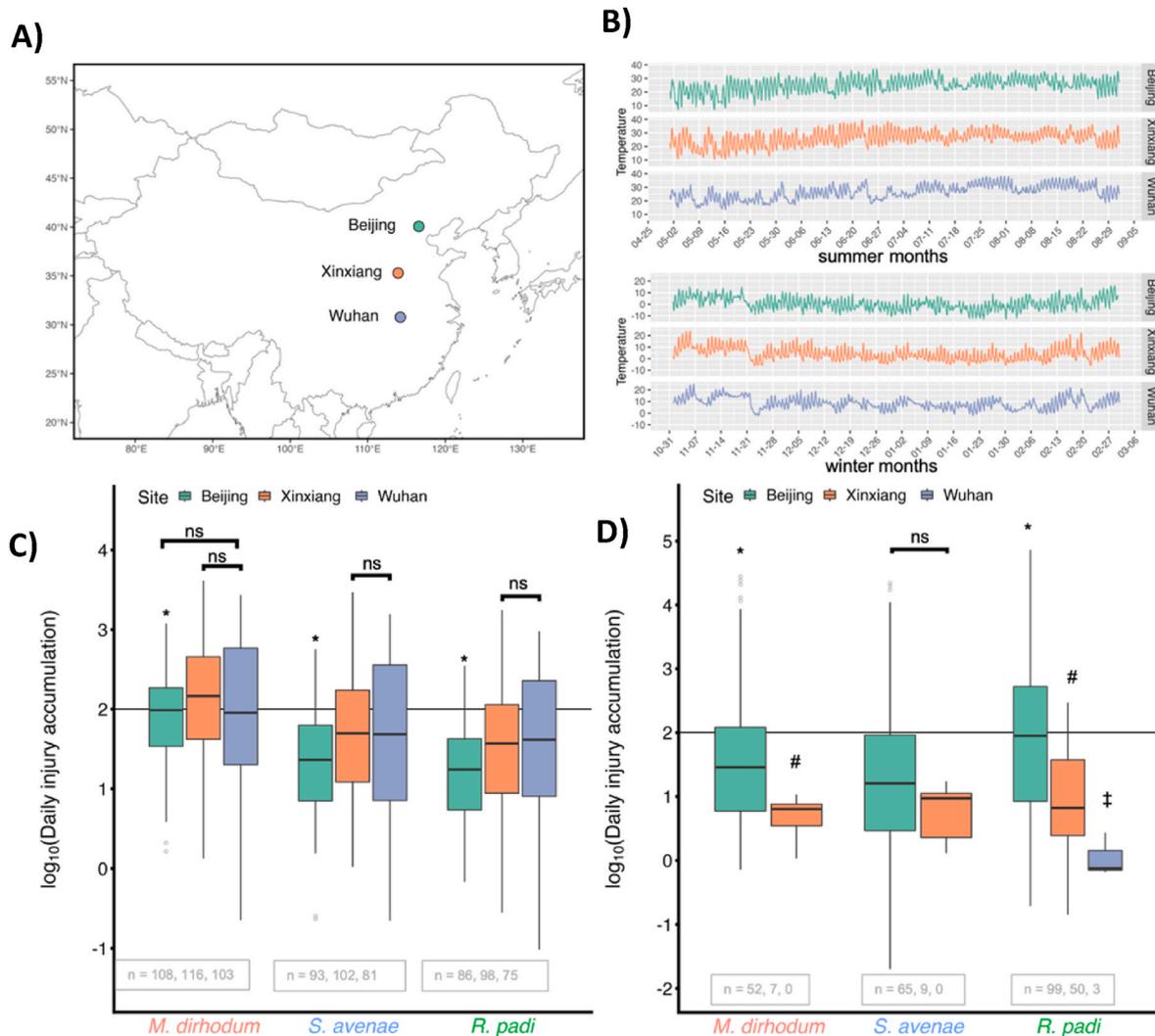


Fig. 4. Temperature profiles and predicted daily injury accumulation based on the TDT parameters for the three species of cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) (age 6 days) under natural temperature fluctuations found in three sites in the summer months (May–August) of 2016 and winter months (November–February) 2016–2017. **A)** Map of the geographic locations of three sites indicated in different colors. **B)** Overview of temperature profiles in the three sites during summer (May–August 2016) (top) and winter months (Nov–Feb 2016–2017) (bottom). **C)** and **D)** showed a comparison of \log_{10} (daily injury accumulation) between sites within species during summer months and winter months respectively. The comparisons were conducted using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction. *, #, and ‡ marked significant differences ($p < 0.05$) among sites. To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as n in the graph) were summarized in the plot. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2021). While in north sites (near Beijing), *S. avenae* was still the most abundant species which accounted for 89.8% in 2015 and 73.0% in 2016, followed by *R. padi* (9.26% and 14.3% in 2015 and 2016) and *M. dirhodum* (0.01% and 11.81% in 2015 and 2016) (Yang et al., 2017). *R. padi* might become the most abundant species, outcompeting *S. avenae* in a warmer future (Zhu et al., 2021). Conversely, *M. dirhodum* is the least abundant species (Yang et al., 2017) owing to its lower tolerance to high temperatures. It appears in the early spring and leaves the wheat field when the temperature warms up (Chen et al., 1994).

It is also interesting to see the differences in the calculated accumulated injuries of aphids between the three sites, which represent a climatic gradient from cold to hot climates. Aphids accumulated more heat injuries in Wuhan, the southmost site, than Xinxiang and Beijing owing to the longest duration of exposure to high temperatures during summer months (the durations $>=30$ °C in Wuhan, Xinxiang, and Beijing were 868h, 736h, and 611h, respectively, see supporting information Table S9). To some extent, this result underlines the importance of exposure duration to stressful temperatures when assessing the impact

of thermal stress.

Winter temperature varied greatly in the three sites: Beijing, the northmost site, had a minimum temperature of -13 °C and an average daily temperature of 0.6 °C whereas winter in Wuhan was mild with an average daily temperature of 8.7 °C (Table S9). Aphids could suffer from cold injury, manyfold the dose resulting in 50% mortality in Beijing, which implied that the three species may not be able to overwinter as nymphs in the winter wheat field here. Indeed, it had been reported that the geographical overwinter limit for *S. avenae* and *R. padi* was the 0 °C isotherm in January (N 35° , near Xinxiang) (Yang and Yang 1991; Luo et al., 1994). Instead, they might be able to overwinter in Wuhan where they hardly get injured during winter and then migrate to the north region at the start of spring (Li et al., 2014).

Over the past 5 decades (1961–2013), China's temperature increased by 1.44 °C (90% confidence interval 1.22 – 1.66 °C) (Sun et al., 2016). There was a general increase in extreme warm indices (such as warm days, warm nights, T_{max}) and a significant reduction of cool nights and cool days during winter (Sun et al., 2016). Thus, the changes in aphid

community abundance that had taken place in the south area would probably push northward in the future.

4.3. Limitations

Although the risk analysis shows very coherent patterns that seem to match with field observations, it is, however, important to recognize that such a model has some limitations, in particular, it does not consider i) the difference between air and insects' body temperature and ii) the capacity of insects to relocate into more permissive microclimates (i.e. avoidance) (Ma and Ma 2012a, 2012b, Woods et al., 2015, Pincebourde and Suppo 2016, Ma et al., 2018b). In addition, the approach does not consider if thermal tolerance changes seasonally with acclimation (Overgaard et al., 2008; Bujan et al., 2020), nor the possibility that changes in feeding behavior could be altered to provide on-time nutritional adjustments aiding physiological changes (for instance: synthesis of compatible solutes, acquisition of antioxidant molecules). Furthermore, we calculated the daily injury accumulation with the assumption that aphids that survived the thermal stress could recover quickly during non-stressful temperature regimes (cooler nights for heat injury and warm noon for cold injury). While this recovery phase is true and well described (see discussion in Colinet et al., 2015), the capacity to fully recover depends on the level of thermal injury and the repair temperature and duration (Colinet et al., 2011; Ma et al., 2018a; Bai et al., 2019; Zhu et al., 2019; Ørsted et al., 2022). Advanced models that include the temperature-dependent repair function have recently been developed to incorporate this recovery capability (Ørsted et al., 2022). Despite these pitfalls, this simple approach based on basal thermal tolerance and cumulative daily thermal stress proved to be rather efficient in discriminating both the most susceptible species and regions. It could be further developed into a fine model to predict the population dynamics of aphid species in the field if integrating the effect of thermal dose on fertility in the future.

5. Conclusion

Our results answered the two questions: 1) interspecific differences in basal thermal tolerance can be a factor driving the community abundance change in aphid communities. The risk assessment under the natural temperature profile seems to fit the field observations that the more tolerant species is becoming more abundant with increasing temperature in the context of climate change. 2) Although the effect of age was not consistently observed in the three aphid species, we found that younger nymphs tended to have a lower thermal tolerance.

Data availability

The raw data and r codes required to reproduce the above findings are available to download from the Dryad Digital Repository doi:10.5061/dryad.mcvdnc4j.

Funding

This work was supported by National Natural Science Foundation of China 31620103914, the Fundamental Research Funds of CAAS (Y2017LM10), and Innovation Program of CAAS (CAAS-ZDRW202012). The study was funded by the International Research Project (IRP) "Phenomic Responses of Invertebrates to Changing Environments and Multiple Stress" (PRICES) supported by INEE-CNRS. This project was supported by ARED PhD grant, provided by Region Bretagne (project climPuce COH20023).

Authors' contributions

Yuan-Jie Li: Investigation; Formal analysis; Writing – original draft; Writing – review & editing, **Si-Yang Chen:** Investigation, **Lisa**

Bjerregaard Jørgensen: Formal analysis; Writing – review & editing, **Johannes Overgaard:** Formal analysis; Writing – review & editing, **David Renault:** Funding acquisition; Supervision; Writing – review & editing, **Hervé Colinet:** Conceptualization; Funding acquisition; Supervision; Writing – review & editing, **Chun-Sen Ma:** Conceptualization; Formal analysis; Funding acquisition; Supervision; Writing – original draft; Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtherbio.2023.103583>.

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Supporting Information

Table S1. Test temperatures, the total number of aphids and the number of exposure times used to assess heat and cold stress tolerance in three aphid species. For each species and temperature, the numbers indicate the total number of individuals assessed, with the number of tested durations indicated in parenthesis. For each temperature x duration combination, there are 2-4 replicates, each consisting of a pool of 7-12 individuals.

Species	Age (days)	High temperature (°C)								Low temperature (°C)							
		34	35	36	37	38	39	40	-11	-10	-9	-8	-7	-6	-5	-4	-3
<i>M. dirhodum</i>	2	218(8)	263(9)	266(9)	252(8)	236(8)	210(7)	183(6)	-	182(7)	216(8)	252(9)	259(10)	274(9)	215(7)	-	-
<i>M. dirhodum</i>	6	273(9)	173(6)	191(6)	264(9)	266(8)	260(8)	240(8)	137(5)	245(9)	222(10)	382(16)	255(10)	205(7)	279(10)	-	-
<i>M. dirhodum</i>	12	383(14)	252(8)	287(9)	231(8)	275(9)	225(7)	280(8)	-	235(8)	294(11)	382(15)	222(8)	225(9)	-	-	-
<i>S. avenae</i>	2	208(5)	148(5)	193(5)	216(6)	200(5)	289(6)	229(5)	279(10)	364(13)	356(12)	271(10)	294(11)	218(7)	180(6)	-	-
<i>S. avenae</i>	6	255(7)	266(6)	298(8)	210(6)	195(6)	176(5)	239(6)	207(8)	280(10)	467(15)	232(8)	374(15)	232(9)	255(8)	-	-
<i>S. avenae</i>	12	201(6)	203(7)	194(6)	292(8)	241(9)	261(10)	209(8)	240(8)	259(8)	236(10)	317(14)	352(12)	200(7)	204(7)	-	-
<i>R. padi</i>	2	298(10)	291(10)	216(8)	175(6)	204(7)	190(7)	192(6)	-	-	-	-	266(12)	185(8)	237(8)	287(11)	286(11)
<i>R. padi</i>	6	272(9)	257(9)	345(11)	229(7)	196(6)	172(5)	212(8)	164(8)	150(7)	204(9)	325(12)	296(12)	410(16)	238(9)	276(10)	-
<i>R. padi</i>	12	284(9)	343(12)	226(9)	204(6)	217(7)	300(10)	206(7)	-	-	123(5)	146(6)	144(7)	196(8)	265(9)	210(7)	167(6)

Table S2. McFadden's pseudo-R² for estimating the goodness of fit of logistic model at each temperature for each species and group.

Species	Age(days)	High temperature (°C)								Low temperature (°C)							
		34	35	36	37	38	39	40	-11	-10	-9	-8	-7	-6	-5	-4	-3
<i>M. dirhodum</i>	2	0.88	0.94	0.88	0.88	0.78	0.95	0.83	-	0.78	0.65	0.71	0.74	0.81	0.82	-	-
<i>M. dirhodum</i>	6	0.85	0.90	0.95	0.90	0.83	0.70	0.88	0.73	0.28	0.79	0.49	0.82	0.71	0.74	-	-
<i>M. dirhodum</i>	12	0.78	0.94	0.84	0.93	0.84	0.80	0.58	-	0.72	0.36	0.66	0.81	0.78	-	-	-
<i>S. avenae</i>	2	0.86	0.87	0.93	0.91	0.91	0.78	0.80	0.57	0.49	0.75	0.92	0.90	0.82	0.77	-	-
<i>S. avenae</i>	6	0.69	0.91	0.52	0.96	0.93	0.88	0.77	0.84	0.17	0.27	0.77	0.85	0.80	0.81	-	-
<i>S. avenae</i>	12	0.94	0.93	0.95	0.89	0.93	0.85	0.91	0.49	0.37	0.67	0.53	0.60	0.79	0.91	-	-
<i>R. padi</i>	2	0.85	0.85	0.91	0.91	0.95	0.92	0.95	-	-	-	-	0.49	0.41	0.81	0.75	0.81
<i>R. padi</i>	6	0.91	0.81	0.87	0.85	0.88	0.86	0.84	0.51	0.68	0.44	0.41	0.51	0.65	0.66	0.62	
<i>R. padi</i>	12	0.84	0.58	0.84	0.82	0.68	0.74	0.87	-	-	0.83	0.76	0.66	0.37	0.39	0.77	0.73

Table S3. Three-way ANCOVA for thermal death time curves from three cereal aphid species of three ages under high temperatures. p -values < 0.05 are marked in bold italics in the table.

Variable	F-value	p -value
Test temperature	2614.58	<i><0.001</i>
Age	8.88	<i><0.001</i>
Species	84.52	<i><0.001</i>
Age \times Species	2.77	<i>0.004</i>
Test temperature \times Age	2.03	0.144
Test temperature \times Species	13.95	<i><0.001</i>
Test temperature \times Age \times Species	1.84	0.137

Table S4. Three-way ANCOVA for thermal death time curves from three cereal aphid species of three ages under low temperatures. p -values < 0.05 are marked in bold italics in the table.

Variable	f	F-value	p -value
Test temperature		607.76	<0.001
Age		16.44	<0.001
Species		136.03	<0.001
Age × Species		13.60	<0.001
Test temperature × Age		0.23	0.80
Test temperature × Species		8.70	<0.001
Test temperature × Age × Species		2.80	0.04

Table S5. Comparison of $\log_{10}(Lt_{50})$ at 37 °C between species and ages. Pairwise p -values from *emmeans* () examining differences in $\log_{10}(Lt_{50})$ at 37 °C between three aphid species from three ages (2, 6 and 12 days). p -values < 0.05 are marked in bold. Light grey cells mark the comparisons between three species with the same age while coloured cells mark the comparisons testing the effect of ages within species.

Age	Species	2 days			6 days			12 days		
		<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>
2 days	<i>M. dirhodum</i>		0.0019	<.0001	0.7424			<.0001		
	<i>S. avenae</i>			0.0056		0.1471			0.0945	
	<i>R. padi</i>						0.0436			0.5296
6 days	<i>M. dirhodum</i>					0.0002	<.0001	0.0001		
	<i>S. avenae</i>						0.0009		0.9649	
	<i>R. padi</i>									0.2879
12 days	<i>M. dirhodum</i>								0.0288	0.0005
	<i>S. avenae</i>									0.1449
	<i>R. padi</i>									

Table S6. Comparison of slope at high temperatures between species and ages. Pairwise p -values from *emmtrends* () examining differences in z ($-1/\text{slope}$) between three aphid species from three ages (2, 6 and 12 days). p -values < 0.05 are marked in bold. Light grey cells mark the comparisons between three species at the same age while coloured cells mark the comparisons testing the effect of ages within species.

Age	Species	2 days			6 days			12 days		
		<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>
2 days	<i>M. dirhodum</i>		0.0803	0.0007	0.6085			0.0015		
	<i>S. avenae</i>			0.0688		0.4568			0.075	
	<i>R. padi</i>						0.7032			0.7118
6 days	<i>M. dirhodum</i>					0.0252	0.0414	0.0105		
	<i>S. avenae</i>						0.9644		0.4974	
	<i>R. padi</i>									0.9999
12 days	<i>M. dirhodum</i>							0.0651	0.4964	
	<i>S. avenae</i>									0.4161
	<i>R. padi</i>									

Table S7. Comparison of $\log_{10}(Lt_{50})$ at $-7\text{ }^{\circ}\text{C}$ between species and ages. Pairwise p -values from *emmeans* () examining differences in $\log_{10}(Lt_{50})$ at $-7\text{ }^{\circ}\text{C}$ between three aphid species from three ages (2, 6 and 12 days). p -values < 0.05 are marked in bold. Light grey cells mark the comparisons between three species at the same age while coloured cells mark the comparisons testing the effect of ages within species.

Age	Species	2 days			6 days			12 days		
		<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>
2 days	<i>M. dirhodum</i>		0.1852	0.0001	0.7806			0.2645		
	<i>S. avenae</i>			0.0006		0.1776			0.3898	
	<i>R. padi</i>						0.263			0.0044
6 days	<i>M. dirhodum</i>					0.7185	<.0001	0.5529		
	<i>S. avenae</i>						<.0001		0.8571	
	<i>R. padi</i>									<.0001
12 days	<i>M. dirhodum</i>								0.2066	<.0001
	<i>S. avenae</i>									<.0001
	<i>R. padi</i>									

Table S8. Comparison of slope at low temperatures between species and ages. Pairwise p -values from *emmtrends()* examining differences in z ($-1/\text{slope}$) between three aphid species from three ages (2, 6 and 12 days). p -values < 0.05 are marked in bold. Light grey cells mark the comparisons between three species while coloured cells mark the comparisons testing the effect of ages within species.

Age	Species	2 days			6 days			12 days		
		<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>
2 days	<i>M. dirhodum</i>		0.1278	0.8663	0.9417			0.3801		
	<i>S. avenae</i>			0.476		0.5397			0.2013	
	<i>R. padi</i>						0.3745			0.0982
6 days	<i>M. dirhodum</i>					0.8262	0.0912	0.2237		
	<i>S. avenae</i>						0.2698		0.7529	
	<i>R. padi</i>									0.3729
12 days	<i>M. dirhodum</i>							0.2713		0.0127
	<i>S. avenae</i>									0.0735
	<i>R. padi</i>									

Table S9. The statistics of temperature in three sites (Beijing, Xinxiang and Wuhan) in summer months and winter months (2016/2017). T_{\max} , T_{\min} and T_{av} are the maximal, minimal and average hourly temperature in the summer/winter months, respectively. $t \geq 30$ °C is the duration when temperature is above 30 °C in the summer months. $t \leq 0$ °C is the duration when temperature is lower than 0 °C in the winter months.

		Beijing	Xinxiang	Wuhan
summer	T_{\max}	37.0 °C	39.5 °C	38.0 °C
	T_{\min}	7.0 °C	10.4 °C	13.4 °C
	T_{av}	25.2 °C	26.1 °C	26.5 °C
	$t \geq 30$ °C	611 h	736 h	868 h
winter	T_{\max}	16.0 °C	24.1 °C	25.0 °C
	T_{\min}	-13.0 °C	-6.2 °C	-2.2 °C
	T_{av}	0.7 °C	4.2 °C	8.7 °C
	$t \leq 0$ °C	1473 h	771 h	67 h

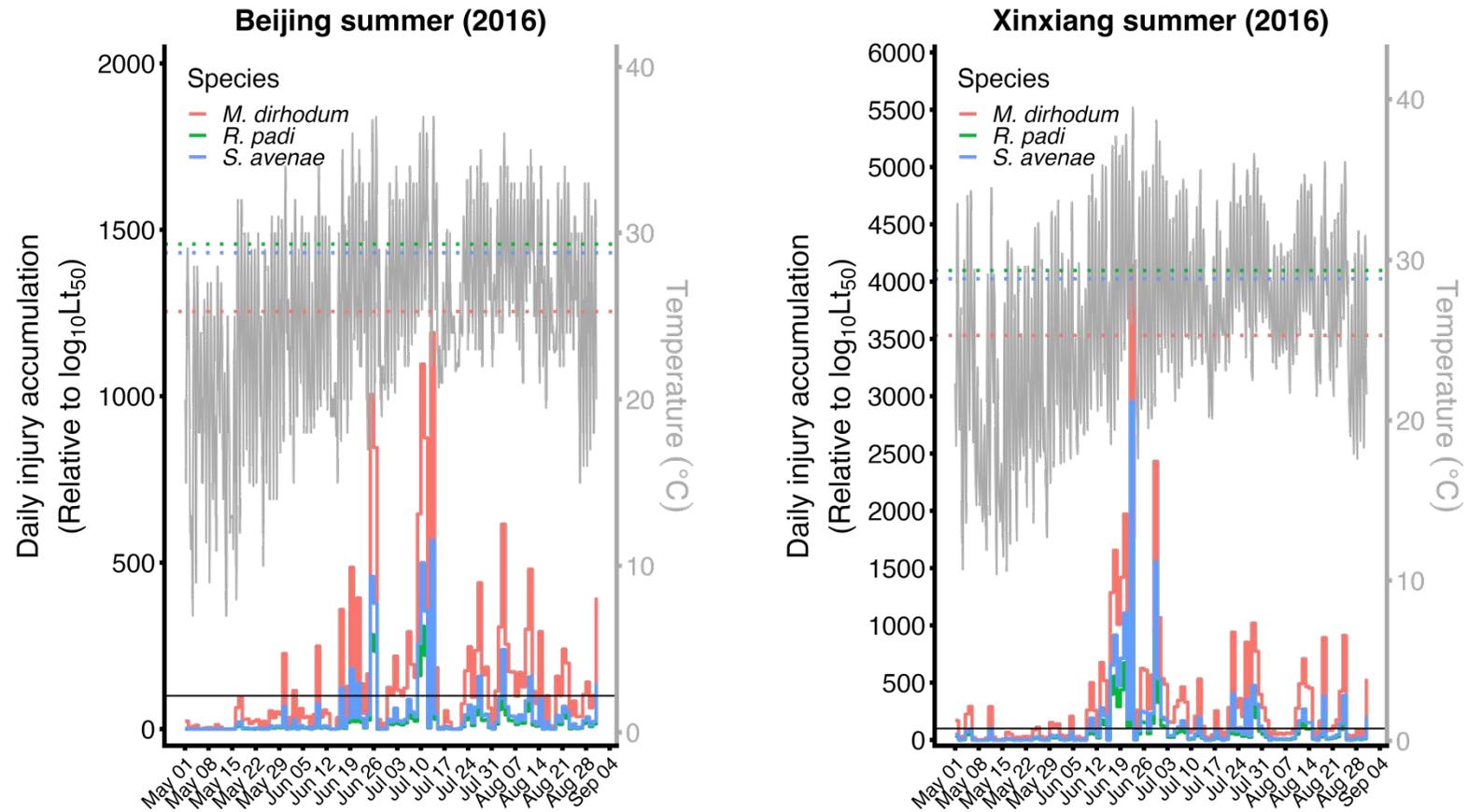


Figure S1. Predicted daily injury accumulation based on the TDT parameters for the three species of cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) (age 6days) under natural temperature fluctuations found in Beijing (A) and Xinxiang (B) in the summer months (May-August) of 2016.

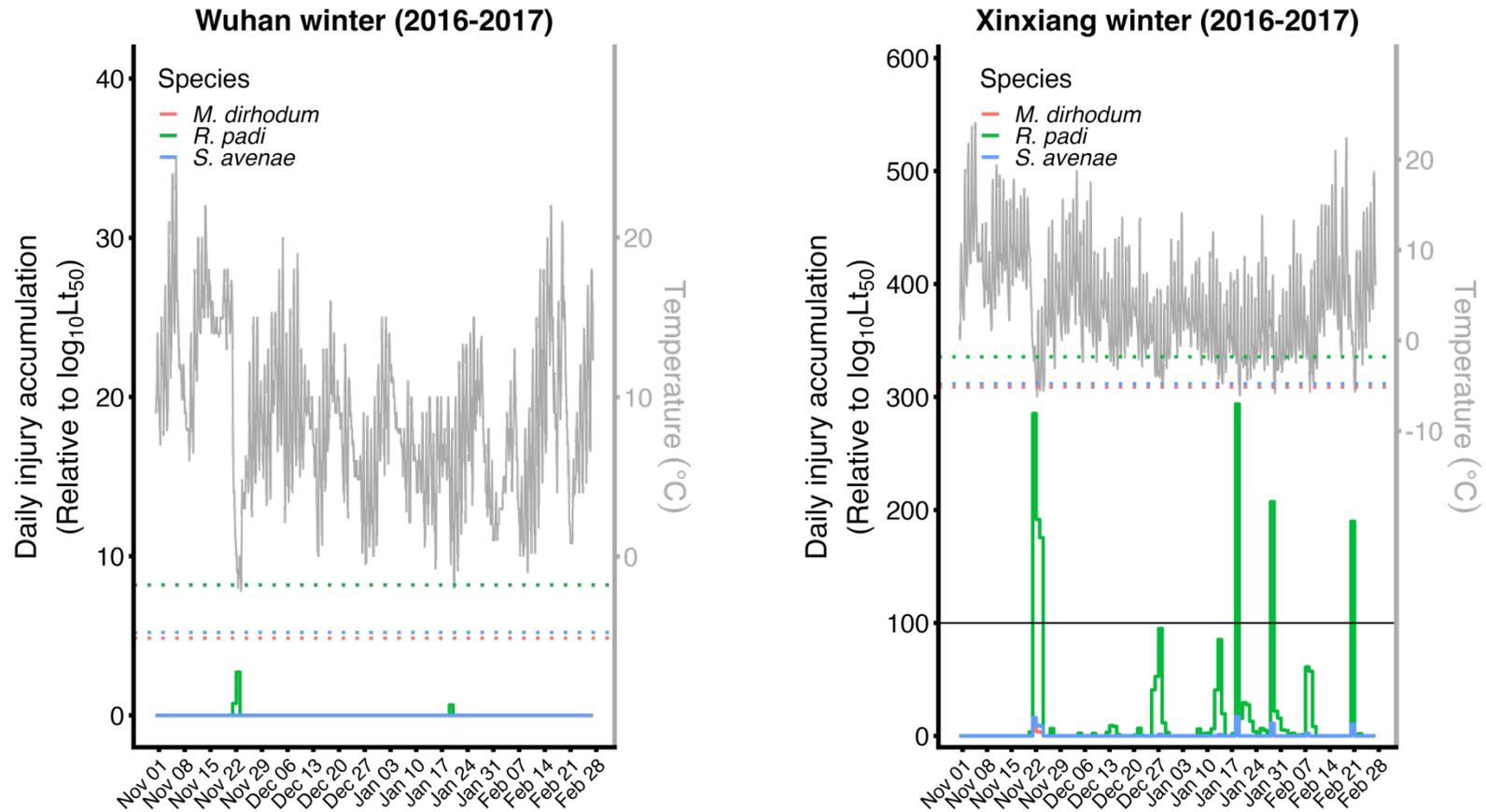


Figure S2. Predicted daily injury accumulation based on the TDT parameters for the three species of cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) (age 6days) under natural temperature fluctuations found in Wuhan (A) and Xinxiang (B) in the winter months (November-February), 2016-2017.

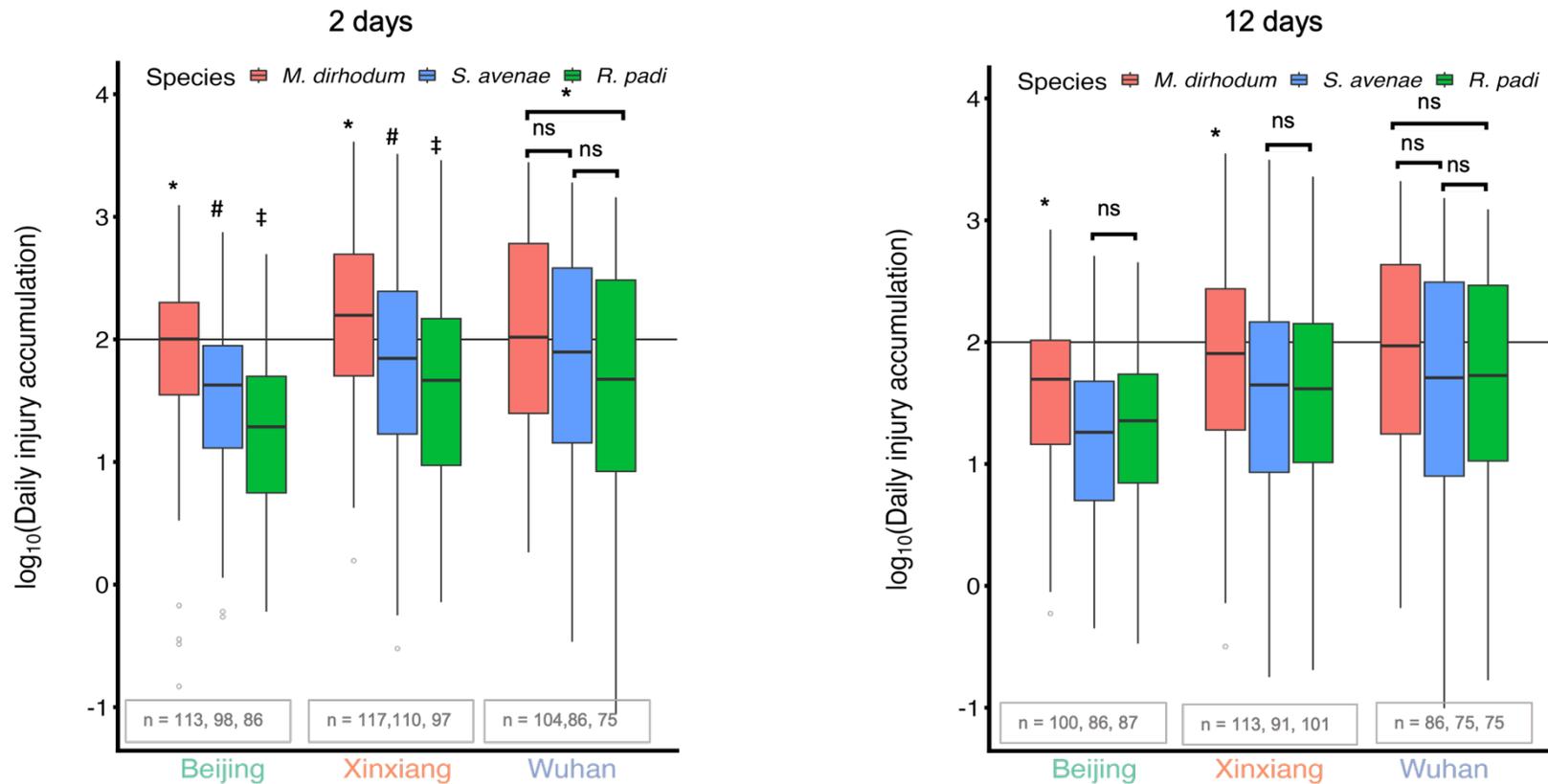


Figure S3. Predicted daily injury accumulation based on the TDT parameters for 2-d-old (A) and 12-d-old (B) cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) under natural temperature fluctuations in the summer (May-August) of 2016. The y-axis was \log_{10} -transformed and the calculated injury accumulation resulting in median lethal (100) was marked by the line at $\log_{10}(\text{Daily injury accumulation}) = 2$. To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as n in the graph) were summarized in this plot. Comparisons between species within sites were examined using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction. *, #, and ‡ mark significant difference ($p < 0.05$) among species.

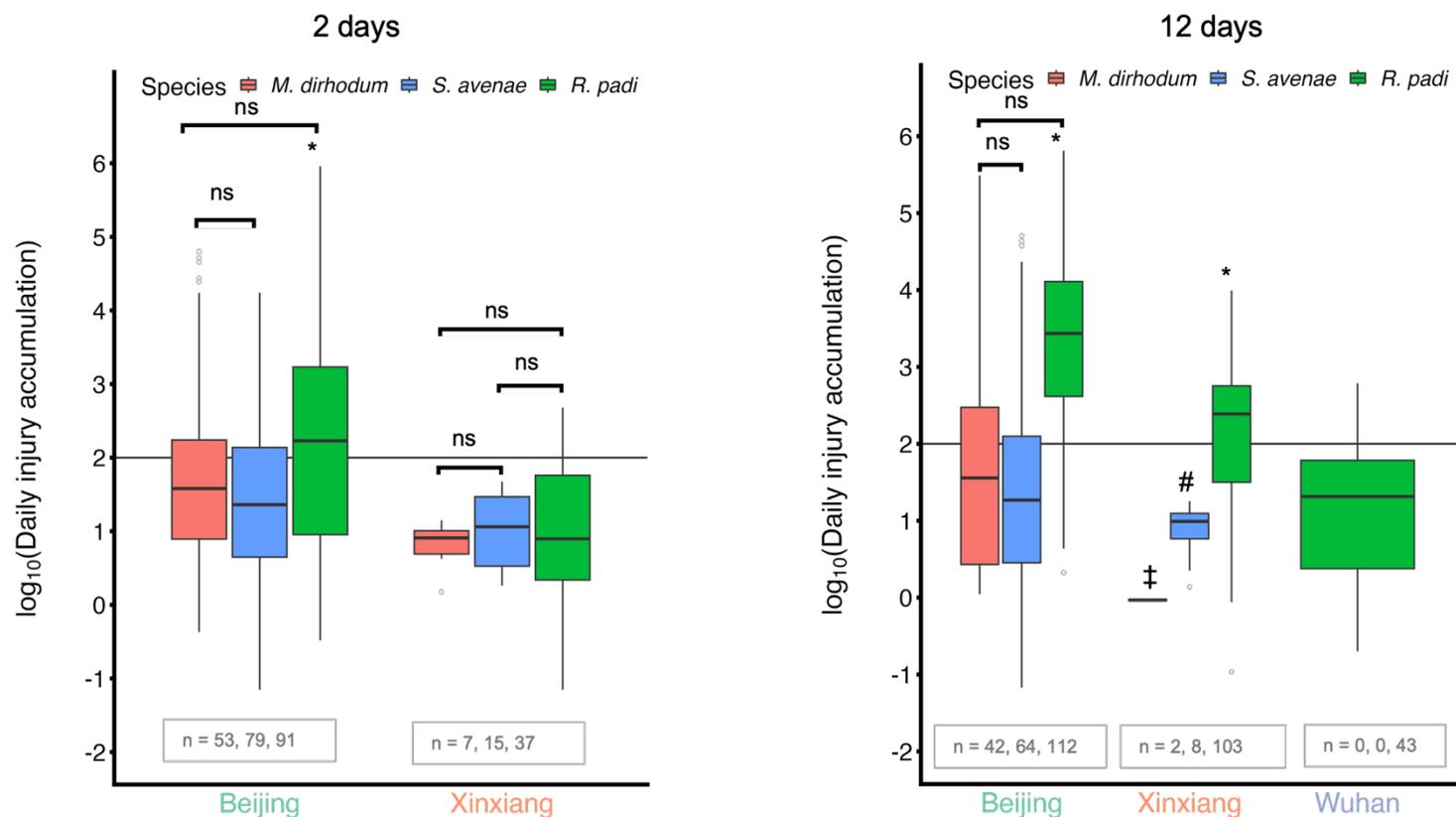


Figure S4. Predicted daily injury accumulation based on the TDT parameters for 2-d-old (A) and 12-d-old (B) cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) under natural temperature fluctuations in the winter (November-February) of 2016-2017. The y-axis was log₁₀-transformed and the calculated injury accumulation resulting in Median lethal (100) was marked by the line at log₁₀(Daily injury accumulation) = 2. To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as n in the graph) were summarized in this plot. Comparisons between species within sites were examined using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction. *, #, and ‡ mark significant difference ($p < 0.05$) among species.

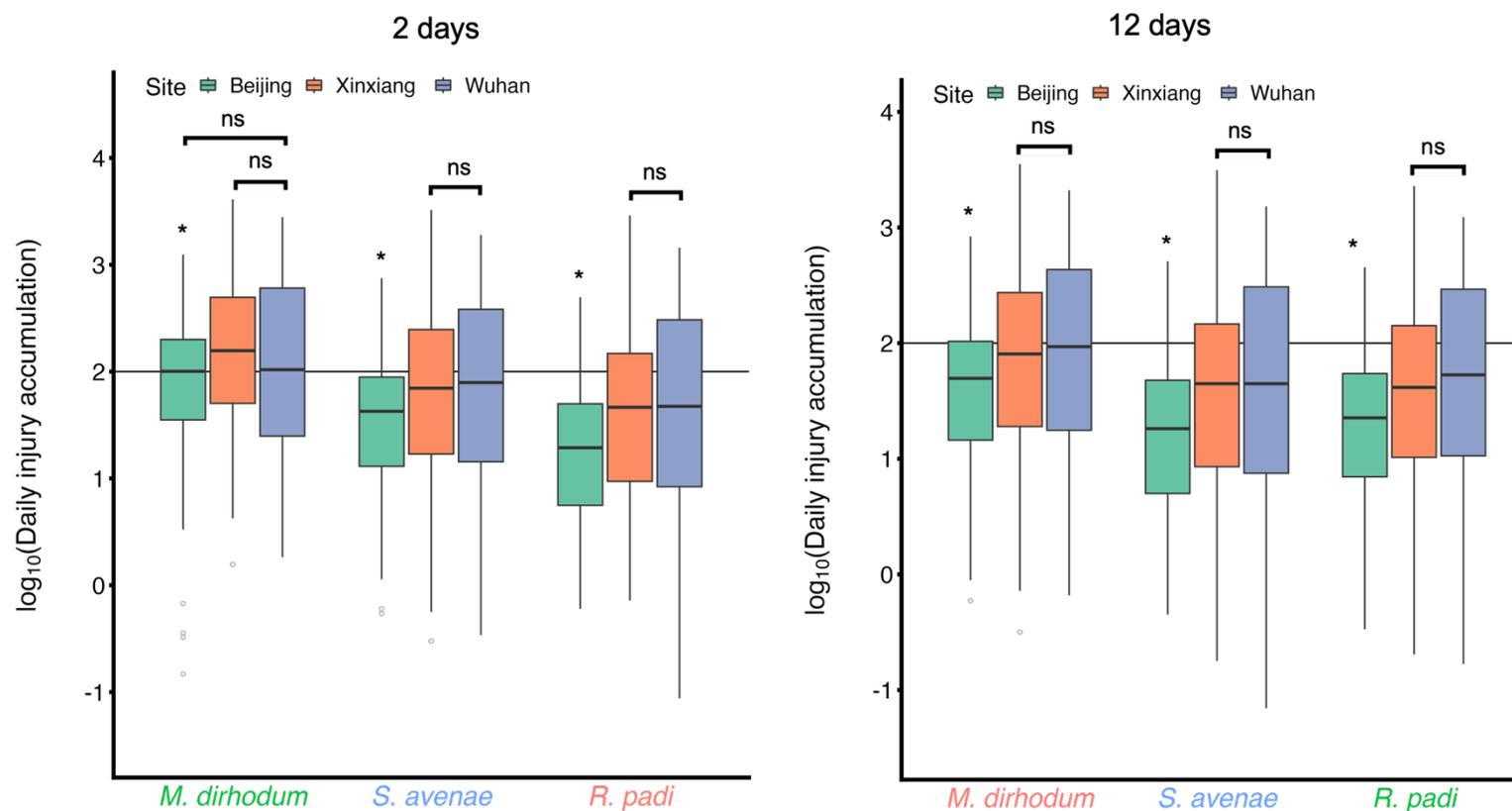


Figure S5. Predicted of daily injury accumulation based on the TDT parameters for 2-d-old (A) and 12-d-old (B) cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) under natural temperature fluctuations in three sites during the summer months (May-August) of 2016. To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as n in the graph) were summarized in this plot. Comparison of $\log_{10}(\text{daily injury accumulation})$ between sites within species during summer months were conducted using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction. *, #, and ‡ mark significant difference ($p < 0.05$) among species.

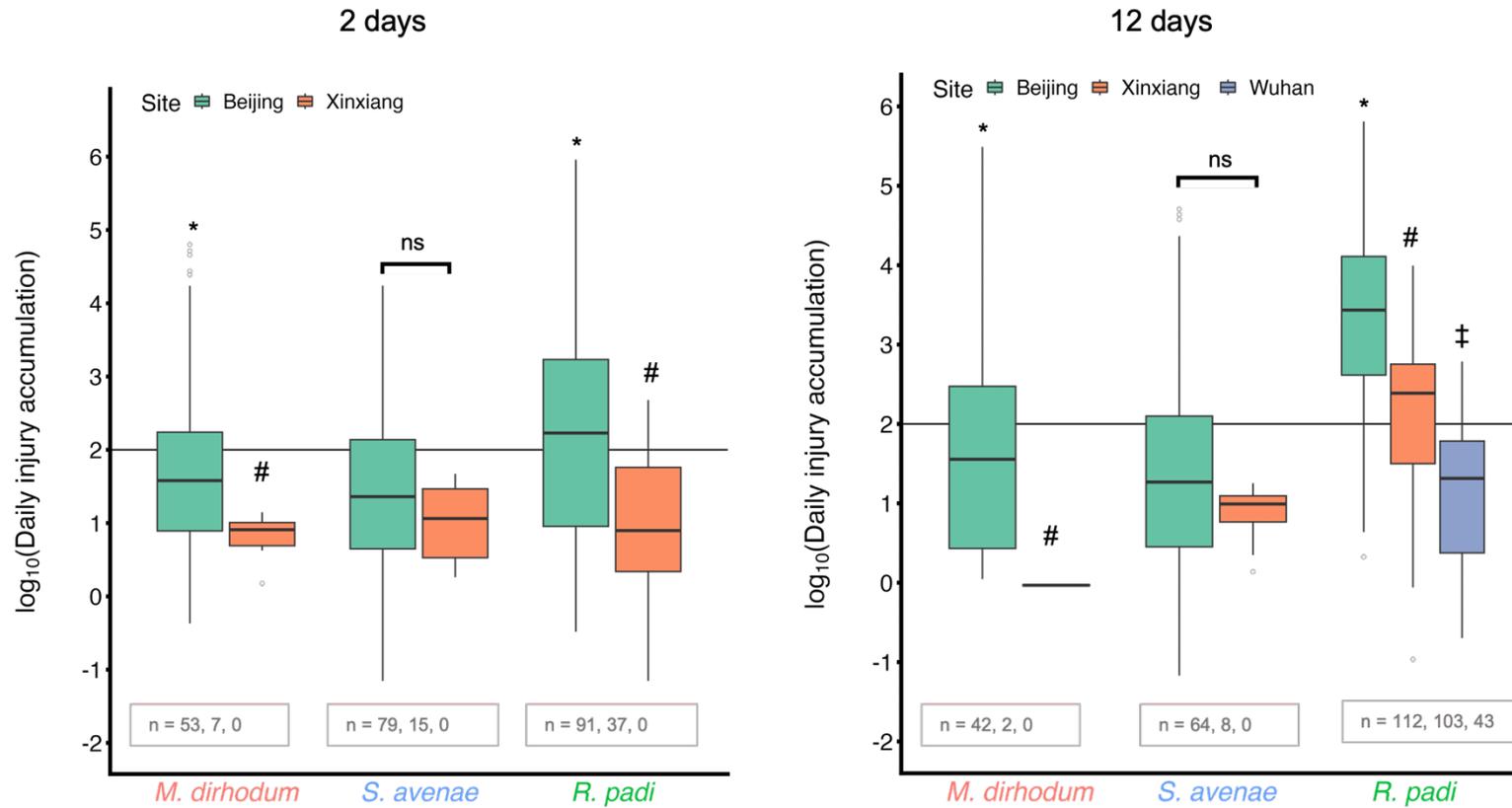


Figure S6. Predicted of daily injury accumulation based on the TDT parameters for 2-d-old (A) and 12-d-old (B) cereal aphids (*M. dirhodum*, *S. avenae*, *R. padi*) under natural temperature fluctuations in three sites during winter months (November-February, 2016-2017). To avoid comparing a lot of days without any injury accumulation, only days with injury accumulation (indicated as n in the graph) were summarized in this plot. Comparison of log₁₀(daily injury accumulation) between sites within species during summer months were conducted using Kruskal-Wallis Rank Sum Tests followed by Pairwise Wilcoxon Rank Sum Tests with a Benjamini-Hochberg correction. *, #, and ‡ mark significant difference ($p < 0.05$) among species.

Chapter 3 Comparative molecular response to thermal stress

Article 2. The interspecific variations in molecular responses to various doses of heat and cold stress: The case of cereal aphids

In Chapter 2, we have demonstrated significant variations in heat and cold tolerances among the three aphid species. One potential explanation for these differences in thermal tolerance is the existence of a distinct molecular response to stress across species. Heat shock proteins (HSPs) are well-known molecular chaperones that play a crucial role in safeguarding against the detrimental effects of thermal stress. Thus, in this Chapter, we investigated the expression patterns of seven *hsp* genes following exposure to equivalent levels of thermal injury and identical durations of exposure.

We hypothesized that:

- 1) In comparison to control conditions, aphids are expected to show high expression levels of *hsps* during/after heat stress. However, they may not express *hsps* during/after cold stress or only moderately express *hsps* during recover from cold stress.
- 2) Species suffering similar stress levels may show similar expression levels of *hsps*.
- 3) The most tolerant species are likely to employ a large network of active molecular chaperones following the same exposure duration to heat and cold stress.

Main findings:

- 1) *Hsp* genes are induced more strongly by heat than cold stress with *hsp70A* being the most up-regulated gene in response to both stressors.
- 2) *R. padi* had more heat inducible genes and significantly higher mRNA levels of *hsp70A*, *hsp10*, *hsp60* and *hsp90* than the other two species after exposure to heat for the similar stress levels or for the same duration.
- 3) *Hsps* ceased to be expressed at 37 °C in *M. dirhodum* and *S. avenae* while expression was maintained in *R. padi*.
- 4) *M. dirhodum* had more cold inducible genes than the other species.



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Journal of Insect Physiology

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The interspecific variations in molecular responses to various doses of heat and cold stress: The case of cereal aphids

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ARTICLE INFO

Keywords:

Cereal pests
Heat and cold shock
Molecular chaperones
hsp genes

ABSTRACT

Insects are currently subjected to unprecedented thermal stress due to recent increases in the frequency and amplitude of temperature extremes. Understanding molecular responses to thermal stress is critically important to appreciate how species react to thermal stress. Three co-occurring cosmopolitan species are found within the guild of cereal aphids: *Sitobion avenae*, *Ropalosiphum padi* and *Metopolophium dirhodum*. Earlier reports have shown that increasing frequency of temperature extremes causes a shift in dominant species within guilds of cereal aphids by differently altering the population's growth. We hypothesize that a differential molecular response to stress among species may partially explain these changes. Heat shock proteins (HSPs) are molecular chaperones well known to play an important role in protecting against the adverse effects of thermal stress. However, few studies on molecular chaperones have been conducted in cereal aphids. In this study, we compared the heat and cold tolerance between three aphid species by measuring the median lethal time (Lt_{50}) and examined the expression profiles of seven *hsp* genes after exposures to comparable thermal injury levels and also after same exposure durations. Results showed that *R. padi* survived comparatively better at high temperatures than the two other species but was more cold-sensitive. *Hsp* genes were induced more strongly by heat than cold stress. *Hsp70A* was the most strongly up-regulated gene in response to both heat and cold stress. *R. padi* had more heat inducible genes and significantly higher mRNA levels of *hsp70A*, *hsp10*, *hsp60* and *hsp90* than the other two species. *Hsps* ceased to be expressed at 37 °C in *M. dirhodum* and *S. avenae* while expression was maintained in *R. padi*. In contrast, *M. dirhodum* was more cold tolerant and had more cold inducible genes than the others. These results confirm species-specific differences in molecular stress responses and suggest that differences in induced expression of *hsp*s may be related to species' thermal tolerance, thus causing the changes in the relative abundance.

1. Introduction

Over the past two decades, temperature extremes have increased in frequency, duration, and intensity due to climate change (Palmer, 2014; Christidis et al., 2015; Masson-Delmotte et al., 2021). Temperature extremes are differentially impacting species living within the same environment, thus causing changes in the species composition and relative abundance (Harvey et al., 2020). This can be the result of species-specific thermal traits and molecular mechanisms for coping with thermal stress (Huang and Kang, 2007; Boucek and Rehage, 2014; Ma et al., 2015; Day et al., 2018). Some tolerant species may be better equipped to survive and reproduce, while others may decline or become

extinct (Chown, 2012; Zhu et al., 2021). Therefore, understanding the thermal tolerance and the molecular response to thermal stress is critical for predicting population dynamics and community structure in the context of climate change.

One of the most important molecular responses to thermal stress is the expression of heat shock proteins (HSPs, plain and uppercase names refer to heat shock proteins, as opposed to italicized and lowercase names which refer to the genes coding for heat shock protein) (King and MacRae, 2015; González-Tokman et al., 2020). Most of the HSPs function as molecular chaperones: they assist in refolding or degrading damaged proteins and/or they prevent the aggregation of proteins damaged by temperature or other proteotoxic stress (Chen et al., 2018).

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<https://doi.org/10.1016/j.jinsphys.2023.104520>

Received 6 March 2023; Received in revised form 17 April 2023; Accepted 2 May 2023

Available online 4 May 2023

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HSPs are divided into several families according to the molecular weight (King and MacRae, 2015). The primary function of HSP70 is to bind unfolded or partially unfolded proteins to prevent their aggregation and to release them for folding (Clare and Saibil, 2013). HSP90 also prevents aggregation of non-native proteins but appears to be more selective for substrates compared to other general chaperones (Clare and Saibil, 2013). HSP60 is primarily localized in the mitochondria (Cheng et al., 1989), where it is involved in the *de novo* folding and refolding of imported proteins (Martin et al., 1992).

In insect, *hsp*s gene expression generally increases during exposure to heat (González-Tokman et al., 2020). *Hsps* are highly conserved and they contribute to heat and cold tolerance in insects (King and MacRae, 2015; Lubawy et al., 2022). Some *hsp*s might be either heat-inducible or cold-inducible, which suggests insects may use different *hsp*s to cope with heat and cold stress, respectively. *Hsp70* and *hsp90* are the commonly activated genes during heat exposure, while *hsp70*, *hsp60* and some small *hsp*s, such as *hsp22* and *hsp23*, are involved in repairing cold injury (Colinet et al., 2010a,b; González-Tokman et al., 2020; Zhao et al., 2021; Lubawy et al., 2022). Examining the response of *hsp*s to heat and cold stress at the same time can provide a holistic view of the roles of *hsp*s.

The degree of *hsp*s induction seems to depend on both the intensity of stress (i.e., temperature) and the exposure time (Huang and Kang, 2007; Li et al., 2017). In studies where expression profiles of *hsp*s have been characterized and compared among species/phenotypes, the protocols generally involved a unique and same stress duration for all experimental groups (Huang and Kang, 2007; Li et al., 2017; Guo and Feng, 2018). However, a given exposure time to stress may induce *hsp*s expression in a species with low tolerance but may not activate expression in another species that is tolerant to the same stress. As such, species-specific exposure times that results in the similar injury level among species (such as Lt_{50} , median lethal time) should be assessed as well. The two treatment sets may provide a complementary perspective on interspecific variation in heat shock reactions. However, such complexity has rarely been considered in *hsp*s expression studies (but Tarapacki et al., 2021).

In this study, we used cereal aphids (*Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum*) as models for our study. These species are important pests worldwide and highly sensitive to temperatures (Hullé et al., 2010; Vereshchagina and Gandrabur, 2021). These three species co-occur in cereal growing regions in China (Li et al., 2021c). Previously, *S. avenae* was commonly considered as the most dominant species (Zhang et al., 2009). With a relatively higher fitness, *R. padi* became favored by heat events leading to a shift in annual relative dominance in some areas (Ma et al., 2015b; Zhu et al., 2021). *Metopolophium dirhodum* seems to prefer cooler areas and leaves the crops at the onset of spring (Hu and Gui 1985; Chen et al., 1994). We hypothesized that the shift in dominant species and the changes in seasonal dynamics might be due to differential basal thermal tolerance and molecular (HSPs) response to thermal stress.

Although *hsp*s have been extensively studied (King and MacRae, 2015; González-Tokman et al., 2020), limited research has been conducted in cereal aphids and mainly on *hsp70*. Previous *hsp* studies on aphids have showed that *hsp70* is induced by heat shock (Wilson et al., 2006; Li et al., 2017; Zhang et al., 2019; Saeidi et al., 2021; Chen et al., 2022). However, expression profiles of the other *hsp*s are unknown in aphids in reaction to thermal stress (heat and cold). In this study, we chose to assess the Lt_{50} (i.e., median lethal time 50%) at high and low temperatures that were often recorded during summer and winter in the temperate winter-growing regions (34 and 37 °C and -5 and -8 °C, respectively). Thereafter, we compared the interspecific differences in mRNA levels of *hsp10*, *hsc70*, *hsp60*, *hsp70*, *hsp75* and *hsp90* after the same exposure durations, but also after the same injury levels (i.e., Lt_{50}). We expected that 1) *R. padi* would be more heat tolerant than the other two species and *M. dirhodum* would be more heat sensitive. 2) Besides *hsp70*, other *hsp*s such as *hsp60*, *hsc70* and *hsp90* are likely to be

activated by heat or cold shocks. 3) There should exist interspecific differences in *hsp*s expression profiles, which may relate to their basal thermal tolerance: the most tolerant species might employ a relatively larger network of active chaperones.

2. Material and methods

2.1. Aphid rearing

Aphids were from the lab population (the original populations were collected in May 2010 from wheat fields near Beijing) (39°48 N, 116°28 E) (Zhao et al., 2014). The colonies were refreshed annually with field-collected aphids. They were then reared on 10–20 cm tall winter wheat seedlings in screened cages (60 × 60 × 60 cm) at 20 ± 0.5 °C, RH 70 ± 5% and a photoperiod of 16L:8D. Seedlings were renewed every week. New molted adult aphids were allowed to reproduce on wheat seedlings for 24 h. Then, new-born nymphs developed for 6 days. Because age can affect the thermal tolerance and *hsp*s expression (e.g., Colinet et al., 2012), all tests were performed using aphids at 6 days old (6-d-old).

2.2. Heat and cold tolerance assays

To reduce handling stress and provide food as well as a water source, we cut off the wheat leaves with aphids and then transferred them directly into glass vials (10 mL). Then, we closed each vial with a dry sponge and placed vials into a glycol bath (Ministat 230-cc-NR; Huber Ltd., Germany, accuracy ± 0.01 °C) pre-set at the experimental temperature. Different groups of aphids were exposed to thermal stress at low (-8 and -5 °C) and high temperature (37 and 34 °C). 34 and 37 °C were frequently recorded temperatures in late spring and summer days in a typical temperate area, such as near Beijing (Ma et al., 2015b). We chose -8 and -5 °C because winter temperatures in temperate regions often drop below -10 °C; and even in the south region, like in Wuhan, the temperature can drop to -5 °C. For each temperature, aphids were exposed to at least five exposure times to get survival rates ranging from 0 to 100%. For each combination of temperature × duration, 3 to 4 replicates were used, each consisting of a pool of 7–12 aphids. After the stress exposure, aphids were removed from the glycol bath and allowed to recover at 20 °C for 24 h before checking the survival of each individual. Aphids were considered alive if the individual moved following a gentle tactile stimulation with a brush. We modeled the survival at each temperature by specifying a generalized linear model (GLM) with logit link function for binary outcome (i.e., dead vs alive aphids) (see the Fig. S1) and estimated Lt_{50} (i.e. the median lethal time 50%) which corresponds to the temperature-specific duration resulting in 50% mortality, using *dose.p* function in R package MASS (Ripley et al., 2013).

2.3. Heat and cold treatments

After obtaining the estimated Lt_{50} for each species at 34, 37, -5, and -8 °C, we sampled aphids exposed to different durations corresponding to the specific Lt_{50} of each species and temperature (i.e., experiments with similar injury levels denoted after as “ Lt_{50} ”) (see Table 1). As mentioned above, to provide a complementary perspective on interspecific variation in heat shock reactions, we also sampled the three aphid species exposed to strictly the same exposure times for each temperature (denoted after as “SE” for same exposure; see Table 1). For each species and treatment, four independent replicates were used for genes' expression, each consisting of a pool of ten 6-d-old aphids. For the two high temperatures (34 and 37 °C), aphids were sampled right at the end of the heat stress exposure. In contrast, for the two low temperatures (-5 and -8 °C), aphids were sampled during recovery phase. Indeed, *hsp*s at cold are known to express during the recovery but not during cold stress itself (Colinet et al., 2010b, Zhang et al., 2015). Therefore, for low temperatures, aphids were sampled after they had recovered for two hours at 20 °C. We named the treatments as follows: 34 °C SE, 37 °C SE,

Table 1
Exposure temperatures and sampling times for three aphid species.

Species	Same injury levels, corresponding to Lt ₅₀ *				Same exposure times (SE)			
	34 °C	37 °C	−5 °C	−8 °C	34 °C	37 °C	−5 °C	−8 °C
<i>M. dirhodum</i>	126 min	23 min	5644 min	179 min	100 min	20 min	600 min	20 min
<i>S. avenae</i>	375 min	45 min	4050 min	141 min	100 min	20 min	600 min	20 min
<i>R. padi</i>	696 min	64 min	542 min	14 min	100 min	20 min	600 min	20 min

* The Lt₅₀ estimates were obtained from survival curves (see Fig. S1).

−5 °C SE, −8 °C SE for the same exposure times and 34 °C Lt₅₀, 37 °C Lt₅₀, −5 °C Lt₅₀, and −8 °C Lt₅₀ for same injury levels (i.e., durations corresponding to the Lt₅₀). As control, four replicates of aphids of the same age (i.e., 6-d-old) were used; they were kept in climatic chambers at 20 °C with 3–4 fresh wheat leaves. The treated and control aphids were transferred into Eppendorf Safe-Lock tubes, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extractions.

2.4. RNA extraction and cDNA synthesis

Aphids were ground to fine powder using pestles in 1.5 mL tubes placed in liquid nitrogen. Samples were mixed with 350 µL of lysis buffer (containing 1% β-mercaptoethanol) from NucleoSpin RNA extraction kit (Macherey-nagel, Germany). RNA extraction and purification were performed using an NucleoSpin spin column following the manufacturer's instructions. Besides the on-column DNA digestion, any potential genomic DNA was removed using an rDNase (Macherey-nagel, Germany). Total RNA was eluted in 40 µL of diethylpyrocarbonate-treated water. The quality and concentration of the obtained RNA was then measured by NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, USA). 1100 ng of total RNA was used in the reverse transcription to cDNA, using the EvoScript Universal cDNA Master (Roche, Switzerland) according to the supplier's instructions. The cDNA was then diluted 3-fold in diethylpyrocarbonate-treated water and stored at −20 °C until use.

2.5. Hsp sequences

Hsp sequences of *R. padi* were obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>) and AphidBase (<https://bipaa.genouest.org/is/aphidbase>) (Table S1.a). For *S. avenae* and *M. dirhodum*, the partial segments of *hsps* were amplified by PCR and sent for sequencing (Tsingke, Beijing, China) (See protocols in Table S1.b). Primers for PCR were designed based on homologous *hsp* gene sequences from closely related aphid species in NCBI (Table S1.b). The transcription levels of seven *hsps* (*hsp10*, *hsp60*, *hsc70*, *hsp70A*, *hsp70B*, *hsp75*, *hsp90*) were measured by real time quantitative PCR (RT-qPCR). For *hsc70*, *hsp60*, *hsp70A*, *hsp90* in *R. padi*, we used primers published in (Li Yuting, 2016). For the remaining *hsp* sequences, we designed primers using primer premier 5.0 (Premier Biosoft International, CA, USA) (Table S1.c) and validated the specificity and efficiency of the primers by the melting curve and standard curve (Table S1.d). *TATA*, *HEL* and *RPL8* were used as the reference genes for *R. padi*, *S. avenae* and *M. dirhodum*, respectively (Li et al., 2020; Li et al., 2021a; Li et al., 2021b).

2.6. RT-qPCR

The RT-qPCR was performed using the Light Cycler® 480 system (Roche Diagnostics, Boulogne-Billancourt, France) with reagent SYBR Green I Master (Roche, France). The PCR reaction mixture contained 10 µL in total, including 5 µL Green I Master mix, 4 µL of gene-specific primers and 1 µL of cDNA template. The thermal cycling conditions were as follows: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. The relative gene expression level of the specific gene was calculated using the 2^{−ΔΔCt} method (Livak and Schmittgen 2001). The expression level of the target genes was

transformed into fold change relative to the untreated control and normalized using the reference gene.

2.7. Statistical analysis

At each temperature, differences in Lt₅₀ values among species were compared with 'ratio_test' function in 'ecotox' package (Wheeler et al., 2006). The expression levels of *hsps* were computed as mean (±standard error) normalized fold change relative to the control. The significance of the differences between treatments and the control was determined using t-tests, and the differences among species were determined using one-way anova followed by Tukey's test for multiple comparisons. The values of *hsp* expression relative to control were log₁₀ transformed in all statistical analyses to reduce residuals and minimize heteroscedasticity. All statistical analyses were performed using in the R software.

3. Results

3.1. Heat and cold tolerance

The differences in Lt₅₀ estimates between species were compared at each temperature using ratio tests (See the detailed result in Table S2). *R. padi* had a significantly higher Lt₅₀ (696 ± 40 min and 64 ± 2 min) than *S. avenae* (375 ± 17 min and 45 ± 1 min) and *M. dirhodum* (125 ± 5 min and 23 ± 1 min) at 34 and 37 °C, respectively (Fig. 1A, *p* < 0.05). In contrast, at −5 °C, *M. dirhodum* (Lt₅₀ = 5644 ± 277 min) survived better than *S. avenae* (4050 ± 136 min) and *R. padi* (542 ± 108 min). The Lt₅₀ estimates of *S. avenae* and *M. dirhodum* were not significantly different at −8 °C (141 ± 8 min and 179 ± 86 min, respectively, *p* = 0.121), and far beyond that of *R. padi* (14 ± 8 min) (Fig. 1B). These results suggest *R. padi* is most tolerant to heat stress, but most cold susceptible. In contrast, *M. dirhodum* is heat sensitive but more cold tolerant.

3.2. Expression of *hsps* during heat stress

We focused on seven *hsps* representing most of the HSP families. All examined genes were somewhat responsive to heat stress, as indicated by the significant differences between stressed and control aphids in Fig. 2A and Fig. 3A. Of the seven genes tested, *hsp70A* was most strongly heat-induced, with fold change variations ranging from 40 to 2000-fold in SE experiments (Fig. 2B) and up to 200-fold in Lt₅₀ experiments (Fig. 3B). *Hsc70*, *hsp10*, *hsp70B* and *hsp60* displayed species-specific modulations and were either upregulated in *R. padi* or downregulated in *S. avenae* (Fig. 2D-G and Fig. 3D-G).

No matter the exposure type, either SE or Lt₅₀, *R. padi* always had stronger expression of heat-inducible *hsp* genes than the two other species. *Hsp90*, *hsc70* and *hsp10* were moderately upregulated in *R. padi* under all heat treatments, except in 37 °C SE, but they remained non-responsive, or only slightly upregulated, in the other species (Fig. 2C-E and Fig. 3C-E). The interspecific variations were more pronounced in *hsp70A*, which was upregulated >2000-fold in *R. padi*, 69-fold in *M. dirhodum* and 42-fold in *S. avenae* (Fig. 2B). At 37 °C, the induced expression of *hsps* ceased in *S. avenae* and *M. dirhodum* but was maintained in *R. padi*. Under 34 and 37 °C Lt₅₀ treatments, *R. padi* also had significantly higher expression levels than *M. dirhodum* and *S. avenae* in

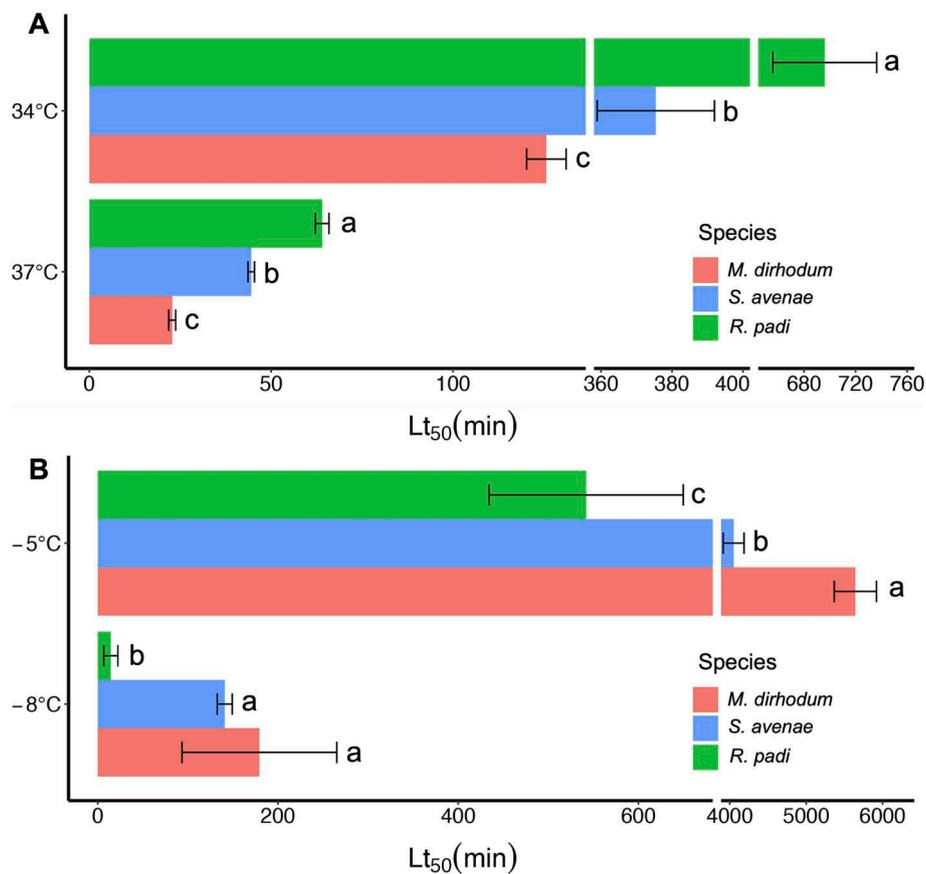


Fig. 1. Lt₅₀ estimates for the three aphid species at high temperatures (34 and 37 °C) (A) and at low temperatures (−5 and −8 °C) (B). The Lt₅₀ values of each species were obtained from logit GLM models and shown as Lt₅₀ ± standard errors. Letters above the bars indicate significant difference among Lt₅₀ values based on ratio tests ($p < 0.05$).

all *hsp*s, except for *hsp75* (Fig. 3). Again, we observed marked interspecific variation in *hsp70A*, which was induced at 34 °C Lt₅₀ and 37 °C Lt₅₀ more than twice as much in *R. padi* than in *M. dirhodum* and *S. avenae* (Fig. 3B).

3.3. Expression of *hsp*s during the recovery from cold stress

In most cases, mRNA levels of *hsp*s in response to cold stress were less than two-fold change, except for *hsp70A*. *M. dirhodum* had more genes that were significantly upregulated than the two other aphid species, especially for the −8 °C SE and −5 °C Lt₅₀ treatments (Fig. 4A and Fig. 5A). *R. padi* displayed significantly higher expression level (40.08-fold) in *hsp70A* than *M. dirhodum* (4.04-fold) and *S. avenae* (1.07-fold) under the −5 °C SE treatment (Fig. 4B). With extended exposure time, *M. dirhodum* and *S. avenae* had increased expression of *hsp70A* (19.47 and 8.11-fold) in the −5 °C Lt₅₀ treatment (Fig. 5D). When the temperature dropped to −8 °C, the upregulation of *hsp70A* in Lt₅₀ treatment was much weaker than at −5 °C (Fig. 4B and Fig. 5B) and in SE treatments, the expression even tended to down-regulate (Fig. 4B). In both experiments (i.e., SE and Lt₅₀), expression of *hsp75* was downregulated in *R. padi* but showed slight upregulation in *M. dirhodum* (Fig. 4H and Fig. 5H).

4. Discussion

4.1. The basal thermal tolerance

With the higher Lt₅₀, *R. padi* proved to survive longer and was the most heat tolerant species, whereas *M. dirhodum* was the most vulnerable (lowest Lt₅₀) at the two high temperatures. Our observations are

supported by a previous study, which suggested that *R. padi* could tolerate higher constant temperatures than *S. avenae* and *M. dirhodum* (Asin and Pons, 2001). Ma et al. (2015b) showed that tolerant aphid species better survived under extreme high temperatures, but they also showed comparatively higher fitness, due to fecundity, life span and adult longevity being less affected than tolerant species. Here, the tested high temperatures (34 and 37 °C) were ecological-relevant, as they were frequently recorded in late spring and summer days in a typical temperate area, such as near Beijing. Therefore, it is possible to link the responses we found here to population dynamics in the field (see below).

The short Lt₅₀ values of the three aphid species at −8 °C suggest a vulnerability to low (freezing) temperatures which clearly induced severe cold stress. It also implies that they are not likely to overwinter as nymphs in temperate regions of China, where winter temperatures frequently drop below −10 °C. In contrast, in west Europe with mild oceanic climate, aphids are active during the whole winter (Alford et al., 2018). Yet, the patterns of cold tolerance of the three aphids in west Europe are comparable to our study (Alford et al., 2014). It showed that *R. padi* was the least cold tolerant species with higher LT₅₀ value (i.e. median lethal temperature) than *S. avenae* and *M. dirhodum* (Alford et al., 2014). But *R. padi* had a lower chill coma temperature and remained attached to the host plants under sub-zero temperatures, which may explain the dominance of this species during winter (Alford et al., 2014; 2016). To understand the patterns of thermal tolerance, comparative studies on behavioral thermal reactions of aphids should be conducted in both China and Europe. Interspecific differences in thermal tolerance have been observed in other species competing for the same resources, as reported in cotton aphids (Gao et al., 2016). In these systems, interspecific variations in thermal tolerance/response have been utilized to elucidate the differences in seasonal abundance and

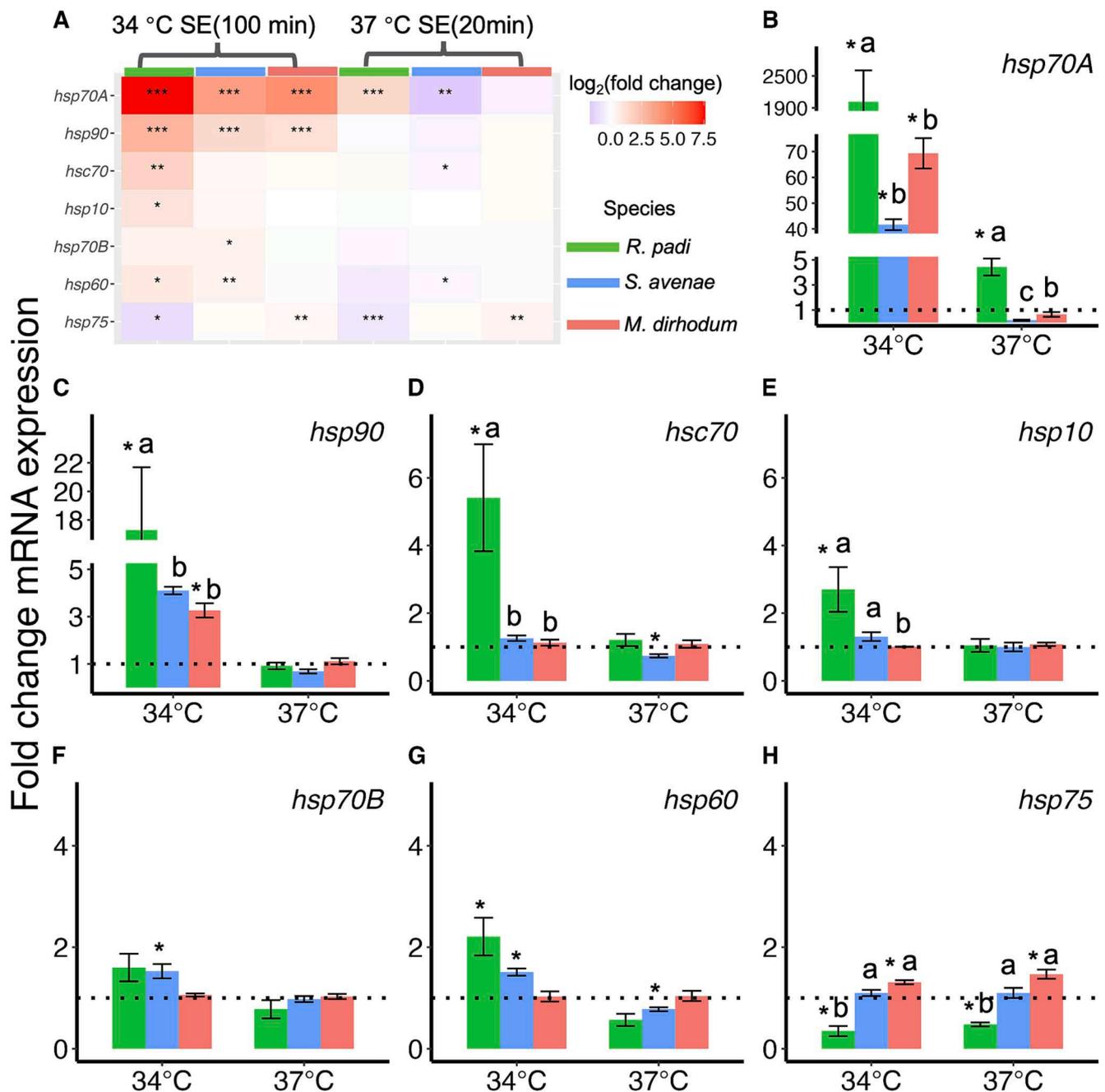


Fig. 2. Expression profile of seven *hsps* of the three aphid species after the same exposure time at 34 and 37 °C (i.e., 100 min at 34 °C and 20 min at 37 °C). The heatmap (panel A) summarizes the log-transformed expression levels of *hsps* in three aphid species after heat treatments with the same exposure time. Asterisks in the cells of heatmap indicate mean values that are different significantly from the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Panel B-H shows the fold change in expression level relative to the control of each *hsp* gene in three aphid species after heat treatments with the same exposure time. Asterisks indicate mean values that are significantly different from the control group ($p < 0.05$). Lower case letters indicate mean values that are significantly different among species ($p < 0.05$).

phenology. As emphasized by Chown (2012), conducting research on interspecific and intraspecific variations in thermal tolerance is essential to better understand the adaptive physiological mechanisms that determine activity limits.

4.2. The expression of *hsps* during heat stress

In accordance with its relative greater heat tolerance, *R. padi* had more heat-inducible *hsp* genes and higher expression level of *hsps* at heat than the two other species, notably *hsp70A*, *hsc70* and *hsp90*, which were the most heat-responsive genes. The induction of *hsps* may provide protection against heat stress, and the levels of *hsps* expression have

been positively related to thermal tolerance (Colinet et al., 2012; Jiang et al., 2012; Lu et al., 2016; Tarapacki et al., 2021).

Our study shows that the expression profile of different family members varied after heat exposure, even within the same family. For instance, *hsp70A* was massively expressed during heat stress, while *hsp70B* was less, or not induced. This is not surprising as HSP70 are encoded by several copies of *hsp70*, supporting the existence of a sub-functionalization in that family (Ramsøe et al., 2020). Sub-functionalization has probably contributed to partition their secondary functions towards different roles, such as ribosome protection (HSP70A) and signaling and cytoskeleton protection (HSP70B) in the Antarctic clam (Ramsøe et al., 2020). In *Drosophila* species, it was clearly showed

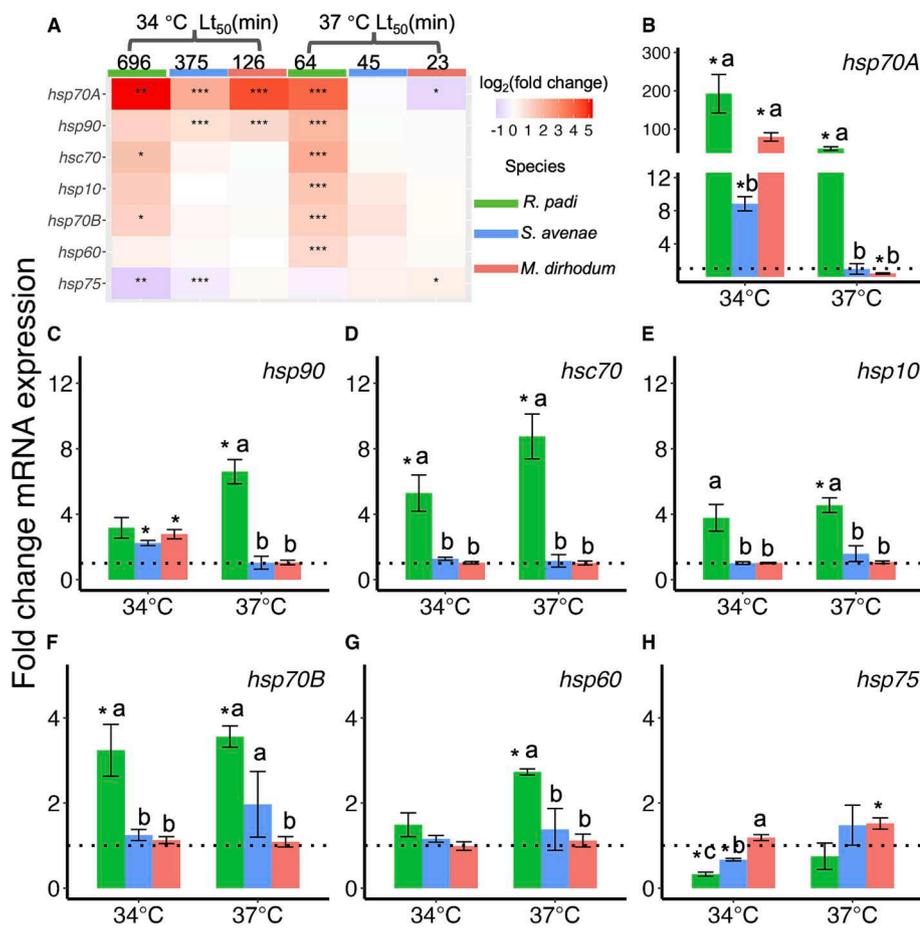


Fig. 3. Expression profile of seven *hsps* of the three aphid species after exposure for the same injury levels at 34 °C and 37 °C (i.e., specific times corresponding to their Lt_{50} values). The heatmap (panel A) summarizes the log-transformed expression levels of *hsps* in three aphid species after heat treatments with the same injury levels. Asterisks in the cells of heatmap indicate mean values that are different significantly from the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Panel B-H shows the fold change in expression level relative to the control of each *hsp* gene in three aphid species after heat treatments with the same injury levels (Lt_{50}). Asterisks indicate mean values that are significantly different from the control group ($p < 0.05$). Lower case letters indicate mean values that are significantly different among species ($p < 0.05$).

that the heat-inducibility of *hsp70A* and *hsp70B* copies was differentially regulated in different cell types (Lakhotia and Prasanth, 2002). Hence, duplications of *hsp70* genes are not just made to produce faster and more chaperones during stress, but these multiple copies truly exert different functions in various cell-specific patterns. Our data shows that, in cereal aphids, *hsp70B* is much less heat-responsive than *hsp70A*, suggesting protective functions under thermal stress in the latter. The large upregulation of *hsp70A* under heat stress suggests an important molecular role as a first line of defense against sub-lethal heat stress, which is consistent with previous studies in insects (Huang and Kang, 2007; Colinet et al., 2013; Cheng et al., 2016; Li et al., 2017; Ashbrook et al., 2022).

Despite being from the same family, we found that *hsc70* and *hsp70* genes (and proteins) differed in their expression patterns, and in their roles in stress response. *Hsc70* is expressed constitutively, and is assumed to be linked to basal thermotolerance, whereas the upregulation of *hsp70* is heat-induced, and is generally associated with acquired thermotolerance (Lindquist, 1980; Feder and Hofmann, 1999). Even though *hsc70* is constitutively expressed, it can also be significantly upregulated in response to heat shock in insects (Wang et al., 2008; Cheng et al., 2016; Li et al., 2017). Interestingly, heat-tolerant desert species of *Drosophila*, as well as species' strains from hot climates, seem to have high constitutive levels of *hsc70* and relatively high induction temperature for *hsp70* (Sørensen et al., 2001; Zatssepina et al., 2001). In our study, *hsc70* gene was the most basally expressed, in the three species, without any stress (i.e., lowest C_p values in the controls, see Fig. S2), which suggests a constitutive high expression. *Hsc70* only showed up-regulation in *R. padi* in both SE and Lt_{50} heat assays. High expression of this constitutively expressed gene in *R. padi* may partially contribute to its relatively higher basal heat tolerance.

HSP90 might cooperate with HSP70 chaperone systems in protein

repair: the former acts to prevent the aggregation of thermally unfolded reporter enzymes (Bose et al., 1999); the latter is responsible for the refolding of these enzymes (Mayer and Bukau, 1999). In our study, following *hsp70A*, *hsp90* was the second most upregulated gene during heat stress, which suggests that HSP90 may be responsible for heat-shocked induced defenses. This pattern was similar to that reported from *D. melanogaster* and other insect species (Xu et al., 2010; Chen et al., 2014; Quan et al., 2022).

The function of HSP60 is dependent upon HSP10 which binds to one end of the HSP60 particle and regulates its ATPase activity and substrate associations (Parsell and Lindquist, 1993). This might explain why *hsp10* and *hsp60* showed very similar expressions during heat stress in our study, as also found in other models (Xu et al., 2014). Besides, the expression of *hsp60* and *hsp10* seems to be species-specific: they were only moderately expressed in *R. padi*. Previous studies also reported that *hsp60* was heat-inducible in some insects (Cai et al., 2017; Cui et al., 2010; Zhang et al., 2015) but not in others (Chen et al., 2015). In insects, information about *hsp10* is scarce. Collectively, our data show that *R. padi* seems to have a large panel of heat-inducible stress genes.

4.3. The expression of *hsps* during the recovery from cold stress

As with heat treatments, *hsp70A* was the most responsive gene among the seven *hsps* during the recovery from cold stress. However, contrary to heat, it was the most intolerant species, *R. padi*, that had the highest expression of *hsp70A* during recovery from cold. This might be due to distinct processes between heat and cold. At heat, we measured the *hsps* right during stress itself, while at cold, we measured gene expression when aphids were recovering after cold stress. *Hsps* during heat stress may relate to direct protective functions (protect against damages), while they may perform indirect reparative functions

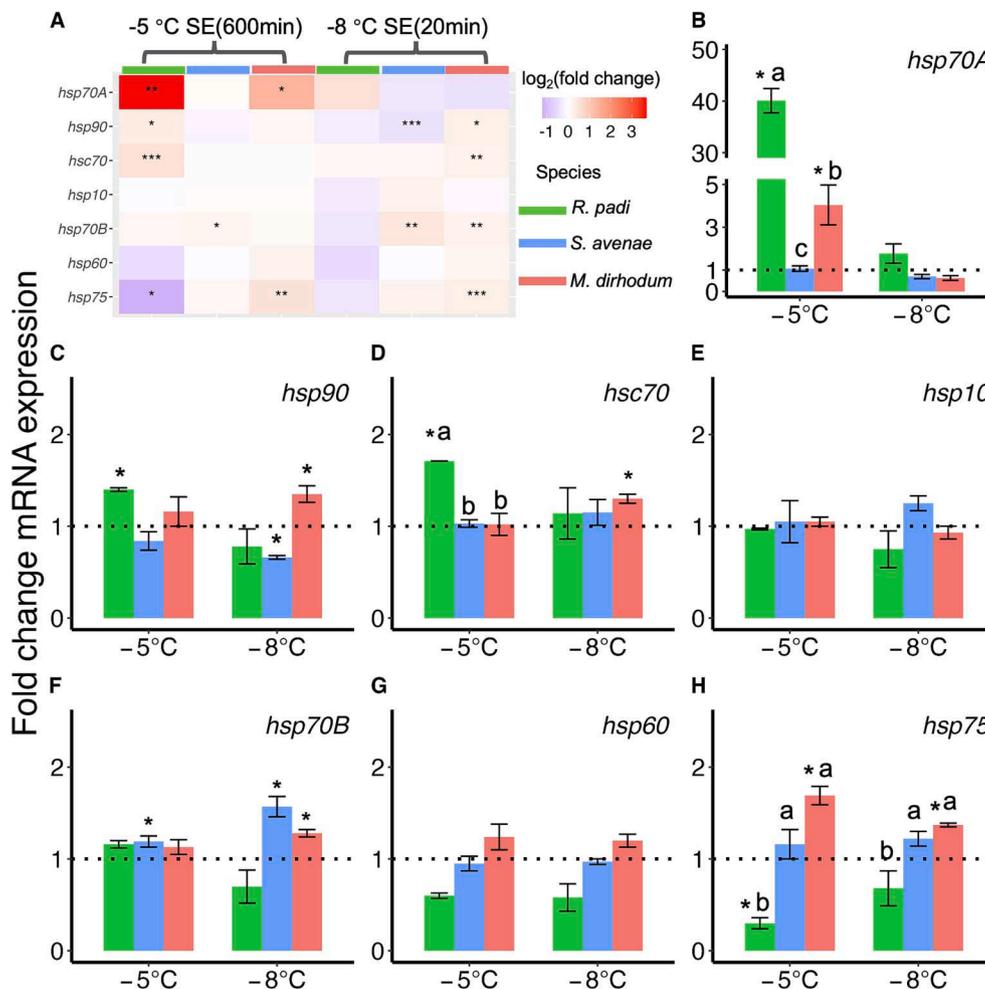


Fig. 4. Expression profile of seven *hsps* of the three aphid species after the same exposure time at -5 and -8°C (i.e., 600 min at -5°C and 20 min at -8°C). The heatmap (panel A) summarizes the log-transformed expression levels of *hsps* in three aphid species after cold treatments with the same exposure time. Asterisks in the cells of heatmap indicate mean values that are significantly different from the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Panel B-H shows the fold change in expression level relative to the control of each *hsp* gene in three aphid species after cold treatments with the same exposure time. Asterisks indicate mean values that are different significantly from the control group ($p < 0.05$). Lower case letters indicate mean values that are different significantly among species ($p < 0.05$).

(neutralization of damages) during recovery from cold (Lubawy et al., 2022). Hence, the species which most suffered at cold should have higher *hsp* genes expression. As evidence, after the same exposure time (600 min) at -5°C , the cold intolerant species, *R. padi*, highly expressed *hsp70A*, but few changes were found in *S. avenae* and *M. dirhodum* (Fig. 4D). For the latter two species, *hsp70A* expressed heavily only after a longer exposure to cold (i.e., an exposure corresponding to their $\text{Lt}_{50\text{s}}$, which is about 5000 min cold exposure, Fig. 5D). Moreover, *M. dirhodum* had more cold responsive genes, for example *hsp70A*, *hsp60* and *hsp75* at -8°C SE and -5°C Lt_{50} ; so, the whole *hsp* machinery seemed to be reactive, while this was less evident in *R. padi*.

We found up to 40-fold expression of *hsp70A* in response to cold in the three aphid species. A strong expression of *hsp70* was also observed in *D. melanogaster* and many other insects (Colinet et al., 2010a,b; Yocum 2001; Štětina et al., 2015; Jiang et al., 2021). The upregulation of *hsp70A* may contribute to the repair of cold injury. After suppressing the *hsp70* up-regulation response to cold in *Pyrrhocoris apterus* adults using RNAi, the proportion of insects that were not able to fully repair cold injury (uncoordinated crawling) increased (Košťál and Tollarová-Borovanská, 2009). In *D. melanogaster*, *hsp70*-null mutants (lacking all copies of *hsp70* gene) showed higher mortality during the recover from severe cold shock than the wild type strain (Štětina et al., 2015).

The upregulation of mitochondrial *hsps*, such as *hsp60* and *hsp75* may help to maintain the homeostasis of mitochondria (Pellegrino et al., 2013; Wang et al., 2015). HSP60 can enhance cold hardiness and promote membrane stability by stabilizing microfilaments in insects (Kayukawa and Ishikawa, 2009). It was slightly upregulated in response to cold exposure in *Belgica antarctica* (Teets et al., 2019), *Monochamus*

alternatus (Cai et al., 2017) and *Galeruca daurica* (Tan et al., 2018). We found *hsp60* also slightly upregulated in cold tolerant species (*M. dirhodum* and *S. avenae*) in -5°C Lt_{50} treatment, while no changes were reported from *R. padi* in all cold treatments. HSP75 would likely facilitate mitochondrial respiration balance in response to cold stress (Jiang et al., 2021). *Hsp75* slightly overexpressed in *M. dirhodum*, which might help to adjust the function of the mitochondria and survive after cold shock.

4.4. Temperature- and treatment-specific patterns

Some temperature-specific patterns can be seen from the differences in *hsps* expression between severe (-8 and 37°C) and moderate (-5 and 34°C) stressful temperatures. At 34°C , all three species could express *hsps*. When the temperature rose to 37°C , the expression of *hsps* ceased in *S. avenae* and *M. dirhodum* but could be sustained in *R. padi*. The heat shock response generally happens within a specific temperature range, and the *hsps* can not be induced, or shut off, if the temperature is below or above this range (Kristensen et al., 2001; Huang and Kang, 2007). Here, 37°C might exceed the limit for *M. dirhodum* and *S. avenae* but not for *R. Padi*, which suggest again the highest heat tolerance capacity of *R. Padi*.

Moderate and severe cold shocks might result in different types of cold injury, expressed as quantitative and/or qualitative differences in the protein denaturation (Štětina et al., 2015). As *hsps* during recovering from cold is likely correlated to repair functions, we expected to see more *hsps* expression in the -8°C treatment where aphids probably suffered more protein damages. Contrary to our expectation, moderate

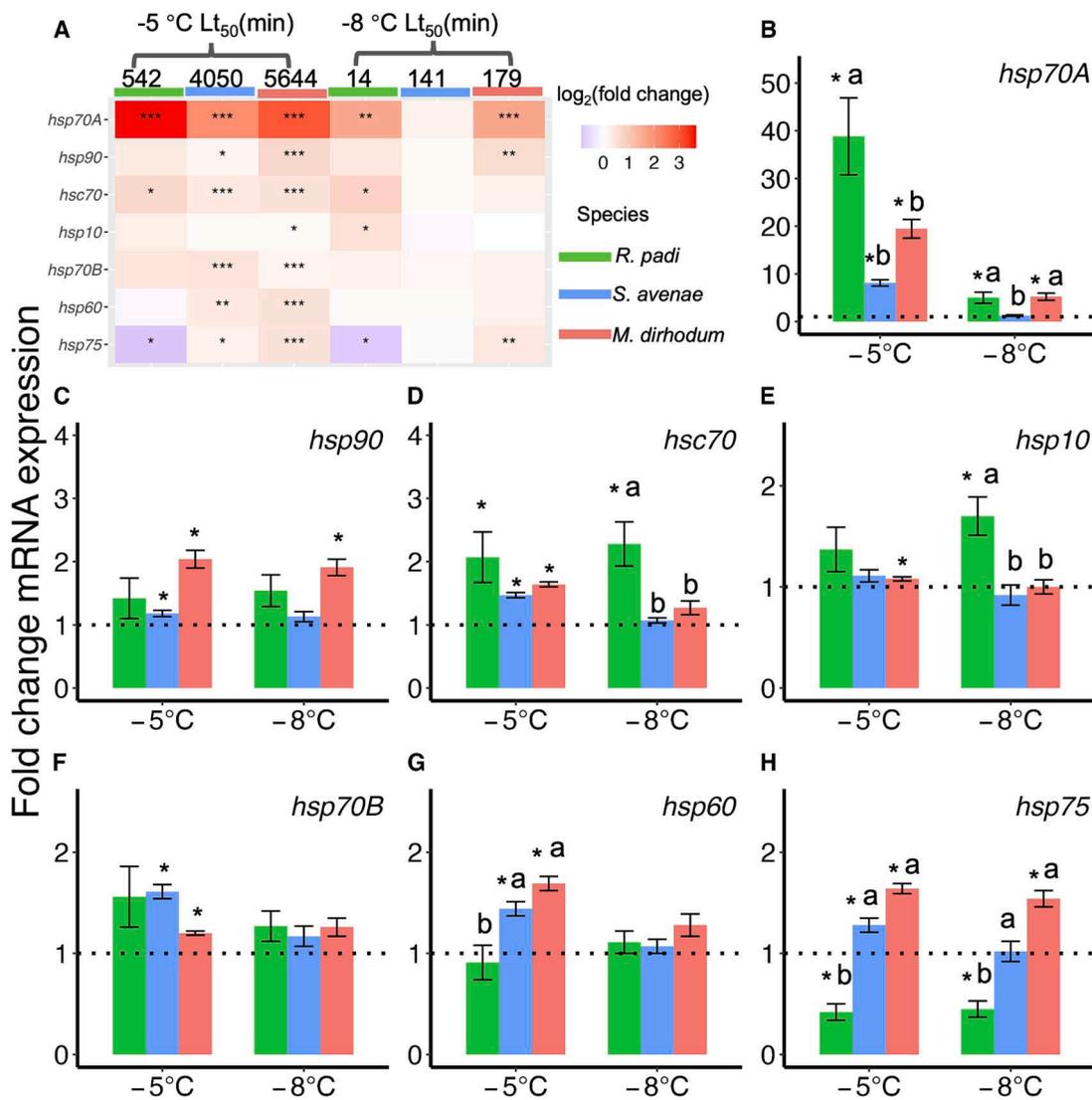


Fig. 5. Expression profile of seven *hsps* of the three aphid species after exposure for the same injury levels at $-5\text{ }^{\circ}\text{C}$ and $-8\text{ }^{\circ}\text{C}$ (i.e., specific times corresponding to their Lt_{50} values). The heatmap (panel A) summarizes the log-transformed expression levels of *hsps* in three aphid species after cold treatments with the same injury levels. Asterisks in the cells of heatmap indicate mean values that are significantly different from the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Panel B-H shows the fold change in expression level relative to the control of each *hsp* gene in three aphid species after cold treatments with the same injury levels (Lt_{50}). Asterisks indicate mean values that are different significantly from the control group ($p < 0.05$). Lower case letters indicate mean values that are significantly different among species ($p < 0.05$).

cold shocks induced more *hsps* expression. We supposed the reason might be 1) 2 h recovery time may be not enough for aphids to launch the *hsp* machinery from a severe cold shock, 2) alternatively, $-8\text{ }^{\circ}\text{C}$ is too low for the cold shock response.

The treatment-specific patterns could be found from the different expression profiles between SE (same exposure time) and Lt_{50} (same injury level), such as $-5\text{ }^{\circ}\text{C}$ SE and $-5\text{ }^{\circ}\text{C}$ Lt_{50} , $37\text{ }^{\circ}\text{C}$ SE and $37\text{ }^{\circ}\text{C}$ Lt_{50} . The two sets of treatments provided a complementary view of interspecific difference in heat shock responses. SE treatments are ecologically relevant, as the three species co-occur temporally and spatially. However, there are shortcomings using only SE treatments: 1) the same thermal dose might be too large for vulnerable species but too small for tolerant species to induce the *hsps*. As shown in $37\text{ }^{\circ}\text{C}$ SE, a 20 min-exposure stopped *hsps* expression of heat-vulnerable species (*S. avenae* and *M. dirhodum*) but was too short to induce the expression of some *hsps* for the tolerant species (*R. padi*). 2) a short thermal exposure might induce expression of some responsive genes but may not be enough to induce some less-reactive genes. For example, *hsp60*, *hsc70*, *hsp70B*, *hsp90* showed no change in $-5\text{ }^{\circ}\text{C}$ SE (600 min) but were slightly

upregulated in $-5\text{ }^{\circ}\text{C}$ Lt_{50} (4050 min and 5644 min) treatments for *S. avenae* and *M. dirhodum*. It could be argued that using a series of exposure times could avoid the above-mentioned problems. With the same injury level, the potential genes (less-reactive genes) and the commonality of the chaperone system of the three species are better revealed. Therefore, combining the treatments after same injury level and at same exposure time might be an efficient way to broadly explore and compare the molecular chaperones' response to stress among species.

4.5. Linking to seasonal dynamics

The capacity to tolerate high/low temperatures plays an important role in the seasonal activities and population growth of aphids (Gao et al., 2016; Ma et al., 2015b; Saeidi et al., 2021). We found that *R. padi* possessed more heat-induced *hsps* and exhibited higher expression levels of those genes than *S. avenae* and *M. dirhodum*, which may contribute to its higher heat tolerance. Species that are tolerant to heat may have a better chance of survival and reproduction compared to those that are

intolerant, thus their demographic variables may be less affected by heat waves. Previous field observations have shown that the relative abundance of *R. padi* increased with extreme high temperature frequency (Ma et al., 2015b; Zhu et al., 2021). In hot conditions, *R. padi* might become the dominant species, outcompeting *S. avenae*, and this pattern may be further detectable in a warmer future (Zhu et al., 2021). Conversely, *M. dirhodum* was comparatively heat vulnerable but had more cold-inducible *hsp*s than *R. padi*. The high susceptibility of *M. dirhodum* to heat is in agreement with its seasonal dynamics and relative abundance. Studies showed that this species thrives in cooler regions and is the least prevalent among the other species found in the field. It usually emerges early in spring and quickly disappears from the wheat field as temperatures rise (Chen et al., 1994; Yang et al., 2017).

5. Conclusion

To summarize, this study showed that *hsp70A* was the *hsp* gene that exhibited the highest up-regulation in response to both heat and cold stress while *hsc70*, *hsp10*, *hsp70B* and *hsp60* displayed species-specific modulations. We propose that the molecular response to thermal stress may be somewhat responsible for the interspecific variation in thermal tolerance. These differences may explain the changes observed in aphid's seasonal dynamics and community abundance, especially in the context of climate change.

Funding

This project was mainly funded by ARED PhD grant, provided by Region Bretagne (project climPuce COH20023). Parts of the study were supported by the International Research Project (IRP) "Phenomic Responses of Invertebrates to Changing Environments and Multiple Stress" (PRICES) supported by CNRS and by International Emerging Actions (IEA - CLIMRESIST) also provided by CNRS. Parts of the work were supported by National Natural Science Foundation of China 31620103914, the Fundamental Research Funds of CAAS (Y2017LM10), and Innovation Program of CAAS (CAAS-ZDRW202012).

CRedit authorship contribution statement

Yuan-Jie Li: Investigation, Methodology, Formal analysis, Data curation, Writing – original draft. **Chun-Sen Ma:** Supervision, Resources, Writing – review & editing. **Yi Yan:** Methodology. **David Renault:** Supervision, Resources, Writing – review & editing. **Hervé Colinet:** Conceptualization, Supervision, Methodology, Validation, Resources, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank Dr. Xiang-Rui Li for assistance in preliminary experiments. We are grateful to Virginie Daburon from PEM technical core facility (UMR CNRS 6553, University of Rennes, France) for training and access to molecular facilities. We would like to thank Joan van Baaren and two anonymous reviewers for their valuable comments, which have significantly enhanced the quality of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jinsphys.2023.104520>.

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Supporting information

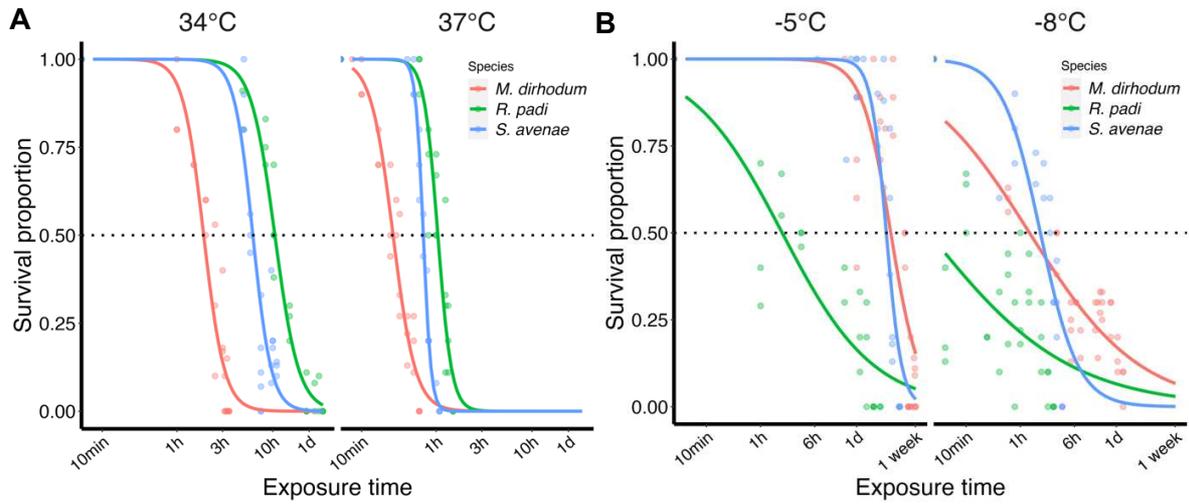


Figure S1. Survival curves (panel A for heat, B for cold) for the three aphid species (*M. dirhodum*, *S. avenae* and *R. padi*). Survival curves was fitted by a generalized linear model (GLM) with logit link function. Each point in panel A and B represents the survival proportion of aphids after the given exposure times in a single replicate. The black dashed line indicates the 50% survival proportion thus the intersections of this dashed line and survival curves are the Lt_{50} estimates of each species at the specific temperature.

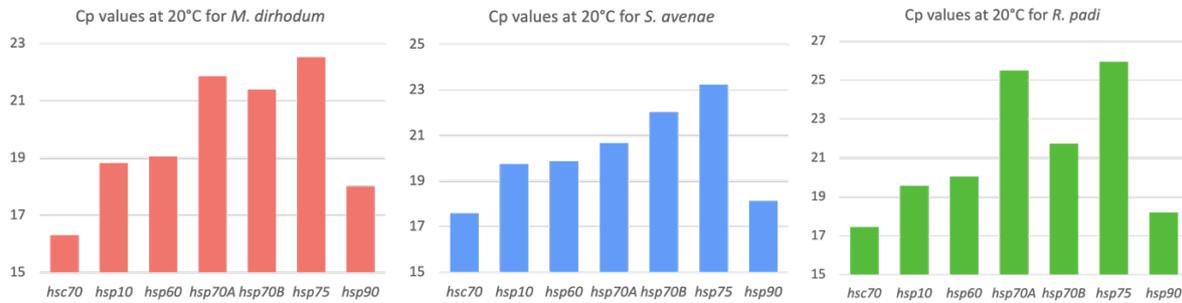


Figure S2. The basal expression level (Cp value) of seven *hsp* genes in *M. dirhodum* (A), *S. avenae* (B) and *R. padi* (C).

Table S1. See the attached Excel file

(<https://www.sciencedirect.com/science/article/pii/S002219102300046X#s0120>)

Table S2. The Z test statistic and p-value (in parentheses) of ratio tests comparing Lt_{50s} at each temperature. p -values < 0.05 are marked in bold.

Temperature	Species			
	Species	<i>M. dirhodum</i>	<i>S. avenae</i>	<i>R. padi</i>
34°C	<i>M. dirhodum</i>		17.7(2.74e-70)	23.7(4.49e-124)
	<i>S. avenae</i>			8.48(2.21e-17)
	<i>R. padi</i>			
37°C	<i>M. dirhodum</i>		14.6(3.29e-48)	20.4(7.12e-93)
	<i>S. avenae</i>			10.3(4.29e-25)
	<i>R. padi</i>			
-5°C	<i>M. dirhodum</i>		5.59(2.2e-8)	11.5(2.23e-30)
	<i>S. avenae</i>			9.99(1.76e-23)
	<i>R. padi</i>			
-8°C	<i>M. dirhodum</i>		1.55(0.121)	4.37(1.27e-5)
	<i>S. avenae</i>			4.16(3.18e-5)
	<i>R. padi</i>			

Chapter 4 Comparative metabolic response to heat stress

Article 3. Metabolomics reveals species-specific metabolic responses to heat stress in three aphid species

Chapter 2 and Chapter 3 have unveiled significant interspecific variations in heat tolerance and molecular responses among the three aphid species. However, it remains unclear whether the variations in heat tolerance among species arise from different metabolic responses to heat stress. Therefore, we compared the metabolic signatures of these species using GC-MS under two experimental conditions: exposure to 34 °C for increasing durations corresponding to their species-specific 1/2 Lt_{50} (median lethal time) and 2/2 Lt_{50} as well as the same exposure duration.

We hypothesized that:

- 1) Aphids exposed to heat stress are expected to exhibit metabolic disorders compared to control conditions. The severity of these metabolic disorders is predicted to vary according to the stress levels, with longer durations of stress (i.e., higher injury level) resulting in increasingly challenging recovery.
- 2) When exposed to the same duration of heat stress, the tolerant species is expected to show fewer metabolic perturbations and more efficient restoration of homeostasis during recovery than the intolerant species.
- 3) Aphids are expected to accumulate cytoprotectants in response to heat stress. Specifically, levels of some polyols, such as mannitol and sorbitol, may increase in response to heat stress.

Main findings:

- 1) The metabolic homeostasis of aphids was disrupted during heat stress, and the degrees of perturbations increased when the exposure duration lengthened.
- 2) While the recovery period enabled full restoration of the metabolic profiles of aphids exposed to a 1/2 Lt_{50} injury level, the metabolic profiles of aphids exposed to a higher injury level (2/2 Lt_{50}) remained distinct from controls.
- 3) Under heat stress, aphids exhibited decreased sucrose levels and an accumulation of TCA cycle intermediates, along with the end products of anaerobic metabolism, namely alanine and lactic acid. These findings indicate a significant reliance on anaerobic metabolism.
- 4) Mannitol emerged as the primary polyol involved in the response to heat stress across all three species. Glycerol only significantly accumulated in the most heat-tolerant species, *R. padi*.
- 5) The most heat-tolerant aphid species, *R. padi*, showed fewer disturbances in metabolic profiles but incredibly higher levels of polyol accumulation compared to other species when exposed to high temperatures for the same time.

Metabolomics reveals species-specific metabolic responses to heat stress in three aphid species

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Abstract: Global warming has led to an increase in extreme heat events, posing significant challenges for insects. To understand how insects tolerate and respond to high temperatures, it is crucial to investigate the underlying physiological mechanisms. Comparative physiology offers valuable insights into these mechanisms and their adaptive significance. In this study, we focused on three common aphid species (*Sitobion avenae*, *Metopolophium dirhodum*, and *Rhopalosiphum padi*) known for causing damage to cereal crops. Our objective was to compare the metabolic signatures of these species under two experimental conditions: exposure to a high temperature (34 °C) for increasing durations corresponding to their 1/2 Lt_{50} (median lethal time) and 2/2 Lt_{50} as well as the same exposure duration. To achieve this, we employed quantified GC-MS profiling to analyze an array of 32 metabolites. The metabolomics analysis revealed disturbances in metabolic homeostasis and alterations in energy metabolism. Aphids exhibited decreased sucrose levels and an accumulation of TCA cycle intermediates under heat stress conditions. The increase in alanine and lactic acid suggested a shift toward anaerobic metabolism as a response to high temperatures. Furthermore, we found that prolonged exposure duration (2/2 Lt_{50}) treatment resulted in more pronounced metabolic perturbations, which were more challenging to recover from within a 24h timeframe compared to mild stress (1/2 Lt_{50}). Mannitol emerged as the primary polyol involved in the response to high temperatures across all three species. Remarkably, the heat-tolerant species, *R. padi*, exhibited fewer perturbations and higher levels of mannitol compared to the less-tolerant species after the same duration of exposure, indicating a potential correlation between mannitol levels and heat tolerance. Additionally, *R. padi* accumulated a species-specific polyol, glycerol, which may contribute to its superior heat tolerance. Overall, this study provides valuable insights into the metabolic responses and heat tolerance mechanisms of cereal aphids.

Keywords: cereal aphids, metabolomics, heat stress, polyols

Introduction

Global warming has led to more frequent and intense extreme heat events in many regions (Meehl and Tebaldi 2004, Ummenhofer and Meehl 2017). Insects heavily rely on ambient temperature for their physiological functions as they have limited ability to regulate their body temperature. Consequently, extreme heat events can affect insects in various ways, ranging from molecular responses to changes in community structure (Vázquez et al. 2017, Gonzalez-Tokman et al. 2020, Harvey et al. 2020, Ma et al. 2021). Predicting the ecological impacts of heat events is challenging because extreme heat events can manifest in different combinations of intensity, duration, and frequency (Ma et al. 2018, Ma et al. 2021). Conducting laboratory studies to simulate these temperature regimes and explore their impact on insects can be a time-consuming and resource-intensive endeavor. However, focusing on elucidating the underlying cellular and physiological processes that enable animals to cope with such heat events could accelerate our understanding of the impact of extreme climate change (Vázquez et al. 2017). This knowledge can be instrumental in predicting and assessing the potential impacts of future climate change scenarios on insect populations. By unraveling the mechanisms of heat tolerance and adaptive responses, researchers can gain insights into how insects are likely to fare in the face of escalating heat events, aiding in the development of effective conservation and management strategies.

Heat tolerance in insects is a complex trait governed by a range of physiological and biochemical mechanisms, such as cellular response and metabolisms (Neven 2000, Gonzalez-Tokman et al. 2020). Heat stress can transiently disturb cellular homeostasis, causing subsequent damage to cellular components such as proteins, membranes, and DNA (Somero 2020). To counteract these effects, cellular homeostatic responses aiming at restoring homeostasis are elicited (Kültz 2005). One important aspect of heat tolerance is the accumulation of compatible solutes in the form of sugars, polyols, or amino acids, which serve as osmolytes to maintain cellular osmotic balance, protect cellular structures and enzymes from denaturation, and stabilize cell membranes (Somero 2020). Heat shock proteins also play vital roles in stabilizing proteins and facilitating their refolding under heat-stress conditions (King and MacRae 2015). All these processes are energy-dependent, but heat exposure challenges energy metabolism and respiration, as high temperatures can impact metabolic processes and cellular energy production (Neven 2000, Zhu et al. 2019, Kuo et al. 2023).

Metabolites, being downstream of both gene transcripts and proteins, can offer valuable insights into the overall integrated response of an organism (Malmendal et al. 2006). Utilizing metabolomics, we can comprehensively identify and quantify the complete set of metabolites in these organisms, providing us with biochemical fingerprints that reveal their coordinated response to stress (Bundy et al. 2008, Hayward and Colinet 2023). Besides, metabolomics, as a powerful tool, also holds the potential to generate new hypotheses and insights in less-studied areas/species.

Comparative physiology has proven to be a valuable approach for advancing our understanding of both physiology and ecology (Chown et al. 2002). Although several comparative metabolomic studies have been conducted on various insect species to investigate their response to thermal stress, these studies have primarily focused on exploring different phenotypes, such as acclimated versus no-acclimated individuals (Enriquez and Colinet 2019), diapause versus non-diapausing morphs (Colinet et al. 2012), young and old specimens (Colinet and Renault 2018). However, there have been limited studies that provided comparative investigations among different insect species, except for research conducted on *Drosophila* flies (Olsson et al. 2016, Mensch et al. 2021), mainly focusing on cold tolerance. Conducting comparative metabolomic analyses on species with varying heat tolerance capacities could significantly enhance our understanding of the physiological mechanism underlying heat tolerance.

This study focuses on three aphid species *Sitobion avenae*, *Metopolophium dirhodum*, and *Rhopalosiphum padi*, which cause damage to cereal crops in temperate regions by feeding on phloem sap and transmitting viruses (Dedryver et al. 2010). These aphids have small body sizes and are highly sensitive to temperatures (Hullé et al. 2010). While these three aphid species coexist in most areas of China, their abundance varies across different regions. *R. padi* tends to prevail in warmer regions characterized by high temperatures, humidity, or frequent heat events (Ma et al. 2015, Zhu et al. 2021). On the other hand, *M. dirhodum* thrives in cooler regions, particularly in the west-northern and alpine areas like the Tibetan plateau (Hu and Gui 1985, Lei et al. 2021). *M. dirhodum* appears in early spring and leaves the wheat field when the temperature warms up in other regions (Chen et al. 1994). *S. avenae* used to be dominant in temperate regions, but the frequent occurrence of hot events has resulted in its displacement by *R. padi* as the prevailing species (Ma et al. 2015). Previous studies have indicated that these three aphid species exhibit different population growth responses to extreme heat events (Ma et al. 2015). Moreover, recent research has unveiled significant interspecific variations in heat tolerances (Li et al. 2023a) and molecular responses (Li et al. 2023b). However, it remains unclear whether the variations in heat tolerance among species arise from different metabolic responses to heat stress.

We hypothesized that: 1) severe heat stress would result in similar but more pronounced metabolic perturbations as mild heat stress, and it is difficult to recover. 2) tolerant species may show lower perturbation levels and restore general homeostasis faster than susceptible ones. 3) the production of polyols, such as mannitol and sorbitol, as potential protective mechanisms during heat stress, as well as the potential shift from aerobic to anaerobic respiration in response to high temperatures. To test these hypotheses, we conducted a comparative study on the metabolic response of the three common cereal aphid species to heat stress. Specifically, we subjected the three aphid species to a high temperature (34 °C) for increasing durations corresponding to their 1/2 Lt_{50} (median lethal time) and 2/2 Lt_{50} as well as the same exposure duration. We employed quantified GC-MS

profiling and principal component analysis to analyze the changes of metabolites during stress and after recovery.

Material and Methods

Aphid rearing

We conducted our experiments on lab populations of three common aphid species, *Sitobion avenae* (English grain aphid), *Metopolophium dirhodum* (Rose grain aphid), and *Rhopalosiphum padi* (Bird cherry-oat aphid). Individuals were originally collected in May 2010 from wheat fields near Beijing (39°48 N, 116°28 E) (Zhao et al. 2014). Since collection, the aphids were reared on 10-20 cm tall winter wheat seedlings at 20 ± 0.5 °C, 70% relative humidity, and a photoperiod of 16: 8 h (Light: Darkness). We used aphids of the same age (6-day-old aphids) for the experiments. Aphids were obtained by allowing newborn nymphs to develop for six days on the winter wheat seedlings.

Median lethal time (Lt_{50})

In a former study, we examined the sensitivity of the three aphid populations to heat exposure by using 6-day-old specimens of *M. dirhodum*, *S. avenae*, *R. padi* exposed at several high temperatures ranging from 34 to 40 °C, and for several different exposure durations (Li et al. 2023a). The experimental design allowed us to obtain survival ranging from 100 to 0% for the three populations, and to further compute median lethal times (Lt_{50}) across a wide range of high temperatures for each species. This former investigation demonstrated the large difference in the level of heat tolerance that the three aphid species can handle, particularly concerning Lt_{50} . Here, we selected a treatment temperature of 34 °C to compare the metabolic profile among species for the following reasons. First, 34 °C is a temperature that is frequently encountered by aphids during the summer period in the field. Second, the former study evidenced noticeable interspecific heat stress responses (Lt_{50}) at this temperature, making it convenient to discern variations in metabolic activity among different species (Li et al. 2023a)

In the former study, we measured the survival curve and estimated Lt_{50} at 34 °C using the following protocol. Aphids were exposed to 34 °C for at least five different exposure durations, which resulted survival rate ranging from 100 to 0%. For each exposure time point, around 10 aphids were tested. To reduce handling stress, and provide the individuals with food as well as water, wheat leaves with aphids were cut and transferred into glass vials (10 mL), which were sealed with a sponge stopper to limit the air circulation and maintain a stable internal environment. The vials were then mounted in a rack, which was merged into a glycol bath (Ministat 230-cc-NR; Huber Ltd., Germany, accuracy ± 0.01 °C) pre-set at 34 °C. After the exposure, test aphids were transferred to a climatic chamber at 20 °C to recover for 24h before checking the survival of each individual. Aphids were considered alive if the individual moved following a gentle tactile stimulation with a brush. We modeled the survival at 34 °C by specifying a generalized linear model (GLM) with a logit link function for the binary outcome (i.e., dead vs alive aphids) (see Figure S1). Lt_{50} , the time which kills

50% of the tested animals at a given temperature, can be used as an index of the inherent/basal physiological thermotolerance in insects (Andersen et al. 2015). We estimated Lt_{50} , which corresponds to the temperature-specific duration resulting in 50% mortality, using the *dose.p* function in R package MASS (Ripley et al. 2013).

Experimental conditions for metabolite profiling

To examine the effects of heat exposure and resilience capacities on the physiology of the three aphid species, we conducted two distinct experiments. *Experiment 1*: aphids were exposed to 34 °C for durations corresponding to 1/2 and 2/2 of the species-specific Lt_{50} values (Table 1), and further allowed to recover for 24h. This experiment allowed us to compare the physiological impacts of two different injury levels on the aphids. *Experiment 2*: aphids were exposed to 34 °C for the same duration of exposure (100min), and further allowed to recover for 24h. We select this duration because it is close to the Lt_{50} of the intolerant species but far below the Lt_{50} of the tolerant species. This experiment permitted us to compare if different physiological effects were incurred among the three species subjected to the same intensity of thermal stress (Table 1). For both experiments, we treated eight replicates, each consisting of a pool of 10 individuals for each exposure time point, and sampled the treated aphids at two time windows: 1) direct sampling (“D”) immediately upon exposure; 2) after 24 hours of recovery (“R”) at 20 °C. Only alive individuals were sampled and were immediately transferred to 2 mL microtubes, and snap-frozen in liquid nitrogen. All samples were then stored at -80 °C until being further processed. Eight biological replicates of ten aphids from the same cohort were promptly frozen and were used as control (“C”). The different experimental treatments of the first experiment corresponding to aphids exposed at 1/2 Lt_{50} , 2/2 Lt_{50} , and the corresponding recovering aphids, were subsequently named “1/2 D”, “2/2 D”, “1/2 R” and “2/2 R”. The different experimental treatments of the second experiment corresponding to aphids exposed to 100min, and the corresponding recovering aphids, were named “D” and “R”, respectively.

Table 1. The experimental conditions

Temperature	Species	Duration of recovery	Increasing injury level		Same exposure duration
			1/2 Lt_{50}	2/2 Lt_{50}	
34 °C	<i>M. dirhodum</i>	0h	63 min	125 min	100 min
	<i>S. avenae</i>	0h	188 min	375 min	100 min
	<i>R. padi</i>	0h	348 min	696 min	100 min
	<i>M. dirhodum</i>	24h	63 min	126 min	100 min
	<i>S. avenae</i>	24h	188 min	375 min	100 min
	<i>R. padi</i>	24h	348 min	696 min	100 min

GC-MS metabolic profiling

Each sample was weighed (Fresh mass, Mettler Toledo UMX2, accurate to 1 μg) before being processed. Samples were prepared as described in Thiébaud et al. (2021) and Baratange et al. (2022) with the following minor modifications. Briefly, after homogenization of the aphids in 400 μL of cold ($-20\text{ }^{\circ}\text{C}$) methanol: chloroform solution (2:1, v:v), followed by an addition of 266 μL of ultrapure water which allowed phase separation, 80 μL of the upper phase was pipetted and transferred into 2 mL glass vials. Then, samples were vacuum-dried (MiVac, Genevac, Ipswich, UK) for 50 min at room temperature. The dry residue was resuspended in 30 μL of 25 mg/mL methoxyamine hydrochloride in pyridine, before incubation under automatic orbital shaking at $40\text{ }^{\circ}\text{C}$ for 60 min. Then, a volume of 30 μL of N, O-bis (trimethylsilyl) trifluoroacetamide was added to each glass vial, and the derivatization was conducted at $40\text{ }^{\circ}\text{C}$ for 60 min under agitation. A CTC CombiPal autosampler (PAL System, CTC Analytics AG, Zwingen, Switzerland) was used, ensuring standardized and on-time sample preparation. Metabolites were separated, identified, and quantified with a 5977B GC/7890 MS platform (Agilent Technologies, CA, US). We used a 30 m fused silica column (HP-5MS Ultra Inert, 0.25mm, 0.25 μm 5%-phenyl-methylpolysiloxane) with helium as the carrier gas at a rate of 1 mL.min⁻¹. One microliter of each sample was injected into the GC/MS, as described in Thiébaud et al (2021). The temperature of the ion source was set at $230\text{ }^{\circ}\text{C}$ and the MS transfer line at $280\text{ }^{\circ}\text{C}$. The oven temperature ranged from 60 to $325\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$ and then remained at $325\text{ }^{\circ}\text{C}$ for 10 min. We completely randomized the injection order of the samples. Detection was achieved using MS detection in electron impact at 70 eV under the full-scan mode from 60 to 600 amu. The detection was achieved by scanning specific ions (1 quantifier and 2 qualifiers at a specific retention time) from 60 compounds. Calibration curves were computed by running each reference compound at 5, 7.5, 10, 20, 40, 80, 100, 200, 300, 400, and 500 μM concentrations, in addition to running three different levels of quality control after 80 samples. Chromatograms were deconvoluted using MassHunter B09.00 software, and metabolite levels were quantified using the quadratic calibration curve for each reference compound.

Statistical analysis

For the comparison of metabolic profiles among the different experimental groups, the main patterns and significance were searched by performing a between-class principal component analysis (PCA). This PCA focused on the differences among the classes defined as qualitative instrumental variables (Dolédec and Chessel 1987). A Monte Carlo test was then performed to examine the significance of the difference between the classes (Romesburg and Marshall 1985). All data were log-transformed before the PCA. All analyses were performed using the ‘ade4’ package in the statistical software RStudio Team (2020) (Dray and Siberchicot 2017).

In experiment 1, we examined the effects of treatment and injury level on the extracted PC1 using two-way ANOVA, including the interaction item, with treatment and injury level as categorical variables. Further, we split the data according to injury level and treatment and individually examine

their effect on PC1 using a one-way ANOVA, followed by Turkey's HSD *Post hoc* test. For the second experiment, the differences in PC2 among treatments were compared using a one-way ANOVA with Turkey's HSD *Post hoc* test.

The concentrations of individual metabolites were presented as fold-change relative to the control group (unexposed aphids). The fold-change of each metabolite relative to control was \log_{10} transformed in all statistical analyses to reduce residuals and minimize heteroscedasticity yet was presented as non-transformed relative values. The treated groups were compared to the control using student's t-test. The difference among treatments or species were examined by one-way ANOVA with Turkey's HSD *Post hoc* test. All statistical analyses were performed in the R software.

Results

Median lethal time (Lt_{50})

At 34 °C, *R. padi* survived better than *S. avenae* followed by *M. dirhodum* (Figure S1). Lt_{50} of the species ranked as follows: *R. padi* (696 ± 40 min) > *S. avenae* (375 ± 17 min) > *M. dirhodum* (125 ± 5 min).

Metabolic profiles

Among the 60 metabolites included in our library, 32 metabolites were accurately identified and quantified, and thus used for subsequent metabolic analyses. Among these metabolites, we found eighteen amino acids, four polyols, four carbohydrates, four metabolic intermediates, one amine, and one phosphorylated compound (see Table S1 for the list of detected compounds).

Effects of similar intensities of heat stress on metabolic profiles and recovering capacities

We characterized the system-wide changes in metabolic profiles of each aphid species using between-classes PCA. For the three aphid species, the treated groups separated from the controls (Monte Carlo test, $p < 0.05$). The impact of heat treatment on metabolic profiles was mainly explained by PC1, which accounted for approximately 50% of the total inertia (46.93%, 58.04%, and 52.5% for *M. dirhodum*, *S. avenae*, and *R. padi*, respectively, Figure 1A). Mannitol, glycine, alanine, inositol, and GABA were the most positively correlated metabolites to PC1 and were more abundant in heat-exposed ("D") aphids (Figure 1C). Conversely, concentrations of phenylamine, isoleucine, and trehalose were negatively correlated to PC1. PC2 opposed the two levels of heat stress intensity we used, i.e., 1/2 versus 2/2 in *M. dirhodum* and *S. avenae*, but separated the recovering group from the control and treated groups for *R. padi*. Some amino acids, in particular asparagine, proline, ornithine, isoleucine, phenylalanine, and valine, were negatively correlated to PC2 and more abundant in recovering specimens of *R. padi*. This axis accounted for 30.65%, 23.87%, and 28.38% of the total inertia for *M. dirhodum*, *S. avenae*, and *R. padi*, respectively (Figure 1A). The temporal changes of individual metabolites of aphids exposed to 1/2 and 2/2 Lt_{50} injury levels are presented in Figures S2 and S3, respectively.

For each experimental group, we extracted the PC1 component and examined the effects of experimental treatment and injury level on the metabolic profiles using two-way ANOVA, including the interaction terms (Figure 1B). The analysis revealed that PC1 was affected by the heat treatment for all aphid species, while the interaction of the terms was only significant for *M. dirhodum* and *S. avenae* (Table S2). To further assess the effects of experimental treatments (effects of heat exposure) and injury level (effects of duration of exposure) separately, we split the dataset by considering the injury level on the one hand, and the experimental treatment on the other hand, and computed one-way ANOVA followed by post hoc comparisons (Table S3). Heat exposure, regardless of the duration of exposure, resulted in significantly higher PC1 scores in comparison to control for the three aphid species ($p < 0.01$). The consideration of increased injury levels, i.e., increased duration of exposure at 34 °C, revealed the distinct degree of metabolic perturbations, as evidenced by higher PC1 scores at 2/2 Lt₅₀ in comparison to 1/2 Lt₅₀. After 24h of recovery, the metabolic profiles of aphids exposed to 1/2 Lt₅₀ were similar to the metabolic of control aphids. Conversely, aphids that experienced higher levels of injury (2/2 Lt₅₀) remained different from controls, except for *M. dirhodum*.

In the univariate analyses, it was found that 13, 21, and 18 metabolites significantly differed in specimens of *M. dirhodum*, *S. avenae*, and *R. padi*, respectively, when the insects were exposed to 1/2 Lt₅₀ and the metabolic profiles measured directly after heat exposure (1/2 D) in comparison to the controls (t-test, $p < 0.05$, Table S4). When the duration of heat exposure was increased to 2/2 Lt₅₀, the number of metabolites that significantly differed between treated and control aphids increased, reaching 17, 20, and 21 in *M. dirhodum*, *S. avenae*, and *R. padi*, respectively (t-test, $p < 0.05$, Table S4). The fold changes of 22 metabolites were significantly altered among the three aphid species, and they were ranked according to their fold change in Figure 2. Mannitol displayed the highest fold change in the three aphid species, and this metabolite was characterized by a significantly higher upregulation in *R. padi* (63-fold) as compared with *S. avenae* (39-fold) and *M. dirhodum* (12-fold) (Figure 2). Specimens of *R. padi* distinguished from the two other aphid species by being the only ones that exhibited a large (38-fold) increase in glycerol amounts. The results revealed that the fold changes of the other metabolites were relatively small, i.e., lower than 4-fold. Yet, it is also important to mention that some amino acids, such as glycine, serine, asparagine, lysine, valine, and proline, had increased concentrations in *R. padi* while they remained stable - or even decreased - in the other two aphid species. Also, *R. padi* and *S. avenae* displayed higher fold changes in metabolites involved in energy metabolism (lactic acid, alanine, citric acid) and putrescine in comparison to *M. dirhodum*.

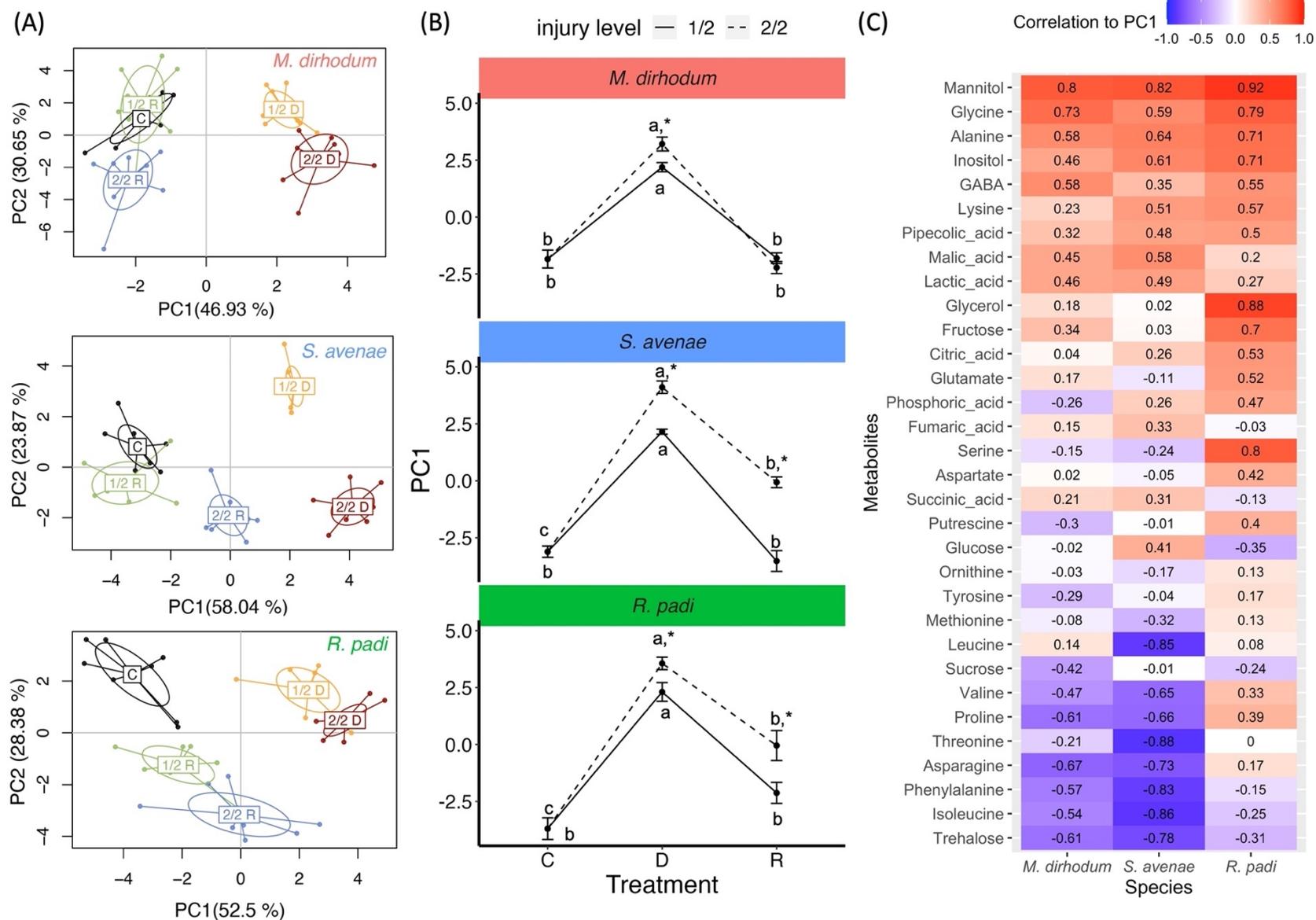


Figure 1. The principal component analysis (PCA) (Panel A), PC1 score for each species (Panel B), and heatmap of metabolites' correlations to PC1 (Panel C) in experiment 1. Panel A shows the projection of the experimental groups on PCA for *M. dirhodum* (top), *S. avenae* (middle), and *R. padi* (bottom). Experimental groups noted as 1/2 D and 1/2 R correspond to aphids, whose metabolic profiles were examined directly at the end of the heat exposure for a duration of 1/2 L_{t50} , or 24h after the heat exposure had stopped, respectively. Similarly, 2/2 D and 2/2 R correspond to aphids, whose metabolic profiles were examined directly at the end of the heat exposure for a duration of 2/2 L_{t50} , or 24h after the heat exposure had stopped, respectively. **Panel B** illustrates individual scores on PC1 according to experimental treatments and injury levels for *M. dirhodum* (top), *S. avenae* (middle), and *R. padi* (bottom). Treatments are control condition ("C"), sampled directly at the end of cold exposure ("D") and sampled after 24-hours of recovery at 20 °C ("R"). Injury levels were 1/2 L_{t50} ("1/2") and 2/2 L_{t50} ("2/2"). Values are mean scores \pm SE. The lowercase letter indicates the significant difference between treatments ($p < 0.05$). Asterisk indicates the significant difference between injury levels ($p < 0.05$). **Panel C** describes the correlation values of each metabolite to PC1 for each aphid species.

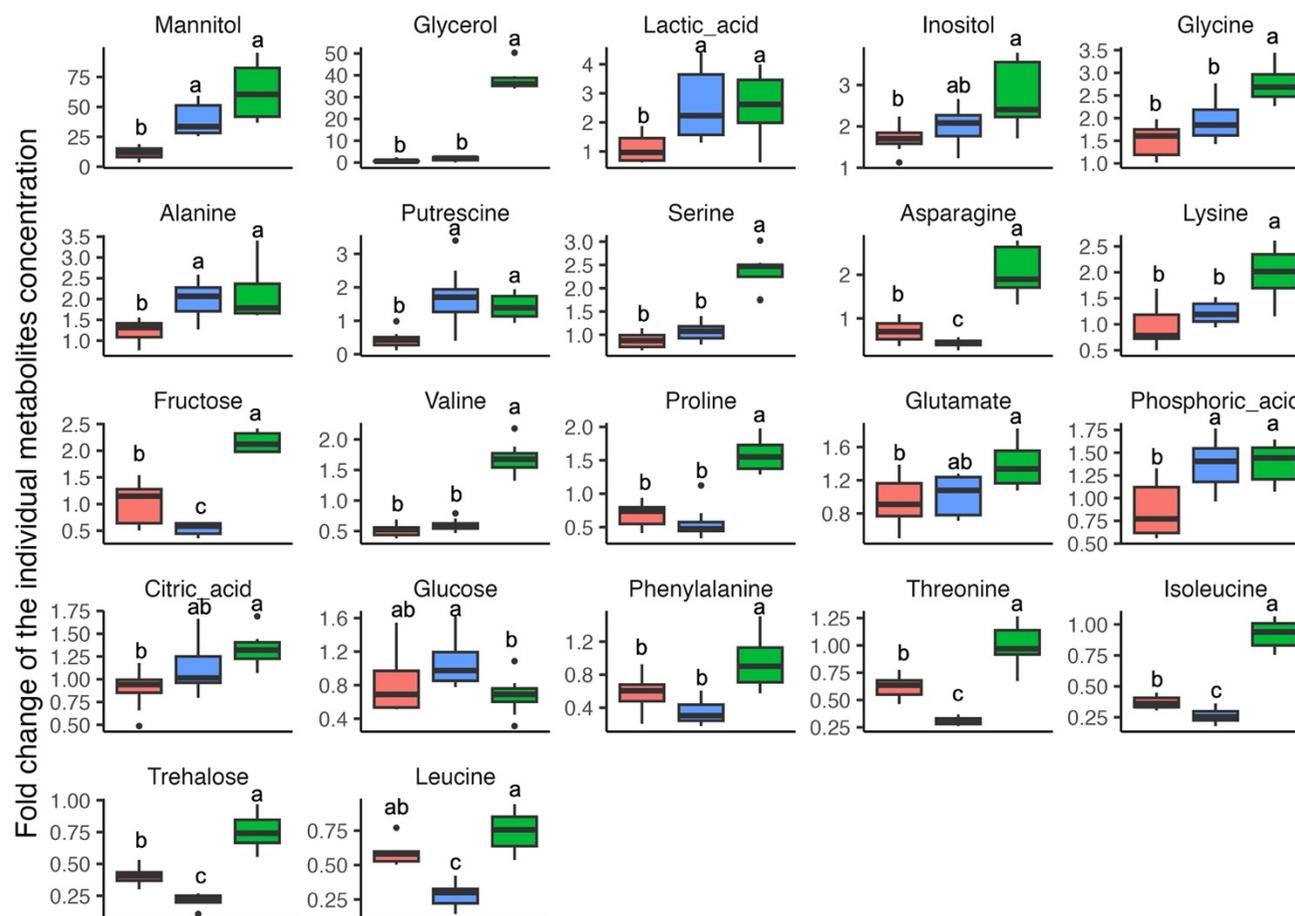


Figure 2. Fold changes relative to untreated control of the individual metabolite concentrations that significantly varied among heat-exposed aphids when they were sampled directly at the end of heat exposure corresponding to their 2/2 Lt_{50} values (“2/2D”). Only metabolites with a significant fold change are presented. The metabolites are ranked by decreasing fold change values. Values represent mean fold changes \pm SE. The lowercase letter indicates the significant level among species ($p < 0.05$).

Effects of identical duration of heat exposure on metabolic profiles

Between-classes PCA analysis showed that the first two principal components (PCs) cumulated 70% of the total inertia (Figure 3A). The differences among aphid species were partly explained by PC1, which accounted for 49.46% of the total inertia. Trehalose, proline, malic acid, and fructose were the metabolites most contributing to PC1 (Figure S4, correlation values to PC1 are shown in Figure S5). Along PC2, which accounted for 21.19% of the total inertia, heat-exposed aphids without recovery (“D”) were opposed to aphids that were allowed to recover for 24h (“R”) and to controls (“C”). For the three aphid species, the ellipses almost totally overlapped for “R” and “C” experimental treatments, thus depicting the existence of similar metabolic phenotypes.

PC2 scores of “D” treatment significantly differed from “R” and “C” treatments for *M. dirhodum* and *S. avenae*, but not for *R. padi* (Figure 3B). This result suggested that the metabolic profile of heat-exposed *R. padi* remained more similar to their relative controls compared with *M. dirhodum* and *S. avenae*. The metabolites that contributed the most to PC2 were asparagine and mannitol (Figure S4, see the correlation values in Figure S6).

The levels of trehalose dramatically decreased, while TCA intermediates such as citric acid, fumaric acid, malic acid, and succinic acid increased in *M. dirhodum* and *S. avenae* but remained stable in *R. padi* (Figure S7). *R. padi* showed higher fold change for glycerol and mannitol than the two other aphid species (Figure S7).

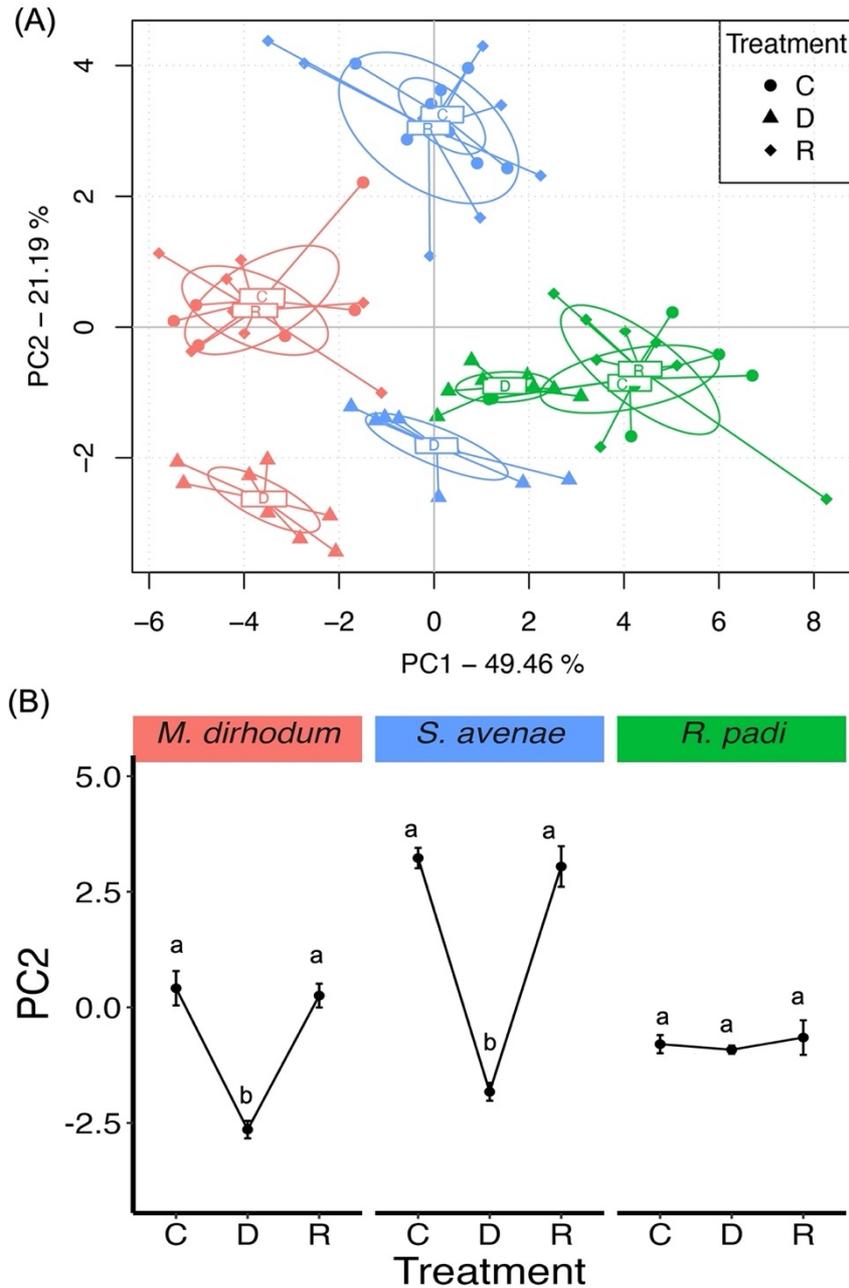


Figure 3. The principal component analysis (PCA) (Panel A) and PC2 scores (Panel B) on all the samples of experiment 2 (i.e., aphids exposed at 34 °C for the same exposure duration (100 min)). **Panel A** shows the PCA of treatments for *M. dirhodum* (red), *S. avenae* (blue), and *R. padi* (green). Treatments are control condition (“C”), sampled directly at the end of cold exposure (“D”) and sampled after 24-hours of recovery at 20 °C (“R”). **Panel B** describes projections of the treatments onto the second principal component plane (PC2) for each species. Values are mean scores \pm SE. The lowercase letter indicates the significant difference between treatments ($p < 0.05$).

Discussion

This study aimed to compare the sensitivity of three aphid populations from China by examining the impact of moderately hot temperatures on the metabolic profiles of the individuals. In a recent study, it has been reported that the three studied aphid species were characterized by significant differences in their ability to handle heat exposures (Li et al. 2023a, Li et al. 2023b). To improve our comprehension of the physiological underpinnings shaping the species-specific heat tolerance, and thus the ability of these insects to handle ongoing warming, we conducted two experiments: in experiment 1, aphids were exposed to high temperature (34 °C) for durations equivalent to 1/2 Lt_{50} or 2/2 Lt_{50} so that the amount of injury should be comparable among the species. By having two distinct durations of exposure at 34 °C, it has also been possible to examine the impacts increased amounts of injuries on metabolic profiles. Our findings demonstrated that the physiological homeostasis of aphids was disrupted during heat stress, and the degree of perturbation increased with increased duration of exposure. While the recovery period allowed the full restoration of the metabolic profiles in aphids subjected to 1/2 Lt_{50} , metabolic profiles remained different from controls for those aphids that experienced a higher injury level (2/2 Lt_{50}). In the second experiment, aphids were exposed to heat stress for the same duration; *R. padi* - the most heat tolerant aphid species (Li et al. 2023a) - displayed fewer metabolic perturbations and high polyol accumulation as compared with the two less heat tolerant aphid species.

Effects of similar intensities of heat stress on metabolic profiles and recovering capacities

The metabolic profiles of the three aphid species displayed significant deviations from the control when exposed to heat for a duration corresponding to 1/2 Lt_{50} and these differences became even more striking when the insects were exposed for a longer duration (Lt_{50}) at 34 °C, particularly in the case of *S. avenae*. These findings are in line with former works which showed that the insects gradually lose homeostatic balance when the duration and intensity of thermal stress were increased (Malmendal et al. 2006), with cascading consequences on a wide range of physiological functions (Neven 2000, Bowler 2018).

The present investigations revealed that energy metabolism was altered in heat-stressed aphids. Sucrose levels were decreased in the three aphid species, suggesting that the energy demand was increased during heat stress. Aphids primarily rely on sucrose for energy production, as this metabolite is particularly abundant in their natural diet made of plant phloem sap; sucrose allows the synthesis of lipids, amino acids, and proteins (Rhodes et al. 1996, Febvay et al. 1999). Aphids efficiently convert dietary sucrose into glucose and fructose, a reaction catalyzed by the enzyme sucrose (Hendrix and Salvucci 1998). In the heat-stressed aphids, TCA cycle intermediates, such as citric acid, fumaric acid, malic acid, and succinic acid also tended to increase, which may indicate higher activity of the TCA cycle. This assumption is consistent with the dramatic decrease in trehalose amounts, and the accumulation of lactic acid and alanine (the end products of anaerobic

metabolism), all suggesting that heat stress prompted carbohydrate mobilization and consumption through anaerobic pathways. The high energy demand posed by heat stress may indeed surpass the capacity of aerobic metabolism, requiring the activation of anaerobic metabolism as a complementary energy source to enhance insect survival at high temperatures near their upper lethal limit (Pörtner 2002). The activation of anaerobic metabolism has been frequently reported from cold-exposed insects (Boardman et al. 2016, Lubawy et al. 2022), but recent investigations have also indicated that anaerobic metabolism under heat stress in certain terrestrial insects (Ju et al. 2014, Zhu et al. 2019, Kuo et al. 2023). The notion that anaerobic metabolism is not prevalent in terrestrial insects due to their efficient oxygen transport through the open tracheal system has been debated (Gonzalez-Tokman et al. 2020). However, recent findings suggested that intracellular oxygen transport may be limited under high temperatures due to decreased oxygen affinity of respiratory proteins (Zhu et al. 2019). Therefore, further investigation is warranted to determine whether anaerobic metabolism during heat stress is a common phenomenon among terrestrial insects.

Metabolomics analysis confirmed the existence of interspecific differences in terms of thermal sensitivity among the three studied aphid species. Alterations of metabolic processes were more pronounced in *S. avenae*, with notable changes in trehalose, alanine, and lactic acid levels, as well as a significant increase in the concentrations of glucose and TCA intermediates such as fumaric acid, malic acid, and succinic acid. Similar patterns were reported by (Verberk et al. 2013), suggesting that anaerobic metabolism is more pronounced in *S. avenae*, and confirming the higher heat sensitivity of this species.

When exposed to relatively short durations of heat stress ($1/2$ Lt_{50}), the three aphid species were able to fully recover, as evidenced by metabolic profiles generally overlapping with those of control aphids. Conversely, when the duration of heat exposure was increased (equivalent to Lt_{50}), specimens of *M. dirhodum* were the only ones that fully recovered. Conversely, *S. avenae* was characterized by limited recovery capacities, with many metabolites from the TCA cycle remaining at higher levels than in controls. This pattern suggested that the experimental condition was stressful for *S. avenae*, likely inflicting physiological damages affecting energetic metabolism. Heat stress has been found to increase the permeability of membranes, causing leakage of K^+ from the cells into the hemolymph, and impairing neurophysiological function (O'Sullivan et al. 2017). Changes in the fluidity of the phospholipid bilayer of membranes may also affect the activity of membrane-bound enzymes (Neven 2000). The alteration of enzyme activity can disturb the respiratory chain and uncoupled oxidative phosphorylation in the mitochondrion, causing a drop in ATP level (Korsloot et al. 2004).

In *R. padi*, metabolic profiles also remained distinct from control aphids after the recovery period. This distinction was mainly due to the higher amounts of certain amino acids, including asparagine, proline, ornithine, isoleucine, phenylalanine, and valine. Our former study showed a significant upregulation of heat shock protein genes in individuals of *R. padi* in response to heat stress

(Li et al. 2023b). The synthesized heat shock proteins during the recovery period may contribute to explaining the high concentrations of these amino acids.

Heat-exposed *R. padi* also accumulated higher levels of polyols in comparison to the other two aphid species. Mannitol was identified as the main polyol in all three aphid species. Previous studies have reported the presence of mannitol in heat-exposed aphid species such as *Aphis gossypii*, *Acyrtosiphon pisum*, and *Schizaphis graminum* (Hendrix and Salvucci 1998, Burke et al. 2010, Saeidi et al. 2021), as well as in other insects (Huang et al. 2022). Six-carbon polyols, like mannitol, play a crucial role as osmotic pressure regulators, as they have limited ability to cross cell membranes easily (Garcia- Perez and Burg, 1991; Hendrix and Salvucci, 1998). Mannitol also functions as a scavenger of increased free radicals caused by exposure to heat, thus prolonging survival time during heat stress (Niu et al. 2003). Furthermore, polyols contribute to protein stabilization by strengthening the pairwise hydrophobic interaction between hydrophobic groups, thus protecting proteins from denaturation under heat stress (Back et al. 1979).

The accumulation of glycerol, another polyol, was observed in *R. padi* only. Glycerol is known to preserve cells from hyperthermic cell death and enhance the stability of thermolabile enzymes, preventing their permanent inactivation under unfavorable conditions (Henle and Warters 1982, Zancan and Sola-Penna 2005). Increased glycerol concentrations also play a role in decreasing the rate of water loss and enhancing dehydration tolerance (Yoder et al. 2006, Benoit et al. 2007). The specific accumulation of glycerol in *R. padi* may contribute to its ability to better withstand heat stress more efficiently. Overall, our results suggested that the accumulation of polyols in *R. padi* represents one of the physiological responses providing thermoprotection.

Heat stress was found to be associated with a general increase in the levels of free amino acids, such as glycine, lysine, and valine, particularly noticeable in *R. padi* (Figure 2). This increase is likely a result of protein breakdown and synthesis induced by the heat treatment. Our former study has shown that *R. padi* exhibited high expression of heat shock protein genes at 34 °C, especially *hsp70* (Li et al. 2023b). Importantly, HSP70 contains very rich glycine, glutamate, aspartate, lysine, phenylalanine, valine, leucine, and alanine (Tangchun et al. 1998).

Both *R. padi* and *S. avenae* exhibited higher levels of putrescine, a polyamine, whose concentrations are often increased in heat-exposed insects (Lima et al. 2022). Increased putrescine amounts represent a reliable indicator of exposure to environmental stress (Renaut et al. 2005). Putrescine has been found to regulate the synthesis of heat shock proteins (Königshofer and Lechner 2002), which aligns with the substantially increased expression of heat shock protein genes in aphids measured in a previous study (Li et al. 2023b). Putrescine may also protect the aphids by scavenging free radicals, enhancing the expression of oxidative stress defense genes (Tkachenko et al. 2001), and altering DNA topology in a manner that promotes survival (Tkachenko et al. 1998).

In contrast, *M. dirhodum* displayed fewer metabolite variations compared to the control group, and the lowest response in terms of polyol levels among the three aphid species. This finding

suggested that *M. dirhodum* had a limited capacity to respond to thermal stress and was in line with the rapid mortality observed in these insects when they were heat exposed.

Effects of identical duration of heat exposure on metabolic profiles

The interspecific differences in metabolic responses to heat stress were particularly apparent when the insects were exposed to 34 °C for the same duration. In *R. padi*, little changes in metabolic profiles were measured, confirming that the species was the most heat tolerant, and better able to maintain physiological homeostasis during heat stress. In contrast, the less tolerant species, i.e., *M. dirhodum* and *S. avenae* were characterized by increased concentrations of TCA cycle intermediates, in particular citric acid, fumaric acid, malic acid, succinic acid, a pattern which was not observed in *R. padi*. The main factors contributing to explain the higher concentrations of TCA cycle intermediates in heat-exposed aphids have been discussed above, and these findings confirmed that *R. padi* has higher upper thermal limits for energy metabolic reactions and/or better thermoprotection mechanisms, as also reported in Li et al. (2023) for the expression of HSPs. A similar pattern was formerly reported from *Drosophila species*, with tolerant species exhibiting more stable metabolic profiles during cold stress, while sensitive species underwent significant metabolic changes (Olsson et al. 2016).

As found in experiment 1, *S. avenae* exhibited greater variation in metabolic profiles during heat exposure, and insects from this population were able to fully recover after 24h (Figure 3B). This indicated again the high thermal sensitivity of the species, whose metabolism was altered even when the duration of heat exposure was moderate (less than 1/2 Lt_{50} for this species). The metabolic profiles suggested that specimens of *S. avenae* may have switched to anaerobic metabolism when they were heat-exposed, ensuring the production of a sufficient amount of ATP. The rapid increase in the amounts of polyols may also protect biomolecules and structures against heat injuries, as discussed above. We hypothesized that the quick response and adaptation to environmental temperatures, along with metabolic flexibility, may contribute to the widespread distribution of *S. avenae* (Wang et al. 2023). Contrary to our expectation, the least tolerant species, *M. dirhodum* was not characterized by large perturbations of the metabolic profiles when they were heat-exposed.

In experiment 2, *R. padi* was again characterized by accumulations of polyols (mannitol and glycerol). The absolute concentration of mannitol ranged from 10 - 40 nmol/mg, which is comparable to the mannitol levels reported from other aphid species during heat acclimation (Saeidi et al. 2021). The fold change of mannitol in the three species ranged from 10-20, indicating that the compound can be synthesized and accumulated rapidly, and likely play an important thermoprotective role (Gekko and Timasheff 1981, Henle and Warters 1982, Zancan and Sola-Penna 2005). These higher levels of polyols may be associated with heat tolerance, as the previous studies on *Drosophila species* with varying chill tolerance also revealed that chill-tolerant species have higher levels of 'cytoprotectants' such as trehalose and proline (Olsson et al. 2016).

Conclusions

In this study, we unraveled the physiological mechanisms that contribute to species-specific heat tolerance so that we could improve our comprehension of the factors shaping aphids in community abundance in the field in response to spikes of heat, which are being more and more frequent with climate change. Our findings revealed system-wide physiological disturbances in the least heat-tolerant species, whereas the most tolerant species was characterized by an accumulation of thermoprotective metabolites. Among the identified compounds, mannitol emerged as the primary polyol, which was accumulated in the response to heat stress across all three aphid species. Furthermore, the heat-tolerant species (*R. padi*) exhibited species-specific polyols, specifically glycerol, which may contribute to its superior heat tolerance. To the best of our knowledge, this study represents the first exploration of comparative metabolomics in insects specifically focused on the response to heat stress. Overall, our research sheds light on the underlying mechanisms governing the physiological responses of aphids to heat stress, thereby contributing to a better understanding of community dynamics under climate change from a physiological perspective.

Supporting information

Table S1: List of targeted and quantified metabolites in the metabolome of three aphid species, grouped by biochemical families. Metabolites that were quantified with GC-MS were indicated in bold.

Amino acids	Amines	TCA intermediates	Organic acids	Carbohydrates	Polyols	Phosphorylated compounds	Nucleobases
Alanine	Putrescine	Citric acid	Lactic acid	Fructose	Glycerol	Phosphoric acid	Cytosine
Glycine	Spermidine	Malic acid	Glyceric acid	Glucose	Mannitol	Glycerol-3P	
Isoleucine	Octopamine	Succinic acid	Quinin acid	Trehalose	Inositol	Gluconic lactone acid	
Leucine	Tyramine	Fumaric acid	Galacturonic acid	sucrose	Adonitol	Glucose-6P	
Proline	Ethanolamine		Pyruvic acid	Arabinose	Arabitol	Fructose-6P	
Valine	Dopamine			Galactose	Erythritol		
Phenylalanine	Triethanolamine			Mannose	Galactitol		
Asparagine	Cadaverine			Maltose	Sorbitol		
Tyrosine				Ribose	Xylitol		
Serine				xylose			
Threonine							
Methionine							
Lysine							
Aspartate							
Glutamate							
GABA							
Ornithine							
Pipecolic acid							

Table S2. Two-way ANOVA, based on the mean scores of the first principal component (PC1) of PCA on experiment 1, testing the effect of treatment (C, D, R) and injury level (1/2 and 2/2), in the three aphid species separately. Treatments are control condition (“C”), sampled directly at the end of cold exposure (“D”) and sampled after 24-hours of recovery at 20 °C (“R”). Injury levels were 1/2 Lt₅₀ (“1/2”) and 2/2 Lt₅₀ (“2/2”).

	Variable	<i>df</i>	F-value	P-value
<i>M. dirhodum</i>	PC1 scores			
	Treatment	2	176.94	< 0.001
	Injury level	1	0.85	0.36
	Treatment × Injury level	2	3.47	0.041
<i>S. avenae</i>	PC1 scores			
	Treatment	2	300.39	< 0.001
	Injury level	1	64.04	< 0.001
	Treatment × Injury level	2	20.39	< 0.001
<i>R. padi</i>	PC1 scores			
	Treatment	2	99.66	< 0.001
	Injury level	1	8.28	0.006
	Treatment × Injury level	2	2.45	0.104

Table S3. Comparison of the first principal component (PC1) scores among treatments and injury levels for each species. Treatments are control condition (“C”), sampled directly at the end of cold exposure (“D”) and sampled after 24-hours of recovery at 20 °C (“R”). Injury levels were 1/2 Lt₅₀ (“1/2”) and 2/2 Lt₅₀ (“2/2”). Pairwise p-values from one-way ANOVA with post hoc Tukey HSD tests. *p*-values < 0.05 are marked in bold. Light grey cells mark the comparisons between injury levels with the same treatment.

Species	Injury level	Treatment	1/2			2/2		
			C	D	R	C	D	R
<i>M. dirhodum</i>	1/2	C	<0.001	0.99	<0.001	0.014	0.26	
		D						
		R						
	2/2	C	<0.001	0.69	<0.001	<0.001	<0.001	
		D						
		R						
<i>S. avenae</i>	1/2	C	<0.001	0.59	<0.001	<0.001	<0.001	
		D						
		R						
	2/2	C	<0.001	<0.001	<0.001	<0.001	<0.001	
		D						
		R						
<i>R. padi</i>	1/2	C	<0.001	0.056	<0.001	0.023	0.021	
		D						
		R						
	2/2	C	<0.001	<0.001	<0.001	<0.001	<0.001	
		D						
		R						

Table S4. Metabolites that showed significant changes from the control in the heat-treated aphids (“1/2 D” and “2/2 D”) of experiment 1. The heat-treated groups were compared to the control using t-tests.

Metabolites	1/2 D			2/2 D		
	<i>R. padi</i>	<i>S. avenae</i>	<i>M. dirhodum</i>	<i>R. padi</i>	<i>S. avenae</i>	<i>M. dirhodum</i>
Alanine	p < 0.05	0.1034	p < 0.05	p < 0.05	p < 0.05	0.0866
Asparagine	0.7269	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Aspartate	p < 0.05	p < 0.05	0.3442	0.5084	0.0824	0.2335
Citric_acid	p < 0.05	0.2245	0.1212	p < 0.05	0.3477	0.209
Fructose	p < 0.05	p < 0.05	0.6267	p < 0.05	p < 0.05	0.774
Fumaric_acid	0.2115	0.1031	0.9532	0.2341	0.2967	0.9889
GABA	p < 0.05	0.7509	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Glucose	p < 0.05	p < 0.05	0.5852	p < 0.05	0.7447	0.0872
Glutamate	p < 0.05	0.2751	0.1489	p < 0.05	0.9872	0.4643
Glycerol	p < 0.05	p < 0.05	p < 0.05	p < 0.05	0.977	p < 0.05
Glycine	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Inositol	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Isoleucine	0.1976	p < 0.05	p < 0.05	0.0791	p < 0.05	p < 0.05
Lactic_acid	0.3483	0.1335	0.3071	p < 0.05	p < 0.05	0.8971
Leucine	0.8902	p < 0.05	0.1507	p < 0.05	p < 0.05	p < 0.05
Lysine	p < 0.05	0.7341	0.7377	p < 0.05	p < 0.05	0.4325
Malic_acid	0.1484	p < 0.05	p < 0.05	0.088	p < 0.05	0.2417
Mannitol	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Methionine	0.2062	p < 0.05	0.6151	0.7906	0.2381	0.1234
Ornithine	0.9374	0.1385	0.7634	0.12	0.3497	0.1677
Phenylalanine	0.5657	p < 0.05	p < 0.05	0.4935	p < 0.05	p < 0.05
Phosphoric_acid	p < 0.05	0.6275	0.9149	p < 0.05	p < 0.05	0.1422
Pipecolic_acid	0.2488	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Proline	p < 0.05	p < 0.05	0.1744	p < 0.05	p < 0.05	p < 0.05
Putrescine	p < 0.05	p < 0.05	p < 0.05	p < 0.05	0.1271	p < 0.05
Serine	p < 0.05	p < 0.05	0.2928	p < 0.05	0.5264	0.0662
Succinic_acid	0.3818	0.7508	0.1834	0.2327	p < 0.05	0.7104
Sucrose	p < 0.05	0.1948	0.0872	0.2517	0.187	p < 0.05
Threonine	0.1914	p < 0.05	0.7041	0.8523	p < 0.05	p < 0.05
Trehalose	0.7623	p < 0.05	0.2613	p < 0.05	p < 0.05	p < 0.05
Tyrosine	0.6267	p < 0.05	0.5199	0.4203	0.3662	p < 0.05
Valine	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05

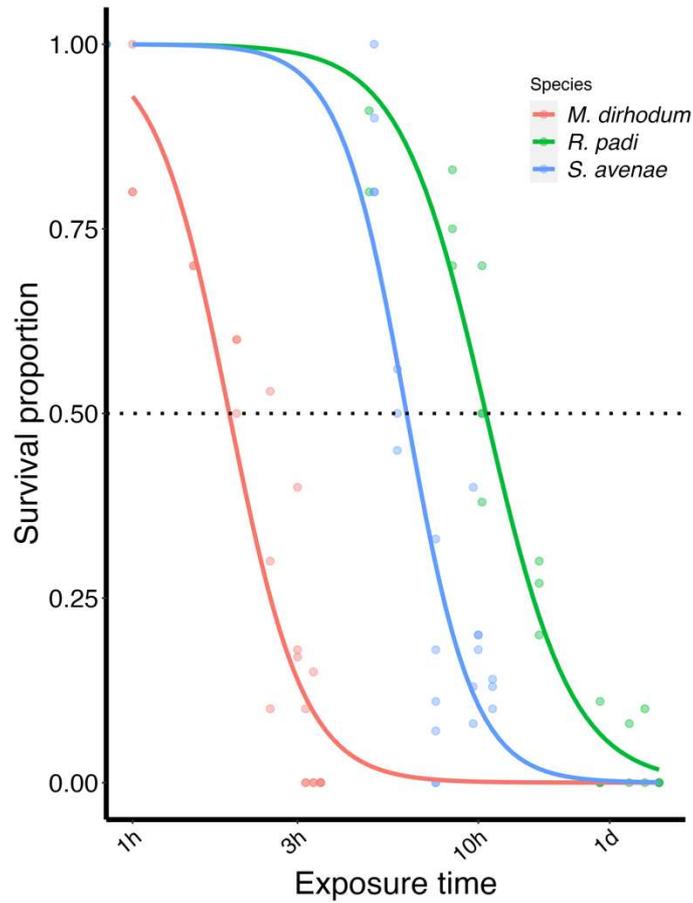


Figure S1. Survival curves at 34 °C for the three aphid species (*M. dirhodum*, *S. avenae*, and *R. padi*). Survival curves were fitted by a generalized linear model (GLM) with a logit link function. Each point represented the survival proportion of aphids after the given exposure times in a single replicate. The black dashed line indicated the 50% survival proportion thus the x-axis of intersections of this dashed line and survival curves were the Lt_{50} estimates of each species.

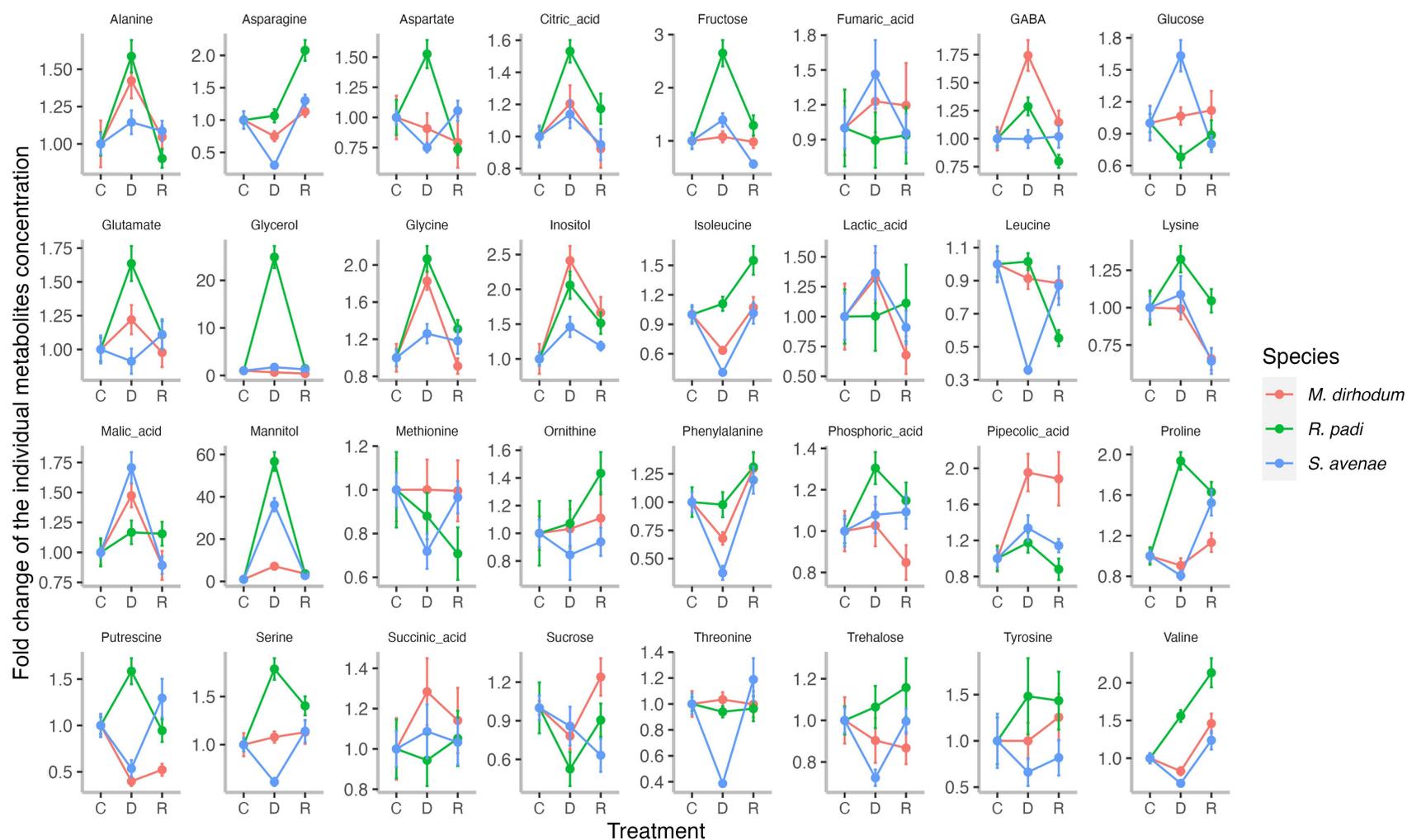


Figure S2. Temporal variations of individual metabolites for treatments at 1/2 injury level. C, D, and R represented the unexposed control, aphids sampled directly at the end of heat exposure and aphids recovered for 24h at 20 °C after heat exposure, respectively. Values were mean \pm SE (n = 8).

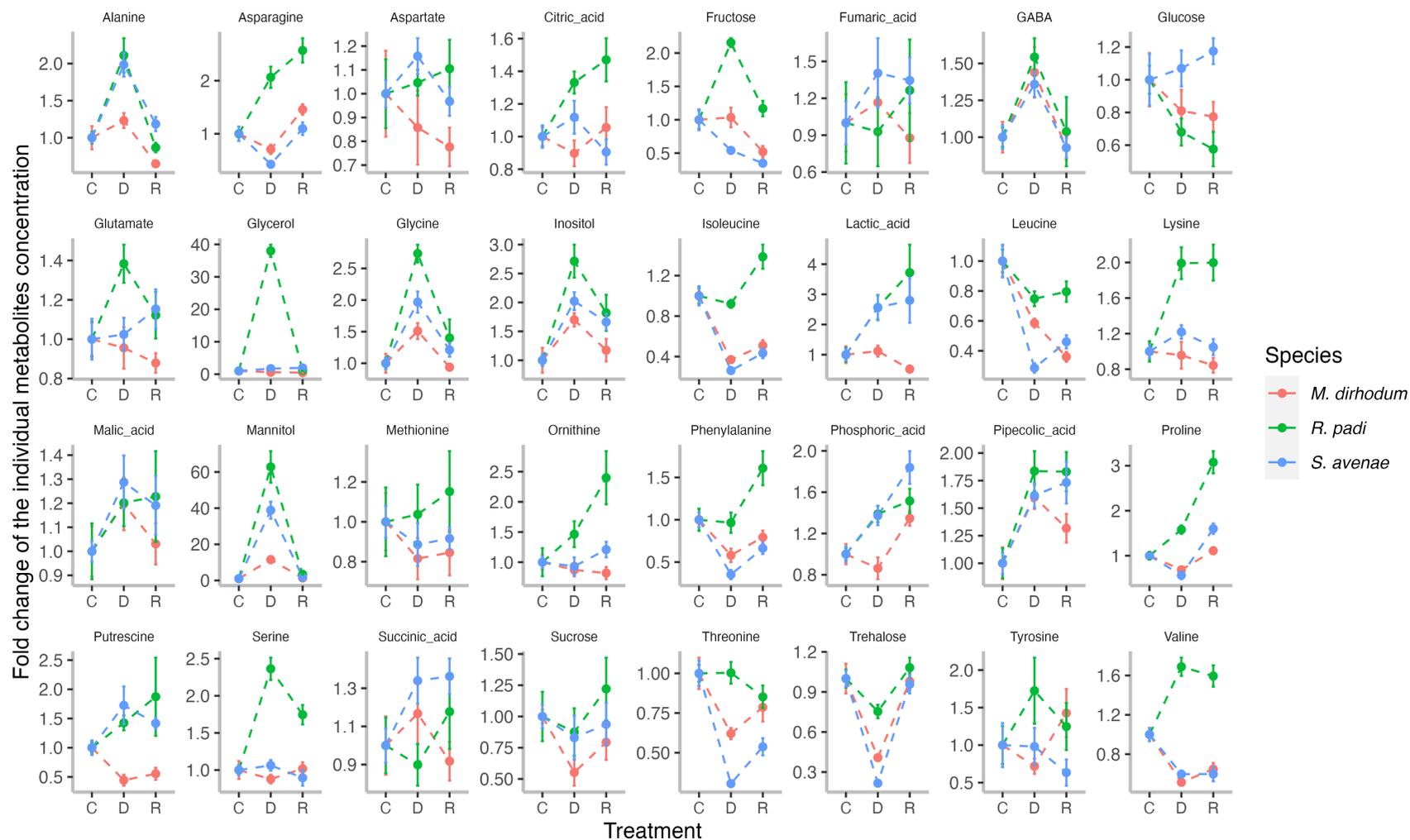


Figure S3. Temporal variations of individual metabolites for treatments at 2/2 injury level. C, D, and R represented the unexposed control, aphids sampled directly at the end of heat exposure and aphids recovered for 24h at 20 °C after heat exposure, respectively. Values were mean \pm SE (n = 8).

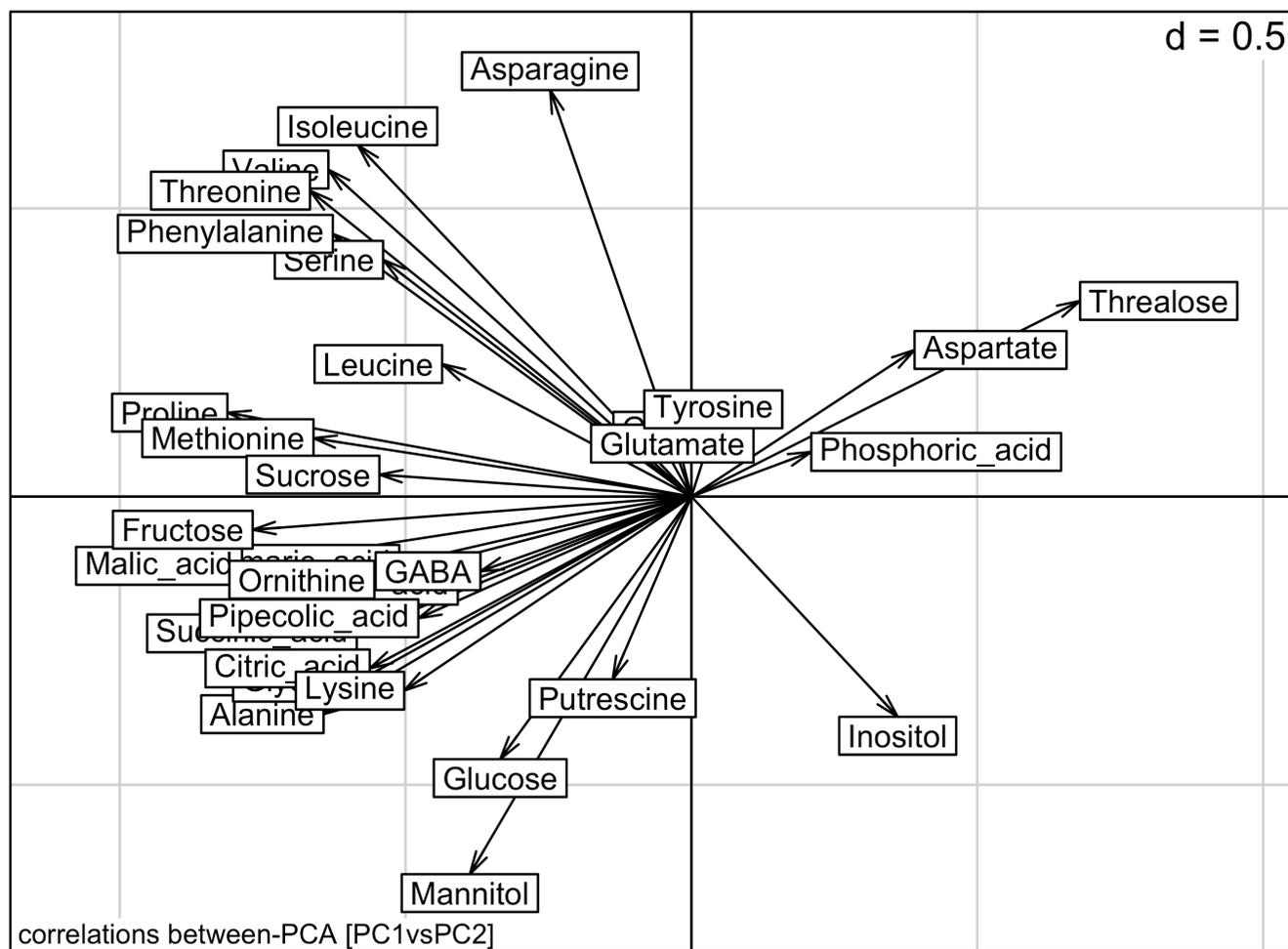


Figure S4. The projections of all metabolites on the first two PCs of PCA on the samples from experiment 2 (i.e., aphids exposed at 34 °C for the same exposure duration (100 min)).

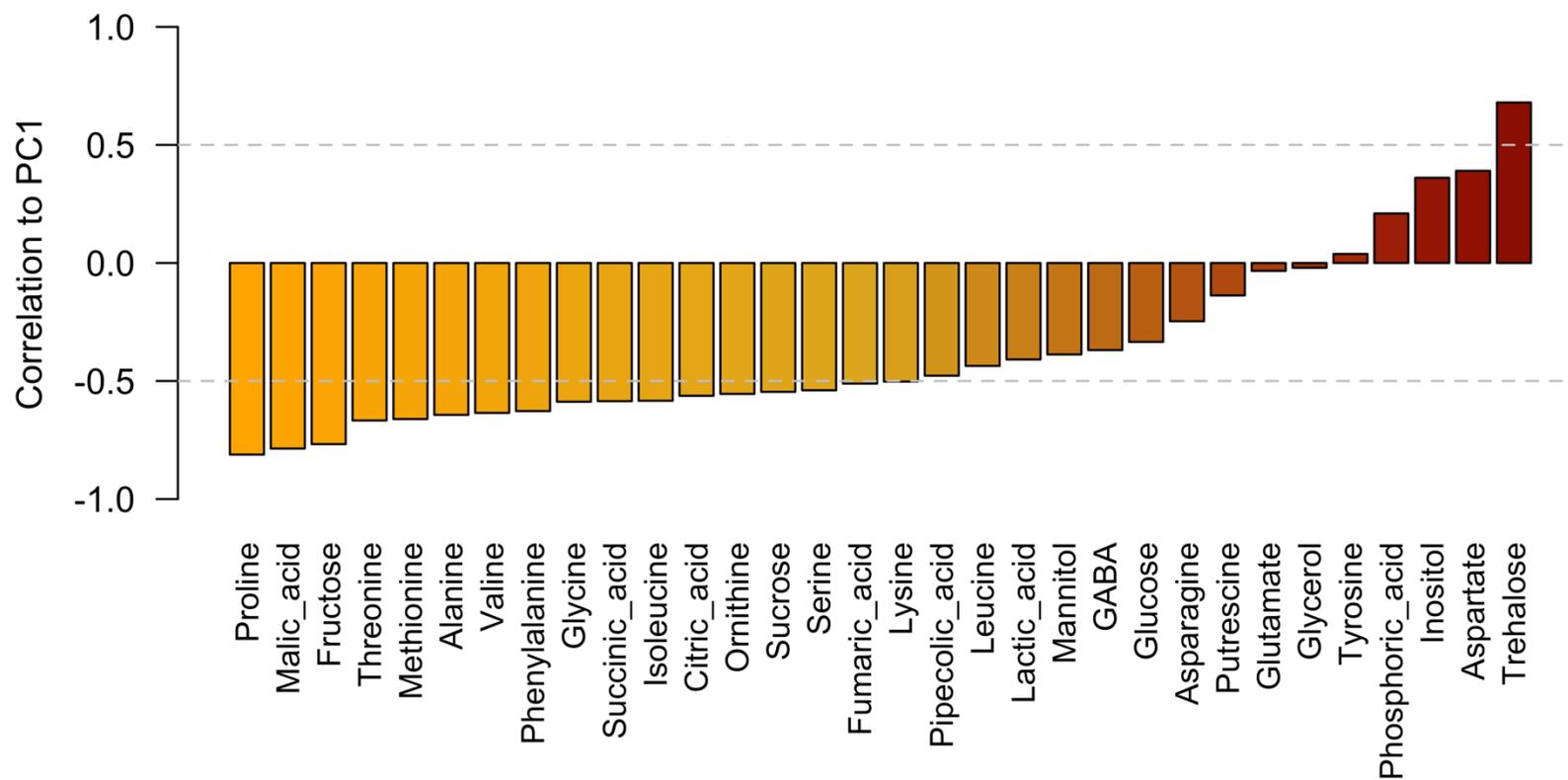


Figure S5. Correlation values of the different metabolites to the first principal component (PC1) of the PCA for experiment 2.

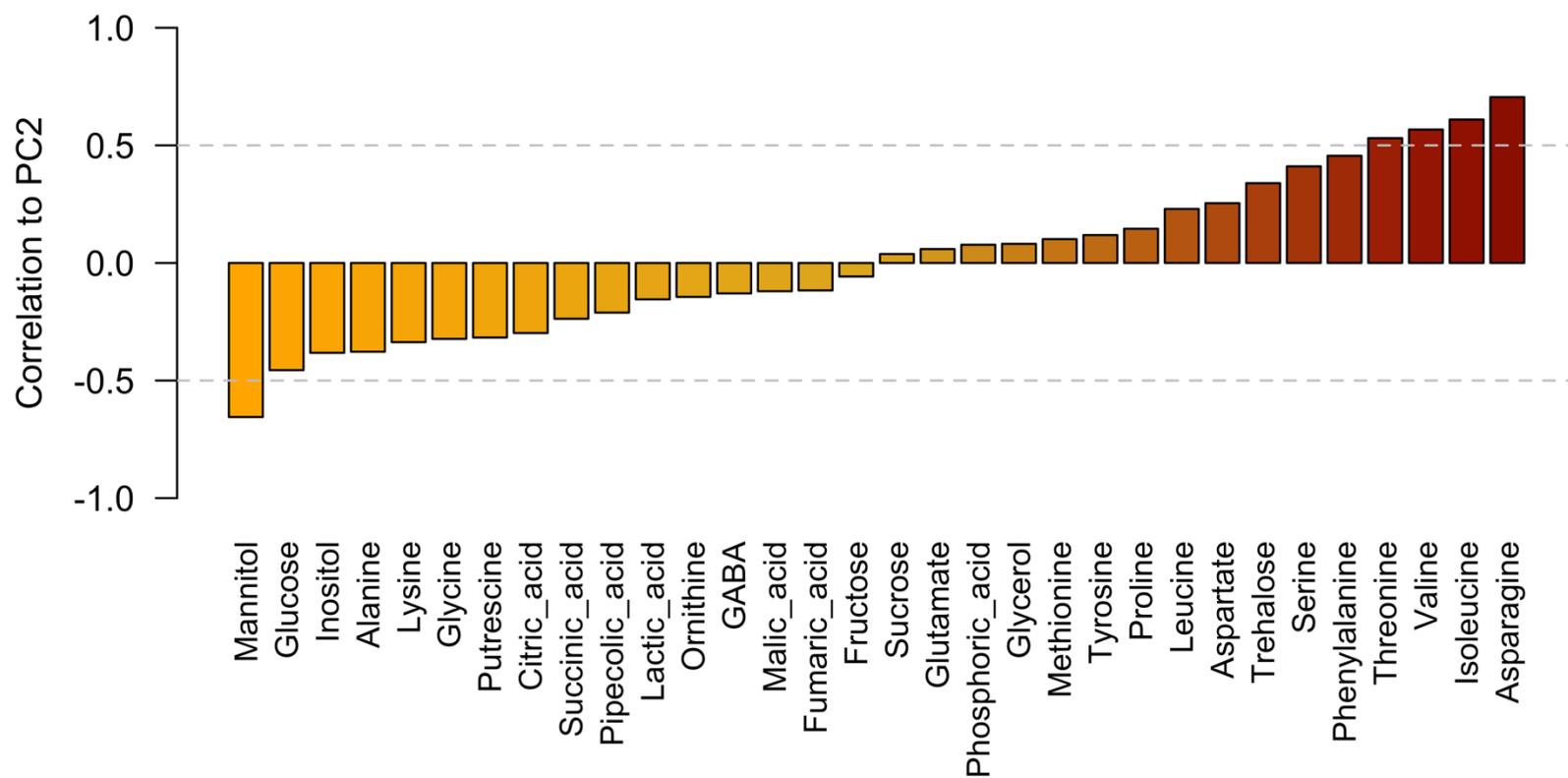


Figure S6. Correlation values of the different metabolites to the second principal component (PC2) of the PCA for experiment 2.

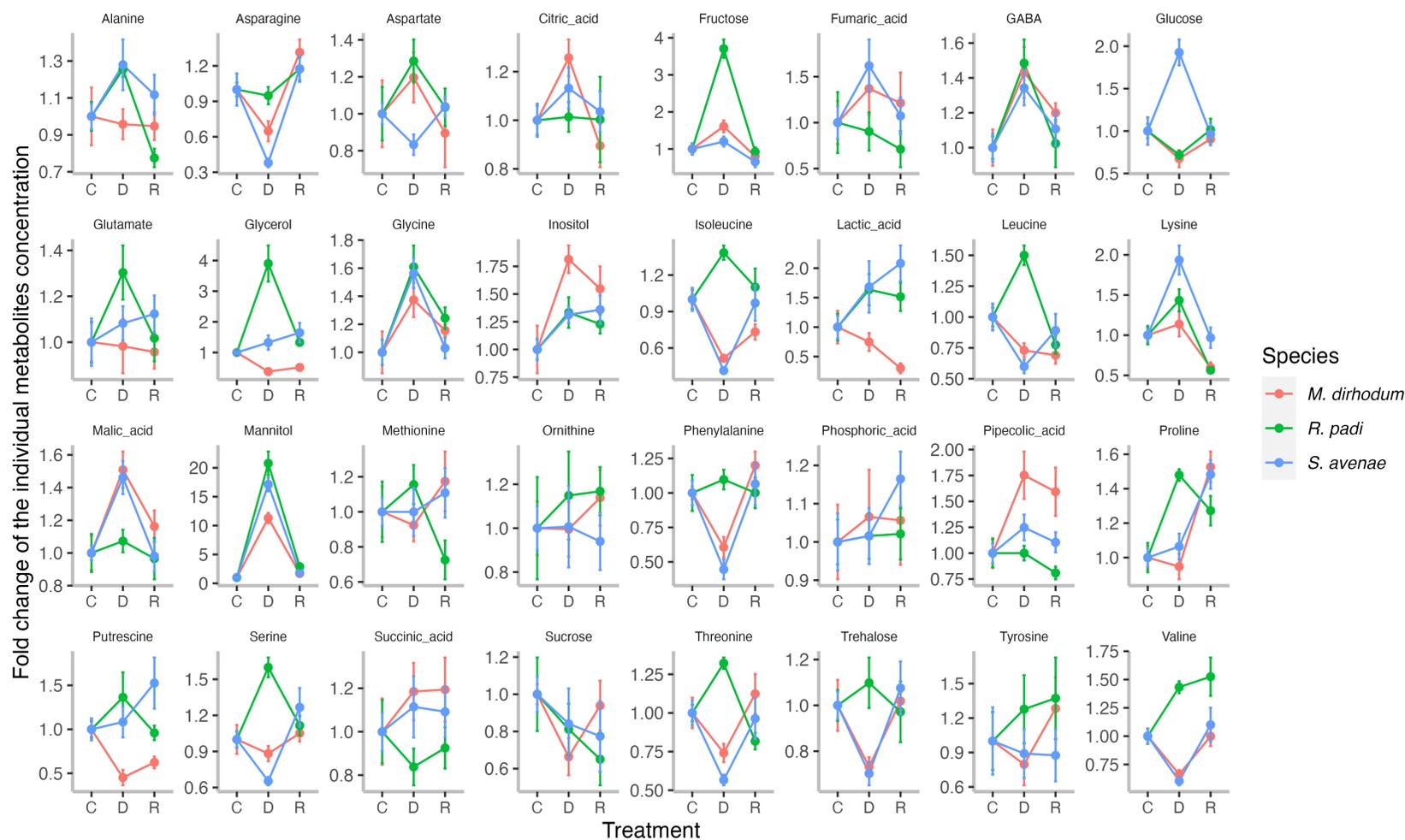


Figure S7. Temporal variations of individual metabolites for treatments of experiment 2, i.e., aphids exposed at 34 °C for the same exposure duration (100 min). C, D, and R represented the unexposed control, aphids sampled directly at the end of heat exposure and aphids recovered for 24h at 20 °C after heat exposure, respectively. Values were mean \pm SE (n = 8).

Chapter 5 Comparative metabolic response to cold stress

Article 4. Metabolic responses to low temperature in aphid species

We have previously shown significant interspecific variations in cold tolerances and molecular responses among the three aphid species (*Metopolophium dirhodum*, *Sitobion avenae*, and *Rhopalosiphum padi*). However, it remains unclear whether the variations in cold tolerance arise from different metabolic responses to cold stress. We conducted a comparative metabolomic analysis using GC-MS on the three aphid species to gain insights into their ability to withstand and recover from cold stress. Specifically, we exposed aphids to a low temperature (-5 °C) for progressively longer periods of time corresponding to 1/2 Lt_{50} (median lethal time) and 2/2 Lt_{50} , as well as the same exposure time.

We hypothesized that:

- 1) Aphids exposed to cold stress are expected to exhibit metabolic disorders compared to control conditions. The severity of these metabolic disorders is predicted to vary according to the stress levels, with longer durations of stress (i.e., higher injury level) resulting in increasingly challenging recovery.
- 2) When exposed to the same duration of cold stress, the tolerant species is expected to show fewer metabolic perturbations and more efficient restoration of homeostasis during recovery than the intolerant species.
- 3) Aphids are expected to accumulate cryoprotectants in response to cold stress. Specifically, trehalose and some amino acids (e.g., proline) may upregulate in response to cold stress.

Main findings:

- 1) Cold stress significantly disturbed the metabolic homeostasis of aphids, with more severe perturbations as the exposure duration lengthened.
- 2) The cold-tolerant species (*M. dirhodum* and *S. avenae*) exhibited similar metabolite profiling during stress, whereas cold-susceptible species (*R. padi*) displayed distinct patterns. The cold-tolerant species exhibited increased levels of cryoprotectants (e.g., trehalose, proline, and asparagine) after recovery from cold stress.
- 3) When exposed to the same duration of cold stress, the cold-susceptible species displayed a higher number of altered metabolites compared to the other two species, which suggested that this species had a poorer ability to maintain metabolic homeostasis, particularly in energy metabolism.
- 4) Generally, cold tolerance was associated with metabolic inertia/robustness.

Metabolic responses to low temperature in aphid species

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Abstract: Most aphids, being chill-susceptible, rely on their ability to maintain physiological homeostasis to determine their cold tolerance at low temperatures. Variations in cold tolerance among species might be manifested by adaptive differences in physiological processes such as osmoregulatory capacity. However, it remains unknown whether variations in cold tolerance are associated with species-specific metabolic responses to cold stress and/or differential mobilization of cryoprotectants. To address this gap, we conducted a comparative metabolomic analysis using GC-MS on three aphid species with varying cold tolerance (*Metopolophium dirhodum*, *Sitobion avenae*, and *Rhopalosiphum padi*) to gain insights into their ability to withstand and recover from cold stress. In our study, we subjected aphids to two different treatments: exposure to a cold temperature (-5 °C) for increasing durations corresponding to 1/2 Lt₅₀ (median lethal time) and 2/2 Lt₅₀, as well as the same exposure time. Results revealed significant disturbances in the metabolotypes of the aphids, with more severe perturbations observed as the exposure duration lengthened. Cold-tolerant species exhibited similar metabolite profiling during stress, whereas cold-susceptible species displayed distinct patterns. The cold-tolerant species increased levels of cryoprotectants (e.g., trehalose, proline, and asparagine) after recovery. Furthermore, when exposed to the same duration of cold stress, the cold-susceptible species displayed a higher number of altered metabolites compared to the other two species, which suggests that this species has a poorer ability to maintain metabolic homeostasis, particularly in energy metabolism. Generally, cold tolerance is associated with metabolic inertia/robustness. In summary, this research provides insights into the metabolic responses of aphid species to cold stress, emphasizing its impact on their physiological processes. Additionally, it sheds light on the specific biochemical compounds associated with cold tolerance and susceptibility.

Keywords: aphids, cold adaptation, cold tolerance, cryoprotectants, metabolic response.

Introduction

Temperature is perhaps the most important environmental variable affecting insects (Skendzic et al. 2021). It directly influences development, reproduction, activity, and survival (Angilletta 2009), as well as their spatial and temporal distribution patterns (Sunday et al. 2012, Warren and Chick 2013). Understanding how insects respond to cold is of utmost importance, as their winter survival plays a crucial role in sustaining ecosystem resilience and ensuring food security for numerous species. (Bale and Hayward 2010). Moreover, studying cold stress response in insects is particularly intriguing because the available timeline to examine stress response pathways is greatly extended compared to studying responses to heat.

In almost all environments, the temperature fluctuates across daily and seasonal timescales, which becomes challenging to insects when thermal conditions extend beyond an individual's 'favorable' range (Colinet et al. 2015). As with all ectotherms, adaptation to variable thermal environments has exerted strong selection pressure, and insects have been proven remarkably successful in coping with this challenge, evolving a complex array of highly plastic responses (Chown and Nicolson 2004, Danks 2006). In response to, or in anticipation of cold (e.g., winter), insects often modulate their metabolic fluxes to regulate their metabolism and energy reserves (Bale and Hayward 2010, Hahn and Denlinger 2011, Teets et al. 2019). Moreover, they often accumulate compatible solutes referred to as cryo/osmoprotectants (Hayward and Colinet 2023). Sugars, polyols, and various amino acids are the main cryoprotectants, which, when accumulated in relatively high amounts, have colligative effects, lowering the supercooling point of their body fluids (Teets and Denlinger 2013). Cryoprotectants also possess stabilizing and protective properties at low concentrations (Yancey 2005, Teets and Denlinger 2013, Kostal et al. 2016, Toxopeus et al. 2019). Indeed, at low concentrations, these cryoprotectants may protect the native macromolecular structures, such as protein and biological membranes by preferential exclusion from their hydration shells, ultimately promoting survival (Arakawa and Timasheff 1985, Timasheff 1992, Košťál et al. 2001, Grgac et al. 2022). Membranes can be protected through homeoviscous adaptation (i.e., remodeling of phospholipids) or by utilizing protective/stabilizing cryoprotectants (Hazel 1995, Hayward et al. 2014, Colinet et al. 2016, Enriquez and Colinet 2019). All these coordinated changes produce systematic remodeling of biomolecules, whose concentrations vary in space (e.g., cells and tissues) and time (Hayward and Colinet 2023). These biochemical changes in cold-tolerant phenotypes can be assessed with metabolomics (including lipidomics). Of course, overwintering insects are taxonomically diverse, and thus, the biochemical adjustments in response to cold also vary among species (Olsson et al. 2016).

Metabolomics can be useful to monitor metabolic trajectories under stress. Studies have found that cold stress generally induces large metabolic disturbance and that cold tolerance, mediated by plasticity or genetic adaptation, is usually associated with metabolic inertia/robustness during both

stress and recovery (Colinet et al. 2012a, Williams et al. 2014, Enriquez et al. 2018, Stetina et al. 2019)). Using metabolomics and lipidomics, a remarkable resemblance in the time-course changes between metabolic and phospholipids networks was found in *Drosophila* flies subjected to thermal fluctuations, suggesting that insects have a very rapid capacity for homeostatic regeneration when cold stress ends (Colinet et al. 2016). Similar rapid metabolic adjustments (i.e., environment-related physiological fine-tuning) also occur in the field with daily thermal variations, as found in the Arctic seed bug (*Nysius groenlandicus*) (Noer et al. 2022). As a result, metabolomics is a useful comparative tool for assessing the rapid perturbations caused by cold stress and the associated homeostatic responses in various species or phenotypes (Hayward and Colinet 2023).

Aphids are a major pest of crops worldwide. Aphids generally utilize two distinct overwintering strategies: cold-hardy eggs and parthenogenetic active females (Van Emden and Harrington 2017). In regions characterized by mild winter temperatures (such as Western Europe or some parts of Asia), the absence of lethal frosts allows aphids to remain active and reproduce parthenogenetically throughout winter (Alford et al. 2014). Sexual lineages predominate in regions with cold winters because of the ecological advantage conferred by overwintering eggs, whereas essentially asexual lineages predominate in regions with mild winters, which support higher fecundity and fitness (Peng et al. 2017).

Wheat plants and other closely related cereals are cultivated all around the world. Aphids are among the most destructive insect pests of cereal crops in temperate regions (Dedryver et al. 2010, Alford et al. 2016). The guild of cereal aphids is very similar in all the parts of the world where cereals are cultivated. It includes *Sitobion avenae* (English grain aphid), *Metopolophium dirhodum* (Rose grain aphid), and *Rhopalosiphum padi* (Bird cherry-oat aphid), which are all globally distributed (Jarošík et al. 2003, Yang et al. 2017, Alford et al. 2018). In China, these species coexist in most wheat-growing regions, but their relative abundance varies across different geographical areas (Hu et al. 2022). *Metopolophium dirhodum* thrives in cooler regions, dominating the west-northern and alpine areas, including the Tibetan plateau (Hu and Gui 1985, Lei et al. 2021), while *R. padi* predominates in warmer regions characterized by high temperatures and humidity, such as the southern regions (Hu et al. 2022). In temperate regions below the latitude 35 degrees of China, all three aphid species can overwinter as nymphs and adults, seeking refuge in the base of wheat stems or soil crevices (Peng et al. 2017, Xu et al. 2020). However, as latitude increases and winters become more severe, only *M. dirhodum* sustains parthenogenetic reproduction (Hu et al. 1995). Remarkably, despite their relatively small population size, *M. dirhodum* exhibits the highest abundance on winter wheat in China (Hu et al. 1995). These species-specific variations in distribution and overwintering strategies suggest differences in cold tolerance among these three aphid species (Li et al. 2023a, Li et al. 2023b).

Most aphids are chill-susceptible as they die at temperatures well above the freezing temperature of their body fluids (Knight and Bale 1986b, Worland et al. 2010). Therefore, their ability

to maintain physiological homeostasis at low temperatures, rather than their ability to resist or tolerate freezing, determines their cold tolerance. Our previous study has shown differential molecular stress responses, with most cold-tolerant species showing more cold-inducible heat shock protein genes (*hsps*) during recovery from cold stress and the least cold-tolerant species showing higher expression levels of *hsps* (Li et al. 2023b). Yet, whether the difference in cold tolerance is associated with species-specific metabolic response to cold stress and/or with differential mobilization of cryoprotectants remained to be explored.

In the present study, we conducted a comparative metabolomic analysis using gas chromatography-mass spectrometry (GC-MS) on the three aphid species (*M. dirhodum*, *S. avenae*, and *R. padi*) to gain insights into their ability to withstand and recover from cold stress. We conducted two experiments: 1) similar and increasing injury levels; To do so, we exposed aphids to durations equivalent to 1/2 and 2/2 of the species-specific Lt_{50} (Median lethal time) values followed by a 24h recovery period, and 2) the same exposure time; To do so, we exposed aphids to the same cold stress duration followed by a 24h recovery period.

We made the following assumptions:

- 1) Stress-specific metabolic disorder. We hypothesized that metabolic alterations triggered by cold stress would vary according to the cold injury level across all aphid species, with a longer/stronger cold stress (i.e., 2/2 Lt_{50}) producing more severe metabolic perturbation than shorter cold stress (i.e., 1/2 Lt_{50}).
- 2) Differential homeostatic response among species with similar cold stress durations. We expect that the metabolic trajectory of the most tolerant species would deviate comparatively less with cold stress than the susceptible species, and we expect they also would be able to restore metabolic homeostasis faster than the susceptible species.
- 3) Maintaining cellular energy homeostasis is a challenge for insects that are exposed to low stressful temperatures (Colinet et al. 2017, Lubawy et al. 2022). Thus, we expect cold stress would result in altered levels of intermediary metabolites involved in energy production.
- 4) We expect cryoprotectants, such as trehalose, polyols, and proline to accumulate in response to cold stress. The tolerant species may have higher fold changes of these cryoprotective molecules.

Material and Methods

Aphid rearing

We conducted our experiments on lab populations of three common aphid species, *Sitobion avenae* (English grain aphid), *Metopolophium dirhodum* (Rose grain aphid), and *Rhopalosiphum padi* (Bird cherry-oat aphid). The original populations were derived from the wild populations collected in May 2010 from wheat fields near Beijing (39°48' N, 116°28' E) (Zhao et al. 2014). Since then, the aphids were reared 10 – 20 cm tall winter wheat seedlings at 20 ± 0.5 °C, 70% relative humidity, and a photoperiod of 16:8 h (Light: Darkness). The colonies were refreshed annually with field-collected

aphids. We used the same age (6-day-old) aphids for the experiments. They were obtained by allowing newborn nymphs to develop for six days on the winter wheat seedlings.

Median lethal time (Lt_{50})

In a former study, we examined the sensitivity of the three aphid populations to cold exposure by using 6-day-old specimens of *M. dirhodum*, *S. avenae*, *R. padi* exposed at several low temperatures ranging from -11 to -3 °C, and for several different exposure durations (Li et al. 2023a). The experimental design allowed us to obtain survival ranging from 100 to 0% for the three populations, and to further compute median lethal times (Lt_{50}) across a wide range of low temperatures for each species. This former investigation demonstrated the large difference in the level of cold tolerance that the three aphid species can handle, particularly concerning Lt_{50} . Here, we chose -5 °C because it is a temperature that is frequently encountered by aphids in most temperate regions during the winter period in the field. Second, former studies evidenced noticeable interspecific differences in cold responses at this temperature, in particular for Lt_{50} , making it convenient to discern variations in metabolic activity among different species (Li et al. 2023a, Li et al. 2023b).

In the former study, we measured the survival curve and estimated Lt_{50} at -5 °C using the following protocol. Aphids were exposed to -5 °C for at least five different exposure durations, which resulted survival rate ranging from 100 to 0%. For each exposure time point, around 10 aphids were tested. To reduce handling stress, and provide the individuals with food as well as water, wheat leaves with aphids were cut and transferred into glass vials (10 mL), which were sealed with a sponge stopper to limit the air circulation and maintain a stable internal environment. The vials were then mounted in a rack, which was merged into a glycol bath (Ministat 230-cc-NR; Huber Ltd., Germany, accuracy ± 0.01 °C) pre-set at -5 °C. After the exposure, test aphids were transferred to a climatic chamber at 20 °C to recover for 24h before checking the survival of each individual. Aphids were considered alive if the individual moved following a gentle tactile stimulation with a brush. We modeled the survival at -5 °C by specifying a generalized linear model (GLM) with a logit link function for the binary outcome (i.e., dead vs alive aphids) (see Figure S1). Lt_{50} , the time which kills 50% of the tested animals at a given temperature, can be used as an index of the inherent/basal physiological thermotolerance in insects (Andersen et al. 2015). We estimated Lt_{50} , which corresponds to the temperature-specific duration resulting in 50% mortality, using the *dose.p* function in R package MASS (Ripley et al. 2013).

Experimental conditions for metabolite profiling

To examine the effects of cold exposure and resilience capacities on the physiology of the three aphid species, two distinct experiments were performed. Experiment 1: aphids were exposed to durations corresponding to 1/2, 2/2 of the species-specific Lt_{50} values (i.e., increasing injury levels, see Table 1). This experiment allowed us to compare the physiological impacts of two different injury levels on the aphids. Experiment 2: aphids were exposed to the same/identical exposure times (see Table 1). We selected 300 min as this exposure duration will cause some mortality to the intolerant

species but not to the tolerant one, which could then be used to compare whether different physiological effects were induced in the three species subjected to the same intensity of cold stress.

For both experiments, we treated eight replicates, each consisting of a pool of 10 individuals for each exposure time point, and sampled the treated aphids at two time windows: 1) direct sampling ("D") immediately upon exposure; 2) after 24 hours of recovery ("R") at 20 °C. Only alive individuals were sampled and were immediately transferred to 2ml Eppendorf Safe-Lock tubes, snap-frozen in liquid nitrogen. All samples were then stored at -80 °C until being processed. Additionally, eight replicates of ten aphids from the same cohort were promptly frozen and were used as untreated control ("C"). The different experimental treatments of the first experiment corresponding to aphids exposed at 1/2 Lt_{50} , 2/2 Lt_{50} , and the corresponding recovering aphids, were subsequently denoted "1/2 D", "2/2 D", "1/2 R" and "2/2 R".

Table 1. The experimental conditions

Temperature	Species	Increasing injury level		
		1/2 Lt_{50}	2/2 Lt_{50}	Same exposure times
-5 °C	<i>M. dirhodum</i>	2822 min	5644 min	300 min
	<i>S. avenae</i>	2025 min	4050 min	300 min
	<i>R. padi</i>	271 min	542 min	300 min

GC-MS metabolic profiling

Each sample was weighed (Mettler Toledo UMX2, accurate to 1 µg) before metabolite extractions. Samples were prepared as described in Thiébaud et al. (2021) and Mensch et al (2021), with the following minor modifications. Briefly, after homogenization in 400 µL of cold (-20 °C) methanol: chloroform solution (2:1, v:v), followed by an addition of 266 µL of ultrapure water, which allowed phase separation, 80 µL of the upper phase was pipetted, transferred into 2 mL glass vials and vacuum-dried (MiVac, Genevac, Ipswich, UK) for 50 min at room temperature. The dry residue was resuspended in 30 µL of 25 mg/mL methoxyamine hydrochloride in pyridine, before incubation under automatic orbital shaking at 40 °C for 60 min. Then, a volume of 30 µL of N, O-bis (trimethylsilyl) trifluoroacetamide was added to each glass vial, and the derivatization was conducted at 40 °C for 60 min under agitation. A CTC CombiPal autosampler (PAL System, CTC Analytics AG, Zwingen, Switzerland) was used, ensuring standardized and on-time sample preparation. Metabolites were separated, identified, and quantified with a 5977B GC/7890 MS platform (Agilent Technologies, CA, US). We used a 30 m fused silica column (HP-5MS Ultra Inert, 0.25mm, 0.25µm 5%-phenylmethylpolysiloxane) with helium as the carrier gas at a rate of 1 mL.min⁻¹. One microliter of each sample was injected into the GC/MS, as described in Thiébaud et al (2021). The temperature of the ion source was set at 230 °C and the MS transfer line at 280 °C. The oven temperature ranged from 60 to

325 °C at 10 °C min⁻¹ and then remained at 325 °C for 10 min. We completely randomized the injection order of the samples. Detection was achieved using MS detection in electron impact at 70 eV under the full-scan mode from 60 to 600 amu. The detection was achieved by scanning specific ions (1 quantifier and 2 qualifiers at a specific retention time) from 60 compounds. Calibration curves were computed by running each reference compound at 5, 7.5, 10, 20, 40, 80, 100, 200, 300, 400, and 500 µM concentrations, in addition to running 3 different levels of quality controls after running 80 samples. Chromatograms were deconvoluted using MassHunter B09.00 software, and metabolite levels were quantified using the quadratic calibration curve for each reference compound.

Statistical analysis

For the comparison of metabolic profiles among the different groups, the main patterns and significance were searched by performing a between-class principal component analysis (PCA) analysis. This PCA focused on the differences among the classes defined as qualitative instrumental variables (Dolédéc and Chessel 1987). A Monte Carlo test was then performed to examine the significance of the difference between the classes (Romesburg and Marshall 1985). All data were log-transformed before the PCAs. All analyses were performed using the ‘ade4’ package in the statistical software RStudio Team (2020) (Dray and Siberchicot 2017).

For experiment 1, we examined the effect of treatment and injury level on the extracted PC1 using two-way ANOVA, including the interaction item, with treatment and injury level as categorical variables. Different treatments at the same injury level and different injury levels within the same treatment were compared using one-way ANOVAs with Turkey’s HSD *Post hoc* tests, respectively.

The concentrations of individual metabolites were transformed into fold change relative to the control group (unexposed aphids). The fold change of each metabolite relative to control was log₁₀ transformed in all statistical analyses to reduce residuals and minimize heteroscedasticity but was presented as non-transformed relative values. The cold-treated groups were compared to the control using t-tests. The differences among treatments or species were examined by one-way ANOVAs with Turkey’s HSD *Post hoc* tests. All statistical analyses were performed using the R software.

Results

Median lethal time (Lt₅₀)

M. dirhodum survived significantly longer at -5 °C than the other two species (Figure S1). Lt₅₀ of the species ranked as follows: *M. dirhodum* (5644 ± 277 min) > *S. avenae* (4050 ± 136 min) > *R. padi* (542 ± 108 min).

General metabolic profile

Among the 60 metabolites included in our library, 32 metabolites were consistently detected in all samples and thus conserved for quantitative metabolic profiling based on the calibration curves of pure standards. Among these, we found eighteen amino acids, four polyols, four carbohydrates,

four metabolic intermediates, one amine and one phosphorylated compound (see Table S1 for the list of compounds).

Metabolic characterization after the increasing injury level (experiment 1)

Through PCA analysis, we were able to cluster all experimental groups for each species separately and found significant differences among classes in all cases (Monte Carlo test, $p < 0.05$). Panel A in Figure 1 illustrates the plot of the first two principal components (PCs), which resulted in clear-cut separations in all cases. The effect of cold exposure was mainly explained by PC1, which accounted for at least 48.69 %, 56.69 %, and 39.19 % of the total inertia for *M. dirhodum*, *S. avenae*, and *R. padi*, respectively (Figure 1A). PC2 revealed the contrast between the control and the recovery groups, which accounted for 26.23 %, 32.52 %, and 30.22 % of the total inertia for *M. dirhodum*, *S. avenae*, and *R. padi*, respectively (Figure 1A). The centroids of aphids sampled directly after stress (D) were far from the control along PC1, while the centroids of aphids that recovered (R) had returned to the initial value, indicating a recovery in the three species and at both injury levels (1/2 and 2/2). The ellipse around the centroids in *R. padi* was much larger, suggesting a relatively greater individual variation within the group, especially for 2/2 D treatment.

The centroids' projections on PC1 were obtained and analyzed to investigate the impact of the cold treatment and the injury level and their interaction terms using two-way ANOVA (Figure 1B). For all species, PC1 was significantly different among treatments and showed significant injury level \times treatment interaction (Table S2). Accordingly, to test the effect of treatment and injury level separately, we split the dataset according to the injury level and the treatment, respectively. Next, we compared the differences in PC1 scores among treatments for each injury level and the differences between injury levels for each treatment using one-way ANOVA, followed by the post-hoc comparisons (Table S3). Irrespective of the injury level, metabolic profile departed significantly from their initial state (i.e., "C") for all species due to cold exposure (i.e., the significant difference between treatments "D" and "C" with $p < 0.01$), showing a strong homeostatic deviation before coming back to control state after recovery period (Figure 1B). In addition, PC1 values differed between injury levels in cold-exposed aphids (D) across the three species ($p < 0.05$). Values dropped and departed from initial values more markedly at 2/2D than at 1/2D level, indicating a greater metabolic disturbance at 2/2D.

The correlation values between each metabolite and PC1 are shown in Figure 1C. Metabolites that positively correlated to PC1 were found to decrease in the exposed group (D) compared to the control (C) and recovery (R) groups. These metabolites included carbohydrates (trehalose, sucrose, and fructose) and asparagine (Figure 1C). Conversely, metabolites that were negatively correlated to PC1 were more abundant in the exposed aphids (D) than in the control (C) and recovery (R) groups. These metabolites included several free amino acids, such as alanine, glycine, glutamate, and leucine (Figure 1C). Hierarchical clustering revealed that *M. dirhodum* and *S. avenae* clustered together, indicating a similar pattern in metabolite changes between these two species (Fig 1C). On the other

hand, *R. padi* showed different and sometimes opposite patterns. For instance, at the end of cold stress (2/2 D), TCA intermediates such as pipecolic acid, citric acid, fumaric acid, and malic acid increased in *M. dirhodum* and *S. avenae* but decreased in *R. padi* (Figure 2).

The correlation values of each metabolite to PC2 of the PCA for *M. dirhodum*, *S. avenae* and *R. padi* are presented in Figure S3-5, respectively, and fold changes in each metabolite at 1/2 and 2/2 level injury are shown in Figure S2 and Figure 2. In *M. dirhodum*, proline, and trehalose exhibited the most negative correlation to PC2 (Figure S3). These compounds showed approximately a two-fold increase compared to the control in the recovery group under both injury levels (Figure S2 and Figure 2). In *M. dirhodum*, a drop in glucose level, concomitant with trehalose increase, was also found after recovery (Figure S2). As for *S. avenae*, the metabolites most correlated to PC2 were asparagine, phosphoric acid, proline, and glucose (Fig S4). In the case of *R. padi*, only serine was strongly correlated to PC2 (Figure S5) and showed relatively increased abundance in the recovery, while other metabolites did not demonstrate notable changes (Figure S2) Yet PC2, did not explain much of the recovery in that species.

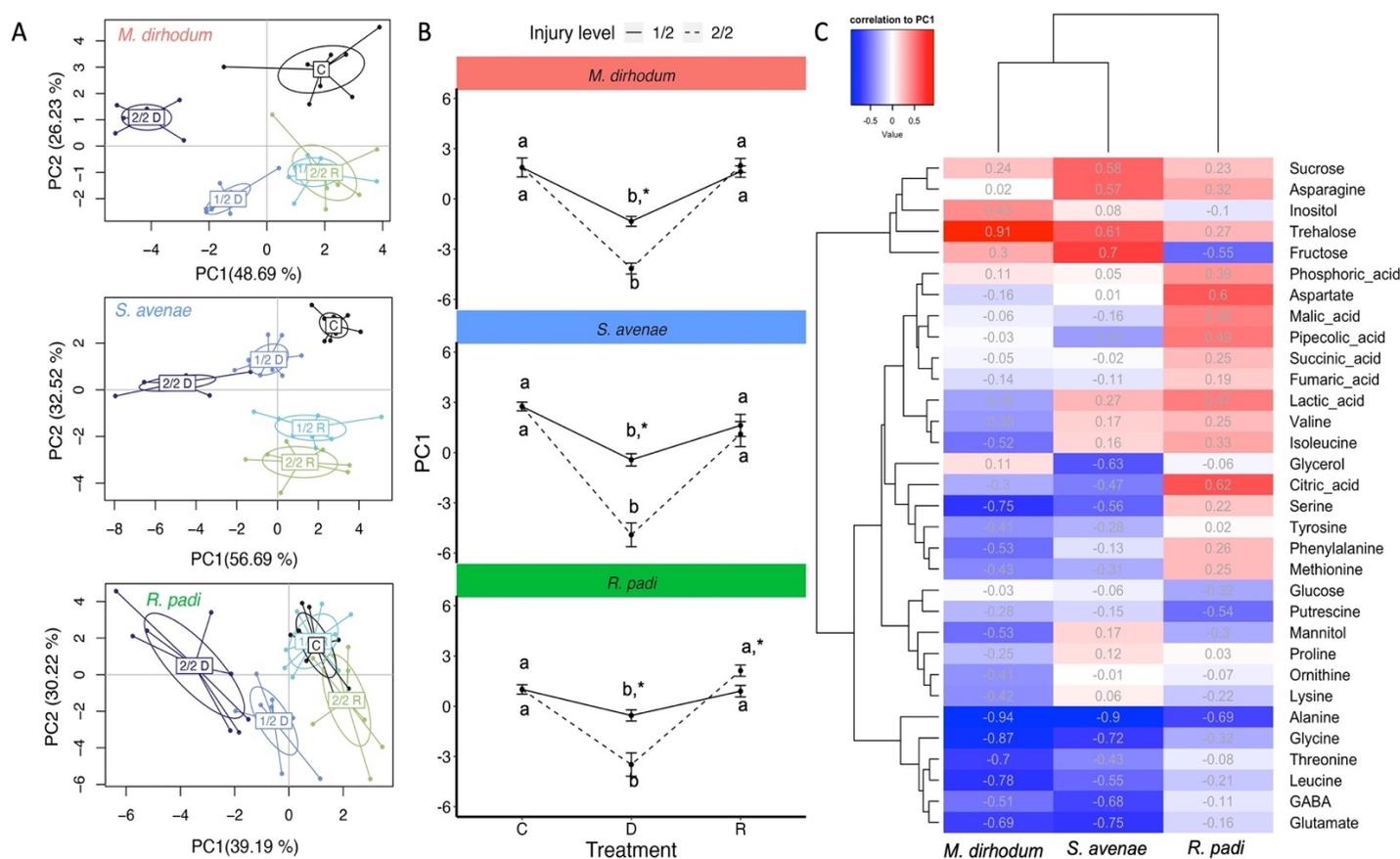


Figure 1. PCA, PC1 score plot and heatmap with hierarchical clustering for the correlation between metabolites and PC1 in experiment 1. Panel A shows PCA (PC1 vs PC2) of all samples for *M. dirhodum* (top), *S. avenae* (middle) and *R. padi* (bottom). **Panel B** illustrates mean PC1 scores according to treatment and injury level for *M. dirhodum* (top), *S. avenae* (middle) and *R. padi* (bottom). Treatments are control condition (“C”), sampled directly at the end of cold exposure (“D”) and sampled after 24-hours of recovery at 20 °C (“R”). Injury levels were 1/2 Lt_{50} (“1/2”) and 2/2 Lt_{50} (“2/2”). Values are mean scores \pm SE. The lowercase letter indicates the significant difference between treatments ($p < 0.05$). Asterisk indicates the significant difference between injury levels ($p < 0.05$). **Panel C** shows the correlation values associated with PC1 for 32 metabolites in each species and clustering tree based on Euclidean distance.

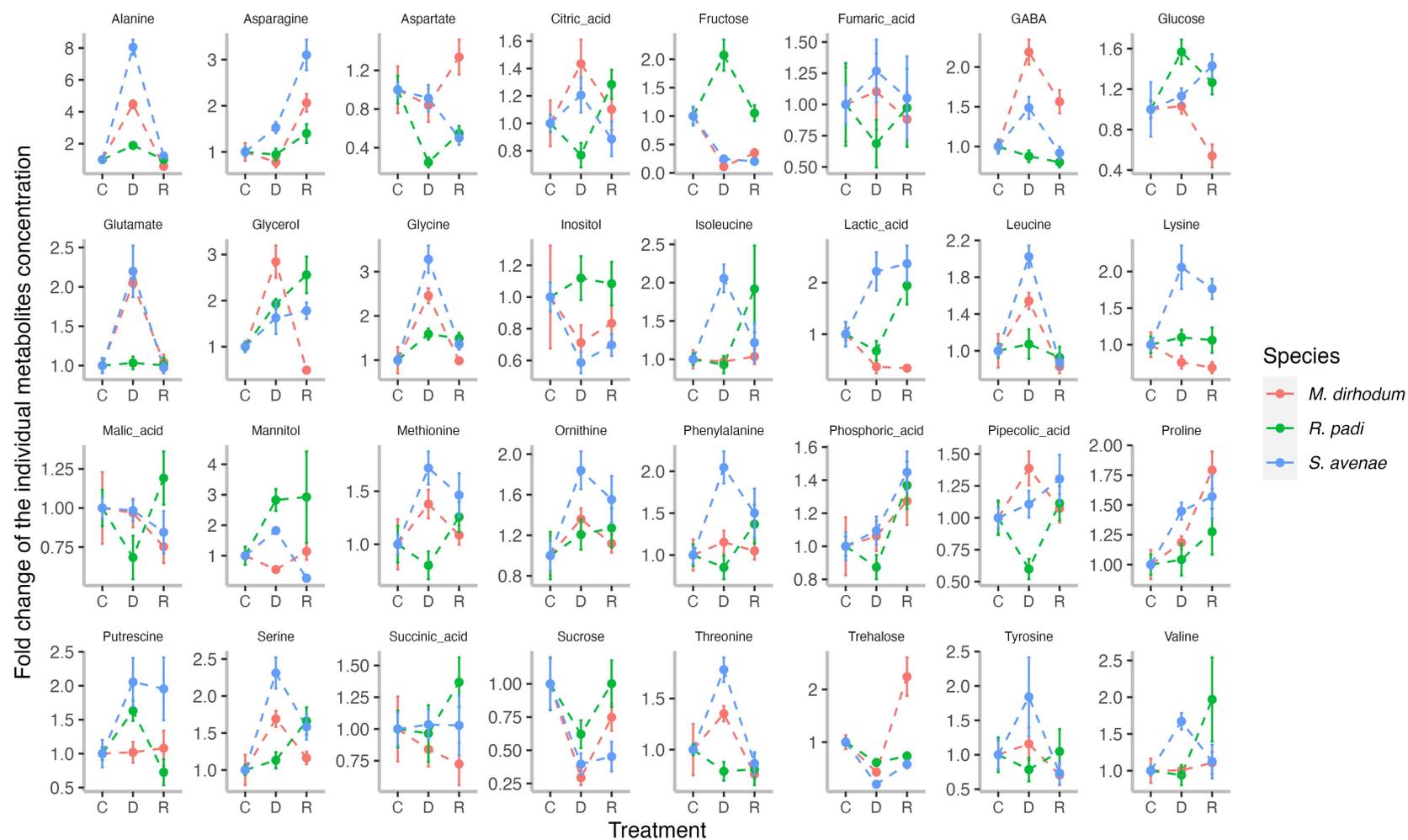


Figure 2. Temporal variations of individual metabolites from samples treated with 2/2 Lt_{50} injury level. C, D, and R represent the unexposed control, aphids sampled directly at the end of cold exposure and aphids recovered for 24 h at 20 °C after cold stress, respectively (see Table 1). Values are means \pm SE (n=8). Temporal variations of individual metabolites from samples treated with 1/2 Lt_{50} injury level are shown in Fig S2.

Metabolic characterization after the same exposure time (experiment 2)

For each species, the ordination of classes of the PCA is presented in Figure 3A. The significance of the differences among classes for each species was confirmed by Monte-Carlo randomizations ($p < 0.05$). The curves with arrows in Fig 3A indicate the temporal trajectory of metabolite changes. In all cases, the effect of cold stress (D) was explained by both PC1 and PC2 as the cold-stressed group deviated from control along both PC1 and PC2. In *M. dirhodum* and *R. padi*, the recovery dynamics were mainly explained by PC2, while in *S. avenae*, it was better explained by PC1, as shown by the arrows in Figure 3A. Indeed, projections of scores showed strong deviations from control (C) when aphids were cold exposed (D) then values tended to go back to initial control values after 24h recovery (R). So, we referred to them as “recovery PC” (Figure 3B). On the other hand, PC1 scores in *M. dirhodum* and *R. padi* and PC2 scores in *S. avenae* were initially high and positive. During stress (D), they dramatically decreased and either further decreased or remained stable after recovery (R). Since these PCs showed no sign of recovery, we denoted them as “no-recovery PC”. The correlations of each metabolite with “recovery PC” and “no-recovery PC” are presented in Figures S6A and B, respectively. In *M. dirhodum*, GABA, pipercolic acid, trehalose; in *S. avenae*, alanine, glucose, lysine, and glycerol; and in *R. padi*, alanine, proline, lysine, leucine, but also fructose and lactic acid positively correlated with “recovery PC” (Figure S6A). The concentrations of these compounds increased during stress and appeared to return to the initial state after recovery (Figure 4). Threonine and valine in *M. dirhodum*, isoleucine and asparagine in *S. avenae*, and glycerol and mannitol in *R. padi* negatively contributed to “no-recovery PC” (Figure S6B). These compounds exhibited an increase after recovery (Figure 4).

Univariate analysis revealed that the concentration of 10, 13, and 16 metabolites were significantly different from the control in the cold-stressed *M. dirhodum*, *S. avenae*, and *R. padi*, respectively (Table S4). Additionally, 18 metabolites showed significant interspecific differences in concentrations among species (Figure 5). Notably, *R. padi* exhibited significantly higher fold change in citric acid, lactic acid, sugars (fructose, sucrose), polyols (mannitol and glycerol), and some amino acids (proline and serine) ($p < 0.05$) (Figure 5). On the other hand, *S. avenae* displayed a higher abundance of specific metabolites such as alanine, glucose, isoleucine, lysine, and putrescine (Figure 5). *M. dirhodum* had higher levels of GABA, glutamate, and trehalose (Figure 5).

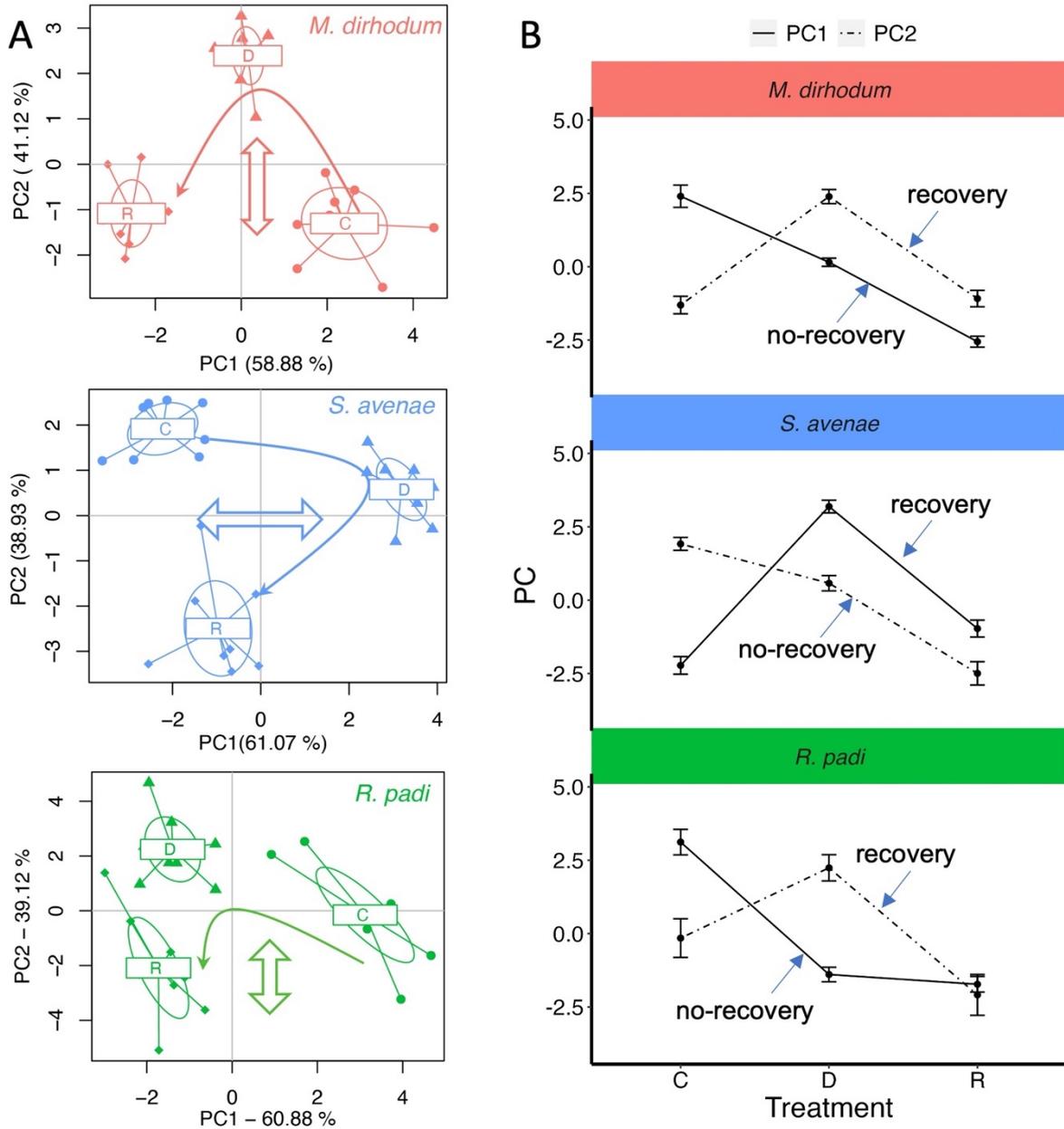


Figure 3. PCA, projections on PCs' scores in experiment 2. Panel A shows PCA (PC1 vs PC2) of samples for *M. dirhodum* (top), *S. avenae* (middle), and *R. padi* (bottom). The curves with arrows indicate the temporal trajectory in metabolite changes. Treatments refer to control ("C"), cold exposure ("D"), and 24 h recovery ("R"). Panel B shows the PC1 and PC2 scores of each treatment for each species. Values are mean scores \pm SE.

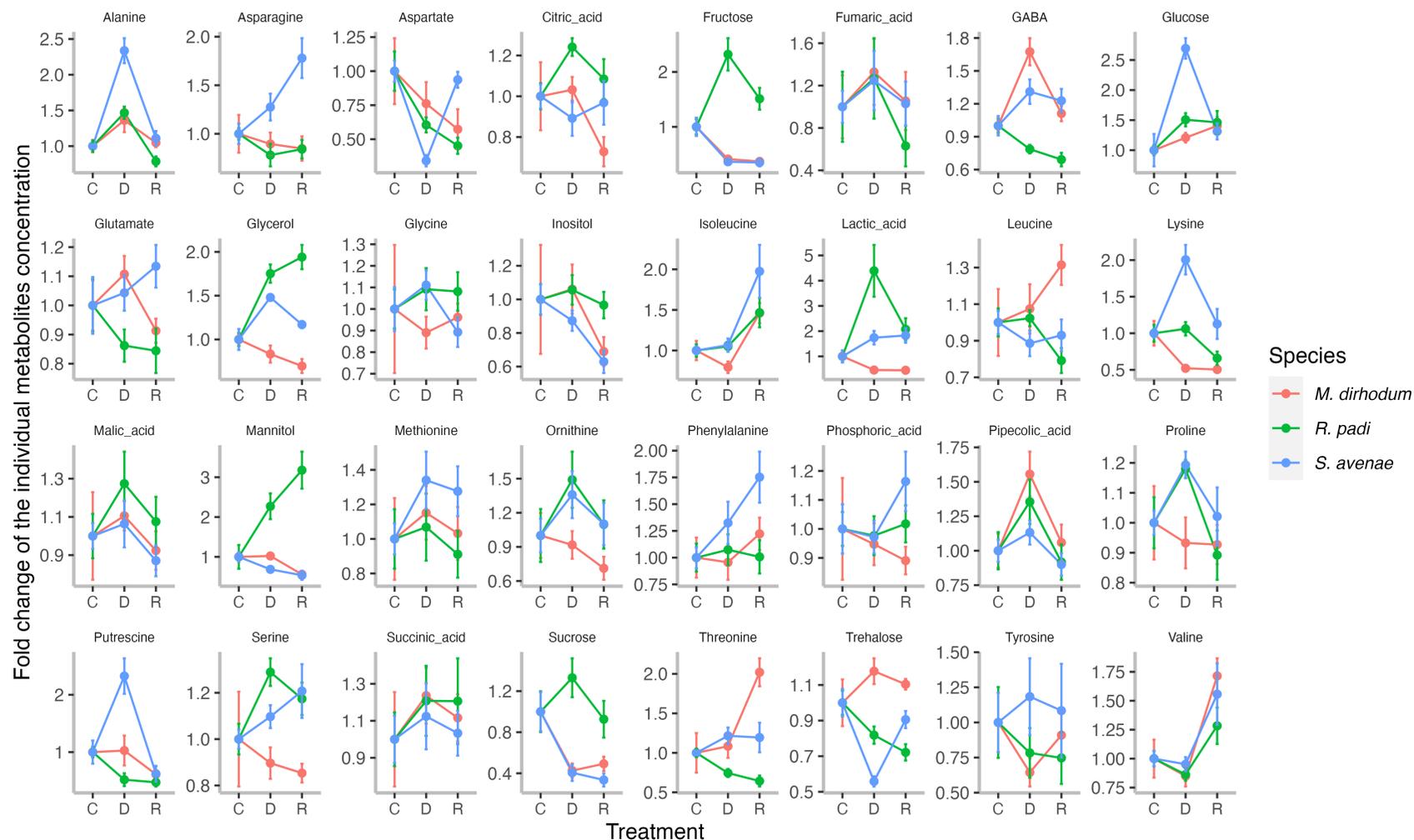


Figure 4. Temporal variations of individual metabolites from samples treated with the same exposure time. C, D, R represent the unexposed control, aphids sampled directly at the end of cold exposure (i.e., 300 min at -5 °C) and aphids recovered for 24 h at 20 °C after cold stress, respectively. Values are means \pm SE (n=8).

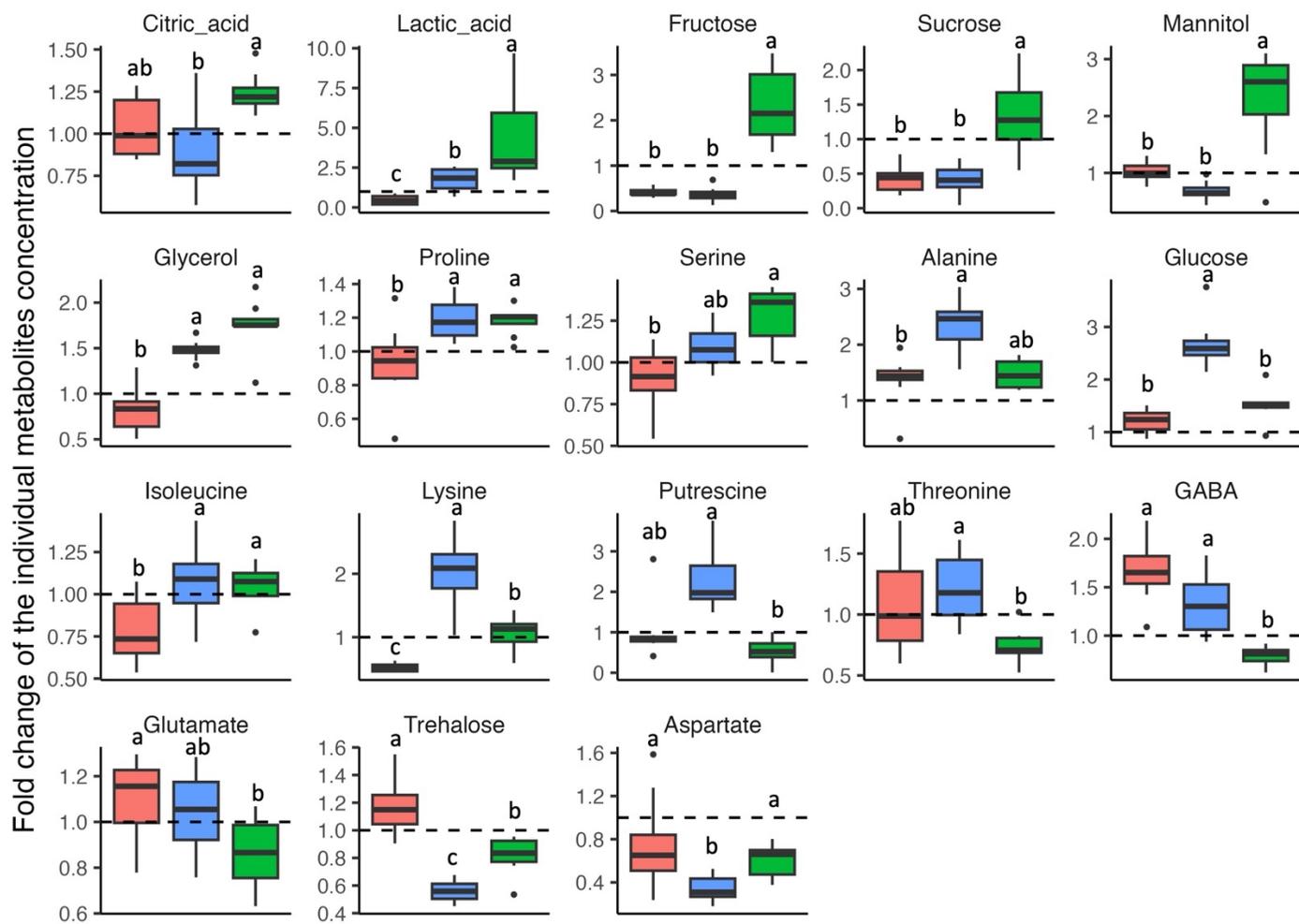


Figure 5. Fold changes relative to untreated control of the individual metabolite concentrations that significantly varied among cold-exposed aphid species when they were sampled directly (D) at the end of cold stress (300 min at -5°C for all). Values represent mean fold changes \pm SE ($n = 8$). The lowercase letter indicates the significant level among species ($p < 0.05$).

Discussion

Our previous studies have found that the three species had significant differences in cold tolerance, with *M. dirhodum* being the most cold-tolerant, followed by *S. avenae* and then *R. padi* (Li et al. 2023a, Li et al. 2023b). This study thus aimed at comparing the physiological mechanisms underlying these contrasted responses by assessing changes in metabolites' levels during cold stress and the subsequent recovery phase. We conducted two different experiments: in experiment 1, aphids were exposed to a cold temperature for durations corresponding to $1/2$ Lt_{50} and $2/2$ Lt_{50} so that the apparent level of injury would be similar between species while testing increasing levels of injury. Our results showed that aphids experienced significant disturbances in their metabolites and more severe perturbations were observed when the exposure duration lengthened (i.e., higher injury level). Contrary to our expectation, perturbations were found to be partially reversible after 24-hours recovery under both exposure durations. Indeed, after 24h recovery, some molecules such as proline, asparagine, or trehalose, had concentrations that remained high or even increased, especially in the cold-tolerant *M. dirhodum* and *S. avenae*. These molecules have been reported as cryoprotectants in other species (Kostal et al. 2016, Grgac et al. 2022, Kučera et al. 2022) and have protective functions on stabilizing proteins and membranes (Yancey 2005). In experiment 2, all three species displayed a disruption of metabolic homeostasis after being exposed to the same duration of cold stress. However, the cold-susceptible *R. padi* exhibited a higher number of altered metabolites compared to the other two species, indicating that this species had a poorer ability to maintain its metabolic balance than the cold-tolerant species. Additionally, our findings revealed an elevation in the levels of polyols (glycerol and mannitol) in the cold-susceptible species. These polyols, commonly known as cryoprotectants in aphids (Durak et al. 2021, Durak and Durak 2021) and other insects (Yoder et al. 2006, Huang et al. 2022), might contribute to survival by non-colligative binding to proteins, preventing denaturation, and safeguarding against membrane damage (Gekko and Timasheff 1981, Tsvetkova and Quinn 1994)

Metabolic characterization after the increasing injury level (experiment 1)

The metabolic profiles of the three aphid species displayed significant deviations from the control when exposed to a duration corresponding to $1/2$ Lt_{50} and these differences became even more pronounced when the insects were exposed for a longer duration ($2/2$ Lt_{50}) at -5 °C. This finding is in line with former works which showed that the insects gradually lose homeostatic balance when the severity of thermal stress is increased (Colinet et al. 2012a), with cascading consequences on a wide range of physiological functions (Overgaard and MacMillan 2017).

When examining the global patterns of metabolite changes during cold stress, *M. dirhodum* and *S. avenae* exhibited a higher similarity, as numerous metabolite levels increased. In contrast, *R. padi* displayed a distinct response, with a significant decrease in many metabolites (Figure 1C). It is noteworthy that *M. dirhodum* and *S. avenae* demonstrated similar cold tolerance, whereas *R. padi*

proved to be sensitive to cold (Figure S1). These findings imply a correlation between the metabolic profile and cold tolerance in general. Interestingly, a previous study also discovered similar metabolic changes in cold-tolerant temperate-origin *Drosophila* flies, whereas the cold-intolerant tropical flies displayed comparable metabolic patterns (Mensch et al. 2021).

Perturbation of the energy metabolism is a common physiological response in insects exposed to low temperatures due to the disruption of cellular respiration enzymes (Denlinger and Lee Jr 2010, Colinet 2011, Lubawy et al. 2022). When aphids were exposed to the durations corresponding to 1/2 Lt_{50} injury level, glycerol, glucose, alanine, metabolites all involved in glycolysis, increased in *R. padi* and *S. avenae*, together with a block of TCA cycle (as suggested by the slight accumulation of TCA intermediates). Furthermore, the accumulations of the glycolytic end product (lactic acid, alanine) suggest these two aphid species rely on anaerobic metabolism during cold exposure. The upregulation of anaerobic metabolites was observed in stressed insects (chilled, supercooled, freeze-stressed, or cryopreserved in liquid nitrogen), suggesting anaerobic respiration might be one of the important processes for energy supply (Robert Michaud et al. 2008, Boardman et al. 2016, Štětina et al. 2018, Lubawy et al. 2022). The exact reason for the increased reliance on anaerobic metabolism during cold stress remains unclear. While it was initially speculated that oxygen limitation could be the primary factor, recent findings challenge this notion (Boardman et al. 2016). Under conditions of hyperoxia, chilling these insects still resulted in elevated levels of alanine. This suggests that factors other than oxygen availability are likely responsible for the shift towards anaerobic metabolism during cold stress (Boardman et al. 2016).

However, when *M. dirhodum* was exposed to the durations corresponding to 1/2 Lt_{50} injury level, carbohydrates (glucose, fructose, and sucrose) and TCA intermediates (fumaric acid, malic acid, succinic acid) dramatically decreased, which may result from their more rapid utilization (i.e., turnover), which was previously reported in cold-acclimated insects with higher mitochondrial density and efficiency (Foray et al. 2013).

With the increasing exposure duration (2/2 Lt_{50}), *R. padi* seemed to experience a significant decline in energy metabolism, as evidenced by a dramatic decrease of intermediates of the TCA cycle (such as citric acid, fumaric acid, and malic acid). Surprisingly, lactic acid and alanine did not accumulate much, suggesting that *R. padi* may not switch to anaerobic metabolism. These observations might be attributed to impaired mitochondrial function in *R. padi*, as indicated by the accumulations of glucose and several derivatives of the glycolytic pathway side-branches (such as fructose, glycerol, and glycine) but this will require further confirmation.

The free amino acid pool in aphids was altered by cold stress with the upregulation of certain amino acids (such as alanine, glycine, and leucine). The elevation of alanine in response to low temperatures has been reported in various insect species under cold hardening (Overgaard et al. 2007), diapause (Michaud and Denlinger 2007), freezing (Robert Michaud et al. 2008), and chill coma (Mensch et al. 2021). This amino acid has been suggested as a reliable marker for low-temperature

stress (Denlinger and Lee Jr 2010). Moreover, alanine plays a protective role in preventing cold inactivation of proteins (Carpenter and Crowe 1988) and may contribute to cold survival by serving as a less toxic end-product of glycolysis during anaerobic conditions than lactic acid (Michaud and Denlinger, 2007). In addition, alanine could potentially serve as an intermediate in pathways involved in the synthesis of cryoprotectants (Mensch et al. 2021). Glycine can stabilize the protein structure against thermal denaturation (Arakawa and Timasheff 1985) and the elevation of glycine has been found in the metabolome of ticks during recovery from rapid cold hardening, yet further injection of glycine had no impact on cold shock survival (Rosendale et al. 2022). In our study, the augmentation of glycine in cold-exposed aphids was potentially the result of increased protein catabolism as the previous research suggested (Rosendale et al. 2022). Although there are no reported non-colligative mechanisms of cold-hardiness inherent to leucine, there has been a report of leucine accumulation in insects during diapause (Wang et al. 2005, Michaud and Denlinger 2007) and cold acclimation (Fields et al. 1998) as well as in the cold-shocked plant, *Arabidopsis thaliana* (Kaplan et al. 2004). Leucine is synthesized from glycolytic intermediates pyruvate (Lu et al. 2014), thus further suggesting that glycolysis is active during cold stress. It is hard to distinguish whether this change correlates with increased cold tolerance or if it is a sign of a disorder/biomarker of stress. As noted by Hayward and Colinet 2023, the observed metabolic changes could reflect either an adaptive biochemical change (e.g., accumulation of Cryoprotectants) that preserves vital functions, or downstream consequences of a disrupted physiological capacity. Since both responses can be found concurrently, it is important to consider, or even test, the adaptive potential of a biochemical change.

After 24h recovery, we observed an increase in proline and trehalose levels in *M. dirhodum*. Similarly, *S. avenae* exhibited higher levels of proline and asparagine during recovery, whereas only serine showed an increase in *R. padi*. Proline, trehalose, and asparagine are well-known cryoprotectants that play a crucial role in cryopreserving freeze-sensitive insects by depressing the melting point of water and reducing the ice fraction (Yancey 2005, Kučera et al. 2022). Additionally, they can stabilize proteins, and lipid bilayers, and maintain membrane integrity during freezing through non-colligative mechanisms (Kostal et al. 2016, Grgac et al. 2022, Kučera et al. 2022). In our study, although the amounts of these compounds increased after recovery, the fold change was relatively small, ranging from 2 to 3. These changes may assist cold injury recovery, such as facilitating the refolding of partially denatured proteins (Lee and Goldberg 1998), aiding in the re-establishment of ion gradient homeostasis (Olsson et al. 2016), scavenging for oxygen radicals (Benaroudj et al. 2001). Further research, targeting these compounds, is needed to determine if the production of these compounds after recovery in *M. dirhodum* and *S. avenae* contributes to their greater cold tolerances compared to *R. padi*.

Metabolic characterization after the same exposure time (experiment 2)

To further compare the physiological effects of thermal stress on three aphid species, we subjected them to the same exposure duration of a low temperature. Interestingly, we found similar

patterns across all three species, where they exhibited both recovery PC and no-recovery PC. However, some metabolites show species-specific patterns, with again *R. padi* displaying notable distinctions. The cold-susceptible *R. padi* had a higher number of altered metabolites (16 metabolites) compared to the tolerant species, *M. dirhodum* (10 metabolites). The exposure time (300 min) was between $1/2$ Lt_{50} and $2/2$ Lt_{50} of *R. padi*, but considerably lower than the $2/2$ Lt_{50} values of *M. dirhodum* (5644 min). Consequently, *R. padi* may have experienced more severe cold injury, resulting in more pronounced changes in metabolites. This indicates that metabolic inertia is generally associated with cold tolerance, which is also found in *Drosophila* flies (Olsson et al. 2016).

Among the altered metabolites in *R. padi*, we observed a higher fold change in citric acid and lactic acid, indicating a potential stalling of the TCA cycle and a greater reliance on anaerobic metabolism. This may indicate that the pathways related to the energy metabolism of *R. padi* may be more susceptible to low temperatures than the other two species.

The reduction in aerobic ATP production is often accompanied by a shift in substrate metabolism to enable the accumulation of cryoprotectants like polyols (Denlinger and Lee Jr 2010). The fold change of mannitol and glycerol in *R. padi* was significantly higher than in *S. avenae* and *M. dirhodum*. Polyols can increase the osmotic potential of the fluids, thus preventing freezing (Storey 1997). In addition, they can contribute non-colligatively to survival by hydrogen-binding to proteins to prevent denaturation (Gekko and Timasheff 1981) and by preventing membrane damage (Tsvetkova and Quinn 1994). Glycerol and mannitol are commonly reported cryoprotectants in overwintering aphids (Saeidi et al. 2017, Durak et al. 2021, Durak and Durak 2021) and other arthropods during diapause, cold acclimation, rapid cold-hardening or freezing (Robert Michaud et al. 2008, Colinet et al. 2012b, Teets and Denlinger 2013, Kojić et al. 2018, Huang et al. 2022). An injection of glycerol into larvae of flesh flies immediately enhanced cold tolerance (Yoder et al. 2006). In our study, the fold changes in glycerol and mannitol in *R. padi* were low (2-3 fold), suggesting that these compounds may protect through non-colligative effects.

Conclusions

This study aimed to investigate the physiological and biochemical mechanisms underlying cold tolerance by comparing the metabolic responses of three aphid species with varying levels of cold tolerance. In line with our hypothesis, we made several key findings: 1) Cold stress led to metabolic disorders, which became more pronounced with longer exposure periods. 2) When subjected to the same duration of cold stress, cold-susceptible species exhibited a higher number of significant metabolite changes relative to the control group compared to the tolerant species. 3) Cold stress disrupted the TCA cycle and resulted in an increased reliance on anaerobic metabolism. 4) The metabolic profile exhibited a general association with cold tolerance, as cold-tolerant species (*M. dirhodum* and *S. avenae*) displayed similar changes in metabolites, whereas *R. padi* exhibited distinct patterns. Furthermore, we observed that cold-tolerant species demonstrated enhanced production of

cryoprotectants such as proline, trehalose, and asparagine. These compounds are suspected to contribute to the protection and repair of cold-induced injuries. In summary, this research sheds light on the metabolic responses of aphid species, highlighting the impact of cold stress on their physiological processes and providing insights into the specific biochemical compounds associated with cold tolerance and susceptibility.

Supporting information

Table S1: List of targeted and quantified metabolites in the metabolome of three aphid species, grouped by biochemical families.

Metabolites that were quantified with GC-MS were indicated in bold.

Amino acids	Amines	TCA intermediates	Organic acids	Carbohydrates	Polyols	Phosphorylated compounds	Nucleobases
Alanine	Putrescine	Citric acid	Lactic acid	Fructose	Glycerol	Phosphoric acid	Cytosine
Glycine	Spermidine	Malic acid	Glyceric acid	Glucose	Mannitol	Glycerol-3P	
Isoleucine	Octopamine	Succinic acid	Quinin acid	Trehalose	Inositol	Gluconic lactone acid	
Leucine	Tyramine	Fumaric acid	Galacturonic acid	sucrose	Adonitol	Glucose-6P	
Proline	Ethanolamine		Pyruvic acid	Arabinose	Arabitol	Fructose-6P	
Valine	Dopamine			Galactose	Erythritol		
Phenylalanine	Triethanolamine			Mannose	Galactitol		
Asparagine	Cadaverine			Maltose	Sorbitol		
Tyrosine				Ribose	Xylitol		
Serine				xylose			
Threonine							
Methionine							
Lysine							
Aspartate							
Glutamate							
GABA							
Ornithine							
Pipecolic acid							

Table S2. Two-way ANOVA, based on the mean scores of the first principal component (PC1) of PCA on the experiment 1, testing the effect of treatment (C, D, R) and injury level (1/2 and 2/2), in the three aphid species separately.

	Variable	<i>df</i>	F-value	P-value
<i>M. dirhodum</i>	PC1 scores			
	Treatment	2	75.54	< 0.001
	Injury level	1	5.38	< 0.05
	Treatment × Injury level	2	8.13	< 0.01
<i>S. avenae</i>	PC1 scores			
	Treatment	2	56.61	< 0.001
	Injury level	1	15.08	< 0.01
	Treatment × Injury level	2	10.71	< 0.001
<i>R. padi</i>	PC1 scores			
	Treatment	2	44.55	< 0.001
	Injury level	1	2.96	0.09
	Treatment × Injury level	2	13.95	< 0.001

Table S3. Comparison of the first principal component (PC1) scores among treatments and injury levels for each species. Pairwise p -values from one-way ANOVA with post-hoc TurkeyHSD tests. p -values < 0.05 are marked in bold. Light grey cells mark the comparisons between injury levels with the same treatment.

Species	Injury level	Treatment	1/2			2/2							
			C	D	R	C	D	R					
<i>M. dirhodum</i>	1/2	C	<0.001		0.91								
		D											
		R											
	2/2	C											
		D										<0.001	
		R											
<i>S. avenae</i>	1/2	C	<0.001		0.22								
		D											
		R											
	2/2	C											
		D										<0.001	
		R											
<i>R. padi</i>	1/2	C	<0.001		0.58								
		D											
		R											
	2/2	C											
		D										<0.001	
		R											

Table S4. Metabolites that showed significant changes from the control in the cold treated aphids (D) of experiment 2 (i.e., same exposure time). The cold-treated groups were compared to the control using t-tests.

Metabolites	<i>p-value</i>		
	<i>R. padi</i>	<i>S. avenae</i>	<i>M. dirhodum</i>
Trehalose	< 0.05	< 0.05	< 0.05
Glucose	< 0.05	< 0.05	< 0.05
Fructose	< 0.05	< 0.05	< 0.05
Sucrose	0.2248	< 0.05	< 0.05
Lactic_acid	< 0.05	< 0.05	< 0.05
GABA	< 0.05	< 0.05	< 0.05
Mannitol	< 0.05	< 0.05	0.8736
Glycerol	< 0.05	< 0.05	0.0892
Aspartate	< 0.05	< 0.05	0.0838
Proline	< 0.05	< 0.05	0.3508
Alanine	< 0.05	< 0.05	0.3318
Threonine	< 0.05	0.0967	0.9143
Serine	< 0.05	0.0955	0.1608
Valine	< 0.05	0.3693	0.1231
Citric_acid	< 0.05	0.1646	0.7611
Glutamate	< 0.05	0.6449	0.1849
Tyrosine	0.0993	0.7635	< 0.05
Lysine	0.7792	< 0.05	< 0.05
Isoleucine	0.4387	0.6098	< 0.05
Putrescine	0.0765	< 0.05	0.5408
Ornithine	0.119	0.1635	0.3037
Asparagine	0.0788	0.1149	0.2403
Phenylalanine	0.9155	0.2528	0.425
Methionine	0.7087	0.1324	0.6692
Malic_acid	0.2705	0.9537	0.2965
Fumaric_acid	0.7744	0.8268	0.6001
Succinic_acid	0.4955	0.8876	0.2565
Glycine	0.5234	0.2034	0.1145
Phosphoric_acid	0.5773	0.5029	0.3564
Leucine	0.714	0.1237	0.9774
Inositol	0.7242	0.0657	0.921

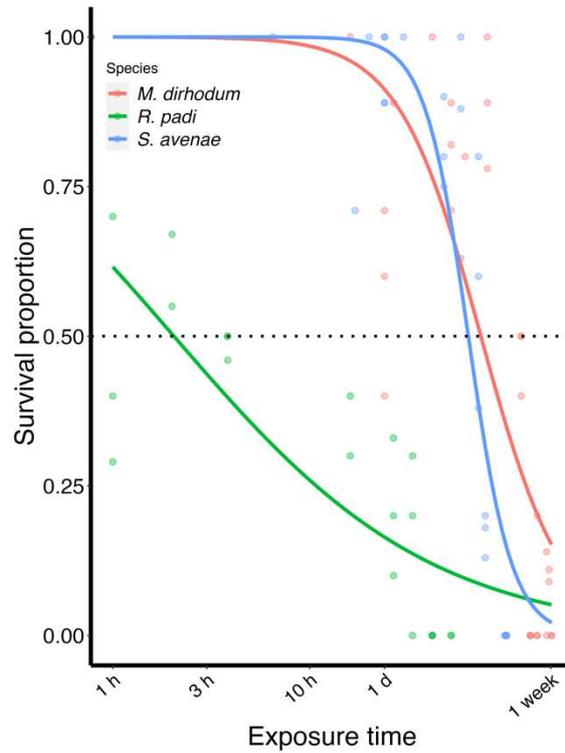


Figure S1. Survival curves at -5 °C for the three aphid species (*M. dirhodum*, *S. avenae* and *R. padi*). Survival curves were fitted by a generalized linear model (GLM) with a logit link function. Each point represented the survival proportion of aphids after the given exposure times in a single replicate. The black dashed line indicated the 50% survival proportion thus the x axis of intersections of this dashed line and survival curves were the Lt_{50} estimates of each species (Li et al. 2023a).

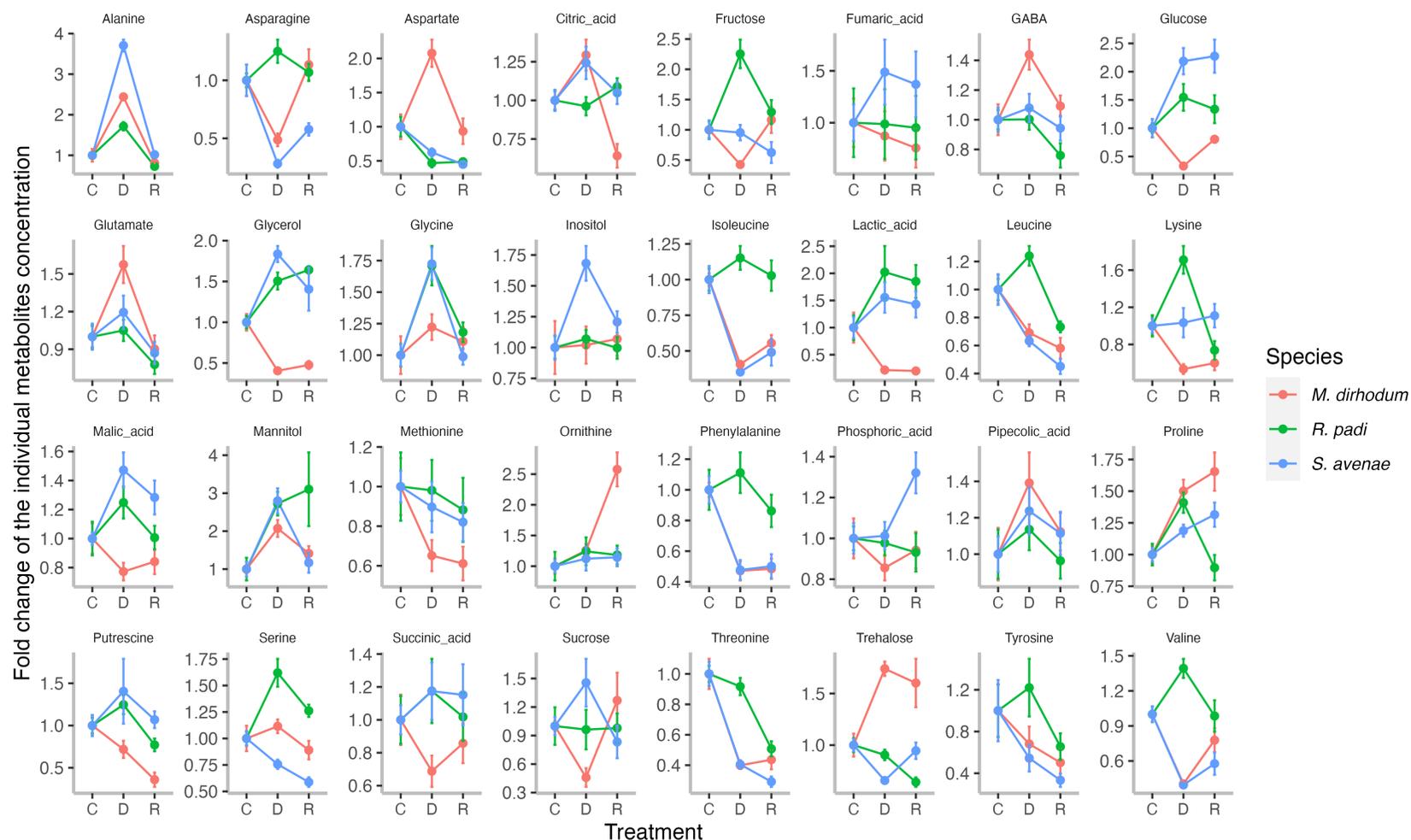


Figure S2. Temporal variations of individual metabolites from samples treated with 1/2 Lt_{50} injury level. C, D, R represent the unexposed control, aphids sampled directly at the end of cold exposure (see table1) and aphids recovered for 24-hours at 20 °C after cold stress, respectively. Values are means \pm SE (n = 8).

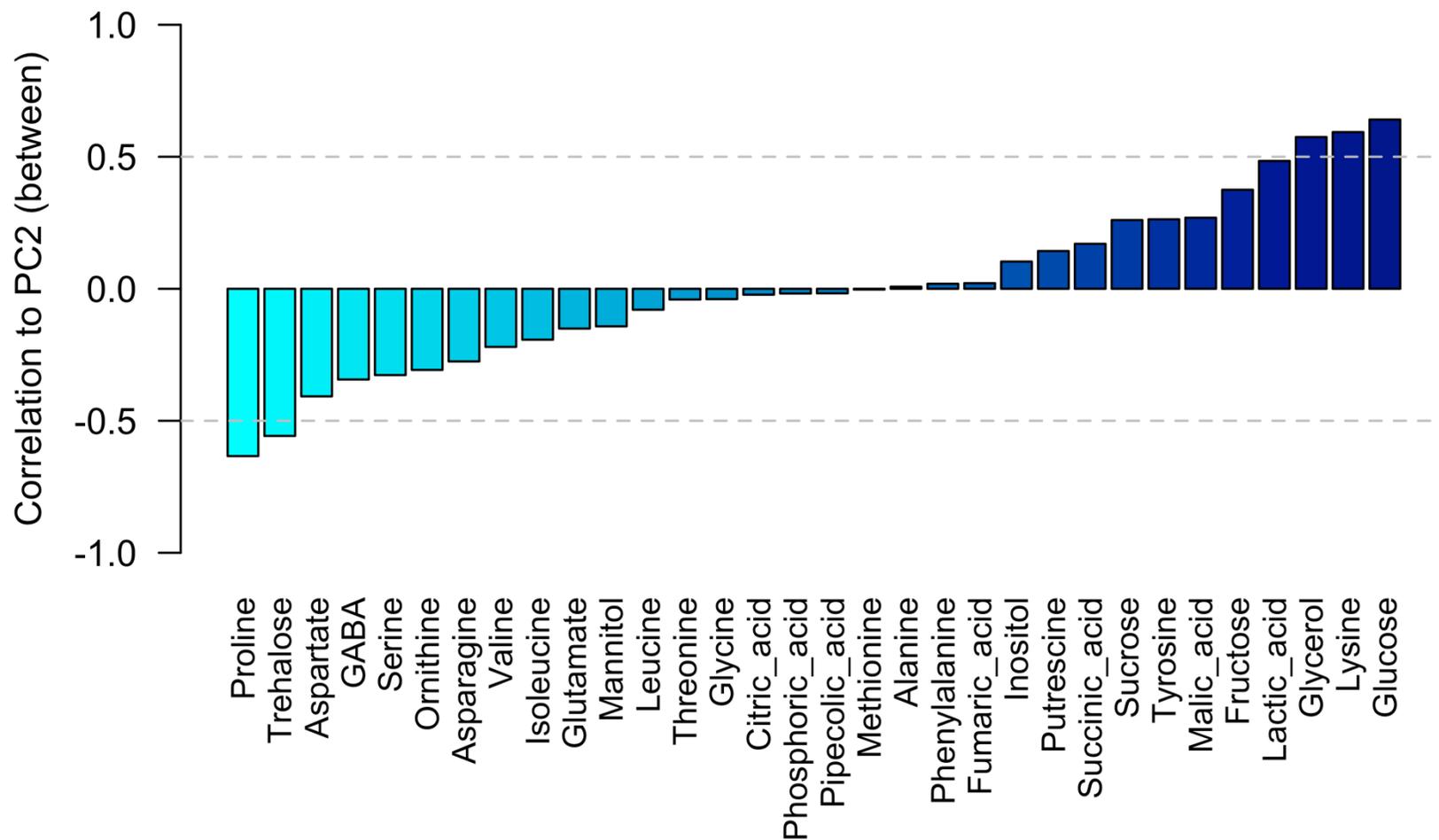


Fig S3. Correlation values of the different metabolites to the second principal component (PC2) of the PCA in *M. dirhodum* for experiment 1 (i.e., increasing injury level)

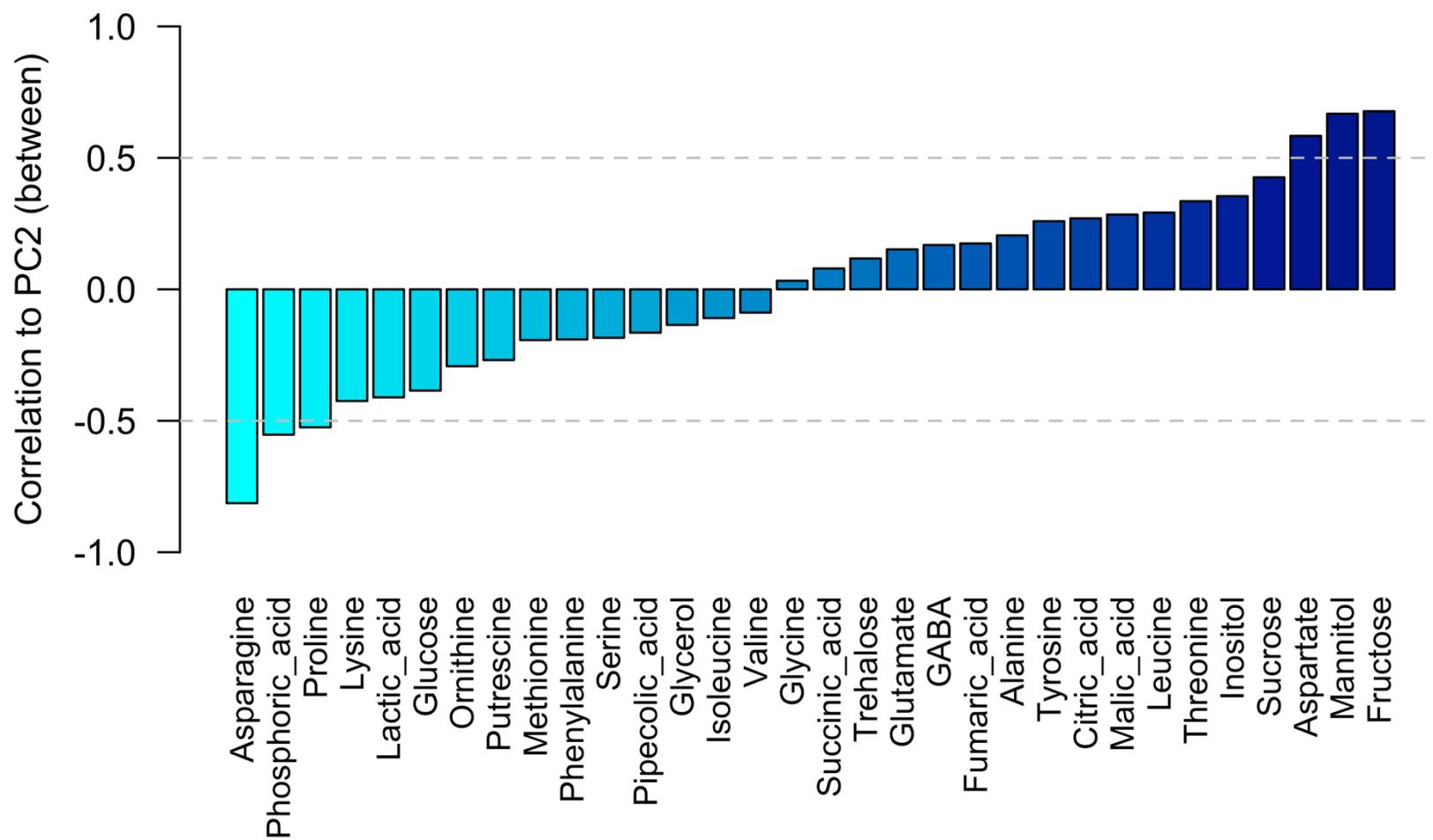


Fig S4. Correlation values of the different metabolites to the second principal component (PC2) of the PCA in *S. avenae* for experiment 1 (i.e., increasing injury level)

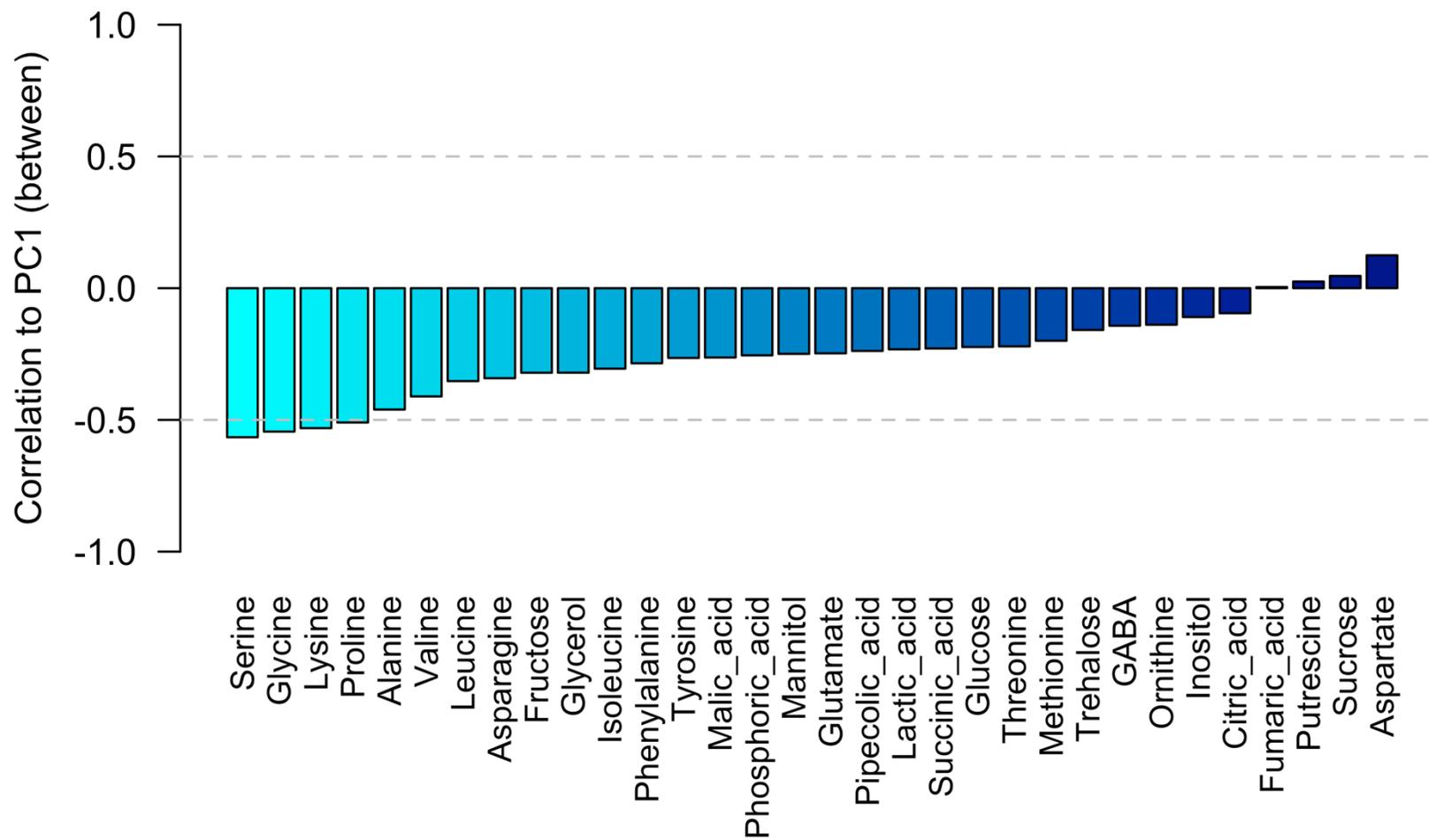


Fig S5. Correlation values of the different metabolites to the second principal component (PC2) of the PCA in *R. padi* for experiment 1 (i.e., increasing injury level)

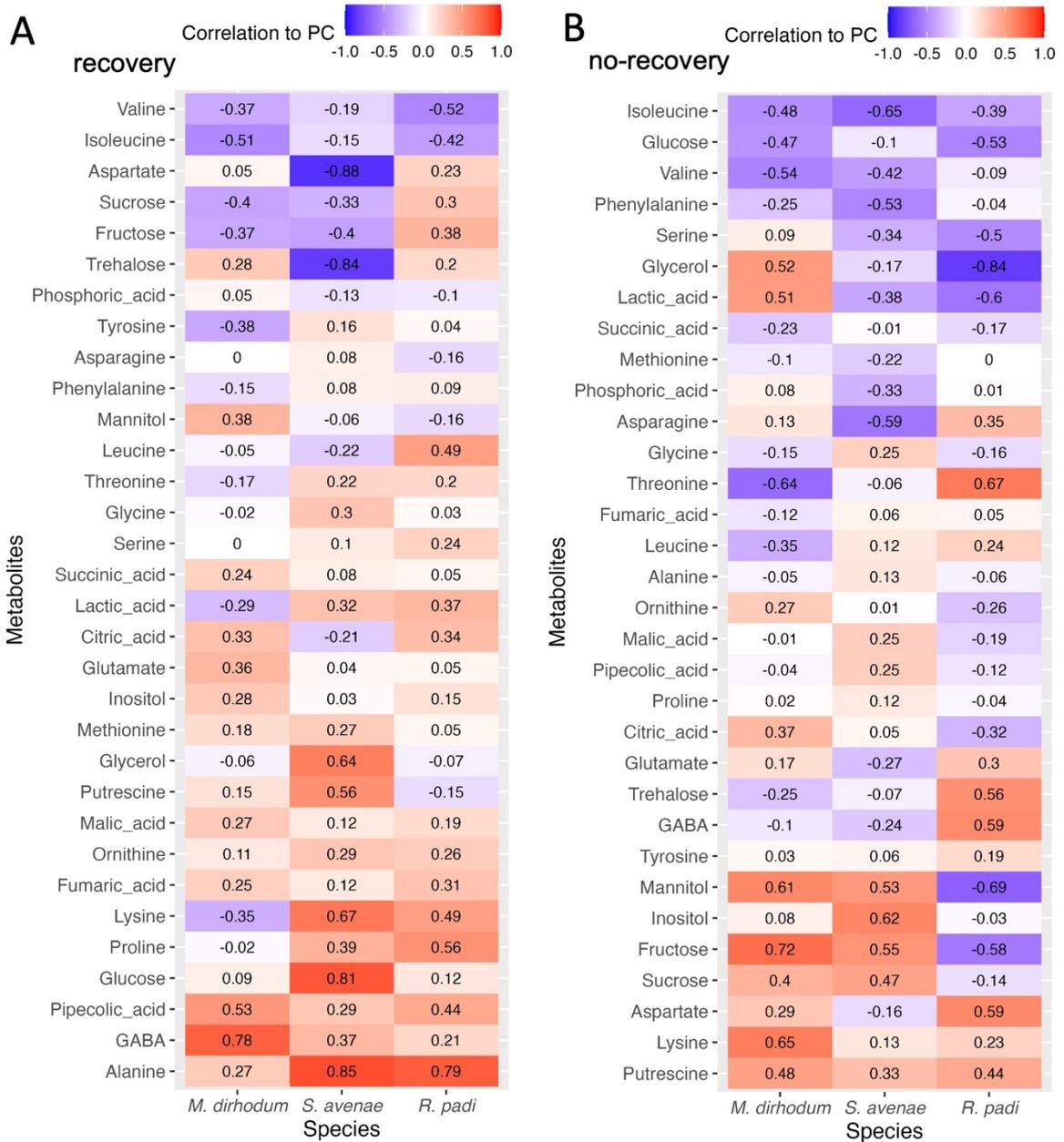


Fig S6. Correlation values of the different metabolites to the recovery PC and no-recovery PC for each species in experiment 2 (i.e., same exposure time)

Chapter 6 General discussion and perspectives

1 General discussion

More frequent, intense, and prolonged temperature extremes in recent years have exposed numerous insect species to unprecedented thermal stress, pushing them to the edge of or even over their adaptive limits (Christidis et al. 2015, Palmer et al. 2017, Harvey et al. 2020). Ecologists face the challenge of understanding how temperature extremes impact individual and population response of insects, as well as the ecological consequences for community structure, and ecosystem functioning (Filazzola et al. 2021, Ma et al. 2021). However, there is a lack of studies that connect the molecular and physiological responses at the sub-individual level to community-level effects, hindering our integrative view of the ecological and evolutionary consequences of stress (Sulmon et al. 2015). To address this gap, we conducted four experiments at various levels of biological organization (from genes to community) using cereal aphids. The guild of cereal aphids includes three main species: *Metopolophium dirhodum*, *Sitobion avenae*, and *Rhopalosiphum padi*. Earlier reports have shown that increasing frequency of temperature extremes caused a shift in dominant species within guilds by differently altering species' population growth (Ma et al. 2015). We hypothesize that this phenomenon is related to the differences in thermal tolerance among species, which may be driven by distinct physiological tolerance (e.g., molecular and metabolic response). To test our hypothesis, we investigated the interspecific variation in thermal tolerance and their metabolic and molecular response to heat and cold stress. We found: 1) The tolerable durations of the three species decreased exponentially as the intensity of stress (temperature) increased. In terms of heat tolerance, the species ranked as follows: *R. padi* > *S. avenae* > *M. dirhodum*. Conversely, for cold tolerance, the ranking was: *R. padi* < *S. avenae* < *M. dirhodum*. During winter, *R. padi* was estimated to accumulate more cold injury than the other two species, while *M. dirhodum* accrued more heat injury during summer. 2) Heat stress induced the up-regulation of several *hsp* genes much more strongly than cold stress. Among the *hsp* genes, *hsp70A* exhibited the highest level of up-regulation in response to both heat and cold stress. *R. padi* had more heat-inducible genes and significantly higher mRNA levels of *hsp70A*, *hsp10*, *hsp60*, and *hsp90* than the other two species. In contrast, *M. dirhodum* had more cold-inducible genes than the other species. 3) The heat-tolerant species, *R. padi*, experienced fewer perturbations in the metabolic profiles and had higher levels of polyols compared to the less-tolerant species after the same duration of exposure to heat stress. 4) Cold stress challenged energy metabolism, which was particularly pronounced in cold-susceptible species, *R. padi*. In contrast, the cold-tolerant species demonstrated increased production of cryoprotectants (proline, trehalose, and asparagine) after recovery, which may contribute to the repair of cold injuries. Collectively, these results suggest the three species exhibit varying levels of thermal tolerance in both heat and cold temperatures, which are likely attributed to their distinct molecular and metabolic responses to thermal stress. Ultimately, the interspecific variations in thermal tolerance drive the shifts in the relative abundance of the species in the context of climate change.

1.1 Linking aphids' response to thermal stress at different levels

Numerous studies have described the biological responses correlated with climate changes (Parmesan 2006, Sambaraju et al. 2012, Kerr et al. 2015), but the underlying causal links have rarely been elucidated. It is crucial to understand the mechanistic connections between environmental stresses caused by climate change and the biological functions of organisms. In this vein, investigating the physiological basis of organism responses to climate change, including their tolerance limits to environmental change, has become increasingly important (Pörtner and Farrell 2008, Somero 2010, Bozinovic and Pörtner 2015). Ecophysiology requires identifying the differential effects of climate change—both sublethal and lethal—within and among species, as a prerequisite for predicting the ecological effects which result from the success or failure of organisms, populations, and species (Bozinovic and Pörtner 2015).

Individuals exposed to adverse climate change may reach a state that is beyond their capacity to maintain homeostasis. The physiological capacities of insects to resist and recover from stress can vary considerably over time, notably through intricate regulatory processes and adaptive mechanisms (Macmillan and Sinclair 2011, Gonzalez-Tokman et al. 2020). Ecophysiology, therefore, plays a pivotal role as a filter, bridging the gap between environmental constraints and the ecological performance of individuals, populations, and species. In line with this general framework, this work contributes to further unraveling the intricate connections between environmental stresses, filling the gap between insect physiology and their community responses to climate change.

In chapter two, we focused on examining the individual-level phenotype of aphids, specifically their survival response to stressful temperatures. Rather than relying on a single critical thermal limit as previous studies have done (Warren and Chick 2013, Birkett et al. 2018), we employed a thermal tolerance landscape approach. This approach allowed us to estimate the temporal dynamic and cumulative impact of environmental extreme temperatures on the survival of the three co-existing aphids. By doing so, we were able to extrapolate the laboratory-based survival response of individual aphids to predict population outcomes in natural settings. Our results revealed that *R. padi* was the heat-tolerant species. It was least affected by high temperatures during summer across the three sites along a latitude gradient. These findings align with field observations that indicate an increased abundance of *R. padi* in areas with frequent occurrences of extremely high temperatures, particularly in southern regions of China (Ma et al. 2015, Zhu et al. 2021). Conversely, *M. dirhodum*, which was cold-tolerant but sensitive to heat, thrived in cooler and alpine areas but gradually disappeared as the temperature rose during spring in the field (Hu and Gui 1985, Lei et al. 2021).

Subsequently, we delved into mechanisms underlying the variations in thermal response among the aphid species. Our investigation focused on two aspects: the molecular chaperones at the molecular level (Chapter 3) and the cytoprotectants at the biochemical level (Chapters 4 and 5).

In Chapter 3, we designed a gene-expression experiment involving two different temperature levels (acute and less acute) and two treatments (same exposure time and same injury level) for both

heat and cold stress. This approach allowed us to broadly explore and compare the response of molecular chaperones to stress among species. In accordance with its higher heat tolerance, *R. padi* had more heat-inducible *hsp* genes and a higher expression level of *hsps* at heat compared to the other two species. Notably, *hsp70A*, *hsc70*, and *hsp90* were the most heat-responsive genes to heat stress. The induction of *hsps* is known to protect against heat stress, and the expression levels of *hsps* have been positively correlated with thermal tolerance (Huang and Kang 2007, Lu et al. 2016). Furthermore, *R. padi*, which suffered the most serious damage from exposure to cold, showed the highest expression of *hsp70A* following recovery from cold stress. HSPs may indirectly contribute to reparation during the cold stress recovery phase by repairing damage (Lubawy et al. 2022). Furthermore, *M. dirhodum* exhibited a greater number of cold-inducible genes, which likely assist in efficient repair mechanisms for cold injuries.

In Chapter 4, we utilized quantified GC-MS profiling to examine the metabolic response of the three aphid species to heat stress. To better revealed the difference among species, we also designed two experiments: exposure to a high temperature (34 °C) for increasing durations corresponding to their 1/2 Lt_{50} (median lethal time) and 2/2 Lt_{50} as well as the same exposure duration. Following exposure durations corresponding to their 2/2 Lt_{50} , all three species displayed disturbances in metabolic homeostasis and alterations in energy metabolism. However, the heat-tolerant species, *R. padi*, demonstrated fewer perturbations and higher levels of mannitol compared to the less-tolerant species after the same duration of exposure. Furthermore, *R. padi* possessed species-specific polyols, glycerol. Polyols have been found to play a crucial role as osmotic pressure regulators (Garcia-Perez and Burg 1991), function as a scavenger of increased free radicals (Niu et al. 2003) and protect proteins from denaturation (Back et al. 1979), thereby prolonging survival time during heat stress. Therefore, we suggest that these polyols may confer greater heat tolerances to *R. padi*.

In Chapter 5, we followed a similar protocol as in Chapter 4 to explore the changes in metabolites of aphids after exposure to the same level of injury and duration of exposure. We discovered that the cold-tolerant species (*M. dirhodum* and *S. avenae*) exhibited similar metabolite profiles under stress, whereas the cold-susceptible species (*R. padi*) displayed distinct patterns. The energy metabolism of *R. padi* completely collapsed after higher levels of stress. Furthermore, we observed that the cold-tolerant species accumulated higher concentrations of certain molecules such as proline, asparagine, and trehalose. These molecules have been reported as cryoprotectants in other species (Kostal et al. 2016, Grgac et al. 2022, Kučera et al. 2022) and have protective functions in stabilizing proteins and membranes (Yancey 2005). These molecules may help to re-establish ion homeostasis, which is critical for insect cold tolerance (Olsson et al. 2016, Overgaard et al. 2021).

As noted by Pörtner, the effects of temperature on higher biological organization levels can be traced back to its direct impact on subcellular physiology and biochemistry (Pörtner et al., 2017). In our study, we observed that the combined molecular and biochemical findings indicated *R. padi* possessed more robust thermoprotective mechanisms against heat stress than the other two species.

This enhanced thermoprotection contributes to the better survival response of individual aphids, ultimately resulting in population advantages within communities facing increasingly frequent and intense high temperature events associated with climate change (Ma et al. 2015, Zhu et al. 2021).

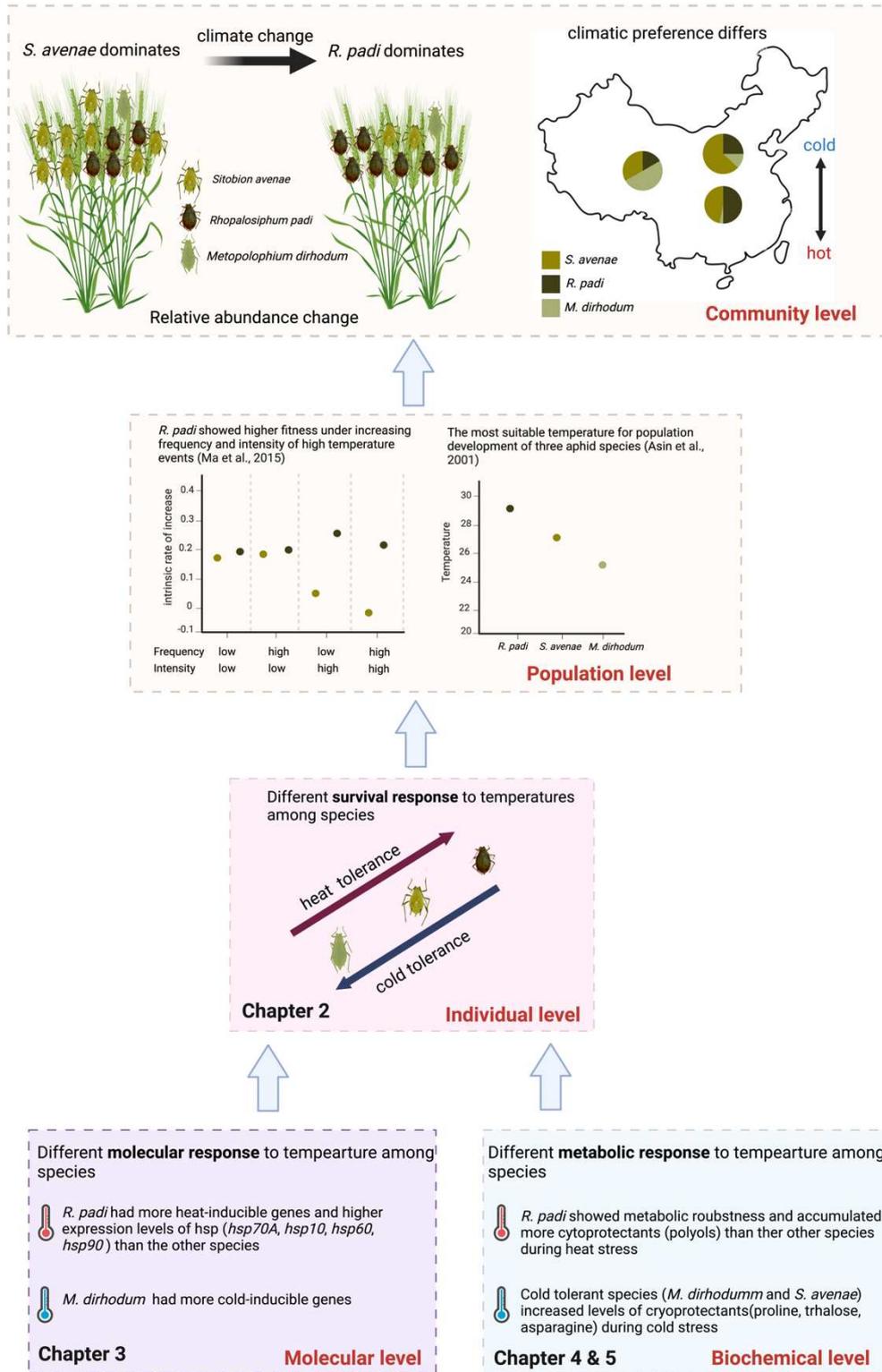


Figure 1. A schematic diagram to summarize the main findings of this thesis.

1.2 Greater variability of cold tolerance than heat tolerance

In our study, we observed greater variations in tolerance to low temperatures compared to high temperatures among the three species. Specifically, the sCT_{max} ranged from 35.21 to 36.97 °C, while the sCT_{min} ranged from -2.84 to -8.94 °C (Table 1-2, Chapter 2). Between species, Lt_{50} at 37 °C (the intermediate of the tested temperatures) showed less than a twofold difference, while Lt_{50} at -7 °C exhibited a difference of over 800-fold (Table 1-2, Chapter 2). These findings align with the previous studies on some terrestrial ectotherms such as *Drosophila* flies (Kellermann et al. 2012), lizards (Grigg and Buckley 2013, Herrando-Pérez et al. 2020) that generally lower thermal limits are far more labile across species, clades, geographical locations than upper limits (Addo-Bediako et al. 2000, Sunday et al. 2011, Kellermann et al. 2012, Hoffmann et al. 2013). Recent studies have extended this pattern to a wide variety of organisms including endotherms and plant species. (Araujo et al. 2013). Therefore, the observation that upper thermal limits are less variable than lower thermal limits holds across the entire tree of life at the global scale.

There are three distinct but non-mutually exclusive mechanisms for this asymmetric variation in heat and cold tolerance. First, “deep-time climate legacies”. On a paleoclimate scale, earth has been predominantly warm with intermittent glaciations, thus most clades may have originated during warm periods and may have limited opportunities to evolve beyond the warm environment conditions in which they arose (Araujo et al. 2013, Bennett et al. 2021). Secondly, “physiological boundary”, asymmetry in conservatism of lower and upper thermal limits is likely to be the consequence of different physiological processes operating near the critical thermal limits (Araujo et al. 2013). In animals, variation in lower thermal limits can be attributed to the thermodynamic effects of temperature on reaction rates, and the regulation of ion homeostasis (Overgaard and MacMillan 2017, Macmillan 2019). On the other hand, variation in heat tolerance is more simply controlled by the ability of organisms to counteract the destabilizing effects of high temperature on membranes and proteins, involving ancient polypeptides like HSPs that are highly conserved across different organisms (Feder and Hofmann 1999, Bowler and Terblanche 2008). Thirdly, “adaptation to current climatic extremes”, Current climatic extremes exert great selection on lower compared to upper thermal limits. The reasons lie in 1) Maximum environmental temperatures tend to be less variable across latitudes compared to minimum temperatures. 2) Behavioral buffering is more likely to reduce selective pressure on heat tolerance relative to cold tolerance. Recently study suggested these three mechanisms all affect species' thermal limits, but their relative importance varies. “Adaptation to current climatic extremes” is the strongest determinant of species' thermal tolerance limits (Bennett et al. 2021).

1.3 Commonalities and differences between heat and cold response

Both heat and cold stress can disrupt cellular membranes and denature macromolecular structures, including enzymes, leading to changes in enzyme reactions and metabolic compensations (Neven 2000, Szyperski et al. 2006, Bowler 2018, Macmillan 2019). Organisms have mechanisms to protect against such damage or repair it through the induction of Heat Shock Proteins (HSPs) and metabolic adjustments.

In our study, we examined the expression of *hsps* in response to cold and heat treatments, and we found distinct differences in these responses. Firstly, the timing of the response varies between heat and cold stress. *Hsps* were expressed during the recovery phase from cold stress, whereas they were upregulated during the actual heat stress. Although we did not directly measure the expression of *hsps* during cold stress, previous research has shown that *hsps* are only expressed during the recovery phase following cold stress (Colinet et al. 2010). Secondly, the expression of *hsps* in response to heat stress was significantly stronger compared to cold stress. In fact, we observed that the expression of *hsps* increased more than 2000-fold in response to high temperatures. In contrast, the maximum fold change in *hsps* expression level in response to low temperatures was only 40-fold (Figure 2 and 5, Chapter 3). This disparity can be attributed to the fact that heat stress poses a more immediate and severe effects to cellular integrity and protein stability, whereas only extremely severe cold stress has the potential to denature proteins.

When it comes to the effects of heat and cold stress on metabolism, our study revealed both similarities and differences. We observed commonalities such as disruptions in energy metabolism and the accumulation of cytoprotectants. Specifically, we found that intermediates and products of the tricarboxylic acid (TCA) cycle, along with anaerobic metabolism-related compounds like lactic acid and alanine, accumulated under both heat and cold stress. These findings indicate disturbances in energy metabolism and an increased reliance on anaerobic pathways. Similar metabolic responses have been reported in other insect models as well (Zhu et al. 2019, Lubawy et al. 2022). Additionally, both heat and cold stress can trigger the accumulation of cytoprotectants, such as polyols, which aid in maintaining membrane integrity and stabilizing proteins under extreme temperatures (Back et al. 1979, Yancey 2005, Yoder et al. 2006).

However, differences emerge when considering the extent of metabolite changes. Heat stress generally leads to an increase in metabolic rates due to accelerated biochemical reactions, whereas cold stress reduces metabolic rates as biochemical reactions slow down and enzyme activity decreases (Macmillan 2019). These variations in metabolic rates contribute to differences in the magnitude of metabolite changes observed. For instance, under heat stress, the highest fold change in metabolites can exceed 70-fold, whereas under cold stress, the highest fold change is typically around 8-fold.

1.4 Limitations

This thesis aims to bridge the individual, molecular, and metabolic responses to temperature with the community-level response observed in the field. However, several limitations in this study hinder its ecological relevance. First, I will discuss the limitations of Chapter 2. 1) The risk assessment model based on TDT curves neglected the capacity of thermal regulation in three aphid species. The test temperatures were based on recorded air temperature, which may not accurately reflect the temperature experienced by insects. Insects can relocate to more suitable microclimates, known as avoidance behavior (Pincebourde and Woods 2020). Previous studies have suggested that behavioral differences may be more important than physiological thermotolerance in explaining resistance to extreme temperatures (Alford et al. 2014). 2) The tolerances measured in this study represent basal tolerance without acclimation or hardening. In nature, the cold tolerance of insect species may change seasonally with acclimation (Overgaard et al. 2008, Bujan et al. 2020). During summer, as the temperature gradually increases to a peak, insects also undergo rapid heat hardening, which may enhance their heat tolerance (Zhao et al. 2017, Sørensen et al. 2019). 3) In this study, survival (Lt_{50}) was measured as the critical thermal limit. While there is evidence that these critical thermal limits for activity and survival are of great importance for animal fitness and biogeography in some taxa (e.g., insects) (Marshall and Sinclair 2012, Overgaard et al. 2014), relying solely on the “easy” survival criteria could strongly overestimate ecologically meaningful survival. Ecologically, only those individuals that retain fitness (the capacity to produce offspring) after thermal stress should be counted (Košťál et al. 2019). This may hinder our ability to translate individual-level processes to population-level outcomes. Furthermore, some argue that the long-term fitness effects of milder temperatures (e.g., acting directly on animal growth or reproduction, or through impairments in biotic interactions) are more ecologically meaningful (Pörtner et al. 2017)

These limitations of Chapter 3 lie in 1) We only examined a subset of *hsp* genes, without considering all chaperones. Besides, other genes such as cuticular protein genes, ion transport-related genes, and cryoprotectant transporters genes were found to be associated with thermal tolerance (Guo et al. 2022, Teets et al. 2022). To gain a holistic understanding of the molecular response, future studies could employ transcriptomic analysis (Enriquez and Colinet 2019b). 2) We did not differentiate between the expression of *hsp* genes in aphid hosts and those in endosymbionts. Aphids' symbionts also express *hsp* genes, which may contribute to thermal stress protection (Burke et al. 2010a, Zhang et al. 2019) (see the below future work section for details) 3) While the up-regulation of HSPs can rapidly confer thermotolerance, it remains unclear whether this thermal plasticity enhances the fitness of genotypes in natural environments. 5) It is worth noting that although heat-tolerant species showed higher expression levels of *hsps*, correlation does not imply causation. Additional research is needed to elucidate the specific function of these *hsps*, which could be accomplished through gene knock-down or RNAi techniques, as previously demonstrated in Colinet et al. 2010.

There are also some limitations in the metabolomic studies (Chapters 4 and 5). Firstly, due to technical constraints, certain molecules with relatively low concentrations, such as bioamines, could not be identified or quantified, resulting in an examination of only a subset of small molecules. Secondly, inferring metabolic pathways based solely on this subset of small molecules carries inherent risks. Some researchers have employed transcriptional approaches at the same time to determine the activity of enzymes involved in metabolic pathways, which provided additional evidence to support such inferences (Zhu et al. 2019). Thirdly, the observed metabolic changes can either indicate an adaptive biochemical adjustment (e.g., the accumulation of cytoprotectants) or they may represent downstream consequences of a disrupted physiological capacity (Hayward and Colinet 2023). In our study, cytoprotectants (specifically polyols) were found to be abundant in heat-tolerant species. However, it remains to be tested whether these metabolites can confer higher thermal tolerance. One possible approach is to artificially increase the levels of certain cryoprotectants in the insect body through diet augmentation, as previous experiments have shown promising results (Yoder et al. 2006, Košťál et al. 2011, Kostal et al. 2016). Furthermore, thermal tolerance has been linked to membrane modifications, such as an increase in the proportion of unsaturated fatty acids and/or short-chain fatty acids in response to cold (Teets and Denlinger 2013, Enriquez and Colinet 2019a). Further studies are required to delve into this aspect.

1.5 Application value for pest management and crop production

Aphids are among the most important agricultural pest insects, reducing crop yields via direct mechanical damage to crops or viral transmission of plant diseases (Brault et al. 2010). Temperature directly affects the development, reproduction, and survival of pests, which in turn determines their population dynamics and potential for causing damage to crops (Secretariat et al. 2021). Thus, investigating pest species' response to temperature is crucial for predicting their population dynamics and managing their impact on agricultural systems (Skendzic et al. 2021). This study measured the survival of three aphid species at different ages under a broad range of extreme temperatures. Based on the survival data, a thermal death time model was developed to assess the mortality risks under the naturally fluctuating temperature regimes. This model enables the calculation of cumulative thermal mortality for aphids at different ages over a specified period ranging from one day to a year. As a result, it could quantitatively estimate the relative abundance of co-existing species. While acknowledging the simplicity and numerous limitations of this model (as discussed in the limitations section), our study establishes a framework that could be enhanced and refined to create a more robust model capable of predicting the population dynamics of aphid species in field conditions. Additionally, our study indicated that under climate warming conditions and the occurrence of more frequent high-temperature events, the competitive advantage of *R. padi* over *S. avenae* in southern areas of China may be further amplified, potentially driving their distribution northward. The relative abundance of different aphid species can have implications for the intensity of virus infection in

cereals, such as the severe viral disease Barley yellow dwarf (BYD) caused by members of the Luteoviridae family (Domier 2008). *R. padi* is responsible for 90% of autumn infections leading to yield losses in barley and wheat (Fabre et al. 2003). While the transmission rate of the other two species remains to be tested in the field (Zhang et al. 1983), changes in the relative abundances of these species can have important ecosystem-wide consequences (Ma et al. 2015). By understanding the response of aphids to temperature and implications for their population dynamics and virus transmissions, farmers and researchers can make informed decisions about the application of control measures, resource allocation, and overall pest management planning. This knowledge helps optimize pest management practices and mitigate the impact of aphids on crop yields and disease transmission.

2 Prospects/ future works

2.1 Compare with European aphid populations

In western France, characterized by mild winter temperatures, the winter aphid community in cereal crops is composed of the same three co-existing species as in our study. However, these species exhibit distinct differences in their winter success. During winter, *R. padi* is the most abundant species, followed by *S. avenae* (Tougeron et al. 2018). On the other hand, *M. dirhodum* has increased in abundance in recent warmer winters but generally remains less abundant than *S. avenae*. As winter progresses into late winter and early spring, the number of *R. padi* decline while *S. avenae* becomes dominant, indicating a shift towards a spring aphid community (Alford et al. 2014). For many years, *S. avenae* has been recognized as the dominant species in the spring, representing more than 80% of the aphids (Andrade et al. 2015). However, in recent times (less than 10 years), populations of *S. avenae* have been declining and are being replaced by *M. dirhodum* (Tougeron et al. 2018). Previous research has suggested that although *R. padi* is the least cold tolerant among the three species, a significantly large number of *R. padi* remain attached to the host plant at extreme sub-zero temperatures than *S. avenae* and *M. dirhodum* (Alford et al. 2014, Alford et al. 2016). This ability of *R. padi* to withstand harsh winter conditions may explain its success during winter (Alford et al. 2014). The increase in *M. dirhodum* populations in spring could be attributed to its sensitivity to the mean temperature, with the current warmer conditions favoring this species more than in the past (Tougeron et al. 2018). However, the response of these three aphids to heat in France remains unknown.

It is fascinating to observe that the dominant species, *S. avenae*, within the aphid community during spring underwent a shift in both France and China, but towards different species. In France, the shift was towards *M. dirhodum*, while in China, it was towards *R. padi*. This phenomenon led to the hypothesis that the variance in thermal tolerance among populations in the two countries, along with dissimilar climates, might account for these contrasting shifts. The original intention of this Ph.D. project was to investigate and verify this hypothesis. Unfortunately, due to time constraints, the planned research could not be conducted. Nevertheless, this aspect of the study holds promise for future exploration and can be anticipated in subsequent research endeavors.

2.2 The role of symbionts

Symbionts can influence the thermal tolerance of their hosts. One well-known example is the algal symbionts that shape the thermal tolerance of corals (Berkelmans and Van Oppen 2006). Similarly, in insects, bacterial symbionts have also been found to alter the thermal tolerance of hosts (Wernegreen 2012, Henry and Colinet 2018). Aphids are a notable example in this regard. All aphids except a few families harbor a primary symbiont, *Buchnera aphidicola* (Chong and Moran 2016) and one or more secondary symbionts (Guo et al. 2017). *B. aphidicola* resides in specialized cells called “bacteriocytes”, which are usually clustered together to form a paired bacteriome organ called the “bacteriome” (Douglas and Emden 2007). The primary symbiont mainly provides aphids with nutrients such as amino acids and vitamins that are lacking in their diet, while secondary symbionts enhance aphids' resistance to biotic and abiotic stress (Douglas 1998, Guo et al. 2017).

Recent studies have also indicated that primary and secondary symbionts may be involved in heat tolerance in aphids (Dunbar et al. 2007, Burke et al. 2010a, Heyworth et al. 2020). *B. aphidicola* is highly susceptible to high temperatures with short-term high temperature treatment significantly reducing its density (Ohtaka and Ishikawa 1991, Chen et al. 2009). The heat sensitivity of primary symbionts may be attributed to their severely reduced genomes and fragile protein structures (Perkovsky and Wegierek 2018). Since *B. aphidicola* is strictly maternally inherited and has a small population size, harmful mutations can easily accumulate, making it a vulnerability for aphids and limiting their thermal tolerance. This may also explain the main distribution of aphids in temperate regions of the northern hemisphere (Perkovsky and Wegierek 2018).

The genotype of the symbiont plays a role in the heat tolerance of aphids. For example, a single-nucleotide deletion in the promoter region of the heat shock gene *ibpA* in *B. aphidicola* (heat-sensitive mutants) resulted in reduced heat tolerance (Burke et al. 2010b). Replacing the heat-sensitive mutant symbiont with a heat-resistant strain enhances the heat tolerance of green peach aphids (Moran and Yun 2015). Furthermore, differences in the heat sensitivity of *B. aphidicola* contributed to variations in heat tolerance among different aphid species (Zhang et al. 2019).

Laboratory studies on aphids and several other insects have shown that secondary symbionts can increase the host's tolerance to temperature and improve their fitness under heat stress (Montllor et al. 2002, Russell and Moran 2006, Brumin et al. 2011, Truitt et al. 2019, Heyworth et al. 2020). Heat shock reduces aphid fecundity, but aphids infected with certain facultative symbionts, such as *Regiella* or *Fukatsuaia*, experience less reduction in fecundity compared to uninfected aphids (Heyworth et al. 2020). The mechanism by which these secondary symbionts confer thermotolerance is not yet fully understood, but they may involve inducing the expression of thermotolerance-related genes in the host (Brumin et al. 2011) or indirectly changing the behavior of the host (Truitt et al. 2019). Secondary symbionts may also protect primary symbionts by lysing themselves to release metabolites, thereby enhancing host thermotolerance (Burke et al. 2010a).

While some studies have explored the relationship between insect symbionts and cold tolerance (Henry and Colinet 2018, Raza et al. 2020), research specifically on the symbionts and aphids' cold tolerance is lacking.

Our study has revealed significant interspecific differences in thermal tolerance among three aphid species, and these differences may be attributed to their symbionts. Therefore, further research is warranted to explore the role of symbionts, particularly secondary symbionts, in determining the thermal tolerance of these aphid species.

3 Conclusions

Throughout this thesis, we have revealed the substantial interspecific variations in thermal tolerance among the three aphid species (*M. dirhodum*, *S. avenae*, and *R. padi*), which may be attributed to their distinct metabolic and molecular responses to thermal stress. By thoroughly examining these responses at the individual, and sub-individual levels (biochemical and molecular levels), our research contributes to a deeper understanding of the mechanistic connections between environmental stresses caused by climate change and the biological functions of organisms. While the complete identification of biochemical and molecular mechanisms governing thermal tolerance in these species remains ongoing, our works have allowed us to propose and refine several hypotheses. Overall, our study sheds light on the intricate relationship between thermal stress and species survival and understanding the ecological dynamics and population resilience in the context of changing climatic conditions. This knowledge has implications for conservation and management strategies in the face of climate change.

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Titre : Impact du changement climatique sur les communautés de pucerons : réponses physiologiques et moléculaires à la température

Mots clés : pucerons des céréales, tolérance thermique, protéines de choc thermique, métabolomique

Résumé : Le changement climatique engendre une augmentation de la fréquence des phénomènes de température extrême, ce qui a des conséquences sur la physiologie des individus et les fonctions des écosystèmes. Des études antérieures ont montré que les phénomènes de température extrême modifient la hiérarchie de dominance de trois espèces de pucerons des céréales coexistantes (*Rhopalosiphum padi*, *Sitobion avenae*, *Metopolophium dirhodum*). Nous émettons l'hypothèse que ce phénomène est étroitement lié à leur tolérance thermique basale différente, résultant de tolérances physiologiques distinctes au stress thermique. L'accumulation de cytoprotecteurs et l'expression de gènes de protéines de choc thermique (*hsps*) sont les mécanismes biochimiques et moléculaires les plus importants pour accroître la tolérance physiologique au stress thermique, respectivement.

Dans cette thèse, nous avons constaté que: 1) les trois espèces de pucerons ont montré des variations interspécifiques significatives en termes de tolérance à la chaleur et au froid, ce qui pourrait entraîner des lésions thermiques différentes sur le terrain. 2) les espèces tolérantes à la chaleur avaient plus d'*hsps* inductibles par la chaleur et des niveaux d'expression plus élevés d'*hsps* pendant le stress que les autres espèces. Les espèces tolérantes au froid avaient plus de gènes inductibles par le froid. 3) les espèces tolérantes à la chaleur ont montré des niveaux incroyablement plus élevés de polyols par rapport aux autres espèces lorsqu'elles étaient exposées à la chaleur pendant la même durée. 4) les espèces tolérantes au froid ont affiché des niveaux accrus de cryoprotecteurs après avoir récupéré du stress causé par le froid.

Title : Impact of climate change on aphid communities: physiological and molecular responses to temperature

Keywords : cereal aphids, thermal tolerance, heat shock protein, metabolomics

Abstract : Climate change leads to more frequent temperature extremes, triggering consequences from individual physiology to ecosystem functions. Previous studies have found that extreme temperature events shift the dominance hierarchy of three co-existing cereal aphid species (*Rhopalosiphum padi*, *Sitobion avenae*, *Metopolophium dirhodum*). We hypothesize that this phenomenon is closely linked to their different basal thermal tolerance, driven by distinct physiological tolerance to thermal stress. The accumulation of cytoprotectants and the expression of heat shock protein genes (*hsps*) are the most critical biochemical and molecular mechanisms for increasing physiological tolerance to thermal stress, respectively.

In this thesis, we found that 1) the three aphid species showed significant interspecific variations in heat and cold tolerance, which may lead to different thermal injuries in the field. 2) the heat-tolerant species had more heat-inducible *hsps* and higher expression levels of *hsps* during stress than the other species. The cold-tolerant species had more cold-inducible *hsps*. 3) the heat-tolerant species showed incredibly higher levels of polyols compared to other species when exposed to heat for the same duration. 4) the cold tolerant species displayed increased levels of cryoprotectants after recovering from cold stress.