How Will Agrochemical Exposure and Climatic Warming Affect Honey Bee Morphology, Foraging Performance, and Heat and Water Balance During Flight?

by

Jordan R. Glass

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Graduate Supervisory Committee:

Jon F. Harrison, Chair
Dale F. DeNardo
Robert Dudley
Jennifer H. Fewell

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ABSTRACT

The alarming decline of insect pollinators is due in part to agrochemical exposure and climate warming. This thesis focuses on understanding how exposure to a commonly used fungicide and high air temperature affect the flight behavior and physiology of the very important commercial pollinator, *Apis mellifera*.

I found that honey bees reared on pollen contaminated with field-realistic levels of a fungicide (Pristine®) commonly applied to almond blossoms before pollination had smaller thoraxes, possibly due to inhibition of protein digestion, plausibly reducing flight capability. By flying unloaded bees in low density air to elicit maximal performance, I found that consumption of high doses of fungicide during development inhibited maximal flight performance, but consumption of field-realistic doses did not.

To understand climatic-warming effects on honey bees, I flew unloaded foragers at various air densities and temperatures to assess the effects of flight muscle temperature (29 to 44°C) on maximal aerobic metabolism. Flight metabolic rate peaked at a muscle temperature of 39°C and decreased by ~2% per degree below and ~5% per degree above this optimum. Carrying nectar loads increased flight muscle temperatures and flight metabolism of foragers flying at air temperatures of 20 or 30°C. Yet, remarkably, bees flying at 40°C were able to carry loads without heating up or increasing metabolic rate. Bees flying at 40°C increased evaporative cooling and decreased metabolic heat production to thermoregulate. High speed video revealed that bees flying at 40°C air temperature lowered their wing beat frequency while increasing stroke amplitude, increasing flight efficiency. My data also suggests that cooler bees use wing kinematic strategies that increase flight stability and maneuverability while generating excess heat

that warms their flight muscle toward optimum. High water loss rates during flight likely limit foraging in dry air temperatures above 46°C, suggesting that CT_{max} measures of resting honey bees significantly overestimate when high air temperature will negatively impact flight and foraging.

DEDICATION

This dissertation is dedicated to my wife, Christine, and our three daughters, Lia, Annie, and Rainey. Thank you for all the laughter, joy, silliness, and love that kept me sane during graduate school.

A special thanks to my wonderful wife – for her love and support. Her advice, “Don’t say ‘no’ until someone else does” is now my personal mantra and has helped get me to where I am today.

I love you.

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CHAPTER 1

OVERVIEW: NEGATIVE EFFECTS OF AGROCHEMCIAL EXPOSURE AND CLIMATE CHANGE ON INSECT POLLINATORS

Insects are declining at an alarming rate across the globe (Rhodes, 2018; Wagner *et al*., 2021; Nath *et al*., 2022), the loss of which will undoubtedly impact ecosystems and the sustainability of human agriculture (Gallai *et al*., 2009; Lever *et al*., 2014; Ramos-Jiliberto *et al*., 2020; van der Sluijs, 2020; Harvey *et al*., 2022). More than 80% of flowering plants (Dicks *et al*., 2021) and 75% of food crops are at least partially dependent on insect pollinators for sexual reproduction (Vanbergen & Initiative, 2013; Ollerton, 2017). The loss of these crucial animals is due in part to factors such as regular exposure to agrochemicals (Johnson *et al*., 2010; Sponsler *et al*., 2019), and climate change (Hickling *et al*., 2006; Williams *et al.*, 2007), will likely have catastrophic impacts (Gallai *et al.*, 2009; Calderone, 2012; Chopra *et al.*, 2015). The pollination services of insects not only contribute significantly to both agricultural (US$14.2−23.8 billion) and industrial sectors (US$10.3−21.1 billion), but also play vital roles in biodiverse ecosystems (Ollerton *et al.*, 2011). The most widely used managed pollinator is the honey bee (*Apis mellifera*), which is necessary for the pollination of many crops including berries, almonds, pome, and stone fruits. Although honey bees are not considered threatened, North American beekeepers are losing more than 40% of their colonies each year (vanEngelsdorp *et al*., 2007, 2011; Steinhauer *et al*., 2014; Kulhanek *et al*., 2017; Bruckner *et al*., 2022), increasing the challenge of keeping up with agricultural demand (Aizen & Harder, 2009). Understanding how environmental factors,

like agrochemicals and climate change, impact honey bee health and foraging performance is imperative for continued food security.

Exposures to insecticides (e.g., neonicotinoids and phenylpyrazoles) have been implicated as a major contributor to insect pollinator decline due to their toxicity, high frequency of use, and persistent accumulation in agricultural foraging environments (Iwasa *et al*., 2004; Vidau *et al*., 2011; Smalling *et al*., 2013; Goulson *et al*., 2015; Zhu *et al*., 2015; Tison *et al*., 2016; Fisher *et al*., 2018). Additionally, fungicides are very commonly encountered by pollinators, and there are studies demonstrating correlations between high levels of exposure to fungicides and poor colony health of honey bees (Mullin *et al*., 2010; Pettis *et al*., 2013). Fungicides are often considered relatively safe for animals, including insect pollinators, due to their high acute contact and oral LD_{50}'s relative to their environmental exposure (Legard *et al*., 2001; Smalling *et al*., 2013; Ostiguy *et al*., 2019). Typically, fungicides are only considered hazardous when paired with other agrochemicals, such as insecticides (Pilling & Jepson, 1993; Pilling *et al*., 1995; Iwasa *et al*., 2004; Johnson *et al*., 2013; Tosi & Neih, 2019). However, little attention has been given to understanding the independent sublethal effects of fungicide exposure on honey bee health (except see: DeGrandi-Hoffman *et al*., 2015; Campbell *et al*., 2016; Liao *et al*., 2019). For example, the widely used fungicide, Pristine®, a known mitochondrial inhibitor of fungal targets, also negatively impacts mitochondrial function of flight muscle *in vitro* and decreased protein digestion, possibly due to damaged midgut epithelia (DeGrandi-Hoffman *et al*., 2015; Campbell *et al*., 2016; da Costa Domingues *et al*., 2020; Tadei *et al*., 2020; Fisher *et al*., 2021). Investigating the

effect of fungicides on the flight metabolism and morphology of honey bees will provide a better assessment of the safety of these commonly used agrochemicals.

Although evidence suggests climate change is contributing to the decline of insect pollinators (Hickling *et al*., 2006; Williams *et al*., 2007; Soroye *et al*., 2020), the magnitude of its impact is still unknown. The effects of temperature likely exceed those of any other abiotic factor, impacting many aspects of the behavior and physiology of these important animals (Hochachka & Somero, 1984; Heinrich, 2013). Most large insect pollinators are endothermic during flight and have evolved thermoregulatory mechanisms to buffer against thermal variation in the environment (Heinrich, 2013). Our current understanding of the effects of high temperatures on the physiology of these important insects is limited (Halsch *et al*., 2021; Janzen & Hallwachs, 2021; Vasiliev & Greenwood, 2021; Johnson *et al*., 2023). We desperately need to identify and determine the behavioral and physiological limitations of insects to high temperature exposure if we are to predict how insect pollinators will fare in the Anthropocene.

Earth is experiencing a general warming trend, with a predicted increase in the frequency, duration, and intensity of extreme heatwave events (Marx *et al*., 2021; Meehl & Tebaldi, 2004; Miller *et al*., 2021; Parmesan *et al*., 2000; IPCC, 2021; Schoof *et al*., 2017; Tewari *et al*., 2019). While some evidence implicates climate warming in these loses of insect pollinators (e.g., Soroye *et al*., 2020), we have a limited understanding of the role and mechanisms of the effects of high temperatures on the physiology of these important insects (Halsch *et al*., 2021; Janzen & Hallwachs, 2021; Vasiliev & Greenwood, 2021). We need to identify and determine the behavioral and physiological

limitations of insects to high temperature exposure if we are to predict how insect pollinators will fare in the Anthropocene.

Honey bees likely evolved in Africa, with considerable exposure to heat. Honey bees have diverse behavioral and physiological mechanisms to cope with flight at high air temperatures (Heinrich, 1980a,b; Cooper *et al*., 1985; Roberts & Harrison, 1999; Feuerbacher *et al*., 2003). For facultative endothermic pollinators, like honey bees, metabolic heat production during flight is a major component of the heat budget, and a potential contributor to overheating at high air temperatures (Roberts & Harrison, 1999). Some insects, such as *Euglossa imperialis* and small-morph male *Centris pallida*, can reduce their flight metabolic rate and wing beat frequency at relatively higher air temperatures to reduce this additional heat gain (Borrell & Medeiros, 2004; Roberts *et al*., 1998). For these species, the reduction in metabolism is the major mechanism of thermoregulation during flight (Parmezan *et al*., 2021). However, whether honey bees reduce flight metabolism when flying at higher air temperatures remains controversial. Heinrich (1980b) and Woods *et al*. (2005) found that flight metabolism of unloaded honey bees was independent of air temperature, which they attributed to the constant lift requirements for flight necessitating the maintenance of high flight muscle temperatures needed for mechanical power production. In contrast, other studies have found that honey bees can decrease their flight metabolic rate and wingbeat frequency when flying at high air temperatures (Harrison *et al.*, 1996; Roberts & Harrison, 1999). Harrison and Fewell (2002) suggest that the effects of air temperature on flight metabolic rate may depend on thoracic temperatures relative to the thermal performance curve. However, this hypothesis has never been quantitatively tested.

Assuming honey bees can decrease flight metabolism and wingbeat frequency, we do not know if the decline in flight metabolic rates observed at high temperatures is due to heat-suppression of behavior and performance, or can be explained by an increase in efficiency that enables high performance while preventing overheating from elevated metabolic heat production. Maximal force production of honey bees declines at thoracic temperatures above 39°C, supporting the hypothesis that maximal power output also declines (Coelho, 1991), but it is not yet clear whether maximal mechanical power output or functional capacities such as load-lifting during flight also declines. Bumble bees can alter kinematics to lift loads while maintaining relatively stable metabolic rates, suggesting that thermal modulation of flight efficiency is possible (Combes *et al*., 2020). Similarly, it has been suggested that euglossine bees can increase elastic energy storage or muscle contraction efficiency as air and thoracic temperatures rise (Borrell & Medeiros, 2004).

Endothermic bees and wasps can also use other physiological mechanisms to avoid thermal stress. When operative temperatures rise above body temperatures, and metabolic heat production cannot be further suppressed, increases in evaporative heat loss can lower body temperatures and prevent lethal overheating. It's been suggested that some bees and wasps utilize evaporative heat loss while nectar foraging to prevent overheating, as their high metabolic rates promote metabolic water production, and foraged nectar may be used for cooling (Nicolson, 2009; Nicolson & Louw, 1982). Evidence to date suggests that relatively few endothermic pollinators use evaporative water loss to avoid overheating during flight (reviewed in Johnson *et al*., 2023), but

honey bee workers can increase evaporative heat loss to thermoregulate when flying at air temperatures above 38°C (Roberts & Harrison, 1999).

For insect pollinators that actively increase evaporative heat loss to thermoregulate, the mechanisms remain poorly understood. Insects are generally thought to lack exocrine glands, such as the sweat glands, that function for cooling in most mammals. However, exocrine glands do occur in cicadas, so it could be possible that they have been overlooked in other insects (Hadley *et al*., 1991). Among insects, increases in ventilatory water loss at high air temperatures has only been verified for grasshoppers (Prange, 1990). However, it would not be surprising to find this mechanism used by pollinating insects, either in flight or at rest. For insect pollinators, a well-demonstrated mechanism of evaporative cooling is regurgitation of fluid from the crop, which has been found in honey bees and wasps (Coelho & Ross, 1996; Heinrich, 1980a). Evaporation of water from the proboscis for thermoregulation is an extension of a behavior used by a variety of bees, including halictid, allodapine, carpenter, stingless, and honey bees, to dehydrate excessively dilute nectar (Nicolson, 2009). However, in a variety of wasps and bees, evaporative water loss has been shown to increase during flight without any observations of regurgitation (Coelho & Ross, 1996; Heinrich & Buchmann, 1986; Johnson *et al*., 2022; Roberts & Harrison, 1999), and there is also evidence that abdominal, as well as head, temperatures can decline below air temperature in both honey bees and wasps (Roberts & Harrison, 1999). Perhaps evaporative water loss can be increased by emission of fluid from the rectum, as occurs in mosquitoes (Lahondère & Lazzari Claudio, 2012; Reinhold *et al*., 2021), or expansion of the abdomen to expose water-permeable intersegmental membranes. In the first chapter, I discuss both the effects

of agrochemical exposure on the morphology and flight metabolism of honey bees. Then, in the following two chapters, I discuss the direct and indirect effects of climatic warming on flight behavior and physiology of honey bees by looking at their flight behavior and performance, flight metabolic and evaporative water loss rate, and flight kinematics.

CHAPTER 2

CONSUMPTION OF FIELD-REALISTIC DOSES OF A WIDELY USED MITO-TOXIC FUNGICIDE REDUCES THORAX MASS BUT DOES NOT NEGATIVELY IMPACT FLIGHT CAPACITIES OF THE HONEY BEE (*APIS MELLIFERA*)

THIS CHAPTER HAS BEEN PUBLISHED IN *ENVIRONMENTAL POLLUTION*

SEE APPENDIX A

Abstract

Commercial beekeepers in many locations are experiencing increased annual colony losses of honey bees (*Apis mellifera*), but the causes, including the role of agrochemicals in colony losses, remain unclear. In this study, I investigated the effects of chronic consumption of pollen containing a widely-used fungicide (Pristine®), known to inhibit bee mitochondria in vitro, which has recently been shown to reduce honey bee worker lifespan when field-colonies are provided with pollen containing field-realistic levels of Pristine®. I fed field colonies pollen with a field-realistic concentration of Pristine® (2.3 ppm) and a concentration two orders of magnitude higher (230 ppm). To challenge flight behavior and elicit near-maximal metabolic rate, I measured flight quality and metabolic rates of bees in two lower-than-normal air densities. Chronic consumption of 230 but not 2.3 ppm Pristine® reduced maximal flight performance and metabolic rates, suggesting that the observed decrease in lifespans of workers reared on field-realistic doses of Pristine®-laced pollen is not due to inhibition of flight muscle mitochondria. However, consumption of either the 230 or 2.3 ppm dose reduced thorax mass (but not body mass), providing the first evidence of morphological effects of

Pristine®, and supporting the hypothesis that Pristine® reduces forager longevity by negatively impacting digestive or nutritional processes.

Introduction

Insect pollinators are in decline globally, due in part to regular exposure to agrochemicals, such as fungicides (Johnson *et al*., 2010; Sponsler *et al*., 2019). Although regulatory agencies of most countries require testing of lethal acute and chronic effects of agrochemical exposure (LD_{50}: Iwasa *et al*., 2004; US EPA, 2014; Tosi & Nieh, 2019), the fitness of pollinators may also be affected by sublethal effects of agrochemicals, which often have not been tested for in currently registered pesticides (Mullin *et al*., 2010). In this study, I investigate the effects of consumption of a widely used fungicide, Pristine®, on the morphology and flight performance of chronically exposed honey bees in field conditions.

The pollination services of insects not only contribute significantly to both agricultural (US$14.2–23.8 billion) and industrial sectors (US$10.3–21.1 billion; Chopra *et al*., 2015), but also play vital roles in biodiverse ecosystems (Ollerton *et al*., 2011), the loss of which will undoubtedly have strong negative economic and ecological impacts (Gallai *et al*., 2009; Calderone, 2012). The most widely used managed pollinator is the honey bee (*Apis mellifera*), which is necessary for the pollination of many crops including berries, almonds, pome, and stone fruits. Although honey bees are not considered threatened, North American beekeepers are losing more than 40% of their colonies each year (vanEngelsdorp *et al*., 2007, 2011; Steinhauer *et al*., 2014; Kulhanek

et al., 2017; Bruckner *et al*., 2022), increasing the challenge of keeping up with agricultural demand (Aizen & Harder, 2009).

Exposures to insecticides (e.g., neonicotinoids and phenylpyrazoles) have been implicated as a major contributor to pollinator decline due to their toxicity, high frequency of use, and persistent accumulation in agricultural foraging environments (Iwasa *et al*., 2004; Vidau *et al*., 2011; Smalling *et al*., 2013; Goulson *et al*., 2015; Zhu *et al*., 2015; Tison *et al*., 2016; Fisher *et al*., 2018). Additionally, fungicides are very commonly encountered by pollinators, and there are studies demonstrating correlations between high levels of exposure to fungicides and poor colony health (Mullin *et al*., 2010; Pettis *et al*., 2013). Fungicides are often considered relatively safe for animals, including insect pollinators, due to their high acute contact and oral LD_{50}'s relative to their environmental exposure (Legard *et al*., 2001; Smalling *et al*., 2013; Ostiguy *et al*., 2019). Typically, fungicides are only considered hazardous when paired with other agrochemicals, such as insecticides (Pilling & Jepson, 1993; Pilling *et al*., 1995; Iwasa *et al*., 2004; Johnson *et al*., 2013; Tosi & Neih, 2019). However, little attention has been given to understanding the independent sublethal effects of fungicide exposure on honey bee health (except see: DeGrandi-Hoffman *et al*., 2015; Campbell *et al*., 2016; Liao *et al*., 2019).

Pristine®, a widely used fungicide, is frequently encountered by foraging honey bees, due to its common application on blooming flowers of nut, stone fruit, and fruit crops prior to obligatory bee pollination (Legard *et al*., 2001; Ostiguy *et al*., 2019). Pristine® has two active ingredients, the anilide fungicide, boscalid, and the strobilurin fungicide, pyraclostrobin, both of which inhibit mitochondrial respiration in fungal

targets (constituting 25.2% and 12.8% of the formulated product by mass, respectively; Avenot & Michailides, 2007). The active ingredients of Pristine® have relatively low contact and oral toxicities for bees relative to the concentrations measured in honey bee hives (Ostiguy *et al*., 2019). However, chronic consumption of pollen containing concentrations of Pristine® similar or lower than those measured in pollen sampled from bees foraging in Pristine®-sprayed orchards reduced worker longevity, colony population size, and overwintering survival (Fisher *et al*., 2021). Additionally, Pristine® consumption in pollen at realistic field doses caused earlier foraging and more pollen foraging (Fisher *et al*., 2021).

The mechanisms of Pristine® effects on worker longevity and behavior are unclear. Because the active ingredients of Pristine® are mitochondrial toxins in honey bees (Campbell *et al*., 2016), they may have wide effects. Pristine® has been shown to reduce pollen digestion (DeGrandi-Hoffman *et al*., 2015), and the earlier foraging and greater pollen foraging documented by Fisher *et al*. (2021) suggests that Pristine® may impair digestive or nutritional processes. In support of this hypothesis, pyraclostrobin has recently been shown to damage the midgut epithelia of honey bees when fed to bees in the lab (da Costa Domingues *et al*., 2020; Tadei *et al*., 2020). However, as yet we lack any direct evidence that Pristine® inhibits honey bee growth, size, or nutritional status. As a mitochondrial inhibitor, Pristine® might also be expected to have negative effects on activities requiring high metabolic rates, such as flight. For honey bees, the highest metabolic rates occur during flight while foraging, and these rates increase with the mass of load carried (i.e., nectar, pollen, or water; Wolf *et al*., 1989; Feuerbacher *et al*., 2003). Plausibly, by inhibiting flight muscle mitochondria, Pristine® might reduce the maximal

flight metabolic rates of workers, impairing foraging or the ability to fly during stressful conditions such as windy or cold weather. In support of this hypothesis, honey bee foragers fed sugar water containing boscalid (10 ppm) exhibited lower wing beat frequencies relative to controls when tethered and flown in an indoor flight treadmill (Liao *et al*., 2019). However, one prior study found no effect of consumption of 6.6 ppm Pristine® on metabolic rate during hovering flight of honey bees reared in the lab (Campbell *et al*., 2016). Because hovering flight in normodense air (i.e., 1.288 kg m^{-3}) does not elicit maximal metabolic performance (Roberts *et al*., 2004), it is plausible that Pristine® has negative effects on maximal flight capacities, which were not tested in the Campbell *et al*., (2016) study. A decrease in maximal metabolic performance induced by an agrochemical could have many potential effects on foraging bees, including reducing maximal load carriage or acceleration, capacities to escape predators, or to fly safely in windy conditions (Dillon & Dudley, 2004; Combes & Dudley, 2009; Buchwald & Dudley, 2010).

Unlike terrestrial or aquatic locomotion, during which graded work effort usually can be elicited by utilizing a treadmill (Seeherman *et al*., 1981) or a swim-flume (Norin & Clark, 2016), a difficulty in investigating the physiology of flight is the challenge of assessing maximal sustained performance (Ellington, 1984, 1985; Dudley and Ellington, 1990; Dickinson & Lighton, 1995; Josephson & Ellington, 1997; Chai *et al*., 1998, 1999; Chai & Dudley, 1999; Roberts *et al*., 2004). Increasing the mass of load carried increases flight metabolic rates (Wolf *et al*., 1989; Feuerbacher *et al*., 2003), but such experiments are time-consuming and poorly suited for ecotoxicology studies. Systematically decreasing air density – achieved by replacing nitrogen with helium in

graded steps – provides an analog of a treadmill to measure increased aerobic performance during hovering flight, because lower air density increases power requirements of hovering for all animals yet tested (Chai & Dudley, 1995, 1996; Dudley, 1995; Chai *et al*., 1996; Dudley & Chai, 1996; Dudley & Winter 2002; Roberts *et al*., 2004). For example, carpenter bees (*Xylocopa varipuncta*) exhibited a 33% increase in flight metabolic rate when air density was decreased by ~64%, (Roberts *et al*., 2004). Because Pristine® has been suggested to inhibit protein digestion or absorption, I tested for developmental effects of chronic consumption of Pristine® on thorax and body mass. To test for the effects of Pristine® consumption on flight capacities, I measured flight metabolic rates and flight quality of honey bees induced to fly in a range of air densities, including low air densities that likely require near-maximal flight performance. I tested the effects of two concentrations of Pristine®, 2.3 and 230 ppm, which represent the lowest concentrations and a value an order of magnitude higher than the highest concentration of Pristine® measured in corbicular pollen of bees pollinating sprayed almond orchards (Fisher *et al*., 2021). The Pristine® was administered in pollen to field colonies, simulating the type of exposure experienced if a colony was pollinating an almond orchard sprayed with Pristine®, over a time period encompassing both larval and the young adult development period when pollen is consumed. Specifically, I wished to partially test two hypotheses for the decreased longevity of honey bee workers in colonies fed field-realistic concentrations of Pristine® in pollen (Fisher *et al*., 2021): 1) Pristine® impairs flight metabolic rate and capacity, and 2) Pristine® impairs growth/size of workers.

Methods

Honey bee colony initiation and maintenance

Details of colony maintenance and experimental design are provided in Fisher *et al*. (2021), with a basic description provided here. Colonies of the Italian honey bee (*Apis mellifera ligustica*) were started from a 1.59 kg bee packages (~10,000 bees) obtained from Pendell Apiaries, Inc. in Stonyford, CA (39.376956, −122.558801). To ensure that colonies were not exposed to comb with previous agrochemical content, each hive was initially stocked with five wooden frames outfitted with a plastic worker cell template foundation, so that workers constructed new comb. All hives were supplied with 30% sugar syrup for the first three weeks after their establishment to assist comb building. Hives were also outfitted with internal pollen traps to restrict access to pollen collected in the surrounding environment (see Hoover & Ovinge, 2018). Hives were maintained with 50 g pollen patties, using pollen collected from desert hives far from agriculture. The pollen patties consisted of a 1:1:1 ratio of dry pollen, sucrose (Great Value) and fondant sugar (ABC Cake Decorating, Phoenix, AZ; 8% inverted); approximately 8% of each pollen patty consisted of deionized H_2O which was added after the dry ingredients were thoroughly mixed. To document the extent to which hives were exposed to other pesticides, I collected bee bread samples from each hive, pooled these into single samples for each treatment, and had these analyzed by the USDA-AMS National Science Laboratory. Pesticide residue analyses found no agrochemicals present above detection levels other than a few herbicides: diuron, fluometuron, and hexazinone that occurred in levels up to 12 ppb. The hives were treated with Amitraz for mites in the month before

our experiments, as is common in U.S. beekeeping, and a metabolite (DMPF) of amitraz was detected at 147 ppb. None of these levels differed among treatments.

Fungicide treatment and dose

The complete experimental design is described in Fisher *et al*. (2021); here I briefly describe the protocols. Doses were based on measurements of the concentrations of boscalid and pyraclostrobin in pollen sampled from bees foraging in California almond orchards in 2010 and 2011 (Fisher *et al*., 2021). Pollen was collected from bees throughout the blooming period, and thus measured levels estimate the average, rather than maximal or minimal values of fungicide which likely vary with time after spray. These measures suggested that bees pollinating almond orchards collect pollen containing 3–24 ppm Pristine® (Fisher *et al*., 2021). To feed colonies specified doses of fungicide, I mixed Pristine® (BASF Corporation, Research Triangle Park, NC) into pollen patties which were fed to colonies equipped with pollen excluders to force the bees to consume the Pristine®-containing pollen. Colonies were reared on these treated pollen patties from May 2018 to November 2019. For this experiment, nine colonies were fed pollen patties containing 0, 2.3 or 230 ppm Pristine®, for a total of three colonies per treatment. All pollen patties were provided *ad libitum*, with a new patty supplied as soon as the previous patty was entirely consumed. If the pollen patty was not completely consumed within one week, it was replaced to maintain freshness. Pollen patties were weighed each week to measure weekly pollen and Pristine® consumption for each hive. To calculate per bee dose from pollen patty consumption, I assessed the number of colony pupal and larval cells and workers during the study for each hive, and used literature values for per larva

and per worker pollen consumption (details in Fisher *et al*., 2021). Bees consume pollen during the latter larval and young adult stage, and cease pollen consumption after initiation of foraging, so age of the forager tested likely did not affect Pristine® dose. The per larvae and per adult doses for each treatment group of Pristine® and the active ingredients, boscalid and pyraclostrobin, are provided in Table 2.1.

Table 2.1. Concentrations of Pristine®, boscalid, and pyraclostrobin in the pollen patties provided to honey bee hives, and the per larva and per adult dose of each compound in the two Pristine® treatments used. Dose calculations are from Fisher *et al*., 2020.

	Pristine®	**Boscalid**	**Pyraclostrobin**
Pollen patty, ppm	**2.3**	**0.6**	**0.3**
Per larva dose, ng	1.0	0.25	0.13
Per adult dose, ng	79.7	20.1	10.2
Pollen patty, ppm	**230**	**60**	**30**
Per larva dose, ng	89.9	22.7	11.5
Per adult dose, ng	7,194	1813	921

Outgoing forager collection

To test for the effects of Pristine® consumption on flight capacities, I measured flight metabolic rates and flight quality of honey bees from three colonies of three of the five treatment groups used in the Fisher *et al*., (2021) study (i.e., 0, 2.3, and 230 ppm; total $N = 9$ hives). Beginning in November 2019, outgoing foragers (Control: $n = 90$; 2.3 ppm: $n = 82$; 230 ppm: $n = 83$) were captured when leaving the colony (between 900

and 1700) by holding an opened plastic bag (~950 ml) approximately 15 cm from the colony entrance. After a single forager flew directly into the opened bag, it was sealed, and the bee was transported within 2 min to a temperature-controlled laboratory room, where air temperature was regulated by a space-heater (36.5 ± 0.5 °C) and using a thermocouple and Expedata (Sable Systems, Las Vegas, NV). Bees were measured immediately after being transported into the laboratory (see below). To control for extraneous possible effects, a random number generator (www.randomizer.org) was used to determine the order and time in which the colonies were sampled.

Measuring flight metabolic rate, flight muscle temperatures and flight behavior at three air densities

Once in the lab, the collected bee was immediately placed into a cylindrical, transparent acrylic flight chamber (350 ml). The flight chamber was sealed and covered with a dark cloth for 2 min, to encourage reduced activity of the bee. The gases from the flow meters delivered air (2 L min^{-1}) sequentially and continuously through a $CaSO_4$ and soda lime column to remove H_2O and CO_2, the reference cell of the LI-COR 6262 CO_2/H_2O analyzer (Lincoln, NE, USA), the flight chamber, a small column of $MgSO_4$ (to remove metabolic water), and the sample cell of the LI-COR. Differential analog output from the LI-COR was digitized (Sable Systems UI-2) and recorded each second (Expedata, Sable Systems, Las Vegas, NV). The LI-COR was calibrated using 252 ppm CO_2 and Ultra-Zero calibration gases, and baseline recordings were taken before and after each measurement period by bypassing the flight chamber.

Foragers were randomly assigned to one of three variable-density gas mixtures [0.441 kg m^{-3} ('heliox'), 0.779 kg m^{-3} ('intermediate'), or 1.288 kg m^{-3} ('normodense') for their flight metabolic rate measures; Table 2.2]. Gas mixtures were created by using cylinders of pure O_2, N_2, and He, which were regulated by a Sable Systems FB8 flow meter (Las Vegas, NV, USA) specifically calibrated for the different gas densities using a soap-film bubble meter (Levy, 1964). The different gas mixtures did not affect the calibration of the LI-COR 6262 CO_2/H_2O analyzer (Lincoln, NE, USA).

Table 2.2. Variable-density gas mixtures used as an aerial treadmill

Gas mixture	% O_2	% N_2	% He	Density ($kg \cdot m^{-3}$)
1	21	79	0	1.288
2	21	31.6	47.4	0.780
3	21	0	79	0.441

While the bee sat in darkness, I flushed the chamber for 2 min prior to the flight trial, allowing CO_2 levels from the chamber to reach a low, stable level. Hovering flight was encouraged for 2 min by shining a 150W dual goose-neck Fiber Optical Illuminator (China) over the chamber. Bees that landed were immediately encouraged to fly or attempt to fly by gently tapping and inverting the chamber. Flight behavior was categorized based on ability, duration, and control (i.e., quality). Flight quality was categorized and ranked as: 1 – no flight, 2 – flapping wings with very brief periods of flight (< 3 s), 3 – intermittent hovering characterized by frequent crashing (i.e., bee usually ends upside down), 4 – intermittent hovering characterized by frequent

controlled landing (i.e., bee gently lands on its feet), or 5 – continual, stable hovering. The zenith duration in Expedata (Sable Systems, Las Vegas, NV) was used to locate and average the 10 s with the highest CO_2 readings during each trial. Flight CO_2 emission rates ($ml \cdot hr^{-1}$) during that highest CO_2 emission period were calculated by multiplying the differential CO_2 fraction by the STP flow rate in through the flight chamber. These values were later converted to milliwatts ($mJ\ sec^{-1}$) by converting the time units, then converting these values to joules (Lighton, 2018), assuming a respiratory quotient of 1 (Rothe & Nachtigall, 1989; Feuerbacher *et al*., 2003). After flight and CO_2 emission rates were measured, the bee was immediately shaken into a plastic bag, which was flattened to restrict the bee's movement. Flight muscle temperature was measured by inserting a Physitemp model MT29/1 hypodermic microprobe (Clifton, New Jersey, USA; 29-gauge, time constant = 0.025 s) through the bag and into the center of the thorax. Temperatures were recorded with a Pico Technology USB TC-08 Thermocouple Data Logger (Tyler, TX, USA). Flight muscle temperatures were measured within 3 s of cessation of flight, and the highest temperature reported by the thermometer was recorded. After measurement, the bee was weighed (±0.1 mg) using an A&D HR-60 Analytical Balance (Tokyo, Japan) and stored at −20 °C. Thorax masses were measured by dissecting the head and abdomen from the thorax and taking its mass. The wings and legs were included in the mass of the thorax to avoid inconsistencies of appendage removal.

Statistical analysis

Data were tested for normality and analyzed using R (3.6.2; R Foundation for Statistical Computing, Vienna, Austria). Two-tailed significance was determined at $\alpha = 0.05$. I used

linear mixed-effects models to test the independent and interactive effects of Pristine® treatment, gas density, and thorax and body mass on flight metabolic rate (milliwatts) and flight muscle temperature. To investigate the effects of the different treatments flown in heliox (0.441 kg m^{-3}; Fig. 2.2A), I used a linear model with a Bonferroni-corrected *post hoc* test. I also used linear mixed-effects models to investigate the effects of Pristine® treatment on the body, head, thorax, and abdominal masses of foraging bees. Linear models were used to investigate the relationship between log-transformed body and thorax masses, as well as the relationship between metabolic rate and thorax mass. I used an ordinal logistic regression model analysis to test the effects of our treatment variables on flight behavior. Foragers heavier than 0.1 g were excluded from these analyses, as these individuals were likely returning or new foragers that had not evacuated their hindguts, as they had large crop and hindgut loads when dissected.

Results

Pristine® consumption significantly reduced thorax masses by approximately 5% (treatment: linear mixed-effects model, $n = 218$, $\chi^2 = 24.85$, $P < 0.0001$; Fig. 2.1), and significantly decreased thorax:body mass ratios (treatment: linear mixed-effects model, $n = 218$, $\chi^2 = 7.14$, $P = 0.008$). Pristine® had no significant effect on body mass (treatment: linear mixed-effects model, $n = 218$, $\chi^2 = 0.14$, $P = 0.71$). Plots of log thorax mass vs. log body mass scaled hypometrically (*t*-test: $n = 218$, $t = 10.11$, $P < 0.0001$), meaning that bees with a heavier body mass had relatively smaller thoraxes ($n = 218$, slope: 0.42, $R^2 = 0.32$).

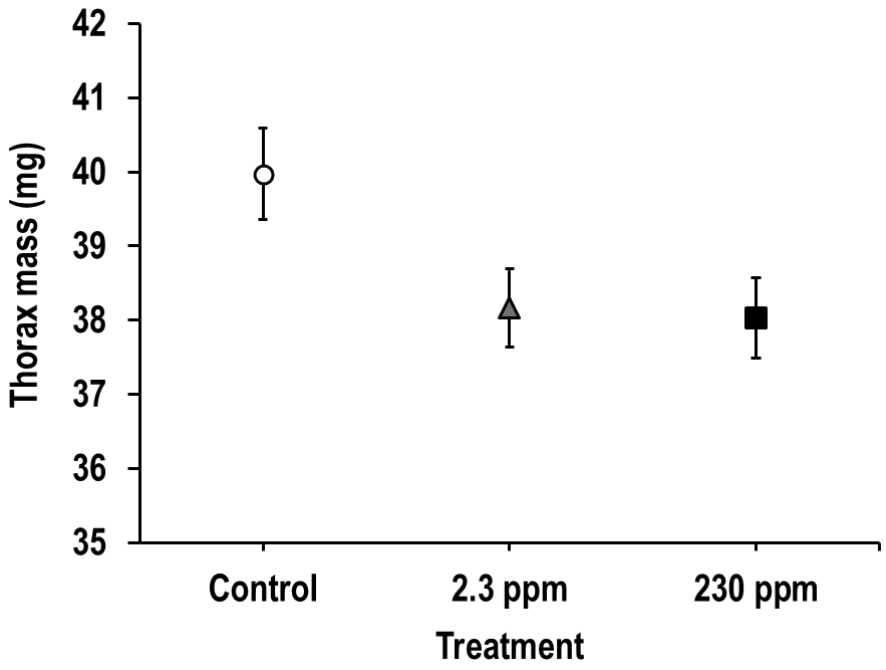

Figure 2.1. Chronic consumption of pollen containing 2.3 ppm or 230 ppm Pristine® reduced thorax masses (linear mixed-effects model: $P < 0.0001$). Each point and accompanying error bars represent the mean±95% CL.

Flight metabolic rates increased with increasing thorax mass and decreasing gas density, with thorax mass becoming less important as gas density decreased (Fig. 2.2; Table 2.3). Similar results were found when body mass was tested as a predictor of flight metabolic rate. Bees with heavier body masses also had significantly higher thorax temperatures relative to lighter bees flying in all gas densities (linear mixed-effects model: $n = 218$, $\chi^2 = 9.19$, $P = 0.002$).

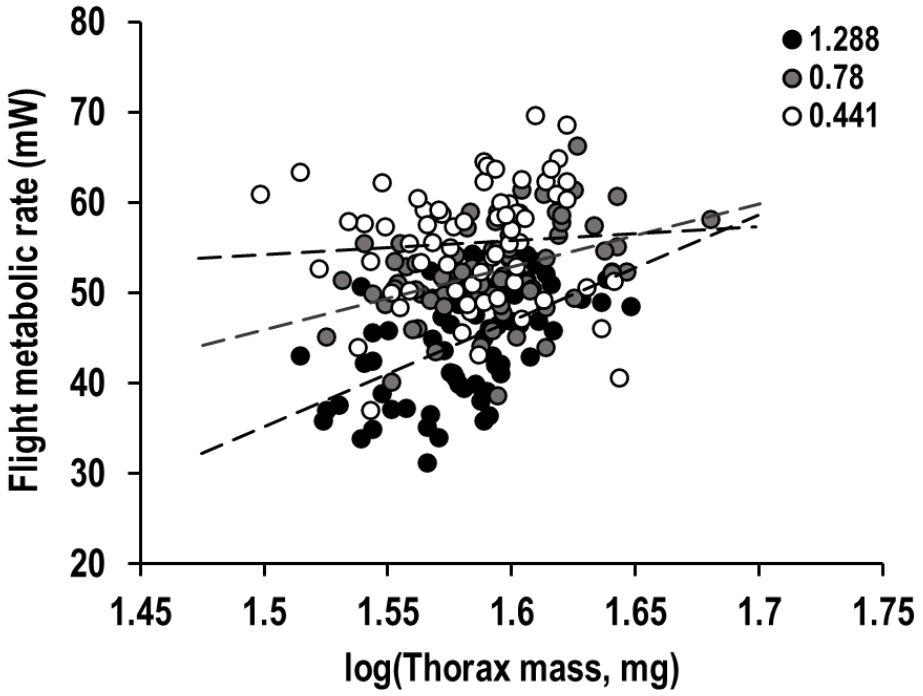

Figure 2.2. Flight metabolic rates increased with thorax mass and in lower density air ($kg \cdot m^{-3}$) in an interactive manner, such that the slope of flight metabolic rate on thorax mass declined at lower densities (linear mixed-effects model: $P = 0.0002$). Each point represents an individually measured bee.

Table 2.3. Linear mixed-effects model results for the independent and interactive effects of gas density ($kg \cdot m^{-3}$), treatment (control, 2.3 ppm, and 230 ppm), and thorax mass (mg) on the flight metabolic rates of honey bees at 36°C.

Variable(s)	χ^2	*P*
Gas density	**137.8**	**< 0.0001 *****
Treatment	0.1	0.76
Thorax mass	**41.6**	**< 0.0001 *****
Gas density x Treatment	**13.7**	**< 0.001 *****
Gas density x Thorax mass	**14.2**	**< 0.001 *****
Treatment x Thorax mass	0.9	0.34
Gas density x Treatment x Body mass	1	0.32

Bees increasingly struggled to fly at the lowest air densities, as shown by the decrease in flight quality score (gas density: ordinal logistic regression, $n = 218$, $t = -3.86$, $P < 0.001$; Fig. 2.3). Consumption of fungicide-treated pollen (the 2.3 and 230 ppm treatments combined) reduced the capacity of bees to fly in low-density air (Fig. 2.3, treatment: ordinal logistic regression model, $n = 218$, $t = -2.05$, $P = 0.04$), but the fungicide treatment effect was not significant if the 230-ppm treatment was excluded (treatment: ordinal logistic regression model, $n = 144$, $t = -1.03$, $P = 0.31$).

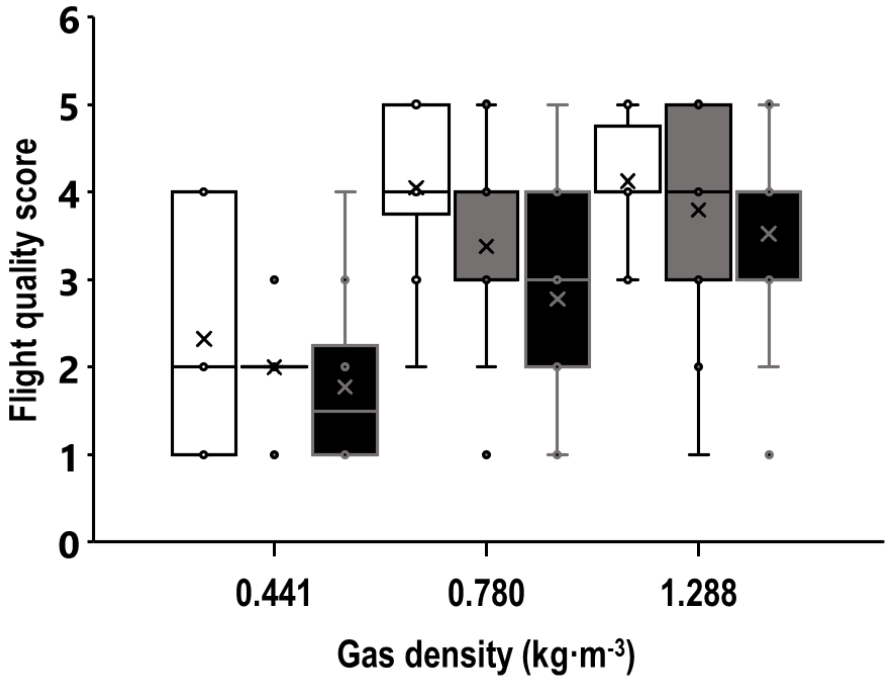

Figure 2.3. Flight quality scores (ranked from 1 = no flight to 5 = stable, continuous hovering) declined in low-density air and for bees fed Pristine®. However, the Pristine® effects on flight quality were only significant if the 230-ppm treatment group was included (ordinal logistic regression: $P < 0.001$). Open, grey, and black boxes represent control, 2.3 ppm, and 230 ppm treatments, respectively. The 'x', solid bar, lower bar, bottom box, top box, and upper bar represent the mean, median,1st quartile, 2nd quartile, 3rd quartile, and 4th quartile, respectively. Note that most bees in the 2.3 ppm-treatment group in the lowest density air displayed one behavior (flight quality: 2), which is why the quartiles are not visible.

For the control bees, flight metabolic rate increased ~1.3-fold with decreasing air density; however, fungicide treated bees were less able to increase flight metabolic rate as air density was decreased (treatment x gas density: linear mixed-effects model, $n = 218$, $\chi^2 = 13.68$, $P = 0.0002$; Fig. 2.4A). Pristine® consumption significantly suppressed the flight metabolic rates of bees flown in heliox (i.e., 0.441 kg m^{-3}; treatment: linear model, $n = 67$, $F = 4.98$, $P = 0.0097$), but not in normodense air (i.e., 1.288 kg m^{-3}; treatment: linear model, $n = 73$, $F = 0.67$, $P = 0.52$, Fig. 2.4A). However,

there was no significant effect of Pristine® on flight metabolic rates when bees from the 230-ppm treatment were excluded from the model (treatment: linear model, $n = 144$, $F = 0.81$, $P = 0.37$; Fig. 2.4A). Maximal metabolic rate of all honey bees during hovering in heliox averaged 56.52 ± 1.85 mJ s^{-1} (mean ± 95% CL; $n = 50$), significantly higher than in the intermediate density air (52.44 ± 1.14 mJ s^{-1}; $n = 75$) and higher than during hovering in normodense air (45.15 ± 1.42 mJ s^{-1}; $n = 71$; linear mixed-effects model: $\chi^2 = 128.44$, $P < 0.0001$; Fig. 2.4A). Thorax temperatures decreased by about 1.5 °C with decreasing gas density (gas density: linear mixed-effects model, $n = 218$, $\chi^2 = 113$, $P < 0.0001$; Fig. 2.4B), but there was no significant effect of Pristine® treatment.

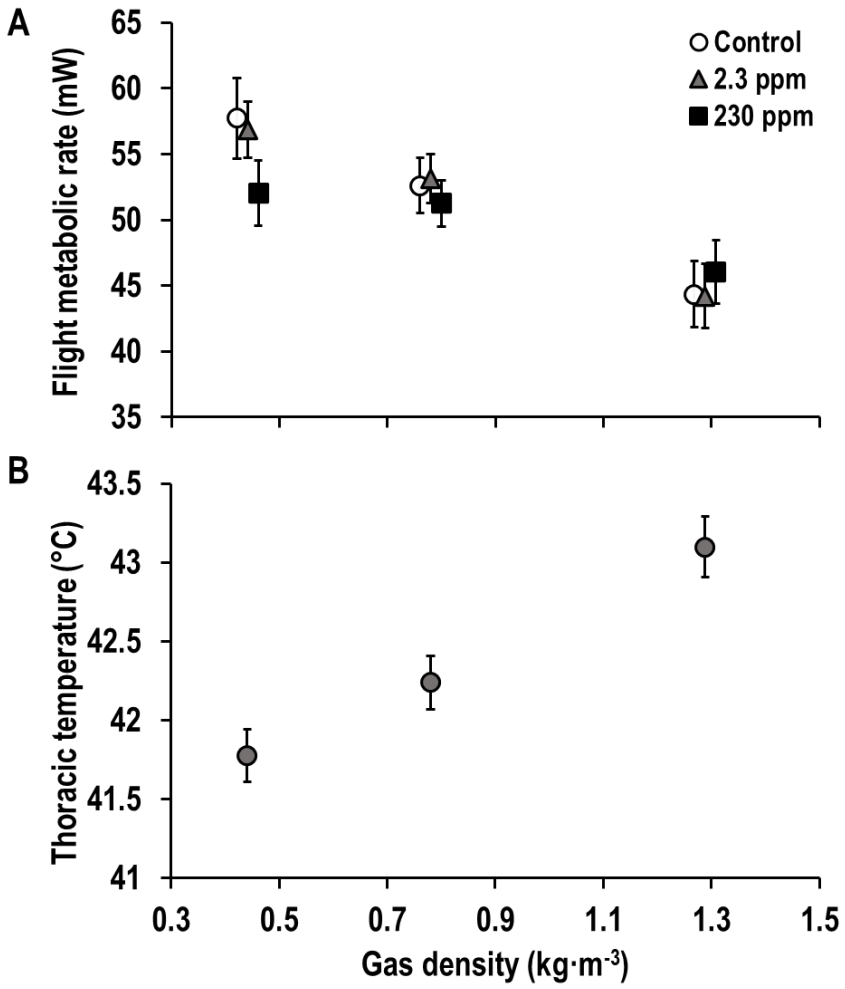

Figure 2.4. (A) Relationship between gas density and flight metabolic rate in honey bees fed pollen with 2.3 ppm or 230 ppm of Pristine® fungicide in pollen (linear mixed-effects model: $P < 0.001$). Control bees were fed pollen without fungicide. Points are staggered for clarity. (B) The independent effect of gas density on the thorax temperature of honey bee foragers during flight at 36°C air temperature (linear mixed effects model: $P < 0.0001$). Pristine® treatment did not significantly affect thorax temperatures. Grey circles denote bees pooled from all treatments. All symbols and accompanying error bars represent the mean±95% CL.

Discussion

Pristine® consumption by honey bee colonies at field-realistic doses decreased thorax masses (Fig. 2.1) and thorax:body mass ratio, but did not affect flight metabolic rates (Fig. 2.4) or the ability of honey bees to fly in low density air (Fig. 2.3). These data suggest that the reduced longevity of honey bees in colonies fed field-realistic doses of Pristine® are likely not due to poisoning of the flight muscle mitochondria. However, the reduced thorax masses provide the first morphological evidence for the hypothesis that consumption of field-realistic doses of Pristine® impairs growth or nutritional status by impairing digestive or absorptive processes.

The conclusion that consumption of field-realistic doses of Pristine® does not impair flight capacities is an important finding given that a mitochondrial toxin might be expected to have its greatest effect when metabolic rates are high, as occurs during flight. While we currently lack data on hemolymph and tissue concentration of boscalid and pyraclostrobin for bees consuming these doses of Pristine®, DeGrandi-Hoffman *et al*. (2013) showed that tissue levels of bees feeding on pollen with a higher dose of Pristine® had whole body concentrations of boscalid and pyraclostrobin that were less than 5% of those measured in pollen. Together these data suggest that the active ingredients of Pristine® either do not readily cross the gut wall or are effectively metabolized. However, an important caveat is that concentrations of Pristine® in pollen have been found to be as high as 24 ppm (Fisher *et al*., 2021), which corresponds fairly closely to the 10 ppm concentration of boscalid shown to inhibit wing beat frequencies of honey bees on a flight mill (Liao *et al*., 2019). Consumption of the supra-field-realistic dose of Pristine® (230 ppm) clearly suppressed flight metabolic rate and the capacity to hover in low-

density air (Fig. 2.3, 2.4). Given that maximal metabolic capacity is strongly linked to maximal physical performance in animals including bees (Wolf *et al*., 1989; Roberts *et al*., 2004; Weibel & Hoppeler, 2005), these data strongly suggest that sufficient Pristine® consumption will reduce the capacities of honey bees to carry loads and fly in severe weather. Future studies should examine effects of these higher concentrations of Pristine® to ensure that field exposures do not negatively impact honey bee flight capacities.

The helium-oxygen mixtures were effective in eliciting higher metabolic rates and for demonstrating effects of pesticide on flight and metabolic function (Fig. 2.1, 2.4A). Flight metabolic rates of honey bees increased by ~36% as air density decreased by ~64% (Fig. 2.4A). The increase in flight metabolic rate I documented in heliox is similar to that shown for bees carrying near-maximal nectar loads (i.e., ~44%; Wolf *et al*., 1989), suggesting that I measured near-maximal flight metabolic rates. Lower air densities also reduced thorax temperatures of flying bees (Fig. 2.4B), likely due to the higher thermal conductance of helium than nitrogen (Reid *et al*., 1987). However, thorax temperatures of all bees were high (over 40 °C) relative to the thermal performance curve for honey bees (Coehlo, 1991), suggesting that this thoracic cooling did not limit metabolic performance. Helium will also increase the diffusivity of oxygen by 2.6-fold (Lide, 2004) in addition to lowering air density, potentially leading to increases in the partial pressure of oxygen at the tissue level. Two hours of exposure to 20% oxygen–80% helium caused mitochondrial swelling of rat myocardial tissue, raising concerns about the toxicity of these treatments (Ślubowski *et al*., 1987). However, there are multiple reasons to suspect that the heliox exposure during flight did not produce a serious physiological

problem in honey bees. First, helium only affects diffusive, not convective transport of oxygen. Bees and other flying insects are known to heavily utilize convection for gas exchange during flight, based on observations of abdominal pumping (Weis-Fogh, 1967), and the fact that the critical PO_2 for flight metabolic rate is similar when PO_2 is changed by altering the fractional content of O_2 in N_2, and when the PO_2 is reduced by lowering barometric pressure (Withers, 1981; Joos *et al*., 1997). If diffusion through the gas-filled tracheae is the major mechanism of oxygen transport during honey bee flight, then lowering barometric pressure should have little effect on oxygen delivery or metabolic rate. Second, unlike most mammals, insects including bees experience substantial variation in tissue PO_2, ranging routinely between 2 and 3 kPa up to near 20 kPa (Komai, 2001; Harrison *et al*., 2020). This reduces the likelihood that a 2-min exposure of tissues to PO_2 levels up to 2.6-fold higher would cause damage. Third, if heliox mixtures damage mitochondria, I would expect to see either an elevation of CO_2 emission rates (due to mitochondrial uncoupling) or a decrease in CO_2 emission rates (due to damage). However, CO_2 emission rates rose to high levels during flight in heliox and fell quickly to resting levels after flight, suggesting that the observed elevation in CO_2 emission rates was completely due to flight and that there was no mitochondrial damage. Fourth, in carpenter bees, the increase in metabolic rates during flight in heliox are proportional to the increase in mechanical power output of the wings (Roberts *et al*., 2004), again suggesting that the mitochondria are undamaged by this treatment. Finally, since all treatment groups experienced the same exposures to helium, even if there is some damage associated with heliox exposure, this is unlikely to change our conclusions regarding Pristine® treatments.

I found a strong effect of chronic ingestion of both 2.3 and 230 ppm Pristine® on the thorax, but not body masses, of foraging adults (Figs. 2.1,2.2), providing important morphological support for the hypothesis that Pristine® impairs honey bee growth. Because earlier foraging can be induced by colonial nutritional stress, and because early foraging is often linked to reduced longevity in honey bee foragers (Rueppell *et al*., 2007), it is plausible that effects of Pristine® on digestive function are responsible for the effects of this pesticide on worker survival (Fisher *et al*., 2021). This hypothesis is further supported by evidence for poor protein digestion by bees fed Pristine® (DeGrandi-Hoffmann *et al*., 2015), and by recent evidence that pyraclostrobin damages the honey bee midgut (da Costa Domingues *et al*., 2020; Tadei *et al*., 2020). Future studies should comprehensively test for effects of Pristine® and its ingredients on digestion, absorption, nutritional status, growth, and size.

In this study, colonies consumed Pristine®-containing pollen for multiple months, whereas in agricultural conditions this is unlikely, raising the concern that though the concentrations of pesticide in pollen were field-realistic (Fisher *et al*., 2021), that the duration of exposure was not. However, it seems unlikely that this affects the magnitude of exposure. As outlined in Fisher *et al*. (2021), bees consume approximately 60 mg of pollen during the larval and adult development. As long as the exposure exceeds 3–4 weeks (the duration of honey bee development), bees developing during the exposure will consume similar amounts of pesticide in pollen. It is true that chronic exposure of the hive provides the potential for additional cuticular exposure, as Pristine® ingredients may accumulate in the wax. However, prior toxicological studies have shown that such cuticular exposures are not toxic except at orders of magnitude higher doses (Ostiguy

et al., 2019). It is also plausible that chronic exposure to Pristine® has other effects on the hive, such as alterations in the various hive microbiomes. Future studies should examine the effects of shorter durations of exposure to Pristine® on field hives, and whether such indirect mechanisms of toxicity exist.

Conclusion

When honey bee colonies consume pollen containing field-realistic doses of Pristine® fungicide, worker longevity decreases (Fisher *et al*., 2021). Here I demonstrated that it is unlikely that the effects of Pristine® consumption on survival arise predominantly from impairment of flight capacity, as might be expected since the active ingredients of Pristine® are mitochondrial toxins and the highest metabolic rates occur during flight. However, Pristine® consumption reduced thorax mass, providing further support for the hypothesis that Pristine® affects digestive and nutritional physiology, impairing growth.

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CHAPTER 3

THE THERMAL PERFORMANCE CURVE FOR AEROBIC METABOLISM IN A FLYING ENDOTHERM

THIS CHAPTER HAS BEEN PUBLISHED IN *PROCEEDINGS OF THE ROYAL SOCIETY: B*

SEE APPENDIX B

Abstract

Performance benefits of stable, warm muscles are believed to be important for the evolution of endothermy in mammals, birds and flying insects. However, thermal performance curves have never been measured for a free-flying endotherm, as it is challenging to vary body temperatures of these animals, and maximal flight performance is difficult to elicit. I varied air temperatures and gas densities to manipulate thoracic temperatures of flying honeybees from 29°C to 44°C, with low air densities used to increase flight metabolic rates to maximal values. Honeybees showed a clear thermal performance curve with an optimal temperature of 39°C. Maximal flight metabolic rates increased by approximately 2% per 1°C increase in thoracic temperature at suboptimal thoracic temperatures, but decreased approximately 5% per 1°C increase as the bees continued to heat up. This study provides the first quantification of the maximal metabolic performance benefit of thermoregulation in an endotherm. These data directly support aerobic capacity models for benefits of thermoregulation in honeybees, and suggest that improved aerobic capacity probably contributes to the multiple origins of endothermic heterothermy in bees and other insects.

Introduction

Why do some animals—including mammals, birds, and some fish and flying insects—thermoregulate at high body temperatures? While there are multiple ultimate hypotheses for the evolution of endothermy (defined as occurring when metabolism generates sufficient heat to significantly raise body temperature above ambient), each hypothesis agrees upon the fact that temperature has a hump-shaped effect on muscle and animal performance. This effect shows performance initially increasing slowly with temperature up to an optimum, and temperatures above this point suppressing performance (Angilletta, 2009; Somero *et al*., 2017. Such patterns, called thermal performance curves, quantitatively define the benefits of maintaining body temperature near optimal, and are well documented for ectotherms (Huey & Stevenson, 1979; Huey & Kingsolver, 1989; Somero *et al*., 2017). Even though *in vitro* and *in situ* physiological studies have shown that the muscular and nervous system of endotherms are affected by temperature, only a few studies of running mammals have quantified the effects of body temperature on whole-body performance in endotherms, and none have measured a broad enough range of temperatures to provide a thermal performance curve (Huey & Kingsolver, 1989; Bennet, 1990; Ranatunga, 1998; Wooden & Walsberg, 2004; Rojas *et al*., 2012). The lack of thermal performance curves for endotherms makes it difficult to define the performance benefits of endothermic homeothermy and to assess the impact of climatic changes that force animals away from their optimal temperature (Levesque & Marshall, 2021). Heterotherms, defined as animals that exhibit substantial variation in body temperature even when active, offer experimental possibilities for assessing thermal performance curves of endotherms, as these animals can function across a relatively

broad range of body temperatures. In this study, I manipulated air temperature and gas density to determine the thermal performance curve of flight metabolic rates and to quantify the benefits of thermoregulation for maximal metabolic performance of the Italian honeybee (*Apis mellifera ligustica*).

The ability to maintain relatively high body temperatures gives several possible advantages to endothermic animals, flying insects included. For example, endothermic homeothermy facilitates success in a broader range of thermal niches, such as improving locomotory performance in cool environments (Block *et al*., 1993), and increasing development rates of offspring (Farmer, 2000). The maintenance of high body temperatures also facilitates high aerobic capacity, muscular power output, and sustained activity (Clark & Pörtner, 2010). Insects, and some vertebrate endotherms, can save energy relative to homeothermic endotherms by allowing body temperatures to decrease under some circumstances, especially when not flying. These facultative endotherms benefit from higher aerobic performance during flight, while their heterothermy reduces overall costs over periods of flight alternating with non-flight. However, there are some disadvantages to endothermy. To support higher rates of metabolic functions, endothermic animals need to eat large quantities of food to meet energetic demands, compared with the intake of similarly sized ectotherms. Moreover, many endothermic animals often experience neurological and muscular pathologies if core body temperatures stray from optimal (Somero *et al*., 2017). The specific selective forces and morphological requirements for the evolution of endothermy remain controversial, partly due to an incomplete fossil record, and partly due to challenges in quantifying the costs and benefits of endothermy (Lovegrove, 2019).

Endothermic flying insects, such as honeybees, bumblebees, dragonflies, and some beetles and moths, are able to fly over a wide range of air temperatures (Heinrich, 2013). In all cases, endothermy is made possible by the high metabolic heat production of the flight muscles. These animals primarily regulate the temperature of the thorax, but thermoregulation is imperfect (Heinrich, 2013). Insect endothermic fliers thermoregulate using a variety of behavioral and physiological mechanisms, including varying evaporative cooling, heat transfer between the thorax and abdomen, and metabolic heat production [Roberts & Harrison, 1999; Heinrich, 2013]. Honeybees have moderate capacities to thermoregulate, with slopes of thoracic temperature on air temperature being reported as 0.18–0.41 (Harrison *et al*., 1996; Roberts & Harrison, 1999; Woods *et al*., 2005). The capacity of honeybees to fly at a wide range of air and flight muscle temperatures makes them an excellent species for assessment of their thermal performance curve.

Measurement of a thermal performance curve requires both variation in body temperature and assessment of maximal performance. For flying insects, maximal performance has been assessed with either load-lifting; flying in graded, low-density gases; or by varying optomotor stimulus (Lehmann, 2001; Dillon & Dudley, 2004; Roberts *et al*., 2004). Such studies have generally found that flight metabolic rate increases linearly with load, lower density air or greater optimotor stimulus (i.e. increasing virtual reality flight stimulation), with maximal metabolic power or mechanical power output values 25–40% higher than measured during unloaded, hovering flight (Lehmann, 2001; Dillon & Dudley, 2004; Roberts *et al*., 2004).

While it is well known that low-density gases increase heat loss rates (Leon & Cook, 1960), no prior studies have used variation in gas density and air temperature to independently manipulate body temperatures and flight power requirements. I hypothesize that the metabolic rates of flying honeybees exhibit a thermal performance curve, with substantial metabolic benefits to thermoregulation at cooler air temperatures, and suppression of metabolic performance at temperatures above optimal.

Methods

I manipulated body temperatures and assessed maximal capacities of bees by flying them in various air densities and temperatures (Tables 3.1 and 3.2). Foragers were collected in random order from three colonies of the Italian honeybee, *Apis mellifera ligustica*, maintained on the third-story balcony of the Interdisciplinary Science and Technology Building 1 at Arizona State University in Tempe, AZ, USA. Unloaded, outgoing foragers were captured when leaving the colony by holding an opened plastic bag (approx. 950 ml) approximately 15 centimeters from the colony entrance. After a single forager flew directly into the opened bag, it was sealed and the bee was quickly transported to a room regulated at 23 ± 0.5°C or 35 ± 0.5°C (EGC, Chagrin Falls, OH, USA) and its flight metabolism was assessed at a single air density.

Table 3.1. Variable-density gas mixtures used as an aerial treadmill at 23°C.

Gas mixture	% O_2	% N_2	% He	Density ($kg \cdot m^{-3}$)
1	21	79	0	1.288
2	21	69.5	9.5	1.186
3	21	60	19	1.084
4	21	50.5	28.5	0.983
5	21	41	38	0.881
6	21	31.5	47.5	0.779

Table 3.2. Variable-density gas mixtures used as an aerial treadmill at 35°C.

Gas mixture	% O_2	% N_2	% He	Density ($kg \cdot m^{-3}$)
1	21	79	0	1.288
2	21	63.2	15.8	1.119
3	21	47.4	31.6	0.949
4	21	31.6	47.4	0.780
5	21	15.8	63.2	0.610
6	21	0	79	0.441

Substituting helium for nitrogen in air lowers its density, requiring bees to generate more lift in order to fly (Dudley, 1995; Roberts *et al*., 2004). This substitution will also increase heat loss rates because helium has a thermal conductivity about six-times higher than nitrogen (Leon & Cook, 1960; Rosenmann & Morrison, 1974; Smith & Dawson, 1985; Glass *et al*., 2021). To further manipulate heat loss, I examined metabolic rates and body temperatures at two air temperatures, 23°C and 35°C. Heat loss rates are

proportional to the thermal gradient between an animal's body and ambient temperature. Thus, I predicted that flight in gases enriched in helium at low air temperatures would induce the greatest heat loss rates and therefore the coolest body temperatures, whereas heat loss would be lowest in nitrox mixtures at 35°C air temperatures.

Respirometry Experiments

Metabolism during free flight was assessed in a cylindrical, transparent acrylic flight chamber (350 ml). After placing the bee in the chamber, it was sealed and covered with a dark cloth to encourage reduced activity of the bee and the chamber was flushed to completely replace atmospheric air and water with the desired gas mixture. Gas mixtures were created by using cylinders of pure O_2, N_2, and He, which were regulated at a total flow rate of 2 l min^{-1} by a multi-channeled Sable Systems FB8 flow meter system (Las Vegas, NV, USA). Each flow meter was calibrated for the different gas densities using a soap-film bubble meter. The gases from the flow meters flowed sequentially through a $CaSO_4$ and soda lime column to remove H_2O and CO_2, the reference cell of a LI-COR 6262 CO_2/H_2O analyzer (Lincoln, NE, USA), the respirometry chamber, a small column of $MgSO_4$ to remove water produced by the bee, and then the sample cell of the LI-COR. Differential analogue output from the LI-COR was digitized (Sable Systems UI-2) and recorded each second (Expedata, Sable Systems, Las Vegas, NV). The LI-COR was calibrated using 252 ppm CO_2 and Ultra-Zero calibration gases at the same flow rate and pressure (761.5–761.8 mm Hg) as during the flight respirometry, and baseline recordings were taken before and after each measurement period.

Flight Quality

Flight was then encouraged for 2 min by shining a 150 W dual goose-neck Fiber Optical Illuminator (China) over the chamber. Bees that landed were immediately encouraged to fly by gently tapping and inverting the chamber. Flight behaviour was categorized based on ability, duration and control (i.e., quality; Glass *et al*., 2021). Flight was categorized and ranked as: 1, no flight; 2, flapping wings with brief periods of flight (less than 3 s); 3, intermittent flight characterized by frequent crashing (i.e., bee usually ends upside down); 4, intermittent flight characterized by frequent controlled landing (i.e. bee gently lands on its feet); or 5, continual, stable flight. Expedata (Sable Systems, Las Vegas, NV) was used to find and average the 10 s with the highest CO_2 readings during each trial. Flight CO_2 emission rates (ml h^{-1}) were calculated by multiplying the decimal CO_2 fraction times the STP flow rate through the flight chamber. After flight CO_2 emission rates were measured, the bee was shaken into a plastic bag, which was flattened to restrict the bee's movement. Thoracic temperature was then measured by inserting a Physitemp model MT29/1 hypodermic microprobe (Clifton, NJ, USA; 29-gauge, time constant = 0.025 s) through the bag and into the center of the thorax. The temperature data were recorded with a Pico Technology USB TC-08 Thermocouple Data Logger (Tyler, TX, USA). Thoracic temperatures were measured within 5 s of cessation of flight, and the highest temperature reported by the thermometer was recorded. After measurement, the bee was weighed (±0.1 mg) using an A&D HR-120 Analytical Balance (Tokyo, Japan) and stored at −20°C.

Statistical Analysis

Data were analysed using R (3.6.2; R Foundation for Statistical Computing, Vienna, Austria). Two-tailed significance was determined at $\alpha = 0.05$. I used a linear mixed-effects model to test the independent and interactive effects of air temperature and gas density on flight metabolic rate (i.e., milliwatts (mJ s^{-1})) and thoracic temperature, with hive included as a random effect. To determine the independent effect of gas density on thorax temperature, I ran a linear model for each separate air temperature. I also ran a similar model for the above independent variables, with body mass included in the model. I used an ordinal logistic regression model analysis to test the independent and interactive effects of air temperature, gas density, and thoracic temperature on flight quality. Models were chosen using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC).

Results

I found that air temperature and gas density had a strong, interactive effect on the flight metabolic rates of unloaded honeybees (linear mixed-effects model: $n = 184$, $\chi^2 = 68.6$, $p < 0.001$; Fig. 3.1A). At 35°C, flight metabolic rates of bees increased linearly—by a magnitude of 1.4 times—as gas density decreased (linear model: $F_{1,65} = 83.9$, $p < 0.001$). By contrast, at 23°C, flight metabolic rates of bees decreased with decreasing air density (linear regression: $F_{1,99} = 11$, $p = 0.001$; Fig. 3.1A). Also, while the ability of bees to hover declined with gas density at both air temperatures, bees flying at 23°C failed sooner as density declined (Fig. 3.2). These differential effects of air temperature and gas density on flight appeared to be mediated by differential effects on thoracic temperature.

Air temperature and gas density had a strong, interactive effect on thoracic temperatures of unloaded honeybees (linear mixed-effects model: $\chi^2 = 41$, $p < 0.001$; Fig. 3.1B). At both temperatures, thoracic temperatures decreased linearly as air density decreased (35°C—linear regression: $F_{1,65} = 31.6$, $p < 0.001$), but the effects were greater at 23°C, likely due to the greater thermal gradient from thorax to air (23°C—linear regression: $F_{1,99} = 60.8$, $p < 0.001$; Fig. 3.1B).

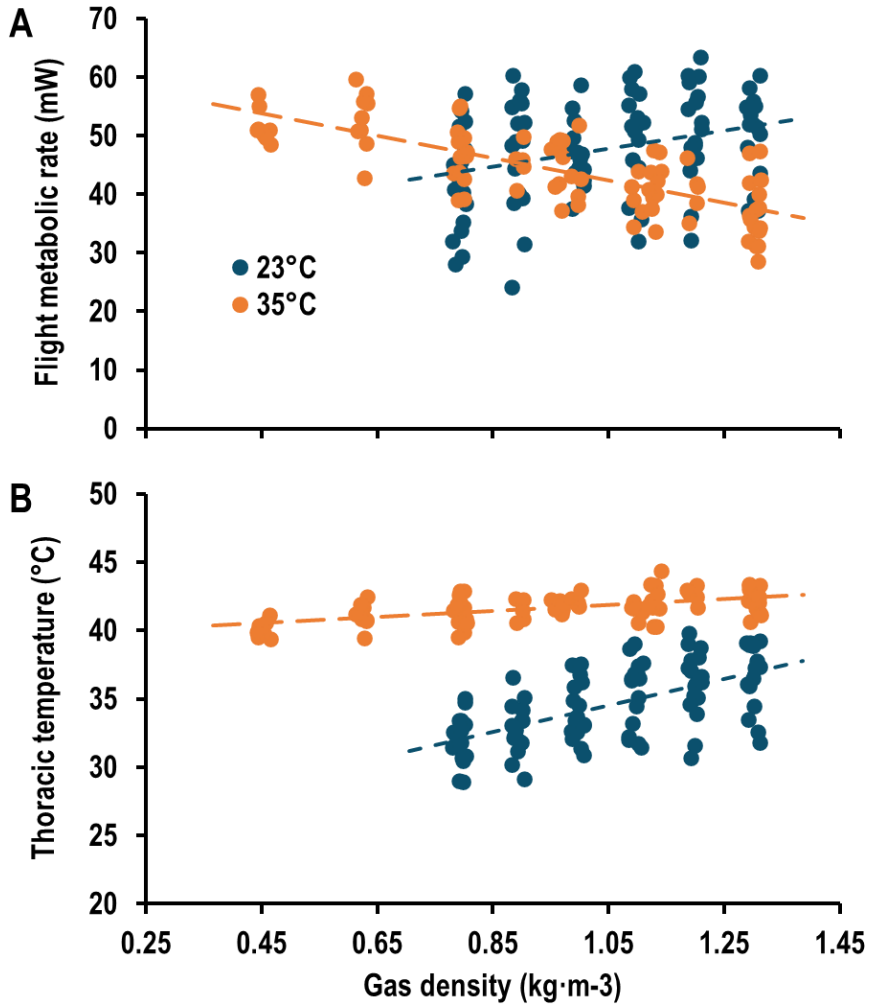

Figure 3.1. (**A**) Gas density significantly influenced flight metabolism of honey bees, but in a temperature-dependent manner (Table 1). (**B**) Decreasing gas density decreased thoracic temperatures of honeybees flown at both air temperatures, but the effect was more pronounced at 23°C (Table 2). Bees were exposed to a narrower range of gas densities at 23°C because honeybees were unable to fly in air densities lower than 0.779 kg m^{-3} at this temperature. Each point represents a single, individually tested bee, with overlapping points being slightly staggered along the *x*-axis to improve data visualization.

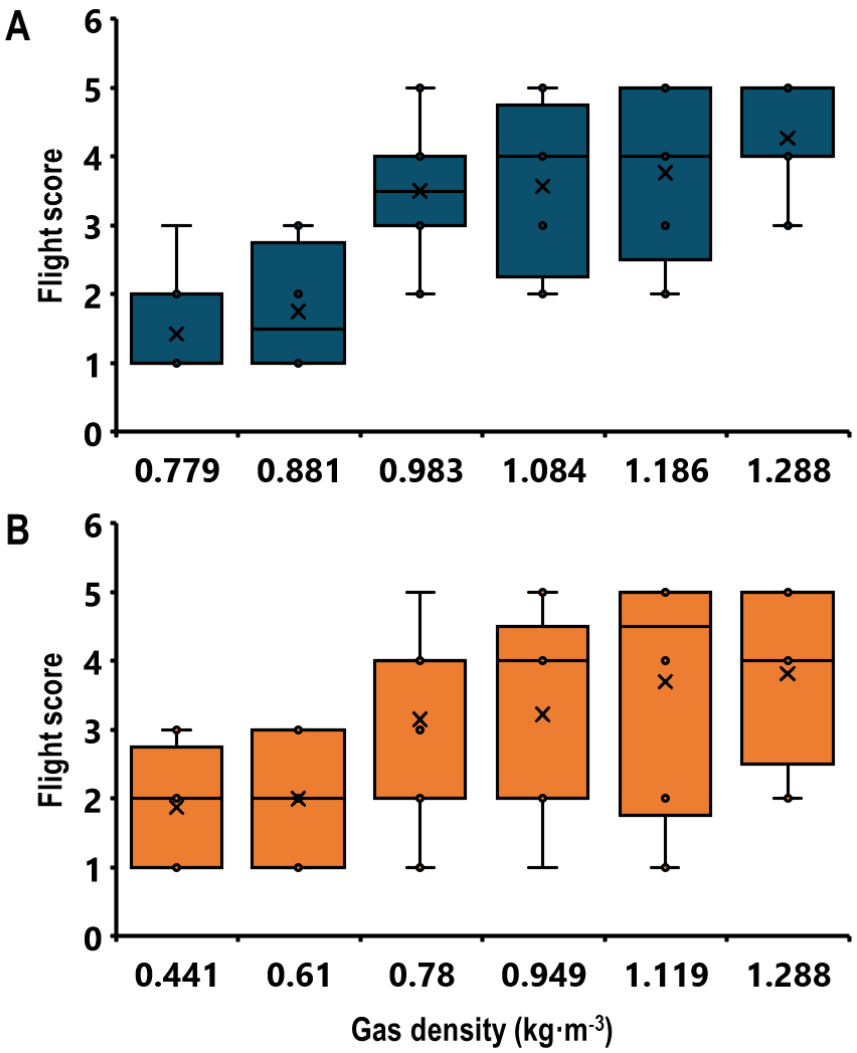

Figure 3.2. (**A**) Flight quality scores of bees flown at 23°C (ranked from 1 = no flight to 5 = stable, continuous flight) declined in low-density air (ordinal logistic regression: n = 99, t = 2.0, p = 0.045) and with declining thoracic temperature (ordinal logistic regression: n = 99, t = 2.2, p = 0.03). Bees flown at 23°C were unable to fly in air densities lower than 0.779 kg m^{-3}. (**B**) Flight quality scores of bees flown at 35°C also declined in low-density air (logistic regression: n = 65, t = 5.7, $p < 0.001$). Flight was categorized and ranked as: 1, no flight; 2, flapping wings with brief periods of flight (less than 3 s); 3, intermittent flight characterized by frequent crashing (i.e. bee usually ends upside down); 4, intermittent flight characterized by frequent controlled landing (i.e. bee gently lands on its feet) or 5, continual, stable flight. The 'x', solid bar, lower bar, bottom bar, top box, and upper bar represent the mean, median, 1st quartile, 2nd quartile, 3rd quartile and 4th quartile, respectively.

Plotting the maximal metabolic value for any bee at each 0.5°C change in thoracic temperature shows a classic thermal performance curve (polynomial linear regression: $y = -0.0168x^3 + 1.5668x^2 - 45.743x + 457.12$; $F_{3,26} = 40.6$, $p < 0.001$; Fig. 3.3). The optimal temperature for flight metabolism and force production (Coelho, 1991) of honeybee workers was 39°C, and maximal flight metabolic rates increased by approximately 2% per 1°C increase in thoracic temperature at suboptimal thoracic temperatures, but decreased approximately 5% per 1°C increase as the bees continued to heat up (Fig. 3.3).

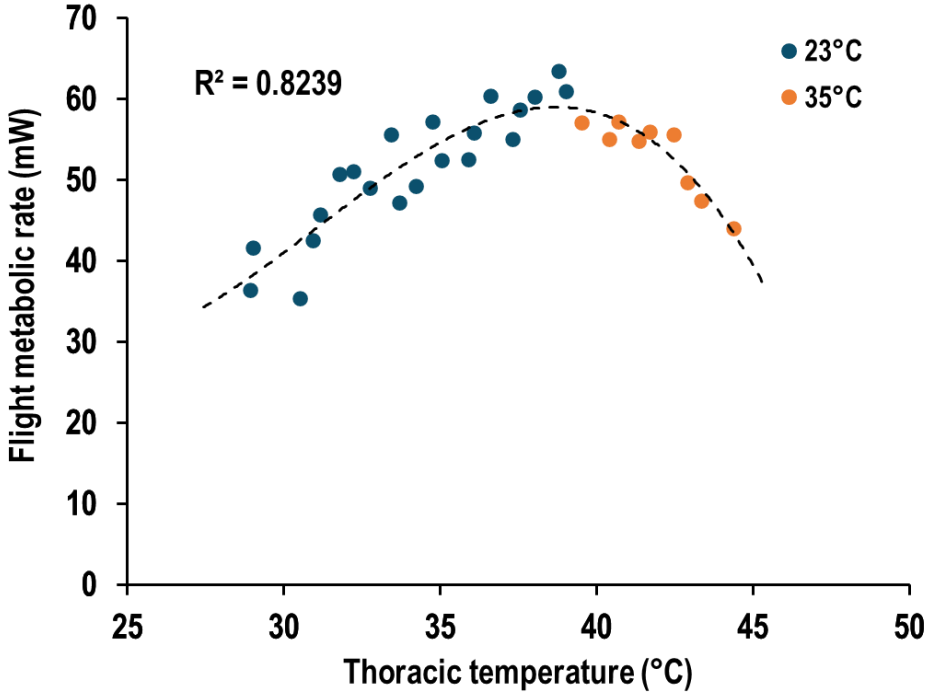

Figure 3.3. Maximal flight metabolic rate as a function of thoracic temperature. Each point represents the maximal value of a single individual bee at each 0.5°C increment (polynomial regression: $y = -0.0168x^3 + 1.5668x^2 - 45.743x + 457.12$; $F_{3,26} = 40.6$, $p < 0.0001$).

Discussion

Our results show that a flying endotherm exhibits a classical thermal performance curve for maximal metabolic rate, with maximal flight metabolic rates measured at an optimal flight muscle temperature of 39°C, and with flight metabolic rates decreasing strongly above and below these body temperatures. An important remaining question is whether mechanical power output during flight shows the same pattern. Metabolic rates often closely correlate with mechanical power output (e.g., Roberts *et al*., 2004), but not always (e.g., in hopping vertebrates; McGowen & Collins, 2018). Force production by the honeybee flight muscle shows a very similar pattern with muscle temperature as I showed for flight metabolism here. Coelho (1991) demonstrated that 39°C was the optimal temperature for force production by honeybee flight muscle, with forces declining above and below 39°C. To confirm that mechanical power output shows a similar thermal performance curve, power outputs could be calculated from measurements of wing kinematics across the range of conditions used here (Vance *et al*., 2014). Another approach would be to assess load-lifting capacity as a function of thoracic temperature (Dillon & Dudley, 2004).

Our data make it possible to quantitatively assess the benefits of endothermic thermoregulation for honeybees. Honeybees can achieve thoracic temperatures up to 17°C higher than air temperature (Fig. 3.1B; Roberts & Harrison, 1999). As an example calculation of endothermic costs and benefits, consider a honeybee forager flying with a thoracic temperature equal to an air temperature of 29°C versus one flying with a thoracic temperature of 39°C. Higher thoracic temperatures come at a cost during maximal performance of about 2 mW per °C elevation in thoracic temperature (Fig. 3.3). Average

foraging trip duration for honeybees is about 30 min (Winston, 1991). If during the return flight they flew at maximal capacity while carrying a heavy load for 15 min, flying with a flight muscle temperature of 39°C at 58 mW rather than at 29°C at 38 mW will increase the cost of the foraging trip by about 20 joules (15 min · 60 s min^{-1} · 20 mjoules s^{-1}). However, the energetic benefit can be substantially higher.

Flight metabolic rate increases linearly with load, by about 40% (approx. 20 mW), as load increases from 0 to 40 mg of nectar (Wolf *et al*., 1989). This is about the same increase as observed in maximal aerobic performance as flight muscle temperature rises from 29°C to 39°C (Fig. 3.3). At 29°C, the flight muscle of honeybees is near the minimal temperature at which these bees can fly (Heinrich, 1979), and so it is unlikely that they can carry a substantial load at this flight muscle temperature. The energetic content of nectar varies, but 9 joules mg^{-1} is an estimated average value from the literature (Winston, 1991). The gross return of energy to the colony for a 40 mg nectar load will be, on average, 360 joules, with a net return of 302 joules (360 – 58 joules). Obviously, the net benefit will depend strongly on the capacity of cool bees to carry loads and on foraging conditions, and endothermy may not be favored if energetic rewards in the field are low. Social bees have been widely shown to modulate thoracic temperatures to reward, with higher temperatures associated with higher energetic content of nectar, suggesting that bees can modulate their body temperatures to maximize net foraging reward (Waddington, 1990). However, these calculations depend on the assumption that efficiency is constant across a range of flight muscle temperatures, something that is still unknown for insect flight.

Substituting helium for nitrogen also affects oxygen diffusivity; might this have influenced our results? Oxygen diffusivity in a gas is inversely proportional to gas density (Lide, 2004). The diffusion rate of oxygen within the tracheae likely increases by slightly more than 2× as gas density decreases from nitrox (79% N_2: 21% O_2) to heliox (79% He: 21% O_2; assuming constant P_{O2} gradients within the tracheae). However, it seems unlikely that variation in oxygen diffusivity explains any of the observed patterns in metabolic rate or flight behavior. Oxygen delivery to unloaded honey bees has a substantial safety margin, as metabolic rates of hovering, unloaded bees are unaffected as air P_{O2} varies between 10 and 39 kPa under normobaric conditions (Joos *et al*., 1997). Admittedly, the safety margin for oxygen delivery is likely to be smaller at maximal performance, where oxygen consumption rates are about 40% higher. However, Withers' finding (Withers, 1981) that metabolic rates of flying honeybees rise with a small decrease in air pressure and then fall linearly with larger decreases in air pressure is inconsistent with diffusion being the major mechanism of gas exchange. In hypobaria, P_{O2} falls, but oxygen diffusivity increases proportionally, so diffusive oxygen delivery should be unaffected. If diffusion is the predominant mechanism of gas exchange, we would expect metabolic rates to continue to rise as air pressure drops up to the point of flight failure due to maintained oxygen delivery as the challenge of generating lift increases. Advective gas exchange declines linearly with air pressure due to the linear decline in oxygen content of air; consistent with Withers' findings (Withers, 1981). As oxygen transport in the gas phase is likely predominantly advective in flying honeybees, it seems unlikely that the rise in flight metabolic rates observed as air density

declines at air temperatures of 35°C is due to improved oxygen transport. This possibility could be tested directly by varying air P_{O2} in different gas densities.

Endothermy and thermoregulation at high body temperatures may expand the thermal niche of foraging bees. As noted above, the minimum flight muscle temperature for flight for honeybees is about 28°C. Honeybees have been observed to forage at air temperatures as low at 12°C (Heinrich, 1979). As nectar and pollen rewards at flowers are usually highest in the early morning, it is plausible that endothermy aids honeybees and other large social bees in competition for nectar and pollen rewards by enabling them to forage during lower morning temperatures (Roubik & Buchmann, 1984). That being said, a rigorous test of the thermal niche expansion hypothesis would compare the air temperatures at which both larger endothermic bees and smaller ectothermic bees can fly. One recent study compared the foraging temperature range of honeybees to *Osmia cornuta*, a smaller bee with limited endothermic capacity. *Osmia cornuta* was able to fly at lower air temperatures and in more inclement weather than *A. mellifera* (Vicens & Bosch, 2000). It appears that rigorous study of thermal niches of endothermic and ecothermic species in a phylogenetic context will be necessary to determine whether endothermy is associated with a broader thermal niche in insects. In addition to increasing aerobic capacity and possibly thermal niche, endothermy has other benefits for some insects. Heat generated by the flight muscle of social bees, such as honeybees and bumblebees, is also used to warm and thermoregulate their offspring, speeding development and possibly improving developmental stability (Jones & Oldroyd, 2006).

The magnitude of cooling caused by exposure to low-density gases depends on the thermal conductivity of a particular gas mixture and air temperature. Convective heat loss (*HF*) can be simply modelled as

$$HF = \frac{-kA\Delta T}{\delta},$$

where k is the thermal conductivity of the gas mixture, A is the surface area of the animal, ΔT is the temperature differential (which in this study represents the difference between the thoracic temperature and air temperature), and δ represents the height of the boundary layer of air around the animal. Because several of these variables are difficult to measure, this equation is often simplified to

$$HF = C_{conv}\Delta T\ ,$$

where C_{conv} represents the thermal conductivity (mW m^{-1} K^{-1}) of the gas mixture. The thermal conductivity of a 79% N_2: 21% O_2 gas mixture (nitrox) is 26 mW m^{-1} K^{-1}, whereas the 79% He: 21% O_2 gas mixture (heliox) has a thermal conductivity of 129 mW m^{-1} K^{-1} (Lide, 2004). For a bee flying with a thoracic temperature of 41°C at an air temperature of 23°C in nitrox air, heat will be lost at a rate of 468 mW, while a bee flying with the identical thermal gradient in heliox will experience a fivefold increase (approx. 2322 mW) in heat loss. However, if a bee with the identical thorax temperature is flying in 35°C nitrox air, heat loss will be decreased threefold, to 156 mw. These combined effects of varying thermal conductivity and air temperature allowed us to manipulate the thorax temperatures of flying honeybees over a wide range.

A crucial question for agriculture is how climatic warming will affect pollinator performance. At cooler locations, seasons, and times of day, warmer air temperatures will

increase flight muscle temperatures toward optimal and increase flight aerobic capacity. However, in warmer locations, seasons, and times of day, high air temperatures and solar radiation may push flight muscle temperatures into the range above the optimal temperature, causing decreasing maximal aerobic performance with increasing body temperature. On hot days, flying honeybee foragers thermoregulate both by increasing water loss rates and by reducing metabolic heat production (Heinrich, 1980; Harrison *et al*., 1996; Roberts & Harrison, 1999). Nonetheless, body temperatures of flying bees rise approximately 0.4°C with each 1°C rise in air temperature, and the highest flight muscle temperature measured for bees flying in the laboratory in dry air at 45°C was approximately 49°C (Roberts & Harrison, 1999), well above the optimal temperature of 39°C (Fig. 3.3). Honeybees flying in desert regions in the field have body temperatures above 40°C, with pollen foragers tending to be hotter due to reduced capacities for evaporative heat loss (Cooper *et al*., 1985). This suggests heat waves associated with climatic warming will negatively impact maximal flight performance and load-carrying capacities in the field for honeybees and possibly other endothermic insects.

This first thermal performance curve for a flying endotherm strongly supports our hypothesis that thermoregulating toward a high temperature (39°C) enhances aerobic capacity, flight capabilities, and foraging performance in honeybees. Because our flight metabolic rates were measured over 10 s, I may have missed spikes in metabolic rate associated with short-term bursts in power output. Therefore, our measures of the effects of flight muscle temperature on maximal power output are, as noted above, probably conservative. To further develop and test aerobic capacity models for the evolution of endothermy in flying insects, it will be important to measure thermal performance curves

for more endothermic insects to determine how general or variable this pattern is, and to determine how maximal aerobic metabolism relates to mechanical power output and load-lifting capacities. Linking physiological with paleontological and systematic research will also be necessary to create a true evolutionary model. In vertebrates, insulation (i.e. fur, feathers) and indices of blood vessel density in bone provide paleontological evidence for endothermy and homeothermy (Lovegrove, 2019). Tests of whether morphological characteristics detectable in fossils, such as thoracic insulation or tracheal dimensions, are linked to endothermy could advance this field.

Why does flight metabolism decrease at higher air temperatures? Several studies have shown that flight metabolism decreases at relatively high air temperatures (Harrison *et al*., 1996; Roberts & Harrison, 1999). However, the mechanisms remain unclear. The decrease in flight metabolism might be due to suppression of flight muscle by thermoregulatory centers in the brain to prevent overheating. Conversely, higher temperatures may be directly inhibiting the flight muscle or motor neurons. For example, high temperatures may increase K^+ leakage in the flight muscle or controlling neurons relative to Na^+/K^+-ATPase activities, causing widespread depolarization and loss of excitable tissue function (O'Sullivan *et al*., 2017). Another possibility is that high temperatures directly inhibit muscle proteins such as myosin ATPase, decreasing the contractile ability of flight muscle.

Endothermy may be ancient within the Insecta, and has been hypothesized to have occurred in the large Protodonata of the Carboniferous (May, 1982). Bees evolved from wasps in the Cretaceous (Michener, 2007), and some larger sphecid wasps are endothermic, suggesting endothermy in bees could have been inherited from wasp

ancestors (Ghazoul & Wilmer, 1994). However, the Mellitidae are the sister taxa to bees, and most, but not all of these, are likely too small to be endothermic (Murray *et al*., 2018), supporting the possibility of an independent origin of endothermy in bees. In any case, miniaturization and enlargement of species is common in lineages of bees (Danforth *et al*., 2019), suggesting that endothermic heterothermy likely evolved multiple times in association with having a sufficiently large body size to enable metabolic heat production to exceed heat loss. Identification of paleontological markers of endothermy could enable rigorous tests of when endothermy occurred. Our findings that endothermy increases both the costs and potential rewards of foraging suggest that the evolution of endothermy in bees should be associated with periods of rich resource availability.

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CHAPTER 4

MECHANISMS AND LIMITATIONS FOR NECTAR-LOADED HONEY BEES FLYING IN THE HEAT

Abstract

Heatwaves are becoming increasingly common due to climate change, making it crucial to identify and understand the capacities for insect pollinators, such as honey bees, to avoid overheating. While critical thermal maxima are commonly used to assess ectotherm upper thermal limits, these likely overestimate the temperatures that limit flight performance. We examined the effect of hot, dry air temperatures on the physiological and behavioral mechanisms honey bees use to fly with nectar loads to assess limitations of overheating or desiccation on foraging. Metabolic rates and flight muscle temperatures increased linearly with load mass at air temperatures of 20 or 30°C, but, remarkably, there was no change in flight muscle temperature or metabolic rate as honey bees carried nectar loads at air temperatures of 40°C. Cost-free load carriage and wing translational power production were accomplished by lowering wingbeat frequency and increasing wing stroke amplitude. At 40°C air temperature, approximately equal decreases in metabolic heat production and increases in evaporative cooling allowed bees to maintain flight muscle temperatures at about 45°C. However, desiccation becomes a serious ecological risk to foraging at air temperatures of 45°C in dry air.

Introduction

Insect pollinators are declining at an alarming rate, due in part to climate change (Halsch *et al*., 2021). Not only is the planet getting warmer, but it is also experiencing increased variation in extreme weather events, such as heatwaves (IPCC, 2021). These higher, more frequent thermal events may push insect pollinators, such as bees, to their thermal limits, potentially contributing to their decline (reviewed in Johnson *et al*., 2023). If we continue to lose our insect pollinators, we will undoubtedly see catastrophic impacts on both human agriculture and ecosystems (Lever *et al*., 2014; van der Sluijs, 2020; Ramos-Jiliberto *et al*., 2020). In this study, I investigate the effects of high air temperature on metabolism, water-balance, and wing kinematics of flying honey bees (*Apis mellifera*) carrying nectar loads to understand at what air temperatures flight becomes heat-limited.

While large, flying insects can thermoregulate, their body temperatures still rise with air temperatures (Heinrich, 1971, 1972a,b, 1980a; Harrison *et al*., 1996; Woods *et al*., 2005, Glass & Harrison, 2022; Johnson *et al*., 2022), potentially increasing their vulnerability to extreme heat. Insects use different physiological and behavioral strategies to avoid overheating. Most insects evade thermal stress by changing when they are active (Cooper *et al*., 1985; Bergman *et al*., 1996; Pyke *et al*., 2011; Di Trani *et al*., 2022), but to ensure the growth and survival of the colony, many eusocial insects, including honey bees, may be obligated to remain active even when the environment heats up. Flying honey bees can stave off overheating by regurgitating fluid from their honey crop to evaporatively cool (Heinrich 1980b; Cooper *et al*., 1985; Roberts & Harrison, 1999). Several insect species, including honey bees, compensate for heat by lowering their wingbeat frequencies and metabolic heat production when flying at high air temperatures

(Chappell, 1984; Cooper *et al*., 1985; May, 1995; Harrison *et al*., 1996; Roberts *et al.*, 1998; Roberts & Harrison, 1999; Borrell & Mederios, 2004). The mechanisms by which flying insects are able to lower metabolic rates during flight at higher air temperatures have been unclear, since, if other aspects of wing kinematics are unchanged, reducing wingbeat frequency should reduce lift and mechanical power generation (Dudley & Ellington, 1990).

Flying animals must increase their aerodynamic force output in order to carry heavier loads (Ellington, 1984), which generally increases metabolic costs for birds, bats, and bees (Chai *et al*., 1997; Iriarte-Diaz *et al*., 2012; Combes *et al*., 2020). Flying insects rely on unsteady mechanisms of force production. Insects that fly with a large stroke amplitude generate the bulk of their force from the wing translation, which is the sweeping motion of the wing during the flapping cycle (e.g., fruit flies: ~140°; Fry *et al*., 2005). In contrast, those insects that fly with much smaller strokes (e.g., mosquitoes: ~40°; Bomphrey *et al*., 2017) rely on the wing-flip at each end of the wing stroke to generate force. Honey bees lie in the middle of this stroke-amplitude range (~90°) and they have been shown to utilize forces associated with both wing translation and rotation to fly, and to increase stroke amplitude to fly in low-density air (Altshuler *et al*., 2005; Vance *et al*., 2014). However, what wing kinematic patterns are used to carry loads remains unknown. Heavily-loaded bumblebees generate the high forces needed by relatively large increases in wing stroke amplitude compared to frequency, reducing the metabolic cost of lifting (Combes *et al*. 2020). These findings for bumblebees suggest that the lower wing beat frequencies previously shown for hotter flying honey bees may provide increased flight efficiency and reduced metabolic heat generation.

Critical thermal maxima (CT_{max}) that identify body temperatures that result in loss of motor control are routinely used to identify temperatures that limit insect survival in the heat (Jørgensen *et al*., 2021). However, ecological function may be limited at lower temperatures than CT_{max}. For flying pollinators, such as honey bees, foraging may be heat-limited by thermoregulatory failure that allows heating to CT_{max}, desiccation due to excessive water loss, or negative effects of high body temperatures on functions such as flight power generation. As yet, we lack the quantitative assessments necessary to determine the environmental conditions that will heat-limit foraging of pollinators, such as honey bees (Johnson *et al*., 2023). To identify and measure the limitations high air temperatures may have on thermoregulation, water-balance, and lift generation during flight, I measured flight muscle temperatures, flight metabolism and water loss rates of honey bees carrying nectar loads at three air temperatures (20, 30, and 40°C), and measured wing kinematics with high-speed video at air temperatures of 25 and 40°C. I then used these data and the prior literature to model heat-limits on honey bee flight. The metabolic thermal performance curve of unloaded flying honey bees suggests that flight muscle temperatures above 40°C will be associated with progressively lower metabolic rates and wing beat frequencies (Harrison *et al*., 1996; Glass & Harrison, 2022), potentially enhancing thermoregulation capacities, but with unknown effects on lifting capabilities. I hypothesized that, like load-lifting bumblebees, hot honey bees generate the forces needed to lift heavier loads primarily by increasing wing stroke amplitude, reducing metabolic heat production and improving thermoregulation. I also predicted that desiccation would limit flight of honey bees in dry air at lower air temperatures than the CT_{max} for honey bees (49-50°C, Kovac *et al*., 2014; Burdine & McCluney, 2019).

Methods

Respirometry Experiment

Study Animals and Location

The experiments measuring the effect of air temperature on body temperatures, metabolic rates, and water balance were conducted with three colonies of honey bees, *Apis mellifera*, maintained on the third-story balcony of the Interdisciplinary Science and Technology Building 1 at Arizona State University (ASU) in Tempe, AZ, USA. Unloaded, outgoing forages were captured when leaving the colony by holding an opened plastic bag (~950 ml) approximately fifteen centimeters from the colony entrance. After a single forager flew directly into the opened bag, the bag was sealed, and the bee was quickly transported into the laboratory. Each bee's pre-fed mass was recorded before being fed a randomized amount of 50% sucrose solution, and its fed mass was recorded after feeding. The bee was then transported into a temperature-controlled Environmental Growth Chamber (set to either 20±0.5, 30±0.5, or 40±0.05°C; Chagrin Falls, Ohio, USA), and the ambient temperature inside the chamber was monitored using a thermocouple integrated with Expedata (Sable Systems, Las Vegas, NV). A random number generator (www.randomizer.org) was used to determine the order and time in which the colonies were sampled.

Metabolic and Water Loss Rate Measurements

Within two minutes after feeding, the bee was transferred to the respirometry system. The bee was placed in a cylindrical, transparent-acrylic flight chamber (350 mL), which was sealed and covered with a dark cloth, to encourage reduced activity of the bee. The air

from the flow meter (Alicat Scientific, Inc., Tucson, AZ) flowed at 2 $L \cdot min^{-1}$ sequentially through a $CaSO_4$ and soda lime column to remove H_2O and CO_2 before flowing to the reference cell of the LI-COR LI-7000 CO_2/H_2O analyzer (Lincoln, NE, USA). The air then flowed to the respirometry chamber, then to the sample cell of the LI-7000. The differential analog output from the LI-COR was digitized (Sable Systems UI-2) and recorded each second (Expedata, Sable Systems, Las Vegas, NV). The LI-7000 was CO_2 calibrated using a 100.4 ppm CO_2 and Ultra-Zero calibration gases at the same flow rate and pressures as during the flight respirometry, and baseline recordings were taken before and after each measurement period. The LI-7000 was also calibrated for H_2O by performing a steady-state volts versus water concentration model.

While the bee sat in darkness, I flushed the chamber for three minutes prior to the flight trial, allowing CO_2 and H_2O levels from the chamber to reach a low, stable level. Hovering flight was then encouraged for six minutes by shining a 150W dual goose-neck Fiber Optical Illuminator (China) over the chamber. Bees that landed were immediately encouraged to fly by gently tapping and inverting the chamber. Bees that refused to fly were discarded from this study. Here, I define 'flight' as free-flight independent of the sides and above the bottom third of the flight chamber. Expedata (Sable Systems, Las Vegas, NV) was used to find and average the ten seconds with the most stable CO_2 and H_2O readings during each trial.

Flight CO_2 emission rates ($\dot{V}_{CO_2}$; $ml \cdot hr^{-1}$) were calculated by multiplying the differential CO_2 fraction times the STP flow-rate through the flight chamber (Lighton, 2018). Then, to calculate the flight metabolism (mW), $\dot{V}_{CO_2}$ was multiplied by the energy yield per amount of CO_2 formed, 21.146 $J \cdot ml^{-1}$ CO_2, assuming simple carbohydrate

catabolism (45-47). Flight water loss rate ($\dot{V}_{H_2O}$, mg $H_2O \cdot h^{-1}$) was calculated by multiplying the fractional concentration of water ($mmol \cdot mol^{-1}$) leaving the chamber by the STP flow rate ($ml \cdot min^{-1}$) and the molar mass of water (18 $g \cdot mol^{-1}$), then dividing by the molar volume of water found in a one-liter container of air at STP (22,400 $ml \cdot mol^{-1}$). I calculated evaporative heat loss (mW) by multiplying $\dot{V}_{H_2O}$ by the latent heat of evaporation of water (2.41 J·mg of $water^{-1}$).

Flight Muscle Temperature Measurements

Flight muscle temperature of each bee was measured immediately after the flight trial by quickly shaking the bee into a plastic bag, which was flattened to restrict the bee's movement, and a Physitemp model MT29/1 hypodermic microprobe (Clifton, New Jersey, USA; 29-gauge, time constant=0.025·s) was inserted through the bag and into the center of the thorax. Flight muscle temperature data was recorded with a Pico Technology USB TC-08 Thermocouple Data Logger (Tyler, TX, USA). Flight muscle temperatures were measured within three seconds of cessation of flight, and the highest temperature reported by the thermometer was recorded. If measurements took longer than five seconds to measure, the bee's temperature measurement was excluded from the analysis. After measurement, the bee was weighed (±0.1 mg) using an A&D HR-120 Analytical Balance (Tokyo, Japan) and stored at -20°C.

Statistical analyses

I used a linear mixed-effects model to test the independent and interactive effects of air temperature (°C), and total body mass (mg) on flight metabolic rate [milliwatts ($mJ \cdot sec^{-}$

^{1})] and water-loss rate (mg $H_2O \cdot min^{-1}$), with hive included as a random effect. I also ran a similar model to investigate the interactive and independent effects of the above independent variables on flight muscle temperature. Any reported interactive or independent effects come from full models [e.g., flight metabolic rate ~ air temperature x total body mass + (1|hive)]. Bees which did not hover continuously in the respirometry chamber were excluded from these analyses. For the respirometry and kinematic experiment, data were analyzed using R (3.6.2; R Foundation for Statistical Computing, Vienna, Austria), specifically using the 'Matrix', lmer', and 'car' packages, and two-tailed significance was determined at $\alpha = 0.05$.

Kinematic Experiment:

Study Animals and Location

The experiments examining the effect of temperature on wing kinematics during loaded flight were conducted with honey bees captured while foraging on flowers (38.541387, -121.753899) on the University of California at Davis campus. Data collection took place from May 29 to June 3, 2022, from approximately 800 to 1800 hrs each day. Single foragers were captured, one at a time, using a 45-ml conical centrifuge tube. The bee was weighed on an Ohaus Explorer EX124 balance (±0.1 mg) within two minutes of capture. Bees were not anaesthetized or chilled and were handled as little as possible to minimize disruptions to normal flight behavior. After taking the bee's mass, the bee was then moved into our custom, temperature-controlled flight chamber set to either 25±0.25 or 40±0.25°C. Immediately after the flight recording, the bee was transferred into a pre-weighed 45-ml conical centrifuge tube and the bee's final, post-flight mass was

measured. The bee was then anesthetized on ice and transferred into a 1.5 ml centrifuge tube to be stored in a -20°C freezer.

Wing Length and Wing Area Measurements

Ten frozen bees were randomly selected from each temperature group and each bee's right wings (i.e., fore- and hindwing) were removed, repositioned to resemble their connected-position during flight, and then photographed. Wings were repositioned as close to their natural, connected position as possible because significant differences were found between wing-area calculations of separate versus overlain wings. Using an image processing program (ImageJ; https://imagej.net/ij/), I measured the average forewing length (mm) and wing area of both fore- and hindwing (mm^2). There was no significant difference between the mean wing length [$t_{(18)} = 0.4$, $p = 0.73$] or wing area [$t_{(18)} = 0.1$, $p = 0.90$] of bees randomly sampled from 25 and 40°C air temperatures.

Flight Chamber and Camera Setup

Bees were flown in a flight chamber (17.8 cm x 16.5 cm x 25.6 cm; width x height x length). The temperature of the chamber was set between trials by allowing cooled or heated air to flow through the chamber until the desired temperature was achieved. A small desk fan (Vornado Air, LLC; Andover, KS, USA) or a ceramic, whole-room heater (Honeywell HZ-445R; Charlotte, NC, USA) were positioned to facilitate movement of cool (25±0.25°C) or warm (40±0.25°C) air, respectively. Each bee was flown in the chamber only once and at a single temperature. The chamber was lit from above and the

side using 23-Watt LED light bulbs (2610 Lumens, 3000K bright white; Great Eagle, Boca Raton, FL, USA).

Flights were filmed with two synchronized, manually triggered Phantom V611 high-speed video cameras (Vision Research, Inc., Wayne, NJ, USA) sampling at 3000 frames·s^{-1} (exposure time: 20 μs) positioned above and in front of the flight chamber setup. Cameras were calibrated using a standard checkerboard calibration method and built-in MATLAB functions (Heikkila & Silvén, 1997; Zhang, 2000). This method captures lens distortion and projective geometry (using the intrinsic parameters), as well as the global positions and orientations of the cameras relative to the flight chamber (via the extrinsic parameters).

Flight Trial and Video Processing

Bees were flown in a dark room, allowing the setup lighting to induce flight. To encourage the bee to fly continuously, the chamber was gently tapped, or a small, plumose feather was waved on the outside of the chamber to elicit flight. Using DLTdv6 (Hedrick, 2008), I tracked four landmarks that were digitized using the two camera views: head, tip of abdomen, one wing hinge, and one wing tip. Which wings of the bee were chosen based on their orientation relative to the cameras. Using the camera calibration, I converted the two-dimensional locations of the points in each view into three-dimensional coordinates of the bee's body and wings. From the digitized videos I was able to calculate wingbeat frequency (Hz) and stroke amplitude (°) of the flying bee. I calculated the arc length of the stroke (m) by converting the stroke amplitude from degrees to radians, then multiplying this by 75% of the wing length (m) to avoid the

portion of the wing that may deform during the stroke (Combes *et al*., 2020). The average wing velocity ($m \cdot sec^{-1}$) was then calculated by multiplying the arc length by 2 (wing stroke includes the up- and downstroke) multiplied by the wingbeat frequency (beats · sec^{-1}). To estimate a "proxy" for the power exerted by the wings on air during translation (P_{tr}), I used the following equation:

$$P_{\mathrm{tr}} = \frac{\rho S {U_w}^2 (2a)}{n} \cdot 1000$$

where S wing area (m^2), U_w^2 is the average wing velocity squared ($m^2 \cdot sec^{-2}$), $2a$ is two times the arc length (m), and n is the average wingbeat frequency (Hz). This "translational power proxy", with units of mW, was used because we did not have the data necessary to calculate rotational forces or the lift coefficient for our bees, so we conservatively do not report mechanical power output or its components, but rather the component of aerodynamic power that we can measure.

Statistical analysis

For the 'Kinematic Experiment' data, I used a generalized linear model to test the independent and interactive effects of air temperature (°C), and total body mass (mg) on the wingbeat frequency and stroke amplitude of flying nectar foragers collected from flowers. Only data from bees that successfully and constantly flew were included in these analyses. I also excluded any videos with poor quality recordings. I ran a 'Type III' ANOVA on the output of the models to control for the interactions and variable-order. Models were chosen using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). I performed linear regressions if there was a significant

interactive effect between two dependent variables in the full models to ensure the slope of the fitted line was different than zero.

Results

Respirometry Experiments

Flight Muscle Temperature and Flight Metabolism

The effect of nectar loading on flight muscle temperatures depended on air temperature (significant air temperature x total body mass interaction term in linear mixed effects models: $n = 141$, $\chi^2 = 86$, $p < 0.0001$). The steepness of the slope of flight muscle temperature on total body mass decreased as air temperature rose (Fig. 4.1A). Flight muscle temperatures increased with increasing total body mass at 20 and 30°C air temperature (Bonferroni-corrected $\alpha = 0.016$; 20°C – linear model: $n = 48$, $F_{1,46} = 9.1$, $p < 0.001$; 30°C – linear model: $n = 46$, $F_{1,44} = 6.9$, $p < 0.01$), but not at 40°C air temperature (Bonferroni-corrected $\alpha = 0.016$; 40°C – linear model: $n = 47$, $F_{1,45} = 0.5$, $p = 0.5$; Fig. 4.1A).

Similarly, flight metabolic rates increased with nectar loading at 20 and 30°C but not 40°C air temperature (Fig. 4.1B). Flight metabolic rates of honey bees increased with nectar-load with nearly-identical slopes at 20 and 30°C air temperature (Bonferroni-corrected $\alpha = 0.016$; 20°C – linear model: $n = 48$, $F_{1,46} = 11.9$, $p < 0.01$; 30°C – linear model: $n = 46$, $F_{1,44} = 13.9$, $p < 0.001$). However, at 40°C air temperature, load mass did not significantly affect flight metabolic rate (Bonferroni-corrected $\alpha = 0.016$; 40°C – linear model: $n = 47$, $F_{1,45} = 3.6$, $p = 0.065$; Fig. 4.1B), and flight metabolic rate was

significantly lower than observed at 20 and 30°C air temperature (linear mixed effects models: $n = 141$, $\chi^2 = 80$, $p < 0.0001$; Fig. 4.1B inset).

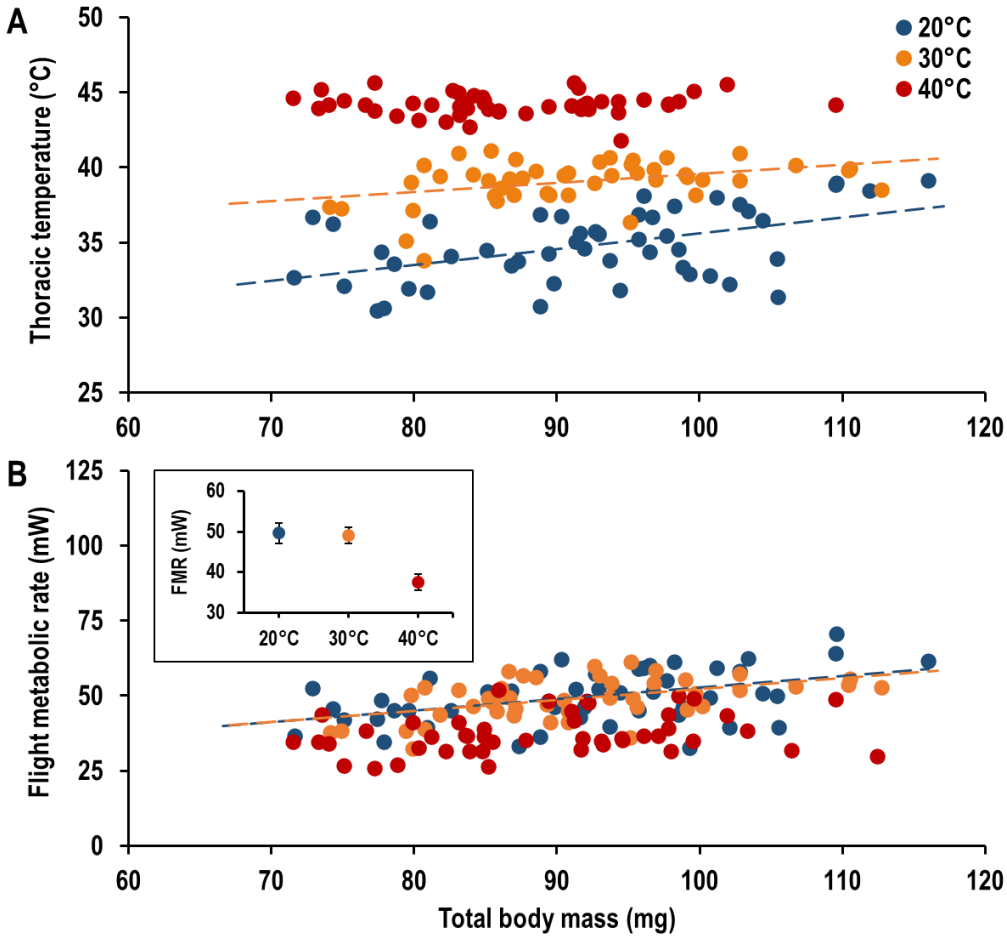

Figure 4.1. Air temperature altered the effect of loading on flight muscle temperature (**A**) and flight metabolic rate (**B**). Each point represents an individually measured bee. Regression lines denote significance; the regression lines for flight metabolic rate at 20 and 30°C air temperature overlap. The points in the inset graph represent the mean ± 95% CL.

Evaporative Water Loss

Air temperature and total body mass had a significant interactive effect on water loss rates (linear mixed-effects model: $n = 141$, $\chi^2 = 8.5$, $p = 0.01$; Fig. 4.2A), with water loss

rates increasing with load for bees flown at 40°C air temperature (40°C – linear model: n = 47, $F_{1,45}$ = 4.2, p = 0.046), but not for those flown at 20 or 30°C air temperature (20°C – linear model: n = 48, $F_{1,46}$ = 0.45, p = 0.51; 30°C – linear model: n = 46, $F_{1,44}$ = 0.001, p = 0.93 Fig. 4.2). Unloaded and loaded bees flying at 40°C air temperature had much higher water loss rates (40°C: 0.33±0.02 mg $H_2O \cdot min^{-1}$; mean±95% CL) than bees flying at 20 and 30°C air temperature (20°C: 0.06±0.002 mg $H_2O \cdot min^{-1}$; 30°C: 0.08±0.004 mg $H_2O \cdot min^{-1}$; linear mixed-effects model: n = 141, χ^2 = 1367.8, p < 0.0001; Fig. 4.2)

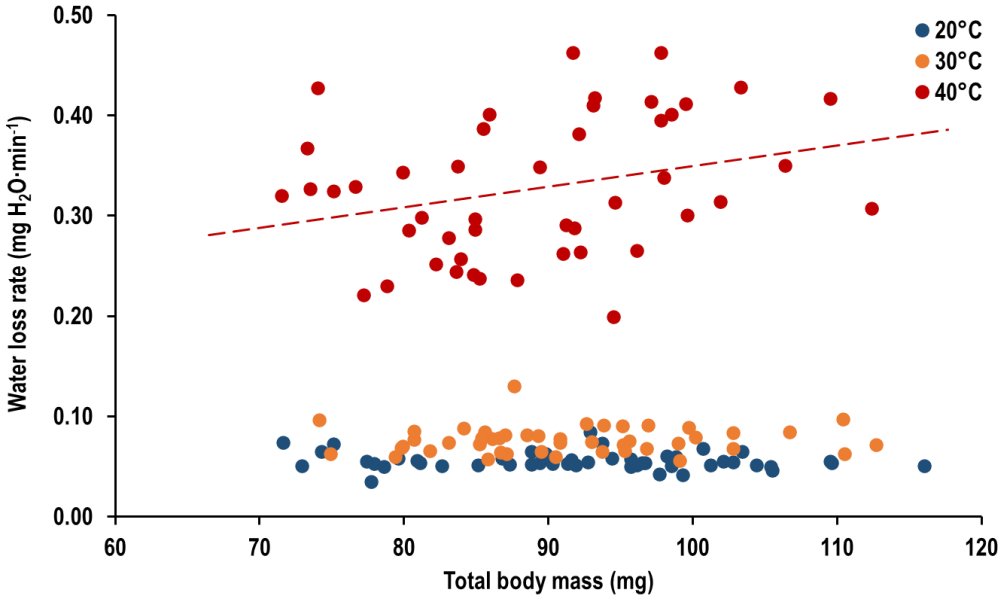

Figure 4.2. Water loss rates of honey bees increased with nectar load at 40°C, but not 20 or 30°C air temperatures. Total body mass is the mass of the bee plus its nectar load. Each point represents an individually measured bee. Regression line denotes significance.

Heat Flux

When data are pooled from all temperatures, there was a strong increase in evaporative heat loss rates when flight muscle temperatures exceeded 40°C (polynomial linear regression: n = 141, $F_{3,137}$ = 327.8, p < 0.0001; Fig. 4.3). Metabolic heat production

increased as the flight muscle temperatures of bees increased up to 39°C, and then decreased at higher flight muscle temperatures (polynomial linear regression: $n = 141$, $F_{2,138} = 40.2$, $p < 0.0001$; Fig. 4.3)

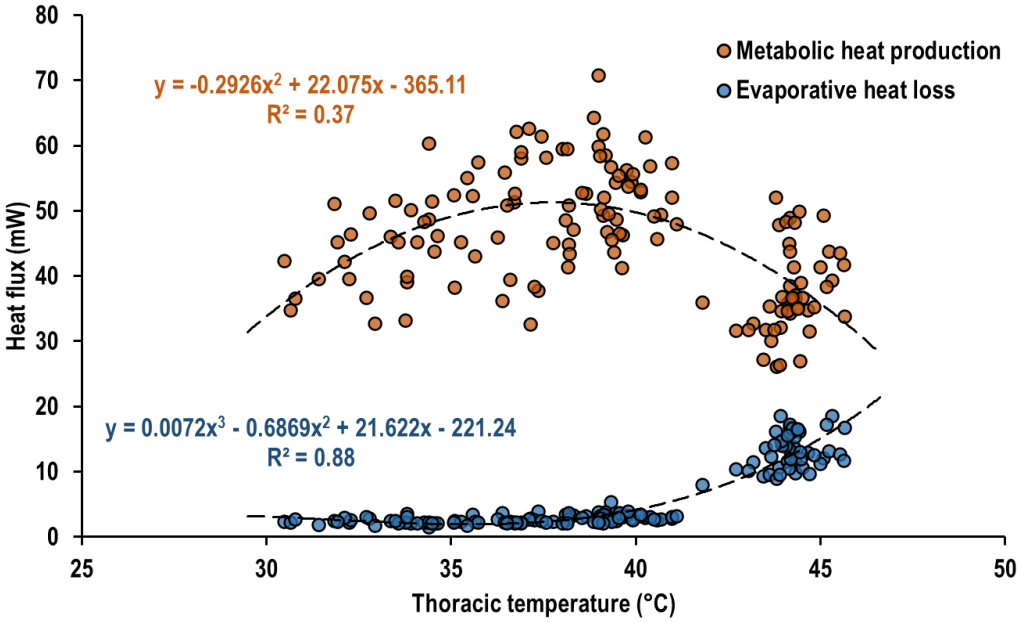

Figure 4.3. The interactive effects of flight muscle temperature on metabolic heat production and evaporative heat loss of pooled loaded and unloaded flying honey bees. Each point represents an individually measured bee.

Desiccation Limitations on Flight Duration

Honey bees flying at high air temperatures are in negative water balance (Roberts & Harrison, 1999), and so bees flying in search of resources will progressively desiccate faster as air temperature rises. To conservatively estimate how long honey bees can fly without finding a water source at various temperatures, I calculated the effect of evaporative water loss (*EWLR*) – balanced with metabolic water production (*MWP*) – on the duration of flight at various air temperatures. Maximum flight duration (MFD; minute) until death by desiccation was calculated as:

$$MFD = \frac{\text{required water stores}}{EWLR - MWP}$$

with required body water stores in mg, and water loss and production rates in mg·min^{-1}. To calculate *EWLR* and *MWP*, I first determined the relationship between air temperature (T_{air}) and flight muscle temperature (T_{thorax}):

$$T_{air} = \frac{T_{thorax} - 25.313}{0.4684}$$

by fitting a line to our pooled data across the three air temperatures tested. Next, I fit a model relating T_{air} to *EWLR* using our pooled data:

$$EWLR = \left(1.84 \cdot 10^{-5} \times {T_{air}}^3\right) - \left(7.73 \cdot 10^{-4} \times {T_{air}}^2\right) + (7.52 \cdot 10^{-3} \times T_{air}) + 0.06$$

Metabolic water production was calculated assuming that one mole of water is produced for each mole of carbon dioxide produced. The relationship between *MWP* and T_{air} in our pooled data was:

$$MWP = \left(-1.46 \cdot 10^{-4} \times {T_{air}}^2\right) + (7.76 \cdot 10^{-3} \times T_{air}) + 0.014$$

Required water stores include both crop contents, hemolymph, and cellular water, and are, in sum, the amount of water honey bees require to live. Nectar- and water-foraging honey bees typically leave the hive to forage with 0.5 to 3 µl of nectar in their crops, with approximately 60% of this nectar being water (Visscher *et al*., 1996; Harano *et al*., 2013). Resting honey bees die at a water content of ~74% (Burdine & McCluney, 2019), suggesting that a 70 mg bee may lose at most 18 mg of water before death, so I used this as our estimate of required water stores.

High air temperatures may limit honey bee foraging due to desiccation. Between 20 and 32°C air temperature, metabolic water production more than compensates for evaporative water loss, allowing honey bees to fly without threat of death by desiccation

(Fig. 4.4). However, in dry air, desiccation-limited flight durations strongly decline as air temperatures rise above 33°C (Fig. 4.4). While foraging in 40°C dry air, a 70 mg honey bee loses water at about 0.3 mg·minute^{-1} (Fig. 4.4), while producing metabolic water at about 0.09 mg·min^{-1}, which means that the water supply in the crop will be exhausted in approximately 0.5 min. A 70 mg forager flying at 40°C air temperature will desiccate to its critical water content (i.e., loss of 18 µl of water; Burdine & McCluney, 2019) after about 1.5 hours (Fig. 4.4). At air temperatures of 46°C, bees will desiccate to death in just over 30 min (Fig. 4.4), near the duration of an average foraging trip for a honey bee (Winston, 1991).

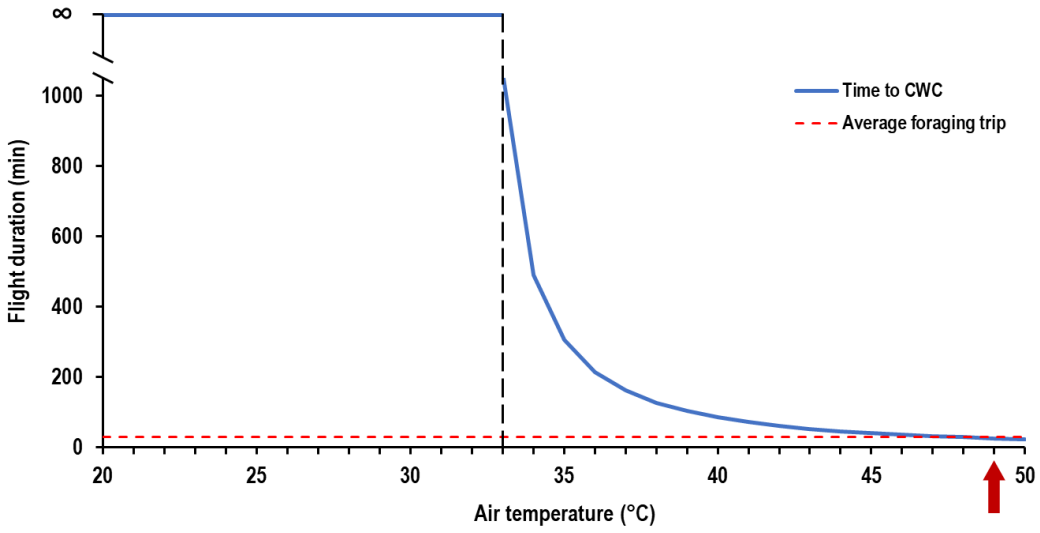

Figure 4.4. The length of time an unloaded forager (70 mg) can fly at a given air temperature before reaching critical water content (CWC) when flying in dry air (blue line). Desiccation-limited flight duration above 33°C air temperature = (18 µl H_2O) · [((-1.46x10^{-4} · T_{air}^2) + (7.76x10^{-3} · T_{air}) + 0.014) – ((1.84x10^{-5} · T_{air}^3) – (7.73x10^{-4} · T_{air}^2) + (7.52x10^{-3} · T_{air}) + 0.060)]$^{-1}$. The red dotted line represents the average foraging trip for a honey bee (30 minutes; Winston, 1991). The red arrow denotes the upper critical thermal limit for honey bees at rest (approximately 49°C; Kovac *et al.*, 2014; Burdine & McCluney, 2019).

Kinematics Experiment

Wingbeat Frequency

Honey bees flying at 40°C air temperature have lower wingbeat frequencies (211.0±4.7 Hz; mean±95% CL) than bees flying at 25°C air temperature (234.7±4.2 Hz; generalized linear model: $n = 89$, $\chi^2 = 3.9$, $p = 0.047$; Fig. 4.5), but only hot bees increase wingbeat frequency to carry heavier nectar loads (25°C – linear regression: $F_{1,41} = 1.7$, $p = 0.2$; 40°C – linear regression: $n = 46$, $F_{1,44} = 13.8$, $p < 0.001$).

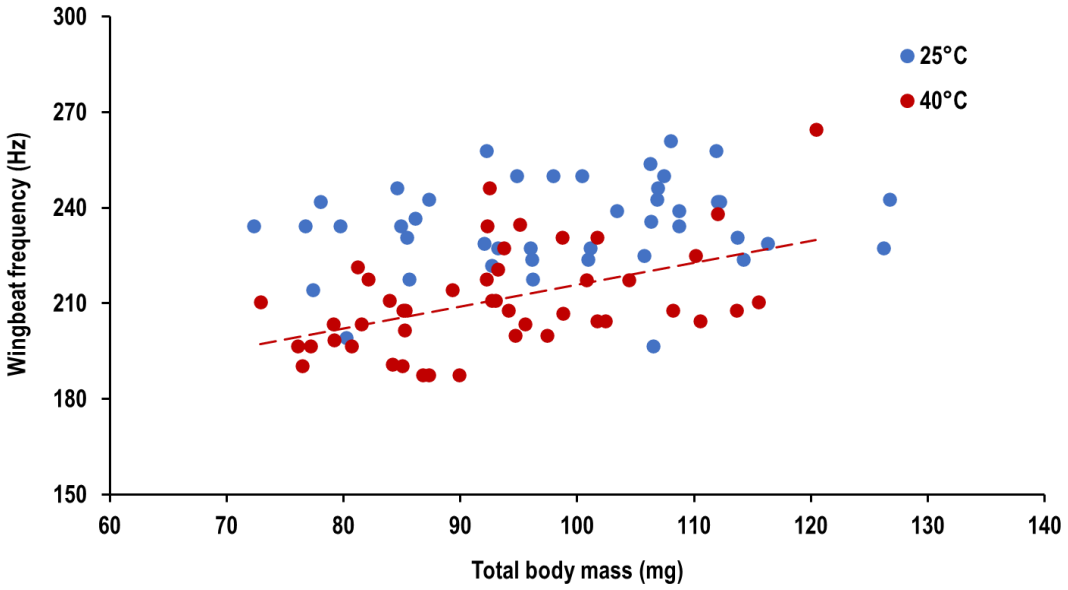

Figure 4.5. Air temperature affected the response of wing beat frequencies to loading. Wingbeat frequency increased with load for bees flying at 40°C, but not at 25°C air temperature. Honey bees flying at 40°C air temperature had lower wingbeat frequencies than bees flying at 25°C. Total body mass = mass of the bee + mass of carried nectar. Each point represents an individually measured bee. Fitted line denotes statistical significance (40°C: y = 0.68x + 147.1, $R^2 = 0.24$).

Stroke Amplitude

Nectar foragers flying at 40°C air temperature had higher stroke amplitudes (mean±95% CL: 90.0±4.1°) than bees flying at 25°C (98.7±3.1°; generalized linear model: $n = 89$, χ^2

= 17.3, $p < 0.001$; Fig. 4.6 inset). Bees flying at both 25 and 40°C air temperature increased stroke amplitude to carry heavier nectar loads (generalized linear model: n = 89, $\chi^2 = 10.4$, $p < 0.01$; Fig. 4.6).

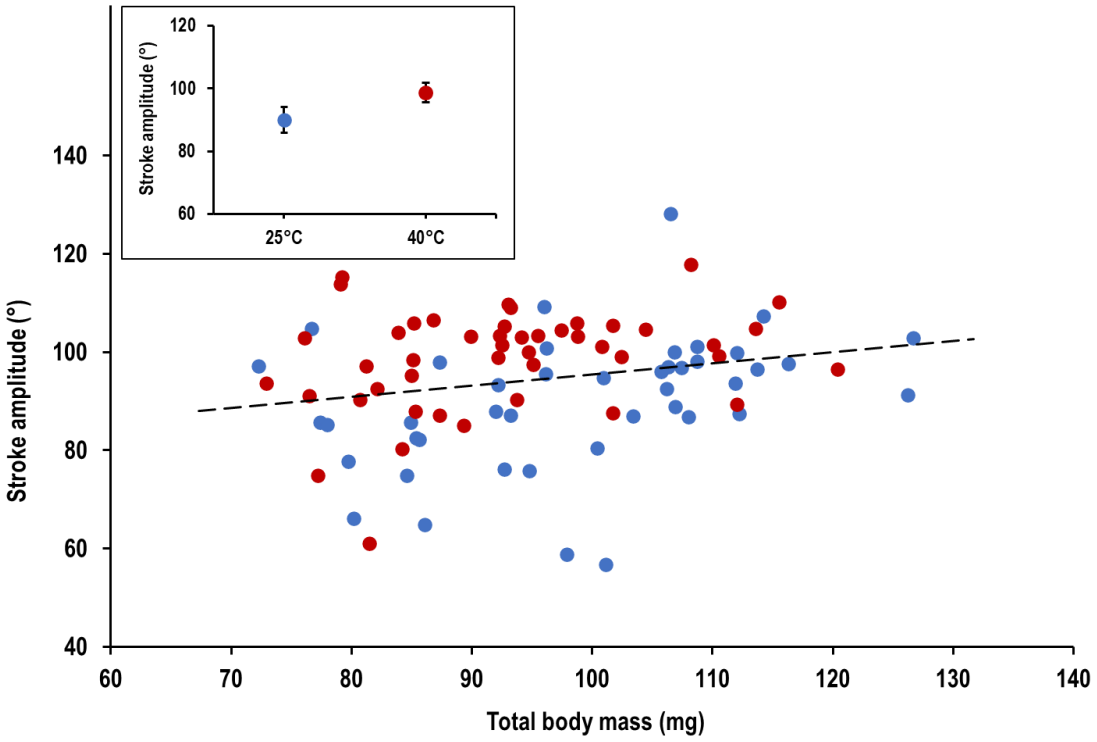

Figure 4.6. Honey bees flying at 40°C air temperature had larger stroke amplitudes (°) than bees flying in 25°C (inset), and bees at both 25 and 40°C increased stroke amplitude (°) to carry heavier nectar loads. Symbols in the inset graph represent the mean ± 95% CL. For the generalized linear regression plot, each point represents an individually measured bee. Fitted line denotes statistical significance (pooled data: y = 0.23x + 72.7; $R^2 = 0.05$).

Translational Power Proxy

Honey bees flying at 40°C air temperature had higher translational power proxies than foragers flying at 25°C air temperature (generalized linear model: n = 89, $\chi^2 = 5.9$, p = 0.02; Fig. 4.7 inset). Nectar foragers flying at both 25 and 40°C air temperatures

increased translational power production to carry heavier nectar loads (generalized linear model: $n = 89$, $\chi^2 = 14.8$, $p < 0.001$; Fig. 4.7).

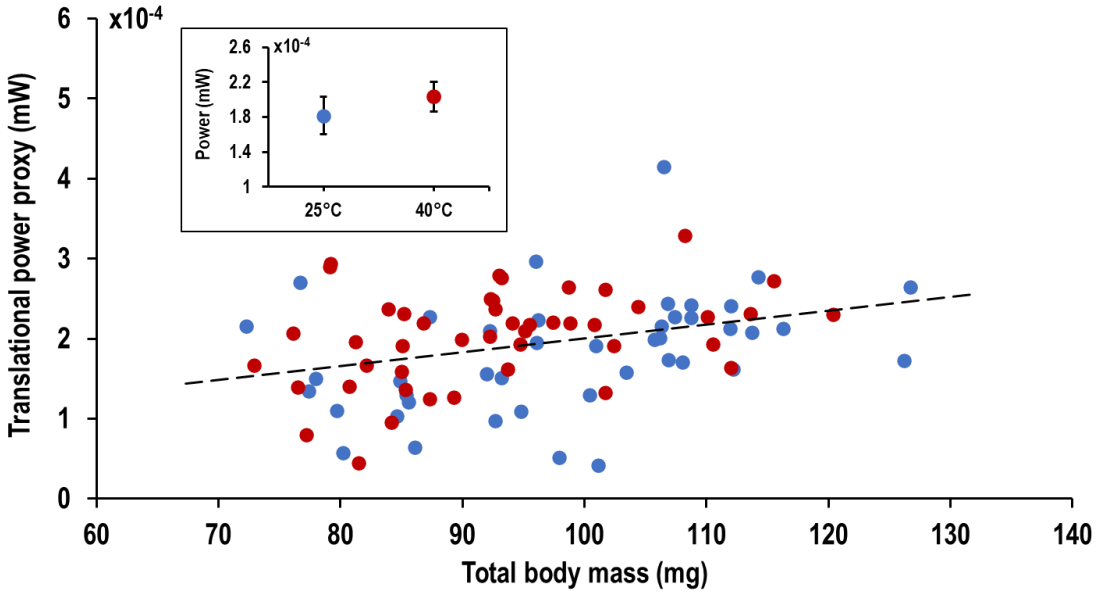

Fig. 4.7. Honey bees flying at 25 and 40°C air temperature both increased translational power production to carry heavy nectar loads (air temperature x total body mass interaction – generalized linear model: $\chi^2 = 0.04$, $p = 0.83$; large graph). Each point represents an individually measured bee. The fitted line denotes statistical significance for the independent effect of nectar load on the translational power proxy (y = 1.7E-06x + 2.7E-05, $R^2 = 0.11$).

Discussion

Honey bees can alter their wing kinematics to reduce metabolic heat production and the risk of overheating (Figs. 4.1, 4.5, 4.6). Depression of wing beat frequency combined with increased stroke amplitude allowed bees to generate more translational power and carry heavy nectar loads with significantly reduced cost or metabolic heat production (Figs. 4.1, 4.7). These mechanisms for reductions in metabolic heat production contribute

approximately equally with increased evaporative heat loss to preventing overheating (Fig. 4.3). Nonetheless, the required high rates of evaporation are likely to begin to limit foraging times in dry air temperatures over 45°C (Fig. 4.4). Thus, while adult honey bees can survive higher temperatures for short periods, their capacities to function as flying pollinators will be restricted to lower temperatures than CT_{max} (Fig. 4.4).

I show that both nectar-loaded and unloaded honey bees suppress wingbeat frequencies and flight metabolic rates at high air and flight muscle temperatures, aiding in thermoregulation (Figs. 4.1B,4.3,4.5). Remarkably, honey bees in the respirometry trials flying at 40°C air temperature were able to carry loads up to 60% of their body mass without heating up or incurring significant metabolic cost (Fig. 4.1). Bees were also able to avoid heating up past 45°C by dramatically increasing evaporative water loss (Fig. 4.2). Foragers flying at an air temperature of 40°C avoided overhearing by decreasing flight metabolic heat production (11.5 mW) and increasing evaporative heat loss (10 mW) (Fig. 4.3). Increasing stroke amplitude (Fig. 4.6) allowed bees flying at 40°C air temperature to maintain power production (Fig. 4.7) to compensate for behaviorally decreasing wingbeat frequency (Fig. 4.5) to reduce metabolic heat gain (Fig. 4.3).

Our data confirm that earlier observations of declining honey bee flight metabolic rate at high flight muscle temperatures, associated with a fall in wingbeat frequency (Harrison *et al*., 1996; Roberts & Harrison, 1999; Fig. 4.5). These data suggest active modulation of flight kinematics to vary metabolic heat production. The observation that wingbeat frequencies are high and invariant with load at an air temperature of 25°C suggests that bees select an inefficient kinematic strategy when flying unloaded in cool air, perhaps to warm themselves toward the optimal temperature for metabolic rate of

39°C (Glass & Harrison, 2022). The depression of metabolic rate is critical for bees to be able to fly for extended periods in the heat. If bees maintained the same metabolic rates at 40°C air temperature as at 30°C air temperature, to keep their flight muscles near 45°C (Fig. 4.1), they would need to evaporatively cool by double the amount. An unloaded honey bee (70 mg) flying at 40°C air temperature that did not depress its metabolic rate would be forced to increase evaporative to 0.6 mg·minute^{-1}, cutting the bee's maximal flight time to about 45 min at 40°C, and below 15 min at 46°C, before lethally exhausting its water reserves (Fig. 4.4).

The mechanisms by which varying flight kinematics translate to varying metabolic heat production remain unclear. Altshuler and colleagues (2005) showed that honey bees generate significant non-steady forces associated with wing rotation that decrease as stroke amplitude increases. In addition, Sane and Dickinson (2001) showed that as stroke amplitude increases, lift-to-drag forces increase. Together these data suggest that the kinematic strategy (i.e., higher stroke amplitude and lower wing beat frequency) shown by hot bees may reduce metabolic costs by reducing the relative importance of rotational relative to translational wing forces and improving lift-to-drag ratios. However, this seems unlikely to be the entire story, since bees flying in low density air at an air temperature of 25°C have increased stroke amplitude and higher flight metabolic rates (Altschuler *et al*., 2005; Glass & Harrison, 2022). Thus, the lower metabolic rates shown by bees flying in 40°C air seem to be associated with both higher stroke amplitude and high flight muscle temperature. Possibly, higher flight muscle temperatures enable higher elastic energy storage and reduction of inertial costs. Resilin exhibits phase changes in vitro above 60°C (Quin *et al*., 2012; Li *et al*., 2015), suggesting

the possibility that some changes in elastic properties occur in the thermal range of hot bees. Fast hopping wallabies show relatively no change in metabolic rate as mass is added to their pouches, likely due to their high conservation of elastic energy storage in their hindleg tendons (Baudinette & Biewener, 1998). Plausibly, the increased stroke amplitude with heavier loads at high air temperatures allows greater elastic energy storage in the flight muscle, thorax, or wing hinge, enabling higher load carriage without metabolic cost.

Even though bees with flight muscle temperatures of 45°C could carry nectar loads up to 60% of their body mass, it remains possible that high air and flight muscle temperatures limit the load-carrying capacity of honey bees. The force production of tethered bees declines as flight muscle temperatures rise above 40°C (Coelho, 1991), and I did not explicitly design these experiments to test whether load-lifting capacity is reduced as temperatures rise about 40°C. Undertaker bees can fly while carrying other bees, likely weighing near their body mass. Nonetheless, our data suggest that metabolic rate and lift production can be, to a substantial extent, uncoupled, and that bees with flight muscle temperatures of 45°C can carry nectar loads up to 50 mg, well above typically observed nectar loads of 30 mg or less (Winston, 1991).

Several caveats must be admitted regarding potential weaknesses of our conclusions. First, CO_2-production rates in the respirometry trials were averaged over 10 seconds of flight, whereas wing kinematics were analyzed over approximately 0.05 second. Plausibly, in the time-averaged respirometry trials, other behaviors may also be varying with temperature, such as the amount of side-to-side movement, or the distance of bees from edges. Moreover, bees in the respirometry trials were flown in a relatively small

chamber (i.e., ~350 ml cylindrical chamber) with relatively high air flow rates through the chamber, creating the possibility of turbulence and edge effects that might alter flight behavior and cost. It is also not impossible that the Arizona and Davis bees differed in their thermal biology, though we found similar flight muscle temperatures and wing beat frequency changes with temperature.

The measurements in this study allowed us to assess thermal and desiccation limits on honey bee foraging. If 49°C is taken as CT_{max} for resting bees (Johnson *et al*., 2023), thorax temperatures are predicted to be 1-2°C above air temperatures at this air temperature (Roberts & Harrison 1999), suggesting that at least brief periods of flight should be possible at air temperatures less than 48°C. However, under dry conditions, at air temperatures of 45°C or higher, bees will desiccate to death at normal foraging trip durations if unable to find nectar or water, suggesting that desiccation can limit foraging at much lower air temperatures than CT_{max} under dry conditions. If humidity approaches 100%, evaporative heat production will be less effective, and the elevation of thorax temperature above air temperature will increase exponentially at air temperatures above 40°C, suggesting that thorax temperatures of flying honey bees will approach CT_{max} at air temperatures as low as 42-43°C. Wind speed and solar radiation will also influence heat limitations on foraging for honey bees and other pollinators. Empirical tests of the interactions between humidity, temperature, solar radiation, and wind speed on flight metabolic rate, thermoregulation, and load carriage capacities will be required to predict honey bee foraging across the full range of environmental conditions. However, our data definitively show that CT_{max} values overestimate the temperatures at which heat will limit foraging. It is also plausible that foraging success may decline at even lower air

temperatures if elevated body temperatures or declining body water content impede the complex behavioral tasks of foraging.

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CHAPTER 5

CONCLUSIONS

The increasing losses of honey bee colonies in North America from agrochemical exposure (Johnson *et al*., 2010; Sponsler *et al*., 2019) and climatic warming (Halsch *et al*., 2021) will undoubtedly have catastrophic consequences on human agriculture. My studies investigating the effects of fungicide exposure and climatic warming emphasize our need to understand the causes and mechanisms for the loss of these crucial animals.

My study of fungicide ingestion on honey bee morphology and metabolic performance (Chapter 2) suggests that Pristine®-consumption, though reducing thorax mass (Fig. 2.1), does not affect flight performance and metabolism at field-realistic concentrations (Figs. 2.3, 2.4). Although we now know that Pristine® has a shocking number of negative, sub-lethal effects on honey bees (DeGrandi-Hoffman *et al*., 2015; Campbell *et al*., 2016; Liao *et al*., 2019; Fisher *et al*., 2021; Fisher *et al*., 2022), my study does not suggest that reduced thorax mass is contributing to the alarming increase in North American honey bee colony loss.

I showed that desiccation may pose a greater threat to honey bees than high air temperatures, especially when flying in dry conditions. Foragers were able to decrease wingbeat frequency (Fig. 4.5) and flight metabolism (Fig. 4.1) to reduce metabolic heat gain while increasing stroke amplitude (Fig. 4.5) to carry significant nectar loads (~60% of their own weight in nectar) with minimal increases in metabolic cost (Fig. 4.1). Honey bees use evaporative cooling to avoid overheating when body temperatures rose above

40°C (Fig. 4.3), but this ability becomes detrimental at air temperatures of 45°C and above (Fig. 4.4).

With the human population growing exponentially and with honey bee colony losses at an all-time high, we literally cannot afford to sit by and deal with the consequences. For the animal responsible for pollinating over a third of what we eat and generating billions of US dollars of revenue, we need to identify and mitigate the factors contributing to honey bee colony losses now to ensure that current and future generations have enough to eat in a changing world.

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APPENDIX A

CONSUMPTION OF FIELD-REALISTIC DOSES OF A WIDELY USED MITO-TOXIC FUNGICIDE REDUCES THORAX MASS BUT DOES NOT NEGATIVELY IMPACT FLIGHT CAPACITIES OF THE HONEY BEE (*APIS MELLIFERA*) PUBLISHED IN *ENVIRONMENTAL POLLUTION*

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Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*)☆

Jordan R. Glass [a, *], Adrian Fisher II [a], Jennifer H. Fewell [a], Gloria DeGrandi-Hoffman [b], Cahit Ozturk [a], Jon F. Harrison [a]

[a] School of Life Sciences, Arizona State University, 427 East Tyler Mall, Tempe, AZ, 85281, USA
[b] USDA-ARS Carl Hayden Bee Research Center, 2000 East Allen Road, Tucson, AZ, 85719, USA

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ABSTRACT

Commercial beekeepers in many locations are experiencing increased annual colony losses of honey bees (Apis mellifera), but the causes, including the role of agrochemicals in colony losses, remain unclear. In this study, we investigated the effects of chronic consumption of pollen containing a widely-used fungicide (Pristine®), known to inhibit bee mitochondria in vitro, which has recently been shown to reduce honey bee worker lifespan when field-colonies are provided with pollen containing field-realistic levels of Pristine®. We fed field colonies pollen with a field-realistic concentration of Pristine® (2.3 ppm) and a concentration two orders of magnitude higher (230 ppm). To challenge flight behavior and elicit near-maximal metabolic rate, we measured flight quality and metabolic rates of bees in two lower-than-normal air densities. Chronic consumption of 230 but not 2.3 ppm Pristine® reduced maximal flight performance and metabolic rates, suggesting that the observed decrease in lifespans of workers reared on field-realistic doses of Pristine®-laced pollen is not due to inhibition of flight muscle mitochondria. However, consumption of either the 230 or 2.3 ppm dose reduced thorax mass (but not body mass), providing the first evidence of morphological effects of Pristine®, and supporting the hypothesis that Pristine® reduces forager longevity by negatively impacting digestive or nutritional processes.

1. Introduction

Insect pollinators are in decline globally, due in part to regular exposure to agrochemicals, such as fungicides (Johnson et al., 2010; Sponsler et al., 2019). Although regulatory agencies of most countries require testing of lethal acute and chronic effects of agrochemical exposure (LD_{50}; Iwasa et al., 2004; US EPA, 2014; Tosi and Nieh, 2019), the fitness of pollinators may also be affected by sub-lethal effects of agrochemicals, which often have not been tested for in currently registered pesticides (Mullin et al., 2010). In this study, we investigate the effects of consumption of a widely used fungicide, Pristine®, on the morphology and flight performance of chronically exposed honey bees in field conditions.

The pollination services of insects not only contribute significantly to both agricultural (US$14.2–23.8 billion) and industrial sectors (US$10.3–21.1 billion; Chopra et al., 2015), but also play vital roles in biodiverse ecosystems (Ollerton et al., 2011), the loss of which will undoubtedly have strong negative economic and ecological impacts (Gallai et al., 2009; Calderone, 2012). The most widely used managed pollinator is the honey bee (*Apis mellifera*), which is necessary for the pollination of many crops including berries, almonds, pome, and stone fruits. Although honey bees are not considered threatened, North American beekeepers are losing more than 30% of their colonies each year (vanEngelsdorp et al., 2007, 2011; Steinhauer et al., 2014; Kulhanek et al., 2017), increasing the challenge of keeping up with agricultural demand (Aizen and Harder, 2009).

Exposures to insecticides (e.g., neonicotinoids and phenylpyrazoles) have been implicated as a major contributor to pollinator decline due to their toxicity, high frequency of use, and persistent accumulation in agricultural foraging environments (Iwasa et al., 2004; Vidau et al., 2011; Smalling et al., 2013; Goulson et al., 2015; Zhu et al., 2015; Tison et al., 2016; Fisher et al., 2018).

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* Corresponding author.
E-mail address: jrglass@asu.edu (J.R. Glass).

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Additionally, fungicides are very commonly encountered by pollinators, and there are studies demonstrating correlations between high levels of exposure to fungicides and poor colony health (Mullin et al., 2010; Pettis et al., 2013). Fungicides are often considered relatively safe for animals, including insect pollinators, due to their high acute contact and oral LD_{50}'s relative to their environmental exposure (Legard et al., 2001; Smalling et al., 2013; Ostiguy et al., 2019). Typically, fungicides are only considered hazardous when paired with other agrochemicals, such as insecticides (Pilling and Jepson, 1993; Pilling et al., 1995; Iwasa et al., 2004; Johnson et al., 2013; Tosi and Neih, 2019). However, little attention has been given to understanding the independent sublethal effects of fungicide exposure on honey bee health (except see: DeGrandi-Hoffman et al., 2015; Campbell et al., 2016; Liao et al., 2019).

Pristine®, a widely used fungicide, is frequently encountered by foraging honey bees, due to its common application on blooming flowers of nut, stone fruit, and fruit crops prior to obligatory bee pollination (Legard et al., 2001; Ostiguy et al., 2019). Pristine® has two active ingredients, the anilide fungicide, boscalid, and the strobilurin fungicide, pyraclostrobin, both of which inhibit mitochondrial respiration in fungal targets (constituting 25.2% and 12.8% of the formulated product by mass, respectively; Avenot and Michailides, 2007). The active ingredients of Pristine® have relatively low contact and oral toxicities for bees relative to the concentrations measured in honey bee hives (Ostiguy et al., 2019). However, chronic consumption of pollen containing concentrations of Pristine® similar or lower than those measured in pollen sampled from bees foraging in Pristine®-sprayed orchards reduced worker longevity, colony population size, and overwintering survival (Fisher et al., 2021). Additionally, Pristine® consumption in pollen at realistic field doses caused earlier foraging and more pollen foraging (Fisher et al., 2021).

The mechanisms of Pristine® effects on worker longevity and behavior are unclear. Because the active ingredients of Pristine® are mitochondrial toxins in honey bees (Campbell et al., 2016), they may have wide effects. Pristine® has been shown to reduce pollen digestion (DeGrandi-Hoffman et al., 2015), and the earlier foraging and greater pollen foraging documented by Fisher et al. (2021) suggests that Pristine® may impair digestive or nutritional processes. In support of this hypothesis, pyraclostrobin has recently been shown to damage the midgut epithelia of honey bees when fed to bees in the lab (da Costa Domingues et al., 2020; Tadei et al., 2020). However, as yet we lack any direct evidence that Pristine® inhibits honey bee growth, size, or nutritional status.

As a mitochondrial inhibitor, Pristine® might also be expected to have negative effects on activities requiring high metabolic rates, such as flight. For honey bees, the highest metabolic rates occur during flight while foraging, and these rates increase with the mass of load carried (i.e., nectar, pollen, or water; Wolf et al., 1989; Feuerbacher et al., 2003). Plausibly, by inhibiting flight muscle mitochondria, Pristine® might reduce the maximal flight metabolic rates of workers, impairing foraging or the ability to fly during stressful conditions such as windy or cold weather. In support of this hypothesis, honey bee foragers fed sugar water containing boscalid (10 ppm) exhibited lower wing beat frequencies relative to controls when tethered and flown in an indoor flight treadmill (Liao et al., 2019). However, one prior study found no effect of consumption of 6.6 ppm Pristine® on metabolic rate during hovering flight of honey bees reared in the lab (Campbell et al., 2016). Because hovering flight in normodense air (i.e., 1.288 kg m^{-3}) does not elicit maximal metabolic performance (Roberts et al., 2004), it is plausible that Pristine® has negative effects on maximal flight capacities, which were not tested in the Campbell et al., (2016) study. A decrease in maximal metabolic performance induced by an agrochemical could have many potential effects on foraging bees, including reducing maximal load carriage or acceleration, capacities to escape predators, or to fly safely in windy conditions (Dillon and Dudley, 2004; Combes and Dudley, 2009; Buchwald and Dudley, 2010).

Unlike terrestrial or aquatic locomotion, during which graded work effort usually can be elicited by utilizing a treadmill (Seeherman et al., 1981) or a swim-flume (Norin and Clark, 2016), a difficulty in investigating the physiology of flight is the challenge of assessing maximal sustained performance (Ellington, 1984, 1985; Dudley and Ellington, 1990; Dickinson and Lighton, 1995; Josephson and Ellington, 1997; Chai et al., 1998, 1999; Chai and Dudley, 1999; Roberts et al., 2004). Increasing the mass of load carried increases flight metabolic rates (Wolf et al., 1989; Feuerbacher et al., 2003), but such experiments are time-consuming and poorly suited for ecotoxicology studies. Systematically decreasing air density - achieved by replacing nitrogen with helium in graded steps - provides an analog of a treadmill to measure increased aerobic performance during hovering flight, because lower air density increases power requirements of hovering for all animals yet tested (Chai & Dudley, 1995, 1996; Dudley, 1995; Chai et al., 1996; Dudley and Chai, 1996; Dudley and Winter 2002; Roberts et al., 2004). For example, carpenter bees (*Xylocopa varipuncta*) exhibited a 33% increase in flight metabolic rate when air density was decreased by ~64%, (Roberts et al., 2004).

Because Pristine® has been suggested to inhibit protein digestion or absorption, we tested for developmental effects of chronic consumption of Pristine® on thorax and body mass. To test for the effects of Pristine® consumption on flight capacities, we measured flight metabolic rates and flight quality of honey bees induced to fly in a range of air densities, including low air densities that likely require near-maximal flight performance. We tested the effects of two concentrations of Pristine®, 2.3 and 230 ppm, which represent the lowest concentrations and a value an order of magnitude higher than the highest concentration of Pristine® measured in corbicular pollen of bees pollinating sprayed almond orchards (Table S1; Fisher et al., 2021). The Pristine® was administered in pollen to field colonies, simulating the type of exposure experienced if a colony was pollinating an almond orchard sprayed with Pristine®, over a time period encompassing both larval and the young adult development period when pollen is consumed. Specifically, we wished to partially test two hypotheses for the decreased longevity of honey bee workers in colonies fed field-realistic concentrations of Pristine® in pollen (Fisher et al., 2021): 1) Pristine® impairs flight metabolic rate and capacity, and 2) Pristine® impairs growth/size of workers.

2. Methods

2.1. Honey bee colony initiation and maintenance

Details of colony maintenance and experimental design are provided in Fisher et al. (2021), with a basic description provided here. Colonies of the Italian honey bee (*Apis mellifera ligustica*) were started from a 1.59 kg bee packages (~10,000 bees) obtained from Pendell Apiaries, Inc. in Stonyford, CA (39.376956, −122.558801). To ensure that colonies were not exposed to comb with previous agrochemical content, each hive was initially stocked with five wooden frames outfitted with a plastic worker cell template foundation, so that workers constructed new comb. All hives were supplied with 30% sugar syrup for the first three weeks after their establishment to assist comb building. Hives were also outfitted with internal pollen traps to restrict access to pollen collected in the surrounding environment (see Hoover and Ovinge, 2018). Hives were maintained with 50 g pollen patties, using pollen collected

from desert hives far from agriculture. The pollen patties consisted of a 1:1:1 ratio of dry pollen, sucrose (Great Value) and fondant sugar (ABC Cake Decorating, Phoenix, AZ; 8% inverted); approximately 8% of each pollen patty consisted of deionized H_2O which was added after the dry ingredients were thoroughly mixed. To document the extent to which hives were exposed to other pesticides, we collected bee bread samples from each hive, pooled these into single samples for each treatment, and had these analyzed by the USDA-AMS National Science Laboratory. Pesticide residue analyses found no agrochemicals present above detection levels other than a few herbicides: diuron, fluometuron, and hexazinone that occurred in levels up to 12 ppb. The hives were treated with Amitraz for mites in the month before our experiments, as is common in U.S. beekeeping, and a metabolite (DMPF) of amitraz was detected at 147 ppb. None of these levels differed among treatments.

2.2. Fungicide treatment and dose

The complete experimental design is described in Fisher et al. (2021); here we briefly describe the protocols. Doses were based on measurements of the concentrations of boscalid and pyraclostrobin in pollen sampled from bees foraging in California almond orchards in 2010 and 2011 (Fisher et al., 2021). Pollen was collected from bees throughout the blooming period, and thus measured levels estimate the average, rather than maximal or minimal values of fungicide which likely vary with time after spray. These measures suggested that bees pollinating almond orchards collect pollen containing 3–24 ppm Pristine® (Fisher et al., 2021). To feed colonies specified doses of fungicide, we mixed Pristine® (BASF Corporation, Research Triangle Park, NC) into pollen patties which were fed to colonies equipped with pollen excluders to force the bees to consume the Pristine®-containing pollen. Colonies were reared on these treated pollen patties from May 2018 to November 2019. For this experiment, nine colonies were fed pollen patties containing 0, 2.3 or 230 ppm Pristine®, for a total of three colonies per treatment. All pollen patties were provided *ad libitum*, with a new patty supplied as soon as the previous patty was entirely consumed. If the pollen patty was not completely consumed within one week, it was replaced to maintain freshness. Pollen patties were weighed each week to measure weekly pollen and Pristine® consumption for each hive. To calculate per bee dose from pollen patty consumption, we assessed the number of colony pupal and larval cells and workers during the study for each hive, and used literature values for per larva and per worker pollen consumption (details in Fisher et al., 2021). Bees consume pollen during the latter larval and young adult stage, and cease pollen consumption after initiation of foraging, so age of the forager tested likely did not affect Pristine® dose. The per larvae and per adult doses for each treatment group of Pristine® and the active ingredients, boscalid and pyraclostrobin, are provided in Table 1.

Table 1
Concentrations of Pristine®, boscalid, and pyraclostrobin in the pollen patties provided to honey bee hives, and the per larva and per adult dose of each compound in the two Pristine® treatments used. Dose calculations are from Fisher et al. (2021).

	Pristine®	Boscalid	Pyraclostrobin
Pollen patty, ppm	2.3	0.6	0.3
Per larva dose, ng	1.0	0.25	0.13
Per adult dose, ng	79.7	20.1	10.2
Pollen patty, ppm	230	60	30
Per larva dose, ng	89.9	22.7	11.5
Per adult dose, ng	7194	1813	921

2.3. Outgoing forager collection

To test for the effects of Pristine® consumption on flight capacities, we measured flight metabolic rates and flight quality of honey bees from three colonies of three of the five treatment groups used in the Fisher et al., (2021) study (i.e., 0, 2.3, and 230 ppm; total $N = 9$ hives). Beginning in November 2019, outgoing foragers (Control: $n = 90$; 2.3 ppm: $n = 82$; 230 ppm: $n = 83$) were captured when leaving the colony (between 900 and 1700) by holding an opened plastic bag (~950 ml) approximately 15 cm from the colony entrance. After a single forager flew directly into the opened bag, it was sealed, and the bee was transported within 2 min to a temperature-controlled laboratory room, where air temperature was regulated by a space-heater (36.5 ± 0.5 °C) and using a thermocouple and Expedata (Sable Systems, Las Vegas, NV). Bees were measured immediately after being transported into the laboratory (see below). To control for extraneous possible effects, a random number generator (www.randomizer.org) was used to determine the order and time in which the colonies were sampled.

2.4. Measuring flight metabolic rate, thorax temperatures and flight behavior at three air densities

Once in the lab, the collected bee was immediately placed into a cylindrical, transparent acrylic flight chamber (350 ml). The flight chamber was sealed and covered with a dark cloth for 2 min, to encourage reduced activity of the bee. The gases from the flow meters delivered air (2 L min^{-1}) sequentially and continuously through a $CaSO_4$ and soda lime column to remove H_2O and CO_2, the reference cell of the LI-COR 6262 CO_2/H_2O analyzer (Lincoln, NE, USA), the flight chamber, a small column of $MgSO_4$ (to remove metabolic water), and the sample cell of the LI-COR. Differential analog output from the LI-COR was digitized (Sable Systems UI-2) and recorded each second (Expedata, Sable Systems, Las Vegas, NV). The LI-COR was calibrated using 252 ppm CO_2 and Ultra-Zero calibration gases, and baseline recordings were taken before and after each measurement period by bypassing the flight chamber.

Foragers were randomly assigned to one of three variable-density gas mixtures [0.441 kg m^{-3} ("heliox"), 0.779 kg m^{-3} ("intermediate"), or 1.288 kg m^{-3} ("normodense") for their flight metabolic rate measures; Table 2]. Gas mixtures were created by using cylinders of pure O_2, N_2, and He, which were regulated by a Sable Systems FB8 flow meter (Las Vegas, NV, USA) specifically calibrated for the different gas densities using a soap-film bubble meter (Levy, 1964). The different gas mixtures did not affect the calibration of the LI-COR 6262 CO_2/H_2O analyzer (Lincoln, NE, USA).

While the bee sat in darkness, we flushed the chamber for 2 min prior to the flight trial, allowing CO_2 levels from the chamber to reach a low, stable level. Hovering flight was encouraged for 2 min by shining a 150W dual goose-neck Fiber Optical Illuminator (China) over the chamber. Bees that landed were immediately encouraged to fly or attempt to fly by gently tapping and inverting the chamber. Flight behavior was categorized based on ability, duration, and control (i.e., quality). Flight quality was categorized and ranked as: 1 — no flight, 2 — flapping wings with very brief periods of flight (<3 s), 3 — intermittent hovering characterized by

Table 2
Variable-density gas mixtures used as an aerial treadmill at 36 °C.

Gas mixture	% O_2	% N_2	% He	Density (kg·m^{-3})
1	21	79	0	1.288
2	21	31.6	47.4	0.780
3	21	0	79	0.441

frequent crashing (i.e., bee usually ends upside down), 4 — intermittent hovering characterized by frequent controlled landing (i.e., bee gently lands on its feet), or 5 — continual, stable hovering. The zenith function in Expedata (Sable Systems, Las Vegas, NV) was used to locate and average the 10 s with the highest CO_2 readings during each trial. Flight CO_2 emission rates (ml·hr^{-1}) during that highest CO_2 emission period were calculated by multiplying the differential CO_2 fraction by the STP flow rate in through the flight chamber. These values were later converted to milliwatts (mJ sec^{-1}) by converting the time units, then converting these values to joules (Lighton, 2018), assuming a respiratory quotient of 1 (Rothe and Nachtigall, 1989; Feuerbacher et al., 2003). After flight and CO_2 emission rates were measured, the bee was immediately shaken into a plastic bag, which was flattened to restrict the bee's movement. Thorax temperature was measured by inserting a Physitemp model MT29/1 hypodermic microprobe (Clifton, New Jersey, USA; 29 gauge, time constant — 0.025 s) through the bag and into the center of the thorax. Temperatures were recorded with a Pico Technology USB TC-08 Thermocouple Data Logger (Tyler, TX, USA). Thorax temperatures were measured within 3 s of cessation of flight, and the highest temperature reported by the thermometer was recorded. After measurement, the bee was weighed (±0.1 mg) using an A&D HR-60 Analytical Balance (Tokyo, Japan) and stored at −20 °C. Thorax masses were measured by dissecting the head and abdomen from the thorax and taking its mass. The wings and legs were included in the mass of the thorax to avoid inconsistencies of appendage removal.

2.5. Statistical analysis

Data were tested for normality and analyzed using R (3.6.2; R Foundation for Statistical Computing, Vienna, Austria). Two-tailed significance was determined at $\alpha = 0.05$. We used linear mixed-effects models to test the independent and interactive effects of Pristine® treatment, gas density, and thorax and body mass on flight metabolic rate (milliwatts) and thorax temperature. To investigate the effects of the different treatments flown in heliox (0.441 kg m^{-3}; Fig. 2A), we used a linear model with a Bonferroni-corrected *post hoc* test. We also used linear mixed-effects models to investigate the effects of Pristine® treatment on the body, head, thorax, and abdominal masses of foraging bees. Linear models were used to investigate the relationship between log-transformed body and thorax masses, as well as the relationship between metabolic rate and thorax mass. We used an ordinal logistic regression model analysis to test the effects of our treatment variables on flight behavior. Foragers heavier than 0.1 g were excluded from these analyses, as these individuals were likely returning or new foragers that had not evacuated their hindguts, as they had large crop and hindgut loads when dissected.

3. Results

Pristine® consumption significantly reduced thorax masses by approximately 5% (treatment: linear mixed-effects model, $n = 218$, $\chi^2 = 24.85$, $P < 0.0001$; Fig. 1), and significantly decreased thorax:body mass ratios (treatment: linear mixed-effects model, $n = 218$, $\chi^2 = 7.14$, $P = 0.008$). Pristine® had no significant effect on body mass (treatment: linear mixed-effects model, $n = 218$, $\chi^2 = 0.14$, $P = 0.71$). Plots of log thorax mass vs. log body mass scaled hypometrically (*t*-test: $n = 218$, $t = 10.11$, $P < 0.0001$), meaning that bees with a heavier body mass had relatively smaller thoraxes ($n = 218$, slope: 0.42, $R^2 = 0.32$; Fig. S1).

Flight metabolic rates increased with increasing thorax mass and decreasing gas density, with thorax mass becoming less important as gas density decreased (Fig. 2; Table 3). Similar results were found when body mass was tested as a predictor of flight metabolic rate (Fig. S2A; Table S2). Bees with heavier body masses also had significantly higher thorax temperatures relative to lighter bees flying in all gas densities (linear mixed-effects model: $n = 218$,

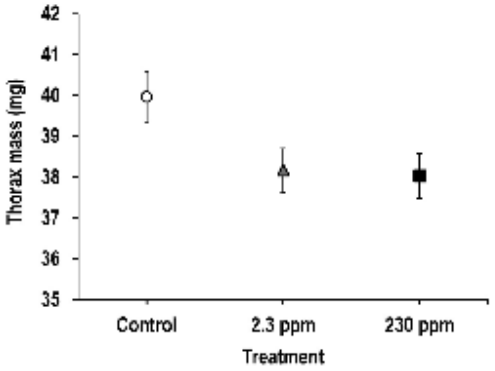

Fig. 1. Chronic consumption of pollen containing 2.3 ppm or 230 ppm Pristine® reduced thorax masses (linear mixed-effects model: $P < 0.0001$). Each point and accompanying error bars represent the mean±95% CL.

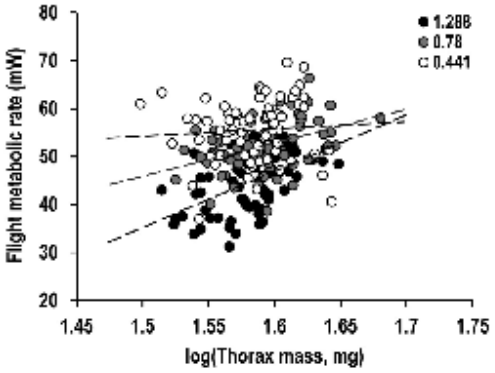

Fig. 2. Flight metabolic rates increased with thorax mass and in lower density air (kg·m^{-3}) in an interactive manner, such that the slope of flight metabolic rate on thorax mass declined at lower densities (linear mixed-effects model $P = 0.0002$). Each point represents an individually tested bee.

Table 3
Linear mixed-effects model results for the independent and interactive effects of gas density (kg·m^{-3}), treatment (control, 2.3 ppm, and 230 ppm), and thorax mass (mg) on the flight metabolic rates of honey bees at 36 °C.

Variable(s)	χ^2	*P*
Gas density	**137.8**	**< 0.0001 *****
Treatment	0.1	0.76
Thorax mass	**41.6**	**< 0.0001 *****
Gas density x Treatment	**13.7**	**< 0.001 *****
Gas density x Thorax mass	**14.2**	**< 0.001 *****
Treatment x Thorax mass	0.9	0.34
Gas density x Treatment x Body mass	1	0.32

linked to maximal physical performance in animals including bees (Wolf et al., 1989; Roberts et al., 2004; Weibel and Hoppeler, 2005), these data strongly suggest that sufficient Pristine® consumption will reduce the capacities of honey bees to carry loads and fly in severe weather. Future studies should examine effects of these higher concentrations of Pristine® to ensure that field exposures do not negatively impact honey bee flight capacities.

The helium-oxygen mixtures were effective in eliciting higher metabolic rates and for demonstrating effects of pesticide on flight and metabolic function (Figs. 2 and 4A). Flight metabolic rates of honey bees increased by ~36% as air density decreased by ~64% (Fig. 4A). The increase in flight metabolic rate we documented in heliox is similar to that shown for bees carrying near-maximal nectar loads (i.e., ~44%; Wolf et al., 1989), suggesting that we measured near-maximal flight metabolic rates. Lower air densities also reduced thorax temperatures of flying bees (Fig. 4B), likely due to the higher thermal conductance of helium than nitrogen (Reid et al., 1987). However, thorax temperatures of all bees were high (over 40 °C) relative to the thermal performance curve for honey bees (Coehlo, 1991), suggesting that this thoracic cooling did not limit metabolic performance. Helium will also increase the diffusivity of oxygen by 2.6-fold (Lide, 2004) in addition to lowering air density, potentially leading to increases in the partial pressure of oxygen at the tissue level. Two hours of exposure to 20% oxygen—80% helium caused mitochondrial swelling of rat myocardial tissue, raising concerns about the toxicity of these treatments (Ślubowski et al., 1987). However, there are multiple reasons to suspect that the heliox exposure during flight did not produce a serious physiological problem in honey bees. First, helium only affects diffusive, not convective transport of oxygen. Bees and other flying insects are known to heavily utilize convection for gas exchange during flight, based on observations of abdominal pumping (Weis-Fogh, 1967), and the fact that the critical PO_2 for flight metabolic rate is similar when PO_2 is changed by altering the fractional content of O_2 in N_2, and when the PO_2 is reduced by lowering barometric pressure (Withers, 1981; Joos et al., 1997). If diffusion through the gas-filled tracheae is the major mechanism of oxygen transport during honey bee flight, then lowering barometric pressure should have little effect on oxygen delivery or metabolic rate. Second, unlike most mammals, insects including bees experience substantial variation in tissue PO_2, ranging routinely between 2 and 3 kPa up to near 20 kPa (Komai, 2001; Harrison et al., 2020). This reduces the likelihood that a 2-min exposure of tissues to PO_2 levels up to 2.6-fold higher would cause damage. Third, if heliox mixtures damage mitochondria, we would expect to see either an elevation of CO_2 emission rates (due to mitochondrial uncoupling) or a decrease in CO_2 emission rates (due to damage). However, CO_2 emission rates rose to high levels during flight in heliox and fell quickly to resting levels after flight, suggesting that the observed elevation in CO_2 emission rates was completely due to flight and that there was no mitochondrial damage. Fourth, in carpenter bees, the increase in metabolic rates during flight in heliox are proportional to the increase in mechanical power output of the wings (Roberts et al., 2004), again suggesting that the mitochondria are undamaged by this treatment. Finally, since all treatment groups experienced the same exposures to helium, even if there is some damage associated with heliox exposure, this is unlikely to change our conclusions regarding Pristine® treatments.

We found a strong effect of chronic ingestion of both 2.3 and 230 ppm Pristine® on the thorax, but not body masses, of foraging adults (Figs. 1 and 2), providing important morphological support for the hypothesis that Pristine® impairs honey bee growth. Because earlier foraging can be induced by colonial nutritional stress, and because early foraging is often linked to reduced longevity in honey bee foragers (Rueppell et al., 2007), it is plausible that effects of Pristine® on digestive function are responsible for the effects of this pesticide on worker survival (Fisher et al., 2021). This hypothesis is further supported by evidence for poor protein digestion by bees fed Pristine® (DeGrandi-Hoffmann et al., 2015), and by recent evidence that pyraclostrobin damages the honey bee midgut (da Costa Domingues et al., 2020; Tadei et al., 2020). Future studies should comprehensively test for effects of Pristine® and its ingredients on digestion, absorption, nutritional status, growth, and size.

In this study, colonies consumed Pristine®-containing pollen for multiple months, whereas in agricultural conditions this is unlikely, raising the concern that though the concentrations of pesticide in pollen were field-realistic (Fisher et al., 2021), that the duration of exposure was not. However, it seems unlikely that this affects the magnitude of exposure. As outlined in Fisher et al. (2021), bees consume approximately 60 mg of pollen during the larval and adult development. As long as the exposure exceeds 3–4 weeks (the duration of honey bee development), bees developing during the exposure will consume similar amounts of pesticide in pollen. It is true that chronic exposure of the hive provides the potential for additional cuticular exposure, as Pristine® ingredients may accumulate in the wax. However, prior toxicological studies have shown that such cuticular exposures are not toxic except at orders of magnitude higher doses (Ostiguy et al., 2019). It is also plausible that chronic exposure to Pristine® has other effects on the hive, such as alterations in the various hive microbiomes. Future studies should examine the effects of shorter durations of exposure to Pristine® on field hives, and whether such indirect mechanisms of toxicity exist.

5. Conclusions

When honey bee colonies consume pollen containing field-realistic doses of Pristine® fungicide, worker longevity decreases (Fisher et al., 2021). Here we demonstrated that it is unlikely that the effects of Pristine® consumption on survival arise predominantly from impairment of flight capacity, as might be expected since the active ingredients of Pristine® are mitochondrial toxins and the highest metabolic rates occur during flight. However, Pristine® consumption reduced thorax mass, providing further support for the hypothesis that Pristine® affects digestive and nutritional physiology, impairing growth.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116533.

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Author contributions (Credit)

Jordan R. Glass: Conceptualization, Data Curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft. Adrian Fisher, II: Validation, Writing – Review & Editing. Gloria DeGrandi-Hoffman: Funding acquisition, Writing – Review & Editing. Jennifer H. Fewell: Funding acquisition, Writing – Review & Editing. Cahit Ozturk: Investigation, Writing – Review & Editing. Jon F. Harrison: Conceptualization, Funding acquisition, Methodology, Resources, Writing – Review & Editing, Supervision.

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APPENDIX B

THE THERMAL PERFORMANCE CURVE FOR AEROBIC METABOLISM IN A FLYING ENDOTHERM, *PROCEEDINGS OF THE ROYAL SOCIETY: B*

I, Jordan R. Glass, confirm that the co-author granted permission to use the following, previously published work in this dissertation.

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Author for correspondence:
Jordan R. Glass
e-mail: jrglass@asu.edu

THE ROYAL SOCIETY PUBLISHING

The thermal performance curve for aerobic metabolism of a flying endotherm

Jordan R. Glass and Jon F. Harrison

School of Life Sciences, Arizona State University, Tempe, AZ, USA

JRG, 0000-0001-7521-2698; JFH, 0000-0001-5223-216X

Performance benefits of stable, warm muscles are believed to be important for the evolution of endothermy in mammals, birds, and flying insects. However, thermal performance curves have never been measured for a free-flying endotherm, as it is challenging to vary body temperatures of these animals, and maximal flight performance is difficult to elicit. We varied air temperatures and gas densities to manipulate thoracic temperatures of flying honeybees from 29°C to 44°C, with low air densities used to increase flight metabolic rates to maximal values. Honeybees showed a clear thermal performance curve with an optimal temperature of 39°C. Maximal flight metabolic rates increased by approximately 2% per 1°C increase in thoracic temperature at suboptimal thoracic temperatures, but decreased approximately 5% per 1°C increase as the bees continued to heat up. This study provides the first quantification of the maximal metabolic performance benefit of thermoregulation in an endotherm. These data directly support aerobic capacity models for benefits of thermoregulation in honeybees, and suggest that improved aerobic capacity probably contributes to the multiple origins of endothermic heterothermy in bees and other insects.

1. Introduction

Why do some animals—including mammals, birds, and some fish and flying insects—thermoregulate at high body temperatures? While there are multiple ultimate hypotheses for the evolution of endothermy (defined as occurring when metabolism generates sufficient heat to significantly raise body temperature above ambient), each hypothesis agrees upon the fact that temperature has a hump-shaped effect on muscle and animal performance. This effect shows performance initially increasing slowly with temperature up to an optimum, and temperatures above this point suppressing performance [1,2]. Such patterns, called thermal performance curves, quantitatively define the benefits of maintaining body temperature near optimal, and are well documented for ectotherms [2–4]. Even though *in vitro* and *in situ* physiological studies have shown that the muscular and nervous system of endotherms are affected by temperature, only a few studies of running mammals have quantified the effects of body temperature on whole-body performance in endotherms, and none have measured a broad enough range of temperatures to provide a thermal performance curve [4–8]. The lack of thermal performance curves for endotherms makes it difficult to define the performance benefits of endothermic homeothermy and to assess the impact of climatic changes that force animals away from their optimal temperature [9]. Heterotherms, defined as animals that exhibit substantial variation in body temperature even when active, offer experimental possibilities for assessing thermal performance curves of endotherms, as these animals can function across a relatively broad range of body temperatures. In this study, we manipulated air temperature and gas density to determine the thermal performance curve of flight metabolic rates and to quantify the benefits of thermoregulation for maximal metabolic performance of the Italian honeybee (*Apis mellifera ligustica*).

The ability to maintain relatively high body temperatures gives several possible advantages to endothermic animals, flying insects included. For example, endothermic homeothermy facilitates success in a broader range of thermal

niches, such as improving locomotory performance in cool environments [10], and increasing development rates of offspring [11]. The maintenance of high body temperatures also facilitates high aerobic capacity, muscular power output, and sustained activity [12]. Insects, and some vertebrate endotherms, can save energy relative to homeothermic endotherms by allowing body temperatures to decrease under some circumstances, especially when not flying. These facultative endotherms benefit from higher aerobic performance during flight, while their heterothermy reduces overall costs over periods of flight alternating with non-flight. However, there are some disadvantages to endothermy. To support higher rates of metabolic functions, endothermic animals need to eat large quantities of food to meet energetic demands, compared with the intake of similarly sized ectotherms. Moreover, many endothermic animals often experience neurological and muscular pathologies if core body temperatures stray from optimal [2]. The specific selective forces and morphological requirements for the evolution of endothermy remain controversial, partly due to an incomplete fossil record, and partly due to challenges in quantifying the costs and benefits of endothermy [13].

Endothermic flying insects, such as honeybees, bumblebees, dragonflies, and some beetles and moths, are able to fly over a wide range of air temperatures [14]. In all cases, endothermy is made possible by the high metabolic heat production of the flight muscles. These animals primarily regulate the temperature of the thorax, but thermoregulation is imperfect [14]. Insect endothermic fliers thermoregulate using a variety of behavioural and physiological mechanisms, including varying evaporative cooling, heat transfer between the thorax and abdomen, and metabolic heat production [14,15]. Honeybees have moderate capacities to thermoregulate, with slopes of thoracic temperature on air temperature being reported as 0.18–0.41 [15–17]. The capacity of honeybees to fly at a wide range of air and flight muscle temperatures makes them an excellent species for assessment of their thermal performance curve.

Measurement of a thermal performance curve requires both variation in body temperature and assessment of maximal performance. For flying insects, maximal performance has been assessed with either load-lifting; flying in graded, low-density gases; or by varying optomotor stimulus [18–20]. Such studies have generally found that flight metabolic rate increases linearly with load, lower density air or greater optimotor stimulus (i.e. increasing virtual reality flight stimulation), with maximal metabolic power or mechanical power output values 25–40% higher than measured during unloaded, hovering flight [18–20].

While it is well known that low-density gases increase heat loss rates [21], no prior studies have used variation in gas density and air temperature to independently manipulate body temperatures and flight power requirements. We hypothesize that the metabolic rates of flying honeybees exhibit a thermal performance curve, with substantial metabolic benefits to thermoregulation at cooler air temperatures, and suppression of metabolic performance at temperatures above optimal.

2. Material and methods

We manipulated body temperatures and assessed maximal capacities of bees by flying them in various air densities and temperatures (tables 1 and 2). Foragers were collected in random order from three colonies of the Italian honeybee, *Apis mellifera ligustica*, maintained on the third-story balcony of the Interdisciplinary Science and Technology Building 1 at Arizona State University in Tempe, AZ, USA. Unloaded, outgoing foragers were captured when leaving the colony by holding an opened plastic bag (approx. 950 ml) approximately 15 centimetres from the colony entrance. After a single forager flew directly into the opened bag, it was sealed and the bee was quickly transported to a room regulated at 23 ± 0.5°C or 35 ± 0.5°C (EGC, Chagrin Falls, OH, USA) and its flight metabolism was assessed at a single air density.

Table 1. Variable-density gas mixtures used as an aerial treadmill at 23°C.

gas mixture	% O_2	% N_2	% He	density (kg m^{-3})
1	21	79	0	1.288
2	21	69.5	9.5	1.186
3	21	60	19	1.084
4	21	50.5	28.5	0.983
5	21	41	38	0.881
6	21	31.5	47.5	0.779

Table 2. Variable-density gas mixtures used as an aerial treadmill at 35°C.

gas mixture	% O_2	% N_2	% He	density (kg m^{-3})
1	21	79	0	1.288
2	21	63.2	15.8	1.119
3	21	47.4	31.6	0.949
4	21	31.6	47.4	0.780
5	21	15.8	63.2	0.610
6	21	0	79	0.441

Substituting helium for nitrogen in air lowers its density, requiring bees to generate more lift in order to fly [19,22]. This substitution will also increase heat loss rates because helium has a thermal conductivity about six-times higher than nitrogen [21,23,24]. To further manipulate heat loss, we examined metabolic rates and body temperatures at two air temperatures, 23°C and 35°C. Heat loss rates are proportional to the thermal gradient between an animal's body and ambient temperature. Thus, we predicted that flight in gases enriched in helium at low air temperatures would induce the greatest heat loss rates and therefore the coolest body temperatures, whereas heat loss would be lowest in nitrox mixtures at 35°C air temperatures.

Metabolism during free flight was assessed in a cylindrical, transparent acrylic flight chamber (350 ml). After placing the bee in the chamber, it was sealed and covered with a dark cloth to encourage reduced activity of the bee and the chamber was flushed to completely replace atmospheric air and water with the desired gas mixture. Gas mixtures were created by using cylinders of pure O_2, N_2, and He, which were regulated at a total flow rate of 2 l min^{-1} by a multi-channeled Sable Systems FB8 flow meter system (Las Vegas, NV, USA). Each flow meter was calibrated for the different gas densities using a soap-film bubble meter. The gases from the flow meters flowed sequentially through a $CaSO_4$ and soda lime column to remove H_2O and CO_2, the reference cell of a LI-COR 6262 CO_2/H_2O

analyzer (Lincoln, NE, USA), the respirometry chamber, a small column of $MgSO_4$ to remove water produced by the bee, and then the sample cell of the LI-COR. Differential analogue output from the LI-COR was digitized (Sable Systems UI-2) and recorded each second (Expedata, Sable Systems, Las Vegas, NV). The LI-COR was calibrated using 252 ppm CO_2 and Ultra-Zero calibration gases at the same flow rate and pressure (761.5–761.8 mm Hg) as during the flight respirometry, and baseline recordings were taken before and after each measurement period.

Flight was then encouraged for 2 min by shining a 150 W dual goose-neck Fiber Optical Illuminator (China) over the chamber. Bees that landed were immediately encouraged to fly by gently tapping and inverting the chamber. Flight behaviour was categorized based on ability, duration and control (i.e. quality; [25]). Flight was categorized and ranked as: 1, no flight; 2, flapping wings with brief periods of flight (less than 3 s); 3, intermittent flight characterized by frequent crashing (i.e. bee usually ends upside down); 4, intermittent flight characterized by frequent controlled landing (i.e. bee gently lands on its feet); or 5, continual, stable flight. Expedata (Sable Systems, Las Vegas, NV) was used to find and average the 10 s with the highest CO_2 readings during each trial. Flight CO_2 emission rates (ml h^{-1}) were calculated by multiplying the decimal CO_2 fraction times the STP flow rate through the flight chamber. After flight CO_2 emission rates were measured, the bee was shaken into a plastic bag, which was flattened to restrict the bee's movement. Thoracic temperature was then measured by inserting a Physitemp model MT29/1 hypodermic microprobe (Clifton, NJ, USA; 29-gauge, time constant = 0.025 s) through the bag and into the center of the thorax. The temperature data were recorded with a Pico Technology USB TC-08 Thermocouple Data Logger (Tyler, TX, USA). Thoracic temperatures were measured within 5 s of cessation of flight, and the highest temperature reported by the thermometer was recorded. After measurement, the bee was weighed (±0.1 mg) using an A&D HR-120 Analytical Balance (Tokyo, Japan) and stored at −20°C.

Data were analysed using R (3.6.2; R Foundation for Statistical Computing, Vienna, Austria). Two-tailed significance was determined at $\alpha = 0.05$. We used a linear mixed-effects model to test the independent and interactive effects of air temperature and gas density on flight metabolic rate (i.e. milliwatts (mJ s^{-1})) and thoracic temperature, with hive included as a random effect. To determine the independent effect of gas density on thorax temperature, we ran a linear model for each separate air temperature. We also ran a similar model for the above independent variables, with body mass included in the model. We used an ordinal logistic regression model analysis to test the independent and interactive effects of air temperature, gas density, and thoracic temperature on flight quality. Models were chosen using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC).

3. Results

We found that air temperature and gas density had a strong, interactive effect on the flight metabolic rates of unloaded honeybees (linear mixed-effects model: $n = 184$, $\chi^2 = 68.6$, $p < 0.001$; figure 1*a*). At 35°C, flight metabolic rates of bees increased linearly—by a magnitude of 1.4 times—as gas density decreased (linear model: $F_{1,68} = 83.9$, $p < 0.001$). By contrast, at 23°C, flight metabolic rates of bees decreased with decreasing air density (linear regression: $F_{1,99} = 11$, $p = 0.001$; figure 1*a*). Also, while the ability of bees to hover declined with gas density at both air temperatures, bees flying at 23°C failed sooner as density declined (figure 2).

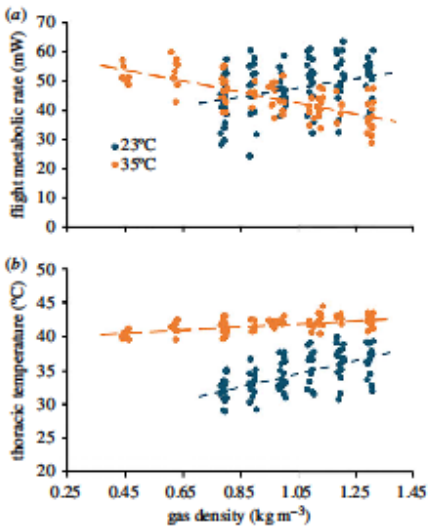

Figure 1. (*a*) Gas density significantly influenced flight metabolism of honey bees, but in a temperature-dependent manner (table 1). (*b*) Decreasing gas density decreased thoracic temperatures of honeybees flown at both air temperatures, but the effect was more pronounced at 23°C (table 2). Bees were exposed to a narrower range of gas densities at 23°C because honeybees were unable to fly in air densities lower than 0.779 kg m^{-3} at this temperature. Each point represents a single, individually tested bee, with overlapping points being slightly staggered along the *x*-axis to improve data visualization. (Online version in colour.)

These differential effects of air temperature and gas density on flight appeared to be mediated by differential effects on thoracic temperature. Air temperature and gas density had a strong, interactive effect on thoracic temperatures of unloaded honeybees (linear mixed-effects model: $\chi^2 = 41$, $p < 0.001$; figure 1*b*). At both temperatures, thoracic temperatures decreased linearly as air density decreased (35°C—linear regression: $F_{1,65} = 31.6$, $p < 0.001$), but the effects were greater at 23°C, likely due to the greater thermal gradient from thorax to air (23°C—linear regression: $F_{1,99} = 60.8$, $p < 0.001$; figure 1*b*). Plotting the maximal metabolic value for any bee at each 0.5°C change in thoracic temperature shows a classic thermal performance curve (polynomial linear regression: $y = -0.0168x^3 + 1.5668x^2 - 45.743x + 457.12$; $F_{3,26} = 40.6$, $p < 0.001$; figure 3). The optimal temperature for flight metabolism and force production [26] of honeybee workers was 39°C, and maximal flight metabolic rates increased by approximately 2% per 1°C increase in thoracic temperature at suboptimal thoracic temperatures, but decreased approximately 5% per 1°C increase as the bees continued to heat up (figure 3).

4. Discussion

Our results show that a flying endotherm exhibits a classical thermal performance curve for maximal metabolic rate, with maximal flight metabolic rates measured at an optimal flight muscle temperature of 39°C, and with flight metabolic rates decreasing strongly above and below these body

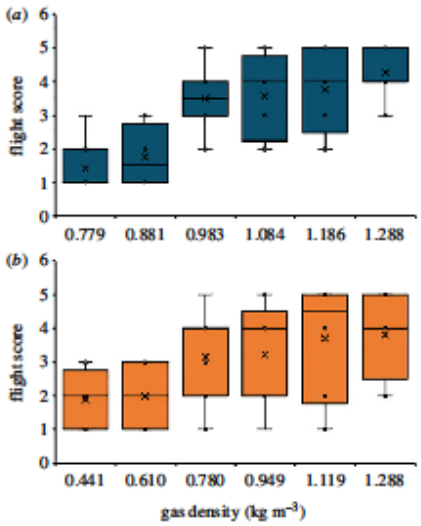

Figure 2. (*a*) Flight quality scores of bees flown at 23°C (ranked from 1 = no flight to 5 = stable, continuous flight) declined in low-density air (ordinal logistic regression: $n = 99$, $t = 2.0$, $p = 0.045$) and with declining thoracic temperature (ordinal logistic regression: $n = 99$, $t = 2.2$, $p = 0.03$). Bees flown at 23°C were unable to fly in air densities lower than 0.779 kg m^{-3}. (*b*) Flight quality scores of bees flown at 35°C also declined in low-density air (logistic regression: $n = 65$, $t = 5.7$, $p < 0.001$). Flight was categorized and ranked as: 1, no flight; 2, flapping wings with brief periods of flight (less than 3 s); 3, intermittent flight characterized by frequent crashing (i.e. bee usually ends upside down); 4, intermittent flight characterized by frequent controlled landing (i.e. bee gently lands on its feet) or 5, continual, stable flight. The 'x', solid bar, lower bar, bottom bar, top box and upper bar represent the mean, median, 1st quartile, 2nd quartile, 3rd quartile and 4th quartile, respectively. (Online version in colour.)

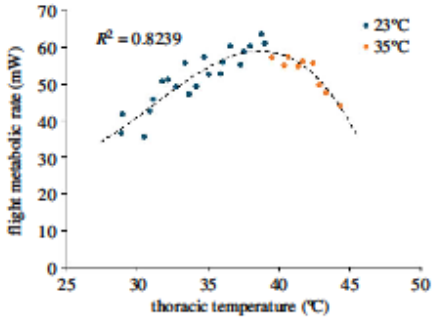

Figure 3. Maximal flight metabolic rate as a function of thoracic temperature. Each point represents the maximal value of a single individual bee at each 0.5°C increment (polynomial regression: $y = -0.0168x^3 + 1.5668x^2 - 45.743x + 457.12$; $F_{3,26} = 40.6$, $p < 0.0001$). (Online version in colour.)

temperatures. An important remaining question is whether mechanical power output during flight shows the same pattern. Metabolic rates often closely correlate with mechanical power output (e.g. [19]), but not always (e.g. in hopping vertebrates, [27]). Force production by the honeybee flight muscle shows a very similar pattern with muscle temperature as we showed for flight metabolism here. Coelho [26] demonstrated that 39°C was the optimal temperature for force production by honeybee flight muscle, with forces declining above and below 39°C. To confirm that mechanical power output shows a similar thermal performance curve, power outputs could be calculated from measurements of wing kinematics across the range of conditions used here [28]. Another approach would be to assess load-lifting capacity as a function of thoracic temperature [18].

Our data make it possible to quantitatively assess the benefits of endothermic thermoregulation for honeybees. Honeybees can achieve thoracic temperatures up to 17°C higher than air temperature (figure 1*b*; [15]). As an example calculation of endothermic costs and benefits, consider a honeybee forager flying with a thoracic temperature equal to an air temperature of 29°C versus one flying with a thoracic temperature of 39°C. Higher thoracic temperatures come at a cost during maximal performance of about 2 mW per °C elevation in thoracic temperature (figure 3). Average foraging trip duration for honeybees is about 30 min [29]. If during the return flight they flew at maximal capacity while carrying a heavy load for 15 min, flying with a flight muscle temperature of 39°C at 58 mW rather than at 29°C at 38 mW will increase the cost of the foraging trip by about 20 joules (15 min * 60 s min^{-1} * 20 mjoules s^{-1}). However, the energetic benefit can be substantially higher.

Flight metabolic rate increases linearly with load, by about 40% (approx. 20 mW), as load increases from 0 to 40 mg of nectar [30]. This is about the same increase as observed in maximal aerobic performance as flight muscle temperature rises from 29°C to 39°C (figure 3). At 29°C, the flight muscle of honeybees is near the minimal temperature at which these bees can fly [31], and so it is unlikely that they can carry a substantial load at this flight muscle temperature. The energetic content of nectar varies, but 9 joules mg^{-1} is an estimated average value from the literature [29]. The gross return of energy to the colony for a 40 mg nectar load will be, on average, 360 joules, with a net return of 302 joules (360 − 58 joules). Obviously, the net benefit will depend strongly on the capacity of cool bees to carry loads and on foraging conditions, and endothermy may not be favoured if energetic rewards in the field are low. Social bees have been widely shown to modulate thoracic temperatures to reward, with higher temperatures associated with higher energetic content of nectar, suggesting that bees can modulate their body temperatures to maximize net foraging reward [32]. However, these calculations depend on the assumption that efficiency is constant across a range of flight muscle temperatures, something that is still unknown for insect flight.

Substituting helium for nitrogen also affects oxygen diffusivity; might this have influenced our results? Oxygen diffusivity in a gas is inversely proportional to gas density [33]. The diffusion rate of oxygen within the tracheae likely increases by slightly more than 2× as gas density decreases from nitrox (79% N_2: 21% O_2) to heliox (79% He: 21% O_2; assuming constant P_{O2} gradients within the tracheae). However, it seems unlikely that variation in oxygen diffusivity explains any of the observed patterns in metabolic rate or flight behaviour. Oxygen delivery to unloaded honey bees has a substantial safety margin, as metabolic rates of

hovering, unloaded bees are unaffected as air P_{O2} varies between 10 and 39 kPa under normobaric conditions [34]. Admittedly, the safety margin for oxygen delivery is likely to be smaller at maximal performance, where oxygen consumption rates are about 40% higher. However, Withers' finding [35] that metabolic rates of flying honeybees rise with a small decrease in air pressure and then fall linearly with larger decreases in air pressure is inconsistent with diffusion being the major mechanism of gas exchange. In hypobaria, P_{O2} falls, but oxygen diffusivity increases proportionally, so diffusive oxygen delivery should be unaffected. If diffusion is the predominant mechanism of gas exchange, we would expect metabolic rates to continue to rise as air pressure drops up to the point of flight failure due to maintained oxygen delivery as the challenge of generating lift increases. Advective gas exchange declines linearly with air pressure due to the linear decline in oxygen content of air; consistent with Withers' findings [35]. As oxygen transport in the gas phase is likely predominantly advective in flying honeybees, it seems unlikely that the rise in flight metabolic rates observed as air density declines at air temperatures of 35°C is due to improved oxygen transport. This possibility could be tested directly by varying air P_{O2} in different gas densities.

Endothermy and thermoregulation at high body temperatures may expand the thermal niche of foraging bees. As noted above, the minimum flight muscle temperature for flight for honeybees is about 28°C. Honeybees have been observed to forage at air temperatures as low at 12°C [31]. As nectar and pollen rewards at flowers are usually highest in the early morning, it is plausible that endothermy aids honeybees and other large social bees in competition for nectar and pollen rewards by enabling them to forage during lower morning temperatures [36]. That being said, a rigorous test of the thermal niche expansion hypothesis would compare the air temperatures at which both larger endothermic bees and smaller ectothermic bees can fly. One recent study compared the foraging temperature range of honeybees to *Osmia cornuta*, a smaller bee with limited endothermic capacity. *Osmia cornuta* was able to fly at lower air temperatures and in more indement weather than *A. mellifera* [37]. It appears that rigorous study of thermal niches of endothermic and ectothermic species in a phylogenetic context will be necessary to determine whether endothermy is associated with a broader thermal niche in insects. In addition to increasing aerobic capacity and possibly thermal niche, endothermy has other benefits for some insects. Heat generated by the flight musde of social bees, such as honeybees and bumblebees, is also used to warm and thermoregulate their offspring, speeding development and possibly improving developmental stability [38].

The magnitude of cooling caused by exposure to low-density gases depends on the thermal conductivity of a particular gas mixture and air temperature. Convective heat loss (*HF*) can be simply modelled as

$$HF = \frac{-kA\Delta T}{\delta},$$

where k is the thermal conductivity of the gas mixture, A is the surface area of the animal, ΔT is the temperature differential (which in this study represents the difference between the thoracic temperature and air temperature), and δ represents the height of the boundary layer of air around the animal. Because several of these variables are difficult to measure, this equation is often simplified to

$$HF = C_{conv}\Delta T,$$

where C_{conv} represents the thermal conductivity (mW m^{-1} K^{-1}) of the gas mixture. The thermal conductivity of a 79% N_2: 21% O_2 gas mixture (nitrox) is 26 mW m^{-1} K^{-1}, whereas the 79% He: 21% O_2 gas mixture (heliox) has a thermal conductivity of 129 mW m^{-1} K^{-1} [33]. For a bee flying with a thoracic temperature of 41°C at an air temperature of 23°C in nitrox air, heat will be lost at a rate of 468 mW, while a bee flying with the identical thermal gradient in heliox will experience a fivefold increase (approx. 2322 mW) in heat loss. However, if a bee with the identical thorax temperature is flying in 35°C nitrox air, heat loss will be decreased threefold, to 156 mw. These combined effects of varying thermal conductivity and air temperature allowed us to manipulate the thorax temperatures of flying honeybees over a wide range.

A crucial question for agriculture is how climatic warming will affect pollinator performance. At cooler locations, seasons, and times of day, warmer air temperatures will increase flight muscle temperatures toward optimal and increase flight aerobic capacity. However, in warmer locations, seasons, and times of day, high air temperatures and solar radiation may push flight muscle temperatures into the range above the optimal temperature, causing decreasing maximal aerobic performance with increasing body temperature. On hot days, flying honeybee foragers thermoregulate both by increasing water loss rates and by reducing metabolic heat production [15,16,39]. Nonetheless, body temperatures of flying bees rise approximately 0.4°C with each 1°C rise in air temperature, and the highest flight muscle temperature measured for bees flying in the laboratory in dry air at 45°C was approximately 49°C [15], well above the optimal temperature of 39°C (figure 3). Honeybees flying in desert regions in the field have body temperatures above 40°C, with pollen foragers tending to be hotter due to reduced capacities for evaporative heat loss [40]. This suggests heat waves associated with climatic warming will negatively impact maximal flight performance and load-carrying capacities in the field for honeybees and possibly other endothermic insects.

This first thermal performance curve for a flying endotherm strongly supports our hypothesis that thermoregulating toward a high temperature (39°C) enhances aerobic capacity, flight capabilities, and foraging performance in honeybees. Because our flight metabolic rates were measured over 10 s, we may have missed spikes in metabolic rate associated with short-term bursts in power output. Therefore, our measures of the effects of flight muscle temperature on maximal power output are, as noted above, probably conservative. To further develop and test aerobic capacity models for the evolution of endothermy in flying insects, it will be important to measure thermal performance curves for more endothermic insects to determine how general or variable this pattern is, and to determine how maximal aerobic metabolism relates to mechanical power output and load-lifting capacities. Linking physiological with paleontological and systematic research will also be necessary to create a true evolutionary model. In vertebrates, insulation (i.e. fur, feathers) and indices of blood vessel density in bone provide paleontological evidence for endothermy

and homeothermy [13]. Tests of whether morphological characteristics detectable in fossils, such as thoracic insulation or tracheal dimensions, are linked to endothermy could advance this field.

Why does flight metabolism decrease at higher air temperatures? Several studies have shown that flight metabolism decreases at relatively high air temperatures [15,16]. However, the mechanisms remain unclear. The decrease in flight metabolism might be due to suppression of flight muscle by thermoregulatory centers in the brain to prevent overheating. Conversely, higher temperatures may be directly inhibiting the flight muscle or motor neurons. For example, high temperatures may increase K^+ leakage in the flight muscle or controlling neurons relative to Na^+/K^+-ATPase activities, causing widespread depolarization and loss of excitable tissue function [41]. Another possibility is that high temperatures directly inhibit muscle proteins such as myosin ATPase, decreasing the contractile ability of flight muscle.

Endothermy may be ancient within the Insecta, and has been hypothesized to have occurred in the large Protodonata of the Carboniferous [42]. Bees evolved from wasps in the Cretaceous [43], and some larger sphecid wasps are endothermic, suggesting endothermy in bees could have been inherited from wasp ancestors [44]. However, the Mellitidae are the sister taxa to bees, and most, but not all of these, are likely too small to be endothermic [45], supporting the possibility of an independent origin of endothermy in bees. In any case, miniaturization and enlargement of species is common in lineages of bees [46], suggesting that endothermic heterothermy likely evolved multiple times in association with having a sufficiently large body size to enable metabolic heat production to exceed heat loss. Identification of paleontological markers of endothermy could enable rigorous tests of when endothermy occurred. Our findings that endothermy increases both the costs and potential rewards of foraging suggest that the evolution of endothermy in bees should be associated with periods of rich resource availability.

Data accessibility. Data available from the Dryad Digital Repository: (doi:10.5061/dryad.xsβtx9jn) [47]. Code for the statistical analysis (R Foundation for Statistical Computing) is available upon request.

Authors' contributions. J.R.G.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing; J.F.H.: conceptualization, funding acquisition, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. Authors declare that they have no competing interests.

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