THESIS

EFFECTS OF GENOTYPE AND ACCLIMATION ON HONEYBEE THERMAL RESPONSES

Submitted by

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ABSTRACT

EFFECTS OF GENOTYPE AND ACCLIMATION ON HONEYBEE THERMAL RESPONSES

As global temperatures rise, animals are increasingly exposed to changing thermal environments that challenge their physiological and behavioral performance. Genetic variation and phenotypic plasticity are two key factors that influence how organisms respond to such environmental change. Understanding the capacity and limitations of these responses is essential for predicting species resilience under climate change. In this thesis, I investigate how thermal responses are shaped by 1) short-term thermal acclimation and 2) genotypic differences at a key metabolic enzyme locus in honeybees, a species of high ecological and agricultural importance. In the first chapter, I assessed the capacity for short-term acclimation to mitigate the effects of thermal stress. Bees were acclimated for 48 hours to either a cool (25°C) or warm (35°C) temperature and subsequently tested at both acclimation and non-acclimation temperatures. Metabolic rate showed evidence of compensatory acclimation, with warm-acclimated bees maintaining stable performance at high temperature. In contrast, activity and learning performance declined following heat exposure, with no evidence of a beneficial acclimation response. These results suggest that energetically demanding traits such as cognition and locomotion may have a more limited capacity for acclimation and higher vulnerability to sustained heat stress. In the second chapter, I examined how genetic variation in a key metabolic enzyme, malate dehydrogenase (MDH-1), influences thermal performance. Bees representing homozygous Slow (SS), homozygous Fast (FF), and heterozygous (SF) genotypes were assayed across four temperatures

and three traits: metabolic rate, locomotor activity, and learning ability. Metabolic rate exhibited a strong genotype-by-temperature interaction; Fast bees consistently had the highest rates, Slow bees the lowest, and heterozygous bees had flexible, intermediate responses. Activity levels varied with both genotype and temperature, while learning performance was influenced by genotype but not temperature. Heterozygotes outperformed both homozygous types in the learning assay, suggesting a potential heterozygote advantage. These results highlight how functional diversity in a key metabolic enzyme can shape trait performance across thermal gradients, with broader implications for colony-level function and honeybee breeding practices. Together, these chapters show that both genetic variation and phenotypic plasticity influence how bees respond to thermal variation, but their effects vary across different performance traits. Genetic variation may support flexible trait expression across environments, whereas short-term acclimation alone may be insufficient to maintain performance in key behavioral traits under thermal stress. These findings emphasize the importance of integrative, trait-based approaches to evaluating thermal responses and have implications for understanding pollinator performance and adaptation in a warming world.

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CHAPTER 1

Short-term acclimation has limited potential to buffer the effects of heat stress on honeybee physiology and behavior

Introduction

Climate change presents new challenges for animal performance and fitness as rising temperatures strain physiology, alter behavior, and disrupt resource availability. Invertebrates are particularly vulnerable since they are largely ectothermic with limited ability to regulate their internal body temperature. The overall capacity of a species to respond to climate change depends on a variety of factors including physiological or behavioral changes attributable to phenotypic plasticity (Hoffmann et al., 2005; McGaughran et al., 2021). One such process is thermal acclimation, a type of physiological plasticity that can stabilize phenotypic responses to prolonged changes in temperature (Rohr et al., 2018; Einum et al., 2019). Reflecting their incredible diversity, insects show great variation in their capacity for thermal acclimation across individuals (Loughland and Seebacher, 2020), populations (Seebacher et al., 2012), and species (Vinagre et al., 2016). Understanding these differences is a necessary step toward developing effective strategies for mitigating the impacts of thermal stress in a species (González-Tokman et al., 2020). Among insects, honeybees are a particularly good model for testing thermal acclimation, as they are ecologically important pollinators that are well-studied in terms of their behavioral and physiological characteristics.

The link between temperature and physiology in ectotherms has been most extensively studied in the context of metabolic rate. Metabolic rate typically increases with temperature up

until a maximum point, beyond which it rapidly declines as molecular processes are destabilized, producing what is known as a thermal performance curve (Gillooly et al., 2001; Dillon et al., 2010; Schulte et al., 2011; Seebacher et al., 2015). Thermal acclimation can shift performance curves for metabolic rate, affecting metabolic rate–temperature relationships (Terblanche et al., 2005; Gray, 2013). These changes in metabolic rate can influence how much energy is available for maintenance, growth, reproduction, and survival, ultimately shaping individual fitness (Gvoždík, 2024). Although many behavioral traits are known to covary with metabolic rate (Réale et al., 2010), relatively little is known about how these traits respond to temperature variation or thermal acclimation. For example, traits like locomotor activity and learning ability depend on energy-intensive neural and muscular processes and may exhibit distinct thermal responses, but studies directly testing this are limited.

Cognitive traits such as learning and memory are key components of behavioral plasticity that shape how animals respond to environmental change (Ducatez et al., 2020). These cognitive traits are critical to exploring and exploiting new food sources as well as to using behavioral thermoregulation to buffer the effects of thermal stress. Cognitive deficits in response to heat stress are well-documented in humans (Hancock & Vasmatzidis, 2003; Gaoua et al., 2010; Cedeño Laurent et al., 2018), but how higher temperatures impact the cognition of non-human animals remains relatively unexplored, particularly in invertebrates (Soravia et al., 2021). Addressing this gap is especially important for a species such as the honeybee in which the cognitive performance of a forager, which is critical to its ability to provision the colony, is highly vulnerable to the negative effects of environmental stressors (Klein et al., 2017). Surprisingly little is known about the effects of temperature on honeybee learning, despite their

status as a classic model of animal cognition. A recent study found that bumblebee workers showed a significant decline in associative learning and memory after just a few hours at 32°C (Gérard et al., 2022). However, this study tested only the acute, immediate effects of heat stress on learning and did not test the prolonged effects of temperature, which can be shaped by active processes such as thermal acclimation.

When evaluating thermal responses, it is important to distinguish between the acute and prolonged effects of temperature. Acute effects of temperature arise from passive biochemical responses while acclimation effects reflect active physiological adjustments to prolonged thermal stress. Distinguishing between these effects that occur at different timescales is critical for understanding the overall thermal performance of an organism (Schulte et al., 2011; Havird et al., 2020). Within the current literature, there are mixed views on how acclimation is expected to affect performance. While it is generally assumed that acclimation to a given temperature should improve performance at that temperature, an idea known as the beneficial acclimation hypothesis (Woods & Harrison, 2002; Wilson & Franklin, 2002), many studies have challenged the idea that acclimation is generally beneficial and emphasized the importance of considering alternative hypotheses (Leroi et al., 1994; Deere & Chown, 2006). One such alternative is the deleterious acclimation hypothesis, which proposes that acclimation, particularly to high temperatures, can lead to reduced performance in certain traits. This may occur due to physiological constraints, cumulative damage, or energetic trade-offs during the acclimation process (Malod et al., 2024). We had two main goals in this study: 1) Test if there is an acute effect of higher temperature on honeybee performance and 2) Test if longer exposure to such temperatures leads to a beneficial, neutral, or detrimental response.

To address these questions, we tested the effects of temperature on honeybee metabolic rate, activity level, and learning performance—three traits that are integral to foraging. We used a factorial experimental design that allows us to distinguish between immediate, acute responses and acclimation responses that arise from longer heat exposure (Seebacher et al., 2015; Havird et al., 2020). While there is no singular definition of heat stress, we used 35°C as the warm temperature because it is commonly used as the threshold for extreme heat by government agencies and climate assessment studies (Marvel et al., 2023), and because it is a temperature at which foraging activity is reduced in honeybees (Bauer et al., 2025). In contrast, 25°C represents a temperature that is widely considered as an optimum for behavioral studies in the laboratory (Williams et al., 2013) as well as one at which honeybees show optimal foraging activity (Burrill and Dietz, 1981; Czekońska et al., 2023). Building on this background, we used an experimental design in which foragers were acclimated for 48 hours to either a cool (25°C) or a warm (35°C) temperature and then tested at one of these two temperatures.

Methods

Animals

The bees used in the experiment came from six source colonies of the honeybee, *Apis mellifera*. Brood frames with mature pupae were collected from these colonies one day prior to adult emergence and kept in an incubator set at 32°C. Newly hatched bees were marked on the abdomen with a dot of colored paint to track their age and introduced into a small nucleus colony with workers and a laying queen to create a common-garden setting for behavioral maturation. These bees were collected from the entrance and outer frames of the colony when they were 21-

25 days old, an age when they normally become foragers, and subjected to the thermal treatments and performance assays. Individual bees were treated as biological replicates, with each bee measured independently under its assigned treatment. A total of 226 bees were tested, with sample sizes approximately equal across the four treatment groups.

Thermal treatment

We created four experimental treatments by acclimating the bees at two temperatures (25℃ [cool acclimated] and 35℃ [warm acclimated]) and testing each of those groups at those same two temperatures. To acclimate the bees, they were individually harnessed into plastic tubes after immobilizing them briefly by placing them on ice, and then randomly assigned to one of the two acclimation temperatures in an incubator kept at 80% relative humidity. They were maintained at these temperatures for 48 hours, during the first 24-30 hours of which they were fed with 30% sucrose solution until satiation, approximately every 8 hours. Bees were then fasted for 18-24 hours to ensure maximum motivation level for the measurement of appetitive learning and to ensure a post-absorptive state for the measurement of metabolic rate. Based on preliminary work showing that bees maintained at a higher temperature exhibit a higher hunger level, we fasted the warm-acclimated group for 18 hours and the cool- acclimated group for 24 hours. These durations resulted in similar survival (~70%) and similar levels of motivation in the two groups, with 92.2% of cool-acclimated bees and 97.3% of the warm-acclimated bees recording a proboscis extension response to a sucrose reward at the end of the fasting period (χ^2 = 2.25, d.f. = 1, p = 0.13). Bees from each acclimation group were then randomly assigned to one of the two testing temperatures, 25℃ and 35℃, to measure metabolic rate, associative learning and activity rate, in that order.

Metabolic rate assay

Metabolic rate was measured with flow-through carbon dioxide respirometry using a FoxBox setup connected to a multiplexer (Sable Systems). Harnessed bees were held immovable inside individual 50 mL plastic respirometry chambers, and seven bees were measured at a time. Ambient air scrubbed free of H2O and CO_2 was run through each chamber at a constant flow rate of 250 mL/min. The CO_2 concentration in the outgoing airflow was measured every second for a total of 8 minutes for each bee and metabolic rate was calculated using the continuous 2-minute period with the lowest variance in CO_2 production. The CO_2 production rates were converted into a power output (in mW) by assuming 21.4 J/mL CO_2 and then weight-corrected.

Associative learning assay

Immediately following the metabolic rate assay, bees were tested for their learning ability using the proboscis extension response (PER) assay, which is an appetitive associative conditioning assay based on olfactory learning (Bitterman et al. 1983). Bees reflexively extend their proboscis in response to sucrose stimulation of their antennae and can be trained to respond to an odor acting as a conditioned stimulus (CS) when it is repeatedly paired with a sucrose reward as an unconditioned stimulus (US). We first tested the motivation of each bee by touching its antennae with 40% sucrose solution and recording whether it extended its proboscis. Any bee that did not show a response was removed from the experiment. Each bee was then presented with the CS (1-hexanol) overlapping with the US (40% sucrose solution) for 6 consecutive trials with a 10-minute inter-trial interval. An extension of the proboscis on

presentation of the CS, prior to presentation of the US, is defined as a conditioned response (CR) and is recorded as a binary variable, 1 or 0, for each trial. The learning score of each bee was calculated as the total number of CR across the six trials.

Activity rate assay

Immediately following the learning assay, bees were released from their harnesses into individual activity monitoring tubes (16mm diameter), which were connected to an automated system consisting of an array of infrared beams that counted the number of passes made by an individual across the tube (TriKinetics System, LAM16). Locomotor activity was monitored for two hours, during which bees had access to ad libitum food (40% sucrose) placed in cotton plugs at both ends of the tube. After this final assay, bees were euthanized by freezing and individually weighed.

Data analysis

All analyses were performed in R. A chi-square test was used to test for a difference in motivation between the two acclimation groups. Two-way ANOVAs were then used to test the effects of acclimation and testing temperature, and Tukey's pairwise comparisons with estimated marginal means were used to test for differences within and between the two acclimation groups. Following the framework of Havird et al. (2020), we assessed acute effects of temperature by comparing performance at the two test temperatures within each acclimation group, and acclimation effects by comparing the two groups when tested at their respective acclimation temperatures. In this framework, a non-significant difference between groups at their acclimation temperatures suggests a compensatory, beneficial acclimation response that stabilizes

performance across thermal conditions. Learning acquisition curves for the four experimental groups were analyzed using repeated measures logistic regression.

Results

Metabolic rate

Acclimation temperature had a significant effect on metabolic rate (Two-way ANOVA $F_{1,221} = 9.76$, $p < 0.01$), as did testing temperature ($F_{1,221} = 11.13$, $p < 0.001$). There was also a significant interaction between acclimation and testing temperatures ($F_{1,221} = 3.90$, $p < 0.05$). Within the cool-acclimated group, there was no difference in metabolic rate across the two testing temperatures (Tukey's test: $t_{221} = 0.96$, $p = 0.77$, Fig. 1.1), showing a lack of acute effect of temperature, while the warm-acclimated group showed an acute effect of temperature by displaying a significant decline in metabolic rate at 35°C compared to 25°C ($t_{221} = 3.74$, $p < 0.01$). The warm-acclimated bees tested at 35°C had metabolic rates near those of the cool-acclimated bees tested at 25°C, indicating an overall stabilizing effect of acclimation ($t_{221} = 0.11$, $p = 0.99$).

Associative learning

Acclimation temperature had a significant effect on associative learning (Two-way ANOVA $F_{1,198} = 5.84$, $p < 0.05$) while testing temperature did not ($F_{1,198} = 2.49$, $p = 0.12$), and there was no interaction between acclimation and testing temperatures ($F_{1,198} = 0.07$, $p = 0.80$). There was no acute effect of temperature within the cool-acclimated group, performance being similar at 25°C and 35°C (Tukey's test: $t_{198} = 0.89$, $p = 0.81$, Fig. 1.2), as well within the warm-acclimated group ($t_{198} = 1.30$, $p = 0.56$). Warm-acclimated bees tested at 35°C had a significantly

lower learning performance compared to cool-acclimated bees tested at 25°C ($t_{198} = 2.81$, $p < 0.05$), which indicates that there was no compensatory beneficial effect of acclimation on learning. All four experimental groups acquired the conditioned response, or learned (Trial number: Wald's $\chi 2 = 91.90$, d.f. = 5, $p < 0.001$), but there was a significant difference among them in their rate of acquisition (Trial Number x Treatment: $\chi 2 = 27.07$, d.f. = 3, $p < 0.001$) with the cool-acclimated group tested at 25°C showing the highest acquisition and the warm-acclimated group tested at 35°C showing the lowest acquisition (Fig. 1.3).

Activity rate

Acclimation temperature had no significant effect on activity rate (Two-way ANOVA $F_{1,222} = 0.02$, $p = 0.88$), but testing temperature did ($F_{1,222} = 16.97$, $p < 0.0001$). There was also a significant interaction between acclimation and testing temperatures ($F_{1,222} = 4.88$, $p < 0.05$). There was no acute effect of temperature in the cool-acclimated group, with no significant difference in activity between the two testing temperatures (Tukey's test: $t_{222} = 1.32$, $p = 0.54$, Fig. 1.4). In contrast, the warm-acclimated group showed an acute effect by displaying a significantly lower activity rate when tested at 35°C compared to 25°C ($t_{222} = 4.48$, $p < 0.001$). Warm-acclimated bees tested at 35°C had a significantly lower activity rate compared to cool-acclimated bees tested at 25°C (Tukey's test: $t_{222} = 2.89$, $p < 0.05$), which indicates that there was no compensatory beneficial effect of acclimation on activity.

Discussion

Thermal acclimation is often proposed as a key mechanism for ectotherms to cope with increasingly challenging thermal environments, but only a few ecologically important traits have

been tested using an acclimation framework. Our results show that traits vary in their short-term acclimation responses and that just two days of heatwave-like temperatures can substantially reduce honeybee performance. Prolonged heat exposure resulted in lower learning performance with no evidence of a beneficial acclimation response, despite the absence of an acute temperature effect. Activity level also did not show evidence of beneficial acclimation; warm-acclimated bees showed elevated activity at 25°C but their activity levels declined past the point of compensation when tested at their acclimation temperature, indicating an overall destabilizing effect. In contrast, acclimation appeared to mitigate the effect of high temperature on metabolic rate in our study. Warm-acclimated bees tested at their acclimation temperature had metabolic rates comparable to the baseline rates of the cool-acclimated group, and this was in contrast to the dramatically elevated metabolic rate seen when they were tested at 25ºC. Overall, prolonged high temperatures led to lower performance in all three traits, but these effects were detrimental only for learning and activity. Whether the observed metabolic rate response was actually due to beneficial acclimation is discussed in more detail below.

Although the capacity for acclimation to heat stress is generally thought to be weak in insects (Sørensen et al., 2016; Weaving et al., 2022), one might expect honeybees to be an exception to this trend because they are considered 'partial endotherms'. Honeybees have several active physiological mechanisms for regulating body temperature, although these come with a high energetic cost (Stabentheiner & Kovac, 2016). For example, forager bees increase evaporative cooling to prevent overheating while flying and shiver to warm their flight muscles under cool conditions (Heinrich, 1979, 1981). Honeybees also have well-known mechanisms for collective thermoregulation at the colony level (Stabentheiner et al., 2010). Some recent work

suggests that honeybee colonies exposed to simulated heatwaves exhibit considerable plasticity at both the individual and colony level; at a temperature of 37°C, no costs were detected in individual foragers, and pollen and nectar collection remained stable (Bordier et al., 2017). In contrast, other studies have found the capacity for thermal plasticity to be more limited across different species of bees, including honeybees, when workers are tested in isolation (Sepúlveda and Goulson, 2023; Gonzalez et al., 2024). Our results support the previous work showing a more limited acclimation capacity in bees, with the important caveat that our study was conducted with individual bees isolated from the colony. It is likely that mechanisms related to colony level thermoregulation can mitigate the effects of heat stress to a larger extent.

Cognitive processes could be particularly vulnerable to the effects of prolonged thermal stress because neurotransmitter activity and neuronal function are known to be highly sensitive to temperature (Wang et al., 2007; van Hook, 2020). Slight deviations from the optimum rearing temperature can lead to learning deficits in honeybees (Tautz et al., 2003; Groh et al., 2004; Jones et al. 2005). Our results also suggest that the temperature for optimal cognitive function is lower than what might be expected in honeybees. Although our warm temperature meets the definition of a heat stress condition, it falls well within the range of temperatures that honeybees experience within the colony and while foraging (Stabentheiner et al., 2010). It is therefore noteworthy that we observed a decline in learning performance with such mild heat stress, suggesting that the rising temperatures expected under climate change could lead to an even more pronounced impact on honeybee cognitive function. These impacts are likely to be the strongest on forager bees which spend considerable time outside the relatively stable temperatures of the colony environment. Environmentally induced changes in physiology, driven

by changes in energy availability, can cause tradeoffs between cognition and other physiological processes, although further research is needed to uncover the causal mechanisms underlying such tradeoffs (Maille and Schradin, 2017). Interestingly, we observed no acute effect of temperature on learning, suggesting that honeybees may not exhibit any decline in their cognitive function under short-term thermal stress. Such impairments are likely to emerge only after longer exposures to higher temperatures.

The observation that acclimation was beneficial for metabolic rate but detrimental for learning and activity is not necessarily incongruent, as different traits often exhibit distinct thermal responses (Kellermann et al., 2019). For example, thermal acclimation has been shown to affect jumping distance but not running speed in crickets (Lachenicht et al., 2010). It is possible that the effects of thermal stress may first be felt on energetically demanding traits such as learning and locomotion, and short-term acclimation may not allow complete compensation of performance in such traits. The detrimental acclimation hypothesis suggests that acclimation to environmental change can come at a cost, potentially reducing performance in some contexts due to energetic tradeoffs, costs of plasticity, or cumulative stress (Leroi et al., 1994). The process of acclimation is itself energetically demanding due to the energetic costs associated with detecting and responding to thermal changes (Angilletta, 2009). Heat shock proteins, which help stabilize molecular responses to heat stress, also consume energy during their function (Krebs and Loeschcke, 1994). It is therefore plausible that the adverse effects of prolonged heat stress on learning performance and activity stem from a reduction in the resources available for non-life sustaining processes. This could be particularly troublesome in the context of foraging, which requires learning and decision-making in a complex and variable environment. Such trade-offs

would be exacerbated by the decreased availability of high-quality resources during heatwaves, further compounding the energetic constraints faced by foraging bees (Vanderplanck et al., 2019; Hemberger et al., 2023).

Several important limitations should be considered here. First, the appropriate duration for thermal acclimation remains debated, and it is possible that 48 hours was insufficient to elicit a strong beneficial acclimation response across all traits. However, smaller organisms with lower body mass and higher metabolic rates are generally expected to acclimate more rapidly due to faster physiological turnover (Rohr et al., 2018), and bees, as small ectotherms, are likely capable of fully acclimating within a few days. A 48-hour period has also been used in prior bee acclimation studies (Gonzalez et al., 2024), though future research should explore longer durations. Second, it is possible that the bees were not entirely at rest during the metabolic rate measurements. If cold, bees may activate their flight muscles to generate heat, which can elevate metabolic rate even when they are physically restrained. This would be especially likely in warm-acclimated bees tested at the cool temperature and could explain the unusually high metabolic rates observed in that group. This complicates interpretation of the metabolic rate trend, as it may not reflect a true beneficial acclimation response. This result should therefore be interpreted with caution. Third, it remains difficult to distinguish between detrimental acclimation responses, in which plasticity leads to maladaptive changes, and generalized stress responses driven by physiological damage. In practice, the two may overlap, as acclimation at extreme temperatures can trigger stress responses that contribute to reduced performance. This challenge has been a major critique of the detrimental acclimation hypothesis (Woods & Harrison, 2002), especially in studies comparing acclimation at an organism's optimal

temperature to one approaching its physiological limits. To limit potential confounding effects, acclimation temperatures should fall within an ecologically relevant range, as we aimed to do in the present study. Nonetheless, laboratory conditions inherently introduce some level of stress, and future acclimation experiments should aim to simulate natural environmental conditions as closely as possible. Finally, while this and most other studies have used constant temperatures to test acclimation, fluctuating temperatures may better reflect natural environments and should be prioritized in future work.

Overall, our findings are consistent with the idea that traits vary in their thermal responses and tentatively suggest that metabolic rate has a higher capacity for acclimation compared to learning performance and activity level in honeybees, although we acknowledge some important limitations above. If cognitive abilities are indeed more vulnerable to prolonged heat exposure, this raises serious concerns, as these functions are essential for foraging and the communication processes that maintain colony homeostasis in honeybees. Cognitive decline during heat stress could further exacerbate the negative effects of heat waves by reducing the efficacy of coordinated thermoregulatory behaviors. There is an urgent need for further research to understand the effects of temperature on cognition in ectotherms and the potential trade-offs between different traits during thermal stress. Our results also underscore the importance of including a wider range of taxa in studies on thermal plasticity, as acclimation responses are likely to vary among different species and environmental conditions. Despite their critical role in pollination and agriculture, honeybees have not been studied as well as some other insects in terms of their acclimation responses, perhaps due to the assumption that their heterothermic abilities allow for greater thermal plasticity than other species. We show that the acclimation

capacity of learning ability and activity is limited in honeybees and conclude that prolonged heat exposure is likely to have a detrimental effect on their foraging performance. As extreme heat events become more frequent and intense with climate change, this may have global implications for the essential services honeybees provide.

Figures

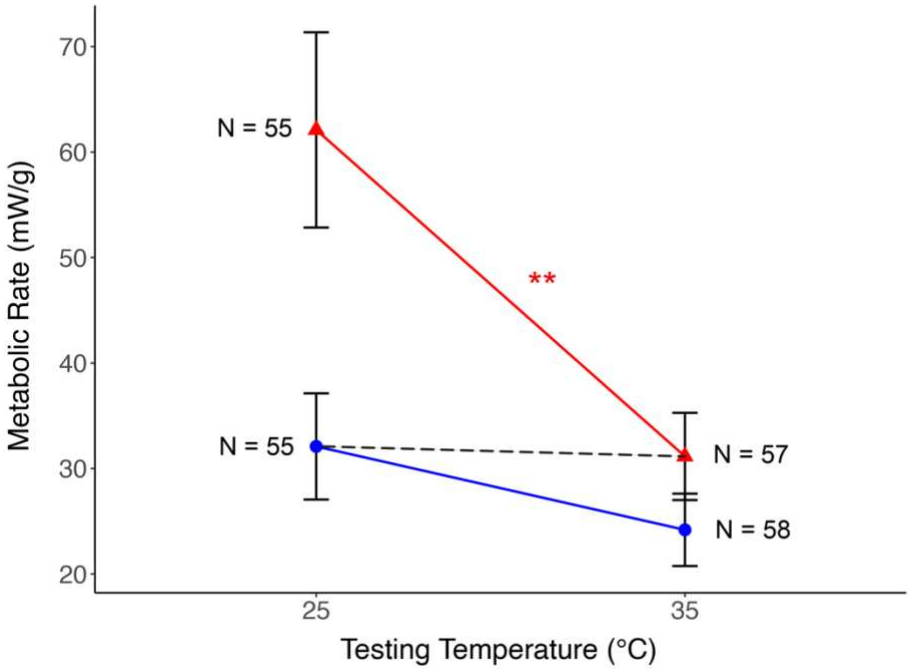

Figure 1.1: Thermal responses of cool (—●—) and warm (—▲—) acclimated bees on resting metabolic rate (n = 225), measured using flow-through carbon dioxide respirometry. Solid lines indicate the acute effect of temperature on performance within each group. Black dashed lines indicate the acclimation effect of temperature on performance, where complete compensation is indicated by a non-significant difference between the two groups. Data are presented as means with standard error bars and statistical significance between two groups is denoted by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001) using Tukey's pairwise comparisons.

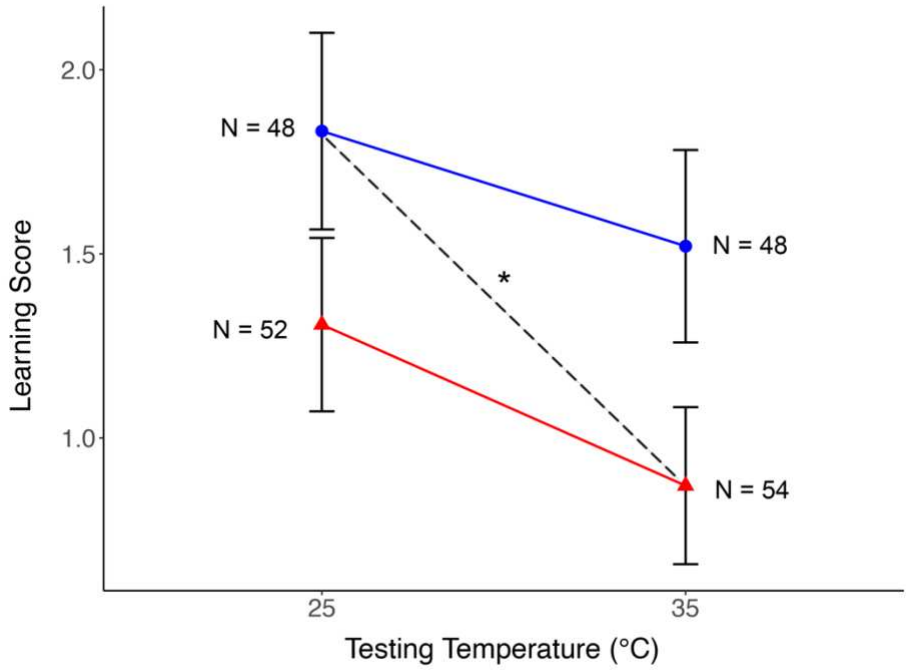

Figure 1.2: Thermal responses of cool (—●—) and warm (—▲—) acclimated bees on learning score (n = 202), measured using a proboscis extension response assay. The learning score for each bee is equal to the total number of trials, out of six, in which a conditioned response was recorded. Solid lines indicate the acute effect of temperature on performance within each group. Black dashed lines indicate the acclimation effect of temperature on performance, where complete compensation is indicated by a non-significant difference between the two groups. Data are presented as means with standard error bars and statistical significance between two groups is denoted by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) using Tukey's pairwise comparisons.

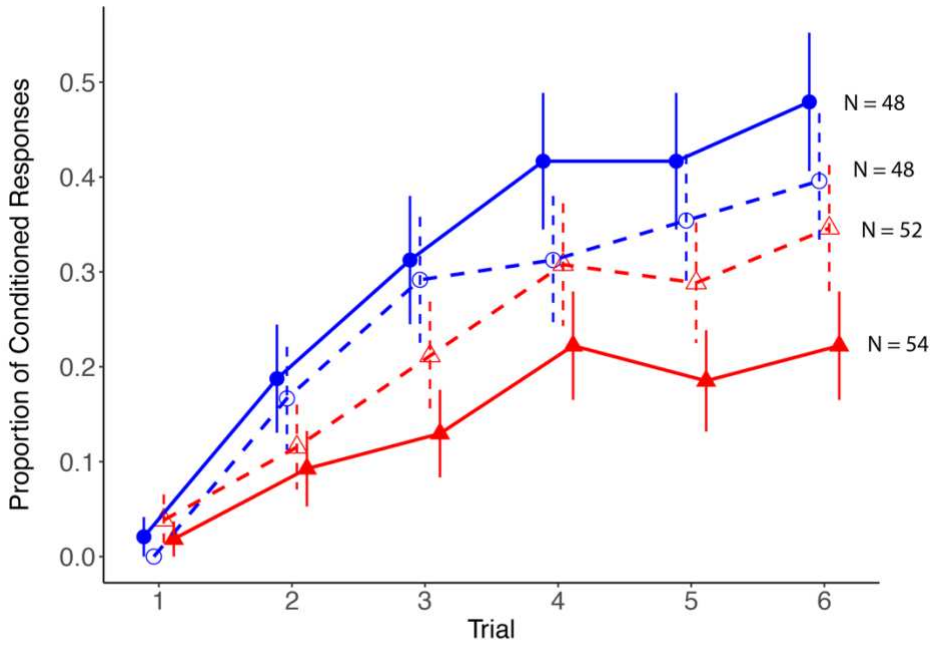

Figure 1.3: Learning, measured as the acquisition of conditioned responses across six trials of PER olfactory conditioning, in cool-acclimated bees tested either at their acclimation temperature (solid line, filled circle, —●—) or at the warm temperature (dashed line, empty circle, --○--), and in warm-acclimated bees tested at either the cool temperature (dashed line, empty triangle, --Δ--) or at their acclimation temperature (solid line, filled triangle, —▲—).

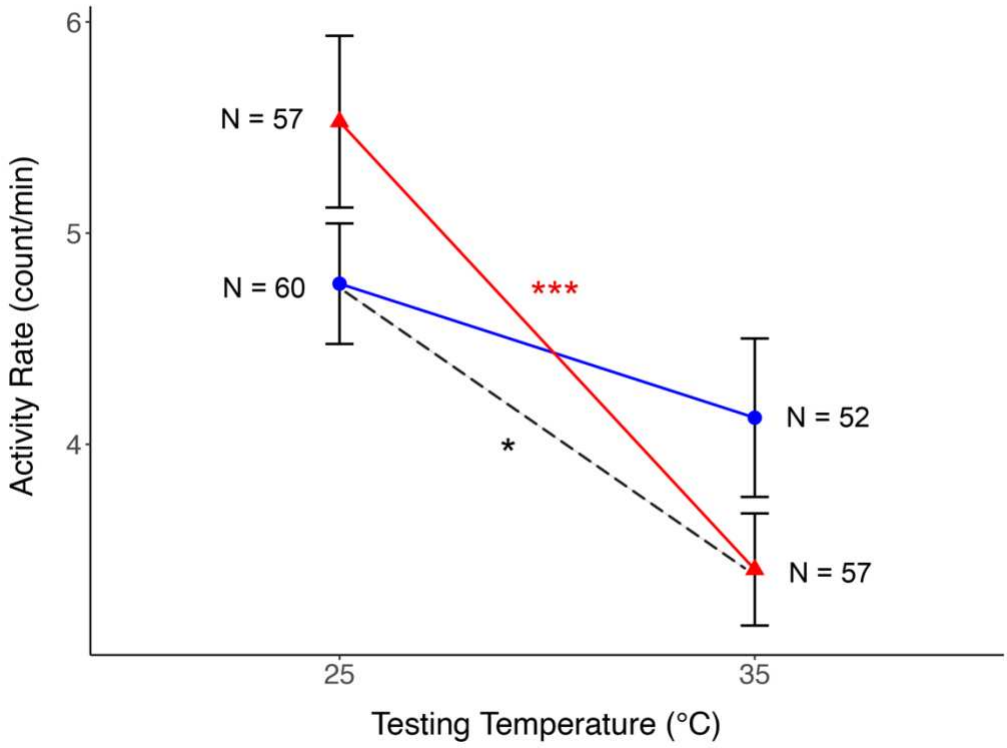

Figure 1.4: Thermal responses of cool (—●—) and warm (—▲—) acclimated bees on activity rate (n = 226) out of 120 minutes using an activity monitoring system. Solid lines indicate the acute effect of temperature on performance within each group. Black dashed lines indicate the acclimation effect of temperature on performance, where complete compensation is indicated by a non-significant difference between the two groups. Data are presented as means with standard error bars and statistical significance between two groups is denoted by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) using Tukey's pairwise comparisons.

CHAPTER 2

Slow-fast metabolic rate phenotypes shape honeybee thermal responses

Introduction

As global temperatures rise, animals must adjust their physiology and behavior to respond to increasingly variable thermal environments. These responses may be shaped by phenotypic plasticity, enabling the modification of traits within an individual's lifetime, or by evolutionary adaptation over longer timescales (Gienapp et al., 2009; Angilletta, 2009; Abram et al., 2017; McGaughran et al., 2021). Life history theory provides a useful framework for understanding animal responses to environmental changes, as species exhibit a spectrum of strategies that influence their survival, growth, and reproduction (Stearns, 1976). More recently, life history strategies have often been categorized along a slow–fast continuum, with metabolic rate often considered to be the fundamental driver of such variation (Réale et al., 2010). In this framework, individuals with low metabolic rate reflect a 'slow' phenotype and are predicted to have slower growth, delayed reproduction, and longer lifespans, while individuals with high metabolic rate represent a 'fast' phenotype exhibiting rapid growth, early reproduction, and shorter lifespans (Le Galliard et al., 2013; Dammhahn et al., 2018).

Given the integral link between temperature and metabolic rate, particularly in ectotherms, recent empirical and theoretical work has suggested that slow-fast phenotypic differences could be related to the thermal physiology of individuals and the ability to cope with different thermal regimes (Struelens et al., 2018; Debecker and Stoks, 2019; Albaladejo-Robles et al., 2023). Under

this framework, it is predicted that behavioral and life history traits are correlated with thermal physiology such that the slow-fast phenotypic continuum is aligned with a cold-hot axis of thermal preference, with fast individuals expected to perform better in warmer environments and slow individuals preferring cooler environments (Goulet et al., 2017; Michelangeli et al., 2018). There is empirical evidence for such an alignment in several ectothermic species, with individuals in warm climates exhibiting fast phenotypes, including higher metabolic rates, activity levels, and exploratory behaviors, compared to conspecifics in cooler climates who exhibit slow phenotypes (Debecker et al., 2019, Tüzün et al., 2022). These phenotypic differences may in part reflect underlying genetic variation in enzymes involved in energy metabolism. One such key enzyme, cytosolic malate dehydrogenase (MDH-1), has been widely documented to show temperature-dependent variation in structural forms across taxa. Populations in warmer environments tend to have MDH-1 allozymes that confer higher thermal stability and greater enzymatic performance at elevated temperatures (Dahlhoff and Somero, 1993; Simon et al., 1983; Smith et al., 1983; Meemongkolkiat et al., 2020).

In honeybees, natural populations in three continents exhibit a thermal cline in metabolic rate that is associated with variation in MDH-1 genotypes (Harrison and Fewell, 2002). Specifically, the MDH-1 gene exhibits distinct allelic variation such that the electrophoretically Slow (S) allele is associated with a lower metabolic rate and the Fast (F) allele is associated with a higher metabolic rate (Coelho and Mitton, 1988; Harrison et al., 1996; Cassano and Naug, 2022). The distribution of these alleles often show latitudinal and thermal gradients (Nielsen et al. 1994; Del Lama et al., 2004). This allelic variation has also been linked to distinct differences in various behavioral and life history traits as well as in their thermoregulatory abilities that align with a slow-

fast phenotypic axis (Mugel and Naug, 2022). Together, these patterns suggest that MDH-1 variation in honeybees is associated with an interaction between thermal physiology and behavioral traits, and understanding this interaction can provide critical insights into adaptive life history strategies under rising global temperatures and increasing thermal variability. Honeybees are vital pollinators that forage in highly variable thermal environments, and their capacity to cope with these conditions is central to maintaining global food systems under climate change (Klein et al., 2017; Zapata-Hernández et al., 2024). While it is therefore important to understand the genetic basis of variation in thermal performance, there are no experimental studies testing the physiological and behavioral responses of these honeybee genotypes across a range of temperatures.

To investigate how MDH-1 genetic variation influences thermal responses in honeybees, we tested the performance of three genotypes (SS and FF homozygotes and SF heterozygotes) across four temperatures, 24°C, 28°C, 32°C, and 36°C, which were selected to span an ecologically relevant range of ambient conditions. We measured three performance traits—metabolic rate, activity level, and learning ability—that have been previously linked to slow–fast phenotypes and are also central to the foraging success of honeybees. We hypothesized an interaction between genotype and temperature, predicting that homozygous Fast and Slow individuals would perform better at high and lower temperatures, respectively, and heterozygous individuals would exhibit an intermediate phenotype. We expected this relationship to be strongest for metabolic rate due to the direct impact of MDH-1 variation on enzyme activity, and potential downstream effects on activity level and learning ability based on previous studies linking these traits within a slow-fast behavioral framework in honeybees (Mugel and Naug, 2020; Tait et al., 2024).

Methods

Establishing SS, SF, and FF colonies

Brother drones of known MDH-1 genotypes were used to artificially inseminate unrelated virgin sister queens of known MDH-1 genotypes to create the following four types of crosses in the honeybee, *Apis mellifera*: S x SS, F x FF (hereafter called SS and FF for the respective types of brood they produce), S x FF, and F x SS (hereafter called SF). Colonies of each genotype (5 FF, 6 SS, and 4 SF) were then set up using worker bees from standard packages (starter colonies) of *A. mellifera* and a queen of a specific genotype (see Mugel and Naug, 2022 for further details). Brood frames at late pupal stage were pulled from these source colonies and placed in a 32°C incubator overnight to hatch. Newly emerged bees were marked on the abdomen with a dot of paint to track each cohort (genotype, source colony and date of emergence) and introduced into a foster colony headed by a wild-type queen to provide a common garden environment.

Experimental treatment

Marked bees were collected at 14-18 days of age, individually harnessed and then placed in a holding room at 30°C (the mid-point of the tested temperature range) for two hours. Bees were then tested for the three parameters of interest at one of the four test temperatures, 24°C, 28°C, 32°C, or 36°C, using a climate-controlled chamber (AC Infinity), within which temperature was maintained within 0.5°C and humidity was kept between 40-50%. A total of 459 bees were tested with each replicate consisting of seven bees from the same cohort tested for all three parameters at a given temperature on a given day.

Metabolic rate

Metabolic rate was measured using flow-through carbon dioxide respirometry with a FoxBox system equipped with a multiplexer (Sable Systems). Each harnessed bee was placed inside a 50 mL respirometry chamber, allowing 20 minutes for acclimatization before data collection began. Ambient air, scrubbed of H_2O and CO_2, was supplied at a constant flow rate of 250 mL/min. The CO_2 concentration in the outgoing airstream was recorded in parallel from all the chambers for 150 seconds at a time in a pseudorandom sequence that repeated three times for a total of 450 seconds per bee. A baseline was measured for each run by recording from an empty chamber for 60 seconds, the mean of which was used to calculate corrected CO_2 output values for each bee. Resting metabolic rate for each bee was then calculated based on the continuous 2-minutes with the lowest variance in CO_2 production. These values were converted to a power output (mW) using an energy equivalence of 21.4 J/mL CO_2 and then corrected for body mass to give a mass-specific metabolic rate.

Activity level

Following the measurement of metabolic rate, bees were released from their restraints and individually placed in activity monitoring tubes (16 mm diameter). Their locomotor activity was recorded for 60 minutes using an automated system (TriKinetics System, LAM16) equipped with infrared beams that detected and counted each time a bee crossed the center of the tube. During this assay, bees had unrestricted access to 40% sucrose provided in cotton plugs positioned at both ends of the tube. To determine the peak activity level for each bee and account for individual

variation in the timing of activity, we used a rolling window to identify the 15 consecutive minutes with the highest total counts for each bee.

Learning performance

Following the activity assay, bees were assayed for their learning ability using an aversive conditioning assay with a shock avoidance apparatus (Figure S1; see also Agarwal et al., 2011), in which bees learn to avoid a color cue (the conditioned stimulus, CS) associated with a mild electric shock (the unconditioned stimulus, US) as punishment. Bees were introduced into the apparatus and allowed to adjust without the conditioned or unconditioned stimuli for 10 minutes. Bees were then subjected to two 5-minute training sessions, during which the US was paired with the CS, separated by a 10-minute inter-training interval (ITI). Following the second training period, bees were tested after 10 minutes for successful avoidance learning in the absence of the US. Bees were placed in the center of the apparatus and by videotaping these test sessions, their position was scored every 15 seconds for 1 minute with respect to whether they exhibited the correct conditioned response (avoidance of the color cue associated with the 'shock side' of the grid). A correct response was scored as 1, and an incorrect response was scored as 0. From this, learning performance for each bee was calculated as the proportion of correct conditioned responses during the test period. At the end of the shock assay, bees were euthanized and weighed to record their body mass.

Statistical analysis

All analyses were performed in R. Separate models were constructed for each of the three response variables: metabolic rate, activity level, and learning performance. The primary predictor variables were genotype (factor with three levels: FF, SF, SS) and temperature (ordered factor with four levels: 24°C, 28°C, 32°C, 36°C). For metabolic rate and activity level, model selection began with a linear model, then diagnostic plots were used to evaluate model assumptions (normality, homoscedasticity) and determine whether transformations or alternative models were necessary. The metabolic rate data exhibited heteroscedasticity, with increasing variance at higher temperatures, and a generalized linear model (GLM) with a gamma distribution and log link provided the best fit. The activity level data were approximately normally distributed and were analyzed using a linear model. Learning performance was analyzed using a GLM with binomial distribution and logit link. Model terms were evaluated using likelihood ratio chi-squared tests. Orthogonal polynomial contrasts were applied to assess linear and quadratic components of the temperature effect. For genotype and temperature effects, estimated marginal means and Tukey-adjusted pairwise comparisons were used for post-hoc analysis. To assess potential effects of source colony on each response variable, additional mixed-effects models were constructed within each genotype, with colony included as a random intercept and temperature as a fixed effect (see Tables S1-3 for details).

Results

Metabolic rate

Metabolic rate was significantly affected by both temperature ($\chi^2 = 59.13$, d.f. = 3, $p < 0.001$) and genotype ($\chi^2 = 21.32$, d.f. = 2, $p < 0.001$), as well as their interaction ($\chi^2 = 15.15$, d.f. = 6, $p < 0.05$). Genotypic differences were observed at 24°C, 28°C, and 36°C (Fig. 2.1; Table S4), with FF bees exhibiting higher metabolic rates than SS bees, and SF bees being intermediate.

Notably, metabolic rates of SF bees shifted from being more SS-like at low temperatures to being more FF-like at high temperatures. The linear component of the temperature effect was significant for all three genotypes (FF: $t = 3.18$, $p < 0.01$; SF: $t = 2.06$, $p < 0.05$; SS: $t = 2.91$, $p < 0.01$), consistent with the increasing metabolic rates observed across the temperature range (Fig. 2.1). However, SS bees also showed a significant quadratic component of the temperature effect ($t = 2.46$, $p < 0.05$), which suggests that the metabolic rate of SS bees may have reached a peak within the tested temperature range—a pattern not observed for SF or FF bees.

Activity rate

Temperature ($F_{3,414} = 2.93$, $p < 0.05$) and genotype ($F_{2,414} = 4.16$, $p < 0.05$) were both significant predictors of activity level, although their interaction was not significant ($F_{6,414} = 1.41$, $p = 0.21$). Genotypic differences were observed only at 32°C (Fig. 2.2, Table S5), with FF bees having the highest activity level and SS bees having the lowest. When averaged over all temperatures, FF bees had higher overall activity levels than SS bees (Tukey's test: 1.89 ± 0.65 SE, $t = 2.89$, $p = 0.01$), while SF bees did not differ significantly from either FF (Tukey's test: 0.56 ± 0.67 SE, $t = 0.84$, $p = 0.67$) or SS bees (Tukey's test: 1.33 ± 0.67 SE, $t = 1.97$, $p = 0.12$). Orthogonal polynomial contrasts revealed a significant linear component of the temperature effect for the FF ($t = 2.72$, $p < 0.01$) and SF genotypes ($t = 2.69$, $p < 0.01$), indicating that FF activity increased and SF activity decreased linearly with temperature. SS activity did not respond significantly to changes in temperature ($t = 1.49$, $p = 0.13$). The quadratic component was not significant for any genotype (FF: $t = 1.32$, $p = 0.19$, SF: $t = 0.01$, $p = 0.98$, SS: $t = 0.38$, $p = 0.69$).

Learning performance

Genotype contributed significantly to learning performance ($\chi^2 = 16.73$, d.f. =2, $p < 0.001$), but temperature did not ($\chi^2 = 3.16$, d.f. = 3, $p = 0.37$), nor did a genotype-by-temperature interaction ($\chi^2 = 4.88$, d.f. = 6, $p = 0.56$). Genotypic differences were observed at 24°C, 28°C, and 32°C; pairwise contrasts indicated that SF bees outperformed SS bees overall, while no significant differences in learning were observed between FF and SF or between FF and SS genotypes (Fig. 2.3, Table S6). When averaged across temperatures, SF bees exhibited higher learning performance than SS bees (Tukey's test: 0.13 ± 0.03 SE, $p < 0.001$), but did not differ significantly from FF bees (Tukey's test: –0.06 ± 0.03 SE, $p = 0.11$). Learning performance also did not differ between FF and SS bees when averaged across temperatures (Tukey's test: 0.07 ± 0.03 SE, $p = 0.10$). There were no significant linear (FF: $z = 1.10$, $p = 0.27$; SF: $z = -1.69$, $p = 0.08$; SS: $z = -0.51$, $p = 0.61$) or quadratic trends (FF: $z = 0.71$, $p = 0.47$; SF: $z = -0.21$, $p = 0.83$; SS: $z = 0.65$, $p = 0.51$) in learning performance across temperatures.

Discussion

This study provides experimental evidence that allelic variation at the MDH-1 locus contributes to temperature-dependent performance differences, with important implications for honeybee foraging. We found that genotypes associated with distinct slow-fast phenotypes responded differently to temperature in terms of their metabolic rates, activity levels, and learning performance. However, the strength and pattern of temperature effects varied by trait, with metabolic rate and activity showing stronger responses to temperature than learning. As predicted, metabolic rate showed a clear genotype-by-temperature interaction, with homozygous Fast

individuals exhibiting consistently higher rates than homozygous Slow individuals and heterozygous bees showing intermediate rates. Notably, heterozygous bees displayed a temperature-dependent shift in metabolic rate, resembling the Slow phenotype at cooler temperatures and the Fast phenotype at warmer temperatures. This pattern could reflect greater thermal sensitivity in the heterozygotes, indicated by a steeper reaction norm, or suggest a flexible strategy for context-dependent trait expression.

Biochemical and structural analyses have shown that the F and S MDH-1 allozymes differ in both catalytic efficiency and thermal stability in honeybees and that SF heterodimers maintain high activity across a broad temperature range (Meemongkolkiat et al., 2020). This broad thermal performance may potentially confer a heterozygote advantage, allowing for higher plasticity in fluctuating thermal environments. Our findings support the general hypothesis that MDH-1 polymorphism contributes to thermal adaptation via differential enzyme stability and function. Given its role in cytosolic redox balance and ATP production, structural variation at this locus can influence metabolic flux and overall energy availability. Although thermal performance curves were not explicitly modeled here, the quadratic trend for metabolic rate observed in homozygous Slow bees but not in homozygous Fast or heterozygous bees implies a metabolic performance peak for Slow bees within the tested temperature range. This pattern is consistent with previous research showing that slow phenotypes have lower thermal optima than fast phenotypes (Goulet et al., 2017; Michelangeli et al., 2018).

Despite the strong genotype-by-temperature interaction observed for metabolic rate, activity level showed no such interaction, although both genotype and temperature independently influenced activity. Previous studies have reported a positive correlation between metabolic rate and activity levels in honeybees (Mugel and Naug, 2020), and Fast foragers with higher metabolic rates have been shown to take shorter trips, suggesting higher flight speeds (Cassano and Naug, 2022). Consistent with these findings, homozygous Fast bees had significantly higher activity levels than Slow bees when averaged across temperatures, and their activity increased linearly with temperature. However, within-temperature differences in activity were only significant at 32°C. It is possible that our lab-based assay for activity level was not able to fully capture the genotypic difference at all temperatures due to the spatial constraints of the activity monitoring tubes. Nonetheless, the strong difference in activity observed at 32°C may translate to genotypic differences in task performance, particularly since this is close to the optimal hive temperature (Kronenberg and Heller, 1982). Interestingly, heterozygotes again represented an intermediate phenotype and were also the only genotype whose activity levels decreased at higher temperatures, potentially suggesting a more flexible behavioral strategy to limit activity under thermal stress. The homozygous Slow bees showed no significant change in activity across temperatures, although their overall activity levels were the lowest. This lower thermal sensitivity is consistent with the idea that individuals with lower thermal optima often exhibit broader thermal tolerance, reflecting a trade-off between thermal breadth and peak performance driven by constraints on enzymatic flexibility and stability (Huey and Kingsolver, 1989). However, empirical evidence for this idea has been mixed, and the opposite trend has also been observed (Knies et al., 2009).

Learning performance was influenced by genotype but not temperature, and we found no evidence of an interaction between the two. Across the temperature range, heterozygous bees outperformed homozygous Slow bees, while homozygous Fast bees showed intermediate learning performance that did not differ significantly from either genotype. Previous research has shown that cognitive differences along the slow–fast continuum in honeybees are associated with faster learning, which in turn correlates with larger brain size and higher whole-animal metabolic rate (Tait et al., 2024). Fast bees had slightly higher learning scores than Slow bees in our study, although this difference was not statistically significant and learning performance showed minimal sensitivity to temperature across all genotypes. It is important to consider that our shock avoidance assay measures aversive learning based on spatial exploration, which could introduce a potential confounding effect of activity on learning performance. Our results contrast with findings that bumblebees experience declines in learning and memory after only a few hours of exposure to high temperatures (Gérard et al., 2022), although these results are based on appetitive conditioning assays, which may in turn be confounded by variation in motivation. Short-term exposure to temperature variation did not strongly influence learning performance in our study, but this does not rule out the possibility that temperature effects might emerge under more extreme or prolonged exposure, or when compounded by other variables such as resource availability (Vanderplanck et al., 2019; Hemberger et al., 2023). Moreover, because learning integrates multiple systems including sensory input, attention, and memory, it may respond more variably to temperature than other classic physiological traits, and more research is needed on this topic.

The fitness of a honeybee colony depends on the coordinated behavior of thousands of individuals. It is well-known that individual-level differences in physiology and behavior can show

complex scaling effects to influence collective functions such as foraging, thermoregulation, and task allocation (Jeanson and Weidenmüller, 2014). We found genotypic differences in all our measured traits, with particularly pronounced differences between the Fast and Slow genotypes and more flexible responses in the heterozygotes. At the colony level, such divergence may support context-dependent effects on task performance where variation among individuals has a significant influence on colony functioning. Genetic diversity has been shown to positively influence group fitness in honeybees (Tarpy, 2003; Jones et al., 2004; Mattila and Seeley, 2007), leading to the common conception that it played a key role in the evolution of sociality (Nonacs and Kapheim, 2007; Oldroyd and Fewell, 2007). However, it is often overlooked that genetic diversity can also produce negative or neutral effects on collective performance by introducing conflicts, inefficiency, or mismatched behavioral responses (Page et al., 1995; Neumann and Moritz, 2000; Arathi and Spivak, 2001; Schmidt et al., 2011). A more detailed understanding of these complex effects is necessary for evaluating how genetic diversity would contribute to colony performance in the face of rising temperatures.

Our results suggest that MDH-1 variation could contribute to colony-level plasticity through its effects on individual thermal responses. Genetically based differences in response thresholds have been shown to stabilize colony-level functions such as thermoregulation, allowing for more effective responses to environmental perturbations (Jones et al., 2004). More flexible metabolic phenotypes have also been associated with enhanced performance under resource-limited conditions (Auer et al., 2015). Our findings support this work by demonstrating that heterozygous bees exhibit intermediate or flexible thermal responses across multiple traits as well as superior performance in an avoidance learning assay, suggesting a potential heterozygote

advantage in fluctuating environments. It is important to note that honeybee populations in North America and Europe are highly managed, and current breeding practices have led to most colonies typically being sourced from a narrow genetic pool (Cobey et al., 2012). As a result, current honeybee populations may not be optimized for their local climates in terms of their slow-fast phenotypes. By experimentally demonstrating that MDH-1 genotypes differ in the thermal sensitivity of traits that are relevant to foraging and colony performance, our findings highlight the importance of studying this genetic variation, not only for addressing fundamental questions in behavioral evolution but also for potentially informing future honeybee breeding practices.

Together, our results support the idea that slow and fast phenotypes exhibit distinct thermal dynamics, at least for traits closely tied to metabolic rate. Our findings extend earlier studies in other taxa regarding the physiological importance of MDH-1 variation in conferring thermal stability under different temperatures (Simon et al., 1983; Dahlhoff and Somero, 1993) and show how genetic variation in metabolic rate contributes to individual-level differences in thermal performance in honeybees. The observed interaction between genotype and temperature raises important questions about how functional diversity influences collective responses. How these gene-environment effects at the individual level scale up to influence collective outcomes and colony performance in different climates are important topics for future studies. Such knowledge could potentially help inform efforts to breed more resilient bees in the face of climate change.

Figures:

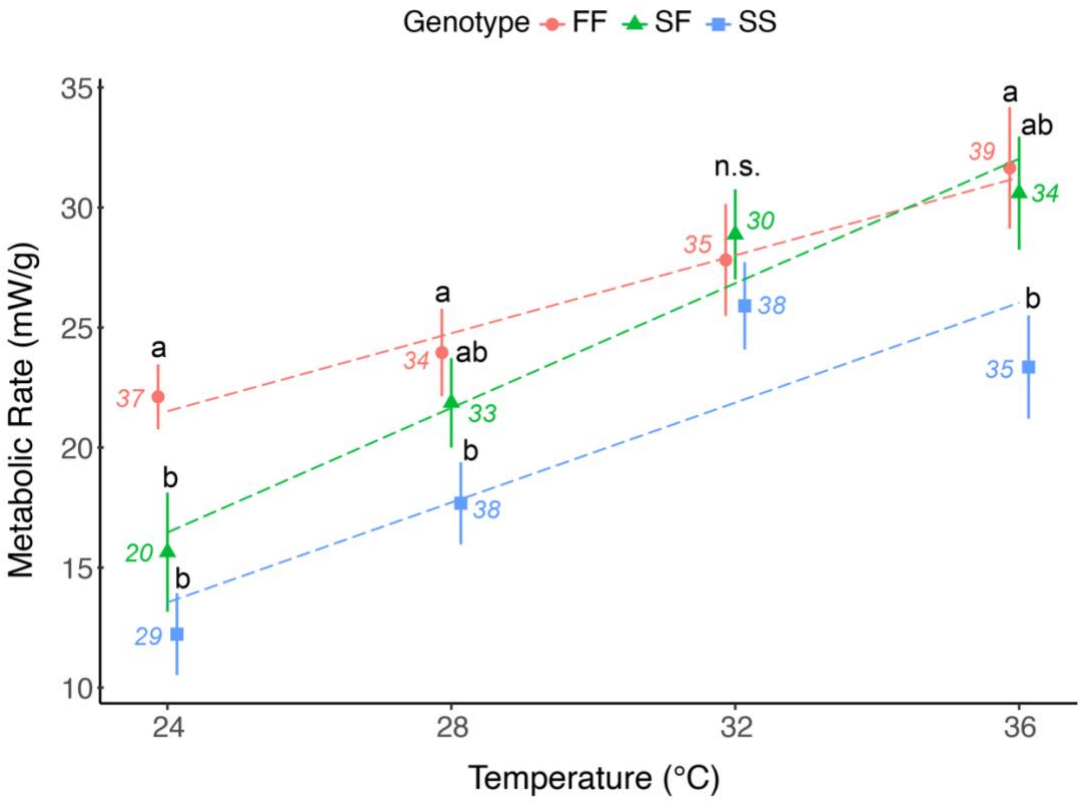

Figure 2.1: Effects of MDH-1 genotype and temperature on mass-specific metabolic rate (mW/g) in honeybees, measured using flow-through respirometry. Data are presented as means with standard error bars and significant differences among genotypes within a temperature are denoted by different letters ($p < 0.05$) using Tukey-adjusted pairwise comparisons of estimated marginal means (see Table S4 for details). Dashed lines show the best-fit linear trends across temperature for each genotype. Sample sizes are reported next to each group.

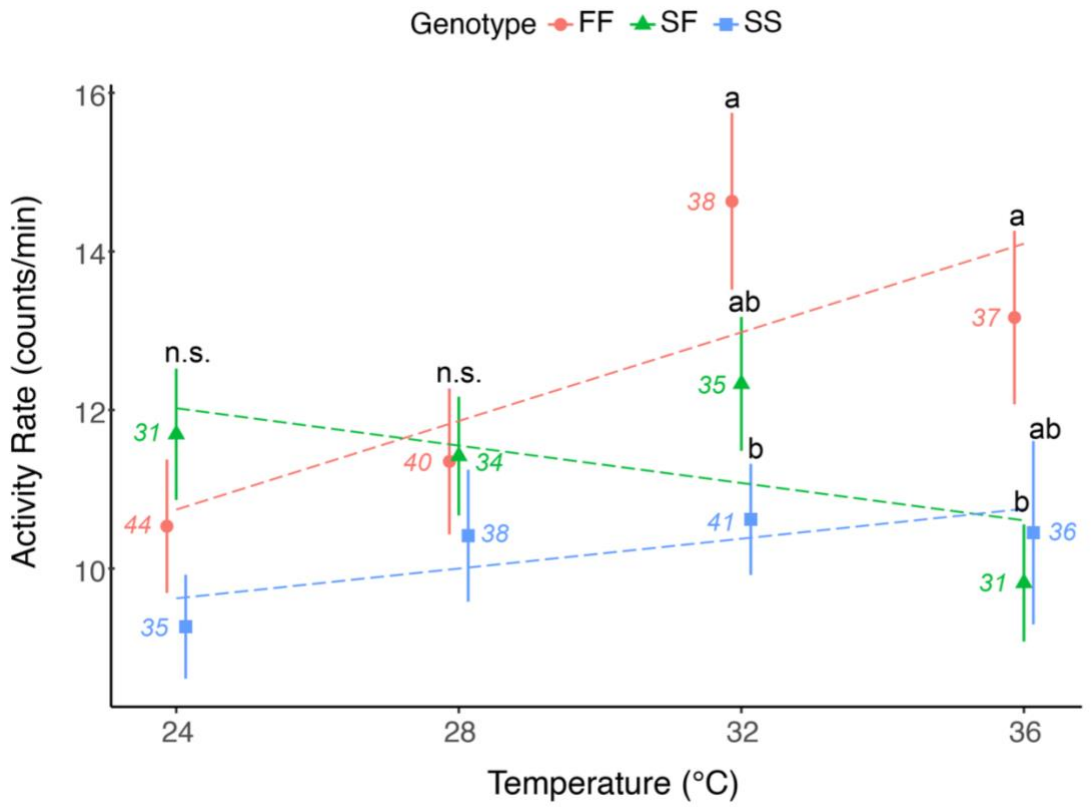

Figure 2.2: Effects of MDH-1 genotype and temperature on activity rate (counts/min) based on the most active 15-minute period for each bee out of 60 minutes using an activity monitoring system. Data are presented as means with standard error bars and significant differences among genotypes within a temperature are denoted by different letters ($p < 0.05$) using Tukey-adjusted pairwise comparisons of estimated marginal means (see Table S5 for details). Dashed lines show the best-fit linear trends across temperature for each genotype. Sample sizes are reported next to each group.

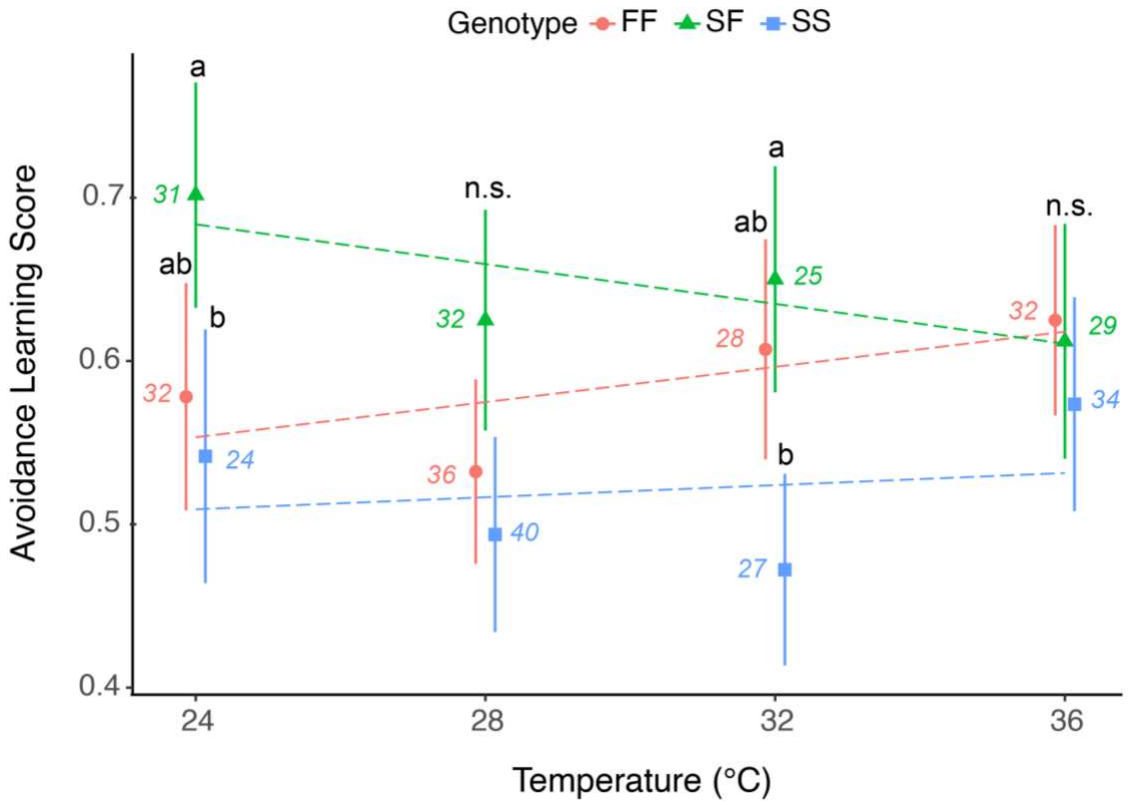

Figure 2.3: Effects of MDH-1 genotype and temperature on learning score in honeybees, calculated as the proportion of conditioned responses during the test phase of a shock avoidance assay in the absence of the unconditioned stimulus, a mild electric shock. Data are presented as means with standard error bars and significant differences among genotypes within a temperature are denoted by different letters ($p < 0.05$) using Tukey-adjusted pairwise comparisons of estimated marginal means (see Table S6 for details). Dashed lines show the best-fit linear trends across temperature for each genotype. Sample sizes are reported next to each group.

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APPENDIX

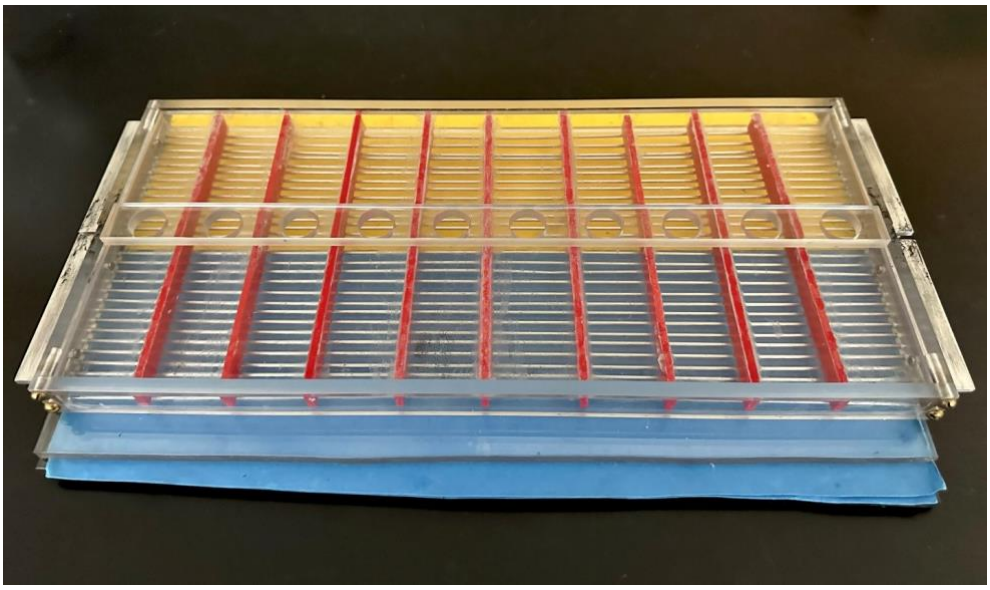

Figure S1: The shock grid apparatus. The shock-grid apparatus consisted of a plexiglass box with two separate halves, each containing a metallic grid of aluminum wires (1.59 mm diameter spaced 3.5 mm apart) such that each half of the box could be independently electrified. The grid was divided into 10 evenly spaced lanes by opaque plastic dividers glued to the plexiglass lid that acted as a ceiling. The ceiling and walls of the apparatus were coated with a thin layer of petroleum jelly to ensure that bees had to walk and stay in contact with the grid. An electric current of 10 V, 40 mA) was applied to one half of the grid such that one half of the chamber provided a punishment in the form of an electric shock (see Agarwal et al., 2011 for more details). After each replicate, the apparatus was wiped down with 95% ethanol to eliminate any scent cues left by the bees.

Table S1: Likelihood ratio tests assessing the contribution of source colony to variation in metabolic rate. Linear mixed models were used to test for potential colony effects within genotypes, treating temperature as a fixed effect and colony as a random intercept within each genotype. Inclusion of colony did not significantly improve model fit for the SS or SF genotypes. However, source colony did have a significant effect on the metabolic rate for the FF genotype.

Genotype	Model with Colony AIC	Model without Colony AIC	Chisq	df	p-value
SS	1077.9	1075.9	0.000	1	1.000
SF	914.3	912.3	0.000	1	1.000
FF	1146.0	1150.2	6.191	1	**0.013**

Table S2: Likelihood ratio tests assessing the contribution of source colony to variation in activity level. Linear mixed models were used to test for potential colony effects within genotypes, treating temperature as a fixed effect and colony as a random intercept within each genotype. Inclusion of colony did not significantly improve model fit for the SF or FF genotypes, but source colony did have a significant effect on the activity level for the SS genotype.

Genotype	Model with Colony AIC	Model without Colony AIC	Chisq	df	p-value
SS	877.91	891.82	9.670	1	**0.002**
SF	814.45	817.62	0.000	1	1.000
FF	961.94	970.69	3.808	1	0.051

Table S3: Likelihood ratio tests assessing the contribution of source colony to variation in learning. Linear mixed models were used to test for potential colony effects within genotypes, treating temperature as a fixed effect and colony as a random intercept within each genotype. Inclusion of colony did not significantly improve model fit for any genotype.

Genotype	Model with Colony AIC	Model without Colony AIC	Chisq	df	p-value
SS	110.76	108.76	0	1	1
SF	115.28	113.28	0	1	1
FF	105.03	103.03	0	1	1

Table S4: Metabolic rate pairwise contrasts between the different genotypes within each test temperature.

Contrast	Temp (°C)	Estimate	SE	df	t-ratio	p-value
FF - SF	24	6.47	2.59	390	2.50	**0.0342**
FF - SS	24	9.89	2.20	390	4.50	**<0.0001**
SF - SS	24	3.42	2.14	390	1.60	0.2484
FF - SF	28	2.09	2.87	390	0.73	0.7468
FF - SS	28	6.28	2.57	390	2.44	**0.0399**
SF - SS	28	4.18	2.45	390	1.71	0.2025
FF - SF	32	-1.07	3.62	390	-0.29	0.9535
FF - SS	32	1.91	3.24	390	0.59	0.8253
SF - SS	32	2.98	3.46	390	0.86	0.6657
FF - SF	36	1.05	3.74	390	0.28	0.9574
FF - SS	36	8.30	3.30	390	2.52	**0.0327**
SF - SS	36	7.24	3.37	390	2.15	0.0814

Table S5: Activity level pairwise contrasts between the different genotypes within each test temperature.

Contrast	Temp (°C)	Estimate	SE	df	t-ratio	p-value
FF - SF	24	-1.61	1.31	413	-1.22	0.4395
FF - SS	24	0.56	1.28	413	0.44	0.9000
SF - SS	24	2.17	1.38	413	1.57	0.2618
FF - SF	28	-0.87	1.31	413	-0.67	0.7838
FF - SS	28	0.94	1.28	413	0.73	0.7439
SF - SS	28	1.81	1.31	413	1.38	0.3535
FF - SF	32	1.79	1.30	413	1.38	0.3546
FF - SS	32	3.33	1.28	413	2.61	**0.0254**
SF - SS	32	1.54	1.29	413	1.20	0.4553
FF - SF	36	2.93	1.40	413	2.09	0.0939
FF - SS	36	2.71	1.39	413	1.95	0.1263
SF - SS	36	-0.21	1.40	413	-0.15	0.9873

Table S6: Learning performance pairwise contrasts between the different genotypes.

Contrast	Temp (°C)	Estimate	SE	z-ratio	p-value
FF - SF	24	-0.12	0.06	-2.06	0.0983
FF - SS	24	0.04	0.07	0.54	0.8495
SF - SS	24	0.16	0.07	2.45	**0.0383**
FF - SF	28	-0.10	0.06	-1.61	0.2415
FF - SS	28	0.04	0.06	0.64	0.7955
SF - SS	28	0.14	0.06	2.36	**0.0479**
FF - SF	32	-0.04	0.07	-0.65	0.7948
FF - SS	32	0.13	0.07	2.03	0.1061
SF - SS	32	0.18	0.07	2.63	**0.0235**
FF - SF	36	0.01	0.06	0.21	0.9765
FF - SS	36	0.05	0.06	0.85	0.6691
SF - SS	36	0.04	0.06	0.62	0.8083