Thermal biology of Australian native stingless bees (*Tetragonula* spp.)

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Adaptive thermal responses in Australian native stingless bees (*Tetragonula spp.*)

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Author Attribution

The work contained in the body of this thesis, except otherwise acknowledged, is the result of my own investigations.

Chapter 2 is being prepared for submission. Inez Ayn Vlasich-Brennan (IVB) collected, conducted, and analysed the data. The co-authors of this study are Ros Gloag (RG), Vanessa Kellermann (VK), Carmen da Silva (CdS), Venkatesh Nagarajan (VN) and Julianne Lim (JL). RG, VK, and CdS contributed to the study design. CdS supplied previously sampled *Tetragonula* thermal tolerance data (accounting for a quarter of all thermal assay data analysed in this chapter) and enabling this study to properly capture *T. hockingsi*'s full distribution into Cape York in Far Northern QLD. Julianne Lim (JL) assisted with species identification analysis in the lab. All co-authors provided valuable feedback and editing and enormous support and encouragement.

Chapter 3 is being prepared for submission. IVB performed the transplants, and collected and analysed all data in this experiment. The co-authors of this study are Ros Gloag, Vanessa Kellerman, Venkatesh Nagarajan, Carmen da Silva, Julianne Lim, Alexander Austin (AA), and Tim Heard (TH). RG, VN, VK, and CdS contributed to the study design. AA and TH contributed beekeeping skills in the field. Julianne Lim (JM) assisted in lab work and genetic analysis. VN and RG assisted in the initial modelling of the data. All co-authors provided valuable feedback and editing.

Statement of Authorship

I certify that this thesis has not been previously submitted for any other degree or purposes nor has it been submitted as part of requirements for a degree.

I also certify that the research described in this thesis is the original work of myself, the author, and that any assistance received in my research work and the preparing and proof reading of my thesis and sources have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in this thesis also.

Inez Ayn Vlasich-Brennan | 28 February, 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Ros Gloag | 28 February, 2023

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Abstract

Eusocial stingless bees (Tribe Meliponini) are widely distributed in the major tropical and sub-tropical geographic regions either side of the equator, including South and Central America, Africa, India, South East Asia, New Guinea and Australia. Over 500 species have been identified worldwide, of which 11 occur in Australia. These bees are key pollinators in many natural ecosystems and are also increasingly used as managed pollinators of tropical fruit and nut crops. This thesis investigates the thermal biology of two endemic Australian species of East Coast stingless bees in the genus *Tetragonula*: *T. carbonaria* and *T. hockingsi*. Both species are broadly distributed along East Australian latitudinal climate gradients. They have predominantly allopatric ranges, occupying different climates and latitudes, though with several areas of overlapping range in Queensland.

I first conducted assays of *Tetragonula* thermal tolerances (critical thermal maxima and minima) across their distributions to understand how these traits varied between species, and whether environmental variation (such as temperature and precipitation) explained variation in these traits **(Chapter 2)**. I found that *T. carbonaria* was more cold tolerant than *T. hockingsi*, while *T. hockingsi* was more heat tolerant than *T. carbonaria*, suggesting the species have partitioned thermal environmental niches. I also found evidence of latitudinal and climatic clines in thermal tolerances.

I next conducted a year-long reciprocal transplant experiment of *T. carbonaria* colonies within their N.S.W. range (temperate to sub-tropical) to better understand how much variation within-species was shaped by environment (i.e., thermal tolerance plasticity) and/or innate genetic variation (consistent with local adaptation) **(Chapter 3).** I found that more temperate-distributed *T. carbonaria* show greater cold tolerance in all environments, consistent with local adaptation. That is, plasticity alone does not seem to explain within-species variation in *T. carbonaria* thermal tolerances, at least for cold tolerance. In all, my research has taken an important step forward in understanding the thermal biology of these important insect pollinators, and highlighted that the thermal sensitivities of these species are likely importantfor predicting how they will respond to a rapidly warming climate.

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Chapter 1

1.GENERAL INTRODUCTION

1.1 Climate change: how will insects respond?

Anthropogenic climate change will be a significant force shaping global biodiversity in the coming decades, with the broad scale redistributions of many species already observed (Pörtner et al., 2021). Fossil records indicate that rapid climate shifts were associated with Permian mass extinctions (260-183 Ma- rapid global warming ~ due to mass volcanic eruption emission), events in which ~90% of all species were wiped out (Benton, 2018). As such, the unprecedented rate of anthropogenic climate change, seeing rapid shifts in global climates, is predicted to poses a similar thereat upon our extant biodiversity, with fears of a sixth mass extinction event (Cowie et al., 2022; Shivanna, 2020).

Recent predictions indicate that under current greenhouse gas emissions and atmospheric CO_2 concentrations, global temperatures will increase by 1.2-1.9°C over the next decade, with upwards of a 5.7°C increase in the next 100 years if emissions remain high (Pörtner et al., 2022). With this, we will continue to see increased frequency and duration of acute weather events such as heat-waves and extreme temperature days. At the same time, traditional cooler seasons will become shorter and warmer (Masson-Delmotte et al., 2021). In Australia for instance, median winter temperatures are predicted to increase by up to 2.3°C within two decades (under a ~2°C warming scenario; Figure 1). Such changes in seasonal temperature cues may particularly affect species whose life cycles depend on fine-tuned phenology with their environment (Forrest, 2016; Stålhandske et al., 2015).

Global climate zones (or macroclimatic zones) are also shifting poleward in both northern and southern hemispheres (Cui et al., 2021; Figure 2). Regions at both higher elevations and latitudes are expected to see the greatest comparative increases in temperatures, thus conferring disproportional thermal stress on species occupying these habitats (Bale & Hayward, 2010). The ability of many species to simply track with 'favourable' environments over time is coming into question, as regional climate shifts threaten to outpace the dispersal or adaptive capabilities of many species (Pecl et al., 2017; Pörtner et al., 2021; Scheffers & Pecl, 2019).

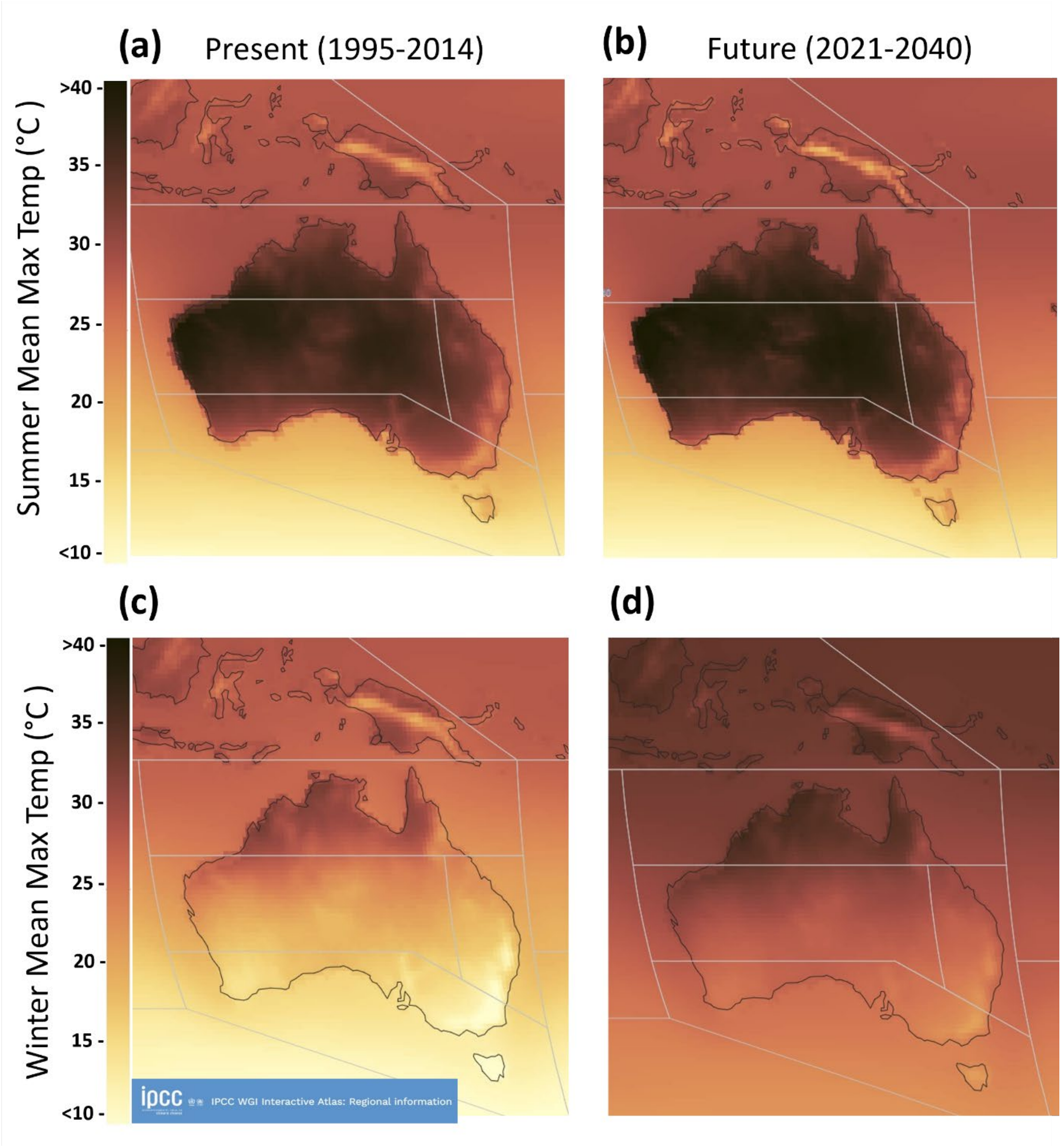

Figure 1. Maps depicting present and future (Mid-Term: 2041-2060) climates of Australia under a moderate climate change scenario ~2°C (CORDEX Australasia - Maximum temperature (TX) ° C). **(a)** Present and **(b)** future summer maximum temperatures; present **(c)** and future **(d)** winter maximum temperatures (June-Aug; RPC4.5). Y-axis indicate the mean temperature (°C) ranging from pale yellow: <10°C, to black: >40°C. Downloaded from IPCC WGI Interactive atlas: Regional information modelling (Gutiérrez et al., 2021; http://interactive-atlas.ipcc.ch/)

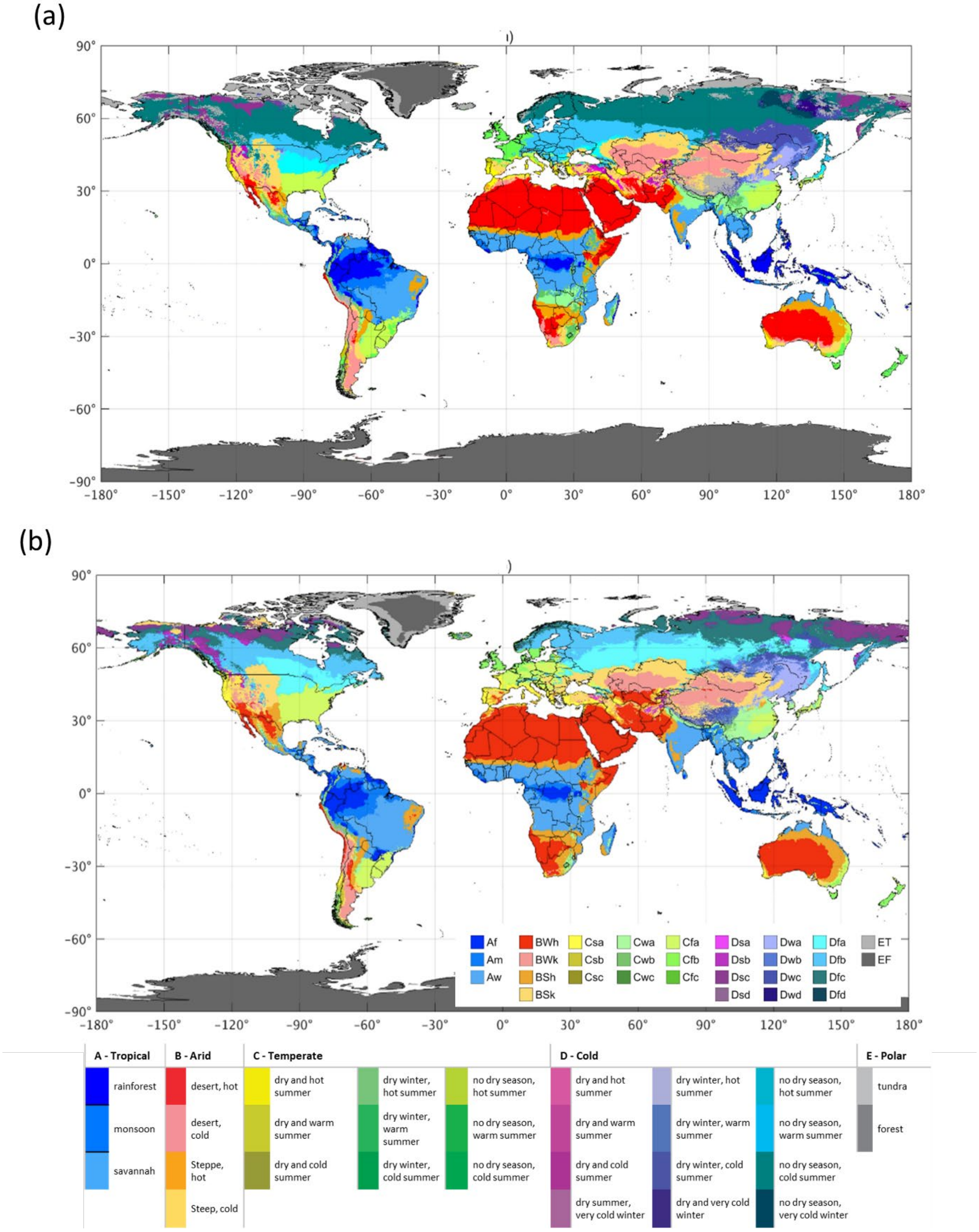

Figure 2. (a) Current (1980-2016) and **(b)** Future (2071-2100: based on high emissions scenario RCP8.5) Köppen-Geiger climate classification maps from Beck et al., (2020) publisher correction. Maps display the the five major climate zones (A-E) and the 30 climate sub-types; see colour key above and *https://doi.org/10.1038/s41597-020-00616-w*

Insects fulfil critical roles across every terrestrial (and some freshwater) ecosystem. They represent the vast majority of extant animal species-level biodiversity (>80% of all described species). Many insect taxa carry out key services, such as primary production (pollination and seed dispersal), ecosystem regulation (natural biological control, and food-wed stability), and nutrient cycling. They also represent some of the most economically important taxa to humans, providing us with natural products (food and pharmaceuticals) and critical auxiliary role in agricultural food security (Prather et al., 2013). Indeed, while some insects are crop pests, agroecosystems overall rely upon insects for pollination, natural pest control and bioturbation to enhance crop yields, economic returns, and long-term soil health (Rawluk & Saunders, 2019).

In insects, as in other ectotherms, temperature has profound direct and indirect influence upon almost all aspects of their biology at both micro and macro scales (e.g., metabolic rate, phenology, reproduction, and population structuring). As such, most insects have a high thermal sensitivity effecting many performance based traits(Huey & Kingsolver, 1989). Temperature fluctuations affect their performance-related traits and in turn should determine species' geographical distributions (Angilletta, 2009; Jørgensen et al., 2022). As such, two broad types of responses to climate change have been predicted for insects.

Firstly, more mobile insects (e.g., some *Hymenoptera* and *Lepidoptera*) may physically shift, expand, or reduce their current geographic ranges. For instance, two consecutive studies by Chen et al. (2009; 2011) found that a tropical moth (*Lepidoptera geometridae*) has been shifting its range to higher elevations in response to regional-scale-warming in Borneo over the last two decades. Such altitudinal shifts are probably already occurring for many other insects, hence are an important mechanism by which some highly-mobile populations can persist (McCain & Garfinkel, 2021). In the case of *L. geometridae*, this moths' 'cool' range boundaries (the leading dispersal front) was advancing substantially faster than its warm boundaries were receding, such that the lagging lower elevation populations were at greatest risk of local extinction (Chen et al., 2011). However, species can only shift their ranges up to a point, and many may be unable to retreat to aid higher elevations in this way.

Second, insects may use adaptive physiological responses (with or without range shifts) to remain in warming habitats (Kellermann & van Heerwaarden, 2019). These physiological responses can occur via adaptive evolution (i.e. innate thermal tolerances are underpinned by adaptive genetic variation and they evolve in response to increasing temperatures; Camus et al., 2017), or via phenotypic plasticity. Phenotypic plasticity is the change in phenotype in response to different environments. This can be adaptive, in that the change in phenotype increases the fitness of an organism in its new environment (Chevin & Hoffmann, 2017; Chevin et al., 2010). For example, increasing of thermal tolerance in warmer environments (Rodrigues & Beldade, 2020). Plasticity therefore allows a rapid response over the course of one lifetime, rather than over multiple generations (Clemson et al., 2016). It may be that successful adaptive changes are first facilitated by plasticity, enabling rapid buffering to increasing rates of warming that maintains populations long enough for any slower evolutionary adaptations to accumulate (Chevin et al., 2010). In this case, plasticity would need to be expressed in the direction which maintains or improves fitness under warmer conditions (Merilä & Hendry, 2014).

The likelihood of a given response to climate change in an insect species depends on a range of factors such as generation time, dispersal capability, the strength of selection imposed by climate change, and the presence of adaptive constraints on key traits. For example, heat tolerance traits in *Drosophila* species appear to be phylogenetically constrained rather than locally-adapted (Kellermann et al., 2012), with strong selection in ancestral environments inhibiting future evolution upon these traits (phylogenetic inertia). Moreover, some insects may already be existing close to their upper physiological limits or tolerated heat capacities (Araújo et al., 2013; Bale & Hayward, 2010; Hoffmann et al., 2013; van Heerwaarden & Kellermann, 2020; van Heerwaarden et al., 2016). While the prevalence of this trend remains debated (van Heerwaard and Kellerman, 2020) for certain insects further adaptation via increasing basal heat tolerance may be unlikely to occur (Sasaki & Dam, 2021; van Heerwaarden & Kellermann, 2020).

Some insect species may fail to adaptively respond adequately to changing climates. Under a 3°C warming scenario, up to 49% of insect biodiversity could be at risk of extinction (Warren et al., 2018). Mid-to high rates of climate change are predicted to outpace many aquatic insect species active dispersal capabilities (Woolway & Maberly, 2020). A key task for biologists therefore is to predict the future responses of species and ecosystems to environmental change, both to facilitate conservation of most at risk species (Scheffers & Pecl, 2019), and better prepare us for the flow on effects impacting food security, disease outbreaks, and pharmaceutical provisioning (Pörtner et al., 2021). An important first step in understanding and mitigating the risks of climate change upon insects is to better understand how past and present climates have shaped their observed distributions and the extent to which populations are locally-adapted to their current regional climates (Kellermann & van Heerwaarden, 2019). In the process, we can improve our understanding of the thermal biology of ectotherms and the macroecological mechanisms that drive global biodiversity.

1.2 **Critical thermal limits**

Critical thermal limits are part of a sweet of key resistance traits defining insects thermal sensitivities to environmental temperatures (Buckley et al., 2022; Sunday et al., 2012), and are increasingly proposed to be key determinants of insects geographical distributions (Bowler & Terblanche, 2008; Colinet et al., 2015) (Angilletta, 2009). For instance, cold tolerance is seen to predictably increase with decreasing local environment temperatures for ~20 *Drosophila* species (Addo-Bediako et al., 2000; MacLean et al., 2019; Sunday et al., 2011). Tropical *Drosophila* species existing in warmer and more climatically stable environments have reduced cold tolerance compared to temperately distributed species of the same genus. Latitudinal variation for heat tolerance has also been reported for some insects (e.g. two *Drosophila* sister species *D. leontia* and *D. kikkawai*; Ranga et al.,2017), though latitudinal clines in heat tolerance are less marked than those of cold tolerance (Kellermann & van Heerwaarden, 2019). In addition, thermal tolerance range (TTR; Figure 3) is predicted to differ between low and high latitude environments, due in part to seasonal temperature variations. More specifically, insects inhabiting higher latitudes should, in theory, have weaker selection upon heat tolerance combined with greater selection on cold tolerance resulting in a wider thermal tolerance range or broader thermal niche (Addo-Bediako et al., 2000; Lancaster et al., 2015; Figure 3). In a large comparative study, Sunday et al. (2011) found strong support for this idea across terrestrial ectotherms, with larger TTR observed for species in habitats with greater seasonal variability in temperature.

Long-term exposure at and beyond critical thermal limits either greatly impair fitness or become irrevocably lethal. Short term exposure at these critical limits can, however, induce plastic shifts in critical thermal limits in some insect species (via adult acclimation/hardening) (Sgrò et al., 2010; van Heerwaarden et al., 2016). Further, exposure to suboptimal temperatures during an insect developmental life stage have also been shown to shift thermal tolerance limits later in life, via developmental plasticity, possibly with longer lasting effects compared to adult acclimation (Kellermann et al., 2017).

Insects' thermal limits can be measured in several ways, each having different benefits and limitations: via thermal performance curves or via dynamic ramping (gradual temperature exposure; (Chown et al., 2009; Jørgensen et al., 2021)). Thermal performance curves (TPCs) measure fitness related traits (i.e., fecundity) across a range of temperatures creating a reaction norm. From these TPC's the temperature at which fitness reaches zero is used to describe the thermal limits of fitness. However, such measures generally require high levels of both environmental and developmental control, and as such are generally limited to measurements in model insect species such as *Drosophila* (Kellermann & van Heerwaarden, 2019). Dynamic ramping approaches estimate the temperature at which insects cease to move (die) and hence represent the absolute bounds of thermal tolerance and tend to be higher than thermal limits estimated from fitness traits. Chown et al. (2009) outlines the use of dynamic ramping to measure insects upper and lower critical thermal limits, with controlled temperature exposures gradually increased or decreased, observing at what temperature and time physiological failure occurs (e.g., loss of all motor functions).

Measures of thermal tolerance in insects can also vary in the endpoints that are measured, which include righting response (reflex correcting body orientation), coma (loss of all motor functions) and death (time to wright an obituary) (Jørgensen et al., 2021). Overall, dynamic assays are often the preferred method for measuring innate thermal tolerance responses in adult insects as they better represent what an individual would naturally experience (gradual abiotic temperature increase or decrease during diurnal or nocturnal activity periods). Furthermore, dynamic ramping methods are highly repeatable and provide rapid assessments of innate adult heat and cold tolerances, making them suitable even for use on non-model insect species in field-based studies. Indeed, in most cases, the best approaches for measuring thermal tolerance limits are set by the biology of the study organism itself. Not surprisingly, much of the current knowledge of thermal tolerance limits stems from *Drosophila* (a well-tested and representative model insect) and other invertebrate species. However, it is essential that we gather thermal tolerance data from diverse insect taxa, as this will enable us to better understand thermal biodiversity from a whole ecosystem perspective, which will be key to future mitigation strategies under climate change.

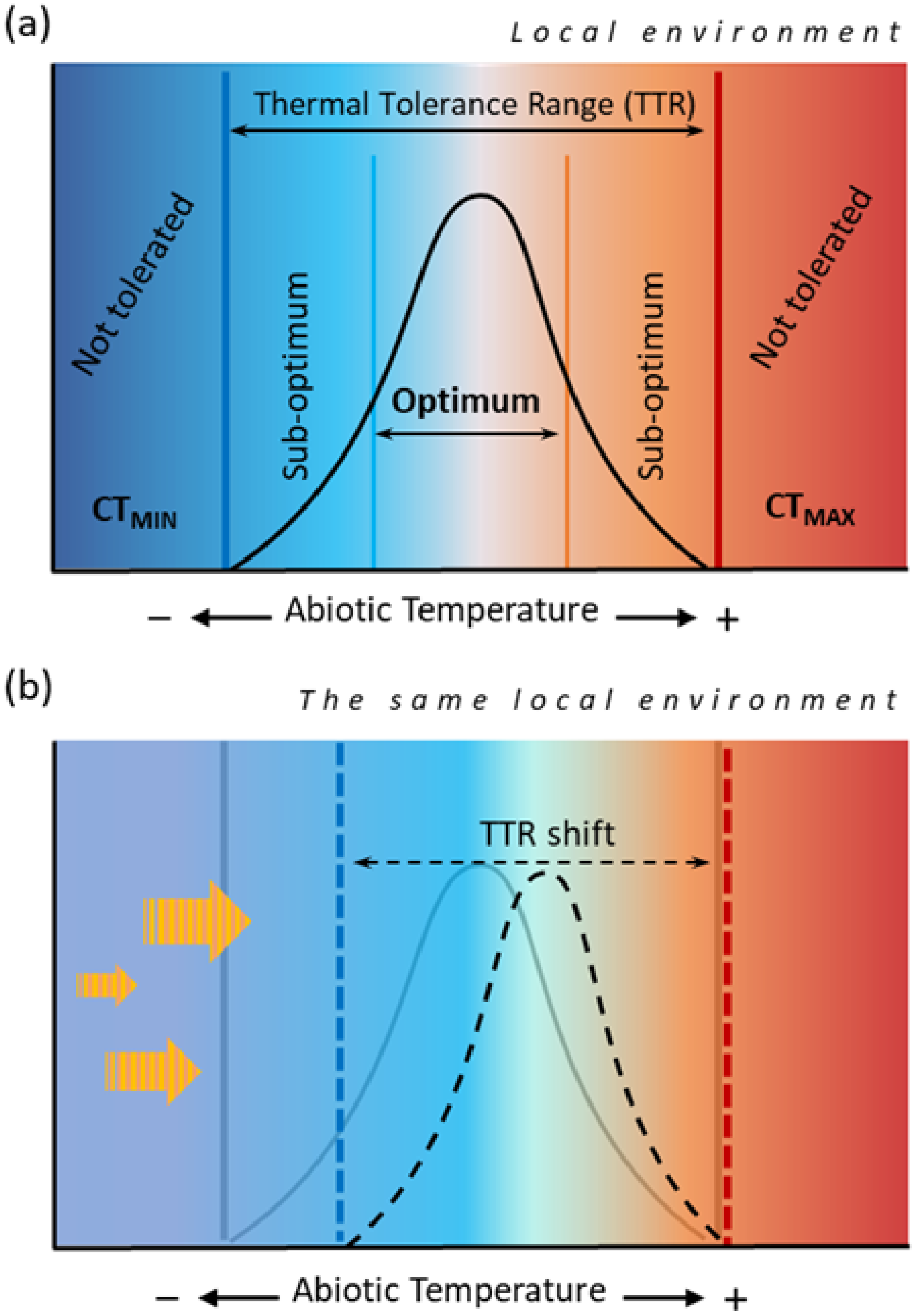

Figure 3. Thermal tolerance range (**TTR**) of organism or population in which (a) **Optimum** temperature range, with **sub-optimal** (stressful) upper and lower tolerated temperatures, into **un-tolerated** or lethal temperature zones: critical thermal maximum (**CT$_{MAX}$**) and critical thermal minimum (**CT$_{MIN}$**); (b) shows possible 'adaptive' shift in **TTR** (dashed lines) responding to an external stressor (orange arrows), e.g., warming environment, to remain in optimal temperature zone.

1.3 **Bees: vital pollinators in natural and agro-ecosystems**

Insect pollinators exist within plant-pollinator networks across a diverse range of natural, agricultural and urban ecosystems(Giannini et al., 2012). Bees (Anthophila) in particular are vital pollinators in these networks, having coevolved with angiosperms for an estimated 130 million years (Cardinal & Danforth, 2013). Bee larvae are obligate pollen feeders; therefore, bees rely on flowering plants for successful reproduction. In turn, many plants rely upon bees to transfer pollen between flowers and facilitate fertilization.

Today, an estimated 80% of all flowering plants are dependent upon insect pollination (Ollerton et al., 2011), with approximately 70% of all agricultural crops likewise reliant upon insect pollination alone (Kremen, 2018). For example, around two thirds of all crops grown in Australia benefit from insect pollination, in crops such as mangos and macadamias (Blanche et al., 2006; Cunningham et al., 2002). Population declines in bees due to direct and/or indirect climate change effects would see a breakdown in the plant-pollinator networks of agroecosystems, and threatens global food security (Potts et al., 2010). Some negative impacts on both commercial honey bee and wild bee populations have already been observed. For example, the direct effects of abiotic temperature increases has led to changes in bumblebee distributions (Pyke et al., 2016), and climate change has indirectly increased frequency of pathogens in global *Apis* populations (Proesmans et al., 2021).

Most of our current knowledge of the thermal biology of bees comes from studies on widespread northern hemisphere species, particularly the Western honey bee (*Apis mellifera*) and several bumble bees (*Bombus* spp) (Dzialowski et al., 2014; Kovac et al., 2014; Sánchez-Echeverría et al., 2019; Scriven et al., 2016). Bees, however, are a diverse clade with an estimated 20,000 species worldwide (Jamieson et al., 2019). *Apis* and *Bombus* species account for <2% of bee species diversity worldwide (*Apis*: ~10spp.; *Bombus*: ~250spp.)(Grüter, 2020a). Further, both *Apis mellifera* and *Bombus* spp. are large-bodied bees capable of facultative ectotherms (Dzialowski et al., 2014; Glass & Harrison, 2022; Stupski & Schilder, 2021); that is they can maintain relatively high body temperatures (in relation to ambient air temperature), whilst also having higher aerobic performance during flight (through heat shunting mechanisms between thorax and head). This enables them to forage and inhabit a broader range of thermal environments (Glass & Harrison, 2022), possibly facilitating greater fitness under dynamic temperatures regimes unlike more thermally sensitive and smaller ectothermic bee species. Southern hemisphere and tropical bee species in particular have received little attention, despite these bees inhabiting regions where global warming will have strong effects.

1.4 **Stingless bees**

Stingless bees (Tribe Meliponini) are a diverse clade of highly eusocial bees (approximately 550 identified species so far), broadly distributed across three major geographical regions: the Neotropics, Afrotropics and the Indo-Malay-Australasia tropics (Grüter, 2020; Figure 4). They are key pollinators in tropical forests and generalist foragers, visiting an estimated 215 different plant families (~60% of all angiosperm families) across their global distribution (Bueno et al., 2021). They are highly eusocial, living in colonies which typically contain one reproductive female (the queen), some males (which do not work) and hundreds or thousands of workers. Workers perform a range of tasks in the colony, including building brood, provisioning brood cells and foraging outside the colony for nectar, pollen and resin (Grüter, 2020b). Most species are observed to nest in tree cavities, or in the ground between tree roots.

Many stingless bee species can be readily kept and propagated in wooden or clay hives. As a result, they are used as managed pollinators for a range of tropical and subtropical crops in many of the regions where they naturally occur (Gonzalez et al., 2022; Heard, 2016), most notably in Brazil. Globally, the Western honeybee (*A. mellifera*) is the dominant managed pollinator of crops, having been introduced to every human-occupied continent in the past few hundred years in association with agriculture and honey production. However, honey be populations have shown instability in recent years due to complex factors including climate change, pests and pathogens (Kremen, 2018; Potts et al., 2010). Therefore there is increasing interest in the use of stingless bees as alternative managed pollinators in the areas where they are native (Cunningham et al., 2002), to reduce reliance on honey bees for this critical service. Indeed, stingless bees are better adapted than honey bees to the warm climates in which many fruit and nut crops are grown, and therefore ideally suited to this role.

There is already some evidence, however, to suggest that stingless bee populations may be negatively impacted by climate change (Maia et al., 2020; Toledo-HernÁNdez et al., 2022). A recent study on the Neotropical stingless bee *Melipona interrupta* in Brazil (Becker et al., 2018) found that a 2°C increase above natural average hive temperatures significantly increased brood mortality and reduced female production (i.e. skewed sex-ratios). Another important consideration is the fact that many stingless bee species are not seen to regulate hive temperatures to the same extent as *Apis* (which will actively keeping brood temperatures between 33-36°C, even in the Northern European winter; Jones & Oldroyd, 2006) . Stingless bee may therefore face an increased risk of global warming as many insects developmental life stages show greater levels of thermal sensitivity (Kingsolver & Buckley, 2020). Whether stingless bee foragers are capable of facultative endothermy is unknown, but a recent study reported that Brazilian stingless bee foragers (*Melipona subnitida*), inhabiting tropical dry forests, experienced overheating in head and abdomen (measured via thermal imaging and previous study estimates of bees critical thermal maximum; Hrncir et al., 2019) when coupled with long distance foraging, when air temperatures were >30°C (Souza-Junior et al., 2020). These recent studies have highlighted our need to better understand how the thermal biology of stingless bees, and their climate change vulnerability (Hrncir et al., 2019; Kingsolver & Buckley, 2020; Souza-Junior et al., 2020; Zhao et al., 2021).

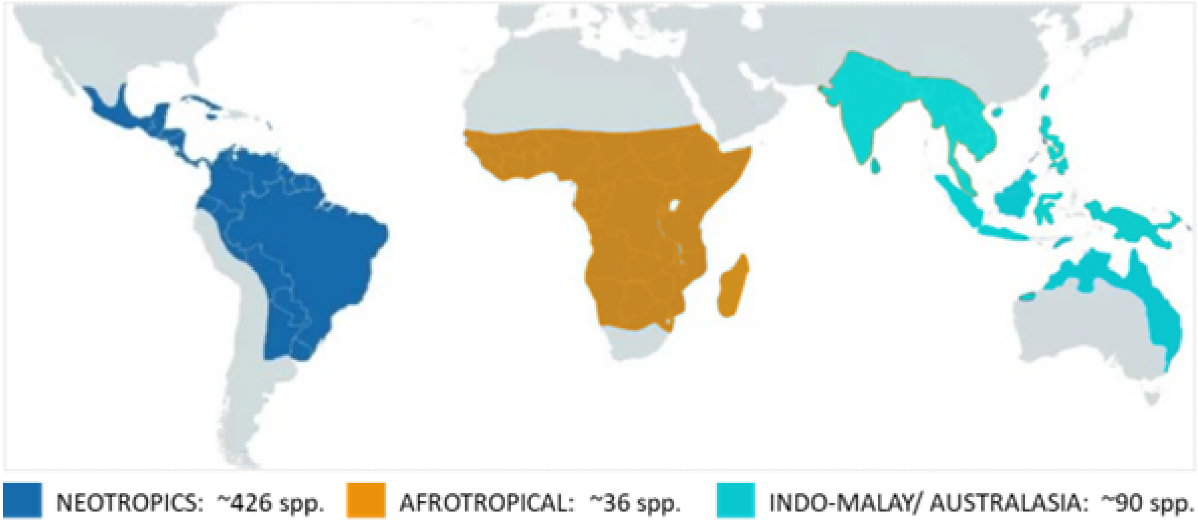

Figure 4. The distribution of tingless bees (Meliponini) as of 2020; Neotropic species distribution (Blue), Afrotropical (Orange), Indo-Mayay (Turquoise). Map adapted from Grüter (2020b)

1.5 **Australian *Tetragonula* spp. stingless bees**

Australia is home to 11 species of stingless bees across two genera: *Tetragonula* and *Austroplebia* (Heard, 2016), with four of the seven *Tetragonula* species endemic to Australia (Figure 5). In this thesis, I focus my research on two endemic species of East Coast *Tetragonula*: *T. carbonaria* and *T. hockingsi*. These cryptic sister species are morphologically identical but differ notably in brood comb structure. Population genetic and behavioural studies have confirmed that they are good species and do not hybridize (Franck et al., 2004; Hereward et al., 2020; Paul, 2023). Both species are today widely propagated by Australian beekeepers for sale as pets and for crop pollination for economically important Australian crops including mango, avocado, blueberry and macadamia (Heard & Dollin, 2000). Better knowledge of the thermal biology of these species is therefore important for understanding the future role of these species in Australian agriculture, and for conserving their wild populations, which perform key pollination services throughout north-eastern Australian forests.

T. carbonaria and *T. hockingsi* are also an ideal study system for investigating both macroecological patterns in insects' thermal traits, and the underlying mechanisms shaping insect thermal tolerance traits. This is because they are distributed across a wide range of latitudinal climates (Figure 6: Present map), with the species displaying both sympatry and allopatry. Furthermore, because these bees can be readily kept and moved around in hives (Brito et al., 2012), they are suitable for reciprocal transplant experiments commonly used to detect gene-by-environment effects on key physiological traits.

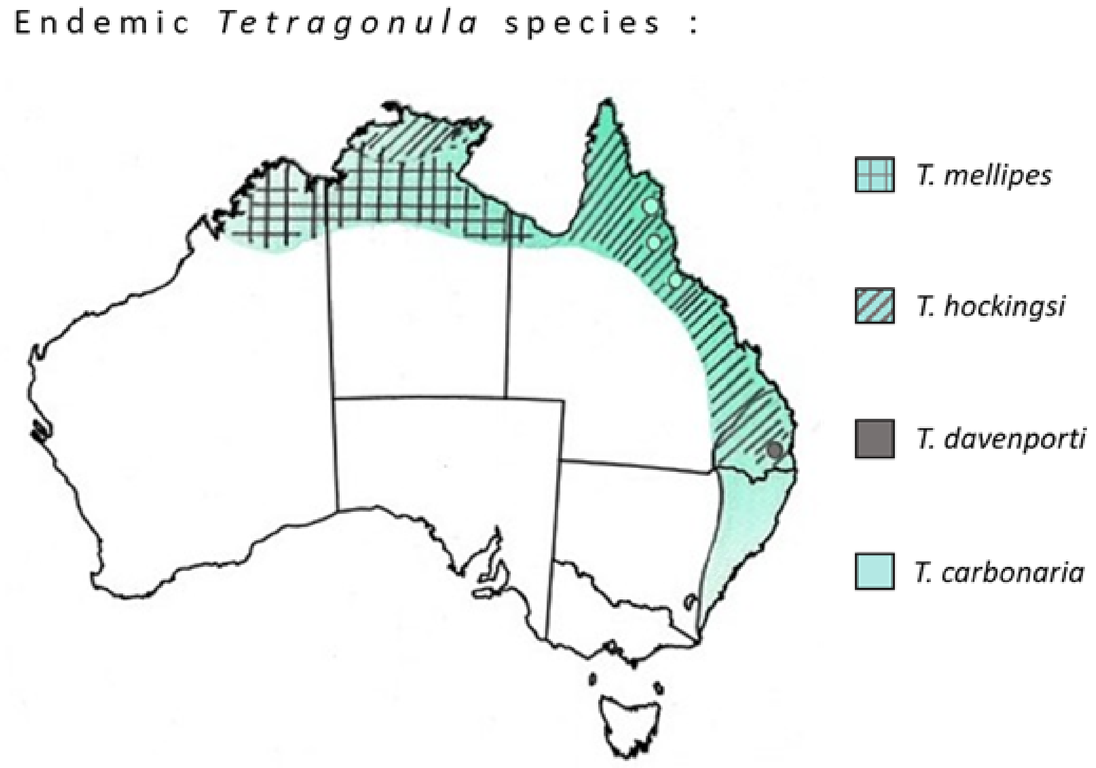

Figure 5. Estimated species distributions for four endemic *Tetragonula* in Australia. These four species form the 'carbonaria' group. *T. mellipes* has a North Coast distribution while *T. davenporti* has a highly restricted distribution on the Sunshine Coast hinterland: these species are not considered in this thesis. There are also two other species of Tetragonula found from Cape York down to Carns QLD (*T. sapiens* and *T. clypearis*), and found in New Guinea also, thus not endemic (not shown).

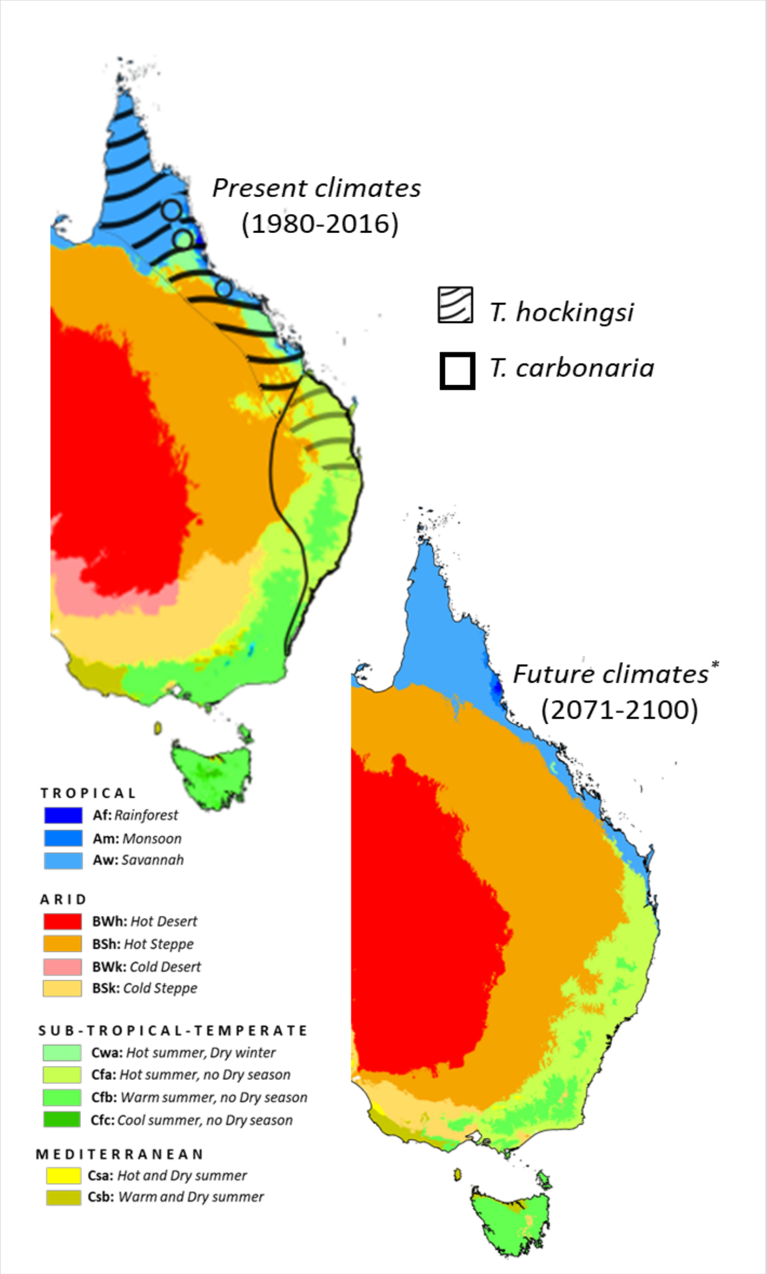

Figure 6. Present and future climate vegetation maps of Eastern Australia showing *T. carbonaria* (black outline and open circles) and *T. hockingsi* (black waves) current species distributions (present map). Maps obtained and adapted from Beck et. al., (2018); Classification classes from Peel et. al, (2007) - updated by *Beck et. al (2018) for future mapping; Climate zone C: Temperate (Greens yellow and gold) further adapted in reference to Bureau of Meteorology (BOM) climate classifications to depicted further climate differences using common name culture, with sub-tropical regions in eastern Australis (~Cwa and Cfa) from temperate zones (~Cfb and Cfc) etc. These climate zones are meant only to display the different climates that these two species currently experience and how their regional climates may change in the future. *Note*: future climate map (Beck et al, 2018) based on high emission scenario RCP8.5.

1.6 **Aims of this thesis**

In this thesis, I investigate the thermal tolerances of the Australian stingless bees *Tetragonula hockingsi* and *Tetragonula carbonaria*, as a first step towards predicting climate change responses in these ecologically and economically important insects.

Specifically, I aim to:
(1) determine the critical thermal limits (heat and cold tolerance respectively) of each species and assess whether they vary with climate and latitude, and
(2) in *T. carbonaria*, determine the contribution of genotype and plasticity to critical thermal limits via a reciprocal transplant experiment.

Chapter 2

The thermal tolerances of Australian stingless bees vary by species and local climate.

2. **Abstract**

Thermal environment is a key predictor of the geographical distributions of many small ectothermic species, such as insects. This is thought in part to be because species must adapt to their thermal environments, such that the temperature extremes that a species can survive (their thermal tolerances) will co-vary bio-climatic variables associated with temperature and show latitudinal clinal variation across an insects' distribution. In this case, we expect different species of the same linage, but inhabiting different thermal environments should display differences in their thermal tolerances. In this study, we investigate how heat and cold tolerance (estimated as critical thermal maxima and minima via ramping assays) vary between two closely related Australian stingless bees *Tetragonula carbonaria* and *Tetragonula hockingsi*, and how the distributions of each vary with climate. These two species are broadly distributed along the north-east Australian cost, with ranges that are mostly allopatric but have some areas of overlap. They are important pollinators in natural bushland, and are also used as managed crop pollinators of a range of tropical and subtropical fruit crops. We found evidence for both within and between species variation in thermal tolerances that were consistent with species and populations having adapted to their local environments. *T. carbonaria* showed greater cold tolerance, consistent with its distribution at lower latitudes and on high altitude refugia, while *T. hockingsi* showed greater heat tolerance. Both climatic and latitudinal clines were observed in heat and cold tolerances of both species. Heat tolerance was far less variable across species and locations however than cold tolerance. In all, our data is consistent with broad trends described for other insects and suggests that the distributions of Australia's two most widespread East Coast *Tetragonula* may be driven by thermal niche partitioning between species.

2.1 **Introduction**

Insects represent a high proportion of global species diversity and perform key ecosystem services, such as nutrient cycling and pollination, across all terrestrial environments on earth (Prather et al., 2013). Like other lineages of small-bodied ectotherms, environmental temperature dictates many aspects of insect biology, including their phenology, abundance and distributions; they will therefore be more acutely effected by temperature fluctuations than endothermic organisms (Bowler & Terblanche, 2008; González-Tokman et al., 2020; Sunday et al., 2012). Anthropogenic activities continue to drive changes in climate: global temperature increases may exceed 1.5°C over the next 20 years and heat waves will increase in prevalence, along with extended warm-seasons and reduced cold-seasons (Legg, 2021). For instance, a north American study found that butterfly species whose immature life stages overwintered at high-latitude range edges were more likely to display population declines, linked to the increasingly warmer and drier winters at these sites (Beed et al., 2013). A later long-term study, looking at the causality behind declining forest-floor beetle (Coleoptera) richness and abundance, reported a strong relationship between observed beetle declines and increased winter temperatures, indicating climate warming as the primary driver (Harris et al., 2019). Indeed, rapid distributional shifts and population declines in insects have been posited in many studies (Gibb et al.,2019; Loboda et al., 2018; Pecl et al., 2017); and reviewed by Halsch et al. (2020)). Thus the global threat to insect diversity and abundance is escalating, with predicted cascading-effects upon ecosystem functionality (Wagner, 2020).

A better mechanistic understanding of how climate drives insect species' distributions, including their capacity to adapt and respond to new thermal environments, is essential to predicting insects responses to climate change (Addo-Bediako et al., 2000; Bowler & Terblanche, 2008; Kellermann & van Heerwaarden, 2019; Kellermann et al., 2009; Perez & Aron, 2020). One key prediction is that species ought to display adaptive variation in their thermal physiological traits, such as heat and cold tolerance, in association with broadscale climate-based variation. That is, different species inhabiting different thermal environments ought to have different critical thermal limits (i.e. the upper and lower temperature bounds for survival); (Chown & Nicolson, 2004; Somero, 2005). Likewise, sympatric species should have similar thermal limits (independent of any phylogenetic signal). This prediction stems from the more general macroecological idea that ecologically meaningful traits are shaped by broad-scale environmental patterns, and typically observed to vary along spatial gradients such as latitude (Addo-Bediako et al., 2000; Sunday et al., 2011; Verheyen et al., 2019). Correlations between thermal tolerance and environmental variation have been reported to date across a range of insect taxa, including *Drosophila* (Kellermann, Overgaard, et al., 2012; Ranga et al., 2017)*, Isoptera* (Janowiecki et al., 2020), *Coleoptera* (Käfer et al., 2020; Moret et al., 2016) and *Bombus* (Scriven et al., 2016). One multi-species *Drosophila* study found that critical thermal limits better predicted species' distributions in this group than other fitness related traits (Overgaard et al., 2014).

Intra-specific variation of thermal tolerance limits or clines (in insects and more broadly ectotherms) are also sometimes observed, with greater cold tolerance towards higher latitudes (in increasingly cooler environments) and greater heat tolerance in more xeric and hotter environments (Buckley et al., 2022; Perez & Aron, 2020). But patterns within species do not always match between species patterns. For instance, while clinal patterns were found for lower thermal limits in *Drosophila melanogaster* along the east-coast of Australia (Overgaard, Hoffmann, et al., 2011), upper thermal limits did not show such within-species trends (Sgrò et al., 2010). Wherever a strong association between thermal tolerances and climate is found however, climate change will presumably drive either adaptive responses in thermal tolerance, or drive insects to shift their geographic ranges (e.g. latitudinal/ altitudinal) into 'new' environments that align with their existing thermal biology (Kellermann & van Heerwaarden, 2019; McCain & Garfinkel, 2021). Therefore, a useful first step in predicting any species' response to climate change is to better understand geographic patterns in its heat and cold tolerances.

Stingless bees (Apidae: Tribe Meliponini) are eusocial bees, occurring throughout the global tropics and subtropics, with over 500 species distributed across South and Central America, Africa, India, Southeast Asia, and Australia (Grüter 2020). They are abundant and important pollinators of flowering plants in these primarily tropical and subtropical ecosystems, visiting the flowers of over 220 plant families (Bueno et al., 2021). Stingless bees are also increasingly being utilised in modern agricultural systems as crop pollinators (Grüter, 2020; Meléndez Ramírez et al., 2018; Perichon et al., 2021). For instance, there is an emerging industry in Australian agriculture utilising endemic *Tetragonula* bees as managed crop pollinators

(Chapman et al., 2018; Cunningham et al., 2014; Heard, 2016). However, we are yet to fully understand how climate shapes the distributions of stingless bees, nor how they might respond as their regional climates change. Unlike other social bees (e.g. honey bees and bumblebees), stingless bees have only a limited capacity to actively thermoregulate their nests or brood (Vollet-Neto et al., 2015). Instead, internal nest temperatures more closely conform with external ambient temperatures, though nests may track around 1°C below ambient temperatures in warmer weather (Ayton et al., 2016; Vollet-Neto et al., 2015). As such, stingless bees may be particularly vulnerable to changes in environmental temperatures. Furthermore, tropical insects (≤ 23°N/S latitudes) are proposed to be at greater risk from climate change than temperate species, due to their narrower thermal safety margins of tropical species (that is, because their current heat tolerances are already close to those of their habitat) (Deutsch et al., 2008).

In this study, we investigate how upper and lower critical thermal limits vary with climate, both inter- and intra-specifically, in Australian stingless bees in the genus *Tetragonula.* We focus on two cryptic *sister* species, *T. hockingsi* and *T. carbonaria*, whose distributions largely differ along Eastern Australia latitudes, though with a few areas of sympatry (Heard 2016; Figure 1). Workers of these two species are morphologically cryptic, and they can only be reliably distinguished via differences in the internal nest architecture (Figure 1) or by molecular means (Franck et al 2004). *T. hockingsi* inhabits the more tropical latitudes of north and central Queensland while *T. carbonaria* is distributed principally in the subtropical to temperate regions of NSW, but the two species ranges overlap in southern Queensland. Additionally, what are believed to be isolated remnant populations of *T. carbonaria* are also found in parts of far northern Queensland, in what are likely altitudinal 'islands' (or wet tropic forest refuges; See Figure 1b) along Queensland's Great Dividing Range (De Deckker et al., 2020).

Given their broad distributions, *T. hockingsi* and *T. carbonaria* are exposed to a continuum of climatic conditions, making them suitable species for investigating how climate shapes critical thermal limits. Furthermore, their partial range overlaps provide the opportunity to investigate variation in thermal tolerance limits between closely-related species in shared environments. Indeed, *T. hockingsi* and *T. carbonaria* are candidates for 'thermal niche partitioning', in which related species specialize in different climates to reduce conflict and competition (Bujan et al., 2022; García-Robledo et al., 2018; Scriven et al., 2016). Here, we are explicitly asking: **(i)** What are the upper and lower thermal limits of *T. hockingsi* and *T. carbonaria*? **(ii)** Do these thermal tolerance limits differ between species, including at sites of sympatry?, **(iii)** Do these thermal tolerance limits also differ between populations of the same species?, and **(iv)** How well does climatic variation explain any inter- and intra-specific variation in thermal tolerance limits?

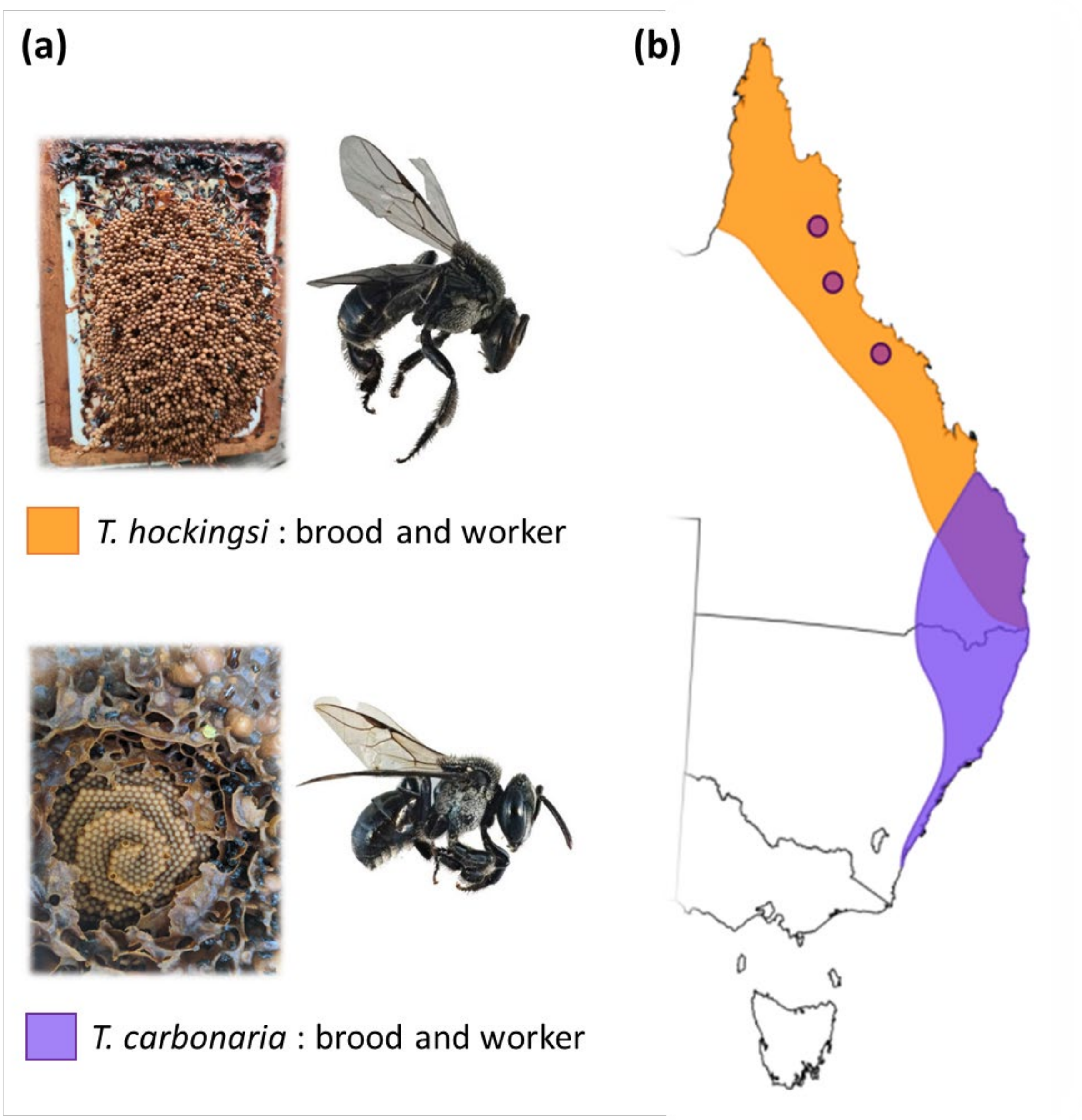

Figure 1. (a) *T. hockingsi* semi-comb brood structure and adult worker (top) and *T. carbonaria* spiral brood structure and adult worker (bottom). **(b)** Map of the estimated range of *T. hockingsi* (orange) and *T. carbonaria* (purple) in Eastern Australia. Worker photographs from Hereward et al. (2020) and species distribution map adapted from Heard (2016).

2.2 **Methods**

2.2.1 **Sample sites and bee handling**

We sampled *Tetragonula* from a total of 21 locations across the Australian East Coast (Figure 2a), during two field trips (November-December 2020 and April-June 2021, permit QLD NP No. P-PTUKI-100080792). These locations ranged from Cape York Peninsula, Queensland (-12.374, 142.196) to Sydney, New South Wales (-33.902, 151.162), spanning ~3,350 km, and cover five out of the six major Köppen vegetation classification groupings that are found in Australia (Stern et al., 2000).

At most locations, we sampled wild foraging *Tetragonula* by netting them at flowers, with collection numbers ranging between 10 – 100 individuals depending on weather conditions (Table *f2*). We recorded the geographical coordinates of all sampling sites using a SPOT Gen4 GPS Satellite device. Based on previous reports of the species' distributions (Heard 2019), we anticipated that at some sites, only one of our target species would occur (either *T. hockingsi* or *T. carbonaria*) while at other sites, both species would occur (Figure 2b, Tabel *f2.*). However, as workers of these cryptic species can only be reliably identified by molecular means (Dollin & Dollin, 1997; Franck et al., 2004), we could not be certain we were adequately sampling both species at known co-occurring sites based on wild-caught samples alone. At these locations therefore, we also sampled returning foragers from locally sourced and managed hives of known species identity (i.e., where species had been determined by beekeepers based on brood morphology), to ensure that we captured representatives of both species (Locations 4 and 13: Figure 2a). Collections from one *T.* hockingsi and two additional *T. carbonaria* sites were also from locally sourced hives (Townsville for *T. hockingsi* and Terranora/Tweed Heads and Sydney for *T. carbonaria*). For both wild and hive samples, we placed bees into holding vials (93mmx22mm; with a maximum of 10 individuals per vial) with paper towel soaked in 1:1 glucose solution and allowed them free access to food for at least 1h prior to thermal assays. Collected bees were retained in holding vials for between 2-6h (depending upon travel distances to and from collection sites), with stress minimised during transportation by keeping vials in a dim box kept at 20°C-25°C, prior to transferring them into individual watertight scintillation glass vials (43x20mm) back at the field laboratory, ready for thermal assays.

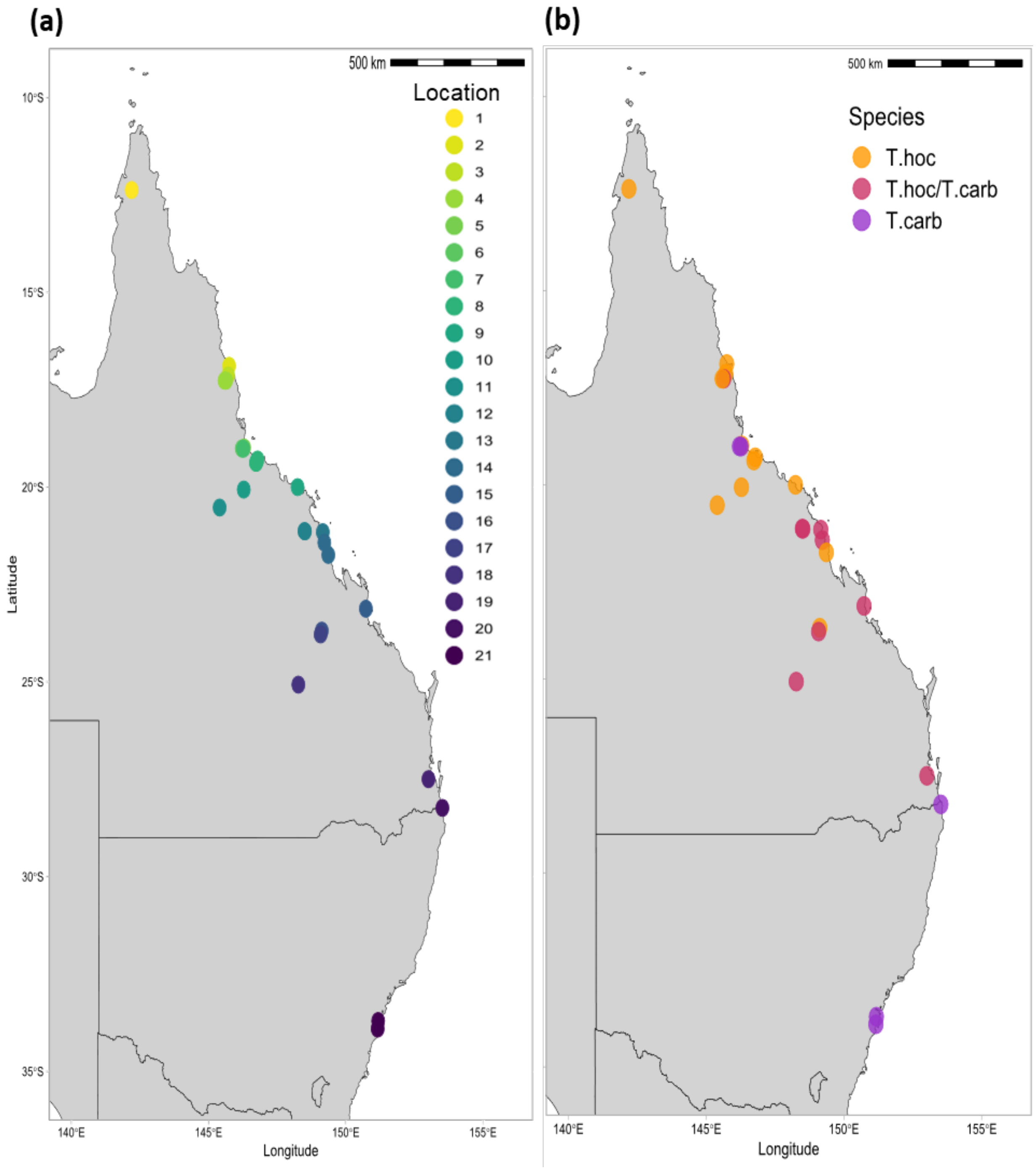

Figure 2. (a) Sampling locations (N=21), with colour gradient indicating latitudinal gradient and site details are given in Table *f*2 below, **(b)** species IDs from post-assay amplification of mt-CO1 indicating at which sites we sampled *Tetragonula carbonaria* only (purple), *T. hockingsi* only (orange) or co-occurrence of species (pink).

Table *f*2. Colour key of sample locations in this study, ordered by latitudinal gradient (lowest latitude : yellow ID = 1 in far northern Queensland; Highest latitude: Dark purple ID = 21 in Sydney, New south Wales); approximate Latitude and Longitude coordinates of sampling locations rounded to 3 d.p; T. hockingsi (T. hoc) and T. carbonaria (T. carb) sampling numbers run in thermal tolerance assays; N/A species columns indicate species not sampled at site. Total = total number of Tetragonula bees assayed. Note: Species and total number of observations in this table represent our data points/observations used in our statistical analysis and subsequent modelling (outliers already removed above 3 Std. deviations), and not actual pre-modelling numbers.

	ID	**Location name**	**Latitude**	**Longitude**	***T. hoc***	***T. carb***	**Total**
🟨	1	Cape York Peninsula	-12.374	142.196	34	*N/A*	34
🟨	2	Cairns	-16.887	145.749	87	*N/A*	87
🟨	3	Little Mulgrave	-17.134	145.714	32	*N/A*	32
🟩	4	Atherton Tablelands	-17.268	145.609	114	141	255
🟩	5	Crystal Creek - low	-18.985	146.295	4	*N/A*	4
🟩	6	Paluma town	-19.010	146.210	2	*N/A*	2
🟩	7	Little Crystal Creek	-19.015	146.266	*N/A*	132	132
🟩	8	Townsville	-19.333	146.757	165	*N/A*	165
🟦	9	Bowen	-20.001	148.243	*N/A*	12	12
🟦	10	Charters Towers	-20.063	146.281	19	*N/A*	19
🟦	11	Pentland QLD	-20.525	145.402	105	*N/A*	105
🟦	12	Eungella/ Finch Hatton	-21.133	148.502	15	62	77
🟦	13	West Mackay	-21.155	149.162	76	62	138
🟦	14	Sarina	-21.584	149.291	31	14	45
🟦	15	Yepoon	-23.122	150.732	36	29	65
🟦	16	Bluff	-23.684	149.125	139	*N/A*	139
🟪	17	Blackdown	-23.783	149.082	9	122	131
🟪	18	Carnarvon Gorge	-25.072	148.271	90	20	110
🟪	19	Brisbane	-27.498	153.012	16	16	32
🟪	20	Terranora	-28.235	153.520	*N/A*	283	283
🟪	21	Sydney	-33.804	151.171	*N/A*	370	370

2.2.2 **Critical thermal limits assays**

We used a standardised dynamic ramping protocol, adapted from Chown et al. (2009) and MacLean et al. (2019), to assess the critical thermal maxima (CT_{max}, hereafter 'heat tolerance') and critical thermal minima (CT_{min}, hereafter 'cold tolerance') of our sampled *Tetragonula*. We randomly assigned each bee to one of our two assays (heat tolerance or cold tolerance). We gave each vial (bee) a unique identifier and positioned them randomly onto Perspex vial racks, where each rack held up to 100 vials. We placed racks into a transparent Perspex water bath for viewing (32cmW x15cmD x33cmH), held at a starting temperature of 26°C (an environmental temperature that bees at all sites would commonly experience when foraging). We then slowly heated (for heat tolerance) or cooled (for cold tolerance) the water bath at a rate of 0.1°C per minute (6°C per hour). Water bath temperature was controlled using an external bipolar heating and cooling thermoelectric controller and high-density bonded-fin heat sink unit with USB interface software (TE Technology, Inc. 2021), with water circulated via the use of a pressure pump (Supplementary Material: equipment Figure S2.1). During assays, we continuously observed specimens and recorded heat tolerance or cold tolerance as their heat or cold knock-down temperature respectively, i.e., the point of which they became unresponsive and lost all motor control. In most cases, both heat tolerance and cold tolerance assays were run, consecutively, on each field-sampling day. Depending upon field collections, anywhere from 50-100 bees were included per assay per sampling day. At the end of assays, all bees were preserved in 80% ethanol for later molecular species identification.

2.2.3 **Molecular species identification**

We identified to species all *Tetragonula* sampled from flowers by amplifying a species-specific fragment of the mitochondrial gene cytochrome oxidase I (COI). We first extracted DNA from the abdominal tissue of each bee in a 5% Chelex solution following the protocol of Walsh, Metzger, and Higuchi (1991). We then performed two PCRs for each sample using different primer sets: one that amplifies only in *T. carbonaria* (*Barhock_F* - CTCCATTGTTACTGGGCATGC and *T_carb_COI_spec_R2* - CAATGAAATTYAGTGACCCT) and one that amplifies only in *T. hockingsi* (*Barhock_R* - AAGGCCGAATCCTGGAAGAA and *T_hock_COI_spec_F5* – GAATTTCATCTATTCTTGGA (Francoso et al., 2019)Paul et al 2023). Amplification conditions were 94°C for 8min, followed by 35 cycles of 94°C, then 55°C (*T. carbonaria* primers) or 60°C (*T. hockingsi* primers), and 72°C for 30s each, followed by 72°C for 9 min. Reactions used 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4µM forward primer, 0.4µM reverse primer and 1U MyTaq polymerase (Bioline, Australia). We then visualized the products of each PCR via gel electrophoresis and assigned species IDs to each sample based on which primer-set produced a band of the expected size (approximately 300 bp), with all samples amplifying for only one primer-set.

2.2.4 **Bioclimatic variables and data analysis**

All statistical analyses were carried out using R Statistical Software (v4.1.2; R Core Team 2021). We firstly identified and removed outliers in our datasets that exceeded preliminary threshold of 3 standard deviations (Smiti, 2020) from the mean via statistical and visual analysis ('qqplot2' package: Wickham, 2016). At three standard deviations, observations below 34°C for heat tolerance (N=33/1319) and above 10°C for cold tolerance (N=105/1067) were removed, with the added rational also being that such bees likely died prematurely due to factors other than experimental thermal stress (e.g., accidental injury during handling into vials). The distribution of heat tolerance data was negatively skewed; however we chose not to transform this asymmetrical data as it was deemed ecologically relevant (DeWitt & Friedman, 1979). Although some small bias is introduced when analysing left-skewed datasets of mean heat tolerances, this is not expected to impact hypothesis-testing in our case (Martin & Huey, 2008) and is preferrable to 'correcting' asymmetry via extreme logarithmic transformation.

Intraspecific analysis: trait variation with climate

To determine if and how the thermal tolerances of each *Tetragonula* species might associate with climate, separate mixed-effect models were built for each species, fitted using the 'lme4' package (Bates et al., 2015), with subsequent linear mixed-effect regression analysis carried out using the lmerTest package (Kuznetsova et al., 2017), considering each species separately. We extracted bio-climatic variable data at each of our 21 sampling locations (using latitude and longitude coordinates of each sampling location) downloaded from WorldClim version 2.0 with 0.5 (30 sec) spatial resolution grided datasets (Fick & Hijmans, 2017), using the raster package (Hijmans et al., 2015). These climatic variables represent annual trends, seasonality and limiting environmental factors for both temperature and precipitation, averaged across 1970-2000 historical climate data. We chose eight variables linked to precipitation and temperature to include in our preliminary models, based on the key variables identified in previous studies on insects (da Silva et al., 2021; Jackson et al., 2020; Käfer et al., 2020; Kellermann, Loeschcke, et al., 2012) and on these variables' likely ecological relevance to *Tetragonula* (Käfer et al., 2020): [BIO1, BIO3, BIO4, BIO5, BIO6, BIO12, BIO13, BIO14 ;see Supplementary Table S2.1 for BCV descriptions].

We then performed commonality coefficient analyses to identify autocorrelation between our eight climatic variables, for heat tolerance and cold tolerance datasets ('yhat' package version 2.0-3; Nimon et al., 2008). Autocorrelation between climatic variables is common and can result in multicollinearity and the overinflation of standard error. These analyses determined the percentage of unique and shared variance explained by all variables, AIC model comparison and Variance Inflation Factors (VIF, 'regclass' version 1.6; with VIF ≥5 rejected), thus allowing stepwise removal of variables given that climatic variables are inherently co-linearly related giving rise to potential confounding effects when modelling.

After removing autocorrelated variables, we were left with four climate variables to model against our bee's thermal tolerance traits: Isothermality (Iso), minimum temperature of the coldest month (Tcold), hottest temperature of the warmest month (Thot), and precipitation of the driest month (Pdry). Isothermality (%) measures the magnitude of diurnal temperature fluctuations relative to a climates' annual summer-to-winter fluctuations, with a hypothetical value of 100 indicating an environment that is seasonally stable (i.e., day-night fluctuations equal summer-winter fluctuations). Values below 100 and decreasing towards 0, indicate increasing variability in seasons' thermal environments. Isothermality is a strong predictor of some ectotherm distributions (Bazzato et al., 2021; Jackson et al., 2020; Ramasamy et al., 2022; Yoon & Lee, 2021), in essence an organism inhabiting an environment with ~100% Iso value will experience the same amount of abiotic thermal variation in one day as it experiences annually. Tcold and Thot relate to the extreme temperatures (°C) and are associated with heat and cold tolerance traits that 'buffer' organisms from these extremes, it should be noted however that Thot and Tcold spatial data are the average temperature extremes for the hottest or coldest month respectively. Pdry (mm) pertains to recorded rainfall averages during the driest month, with precipitation a major driver of some species distributions and often associated with heat tolerance (Kellermann & Sgrò, 2018).

We ran each model (models for heat and cold tolerance per species) with two random effects: location (i.e., 21 locations; Figure 2) and year of sampling (2020 or 2021). Year of sampling also accounted for possible differences in experimenter (CdS in 2020 and IVB in 2021). Random effect variance was assessed to determine inclusion in final models.

Finally, we investigated the relationship between latitude and heat and cold tolerance for both species. Latitude could not be included together with bioclimatic variables due multicollinearity, however it is often a variable of interest when trying to understand geographic patterns in traits. We conducted linear mixed-effect models for each species (species and associated thermal response modelled separately as above), with each analysis assessing thermal response covariation with latitude, and location and year controlled for as random effects. All linear mixed effect modelling used the 'lme4' package (Bates et al. 2014).

Interspecific analysis

To investigate differences in the thermal tolerance between species, we considered data from locations where both species co-occurred (Locations: L4, L13, and L18; Figure 2a). Any differences identified between species at these locations cannot be due to plasticity in thermal traits but rather must be intrinsic species differences. We modelled heat tolerance and cold tolerance (separately as above) for the three locations where species co-occurred with comparable sample numbers ($N \geq 5$). Species comparison and location interaction modelling was conducted using linear modelling (stats package version 3.6.2; R Core Team 2022), with regression analysis using Type-III Analysis of Variance (ANOVA); (car package version 3.1-1; Fox et al., 2022).

We used 'ozmaps' version 0.4.5 and 'ggplot2' version 3.4.0 packages to plot the distribution of each species across our sample locations. All intext model contrast results are given with ± *SE* (standard error) unless otherwise specified.

2.3 **Results**

2.3.1 **Cold tolerance**

Cold tolerance across climate and latitude

We first examined whether cold tolerance in *T. hockingsi* (Table 1a) and *T. carbonaria* (Table 1b) predictably varied along climate gradients. For each species, we found that variation in cold tolerance response was best explained by linear mixed-effect models that included three climate variables: Isothermality (Iso), minimum temperature of the coldest month (Tcold), and precipitation of the driest month (Pdry); Figure 3. Of the two species however, this relationship with climate was a significant trend only for *T. carbonaria*, while climate variables were only weakly associated with variation in cold tolerance for *T. hockingsi* (Table 1a; Figure 3b,d,e).

For *T. carbonaria*, cold tolerance was best explained by the bioclimatic variables of Iso (F = 59.014, p = <0.001; Table 1a and Figure 3a) and Pdry (F = 83.631, p = <0.001; Table 1a and Figure 3e), with Tcold the least predictive variable (F = 83.631, p = 0.169; Table 1a and Figure 3c). Specifically, *T. carbonaria* cold tolerances displayed a strong positive trend with Iso (Estimate of β (slope) = 0.282 ± 0.036 SE, $t_{3.66}$= 7.906, p <0.001; Figure 3a), such that bees from locations with higher Iso (% index) values (i.e., sites with more thermally stable seasonal climate oscillations) were less cold tolerant. For Pdry (mm), *T. carbonaria* cold tolerance exhibited a moderate and negative association (Estimate of β = -0.077 ± 0.008 SE, $t_{4.33}$= -9.523, p <0.001; Figure 3e) such that bees were predictably less cold tolerant in environments that received less rain during their driest month. Lastly, minimum temperature of the coldest month was not a significant predictor of either species cold tolerance variation (Figure 3c and d).

Table 1. Results from linear mixed-effect regression analysis of clinal variation of Cold tolerance with Bioclimatic variables for (a) *T. hockingsi* and (b) *T. carbonaria* models.

(a) *T. hockingsi* bioclimatic model

Fixed effects	$F_{\text{df (num,den)}}$	***p-value***
Isothermality	$1.468_{1,\ 6.594}$	0.267
Minimum Temperature of Coldest Month	$1.059_{1,\ 6.369}$	0.341
Precipitation of Driest Month	$0.878_{1,\ 6.015}$	0.385
Random effects	**SD**	***p***
Location	0.274	<0.001
Residual	0.896	

(b) *T. carbonaria bioclimatic* model

Fixed effects	$F_{\text{df (num,den)}}$	***p-value***
Isothermality	$59.014_{1,2.913}$	<0.001
Minimum Temperature of Coldest Month	$2.385_{1,6.592}$	0.169
Precipitation of Driest Month	$83.631_{1,3.567}$	<0.001
Random effects	**SD**	***p***
Location	0.132	0.454
Residuals	1.250	

Note: Separate mixed-effect models were built for each species' cold tolerance variation with Bioclimatic variables; *T. hockingsi* model **(a)** and *T. carbonaria* model **(b)**, with both models dropping R.effect of Year as variance approx. zero. Above variable estimates of fixed effects applied restricted maximum likelihood estimation method along with Type-III F-test using Kenward–Roger's approximation of degrees of freedom. *F -value* (*F*) with subscript numerator(num) and denominator (den) degrees of freedom using Kenward-Roger's method. Random effect of year with two levels, p-value (*p*) from random effects anova results.

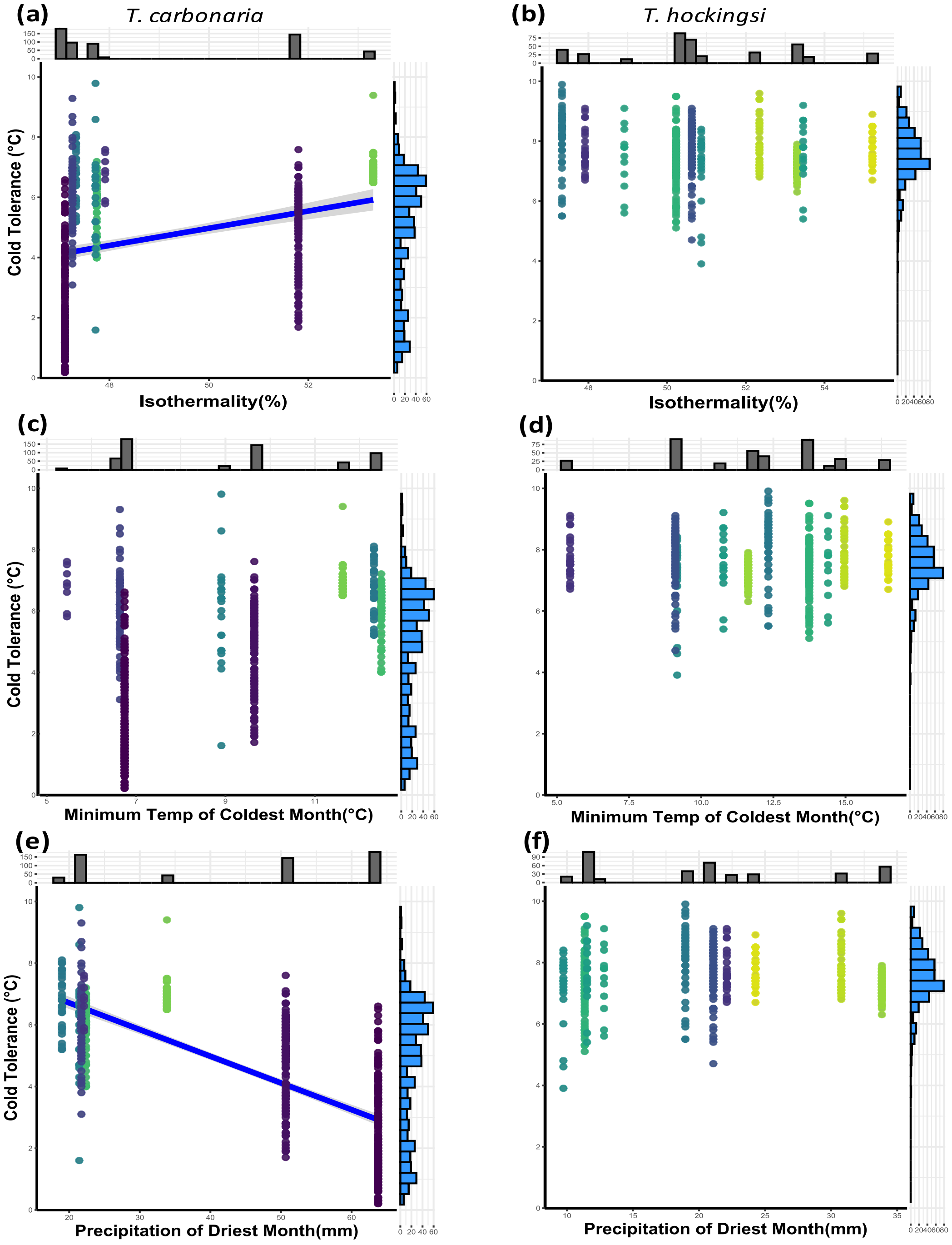

Figure 3. Linear relationships between cold tolerance for T. carbonaria (a, c, e) and T. hockingsi (b, d, f) and three climatic variables determined to be the key predictors of cold tolerance based on linear models: Isothermality (%), Minimum temp. coldest month (°C), Precipitation. driest month (mm). Scatter plot point colours indicate sampling locations (see Figure 1a). Blue histograms: cold tolerance distributions for each species; Grey histograms: number of observations/datapoints per location; Blue lines: significant linier regression with ± SE (grey).

Modelling latitudinal effects for each species found that cold tolerance only predictably varied with latitude for *T. carbonaria* (F = 706.86, p <.0001; Table 2b), consistent with a latitudinal cline in this trait (Figure 4a). *T. carbonaria* cold tolerance significantly increased towards higher latitudes (Estimate of β = 0.273 ± 0.010 SE, $t_{556.1}$= 27.59, p <.0001). Conversely, regression analysis of *T. hockingsi* cold tolerance found no notable trend in association with latitudinal distribution, finding that neither the reduced model (with only the random effect of year) nor the full model explained cold tolerance variation (Table 2a; Figure 4b).

Table 2. Results from linear mixed-effect regression analysis of latitudinal effect on Cold tolerance for (a) *T. hockingsi* and (b) *T. carbonaria*.

(a) Latitudinal effect on *T. hockingsi* cold tolerance

Fixed effects	$F_{\text{df (num,den)}}$	***p-value***
Latitude	$1.69_{1,383.31}$	0.194
Random effects	**SD**	***p***
Year	0.000	1
Residual	0.859	

(b) Latitudinal effect on *T. carbonaria* cold tolerance

Fixed effects	$F_{\text{df (num,den)}}$	***p-value***
Latitude	$760.86_{1,556.12}$	<.0001
Random effects	**SD**	***p***
Year	1.123	0.004
Residuals	1.364	

Note: Separate linier mixed-effect models were built for each species' cold tolerance response. Above variable estimates of fixed effects applied restricted maximum likelihood estimation method along with Type-III F-test using Kenward–Roger's approximation of degrees of freedom. *F -value* (*F*) with subscript numerator(num) and denominator (den) degrees of freedom using Kenward-Roger's method. Random effect of year with two levels, p-value (*p*) obtained via random effects anova results.

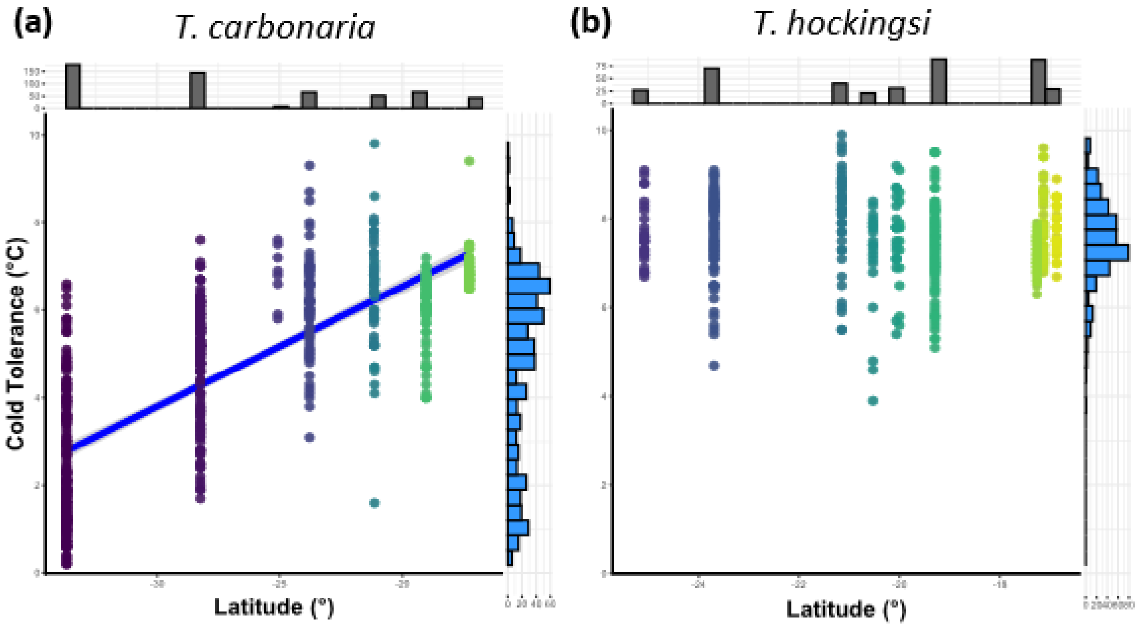

Figure 4. The relationship between cold tolerance and latitude for a) *T. carbonaria*; b) *T. hockingsi.* Colours of data points represent Locations (see Figure 1a) Blue histograms indicate cold tolerance data spread for each species; grey histograms indicate number of observations a sampling location. Blue line: significant trend with ± SE (grey); only significant trends are plotted.

Interspecific variation in cold tolerance at co-occurring locations

We found support for interspecific variation in cold tolerance when comparing the species at locations where they co-occurred (N= 3 locations; AIC_{null} = 520.17, AIC_{full} = 459.23). These locations were Lake Barrine (Atherton Tablelands), West Mackay and Carnarvon Gorge (Table 3; Figure 5). Mean cold tolerance was greatest in *T. carbonaria* (6.82°C ± 0.10) compared to *T. hockingsi* (7.64°C ± 0.07); that is, *T. carbonaria* was the more cold tolerant species (mean difference between species: 0.82°C ± 0.13 , *t* = -6.591, *p* <.0001). Further there was also a location-specific interaction effect (location x species). *Post-hoc* comparisons (estimated marginal means) revealed that cold tolerace varied most between the species in West Mackay, with *T. carbonaria* displaying greater cold tolerance than *T. hockingsi* by an estimated 1.32°C (± 0.18, t = -7.463, *p* <.0001; Figure 5b). *T. carbonaria* also displayed greater cold tolerance than *T. hockingsi* at Carnarvon Gorge (estimated difference: 0.90°C ± 0.29, t = -3.049, *p* = 0.003; Figure 6c), with no significant diffrence between species' cold tolerance detected at Lake Barrine (Atherton Tabelands); Figure 6a.

Table 3. Analysis of variance (ANOVA) Type III test of the effects of species and location on cold tolerance variation in *Tetragonula* for three locations (sites) where *T. carbonaria* and *T. hockingsi* co-occur. *SS* = type III sum of squares.

Source of variance	***SS***	***df***	***F***	***p***
(Intercept)	1353.41	1	2529.838	<.0001
Species	29.79	1	55.693	<.0001
Location	0.92	2	0.863	0.423
Species x Location	11.72	2	10.956	<.0001
Residuals	105.93	198		

Model residual standard error: 0.731 on 198 *df.* Multiple R^2: 0.294; Adjusted R^2: 0.276, *F* (5,198*df*) =16.47; *p* <.0001.

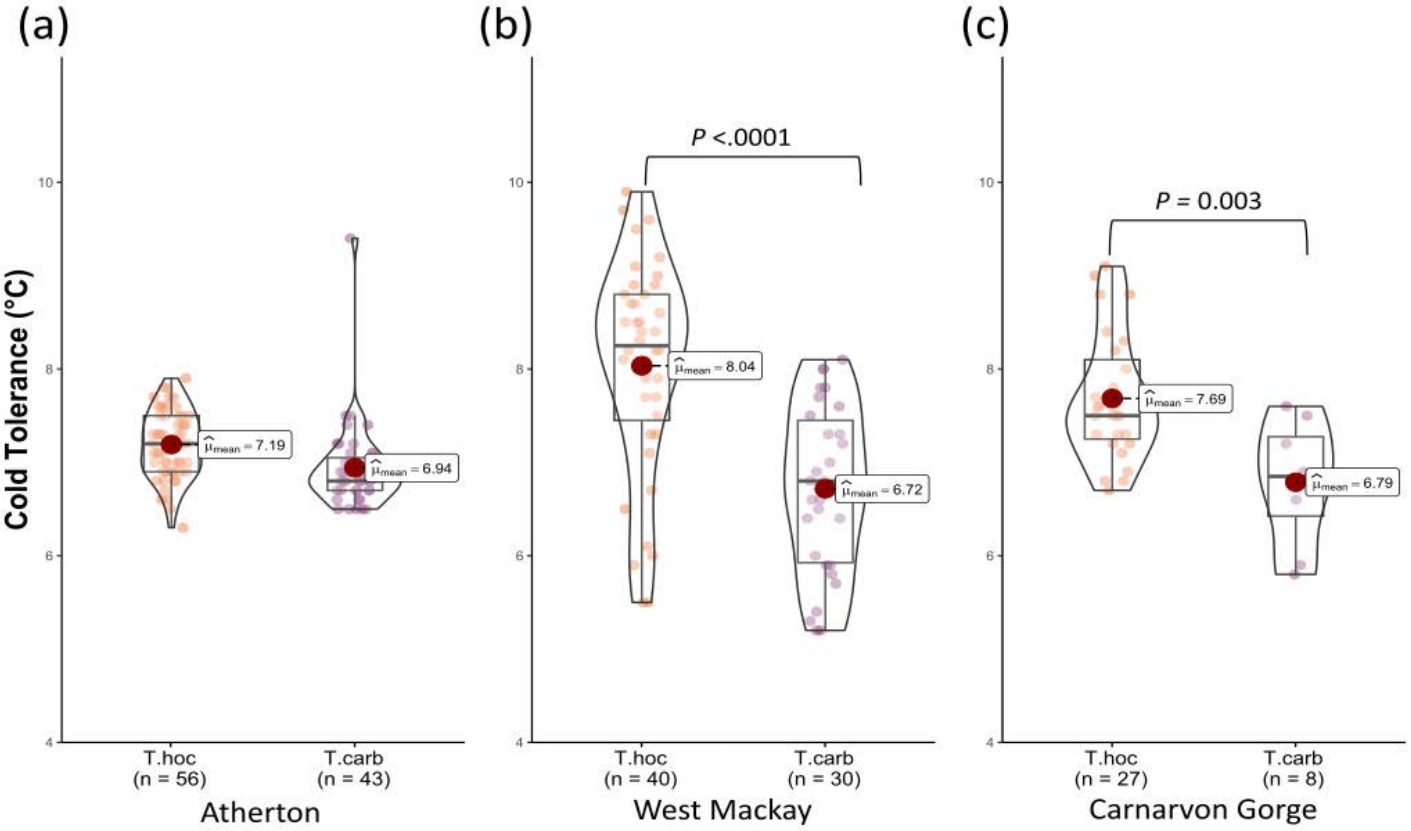

Figure 5. Mean cold tolerance for *T. carbonaria* (T.carb: purple) and *T. hockingsi* (T.hoc: orange) assayed at three locations in Queensland where the species co-occurred and could be assayed with sufficient sample sizes: (a) Atherton (b) West Mackay, (c) Carnarvon Gorge. Red dot: group mean with value given in boxes to the left. Significant species differences denoted by P-value.

2.3.2 **Heat tolerance**

Heat tolerance across climate and latitude

Heat tolerance variation in both *Tetragonula* species was best explained by models containing three climate variables (Iso, Thot: maximum temperature of warmest month, and Pdry; Table 4). Overall, only *T. hockingsi* displayed a significant relationship, and only with the climate variable Thot (F = 5.525, p = 0.043; Table 4a), with a minor but significant positive liner trend (Estimate of β = 0.541 ± 0.229 SE, $t_{2.36}$= -9.523, p = 0.043; Figure 6d), with heat tolerance increasing in warmer environments. In case of *T. carbonaria*, the random effects of location and year explained more variation than all three bioclimatic variables (Table 4b).

Table 4. Results from linear mixed-effect regression analysis of variation in heat tolerance with bioclimatic variables for (a) *T. hockingsi* and (b) *T. carbonaria* models.

***(a)** T. hockingsi bioclimatic model*		
Fixed effects	$\mathbf{F}_{\mathbf{df\ (num,den)}}$	***p-value***
Isothermality	$2.300_{1,\ 7.830}$	0.169
Maximum Temperature of Warmest Month	$5.525_{1,\ 9.155}$	0.043
Precipitation of Driest Month	$0.257_{1,\ 9.395}$	0.624
Random effects	**SD**	***p***
Location	0.827	<.0001
Year	1.540	<0.001
Residual	2.078	
(b) *T. carbonaria bioclimatic* model		
Fixed effects	$\mathbf{F}_{\mathbf{df\ (num,den)}}$	***p-value***
Isothermality	$1.854_{1,6.697}$	0.217
Maximum Temperature of Warmest Month	$1.286_{1,9.286}$	0.285
Precipitation of Driest Month	$0.586_{1,7.188}$	0.468
Random effects	**SD**	***p***
Location	0.997	<.0001
Year	2.660	<.0001
Residuals	1.464	

Note: Separate mixed-effect models were built for each species heat tolerance variation with Bioclimatic variables. Estimates of fixed effects applied restricted maximum likelihood and Type-III F-test using Kenward–Roger's approximation of degrees of freedom. *F -value* (*F*) with subscript numerator(num) and denominator (den) degrees of freedom using Kenward-Roger's method. Random effect of year with two levels, p-value (*p*) from random effects anova results.

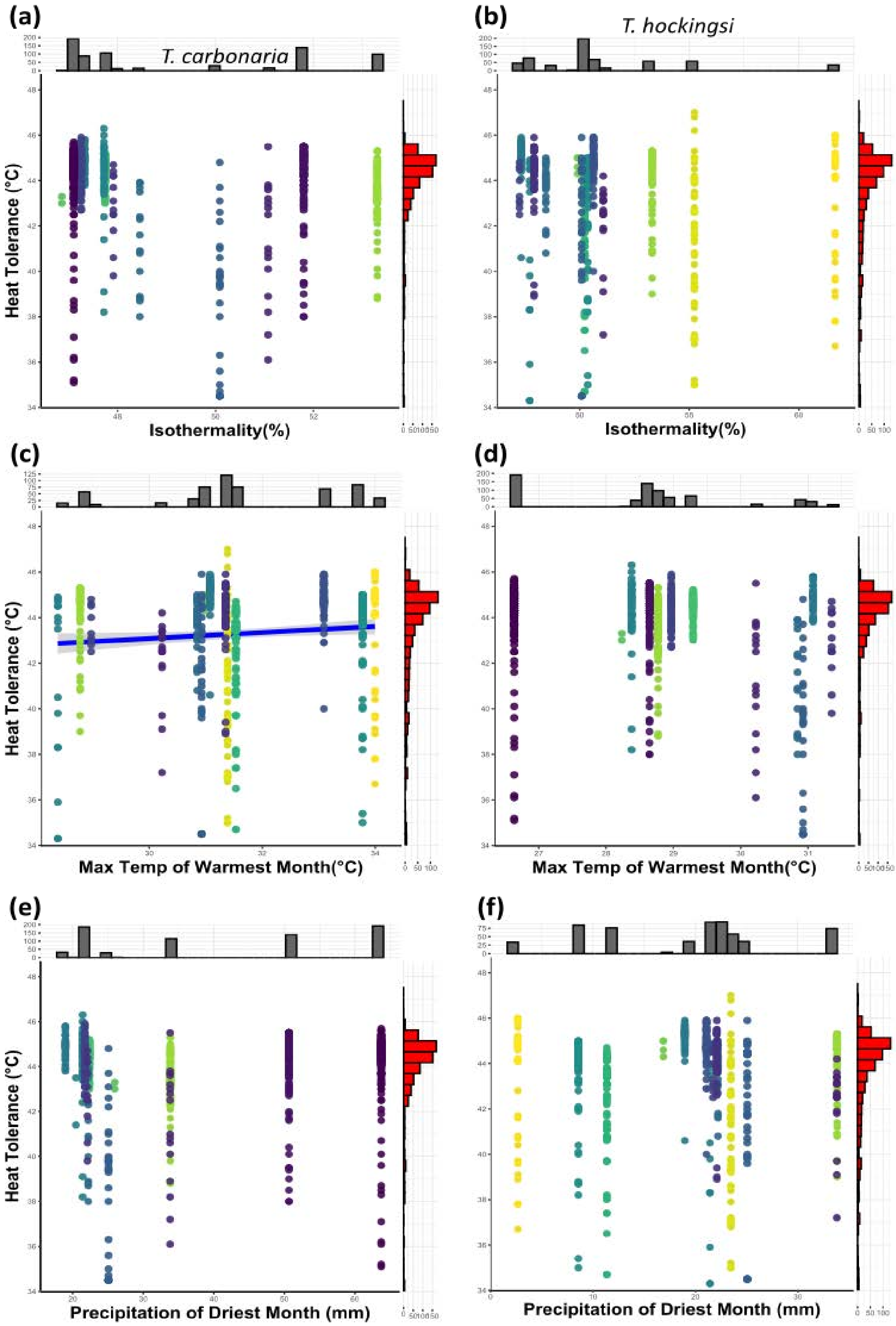

Figure 6. Linear relationships between Heat tolerance for *T. carbonaria* (a, c, e) and *T. hockingsi (*b, d, f) and three climatic variables determined to be the key predictors of Heat tolerance: Isothermality (%); a and b, Maximum temperature of the warmest month (°C); c and d, Precipitation of the driest month (mm); e and f. Scatter plot point colours indicate sampling locations: see Figure 1(a). Grey histograms: number of observations at each location; Red Histogram: Heat tolerance of species data spread. Blue line: significant linier regression with ± SE (grey).

When considering the relationship between heat tolerance and latitude, we found that latitude was a significant predictor of variation in this trait for both *Tetragonula* species (Figure 7, Table 5). Both species showed reduced heat tolerance towards lower latitudes (that is, a negative cline was observed for heat tolerance at latitudinal locations towards their northern range edges), though this trend was more pronounced for *T. hockingsi* (Estimate of $\beta = -0.111 \pm 0.03$ SE, $t_{586.08} = -4.107$, $p < .0001$; Table 5a, Figure 7b) than *T. carbonaria* (Estimate of $\beta = -0.028 \pm 0.01$ SE, $t_{691.0} = -2.844$, $p = 0.005$; Table 5b, Figure 7a).

Table 5. Results from linear mixed-effect regression analysis of the association. Between latitude and heat tolerance for (a) *T. hockingsi* and (b) *T. carbonaria* models.

(a) Latitudinal effect on *T. hockingsi* heat tolerance		
Fixed effects	$F_{\text{df (num,den)}}$	***p-value***
Latitude	$16.87_{1,\ 586.09}$	<.0001
Random effects	**SD**	***p***
Year	1.201	<.0001
Residual	2.238	
(b) Latitudinal effect on *T. carbonaria* heat tolerance		
Fixed effects	$F_{\text{df (num,den)}}$	***p-value***
Latitude	$8.09_{1,691}$	0.005
Random effects	**SD**	***p***
Year	2.613	<.0001
Residuals	1.576	

Note: Separate mixed-effect models were built for each species' heat tolerance response. Above variable estimates of fixed effects applied restricted maximum likelihood estimation method along with Type-III F-test using Kenward–Roger's approximation of degrees of freedom. *F -value* (*F*) with subscript numerator(num) and denominator (den) degrees of freedom using Kenward-Roger's method. Random effect of year with two levels, p-value (*p*) from random effects anova results.

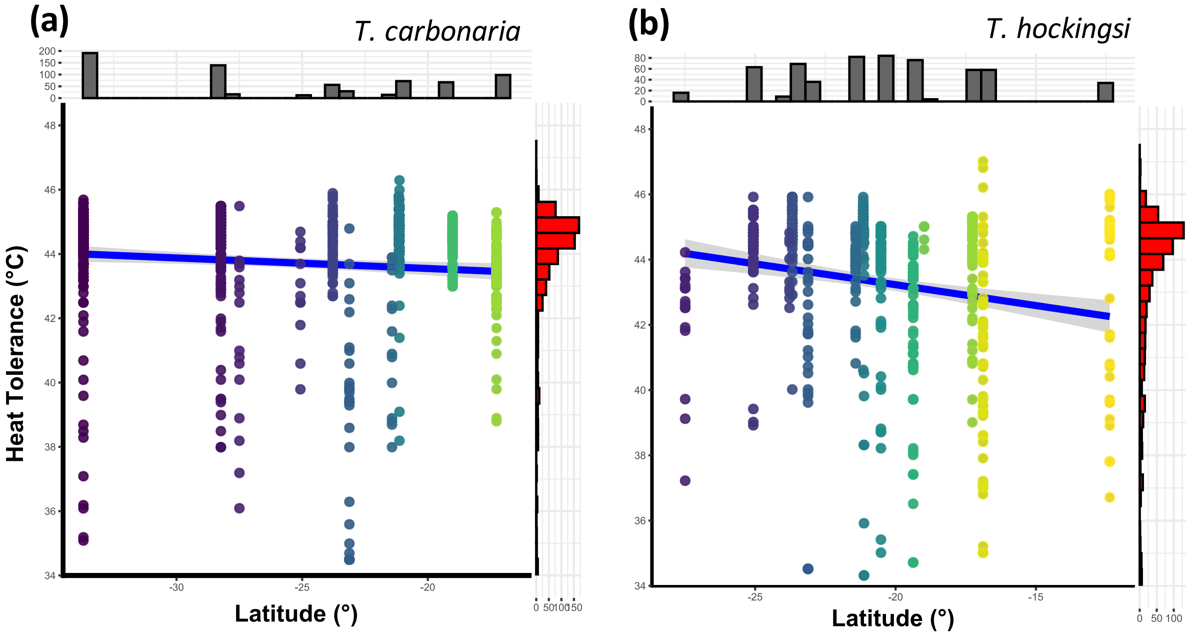

Figure 7. Heat tolerance variation along latitude for *Tetragonula* bee species **a)** *T. carbonaria*, **b**) *T. hockingsi*. Red histograms: species heat tolerance data spread; Grey histograms: number of observations at each location; Red Histogram: species Heat tolerance data spread. Blue line: significant linier regression with ± SE (light grey). Data point colours indicate sampling location (see Figure 1a).

Interspecific variation in heat tolerance at co-occurring locations

There were minor interspecific differences in heat tolerance across the three locations at which both species co-occur (Figure 8; Table 6), with our full interaction model explaining this variation much better than a reduced (null) model (AIC_{null} 1038.18, AIC_{full} 979.54). However, ANOVA results indicated that species was a significant predictor of heat tolerance variation only via interaction with location (species x location) (Table 6; mean species difference averaged over locations : 0.22°C ± 0.30, *t* = 0.731, *p* = 0.466). *T. hockingsi* had the slightly greater heat tolerance than *T. carbonaria*, but only at two of the three locations (Atherton species difference: 0.53°C ± 0.20, *t* = -2.611, *p* = 0.01; and Carnarvon Gorge species difference: 1.41°C ± 0.39, *t* = -3.632, *p* <0.001); Figure 8. Overall, location was the best predictor of heat tolerance variation (*F* = 18.945, *p* <.0001; Table 6). Pairwise comparisons between locations found significant differences between West Mackay - Atherton (difference estimate: 1.39°C ± 0.24, *t* = 5.704, *p* = <.0001) and Carnarvon Gorge – West Mackay (difference: 1.258°C ± 0.18, *t* = 6.921, *p* = <.0001) but not between Carnarvon Gorge – Atherton (difference estimate: 0.14°C ± 0.22, *t* = -0.657, *p* = 0.789).

Table 6. Analysis of variance (ANOVA) Type III test of the effects of species and location on heat tolerance variation in *Tetragonula* for three locations (L.4, L.13, L.18) where *T. carbonaria* and *T. hockingsi* co-occur. *SS* = type III sum of squares.

Source of variance	***SS***	***df***	***F***	***p***
Intercept	64333	1	42625.33	<0.0001
Species	1	1	0.53	0.466
Location	57	2	18.95	<0.0001
Species x Location	9	2	3.04	0.049
Residuals	442.2	293		

Model residual standard error: 1.229 on 293 *df.* Multiple R^2: 0.205; Adjusted R^2: 0.192, F (5,293*df*) =15.12; *p* <.0001.

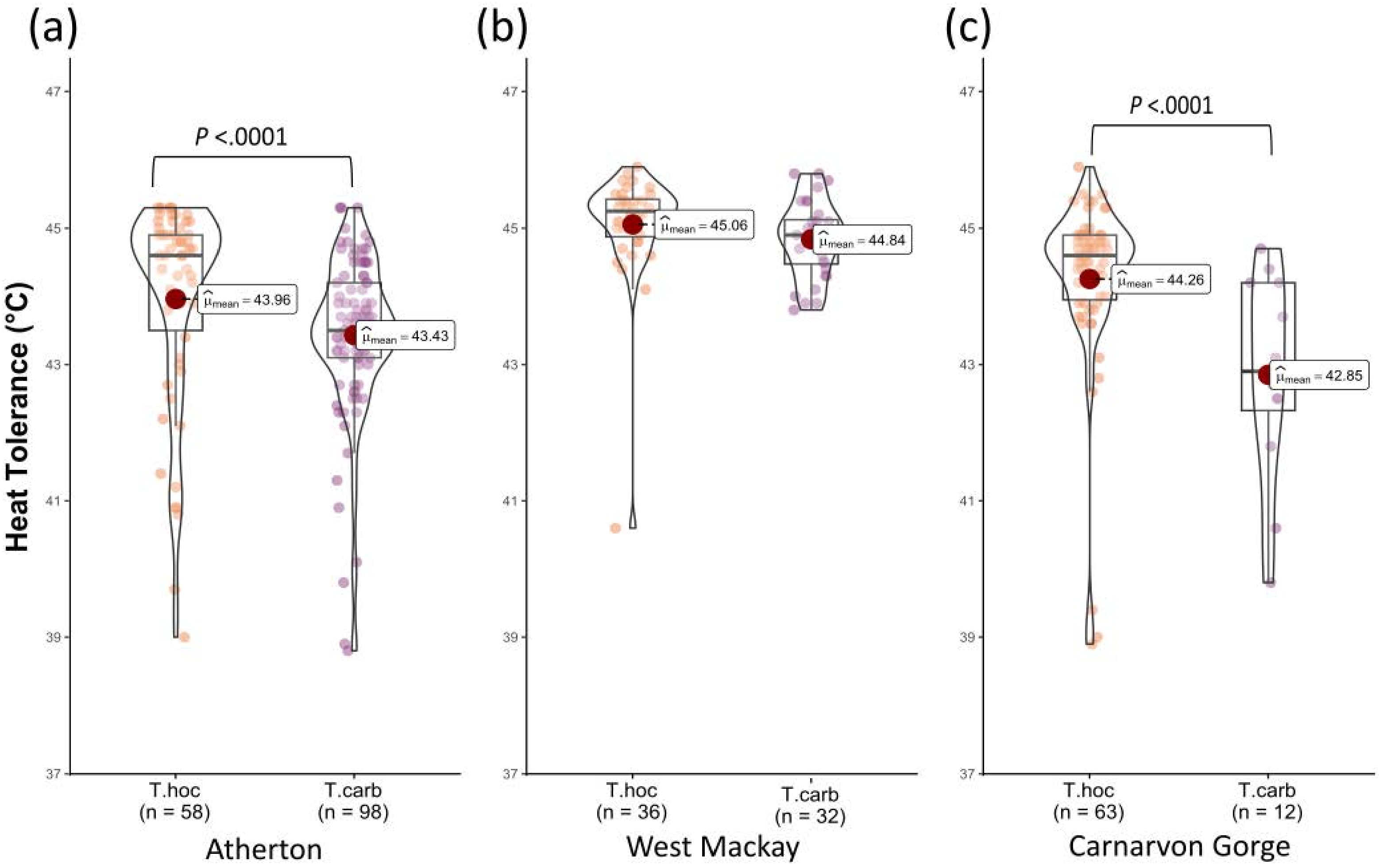

Figure 8. Mean heat tolerance for *T. carbonaria* (T.carb: purple) and *T. hockingsi* (T.hoc: orange) assayed at three locations in Queensland where species co-occurred and could be assayed with sufficient sample sizes: **(a)** Atherton – Location 4, **(b)** West Mackay- Location 13, **(c)** Carnarvon Gorge – Location 18. Significant differences denoted by p-values.

2.4 **Discussion**

In this first investigation of the thermal biology of Australian *Tetragonula* stingless bees, we find, broadly speaking, that the widespread Australian East Coast Species *T. carbonaria* and *T. hockingsi* differ in both heat and cold tolerance, that these thermal tolerances display latitudinal clines within each species, and that thermal tolerances in both species are to some extent associated with climate variation. In our study heat tolerance (measured as the upper critical limit) showed limited variation between species and locations, while cold tolerance (measured as the lower critical limit) showed marked variation both between species and locations. Latitude was an effective predictor of thermal tolerances, with bees both more heat tolerant (both species) and more cold tolerant (only *T. carbonaria*) towards higher latitudes i.e., towards southern ends of their distributions. *T. carbonaria*, the species with the larger latitudinal range (spanning 'remnant' populations in tropical Far North Queensland and in temperate Sydney, most southern sight in study), showed stronger associations between lower critical limits and climate variability trends than did *T. hockingsi*, whose distribution is confined to tropical and subtropical regions. In all, the variation observed in *Tetragonula*'s critical thermal limits within and between species was therefore broadly consistent with local adaptation in these key thermal traits, and thus consistent with the idea that climate plays an important role in shaping the current distributions of these bees.

The patterns we observed within *Tetragonula* for both heat and cold tolerance bore similarities to those seen in other insect studies (Overgaard et al., 2014). Cold tolerance has been found to increase with increasing latitude or elevation in a variety of insects (Bozinovic et al., 2014; Gaston & Chown, 1999; Gonzalez et al., 2022; Hoffmann et al., 2002; Overgaard, Hoffmann, et al., 2011; Overgaard et al., 2014; Overgaard, Kristensen, et al., 2011), similar to the trends found here for *T. carbonaria*. Indeed, climate appears likely to have been important in shaping cold tolerance and the distribution of *T. carbonaria*. This association has also been reported by a recent study looking at neotropical stingless bee species inhabiting different elevational gradients (Gonzalez et al., 2022), finding that cold tolerance increased with increasing elevation whilst heat tolerance did not differ among populations. Further, a study in *Apis mellifera* also reported cold tolerance variation between rural verses urban bee populations, yet equivalent heat tolerances (Sánchez-Echeverría et al., 2019). Yet the lack of either a latitudinal nor climatic variable association for cold tolerance in *T. hockingsi* may suggest that either cold tolerance was not 'ancestrally' important for shaping the distribution of this species, or that

T. hockingsi face some evolutionary constraint with respect to cold tolerance that has prevented them from expanding beyond their current thermal environments (Bennett et al., 2021; Kellermann et al., 2009).

Heat tolerance has been found to be less variable than cold tolerance across a range of ectotherms (Bozinovic et al., 2014). Both *Tetragonula* species showed only a weak latitudinal cline in heat tolerance, with decreasing heat tolerance trending towards lower latitudes. In wild populations of *Drosophilia*, upper critical limits (heat tolerance) also have relatively little power to explain differences in the distributions of these species (Kellermann, Overgaard, et al., 2012). Why heat tolerance should vary less than cold tolerance across space is unclear. It is frequently proposed that heat tolerance is evolutionarily conserved in terrestrial ectotherms, accounting for the limited variability observed in this trait (Sunday et al. 2010). Such a constraint arises because ectotherms cannot increase heat tolerance without trade-offs with other key physiological traits, an idea with some support in insects (Barley et al., 2021; González-Tokman et al., 2020; Jørgensen et al., 2019; van Heerwaarden et al., 2016). Alternatively, climatic variability may simply impose different selective pressures upon species climate tolerance traits, with precipitation (aridity) and temperature perturbations (current and ancestral climate temperature extremes; Bennett et al., 2021) acting differently on heat and cold tolerances at local climate scales (Baudier et al., 2018; Bazzato et al., 2021). For heat tolerance in particular, there may be a complex and important relationship with traits involved in desiccation resistance, given that precipitation is a key driver of heat tolerance in some insects (Bujan et al., 2016; Kalra et al., 2017; Kellermann & van Heerwaarden, 2019). Whatever the cause, the idea of a fixed upper thermal ceiling in heat tolerance has raised alarms for tropical and sub-tropical insect species and may apply also for *Tetragonula*. This is because many of these species appear to be already existing near to their upper limits, and only small increases in temperatures may push species beyond their current heat tolerance (Deutsch et al., 2008; Kellermann, Overgaard, et al., 2012; Sunday et al., 2014). Though tropical and temperate insects may actually possess quite similar climate risks when differential activity periods are accounted for (i.e., temperate species have narrower activity periods; Johansson et al. (2020)).

The variation in *Tetragonula* thermal tolerances observed in this study, both within and between species, are consistent with species and populations having adapted to their local environments. However, both adaptation (genotypic change) and plasticity might contribute to the critical thermal limits of *Tetragonula* (Gao et al., 2018; Kellermann & van Heerwaarden, 2019; Rodrigues & Beldade, 2020; Valladares et al., 2014), and the relative role of genotype and environment on these traits remains unclear (Chung & Schulte, 2020; Jørgensen et al., 2021). In *Tetragonula*, the direction of the weak observed clines in heat tolerance are opposite to what would be expected if warmer environments produced more heat tolerant individuals; this would seem to suggest that plasticity is unlikely to explain the patterns observed in heat tolerance in these species. Furthermore, the between-species differences in heat and cold tolerance we observe at co-occurring sites, where both species are exposed to the same environmental conditions must be driven by innate differences between the species (because otherwise we would expect all individuals at the same location to have the same trait values). However, the capacity for plasticity can itself be a species-specific trait. That is, plasticity in critical limits is a complex trait, which is itself under selection and can thus be encoded in genotypes (Kelly, 2019; Scheiner, 1993). For example, the lower cold tolerance observed in *T. carbonaria* may arise because this species has greater *plasticity* in cold tolerance than *T. hockingsi* (rather than a *fixed* lower cold tolerance).Further investigation of the extent to which critical limits in *Tetragonula* vary across space and time within species is now needed to better understand the extent to which these limits reflect local adaptation, and in turn whether they are thus likely to constrain species' climate change responses.

In addition to climate, interspecific competition is often an important factor determining the distribution of species. *T. carbonaria* and *T. hockingsi* have largely allopatric distributions, except for a few regions in Queensland. They are ecologically similar species that are likely to compete for nest sites and food resources where they co-occur (Heard et al 2016). The differences in thermal tolerance between the species we observe is consistent with competition driving thermal niche partitioning in these species, such that *T. hockingsi* dominates in hotter and drier locations, while *T. carbonaria* occupies cooler climates, including higher altitude areas in norther Queensland. One explanation for their current distribution is that *T. carbonaria* was previously widespread throughout Queensland but was forced onto wet tropic rainforest mountain refugium during the Pliocene due to changing climates, and possibly also direct competition with *T. hockingsi*. Indeed, *T. hockingsi* is generally considered the more aggressive species by beekeepers and is known to usurp the nest sites of *T. carbonaria* in Brisbane where the two species co-occur (Cunningham et al. 2014; Xia 2022). Similar restricted distributions in Queensland highlands are known for other cool-rainforest adapted species (Hilbert 2007). In this scenario, *T. hockingsi* is the more competitive species in warmer climates, but it's narrower thermal envelope (i.e., lower cold tolerance) prevent it from out-competing *T. carbonaria* in cooler regions. Phylogenetic models estimating the relative divergence times of different *Tetragonula* populations, relative to past climate change, are needed to further test these ideas of thermal niche partitioning

Can we predict how Australia's East Coast *Tetragonula* will respond to changing climates? One possibility is that species will shift their ranges as local climates change, to remain within their optimal thermal envelopes. This would suggest that new areas of New South Wales become suitable habitat for each species (south coast NSW for *T. carbonaria* and north coast NSW for *T. hockingsi*). Interestingly, *T. hockingsi* has already shifted its range southwards in recent decades due to beekeeper activities (Paul et al. 2023). For example, while it currently co-occurs in Brisbane with *T. carbonaria*, it was considered rare or even absent from this location only a few decades ago (Heard, pers. comm). Other adaptive responses to changing climates (e.g., evolution of thermal limits themselves) will depend on the adaptive capacity of these traits in *Tetragonula*. In some parts of their range, the environmental temperatures of periodic heat waves already exceed the critical thermal limits of *Tetragonula* workers we observe here (see also Chapter 3). However, under natural conditions, social insects might have considerable resilience to heat stress events due to social behaviours that can mitigate thermal stress (Menzel & Feldmeyer, 2021). At a bare minimum, stingless bee foragers can presumably remain in the nest and avoid foraging on days of extreme heat. In all, further work is now needed therefore to understand the mechanisms underlying thermal limits in Tetragonula, their possible mitigating behaviours, and therefore the likely climate change responses in this important group of Australian pollinators.

Chapter 3

A first indicator that critical thermal limits are shaped by genotype and environment in a social bee: A reciprocal transplant experiment.

3. **Abstract**

Warming and changing climates are predicted to drive changes in insect abundances and distributions globally. As such, predicting how a given insect population will respond to climate change depends in part upon understanding the mechanisms that determine its critical thermal limits (or thermal tolerances). Species with high phenotypic plasticity in these key tolerance traits may be better equipped, in the short term, to tolerate changes in their thermal environment. In this study we aimed to assess the relative contributions of genes (local adaptation) and environment (plasticity) to within-species variation in heat and cold tolerance in the Australian stingless bee *Tetragonula carbonaria*. This species is an important insect pollinator in natural and agro-ecosystems on Australia's North-East coast.

We conducted a year-long reciprocal transplant experiment between hived colonies sourced from their temperate range edge (Sydney, N.S.W.) and their mid-sub-tropical range (Tweed Heads, N.S.W.). Bees were assayed for heat and cold tolerance pre-transplant and then again three times post-transplant (Autumn, Spring and Summer). We found that bees heat tolerance was largely invariant across locations and between populations, but did vary marginally across seasons. Variation in cold tolerance meanwhile was consistent with being shaped more by local adaptation (i.e., population-of-origin effects) than current location, with temperate-origin bees always tolerating lower temperatures, as well as showing greater seasonal plasticity in cold tolerance than subtropical-origin bees.

In all, our data show that both genotype and environment (and their interaction) contribute to thermal tolerances in *T. carbonaria,* though to differing degrees. This data contributes to our efforts to understand likely future shifts in Australian native pollinator distributions.

3.1 **Introduction**

In insects and other ectotherms, thermal limits (or tolerances) – the upper and lower temperatures at which the animal can function – are thought to be key physiological traits associated with species' distributions (García-Robledo et al., 2016; Overgaard et al., 2014) (Chown & Nicolson, 2004). Many insect species show variation in these traits aligning with variation in local thermal environments, including *Drosophila* (Kellermann, Loeschcke, et al., 2012; Schiffer et al., 2013), termites (Janowiecki et al., 2020) and bumble bees (Jackson et al., 2020). In particular, thermal tolerances are predicted to align to the temperature extremes an organism will experience in its habitat (Bennett et al. (2021), such that species are rarely exposed to temperatures that exceed their upper or lower thermal limits ((Overgaard et al., 2014).

Variation in thermal tolerance may also exist within a single species across its distributional range. As with the variation seen between species, intra-specific variation in these traits is often associated with environmental differences. For example, populations of burying beetle (*Nicrophorus nepalensis*) inhabiting mountains of different altitudes and latitudes (and thus climates) vary in their thermal tolerances (Tsai et al., 2020). Similarly, intra-specific thermal tolerance variation is also observed in some *Drosophila* species (Ranga et al., 2017); heat tolerance was observed to be higher in *D. melanogaster* individuals sampled from lower (more tropical) latitudes (Sgrò et al., 2010). An understanding of the mechanisms that enable insects' thermal traits to vary intraspecificaly, and thus match incongruent thermal environments across space, is important for understanding how insect populations might respond to changing thermal environments across time, determining the relative risk that climate change may pose to insects and ecosystem services (Legg, 2021).

Broadly-speaking, two mechanisms may account for a species' thermal tolerance being suitably matched to their thermal environment: local adaptation (intrinsic adaptive genetic variation) and phenotypic plasticity (phenotypic variation induced by the environment) (Gao et al., 2018; Kellermann & van Heerwaarden, 2019; Rodrigues & Beldade, 2020; Valladares et al., 2014). Locally adapted thermal tolerance arises from innate genetic variation specific to the unique habitat of the population (or species), having evolved *in situ* in that habitat. In this case, the trait will have significant additive genetic variance underpinning it, for selection to act upon (Falconer & Mackay, 1996). Conversely, phenotypic plasticity in thermal tolerance is generally defined on an individual level (i.e. within a lifetime rather than over generations) as the capacity of individuals to rapidly alter their thermal tolerance in the direction of an abiotic temperature stress (Schulte et al., 2011). This may be during development or as an adult (developmental plasticity and adult thermal acclimation respectively). One nice example of thermal plasticity in an ectotherm is observed in two intertidal *Acartia* copepod species (*A. tonsa* and *A. hudsonica*), which display cyclic thermal tolerances' in response to seasonal temperature fluctuations (Sasaki & Dam, 2020). As is often the case, these adaptive mechanisms (local adaptation and plasticity) are not mutually exclusive. Rather, variation in thermal tolerances can be influenced by both genetic variation within a population and environmental variation, i.e. gene-by-environment interactions (Culumber et al., 2015). Furthermore, plasticity itself is likely heritable and may also evolve, potentially making some populations more capable of responding rapidly to temperature perturbations than others (Kellermann et al., 2017).

Reciprocal transplant experiments are a powerful means to test the relative contribution of genotype (local adaptation) and environment (phenotypic plasticity) to spatial variation in thermal traits (Johnson et al., 2022; Nooten & Hughes, 2017). Comparing the traits of individuals in their native ('home') environment versus those transplanted into novel ('away') environments allows us to assess the strength of genotype-by-environment interaction effects and thus to what extent these traits may be locally adapted or plastic in their response. In the context of thermal tolerance traits, field-based transplant experiments have been widely used for sedentary organisms such as plants (Anderson et al., 2015; Gao et al., 2018), corals (Chakravarti & van Oppen, 2018; Klepac & Barshis, 2020) and other intertidal invertebrates (Gleason et al., 2018; Sasaki & Dam, 2021). However, few mobile terrestrial species are amenable to these types of experiments, with the exception of some ants (Bujan et al., 2022; Diamond et al., 2022; Martin et al., 2021). In insects, such experiments of gene-by-environment effect on thermal tolerances are commonly restricted to keeping individuals within cages or other controlled spaces, with the majority of thermal tolerance research conducted upon the model insect *Drosophila* (Camus et al., 2017; Davis et al., 2021; Hoffmann, 2010; Jørgensen et al., 2019; Kellermann, Loeschcke, et al., 2012; Kellermann, Overgaard, et al., 2012; Kellermann et al., 2017; Kellermann et al., 2009; MacLean et al., 2019; Ørsted et al., 2019; Overgaard, Hoffmann, et al., 2011; Overgaard, Kristensen, et al., 2011; Ranga et al., 2017; Sgrò et al., 2010; van Heerwaarden et al., 2016). While this body of work has delivered remarkable insights, studies on non-model insect species, and in more ecologically authentic contexts, are also needed.

In this study, we perform a reciprocal transplant experiment with the eusocial stingless bee *Tetragonula carbonaria* to investigate the extent to which plasticity shapes its thermal tolerance traits. Stingless bees (Tribe Meliponini) are suitable for investigating thermal tolerance via reciprocal transplant experiments as they are central place foragers that return daily to the nest, and many species can be kept and managed in readily movable wooden hives. *T. carbonaria* naturally inhabits a dynamic climatic gradient on the Australian East Coast, from isolated parts of tropical Queensland down to the temperate New South Wales south coast (Figure 1). Stingless bees are principally distributed in the world's tropics, and the southern range of *T. carbonaria* give it the most temperate distribution of any stingless bee (Garcia Bulle Bueno et al., 2022; Heard, 2016). Furthermore, recent evidence shows that this species shows variation in heat and cold tolerances across its broad range (Chapter 2).

We transplanted *T. carbonaria* between the northern (subtropical) and southern (temperate) ends of a continuous population spanning 900 km along Eastern NSW. Stingless bees have low female dispersal (Bueno et al. 2022) and evidence from both nuclear and mitochondrial markers indicate that there is strong genetic structure across this range (Chapman et al., 2018; Nacko, 2023), indicating that these bees may be local adapted to these sub-tropical and temperate environments. We maintained colonies for a one-year period pre- and post-transplant, and assayed heat and cold tolerances (critical thermal maxima and minima respectively) of foraging workers (females) across three seasons (Autumn, Spring and Summer). We then assessed the extent to which population-of-origin (subtropical or temperate) and environment (location and season) predicted variation in these thermal traits. Lastly, we examined thermal tolerance limits in our study populations of *T. carbonaria* as they relate to the current environmental temperature extremes they experience (i.e. their thermal safety margins (Bennett et al., 2021; Leclair et al., 2020).

Throughout this experiment, we specifically asked; (i) Do *T. carbonaria* thermal tolerances show signatures of local adaptation? If so, we expected that transplant treatment would have little effect upon the tolerance of 'away' colonies; (ii) Are thermal tolerances shaped in part by plasticity? If so, then we expected a strong signature of current environment, with the tolerances of 'away' colonies matching those of 'home' colonies in a shared environment; (iii) Do we detect decreased survival of 'away' colonies relative to 'home' colonies? If local adaptation in thermal traits was essential for survival, then we might expect higher mortality in transplanted 'away' colonies than 'home colonies in their original environment; and (iv) Do *Tetragonula*'s thermal limits match their current climates' extreme temperatures across seasons?

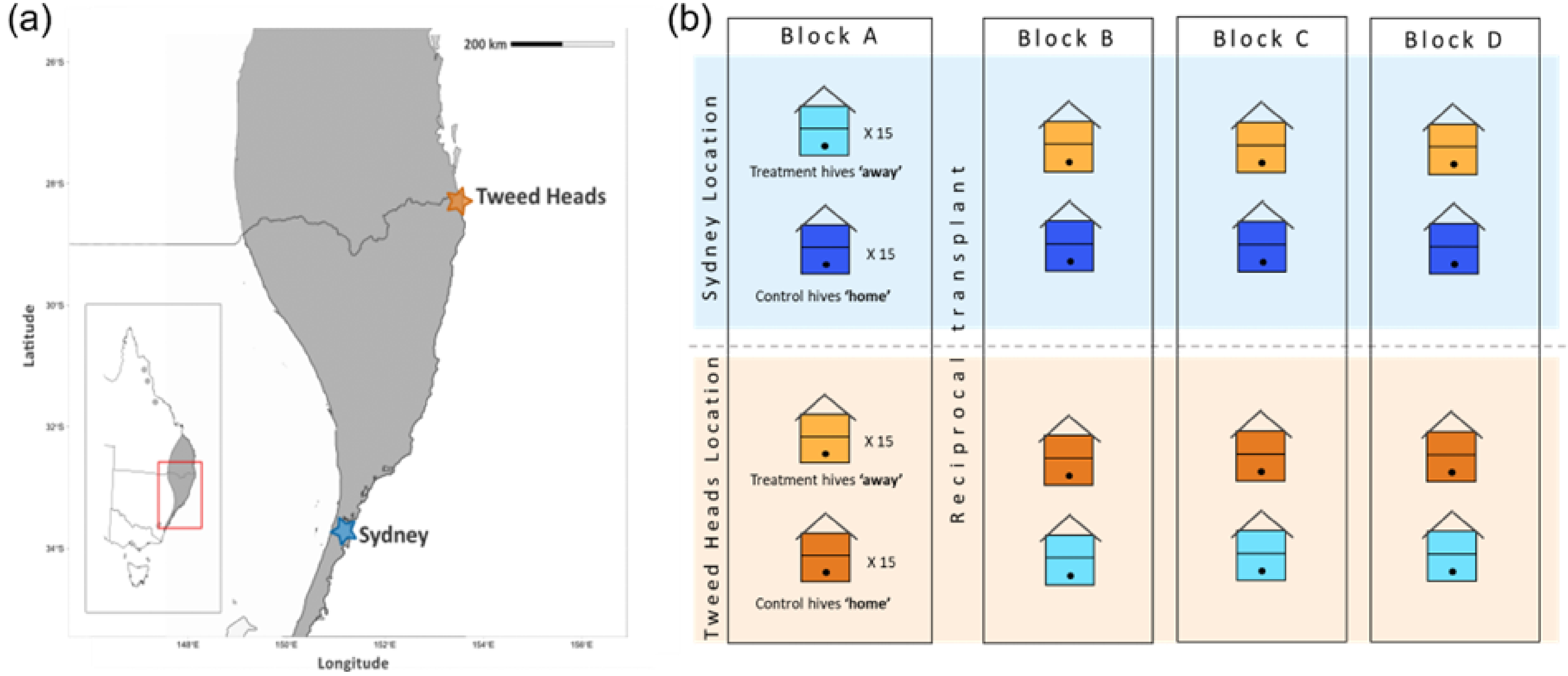

Figure 1. (a) Temperate (Sydney) and subtropical (Tweed Heads) locations within the NSW range (grey) of *T. carbonaria*, and **(b)** A reciprocal hive transplant experiment conducted from February 2021 to February 2022; n = number of observations per block (90 individuals tested per hive x 30 hives per location = 2,700 x two locations = 5,400 number of observations per testing block). Thermal tolerances were assayed at four-time blocks: Block A – pre transplant (Summer); Blocks B (Autumn), C (Spring), D (Summer) – post transplantation.

3.2 **Methods**

3.2.1 **Reciprocal transplant experiment**

To assess the contribution of local adaptation (genetic variation) and local environment (plasticity) to the thermal tolerance of *T. carbonaria* workers, we performed a reciprocal transplant experiment of one year duration (summer 2021- summer 2022) with hived colonies. We translocated hives between two sites within the native range of *T. carbonaria* along the New South Wales coast, approximately 800km apart; Figure 1. The southern site was located at Ku-ring-gai Council Wildflower Nursery, Sydney, N.S.W. (-33.707017, 151.179709); this site is close to the southern edge of the species distribution and has a temperate climate. The northern site was a private property in Terranora, Tweed Heads, N.S.W. (-28.232319, 153.528099); this site has a humid subtropical climate, with warmer winters than the southern site (Köppen-Geiger climate zones from Peel et al., 2007 and Meteorology (2022); Supplementary Tables S1-S2).

We sourced thirty *T. carbonaria* colonies from bee breeders at each site (N = 60 total colonies). Southern colonies (N = 30) were sourced directly from Ku-ring-gai Council, in Sydney, NSW, where they are propagated. Northern colonies (N = 30) were sourced from Indooroopilly Brisbane, QLD (124km north of our northern site) and allowed to acclimate on site for two months prior to the experiment. The colonies at both sites were housed in similar wooden hive boxes (internal volumes: Syd ~14,100 cm3, and Tweed: ~10,000 cm3).

After acclimation both populations colonies had their thermal tolerance assayed in-situ (whilst all colonies were residing in their 'home' locations) prior to translocation. We then translocated fifteen colonies from each site to the alternative site, such that each site contained 15 'Home' colonies (i.e., control treatment: hives remain in their local environment) and 15 'Away' colonies (i.e., translocation treatment: hives are moved to a new environment); Figure 1. For all colonies, we assessed (i) worker critical thermal maxima and minima, and (ii) colony strength and survival, at four time points (Block A Summer: Feb-Mar 2021, Block B Autumn: May-June 2021, Block C Spring: Oct-Nov 2021, and Block D Summer: Feb 2022). We retained all bees post thermal assays on 100% ethanol.

3.2.2 **Thermal tolerance assays**

We estimated the critical thermal maxima and minima limits (hereafter heat tolerance and cold tolerances respectively) of *T. carbonaria* foragers using a standard dynamic ramping protocol, adapted from previous studies of small insects (Chown et al., 2009; Kellermann & van Heerwaarden, 2019; MacLean et al., 2019; Overgaard et al., 2014). We first collected foragers at hive entrances between 9-11 AM and placed them individually into glass holding vials (93mm x 22mm) with free access to food (1cm cotton balls soaked in 1:1 glucose solution) for at least 1 hour prior to individual transfer into glass assay 7ml vials without food. We labelled each assay vial with a unique identifier and then positioned them randomly into a 100-vial Perspex holding rack. The rack with vials was then submerged into a 26°C circulating water bath (Perspex tank dimensions: 32cmWx15cmDx33cmH) that allowed continual observation of the bees in their vials throughout the assay period.

We slowly heated (for heat tolerance) or cooled (for cold tolerance) the water bath at a ramping rate of 0.1°C per minute and recorded the critical thermal limit of individual foragers as the temperature in which all motor function ceased. To achieve precise water bath temperatures, we used a bipolar heating and cooling thermoelectric temperature controller and high-density bonded-fin heat sink unit with USB interface software (TE Technology, Inc. 2021) and a circulating pump. For cold tolerance assays, we added 1:1 glycol to water to prevent freezing. Assays lasted 4- 5 hours until all bees had reached critical limits.

We set out to test each populations' thermal tolerances measured in separate thermal tolerance assays (Cold tolerance assays and heat tolerance assays; see above), across four temporal time blocks (~ 3months apart) over the course of a year. In each testing block (A, B, C and D), both populations (Sydney and Tweed Heads) had 12 workers assayed (across four replicate thermal assays) from each of their 30 colonies, per population (n = 360 total bees assayed per testing block) for each thermal tolerance assay (heat and cold). This gave a total of 720 assayed bees per testing block ($n = 360_{\text{population}}$ x 2 populations), 2,880 assayed bees per thermal tolerance trait across the whole experiment ($360_{\text{population}} \times 4_{\text{Test Blocks}} = 2880_{\text{obs}}$).

3.2.3 **Colony survival**

We recorded a colony death during the experiment if foraging activity completely ceased; colonies meeting this criterion were opened to confirm they contained no queen and little to no brood or surviving adults. We also measured two proxies of colony health that can be indicative of failing colonies at each of our four-time blocks: colony weight and forager count. We weighed hives to the nearest 100g, and then compared proportional change in colony weight since the start of the experiment (where colony weight = hive weight – weight of empty hive box). The weight of a stingless bee colony is the combined weight of brood, bees and resources and its relationship to fitness is complex. This is because colonies that are actively reproducing (i.e., establishing a daughter colony in a new location) may lose weight in the short term, as workers and stores are transferred to the new site. However, consistent weight loss over an extended period of many months is typically indicative of a colony in poor health (Heard, 2016).

3.2.4 **Queen survival**

We assessed queen survival per colony over the one year of the experiment for two reasons: (1) to confirm that we were assaying full sisters across each testing block, and (2) because elevated rates of queen death may indicate colony stress, and thus are another fitness measure. In *T. carbonaria*, virgin queens are reared by colonies all year round (Bueno et al., 2022). When resident queens die, a new queen is selected among these available virgin queens. Swarms of males from colonies in the area aggregate outside colonies which have unmated queens; these queens then leave the colony briefly to mate (Bueno et al., 2022; Heard, 2016). Queens mate with a single male from the swarm and return home. “Away” colonies that requeened in their new environments are therefore likely to have mated with local males.

We genotyped starting (Block A) and final (Block D) worker samples per colony to establish whether requeening had occurred during the experimental period. We extracted DNA from whole abdomens per worker in a 5% Chelex solution (Walsh et al., 1991). We then amplified each sample at five fluorescently-labelled microsatellite marker loci: Tc3. 155, Tc4. 302, Tc4.

214, Tc4. 287 and Tc4. 63 following (Green et al. 2001). We analysed the subsequent products using a 30130xl Genetic Analyzer and Genemapper Version.5 (Applied Biosystems, United States). We manually inferred the maternal (queen) and paternal alleles for each colony based on shared alleles of the 12 workers per colony per time block. We then determined that a colony had requeened if the alleles at any one locus indicated a new maternal and paternal genotype. For any colonies that showed this change, we then genotyped a further 12 workers from each of Block's B and C to establish the time of requeening.

3.2.5 **Data analysis**

All analyses were performed using R statistical software (Team, 2022: R version 4.2.1 (2022-06-23 ucrt)).

Colony and queen survival

We compared re-queening rates between pairs of home and away colonies using Fisher's Exact Tests. Further, proportional hive weight changes were assessed for both populations and their corresponding treatment groups ('Home' and 'Away'). We used linear regression to assess proportional change in hive weight during the year between home and away colonies (i.e., weight change from time Block A to Block D as a proportion of Block A weight).

Heat and cold tolerance

Prior to statistical analysis, critical thermal limit data sets were tested for normality via QQ plots (quantile-quantile plot) and histograms. Normality assumptions were not met in the case of our heat tolerance data, as observed in heat tolerance assays in other studies (Martin & Huey, 2008). Invasive log transformations were unadvisable and this skew is likely to be ecologically significant (DeWitt & Friedman, 1979; Martin & Huey, 2008), thus only outliers outside of two standard deviations in our heat tolerance data sets were removed. Outliers were identified as bees that ceased movement ≥ 9°C for cold tolerance (N=27 from 2634 total) and ≤37°C for heat tolerance (N=32 from 2605 total).

We also removed from our dataset any workers from colonies that had re-queened and which were therefore not offspring of our starting queens. To determine the effect of population-of-origin, location and testing season on thermal limits, we used linear models and linear mixed-effects models (LMM) (stats package (version 3.6.2); lme4 package (version 1.1.31; Bates et al., 2014); lmerTest package (version 3.1; Kuznetsova et al. 2016). LMM were fit using Restricted Maximum Likelihood (REML) coupled with type III analysis of variance (ANOVAs using Kenward-Roger method to compute denominator d.f). In all cases, upper and lower critical limit data were modelled separately. We used comparative regression analysis (fitted using maximum likelihood) to compare null and full model fits via Akaike information criterion (AIC) and log likelihood ratios (logLink) metrics. Inferential summary statistics were also used to determine LMM and effect variances, along with R^2 values (Nakagawa & Schielzeth, 2013).\. We then performed *Post hoc* t-testing upon ‘statistically significant’ interaction effects using the emmeans package (version 1.8.3, Lenth, 2021).

In all, we used two sets of models for heat and cold tolerance responses respectively. First, to determine whether our initial experimental populations (i.e., pre-transplant; Block A timepoint) varied in critical thermal limits, we used simple linear regressions. Second, to determine the effect of genotype and environment (location and season) on thermal tolerances, we ran linear mixed models of post-transplant data (Blocks B-D) using Population-of-origin, Transplant Treatment group (‘Away’ or ‘Home’) and Season (Testing block) as fixed effects. In these models, random effects were nested, with experimental day (test block x location x day) and assay run number (block x location x day x run) included as random effects. We also estimated thermal tolerance breadth at the colony-level (i.e., the temperature range between the mean heat and cold tolerance values for all workers assayed from a given colony) for ‘home’ populations (N=30 total colonies). We visualized data using ggstatsplot (version 0.9.5; Patil 2021), ozmaps (Sumner et al., 2021) and ggplot2 (version 3.4.0; Wickham 2016).

Association of thermal tolerances with monthly temperature extremes

We used linear regression to assess whether the heat and cold tolerances of *T. carbonaria* from ‘home’ populations fluctuated in accordance with the temperature of the testing months (the highest or lowest recorded monthly temperature in their home location:

Australian Bureau of Meteorology; Tables S3.1-S3.2). Models included population-of-origin, Max/Min recorded monthly temperatures and their interaction upon Heat or cold tolerance response's respectively.

3.3 Results

3.3.1 **Colony and queen survival**

In all, we found no differences in colony or queen survival between local and transplanted colonies that would suggest higher fitness for local colonies. All 30 home colonies, and 29 of the 30 away colonies, survived the one-year duration of the experiment; one translocated colony (from Tweed Heads to Sydney) perished between the autumn and spring assay periods. Transplanted colonies were essentially no more likely to lose weight over the course of the year than home colonies at the same site; indeed, at our southern site, 'Away' colonies increased more in proportional weight than local colonies (9.02% ± 3.48 *SE, t* = -2.59, *p* = 0.012; Mean proportional weight change: Sydney 'Home' = 4.04%, Tweed 'Away' = 11.66%). This likely arose because the southern site was adjacent to National Park and particularly resource rich, as all colonies at the southern site increased more in weight (relative to starting weights) than those at the northern site (7.86% ± 3.26 *SE*, *t* = -2.409, *p* = 0.02; Mean weight change: Tweed 'Home' = -1.10%, Sydney 'Away' = -2.96%); Figure 2; Supplementary Table3). Most queens also survived the duration of the experiment (50 of 60 queens). Of the 10 colonies that did requeen, the majority were from the Tweed Heads population (9 of 10; Fisher's Exact Test: p=0.012; χ^2 = 5.88, *p* = 0.015). However, translocation did not impact the rate of requeening, with 'Away' colonies no more likely to re-queen than those from the same population that remained home (Sydney 'Home': n = 0 vs. Sydney 'Away': n = 1, Fisher's Exact Test: p=1.000; Tweed Heads 'Home': n = 6 vs. Tweed Heads 'Away': n=3 Fisher's Exact Test: p=0.427; Supplementary Table S3.4).

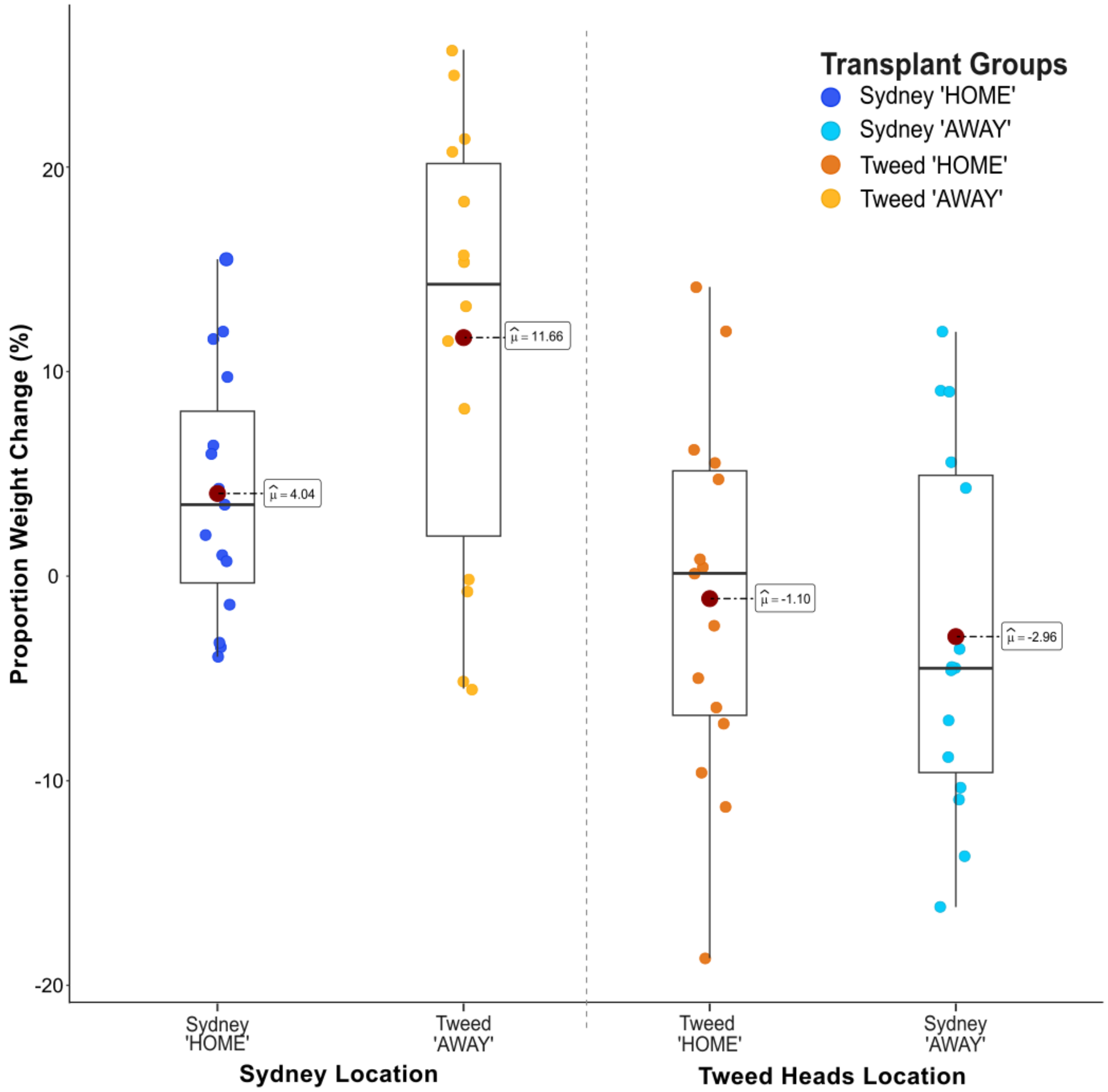

Figure 2. Proportional change in hive weight between the start and end of the transplant experiment for *T. carbonaria* originating from Sydney of Tweed Heads that were either transplanted ('Away') or not ('Home').

3.3.2 **Heat tolerance**

Prior to transplants, *T. carbonaria* at our subtropical site (Tweed Heads) had marginally higher critical upper thermal limits (heat tolerance) than those at our temperate site (Sydney); mean difference: 0.42°C ± 0.08 *SE*, $t = 5.446$, $p < .0001$; linear regression analysis, Block A; Model fitting: H_0 AIC= 2098, H_a AIC= 2071; R^2(Multiple) = 0.040, R^2(Adjusted) = 0.039; ANOVA, $F(1,712_{df}) = 29.66$, $p < 0.0001$.).

In all, however, we found that neither population-of-origin nor environment (i.e., transplant treatment or season) were strong predictors of heat tolerance across the full year of our experimental period (Figure 3). Linear mixed-effect modelling indicated that a full model accounted for only 5% of variation in heat tolerance (H_a: population x treatment group x season; R^2(Marginal) = 0.050) and did not explain variation in heat tolerance any better than a reduced (null) model with intercept only (H_0) (Model fitting: H_0 AIC = 6187.0, H_a AIC = 6181.3; Table 1). Mean values of heat tolerance for all treatment groups lay between 44.1 – 45.3°C (Figure 3).

Regression analysis of the full model did indicate some effect of season on heat tolerance, driven principally by variation in the heat tolerance of temperate-origin *T. carbonaria* (Sydney) between Autumn and Summer (August mean: 44.5 ± 0.16 *SE*, Summer mean: 45.3 ± 0.16 *SE*; difference: 0.80°C ±0.22 *SE* ($t(9.65_{df}) = -3.570$, $p = 0.045$).

Table 1. Analysis of variance (ANOVA) Type III) test of the effects of population-of-origin (Sydney or Tweed Heads) and Transplant Treatment group (Tx group: 'Home' vs. 'Away') and Season (Testing Blocks B-Autumn, C-Spring, D-Summer), plus their interactions, on heat tolerance variation in *T. carbonaria*. SS: Type III sum of squares; *MS*: Type III mean of squares; Den*df*: denominator degrees of freedom - Kenward-Rodger.

Type III - ANOVA

Source of variance	***SS***	***MS***	***df***	**Den*df***	***F***	***p***
Population	0.03	0.03	1	1792.03	0.020	0.889
Tx group	2.39	2.39	1	1792.03	1.409	0.235
Season	21.03	10.51	2	4.56	6.195	0.050
Population x Tx group	5.48	5.48	1	4.86	3.231	0.134
Population x Season	1.33	0.66	2	1792.03	0.391	0.676
Tx group x Season	13.63	6.81	2	1792.03	4.016	0.018
Population x Tx group x Season	12.78	6.39	2	4.86	3.764	0.103

Model Residual Variance = 1.696, *SD* = 1.302, REML criterion of 6176.3, R^2(Marginal) = 0.050, R^2(Conditional) = 0.0817, N = 1822

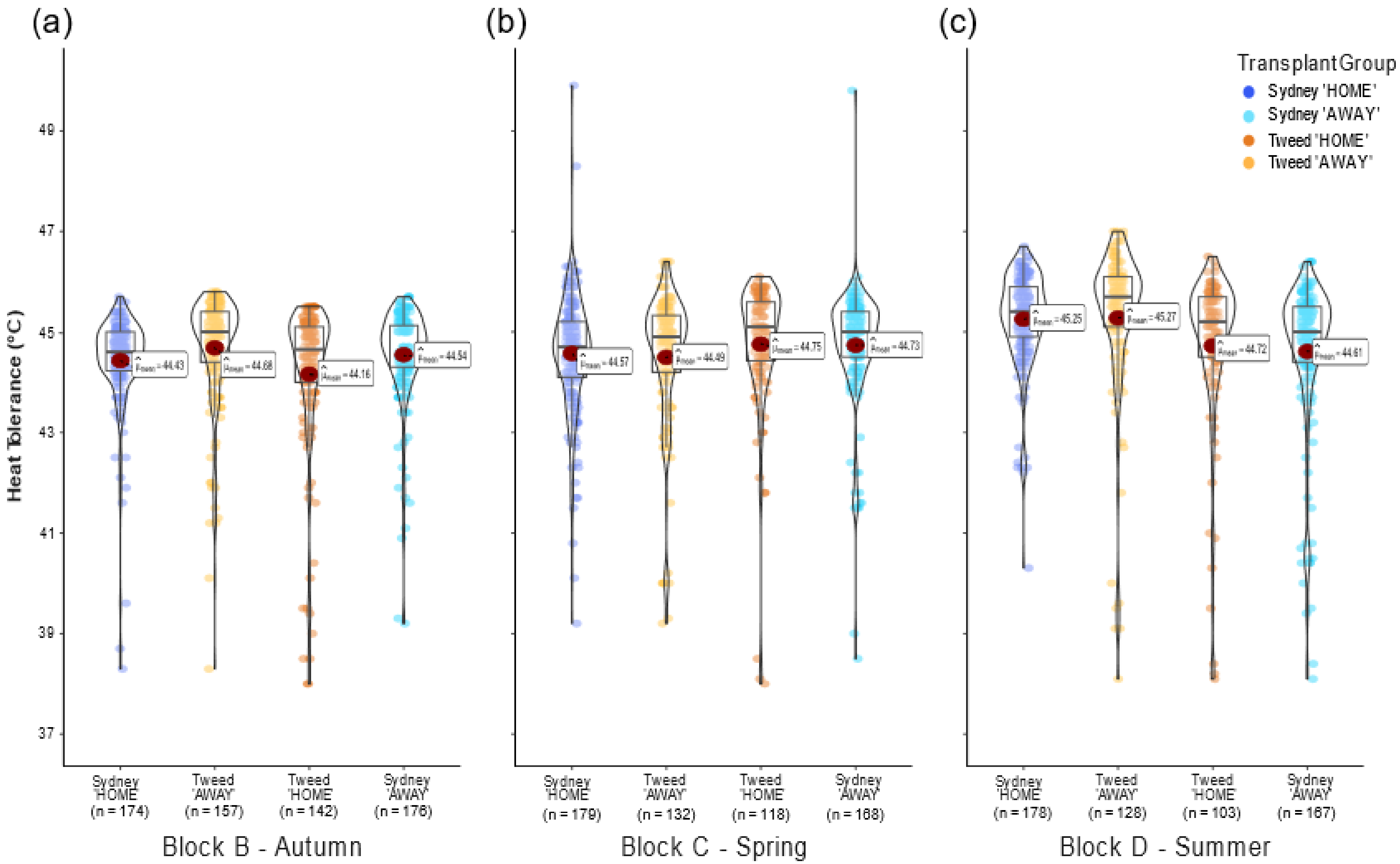

Figure 3. Heat tolerance measured as CT_{max} (°C) for post-transplant *T. carbonaria* in four transplant treatment groups (Sydney 'Home'- dark blue, Sydney 'Away'-light blue, Tweed Heads 'Home'-dark orange, and Tweed Heads 'Away'-yellow) assayed at each of three testing blocks: **(a)** Block B – Autumn 2021; **(b)** Block C – Spring 2021; and **(c)** Block D – Summer 2022. Red circles within each Transplant treatment group violin plot indicate group heat tolerance means with values given in text boxes (estimated means of Transplant Tx groups). Number of observations per Tx group (n =).

3.3.3 **Cold tolerance**

Pre-transplant, the local *T. carbonaria* populations of our temperate and subtropical sites differed in their cold tolerance (critical thermal minima), with the temperate (Sydney) population tolerating the lowest temperatures (difference: -0.95°C ± 0.06 *SE*, *t* = 15.51, *p* <.0001; linear regression analysis, Block A; Model fitting: *N* = 708; H_0 AIC = 623.30, H_a AIC = 464.95; R^2(Multiple) = 0.254, R^2(Adjusted) = 0.253; ANOVA, *F*($1,706_{df}$) = 240.46, *p* <.0001.). Post-transplant, variation in cold tolerance was consistent with this trait being shaped by both genotype and environment. That is, both population-of-origin and local environment (location and season) contributed to observed variation in the cold tolerance of *T. carbonaria* workers (Figure 4). Linear mixed effect model fitting indicated that a full interaction model (including Population, Transplant Treatment and Season) captured the observed variation in cold tolerance better than the null model (H_0 AIC = 6630.0, H_a AIC = 6427.0; Figure 4; Table 2).

Population-of-origin (i.e., genotype) in particular was a key explanatory predictor of CT_{min} variation (A; (Table 2; Supplementary Table S4); indeed, a model lacking Population as a key predictor (i.e. Transplant Treatment group and Season only: H_a AIC = 6585.5) performed worse than a model having Population as the only predictor (H_a AIC = 6455.7). Those bees originating from the more temperate population (Sydney) consistently tolerated lower temperatures than those from the subtropical population (Tweed Heads), regardless of location or season (Figure 4; on average, they tolerated temperatures 0.80°C ± 0.064 *SE* lower; (*t*(1812_{df}) = -12.541, *p* <.0001; N=1047 Sydney, N=795 Tweed Heads). Further, the combined effect of Population and Transplant Treatment group (averaged across all seasons) revealed the same trend in between-population cold tolerance variation for pairs of home and away treatments inhabiting the same location; namely, bees in the Sydney 'Home' treatment were more cold tolerant than the co-located Tweed-origin 'Away' bees (difference: 0.82°C ± 0.089 *SE* (*t*(1812.10_{df}) = -9.289, *p* <.0001; temperate location), and Sydney-origin 'Away' bees were more cold tolerant than bees of the co-located Tweed 'Home' group (difference: 0.78°C ± 0.092 *SE* (*t*(1812.03_{df}) = -8.468, *p* <.0001; subtropical location); Figure 4.

Nevertheless, a two-way interaction between location (Transplant Treatment group) and testing season also contributed to our best-fit model, indicating that cold tolerance in *T. carbonaria* does have a plastic component; Table 2; Supplementary Table S4). This effect was most prominent between treatment groups ('Home' and 'Away') during Autumn, in which bees in the Away treatment at both sites were less tolerant too cold than those of the 'home' treatment (average 0.47°C ± 0.10 *SE* ($t(1812_{df}) = 4.417$, $p < .0001$; averaged over Population). In particular, within-location variation was driven by the cold tolerance displayed in autumn by temperate-origin (Sydney) 'Home' bees, which was lower than that of the temperate-origin colonies transplanted north (location effect; 1.98°C ±0.47; $t\ (10.3_{df}) = 4.213$, $p = 0.007$) and also lower than that of the same colonies assayed in spring (seasonal effect; 2.02°C ±0.49; $t = -4.141$, $p = 0.008$); Figure 4).

Given that heat tolerance varied little between populations and across seasons, the overall thermal tolerance range (TTR) seen in *T. carbonaria* colonies was primarily determined by variation between colonies in cold tolerance (ANOVA: $F_{1,72} = 63.485$, $p < .0001$; Figure 5). Consistent with the thermal requirements of living in regions of greater seasonality, *T. carbonaria* originating from the temperate climate (i.e., Sydney 'Home') showed a larger thermal tolerance range ($\mathrm{EMM}_{\mathrm{sydney}}$ 41.3 ± 0.27 *SE*) than that of the sub-tropical population ($\mathrm{EMM}_{\mathrm{Tweed}}$ 39.5± 0.29 *SE*), estimated difference of 1.82°C (± 0.23 *SE*, $t = 7.968$, $p < .0001$).

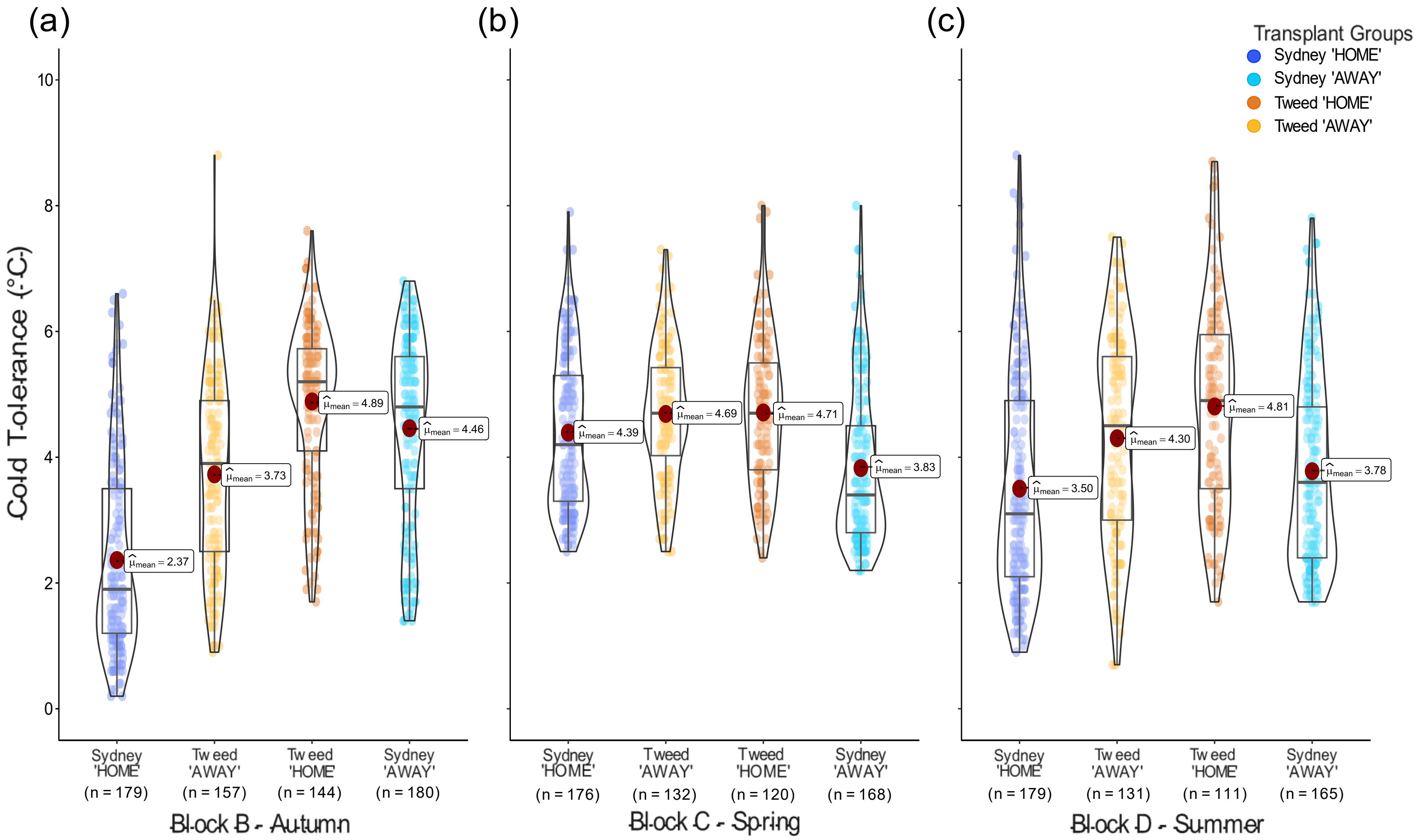

Figure 4. Cold tolerance measured as CT_{min} (°C) for post-transplant *T. carbonaria* in four transplant treatment groups Sydney 'Home'- dark blue, Sydney 'Away'-light blue, Tweed Heads 'Home'-dark orange, and Tweed Heads 'Away'-yellow) assayed at each of three testing blocks: **(a)** Block B – Autumn 2021; **(b)** Block C – Spring 2021; and **(c)** Block D – Summer 2022. Red circles within each Transplant treatment group violin plot indicate group heat tolerance means with values given in text boxes (estimated means of Transplant Tx groups). Number of observations (n =).

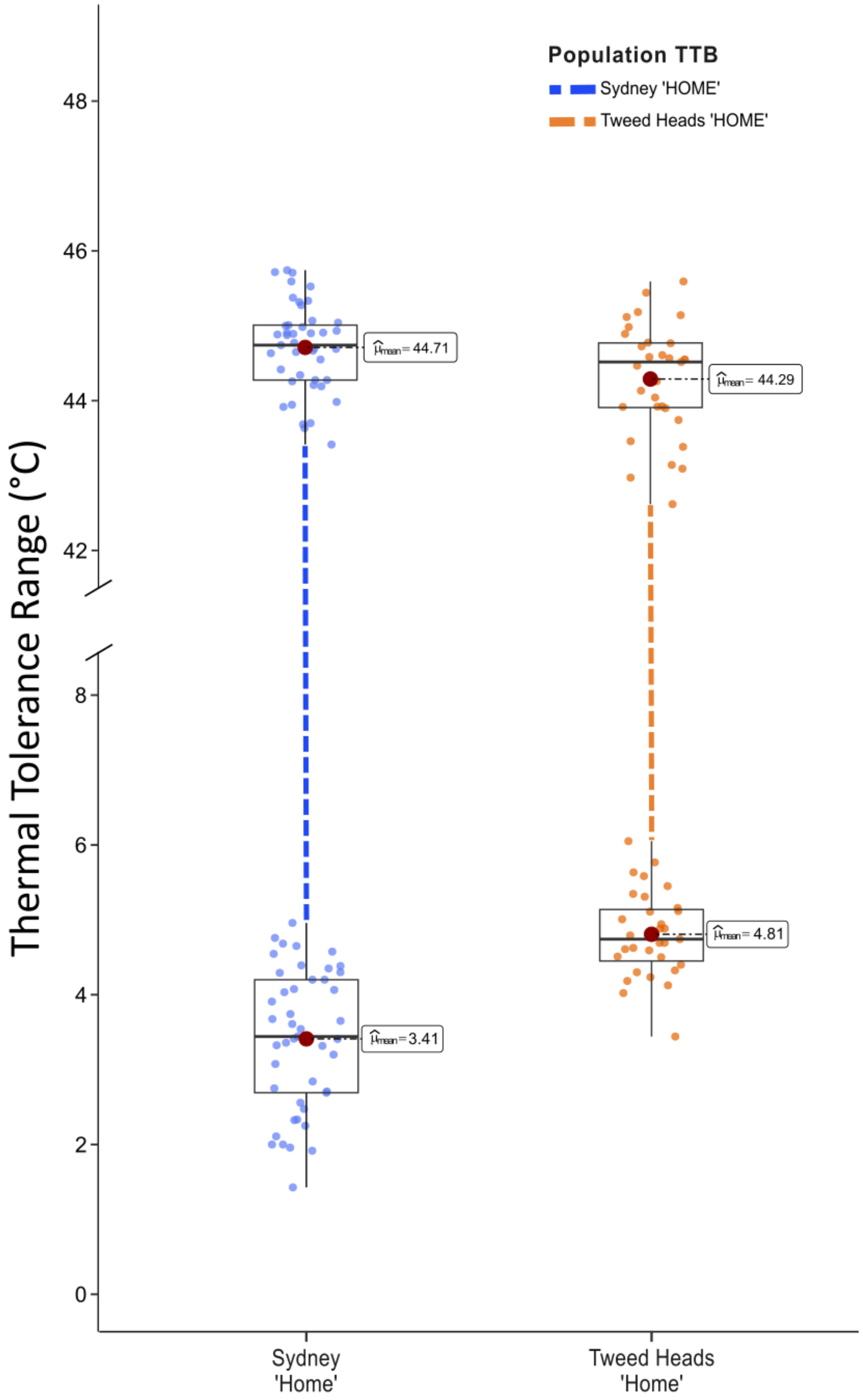

Figure 5. Thermal tolerance range (limits) estimation (the range of temperatures within which motor funcction is maintained – predicted to be wider than expected thermal fitness limits) for *T. carbonaria* 'Home' populations from temperature (Sydney) and subtropical (Tweed Heads) locations.

3.3.4 **Association of thermal tolerances with monthly temperature extremes**

The heat and cold tolerances of *T. carbonaria* in both temperate and subtropical locations easily exceeded the observed maximum and minimum environmental temperatures recorded in assay months at those locations; Figure 6. However, thermal tolerances of *T. carbonari*a in Sydney were close to the temperature extremes reported from the last 15 + years at this location (Highest recorded temperature: 44.5°C; Lowest recorded temperature: 0.2°C) (Supplementary Tables S3.1, S3.2), consistent with small thermal safety margins with respect to rare extreme temperature events for this population.

Seasonal variation in cold tolerances of *T. carbonaria* from our temperate (Sydney) population correlated over time with the seasonal variation in environmental temperature (minimum monthly temperature extremes) in Sydney (Table 4; Figure 6). However, we found no equivalent association for *T. carbonaria* of our subtropical population (Tweed Heads). There was also an association between heat tolerance and the maximum environmental temperature during assay months for *T. carbonaria* in both populations (Table 4; Figure 6)

Table 4. Pearson's Correlation tests of the association between heat and cold tolerances in *T. carbonaria* from each 'home' population (Sydney and Tweed Heads) in relation to the minimum and maximum recorded temperatures experienced across post-transplant testing blocks (Loc. Temp: Min and Max Temp respectively).

Pearson's Correlation

Population	**Loc. Temp**	**Tolerance**	***r***	**95% *CI***	***t***	***df***	***p***
Sydney	Min Temp	cold	0.26	[0.18, 0.34]	6.25	532	<0.001
Tweed Heads	Min Temp	cold	0.02	[-0.12, 0.09]	0.31	373	0.756
Sydney	Max Temp	heat	0.22	[0.14, 0.30]	5.18	529	<0.001
Tweed Heads	Max Temp	heat	0.18	[0.08, 0.28]	3.42	316	<0.001

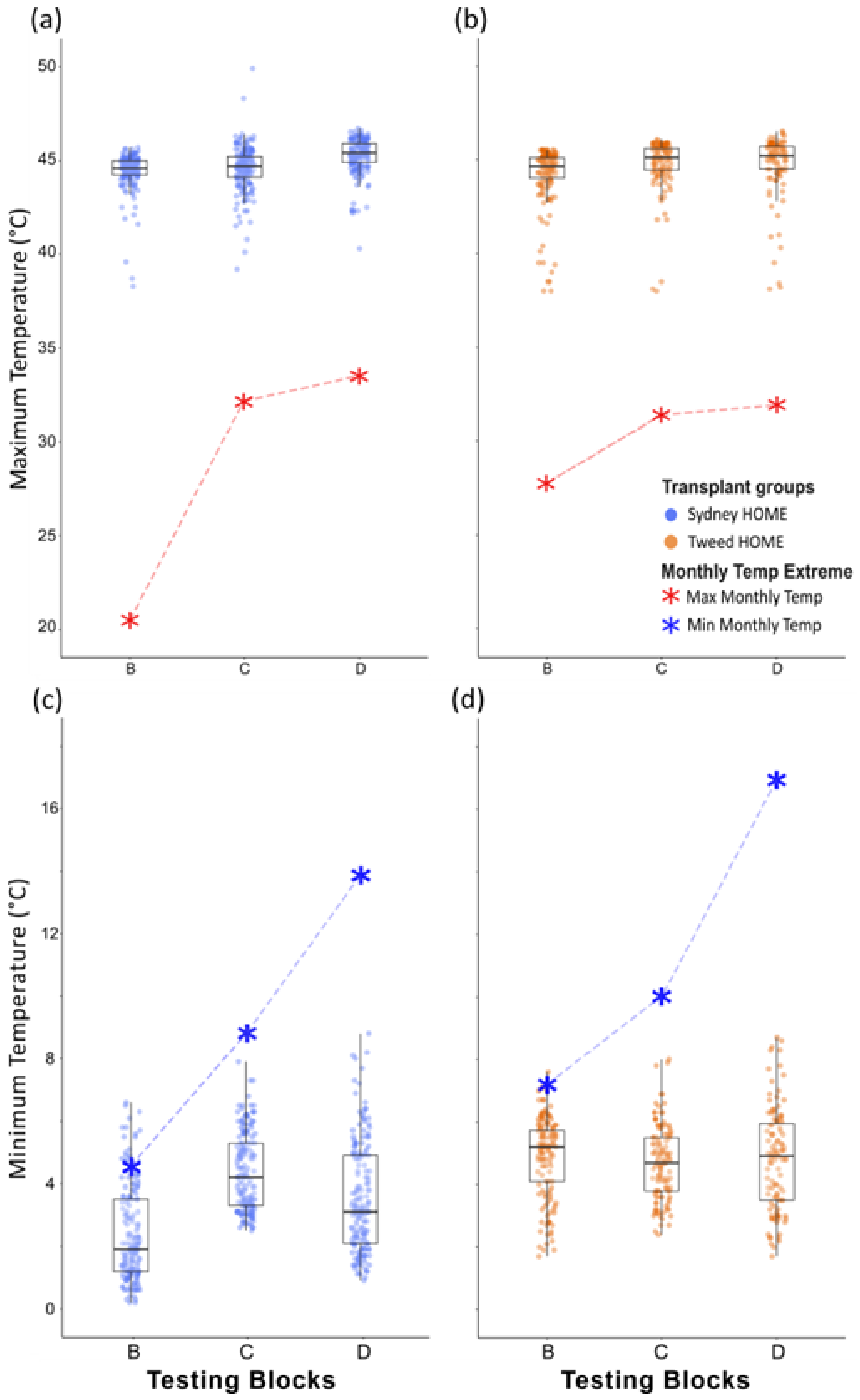

Figure 6. Seasonal variation in cold and heat tolerances of *T. carbonaria* in association with variation in environmental temperatures during the assay months.

3.4 **Discussion**

Using a reciprocal transplant experiment, we find that the thermal tolerance of the Australian stingless bee *T. carbonaria* is determined in part by their current environment (consistent with trait plasticity) and in part by the population from which the bees originated (consistent with local adaptive genetic variation and/or gene-by-environment effects). Patterns in the variation of these traits differed however between heat and cold tolerances, with upper thermal limits comparatively stable across time and space, showing only weak seasonal plasticity, while lower thermal limits were more variable and had a stronger population-of-origin signal. In all, these results highlight that there are differences in either the mechanisms underlying heat versus cold tolerance, or that selection may act differently on these respective thermal limits, as proposed in other insects (Addo-Bediako et al., 2000; Bozinovic et al., 2014). They also provide an initial guide for predicting likely responses of *T. carbonaria* to changing climates, and thus the resilience of the pollination services this species provides in Eastern Australia.

Cold tolerance shows significant intraspecific variation in a range of insects studied to date (Käfer et al., 2020; Sunday et al., 2014); Bozinovic et al. (2014), including *T. carbonaria* (Chapter 2). Across both transplanted and 'home' colonies in our experiment, we found that population-of-origin was the primary predictor of the cold tolerance observed in *T. carbonaria*, consistent with a strong effect of genotype (or gene-by-environment interaction) on this thermal trait. *T. carbonaria* sourced from higher latitudes within the species range (33°S to 35°S; temperate Sydney), were capable of tolerating colder temperatures than those originating from lower latitudes (≤28°S; subtropical Northern NSW to QLD). This strong population-specific signal possibly reflects local genetic adaptation in this thermal trait, with *T. carbonaria* in more temperate regions under stronger selection to remain active in winter. Notably, while plasticity in cold tolerance was also evident, this too was closely-associated with a population-of-origin effect. That is, *T. carbonaria* native to the temperate end of their range (Sydney) showed a greater capacity to shift their cold tolerance in association with season throughout the one year of our experiment, relative to the *T. carbonaria* of the subtropics. This too may reflect local adaptation, with the lower latitude population having greater adaptive plasticity in cold tolerance in response to the greater annual variability in minimum environmental temperatures this population experiences. Ultimately however, evidence of local genetic adaptation in cold tolerance will require further linking of cold tolerance to fitness. Over the one year of our experiment, our *T. carbonaria* colonies of temperate and subtropical origin were equally likely to survive in either climate; that is, we failed to detect any strong fitness benefit upon being locally adapted in cold tolerance. Temperate-origin colonies transplanted into the sub-tropics (Sydney 'away') were marginally slower to gain weight than their neighbouring Tweed Heads 'home' colonies, which might indicate a weak signal of reduced performance in a novel environment. Ultimately, however, the biology of social bees, in which colonies are long-lived and reproductive output (daughter colonies) is hard to measure, meaning that we had a limited ability to measure robust lifetime fitness in *T. carbonaria*. The benefits of being cold-adapted may manifest only in particularly cold years, or only cumulatively over a period of many years.

Heat tolerances, or upper critical thermal limits, show only modest variation both within and between species among insects and other ectotherms (Araújo et al., 2013; Bennett et al., 2021; Bozinovic et al., 2014; Buckley et al., 2022; Kellermann, Overgaard, et al., 2012). This has led to the suggestion that heat tolerance may be phylogenetically constrained (Araújo et al., 2013; Halsch et al., 2021; Kellermann, Overgaard, et al., 2012). Furthermore, at least in insects, this limited variation in heat tolerance generally displays only weak associations with latitudinal clines and environmental gradients such as maximum temperature (Sunday et al. 2011; Hoffmann et al. 2021; Kellerman et al. 2012). For example, in a multi-population study of the widely distributed species *Nicrophorus nepalensis* (Asian burying beetle), all populations displayed similar heat tolerance limits (between 38.2 -39°C) despite diversity in their thermal environments (Tsai et al. (2020)). This appears to be the case also for Australian *Tetragonula*, with respect to both interspecific and intraspecific variation (Chapter 2; this Chapter). For example, in the transplant experiment of this study, our two *T. carbonaria* populations maintained critical thermal upper limits of around 44.7°C throughout the experimental year, though pre-transplant thermal assays did show an initial difference. That is, we also find little evidence that upper thermal limits are locally-adapted in our temperate and subtropical *Tetragonula* study populations.

Rare extreme heat events likely provide an important selective pressure on thermal tolerances in insects and may be key to understanding whether a given species is at risk from climate change (Bennett et al., 2021; Leclair et al., 2020). We find that for *T. carbonaria* in both our subtropical and temperature populations, heat tolerances are 5°C to 7°C higher than their local climate's mean highest temperature extremes, indicating a comfortable "thermal safety margin" (Table S2). However, if we take into account the highest recorded temperatures in each location over the past 15 years, we see that *T. carbonaria* at the southern edge of their range (i.e., temperate population) have heat tolerances very close to this rare temperature extreme (Sydney's highest recorded temperature 2010-2023: 44.5°C). We suggest therefore that *T.carbonaria* may be vulnerable to changing climates in at least some parts of their range for three reasons: (i) periodic extreme heat events already exceed the upper critical thermal limits detected in this study, (ii) such extreme heat events are predicted to increase in both frequency and severity in the coming decades under current climate change predictions (Legg, 2021), and (iii) bee survival and reproduction is likely to be impacted long before lethal upper limits are reached, due to their life history. In particular, bees must forage during the daytime to collect food and nest materials. As flight produces metabolic heat, bees in flight must endure body temperatures higher than the ambient air temperatures. Although some large-bodied bees are known to be capable of physiological thermoregulation during flight (e.g. facultative endothermy in bumblebees, in which heat from thoracic muscles is shunted to other parts of the body: Dzialowski et al., 2014; Scriven et al., 2016b), it remains unknown whether most bees (including *T. carbonaria*) can adjust body temperatures during flight in response to high environmental temperatures. Indeed, some evidence points to the contrary for stingless bees; in some Brazilian stingless bee species, foragers experience increased heat stress during foraging flights were more likely to overheat during days where temperatures exceeded 30°C (Souza-Junior et al., 2020).

Plasticity in thermal traits has been proposed to be an important mechanism by which insects and other organisms can persist *in situ* as climates change, rather than shifting ranges to match their thermal tolerance limits (Kellermann & Sgrò, 2018; Lande, 2014; Martin et al., 2019; Merilä & Hendry, 2014). Plasticity could ensure short-term survival and thus buy populations more time for subsequent evolutionary adaptation to new thermal extremes and climate perturbations. Given that we observe very limited plasticity in heat tolerance in our study populations of *T. carbonaria*, it is unlikely that plasticity in this thermal trait will buffer future population change.

The global distribution of stingless bees is almost exclusively tropical and subtropical. As such minimum environmental temperatures are presumably a key factor determining their present-day distributions (Grüter, 2020a), with cold generally seen to be a limiting factor upon insect development (Chown et al., 2002). This is presumably in part because they lack complex nest thermoregulatory behaviours that maintain stable brood developmental temperatures (such as seen in honey bees, *Apis*; Jones et al., 2004). *T. carbonaria* has among the most southerly (highest latitude) distribution of any stingless bee species worldwide (Grüter, 2020b), suggesting that its ability to tolerate cold (and/or seasonal variation in low temperatures) is a significant adaptation determining its species range. Species distribution modelling under future climate change predictions is needed to shed light on whether range expansions in parts of New South Wales are likely for *T. carbonaria*. In addition, future studies will need to investigate the extent to which any plasticity in cold tolerance in *T. carbonaria* is the product of developmental plasticity (i.e., workers must develop within a set temperature so as to express the observed tolerance; developmental plasticity) or rapid adult acclimation.

Social bees are well-suited to reciprocal transplant experiments because they can be readily relocated in hives. However, a eusocial life history and haplodiploidy (females are diploid, males are haploid) also adds some complications to these experiments. In this study, we assayed the thermal tolerances of workers. These workers were the progeny of queens and males from a single given population (population-of-origin) and developed from eggs laid in the test location (either home or away). The queens of each colony however lived throughout the one-year of the experiment (as workers born of new queens were excluded from our analysis). Could queens therefore show adult acclimation between locations or seasons? And may queens affect the thermal physiology of their offspring via maternal effects? *T. carbonaria* queens live an estimated 1-3 years (Heard, 2016; Xia, 2022). Virgin Queens mate within the first two weeks of life and thereafter do not leave the nest (Bueno et al. 2022). They therefore experience more consistent thermal microclimate than foraging workers (which leave the nest daily) but nevertheless would be exposed to variation in nest temperature with changing environmental temperature. Further work is needed to understand the thermal tolerances of different female castes (queen vs worker) and different sexes that forage outside of the nest (males vs workers), and the implications for *T. carbonaria* populations under changing climates.

Chapter 4

4.GENERAL DISCUSSION

This thesis advances our understanding of the thermal biology of two stingless bees of ecological and economic importance in Australia: *Tetragonula carbonaria* and *Tetragonula hockingsi*. Specifically, I have investigated how their thermal limits vary across the climatic landscape of their distributions (**Chapter 2**) and the extent to which plasticity shapes their thermal tolerances (**Chapter 3**).

I hope that together these studies help to inform the conservation and management of these incredible bees under climate change and that it has highlighted the importance of understanding thermal biology in non-model insects.

Below I briefly recap each chapter's findings. I then discuss some promising future research directions.

4.1 **Two species, two thermal niches**

The thermal tolerances of many insects are predicted to match the thermal environments in which they live. In **Chapter 2** I tested this prediction in two cryptic species of East Coast *Tetragonula* that have largely allopatric distributions, but with a few areas of sympatry. Among *Tetragonula* beekeepers, *T. hockingsi* have a reputation for being the "hot-adapted" species and *T. carbonaria* for being the "cold-adapted" species. This is based on the anecdotal observation that in South East Queensland, where many beekeepers regularly keep both species, *T. carbonaria* is seen to forage at cooler temperatures than *T. hockingsi.* Furthermore, only *T. carbonaria* is naturally found in N.S.W, and thus has a unique distribution extending into more temperate regions. My estimates of heat and cold tolerances (measured as critical thermal maxima and minimum limits) for each species supports this general view of inter-specific differences, characteristic of inhabiting different climates. That is, *T. carbonaria* has the greater cold tolerance, aligning with its distribution at higher elevations throughout Queensland and the lower latitudes of NSW. *T. hockingsi* in contrast displayed greater heat tolerance (though difference was marginal), aligning with its distribution in warmer and drier areas of Queensland. Over time, these two species may have diverged therefore to occupy distinct thermal niches across their range, such that one species is able to outcompete the other in each respective thermal habitat. That is, the combination of competition and divergent thermal adaptation may have led to the largely non-overlapping distributions of these species. Indeed, *T. hockingsi* and *T. carbonaria* are known to compete for nest sites in at least some parts of their range (Cunningham et al., 2014; Lau et al., 2022). Presumably, the ranges of the two species have been in flux over time, as climates have changed in the past. The elevated regions of Queensland, currently occupied by *T. carbonaria,* are well-documented as refugia for many species adapted to the wet-tropic climates that were widespread up until the late Pleistocene (see Hilbert et al., 2007). *T. carbonaria's* past range therefore may have been far greater in Queensland, and their thermal biology today continues to reflect the "thermal history" of the species (Bennett et al., 2021; Kellermann et al., 2017).

Overall, I found that climatic variation explained variation in the thermal tolerances of *Tetragonula* both within and between species. Importantly however, temperature is likely to be just one of the key abiotic drivers of *Tetragonula* distributions and thermal physiologically traits. Particularly, I found that rainfall (Bioclimatic variable BIO14: precipitation of the driest month) was also a significant predictor of their thermal tolerances when considering my chapter 2 clinal study, specifically their heat tolerance. Rainfall has been found to be similarly important in shaping these traits in other insects (Amundrud & Srivastava, 2020; Jørgensen et al., 2019; Kellermann & van Heerwaarden, 2019).

4.2 **What determines thermal tolerances: local adaptation or plasticity?**

Reciprocal transplant experiments have a long history as tools for the study of phenotypic variation (Faske et al., 2019; Martin et al., 2021; Nooten & Hughes, 2017; Tsai et al., 2020). In Chapter 3, I transplanted *T. carbonaria* colonies between two locations (‘populations’) in their natural range that differed in climate: humid subtropical north-eastern NSW and temperate coastal Sydney. I then assayed the heat and cold tolerances of both ‘Home’ and ‘Away’ colonies at three timepoints throughout a year, to better understand how the current environment shaped their thermal tolerance traits. I found that heat tolerance was largely invariant between the two populations, though some slight variation may exist in accordance with seasonal fluctuations. This aligns to the broadly observed conservatism in heat tolerance for many ectothermic species (Bozinovic et al., 2014). Cold tolerance, meanwhile, was far more variable, with *Tetragonula* originating from the more-temperate end of their range displaying greater cold tolerance, including seasonal acclimation (seasonal plasticity) in cold tolerance, than those originating from the subtropical site. These findings are consistent with the idea that cold tolerance (though perhaps not heat tolerance) is partly due to local genetic adaptation in *T. carbonaria.* Like heat, cold is also seen as a strong abiotic selective pressures limiting small ectotherms from inhabiting cooler climates. As such this may also be why we don’t find evidence of *T. carbonaria* at latitudes beyond ~35-36°S, i.e. further cold tolerance may be ‘evolutionarily constrained, preventing *T. carbonaria*’s ‘cooler’ range edges from expanding further south under current climate conditions.

New South Wales will experience a predicted 1.7-2.5°C regional increase in mean annual temperature in coming decades (Long Term 2°C Global warming level: Grose et al., 2023). Whether this is problematic for populations of T. carbonaria in northern NSW is not yet clear and may depend on the sub-lethal effects of increased temperate (see **Chapter 3.1** and **3.4**). It does seem likely though that *T. carbonaria* will shift their current distribution to track with their optimal thermal environments, including expanding its range south as the current “cold barrier” to their distribution retreats (Beck et al., 2018; Cui et al., 2021). Range changes however may be quite slow. As eusocial insects, Tetragonula are ‘quasi-sessile’, and female dispersal rate is limited due to nesting and social behaviour (Bueno et al. 2022).

Overall, the results of my reciprocal transplant experiment indicate that the within-species variation in cold tolerance observed in Chapter 2 is likely to reflect, at least in part, local adaptation and not simply plasticity.

4.3 **Directions for future research**

This thesis has not only determined the relative thermal tolerance limits of *T. hockingsi* and *T. carbonaria*, but it has also uncovered distinct signatures of bio-climatic drivers of their broad scale distributions, along with a first look at understanding the mechanisms behind these important thermal traits. As such, it is no surprise that many interesting questions have arisen as a direct result from the work that I have done in this thesis. Therefore, I propose three main directions for future research.

1. Thermal tolerances across life stages, castes, and sexes

Temperature is known to affect organisms differently at different life-history stages. For example, larva may be more sensitive to high temperatures than adults (Kellermann & van Heerwaarden, 2019; Kingsolver & Buckley, 2020). This is the case for at least some stingless bees in the Neotropics (Vollet-Neto et al., 2015). Juvenile life stages may also be more plastic than adult life stages for some thermal tolerance traits (reported extensively in *Drosophila*; (Hector et al., 2022; Kellermann & Sgrò, 2018; Kellermann et al., 2017; MacLean et al., 2019; Seebacher & Little, 2021; Sgrò et al., 2016; van Heerwaarden et al., 2016). Further study of these differences in *Tetragonula* could be investigated by rearing brood comb at different temperatures in incubators to assess relative brood mortality, as well as then assaying the resulting adults' thermal tolerances. This could be achieved not only for workers but also for developing queens, and therefore used to understand how thermal tolerances may vary both across life stages and between social castes in *Tetragonula* colonies. An understanding of the responses of queens is important for understanding responses of this species to climate change. This is because any negative effects of thermal stress on queens will impact the overall persistence of the colony; for example, reduced fecundity could result in colonies with an insufficient worker- force to maintain colony function. Thermal stress has been observed to negatively affect fecundity in *Drosophila* females (Green et al., 2019; Sales et al., 2021).

In addition, thermal biology may vary importantly between the sexes (Hangartner et al., 2022; van Heerwaarden & Sgrò, 2021). In this thesis, I consider only the workers (non-reproductive females). However, *Tetragonula* males have very different life histories to their sisters, and therefore might be predicted to have evolved sex-specific thermal responses. Male *Tetragonula* leave the nest when mature (a few weeks of age) and never return to the nest (Bueno et al. 2022). They therefore do not have recourse to retreat to the nest if environmental conditions become adverse. New evidence from Australian solitary bees suggests that a species' nesting biology is a key predictor of its thermal tolerance, with stem-nesting bees having higher thermal tolerances than those nesting in the ground or in trunks like eusocial stingless bee workers (unpublished data; da Silva, Gloag & Kellerman). As an interesting next step therefore, it would be informative to assay the thermal tolerances of males and workers from the same colonies, to understand sex-specific differences in *Tetragonula* thermal biology.

2. Behavioural thermoregulation

Some social insects are capable of collective forms of thermoregulation that allow them to heat or cool the temperature inside their nests. For example, *Apis mellifera* can thermoregulate the temperature of developing brood within the nest to maintain it between 32-36°C; this active thermoregulation is achieved via metabolic heat, direct incubation and fanning (Becher et al., 2009). Conversely, most stingless bees "thermoconform" and only passively regulate hive temperature through behaviours such as nest site selection (Jones & Oldroyd, 2006). To date, there are only two documented exceptions among the neotropical species, which appear to maintain brood temperatures between 29-39°C (Engels et al., 1995). However, active thermoregulation in one of these species (*Scaptotrigona depilis*) via fanning behaviour and water collection was not enough to prevent pupae from lethal effects of very high environmental temperate (38°C; Vollet-Neto et al., 2015). In Australia the stingless bee *Austroplebia essingtoni*, which inhabits the arid tropics of Australia (hot wet summers and cool dry winters), does not actively thermoregulate their hives, but can regulate hive relative humidity, which may be crucial for brood survival during the cool and dry seasons (Ayton et al., 2016). Given that even small temperature increases during extreme weather events can negatively affect bee brood development, the thermoregulation capacity within nests may determine the extent to which eusocial insects are vulnerable to climate change (Vollet-Neto et al., 2015).

Preliminary evidence suggests that Australian *Tetragonula* do not actively thermoregulate their nests or brood (Heard et al 2016). However, they presumably can exercise choice of nest site, which is a form of behavioural thermoregulation. Nest site choice remains a poorly understood feature of Australian stingless bees. Nests are often located in tree hollows close to the ground or man-made structures (fence posts, walls of houses etc), but this is likely a detection-bias as nests in those locations are more readily observed. One avenue for future research therefore may be to better document the types of nest sites used by wild *Tetragonula* in Australia in different climates, and how these relate to the thermal insulation for the nest (and bees and brood inside). This information could then be used to help design better hives for managed colonies of *Tetragonula*.

Also, interesting to consider with respect to behavioural nest thermoregulation in *Tetragonula* is the differences between species in the form of the brood comb. Could the spiral brood shape of *T. carbonaria* somehow relate to the thermal biology and climate niche of this “cold-adapted” stingless bee? Workers often stand on the brood when cold, thus one possibility is that the spiral brood allows workers to “incubate” brood using their metabolic heat. Bumble bee queens are known to incubate their brood in this way when they first start their nests (before workers have hatched; (Heinrich, 1972), but whether such incubation occurs in other social bees is poorly understood and an interesting direction for future research.

3. The genetic basis of thermal traits – are mito-nuclear interactions involved?

The adaptive potential of traits in response to changing climates depends in part on the genetic architecture underpinning those traits. One interesting possibly in the case of *Tetragonula* is that thermal traits may be determined in part by genetic variation in the mitochondrial genome. This is because the mitochondrial genomes of *Tetragonula* in the "carbonaria complex" are known to have very high evolutionary rates (c. 5-fold more than seen in most insects; Gloag et al. unpublished data; Allio et al., 2017; Francoso et al., 2019). For example, the mitogenomes of *T. hockingsi* and *T. carbonara* show pairwise divergence of 22%, far higher than is typically observed in closely related species (in contrast, neutral regions of the nuclear genome show only 1% divergence; Hereward et al., 2020). *Tetragonula* also show high within-species between-population variability in mito-haplotypes across latitudes (Brito et al., 2014 ; Gloag et al., unpublished data); for example, populations of *T. hockingsi* in Far-Northern Queensland (Cape York) have haplotypes that show 11% pairwise divergence from those in the south.

Recent evidence from diverse sources suggests that inter- and inter-specific mitochondrial variation may reflect adaptation to an ectotherms local abiotic environments, as the expression of many key fitness traits, linked to mitochondria, are often dependent upon the thermal environment (Camus et al., 2017; Chung & Schulte, 2020). As mitochondrial function is temperature sensitive, thermal adaptation may involve selection on mitochondrial genes and the nuclear genes with which they interact. Previous efforts in insects (largely *Drosophila spp.*) have found strong evidence to suggest that failure of specific mito-respiratory chain proteins (when exposed to extreme temperatures) may negatively affect insect flight ability (Jørgensen et al., 2021). An earlier study by Arnqvist et al. (2010) found that variation in mitochondrial and nuclear substrate interactions effected metabolic rates of a seed beetle (*Callosobruchus maculatus*) under differential temperature regimes.

Furthermore, mitochondrial haplotypes covary across latitude in a wide range of species, though how often such variation is adaptive (rather than due to the chance processes of genetic drift) is unclear. In Australian *Drosophila* at least, experimental evidence suggests that spatially distinct insect populations current thermal climate imposes selective pressure on standing variation in mitogenomes (Mitochondrial climatic adaptation hypothesis: Camus et al., 2017; Dowling, 2014; Koch et al., 2021). Future research could aim to determine whether variation in thermal limits in *Tetragonula* correlates to variation in mitochondrial haplotypes, as a first step towards understanding whether climate-adaptation may be contributing to the extreme divergence in the mitogenomes of species in this group.

4.4 **Concluding remarks**

Understanding the biology of Australia’s native bees is critical to inform future management strategies in preparation for an increasing intensity and duration of thermal extremes and other climate shifts. My research has added to a global endeavour to better understand the thermal sensitivities of pollinator insects (Tomlinson et al., 2015). I hope that my thesis serves as a steppingstone for future research that will tackle the important questions of wild bee resilience or vulnerability in response to climate change

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6. Appendix

Chapter 2. Supplementary

Table S2.1 The Eight bioclimatic variables (BCV's; WorldClim version 2.0 gridded database at 30 seconds /~1 km^2 spatial resolution; Fick and Hijmans (2017)) used for initial Commonality analysis of Heat and Cold tolerance data sets. As climate data is inherently collinear, BCV's were assessed for both unique and shared variance, to partition out potential confounding effects caused by multicollinearity in our final linier mixed effect modelling. BCV in final thermal tolerance models: * = Cold tolerance, ▲ = Heat tolerance.

BCV Code	**Variable Type**	**Description**	**Unit**
BIO1	Temperature-related	Annual Mean Temperature	°C
BIO3 *▲	Temperature-related	Isothermality: (BIO2/BIO7) (x 100)	Index
BIO4	Temperature-related	Temperature Seasonality (standard deviation x 100)	Index
BIO5 ▲	Temperature-related	Maximum Temperature of Warmest Month	°C
BIO6 *	Temperature-related	Minimum Temperature of Coldest Month	°C
BIO12	Rainfall-related	Annual Precipitation	mm
BIO13	Rainfall-related	Precipitation of Wettest Month	mm
BIO14 *▲	Rainfall-related	Precipitation of Driest Month	mm

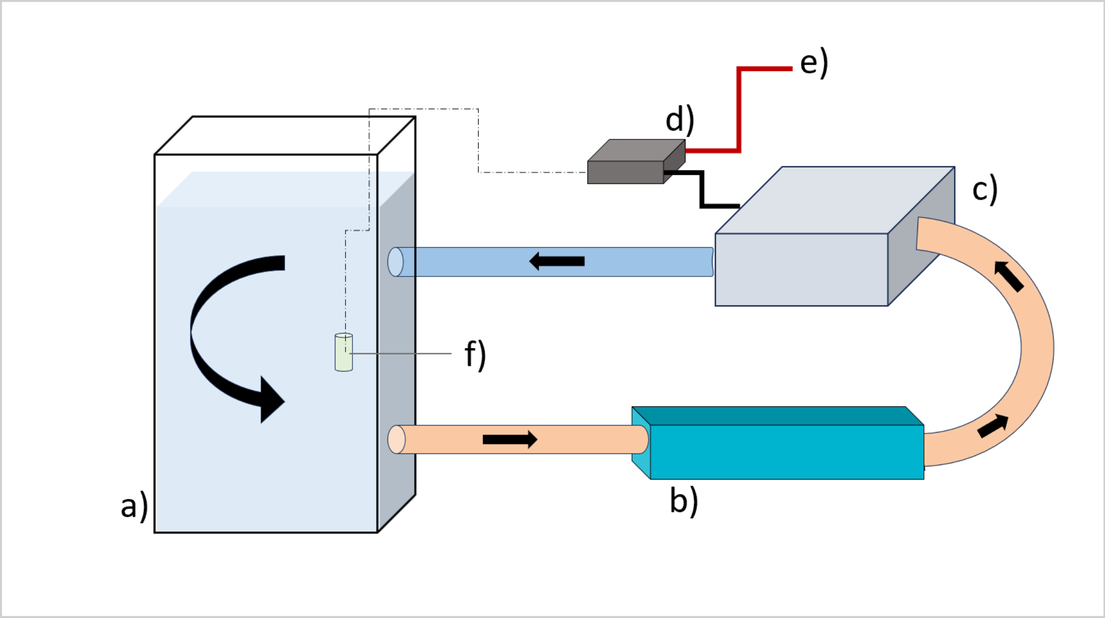

Figure S2.1 Thermal tolerance assay experimental equipment setup: **a)** perspex water bath; **b)** water pressure pump; **c)** Thermoelectric Liquid Cooler/ heater depending on tolerance tested; **d)** external TC-720 thermoelectric temperature controller (with USB communications allowing program control via a computer), connected to **c)** and interfacing with **f)** temperature gage inside water bath. Water is circulated through the water bath via the pressure pump, arrows indicate circulation direction with diagram depicting cold tolerance assay with water cooled.

Chapter 3. Supplementary

SUPPLEMENTARY TABLES

Table S3.1. Monthly temperature extremes (max and min temperatures) records from Australian Bureau of Meteorology; climate observations of months that *T. carbonaria* workers were assayed in this study. Climate data sourced from Terry Hill AWS (reporting period: 2004-present) for Sydney location (~5 km from site) and Coolangatta AWS (reporting period: 1992-present) for Tweed Heads location (~4Km from site).

Sydney Location – St Ives	BLOCK A	**BLOCK B**	**BLOCK C**	**BLOCK D**
Month Tested	*Feb 21'*	*Jun*	*Oct*	*Feb 22'*
Minimum Temp of Month (°C)	14.9	4.4	8.8	13.6
Lowest Monthly Mean (°C)	14.1	4.3	8.2	14.1
Maximum Temp of Month (°C)	31.1	20.4	31.8	32.9
Highest Monthly Mean (°C)	34.1	19.9	33.0	34.1
Tweed Heads Location -Terranora				
Month Tested	*Mar 21'*	*May*	*Nov*	*Feb 22'*
Minimum Temp of Month (°C)	16.4	7.1	10.0	16.9
Lowest Monthly Mean (°C)	15.9	7.2	12.7	17.6
Maximum Temp of Month (°C)	31.4	27.6	31.2	31.6
Highest Monthly Mean (°C)	30.8	26.4	30.7	31.6

Table S3.2. Highest and lowest recorded extreme temperatures and Annual Mean of highest and lowest temperature records, at our temperature and subtropical study locations: Sydney and Tweed Heads. Historical Climate data sourced from the Australian Bureau of Meteorology climate data taken from reporting periods 1992-2022; Australian Weather stations and reporting periods: Terry Hills AWS (for Sydney): 2004-2023, Coolangatta AWS (for Tweed Heads): 1992-2023, Brisbane AWS (Tweed ancestral location): 1999-2023.

Southern Population

Sydney location – Terrey Hills AWS	**Temperature (°C)**
Lowest recorded Temperature Extreme	**0.2**
Mean lowest Temp	2.7
Highest recorded Temperature Extreme	**44.5**
Mean highest Temp	39.5

Northern Population

Tweed Heads Location - Coolangatta AWS	**Temperature (°C)**
Lowest recorded Temperature Extreme	**-0.1**
Mean lowest Temp	2.5
Highest recorded Temperature Extreme	**40.0**
Mean highest Temp	34.5

Brisbane /Original Northern Location – Brisbane AWS	**Temperature (°C)**
Lowest recorded Temperature Extreme	**2.6**
Mean lowest Temp	5.1
Highest recorded Temperature Extreme	**41.7**
Mean highest Temp	37.4

Table S3.3. Type III ANOVA (Analysis of Variants) results of the main effects of Population and Location upon bee colonies proportional hive weight changes (Block A vs Block D) and possible interaction between the two effects. *SS* (Sum of Squares).

Type III ANOVA - *Kenward-Roger method*

Source of variance	*SS*	*df*	*F*	*p*
Intercept	245.0	1	3.183	0.081
Population	516.5	1	6.711	0.013
Location	446.8	1	5.805	0.020
Population x Location	121.3	1	1.576	0.213
Residuals	3463.7	45		

Table S3.4. Contingency tables and Fishers Exact Testing (FET) of colony requeening during Transplant experiment. * Indicates significant *p*-value

Requeening of novel environment on population 'Away' colonies

	Did requeen	Did not requeen
Tweed Heads 'Away'	3	12
Sydney 'Away'	1	14

FET: *p* = 0.598 with 95% CI [0.234, 196.368], odds ratio = 3.364

Requeen rate of Tweed Heads population ‘Hove’ vs ‘Away’

	Did requeen	Did not requeen
Tweed Heads 'Home'	3	12
Tweed Heads 'Away'	6	9

FET: *p* = 0.704 with 95% *CI* [0.2323265, 11.6914824], odds ratio = 1.480

Population comparison of all transplant treatment groups

	Did requeen	Did not requeen
Tweed Heads all	9	21
Sydney all	21	29

FET: *p* = 0.012*, with 95% *CI* [1.468793, 561.386814], odds ratio = 11.984

Table S3.5. [Supplementary t-tests] Summary of Linear Mixed-Effect t-test results of the main effects of *T. carbonaria* Population, Tx group (Transplant treatment groups – location effects), Season (Testing blocks B, C, and D), with both their two- and three-way effects upon cold tolerance. Statistical significance effects *, taken at ≤ 0.050, calculated using Satterthwaite *df.*

Fixed effects	***Est.***	***SE***	***df***	***t***	***p***
Intercept	4.35	0.32	10.41	13.245	<.0001
Population Tweed	-0.62	0.47	10.55	-1.330	0.211
Tx group 'Home'	-1.98	0.46	10.41	-4.264	0.002*
Season 'C'	-0.53	0.49	8.40	-1.080	0.310
Season 'D'	-0.58	0.49	8.41	-1.182	0.270
Population Tweed x Tx group 'Home'	3.03	0.91	9.54	3.329	0.008*
Population Tweed x Season 'C'	1.48	0.69	8.56	2.144	0.062
Population Tweed x Season 'D'	1.50	0.69	8.57	1.663	0.132
Tx group 'Home' x Season 'C'	2.55	0.69	8.38	3.707	0.006*
Tx group 'Home' x Season 'D'	1.70	0.69	8.38	2.470	0.037*
Population Tweed x Tx group 'Home' x Season 'C'	-3.57	1.35	7.76	-2.649	0.030*
Population Tweed x Tx group 'Home' x Season 'D'	-2.23	1.35	7.77	-1.655	0.138

Random effects	***Variance***	***SD***
Exp. Assay Run (24 levels)	0.197	0.444
Exp. Testing Day (14 levels)	0.137	0.370
Residual	1.837	1.355

Model REML criterion at convergence = 6412.1, R^2 (Marginal)= 0.182, R^2 (Conditional) = 0.308, N = 1842.

Table S3.6. Summery estimates of linier modelling (OLS linier regression), main effects of Population and recorded Monthly temperature extremes (hottest and coldest recorded day during testing Month- Blocks B-D), and their two-way interaction effects upon 'Home' colonies mean critical thermal limits. Both the heat and cold linier model coefficient results are displayed below.

Heat model - *Coefficients*

	Est.	***SE***	***t***	***p***
Intercept	43.504	0.292	148.74	<.0001
Population - Tweed	-3.527	1.147	-3.07	0.002
Minimum Temp of Month	0.044	0.01	4.35	<.0001
Population Tweed x Minimum Temp of Month	0.108	0.038	2.81	0.005

Cold model - *Coefficients*

	Est.	***SE***	***t***	***p***
Intercept	2.353	0.171	13.724	<.0001
Population - Tweed	2.513	0.286	8.799	<.0001
Minimum Temp of Month	0.119	0.018	6.714	<.0001
Population Tweed x Minimum Temp of Month	-0.214	0.026	-4.702	<.0001

Heat model fit: Residual ($890_{d.f}$) *SS* =2144.52 ± 1.308 *SE*, Multiple R^2 = 0.046, Adjusted R^2 = 0.043, *F* ($3,890_{d.f}$) = 14.32, *p* <.0001, N = 894; Cold model fit: Residual ($905_{d.f}$) *SS* =2144.52 ± 1.539 *SE*, Multiple R^2 = 0.199, Adjusted R^2 = 0.197, *F* ($3,905_{d.f}$) = 75.24, *p* <.0001, N = 909.