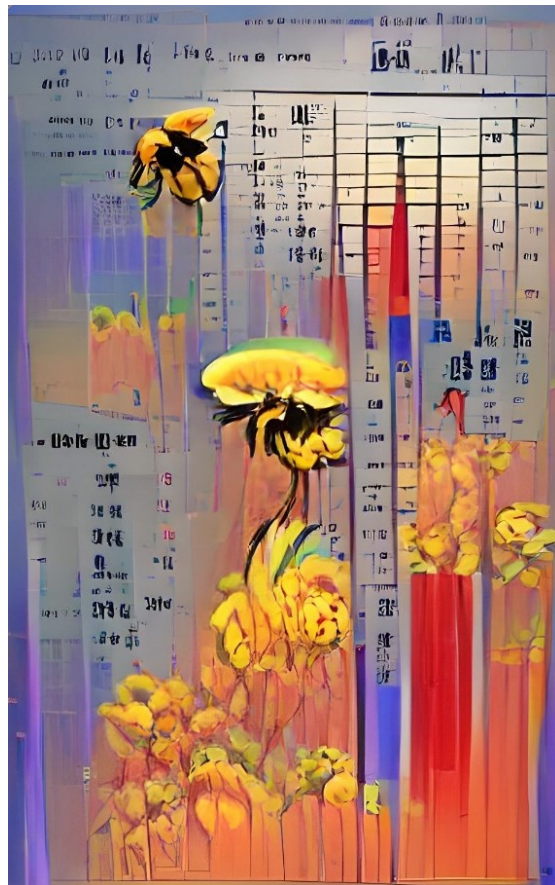


Impacts of insecticide exposure on bumblebee behaviour in a warming world

Daniel Kenna



Imperial College London

Department of Life Sciences

A thesis submitted for the degree of *Doctor of Philosophy* in Applied Ecology

January 2022

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Statement of Originality

This thesis is the product of my own work with the following acknowledgements:

Cover image on page 1 was generated through WOMBO (<https://www.wombo.art/>) and the image on page 6 was painted by Freja Gjerstad.

All chapters have been put together by myself, with support and feedback from my supervisor, Dr Richard Gill. The experimental work in Chapter 2 was undertaken by a previous undergraduate student, Hazel Cooley, as part of her final year dissertation project and supervised by Dr Richard Gill. However, all aspects of the data analysis, including data cleaning, filtering, visualisation and statistical analysis, in addition to interpretation and study write-up has been conducted by myself. All experimental work in Chapters 3 & 4, along with all data analysis and study write-up, was conducted by myself. This is with the exception of experiment 1 in Chapter 4, in which Peter Graystock (Imperial Research Fellow) assisted with preparing the experimental setup (~10% of work), conducted monitoring on one day of the experimental trial, and provided insightful guidance on Cox proportional hazard models. Additionally, the R script used to clean and filter flight mill data was produced in collaboration with previous Masters students Chloe Sargent and Freja Gjerstad

The study aims in Appendix D were formulated by me, the subsequent experimental work was designed in collaboration between myself and MSc project student Freja Gjerstad, with Freja (70%) conducting the majority of experimental work and data analysis with assistance by me (30%). The study aims in Appendix E were formulated by me, extensive pilot studies were performed by me to test and validate an experimental protocol, and the subsequent experimental work was designed in collaboration between myself and MRes project student Connor Lovell. The majority of experimental work was conducted by Connor (80%) with assistance by me (20%). The data analysis and study write-up were conducted by Connor with close supervision by myself.

The work presented in Chapter 2 was published in the peer-reviewed journal *Ecology & Evolution* on 29th April 2019: Kenna, D., Cooley, H., Pretelli, I., Ramos Rodrigues, A., Gill, S. D., & Gill, R. J. (2019). Pesticide exposure affects flight dynamics and reduces flight endurance in bumblebees. *Ecology and Evolution*, 9(10), 5637–5650. <https://doi.org/10.1002/ece3.5143>

The work presented in Chapter 3 was published in the peer-reviewed journal *Functional Ecology* on 17th August 2021: Kenna, D., Pawar, S., & Gill, R. J. (2021). Thermal flight performance reveals impact of warming on bumblebee foraging potential. *Functional Ecology*, 35(11), 2508-2522. <https://doi.org/10.1111/1365-2435.13887>

Acknowledgements

This PhD was funded by a NERC (National Environment Research Council) studentship as part of the Grantham Institute Science and Solutions for a Changing Planet (SSCP) Doctoral Training Programme.

Thanks to the SSCP for the learning and development opportunities over the first few years, support and guidance in undertaking an internship, and for their understanding and help during the Covid pandemic.

Thanks to Kew Gardens, and Sam Pironon in particular, for having me on a 3-month internship. It was a fantastic learning experience and I appreciate the patience whilst I very slowly tried to grasp species distribution modelling.

Thanks to Samraat as my co-supervisor, and to Joe Tobias and Rob Ewers as my review panel, who's comments help shape the direction of my PhD. Thanks also to my thesis examiners Natalie Hempel de Ibarra and Tom Bell, for your valuable feedback and insightful comments, and for making my viva a genuinely enjoyable experience! And a special mention to Paul Beasley and Martin Selby, who have been absolutely vital to me making it through my experimental work, constantly dealing with my requests to build and fix things on top of all the actual work they need to do.

Thanks to the Silwood community and all the friends I made along the way, who have made my PhD experience so enjoyable, and thank you to the lab group (Ana, Andres, Peter, Dylan, Jacob, Aoife and Monika) for advice and friendship throughout. I would like to thank the master's students, Freja Gjerstad and Connor Lovell, who I supervised on projects. You both did amazing jobs, and it was a pleasure to work with you. And of course, special thanks to my supervisor, Richard Gill, for all the support, advice, and entertainment throughout!

Thanks to my family for encouraging me to apply for a PhD in the first place and never doubting my ability to make it through. But most of all thank you to Flo, for being so unbelievably patient over these last four years and continually feigning interest when I talk about bees.



Abstract

With ongoing agricultural land-use intensification and climate change, important functional groups of organisms, including insect pollinators, are being simultaneously and ubiquitously exposed to both pesticides and changing temperatures. Given the notable ongoing declines in insect pollinators, understanding how these stressors affect aspects of key functional behaviours underpinning fitness and pollination service provision is of global ecological and economic importance. Importantly, a critical but understudied step in evaluating pesticide risk is to understand how environmental temperature modulates pesticide effects on these behaviours. Through empirical lab experiments on a key wild pollinator, the bumblebee *Bombus terrestris*, I quantified the temperature dependent effects of insecticide exposure on individual flight and general mobility. Neonicotinoid exposure dramatically impaired flight motivation and endurance, and these same performance traits were also found to be highly sensitive to environmental temperature. However, the effect of neonicotinoid exposure varied depending on post-exposure temperature, with impacts on flight being enhanced under warmer conditions and impacts on food consumption, walking, and general responsiveness being enhanced under cooler conditions. The hazard posed by insecticides to insect pollinators may therefore vary both temporally and spatially, with impairment to flight and mobility traits likely constraining colony foraging potential and diminishing pollination service capabilities. Interestingly, the same effects were not seen across a different class of insecticide, with the new to market sulfoximine insecticide, sulfoxaflor, showing no detectable effect on any measure of mobility regardless of temperature when applied at comparable concentrations. The findings of this thesis suggest both the choice of insecticide and timing of exposure need to be considered in farming practices. Additionally, my findings provide crucial information for advising insecticide risk assessment protocols, developing mitigative and conservation action, and above all understanding which possible climate regions in the world insect pollinators are most at risk from insecticide exposure.

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CHAPTER 1

General Introduction

1.1 | Importance of insect pollinators and concerns over declines

Human activities have drastically altered the global environment over the past millennia, leading many to suggest we are in a new geological epoch - the Anthropocene (Lewis & Maslin, 2015; Steffen et al., 2007). In particular, the past century has seen high rates of biodiversity loss globally (Dirzo et al., 2014; Pimm et al., 2014; Tittensor et al., 2014), with land use change, pollution and climate change considered as the greatest human-related drivers (Bellard et al., 2012; Ceballos et al., 2017; Newbold et al., 2015; C. Thomas et al., 2004). Though a paucity of long-term data precludes a reliable worldwide assessment, insects appear to be no exception to these losses (Saunders et al., 2020; J. Thomas et al., 2004; Wagner et al., 2021). This is of particular concern given the variety of ecosystem services they support, such as decomposition, biological control, and pollination (Losey & Vaughan, 2006; Schowalter et al., 2017).

One group of insects for which there is considerable apprehension surrounding current and future conservation status are insect pollinators, and evidence of widespread reductions in pollinator abundance and diversity is mounting (Biesmeijer, 2006; Burkle et al., 2013; Grixti et al., 2009; Kosior et al., 2007; Ollerton et al., 2014; Powney et al., 2019). For example, over the past half-century there have been reports of sharp declines in global bee species richness (Potts et al., 2016; Zattara & Aizen, 2021), with >20% of bumblebee species threatened in each of North America, Mesoamerica and Europe (Forister et al., 2019). Furthermore, multidecadal studies have revealed sweeping declines in formerly abundant butterfly species across Europe (Maes & Van Dyck, 2001; Warren et al., 2021), with reductions also observed in North America (Forister et al., 2018, 2010). Similarly in the UK, substantial declines in abundance of butterflies and moths have repeatedly been shown over the last few decades (Fox, 2013; Thomas, 2005; J. Thomas et al., 2004), accompanied by declines in bee, wasp, and hoverfly diversity (Biesmeijer, 2006; Powney et al., 2019; Senapathi et al., 2015).

Such reports are worrying because insect pollinators are essential for maintaining ecosystem health and global food security (Goulson et al., 2008; Potts et al., 2010). Mutualistic plant-pollinator interactions confer fitness advantages to rarer plant species, and insect pollinators contribute to the reproduction of approximately 85% of wild plants worldwide, highlighting the vital importance of this functional group in conserving plant diversity (Ollerton et al., 2011; Wei et al., 2021). This critical

pollination service also underpins the productivity of agricultural systems, with >75% of global crop species showing enhanced yield or quality from insect visitations (Kleijn et al., 2015), equating to around 35% of food production worldwide (Klein et al., 2007). These pollinator dependent flowering crops are important sources of micronutrients that are vital for healthy and balanced human diets (Eilers et al., 2011; Smith et al., 2015). Consequently, this ecosystem service has been estimated to have a global annual market value >€150 billion (Gallai et al., 2009; Potts et al., 2016), and economic benefits of over £500 million per year in the UK alone (Garratt et al., 2014; Steele et al., 2019; Vanbergen et al., 2014).

With the global human population set to exceed 9.5 billion by 2050 (UN DESA, 2015), increased food consumption and dietary changes are placing unprecedented demands on agricultural practices to become more efficient (Cirera & Masset, 2010; Crist et al., 2017; Foley et al., 2011; Pelletier & Tyedmers, 2010). Indeed, the cultivation of insect pollinator dependent crops has rapidly increased across the world in the last 50 years (Aizen et al., 2008), and pollination demand has quickly outstripped supply leading to an increasing pollination deficit (Aizen & Harder, 2009; Breeze et al., 2014; Garibaldi et al., 2011). To keep pace it has been suggested that current crop yields will need to considerably increase, perhaps even double (Clay, 2011; FAO, 2009; Foley et al., 2011). As such, there is an urgent need to protect any biotic processes that can enhance crop quantity and quality (Bommarco et al., 2013). Safeguarding insect pollinators is therefore of global importance (Gill et al., 2016; Potts et al., 2016). To inform and improve our current safeguarding strategies, it is essential we understand the factors that threaten insect pollinators and the mechanisms by which these stressors impact behaviour, health, and ultimately fitness. By understanding the mechanistic basis of harmful effects through controlled studies, we can formulate targeted action to mitigate these effects under future scenarios (Gill et al., 2016; Goulson et al., 2015).

1.2 | Two key drivers implicated in insect pollinator declines

Multiple anthropogenic stressors are considered to be driving insect pollinator declines (Dicks et al., 2021; Forister et al., 2019; Vanbergen et al., 2021). Two of the most heavily cited, which are multifaceted and can act individually or in combination (Oliver & Morecroft, 2014; Vanbergen & Garratt, 2013), are pesticide use and climate change (reviewed in Forister et al., 2019; Goulson et al., 2015; Wagner et al., 2021). These have recently been ranked as number 3 and 4 respectively in a list of the most important threats to pollinators globally, as decided by a panel of experts (Dicks et al., 2021).

1.2.1 | *Pesticide exposure*

Within the last century, there has been a dramatic shift in agricultural practices from low-input, small-scale farming styles to intensive, industrial-scale production (Bambaradeniya & Amerasinghe, 2003; Ollerton et al., 2014). Crop monocultures and removal of hedgerows to aid mechanisation have created more suitable conditions for crop pests to increase in population density (Meehan et al., 2011; Sánchez-Bayo & Wyckhuys, 2019). This has contributed to the systematic and widespread use of chemical insecticides, herbicides, and fungicides (Fernandez-Cornejo & Vialou, 2014; Jeschke et al., 2010; Lamichhane et al., 2016; Oerke & Dehne, 2004), changing the chemical landscape that pollinators operate in (Goulson, 2020; Goulson et al., 2008). For example, in the UK there is approximately 4.5 million hectares of cropped land, and whilst this has remained relatively consistent since 1990, the total area treated with pesticides has approximately doubled, with a report suggesting fields are now treated an average of 17 times per year (Goulson, 2020). As agriculture covers as much as 70% of the UK (Ollerton et al., 2014), and >10% of the total land surface of the planet (FAO, 2009), intensive pesticide application to crops has the potential to affect a large diversity of the world's insect pollinator species (Dudley & Alexander, 2017; Ramankutty et al., 2018). In addition, whilst the most intensive application of pesticides is on arable land, pesticides are also used in a variety of other agricultural settings such as grasslands and orchards, as well as amenity settings such as parks and golf courses (PUSSTATS., 2022). Organic farming practices involve a much reduced and restricted application of pesticides, but there are still around 30 approved for use (both naturally derived and synthetic products) in the EU and UK (EU Commission Regulation No 889/2008), and even with shifts towards integrated pest management approaches more generally, pesticides remain important for targeted application (Creissen et al., 2021). Consequently, pesticides do, and will continue to, pose a potential risk to pollinators across the globe.

Given the scale of pesticide application globally (Friedle et al., 2021; Mitchell et al., 2017; Tang et al., 2021; Traynor et al., 2021), the potential for these compounds to negatively affect non-target insect pollinators must be considered, as the costs of losing these beneficial species has the potential to outweigh the gains in pest reduction (Budge et al., 2015). Whilst pesticides are assessed in terms of their toxicity to non-target insects before being approved for use (Sanchez-Bayo & Goka, 2014), there is a growing understanding that they can have a range of unintended direct and indirect effects on insect pollinators (Brittain et al., 2010; Steele et al., 2019). For example, there is evidence supporting an association between pesticide use and bee population declines (Ellis et al., 2010; Hallmann et al., 2017; Ollerton et al., 2014; Woodcock et al., 2016), and recent field studies investigating the effects of insecticide exposure on insect pollinator communities located near to

pesticide treated fields have supported an association with reduced wild bee density and species richness (Kennedy et al., 2013; Park et al., 2015; Rundlöf et al., 2015).

Neonicotinoids

Over recent years, considerable attention has been focused on the impacts of the neonicotinoid class of insecticides. They have been explicitly implicated as a key driver of insect pollinator declines across the globe (Godfray et al., 2014; Van Der Sluijs et al., 2015), and range contractions in wild bees and hoverflies throughout the UK appear to coincide with their introduction to agricultural pesticide regimes (Powney et al., 2019; Woodcock et al., 2016). Neonicotinoids are a class of neurotoxic insecticide, consisting of three chemical groups; nitroguanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran), cyanoamidines (acetamiprid and thiacloprid), and nitromethylenes (nitenpyram) (Matsuda et al., 2001; Simon-Delso et al., 2015; Tomizawa & Casida, 2005; Zhang et al., 2021). Since their initial discovery in the 1980's, they have become the most widely used class of insecticide on the global market due to their: i) selective toxicity to arthropods and perceived safety compared to other insecticides such as pyrethroids and organophosphates; ii) systemic nature; and iii) high persistence in the environment (Godfray et al., 2014; Jeschke & Nauen, 2008; Riedo et al., 2021; Simon-Delso et al., 2015; Woodcock et al., 2021).

Neonicotinoids are agonists of nicotinic acetylcholine receptors (nAChR) found in the central nervous system of animals, mimicking the natural neurotransmitter acetylcholine and mediating fast excitatory synaptic transmission (Déglise et al., 2002; Tomizawa & Casida, 2005). They are selectively more toxic to insects than vertebrates due to a higher affinity for insect nAChRs (Tomizawa & Casida, 2005). At sufficiently high concentrations, exposure can lead to neural over-excitation leading to paralysis and death, but even low concentrations can cause nervous stimulation, and thus sublethal exposure to concentrations of these chemicals commonly used in agriculture has the capacity to impact insect pollinator behaviour (Matsuda et al., 2001; Moffat et al., 2016; Suchail et al., 2001). As neonicotinoids are highly water-soluble, they can be applied to plants via a range of forms including soil drench, foliar spray, and seed treatment, allowing application methods to be both flexible and targeted (Bonmatin et al., 2015; Jeschke et al., 2010). Seed treatments have increased in popularity with farmers around the world, whereby the systemic nature of neonicotinoids means the active ingredient is absorbed by the plant and translocated across all plant tissues. This not only provides long-term whole-organism protection for the plant, thus maximising exposure to target pests, but also reduces the need for repeated applications (Elbert et al., 2008; Jeschke et al., 2010).

The benefits of neonicotinoids for crop protection do, however, have inadvertent costs to beneficial organisms, such as insect pollinators. Residues can be present in the pollen, nectar and guttation fluid of treated plants, providing multiple routes of exposure to non-target insect pollinators (Blacquière et al., 2012; Chauzat et al., 2006; David et al., 2016). In addition, these compounds can degrade slowly, persisting in the environment for long periods and leaching into water sources and nearby fields (Goulson, 2013; Krupke et al., 2012; Lundin et al., 2015), with studies reporting detection of neonicotinoids in wild flowers (Botías et al., 2016) and garden plants (Lentola et al., 2017). Consequently, insect pollinators are known to frequently encounter these chemicals when foraging in treated landscapes, as supported by residue analysis from the bodies of pollinators and their collected food (Botías et al., 2017; Hladik et al., 2016; Mitchell et al., 2017; Woodcock et al., 2018). Furthermore, studies have suggested that bees will not actively avoid neonicotinoid contaminated food (Kessler et al., 2015; Muth et al., 2020) and may even acquire a preference for it over time (Arce et al., 2018). Whilst foraging insect pollinators are rarely exposed to neonicotinoid concentrations high enough to cause direct mortality in the field (Tapparo et al., 2012), there is strong evidence that exposure to field-realistic concentrations can have a range of detrimental sublethal impacts on behaviour, fitness and the pollination service being provided (Arce et al., 2017; Baron, Raine, et al., 2017; Crall et al., 2018; Goulson, 2015; Pisa et al., 2014; Siviter, Richman, et al., 2021; Whitehorn et al., 2012). Following the growing evidence for widespread environmental contamination and damaging impacts of neonicotinoids on non-target organisms (Wood & Goulson, 2017), in 2018 the European Commission extended a 2013 moratorium and prohibited the use of the three most commercialised and toxic neonicotinoids (imidacloprid, thiamethoxam, and clothianidin) outside of closed greenhouses (EFSA, 2018; Goulson, 2020; Sgolastra et al., 2020).

Sulfoximines

Rising pest resistance to neonicotinoids (Bass et al., 2015), combined with these new restrictions in the EU, has led to the development and registration of new chemicals to control sap-feeding insect pests (Siviter & Muth, 2020). One of the most promising of these replacements is the systemic sulfoximine class, and in particular the pesticide sulfoxaflor, which is the first marketed insecticide of the sulfoximines (Sparks et al., 2013). Sulfoxaflor exhibits a high potency to insect pests and resembles the neonicotinoids in that it is an agonist of insect nicotinic receptors. Indeed, when it first came to attention, similarities in pharmacological profile and insecticidal activity prompted some to ask that sulfoxaflor be considered a neonicotinoid (Cutler et al., 2012). However, it differs sufficiently in structure to prevent insecticidal cross-resistance, which appears to be the result of differences in susceptibility to oxidative metabolism (Longhurst et al., 2013; Shao et al., 2013; Sparks

et al., 2013; Zhu et al., 2011). Nevertheless, the similarities in mode of action with the neonicotinoids has raised concerns surrounding the toxicity of sulfoxaflor towards non-target insects (Gauthier & Mabury, 2021; Linguadoca et al., 2021; Siviter et al., 2020; Zhu et al., 2017). Whilst studies have suggested sulfoxaflor to be less toxic to insect pollinators, and thus useful as a replacement to mitigate declines of non-target insects (Parkinson et al., 2020; Tamburini, Wintermantel, et al., 2021), a study by Siviter et al (2018) demonstrated that exposure to sulfoxaflor, at concentrations below the maximum residue limits set by the Environmental Protection Agency (EPA) in the US, negatively impacted bumblebee colony fitness by reducing the numbers of workers produced and the subsequent reproductive output. Additionally, two recent studies exposing bees to field realistic / recommended application rates of sulfoxaflor found that oral exposure had negative impacts on bumblebee fecundity (Linguadoca et al., 2021) and contact exposure had dramatic impacts on honey bee mortality (Chakrabarti et al., 2020).

Since being registered in 2016, the EPA has approved the use of sulfoxaflor on hundreds of crops, and this has been accompanied by rapid registration for widespread crop use across the globe (Centner et al., 2018; Siviter & Muth, 2020). Whilst it was initially formulated for use as a foliar spray, a method that allows a greater control on application timing, it is now being developed for use as a seed treatment for a number of bee attractive crops, which may facilitate their replacement of neonicotinoids over vast areas (Brown et al., 2016; Centner et al., 2018; Simon-Delso et al., 2015). This raises further concern about potential impacts to non-target organisms that feed off nectar and pollen of treated plants, making urgent the need to understand the comparative toxicity of this new neuroactive insecticide (Brown et al., 2016; Linguadoca et al., 2021). Moreover, evidence is needed to better inform future application guidance, so that we can avoid a situation whereby widespread rollout is undertaken prior to being fully aware of the sublethal impacts posed to beneficial insects, which was arguably the case for the heavily debated neonicotinoids (Sgolastra et al., 2020).

1.2.2 | *Climate change*

The global span of insect pollinators mean they inhabit a wide range of climate regions and often experience thermally dynamic environments. Indeed, particularly in temperate environments, populations have to cope with changing thermal regimes both during the day and through the foraging season (Germ et al., 1997; Stabentheiner, 2001). For insects, ambient temperature acts as both a phenological cue and a physiological limitation by directly affecting chemical and physical processes such as gene function, energy metabolism, and activity rates (Brown et al., 2004; Henry et al., 2014; King & Macrae, 2015; Schulte, 2015; Stemkovski et al., 2020). Climate therefore plays a key role in the

geographic distribution of insect species and their functioning within ecosystems (Easterling et al., 2000; Kerr et al., 2015; Parmesan et al., 1999; Sunday et al., 2011; Thomas, 2010).

Alongside agricultural intensification, the past century has seen increased rates of climate change, with the annual global mean air temperature now around 1°C higher than pre-industrial levels (WMO, 2020). Whilst the Paris Agreement took an important step in trying to limit rising temperatures to below 1.5°C above pre-industrial levels, global greenhouse gas emissions are projected to be about the same in 2030 as they are today, which would mean global temperatures could pass the 1.5°C warming target by the mid-2030's, would pass 2°C warming by 2050-2070, and could result in a rise of 3-4°C by the end of the 21st century (IPCC, 2018; WWF, 2020). These average temperature increases are expected to be coupled with an increase in the frequency and duration of heatwaves in most terrestrial regions (IPCC, 2018). Such a dramatic shift in the climate is anticipated to have major impacts on insect species distribution, abundance, and diversity, as well as the ecological roles and interactions that support the functioning of healthy ecosystems (Forister et al., 2019; IPBES, 2019; Vanbergen et al., 2021; Wagner et al., 2021; Warren et al., 2018). We have already witnessed well documented responses to climate change over the past few decades, such as shifts in the seasonal timing of biological events (Cohen et al., 2018; Thackeray et al., 2016), which can disrupt life cycles and interactions between species and cause potential mismatches in the phenology of insect pollinators and their host plants (Burkle et al., 2013; Herbertsson et al., 2021; Petanidou et al., 2018; Stemkovski et al., 2020). Furthermore, there is growing evidence that the warming climate is driving changes in insect pollinator abundances (Lister & Garcia, 2018) and distributions (Breed et al., 2013; Forister et al., 2010; Jacobson et al., 2018; Kerr et al., 2015; Parmesan et al., 1999; Parmesan & Yohe, 2003), bringing new threats to species and potentially exacerbating the impact of other stressors (Goulson et al., 2015; Holmstrup et al., 2010; Warren et al., 2021). Indeed, global warming has been attributed to insect species range shifts towards poles and higher elevations as they seek to stay within suitable thermal limits (Chen et al., 2011; Herrera et al., 2014; Marshall et al., 2020; Sunday et al., 2012). Additionally, a variety of insect pollinator species are suffering range contractions at their southern tips without compensating with expansion at their northern edges (Breed et al., 2013; Kerr et al., 2015). Together, these findings raise serious concerns about further decline and localised extinction risk under future climate change projections (Halsch et al., 2021; Janzen & Hallwachs, 2021; Minter et al., 2020; Powney et al., 2019; Soroye et al., 2020) and emphasize the need to better understand how insect pollinator species respond to changing thermal conditions across their range (Hamblin et al., 2017; Pimsler et al., 2020).

The sensitivity of insect pollinator species to climate change appears to be linked to their thermal tolerances, with declining species demonstrating narrower thermal limits (Deutsch et al.,

2008; Herrera et al., 2018; Martinet et al., 2015; Williams et al., 2007). Whilst critical thermal limits have been characterised for many insect pollinator species, these limits will rarely be surpassed in natural settings, even under temperature extremes (Klockmann et al., 2017; Martinet et al., 2015; Oyen & Dillon, 2018; Zambra et al., 2020). Instead, detrimental sublethal effects within such limits warrant further investigation (Cameron & Sadd, 2020). The shapes of the relationships between temperature and an organism's performance traits are crucial for understanding the functional responses of populations and communities to climatic fluctuations (Angilletta, 2006; Dell et al., 2011; Huey & Stevenson, 1979). Quantifying these relationships will allow us to understand where insect pollinator species are operating in respect to their thermal limits, helping predict responses to projected temperature increases and/or erratic weather events. It is therefore important to gain better data not only on the thermal tolerance of insect pollinators, but also on thermal performance, as behavioural thermal responses will have direct consequences for organism fitness and how global pollination networks will be affected under future scenarios of climate change (Rader et al., 2013; Steele et al., 2019).

1.3 | Evidence for how temperature modulates insecticide toxicity on pollinator behaviours is lacking

Experiments exposing non-target beneficial organisms to field-level insecticide concentrations, despite being sublethal, have shown impairment of key behaviours important for organism fitness and ecosystem service provision (Gill et al., 2012; Rundlöf et al., 2015; Stanley et al., 2015). Such studies have proven important for raising awareness concerning the hazards posed by such pesticides. Assessment of whether a pesticide poses an unacceptable risk, however, can be particularly difficult. This is because the degree of toxic effect appears to show significant variation across studies, even when considering the same species and pesticide (Arena & Sgolastra, 2014; Decourtye et al., 2013; Godfray et al., 2014; Heard et al., 2017). A possible explanation for these discrepancies is that the environmental context in which lab and field studies are conducted is often not appropriately considered (Cameron & Sadd, 2020; Crall et al., 2018; Holmstrup et al., 2010). For example, exposure to field-realistic levels of common neonicotinoids and nutritional stress (reduced nectar provision) can synergistically interact to reduce bee survival by as much as 50% (Tosi, Nieh, et al., 2017), suggesting the nutritional quality of the landscape may influence the toxicity of insecticides to insect pollinators (Dance et al., 2017; Tong et al., 2019). Furthermore, co-exposure with azole fungicides has been shown to exacerbate the toxicity of a range of pyrethroid, organophosphate, and neonicotinoid insecticides (Biddinger et al., 2013; Pilling & Jepson, 1993; Tsvetkov et al., 2017), and the impacts of neonicotinoids on individual bee activity and colony health can be enhanced in the

presence of parasites (Blanken et al., 2015; Botías et al., 2021; Goulson et al., 2015). Similarly, it has been suggested that the effects of habitat loss, invasive species, and nutritional stress can all be temperature dependent (Vanbergen et al., 2021; Vanderplanck et al., 2019). Yet, despite the co-occurrence of increasing pesticide usage and rising climatic temperatures over the past century, and despite the remarkable media attention surrounding pesticide and climate change impacts on insect pollinators, there is limited work investigating how environmental temperature modulates the impacts of pesticide exposure on these organisms (but see Henry et al., 2014; Kolano et al., 2021; Monchanin et al., 2019; Zaragoza-Trello et al., 2020). In particular, the quantification of impacts on sublethal behavioural endpoints is largely undescribed, yet these behaviours determine individual and colony level fitness as well as ecosystem service provision (Greenop et al., 2020; Noyes & Lema, 2015).

Understanding how temperature can module the impacts of pesticide is important, because the seasonal pattern of pesticide application to agricultural landscapes makes exposure to insects under wide thermal ranges a very realistic scenario (Holmstrup et al., 2010). Additionally, future landscapes are predicted to experience increased rates of pesticide application along with warmer and more extreme climates, increasing the likelihood that insect pollinators will experience these stressors simultaneously (Deutsch et al., 2018; IPCC, 2018; Tang et al., 2021; Zhang, 2018). This co-exposure may be further amplified as warming environmental temperatures are expected to increase the reproductive rate and migratory capacity of pests, which could thus place an even greater reliance on control by chemical pesticides (Deutsch et al., 2018). Understanding the interactions between temperature and pesticide stress is thus crucial for improving our ability to safeguard insect pollinator populations and ensure pollination services into the future (Botías et al., 2021; Goulson et al., 2015; Potts et al., 2016; Schweiger et al., 2010).

1.3.1 | *Mechanisms and evidence from other insects*

When considering lethal endpoints, temperature-modulated pesticide toxicity has been observed across insect crop pests and aquatic organisms (Boina et al., 2009; Camp & Buchwalter, 2016; Noyes et al., 2009; Op de Beeck, Verheyen, Olsen, et al., 2017; Tsaganou et al., 2021). In light of climate change projections, a potentially alarming finding for insect pollinators is that pesticides often appear to increase in toxicity with environmental warming and extreme heat events (Holmstrup et al., 2010; Lydy et al., 1999; Macaulay et al., 2021). These interactions, however, appear complex and can be pesticide specific, with some pesticides increasing in toxicity under cooler conditions (Khan & Akram, 2014; Noyes et al., 2009; Op de Beeck, Verheyen, & Stoks, 2017).

The physiological basis of this phenomenon is becoming better understood (Hooper et al., 2013), with ambient temperature likely playing an important role in several processes that determine toxicity such as pesticide consumption, distribution, receptor binding efficiency, metabolism and excretion (Boina et al., 2009; Camp & Buchwalter, 2016; Harwood et al., 2009; Holmstrup et al., 2010; Khan & Akram, 2014; Lydy et al., 1999; Noyes et al., 2009; Weston et al., 2009). For example, ambient temperature directly affects metabolic and other physiological rates in insects (Gillooly et al., 2001; Huey & Stevenson, 1979). As such, warmer environments can place increased energetic demands on individuals, thus driving increased consumption of potentially contaminated food, enhancing the level of pesticide exposure and providing a mechanistic basis to observed synergistic interactions between environmental warming and pesticide exposure on insect mortality (Camp & Buchwalter, 2016; Holmstrup et al., 2010; Lydy et al., 1999; Tsaganou et al., 2021). However, as the surrounding environmental temperature can determine metabolic and enzymatic activity rates, it can thus influence the rate of metabolism and detoxification of a pesticide once ingested by an exposed individual (Gerba, 2019; Khan & Akram, 2014; Noyes et al., 2009). Indeed, the toxicity of pyrethroid insecticides have been observed to increase under colder post-exposure temperatures, due to a reduced elimination rate and thus increased accumulation of the pesticide within individuals (Harwood et al., 2009; Weston et al., 2009). Whilst pesticides can be metabolised at a faster rate at higher temperatures, previous studies have shown this does not always increase the rate of detoxification, because the metabolites of some pesticides, either by chance or by design, are more toxic than the parent compound (Gerba, 2019; Holmstrup et al., 2010; Lydy et al., 1999). For example, organophosphates have been observed to become more toxic to insects under warmer environmental temperatures due to an increase in the rate at which they are broken down into their more toxic metabolites (Glunt et al., 2013; Harwood et al., 2009; Khan & Akram, 2014). These mechanisms can provide useful insights, allowing us to hypothesize how insect pollinator behavioural traits may be affected.

The temperature-toxicity relationship for a given insecticide can be designated by a temperature coefficient (TC) (Khan & Akram, 2014; Muturi et al., 2011). Insecticides with a positive TC become more toxic with increasing temperature, whereas those with a negative TC become more toxic with decreasing temperature (Glunt et al., 2013; Mansoor et al., 2015). In the farming industry, assigning TC's is important to ensure maximum efficacy and return for investment when deciding when to apply pesticides to agricultural fields to protect against insect pests (Musser & Shelton, 2005; Tsaganou et al., 2021). Understanding these relationships in pollinators could help achieve the optimum trade-off between effective pest-control and limiting exposure effects to beneficial insect pollinators.

1.3.2 | *Implications of temperature-toxicity relationships for insect pollinators*

Whilst temperature-toxicity relationships have been examined for insect pests and some aquatic insects, they almost exclusively focus on lethal effects of pesticides (Op de Beeck, Verheyen, Olsen, et al., 2017; Tran et al., 2019). Moreover, there is a scarcity of literature relating to these relationships in insect pollinators (but see Belzunces et al., 2012; Gooley & Gooley, 2020; Kolano et al., 2021; Manzi et al., 2020; Saleem et al., 2020). This is an evidence gap that may have fundamental implications for current pesticide regulatory guidelines and risk assessments. Before being approved for commercial agricultural use, pesticides are assessed in terms of their toxicity to non-target organisms, and these risk assessments inform recommended pesticide application rates (Sanchez-Bayo & Goka, 2014; Sgolastra et al., 2020). Guidance on these risk assessments in the European Union (EU) is provided by the European Food Safety Authority (EFSA). When assessing the risk to insect pollinators, they evaluate whether the pesticide is likely to have any harmful effects on bees, specifically the honey bee *Apis mellifera*, the bumblebee *Bombus terrestris*, and two solitary mason bees *Osmia cornuta* and *Osmia bicornis* (EFSA, 2013). The EFSA recommends protocols for laboratory, semi-field, and field studies to assess pesticide effects on these bee species, and in order to ensure that risk assessments are robust, the recommended protocols follow internationally agreed OECD (Organisation for Economic Co-operation and Development) guidelines that aim to provide reliable and repeatable results (EFSA, 2013). Whilst it is not possible to control temperature in field studies, OECD guidelines recommend laboratory studies be conducted at standardised temperatures. For honey bees this temperature is $33\pm 2^{\circ}\text{C}$, but for bumblebees this temperature drops to $25\pm 2^{\circ}\text{C}$ when using standard queenright colonies and $28\text{--}30^{\circ}\text{C}$ when using queenless microcolonies, and for solitary bees the temperature drops again to 22°C (EFSA, 2013). Whilst the need for standardisation is important, this does not account for the fact that environmental temperature varies spatially over latitudes and elevations and also temporally within season and even daily cycles for single species (Oyen et al., 2016; Stabentheiner, 2001). In Europe, for example, mean midday temperatures throughout May - a month in which pesticide application is high and bees are actively foraging (Garthwaite et al., 2018) - vary widely across the continent and often fall considerably below the 33°C and 25°C recommended testing temperatures for honey bees and bumblebees respectively (Figure 1.1; Huld et al., 2006).

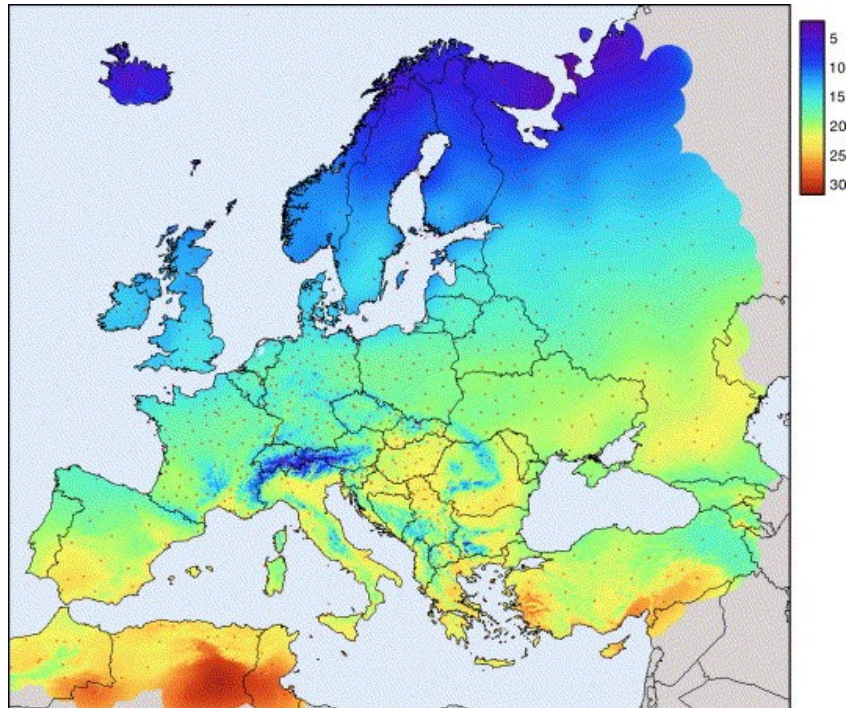


Figure 1.1. Map of average temperature at 12:00 hours GMT in May [°C] between 1995 to 2003. Figure reproduced from Hudl et al., 2006 with permission from Elsevier. The red points indicate the position of the meteorological stations from which data were gathered.

If the toxicity of a specific pesticide is altered by temperature, then the current pesticide application rate guidance may be placing insect pollinators at higher risk in one geographic region over another and/or at one time of the year more than another. Additionally, as it is recommended to test different bee species under different standardised temperatures, this may render comparisons between species inappropriate and could contribute to previously observed findings of differential sensitivity of species to the same insecticide (Decourtye et al., 2013). Gaining an understanding of how pesticide and temperature interact to determine sublethal effects, the importance of which are becoming increasingly recognised by decision-makers in addition to the lethal effects that are traditionally focussed on in risk assessments, will help inform how future pesticide application guidance can be tailored for the UK's and the world's different climatic regions.

1.4 | Bumblebees as an insect pollinator study system

Bees represent the most important group of insect pollinators for agricultural pollination worldwide (Breeze et al., 2011; Kearns et al., 1998; Klein et al., 2007; Ollerton et al., 2011; Potts et al., 2016), and are the representative group used to assess the risks that pesticides pose to pollinators by

policymakers (EFSA, 2013; EPA, 2014). The western honeybee, *Apis mellifera*, is the archetypal insect pollinator, that whilst native to Eurasia and Africa, has been introduced almost worldwide for crop pollination and honey production (Crane, 1999). It has traditionally been assumed to provide the majority of pollination services to agriculture (Breeze et al., 2011), yet increased awareness of the importance of non-*Apis* bees for crop pollination has brought this assumption under question (Garibaldi et al., 2013; MacInnis & Forrest, 2019; Mallinger & Gratton, 2015). Non-*Apis* bees have been estimated to contribute >20% of pollination services in agricultural production (Losey & Vaughan, 2006; Powney et al., 2019), demonstrating superior efficiency in the pollination of key crops such as strawberries compared to honey bees (Horth & Campbell, 2018; MacInnis & Forrest, 2019). Honeybees often cannot replace many of the specialist mutualistic relationships of non-*Apis* bees and plants, and they are rarely the most effective pollinators of a plant, thus appearing ‘imperfect’ substitutes for wild pollinators (Page et al., 2021; Woodcock et al., 2013). Wild bee diversity in agricultural landscapes is important for stabilizing pollinator persistence across years, protecting both biodiversity and crop pollination services, and conferring ecological stability (Senapathi et al., 2021). Moreover, greater bee species diversity has been shown to increase the temporal and spatial range of pollination activity (Brittain et al., 2013; Fründ et al., 2013), increase fruit set and crop yields (Greenleaf & Kremen, 2006; Hoehn et al., 2008; Kremen et al., 2002), and for many agricultural crops successful pollination is consistently increased with non-*Apis* bee but not honeybee abundance (Garibaldi et al., 2013). Non-*Apis* bees may therefore be able to compensate for honeybee colony losses, but this may not be the case vice versa (Tylianakis, 2013).

Attempts to combat global pollination deficits have resulted in a ~45% increase in beekeeping over the past half century (Aizen & Harder, 2009; Jaffé et al., 2010), resulting in steep global increases in managed honey bee colony numbers over the past decade (IPBES, 2016; Steele et al., 2019; Vanbergen et al., 2014). In contrast, non-*Apis* bee species have been reported to have suffered some of the greatest declines relative to other insects in the last few decades, with a number of species having gone extinct (Bennett & Isaacs, 2014; Bommarco et al., 2012; Nieto et al., 2014; Ollerton et al., 2014; Sánchez-Bayo & Wyckhuys, 2019). Indeed, a recent national-scale study in the UK found that wild bees had been lost from a quarter of the places they were found in 1980, and that a third of species now occupy smaller ranges (Powney et al., 2019). These findings support previous reports of declining population trends and range reductions of non-*Apis* pollinators in the UK (Carvalho et al., 2013; Shortall et al., 2009; Steele et al., 2019; Vanbergen et al., 2014; Williams, 1982). Worryingly, increases in honey bee densities can lead to the gradual replacement of wild bees and disruption to the structure and functionality of plant-pollinator networks (Garibaldi et al., 2013; Herrera, 2020; Valido et al., 2019). All this highlights that the current reliance on honeybee pollination services for

food production is both risky and inefficient (MacInnis & Forrest, 2019; Park et al., 2015; Winfree et al., 2008). Yet in spite of this, *Apis mellifera* has been considered the model bee pollinator species for many years, with the vast majority of research and ecotoxicological testing taking place on this species (Decourtye et al., 2013; Heard et al., 2017).

The response of all bee species to changing environmental conditions cannot be assumed to be equal (Wood et al., 2020). Indeed, Hamblin et al (2017) found differential sensitivity to urban heat-island effects between species, with bumblebees having a higher sensitivity compared to honeybees, suggesting they may be at greater risk from future climate warming. Furthermore, Decourtye (2013) drew attention to 42 studies reporting deleterious side effects of registered pesticides on other bee species despite these pesticides passing risk assessments based on the perceived lack of effect on honeybees. This review has been compounded by numerous more studies from both the lab and field highlighting differential pesticide sensitivity between species (Almeida et al., 2021; Arena & Sgolastra, 2014; Cresswell et al., 2012; Heard et al., 2017; Rundlöf et al., 2015; Tomé et al., 2017). In light of this, it is critically important we improve our understanding of the thermal and pesticide sensitivity of non-*Apis* species, if we are to truly appreciate the impact these environmental stressors are having on our pollinators and the services they provide (Dicks, 2013; Schmolke et al., 2021; Siviter et al., 2021; Wood et al., 2020).

Bumblebees

Bumblebees (*Bombus* sp.) are a globally important non-*Apis* pollinator for wild flowers and crops, and primary pollinators of legume and orchard crops that are predicted to increase in cultivation under projected future climatic and socioeconomic scenarios (Aizen et al., 2008; Potts et al., 2016; Smith et al., 2015). They are a particularly suitable study system to address questions in this thesis for two primary reasons:

1) Bumblebees have been shown to be sensitive to climate change and are demonstrating population range contractions (Kerr et al., 2015; Oyen & Dillon, 2018; Soroye et al., 2020). Shifts in elevational and latitudinal ranges have recently been documented for bumblebees across Europe and North America, which appear to be correlated with changes in climate (Kerr et al., 2015). These shifts are predicted to continue into the future, with a recent study by Soroye (2020) suggesting bumblebees are likely to experience range contractions and declines over time due to species more frequently experiencing temperatures that exceed their thermal tolerance. Whilst species can colonise areas that become climatically suitable, it has been suggested that bumblebee dispersal rates may be insufficient to track rates of environmental warming as projected under any one of the IPCC scenarios (IPBES,

2016; Sirois-Delisle & Kerr, 2018). Moreover, evidence suggests they may be evolutionarily constrained in their thermal adaptation, which may increase their sensitivity to warming (Pimsler et al., 2020; Radchuk et al., 2019). Thus, colonisation of new thermally suitable habitat may be outweighed by declines (Soroye et al., 2020), and consequently there is an urgent need to understand the thermal sensitivity of key bumblebee traits.

2) Bumblebees show large intra-specific variation in worker body size (Couvillon, Jandt, et al., 2010; Jandt & Dornhaus, 2009), which has the added benefit of allowing the testing of hypotheses regarding size variation whilst controlling for taxonomic differences in life-history (see section 1.4.1). This can help to shed light on how body size can mediate responses to stressors, with implications for understanding how small versus large species may be environmentally filtered from communities or selected against across different landscapes.

For this thesis, the bumblebee species *Bombus terrestris* will be used. This species is commercially available and easy to rear in the lab, and as such lend themselves as an excellent study species if wanting to acutely or chronically expose either individual bees or whole colonies (Gill et al., 2012; Heard et al., 2017). Additionally, they are a species that are ubiquitous across Eurasia so naturally experience a range of climates (Rasmont et al., 2015), and are abundant agricultural pollinators so are commonly exposed to a diverse collection of pesticides (Herbertsson et al., 2021), making questions surrounding temperature-pesticide interactions particularly pertinent.

1.4.1 | *Bumblebee alloethism*

Like many eusocial insects, bumblebees exhibit large intra-colony size variation in the worker caste (Figure 1.2: Alford, 1975; Goulson et al., 2002). The division of labour among workers is a key feature underpinning the ecological success of eusocial insects (Wilson, 1990), and bumblebees appear to demonstrate alloethism, whereby worker body size correlates with division of colony tasks (Goulson et al., 2002; Herrmann et al., 2018; Peat, Tucker, et al., 2005). Smaller workers tend to stay in-hive and rear brood, whereas larger workers are more likely to forage (Jandt & Dornhaus, 2009; Spaethe & Weidenmuller, 2002). Body size appears to be related to how quickly workers can learn tasks associated with foraging (Worden et al., 2005), with larger workers being more discriminatory and preferentially memorising the features of richly rewarding flowers (Frasnelli et al., 2021). Additionally, evidence suggests that both foraging rate and distance increase with body size (Greenleaf et al., 2007; Kapustjanskij et al., 2007; Spaethe & Weidenmuller, 2002). Together, these traits appear to underpin the observation that foraging efficiency (nectar collected per unit time) is positively related with bumblebee worker body size (Goulson et al., 2002). Whilst the benefits to the

colony of larger workers are commonly explained through foraging ability, less is understood about the benefits of smaller workers. However, when body size variation within colonies is experimentally reduced whilst keeping the same mean body size, colonies appear to perform less well, suggesting that size variation in the worker caste is advantageous in at least some scenarios (Holland et al., 2021). Indeed, intraspecific variation in worker body size appears to make bumblebee species less susceptible to decline (Austin & Dunlap, 2019). Recent evidence suggests size variation may allow colonies to cope with changing or unpredictable landscapes (Austin & Dunlap, 2019; Kelemen et al., 2020; Theodorou et al., 2021). For example, Couvillon & Dornhaus (2010) demonstrated that small workers are hardier against starvation than their larger sisters, and speculated that their presence may provide robustness to bumblebee colonies, allowing them to survive periods of nectar shortage and high colony stress.

There is tentative evidence suggesting that larger workers are more susceptible to the effects of neonicotinoids than smaller nestmates (Samuelson et al., 2016), raising the possibility that having a range of worker sizes could buffer a colony against pesticide exposure. In addition, smaller workers may be better able to maintain body temperature at the required level for flight during warmer ambient temperatures, as they have a lower surface area to volume ratio and can consequently lose heat to the environment at a faster rate (Bishop & Armbruster, 1999; Peat & Goulson, 2005). Considering the wide thermal range that bees are subject to in temperate environments, this may allow colonies to more efficiently exploit resources throughout the season and may also be important when predicting responses to a warming global climate. For bumblebees, climate change and pesticide application across landscapes may therefore act as environmental filters and provide selection pressures towards certain sized individuals. Understanding size-dependent effects on key behavioural traits will help elucidate this, and findings could also be applied across taxa to predict species at risk and inform questions regarding the fitness and service provision of contemporary insect pollinator assemblages under current and future scenarios (Kelemen et al., 2020). Investigation into size-specific responses is timely, as: i) increasing environmental temperature can lead to increased worker size variation (Kelemen & Dornhaus, 2018), smaller mean size (Ohlberger, 2013; Zaragoza-Trello et al., 2021), and changes to morphology (Gerard et al., 2018); and ii) resource limitation and declines in habitat quality arising from agricultural land use change and urbanisation appear to be driving reductions in bee body size across multiple bee taxa (Oliveira et al., 2016; Renauld et al., 2016; Theodorou et al., 2021; Zaragoza-Trello et al., 2021).



Figure 1.2. Variation in *Bombus terrestris* worker body size within a colony can vary dramatically. All workers shown above (first 6 individuals from the left) are from the same colony, with the wet mass of the smallest worker being just 0.043g compared to 0.269g for the largest worker. The queen (far right) had a wet mass of 0.752g, weighing over 17 times that of the smallest worker. Photo taken by Daniel Kenna.

1.5 | How might insecticide exposure and temperature variation, both individually and in combination, affect bumblebee behaviour?

Growth and ultimately fitness of eusocial bee colonies relies on the behavioural performance of many workers contributing towards colony-level functions such as nest maintenance, brood care and foraging tasks (Oster & Wilson, 1978). An accumulation of behaviourally impaired workers could lead to degraded overall colony function, leading to reduced fitness and eventually colony collapse (Bryden et al., 2013). This thesis will explore how temperature variation and pesticide exposure, both individually and in combination, affect distinct behaviours known to be important for colony function and ecosystem provision, namely aspects of locomotion.

1.5.1 | How might insecticides and ambient temperature affect flight and other locomotory activity?

Flight capability represents a behaviour integral for survival of the majority of insect pollinator species, especially bees. The ability of bees to move around the landscape determines the dispersal capacity of sexuals and the efficiency with which workers can exploit resources required for growth and reproduction (Greenleaf et al., 2007; Kremen, 2005; Kremen et al., 2007; Waser et al., 1996). Bees forage for both nectar and pollen, which promotes a high floral resource use. Adults rely on nectar as their carbohydrate energy source with bee offspring heavily reliant on pollen provisions as a protein source (Couvillon et al., 2015; Pelletier & McNeil, 2003). The emergence of intensive agricultural practices has led to widespread habitat loss and fragmentation, increasing the foraging and dispersal demands placed on bees (Senapathi et al., 2017; Viana et al., 2012; Winfree et al., 2009). Farmland

has been found to have a significantly lower nectar diversity than urban areas (Tew et al., 2021), and the decrease in crop and floral resource diversity over the last half-century due to ever more intense farming has been associated with declines in bee species (Hemberger et al., 2021). In agricultural landscapes where resource availability is low and sporadic, the ability of bees to forage effectively and efficiently over great distances may therefore be vital (Jha & Kremen, 2013; Redhead et al., 2016; Steffan-Dewenter & Tschardt, 1999). This poses a particular challenge for bees such as bumblebees, which are central place foragers having a fixed nest site, so are unable to track changing resources across landscapes (Williams et al., 2012). Workers must undertake return foraging trips from this set nest location, and consequently any habitat discontinuity may require workers to fly longer distances to find the volume, quality and complementarity of resources they require for growth and survival (Gervais et al., 2020; Goulson et al., 2008; Jha & Kremen, 2013; Redhead et al., 2016; Schmid-Hempel & Schmid-Hempel, 1998). Hence any stressor lowering individual worker flight ability could translate to negative colony level impacts (Gill et al., 2012), with implications for the crucial ecosystem services they provide (Delaplane & Mayer, 2000; Garibaldi et al., 2013; Greenleaf & Kremen, 2006; Potts et al., 2010; Winfree et al., 2008).

Pesticide exposure likely represents one such stressor. For instance, controlled neonicotinoid exposure experiments have demonstrated negative effects on bee homing ability (Bortolotti et al., 2003; Fischer et al., 2014; Henry et al., 2012, 2014) and foraging efficiency, including less frequent but longer foraging trips and a reduction in pollen collected per trip (Feltham et al., 2014; Gill & Raine, 2014; Gill et al., 2012; Kolano et al., 2021; Schneider et al., 2012; Siviter, Johnson, et al., 2021; Stanley & Raine, 2016). Furthermore, a handful of recent studies have highlighted potential negative effects of neonicotinoid exposure on bumblebee foraging motivation using foraging arena setups, with exposed individuals being slower to initiate foraging trips and visiting less flowers when they do (Lämsä et al., 2018; Muth & Leonard, 2019), and with exposed colonies committing less workers to becoming foragers (Crall et al., 2018). Similar findings arose from the only available study examining the effects of sulfoxaflor on bee foraging behaviour, with exposed solitary bees found to have a reduced flower visitation rate and being more likely to walk to flowers than fly in foraging arenas (Boff et al., 2021).

The mechanistic basis for the aforementioned observations from pesticide exposure is not fully known but could involve impairments to motor function (Fischer et al., 2014; Stanley et al., 2016; Williamson et al., 2014), such as flight (and other locomotory) endurance and motivation. However, difficulties in tracking and quantifying movement for highly mobile organisms means this has been little investigated. To my knowledge, only three studies have directly investigated the effect of pesticide exposure on flight endurance to date, all using honey bees and with mixed results. A study

by Tosi et al. (2017) found detrimental effects on flight endurance and velocity following chronic neonicotinoid exposure, but beneficial effects on endurance following acute exposure of the same chemical. In contrast, a study by Blanken et al. (2015) found no effect of chronic neonicotinoid exposure on flight unless bees were also infected with the parasitic *Varroa* mite. More recently, Tong and colleagues (2019) found that chronic exposure of a new systemic butenolide insecticide caused reduced flight success but increased flight velocity, highlighting that novel insecticides can impact flight but in potentially different ways to the established neonicotinoids. Clearly, more research is needed to elucidate the impact of neurotoxic insecticides under realistic exposure scenarios on bee flight performance.

When considering the effects of pesticides on bumblebee flight and foraging performance, it is essential that we consider how temperature places energetic limitations. Bees are heterothermic, meaning that they are able to transition between ectothermic states, where body temperature is reliant on environmental heat sources and follows ambient temperature closely, and endothermic states, where body temperature is regulated and reliant on heat from internal metabolic processes (Esch et al., 1991; Heinrich, 1975; Stabentheiner et al., 2012). As heterotherms, they do not solely rely on thermal energy from the environment to warm themselves and can generate heat endogenously when required through tetanic shivering of flight muscles in a process known as non-flight thermogenesis (Esch et al., 1991; Heinrich, 1993; Potts et al., 2018). This endothermic ability to raise body temperature above the ambient temperature is vital for colony thermoregulation, but also for tasks such as flight and foraging (Heinrich, 1975; Kovac & Stabentheiner, 2011; Stabentheiner & Kovac, 2014). In order to generate sufficient force to support continuous flight, bees must attain and maintain a minimum thoracic temperature typically above that of their surroundings (Heinrich, 1972, 1993; Nieh et al., 2006). In cold conditions this can place a large energetic strain on individuals whilst foraging, as they need to generate the required heat endogenously, and must maintain the required thorax temperature throughout the whole foraging cycle, in order to promote the fast exploitation of resources whilst always staying prepared for immediate flight (Heinrich, 1979; Kovac & Stabentheiner, 2011; Stabentheiner & Kovac, 2016). Due to their small size, this endothermic behaviour requires enormous energetic input, in order to compensate for rapid heat loss because of their large surface area to volume ratio (Bishop & Armbruster, 1999). For example, in honeybees this effort can require around a 120-fold increase from their resting metabolic rate (Stabentheiner et al., 2012). In flying insects, activity rates are a direct function of thoracic temperature (Heinrich, 1974b), with muscle performance, wing-beat frequency, flight force production, and oxygen consumption all appearing to be positively correlated with increasing body temperature up to tipping points (Coelho, 1991; Kammer & Heinrich, 1974; Woods, 2005). Ambient temperature should therefore influence bumblebee flight

performance and foraging efficiency, but to date there is surprisingly little direct empirical evidence to quantify this (Coelho, 1991; Heinrich, 1972; Kwon & Saeed, 2003; Peat & Goulson, 2005).

1.5.2 | *How might bumblebee workers be exposed to insecticides under variable thermal conditions?*

Workers in bumblebee colonies can naturally be exposed to insecticides under a wide range of thermal conditions. Workers experience a wide thermal gradient, both spatially and temporally, when foraging outside the nest, and climate change is warming the landscapes with more frequent and intense extreme temperature events (Deutsch et al., 2018; Germ et al., 1997; Tang et al., 2021). Thermoregulatory behaviour in bumblebees allow colonies to maintain consistent nest temperatures around 30°C (Crall et al., 2018; Heinrich, 1979; Potts et al., 2018), hence insecticide exposure whilst in the nest will likely come at widely different ambient temperatures to exposure whilst on the wing foraging (Stabentheiner et al., 2010; Vogt, 1986). Additionally, neonicotinoids can impair thermoregulatory behaviour, both at the individual and colony level (Potts et al., 2018; Tosi et al., 2016), creating individuals and nests with greater internal thermal fluctuation and thus shaping the thermal environments in which workers are exposed (Manzi et al., 2020). This can be accentuated in agricultural landscapes, as low resource availability and nutrition can cause a greater proportion of workers to be allocated to foraging rather than nest thermoregulation duties, compromising the stability of the nest temperature (Stewart et al., 2021). Neonicotinoid exposure has also been found to disrupt circadian rhythms, with consequences such as foragers leaving the colony at suboptimal times exposing them to more extreme temperatures (Tackenberg et al., 2020; Tasman et al., 2020).

1.5.3 | *How might temperature modulate pesticide toxicity effects on flight and other locomotory activity?*

Due to their heterothermic nature and ability to closely regulate internal body temperature independent of ambient temperature, the response on bumblebees to insecticides under thermally variable environments may differ from that of the predominantly ectothermic insect pests for which pesticide temperature coefficients are commonly assessed (Tsaganou et al., 2021). For example, studies in insect pests commonly suggest that neonicotinoids are positive temperature coefficient insecticides, meaning that they show enhanced toxicity as ambient temperature increases (Mansoor et al., 2015; Tsaganou et al., 2021). However, when investigating effects in bumblebees, Crall et al. (2018) found that the detrimental impacts of imidacloprid on both colony-level thermoregulation and individual worker behaviour were more severe at night as opposed to day, suggesting that

neonicotinoids show enhanced toxicity to bees as ambient temperature decreases. Similarly, Saleem et al. (2020) found that the effect of both imidacloprid and thiamethoxam on honey bee mortality rates were significantly greater at lower (24°C) compared to higher (35°C) temperatures.

Recent work has shown that the neonicotinoid class of insecticides can disrupt non-flight thermogenesis in both honey bees (Tosi et al., 2016) and bumblebees (Potts et al., 2018), which may compromise the ability of exposed bees to attain or maintain the required temperature for flight or other locomotory behaviours (Azpiazu et al., 2019). In addition, neonicotinoids can also disrupt the ability of bumblebee colonies to effectively thermoregulate their nests, with implications for nest behaviour and brood development (Crall et al., 2018). These disruptions have the potential to be more restricting to individual and colony level function under colder conditions, due to the increased energetic requirements involved in thermoregulation, which may explain the results found by Crall et al. (2018). Indeed, the impact of pyrethroid insecticides on thermogenesis has been discussed as the mechanism underpinning the enhanced toxicity of these chemicals to honeybees under lower temperatures (Belzunces et al., 2012). Additionally, studies addressing the negative impacts of both thiamethoxam on honey bee homing ability and clothianidin on bumblebee foraging duration, have found that impairments become increasingly aggravated as environmental air temperature decreases (Henry et al., 2014; Kolano et al., 2021; Monchanin et al., 2019).

A study by Gooley & Gooley (2020) interestingly found that imidacloprid exposed honey bees had elevated metabolic rates when exposed at low (25°C) temperatures but not at higher temperatures (30 & 35°C). These disrupted energy costs at lower temperatures may be because workers needed extra energy to detoxify the pesticide, achieved by raising body temperature to speed up the rate of enzyme synthesis or activity (Rand et al., 2015). Furthermore, Manzi et al. (2020) found that the expression of genes involved in motor rhythms, metabolic state, and flight were increased following imidacloprid exposure, and this overexpression was further intensified at 20°C but not at warmer temperatures of 28°C or 36°C. They also found that heat shock proteins were under expressed in exposed bees but only at the highest temperature of 36°C. Together, these studies provide potential mechanistic pathways for temperature-modulated toxicity in bumblebees. However, these findings stand in slight contrast to two recent studies exploring combined stressors in bumblebees, which found no detectable interaction between temperature and neonicotinoid exposure on measures of either colony success (Zaragoza-Trello et al., 2020) or pollination service (Greenop et al., 2020). Interestingly, these studies attempted to alter the temperature of the nest rather than exploring variation in the air temperature of the foraging landscape, and measured colony-level rather than individual responses, which may provide useful insights into why pesticide-temperature interactions were not detected.

Whilst there is no known information available evidencing how temperature may modulate the effects of sulfoxaflor, this insecticide has been shown to effect foraging behaviour in solitary bees (Boff et al., 2021), and another new emerging insecticide, flupyradifurone, has been shown to impair key locomotory behaviours such as flight success and velocity as well as thermoregulation in honey bees. This highlights that new insecticides to market can have detrimental sublethal impacts on bumblebee behaviour with potential to be temperature dependent (Tong et al., 2019).

1.6 | Thesis aims

1.6.1 | *Key objectives*

The overarching aim of this thesis is to assess how insecticide exposure and surrounding environmental temperature, both individually and in combination, affect locomotory behaviours in individual bumblebee workers; behaviours that underpin organismal function, fitness and pollination provision.

From controlled and complementary flight performance experiments, I address this aim by exploring how neonicotinoid exposure (Chapter 2) and environmental temperature (Chapter 3) individually influence flight behaviour. I utilised a novel flight mill setup, allowing questions surrounding flight performance and behaviour to be investigated efficiently and repeatably under controlled conditions (Minter et al., 2018). Importantly, these experiments examined responses across a full spectrum of worker body sizes found within colonies providing: i) insights into the basis of task division in bumblebees; ii) indication of how variation in individual level responses scale-up to colony level effects; and iii) predictions to be made about how smaller versus larger insect pollinator species may respond to stressors. Findings were then used to inform the pesticide choices / concentrations and temperatures used in Chapter 4, in which I examined the combined effects of insecticide exposure (a neonicotinoid and a sulfoximine) and variable temperature on both lethal and sublethal behavioural endpoints.

1.6.2 | Thesis structure

In chapter 2, the aforementioned tethered flight mill setup was employed to investigate how oral exposure to a field realistic acute dose of the neonicotinoid imidacloprid affects aspects of flight performance, namely distance, duration, and velocity, of bumblebee workers.

In chapter 3, I used insights gained from Chapter 2 to develop and refine the flight mill protocol and used this setup to quantify how the flight performance of bumblebee workers varied over a 12-30°C

temperature range. By incorporating results from individual workers on flight endurance and likelihood of flight, I calculated a simple metric termed 'temperature-mediated foraging potential' to gain a clearer understanding of how temperature may constrain colony foraging.

In chapter 4 I conducted a multi-experimental study to explore temperature-modulated toxicity of insecticides to a key insect pollinator. Examining flight behaviour, walking behaviour, consumption and survival, here I tested how individual bumblebees exposed to either imidacloprid or sulfoxaflor were differentially affected across three different ambient temperatures.

In chapter 5 I discuss the findings from this thesis and how it can help to: i) provide a mechanistic explanation for recent reports of insect pollinator distributional changes associated with temperature range limits (Kerr et al., 2015; Soroye et al., 2020); ii) highlight additional burdens associated with pesticide usage, and provide insights as to which crop growing regions may suffer from future [bumblebee] pollination deficits; iii) understand which possible climate regions in the world are insect pollinators most at risk from pesticide exposure; and iv) inform how policy and the farming industry should assess risk and guide the assessment of when best to apply pesticides in the environment in order to prevent pollinator population tipping points and maintain stable wild and agri-ecosystems.

In Appendix D & E, I provide further preliminary studies that I designed, supervised, and helped conduct, which build on the data chapters presented in this thesis, but which require additional work.

CHAPTER 2

Neonicotinoid exposure affects flight dynamics and reduces flight endurance in bumblebees

2.1 | Introduction

The extent to which insects move across landscapes has significant implications for human welfare. Highly mobile species can potentially cause detrimental insect pest outbreaks (Mazzi & Dorn, 2012; Sharov & Liebhold, 1998), invasions (Myers et al., 2000; Renault et al., 2018) or the spread of vector borne diseases (Dujardin et al., 2008; Estrada-Peña et al., 2014; Githeko et al., 2000; Rogers & Packer, 1993). Yet insect movement can also underpin beneficial ecosystem service provision. For example, the majority of angiosperms, including around $\frac{3}{4}$ of our crop species, are to some degree reliant upon the extensive movement of foraging insect pollinators (Gill et al., 2016; Kleijn et al., 2015; Klein et al., 2007; Ollerton et al., 2011). It is therefore important we understand which, and to what extent, stressors affect insect pollinator flight performance if we are to mitigate threats to a global pollination service valued at >€150 bn annually (Benaets et al., 2017; Fischer et al., 2014; Gallai et al., 2009; Gill & Raine, 2014; Stanley et al., 2015; Wolf et al., 2014).

The emergence of intensive agriculture can cause loss and fragmentation of suitable foraging habitats, leading to resources becoming increasingly sparse and isolated within an insect's foraging range (Didham et al., 1996; Hadley & Betts, 2012; Steffan-Dewenter & Tscharntke, 1999; Tscharntke & Brandl, 2004; Zurbuchen et al., 2010). This may pose a considerable challenge for eusocial bees, which are central place foragers having a fixed nest site. Workers must undertake return foraging trips from this set nest location, and consequently any habitat discontinuity may require workers to fly longer distances to find and bring back resources, such as pollen and nectar (Goulson et al., 2008; Jha & Kremen, 2013; Pelletier & McNeil, 2003; Schmid-Hempel & Schmid-Hempel, 1998). Hence any stressor lowering individual worker flight ability could translate to negative colony level impacts (Gill et al., 2012), with implications for the crucial ecosystem services they provide (Delaplane & Mayer, 2000; Garibaldi et al., 2013; Greenleaf & Kremen, 2006; Potts et al., 2010; Winfree et al., 2008).

Insecticides are commonly applied in agricultural landscapes as a pest management strategy (Fernandez-Cornejo & Vialou, 2014; Ramankutty et al., 2018), with neonicotinoids being one of the most widely used classes worldwide (Simon-Delso et al., 2015). However, neonicotinoids have been implicated as a threat to eusocial bees (Gill et al., 2012; Goulson, 2013; Lundin et al., 2015; Tsvetkov

et al., 2017; Whitehorn et al., 2012; Woodcock et al., 2017). Foraging eusocial bees are frequently exposed to neonicotinoids in treated landscapes (Botías et al., 2017, 2015; David et al., 2016; Mitchell et al., 2017; Rolke et al., 2016), and controlled exposure experiments have demonstrated both impaired homing ability (Fischer et al., 2014; Stanley et al., 2016) and foraging efficiency of workers, including longer foraging trips and reduced rate of pollen collection (Feltham et al., 2014; Gill & Raine, 2014; Stanley & Raine, 2016). A possible explanation for these reported impairments is that certain aspects of foraging flight dynamics, such as endurance and speed, are affected by neonicotinoid exposure. However, to date only two studies have specifically tested this and have reported mixed findings. One study found acute neonicotinoid (118 ppb thiamethoxam) exposure increased flight endurance, with the opposite effect shown following chronic (32.5 – 45 ppb thiamethoxam) exposure (Tosi, Burgio, et al., 2017). The other study detected a negative effect of chronic (6 ppb imidacloprid) exposure on flight distance, but only when provided to individuals in combination with the parasitic varroa mite (Blanken et al., 2015). Hence, further investigation is needed to understand the generality of the effects of exposure on bee flight, whilst also: i) ensuring that a concentration within the field realistic range is used; ii) gaining a more in-depth analysis of the dynamics of flight during testing; iii) investigating a representative species of wild bee, given there can be differential responses to pesticide exposure among insect pollinator species (Cresswell et al., 2012; Heard et al., 2017; Rundlöf et al., 2015); and iv) considering variation in worker body size, given this can modulate flight capability, can be associated with variation in foraging behaviours within bumblebee colonies (Goulson et al., 2002; Spaethe & Weidenmüller, 2002), and size-specific energetic demands show a non-linear positive relationship (Greenleaf et al., 2007; Kaufmann et al., 2013).

To date, studying the physical flight of small mobile insects has proved challenging. When examining flight behaviour under field or semi-field setups it is difficult to isolate the effects of pesticides from other confounding / covarying factors (Henry et al., 2014). Additionally, current tracking technologies typically cannot record a full foraging bout, with RFID tagging limited to recording bees moving past a fixed location, providing little information on what occurs during the flight trip (e.g. Gill & Raine, 2014). Harmonic Radar can track a bee over a foraging bout, but only in uncluttered landscapes and heavy tags likely confine potential foraging ranges, which would have a disproportionate effect on smaller bees (O’Neal et al., 2004; Osborne et al., 1999). To help address this challenge, a tethered flight mill setup (Minter et al., 2018; Tosi, Burgio, et al., 2017) was used under controlled environment conditions to investigate the effects of acute oral neonicotinoid exposure on different aspects of bumblebee (*Bombus terrestris audax*) flight performance (Figure 2.1). For this study, individual workers were exposed to the neonicotinoid imidacloprid at a concentration of 10 ppb as it is: i) a neonicotinoid registered for use in over 100 countries (Jeschke et al., 2010) and

one of the most popular pesticides for the control of sucking insect pests worldwide (Casida, 2018; Raymann et al., 2018; Zhang, 2018; Zhu et al., 2017); ii) a concentration that can be found inside social bee colonies, on return foraging workers, and in the pollen and nectar of individual flowers (Blacqui re et al., 2012; Bot  as et al., 2016; Cresswell, 2011; Dively & Kamel, 2012; Goulson, 2013; Hladik et al., 2016); iii) known to impair foraging performance after exposure (Godfray et al., 2014; Pisa et al., 2017); and iv) a neonicotinoid under current scrutiny by policy makers and regulators (Cressey, 2017), resulting in a recent EU ban from agricultural use outside of closed greenhouses. As the neonicotinoid with the longest history of use across the largest geographic area (Casida, 2018; Mitchell et al., 2017; Simon-Delso et al., 2015), testing the effects of imidacloprid can help reveal why both past and future insect pollinator populations were / are under threat. The propensity of individual bees to fly was tested, followed by measures of flight distance, duration, and the dynamics of velocity over the course of the flight test, and the interaction between neonicotinoid exposure with worker body size on these performance measures was investigated.

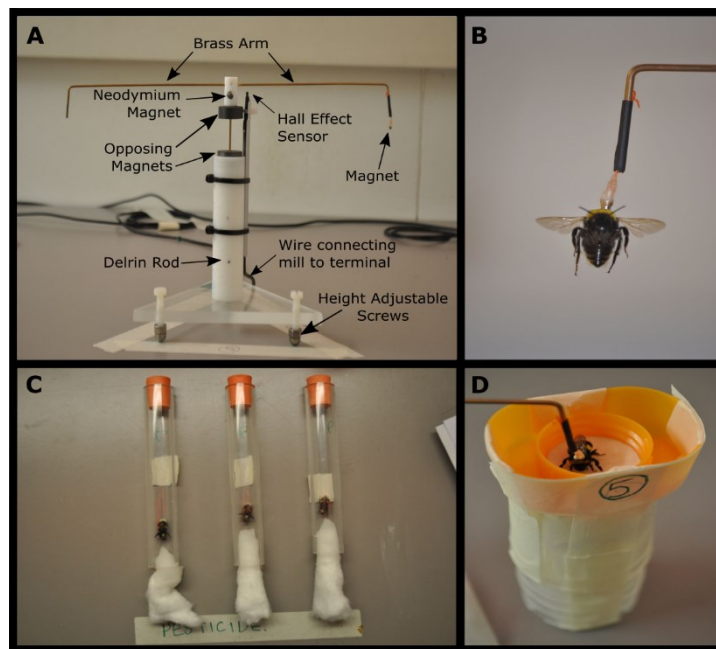


Figure 2.1. Flight mill setup and associated experimental procedures. Panels show A) flight mill used in the study (the ‘height adjustable screws’ ensured the mill could be horizontal with an attachable bubble level used to ensure this); B) tethering of an individual worker bumblebee to the flight mill magnet; C) feeding procedure in which workers were placed in banded tubes with one end consisting of cotton wool lightly soaked in 50% sucrose solution (with or without 10 ppb imidacloprid); D) support stands used to hold workers prior to flight tests and following a stop in flight.

2.2 | Methods

2.2.1 | *Bee husbandry*

Three bumblebee *Bombus terrestris audax* colonies, containing a queen and between 130-150 workers, were supplied by a commercial company (Agralan Ltd). Each colony was delivered in a separately housed plastic nest box (29 x 22.5 x 13 cm) and kept in a controlled environment room (25°C) under red light. From the point of arrival, colonies were provisioned with 4g of pollen daily and supplied with ad libitum 10/90% sucrose/water solution via a connected reservoir. A 10% sucrose concentration falls within the range of many flower species (Pierre et al., 1999; Pyke & Waser, 1981), and was sufficient to ensure that provisioned colonies did not suffer from dehydration or starvation. However, the primary justification for using this relatively low concentration in the nest was to increase motivation of individual workers to feed when provided with a higher concentration of sucrose solution during the acute exposure setup. This was necessary to facilitate sufficient uptake of pesticide active ingredient in exposed workers and increased the likelihood that all workers (control or treatment) would feed to satiation prior to flight.

2.2.2 | *Flight mill setup and bee tethering*

Six flight mills were set up in a separate adjoining room under the same environmental conditions as the housing room (constant 25°C temperature), but with the option to switch between red (Philips TLD 58W Red 1SL/25; mean 660 nm) and white light (Philips TLD 58W 840). Flight mills were adapted from a previous design (Smith & Jones, 2012), consisting of a revolving brass wire, with a magnet hanging from one end designed to attach to a metal tag glued to the bee's thorax through magnetic attraction (Figure 2.1). The revolving brass wire was suspended over a central Delrin rod by the repulsive forces of two magnets, preventing friction during arm rotation to allow fluid motion. The Delrin rod was positioned vertically (90° perpendicular) on a horizontally flat triangular Perspex base. A digital Hall-effect sensor placed on one side of the mill detected each complete revolution by the passage of a neodymium magnet (Figure 2.1a) and sent an impulse to a Raspberry Pi computer (model B) via a copper wire connector. From here a Python script recorded the time (in seconds) between each impulse, with each revolution defined as a 'circuit' from hereon.

When the colonies arrived, 110 workers per colony (total=330) were randomly selected and under red light a circular galvanised iron tag (diameter = 2 mm, thickness = 0.4 mm) was attached to the thorax of each worker using super glue (Figure 2.2), allowing each individual to be tethered to the hanging flight mill magnet (Figure 2.1b,d). I am confident that tag mass did not cause any significant impairment to bee flight performance, because: i) magnetic attraction of tag to mill allows suspended

bees to fly without carrying the load of the tag; and ii) mean ($\pm$ s.e.m) tag mass was only 18 ± 0.3 mg (calculated from weighing 30 tags), equating to just 7.5% of the mean worker wet mass of all individuals tested in this study (240 ± 5 mg). Indeed, bumblebees are capable of carrying $> 50\%$ of their own body mass in nectar alone when foraging (Brian, 1954). Each tag was placed at the centre of the thorax, with the tag leading edge touching the back of the first thoracic stripe (Figure 2.2). Care was taken to ensure tag attachment would not interfere with wing movement, with the small tag size easily fitting between the wing joints of even the smallest tested worker with no obstruction. The meticulous nature of tagging each bumblebee meant that a tag rating system was designed, whereby tag positions were scored as 1 = ideal, 2 = unideal or 3 = unacceptable (Figure 2.2). Bees with scores 1 and 2 were considered acceptable to experimentally test, but bees with score 3 were excluded from further use as these placements had potential to impede wings or affect flight posture. In total, 74 workers per colony were experimentally tested (total = 222).

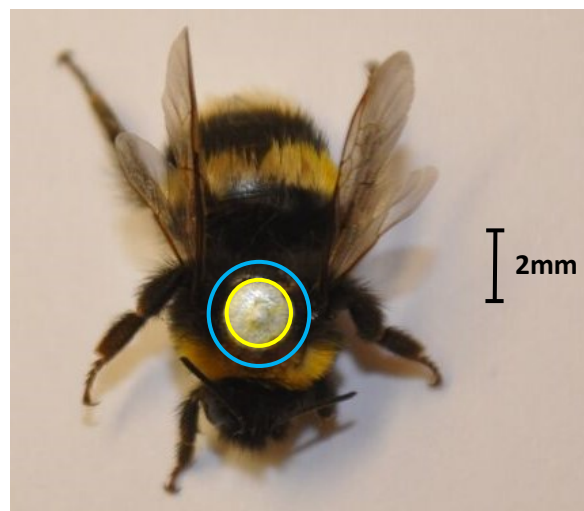


Figure 2.2. Example of the ideal positioning of a metal tag (tag score 1) on the thorax of a *Bombus terrestris audax* bumblebee worker. If the tag positioning was unideal (tag score 2) the metal tag would overlap the yellow circle but remain inside the blue circle. If positioning was unacceptable (tag score 3) it would overlap the blue circle.

2.2.3 | Pesticide preparation

A 10 ppb imidacloprid working solution was produced to supply the acute pesticide exposure to worker individuals. A previously made stock solution of imidacloprid dissolved in acetone at a 1 ppt concentration was used, which was stored in a freezer wrapped in aluminium foil to prevent light degradation (Soliman, 2012). Aliquots of the stock solution were diluted by addition of a 50%

sucrose solution to create the working 10 ppb *pesticide* treatment solution. The control solution was made by adding the respective volume of acetone to 50% sucrose solution to produce a 10 ppm acetone *control* working solution.

2.2.4 | *Experimental procedure*

Pilot studies were first conducted in September 2015 and March 2017 to trial and verify the experimental setup and procedures, with the main experimental study conducted in April 2017. The main experimental testing started 12 hours after tagging was completed, and testing took place over an 8-day period. Workers were tested in bouts, with 5-6 bouts undertaken per day. Six workers were sampled per bout (one per flight mill), consisting of two workers sampled per colony, with one worker randomly assigned to the *treatment* and the other to the *control*. This ensured that all three colonies and both treatment groups were represented equally in each bout and over the totality of the experiment (total: $n = 37$ bees per treatment per colony; $n = 111$ *control* & 111 *pesticide*). Once removed from the colony, each worker was directly transferred to a separate transparent horizontally laid Perspex tube (length = 150 mm, internal diameter = 19 mm). The tube had a rubber bung at each end creating a holding compartment for the bee, with individuals left to acclimatise for a resting period of 3 minutes. After this resting period, one of the bungs was replaced with cotton wool lightly soaked in the *control* or *pesticide* treatment sucrose solution (Figure 2.1c). This already piloted method ensured that 94.1% of workers fed. The assumption was made that spiking the sucrose solution with the neonicotinoid would not deter feeding if given no other option, as supported by pilot observations and previous studies (Arce et al., 2017; Gill et al., 2012; Kessler et al., 2015). Pilot studies also indicated that workers took a mean ($\pm$ s.e.m) duration of 50 ± 13 seconds to commence feeding, defined as prolonged (>2 seconds) proboscis extension on to the cotton wool, and fed for a mean ($\pm$ s.e.m) duration of 213 ± 24 seconds before stopping, with subsequent feeds being rare, sporadic, and short (<10 seconds). Workers could access the provisioned sucrose-soaked cotton wool for 10 minutes, after which the cotton wool was removed and original bung replaced, followed by a 5-minute resting period inside the tube. Whilst this protocol meant the precise dosage of imidacloprid consumed by each worker could not be determined, which might have improved the predictive power of statistical models, it did allow workers to feed to satiation, which is a state likely to occur in the field during foraging bouts and allowed consumption volume to vary proportionately to individual worker size (Free & Butler, 1959; Goulson et al., 2002).

The workers that fed were then removed carefully using tweezers and tethered to the flight mill. The 5.9% of workers that did not feed were immediately frozen (-20°C) and weighed along with

all other bees after all flight tests had been completed. All of this was carried out under red light conditions, but once workers were tethered to the mills the room was switched to white light. Each mill had a separate height-adjustable stand which was erected once the bee was tethered and used to hold the worker in place (Figure 2.1d). Prior to initiating the flight test, workers were held in place for a period of 10 minutes for two primary reasons. Firstly, pilot observations demonstrated that some bees were initially irritated by attachment to the mill and would attempt to dislodge themselves from the magnet using middle and hind legs, which discouraged flight. A 10-minute acclimatisation period allowed irritation to subside, and all bees in the pilot study had withdrawn legs from the magnet at this point. Secondly, a balance was sought between giving workers time to metabolise the neonicotinoid and preventing de-motivation to fly through having them separated from their natal colony for too long. Honey bees metabolise imidacloprid and other neonicotinoids quickly, with a 100 $\mu\text{g kg}^{-1}$ dose of imidacloprid showing the greatest levels of presence in the thorax and abdomen after just 20 minutes from ingestion (Suchail et al., 2004), and >50% of a 100 $\mu\text{g kg}^{-1}$ dose of acetamiprid being metabolised in less than 30 minutes (Brunet et al., 2005). In this study, a total of 25 minutes passed from starting the feeding trial to starting the flight test, which I am therefore confident represents enough time for absorption and metabolism of some of the imidacloprid consumed.

Immediately after the 10-minute acclimatisation period, the support stand was removed quickly from beneath the bee to stimulate flight. Prior to removal, the stand was rotated to ensure the worker had a forward-facing orientation. Stand removal caused loss of tarsal contact with the stand surface, which can trigger flight as evidenced in pilot studies and other previous studies (Blanken et al., 2015; Brodschneider et al., 2009; Tosi, Burgio, et al., 2017). However, if the worker did not initially start flying the flat side of the stand was used to gently tap the legs in order to generate a sharp loss of tarsal contact. Up to three taps were allowed in this first flight attempt, with individuals being removed from the flight test if no flight was initiated.

Workers that successfully flew in the first attempt were monitored for any subsequent flight stoppages. Each flight stoppage was noted, and each worker was permitted five stoppages before their flight test was terminated. Immediately following a flight stoppage, the individual would be held in the stand to ensure tarsal contact for a 20-second rest period before removal of the stand again. Therefore, in the subsequent data analysis, flight stoppages were identified as circuits with a duration >20 seconds. After a stoppage, workers were only permitted one tap of the legs in an attempt to trigger flight; otherwise their flight test was terminated. Any stoppages that occurred on the first circuit were discounted as genuine stoppages, as this was deemed an acclimatisation circuit for bees to familiarise themselves with the experimental setup. All workers were given the opportunity to fly for up to 60 minutes, including all stops, after which the flight test was terminated. Each individual

worker was allowed a maximum of five flight stoppages, essentially allowing each bee five chances to continue its flight until the 60-minute end point. This provided a better representation of field conditions and a more realistic prediction of foraging distances, as foraging bees do not fly continuously during foraging bouts but will stop at flowers periodically to feed and rest (Woodgate et al., 2016). Additionally, it allows individuals to acclimatise to the conditions of the flight mill and decreases the possibility of excluding individuals from testing that are initially demotivated to fly due to the experimental setup.

Following each flight test, workers were placed in separate labelled tubes and frozen (-20°C). After completion of the whole experiment, for each individual worker the following measurements were taken: i) wet body mass (including the attached metal tag; accuracy 0.1 mg); and ii) intertegula span (ITS; accuracy 0.01 mm) taken using a digital calliper (Workzone 150 mm), with the mean of three repeated measurements being used. For data analysis, ITS was taken as a proxy for worker body size (Cane, 1987; Greenleaf et al., 2007). This is more appropriate than considering worker wet mass, as wet mass will vary according to both the volume of sucrose solution consumed and the duration of flight, as individuals gain mass through feeding and lose it through energy metabolism during flight.

2.2.5 | Data filtering and statistical analysis

Frequency distribution plots revealed a spike in the number of workers that terminated the flight test before completing 100 m (118 circuits; Figure 2.3). Workers that did not fly over this threshold distance were excluded from the endurance and velocity analysis as a precautionary measure to discount individuals whose flight mill performance is not representative of actual flight capacity. For each worker flying beyond the 100 m threshold, I calculated the following: i) total distance flown during the flight test, by taking the total number of circuits flown multiplied by the circuit circumference (0.848 m); ii) total duration of the flight test, by summing all circuit interval times; and iii) velocity of each circuit, by taking the circuit circumference and dividing it by the respective circuit interval time. I took a simple calculation for mean velocity, calculated as the total distance flown divided by the total duration flown, and maximum velocity was taken from the circuit showing the highest velocity attained across the flight test.

The velocity calculations for each individual flight test were carried out on filtered data in which the following circuits were excluded from the analysis: i) first five circuits of the first flight attempt; ii) first five circuits directly following a flight stoppage; iii) the circuit directly preceding a flight stoppage. It was noted from pilot observations and the main study that removal of the support stand or tapping of the legs would often stimulate strikingly high velocities. It is likely this behaviour

is a reaction to stimulatory stress, so I felt actions i) and ii) were justified as a precautionary measure to ensure I only considered circuits representative of normal continuous flight. Similarly, in justification of action iii), the minimal rotational resistance of the mill means that when a worker stops flying it does not equate to an abrupt stop, but the brass arm continues to rotate and slows gradually.

When considering total duration flown it was noted that the data were bimodally distributed (Figure 2.3b); therefore, I converted the results to a binary response variable categorised as having or having not flown >2000 seconds, with this duration value decided on as it fell at the bottom of the bimodal concave.

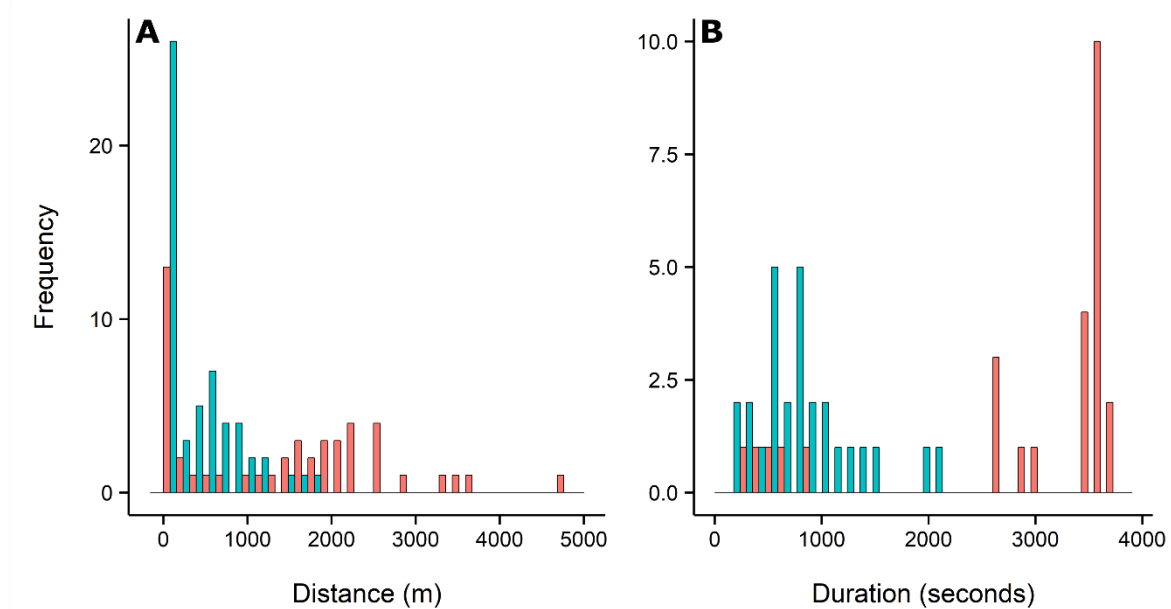


Figure 2.3. Frequency distributions of distance (A) and duration (B) flown by workers during the flight trials, considering both *control* (red) and *pesticide* exposed (blue) workers. Distributions highlight a spike in the number of workers that terminated flight within the first 100m (A) and demonstrate a bimodal distribution when considering total duration flown (B). For distance flown, all workers that initiated flight were considered (n=103, Table 2.1 – filter step 5), consisting of 47 *control* and 56 *pesticide* treated workers. For duration flown, data shown is for the subset of bees used (n=53, Table 2.1 – filter step 7), consisting of 26 *control* and 27 *pesticide* treated bees, but the same distribution was observed when considering the full dataset (n=67, Table 2.1 – filter step 6).

Statistical analyses were conducted using the ‘lme4’ (Bates et al., 2015) package in R v3.2.0 (R Core Team, 2015), with summary statistics generated using the package ‘psych’ (Revelle, 2015) and results reported using the package ‘lmerTest’ (Kuznetsova et al., 2017). A linear model was used to compare variation in *ITS* (body size) between treatments, with *treatment* (*control* or *pesticide*) as the only fixed effect. For all other analyses, mixed effects models (fitted by maximum likelihood) were initially used with *colony* included as a random factor. Unless otherwise stated, fixed effects in each analysis included *treatment* (*control* or *pesticide*), *ITS* and the associated interaction term. Where response variables were binary (propensity to feed, propensity to fly, flight over 100 m, flight longer than 2000 seconds) the data were analysed using a generalised linear mixed model (GLMM) function under a binomial family distribution, with a linear mixed model (LMM) function used for all other responses (feeding time, total distance flown, mean velocity, maximum velocity). However, where the random effect of *colony* explained none of the variance in the data, it was removed from the model, and the model reverted to either a generalised linear model (GLM) or linear model (LM), with the type of model used indicated with each result. To examine whether an unideal (score 2) tag fitting inhibited flight or impeded movement, I compared the propensity to fly (GLM) and distance flown (LM) between tag ratings for both *treatment* groups separately. Here, the fixed effects were *tag rating* (score of 1=ideal or 2=unideal), *ITS* and the interaction between the two. Flight velocity over time (considering flight over the first 900 circuits) was analysed using an LMM function with the random effect structure nesting individual bee ID within circuit to account for individual repeated measures over time, and fixed effects including *treatment*, *ITS*, *circuit*, and the interaction term between *treatment* and *circuit*. The model suffered from high eigenvalues and had trouble converging when considering all 900 repeated measures, therefore, to enhance model fit and convergence I scaled the *circuit* variable and considered the average velocity of every 50th circuit (i.e., each bee had a mean per circuit velocity for circuits 1 to 50, 51 to 100, 101 to 150 and so on) resulting in 18 repeated measures. In all cases, model residuals were plotted to confirm the data met the parametric assumptions of the tests used. Where appropriate, normality tests were used to reveal distributions of the data, and those which appeared non-normal were suitably transformed, with details of these found in the Appendix A. For all results, sample sizes are stated where appropriate, and as part of the statistical outputs I provide in subscript the residual degrees of freedom from the respective model.

Table 2.1. An overview of the filter steps used when cleaning the data for analysis of flight performance, outlining the number of workers removed from each treatment at each stage.

	Control	Pesticide	Total
Total bees at start	111	111	222
Filter Step 1			
Did not feed	9	4	13
Fed	102	107	209
Filter Step 2			
Fed <60 seconds	16	13	29
Fed >60 seconds	86	94	180
Filter Step 3			
Technical difficulties	2	2	4
Used in flight mill study	84	92	176
Filter Step 4			
Tag Rating 2	18	18	36
Tag Rating 1	66	74	140
Filter Step 5			
Did not fly	19	18	37
Flew	47	56	103
Filter Step 6			
Flew <100m	12	24	36
Flew >100m	35	32	67
Filter Step 7			
Removed top and bottom 10% sized individuals	9	5	14
Remaining bees for final analysis	26	27	53

2.3 | Results

2.3.1 | Feeding behaviour

I found no significant effect of treatment on the propensity to feed ($n = 9$ control & 4 pesticide workers did not feed; GLM: $z_{219}=1.3$, $p=0.20$). In concordance with pilot observations, I found that any feeds following the first were sporadic and short, suggesting workers fed to relative satiety on their first feed. Therefore, I used the length of first feeding time as a reliable proxy for total feeding time. Of the 209 workers that fed, the mean ($\pm$ s.e.m) time spent feeding was 138 ± 9.0 seconds ($n=102$) and 127.2 ± 7.8 seconds ($n=107$) for control and pesticide workers respectively, with no significant difference between treatments (LMM: $t_{204}=-0.8$, $p=0.44$; Figure A1). I found that whilst body size was

not a significant predictor of feeding time (LMM: $t_{204}=1.4$, $p=0.17$), the propensity to feed increased with increasing body size (GLM: $z_{219}=2.6$, $p=0.008$). For the main flight analysis, I decided to include only those workers that fed for >60 seconds (*control* = 86, *pesticide* = 94, total = 180; Table 2.1), because I wanted to increase the likelihood that each worker had fed to satiation. Additional analyses were run including all bees that fed (regardless of feeding time), which showed the same pattern of results to the main flight analyses detailed below, and these are reported in the Appendix A (Table A1).

2.3.2 | *Flight behaviour*

The flight data from 140 of the 180 bees tested on the flight mill were analysed (Table 2.1), as four workers were not considered due to flight mill technical difficulties, and 36 not considered because unideal (score 2) tag application appeared to affect aspects of flight performance (please see below for justification).

2.3.2.1 | *Effect of tag fitting*

The propensity of workers to fly was not significantly affected by tag rating (GLM: *control*; $z_{81}=-1.0$, $p=0.33$; *pesticide*; $z_{89}=-0.04$, $p=0.97$), although it is interesting that a higher percentage of tag rating 1 (ideal fitting) workers flew compared to tag rating 2 (unideal fitting) workers in both the *control* (71% vs 61%) and *pesticide* (76% vs 72%) groups. When considering total distance flown, however, tag rating 2 workers flew a significantly shorter mean distance compared to tag rating 1 bees in the *control* group (640 vs 1436 m respectively; LM: $t_{55}=-2.2$, $p=0.03$), with a similar, although non-significant, trend observed in the *pesticide* group (191 vs 415 m; LM: $t_{66}=-1.6$, $p=0.11$). Furthermore, I saw similar patterns in other flight metrics with tag rating 2 bees showing lower total duration flown (*control* = 1114 vs 2132 seconds; *pesticide* = 272 vs 553 seconds) and slower mean velocity (*control* = 0.562 vs 0.657 m/s; *pesticide* = 0.618 vs 0.744 m/s). It was therefore decided to exclude all 36 tag-rated 2 workers (*control* = 18, *pesticide* = 18; Table 2.1) from analyses, to avoid potential artefactual results.

2.3.2.2 | *Initial flight behaviour*

Flight was initiated by 103 workers, comprising 71% of *control* ($n=47$ of 66) and 76% of *pesticide* workers ($n=56$ of 74), revealing a similar propensity to fly between treatments (GLM: $z_{137}=0.5$, $p=0.62$, Table 2.2). Body size was a significant predictor of propensity to fly, with the

likelihood of flying increasing with *ITS* (GLM: $z_{137}=2.2$, $p=0.03$; Table 2.2). This translated to an estimated probability of *control* workers initiating flight of 0.49, 0.77 and 0.92 for workers with a 4 mm, 5 mm, and 6 mm *ITS* respectively, with a similar pattern observed for *pesticide* workers (Figure 2.4).

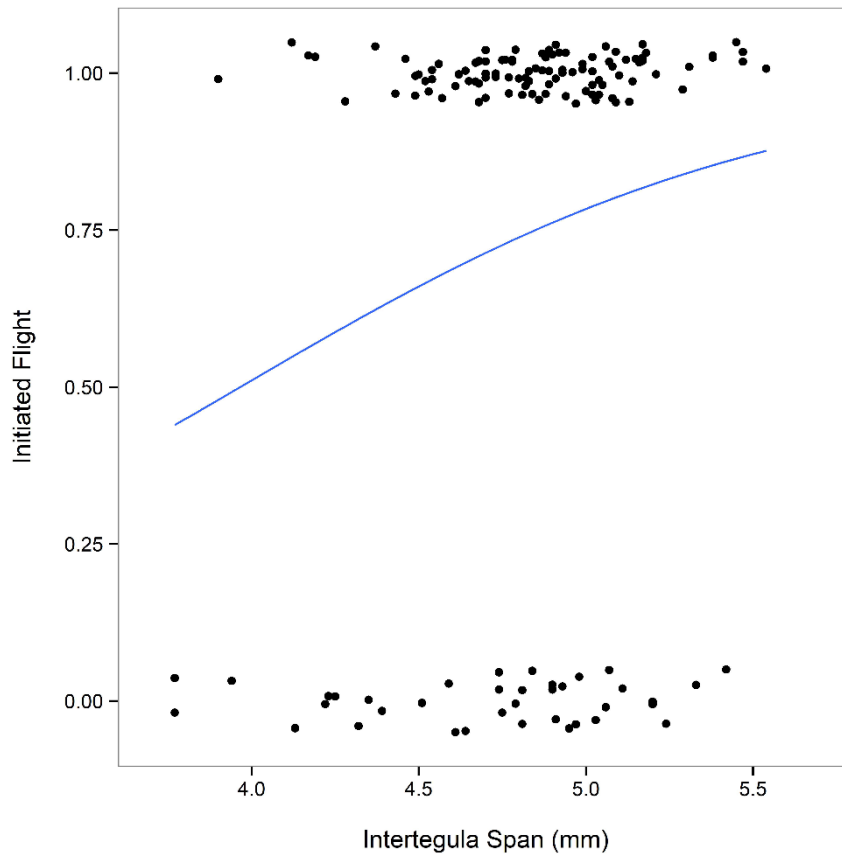


Figure 2.4. Logistic regression plot showing the effect of body size (*ITS*) on the propensity to fly. All workers from both treatments were pooled ($n=140$) and could either have initiated flight ($= 1$) or refused to fly ($= 0$; Table 2.1 – filter step 4). Jitter has been added in the y-plane so that individual data points are clearly identifiable.

I found that 43% ($n=24$ of 56) of *pesticide* workers terminated the flight test within the 100 m threshold compared to just 26% ($n=12$ of 47) of *control* workers, representing a significantly higher flight termination by *pesticide* compared to *control* workers within the first 100 m distance of the test (GLM: $z_{100}=-2.1$, $p=0.04$; Table 2.2). For instance, a *control* worker with 5 mm *ITS* had an estimated probability of 0.81 of flying >100 m, compared to just 0.62 for a *pesticide* worker of the same *ITS*. I further found that larger *ITS* significantly increased the probability of flying >100 m (GLM: $z_{100}=2.3$, $p=0.02$), with no clear significant difference in this relationship between treatments (GLM: $treatment*ITS$: $z_{99}=1.9$, $p=0.06$).

Table 2.2. Generalised linear model summary output for the effect of neonicotinoid (*pesticide*) exposure, worker body size (*ITS*) and the interaction between these two variables on the propensity of individuals to initiate flight and to fly > 100m threshold distance.

		<i>Estimate</i>	<i>Std.Error</i>	<i>z value</i>	<i>P value</i>
<i>Propensity to Fly</i> <i>(n=140)</i>	<i>Intercept</i>	-4.981	2.723	-1.829	0.067
	<i>Treatment - Pesticide</i>	0.194	0.391	0.496	0.620
	<i>ITS</i>	1.233	0.570	2.163	0.031
	<i>Treatment * ITS</i>	0.930	1.151	0.808	0.419
<i>Flight > 100m</i> <i>(n=103)</i>	<i>Intercept</i>	-7.620	3.738	-2.038	0.042
	<i>Treatment - Pesticide</i>	-0.959	0.454	-2.115	0.035
	<i>ITS</i>	1.816	0.784	2.318	0.020
	<i>Treatment * ITS</i>	3.169	1.703	1.862	0.063

2.3.2.3 | *Flight endurance & velocity*

Inspection of the 67 bees that flew >100 m showed an uneven *ITS* distribution between treatments, with a significant bias of larger *pesticide* workers (mean *ITS* of 4.83 ± 0.05 mm vs 4.99 ± 0.04 mm for *control* vs *pesticide* workers respectively; LM: $t_{65}=2.4$, $p=0.02$). I therefore took a conservative approach and ran two separate analyses on: i) the full dataset including all 67 bees (*control* = 35, *pesticide* = 32); and ii) a subset of the data (*control* = 26, *pesticide* = 27) in which I attempted to normalise the worker *ITS* distribution by removing the smallest 10% ($n = 6$ *control* & 1 *pesticide*) and largest 10% ($n = 3$ *control* & 4 *pesticide*) of workers; resulting in no significant difference in worker *ITS* between treatments (4.86 ± 0.03 mm vs 4.94 ± 0.03 mm for *control* vs *pesticide* workers respectively; LM: $t_{51}=1.8$, $p=0.08$). Normalising the dataset allowed me to better meet the assumptions of linear models; therefore, here I present the analysis using the data subset, and provide the results using the full dataset in the Appendix A (Figure A2 & Table A1), which showed the same directional pattern in flight performance between treatments.

Pesticide workers flew a significantly lower mean ($\pm$ s.e.m) total distance at just 659.1 ± 78.7 m compared to $1,833.9 \pm 207.6$ m for *control* (LMM: $t_{48}=-5.6$, $p<0.001$; Figure 2.5a, Table A1). The effect of pesticide exposure on distance flown was mirrored in the effect on duration flown, with a mean ($\pm$ s.e.m) flight duration of just 822.0 ± 90.8 seconds for *pesticide* exposed workers being considerably shorter than $2,852.2 \pm 234.4$ seconds for *control* workers (Figure 2.5b, Table A1). Durations flown across all workers (Figure 2.5b) showed a striking difference between treatments, with a proportion of just 0.04 of *pesticide* workers flying >2000 seconds, whilst 0.81 of *control* workers

surpassed this duration (GLMM: $z_{49}=-4.0$, $p<0.001$, Table A1). Furthermore, a proportion of 0.65 of *control* workers flew for the full 60 minutes permitted, whereas critically not one *pesticide* exposed worker achieved this.

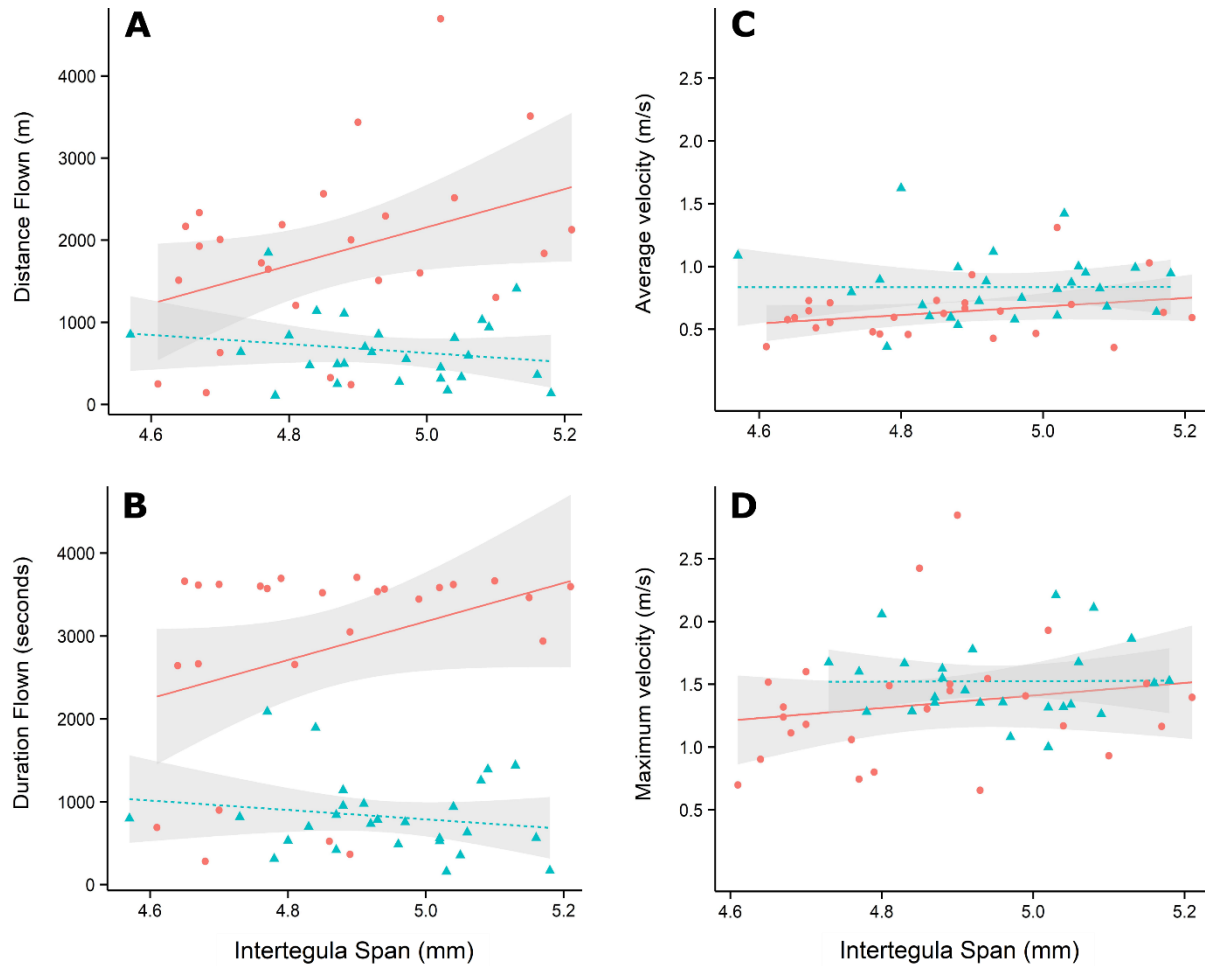


Figure 2.5. Scatterplot showing key flight performance indicators of endurance (distance flown in metres (A); duration flown in seconds (B)) and average and maximum velocity in meters per second (C-D) against worker body size (*ITS*) for both the *control* (red circle) and *pesticide* treated (blue triangle) groups. Data plotted are for the subset of bees with normalised *ITS* between treatments (number of workers = 26 *control*; 27 *pesticide*), and linear fitted lines (*control* = solid red, *pesticide* = dashed blue) with associated standard error (shaded bands) are estimates of linear models.

Interestingly, the effect of worker body size on distance flown appeared to differ between *pesticide* and *control* groups, as indicated by a significant *treatment*ITS* interaction (LMM: $t_{47}=-2.2$, $p=0.03$; Figure 2.5a, Table A1). Separate analysis of each treatment group showed that whilst

increasing *ITS* resulted in significantly higher total distances for *control* workers (LMM: $t_{22}=2.2$, $p=0.04$), this relationship was not found for *pesticide* exposed workers (LMM: $t_{23}=-1.0$, $p=0.31$; Figure 2.5a). The effect of *ITS* on total duration flown showed the same general trend as that found for distance (Figure 2.5b, Table A1); however, the difference in effect between treatments was less strong (GLMM: $z_{48}=-1.7$, $p=0.09$). Separate analyses for each treatment group found no significant relationship between *ITS* and the proportion of bees flying >2000 seconds for both treatments (GLMM: *control*: $t_{23}=1.5$, $p=0.13$; *pesticide*: $t_{24}=-1.1$, $p=0.26$).

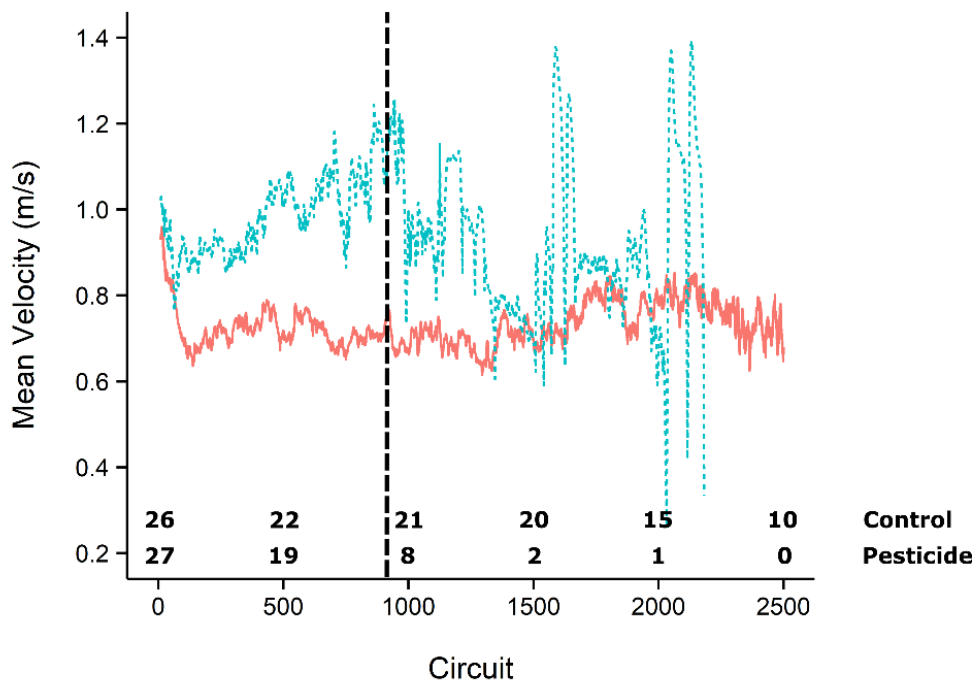


Figure 2.6. Mean velocity (m/s) flown by each treatment group (*control* = solid red, *pesticide* = dashed blue) plotted for each consecutive circuit for just the first 2500 circuits. Numbers at the bottom of the graph refer to the number of bees still flying on the corresponding circuit, and the data plotted is for the subset of bees with normalised *ITS* between treatments (starting number of workers = 26 *control*; 27 *pesticide*). Vertical line represents the first 900 circuits used in the analysis for initial individual velocity, and the associated error per mean circuit velocity is not shown.

When considering the velocity of individuals across the total flight period, I found *pesticide* exposed workers attained a significantly higher mean ($\pm$ s.e.m) velocity of 0.84 ± 0.05 m/s per worker compared to 0.63 ± 0.04 m/s for *controls* (LMM: $t_{48}=3.0$, $p=0.005$; Figure 2.5c, Table A1). For maximum

velocity I found no significant difference between treatments (LMM: $t_{47}=1.6$, $p=0.12$; Figure 2.5d, Table A1). However, it was intriguing that the average maximum velocity for *pesticide* exposed workers was higher than *controls* (mean $\pm$ s.e.m = 1.52 ± 0.06 m/s vs. 1.34 ± 0.09 m/s). This observation is consistent with the previous analysis looking at mean velocity over the total flight period and motivated me to examine at what stages in flight these differences in velocity may occur. *Pesticide* workers appeared to maintain a higher velocity compared to *controls* during the initial phase (the earlier circuits) of the flight test (see Figure 2.6). *Pesticide* workers also showed a sharp decline in velocity around 900 circuits (760 m) as a large proportion terminated flight. Therefore, focusing on the first 900 circuits, I reveal that *pesticide* workers did fly significantly faster compared to *control* workers (LMM: $t_{753}=3.5$, $p=0.001$; Table 2.3), with this difference between treatments maintained over these circuits (*treatment*circuit* interaction: $t_{752}=1.9$, $p=0.07$). Neither mean or maximum velocity was significantly predicted by worker *ITS* (LMM: $t_{48}=1.6$, $p=0.12$ & $t_{47}=1.0$, $p=0.32$ respectively; Figure 2.5c,d, Table A1), and there appeared to be no effect of *ITS* on velocity over the first 900 circuits (LMM: $t_{753}=0.5$, $p=0.62$, Table 2.3).

Table 2.3. Linear mixed model summary output for flight velocity over time: initial velocity over the first 900 circuits was significantly affected by treatment, with *pesticide* treated bees flying faster over this period than *control* bees.

	Full model (value for all 900 circuits)		Final model (average velocity taken for bands of 50 circuits)	
	T-value	p-value	T-value	p-value
<i>Treatment - pesticide</i>	2.803	0.007	3.459	0.001
<i>Circuit</i>	-0.581	0.564	-0.328	0.745
<i>ITS</i>	0.085	0.933	0.502	0.618
<i>Treatment * Circuit</i>	1.735	0.089	1.862	0.072

Note: The model suffered from high eigenvalues and had trouble converging when considering all 900 repeated measures (Full model). Therefore, to enhance model fit and convergence I scaled the *circuit* variable and considered the average velocity of every 50th circuit (i.e., each bee had a mean per circuit velocity for circuits 1 to 50, 51 to 100, 101 to 150 and so on) resulting in 18 repeated measures (Final model). This analysis considered the subset of workers (*control* = 26, *pesticide* = 27; see Table 2.1 – filter step 7).

2.4 | Discussion

Despite the importance of bumblebee foraging ability in providing a key pollination service (Garibaldi et al., 2013; Kleczkowski et al., 2017; Stanley et al., 2015), this study, to the best of my knowledge (at the point of publication), is the first to test how a specific stressor directly affects the different properties of flight in bumblebees. My findings demonstrate that acute exposure to the neonicotinoid pesticide, imidacloprid, was sufficient to significantly impact overall flight endurance, reducing flight distance and duration to around a third of what *control* workers were able to achieve. Whilst both *control* and *pesticide* exposed workers were equally motivated to fly initially, *pesticide* exposed workers had a higher probability of terminating flight before the end of the 60-minute flight test, which was even evident within the first 100 m. Intriguingly, *pesticide* workers exhibited a higher mean velocity compared to *control* workers, which was underpinned by faster flight speeds over the course of the first $\frac{3}{4}$ km, both during and after which I observed a considerable proportion of pesticide workers terminating their flight. Furthermore, my results suggest that pesticide exposure may negate the capability of larger workers to fly longer distances than their smaller sister workers.

The degree of impact that an acute neonicotinoid exposure had on reducing bumblebee worker flight endurance observed in this study did come as a surprise, as a previous honey bee study showed acute exposure to thiamethoxam increased flight endurance. One possible explanation for these contrasting results is the structural differences between thiamethoxam and imidacloprid compounds, which bind to different sites on nicotinic acetylcholine receptors (nAChRs) with variable affinity (Iwasa et al., 2004; Kayser et al., 2004; Marletto et al., 2003; Wiesner & Kayser, 2000). Indeed, studies have previously shown bumblebees to be less sensitive to thiamethoxam compared to imidacloprid when considering effects on brood production and food consumption (Heard et al., 2017; Laycock et al., 2014), and have found contrasting dose-dependent responses when considering effects on bumblebee non-flight thermogenesis (Potts et al., 2018). That said, the only other flight mill study to test neonicotinoid effects on honey bee flight capacity also used imidacloprid (as in my study), yet found no effect on workers free from infection with *Varroa destructor* (Blanken et al., 2015). Therefore, this reinforces the view that responses to pesticide exposure can vary even between closely related genera. Indeed, both lab (Cresswell et al., 2012) and field (Rundlöf et al., 2015) studies have highlighted differences in neonicotinoid effects between honey bees and bumblebees, with large interspecific differences in the toxicity of pesticides over time (Heard et al., 2017). Together this emphasises the growing appreciation that reported effects on honey bees cannot always be extrapolated to other wild bees, and highlights the danger of using honey bees as lone indicator

species for insect pollinator responses to pesticides (Gill et al., 2016; Heard et al., 2017; Raine & Gill, 2015).

Flight tests suggest that imidacloprid exposed bumblebee workers experienced a rapid demotivation to fly as the test progressed and/or tired quickly leading to premature physical exhaustion. This study was not designed specifically to test these two non-mutually exclusive explanations; however, given that only 4% of pesticide exposed workers flew >2000 seconds (control = 81%) and that not one individual completed the 60-minute test (control = 65%), these findings suggest that physical ability may have been affected, which could then have subsequently led to demotivation. I found no difference in initial motivation to fly and in fact pesticide exposed workers flew faster than control workers, implying that immediate motor function was not impaired *per se*, but instead that imidacloprid reduced flight stamina. Neonicotinoids have been implicated in affecting honey bee energy metabolism (Derecka et al., 2013), and imidacloprid has been shown to reduce mitochondrial activity, impairing respiratory processes and causing rapid mitochondrial depolarization in neurons of bumblebees and honey bees (Moffat et al., 2015; Nicodemo et al., 2014). Given the high energy expenditure required during flight, a reduction in mitochondrial functioning and the consequent inhibition of ATP production in flight muscles could lead to rapid muscle exhaustion, which might explain my findings of significantly reduced endurance. Additionally, neonicotinoids can impair non-flight thermogenesis in bees (Tosi et al., 2016), with bumblebee exposure to imidacloprid at a concentration of just 5 ppb resulting in a drop in thoracic temperature by around 1.5 – 2°C, equating to a subsequent 15-20% reduction in metabolic rate (Potts et al., 2018). This impact on bumblebee thermogenesis and metabolism may consequently compromise the performance of thoracic flight muscles, which must be raised to and maintained at approximately 35°C for successful flight (Heinrich, 1975; Potts et al., 2018). Imidacloprid can also induce the down-regulation of genes involved in sugar metabolising pathways in honey bee larvae (Derecka et al., 2013), which if true for bee adults could seriously impact flight performance that requires muscles to function at high glycolytic rates (Staples & Suarez, 1997). It is interesting to note that buzz pollination by bumblebees, whereby the creation of resonant vibrations from the flight muscles dislodges pollen from anthers (Morgan et al., 2016), has also been reported to be impaired by neonicotinoid exposure (Whitehorn et al., 2017).

Neonicotinoids are agonists of insect nicotinic acetylcholine receptors (Déglise et al., 2002) and can acutely increase neuronal activity (Matsuda et al., 2001; Moffat et al., 2016). A resultant effect of this may be individual hyperactivity of specific tasks, which could explain observations of higher velocity in exposed workers during the initial phase of the flight test, and has been previously suggested to underpin neonicotinoid effects on honey bee flight and locomotor activity (Lambin et al., 2001; Suchail et al., 2001; Tosi, Burgio, et al., 2017). Bumblebee colony level exposure to imidacloprid

also leads to a higher number of workers going out to forage (Gill et al., 2012), a pattern that could be an adaptive response to filling a foraging deficit, but could also be maladaptive hyperactive behaviour. Interestingly, Crall et al. (2018) found that whilst chronic exposure to imidacloprid increased movement speed in nest bumblebee workers, it actually decreased the amount of time workers were active, showing similar patterns of effects to my own findings. Similarly, when investigating neonicotinoid effects on non-flight thermogenesis in bumblebees, Potts et al. (2018) reported that the dose-response relationship for dietary imidacloprid exposure exhibited a biphasic hormesis, whereby a low-dose stimulated warming rates and a high-dose led to inhibition. Heat generation in bumblebees is attained through contraction of thoracic flight muscles. Hence, the dose-response relationship demonstrated by Potts et al. (2018) may corroborate my findings of stimulatory behaviour at the start of the experiment followed by inhibition of flight as the trials progressed, with neonicotinoid active ingredient accumulating at the target site during the course of the trial. Alternatively, this study may suggest a potential cost to hyperactivity, as exposed workers terminated flight prematurely, which may have been due to increased energy expenditure during the initial phase leading to faster muscle fatigue and energy depletion, but further testing would be needed to understand this. In sum, my results highlight the importance of looking at the pattern of flight dynamics, rather than experimental end-points, to better understand the mechanisms behind how neonicotinoids act and their temporal effects (Suchail et al., 2001; Wen & Scott, 1997).

Bumblebees reportedly exhibit a certain degree of alloethism, whereby worker body size can determine divisions in colony tasks (Goulson et al., 2002; Herrmann et al., 2018; Peat, Tucker, et al., 2005). Larger workers of a colony are considered more likely to become committed foragers (Jandt & Dornhaus, 2009; Spaethe & Weidenmuller, 2002), and there have been reports of foraging rate, distance and efficiency (nectar collected per unit time) increasing with body size (Goulson et al., 2002; Greenleaf et al., 2007; Jandt & Dornhaus, 2009; Kapustjanskij et al., 2007; Spaethe & Weidenmuller, 2002; Worden et al., 2005). Whilst this study found no clear relationship with flight velocity and body size, I did find that both the propensity to fly and total flight distance were positively related in *control* workers, which might provide a mechanistic explanation as to why foragers tend to be the larger colony workers. Critically, however, I found no such significant relationships in *pesticide* exposed workers, suggesting that the negative effect of neonicotinoid exposure on flight actually increased in magnitude as workers increased in body size. Intriguingly, a previous study showed that neonicotinoid induced impairment to spatial learning behaviour in bumblebees appeared to be exhibited more highly in the largest colony workers (Samuelson et al., 2016). Together these findings raise the question as to whether larger bumblebee workers are more susceptible to sub-lethal behavioural pesticide effects. Further investigation is required to look at this, however, as whilst the interactive

effect of pesticide and body size was detected in the subset of workers analysed in the current study, this effect seemed to be lost when considering the full dataset, a discrepancy that may stem from biases in worker size between treatments as a consequence of the flight trial filtering process.

Bumblebee foraging ranges are difficult to accurately measure, and further knowledge of this important behaviour is critical for predicting colony success and pollination services in changing landscapes. The flight mill setup used in this study showed *control* workers to fly a mean total distance of 1.8 km, which sensibly conforms to other estimates of bumblebee foraging ranges. Estimated foraging ranges using different techniques including harmonic radar (Osborne et al., 1999), mark-recapture (Kreyer et al., 2004; Osborne et al., 2008), and use of microsatellite genetic markers (Darvill et al., 2004) for *Bombus terrestris* vary from 0.34 km to 2.2 km. As bumblebees are central place foragers, foraging trips require not only reaching a resource, but also returning to the nest site after collection of food or other resources. The minimum round-trip flight distances associated with the above foraging ranges could therefore span from around 0.68 km to 4.4 km. Measures for control workers in this study fall in the middle of these foraging range estimates, giving confidence that the flight mill test setup can provide meaningful insights into the effects of stress on flight capabilities that can occur in the field. Pesticide exposed workers flew less distance than the lower limit of this estimated foraging range, with imidacloprid exposure reducing total flight distance by nearly 1.2 km on average. This corresponds to a 64% reduction in comparison to the *control*, which would lead to a notable 87% decline in the total foraging area accessible to a colony (using the colony as the epicentre). Pesticide exposure will therefore place increased stress on bumblebee colonies, with foragers potentially being unable to reach resources they previously could, or unable to return to the nest following exposure feeding on contaminated flowers. Not only would this reduce the abundance, diversity, and nutritional quality of food available to a colony, but could also reduce the pollination service the colony is able to provide (Blanken et al., 2015; Tosi, Burgio, et al., 2017; Van der Sluijs et al., 2013).

While this study examined the impacts of acute neonicotinoid exposure on flight, looking at the effects of chronic exposure would provide further insights, as foraging bees in the wild would likely be exposed to treated or contaminated flowering plants throughout the season (Simon-Delso et al., 2015; Stanley et al., 2013; Tison et al., 2016), and if this food is brought back and deposited in the nest, this also provides a potential route of continual exposure to larvae and other individuals within the nest (Blacqui re et al., 2012; David et al., 2016; Rortais et al., 2005). Furthermore, workers can be exposed to multiple pesticides concurrently when foraging (Bot  as et al., 2017; David et al., 2016; Hladik et al., 2016), and a diversity of pesticides can build up inside nests, with studies showing over 10 different active ingredients can be found (Calatayud-Vernich et al., 2018; Friedle et al., 2021;

Mitchell et al., 2017; Traynor et al., 2021). However, an often-overlooked issue is that of possible interactive effects between chemicals (Iwasa et al., 2004; Sgolastra et al., 2017; Siviter, Bailes, et al., 2021), particularly when considering sublethal endpoints (Gill & Raine, 2014; Gill et al., 2012). An important next step, therefore, is to investigate interactive effects of commonly used pesticides ('cocktail effects') on the dynamics of bee flight, to gain a more holistic understanding of the impacts of whole pesticide regimes. An example of this can be found in Appendix D, where I designed an experiment to explore how a neonicotinoid insecticide (thiacloprid) affected bumblebee flight when applied in isolation or in conjunction with a fungicide (tebuconazole). In addition to being simultaneously exposed to other pesticides in the environment, foraging bees can be susceptible to a range of other environmental factors. For example, given questions surrounding climate change, we can use the setup described in this study to understand how factors such as temperature can affect flight performance, and this is explored in Chapter 3.

CHAPTER 3

Thermal flight performance reveals impact of warming on bumblebee foraging potential

3.1 | Introduction

For the majority of insect species, flight is critical in facilitating important life-history traits including dispersal and resource exploitation (Zera & Denno, 1997). The influence that the dynamic surrounding environment has on flight behaviour and performance can therefore be a primary determinant of organism fitness and ecosystem functional roles (Greenop et al., 2020; Kremen et al., 2007; Niitepõld et al., 2009; see Chapter 2). Understanding how temperature mediates insect flight performance, for example, can reveal species success across different geographic regions. Indeed, with regional temperature increases associated with insect population range shifts (e.g., Herrera et al., 2014; Jacobson et al., 2018; Kerr et al., 2015; Powney et al., 2019) and localised extinction risk (e.g., Soroye et al., 2020), there is a need to understand the thermal limits of insect flight. Furthermore, such studies will help us predict spatial movement of key insect functional groups under different scenarios of climate change and the subsequent impacts on ecosystem service provision (Rader et al., 2013). For insect pollinators, sensitivity of flight to temperature fluctuations could affect foraging and subsequent reproductive success, whilst simultaneously affecting pollination of flowering wild plants and crops with major ecological and economic implications (Duchenne et al., 2020; Gill et al., 2016; Potts et al., 2016). To date, however, we lack quantification of the degree to which flight performance changes over a thermal gradient for insect pollinators, or even insects in general (but see Niitepõld et al., 2009; Spiewok & Schmolz, 2006).

The shapes of the relationships between temperature and an organism's performance traits - thermal performance curves (TPC) - are crucial for understanding the functional responses of populations and communities to climatic fluctuations (Angilletta, 2006; Dell et al., 2011; Huey & Stevenson, 1979). By quantifying the TPC of flight performance, we can determine where insect pollinators are operating in respect to their thermal limits, and better understand flight responses to projected temperature change (IPCC, 2014). Understanding how individual mobility traits such as flight motivation, endurance, and velocity respond to temperature can highlight the capabilities of foraging individuals to reach increasingly patchy floral resources under projected climate change (Jha & Kremen, 2013; Senapathi et al., 2017). This is particularly relevant to eusocial bees such as

bumblebees, which as central place foragers (have a fixed nest site), are unable to adaptively move to track within-season floral resource turnover and variation in micro-climatic conditions (Bladon et al., 2020; Raine & Gill, 2015).

Bumblebees are keystone species in many terrestrial ecosystems (Goulson et al., 2011) and represent a group of insects that are critical for the pollination of many wild and cultivated plants (Breeze et al., 2011; Goulson, 2003; Velthuis & Van Doorn, 2006). It is thus concerning that recent research indicates them to be under threat from rising temperatures and frequent extreme heat events associated with climate change, pushing species more regularly towards their critical thermal (CT) limits (Sirois-Delisle & Kerr, 2018; Soroye et al., 2020; Vanderplanck et al., 2019). At present, these thermal limits are often determined by measures of tolerance or mortality (e.g. CT_{max} / CT_{min}), where studies identify temperatures at which individuals enter unresponsive states or die (Hamblin et al., 2017; Oyen & Dillon, 2018; Pimsler et al., 2020; Zambra et al., 2020). However, the extent to which temperature constrains behavioural functions such as foraging, that likely operate within narrower thermal limits, are not well documented (Rader et al., 2013). This is despite us knowing that changes to flight performance alters the amount of food brought back for colony growth and hence fitness (e.g., Gill et al., 2012). To predict colony success under warming, we must therefore understand how individual flight across the collective foraging workforce is impacted by temperature.

As for most flying insects, bee flight muscle activity rates are a direct function of muscle temperature (Coelho, 1991; Heinrich, 1975; Woods, 2005). Bumblebees are heterothermic, meaning they can transition between ectothermic and endothermic states. Through endogenous heat production, individuals can raise their thoracic temperature to facilitate the required muscle temperatures for flight (Heinrich, 1975). The temperature of the surrounding environment will determine the energy expenditure of this thermogenic process, and thus ambient temperature should place constraints on bumblebee flight performance (Bishop & Armbruster, 1999; Dudley, 2000; Harrison & Roberts, 2000; Woods, 2005). This is supported by observations of bumblebee foraging activity changing in response to daily and/or seasonal variation in ambient temperature (Arce et al., 2017; Kwon & Saeed, 2003; Rader et al., 2013). What remains unclear, however, is which components of physical flight are being affected by temperature to produce such effects. Moreover, when investigating these questions in social bumblebees, we need to consider that workers exhibit large intra-colony variation in body size, thought to be important for task partitioning (Couvillon, Fitzpatrick, et al., 2010; Goulson et al., 2002). Flight performance across the colony workforce is therefore unlikely to be uniform because key flight determinants, such as morphology and wing loading, are dependent on body mass (Billardon & Darveau, 2019; Bishop & Armbruster, 1999; Skandalis & Darveau, 2012). Indeed, worker body size has been reported to positively correlate with both foraging rates and range

(Goulson et al., 2002; Greenleaf et al., 2007; Kapustjanskij et al., 2007; Spaethe & Weidenmuller, 2002; Worden et al., 2005). Size-dependent traits, such as thermogenic capacity, may also influence the thermal sensitivity of foraging workers. For example, the large body mass of alpine bumblebees is believed to allow individuals to forage in temperatures down to 5°C (Bishop & Armbruster, 1999; Corbet et al., 1993; Lundberg, 1980). To reveal colony-level foraging potential, therefore, we must elucidate the size-dependence of flight performance.

Here I use the benefits of a tethered flight mill setup (developed from Chapter 2) under controlled environmental conditions to quantify how different components of flight for individual bumblebees (*Bombus terrestris*) respond over a thermal gradient, and determine how these relate with worker body mass. I report how motivation to fly, flight ‘endurance’ (distance and duration), and flight velocity dynamics vary over a 12-30°C range. Finally, I use these individual level flight performance results to develop a simple yet novel measure of ‘temperature-mediated foraging potential’ to gain a clearer understanding of how temperature may constrain foraging at the colony level. I discuss how my findings reveal the ways that future change in ambient temperature could affect the foraging potential and pollination service of bumblebee colonies, and the implications of body mass specific performance for understanding responses of small versus large bodied insects.

3.2 | Methods

3.2.1 | *Bumblebee husbandry and tagging*

Eight *Bombus terrestris audax* colonies, each containing 80-100 workers, were supplied by Biobest (distributed by Agralan Ltd). On arrival, colonies were kept under red light in a controlled environment (CE) ‘holding’ room at 60% relative humidity (RH) and 21°C (mid-point temp. of tested thermal gradient). Colonies were fed 40% w/w sucrose/water solution ad libitum, which is a marked change from Chapter 2 in which colonies were fed 10% sucrose solution to promote pesticide uptake immediately prior to the flight. In this chapter, however, I deemed it appropriate to match sucrose concentrations between colonies and feeding trials to limit any differences in endogenous energy stores between workers. I also provided colonies with 3g of honey bee collected pollen daily (Agralan Ltd) as was also done in chapter 2. During the five days after arrival, I applied the tagging protocol described in Chapter 2 to 60 workers per colony. However, for any unsuitable tag placements (i.e., a score = 2 or 3 from Chapter 2), the respective bee was removed from the experiment (< 5% of bees per colony) in order to alleviate issues arising from substandard tag placement. Tagged workers were then placed in separate plastic pots for 60 minutes to rest, after which they were returned to the natal colony.

3.2.2 | *Experimental design*

Individual worker flight performance was assessed at seven evenly distributed temperatures (12, 15, 18, 21, 24, 27, 30°C; all at 60% RH) representing a realistic temperature gradient experienced by workers of temperate bumblebee spp. during the typical flight period months of April to September (Figure B1 for UK data; also see Fick & Hijmans, 2017; Hooker et al., 2018). The experiment was split into two blocks (n=4 colonies per block), which allowed an adequate number of workers to be tested whilst limiting potential differences in age and development. Per block, each temperature was tested on a single day. To try and account for any impacts of age or disturbance on normal behaviour caused by the tagging process, the order of testing was swapped between blocks. For block-1, temperatures were tested sequentially in ascending order over a 7-day period (i.e., 12°C on day-1, 15°C on day-2, and so forth), whereas block-2 was tested in a descending temperature order starting at 30°C. Per temperature, a cohort of 48 workers were tested (n=24 workers per block), consisting of six workers per colony.

Workers were selected for testing such that the distribution of worker body mass within each temperature cohort was symmetric about a similar mean, as bumblebee colonies tend to produce workers with normally distributed body sizes throughout the colony life cycle under standard conditions (Couvillon, Jandt, et al., 2010). This was achieved by assigning workers to cohorts based on their wet mass weight directly following removal from the natal colony.

3.2.3 | *Flight mill setup*

The flight mill apparatus was adopted from Chapter 2, with each full rotation of the mill arm (from here-on termed 'circuit') recorded by an attached Raspberry Pi 3 computer (Model B). A magnet hanging from one end of the mill arm allowed a metal tagged worker to be attached through magnetic attraction, allowing the suspended bee to fly without carrying the load of the tag and subsequent flight circuits recorded. Wet mass of each tested bee was measured again directly preceding the flight trial, and subsequently a respective small (150 mg), medium (225 mg) or large (300 mg) counterweight (made of modelling clay) was attached to the end of the opposing arm of the mill based on which size was closest to the worker's mass, in order to more accurately balance the mill arm during rotations (Figure 3.1). The mills were setup in a separate CE 'testing' room set at the experimental ambient temperature (Table 3.1) and with the option of being under red (Philips TLD 58W Red 1SL/25; mean 660 nm) or white (Philips TLD 58W 840) light. Each mill was placed on non-glare green card, and providing a gap of 60 mm from the end of the mill arm (mill radius = 135 mm), each mill was encircled with a vertical border (border radius = 195 mm) of alternating black and white vertical stripes (Tosi,

Burgio, et al., 2017; diameter of each stripe = 35mm; Figure 3.1). The border: i) prevented potential interfering air currents, as wind speed has been shown to affect the hesitancy for worker flight take-off (Hennessy et al., 2020); ii) allowed relative isolation from neighbouring mills; and iii) presented an identical visual stimulus to each tested worker, which can be a contributing factor in determining flight velocity (Baird et al., 2005).

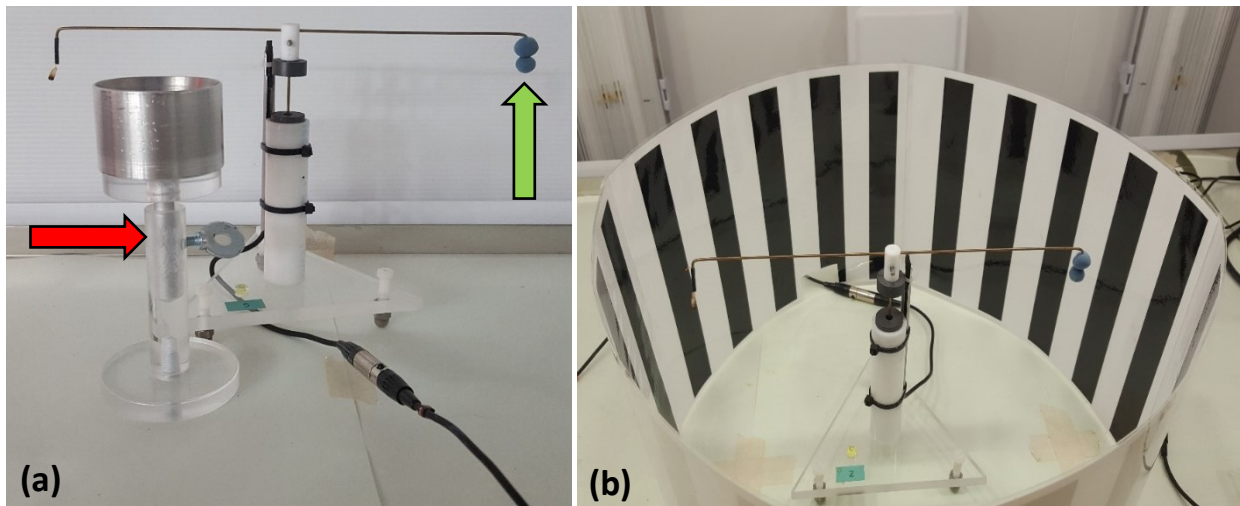


Figure 3.1. Changes to the flight mill apparatus from Chapter 2. (a) height adjustable support stands (red arrow) were used to hold workers prior to flight tests and following a stop in flight. The flight mill used for tethered flight is in the background, with counterweights (green arrow) added to the non-attachment end of the flight mill arm; (b) flight mill used in the study, surrounded by border with vertical black and white stripes. The green floor that was used during the trials of this study are not shown in this photo.

3.2.4 | *Flight trials*

On each day of testing, four flight performance ‘bouts’ were run, with each bout consisting of six flight mills running simultaneously. The six workers per bout were removed from colonies (one worker from two colonies, and two workers from the remaining two colonies; Table 3.2) and placed in feeding tubes setup in the same 21°C holding room (Figure 3.2). In the tubes, each bee was presented with a droplet (minimum 90 mg) of 40% w/w sucrose solution placed on a microscope coverslip and allowed to feed for a 5-minute period whilst being observed under red light. Each worker was allowed to feed multiple times within this period to increase the likelihood that workers had fed to satiation (please see Appendix B for support), with the aim of limiting any proportional differences in endogenous energy stores between workers prior to flight trials (N.B. no worker consumed the entire droplet). Feeding tubes consisted of a Perspex tube (length = 100 mm, internal diameter = 19 mm) with a rubber bung at one end, which was then placed inside a queen marking cage such that the

open-end of the Perspex tube was placed against the mesh-end of the marking cage, allowing the worker to feed through the mesh (Figure 3.2). Direct feeding was identified as the proboscis extending into the sucrose droplet, with the length of time that each worker fed recorded through direct observation. The coverslip was weighed with the droplet before and after feeding to calculate the mass of sucrose solution consumed. With workers varying in the volume of sucrose consumed over the 5-minute period, I produced a 'mass-specific consumption' value per worker, calculated as the total mass of sucrose consumed (mg) per unit of body mass (mg of dry mass). Any bees observed not to feed within the 5-minute feeding period were removed from further testing.

Table 3.1. All flight mills were housed in the same 'testing' controlled environment (CE) room, which was able to consistently hold the desired test temperatures (°C) and 60% relative humidity (RH). Temperature and RH recordings were taken every minute throughout the course of the trials, with the number of readings depicting the number of minutes it took to run all trials at each experimental temperature (i.e., the higher number of readings is indicative of the workers flying longer at higher versus lower temperatures).

Tested Temp (°C)	Mean Temp (°C)	SD Temp	Mean Humidity (RH)	SD Humidity	Number of Readings
12	12.011	0.134	60.309	1.152	642
15	15.004	0.108	60.542	0.969	896
18	18.002	0.078	61.219	0.869	1151
21	21.005	0.073	60.206	0.687	1303
24	24.036	0.151	60.641	1.191	1304
27	27.005	0.060	60.016	0.320	1297
30	29.983	0.140	59.982	0.444	1164

Table 3.2. Each parent colony had their workers tested equally across all six mills. Four bouts were run per day at one temperature, with six workers used in each bout (one per mill). The table below shows how colony workers were assigned to the mills over the four bouts on each day. The blue italic numbers in the table refer to the four parent colonies that the workers came from.

Bout	Mill 1	Mill 2	Mill 3	Mill 4	Mill 5	Mill 6
One	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>1</i>	<i>2</i>
Two	<i>3</i>	<i>4</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>
Three	<i>2</i>	<i>3</i>	<i>4</i>	<i>1</i>	<i>2</i>	<i>3</i>
Four	<i>4</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>1</i>

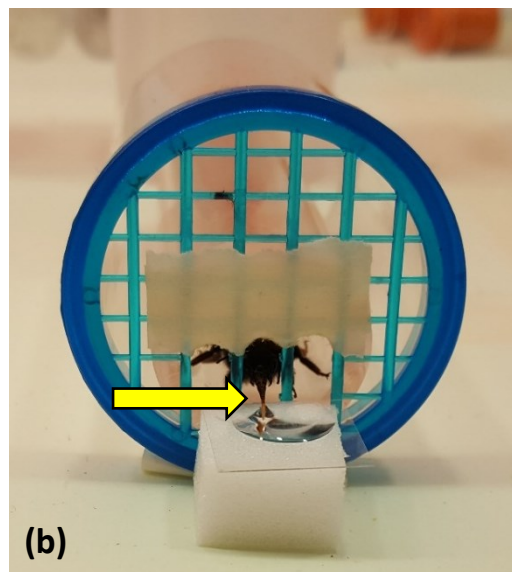
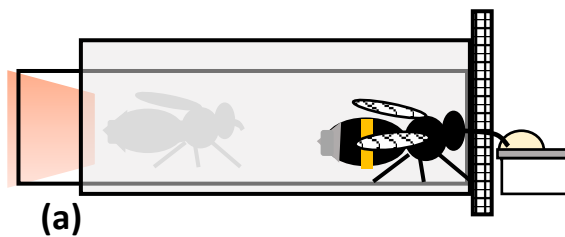


Figure 3.2. Feeding setup prior to flight mill testing, whereby a microscope cover slip with droplet of sucrose solution (of known mass) was placed on a foam stand in front of the feeding tube. The contained bee was free to feed on the droplet during a 5-minute period by protruding its proboscis (yellow arrow) through the mesh. Panel a) shows animated representation of side view of feeding apparatus, and panel b) is an image taken during a feeding event. The image in panel b) is taken under white light to provide detail, but the feeding assays were conducted under red light.

Of the 336 workers tested, 24 were removed from testing as five were visibly unhealthy or possessed damaged wings and 19 did not initiate feeding (Table 3.3). Considering all 312 workers that successfully fed, there was no difference in dry mass mean (ANOVA, $p > 0.1$ for all pairwise comparisons) or variance (Bartlett's $K^2 = 5.95$, $df = 6$, $p = 0.43$) between temperature cohorts. Once the feeding trial had ended, successfully fed workers were removed from their respective feeding tubes, placed into separate plastic holding pots (120 mL) and transported to the neighbouring testing room. Still under red light, workers were left to rest inside the pots for 5 minutes and immediately after were removed and magnetically attached to each respective flight mill. Once all were attached and following the same procedures in Chapter 2, the room was switched to white light and a support stand was used to hold workers to rest for a 10-minute acclimatisation period deemed necessary to allow workers to settle whilst being held on the mill. The support stands differed from those in Chapter 2, being height-adjustable to allow workers to sit comfortably in the stand regardless of body size (Figure 3.1). Immediately after, the bee was manoeuvred (if required) to a forward-facing orientation and the support stand was quickly removed to stimulate loss of tarsal contact and initiate flight. If removal of the stand did not stimulate flight, then either: i) the tarsi would be gently tapped with the stand if all legs were hanging down; or ii) plastic tweezers would be used to gently flick the legs off the tether if workers had their front or middle legs touching the tag attachment. This would be attempted twice per method, and if no flight response was exhibited this was considered a 'strike' and the stand would be placed back under to support the bee. Workers that successfully flew on this first flight attempt were monitored for any subsequent stoppages in flight. Following a stoppage, the stand was placed under the worker and this was again considered a 'strike'. For both these 'strike' scenarios, after 30 seconds of the worker resting the stand would again be removed and the process repeated. If a bee was deemed to be poorly orientated on the mill during its flight (i.e., if it was not facing directly forward), then the mill was manually stopped, and the bee was re-orientated. This was marked down as an 'orientation' circuit and the circuit number was recorded, but it was not classified as a strike.

Workers were not capped in terms of how long they could fly for, but were only allowed three 'strikes' in total, with their flight trial terminated on the third strike. The decision to have no cap for flight duration was informed by Chapter 2, in which a high proportion of non-exposed workers flew for the full 60-minute limit that was used in that study. Removing the flight cap allowed workers to fly to their maximum capacity, increasing the capacity to discern any subtle effects of temperature on flight performance. The decision to allow three 'strikes' was again supported by Chapter 2, which showed that the majority of flight periods after a third stoppage were short-lived, indicating flight motivation or capacity had reached a limit by this point. After the third strike, workers were removed from the mill and frozen at -20°C , followed by a measure of dry mass by weighing the worker after

oven warming at 80°C for 48 hours. Workers were weighed with their metal tag attached, which did not bias any body mass measurements as tags were of standardised mass (18 ± 0.1 mg).

Table 3.3. An overview of the filter steps used when cleaning the data for analysis of flight performance, outlining the number of workers removed from each temperature cohort at each stage.

Temp (°C)	Started the experiment	Looked unhealthy / issues with wings	Tested in feeding trials	Did not feed	Tested on flight mills	Did not fly	Initiated flight	Flew <100m	Successful fliers (flew ≥ 100 m)
12	48	0	48	4	44	8	36	27	9
15	48	2	46	1	45	0	45	28	17
18	48	0	48	5	43	0	43	21	22
21	48	2	46	2	44	0	44	20	24
24	48	1	47	2	45	0	45	19	26
27	48	0	48	2	46	0	46	11	35
30	48	0	48	3	45	0	45	14	31
Total	336	5	331	19	312	8	304	140	164

3.2.5 | Data and statistical analyses

Flight data for each trial was initially screened to check for consecutive strike circuits, with strike circuits identified as any circuit > 15 seconds in duration. Unless these were noted during the trials as being two genuine strikes, these consecutive strike circuits were merged and treated as a single strike. These events were relatively common and usually arose from the time taken handling the mills and reorientating bees. After ensuring that the data for each trial was terminated at three strikes, the data was filtered by excluding the following circuits: (i) the first five circuits of the first flight attempt; (ii) any flight stoppage circuit or orientation circuit; (iii) the three circuits directly preceding a flight stoppage or orientation circuit; (iv) the five circuits directly following a flight stoppage or orientation circuit. As noted in both this study and Chapter 2, removal of the support stand or leg tapping would often stimulate high velocities. Additionally, unintentional circuits could be triggered from handling of the mill when placing bees in support stands or reorientating following a stoppage. Therefore, I felt actions (i) and (iv) were justified as a precautionary measure to ensure that observed flight velocities were not an artefact of my experimental setup. Similarly, in justification

of action (iii), the minimal rotational resistance of the mill means that when a worker stops flying it does not equate to an abrupt stop, but the brass arm continues to rotate and slows gradually. All data filtering was conducted in R v3.6.2 (R Core Team, 2019) using the “dplyr” (Wickham et al., 2019) and “magicfor” (Makiyama, 2016) packages.

When examining worker flight endurance for all tested workers, frequency distribution plots revealed a right skew to the data, with many workers terminating flight before completing 100 m (Figure 3.3). As highlighted in Chapter 2, bees that fly under approximately 100 m appear to exhibit a distinctly different behaviour from those flying over this distance, with flight bouts < 100 m consisting of short and intermittent flight periods with no periods of sustained flight, indicating low motivation to fly or lack of physical ability to initiate and sustain flight. As such, I only considered ‘successful fliers’ as those flying ≥ 100 m. This threshold served the dual purpose of allowing: (1) investigation into drivers of flight motivation / success; and (2) analysis of workers that are seemingly motivated to fly and to use normalised data to measure flight endurance allowing parametric linear models to be run.

For successful fliers, I calculated: (a) total distance flown during the flight test, calculated as the total number of circuits flown multiplied by the circuit circumference (0.848 m); (b) total duration of the flight test, calculated by summing all circuit interval times; (c) velocity of each circuit flown, calculated by taking circuit circumference and dividing by the respective circuit interval time; (d) mean velocity, calculated as the total distance flown divided by the total duration flown; and (e) maximum velocity, by taking the highest velocity circuit that was recorded.

Statistical analyses were conducted using the “lme4” (Bates et al., 2015) package in R v3.6.2 (R Core Team, 2019), with results reported using the package “lmerTest” (Kuznetsova et al., 2017). For all analyses, the effect of body mass was examined using log-transformed dry mass (mg).

Bartlett’s test was used to compare the distribution of body mass between temperature cohorts, whilst ANOVA with post-hoc Tukey tests (implemented using the ‘TukeyHSD’ function in base R “stats” package) were used for pairwise comparisons of mean mass between temperature cohorts. Linear regression was used to examine the relationships between body mass and both the total sucrose consumption (mg consumed by end of the 5-minute feeding trial) and rate of sucrose consumption ($\text{mg}\cdot\text{sec}^{-1}$ during observed feeding), with the latter response variable being log-transformed to meet model assumptions (see Appendix B for results; Figure B2).

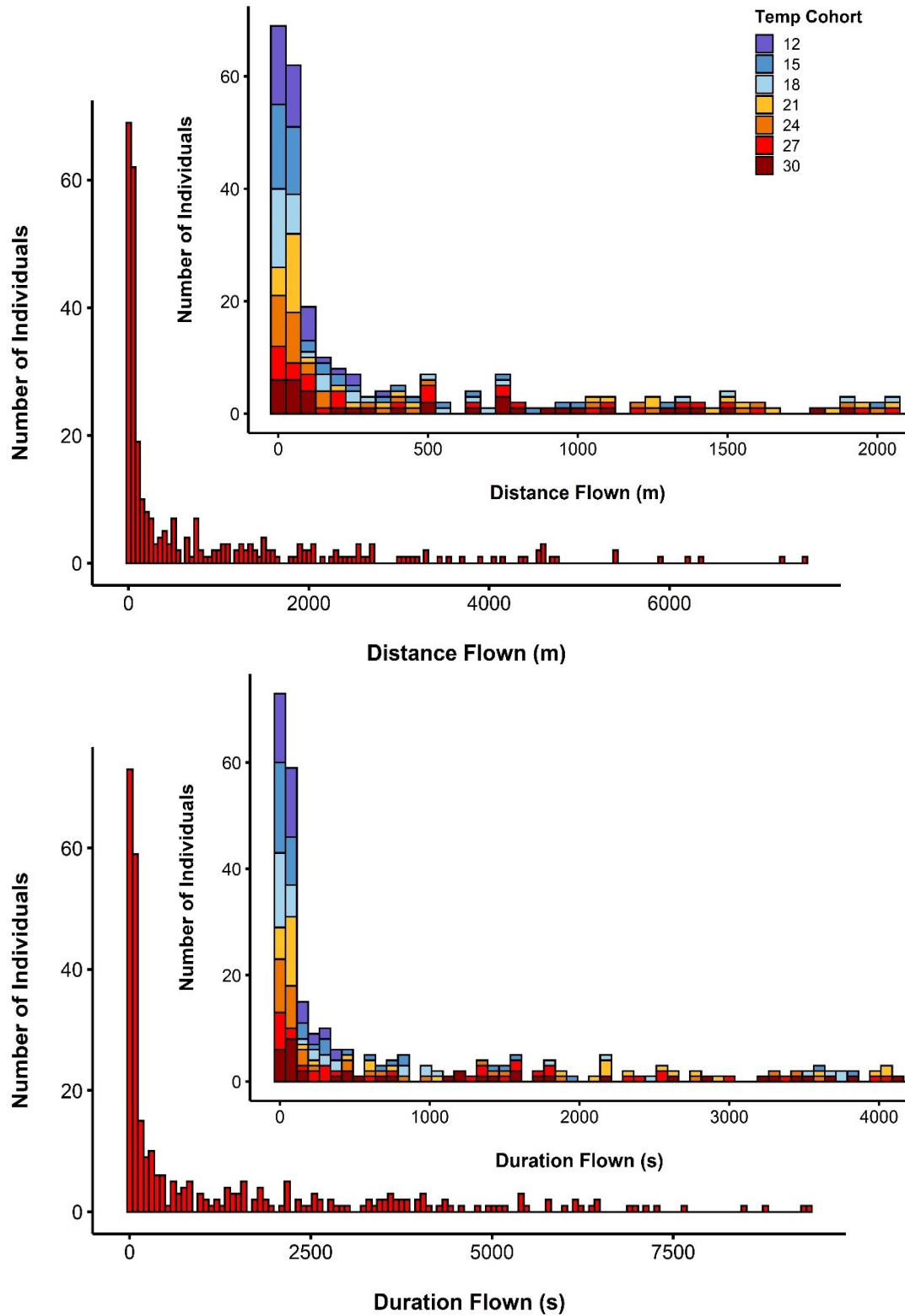


Figure 3.3. Frequency distribution of the flight distance and duration data for all workers that initiated flight (n=304). Each bar represents binning at a 50m distance (top pair) or 75 second duration (bottom pair). There is a spike in bees that flew under 100m (~150 seconds), as previously observed in Chapter 2, and this held true across all temperature cohorts.

For all analyses of the likelihood of successful flight (flight > 100 m), flight distance (m), flight duration (secs), mean velocity (m/s) and maximum velocity (m/s), linear mixed-effects models were used, including colony as a random intercept to account for inherent colony level differences. However, when examining the likelihood of successful flight, the random effect variance estimate was close to zero, and so colony was removed to avoid model overfitting and non-convergence. In all cases, the Gaussian distribution was used, except for when examining likelihood of successful flight, which was modelled as a binomial family distribution (flight < 100 m = 0, flight > 100 m = 1). Analyses examining both flight distance and duration were carried out on log-transformed data so that distributions met model assumptions. All models were initially constructed considering the main effects of temperature, body mass, mass-specific consumption and the interactions temperature x body mass and temperature x mass-specific consumption, with all main effects modelled as continuous variables. Models were then simplified through stepwise removal of non-significant terms, checking after each removal that the simplified model had not significantly decreased explanatory power.

Visualisation of the relationship between both flight distance and flight duration with temperature revealed a unimodal curve (see results), which was best explained by fitting a 2nd order polynomial relationship between temperature and flight distance and duration, whilst assuming both body mass and mass-specific consumption to be linear predictors of the response variables. Thermal performance curves were constructed using these model predictions.

Assigning a mean velocity value calculated across the whole flight period may not fully encapsulate what occurred during the flight trial and could overlook the dynamics of flight. From visualising velocity flown by workers per temperature cohort over time (see results), workers appeared to fly at comparatively high velocities immediately following the start of the flight mill test ('Immediate'), which is likely a stimulatory response to the withdrawal of the support stand. Following this, workers rapidly declined in speed ('Initial'); a response that appeared standard across all temperature cohorts after which flight velocity levelled-off and remained relatively steady over the remainder of the trial ('Steady'). Focussing on these characteristic periods of flight, and using linear mixed-effects models as previously described, I additionally examined mean velocity flown by workers per cohort for: (a) immediate flight, encompassing the first 30 seconds of flight; (b) initial flight, encompassing flight > 30 to ≤ 300 seconds post flight initiation; (c) steady flight, encompassing flight > 300 to ≤ 600 seconds post flight initiation. I capped steady flight at 600 seconds to ensure I could analyse a sufficient number of workers within each cohort to allow statistically suitable comparisons (Figure 3.4).

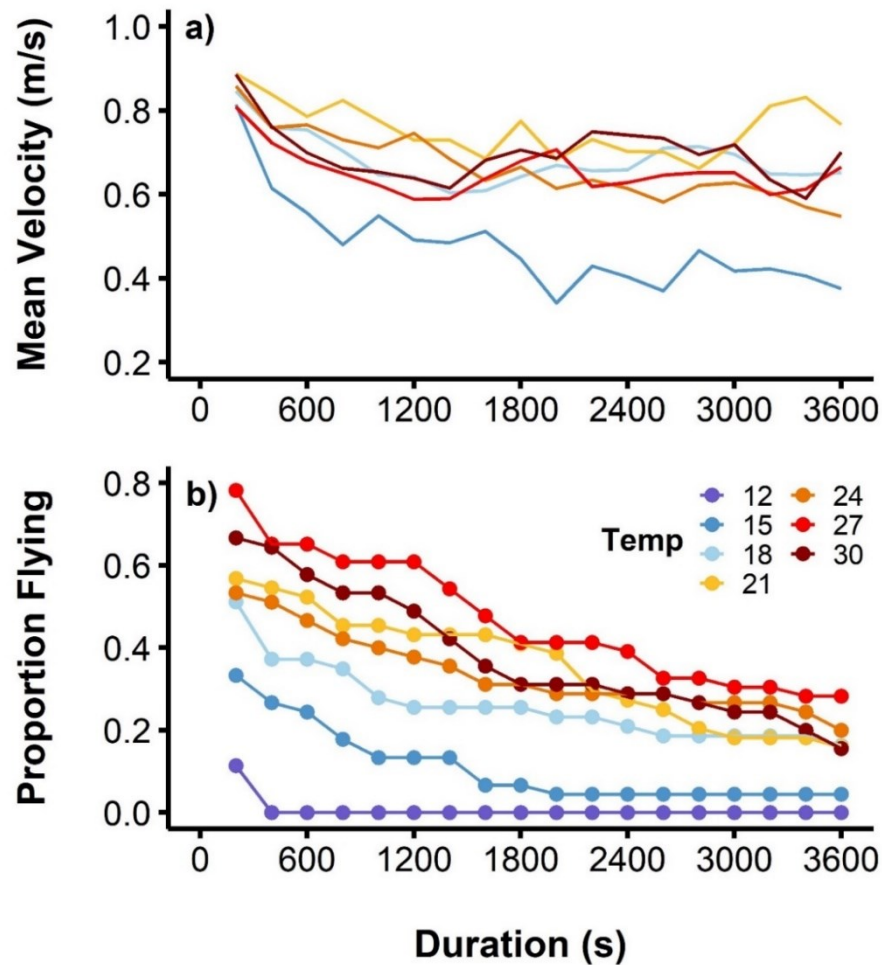


Figure 3.4. Measures of worker flight performance over the course of the flight trials per temperature cohort. a) mean velocity (m/s), with mean values calculated for every 200 second binned period, capped at 60 minutes and omitting standard error bars for aiding visualization; b) proportion of bees still flying after each 200 second period relative to the number of bees tested on the flight mill to visualize the temporal pattern of cumulative worker drop-out during the trial.

For all analyses examining flight metrics, additional linear mixed models were fitted in which temperature was modelled as a fixed factor, allowing pairwise comparison in flight metric means between temperature cohorts through Tukey contrasts. For this analysis I report adjusted p-values which corrected for multiple testing through the single-step implementation in the “multcomp” R package (Hothorn et al., 2008).

As a final analysis, I took individual flight performance across workers to produce a comparative metric of how ambient temperature determined ‘temperature-mediated foraging potential’. To better understand a colony’s potential foraging range and capability to exploit resources across a given landscape, we must incorporate not only flight endurance per worker but also the

number of successful fliers constituting the colony workforce. With no significant difference in worker mass distributions between temperature cohorts, for each cohort the mean distance flown (m) was multiplied by the proportion of successful fliers (relative to those that initiated flight) to provide a relative value for temperature comparisons. I used mean flight distance as my measure of flight endurance in the calculation as it directly incorporates both flight velocity and flight duration.

3.3 | Results

3.3.1 | *Likelihood of flight*

Ninety-seven per cent of workers ($n=304$) initiated flight, with all eight workers that did not fly coming from the 12°C cohort (Table 3.3; Fisher's Exact Test: $p<0.01$).

Across all temperature cohorts, 54% of workers ($n=164$) were classified as successful fliers (flew $>100\text{m}$; Table 3.3), with the likelihood of successful flight showing an overall positive correlation with ambient temperature (GLM: $z_{299} = 2.18$, $p = 0.029$; Figure 3.6a, Table 3.4). The probability of a worker being a successful flier significantly increased with body mass (GLM: $z_{299} = 3.40$, $p < 0.001$; Figure 3.5a), but this relationship was influenced by ambient temperature (GLM: '*Temp x Body Mass*': $z_{299} = -2.01$, $p = 0.044$). Specifically, the negative estimate from the interaction term (est = -0.34) indicated that as temperatures increased there was a reduction in the slope gradient. The reduced flight success at lower temperatures was therefore more severe for smaller workers (Figure 3.5a). Considering the 164 workers classed as successful fliers, there was an even variance in dry mass values between temperature cohorts (Bartlett's $K^2 = 4.51$, $df = 6$, $p = 0.61$). However, successful fliers in the 12°C cohort had a significantly higher mean dry mass compared to successful fliers in the 24, 27 and 30°C cohorts ($t_{157} \geq -2.40$, $p \leq 0.02$ in all cases; Figure 3.5b).

Mass-specific consumption showed a positive association with the probability of being classed as a successful flier, but this parameter was not detected as significant (GLM: $z_{298} = 1.84$, $p = 0.07$; Figure B3).

Pairwise comparisons between temperature cohorts revealed that at 12°C, workers showed a significantly lower likelihood of being a successful flier relative to 24, 27 and 30°C ($p \leq 0.029$ in all cases; Figure 3.6a Table B1). Additionally, the 15°C cohort were less likely to be a successful flier relative to 27°C ($p = 0.004$; Figure 3.6a).

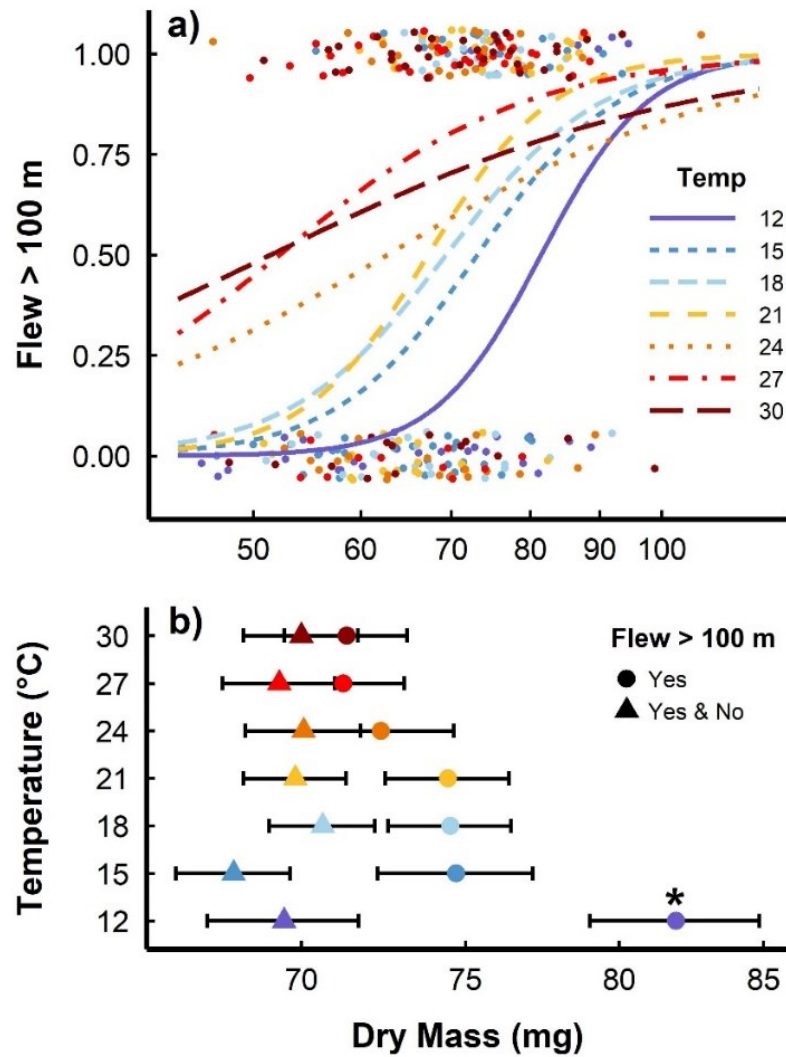


Figure 3.5. Relationships between worker dry body mass and successful flight for each temperature cohort. a) Effect of dry mass on the likelihood of successful flight (≥ 100 m) shown in a logistic regression plot (unsuccessful flight = 0, successful flight = 1) and b) comparison of worker mean ($\pm$ S.E.M.) dry mass per temperature cohort when considering all workers tested on the flight mills ($n=312$, triangles) or just successful fliers ($n=164$, circles); In both panels, dry mass has been plotted on a $\log_{10}$ scale. In panel a) jitter has been added in the y-plane and confidence intervals have not been plotted around model lines to aid visualization. In panel b) the asterisk (*) indicates that worker dry mass of successful workers in the 12°C cohort significantly differed from the 24, 27, and 30°C cohorts ($p < 0.05$).

Table 3.4. GLM model output (binomial family) testing the likelihood of successful flight (flight > 100 m) as a function of Temperature (°C), Dry Body Mass (mg)*, Mass-Specific Consumption (mg /mg; MSC), Temperature x Dry Body Mass interaction, and Temperature x MSC interaction. Non-significant variables were removed by step-wise removal leaving a final simplified model (output furthest left in table) containing just significant variables showing no decrease in explanatory power. *variable $\log_{10}$ transformed.

	<i>Estimate</i>	<i>std. Error</i>	<i>p</i>	<i>Estimate</i>	<i>std. Error</i>	<i>p</i>	<i>Estimate</i>	<i>std. Error</i>	<i>p</i>
<i>Intercept</i>	-58.86	16.68	<0.001	-58.45	16.85	<0.001	-61.01	17.06	<0.001
Temperature	1.56	0.71	0.029	1.54	0.72	0.032	1.65	0.73	0.023
Dry Body Mass	13.30	3.91	<0.001	12.99	3.95	0.001	14.01	4.06	<0.001
Temperature x Dry Body Mass	-0.34	0.17	0.044	-0.33	0.17	0.050	-0.38	0.17	0.030
Mass-Specific Consumption				0.83	0.45	0.066	-1.33	1.75	0.448
Temperature x MSC							0.11	0.08	0.203

3.3.2 | Flight endurance

Flight endurance showed a unimodal trend over the temperature gradient (Figure 3.6c,d). Mean ($\pm$ s.e.m.) flight distance at first increased from a minimum of 176.8 ± 27.3 m at 12°C to a maximum of 2046.4 ± 374.1 m at 24°C, with a significant positive relationship between temperature and flight distance across the entire temperature gradient (LMM: ‘Temp’, $t_{158} = 5.16$, $p < 0.001$; Figure 3.6c, Table 3.5). As ambient temperature increased above 24°C, mean distance started to decrease falling to a mean of 1816.0 ± 249.5 m at 27°C and then 1540.1 ± 261.9 m at 30°C; a pattern best described by fitting a quadratic relationship (LMM: ‘Temp²’, $t_{158} = -4.92$, $p < 0.001$; Figure 3.6c). This unimodal trend was mirrored in the relationship between temperature and flight duration. Mean flight duration ranged from a minimum of 227.0 ± 31.1 seconds at 12°C up to a maximum of 3243.9 ± 567.9 seconds at 24°C, showing a significant positive relationship between temperature and flight duration across the entire temperature gradient (LMM: ‘Temp’, $t_{158} = 5.22$, $p < 0.001$). The quadratic temperature term provided additional significance (LMM: ‘Temp²’, $t_{158} = -5.06$, $p < 0.001$), indicating that as temperature increased above 24°C, flight duration started to decrease, with mean duration falling to 2831.7 ± 335.5 seconds at 27°C and 2289.8 ± 305.5 seconds at 30°C (Figure 3.6d).

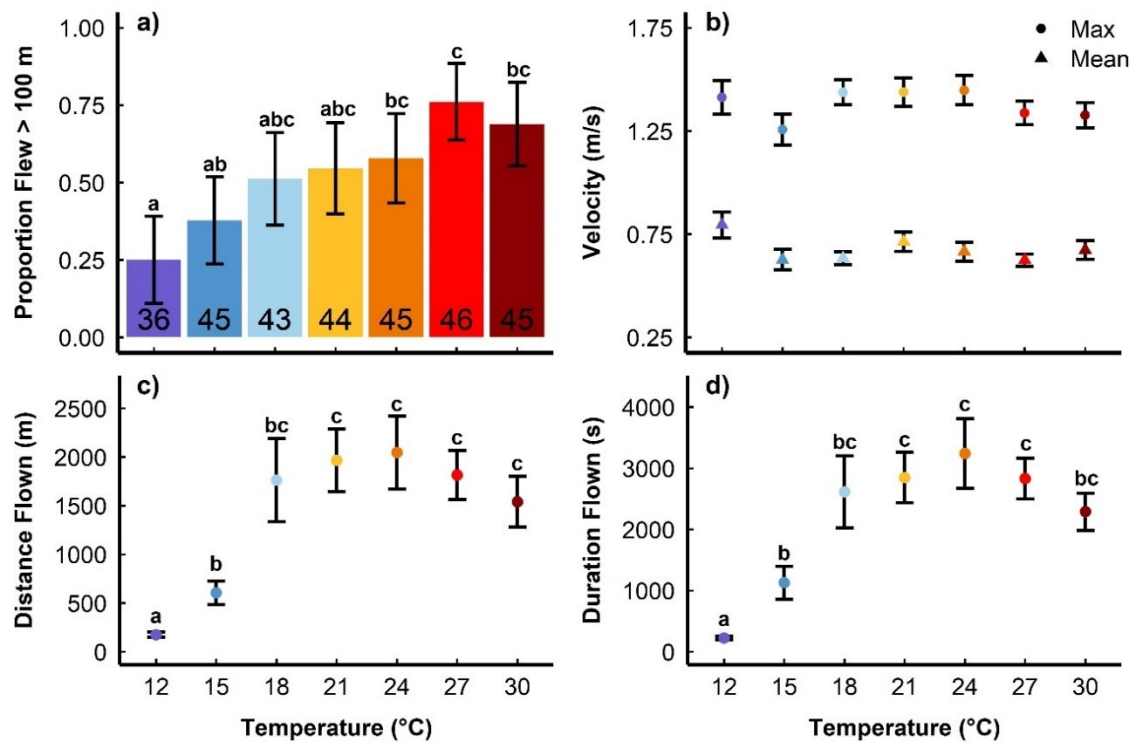


Figure 3.6. Effect of temperature on a selection of flight metrics. a) Proportion of successful fliers (flight $\geq 100\text{m}$) relative to the number of workers that physically initiated flight (bottom of each bar); b) mean (triangle) and maximum (circle) velocity (m/s) flown per worker over the whole flight trial; c) total distance (m) flown per worker; and d) total duration (s) flown per worker. In panel a) each bar is plotted with associated 95% confidence intervals calculated using the ‘Wald’ method. Panels b), c) and d) only consider successful fliers, where points represent mean values plotted with associated standard error bars and treatment pairs displaying the same letter are not significantly different ($\alpha = 0.05$).

Body mass had a significant positive relationship with flight distance and duration (LMM: $t_{158} \geq 2.73$, $p \leq 0.006$). I found no significant change in the relationship between either flight distance or duration and body mass across the temperature gradient, although slope estimates were lowest for the 30°C cohort and greatest for 12 and 15°C cohorts (Figure 3.7a,b). In fact, 30°C was the only temperature that flight duration showed a negative relationship with body mass (Figure 3.7b). Mass-specific consumption was found to have no significant effect on either flight distance or duration (LMM: $t_{157} \leq 1.38$, $p \geq 0.17$; Figure B4).

Pairwise comparisons highlighted that the 12°C cohort flew a significantly shorter distance and duration relative to all other temperatures ($p \leq 0.045$ in all cases; Table B2). The 15°C cohort flew significantly less distance relative to 21, 24, 27 and 30°C ($p \leq 0.038$ in all cases), and a significantly shorter duration relative to 21, 24 and 27°C cohorts ($p \leq 0.034$ in all cases).

Table 3.5. LMER model output testing a) Flight Distance (meters)* and b) Flight Duration (seconds)* as a function of Temperature (°C), Temperature², Dry Body Mass (mg)*, Mass-Specific Consumption (mg /mg; MSC), Temperature / Temperature² x Dry Body Mass interactions, and Temperature / Temperature² x MSC interactions. Non-significant variables were removed by step-wise removal, leaving a final simplified model (output furthest left in tables) containing just significant variables showing no decrease in explanatory power.

*variable log₁₀ transformed.

a)		<i>Estimate</i>	<i>std. Error</i>	<i>p</i>	<i>Estimate</i>	<i>std. Error</i>	<i>p</i>	<i>Estimate</i>	<i>std. Error</i>	<i>p</i>
	<i>Intercept</i>	-1.83	2.46	0.457	-2.05	2.48	0.407	-2.39	2.50	0.340
	Temperature	5.34	1.04	<0.001	5.50	1.06	<0.001	31.14	33.74	0.356
	Temperature ²	-4.98	1.01	<0.001	-5.06	1.02	<0.001	23.98	31.86	0.452
	Dry Body Mass	2.01	0.57	<0.001	2.03	0.57	<0.001	2.12	0.58	<0.001
	Mass-Specific Consumption				0.18	0.28	0.514	0.16	0.30	0.592
	Temperature x Dry Body Mass							-6.34	7.74	0.413
	Temperature ² x Dry Body Mass							-5.69	7.39	0.441
	Temperature x MSC							1.30	3.92	0.740
	Temperature ² x MSC							-5.28	3.76	0.160

b)		<i>Estimates</i>	<i>std. Error</i>	<i>p</i>	<i>Estimates</i>	<i>std. Error</i>	<i>p</i>	<i>Estimates</i>	<i>std. Error</i>	<i>p</i>
	<i>Intercept</i>	0.51	2.47	0.837	0.03	2.49	0.992	-0.44	2.48	0.858
	Temperature	5.45	1.04	<0.001	5.79	1.07	<0.001	41.62	33.38	0.212
	Temperature ²	-5.16	1.02	<0.001	-5.33	1.02	<0.001	46.27	31.52	0.142
	Dry Body Mass	1.58	0.58	0.006	1.61	0.57	0.005	1.73	0.57	0.002
	Mass-Specific Consumption				0.39	0.28	0.167	0.36	0.30	0.218
	Temperature x Dry Body Mass							-8.89	7.66	0.246
	Temperature ² x Dry Body Mass							-10.55	7.31	0.149
	Temperature x MSC							1.86	3.87	0.630
	Temperature ² x MSC							-7.16	3.72	0.054

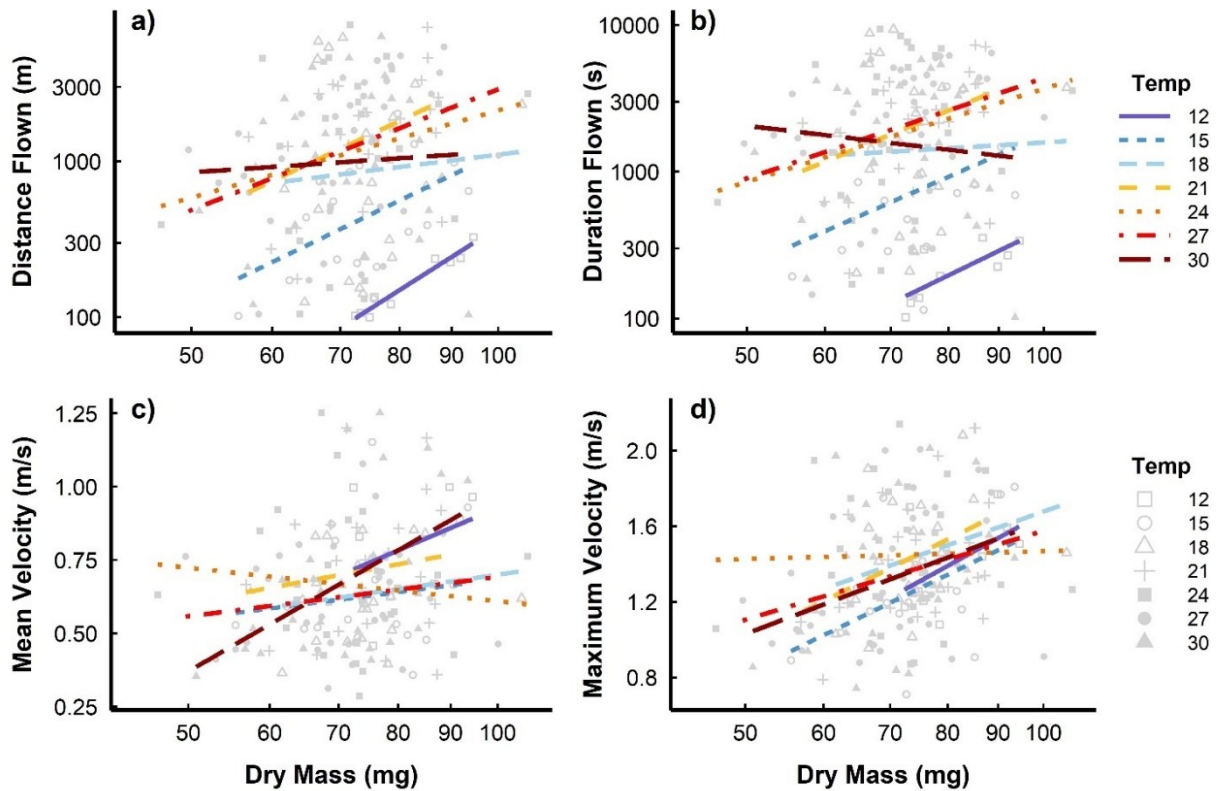


Figure 3.7. Relationships between worker dry body mass and a selection of tested flight metrics for each temperature cohort. Scatter plots with linear best fit lines for each temperature cohort show the relationship between dry mass and a) flight distance (m); b) flight duration (s); c) mean velocity (m/s) flown over the whole flight trial; and d) maximum velocity (m/s) flown over the whole flight trial. In all panels, dry mass has been plotted on a log₁₀ scale and confidence intervals have not been plotted around model lines to aid visualization. In panels a) and b) respectively, distance and duration flown have been plotted on a log₁₀ scale, as these variables were log-transformed in the data analysis.

3.3.3 | Flight velocity & dynamics

I found no clear relationship between ambient temperature and velocity when measured over the whole flight trial (LMM: mean: $t_{158} = -0.61$, $p = 0.54$ & maximum: $t_{158} = 0.43$, $p = 0.67$; Figure 3.6b, Table 3.6), with pairwise comparisons between temperature cohorts showing no significant differences (Table B3). Body mass had a significant positive effect on both mean and maximum velocity when considering flight across the whole trial (LMM: $t_{158} \geq 2.63$, $p \leq 0.009$ in both cases; Figure 3.7c,d, Table 3.6), with no clear change in the slopes of these relationships across the temperature gradient (LMM: 'Temp $\times$ Body Mass': $t_{155} \leq 1.23$, $p \geq 0.22$ in both cases). I found mass-specific consumption showed no relationship with maximum velocity (LMM: $t_{158} = -0.42$, $p = 0.67$), however,

it was negatively related with mean velocity (LMM: $t_{158} = -2.48$, $p = 0.013$), indicating workers that fed more relative to their body mass flew slower on average (Figure B4).

I found no difference in mean velocity between temperature cohorts when considering both 'Immediate' (0 to 30 seconds) and 'Initial' (> 30 to ≤ 300 seconds) flight ($p > 0.05$ in all cases). However, when considering 'Steady' (> 300 to ≤ 600 seconds) flight, I found that the 15°C cohort had a significantly lower mean velocity compared to 18°C (LMM: $t_{117} = 2.05$, $p = 0.043$), 21°C (LMM: $t_{117} = 2.70$, $p = 0.008$) and 24°C (LMM: $t_{117} = 2.22$, $p = 0.028$; Figure 3.8 & 3.9).

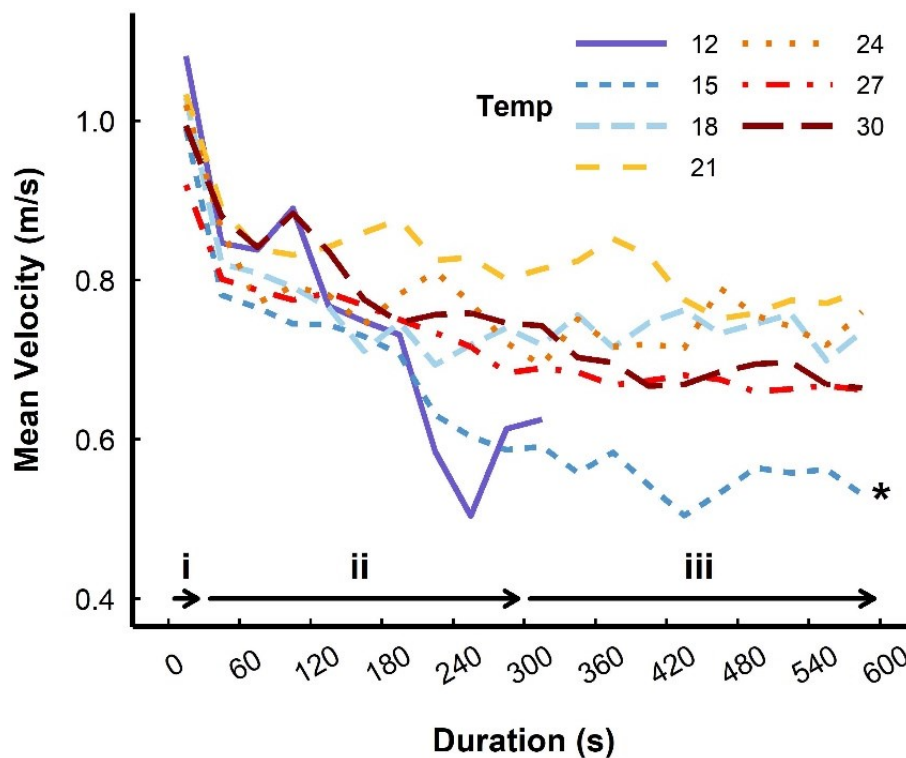


Figure 3.8. Mean velocity (m/s) flown by each temperature cohort over the first 600 seconds of flight. The mean velocity for each temperature cohort was calculated for each 30 second period from 0 until 600 seconds, with values then plotted on the mid-point of the period (e.g., mean velocity for period of 0-30 seconds plotted on $x = 15$ seconds), and standard error bars have been omitted for visualization purposes. Arrows indicate three time periods representing different velocity signatures: i) Immediate Flight, encompassing the first 30 seconds, characterized by comparatively high velocities; ii) Initial Flight, > 30 to ≤ 300 seconds, characterized by decreasing velocities; iii) Steady Flight, > 300 to ≤ 600 seconds, characterized by more steady velocities. *Examining all three time periods, the 15°C cohort significantly differed from the 18, 21, and 24°C cohorts ($p < 0.05$) in steady flight mean velocity, but there were no other detected differences.

Table 3.6. LMER model output testing a) Mean Velocity (m/s) and b) Maximum Velocity (m/s) as a function of Temperature (°C), Dry Body Mass (mg)*, Mass-Specific Consumption (mg /mg; MSC), Temperature x Dry Body Mass interaction, and Temperature x MSC interaction. Non-significant interaction terms were removed, leaving a final simplified model (output furthest left in table) just containing the main effects showing no decrease in explanatory power. *variable $\log_{10}$ transformed.

<i>a)</i>		<i>Estimates</i>	<i>std. Error</i>	<i>p</i>	<i>Estimates</i>	<i>std. Error</i>	<i>p</i>
	<i>Intercept</i>	-0.45	0.51	0.376	1.87	2.18	0.391
	Temperature	0.00	0.00	0.544	-0.10	0.09	0.266
	Dry Body Mass	0.29	0.11	0.009	-0.30	0.50	0.547
	Mass-Specific Consumption	-0.14	0.05	0.013	0.16	0.23	0.499
	Temperature x Dry Body Mass				0.03	0.02	0.220
	Temperature x MSC				-0.01	0.01	0.186
<i>b)</i>		<i>Estimates</i>	<i>std. Error</i>	<i>p</i>	<i>Estimates</i>	<i>std. Error</i>	<i>p</i>
	<i>Intercept</i>	-1.74	0.80	0.029	-0.93	3.46	0.789
	Temperature	0.00	0.00	0.665	-0.03	0.14	0.822
	Dry Body Mass	0.72	0.18	<0.001	0.63	0.80	0.428
	Mass-Specific Consumption	-0.04	0.09	0.673	-0.51	0.36	0.158
	Temperature x Dry Body Mass				0.00	0.03	0.919
	Temperature x MSC				0.02	0.02	0.178

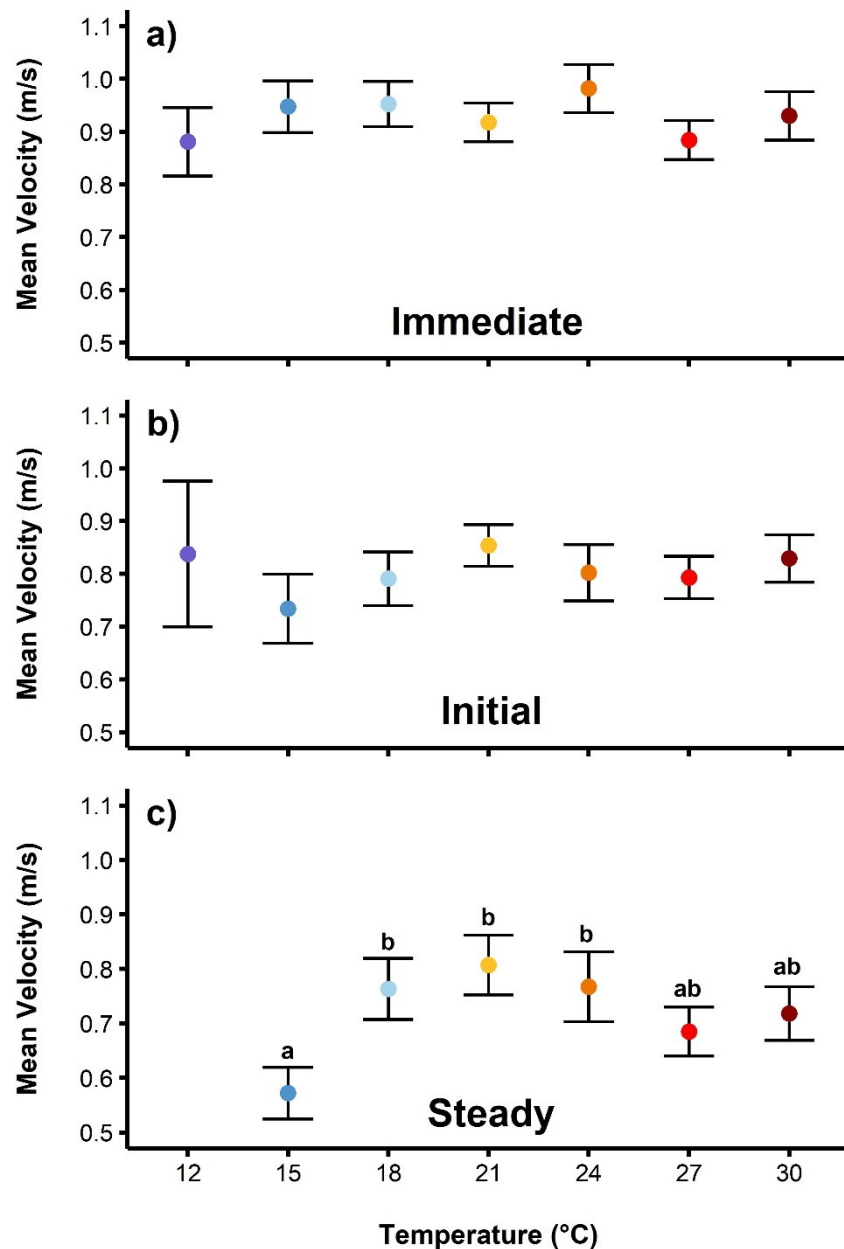


Figure 3.9. Mean velocity (m/s) flown by workers in each temperature cohort over the first 10 minutes (600 seconds) of flight examined over three time periods. a) Immediate Flight, encompassing the first 30 seconds of flight following flight initiation, characterized by comparatively high velocities; b) Initial Flight, encompassing flight > 30 and $\leq$ 300 seconds, characterized by decreasing velocities; and c) Steady Flight, encompassing flight > 300 and $\leq$ 600 seconds, characterized by more steady velocities, which continued on past the 600-second mark. Error bars represent standard error around the mean. In panel c), treatment pairs displaying the same letter are not significantly different ($\alpha = 0.05$) in terms of mean velocity. In panels a) and b) no treatments significantly differed from another.

3.3.4 | *Thermal performance curve*

From the final fitted regression model used for analyzing distance flown (Table 3.5; flight duration results mirror these outputs), I plotted model predictions of flight distance over the temperature gradient 10 to 35°C (Figure 3.10). This regression model was used to find the temperature at which distance was at its maximum, with the thermal optimum determined to be 24.7°C (Figure 3.10). As dry body mass was significantly positively related with flight distance (Figure 3.7a), the peak distance flown predicted by the thermal performance curve increased with body mass (Figure 3.10). However, since I detected no change in the slope of this relationship across the temperature gradient (Figure 3.7a), the predicted thermal optimum presented here was the same across the workforce (Figure 3.10).

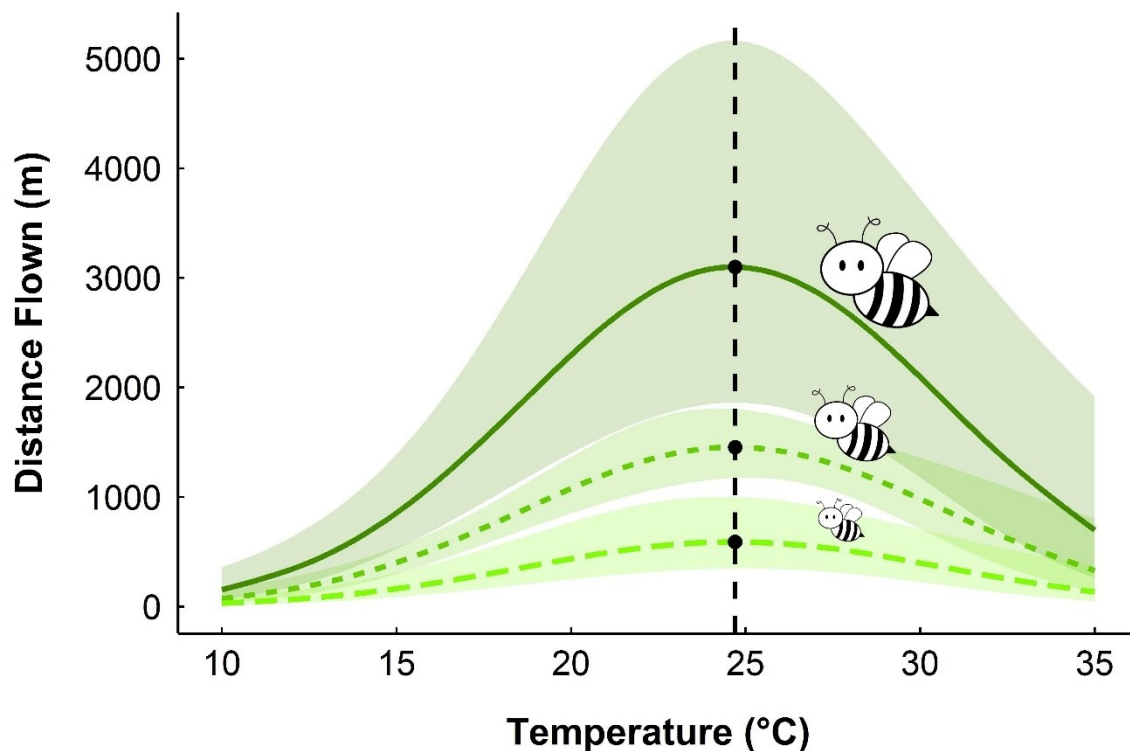


Figure 3.10. Thermal performance curves (TPCs) for workers spanning a range of body size. Three TPCs corresponding to mean (73.3 mg; middle line, short-dashes), minimum (46.7 mg; bottom line, long-dashes), and maximum (106.9 mg; top line, no dashes) dry body mass of all successful fliers. Black circles indicate the thermal optimum for each individual and dashed black line indicates the corresponding temperature (24.7°C). The curves are formed from predictions of my final linear mixed model (Table 3.5), each plotted with 95% confidence intervals.

3.3.5 | *Temperature-mediated foraging potential*

Incorporating both the mean distance flown and the proportion of successful fliers (Table B4), my calculated metric of temperature-mediated foraging potential for each temperature cohort highlighted 27°C to show the highest potential (see Figure 3.11).

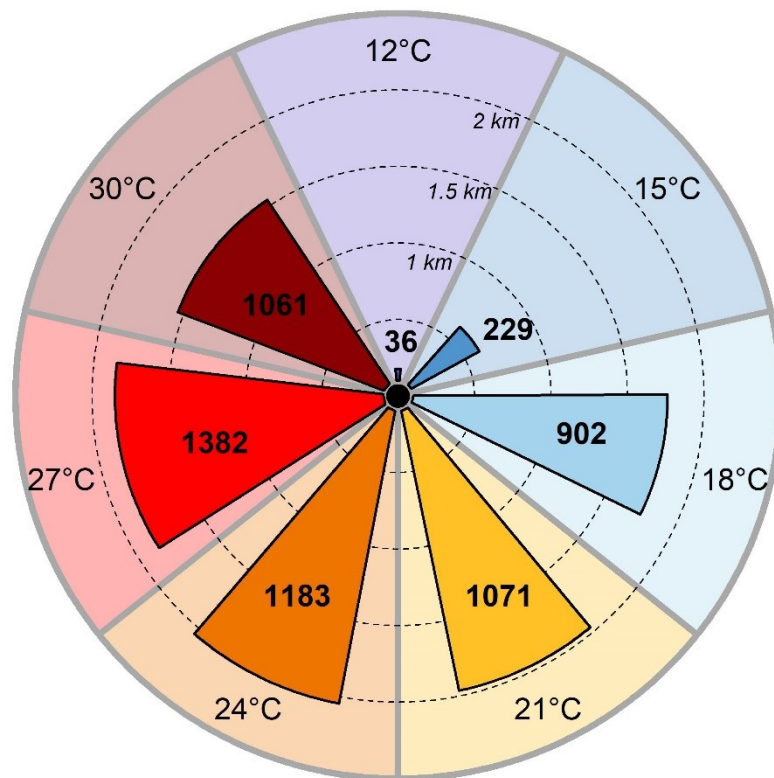


Figure 3.11. Temperature-mediated foraging potential for each tested temperature. The black central spot indicates a hypothetical colony and dashed lines represent potential flight ranges from this fixed nest site. For each temperature cohort, there is a bordered darker coloured segment in which the length is determined by the mean distance flown (m) by successful fliers from that respective cohort, the angle and subsequent area is determined by the proportion of successful fliers relative to those that initiated flight in that cohort, and the number in bold depicts the relative temperature-mediated foraging potential value (calculated as the mean flight distance multiplied by the proportion of successful fliers; see Table B4 for full results). For each temperature cohort, the angle of the lighter shaded segment represents the maximum area available to the cohort if all tested workers were successful fliers.

3.4 | Discussion

Using a bespoke tethered flight mill, I investigated how the flight performance of bumblebees varied over a thermal gradient. My study showed that bumblebee workers were capable of initiating and sustaining flight over a wide thermal range, illustrating the capability of this key insect pollinator group to exploit thermally dynamic foraging landscapes experienced within a daily cycle and across the season (Arce et al., 2017; Corbet et al., 1993; Couvillon, Fitzpatrick, et al., 2010; Peat & Goulson, 2005). Both flight distance and duration showed a concave unimodal trend over the 12-30°C range (Figure 3.6c,d), with my model estimating 24.7°C as optimal for distance flown (Figure 3.10). Mean flight velocity over the entire flight trial, however, was not affected by temperature (Figure 3.6b). The longer distances flown at more optimal temperatures were therefore a result of increased flight duration rather than increased flight speed. My findings further revealed variation in flight capacity across the colony workforce. Larger workers not only flew further, longer, and faster than their smaller nestmates, but were also more likely to fly, with this latter finding particularly striking at low ambient temperatures.

3.4.1 | *Individual-level flight performance*

There was little difference in mean flight velocity (for whole flight trial) across the tested temperature gradient – a finding that contrasts with Hrassnig & Crailsheim (1999) when testing tethered honey bees in a similar flight mill setup. It seems unlikely this finding is due to flight mill limitations on velocity potential, as mean flight velocity was around half the recorded maximum velocities, demonstrating scope for mean velocity to increase. A more plausible explanation centres around thermoregulation. For bees, flight muscle performance is positively associated with muscle temperature up to certain tipping points (Coelho, 1991; Heinrich, 1975; Woods, 2005). As a result, wingbeat frequency, and thus flight velocity, should be driven by the temperature of the thorax (Hrassnig & Crailsheim, 1999; Tong et al., 2019). During flight, bumblebee workers are able to effectively regulate thorax temperature to optimise muscle performance and prevent overheating (Dudley, 2000), which may explain the similar mean flight velocities between temperature cohorts (Spiwok & Schmolz, 2006; Figure 3.6b). This constancy of muscle temperature during flight is achieved by a dynamic balance of heat gain and loss, with workers gaining heat from the environment or through endogenous heat production (Heinrich, 1975; Rubalcaba & Olalla-Tarraga, 2020), and losing heat through processes such as convective cooling and forced transfer of heat from thorax to abdomen (Harrison & Roberts, 2000; Heinrich & Esch, 1994). These processes provide an explanation for the observed concave curves with respect to flight endurance. For example, lower ambient

temperatures may demand a greater energy expenditure as workers will need to generate more heat to maintain a sufficient thorax temperature for flight (Heinrich, 1975). Conversely, at higher ambient temperatures, workers may not be able to lose heat to the environment at a fast-enough rate to prevent thorax overheating (Heinrich, 1975), which could explain the observed decrease in flight endurance past 24°C. This latter point could support why field studies have observed higher bumblebee activity on hot days occurring primarily in the early morning and late afternoon (Arce et al., 2017; Kwon & Saeed, 2003).

Whilst bumblebees demonstrate heterothermy, the majority of non-bee insect pollinators, such as most butterflies and *Dipterans*, are ectotherms. As such, they rely on solar radiation and other environmental heat sources to warm up flight muscles rather than endogenous heat production (Wickham, 2009), and consequently low ambient temperatures could be more limiting for these non-bee pollinating insects compared to bumblebees. Interestingly however, the thermal optimum estimate for flight distance in this study aligns closely with the thermal optimum estimated for dispersal of a genotype of the Glanville fritillary butterfly (~24°C) using harmonic radar in the UK (Niitepöld et al., 2009), which to my knowledge is the only other study to look at insect flight TPCs.

Dependent on resource availability in the landscape, bumblebee foraging ranges are estimated to reach around 2 km from the nest (Osborne et al., 2008; Walther-Hellwig & Frankl, 2000), equating to a foraging round trip of 4 km. Whilst the tethered nature of the flight mill setup meant that workers had a limited requirement to generate lift (Riley et al., 1997), the vast majority of individual flight distances fell within the 4 km mark with only 6% of workers surpassing this distance. This demonstrates that 4 km was attainable, but that the mill setup was not allowing bees to fly longer than they would naturally. Whilst my results are primarily intended as comparative estimates of flight performance across a temperature gradient, this highlights that my results are providing ecologically relevant insights into bumblebee flight endurance.

3.4.2 | Relationship between worker body mass and flight

Worker body mass was positively related with all measured flight metrics (Figure 3.7; body mass explained between 3.4 - 9.2% of the variation in the responses). For instance, for every 2-fold increase in bumblebee body mass there was a predicted 3-fold increase in flight duration and a 0.2 m/s increase in mean flight velocity (across whole flight trial), supporting the theory that energy efficiency of insect flight increases with body mass (Billardon & Darveau, 2019; Darveau et al., 2005; Skandalis & Darveau, 2012). These positive relationships, which would be expected under metabolic scaling theory (Brown et al., 2004), may also be partially explained by differences in morphological

traits and flight kinematics, as wingbeat frequency in insects directly relates to energy consumption and is inversely related to body mass and wing surface area (Billardon & Darveau, 2019; Darveau et al., 2005; Santoyo et al., 2016). Another important consideration here is the capacity of individuals to store food. Across insects, whilst the absolute energy demand of flight is generally greater in larger bodied individuals, this can be more than compensated for by greater baseline endogenous energy stores, which results in flight endurance capacity increasing with body size (Kaufmann et al., 2013).

Foraging bumblebee workers are known to collect up to 60-80% of their own body mass in nectar and pollen on a single foraging trip (Combes et al., 2020). Interestingly, I found that workers with higher mass-specific sucrose consumption flew slower on average, which could indicate that carrying a heavier load places constraint on flight velocity or could indicate a determined kinematic response to cope with the heavier load (Combes et al., 2020; Heinrich, 1975). Whilst I tried to ensure that each bee had fed to satiation prior to flight, there was no way to definitively control this, and therefore the variation in flight performance between workers of similar body mass (Figure 3.5 & 3.7) could stem from variable endogenous energy stores prior to flight (Kaufmann et al., 2013). This would seem plausible as flying insects are believed to have metabolic enzymes working close to maximum flux rates, and thus the only way for an individual to improve flight capacity is via increased energy consumption (Niitepõld et al., 2009; Suarez, 2000).

Ultimately, my controlled lab findings imply that larger individuals are able to reach flowering resources more quickly and over a greater distance than their smaller counterparts, supporting field observations of foraging scope and efficiency increasing with body size (Goulson et al., 2002; Greenleaf et al., 2007; Spaethe & Weidenmuller, 2002; Willmer & Finlayson, 2014) and providing a further mechanistic explanation for observed alloethism in bumblebees (Herrmann et al., 2018; Holland et al., 2021). Interestingly, agricultural intensification and associated declines in habitat quality and quantity appear to be leading to shrinkages in bee body size, not just in *Bombus terrestris* but also across other social and solitary species (Oliveira et al., 2016; Renauld et al., 2016). Applying my findings on size-mediated flight performance across taxa therefore suggests this trend of declining bee body size could have negative consequences for pollination service delivery by contemporary bee communities.

Furthermore, I found that the effect of body mass on flight success (flight ≥ 100 m) became stronger as temperature decreased (Figure 3.5a). This can potentially be explained by considering how thermoregulatory capacity varies with body mass, with the lower surface area to volume ratio of larger individuals resulting in a slower rate of heat transfer to the environment, allowing them to more effectively retain heat (Bishop & Armbruster, 1999; Heinrich, 1975; Peat et al., 2005).

3.4.3 | *Temperature-mediated foraging potential*

When applying my individual flight performance results to the context of temperature-mediated foraging potential, I considered not only flight distance but also the proportion of tested workers motivated or physically able to fly at the respective temperatures. Subsequently, whilst mean distance was highest in the 24°C cohort, there were more successful fliers in the 27 and 30°C cohorts, meaning that foraging potential at the colony-level may actually be maximized at temperatures closer to 27°C (Figure 3.11).

At the other end of the temperature scale, successful fliers in the 12 and 15°C cohorts exhibited a mean flight distance of just 177 and 605 m respectively, with nearly two thirds of the workers classed as unsuccessful fliers (not flying past 100 m; Figure 3.6a,c). On cold days, foraging bees can use direct solar radiation to assist in warming (Rubalcaba & Olalla-Tarraga, 2020; Stabentheiner & Kovac, 2014), which was not available to workers under my controlled flight mill setup. This should be noted when extrapolating my findings directly to real-world scenarios, as bumblebees are known to forage in temperatures of $\leq 12^{\circ}\text{C}$ (Bishop & Armbruster, 1999; Corbet et al., 1993; EFSA, 2013). Additionally, the workers tested in this study were from commercial colonies with no prior flight experience. This allowed us to standardise flight comparisons between temperature cohorts, but should be considered when comparing flight distances on the mill with foraging distances in the field, as flight experience has been reported to influence aspects of foraging performance (Peat & Goulson, 2005). However, foraging *Bombus terrestris* workers often fly around 600 m from the nest to exploit floral resources (Darvill et al., 2004; Osborne et al., 1999), and have been noted to fly up to 2 km in agricultural landscapes (Osborne et al., 2008; Walther-Hellwig & Frankl, 2000), so my work suggests that temperatures down to 12 and 15°C may prevent colonies from reaching resources they would otherwise forage on under warmer conditions. In addition, when examining the dynamics of flight velocity over the flight trials, I did find that workers flew slower at 15°C compared to warmer temperatures once they had settled into a steady rhythm of flight (Figure 3.8 & 3.9). If such conditions translate to workers taking longer to reach and move between flower patches, this may subsequently increase the energetic cost of foraging trips, exacerbating the challenge faced at the colony-level.

Consistent temperatures of $\leq 15^{\circ}\text{C}$ are only likely to affect colonies of temperate bumblebee species in their first stages of development (early spring months), when the predominant foragers will be larger bodied queens that are capable of foraging in cooler conditions relative to workers (Bishop & Armbruster, 1999; Lundberg, 1980). Recent and projected extreme weather events, however, do suggest that erratic and longer-term cold spells may become more common within bumblebee ranges early in the year. As colonies tend to produce smaller workers earlier in the season (Hefetz &

Grozinger, 2017), this could potentially lead to an unmotivated and ineffective workforce (Cohen et al., 2018; Francis et al., 2018; Kretschmer et al., 2018). These results highlight the need for ample flowering resources close to nests early in the year to allow shorter and less demanding foraging trips (Gervais et al., 2020; Westphal et al., 2006), which may be an issue with co-occurring increases in human induced fragmentation of natural floral resources (Jha & Kremen, 2013; Osborne et al., 2008; Redhead et al., 2016). With bumblebees being central place foragers, this is of particular concern, as a fixed nest site prevents within-season relocation of the 'central place' to track floral resource turnover or more favourable thermal microclimates (Bladon et al., 2020; Raine & Gill, 2015).

Our temperature-mediated foraging potential values were calculated based on a colony with a normal distribution of worker body mass, but it would be useful to understand how colonies with different worker mass distributions determine variation in colony foraging potential. My results suggest that a workforce consisting of a higher proportion of larger bodied workers would benefit a colony under cold conditions. However, producing larger workers can come at a cost to a colony as they are more energetically expensive to rear and maintain, and larger workers have been reported to exhibit lower longevity and be less resistant to starvation than their smaller sisters (Couvillon & Dornhaus, 2010; Kerr et al., 2019). Furthermore, in hot conditions, large workers may be more at risk of overheating during flight (Dudley, 2000; Harrison & Roberts, 2000; Rubalcaba & Olalla-Tarraga, 2020). Therefore, large body mass may not confer such an advantage to a colony under hotter conditions, and it is interesting that a global decline in the body size of many ectothermic taxa has been associated with climate warming (Gardner et al., 2011). On this note, my observations showed that the correlation between flight distance and body mass was least positive at the highest tested temperature of 30°C, with flight duration correlating negatively with body mass at the same temperature (Figure 3.7a,b). In other words, smaller workers appeared to perform on average comparatively better than larger workers at the highest temperature studied. If workers of different mass are more suited to forage in different thermal conditions and thus at different times of the day, it may better allow bumblebee colonies to temporally exploit their foraging landscape (Couvillon, Fitzpatrick, et al., 2010; Peat & Goulson, 2005). Given that ambient temperatures throughout temperate bee species' ranges are predicted to more commonly rise above 30°C in the coming years (IPCC, 2014), it would be useful to expand body mass vs flight performance relationships to higher temperatures to better predict how intraspecific worker size variation may mediate colony-level responses to climate warming. Additionally, by applying these findings across insect taxa with similar thermal mechanisms, we may be able to gain deeper insights into how the foraging capacity and pollination service delivery of whole insect pollinator assemblages will vary under future environmental temperature scenarios.

3.4.4 | *Impacts of climate change*

When considering the geographic range of the study species *B. terrestris*, it is interesting that temperatures experienced throughout the foraging season mostly fall on the ascending slope of the predicted thermal performance curve (Figure 3.10; Figure B1). Species of terrestrial insects in higher latitudes have been shown to occupy areas with mean temperatures below their thermal optimum (Buckley et al., 2013; Kingsolver et al., 2013), and well below their thermal limits (Deutsch et al., 2008), which is seen as a mechanism to buffer against seasonal temperature variation and the fitness consequences associated with overheating (Buckley et al., 2013; Martin & Huey, 2008). Indeed, in Europe, the probability of bumblebee occurrence is greater when seasonal thermal variation is low (Ghisbain et al., 2020).

Given that mean daily temperatures are projected to increase across much of the temperate range of bumblebees (i.e. ca. 2-4°C rise by 2100; IPCC, 2014), my findings suggest global warming may actually push bumblebee colonies closer to their flight thermal optimum, potentially improving foraging returns and pollination delivery (Deutsch et al., 2008; Herbertsson et al., 2021; Sánchez-Bayo & Wyckhuys, 2019). However, my results also suggest that if ambient temperatures more frequently pass 30°C from extreme climatic events such as extended heatwaves, this could start to have detrimental effects on key behaviours underpinning colony fitness (Meehl & Tebaldi, 2004). Further study on this could offer a potential explanation for reported bumblebee losses at southern range limits (Kerr et al., 2015; Sirois-Delisle & Kerr, 2018; Soroye et al., 2020). Conversely, although bumblebees are commonly considered an insect pollinator group that can cope well with cold conditions (Hines, 2008), my data further suggests that prolonged cold snaps, as more commonly predicted in the early months of the year under climate change, will be detrimental to the foraging capacity of developing bumblebee colonies which commonly do not store sufficient food to last for extended periods (Couvillon & Dornhaus, 2010).

3.4.5 | *Conclusion*

Our results suggest that climate warming may favour aspects of bumblebee flight performance, such as motivation and endurance, across large parts of their geographic range. However, sensitivity to climate change can vary widely both between bumblebee species and across insect groups more generally (Arribas et al., 2012; Hamblin et al., 2017; Sunday et al., 2014; Zambra et al., 2020), and my results suggest that even within the same species, responses to temperature vary with body mass and thus likely affect smaller and larger bodied taxa differently. It is therefore important we quantify and compare flight and other key behavioural thermal response curves for

Chapter 3 | Thermal flight performance reveals impact of warming on bumblebee foraging potential

species across the insect phylogeny in order to understand spatio-temporal risks and identify conservation priorities of similar and different functional groups.

CHAPTER 4

Environmental temperature modulates pesticide effects on bee activity and survival

4.1 | Introduction

Insect pollinator declines are a worldwide issue (Potts et al., 2016; Zattara & Aizen, 2021), making identifying the drivers a research priority and crucial for developing mitigative strategies (Gill et al., 2016; Goulson et al., 2015; Wagner et al., 2021). The widespread use of pesticides in the environment is one proposed driver. For example, studies have shown impeded development and impaired behaviour in insects exposed to field realistic concentrations (Chapter 2; Crall et al., 2018; Siefert et al., 2020; Smith et al., 2020; Wood & Goulson, 2017), which can translate to reduced fitness (Arce et al., 2017; Baron, Jansen, et al., 2017; Whitehorn et al., 2012; Woodcock et al., 2017). Another increasingly scrutinised driver is climate change. For instance, environmental warming and increasingly common extreme heat events over the past century have been associated with insect population range shifts (e.g., Halsch et al., 2021; Kerr et al., 2015; Powney et al., 2019) and species' extinction risk (e.g., Janzen & Hallwachs, 2021; Soroye et al., 2020), as temperatures have more frequently exceeded historical thermal tolerances. To date, however, the interplay between these two drivers has been little investigated, despite them co-occurring in nature and despite risks to beneficial insects considered to increase through multi-stressor interactions (Cameron & Sadd, 2020; Vanbergen & Garratt, 2013: but see Henry et al., 2014; Kolano et al., 2021; Monchanin et al., 2019). Hence, determining how temperature can modulate pesticide exposure impacts is a pressing issue, as future landscapes are likely to experience increased rates of pesticide application that will coincide with warmer and more extreme temperature regimes under climate change (Deutsch et al., 2018; IPCC, 2018; Tang et al., 2021; Zhang, 2018). Moreover, gaining such insights will provide a greater understanding of the spatio-temporal risks to wild insect populations, such as where and when across the world's climate regions insects are, and will be, most at risk from pesticide exposure.

When studying the impacts of pesticide exposure, it is important we quantify sublethal effects, as exposure to field-level concentrations is often not acutely lethal (Bryden et al., 2013; Desneux et al., 2007; Gill et al., 2012). Studies assessing sublethal impacts from insecticide exposure on insect behaviour, however, have often reported mixed responses or varying effect sizes; even when

considering the same species and pesticide (Arena & Sgolastra, 2014; Decourtye et al., 2013; Godfray et al., 2014; Heard et al., 2017). A possible explanation for such discrepancies is that the environmental context has not been appropriately considered, such as temperature variation between and within different lab and field studies (Holmstrup et al., 2010). Variation in ambient temperature can affect insect metabolic and physiological rates (Gillooly et al., 2001; Huey & Stevenson, 1979), such as consumption (Camp & Buchwalter, 2016; Holmstrup et al., 2010; Noyes et al., 2009) and chemical detoxification and excretion (Harwood et al., 2009; Khan & Akram, 2014; Lydy et al., 1999; Weston et al., 2009), as well as altering pesticide binding efficiency at target-receptors (Boina et al., 2009). Hence, studies quantifying how temperature modulates pesticide effects on both lethal and sublethal endpoint behaviours are urgently needed. Furthermore, studying a range of functional behaviours under a single study framework is important, as this provides a holistic understanding of organismal responses, helping to predict how ecosystem service provision may be affected and population tipping points reached (Bryden et al., 2013; Moore, 2018).

To describe the temperature dependency of pesticide effects on behaviour, we can elucidate the direction of the interactive relationship by designating a temperature coefficient (TC) (Boina et al., 2009; Khan & Akram, 2014; Muturi et al., 2011). Insecticides with a positive TC become more toxic with increasing temperature, whereas a negative TC becomes more toxic at decreasing temperatures (Glunt et al., 2013; Mansoor et al., 2015). Conducting experiments to reveal these relationships has fundamental implications for current pesticide regulatory guidelines and risk assessments, which often recommend carrying out single response studies at standardised temperatures and primarily focus on lethal (not sublethal) endpoints (EFSA, 2013). Here I studied the TCs for multiple behavioural measures in a model insect pollinator – the bumblebee *Bombus terrestris* – exposed to either imidacloprid (neonicotinoid) or sulfoxaflor (sulfoximine) insecticides across three different ambient temperatures. A neonicotinoid was considered in this study for a number of reasons: i) neonicotinoids represent the most widely used insecticide class on the global market (Godfray et al., 2014; Jeschke & Nauen, 2008; Simon-Delso et al., 2015); ii) they are systemic and are thus frequently encountered by insect pollinators, including oral exposure by consuming / collecting contaminated pollen and nectar (Blacquière et al., 2012; David et al., 2016; Elbert et al., 2008; Jeschke et al., 2010); iii) exposure can impair thermogenesis (Azpiazu et al., 2019; Potts et al., 2018; Tosi et al., 2016), which given is a temperature dependent process, indicates toxicity effects will alter under temperature change; iv) nest activity and thermoregulation of bumblebees has been reported to be differentially affected by exposure under warmer days versus cooler nights (Crall et al., 2018); and iv) recent evidence suggests effects of exposure on honey bee metabolic rate and gene expression are temperature dependent (Gooley & Gooley, 2020; Manzi et al., 2020). Rising pest resistance to neonicotinoids (Bass et al., 2015),

combined with new restrictions on the use of nitroguanidine neonicotinoids in the EU (EFSA, 2018; Sgolastra et al., 2020), has led to the development and registration of new chemicals to control sap-feeding insect pests (Siviter & Muth, 2020). Hence I also considered sulfoxaflor as it represents one of the most promising replacements of neonicotinoids across the globe (Sparks et al., 2013). Moreover, recent work reported exposure to sulfoxaflor, at concentrations below the maximum residue limits set by the Environmental Protection Agency (EPA) in the US, negatively impacted bumblebee colony development (Siviter et al., 2018).

I undertook three complementary experiments and investigated a total of seven different responses representing important bee life-history and ecological functions (see Figure 4.3). Specifically, I investigated how ambient temperature modulated the effect of: 1) chronic exposure to a high (150 µg/L) concentration of each insecticide on the likelihood of survival and immobility (together termed responsiveness – important for within nest activity); 2) chronic exposure to a mid (40 µg/L) or low (10 µg/L) concentration of each insecticide on the likelihood of movement, walking rate, and consumption rate (behaviours important for nesting duties, colony thermoregulation, meeting energy demands, and food collection); 3) acute exposure to a low (10 µg/L) concentration of each insecticide on flight endurance and velocity (behaviours crucial for foraging performance). Using a fully factorial design with appropriate control groups, each experiment for each insecticide was conducted at a relative low (21°C), mid (27°C), and high (30°C) temperature, with these values informed by the temperature-mediated foraging potential values reported in Chapter 3 (see Methods 4.2.3). Here I tested whether insecticide exposure shows a negative or positive TC, investigating how this relationship varied between each insecticide and if this differed dependent on the behavioural endpoints. For measures of flight, I additionally investigated how body size affected these relationships. Given that neonicotinoids are generally considered as positive TC insecticides (Mansoor et al., 2015; Tsaganou et al., 2021), and that neonicotinoids and sulfoximines have a similar chemical structure and mode of action (Sparks et al., 2013), an expectation would be that both insecticides would lead to greater impairment at the higher temperatures. However, recent studies have suggested that the impacts of neonicotinoids on bee survival and thermoregulatory behaviour increase in severity as temperatures get cooler (Crall et al., 2018; Saleem et al., 2020), with similar findings from pyrethroids on honey bees (Belzunces et al., 2012). Therefore, I would predict that both imidacloprid and sulfoxaflor would have greater impacts on bumblebee responses at the lower temperature.

4.2 | Methods

4.2.1 | *Colony husbandry*

All colonies from which workers were tested were commercially supplied by Biobest (distributed by Agralan Ltd). On arrival each colony was checked for good health and censused, ensuring healthy queen presence and removal of any dead individuals. Any sugar feeders and pollen patties that arrived with the colonies were removed. Colonies were provisioned with freshly made ad-libitum 40/60% (w/w) sucrose/water solution weekly, and with irradiated honey bee collected pollen (supplied by Agralan Ltd) on Mondays (6g), Wednesdays (6g) and Fridays (9g). Colonies were kept in a controlled environment (CE) chamber at 21°C and 60% relative humidity (RH) under constant red light. Testing of workers was conducted in separate CE chambers at the respective testing temperatures also under 60% RH (Table C1). No one colony was used across the multiple experiments.

4.2.2 | *Choice of insecticides & concentrations tested*

In addition to the points raised in Chapter 2, imidacloprid was decided on for this study for two primary reasons: i) it has previously been shown to affect survival, mobility, flight (please see Chapter 2), and foraging performance in bees at similar concentrations to the ones used in this study (e.g., Gill & Raine, 2014; Iwasa et al., 2004; Williamson et al., 2014), and therefore it effectively acts as a positive control to understand whether temperature influences the toxicity of a given insecticide; and ii) it is regarded as the most toxic of the neonicotinoid insecticides (Iwasa et al., 2004), providing a worst-case scenario benchmark with which to compare sulfoxaflor against. As outlined in Chapter 1, sulfoxaflor is the first commercially marketed sulfoximine insecticide (Sparks et al., 2013) and is likely to replace neonicotinoids across large areas globally (Simon-Delso et al., 2015). Although purported to be chemically distinct from the neonicotinoids, it has a similar mode of action and is an agonist of nicotinic acetylcholine receptors (Ulens et al., 2019), which has raised concerns around the potential impacts of exposure to insect pollinator health (Brown et al., 2016; Linguadoca et al., 2021; Siviter & Muth, 2020), and consequently there is a pressing need to understand the comparative toxicity of this neuroactive insecticide.

I mirrored the concentrations between imidacloprid (I) and sulfoxaflor (S) in order to facilitate direct comparisons of the two insecticides, and exposed individual workers via a spiked nectar substitute (40% w/w sucrose/water solution). For experiment 1, I examined the probability of survival and immobility (collectively termed 'responsiveness') when exposed to pesticide concentrations of 150 µg/L (termed I150 & S150). This concentration falls within the wide range of previously calculated imidacloprid LC₅₀ values for bees over similar exposure periods (Mommaerts et al., 2010; Suchail et

al., 2000, 2001), and neonicotinoid concentrations over this level can be experienced in agricultural fields (Zioga et al., 2020), especially immediately following pesticide application (Tapparo et al., 2012), and can accumulate to such levels inside colonies (Ellis et al., 2017; Mullin et al., 2010). For experiment 2, I examined movement and walking behaviour in response to concentrations of either 40 µg/L (termed I40 & S40) or 10 µg/L (termed I10 & S10), which represent sublethal concentrations of imidacloprid that have been found in pollen and nectar of treated plants and within bee material (Blacqui re et al., 2012; Bonmatin et al., 2015; Bot  as et al., 2015; Mitchell et al., 2017; Zioga et al., 2020). For experiment 3, I examined flight behaviour in response to 10 µg/L. Due to its comparatively shorter time as a commercial option, considerably less is known about concentrations of sulfoxaflor present in the environment. However, recent studies examining the nectar and pollen of a variety of treated crops after application at label recommended rates have found sulfoxaflor residues regularly above the 10 µg/L concentration used in this study, and above 150 µg/L when applied close to blooming period, highlighting that the chosen concentrations represent both realistic and conservative exposures (EFSA et al., 2019; EPA, 2019; Jiang et al., 2020; Linguadoca et al., 2021). In each experiment I focused on oral pesticide exposure since evidence suggests this is often the most relevant and the most conservative approach for bees (Heard et al., 2017).

Separate stock solutions (100 mg/L) were prepared by taking dry powder forms of imidacloprid (Sigma-Aldrich, Pestanal; 100% purity) and sulfoxaflor (Dr Ehrenstorfer GmbH; 99.4% purity) and dissolving in acetone, with these stock solutions then stored at -80 C between experiments. Pesticide working solutions were made by diluting the respective pesticide stock solution in 40% w/w sucrose/water solution. For each experiment, the control working solution consisted of 40% w/w sucrose/water solution with an identical concentration of acetone added to that which was used in the pesticide working solutions. All dilutions were carried out in light restricted rooms with containers wrapped in foil to prevent light degradation (Soliman, 2012). Working solutions were made directly prior to each experiment. For experiment 1 & 2, a sufficient number of feeding tubes to cover the duration of the experiments were aliquoted. For experiment 3, a sufficient number of falcon tubes to cover the duration of the experiment were each individually filled with enough working solution for one days' worth of trials. These working solutions were stored in a cardboard box to prevent light degradation and were frozen at -20 C, with the required number for use being removed and defrosted on the morning of each trial.

4.2.3 | Rationale for temperatures tested

Biological rates respond to temperature change in a non-linear fashion (Huey & Stevenson, 1979), hence I avoided selecting evenly spaced temperatures. Instead, each experiment was conducted across a low (21°C), mid (27°C) and high (30°C) temperature based on the reported thermal performance of *B. terrestris* workers in Chapter 3, which used the same flight mill setup described in experiment 3 of the current study. The mid temperature represents peak performance (when considering motivation to fly and flight endurance), with low and high temperatures representing an approximate 25% performance reduction either side (Chapter 3 – Figure 3.11). This temperature range is also realistic of air temperatures experienced by temperate bee species during the summer months (Fick & Hijmans, 2017; Hooker et al., 2018).

4.2.4 | Experiment 1: Survival & responsiveness

Experimental Protocol

On arrival and for each of five colonies, 81 workers were removed under red light and cooled at 4°C to decrease activity for easy handling. The age or previous activity of workers within the nest was unknown. Workers were then distributed into 340 ml circular plastic feeding cages (base dimensions = 110 mm diameter, 60 mm depth), with each cage containing three workers from the same natal colony (27 cages per colony; total = 405 bees across all colonies). Based off visual inspection, I ensured each feeding cage contained workers spanning a range of body sizes and consequently there was no difference in intertegula span (ITS; proxy for body size) mean (ANOVA: $p > 0.1$ for all pairwise comparisons) or variance (Bartlett's $K^2 = 5.49$, $df = 8$, $p = 0.70$) between treatments. Using groups of three workers is consistent with previous chronic exposure tests using feeding cage designs (e.g., Heard et al., 2017) and reflects the social nature of *Bombus terrestris*, allowing social contact even if one worker died. Feeding cages had a ventilated lid, circular piece of filter paper (diameter = 100 mm) on the floor to soak up any sucrose solution spillage and bee excrement, and two holes on opposing sides in which perforated Eppendorf 'feeding' tubes were inserted to provision set volumes of the sucrose solution.

Using a factorial experimental design, I evenly assigned feeding cages to a chronic pesticide exposure (untreated, I150 or S150) and temperature (low, mid or high) combination, resulting in nine treatments and 15 feeding cages ($n = 45$ workers) examined in total per treatment. For each colony, a further nine workers were then removed and distributed evenly across three reserve cages (same design as feeding cages), with one cage assigned to each temperature. All cages (feeding and reserve) were initially provisioned with two feeding tubes each containing 1.5 ml of untreated sucrose solution

and were moved to their respective controlled temperature chamber before the end of the first day to allow acclimation. The following morning (06:00-07:00), feeding cages were inspected and if any workers had died these were replaced by workers taken from reserve cages of the corresponding natal colony and temperature. Directly following this (07:00-08:00), each cage had its feeding tubes replaced with tubes each containing 1.5 ml of assigned sucrose solution treatment. These tubes were subsequently replaced every 48 hours and collectively weighed before and after use to calculate average consumption rates per treatment, with the filter paper in each cage replaced at the same time.

Workers were exposed to their respective treatments for 144 hours whilst held under red light throughout. Feeding cages were monitored every 12 hours (07:00-08:00 and 19:00-20:00) using the following protocol: 1) the cage was tapped gently three times, and individuals observed walking (clear movement of both thorax and legs) within the following 10 seconds were recorded as 'mobile'; 2) individuals that did not walk were turned onto their back using a pair of metal forceps via the hind leg and monitored for 30-seconds. If the individual successfully self-righted within this time then it was recorded as 'mobile', but if unable it was classed as 'immobile'; 3) any 'dead' individuals were recorded, transferred to an Eppendorf tube, and frozen (-20°C); 4) any individual classed as immobile was righted using forceps and left in the feeding cage so they could be further monitored. A bee was never observed to revert from being immobile to mobile during the course of the trials. Two distinct datasets were subsequently produced that outlined the time to event for every individual bee when classifying the event as either: i) death, or ii) unresponsiveness. 'Unresponsive' is here defined as a worker being either immobile or dead, whichever occurred first.

On days where sucrose solution was replenished, monitoring was conducted beforehand. After the last monitoring event all workers were placed in separate Eppendorf tubes and frozen (-20°C). As in Chapter 2, ITS was here used as a proxy for body size instead of body mass, because workers were frozen the day after being removed from food due to logistical reasons, and therefore taking mass measurements may not be reliable indicators of body size due to starvation. ITS (accuracy 0.01 mm) was taken using a digital calliper (Workzone 150 mm), with the mean of three repeated measurements being used in data analysis (Cane, 1987; Greenleaf et al., 2007).

4.2.5 | Experiment 2: Walking & consumption

On arrival and for each of nine colonies, 90 workers were removed using long forceps, placed on ice to cool and immobilise (Okubo et al., 2020), and subsequently had a small (diameter = 2mm) circular uniquely numbered plastic tag (Abelo Ltd) attached to the thorax using superglue. Tagged

workers were then placed in separate plastic pots for 60 minutes to rest before being returned to their natal colony. The age or previous activity of workers within the nest was unknown.

Following a factorial experimental design, workers were placed in feeding cages assigned to a chronic pesticide exposure (untreated, I40, I10, S40, or S10) and temperature (low, mid or high) combination, resulting in 15 treatments. Feeding cage setups were the same as in experiment 1, each having three workers from the same natal colony with pilot studies supporting this decision (see Appendix C for support). However, this time only a single hole was present for the provision of a 2 ml perforated Eppendorf feeding tube, which was deemed sufficient based off pilot experiments as feeders were replaced every 24 hours instead of 48 hours (see Appendix C for support). With tagging being time consuming and pilot data suggesting no effect of tags on behaviour, workers were distributed such that each feeding cage consisted of two tagged and one non-tagged worker allowing every worker to be uniquely identifiable. As in experiment 1, I ensured each feeding cage contained workers spanning a range of body sizes resulting in no significant difference in dry mass mean (ANOVA: $p > 0.1$ for all pairwise comparisons) or variance (Bartlett's $K^2 = 23.4$, $df = 14$, $p = 0.054$) between treatments.

The experiment was run in two blocks in a staggered manner, with 810 workers tested in total across the 15 treatments. In the first block, one feeding cage ($n = 3$ workers) per colony was used for each treatment from eight of the nine colonies, resulting in eight cages per treatment ($n = 24$ workers). For the second block, which started 2 days after the completion of the first block, for each of the original eight colonies one feeding cage was again used for each treatment, but two feeding cages were used for each treatment from the ninth colony. This resulted in 10 feeding cages per treatment for the second block ($n = 30$ workers). This staggered design was implemented to ensure there was always an even number of feeding cages within each treatment in each block, which facilitated a more efficient use of time as feeding cages were filmed in pairs.

Feeding cages were provisioned with a feeding tube containing 2 ml of untreated sucrose solution and moved to their respective controlled temperature chamber before the end of the first day. The following morning, feeding cages were inspected for any dead workers and these were replaced by healthy workers from reserve cages of the corresponding natal colony and temperature following the same protocol as was outlined for experiment 1. After letting workers settle for 4 hours, 'pre-exposure' recordings were taken by filming feeding cages to establish baseline worker behaviour. Cages were filmed in pairs using a Panasonic HC-V160 video camera held in a support stand (Figure 4.1), with a ruler included in each video to allow calibration of distances in later analysis (see Appendix C for support). Videos were taken under two sequential stimulation scenarios: i) First a 'no-stimulation' trial where cages were filmed under red light; ii) followed by a 'light-stimulation' trial,

where cages were filmed after they were transitioned into a full spectrum white light environment provided by a height adjustable LED lamp (SCHOTT AG). The cages being filmed were surrounded by a circular border (height = 40 cm), and the lamp was directed down at the pots so that all surrounding cages not being filmed at that time were not exposed to the white light. Video recordings started 10 seconds after switching on the lamp, thus allowing a period of time for workers to respond to the stimulus. For both stimulation scenarios, behaviour was recorded for 30 seconds. The no-stimulation scenario videos were taken first, starting with the low temperature treatments, followed by the mid temperature treatments and finally the high temperature treatments. The entire process of filming all cages for the no-stimulation trials took ca. 90 minutes, with the light-stimulation filming starting immediately after and repeating the same treatment order. At the start of every video recording, the position of each worker ID was mapped, which was then cross-referenced with the video file when analysing behaviour.

On finishing the 'pre-exposure' video recordings, the feeding tube for each cage was replaced with a tube containing 2 ml of assigned treatment sucrose solution treatment, which was weighed prior to being provisioned. After 20 hours post onset of exposure, cages were monitored, and workers were classified as either alive or dead. Dead workers were removed from their respective cages, placed in separate Eppendorf tubes, and frozen (-20°C). Feeders were then replaced, with the mass of both the new and used feeders being recorded. After four hours (24 hours since onset of exposure), filming of cages began under the two stimulation scenarios as described for the pre-exposure recordings. This protocol of removing any dead workers, replenishing feeders, and video recording cages, was repeated every 24 hours, with the last recording made 120 hours post onset of pesticide exposure. On day 6, workers were checked to classify as dead or alive, feeders were removed and weighed, and cages were frozen (-20°C). Dry mass was obtained for each frozen worker by placing in an oven at 80°C for 48 hours, with tag mass (standard weight of 0.003g after oven heat) subtracted when applicable.

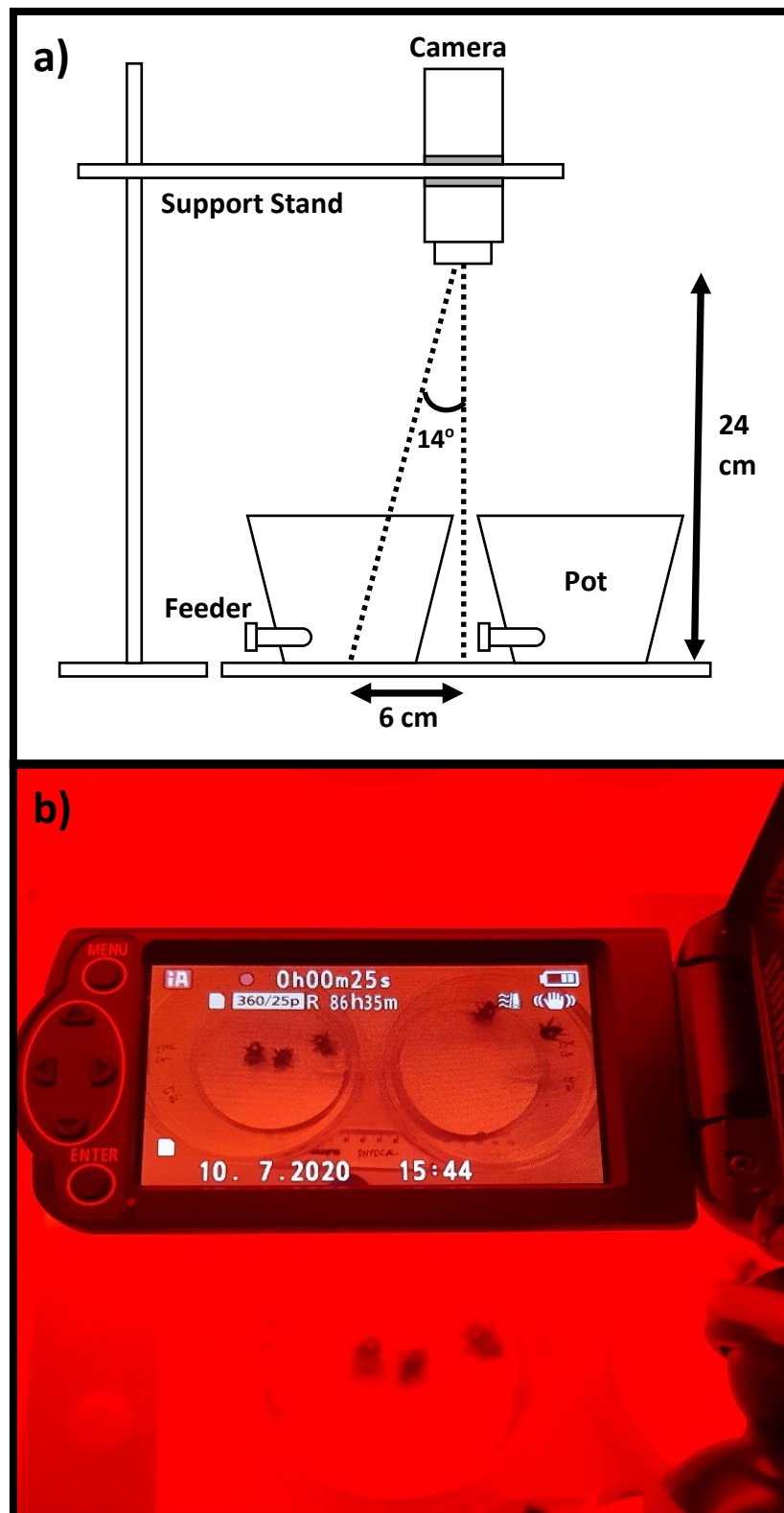


Figure 4.1. Experiment 2: a) diagram of camera setup and b) two pots could fit in frame whilst allowing clear view of each worker within each pot.

For both the 'no-stimulation' and 'light-stimulation' scenarios, an initial round of monitoring was carried out on the video recordings covering the first 96 hours of the trials. Videos were examined within the video tracking software Kinovea (version 0.8.15) and a set of key behaviours were recorded for each individual over a specific time window. For the no-stimulation scenario this time window was 20 seconds running from 4 - 24 seconds into each video, whereas for the light-stimulation it was 15 seconds running from 4 - 19 seconds into each video due to some videos being cut below 24 seconds duration through technical issues. Using these particular timeframes gave buffer zones of a few seconds duration at the start and end to account for any shaking of the video that could potentially occur from touching the camera to start and end the recording. During this time window, each individual was visually observed and assessed using the following ethogram:

- 1) Walking: movement of a leg to a new position accompanied by movement of the thorax. This movement has to be by the bee's own accord, for example if a bee only moves because another bee has knocked in to it, then no walking is recorded at that point.
- 2) Flight: rapid beating of wings followed by movement up the side of the pot (where legs are allowed to be touching the surface of the pot wall) or across the pot (where legs must be off the floor of the pot).
- 3) Feeding: proboscis extension towards the feeding hole in the Eppendorf feeder (proboscis extension easily identifiable as feeding hole was on upper side of Eppendorf feeder).
- 4) Movement: displaying any of walking, flight, or feeding behaviours.

Following this initial round of monitoring, a more in-depth round of 'walking tracking' was carried out for the 'no-stimulation' scenario on the video recordings covering the first 72 hours of the trials (N.B. walking tracking was not conducted for the 'light-stimulation' scenario due to the highly elevated flying rates; N.B. walking tracking not done at 96 hours due to time constraints). The initial monitoring round was carried out on original MP4 videos produced directly from video recordings, which had a rate of 25 frames per second (fps), whereas the walking tracking round was carried out on condensed 6.25 frames per second AVI videos that were produced using a bespoke MATLAB script (version R2020a). The higher quality 25 fps videos allowed easier detection of movement in the initial monitoring round, whereas the condensed 6.25 fps videos facilitated a dramatic reduction in time taken to track individual workers without suffering a significant loss in tracking accuracy (see Appendix C for support). Using the tracking functionality within the Kinovea software, each individual had its total distance walked (cm) through the arena assessed over the same 20-second time window used during the monitoring round outlined above (Figure 4.2; see Appendix C for full protocol). Tracking was paused when an individual was either not fully visible (i.e., when it had crawled under the filter paper), when it was flying, or when it was feeding, and the duration of these events was recorded for

each individual. A coordinate output text file was generated for each worker, from which a measure of walking distance (cm) was calculated by creating a SpatialLines object in R v3.6.2 (R Core Team, 2019) and using the SDraw package (McDonald & McDonald, 2020) to determine the objects length. Walking rate (cm/s) was calculated as the total walking distance (cm) divided by the duration the bee was visible (i.e., above the filter paper) and not flying or feeding (s). Walking speed (cm/s) was calculated as the total walking distance (cm) divided by the total duration the bee actually walked during the 20-second recording period (s). To calculate the duration an individual bee walked, its coordinate text file had all frames extracted in which the individual moved, classed as all the frames where there was a change in the tracking coordinates (in either the x or y plane) relative to the previous frame. As the duration of each frame was known to be 0.16 seconds, the duration each bee walked could thus be calculated by multiplying the number of frames by 0.16 seconds.

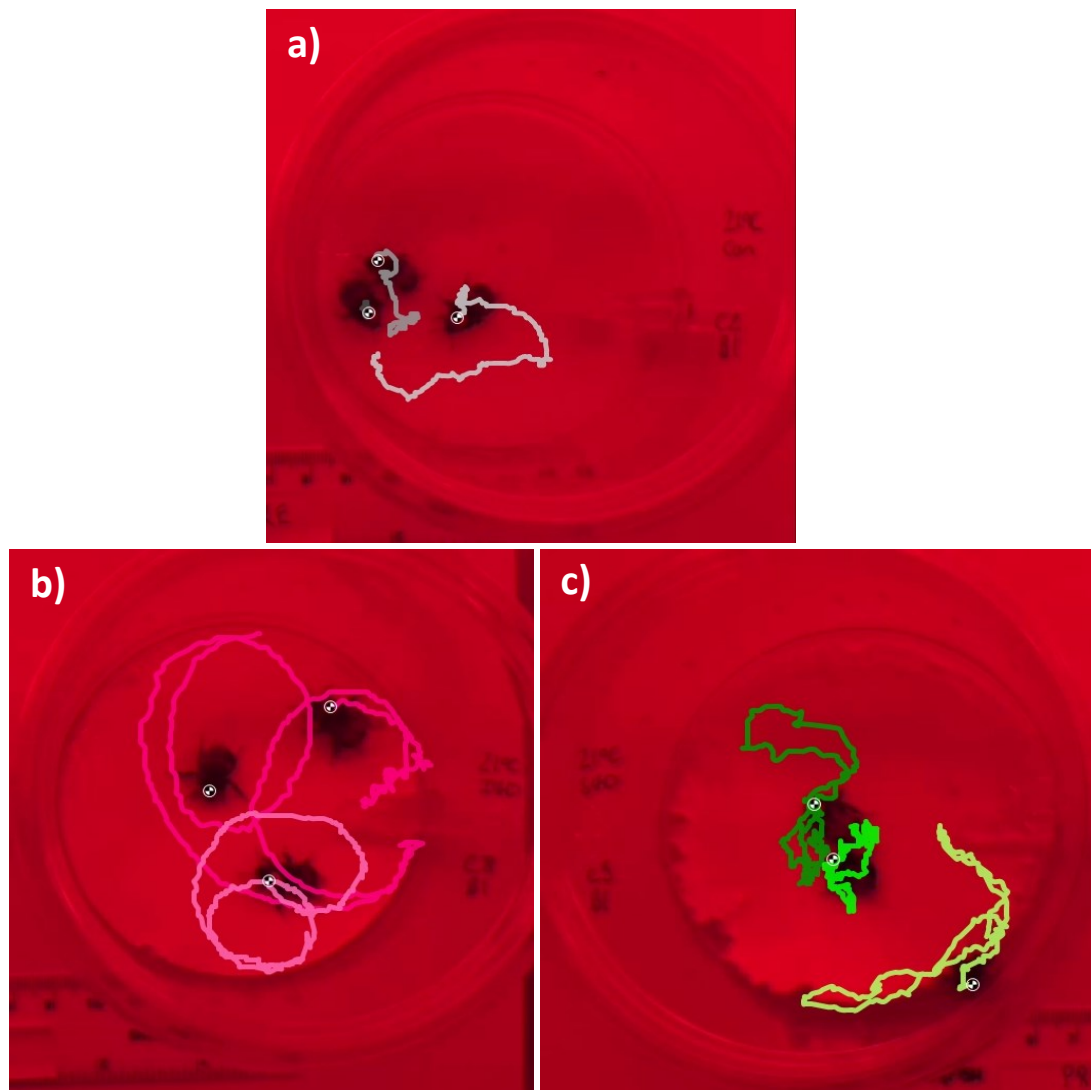


Figure 4.2. Experiment 2: Example walking paths for a set of three workers from a feeding cage in each of: a) Control, b) I40, and c) S40 treatments at the low temperature at the 24-hour time point.

4.2.6 | *Experiment 3: Flight distance & speed*

This experiment was run in two consecutive blocks, with each block following the same protocol and using three colonies each containing ~150 workers (total = 6 colonies). During the three days following colony arrival, 120-125 workers per colony were randomly removed, placed on ice to lower activity (Okubo et al., 2020), and a small circular galvanized iron tag (diameter = 2 mm) was attached using super glue to the centre of each worker's thorax (allowing later attachment to the flight mill magnet) following the protocol described in Chapters 2 & 3. Each tagged worker was then allowed to rest in a separate plastic pot for 60 minutes before being returned to their natal colony. The leading edge of the tag was placed so that it touched the back of the thoracic stripe, in order to ensure wing movement was unimpeded. For any substandard tag placement, the respective bee was removed from the experiment (< 2% of bees).

Workers remained in their natal colonies until being removed for flight tests. All workers were naïve fliers as they had not previously left the colony box. Flight testing was run in bouts, with 3 or 4 bouts run daily. Each bout consisted of six workers (two workers per colony) tested in parallel on individual flight mills using the apparatus described and justified in Chapter 3. Flight mills were setup in a separate CE 'testing' room to where colonies were housed, set at the respective experimental ambient temperature, and had the option of being under red or white light.

When removing workers from their natal colony for testing, each was assigned to an acute pesticide exposure (untreated, I10 or S10) and temperature (low, mid or high) combination, resulting in nine treatments with 60 workers tested per treatment pooled across both blocks (10 workers per colony). To ensure there was an equal distribution of worker body sizes across treatments, workers were assigned based on their wet mass directly following removal from the natal colony, resulting in no significant difference in dry mass mean (ANOVA: $p > 0.1$ for all pairwise comparisons) or variance (Bartlett's $K^2 = 6.31$, $df = 8$, $p = 0.61$) between treatments. No two workers from the same natal colony were assigned the same treatment when in the same bout, and each colony and each treatment were tested an equal number of times on each of the six different flight mills. Temperature treatments were tested on a daily rotational basis (i.e., Day 1 = low, Day 2 = mid, Day 3 = high, then repeat; see Table C2 for schedule of tested workers).

Prior to flight testing, each worker was placed inside a self-contained Perspex feeding tube with one end sealed using a mesh, as described in Chapter 3. Each worker was left to acclimatise for 2 minutes, immediately after which a droplet (minimum 100 mg) of the untreated or treated (I10 or S10) sucrose solution was presented on a cover slip on the outer side of the mesh allowing the bee to reach the droplet with its antennae and proboscis. Following the same protocol as Chapter 3, the

cover slip and droplet were presented for 5-minutes and were weighed immediately before and after this presentation period to calculate the mass of sucrose solution consumed. With workers varying in the mass consumed over the 5-minute period, I produced a 'mass-specific consumption' value per worker, calculated as the total mass of sucrose consumed (mg) per unit of body mass (mg of dry mass). Feeding trials were run in the same 21°C room that natal colonies were housed in, and any possibility of cross-contamination was eliminated by using feeding tubes exclusive to a treatment. Any bees observed not to feed within the 5-minute feeding period ($n=22$) or were visibly unhealthy or possessed damaged wings ($n=3$) were removed from further testing. Additionally, there were mill technical issues for six workers resulting in these also being excluded from the analyses (Table C3).

Once the feeding trial had ended, workers were removed from their respective feeding tubes, placed into separate plastic holding pots (120 mL), and transferred to the testing CE room set to the treatment temperature (total of 509 workers tested). Still under red light, each bee was left to rest inside the pot for 5 minutes and immediately after was removed and magnetically tethered to its respective flight mill. As previously described in Chapter 3, counterweights were used to balance the mill against the weight of the bee. Once tethered, the room was switched to white light and a support stand was used to hold the bee in place for a 10-minute period to allow metabolism of the ingested pesticide (Chapter 2; Suchail et al., 2004), after which the support stand was removed to stimulate flight. The flight trial protocol was identical to that described in Chapter 3, with the exception that trials were capped at a 90-minute time limit, at which point the trial was terminated. However, if a worker reached its third strike before this 90-minute point, then its flight trial was immediately terminated. The 90-minute cap was implemented because the results from Chapter 3, which included the three tested temperatures used in the current study, showed that > 90% of bees did not fly past this duration. The cap therefore improved experimental logistics by providing a known endpoint, without compromising the ability to address hypotheses. Once the flight trial was terminated, each bee was untethered from the mill, placed in a labelled Eppendorf tube, and frozen at -20°C. This was followed by a measure of dry mass by weighing the worker after oven warming at 80°C for 48 hours. Workers were weighed with their metal tag attached, which did not bias any of the body mass measurements as tags were of standardised mass (18 ± 0.1 mg).

Flight data was initially screened to check if any trial ran past 90 minutes, and if so the data was cut at the 90-minute point. Following the same process as was carried out for Chapter 3, flight data for all trials was then filtered to remove strike and orientation circuits along with buffer circuits either side of these (three preceding and five following circuits). All 509 bees tested on the flight mills initiated flight (classified as beating wings and rotating mill at least once). However, following the data filtering process there were 13 bees that had a 'filtered' flight distance of zero metres, which equated

to just 2.5% of all bees tested on the flight mills. The 496 bees that had a filtered flight distance > 0 m (termed 'positive fliers') were used in data analysis.

4.2.7 | Data analysis

All statistical analyses were conducted in R v3.6.2 (R Core Team, 2019).

4.2.7.1 | Experiment 1

The Cox proportional hazards model (Therneau, 2020) was used to conduct two separate event analyses, investigating the effect of pesticide, temperature, and associated interaction on the probability of i) death, and ii) responsiveness over time. For both event analyses, Firth's penalised maximum likelihood bias reduction method was implemented using the 'coxphf' package (Heinze et al., 2020), due to some treatment combinations having zero events throughout the 6-day trial. Natal colony was included as an additional covariate to account for inherent differences between colonies. Examination of Schoenfeld residuals showed that the assumption of proportional hazards was supported for all independent variables across both event analyses. Hazard ratios (ratio of the probability of the event occurring in the exposed versus control group), are reported for each pesticide-temperature combination. A hazard ratio of 1 indicates no detected association, whereas >1 indicates a pesticide is positively associated with the event probability. Kaplan-Meier curves were produced using the 'survminer' package (Kassambara et al., 2020) to show event probability over the 6-days.

To account for workers dying during the trial, for each pesticide-temperature treatment the mass of sucrose consumed (g) over each 48-hour period was divided by the number of bees that were alive at the end of the 48-hour period to get a proxy for mean sucrose consumption per bee (g / 48 hrs). Pooling data across temperatures, a linear mixed effects model was then used to examine consumption with respect to pesticide, whilst including pesticide-temperature combination as a random effect to account for repeated measures over time.

4.2.7.2 | Experiment 2

Statistical analyses were conducted using the "lme4" (Bates et al., 2015) package, with results reported using the package "lmerTest" (Kuznetsova et al., 2017) and pairwise contrasts using the Tukey method to account for multiple testing performed with the package "lsmeans" (Lenth, 2016). Diagnostic residual plots were examined using the "DHARMA" package (Hartig, 2021) to ensure models met all assumptions. Please see Appendix C for model selection process.

Likelihood of movement

The likelihood of movement for the no-stimulation and light-stimulation data were examined separately, fitting generalised linear mixed models under a binomial family distribution (moved = 1, didn't move = 0). Data was analysed for all live bees, with any bees not visible during the recording period (due to crawling under the filter paper) being excluded.

Models were fit with separate random intercepts, including bee ID to account for repeated measures of each individual over time, and feeding cage ID to account for inherent colony level differences as well as for non-independence of the response variable between workers in a given feeding cage (as one worker's behaviour can potentially influence the others). Colony ID was not retained as a random effect in the model to prevent overfitting and because the variance was close to zero. Models were fit for each pesticide examining the main effects of temperature, time, and the interaction between these two categorical variables, with dry body mass removed as it was a non-significant term and inclusion did not significantly improve model explanatory power. Modelling time as a categorical predictor allowed comparison of the likelihood of movement after each of 24, 48, 72, and 96 hrs against the pre-exposure likelihood, with the interaction with temperature allowing examination into whether the effect of pesticide differed between the three temperature treatments at each time point.

Walking Rate

For walking rate (cm/s), I fitted linear mixed effects models under a Gaussian distribution, with bee ID and feeding cage ID again included as random intercepts. Models were fit for each pesticide examining the main effects of temperature, time, and the interaction between these two categorical variables, with dry mass included as a continuous covariate. For each model the response variable of walking rate was transformed according to Box-Cox estimates calculated through the 'EnvStats' package (Millard, 2013), which resulted in a square root transform in all instances except when examining I40 for which the response variable was raised to the power 0.8. Visualisation of walking speed (cm/s) across treatments showed almost identical patterns to walking rate and so was not statistically analysed (Figure C4).

Sucrose Consumption

I calculated per capita consumption rate of sucrose solution per 24-hour period by taking the total mass of sucrose consumed divided by the number of bees alive in the respective feeding cage at the end of the respective 24-hour period. To examine the effects of treatments, I fitted a generalised linear mixed effects model under a Gaussian distribution, with the response variable square-root

transformed so that residual distributions met model assumptions. Consumption was examined with respect to the main categorical predictors of pesticide, temperature, and time, as well as pesticide x temperature and pesticide x time interactions. I included feeding cage ID as a random intercept, with the slope of the relationship between time and sucrose consumption allowed to vary between feeding cages to account for repeated measures over time. Colony ID was included as a random intercept to explicitly account for inherent colony level differences.

4.2.7.3 | Experiment 3

Flight Metrics

For each positive flier ($n = 496$), I calculated total distance (m) and duration (s) flown over the whole flight test. Visualising the rate of flight termination along with the velocity flown by workers over time (Figure 4.11), I found distinct patterns of flight over the course of the trials. Subsequently, mean and maximum flight velocity (m/s) were determined for each bee over four periods of flight: i) 0-100m; ii) 100-500m; iii) 500-1000m; and iv) 1000-2000m (see Appendix C for details).

Models

All statistical packages in R used in experiment 2 were also used here. For all analyses of flight distance (m), mean velocity (m/s), and maximum velocity (m/s), generalised linear mixed effects models were fit under a gamma family with log link function due to skewed error distributions. Analyses were performed on all 'positive' fliers (flew > 0 m after filtering) rather than just 'successful' fliers (flew > 100 m) as was done in Chapters 2 & 3. This decision was made because the temperatures tested in this study were decided upon based on the temperature-mediated foraging potential metric in Chapter 3, which is a metric that incorporated both the proportion of bees that 'successfully' flew and the mean flight distance of the bees that 'successfully' flew. To explore essentially the same metric here without splitting the data and running multiple analyses, the analysis in the current chapter was performed on all 'positive' fliers. However, to further explore the dynamics of flight, I ran analyses examining the likelihood of flying past two 'milestone' distances, 100m and 1000m, by fitting generalised linear mixed effects models under a binomial family distribution (flight surpassing milestone distance = 1, flight not reaching milestone distance = 0). All models were initially constructed considering the main effects of pesticide, temperature, dry body mass, mass-specific consumption and the interactions pesticide x temperature, pesticide x dry mass, temperature x dry mass, and pesticide x mass-specific consumption, with pesticide and temperature modelled as categorical variables and dry mass and mass-specific consumption modelled as continuous variables.

Additionally, colony ID was included as a random intercept to account for inherent colony level differences. Models were then simplified through stepwise removal of non-significant terms, checking after each removal that the simplified model had not significantly decreased in explanatory power.

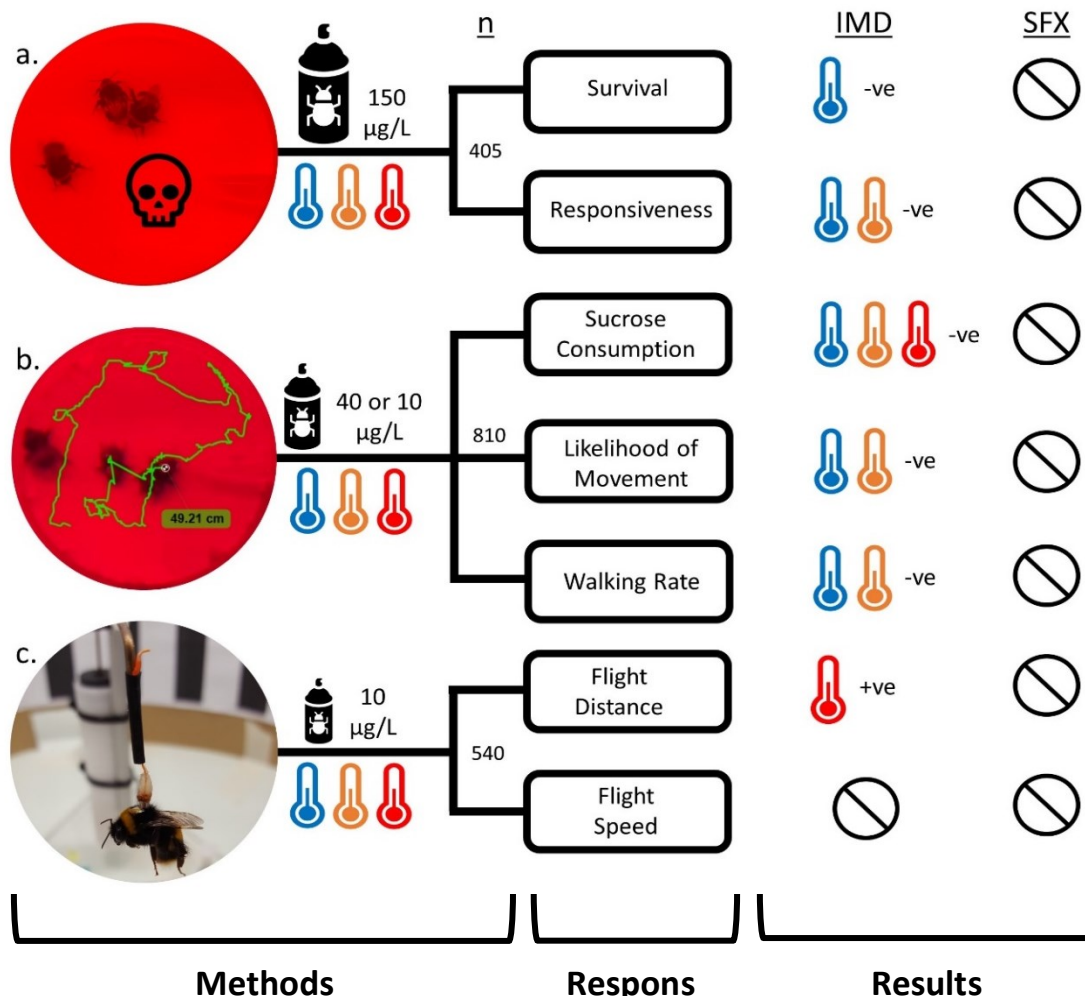


Figure 4.3. Overview of the setups, treatments, sample sizes, response variables measured and key results for the three reported experiments. Thermometers represent the three temperatures tested (blue = low[21]°C, orange = mid[27]°C, red = high[30]°C). If the insecticide (IMD = imidacloprid, SFX = Sulfoxaflor) did have an observed impact on the response variable, then we provide the temperatures at which there was an effect in the final two columns (e.g., blue thermometer = observed effect at low temperature), and we also indicate whether there was evidence of a negative (-ve) or positive (+ve) temperature coefficient (e.g., evidence of a significantly greater effect at the lower compared to higher temperatures). The  symbol indicates no effect of the insecticide on the response variable at any temperature. Experiment 1 (panel a) was conducted solely under red light, experiment 2 (panel b) had both a no-stimulation (red light) and light-stimulation (white light) scenario, and experiment 3 (c) had flight responses measured under white light. Note that the likelihood of movement results refer to the no-stimulation scenario rather than the light-stimulation scenario to allow comparison with walking rate results that were under a no-stimulation scenario.

4.3 | Results

4.3.1 | *Experiment 1: Survival & responsiveness*

4.3.1.1 | *Survival*

For non-exposed bees, temperature had a significant impact on survival, with the risk of death being identical at the mid compared to the low temperature (Hazard Ratio (HR) = 1, $p = 1$), but being around nine times higher at the high compared to the low and mid temperatures at any point in time (HR = 9.52, $p = 0.048$). However, when considering bees exposed to imidacloprid, the greatest risk of death was found in bees kept under the low temperature, where exposed bees were 14 times more likely to die compared to non-exposed bees (Figure 4.4 & 4.5). Whilst imidacloprid exposure led to a significantly increased risk of death at the low temperature (HR = 14.10, $p = 0.010$), the same effect was not seen at either the mid (HR = 5.18, $p = 0.22$) or the high (HR = 0.76, $p = 0.70$) temperature, with the risk of death from imidacloprid significantly greater (~19 times higher risk) at the low temperature compared to the high temperature (HR = 0.054, $p = 0.027$). Contrastingly, there was no detected effect of sulfoxaflor on the risk of death at any temperature (Figure 4.5).

4.3.1.2 | *Responsiveness*

Imidacloprid exposed bees were found to be 113 and 16 times more likely to become unresponsive (defined as dead or immobile) compared to controls at the low (HR = 112.54, $p < 0.001$) and mid (HR = 15.85, $p = 0.005$) temperatures respectively (Figure 4.4 & 4.5). However, I detected no significant effect of imidacloprid compared to controls at the high temperature (HR = 1.01, $p = 0.99$). Consequently, the risk of unresponsiveness from imidacloprid was significantly greater at the low (HR = 0.009, $p < 0.001$) and mid (HR = 0.064, $p = 0.033$) temperatures compared to the high temperature. As there was only one instance of a sulfoxaflor treated bee being classed as immobile, the responsiveness results mirrored those when examining survival, with no detected effect of sulfoxaflor on the risk of becoming unresponsive at any temperature (Figure 4.5).

4.3.1.3 | *Sucrose consumption*

Imidacloprid exposed workers appeared to continually feed less than control and sulfoxaflor treated workers across the duration of the trial at all temperatures (Figure 4.6a). When pooled across all temperatures and time points (to provide adequate sample size for statistical analysis), imidacloprid exposed workers were found to have fed significantly less relative to control workers (t

= -3.78, $p = 0.009$; Figure 4.6b). In contrast, sulfoxaflor exposed workers showed no detectable difference relative to control workers ($t = -0.08$, $p = 0.94$; Figure 4.6b).

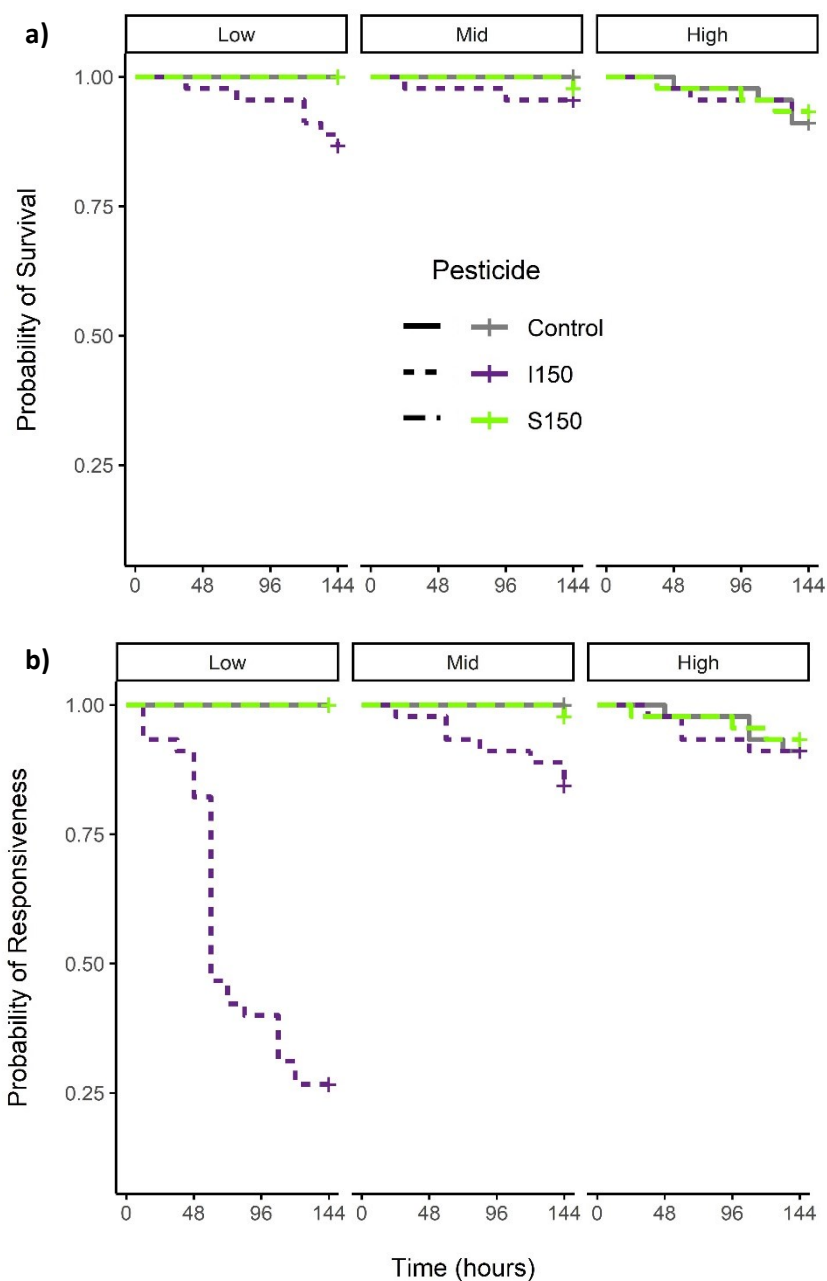


Figure 4.4. Experiment 1: Kaplan-Meier survival curves showing the probability of a) survival and b) responsiveness every 12 hours over the 144-hour trial period for each temperature-pesticide treatment. Note that in both panels the control and sulfoxaflor lines overlap for the majority of the plot at the low and mid temperature whilst there were no events. Confidence intervals are omitted to allow clear distinction between treatment lines. There were 15 feeding cages ($n = 45$ workers) examined in total per treatment.

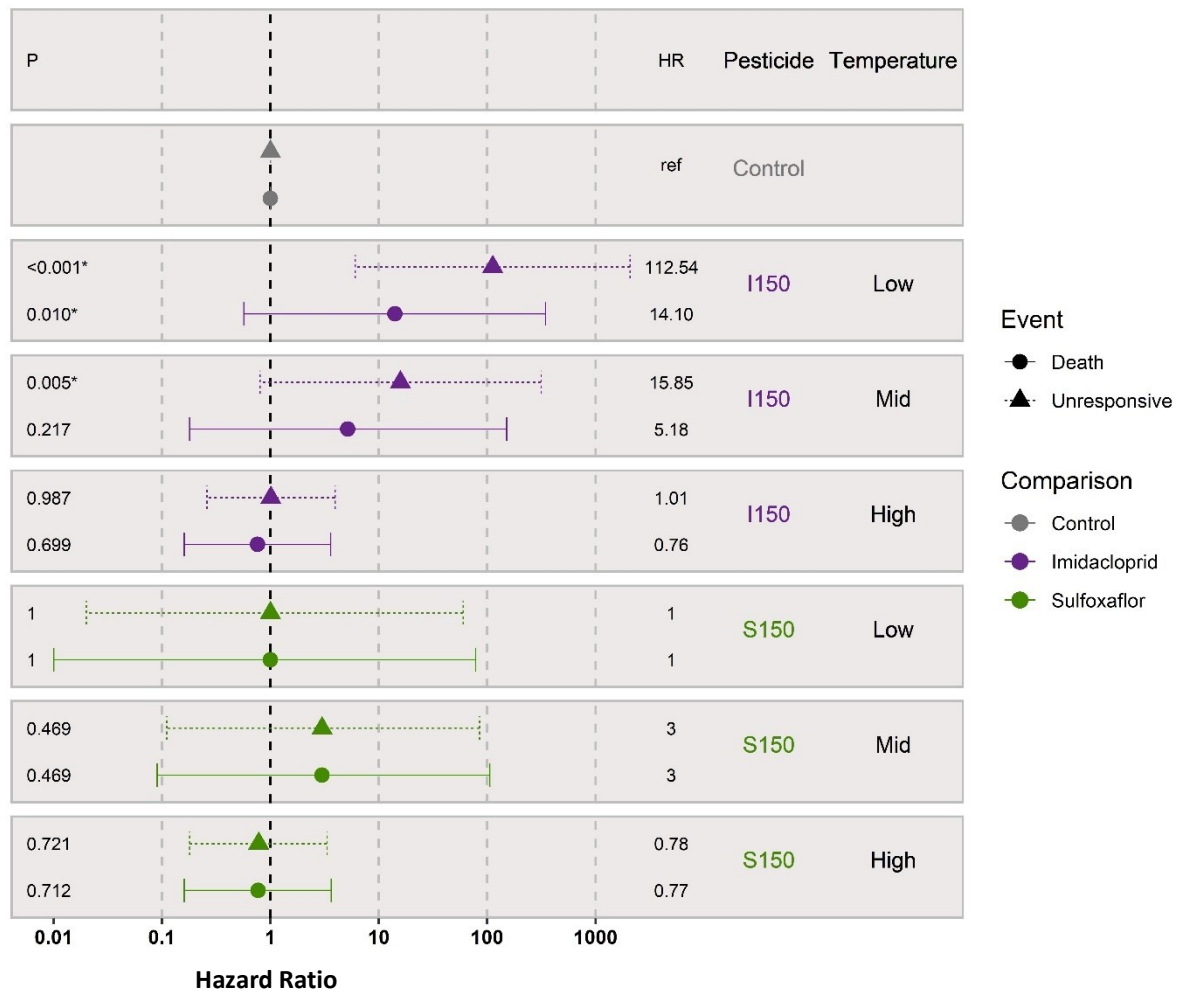


Figure 4.5. Experiment 1: Forest plot showing the hazard ratios (HR) of each pesticide at each of the three tested temperatures, when considering time to death and time to unresponsiveness. Dashed lines either side of points represent 95% confidence intervals.

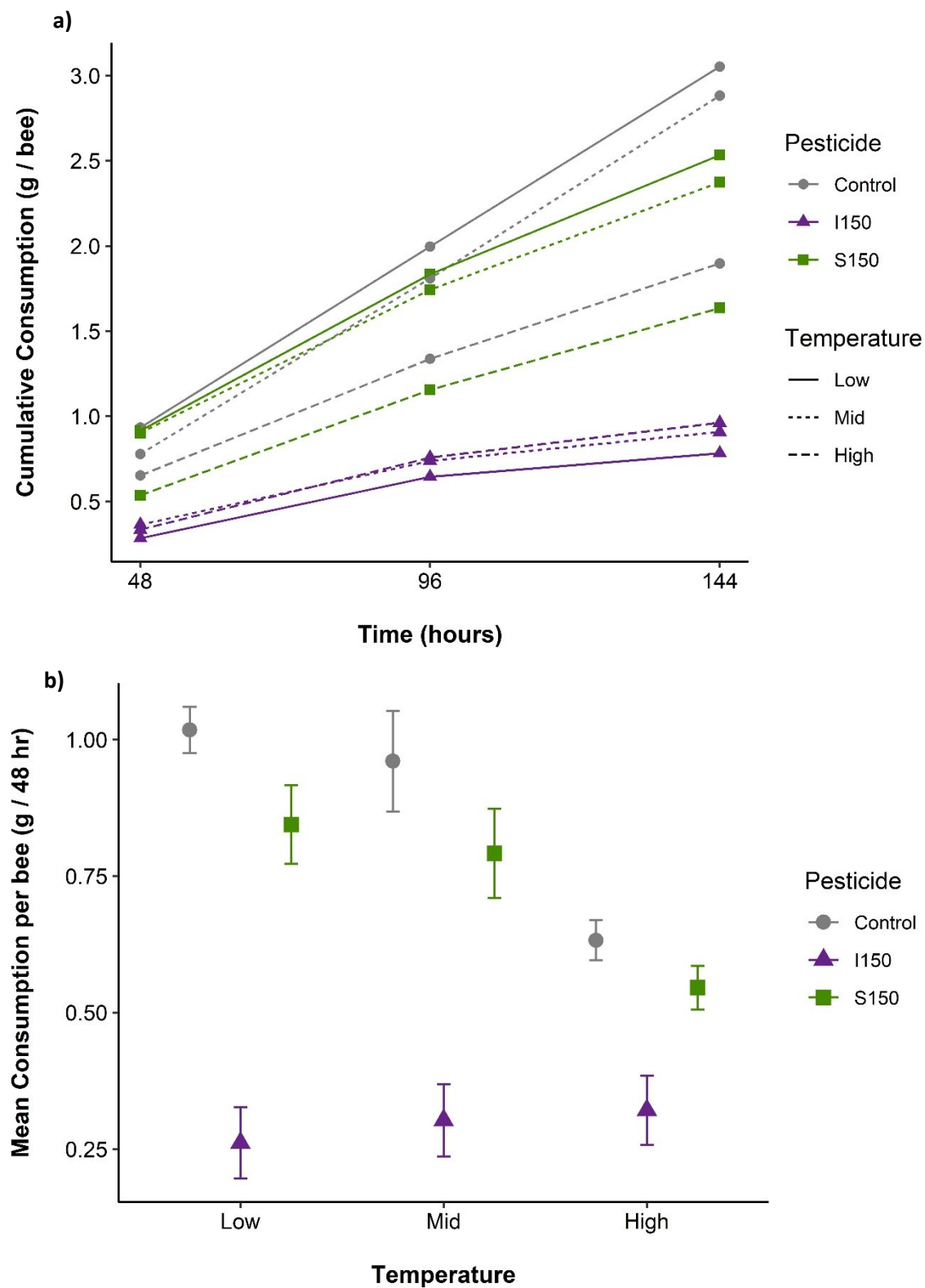


Figure 4.6. Experiment 1: Sucrose consumption per 48-hour period for each pesticide-temperature treatment: a) plotted cumulatively over the 144-hour period; and b) averaged over the 144-hour trial with associated standard error.

4.3.2 | *Experiment 2: Walking & consumption*

As expected, due to sublethal pesticide concentrations being tested, mortality was low in all pesticide treatments across all temperatures over the 6-day trial, with never more than three deaths out of 54 total workers per treatment (Table C4).

4.3.2.1 | *Likelihood of movement*

When pooled across all treatments and time points, the likelihood of movement in the no-stimulation and light-stimulation scenario was 69.1% and 68% respectively, and the likelihood of workers to initiate flight was 16.1% and 25.3% respectively.

Under the no-stimulation scenario (see Tables C5 to C9 for all results tables), workers exposed to I10 at the low temperature had a significantly reduced likelihood of movement after 72hrs (GLMER: $z = -3.38$, $p = 0.006$) and 96hrs ($z = -3.47$, $p = 0.005$) relative to pre-exposure responses (Figure 4.7a). However, there was no detectable effect of I10 on the likelihood of movement at the mid or high temperature at any time point ($p > 0.08$ in all cases). Workers exposed to I40 demonstrated a significantly reduced likelihood of movement at all time points at low and mid temperatures, except after 48hrs at the mid temperature ($z = -1.44$, $p = 0.60$), with the effect of I40 after 48hrs of exposure being significantly greater at the low relative to the mid temperature ($z = 2.07$, $p = 0.038$). At the high temperature, a reduced likelihood of movement was not observed until 96hrs after exposure ($z = -2.98$, $p = 0.024$), with the effect of I40 at the low temperature significantly more severe after both 24hrs ($z = 2.16$, $p = 0.031$) and 48hrs ($z = 2.31$, $p = 0.021$) of exposure relative to the high temperature.

Under the light-stimulation scenario (see Tables C10 to C14 for all results tables), workers exposed to I10 at the low and mid temperatures had a significantly reduced likelihood of movement after 72hrs (low: $z = -4.04$, $p < 0.001$; mid: $z = -3.15$, $p = 0.016$) and 96hrs (low: $z = -4.14$, $p < 0.001$; mid: $z = -3.72$, $p = 0.002$) relative to pre-exposure responses (Figure 4.7c). Workers exposed to I10 at the high temperature had a significantly reduced likelihood of movement only after 96hrs of exposure ($z = -2.95$, $p = 0.027$), with the effect of I10 after 72hrs of exposure being significantly greater at the low relative to the high temperature ($z = 2.37$, $p = 0.018$). Workers exposed to I40 demonstrated a significantly reduced likelihood of movement at all time points at all temperatures, except after 24hrs at the mid temperature ($z = -2.49$, $p = 0.09$), with no detectable significant difference in the effect of I40 across the temperature range ($p > 0.06$ in all cases).

Considering both the no-stimulation and light-stimulation scenarios, there were no detectable effects of either S10 or S40 on the likelihood of movement at any temperature ($p > 0.17$ in all cases; Figure 4.7a,c).

4.3.2.2 | *Walking rate*

After 24 hrs, workers from the I10 treatment at the low temperature significantly increased their walking rate compared to pre-exposure levels (LMER: $z = 2.83$, $p = 0.026$), with this effect lessening in magnitude as the experiment progressed (Figure 4.7b). However, workers exposed to I10 at mid and high temperatures showed no detectable change in walking rate over the course of the trials ($p > 0.58$ in all cases). After 24 hrs, workers from the I40 treatment significantly increased their walking rate compared to pre-exposure levels at low (LMER: $z = 3.73$, $p = 0.001$) and mid ($z = 3.03$, $p = 0.014$) temperatures, with this effect again lessening in magnitude as the experiment progressed (Figure 4.7b). In contrast, I40 workers at the high temperature showed no detectable change in walking rate over the course of the trials ($p > 0.57$ in all cases). Accordingly, the effect of I40 on walking rate after 24hrs was significantly greater at the low relative to the high temperature ($t = -2.06$, $p = 0.040$). There was no detectable effect of S10 on walking rate at any temperature ($p > 0.36$ in all cases), but S40 workers showed a significantly increased walking rate after 24 hours of exposure (LMER: $z = 3.38$, $p = 0.005$), with this effect then being lost after further exposure (Figure 4.7b; see Tables C15 to C19 for all results tables).

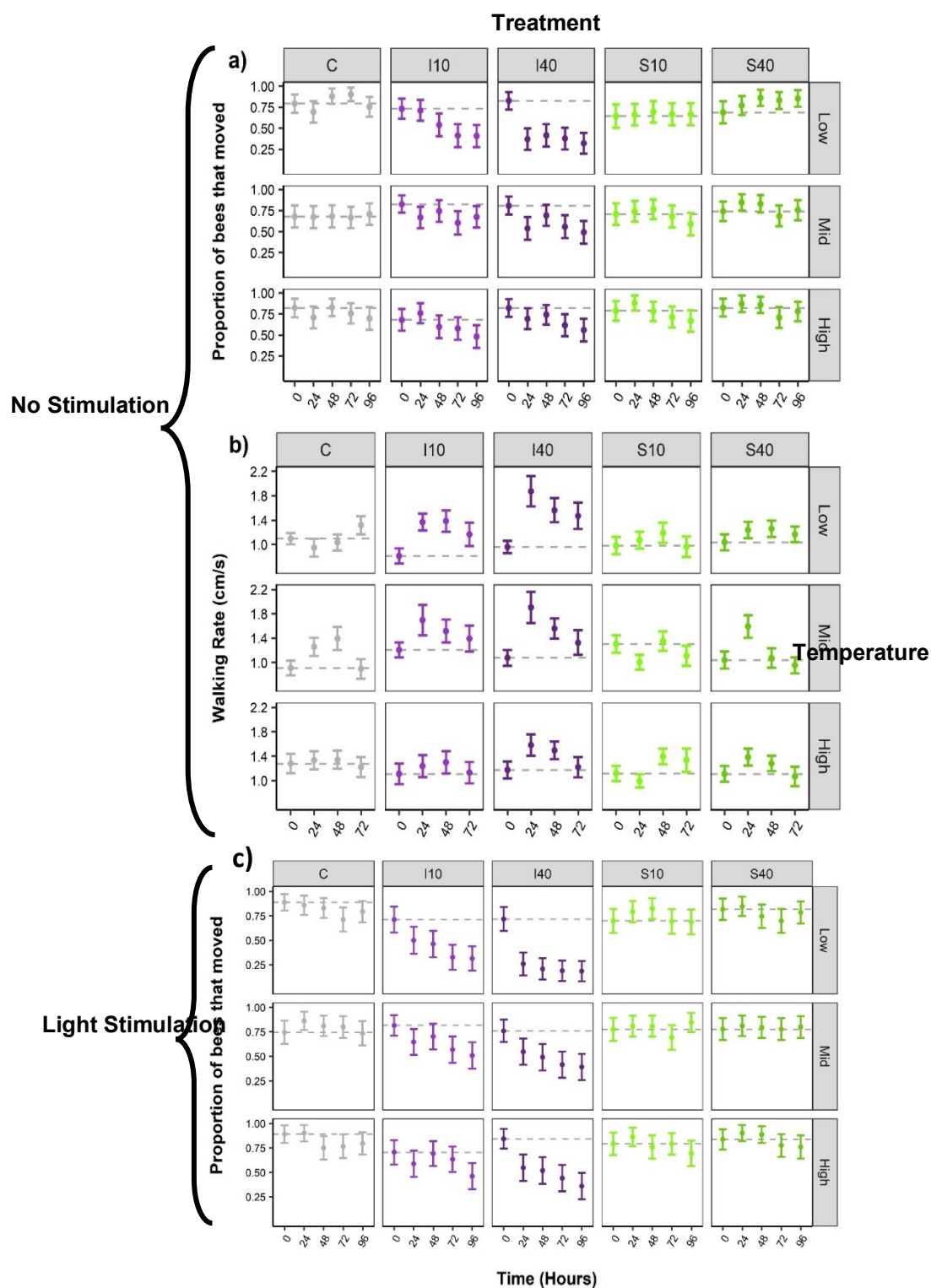


Figure 4.7. Experiment 2: Movement results showing a) the proportion of bees that moved at each 24-hour monitoring period over the 96-hour trial period in the no-stimulation scenario; b) walking rate (cm/s) of bees that initiated walking at each tracking period over the 72-hour trial period in the no-stimulation scenario; and c) the proportion of bees that moved at each 24-hour monitoring period over the 96-hour trial period in the light stimulation scenario. In panels a) & c), error bars represent 95% confidence limits, and in panel b) error bars represent standard error. For all panels, the grey dashed line represents the response value for the pre-exposure (time = 0 hours) recordings, for easy comparison across the time periods.

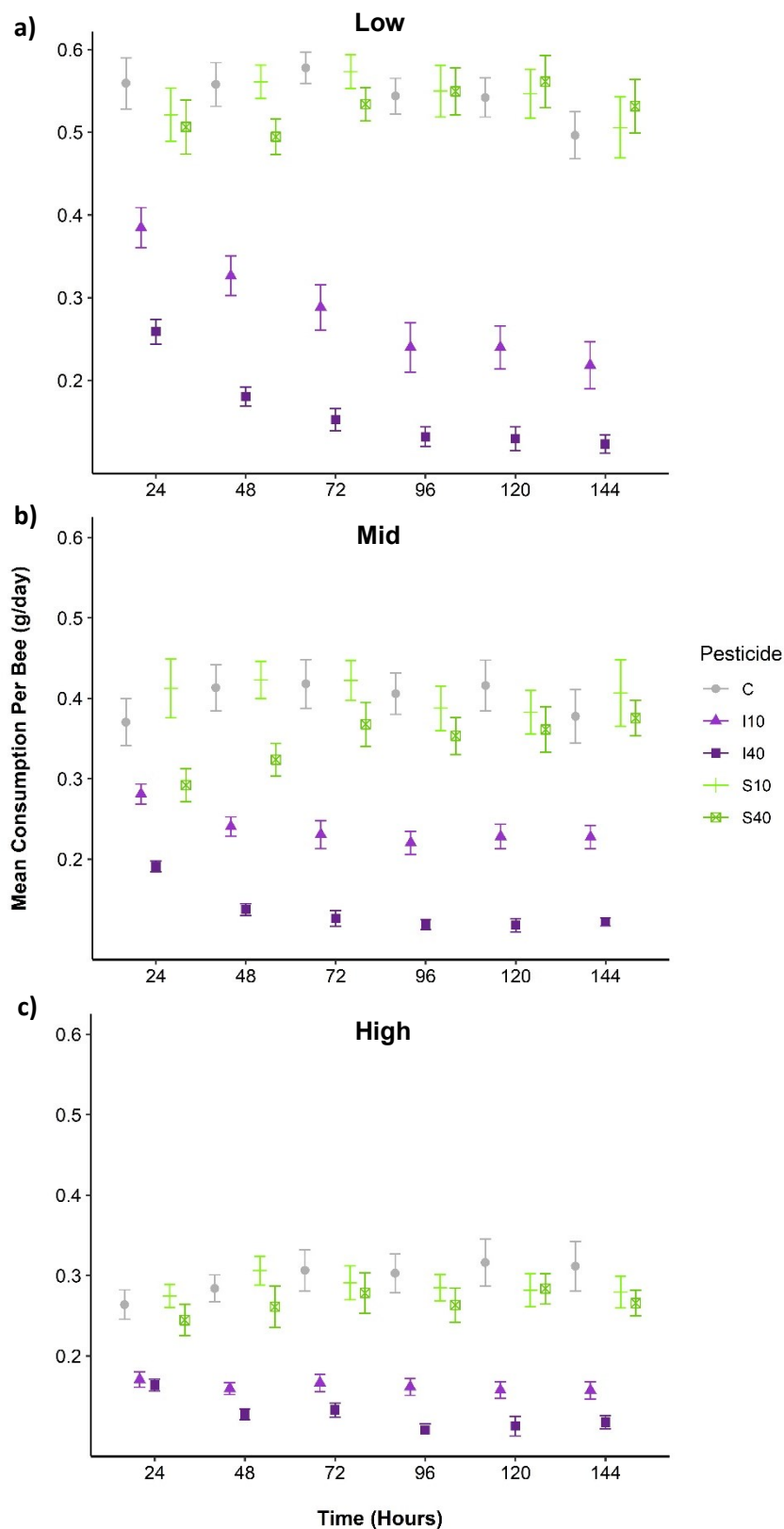


Figure 4.8. Experiment 2: Per capita consumption rate of sucrose solution per 24-hour period for each pesticide treatment at each of a) Low; b) Mid; and c) High temperature. For each feeding cage, the total mass of sucrose consumed was divided by the number of bees alive in the cage at the end of the respective 24-hour period (to account for dead bees), and then the mean was taken across all feeding cages. Error bars represent standard error around the mean.

4.3.2.3 | *Sucrose consumption*

When pooled over all pesticide treatments, sucrose consumption decreased as temperatures got warmer ($p < 0.001$ for all contrasts; Table C20). Bees exposed to I10 or I40 fed significantly less relative to control bees at all temperatures ($p < 0.001$ in all cases; Figure 4.8). However, the difference in consumption between control workers and imidacloprid exposed workers was significantly greater at the low temperature relative to mid (LMER: I10: $t = 2.26$, $p = 0.025$ & I40: $t = 2.78$, $p = 0.006$) and high (I10: $t = 2.41$, $p = 0.017$ & I40: $t = 5.77$, $p < 0.001$) temperatures.

There was no detectable effect of time on mean consumption for control bees when pooled across all temperatures (LMER: $t = -0.05$, $p = 0.96$). However, whilst control workers seemed to feed consistently over the course of the trials, the consumption rate of imidacloprid exposed bees decreased (Figure 4.8), and the slope of the time vs consumption relationship significantly differed between control and imidacloprid exposed bees (I10: $t = -3.88$, $p < 0.001$; I40: $t = -5.00$, $p < 0.001$). Conversely, whilst there was no detectable difference in sucrose consumption between control and sulfoxaflor exposed bees at any temperature ($p > 0.13$ in all cases), the slope of the time vs consumption relationship did significantly differ between control and S40 workers (LMER: $t = 2.20$, $p = 0.029$), with exposed bees feeding more over time relative to controls (Figure 4.8).

4.3.3 | *Experiment 3: Flight distance & speed*

4.3.3.1 | *Flight distance*

Flight distance at the low temperature for control bees was a mean ($\pm$ s.e.m.) of 1368.2 ± 189.6 m (Figure 4.10), with imidacloprid and sulfoxaflor treated bees exhibiting a non-significant reduction of 14.9% (GLMER: $z = -0.54$, $p = 0.852$) and 8.6% ($z = -0.58$, $p = 0.832$), respectively (Table C21). Similarly, mid temperature control bees flew a mean ($\pm$ s.e.m.) distance of 1322.7 ± 202.9 m, with imidacloprid treated bees exhibiting a non-significant 5.6% reduction in flight distance ($z = -0.53$, $p = 0.859$) and sulfoxaflor a non-significant 0.7% increase ($z = 0.12$, $p = 0.992$). At the high temperature, however, imidacloprid treated bees showed a dramatic and significant 53.2% reduction in flight distance (GLMER: $z = -3.07$, $p = 0.006$), and sulfoxaflor showed a larger (than previous), although non-significant, 24.2% reduction ($z = -1.42$, $p = 0.326$) relative to control bees that flew 1503.8 ± 192.2 m. Accordingly, the negative effect of imidacloprid was greater at the high relative to the mid temperature ($t = -2.52$, $p = 0.012$). When data was pooled across all treatments, dry mass was a significant positive predictor of flight distance ($t = 3.23$, $p = 0.001$), and this effect was consistent across all temperatures and pesticides (Figure 4.9; Table C21), whereas mass-specific consumption had no

detectable significant effect ($t = 1.68$, $p = 0.094$). Flight duration was not statistically analysed as visualisation of the data showed almost identical patterns to flight distance (Figure C5).

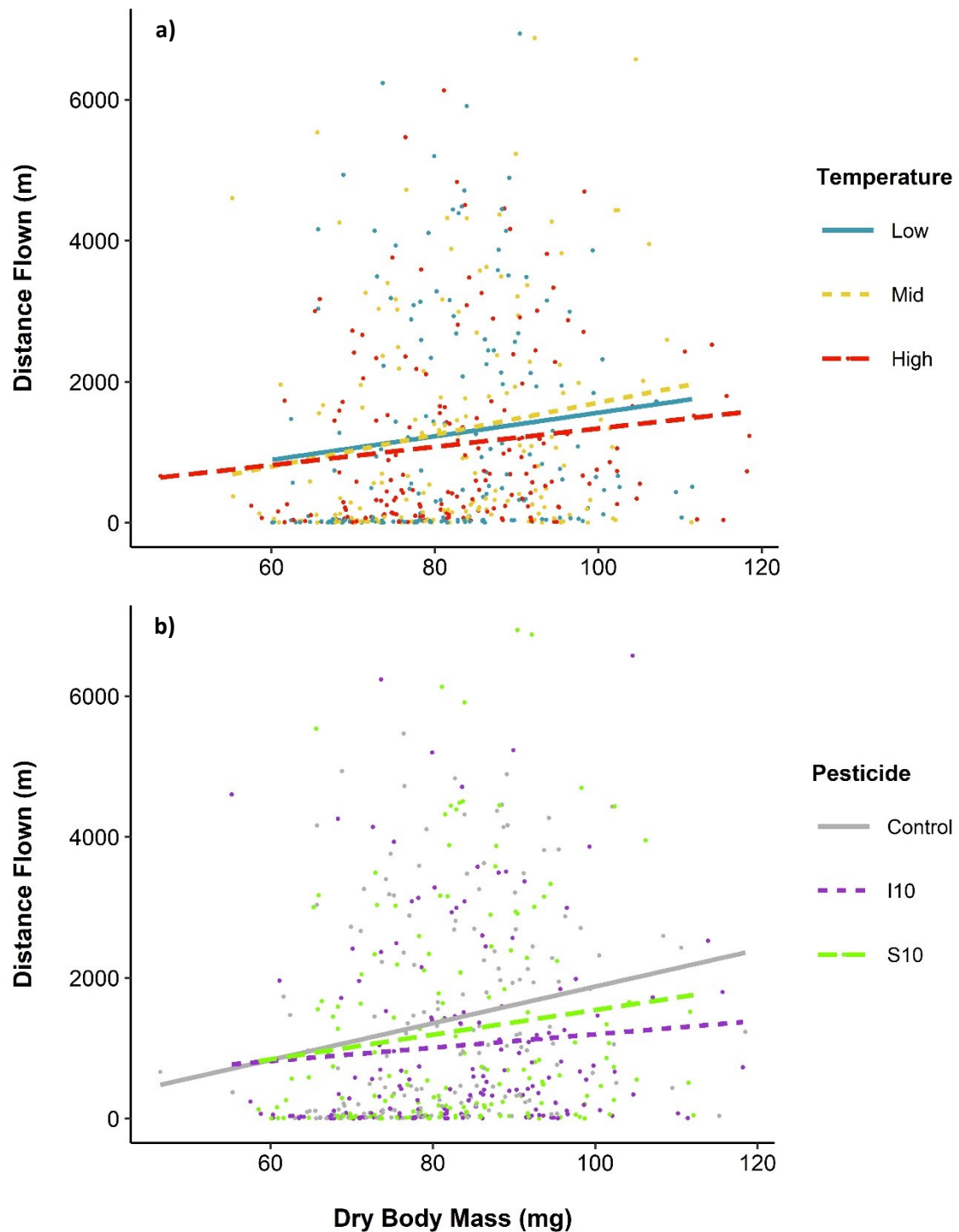


Figure 4.9. Experiment 3: Relationship between dry body mass (mg) and total distance flown (m) for all ‘positive’ fliers (i.e., had filtered flight distance > 0 m), split by a) temperature and b) pesticide, with associated standard error.

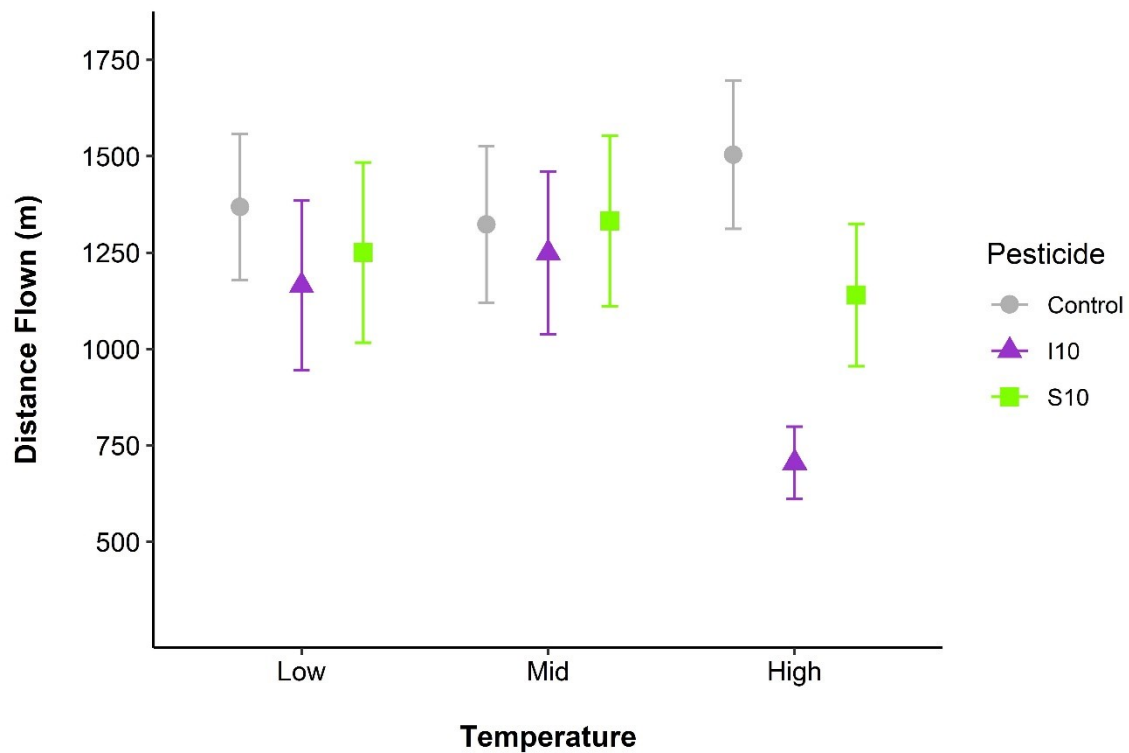


Figure 4.10. Experiment 3: Mean flight distance (m) for each temperature-pesticide treatment, considering all workers that were classed as positive fliers (i.e., had filtered flight distance > 0 m), with associated standard error.

4.3.3.2 | *Flight speed*

There was no detectable effect of imidacloprid or sulfoxaflor on either mean or maximum velocity (Figure 4.12), and this remained consistent over all three temperatures and all four periods of flight (see Tables C22 to C29). When data was pooled across all treatments, dry mass was a significant positive predictor of both mean velocity (GLMER: 0-100m: $t = 3.68$, $p < 0.001$; 100-500m: $t = 2.42$, $p = 0.016$) and maximum velocity (0-100m: $t = 2.89$, $p = 0.004$; 100-500m: $t = 2.22$, $p = 0.028$) over the first 500m of flight, whereas there was no detectable significant effect of mass-specific consumption over any period of flight (see Tables C22 to C29).

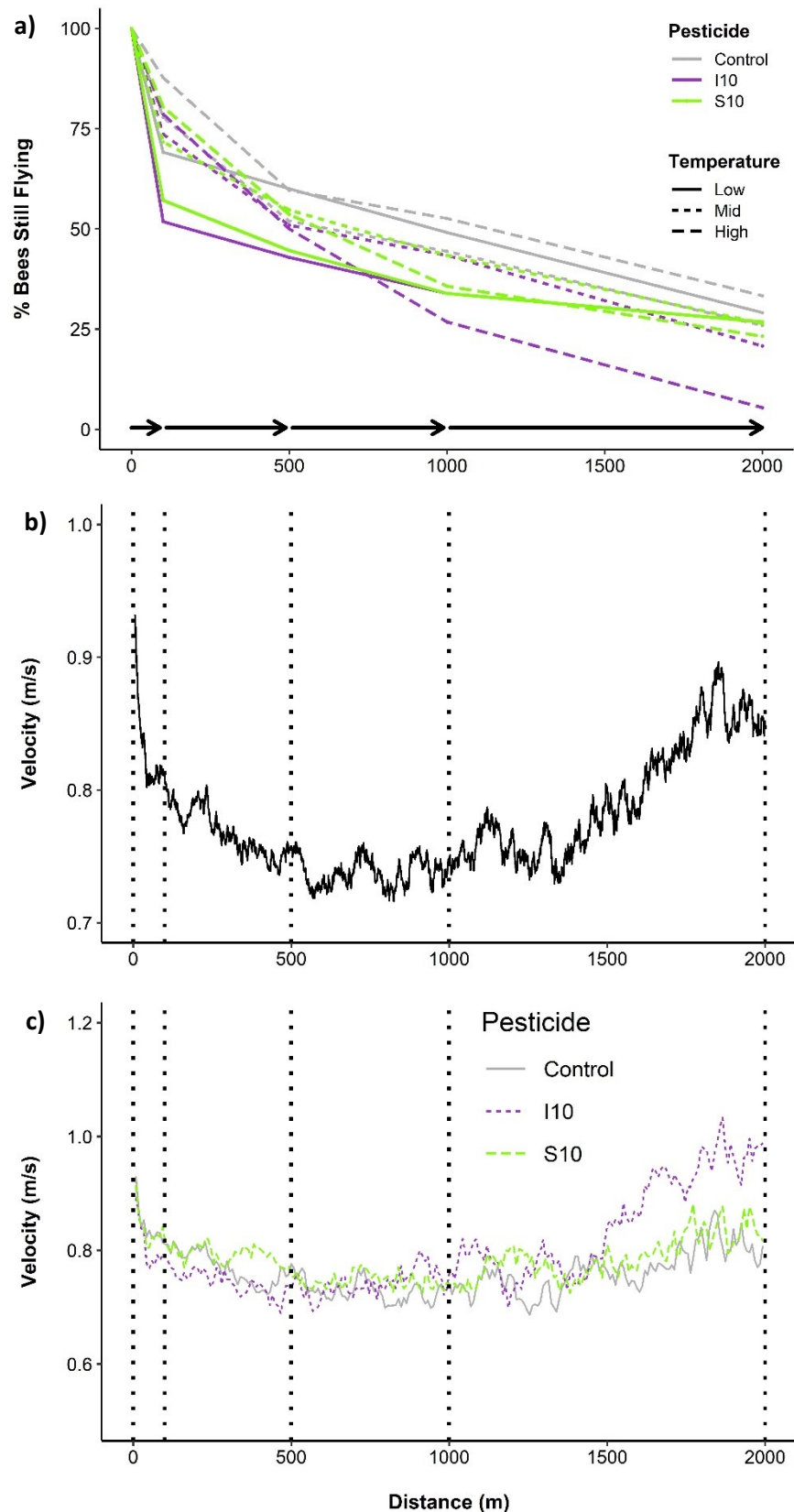


Figure 4.11. Experiment 3: Flight dynamics over time, with horizontal dashed black lines delineating four distinct periods of flight (0-100 m, 100-500 m, 500-1000 m, 1000-2000 m). Panels show: a) percentage of bees still flying after each period of flight; and mean flight velocity (m/s) averaged over b) all positive fliers pooled across treatments and c) all positive fliers split by pesticide treatment.

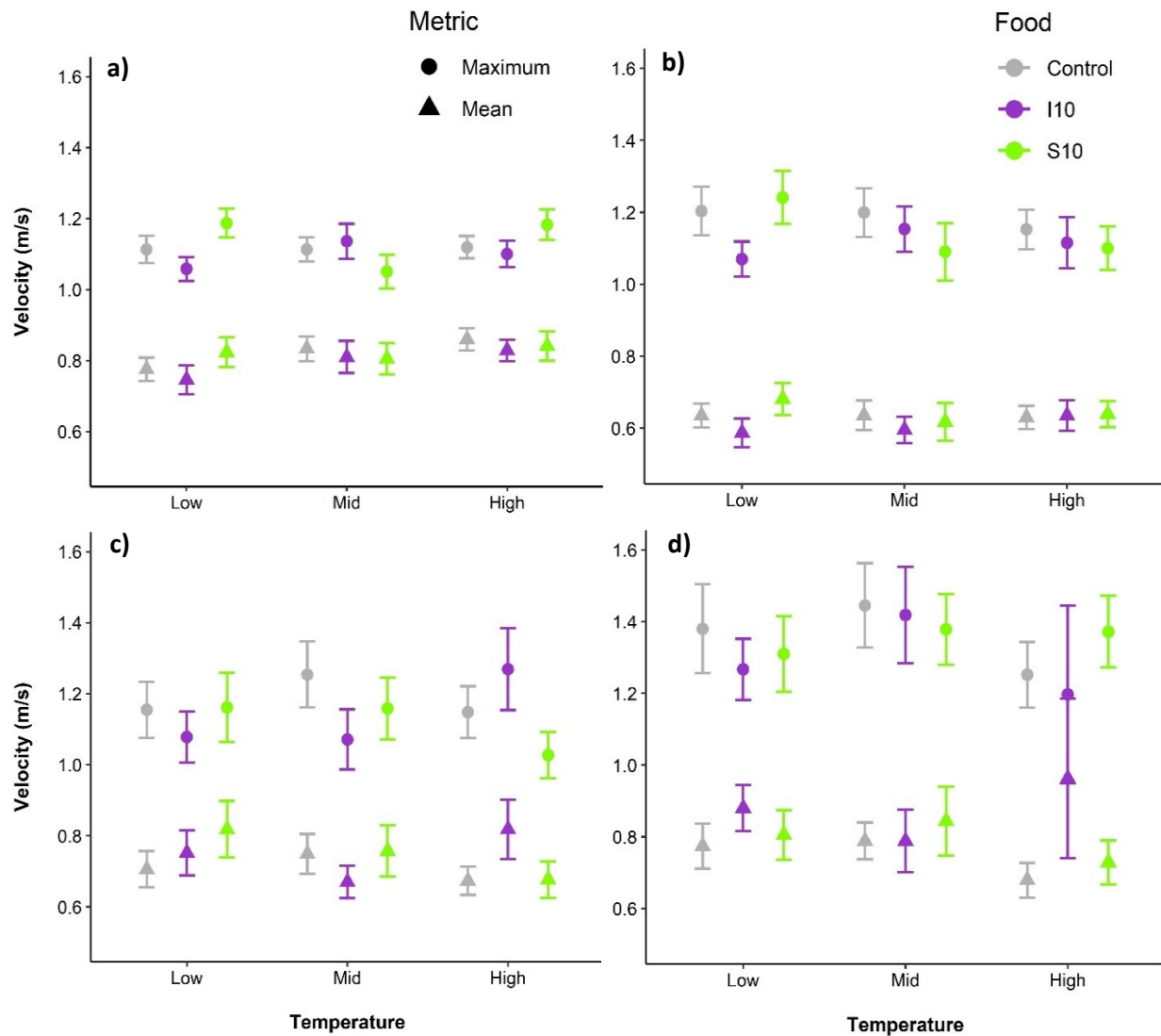


Figure 4.12. Experiment 3: Mean and max velocity (m/s) flown by workers for each temperature-pesticide treatment over four distinct periods of flight: a) 0 – 100 m; b) 100 – 500 m; c) 500 – 1000 m; and d) 1000 – 2000 m. Points are means with standard error bars added.

4.3.3.3 | Likelihood of reaching milestones

The likelihood of reaching the 100 m milestone did not differ between control and imidacloprid treated bees across any of the temperatures (Figure 4.13), which was also found when looking at the ability to reach 1,000 m at low and mid temperatures (Table C30 & C31). However, when at the high temperature, imidacloprid did significantly reduce the likelihood of reaching 1,000m relative to controls (GLMER: $z = -2.93$, $p = 0.009$). Accordingly, the negative effect of imidacloprid at the high temperature was significantly greater than at the mid temperature ($z = -2.02$, $p = 0.043$). There was no detectable effect of sulfoxaflor on the likelihood of reaching either milestone distance across any temperature (Table C30 & C31).

Dry mass was a significant positive predictor of likelihood to reach the 100 m milestone when all data was pooled (GLMER: $z = 3.25$, $p = 0.001$), with this effect being consistent across the pesticide treatments (Figure 4.13). However, whilst dry mass was still a significant positive predictor of likelihood to reach the 1,000 m milestone when all data was pooled ($z = 3.77$, $p < 0.001$), the slope of the dry mass vs likelihood of flight relationship was significantly less positive for both the imidacloprid ($z = -2.22$, $p = 0.027$) and sulfoxaflor ($z = -2.12$, $p = 0.034$) treatments relative to controls (Figure 4.13). There was a positive, but non-significant, effect of mass-specific consumption on the likelihood of reaching 100 m (GLMER: $z = 1.48$, $p = 0.138$) and 1,000 m (GLMER: $z = 1.86$, $p = 0.063$) milestones.

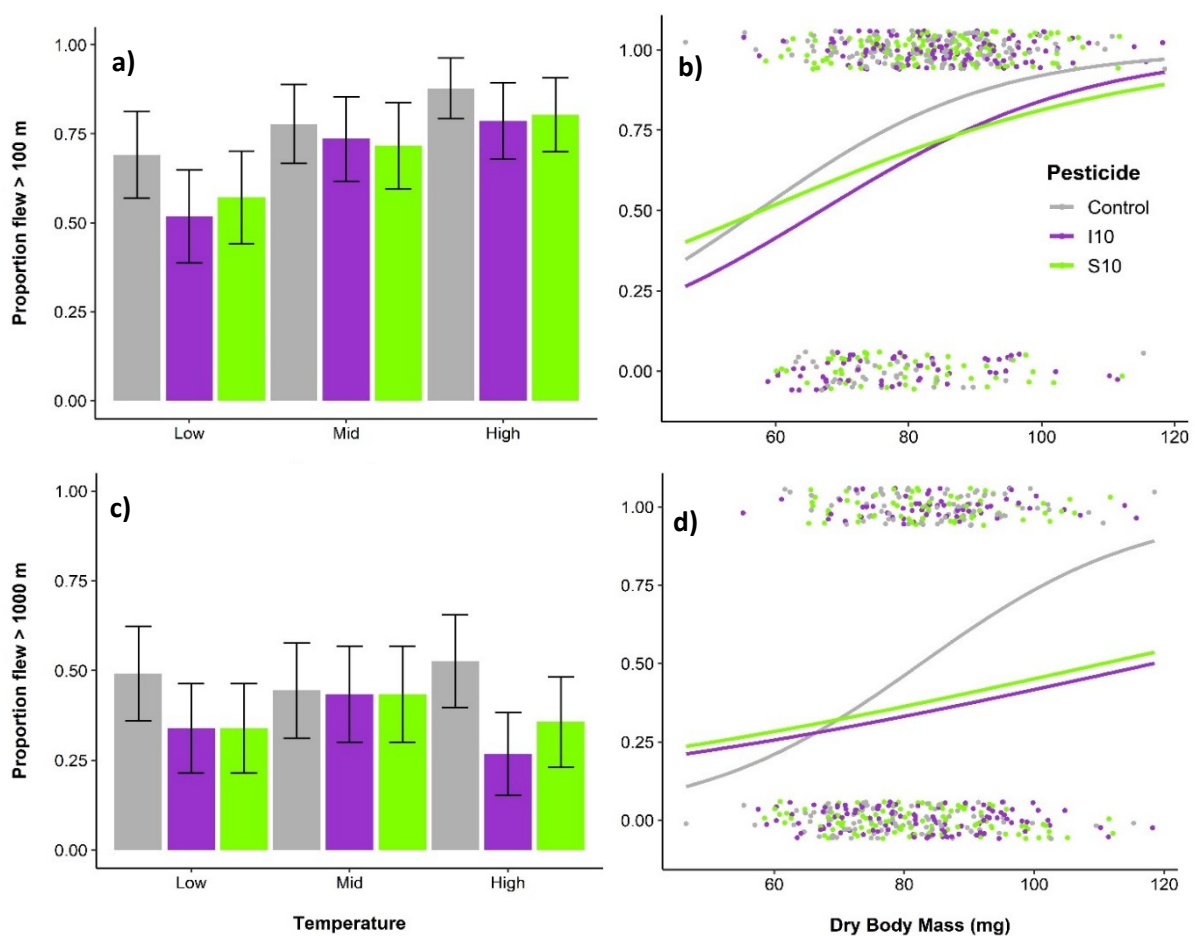


Figure 4.13. Experiment 3: Likelihood of flight over 100 m and 1000 m milestones. For each of the temperature-pesticide treatments, panels a) and c) show the proportion of bees (with associated 95% CI) that flew over the 100 m and 1000 m milestone respectively. Panels b) and d) show logistic regression plots (flew under the milestone = 0, flew over the milestone = 1) examining the effect of dry body mass (mg) on the likelihood of flight over the 100 m and 1000 m milestone respectively, with data split by pesticide treatment. In panels b) and d) jitter has been added in the y-plane and confidence intervals have not been plotted around model lines to aid visualization.

4.4 | Discussion

Here I quantified the degree to which lethal and sublethal responses from oral exposure to either a neonicotinoid or sulfoximine insecticide are temperature dependent. My results reveal that temperature can modulate the impact of insecticide exposure on multiple functional behaviours of a key insect pollinator (Figure 4.3). Specifically, it was the toxicity of the neonicotinoid imidacloprid that was strongly influenced by temperature, but whilst a negative temperature coefficient (TC) was detected for responsiveness and walking and consumption rates, contrastingly a positive TC was found for flight performance. This highlights the importance of considering a set of distinct sublethal behaviours when assessing insecticide risk. Comparatively, I found little effect of sulfoxaflor on sublethal behaviour, indicating that over the tested temperature range a sulfoximine could be a preferable option if similar concentrations used in the study could provide effective pest control. Perhaps most importantly, my findings support that just a small temperature change (as little as 3°C) towards the higher end of a bees tolerance range can induce relatively dramatic changes to insect pollinator behaviour from pesticide exposure, which is especially pertinent given current climate predictions suggest average temperatures are likely to rise above 2.5°C over the next century (IPCC, 2018).

4.4.1 | *Responsiveness & mobility*

In experiments 1 & 2, the response to imidacloprid exposure at every concentration was strongly temperature dependent – showing a negative TC. Exposure to I150 presented a greater risk to survival and responsiveness at the low compared to the high temperature, and just a 3°C drop from the high to mid temperature also led to a significant reduction in responsiveness for imidacloprid exposed bees. Additionally, exposure to I10 led to a reduced likelihood of movement only at the low temperature, and the effects of I40 on likelihood of movement were more severe and quicker to manifest at the low and mid temperature relative to the high temperature. Interestingly, chronic neonicotinoid ingestion has been shown to affect honey bee locomotor responses to light (phototaxis), and I observed that the impacts of I40 and I10 were similar when bees were provided with a light stimulus compared to when they were provided with no stimulus, indicating that even when presented with a natural light cue, exposed workers in colonies may still demonstrate abnormal responses and lower task motivation in certain thermal environments (Tosi & Nieh, 2017). These findings highlight the importance of considering ambient temperature when aiming to quantify the risks posed to pollinators by a given pesticide. For example, if I had followed standard toxicology guidelines and only tested between 25-30°C, I would have wrongly concluded that I150 has no effect on bumblebee survival and that I10 has no effect on mobility (EFSA, 2013; Nauen et al., 2001).

Furthermore, studies predominantly centred around lethality tests on insect pests have generally designated neonicotinoids as positive TC insecticides (Mansoor et al., 2015; Tsaganou et al., 2021). My findings suggest that conclusions drawn from such studies cannot be directly applied to insect pollinators and demonstrate the need to assess sublethal behavioural endpoints in addition to lethal ones as temperature modulation may vary across responses. Studying behaviours that represent a larger spectrum in response, rather than a binary dead or alive, and which underpin colony fitness and ecosystem service provision, is important if we are to understand and quantify impairment tipping points particularly when considering interacting multi-stressor impacts.

Intriguingly, whilst chronic exposure to I10 and I40 led to less bees initiating movement in experiment 2, those bees that did move walked at a faster rate following exposure, and again this effect was temperature dependent. Increased walking speeds were observed in I40 exposed workers at both the low and mid temperature, but not the high temperature, and it was only the low temperature at which I10 elicited faster walking rates. Neonicotinoids are agonists of insect nicotinic acetylcholine receptors and can therefore stimulate neuronal activity (Moffat et al., 2016; Tomizawa & Casida, 2005), potentially leading to hyperactive behavioural responses (e.g., Alkassab & Kirchner, 2018; Tosi, Burgio, et al., 2017). However, continued exposure can lead to neuronal dysfunction and trigger oxidative stress (Martelli et al., 2020; Palmer et al., 2013), cause mitochondrial damage that perturbs energy metabolism (Lu et al., 2020; Moffat et al., 2015; Sargent et al., 2021), and alter the regulation of genes involved in muscle function (Manzi et al., 2020; Wu et al., 2017). This could have direct consequences for muscular activity and ultimately lead to the decreased responsiveness that I observed (Almeida et al., 2021; Williamson et al., 2014). My results of both hyper- and hypo-active behaviour occurring within the same trial may therefore reflect dose-dependent effects (Alkassab & Kirchner, 2018; Muth & Leonard, 2019), and previous studies have shown similar biphasic responses to neonicotinoid exposure, with an initial short-term stimulation followed by inhibition of the same response after prolonged exposure (Potts et al., 2018; Tosi et al., 2016).

Interestingly, Crall et al. (2018) found that whilst chronic exposure to imidacloprid increased movement speed in nest bumblebee workers, it actually decreased the amount of time workers were active, which suggests my findings would translate to colony level impacts. With regards to the temperature-dependency of these effects, insights can be gained from Boina et al. (2009) who demonstrated that imidacloprid was more toxic to a hemipteran pest at 17°C compared to 27°C, and attributed this to the greater binding efficiency of imidacloprid to neuronal membranes at lower temperatures (Wellmann et al., 2004). Further to this, a recent study by Manzi et al. (2020) demonstrated that imidacloprid can lead to changes in the expression of genes in honeybees relating to both stress response and ionic channels in the brain that are key in neuronal excitability. Expression

of these genes was increased following imidacloprid exposure, and this overexpression was further intensified at 20°C but not at warmer temperatures of 28°C or 36°C, which may help provide a mechanistic basis for the hyperactive behaviour and subsequent depressive states seen at the low temperature in my study.

Furthermore, bumblebees are heterothermic, and as such they don't solely rely on thermal energy from the environment to warm themselves up, generating heat endogenously when required through tetanic shivering of flight muscles in a process known as non-flight thermogenesis (Esch et al., 1991; Potts et al., 2018). When provided with an unlimited food supply, as was done in experiments 1 & 2 in the current study, it has been shown that bees will maintain elevated thoracic temperatures that promote the fast exploitation of resources and allows workers to remain active (Azpiazu et al., 2019; Holder et al., 2018; Nieh et al., 2006; Stabentheiner & Kovac, 2014, 2016). Neonicotinoids have been shown to impair thermoregulation (Crall et al., 2018), and in particular the ability for non-flight thermogenesis in both honey bees (Tosi et al., 2016) and bumblebees (Potts et al., 2018; also see Appendix E). In fact the same effect from pyrethroids has been discussed as the mechanism underpinning their enhanced toxicity to honeybees under lower temperatures (Belzunces et al., 2012). Effects on thermoregulation may be especially pertinent, as it will create both individuals and nests with greater internal thermal fluctuation, thus shaping the thermal environments in which workers are exposed (Manzi et al., 2020).

Imidacloprid exposure, but not sulfoxaflor exposure, elicited an anti-feeding response in experiment 2 at both tested concentrations, and the magnitude of this effect was greatest at the low temperature. Feeding suppression following exposure to imidacloprid has been observed across a range of bee species (Cresswell et al., 2012; Laycock et al., 2012; Muth et al., 2020; Zhu et al., 2017), which can compromise energy balance and consequently has been shown to be associated with decreased thoracic temperatures and apathy (Azpiazu et al., 2019). Additionally, at the low temperature, as bees became immobile this may have rendered them unable to feed, further intensifying the impacts on mobility (Nauen et al., 2001). Prolonged exposure to imidacloprid, and the consequent anti-feeding effects and potential impairment to thermogenesis, may have led to the observations of reduced responsiveness and likelihood of movement at the low temperature (Azpiazu et al., 2019), and may in part explain why I observed a negative TC for imidacloprid when previous studies on ectothermic insect pests have found a positive TC (Arthur et al., 2004; Mansoor et al., 2015; Tsaganou et al., 2021).

4.4.2 | *Flight performance*

In chapter 2, it was found that neonicotinoids can impair aspects of flight performance, such as endurance and speed, in bumblebee workers. Whilst the underlying drivers of these effects are not fully known, one possibility is impairment to thermogenesis. Bees must attain and maintain a minimum thoracic temperature typically around 35°C to support take-off and continuous flight (Heinrich, 1972, 1975; Nieh et al., 2006), which is typically above the temperature of the surrounding environment when foraging (Potts et al., 2018). Additionally, Manzi et al. (2020) found that octopamine expression was increased following imidacloprid exposure at relative low (20°C) but not high (28°C or 36°C) temperatures. Octopamine is a neurotransmitter with an important role in modulating a variety of physiological and behavioural processes in insects, such as metabolic state and motor rhythms, which are both integral for flight (Brembs et al., 2007; Pflüger & Duch, 2011). However, despite these points raising the expectation of reduced flight initiation and quicker energy exhaustion in imidacloprid exposed bees at cooler temperatures (Ahmed et al., 2015; Manzi et al., 2020), instead I observed no effect of acute I10 exposure on any aspect of flight performance at the low or mid temperatures. In fact, flight distance was significantly impaired by I10 exposure at the high temperature, and whilst I10 exposed workers at this temperature did not show any detectable difference from non-exposed workers in initiating flight and flying past the 100m milestone, they did show a significantly lower likelihood in flying past the 1000m milestone. This result shows that for flight performance, neonicotinoids have a positive TC effect, which is perhaps surprising given previous insect pollinator studies found that the effects of different neonicotinoids (thiamethoxam and clothianidin) on honeybee homing success and bumblebee foraging behaviour (respectively) became more pronounced under colder conditions (Henry et al., 2014; Kolano et al., 2021).

The above differences raise some interesting points. Firstly, controlled lab studies such as this may be important in gaining insights into effects under future scenarios. Whilst Kolano et al. (2021) found that impairment to bumblebee foraging duration from clothianidin diminished as temperatures got warmer, the study never recorded any ambient temperatures over 28°C. In the current study, I observed no effect of imidacloprid at 27°C, and it was only at 30°C where impacts on flight distance became apparent, highlighting the importance of considering more extreme temperatures that may become more common under current climate change predictions, and indeed extreme heat events have already been shown to have synergisms with imidacloprid exposure in freshwater insects leading to impairment of mobility (Macaulay et al., 2021).

Secondly, not all species of bee may respond to stressors equally, and this is a key point underpinning the need to expand risk assessments to include a wider range of pollinator species (Arena & Sgolastra, 2014; Decourtye et al., 2013). Bumblebees utilise complex thermoregulatory processes to prevent overheating during flight (Dudley, 2000; Heinrich, 1975), but recent research

suggests that bumblebees are moving towards their thermal limits for flight at 30°C (see Chapter 3). As neonicotinoids are known to have detrimental impacts on other thermoregulatory processes in individuals (Potts et al., 2018; Tosi et al., 2016), it is possible 110 exposed workers edged too close to their thermal limits leading to earlier termination of flight. This impairment seemed to stem from larger workers prematurely terminating flight, which would make sense, as larger workers are less able to dissipate heat to the environment due to their larger surface area to volume ratio, and thus would be more at risk from overheating compared to smaller nestmates (Harrison & Roberts, 2000; Rubalcaba & Olalla-Tarraga, 2020). Responses of bumblebees to insecticides under variable temperature scenarios cannot therefore be assumed to be the same as for honeybee workers, which are uniform in size, but may help elucidate how bee species of different sizes will respond to pesticide-temperature interactions in the future.

Lastly, all pesticides may not show the same temperature coefficients when measuring behavioural impairment, even if they have a similar mode of action (Boina et al., 2009; Khan & Akram, 2014; Muturi et al., 2011; Reid et al., 2020). Once ingested, xenobiotic chemicals such as pesticides are enzymatically modified to facilitate excretion from the body in a process known as biotransformation (Gerba, 2019). Given metabolic rate in insects is highly temperature dependent, and since insect body temperature is influenced by the ambient temperature, this would intuitively suggest that imidacloprid should be detoxified at a faster rate, and thus be less toxic under warmer environmental conditions (Khan & Akram, 2014; Noyes et al., 2009). For instance, the toxicity of pyrethroid insecticides have been observed to increase under colder post-exposure temperatures, due to a reduced elimination rate and thus increased accumulation of the pesticide within individuals (Harwood et al., 2009; Weston et al., 2009), which could help explain my results for experiment 1 & 2. However, biotransformation can sometimes yield toxic metabolites in a process known as bioactivation (Gerba, 2019). One of the first metabolites of imidacloprid in bees is an olefin compound, which is more toxic to insects than the parent compound (Nauen et al., 2001; Seifrtova et al., 2017; Simon-Delso et al., 2015; Suchail et al., 2004). Under a one-shot acute dose of imidacloprid in experiment 3 (flight), where there was no time for imidacloprid to accumulate within individuals like there was in experiments 1 & 2 (responsiveness & walking), conversion of imidacloprid to olefin at a faster rate provides a rationale for impairment to flight performance at the high temperature but not at the low or mid temperature. Indeed, a similar process has been proposed to explain the increased mortality of insect pests from organophosphates at warmer post-exposure temperatures (Khan & Akram, 2014; Lydy et al., 1999).

4.4.3 | Imidacloprid vs sulfoxaflor

The slow detoxification of neonicotinoids within exposed bees has been proposed as a reason for their high toxicity compared to other insecticides, providing a plausible mechanism for the observed difference in toxicity between imidacloprid and sulfoxaflor in this study (Zhang et al., 2021). Furthermore, one of the primary reasons sulfoxaflor is deemed to be a safer alternative to the neonicotinoids is its more rapid degradation rate in nectar and pollen (EPA, 2019; Linguadoca et al., 2021). In addition to generally having a lower LD₅₀, this may explain why I observed a comparatively smaller effect of sulfoxaflor on flight distance at the high temperature (non-significant 24% reduction in distance vs controls) relative to imidacloprid (significant > 50% reduction in distance vs controls), and why I didn't observe any negative effects of sulfoxaflor on survival or mobility in experiments 1 & 2 (Siviter et al., 2019). Tamburini (Tamburini, Wintermantel, et al., 2021) found that sulfoxaflor application at label recommended rates as part of a commercial formulation had no adverse effects on honeybees when applied six days before bloom as recommended. On the other hand, recent studies have found significant effects of sulfoxaflor exposure on bumblebee reproduction, fecundity, and fitness (Linguadoca et al., 2021; Siviter et al., 2018), although concentrations used were above the 150 ug/L tested in the current study. Thus, close attention needs to be paid to the recommended application rates and how this translates to the level of actual exposure across landscapes (Siviter & Muth, 2020; Zioga et al., 2020), as my study suggests that sulfoxaflor concentrations below 150 ug/L are a lower risk to bumblebees and likely other beneficial insects relative to nitrimine-based neonicotinoids.

4.4.4 | Conclusion

With the growing and widespread use of systemic cholinergic pesticides, alongside increasing temperatures and extreme weather events associated with climate change, my results highlight concern for future insect pollinator function and survival (Deutsch et al., 2018; Goulson, 2020; IPCC, 2018; Tang et al., 2021). Phenomena such as cold snaps during crop growing months where pesticide applications are heavy, may impair rudimentary but central behaviours underpinning pollinator life-histories and survival. Climate warming could thus be seen as beneficial to some pollinators under pesticide contaminated landscapes, however, my findings show that more complex behaviours, such as flight, may adversely be affected by pesticides when at higher temperatures. Understanding how my mobility and flight results translate to effects on foraging activity (Kolano et al., 2021; Muth & Leonard, 2019) and pollination service (Abdel-Raziq et al., 2021; Greenop et al., 2020) are important next steps, as this has obvious implications for colony fitness and food security. With behaviours like

foraging subject to environmental conditions that vary through the year, the timing of pesticide applications will be important in terms of determining the impact of ingested pesticides on foraging individuals. This has significant implications when thinking about the optimal timing of pesticide exposure from both a conservation and a pest management strategy perspective (Mansoor et al., 2015). It also highlights that these risks will vary dependent on the different climate regions of the world, particularly those regions where insects are already functioning close to their thermal limits (Chapter 3; Soroye et al., 2020). My work thus supports the urgent need to: i) conduct further study to understand how wider temperature ranges modulate sublethal pesticide toxicity to non-target beneficial organisms on flight and other behaviours and physiological functions such as thermoregulatory capacity; ii) modify risk assessments to consider this across a range of organisms; iii) account for differences in temperature coefficients between pesticides when recommending application rates and which pesticides pose the lowest risk to non-target organisms for a given climatic region; and iv) take into consideration multi-stressor interactions when predicting future insect pollinator population trends, and when projecting spatio-temporal pollination service provision.

CHAPTER 5

General Discussion

5.1 | Summary of findings

The work in this thesis has shown that insecticide exposure and environmental temperature are both key factors in determining performance of key functional behaviours in a flagship wild insect pollinator. In chapter 2, I investigated how exposure to a field realistic (10 ppb) acute dose of the neonicotinoid imidacloprid affected worker flight performance of the widespread bumblebee, *B. terrestris*. Intriguingly, observations showed exposed workers flew at a significantly higher velocity over the first $\frac{3}{4}$ km of flight. This apparent hyperactivity, however, may incur a cost because exposed workers showed dramatically reduced flight distance and duration to around a third of what control workers were capable of achieving. In chapter 3, I quantified how flight performance of *B. terrestris* workers varied over a temperature range (12–30°C). Workers struggled to fly over a few hundred metres at the lowest tested temperature of 12°C; however, flight endurance increased as temperatures rose, peaking around 25°C after which it declined. By incorporating both flight endurance and likelihood of flight, I calculated a simple metric termed ‘temperature-mediated foraging potential’ to gain a clearer understanding of how temperature may constrain colony foraging. Of the tested temperatures, 27°C supported the highest potential, indicating that for much of the range of this species, higher mean daily temperatures as forecasted under climate warming will push colonies closer to their thermal optimum for flight. Together the findings of these two chapters would suggest that any increases in pesticide exposure alongside more extreme temperatures being experienced under climate change would have negative implications for bumblebee flight performance. Given that bumblebees are social and central place foragers, impairment to flight motivation and endurance could translate to a decline in potential workforce size and forage area, decreasing the abundance, diversity, and nutritional quality of available food, while potentially diminishing pollination service capabilities.

Interestingly, across chapters 2 & 3, I found body size to have a mediating effect on flight responses. My findings revealed variation in flight capacity across the workforce, with larger workers flying further, longer, and faster than their smaller nestmates. However, my results suggest that pesticide exposure may negate the capability of larger workers to fly longer distances than their smaller sister workers. Furthermore, whilst body mass was positively related with the likelihood of

flight, importantly this relationship became stronger as temperatures cooled, such that at 12°C only the largest workers were successful fliers. My results thus highlight that colony foraging success under variable pesticide and thermal environments may be dependent on the body mass distribution of constituent workers, and more broadly suggests that smaller-bodied insects may benefit disproportionately more from warming than larger-bodied ones in terms of flight performance.

The seasonal pattern of pesticide application to agricultural landscapes makes exposure to insects under wide thermal ranges a very realistic scenario (Holmstrup et al., 2010). Additionally, future landscapes are predicted to experience increased rates of pesticide application along with warmer and more extreme climates, increasing the likelihood that insect pollinators will experience these stressors simultaneously (Deutsch et al., 2018; IPCC, 2018; Tang et al., 2021; Zhang, 2018). In chapter 4 I conducted a multi-experimental study, exploring how exposure to varying concentrations of either a neonicotinoid or sulfoximine insecticide affected different aspects of bumblebee behaviour across a temperature range. The effect of neonicotinoid exposure varied depending on post-exposure environmental temperature, with impacts on flight being enhanced under warmer conditions and impacts on food consumption, walking, and general responsiveness being enhanced under cooler conditions. The hazard posed by insecticides across landscapes may therefore vary both temporally and spatially. Interestingly, the same effects were not seen for a different class of insecticide, with the new to market sulfoximine insecticide, sulfoxaflor, showing no detectable effect on any measure of mobility regardless of temperature when applied at comparable concentrations. The findings of this study suggest both the choice of insecticide and timing of exposure need to be considered in farming practices, and provide crucial information for advising pesticide risk assessment protocols, understanding how to develop mitigative and conservation action, and understanding which possible climate regions in the world insect pollinators are most at risk from pesticide exposure.

5.2 | Implications

The findings from this thesis have implications for how past, current, and future pesticide and climate landscapes may affect important behavioural functions both within and outside the nest in social bee pollinators.

A) Within nest function

The effects of neonicotinoid exposure at field-realistic concentrations on bumblebee responsiveness and mobility in chapter 4 have potential implications for within-nest behaviours such as nursing and nest construction. Neonicotinoids have been shown to disrupt nest activity and social

networks in bumblebee colonies (Crall et al., 2018), with impacts on nurse worker behaviour that can ultimately lead to retarded larval development (Siefert et al., 2020), which may partly stem from impacts on bee motor function and locomotory activities related to these nest behaviours (Alkassab & Kirchner, 2018; Almeida et al., 2021; Azpiazu et al., 2019; Tosi & Nieh, 2017; Williamson et al., 2014). Importantly, the results presented in this thesis demonstrate that these impacts may be enhanced under colder conditions. Within bumblebee colonies, workers exhibit collective thermoregulatory behaviours, maintaining relatively stable internal nest temperatures to support larval development (Gardner et al., 2007; Yoon et al., 2002). Workers found inside colonies are thus less likely to experience large thermal fluctuations compared to nestmates on the wing (i.e., when leaving the colony to forage). That said, the changing temperatures associated with climate change are inevitably going to place changing demands on bumblebee colony thermoregulation (Ryding et al., 2021), and importantly, neonicotinoids can impair both individual and colony level thermoregulation in bees (Azpiazu et al., 2019; Potts et al., 2018; Tosi et al., 2016). Indeed, a project conducted during my PhD, in which bumblebee individuals were thermally imaged (please see Appendix E), did indicate that neonicotinoid exposed individuals may be poorer at maintaining non-active body temperature (non-flight thermogenesis) and that this impairment can be exaggerated under a low versus high ambient temperature (Appendix E – Figure 7). In cooler ambient conditions, such as early spring months in the UK, impairment to individual thermoregulation from insecticide exposure may thus compromise the thermoregulatory capacity of the colony. These months are also when most bumblebee colonies are founding and developing, and as such may be more susceptible to temperature stress due to less workers available to maintain social homeostasis (Vanderplanck et al., 2019). Ultimately, this could create thermally variable nest environments across which susceptibility to insecticide exposure may then vary.

B) Outside nest function

In chapter 2, I showed that exposure to neonicotinoids at field-realistic concentrations can dramatically impair flight motivation and endurance. This has implications for foraging capacity across past, current, and future landscapes, especially given that over the last 50 years natural and semi-natural habitat has been replaced by pasture and cropland across vast areas, resulting in the clearance, homogenisation, and fragmentation of floral resources (Fahrig, 2003; Kremen et al., 2002; Ramankutty et al., 2018; Senapathi et al., 2017). Increasingly intensive agriculture with more arable land leads to increased foraging distance requirements for bumblebee workers (Gervais et al., 2020; Redhead et al., 2016). Exacerbating this effect may also be that agricultural landscapes have been shown to provide a lower diversity and quality of food for pollinators to exploit (Tew et al., 2021). Lower quality of floral resources can lead to colonies recruiting more workers to foraging to avoid starvation (Stewart et al.,

2021). My results that neonicotinoid exposure can drastically reduce flight motivation are thus highly worrying.

The risks that pesticides such as neonicotinoids pose to pollinators, however, are likely to vary across the season given the seasonal timing of crop growing and pesticide application. For example, high pesticide input during the spring months when crops are grown (Garthwaite et al., 2018) coincides with colder weather (relative to summer), under which my work in chapter 3 shows foragers may have reduced flight performance. Warmer temperatures, on the other hand, may be beneficial for pollinators in terms of flight performance, as my work showed that both flight motivation and endurance increased with temperature up until tipping points around 24-27°C. Interestingly, urbanisation has been proposed to provide benefits to some bee species as they act as so-called 'heat islands', providing warmer and more thermally stable local environments (Hamblin et al., 2017). However, my findings in chapter 3 suggest that if environmental temperatures get too high (~30°C) this may actually cause flight performance to be reduced as workers tip onto the declining slope of their thermal performance curve, and results from chapter 4 suggest that the effects of insecticide exposure on flight are most prominent at similar high temperatures. In the summer months, therefore, both temperature stress and insecticide exposure may have greater impacts on the ability of colonies to collect the volume and diversity of food required to meet energetic and dietary needs.

5.2.1 | *Informing agricultural practices and pesticide risk assessments*

Ultimately, the findings in this thesis suggest attention should be given to temperature variation as a key factor in decisions surrounding the choice of insecticide and the timing of insecticide application in agricultural management practices. Findings could inform advice given to pesticide users on recommended application timing based on predicted thermal conditions alongside known pollinator susceptibility windows (e.g., when colonies are being founded; when nutritional landscapes are limiting). Importantly, temperature coefficients may differ between pests and pollinators, as insect pests seem to show positive temperature coefficients for neonicotinoids, whereas the majority of responses I examined suggest neonicotinoids have a negative temperature coefficient for bumblebees, which may in part be due to differences in thermal control mechanisms (Mansoor et al., 2015; Tsaganou et al., 2021). This needs to be explored further, as it may highlight exposure windows that can be exploited by applying chemical pesticides at times that have high efficacy against pests but low toxicity to pollinators. Farmers could thus mitigate risks to beneficial insect pollinators whilst ensuring effective control of damaging insect pests.

The most effective insecticide against a pest may vary with environmental conditions. In situations where comparable products from multiple insecticide classes are available, temperature should be included as a factor in the decision-making process (Boina et al., 2009; Musser & Shelton, 2005). It is possible that insecticides could be used on a rotational basis, using knowledge of temperature coefficients to limit harm to beneficial insect pollinators, and this targeted approach to pesticide use could be incorporated into integrated pest management (IPM) strategies currently being developed and promoted (Creissen et al., 2021). Farming appears to be moving towards integrated pest management approaches, with agri-environment schemes promoting creation of florally rich habitats, which will reduce both foraging distance requirements and the application of chemical pesticides to fields (Gagic et al., 2021; Marja et al., 2021; Ricciardi et al., 2021; Siviter & Muth, 2020). Insect pollinator conservation and agricultural production can be compatible, with increasing on-farm and landscape-level crop diversity predicted to have positive effects on bumblebees (Hemberger et al., 2021). Schemes like the Sustainable Farming Incentive (SFI), which is a new land management scheme in England that will pay farmers to produce public goods, such as biodiversity and climate change mitigation, alongside food production, will be an important tool for driving landscape level change to benefit pollinator populations over the coming years (DEFRA, 2021).

Translating the potential issue of increased pesticide risk under different temperature scenarios into decision-making will be challenging. One recommendation for policy is that risk assessments should be updated, as they form a key component of the regulatory process when authorising the usage of pesticides. Risk assessments could be expanded to test pesticide impacts over a temperature gradient, with climate data used to inform this gradient, allowing approvals and recommended application rates to be tailored towards specific regions. Furthermore, assessments should more routinely evaluate and quantify sublethal impacts, as the development of acceptable threshold values for sublethal endpoints could help prevent the unwanted approval of pesticides with detrimental impacts on insect pollinators (Azpiazu et al., 2019; Sgolastra et al., 2020; Siviter, Bailes, et al., 2021). For pesticides already approved for use, such as sulfoxaflor in the UK, one of the single most effective changes that could be made to the regulatory process is to include large-scale and long-term post-licensing monitoring of the impacts of application on pollinator populations (Siviter, Bailes, et al., 2021). This is a key feature in public health assessments following pharmaceutical chemical release but is still not systematically carried out for agrochemicals (Milner & Boyd, 2017).

It is important to note that this thesis focused on the direct ways environmental temperature could influence the impacts of pesticides on insect pollinators. However, climate change could have a range of indirect impacts on pesticide use and exposure dynamics. For example, the area of crops treated with pesticides, as well as the volume of pesticides applied, are consistently growing both in

the UK (Goulson, 2020) and globally (Tang et al., 2021; Zhang, 2018), and this trend is anticipated to continue as pesticides are used to tackle predicted rises in pest invasions under a warming climate (Deutsch et al., 2018). Additionally, it is interesting to consider that rising temperatures across the UK, coupled with other changes to climatic conditions such as rainfall patterns, are predicted to lengthen growing seasons for certain crops, accelerate plant growth, but increase drought and heat risks (Arnell & Freeman, 2021). Climate change may therefore alter the potential spatial and temporal forage availability for insect pollinators and may thus counteract or compound impairments on pollinator foraging from pesticide exposure, which should be factored in to future decision making.

5.2.2 | *Impacts of insecticide exposure around the globe*

In this thesis I examined the effects of the neonicotinoid insecticide imidacloprid, which is restricted for use in the EU and UK outside of closed greenhouses (EFSA, 2018; Sgolastra et al., 2020). Whilst it can persist in the environment for years after application, once application has ceased the concentrations found in subsequent years are generally below the levels tested here or considered damaging to bees (Riedo et al., 2021; Woodcock et al., 2018, 2021). However, that does not mean that the neonicotinoid specific findings from this thesis are not of benefit to current decision making or pollinator conservation in these regions. The laws on neonicotinoid use in the EU (and UK) have an article written in that allows EU member states to grant emergency derogation; that is, they can approve the temporary use of neonicotinoids for a period of 120 days if there are dangers to crops that cannot be contained by any other reasonable means (Article 53 of Regulation No 1107/2009). Whilst this allows for flexibility for farmers when faced with otherwise challenging situations and risks to crop yields, it means that some countries have been using imidacloprid on certain crops every year since the ban came into force (European Commission, 2022). In the UK in 2021, the government approved the use of thiamethoxam on sugar beet to control aphids that transmitted virus yellows, but the emergency authorization was ultimately not put into action as a harsh cold winter controlled most of the aphid population without the need for chemical intervention. The risks posed by neonicotinoids are therefore still relevant across Europe, and results from this thesis can feed into future decision-making regarding derogations.

Despite rapid increases in pesticide use across middle-income countries of South America, Asia Pacific, and Africa, pesticide regulations are generally weaker in the global south, and imidacloprid is still used heavily across many of these regions (Dicks et al., 2021; IPBES, 2016; NASAC, 2019; Schreinemachers & Tipraqsa, 2012). In Brazil, it is one of the major insecticides still in use (Almeida et al., 2021), it is one of the biggest insecticide exports from China (Zhang, 2018) and is one

of the most popular insecticides across African nations (Bertrand, 2019). My findings on imidacloprid effects therefore have significant implications for the global plight of insect pollinators. Importantly, these regions in the global south are where there is a greater reliance on pollinator dependent crops for food production, trade, and livelihoods (Chaplin-Kramer et al., 2014; Dicks et al., 2021; Hipólito et al., 2018; Lautenbach et al., 2012). However, published evidence for trends in pollinator numbers and links to insecticide use is largely from high-income countries across North America and Europe (Dicks et al., 2021; IPBES, 2016; Potts et al., 2016). There is an urgent need to investigate these trends and relationships in regions in the global south, which are thought to be most vulnerable to decreases in pollinator diversity and pollination services (Breeze et al., 2016; Teichroew et al., 2017). Of concern is that these are regions where neonicotinoids are still routinely applied and are some of the most vulnerable to climate impacts, which is especially pertinent given that my findings indicate insecticide toxicity may be dependent on environmental temperature (Dicks et al., 2021; IPBES, 2016). In these regions, there may be a greater need to consider non-insecticide approaches to pest management.

5.2.3 | Sulfoxaflor as an alternative to nitroguanidine neonicotinoids

Neonicotinoids are coming under tighter regulation globally, and combined with emerging resistance to current insecticides from certain pest species, there are gaps in the market for new and novel insecticides with less collateral damage to beneficial insects (Bass et al., 2015; Sgolastra et al., 2020; Siviter & Muth, 2020). One such potential insecticide is sulfoxaflor, the first marketed pesticide in the sulfoximine class (Sparks et al., 2013). In chapter 4, regardless of the surrounding environmental temperature, sulfoxaflor exposure was found to have no detectable effect on any of the lethal or sublethal responses measured, which stood in contrast to the observed detrimental effects of imidacloprid exposure on flight, mobility, responsiveness and survival. Whilst sulfoximine insecticides share a similar mode of action with neonicotinoids, my findings highlight that toxicity cannot be assumed based on this, and the impacts of sulfoxaflor on non-target insects need to be investigated independently and thoroughly (Reid et al., 2020; Sparks et al., 2013). These findings support previous studies that have demonstrated sulfoxaflor to be comparatively less toxic to insects than imidacloprid and the other nitroguanidine based neonicotinoids (Azpiazu et al., 2021; Parkinson et al., 2020), and suggest that sulfoxaflor could be a preferable insecticide choice to these neonicotinoids if similar concentrations to those used in my study provide effective pest control. However, the comparably lower toxicity will likely mean recommended application rates will be higher. Indeed, datasets compiled by the EFSA (Europe) and EPA (USA) show that residues in the environment under realistic application scenarios are above the 150 ug/L concentration used in my study, with worse case

scenarios suggesting bees will come into contact with concentrations around 1000 ppb and above (EFSA et al., 2019; EPA, 2019; Linguadoca et al., 2021; Siviter & Muth, 2020), which is greater than known concentrations of neonicotinoids found in nectar and pollen of treated plants and bee material (Blacquière et al., 2012; Bonmatin et al., 2015; Botías et al., 2015; Mitchell et al., 2017; Zioga et al., 2020). If wanting to establish environmental risk, a direct comparison between sulfoxaflor and imidacloprid at the same concentrations, as was done in this thesis, may not be fully appropriate if sulfoxaflor is applied to crops at a greater rate due to the assertion it is less toxic. Whilst data on field-realistic sulfoxaflor concentrations were lacking when this thesis was first undertaken, the data is improving as sulfoxaflor is adopted by more pesticide users, and an improvement to this thesis could come from using field data to inform tested concentrations.

Worryingly, studies have found significant effects of sulfoxaflor on bumblebee reproduction, fecundity, and fitness from exposure to concentrations above the 150 ug/L tested in this study (Linguadoca et al., 2021; Siviter et al., 2018, 2020). Furthermore, Boff (2021) found that sulfoxaflor exposure at 50 ppb led to impaired flight and foraging in a solitary bee, with exposed individuals more likely to walk than fly to flowers compared to non-exposed bees and visiting less flowers on average as well. Interestingly, however, individuals exposed to just 10 ppb, similar to the concentration used in this thesis, demonstrated no impairment to behaviour. An additional concern is that whilst sulfoxaflor treated seeds are becoming more widely available (Centner et al., 2018; Gauthier & Mabury, 2021), it is still often applied as a foliar spray, and thus contact exposure is a more common route of exposure compared to neonicotinoids (Brown et al., 2016), which has been shown to lead to dramatic effects on honey bee mortality from field recommended application rates (Chakrabarti et al., 2020). Close attention therefore needs to be paid to both the application method and rates recommended for sulfoxaflor going forward, how this translates to the level of actual exposure across landscapes, and the impacts of this level of exposure for individual and colony fitness (Siviter & Muth, 2020; Zioga et al., 2020).

5.2.4 | *Is body size an important trait in determining sensitivity to temperature and pesticide stress?*

Bee species exhibit appreciable interspecific variation in life-history traits, and even within bumblebee genera colony and behavioural characteristics vary widely (Gradish et al., 2019). Worker body size has previously been implicated as an important trait in determining bumblebee foraging efficiency (Goulson et al., 2002), and studies show that higher mean body size and greater intra-colony size variation lead to increased colony performance (Cnaani & Hefetz, 1994; Herrmann et al., 2018; Holland et al., 2021). Austin & Dunlap (2019) suggest that higher intraspecific variation in worker body

size is associated with bumblebee species being less susceptible to decline in rapidly altered environments in North America. My results provide tentative evidence for why size variation may buffer species against pesticide contaminated and thermally dynamic landscapes. Whilst flight performance increased with body size for non-pesticide exposed workers over a wide temperature range (Chapters 2, 3 & 4), larger workers seemed disproportionately affected by neonicotinoid exposure (Chapter 2) and there were hints that smaller workers became more efficient fliers than their larger sisters at high temperatures of 30°C (Chapter 3). Current environmental changes, such as intensive pesticide application and increasing and more unpredictable air temperatures may therefore provide selection pressures for bumblebees. Studies linking historical shifts in body size with landscape level changes (e.g., climate, pesticide usage, land-use) over time will be valuable for predicting this. Indeed, Theodorou et al. (2020) found that increasing air temperatures were associated with smaller mean body size of *Bombus terrestris* workers and an increase in variation in body size in multiple other bumblebee species. Environmental change could thus have cascading effects on pollination and ecosystem services by driving shifts in body size distributions.

5.3 | Limitations

5.3.1 | *Automation of walking tracking*

In chapter 4, I used the software Kinovea to examine walking behaviour in feeding cage trials, as has been done previously by Alkassab et al. (2018) when investigating the effects of insecticides on honey bee mobility. This software provides flexibility in tracking by allowing manual supervision and readjustment of the tracking marker at each frame. This meant that a bee could be reliably tracked through every single frame of a recording, allowing highly accurate tracking to be achieved with minimal error overall. However, as the tracking was not automated, this meant that tracking had to be done for each bee individually, resulting in a time intensive process even after videos had been condensed down to a lower frame rate. Overall, across all time periods and stimulation scenarios, there were 8100 bees to monitor behaviour for (e.g., to assess if moved / walked / fed / fly), and 3240 bees to track walking paths for. As a result, walking behaviour was only examined over 20-second windows due to time limitations, which still equated to around 300 hours of work. Ultimately, examining behaviour over a short snapshot like this has its limitations in terms of how representative it is of each individual's actual behaviour. Fully automating the tracking process to facilitate tracking over minutes or even hours would have improved comparative measures of activity. Prior to the study I tested out the feasibility of using existing automatic tracking software, such as Ctrax (Kimura et al., 2014) and BeeTag (Crall, Gravish, et al., 2015). However, due to Covid-19 restrictions and the urgency

of fitting in experiments before future lockdowns, these methods couldn't be optimised in time to be used. In future work, tracking through BeeTag should be explored further. As bees are automatically detected by a unique barcode placed on the thorax, this not only supports automated tracking but also provides information on orientation and thus can be used to identify social interactions between individuals, which could allow investigation into the effect of temperature and insecticide exposure on social networks and nest behaviours (Crall et al., 2018).

5.3.2 | *Extrapolating flight mill results to foraging behaviour*

When attempting to extrapolate the flight results from this thesis to foraging behaviour and performance, it needs to be considered that some aerodynamic and physical requirements of flight, such as the requirement to generate lift when in free flight, will not be fully accounted for when using a flight mill setup (Riley et al., 1997). Workers were also naïve fliers, in order to control for any effects of experience, which can influence foraging rate (Peat & Goulson, 2005). Foraging is also dependent on a range of complementary behaviours such as navigation, memory, motor skills, and olfactory and visual acuity (Almeida et al., 2021; Fischer et al., 2014; Spaethe et al., 2007, 2001; Stanley et al., 2016). In addition, a range of environmental conditions, such as humidity, wind, rain, and physical clutter, which have been controlled for in the lab experiments described in thesis in order to assess the effects of specific stressors, will affect foraging in real-world environments (Crall et al., 2020, 2017; Crall, Ravi, et al., 2015; Hennessy et al., 2020; Peat & Goulson, 2005; Ravi et al., 2013). I found that bumblebee workers struggled to initiate flight at 12°C, and whilst frequency of foraging trips in wild colonies is known to be reduced at these temperatures, workers are seen to fly in these temperatures suggesting other factors may be at play (Bishop & Armbruster, 1999; Corbet et al., 1993). For example, in the field, solar radiation is known to play an important role in heat gain for insects (Rubalcaba & Olalla-Tarraga, 2020; Stabentheiner & Kovac, 2014), and in social bee's, individual and collective thermoregulation facilitates nest temperatures being maintained at elevated temperatures above that of the surrounding air, which should aid in initial heat gain for flight initiation (Esch et al., 1991; Heinrich, 1974a). Whilst the tested workers in this thesis did fly distances on the flight mills that do seem to match with known foraging distances of bumblebees in the wild, the flight mill setup cannot fully replicate real-world settings (Greenleaf et al., 2007; Osborne et al., 2008). Whilst my results provide comparative estimates and allow us to assess the relative effects of temperature and pesticide exposure on flight performance, they may not precisely reflect how far bumblebees can or do fly in the wild. Hence, my results should be used as a proxy for potential foraging distances rather than any absolute measure of foraging performance or capacity.

5.3.3 | *Comparing impacts of imidacloprid between chapters*

Differences in the exposure method between chapters 2 and 4 may explain some discrepancies between results, hindering direct comparisons between these studies. In chapter 2, flight trials were run at 25°C, and therefore an expectation would be that the observed effect of imidacloprid on flight performance would fall in between the observed effect found in chapter 4 when exposed bees were flown in 21°C and 27°C. However, whilst there was no detectable effect of imidacloprid on flight at these temperatures in chapter 4, there was a dramatic impairment to flight resulting from imidacloprid exposure in chapter 2. As most other elements of the experimental setup were consistent across the two studies, a possible reason for this discrepancy is the feeding method. Workers in chapter 2 were fed from soaked cotton wool, and therefore exposure could be both oral and topical as bees would rest on the cotton wool whilst feeding. Workers in chapter 4, however, were fed through a feeding tube, which I designed to be able to directly quantify consumption (and thus active ingredient dosage intake), confining exposure to be purely oral. These differences may highlight that topical exposure to neonicotinoids provides a considerable risk to bumblebees. Considering sulfoxaflor is often applied as a foliar spray (Brown et al., 2016), the topical exposure effects of this chemical on behavioural responses should be examined.

5.3.4 | *Thermal adaptation and acclimation may affect responses*

When using the results in this thesis to make predictions about bumblebee responses to climate change, it is important to consider that thermal tolerance can vary geographically both between species and within species (Rubalcaba & Olalla-Tarraga, 2020; Sunday et al., 2014). There is evidence for geographical differences in tolerance of temperature extremes in bumblebees, with high altitude species found to be more cold tolerant and less heat tolerant than low altitude species (Oyen et al., 2016). In addition, Pimsler et al. (2020) revealed that local adaptation in thermal limits may facilitate cold tolerance across a species range, with *Bombus vosnesenskii* workers originating from populations in colder higher latitudes found to have reduced CT_{min} compared workers originating from populations in warmer lower latitudes. My findings in Chapter 3 suggest that whilst rising air temperatures may push bumblebee workers closer towards their thermal optimum for flight in temperate regions, at low latitudes climate warming and extreme heat events may tip species towards the declining slope of their thermal performance curves, thus constraining resource acquisition with implications for colony fitness and pollination service delivery (Deutsch et al., 2008; Herbertsson et al., 2021). However, a limitation of my results is that they may not be directly translatable to species / populations in these climate regions, which may be adapted to cope with higher temperatures.

In addition to considering longer-term adaptation of species or populations, an individual's thermal experience over the short-term can lead to acclimatisation to certain temperatures or influence development. For example, many insects are phenotypically plastic and have the capacity to adjust their physiology and behaviour to modify or tolerate their thermal environment (Abram et al., 2017). Thermal experiences during development can also influence adult phenotype, with Soule et al. (2020) finding that rearing butterflies at a higher temperature caused a change to wing-aspect ratio. This consequently appeared to reduce flight performance when tested on a mill similar to the one used in this thesis. Interestingly, pesticide exposure at the larval stage can also lead to impaired development (Smith et al., 2020) and asymmetry in wing shape and structure in the adult stage (Friedli et al., 2020). Thermal experience may subsequently influence the response to pesticide exposure, with both Tong et al. (2019) and Tosi & Nieh (2019) finding that bees sampled at different times of year, with different experienced thermal histories, show different responses to pesticide exposure on flight mills. Consequently, a limitation of the work presented in this thesis is that I reared every worker under the same thermal conditions. Rearing workers at a temperature other than 21°C in chapter 3 may have shifted the predicted thermal response curve left or right, and in chapter 4 may have influenced the direction or magnitude of the temperature coefficient. Future work should address this and is discussed below.

5.4 | Future work

5.4.1 | *Determining the mechanisms underpinning behavioural responses*

The findings from this thesis suggest that effects of insecticide exposure on bumblebee behavioural responses vary with temperature. An important next step is to investigate what mechanisms underpin such effects by examining responses at smaller biological scales. Two potential mechanisms relate to the rate at which pesticides breakdown, either in the environment (pre-exposure) or within an individual bee (post-exposure). Environmental temperature can play a crucial role in dictating the rate of degradation in the environment prior to exposure (Liu et al., 2006; Moza et al., 1998). Previous work has attributed observed decreases in efficacy of both organophosphate and pyrethroid insecticides under warmer conditions to the reduced residual life of the respective insecticides (Arthur et al., 1992; Musser & Shelton, 2005). In experiments 1 & 2 of chapter 4, feeders were changed every 48 and 24hrs, respectively, to limit pesticide degradation throughout the 6-day trials, and feeding cages were all held under red light to limit photodegradation (Liu et al., 2006). However, different rates of pesticide degradation across the three temperature treatments still remains a possibility and could help explain why there were no observed effects of imidacloprid on

behaviour or survival at the high temperature. The quantification of exposure concentrations across the trial period through liquid chromatography and mass spectrometry (Linguadoca et al., 2021) could be incorporated into future studies to assess this. Interestingly, one of the primary reasons sulfoxaflor is deemed to be a safer alternative to the neonicotinoids is its more rapid degradation rate in nectar and pollen (EPA, 2019; Linguadoca et al., 2021). This may be why I didn't observe any negative effects of sulfoxaflor on survival or mobility in chapter 4. If applying sulfoxaflor to crops an adequate amount of time before the blooming period, as is recommended in Europe, then this may prevent the worst effects of sulfoxaflor in non-target insects. For example, Tamburini, Wintermantel, et al. (2021) found that sulfoxaflor application at label recommended rates as part of a commercial formulation had no adverse effects on honey bees when applied six days before bloom as recommended. Allowing a safety period between sulfoxaflor application and crop bloom may be crucial to limit effects on bees, however, doing this is difficult for crops with indeterminate blooming periods (Linguadoca et al., 2021).

Once ingested, xenobiotic chemicals such as pesticides are enzymatically modified to facilitate excretion from the body in a process known as biotransformation (Gerba, 2019). As the surrounding environmental temperature can determine metabolic and enzymatic activity rates in insects, it can thus influence the rate of metabolism and detoxification of a pesticide once ingested by an exposed individual (Gerba, 2019; Khan & Akram, 2014; Noyes et al., 2009). To establish whether rates of biotransformation underpinned my results in chapter 4, a useful next step would be to run similar trials but with marked [^{14}C] imidacloprid to assess accumulation rates in the tissues of exposed individuals across a thermal range (Camp & Buchwalter, 2016). This may also shed light on the apparent difference in toxicity between imidacloprid and sulfoxaflor, as the nitroguanidine neonicotinoids are believed to demonstrate high toxicity compared to the cyanoamidine neonicotinoids due to their relatively long degradation rate within exposed individuals (Zhang et al., 2021). Understanding pesticide degradation rates, in both the environment and individual organisms, is critical in understanding the exposure risks to pollinators, and this should be coupled with assays elucidating the physiological and behavioural sublethal effects of these chemicals.

With insect energy metabolism modulated by ambient temperature (Abram et al., 2017; Gillooly et al., 2001; Woods, 2005), and evidence showing that pesticide exposure can cause bee mitochondrial dysfunction (Moffat et al., 2015; Nicodemo et al., 2014; Sargent et al., 2021), there is a strong rationale to expect an interactive effect between temperature and pesticides on metabolic rate, which may provide a physiological mechanism contributing to the findings in chapter 4. However, to my knowledge this has not been explicitly tested or quantified in any animal. Furthermore, impairment to metabolic rate is likely to influence individual endothermic performance (internal

thermoregulation) - a process underpinning behaviours such as heating-up for flight (Heinrich, 1975; Potts et al., 2018; Tosi et al., 2016) and, in social bees, colony thermoregulation (Crall et al., 2018; Esch et al., 1991). An impairment to thermoregulation could be more or less restricting dependent on the ambient temperature, as this determines the internal energetic input required for reaching a specific body temperature. This may have been a predominant factor in my finding in chapter 4 that bees chronically exposed to imidacloprid became less responsive and moved less frequently at 21°C but not at higher temperatures of 27°C and 30°C. In order to generate sufficient force to support continuous flight, bees must attain and maintain a minimum thoracic temperature typically around 35°C (Heinrich, 1972, 1975; Nieh et al., 2006), which is very often above the temperature of the surrounding environment when foraging (Potts et al., 2018). Whilst I did not see a statistically significant effect, there was a noticeable drop in the proportion of bees that flew over the 100m milestone when exposed to imidacloprid at the low temperature in chapter 4 (Figure 4.13). This may potentially stem from the neonicotinoid impairing non-flight thermogenesis and the ability to adequately heat flight muscles (Potts et al., 2018; Tosi et al., 2016), and a next step could be to implement thermal imaging to quantify thorax temperature prior to flight. I started to explore these ideas in a Masters student project that I designed and supervised (please see Appendix E). However, a complete set of assays are now needed to quantify how pesticide exposure may be affecting metabolic rate at different temperatures and further measure the subsequent effects this has on thermoregulatory performance.

5.4.2 | *Testing pesticide impacts in realistic thermally fluctuating environments*

All the experiments presented in this thesis were conducted in controlled, thermally static environments, which does not necessarily represent the typically fluctuating environmental conditions bees face in the wild (dependent on the timeframe being tested). By taking data on ambient temperatures from the field, the impacts of pesticide exposure on colony fitness traits, such as reproductive capacity, could be assessed in controlled thermally fluctuating environments in lab settings. For example, controlled temperature rooms could be set to assess if the impacts of pesticide exposure differ between high or low temperature amplitudes, and how day or night conditions place demands (expanding on work by Crall et al. 2018). One interesting application could be to assess if impacts differ between thermal regimes experienced in spring months vs summer months, and this could be assessed over colonies differing in the number of workers and size distribution of the workforce.

5.4.3 | *Scaling-up results to explain real world scenarios*

Laboratory studies provide the benefit of being able to tightly control conditions and have thus allowed me to directly test the effect of both insecticide exposure and changes in ambient temperature on bee flight performance. This would not have been possible in semi-field setups due to the spatial scale needed to assess flight behaviour, and similarly would not have been possible in field settings where temperature can constantly fluctuate, pesticide contamination in the wider environment is unknown, and a whole range of additional environmental conditions known to affect flight are not able to be controlled (e.g., wind, humidity, predators). However, understanding how my mobility and flight results translate to effects on foraging activity (Kolano et al., 2021; Muth & Leonard, 2019) and pollination service (Abdel-Raziq et al., 2021; Greenop et al., 2020) are important next steps, as this has obvious implications for colony fitness and food security. Understanding the link between how impaired individual performance translates to impacts at the colony and population level is critical to understanding how tipping-points can be avoided (Bryden et al., 2013; Moore, 2018). However, monitoring impacts on wild populations in complex environments over a feasible timeframe and budget has proved extremely challenging, and consequently there has been limited research investigating the impacts of insecticide exposure on insect pollinator behaviour and colony fitness under different thermal conditions in the field.

A handful of studies have examined how neonicotinoid effects on bee homing capacity and foraging behaviour (two responses underpinned by flight) vary according to air temperature, with results appearing to contrast to the flight results outlined in Chapter 4 of this thesis. Both Henry et al. (2014) and Monchanin et al. (2019) found homing failure of honey bees when exposed to thiamethoxam to be more pronounced at lower temperatures, and Kolano et al. (2021) found that impairment to bumblebee foraging duration from clothianidin exposure increased as temperatures got cooler. The negative temperature coefficient observed in these studies, compared to the positive temperature coefficient found for flight in chapter 4, may suggest that traits other than flight were being affected by neonicotinoid exposure (e.g., navigation). Alternatively, these studies all used different neonicotinoids to the one used in this thesis, which could suggest that the direction of the temperature effect can be chemical dependent as outlined in Chapter 4. It will thus be important to expand the work presented in this thesis to explore the temperature coefficients of a wider range of commonly used pesticides and examine different behavioural endpoints to provide a more holistic understanding of individual and colony responses. It is of note that the study by Kolano et al. (2021) never experienced any air temperatures over 28°C, and whilst the full air temperature data is not available for the other two studies, Monchanin et al. (2019) classed high temperature as 24-26°C and Henry et al. (2014) classed high temperature as 28°C. The flight results in Chapter 4 suggest that

imidacloprid exposure, at doses similar to the doses used in the aforementioned studies, has no detectable effect on bumblebee flight performance at 27°C. Additionally, the thermal performance results in Chapter 3 suggest that bumblebees may be tending towards the declining side of their thermal performance curve at temperatures of 30°C and above. With the most recent IPCC report (IPCC, 2021) highlighting with even more certainty that intense and prolonged heatwaves will become more common, an important next step will be for field and semi-field studies to explore pesticide effects at temperatures above the norm.

New technologies are being developed which may aid in answering questions surrounding flight and foraging behaviour in field settings. For example, Williams et al. (2021) are developing machine learning approaches that can predict if bees leaving the nest are undertaking a foraging or an exploration trip based off measurements such as the angle and speed it leaves the nest, Shearwood et al. (2021) have presented proof-of-concept miniature transmitter and drone technology to autonomously track foraging bees across landscapes, and Abdel-Raziq et al. (2021) have used miniature bee-mounted sensors to infer honey bee colony-level pollination activity in orchards. However, complexities arise in field studies owing to the uncontrollable and unpredictable nature of weather, and disentangling individual and interacting effects of multiple stressors in field settings is often not feasible. One approach to bridge this gap could be to employ agent-based modelling of the effects of temperature and pesticide exposure on the likelihood of foraging success across landscapes of varying floral abundance and configuration. Indeed, models such as *Bumble-BEEHAVE* already exist, which aim to simulate bumblebee colony development in realistic landscapes in order to study how multiple stressors affect population dynamics, and the findings from this thesis could be used to parameterize and improve model predictions (Becher et al., 2018).

5.4.4 | Accounting for thermal histories of individuals

When assessing the effect of stressors on flight and foraging in lab experiments, thermal history should be taken into consideration, and it would be interesting to see if responses to pesticide exposure under different thermal regimes vary based on thermal tolerances. In chapter 3, the colonies used in this study were factory-reared, likely in optimal growth conditions where ambient temperatures were artificially kept around or above 25°C. But on arrival at the lab, colonies were housed in controlled environment rooms at 21°C, as this was the midway value between the lowest (12°C) and highest (30°C) temperature tested. This thermal history may have influenced the flight performance results of tested workers, and it would thus be interesting to construct thermal performance curves from workers reared in colonies at different ambient temperatures to gain

insights into how colonies will perform in different climate regions or at different times of the year. An important next step if wanting to more reliably use the findings of this thesis to directly inform management practices in the UK will be to replicate the trials on wild-caught *B.terrestris audax* workers, to see if results match up.

5.4.5 | Explore how declining species respond to stressors

Despite evidence for species specific declines in bumblebees (Guzman et al., 2021; Pilotto et al., 2020; Wagner et al., 2021), most research is carried out on two species, *Bombus terrestris* and *Bombus impatiens*, yet these are both widespread and abundant (Huml et al., 2021; Rasmont et al., 2015; Vanderplanck et al., 2019). *Bombus terrestris* have been found to be less sensitive to hyperthermic stress compared to declining bumblebee species (Zambra et al., 2020), and this relatively high thermal tolerance and high resilience to extreme events may be a factor in the geographic range they inhabit (Rasmont et al., 2015; Vanderplanck et al., 2019). Indeed, recent observations suggest *Bombus terrestris* are expanding in range and increasing dominance across landscapes under climate warming (Herbertsson et al., 2021). Hamblin et al. (2017) showed that thermal limits vary widely between bumblebee species, and these differences may in part explain how species respond to temperature change and where they can and could exist (Oyen & Dillon, 2018). Declining species have been shown to have narrower climate niches, and climate niche breadth appears to be informative for predicting species responses to climate change more broadly (Cameron & Sadd, 2020; Herrera et al., 2018). In addition to differences in thermal sensitivity, numerous studies from both the lab and field have highlighted differential pesticide sensitivity between bee species (Arena & Sgolastra, 2014; Cresswell et al., 2012; Heard et al., 2017; Rundlöf et al., 2015). Wild bees, such as solitary bees and bumblebees, are critically important for pollination of certain plants and crops, such as fruits, and for ecosystem stability (Page et al., 2021; Senapathi et al., 2021), but often show increased sensitivity to insecticides compared to honey bees (Gradish et al., 2019) and can be overlooked in risk assessments. As with the neonicotinoids, studies suggest that effects of sulfoxaflor exposure may also be species specific, with Azpiazu (2021) suggesting bumblebees to be less sensitive to sulfoxaflor compared to honey bees or mason bees when considering lethal endpoints, but Tamburini et al. (Tamburini, Pereira-Peixoto, et al., 2021; Tamburini, Wintermantel, et al., 2021) suggesting bumblebees to be more susceptible compared to honey bees when considering sublethal endpoints such as foraging performance and colony growth. It is thus clear that some bee species may be more susceptible to climate change or pesticide exposure than others (Deutsch et al., 2008; Huml et al., 2021; Zambra et al., 2020). In light of this, it is critically important we improve our understanding

of the thermal and pesticide sensitivity of declining bee species in addition to widespread and easy to study species, and identify the traits underpinning the sensitivity to stressors (e.g., body size as discussed previously), if we are to: i) truly appreciate the impact these environmental stressors are having on our pollinator communities and the services they provide; ii) highlight species most vulnerable to environmental change; and iii) produce more targeted mitigation and conservation strategies.

5.4.6 | *Translating effects on flight performance to dispersal capabilities*

In contrast to observed and predicted responses to climate change for other terrestrial animal taxa, there appears to be no change in the northern range limits for many bumblebee species across Europe and North America. Latitudinal range shifts towards the poles appears to be accelerating for most species' groups, however, bumblebees seem to be failing to track the substantial warming in these regions (Chen et al., 2011; Kerr et al., 2015; Sunday et al., 2012), and consequently colonisation of new thermally suitable habitat in high latitudes may be outweighed by declines at low latitudes (Soroye et al., 2020). It has been suggested that bumblebee dispersal rates may be insufficient to track rates of environmental warming as projected under any one of the IPCC scenarios (IPBES, 2016; Sirois-Delisle & Kerr, 2018). Owing to their reproductive social structure, population expansion is dependent on queen dispersal both before and after over-wintering. Using the flight mill setup outlined in this thesis to quantify queen (and drone) flight capacity under thermal gradients could provide useful indications of dispersal limits to help predict population trends under climate change. Interestingly, the allometric scaling relationships of body size with morphological and physical traits associated with flight in workers have been shown to predict trait values of larger queens (Billardon & Darveau, 2019). My findings on how worker body mass affected flight performance in Chapter 3 could therefore be useful in predicting queen dispersal capabilities under different thermal regimes.

5.4.7 | *Extrapolating findings to other insect taxa*

In addition to predictions within and across bee species, the relationships between body mass and flight performance across thermal gradients reported in Chapter 3 may have the potential to predict responses across insect taxa with similar thermal control mechanisms, providing useful insights into how the pollination service delivery of insect assemblages may respond to climate change. In addition to beneficial ecosystem services, the extent to which insects move across landscapes can also have dramatic implications for human health. For example, flight capacity

underpins the spread of insect-borne diseases, as well as insect pest outbreaks that threaten agricultural systems (Dujardin et al., 2008; Khan & Akram, 2014; Mazzi & Dorn, 2012; Muturi et al., 2011). Methods to predict flight capacity across insect taxa therefore represent valuable multi-disciplinary tools. However, care must be taken when extrapolating findings to wider insect taxa, because bumblebees are generally cold adapted species owing to their thermogenic capacity and insulated hairy bodies, whereas other insect pollinators such as butterflies have body temperatures that more closely acclimate to the surrounding air temperature and rely on basking and solar radiation absorbed to generate the required heat to fly (Heinrich, 1975; Wickham, 2009). It is therefore important that future work aims to quantify thermal responses of functional traits, such as flight performance, across other insect genera, in order to build a clearer picture of how pollinator communities as a whole will respond to environmental change.

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APPENDICES

Appendix A | Chapter 2 Supporting Information

Statistical Analysis

In all cases, model residuals were plotted to confirm the data met the parametric assumptions of the tests used, and model fit was assessed and optimised where possible. Where appropriate, normality tests were used to reveal distributions of the data, and those which appeared non-normal were suitably transformed as outlined below:

Feeding time

The feeding time response variable was Box-Cox transformed (Venables & Ripley, 2002) to the optimal exponent.

Effect of tag fitting on flight behaviour

The effect of tag fitting on flight behaviour was examined separately for both treatments. The effect of tag fitting on total distance flown was analysed using a linear model, with the response variable being square root transformed when considering the *pesticide* treatment, but being left untransformed when considering the *control* treatment.

Average and maximum velocity

Prior to maximum velocity analysis, one outlier was identified in the *pesticide* treatment group with a maximum velocity of 14 m/s. This value was around 12 m/s higher than any other individual, and being considered a reaction to stimulation as opposed to normal flight behaviour this value was removed from further analysis. When considering the full dataset of 67 bees (Table 2.1 – filter step 6), the average velocity and maximum velocity values were square root and cube root transformed respectively. When considering the additional dataset of 72 bees and the subset of 53 bees (Table 2.1 – filter step 7, Table A1), both the average velocity and maximum velocity values had to be square root transformed.

Total distance flown

For analysis of all datasets, the total flight distance response variable was square root transformed.

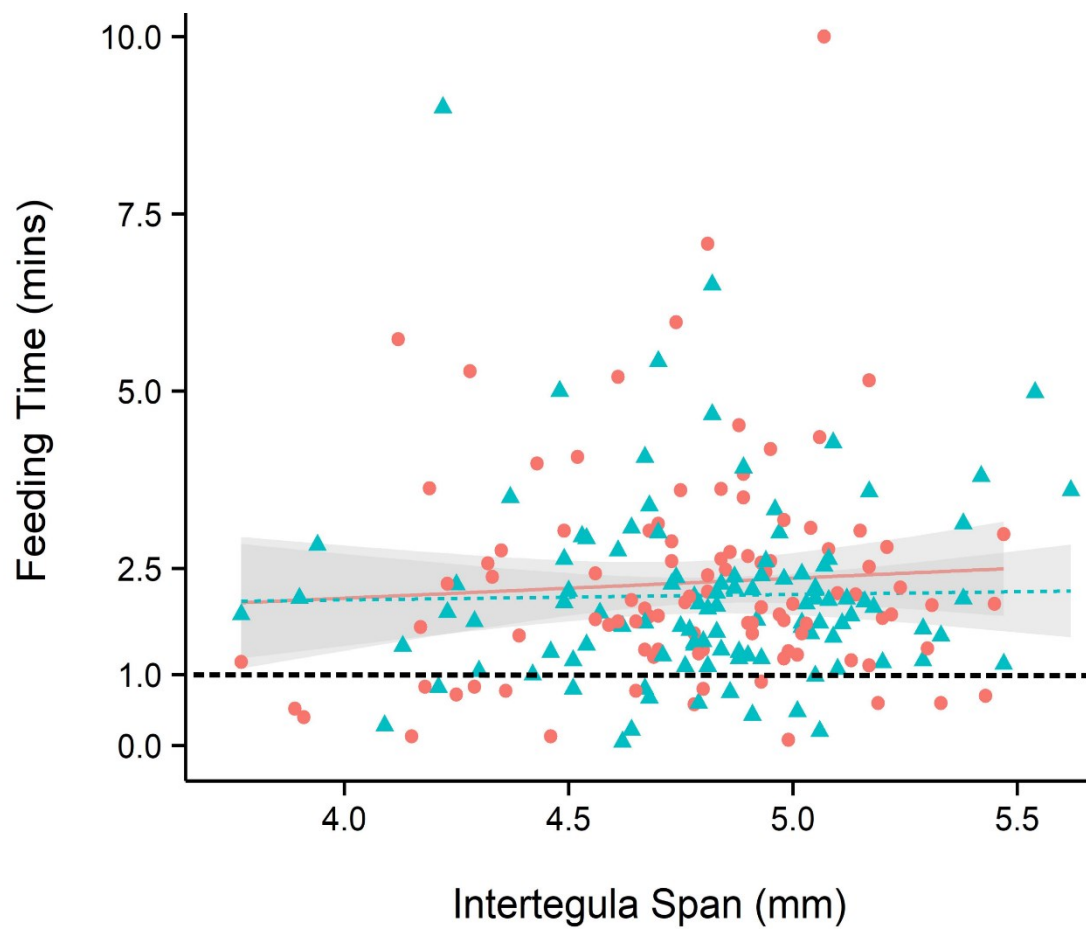


Figure A1. Scatter plot showing no significant relationship between length of feeding time and body size (*intertegula span*) for *control* (red circle) and *pesticide* exposed (blue triangle) workers. There were 222 bees selected for testing (*control* = 111, *pesticide* = 111), of which 209 initiated feeding (*control* = 102, *pesticide* = 107). The dashed black line corresponds with a feeding time of 60 seconds, and all bees that fed below this (*control* = 16, *pesticide* = 13) were not considered in the main flight analysis. Linear fitted lines (*control* = solid red, *pesticide* = dashed blue) with associated standard error (shaded bands) are estimates of linear models.

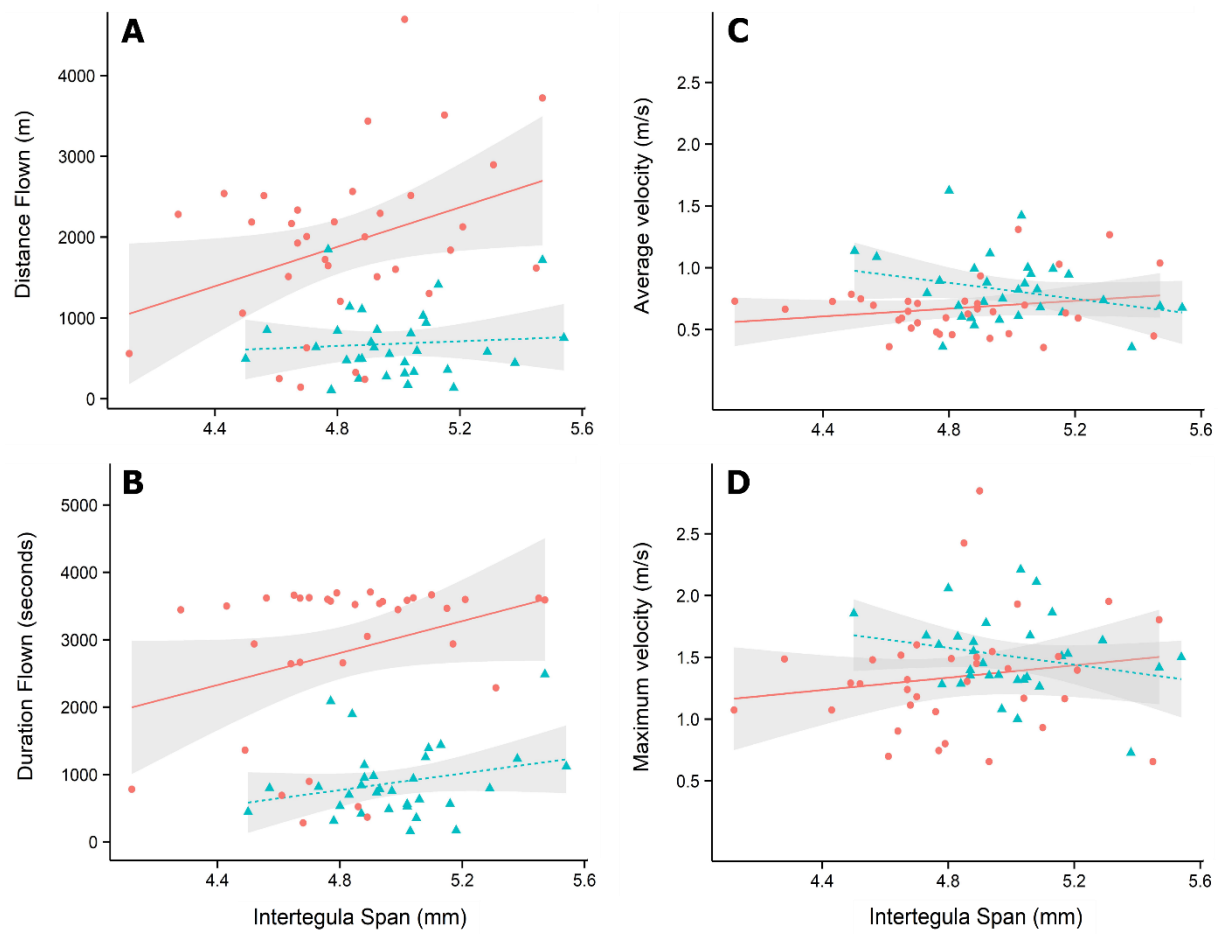


Figure A2. Scatterplot showing key flight performance indicators of endurance (distance flown in meters (A); duration flown in seconds (B)) and average and maximum velocity in meters per second (C-D) against worker body size (*interegula span*) for both the *control* (red circle) and *pesticide* treated (blue treatment) groups. Data plotted are for the full dataset of bees ($n = 35$ *control*; 32 *pesticide*; see Table 2.1 – filter step 6), and linear fitted lines (*control* = solid red, *pesticide* = dashed blue) with associated standard error (shaded bands) are estimates of linear models.

	Distance Flown			Duration Flown			Average Velocity			Maximum velocity			
	Estimate	t value	P value	Estimate	z value	P value	Estimate	t value	P value	Estimate	t value	P value	
Additional Dataset (n=72)	Intercept	-8.347	-0.393	0.695	-14.209	-2.044	0.041	0.792	2.968	0.004	0.947	2.774	0.007
	Treatment - Pesticide	-17.876	-7.179	<0.001	-4.809	-7.729	<0.001	0.078	2.483	0.015	0.062	1.548	0.126
	ITS	10.250	2.344	0.022	3.250	2.208	0.027	0.003	0.051	0.959	0.040	0.568	0.572
	Treatment * ITS	-7.288	-0.829	0.411	-0.059	-0.168	0.866	-0.134	-1.223	0.226	-0.048	-0.334	0.739
Full Dataset (n=67)	Intercept	-8.14	-0.347	0.735	-14.18	-1.951	0.051	0.860	2.936	0.005	1.060	4.511	<0.001
	Treatment - Pesticide	-18.50	-6.903	<0.001	-5.10	-6.609	<0.001	0.077	2.296	0.025	0.050	1.855	0.068
	ITS	10.337	2.135	0.036	3.77	2.120	0.034	-0.010	-0.166	0.868	0.006	0.124	0.902
	Treatment * ITS	-12.45	-1.236	0.222	-0.91	-0.255	0.799	-0.226	-1.813	0.074	-0.149	-1.448	0.152
Subset (n=53)	Intercept	-23.85	-0.522	0.600	-13.59	-0.938	0.348	-0.065	-0.124	0.902	0.419	0.578	0.566
	Treatment - Pesticide	-17.19	-5.618	<0.001	-5.11	-5.016	<0.001	0.103	2.954	0.005	0.074	1.580	0.120
	ITS	13.26	1.412	0.164	3.60	0.031	0.303	0.174	1.603	0.115	0.148	0.997	0.324
	Treatment * ITS	-40.96	-2.242	0.029	-14.16	-1.720	0.085	-0.166	-0.764	0.448	-0.227	-0.722	0.474

Note: In all cases, bees were only considered in analyses if they had an 'ideal' tag fitting (tag rating 1) and (Additional Dataset) considered all bees that fed, regardless of how long they fed for (*control* = 37, *pesticide* = 32). The bottom row considered all bees that fed > 60 seconds (*control* = 35, *pesticide* = 32). The bottom row considered all bees that fed > 60 seconds with the smallest 10% and largest 10% of workers removed (*control* = 26, *pesticide* = 26).

summary output for the effect of neonotid pesticide exposure on worker food between these two variables on key flight performance metrics.

Appendix B | Chapter 3 Supporting Information

Feeding protocol justification

The feeding setup was designed so that bees had an *ad libitum* sucrose supply when in the feeding tube with the opportunity to feed multiple times over the 5-minute feeding period. This is similar to previous feeding setups aiming to achieve satiation (Ben-Shahar & Robinson, 2001). Furthermore, when examining *Bombus terrestris* workers foraging in natural conditions, Goulson and colleagues (2002) found that workers collected an average load mass of nectar equal to 23% of unladen worker body mass. In my study, the mean mass of sucrose consumed was 56 mg and the mean wet mass of workers before feeding was 232 mg, which corresponds to workers feeding 24% of their body mass. My results are thus suggestive that workers did feed to satiation as intended.

Feeding results

Sucrose consumption per worker significantly increased with body mass (LM: total: $t_{310} = 10.04$, $p < 0.001$ & rate: $t_{310} = 7.99$, $p < 0.001$ respectively; Figure B2), with a mean ($\pm$ s.e.m) of 55.6 ± 1.38 mg consumed per worker over the 5-minute feeding period. Sucrose consumption rate scaled with worker dry mass (M) to the power 0.94 ($M^{0.94}$; Figure B2), with 95% confidence limits around this exponent ranging from 0.71 to 1.17, which encompasses the 0.75 scaling exponent predicted under metabolic scaling theory (J. Brown et al., 2004; West et al., 1997).

Month	Mean Air Temperature (°C)	Minimum Air Temperature (°C)	Maximum Air Temperature (°C)
April	11.17	-4.05	28.09
May	14.17	0.45	27.86
June	17.33	5.73	32.49
July	19.87	6.27	35.67
August	18.49	5.03	32.34
September	15.70	-1.01	29.95

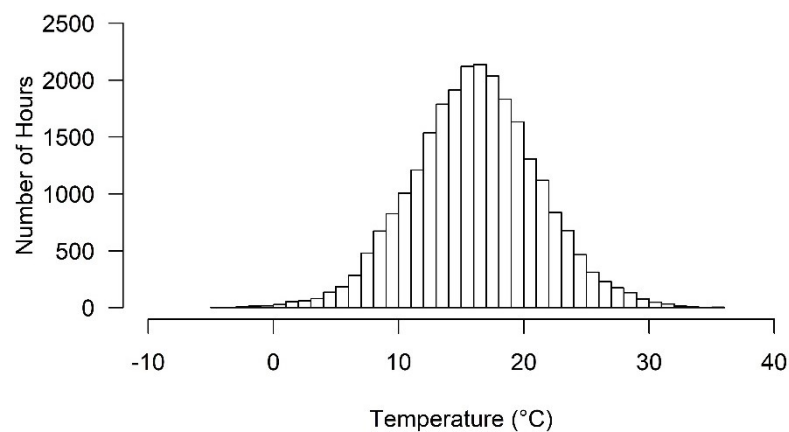


Figure B1. (*Top panel*) Overview of air (ambient) temperature during the months April to September (foraging season for workers of most temperate bumblebee spp.) at Silwood Park (Ascot, UK) over ten-year period from 2010 to 2019, considering only the hours 07:00-19:00 (foraging period). (*Bottom Panel*) Histogram of the number of hours in which temperatures were experienced during the same period. The mean temperature over this entire period was 16.14°C.

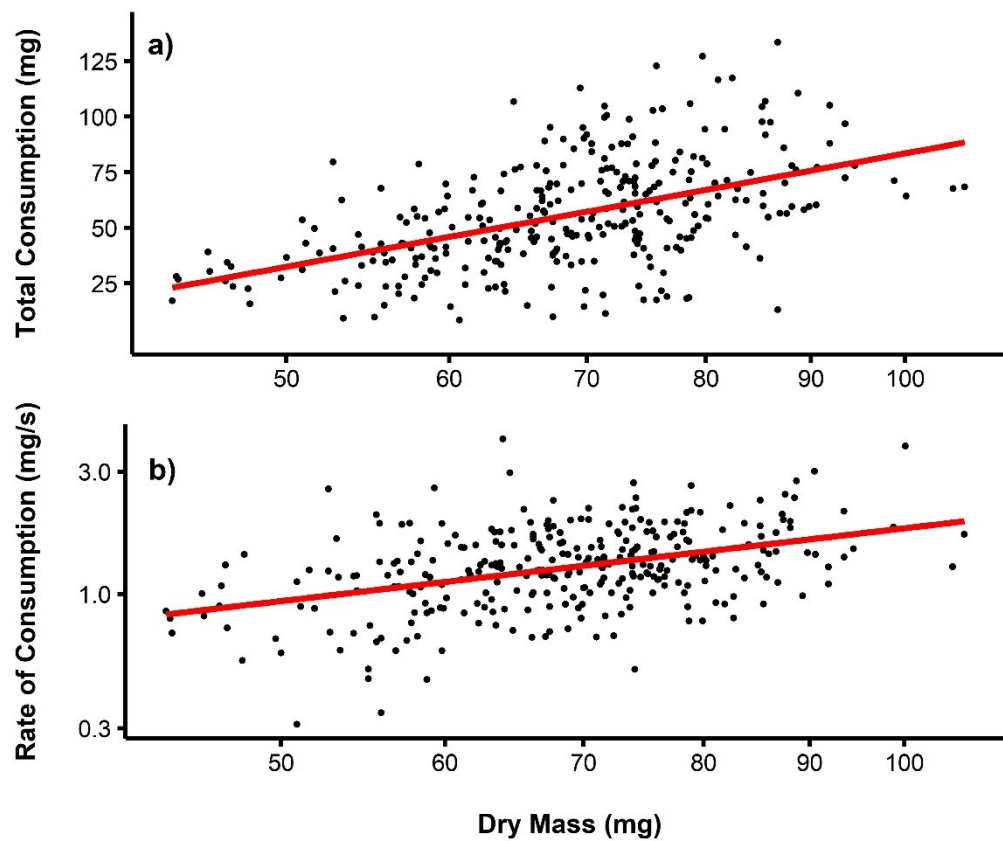


Figure B2. Scatter plot showing the relationship between worker dry mass (mg) with feeding behaviour. a) total sucrose consumption (mg) per worker over the course of the 5-minute feeding trial; b) sucrose consumption rate (mg of sucrose consumed per second of observed feeding). Dry mass and rate of sucrose consumption have been plotted on $\log_{10}$ scale, as these variables were log-transformed in the data analysis, Red lines represent linear model (LMM) regression lines ($p < 0.05$ in both cases).

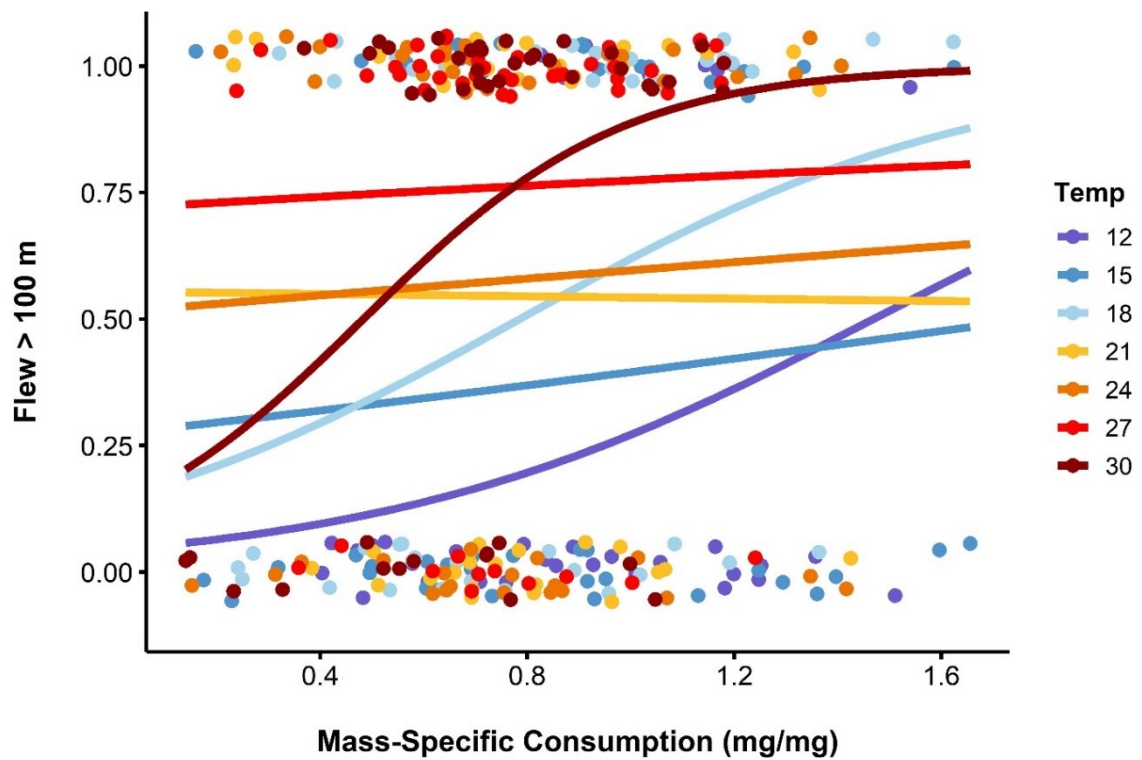


Figure B3. Effect of mass-specific consumption on the likelihood of successful flight (flew $\geq$ 100 m) for each temperature cohort, shown by a logistic regression plot (successful = 1; unsuccessful = 0). Jitter has been added in the y-plane so that individual data points are clearly identifiable.

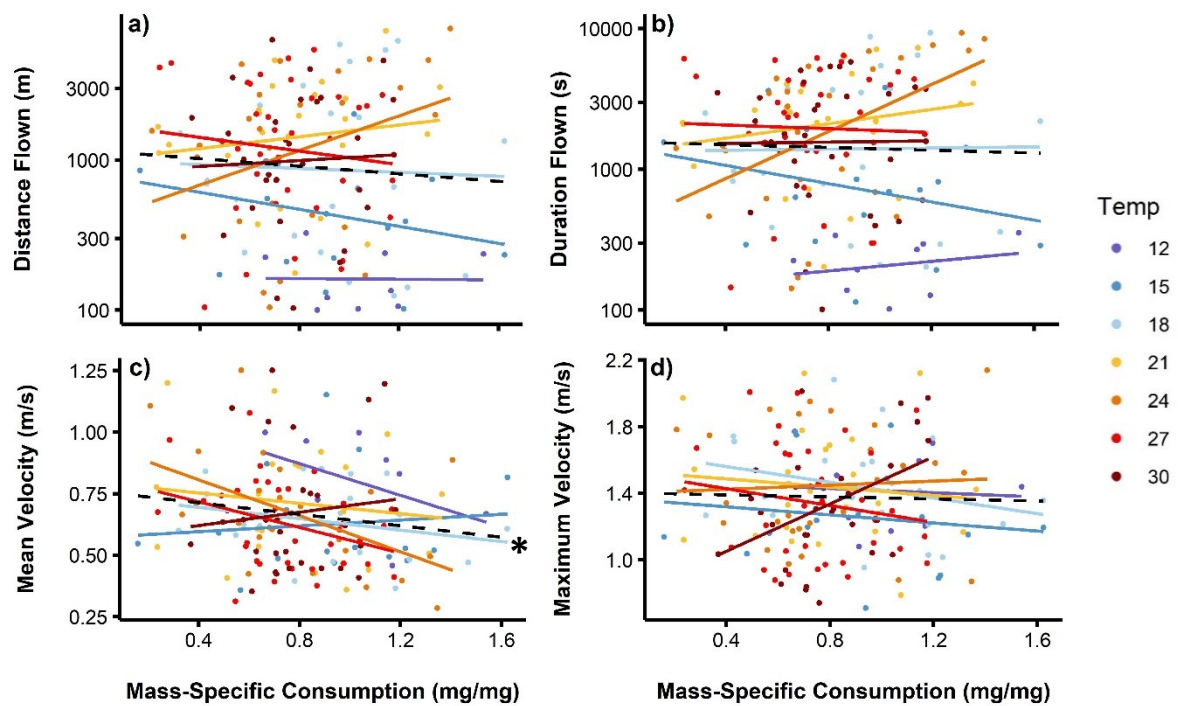


Figure B4. Relationships between mass-specific consumption and the different flight metrics measured at each tested ambient temperature. a) flight distance (metres); b) flight duration (seconds); c) mean velocity (m/s) and d) maximum velocity (m/s) flown over the whole flight trial. In all cases, dashed black lines indicate fitted lines from linear models, with an asterisk highlighting a significant ($p < 0.05$) relationship. In panels a) and b), distance and duration flown have been plotted on a log₁₀ scale, as these variables were log-transformed in the data analysis. Confidence intervals have not been plotted around model lines to aid visualization.

Table B1. Pairwise comparisons by Tukey contrasts, testing the likelihood of successful flight (flight > 100 m) between temperature cohorts. Adjusted p-values are reported, correcting for multiple testing through the single-step method. Tukey contrasts were derived from GLM examining main effects of Temperature (°C) and Dry Body Mass (mg)*. *variable log₁₀ transformed.

Pairwise Comparison	<i>Estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
12 – 15	0.892	0.546	1.632	0.659
12 – 18	1.330	0.542	2.454	0.175
12 – 21	1.556	0.544	2.860	0.063
12 – 24	1.708	0.546	3.127	0.029
12 – 27	2.787	0.590	4.727	<0.001
12 – 30	2.293	0.566	4.055	<0.001
15 – 18	0.438	0.471	0.931	0.967
15 – 21	0.664	0.470	1.412	0.794
15 – 24	0.816	0.472	1.728	0.595
15 – 27	1.895	0.514	3.691	0.004
15 – 30	1.402	0.490	2.859	0.064
18 – 21	0.226	0.465	0.486	0.999
18 – 24	0.378	0.467	0.809	0.984
18 – 27	1.457	0.508	2.870	0.062
18 – 30	0.963	0.485	1.987	0.421
21 – 24	0.152	0.465	0.326	1.000
21 – 27	1.231	0.504	2.442	0.180
21 – 30	0.737	0.482	1.529	0.726
24 – 27	1.080	0.505	2.136	0.330
24 – 30	0.586	0.484	1.211	0.889
27 – 30	-0.494	0.517	-0.955	0.963

Table B2. Pairwise comparisons by Tukey contrasts, testing a) Flight Distance (meters)* and b) Flight Duration (seconds)* between temperature cohorts. Adjusted p-values are reported, correcting for multiple testing by the single-step method. Tukey contrasts were derived from LMER examining main effects of Temperature (°C) and Dry Body Mass (mg)*. *variable $\log_{10}$ transformed.

a) Flight Distance				
Pairwise Comparison	<i>Estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
12 – 15	1.239	0.417	2.967	0.045
12 – 18	1.922	0.401	4.792	<0.001
12 – 21	2.405	0.396	6.079	<0.001
12 – 24	2.261	0.395	5.725	<0.001
12 – 27	2.329	0.384	6.062	<0.001
12 – 30	2.156	0.389	5.538	<0.001
15 – 18	0.683	0.323	2.113	0.337
15 – 21	1.166	0.317	3.675	0.004
15 – 24	1.022	0.313	3.268	0.018
15 – 27	1.090	0.298	3.663	0.004
15 – 30	0.917	0.303	3.027	0.038
18 – 21	0.483	0.295	1.636	0.651
18 – 24	0.339	0.291	1.166	0.904
18 – 27	0.407	0.274	1.486	0.747
18 – 30	0.234	0.280	0.835	0.981
21 – 24	-0.144	0.284	-0.508	0.999
21 – 27	-0.076	0.267	-0.286	1.000
21 – 30	-0.249	0.273	-0.911	0.970
24 – 27	0.068	0.259	0.263	1.000
24 – 30	-0.105	0.266	-0.393	1.000
27 – 30	-0.173	0.247	-0.699	0.992

b) Flight Duration				
Pairwise Comparison	<i>Estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
12 – 15	1.455	0.420	3.468	0.009
12 – 18	2.119	0.403	5.258	<0.001
12 – 21	2.502	0.398	6.293	<0.001
12 – 24	2.419	0.397	6.096	<0.001
12 – 27	2.528	0.386	6.549	<0.001
12 – 30	2.301	0.391	5.878	<0.001
15 – 18	0.665	0.325	2.046	0.378
15 – 21	1.047	0.319	3.282	0.017
15 – 24	0.964	0.314	3.069	0.034
15 – 27	1.073	0.299	3.589	0.006
15 – 30	0.846	0.304	2.779	0.077
18 – 21	0.382	0.297	1.288	0.853
18 – 24	0.300	0.292	1.027	0.946
18 – 27	0.409	0.275	1.487	0.746
18 – 30	0.181	0.281	0.644	0.995
21 – 24	-0.082	0.285	-0.289	1.000
21 – 27	0.027	0.268	0.100	1.000
21 – 30	-0.201	0.275	-0.731	0.990
24 – 27	0.109	0.260	0.420	1.000
24 – 30	-0.118	0.268	-0.443	1.000
27 – 30	-0.228	0.248	-0.917	0.969

Table B3. Pairwise comparisons by Tukey contrasts, testing (a) Mean Velocity (m/s) and (b) Maximum Velocity (m/s) between temperature cohorts. Adjusted p-values are reported, correcting for multiple testing by the single-step method. Tukey contrasts were derived from LMER examining main effects of Temperature (°C), Dry Body Mass (mg)*, and Mass-Specific Consumption (mg/mg). *variable $\log_{10}$ transformed.

a) Mean Velocity				
Pairwise Comparison	<i>Estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
12 – 15	-0.155	0.082	-1.892	0.477
12 – 18	-0.158	0.079	-2.010	0.399
12 – 21	-0.097	0.078	-1.240	0.874
12 – 24	-0.127	0.078	-1.627	0.657
12 – 27	-0.169	0.077	-2.208	0.283
12 – 30	-0.116	0.078	-1.499	0.739
15 – 18	-0.003	0.063	-0.047	1.000
15 – 21	0.058	0.062	0.933	0.966
15 – 24	0.028	0.061	0.453	0.999
15 – 27	-0.014	0.058	-0.241	1.000
15 – 30	0.039	0.059	0.653	0.995
18 – 21	0.061	0.058	1.052	0.940
18 – 24	0.031	0.057	0.539	0.998
18 – 27	-0.011	0.054	-0.207	1.000
18 – 30	0.042	0.055	0.757	0.988
21 – 24	-0.030	0.055	-0.547	0.998
21 – 27	-0.072	0.052	-1.388	0.803
21 – 30	-0.019	0.053	-0.362	1.000
24 – 27	-0.042	0.050	-0.832	0.981
24 – 30	0.011	0.052	0.211	1.000
27 – 30	0.053	0.048	1.095	0.927

b) Maximum Velocity				
Pairwise Comparison	<i>Estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
12 – 15	-0.053	0.130	-0.412	1.000
12 – 18	0.113	0.124	0.906	0.971
12 – 21	0.105	0.124	0.847	0.979
12 – 24	0.146	0.124	1.177	0.900
12 – 27	0.047	0.121	0.384	1.000
12 – 30	0.044	0.123	0.355	1.000
15 – 18	0.166	0.099	1.670	0.628
15 – 21	0.158	0.098	1.615	0.665
15 – 24	0.199	0.097	2.062	0.367
15 – 27	0.100	0.092	1.080	0.932
15 – 30	0.097	0.094	1.037	0.943
18 – 21	-0.008	0.091	-0.082	1.000
18 – 24	0.033	0.090	0.369	1.000
18 – 27	-0.066	0.085	-0.775	0.987
18 – 30	-0.069	0.087	-0.793	0.985
21 – 24	0.041	0.087	0.467	0.999
21 – 27	-0.059	0.082	-0.714	0.991
21 – 30	-0.061	0.084	-0.731	0.990
24 – 27	-0.099	0.080	-1.248	0.871
24 – 30	-0.102	0.082	-1.248	0.871
27 – 30	-0.003	0.076	-0.038	1.000

Table B4. Colony-level flight potential values for each tested temperature, which is a metric that encapsulates both the distance workers can fly (i.e., resources a worker could reach in a landscape) and the likelihood of flight (i.e., size of the foraging workforce). To this end, colony-level flight potential was calculated as the proportion of workers that successfully flew (flew ≥ 100 m) multiplied by the mean distance flown (m) by workers at that temperature.

Temperature (°C)	No. of workers tested on mills	No. of Successful fliers	Proportion of workers that successfully flew	Mean Distance of Successful fliers (m)	Colony-level flight potential
12	44	9	0.205	176.76	36.24
15	45	17	0.378	604.87	228.64
18	43	22	0.512	1762.18	902.24
21	44	24	0.545	1965.17	1071.02
24	45	26	0.578	2046.35	1182.79
27	46	35	0.761	1815.96	1381.95
30	45	31	0.689	1540.05	1061.09

Appendix C | Chapter 4 Supporting Information

Experiment 2: Piloting the feeding cage setup

A pilot trial was run to determine if feeding cages should contain solitary or multiple individuals, whilst also determining the volume of sucrose solution different worker densities would need throughout the main trials to ensure *ad libitum* provision. Using 27 feeding cages, I monitored nine cages each containing one worker, nine each containing two workers, and nine each containing three workers. Workers selected for the trial were from the same nine colonies used in the main experimental trial, and of similar body size based off visual inspection. The feeding cages were held under the same light and humidity conditions as the main trials, and at 21°C as workers would have the greatest demand for sucrose solution at this ambient temperature (based off results of experiment 1). Each feeding cage was provided *ad-libitum* untreated 40% w/w sucrose/water solution, with consumption recorded every 24 hours.

Following the same protocol used in the main trials, feeding cages were filmed every 24 hours for a total period of 120 hours under a 'no-stimulation' scenario, with 20 seconds of video recordings examined using Kinovea (version 0.8.15). From this I assessed (yes/no) whether a key set of behaviours (movement, flight, and feeding) for each worker occurred, alongside calculating walking distance (cm).

Workers kept in feeding cages individually had a higher mean walking distance compared to workers kept in feeding cages in groups of two or three (Figure C1c). However, solitary workers were considerably more likely to fly relative to three worker cages (40-50% vs 20-25% respectively; Figure C1b). In terms of walking distances, workers kept in cages in groups of three showed less variation and remained consistent in their behaviour over time (Figure C1c). Furthermore, workers kept in cages of three showed little difference in behaviour if one bee died (Figure C1). Given my intention to track walking behaviour, it was deemed appropriate to use three worker cages. The pilot trial showed workers to feed between 0.4 - 0.5 ml of sucrose solution per day, justifying the decision to provide a 2 ml feeder filled to maximum capacity with sucrose solution every 24 hours in the main trial. Indeed, during the main trial, no feeder ran dry.

Experiment 2: Piloting the camera setup

To feasibly record all cages daily, I needed to film two pots concurrently within each video. The video camera used (Panasonic HC-V160) had an image resolution of 3968 x 2232 pixels (width x height), meaning that two pots could comfortably fit in the frame when the camera was held at a height that

allowed clear view of the bees in the pots. This meant that the central point of each pot was at an angle of 14° from the central point of the camera lens (Figure 4.1). As filming on an angle could potentially distort the length of a line, checks were run to see how much error would be introduced taking this approach. Following the 'Tracking Protocol' (described in main methods and section below), I measured the distance of a series of lines that spanned various directions across the bottom surface of a pot with a known total distance of 350mm (estimated maximum distance a bee would walk in the trials based on pilot observations). These measurements were taken on images of a pot under two scenarios: 1) when the pot was placed directly beneath the camera lens; and 2) when the centre of the pot was at an angle of 14° to the center of the camera lens (Figure C2). I found the difference in total measured line distance between the two scenarios to be negligible (mean $\pm$ 95% CI = $0.3 \pm 0.6\%$), justifying the protocol of filming two pots simultaneously.

Experiment 2: Tracking Protocol

The following gives a detailed stage-by-stage overview of how walking paths were tracked (only for bees recorded to have moved) using the video tracking software Kinovea (version 0.8.15), and subsequently how measures of walking distance were calculated.

The tracking process was as follows: 1) calibrate videos by assigning a distance (cm) value to each pixel based off a known standard. For this purpose, each video had a ruler included as a scale; 2) cross-reference the first image in the video with the manual note of worker positions made during the trials to ascertain identities of all workers in the video. Note that a picture (jpeg) was also taken prior to the video recording using the same Panasonic HC-V160 camera that video recordings were made with. The enhanced resolution of the .jpeg image meant that tag numbers were visible and could be used to identify workers as a backup in case manual notes weren't sufficient; 3) play the video to the 4-second mark (frame 26 when run at 6.25 fps) whilst visually monitoring each individual and mark down the positions; 4) set the working zone in Kinovea from frame 26 (4:00 time) to frame 127 (20:16 time), because the track path software always terminates the tracked distance on the frame preceding the end of the working zone (i.e., frame 126); 5) place a tracking marker in the centre of the thorax on the bottom edge of the thoracic stripe; 6) commence tracking by starting the video, manually moving the tracking marker back to the correct position on the thorax for any frame necessary, and run the tracking through to the end of the 20-second window at which point the distance walked value can be displayed and recorded; 7) if the bee is flying to begin with then start the tracking from the point at which it stops. If the bee starts to fly during the tracking, then stop the track path at the point it starts flying and start a new track path once it stops flying. If the bee goes under the filter paper or starts to

feed at any point then follow the same process as above relating to flying; 8) save the tracking coordinates as a text document.

Note that workers that had multiple tracking coordinate text files for a single video, due to their tracking being broken up into multiple phases by flying, feeding, or going under filter paper, had one walking distance, one rate, and one speed value that incorporated all phases of their tracking.

Walking distance (cm) was calculated from this tracking coordinate text file by creating a SpatialLines object in R v3.6.2 (R Core Team 2019) and using the SDraw package (McDonald 2020) to determine the objects length. It is important to note that the distance calculated using the SDraw package differs very slightly from the Kinovea distance display output due to different calculation methods. The SDraw distances were used for all analyses in this study because these were able to be repeatedly calculated using the saved coordinate text files. To support this decision, the SDraw distances were compared with the Kinovea distances to verify that there were no obvious errors.

Experiment 2: Video Processing

When carrying out walking tracking, videos were condensed down from 25 fps MP4 files to 6.25 fps AVI files using a bespoke MATLAB script (version R2020a), which worked by extracting every 4th frame out of original 25 fps videos and then stitching the remaining frames back together. Due to the manual supervision required for the tracking protocol, reducing the frame rate was highly beneficial as is dramatically lowered time demands. However, reducing the frame rate lowers the definition, and by cutting out frames you potentially lose information which can affect the overall accuracy of measurements. Therefore, a sample of eight workers from the main trials had their walking distances tracked (following the same protocol as described in the 'Tracking Protocol' section above) over a 20-second period at both 25 and 6.25 fps. These eight videos were selected such that the workers were observed not to fly or crawl under the filter paper during the course of the video recordings, and such that a range of walking distances were tested spanning the range of walking distances observed in the study.

In Figure C3a, the red dashed line shows a 1:1 line (i.e., slope of 1 and intercept of 0), which represents the ideal scenario where tracked distances are identical at both frame rates. The blue line is the regression line for the actual tracking data. For the 6.25 fps videos, the slope is close to 1 (the 95% confidence limits actually cover 1) and the r^2 is almost indistinguishable from 1 (indicating a linear relationship as opposed to an exponential relationship), which would suggest that over the range of

distances workers are likely to walk in this study, there is no loss of accuracy using videos condensed down to 6.25 frames per second.

Tracking measurements were then examined for repeatability. The 6.25 fps videos were tracked for a second time, and the walking distances from these two tracking runs were compared against each other. In Figure C3b, the dashed red line indicates the ideal scenario (slope=1, intercept=0) where each workers' tracked distances were identical for both runs. The regression line has a slope close to 1 and an r^2 of 0.999, indicating that the walking distances matched up well and there was no variation in error as walking distances increased. Based on these results, tracking in the main trials was carried out on videos condensed down to 6.25 fps, and within every video each worker was tracked just once as repeated measurements were not deemed necessary.

Experiment 2: Model Selection

Likelihood of movement

The maximal biologically relevant model was initially constructed considering the main effects of pesticide, temperature, time, all possible interactions between these three categorical variables, and the continuous covariate of dry body mass. Due to non-convergence, however, the maximal model was simplified to examine each pesticide separately, which was deemed appropriate as the control treatments showed no significant change in likelihood of movement over time (Table C5 & C10).

Walking rate

The maximal biologically relevant model was again initially constructed considering the main effects of pesticide, temperature, and time, every interaction possible between these three categorical variables, and also the additional continuous covariate of dry mass. However, even when applying a square root transform to the response variable, which was estimated as the optimal transformation (exponent of 0.47) from the Box-Cox test in the 'EnvStats' package (Millard 2013), key assumptions of the model were broken (normality of residuals and homogeneity of variance), and no other distribution was found to adequately explain the variance in the data. As such, and consistent with the likelihood of movement analysis, the maximal model was again simplified to examine each pesticide separately, which was deemed appropriate as the control treatments showed no significant change in likelihood of movement over time (Table C15).

Experiment 3: Calculating Flight Metrics

Total distance flown during the whole flight test was calculated as the total number of circuits flown multiplied by the circuit circumference (0.848 m). Total duration of the whole flight test was calculated by summing all circuit interval times

Mean flight velocity, calculated as the total distance flown divided by the total duration flown, and maximum flight velocity, calculated by taking the highest velocity circuit that was recorded, were determined for each bee over four periods of flight. These periods were: i) 0-100m, characterised by comparatively high velocities immediately following the start of the flight mill test, which was followed by rapid termination of flight and a rapid decline in velocity; ii) 100-500m, characterised by a decrease in rate of flight termination and a steady decline in flight velocity; iii) 500-1000m, characterised by velocity levelling off and settling into a steady rhythm of flight; and iv) 1000-2000m, characterised by a low number of workers still in flight and exhibiting increasing and more erratic flight velocity (Figure 4.11). For each flight period, bees only had their velocity calculated if they had flown the full flight period (i.e., only bees that had flown ≥ 500 m would have their velocity calculated for the 100-500 m analysis), in order to allow fair comparison (Table C3). The fourth flight period only went up to 2000 m to ensure that enough workers were still flying within each pesticide-temperature treatment, and thus could be included in our analysis, to allow statistically suitable comparisons.

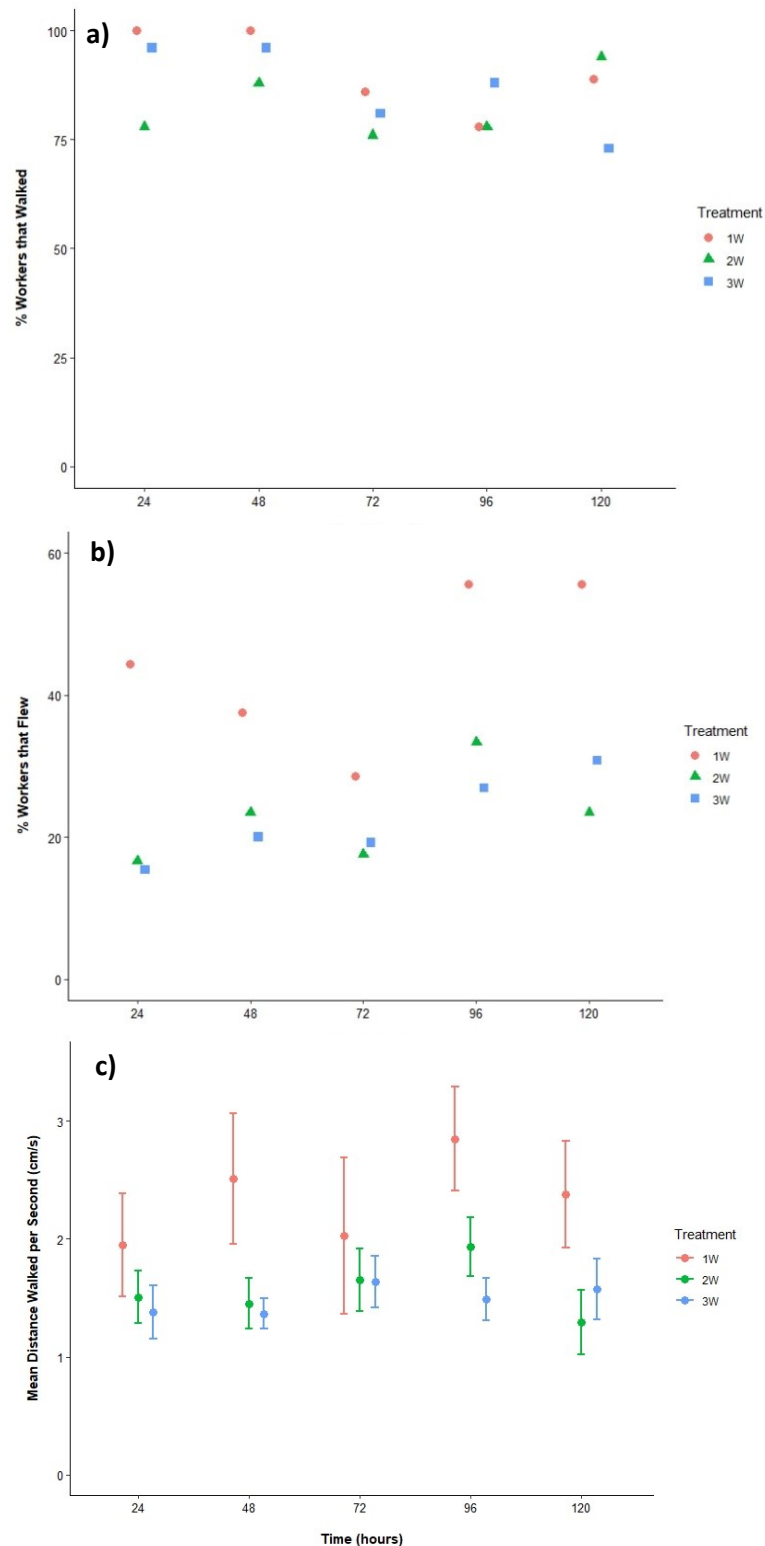


Figure C1. Experiment 2: The percentage of workers that were recorded to have walked (a) and flown (b) in the pilot trials, as well as the mean walking rate (cm/s) of workers in pilot trials (c). Mean distances calculated only from workers that did walk (i.e., excluded bees that remained completely stationary over whole period) and were visible during the trial (i.e., weren't under the filter paper). Error bars show standard error.

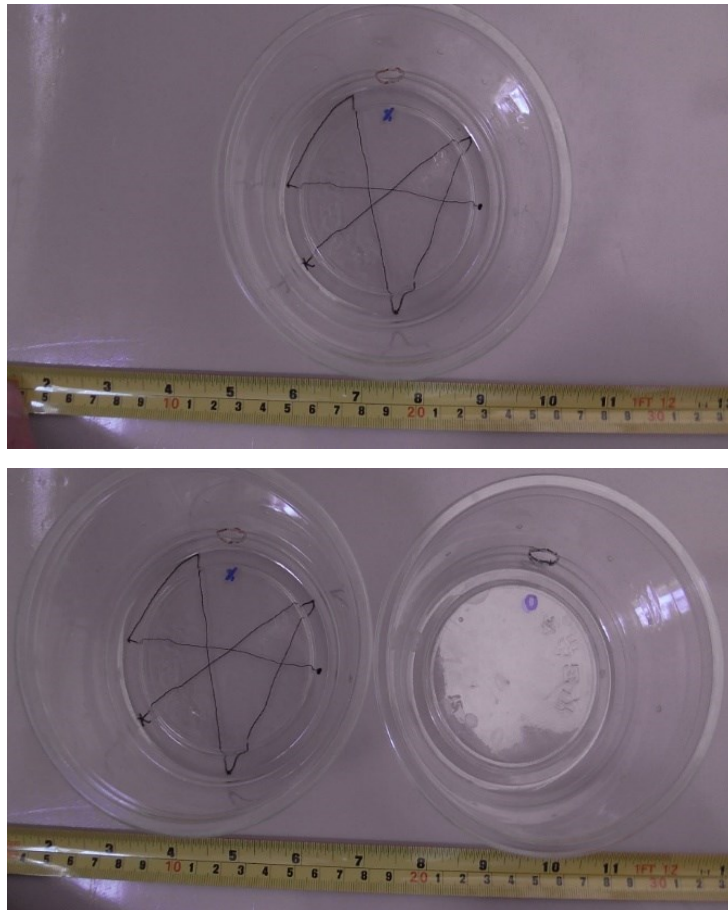


Figure C2. Experiment 2: Images used to assess if pot placement affected the measured distance of identical length lines.

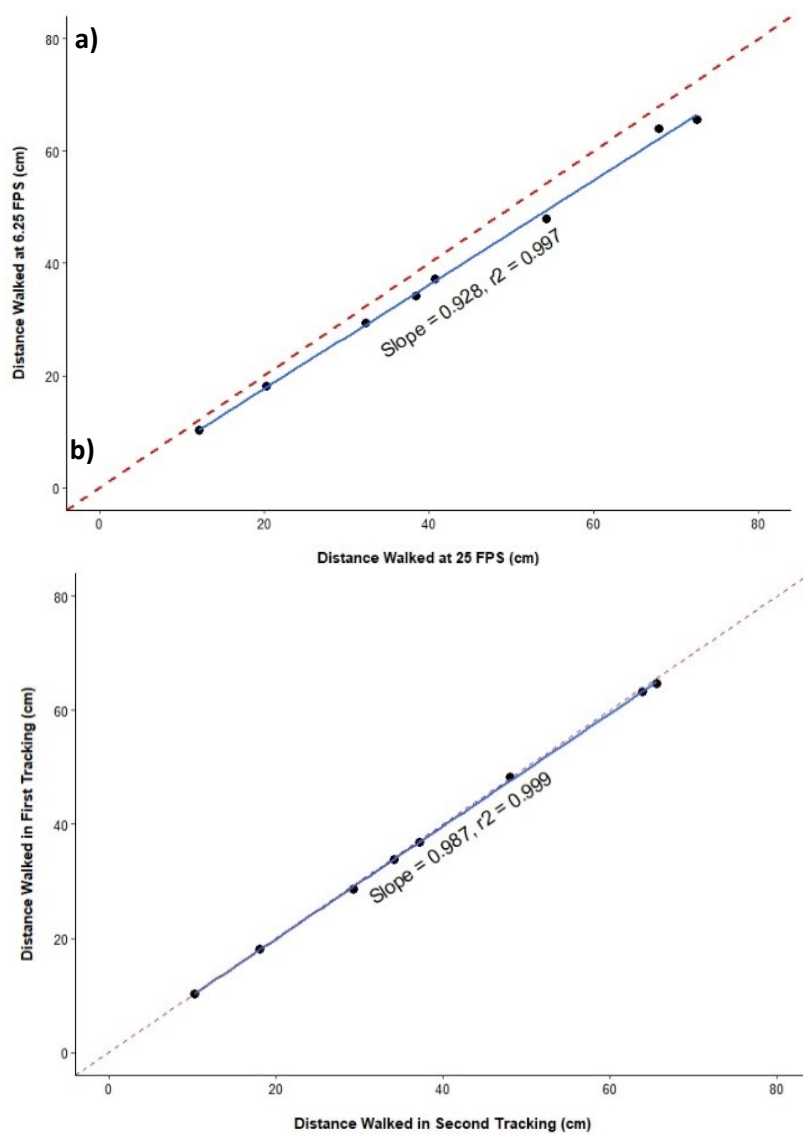


Figure C3. Experiment 2: Pilot data to validate video processing method. The walking distances of 8 workers were measured: a) at 25 fps vs 6.25 fps; and b) at 6.25 fps from two repeated measurements.

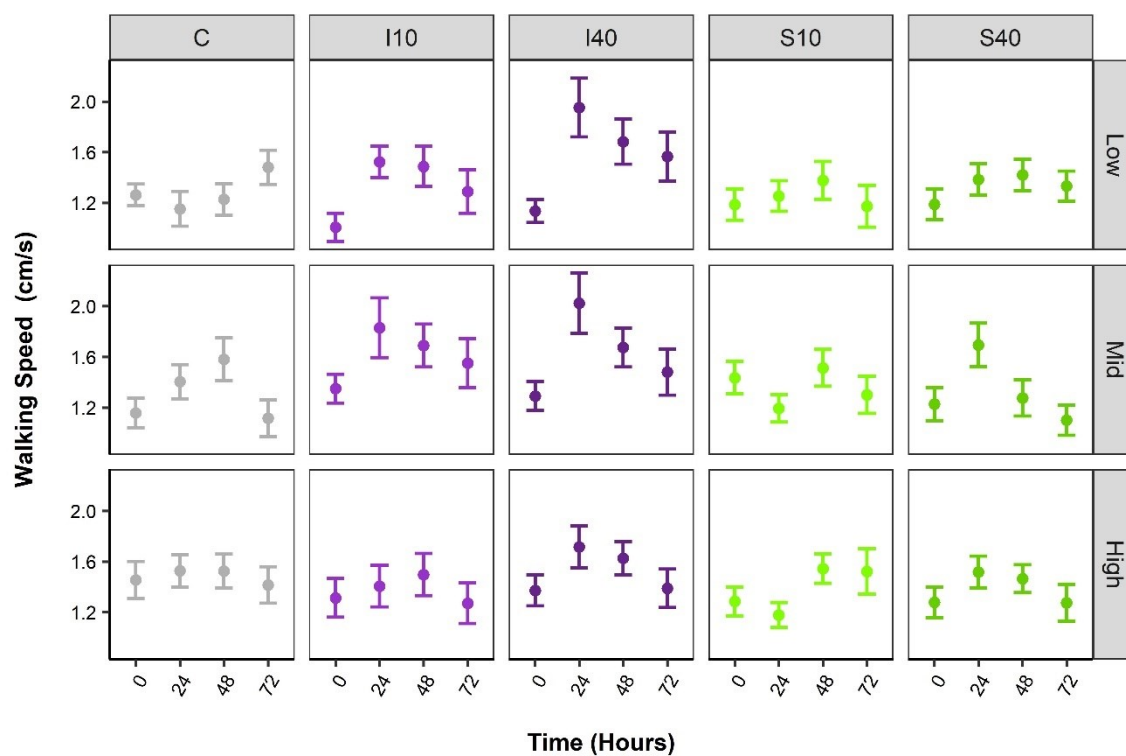


Figure C4. Experiment 2: Mean walking speed (cm/s) over time with associated standard error bars. Walking speed was calculated as distance walked per second of being active rather than per second of being visible (with the latter being termed walking rate).

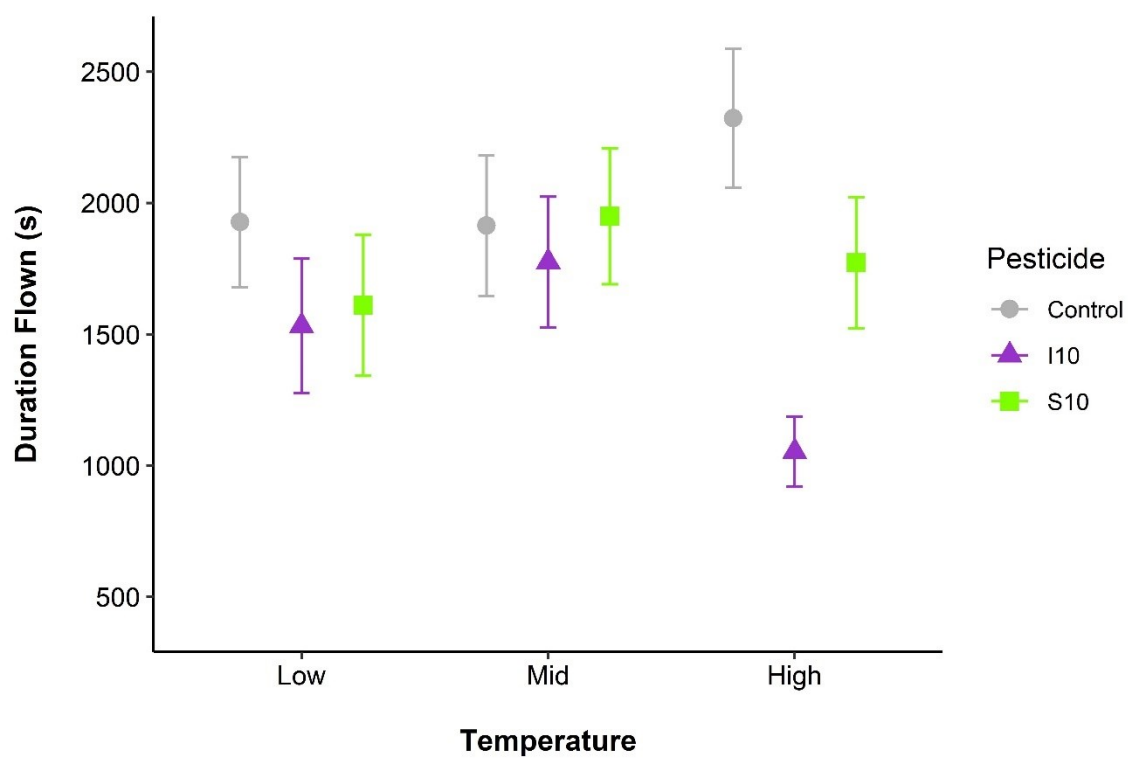


Figure C5. Experiment 3: Mean flight duration (secs) for each temperature-pesticide treatment, considering all workers that were classed as 'positive' fliers (i.e., had filtered flight distance > 0 m), with associated standard error.

Table C1. For each study, trials were conducted in controlled environment (CE) rooms, which were able to consistently hold the desired test temperatures (°C) and 60% relative humidity (RH). Temperature and RH recordings were taken every minute throughout the course of the trials, allowing constant monitoring of conditions.

Study	Target Temp (°C)	Mean Temp (°C)	SD Temp	Mean Humidity (RH)	SD Humidity
Responsiveness	21	21.000	0.041	60.004	0.160
	27	26.998	0.048	59.999	0.186
	30	30.000	0.043	59.998	0.0161
Walking	21	21.000	0.059	61.021	2.173
	27	27.000	0.094	59.999	0.698
	30	30.000	0.039	59.919	1.399
Flight	21	21.006	0.057	60.083	0.698
	27	27.001	0.060	60.003	0.244
	30	30.003	0.070	60.002	0.331

Table C2. Experiment 3: The order of testing for flight trials (order was identical for each temperature). Red numbers refer to the colony that the bee is coming from.

Day 1	Bout	Control		Imidacloprid		Sulfoxaflor	
		Mill 1	Mill 2	Mill 3	Mill 4	Mill 5	Mill 6
21°C	1	1	2	1	3	2	3
	2	3	1	2	3	1	2
	3	2	3	1	2	3	1
	4	2	1	3	1	3	2

Day 2 = 27°C, Day 3 = 30°C

Day 4	Bout	Control		Imidacloprid		Sulfoxaflor	
		Mill 5	Mill 6	Mill 1	Mill 2	Mill 3	Mill 4
21°C	1	1	3	2	3	1	2
	2	2	3	1	2	3	1
	3	1	2	3	1	2	3
	4	3	1	3	2	2	1

Day 5 = 27°C, Day 6 = 30°C

Day 7	Bout	Control		Imidacloprid		Sulfoxaflor	
		Mill 3	Mill 4	Mill 5	Mill 6	Mill 1	Mill 2
21°C	1	2	3	1	2	1	3
	2	1	2	3	1	2	3
	3	3	1	2	3	1	2
	4	3	2	2	1	3	1

Day 8 = 27°C, Day 9 = 30°C

Day 10	Bout	Control		Imidacloprid		Sulfoxaflor	
		Mill 1	Mill 2	Mill 3	Mill 4	Mill 5	Mill 6
21°C	1	1	2	3	1	2	3
		3	1	2	3	1	2
	2	3	1	2	3	1	2
		1	2	3	1	2	3
	3	3	1	2	3	1	2
		1	2	3	1	2	3

Day 11 = 27°C, Day 12 = 30°C

Table C3. Experiment 3: Overview of the number of bees that were used at each stage of the experiment and analysis in each temperature-pesticide combination (Temp: Low = 21°C, Mid = 27°C, High = 30°C; Pesticide: Control = Untreated, I10 = Imidacloprid 10 µg/L, S10 = Sulfoxaflor 10 µg/L).

Filter Stage	Low Control	Low I10	Low S10	Mid Control	Mid I10	Mid S10	High Control	High I10	High S10	Total
Total at start	60	60	60	60	60	60	60	60	60	540
Visibly Unhealthy	0	0	0	2	1	0	0	0	0	3
Didn't feed	4	3	2	2	2	4	2	1	2	22
Technical issues	0	0	0	2	2	2	0	0	0	6
Flight data acquired	56	57	58	54	55	54	58	59	58	509
Cleaned flight = 0m	1	1	2	0	2	1	1	3	2	13
Bees that flew > 0m	55	56	56	54	53	53	57	56	56	496
Flew > 100m	38	29	32	42	39	38	50	44	45	357
Flew > 500m	33	24	25	28	27	29	34	28	30	258
Flew > 1000m	27	19	19	24	23	23	30	15	20	200
Flew > 2000m	16	15	15	14	11	14	19	3	13	120

Table C4. Experiment 2: Cumulative mortality over the course of the trial for each treatment. The numbers represent the total number of bees that were classed as dead within each treatment after each period (i.e., total dead at that time, not the number of new dead bees within that time window).

Treatment		Hour					
Temperature	Pesticide	20	44	68	92	116	140
Low	Control	0	0	0	0	0	0
Mid	Control	0	0	1	1	1	2
High	Control	0	0	0	2	3	3
Low	I10	0	0	0	0	0	0
Mid	I10	0	0	0	0	0	0
High	I10	0	0	0	1	1	2
Low	I40	0	0	0	0	0	1
Mid	I40	0	0	0	1	1	1
High	I40	0	0	0	1	3	3
Low	S10	0	0	0	0	0	0
Mid	S10	0	0	0	0	1	2
High	S10	0	0	0	0	0	1
Low	S40	0	0	0	1	1	1
Mid	S40	0	0	0	2	3	3
High	S40	0	0	1	2	3	3

Table C5. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for Control workers under the no-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-0.643	0.491	-1.310	0.685
0 - 48 hours	0.752	0.577	1.305	0.688
0 - 72 hours	0.948	0.603	1.573	0.515
0 - 96 hours	-0.254	0.494	-0.515	0.986

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.099	0.472	0.209	0.999
0 - 48 hours	0.168	0.479	0.351	0.997
0 - 72 hours	0.035	0.466	0.075	1.0000
0 - 96 hours	0.214	0.480	0.446	0.992

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-0.668	0.541	-1.233	0.732
0 - 48 hours	0.113	0.570	0.199	0.999
0 - 72 hours	-0.404	0.550	-0.734	0.949
0 - 96 hours	-0.778	0.545	-1.428	0.609

Table C6. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for I10 workers under the no-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-0.089	0.462	-0.192	0.999
0 - 48 hours	-0.942	0.446	-2.114	0.214
0 - 72 hours	-1.536	0.454	-3.384	0.006
0 - 96 hours	-1.556	0.448	-3.472	0.005

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-1.002	0.500	-2.005	0.264
0 - 48 hours	-0.610	0.538	-1.132	0.790
0 - 72 hours	-1.293	0.502	-2.574	0.075
0 - 96 hours	-0.953	0.499	-1.910	0.312

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.378	0.477	0.791	0.933
0 - 48 hours	-0.426	0.445	-0.956	0.875
0 - 72 hours	-0.485	0.445	-1.091	0.812
0 - 96 hours	-0.965	0.445	-2.167	0.192

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.913	0.681	-1.342	0.180
21°C 0-24 hours : 30°C 0-24 hours	0.466	0.664	0.702	0.483
27°C 0-24 hours : 30°C 0-24 hours	1.380	0.691	1.997	0.046
21°C 0-48 hours : 27°C 0-48 hours	0.332	0.698	0.476	0.634
21°C 0-48 hours : 30°C 0-48 hours	0.516	0.629	0.820	0.412
27°C 0-48 hours : 30°C 0-48 hours	0.184	0.698	0.264	0.792
21°C 0-72 hours : 27°C 0-72 hours	0.244	0.673	0.362	0.718
21°C 0-72 hours : 30°C 0-72 hours	1.051	0.634	1.658	0.097
27°C 0-72 hours : 30°C 0-72 hours	0.808	0.670	1.206	0.228
21°C 0-96 hours : 27°C 0-96 hours	0.603	0.668	0.903	0.367
21°C 0-96 hours : 30°C 0-96 hours	0.591	0.629	0.939	0.348
27°C 0-96 hours : 30°C 0-96 hours	-0.013	0.667	-0.019	0.985

Table C7. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for 140 workers under the no-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-2.376	0.498	-4.770	<0.001
0 - 48 hours	-2.161	0.495	-4.363	<0.001
0 - 72 hours	-2.358	0.500	-4.720	<0.001
0 - 96 hours	-2.643	0.508	-5.204	<0.001

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-1.535	0.487	-3.152	0.014
0 - 48 hours	-0.713	0.495	-1.439	0.602
0 - 72 hours	-1.384	0.485	-2.852	0.035
0 - 96 hours	-1.747	0.486	-3.592	0.003

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-0.857	0.502	-1.706	0.430
0 - 48 hours	-0.525	0.506	-1.037	0.838
0 - 72 hours	-1.194	0.494	-2.415	0.111
0 - 96 hours	-1.469	0.492	-2.983	0.024

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	0.841	0.691	1.217	0.224
21°C 0-24 hours : 30°C 0-24 hours	1.519	0.704	2.158	0.031
27°C 0-24 hours : 30°C 0-24 hours	0.679	0.697	0.974	0.330
21°C 0-48 hours : 27°C 0-48 hours	1.448	0.699	2.073	0.038
21°C 0-48 hours : 30°C 0-48 hours	1.636	0.707	2.314	0.021
27°C 0-48 hours : 30°C 0-48 hours	0.188	0.708	0.266	0.791
21°C 0-72 hours : 27°C 0-72 hours	0.974	0.692	1.408	0.159
21°C 0-72 hours : 30°C 0-72 hours	1.164	0.699	1.666	0.096
27°C 0-72 hours : 30°C 0-72 hours	0.190	0.690	0.275	0.783
21°C 0-96 hours : 27°C 0-96 hours	0.896	0.696	1.288	0.198
21°C 0-96 hours : 30°C 0-96 hours	1.174	0.702	1.672	0.095
27°C 0-96 hours : 30°C 0-96 hours	0.278	0.688	0.404	0.687

Table C8. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for S10 workers under the no-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.184	0.459	0.400	0.995
0 - 48 hours	0.406	0.474	0.856	0.913
0 - 72 hours	0.237	0.464	0.510	0.986
0 - 96 hours	0.200	0.463	0.432	0.993

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.148	0.476	0.311	0.998
0 - 48 hours	0.326	0.481	0.678	0.961
0 - 72 hours	-0.158	0.464	-0.340	0.997
0 - 96 hours	-0.607	0.452	-1.342	0.665

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.695	0.581	1.195	0.754
0 - 48 hours	-0.074	0.515	-0.143	0.999
0 - 72 hours	-0.499	0.493	-1.012	0.850
0 - 96 hours	-0.714	0.488	-1.463	0.587

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.036	0.661	-0.054	0.957
21°C 0-24 hours : 30°C 0-24 hours	0.511	0.740	0.690	0.490
27°C 0-24 hours : 30°C 0-24 hours	0.547	0.751	0.728	0.467
21°C 0-48 hours : 27°C 0-48 hours	-0.080	0.675	-0.119	0.906
21°C 0-48 hours : 30°C 0-48 hours	-0.480	0.701	-0.685	0.493
27°C 0-48 hours : 30°C 0-48 hours	-0.400	0.705	-0.567	0.570
21°C 0-72 hours : 27°C 0-72 hours	0.394	0.656	-0.601	0.548
21°C 0-72 hours : 30°C 0-72 hours	-0.736	0.678	-1.086	0.278
27°C 0-72 hours : 30°C 0-72 hours	-0.341	0.677	-0.505	0.614
21°C 0-96 hours : 27°C 0-96 hours	-0.807	0.648	-1.246	0.213
21°C 0-96 hours : 30°C 0-96 hours	-0.914	0.673	-1.358	0.175
27°C 0-96 hours : 30°C 0-96 hours	-0.107	0.665	-0.161	0.872

Table C9. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for S40 workers under the no-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.496	0.475	1.045	0.835
0 - 48 hours	1.181	0.532	2.218	0.173
0 - 72 hours	0.904	0.501	1.806	0.370
0 - 96 hours	1.001	0.533	1.881	0.328

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.747	0.512	1.457	0.590
0 - 48 hours	0.566	0.498	1.136	0.788
0 - 72 hours	-0.305	0.449	-0.681	0.961
0 - 96 hours	0.127	0.476	0.266	0.999

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.394	0.581	0.678	0.961
0 - 48 hours	0.279	0.559	0.499	0.988
0 - 72 hours	-0.684	0.494	-1.386	0.637
0 - 96 hours	-0.270	0.515	-0.525	0.985

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	0.250	0.698	0.359	0.720
21°C 0-24 hours : 30°C 0-24 hours	-0.102	0.750	-0.136	0.892
27°C 0-24 hours : 30°C 0-24 hours	-0.353	0.775	-0.455	0.649
21°C 0-48 hours : 27°C 0-48 hours	-0.615	0.728	-0.844	0.399
21°C 0-48 hours : 30°C 0-48 hours	0.902	0.772	-1.169	0.243
27°C 0-48 hours : 30°C 0-48 hours	-0.287	0.749	-0.383	0.702
21°C 0-72 hours : 27°C 0-72 hours	-1.209	0.673	-1.798	0.072
21°C 0-72 hours : 30°C 0-72 hours	-1.588	0.703	-2.258	0.024
27°C 0-72 hours : 30°C 0-72 hours	-0.379	0.667	-0.568	0.570
21°C 0-96 hours : 27°C 0-96 hours	-0.875	0.714	-1.225	0.221
21°C 0-96 hours : 30°C 0-96 hours	-1.272	0.741	-1.717	0.086
27°C 0-96 hours : 30°C 0-96 hours	-0.397	0.701	-0.566	0.571

Table C10. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for Control workers under the light-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-0.261	0.616	-0.423	0.993
0 - 48 hours	-0.548	0.589	-0.931	0.885
0 - 72 hours	-1.320	0.559	-2.363	0.126
0 - 96 hours	-0.810	0.573	-1.414	0.619

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.850	0.528	1.611	0.490
0 - 48 hours	0.418	0.488	0.856	0.913
0 - 72 hours	0.345	0.490	0.703	0.956
0 - 96 hours	-0.054	0.471	-0.114	1.000

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.085	0.680	0.125	0.999
0 - 48 hours	-1.100	0.591	-1.861	0.339
0 - 72 hours	-0.962	0.605	-1.591	0.503
0 - 96 hours	-0.786	0.609	-1.290	0.698

Table C11. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for I10 workers under the light-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-1.191	0.513	-2.320	0.138
0 - 48 hours	-1.380	0.509	-2.713	0.052
0 - 72 hours	-2.134	0.529	-4.037	<0.001
0 - 96 hours	-2.174	0.525	-4.139	<0.001

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-1.114	0.512	-2.176	0.200
0 - 48 hours	-0.883	0.528	-1.672	0.466
0 - 72 hours	-1.611	0.511	-3.154	0.016
0 - 96 hours	-1.892	0.508	-3.722	0.002

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-0.673	0.481	-1.399	0.628
0 - 48 hours	-0.092	0.489	-0.187	0.999
0 - 72 hours	-0.440	0.483	-0.911	0.893
0 - 96 hours	-1.423	0.483	-2.945	0.027

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	0.073	0.728	0.100	0.920
21°C 0-24 hours : 30°C 0-24 hours	0.518	0.702	0.738	0.460
27°C 0-24 hours : 30°C 0-24 hours	0.444	0.696	0.638	0.524
21°C 0-48 hours : 27°C 0-48 hours	0.494	0.737	0.671	0.502
21°C 0-48 hours : 30°C 0-48 hours	1.289	0.706	1.826	0.068
27°C 0-48 hours : 30°C 0-48 hours	0.792	0.715	1.108	0.268
21°C 0-72 hours : 27°C 0-72 hours	0.517	0.733	0.705	0.481
21°C 0-72 hours : 30°C 0-72 hours	1.695	0.714	2.374	0.018
27°C 0-72 hours : 30°C 0-72 hours	1.173	0.696	1.685	0.092
21°C 0-96 hours : 27°C 0-96 hours	0.274	0.727	0.377	0.706
21°C 0-96 hours : 30°C 0-96 hours	0.751	0.706	1.063	0.288
27°C 0-96 hours : 30°C 0-96 hours	0.474	0.690	0.687	0.492

Table C12. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for 140 workers under the light-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-2.304	0.477	-4.829	<0.001
0 - 48 hours	-2.636	0.498	-5.288	<0.001
0 - 72 hours	-2.775	0.507	-5.469	<0.001
0 - 96 hours	-2.784	0.506	-5.498	<0.001

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-1.135	0.456	-2.486	0.094
0 - 48 hours	-1.389	0.457	-3.042	0.020
0 - 72 hours	-1.735	0.461	-3.763	0.002
0 - 96 hours	-1.850	0.468	-3.954	<0.001

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	-1.773	0.525	-3.377	0.007
0 - 48 hours	-1.940	0.528	-3.675	0.002
0 - 72 hours	-2.268	0.532	-4.267	<0.001
0 - 96 hours	-2.669	0.545	-4.895	<0.001

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	1.170	0.655	1.785	0.074
21°C 0-24 hours : 30°C 0-24 hours	0.531	0.702	0.757	0.449
27°C 0-24 hours : 30°C 0-24 hours	-0.638	0.692	-0.923	0.356
21°C 0-48 hours : 27°C 0-48 hours	1.247	0.670	1.861	0.063
21°C 0-48 hours : 30°C 0-48 hours	0.696	0.717	0.971	0.332
27°C 0-48 hours : 30°C 0-48 hours	-0.551	0.693	-0.795	0.427
21°C 0-72 hours : 27°C 0-72 hours	1.040	0.677	1.535	0.125
21°C 0-72 hours : 30°C 0-72 hours	0.506	0.724	0.700	0.484
27°C 0-72 hours : 30°C 0-72 hours	-0.534	0.697	-0.766	0.444
21°C 0-96 hours : 27°C 0-96 hours	0.934	0.680	1.372	0.170
21°C 0-96 hours : 30°C 0-96 hours	0.115	0.732	0.157	0.875
27°C 0-96 hours : 30°C 0-96 hours	-0.819	0.710	-1.154	0.249

Table C13. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for S10 workers under the light-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.523	0.461	1.134	0.788
0 - 48 hours	0.743	0.483	1.538	0.537
0 - 72 hours	-0.028	0.435	-0.063	1.000
0 - 96 hours	-0.059	0.436	-0.136	0.999

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.224	0.500	0.447	0.992
0 - 48 hours	0.223	0.500	0.446	0.992
0 - 72 hours	-0.430	0.466	-0.922	0.888
0 - 96 hours	0.493	0.523	0.943	0.880

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.506	0.549	0.922	0.889
0 - 48 hours	-0.209	0.497	-0.421	0.993
0 - 72 hours	-0.022	0.501	-0.043	1.000
0 - 96 hours	-0.556	0.484	-1.148	0.781

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.300	0.680	-0.441	0.659
21°C 0-24 hours : 30°C 0-24 hours	-0.017	0.717	-0.024	0.981
27°C 0-24 hours : 30°C 0-24 hours	0.283	0.742	0.381	0.703
21°C 0-48 hours : 27°C 0-48 hours	-0.520	0.695	-0.749	0.454
21°C 0-48 hours : 30°C 0-48 hours	-0.952	0.693	-1.374	0.170
27°C 0-48 hours : 30°C 0-48 hours	-0.432	0.705	-0.613	0.540
21°C 0-72 hours : 27°C 0-72 hours	-0.402	0.637	-0.631	0.528
21°C 0-72 hours : 30°C 0-72 hours	0.006	0.664	0.009	0.993
27°C 0-72 hours : 30°C 0-72 hours	0.408	0.684	0.596	0.551
21°C 0-96 hours : 27°C 0-96 hours	0.552	0.681	0.811	0.417
21°C 0-96 hours : 30°C 0-96 hours	-0.496	0.652	-0.762	0.446
27°C 0-96 hours : 30°C 0-96 hours	-1.049	0.713	-1.471	0.141

Table C14. Experiment 2: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of movement for S40 workers under the light-stimulation scenario, as a function of temperature, time, and the interaction between these two categorical variables. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of movement at each time point against pre-exposure likelihood for each temperature.

Low Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.241	0.571	0.421	0.993
0 - 48 hours	-0.494	0.530	-0.932	0.885
0 - 72 hours	-0.830	0.519	-1.599	0.498
0 - 96 hours	-0.257	0.543	-0.473	0.990

Mid Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.232	0.501	0.462	0.991
0 - 48 hours	0.125	0.492	0.255	0.999
0 - 72 hours	<0.001	0.485	0.000	1.000
0 - 96 hours	0.198	0.504	0.393	0.995

High Temp	Estimate	Std Error	Z-value	P-value
0 - 24 hours	0.652	0.628	1.037	0.838
0 - 48 hours	0.446	0.601	0.743	0.946
0 - 72 hours	-0.544	0.545	-0.998	0.857
0 - 96 hours	-0.580	0.538	-1.079	0.818

Pooled	Estimate	Std Error	Z-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.009	0.760	-0.012	0.990
21°C 0-24 hours : 30°C 0-24 hours	0.411	0.849	0.484	0.628
27°C 0-24 hours : 30°C 0-24 hours	0.420	0.804	0.523	0.601
21°C 0-48 hours : 27°C 0-48 hours	0.620	0.723	0.856	0.392
21°C 0-48 hours : 30°C 0-48 hours	0.941	0.801	1.174	0.241
27°C 0-48 hours : 30°C 0-48 hours	0.321	0.776	0.414	0.679
21°C 0-72 hours : 27°C 0-72 hours	0.830	0.710	1.168	0.243
21°C 0-72 hours : 30°C 0-72 hours	0.286	0.752	0.381	0.703
27°C 0-72 hours : 30°C 0-72 hours	-0.544	0.729	-0.745	0.456
21°C 0-96 hours : 27°C 0-96 hours	0.455	0.741	0.614	0.539
21°C 0-96 hours : 30°C 0-96 hours	-0.323	0.764	-0.423	0.673
27°C 0-96 hours : 30°C 0-96 hours	-0.778	0.737	-1.056	0.291

Table C15. Experiment 2: Statistical output for the linear mixed model examining walking rate for Control workers under the no-stimulation scenario, as a function of temperature, time, the interaction between these two categorical variables, and dry mass. Pairwise contrasts were conducted using the Tukey method to compare walking rate at each time point against pre-exposure walking rate for each temperature.

Low Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	-0.114	0.094	-1.215	0.618
0 - 48 hours	-0.093	0.088	-1.059	0.715
0 - 72 hours	0.040	0.087	0.455	0.969

Mid Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.184	0.102	1.799	0.276
0 - 48 hours	0.210	0.101	2.083	0.161
0 - 72 hours	-0.044	0.099	-0.448	0.970

High Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.031	0.100	0.307	0.990
0 - 48 hours	0.018	0.095	0.190	0.998
0 - 72 hours	-0.021	0.097	-0.210	0.997

Table C16. Experiment 2: Statistical output for the linear mixed model examining walking rate for I10 workers under the no-stimulation scenario, as a function of temperature, time, the interaction between these two categorical variables, and dry mass. Pairwise contrasts were conducted using the Tukey method to compare walking rate at each time point against pre-exposure walking rate for each temperature.

Low Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.283	0.100	2.832	0.026
0 - 48 hours	0.256	0.107	2.378	0.084
0 - 72 hours	0.186	0.118	1.583	0.390

Mid Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.127	0.100	1.270	0.583
0 - 48 hours	0.087	0.101	0.860	0.826
0 - 72 hours	0.014	0.102	0.140	0.999

High Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.063	0.105	0.595	0.934
0 - 48 hours	0.108	0.110	0.985	0.758
0 - 72 hours	0.046	0.112	0.410	0.977

Pooled	Estimate	Std Error	t-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.156	0.141	-1.107	0.269
21°C 0-24 hours : 30°C 0-24 hours	-0.220	0.145	-1.519	0.130
27°C 0-24 hours : 30°C 0-24 hours	-0.064	0.145	-0.442	0.659
21°C 0-48 hours : 27°C 0-48 hours	-0.169	0.147	-1.152	0.250
21°C 0-48 hours : 30°C 0-48 hours	-0.147	0.153	-0.960	0.338
27°C 0-48 hours : 30°C 0-48 hours	0.022	0.149	0.147	0.883
21°C 0-72 hours : 27°C 0-72 hours	-0.172	0.155	-1.106	0.270
21°C 0-72 hours : 30°C 0-72 hours	-0.140	0.161	-0.869	0.386
27°C 0-72 hours : 30°C 0-72 hours	0.031	0.151	0.208	0.835

Table C17. Experiment 2: Statistical output for the linear mixed model examining walking rate for 140 workers under the no-stimulation scenario, as a function of temperature, time, the interaction between these two categorical variables, and dry mass. Pairwise contrasts were conducted using the Tukey method to compare walking rate at each time point against pre-exposure walking rate for each temperature.

Low Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.640	0.172	3.733	0.001
0 - 48 hours	0.444	0.166	2.676	0.039
0 - 72 hours	0.348	0.176	1.971	0.201

Mid Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.474	0.157	3.025	0.014
0 - 48 hours	0.343	0.145	2.362	0.087
0 - 72 hours	0.176	0.155	1.137	0.667

High Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.183	0.142	1.288	0.571
0 - 48 hours	0.177	0.137	1.289	0.571
0 - 72 hours	-0.024	0.145	-0.162	0.999

Pooled	Estimate	Std Error	t-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.167	0.232	-0.719	0.473
21°C 0-24 hours : 30°C 0-24 hours	-0.457	0.222	-2.061	0.040
27°C 0-24 hours : 30°C 0-24 hours	-0.291	0.211	-1.380	0.169
21°C 0-48 hours : 27°C 0-48 hours	-0.101	0.220	-0.461	0.645
21°C 0-48 hours : 30°C 0-48 hours	-0.267	0.215	-1.244	0.215
27°C 0-48 hours : 30°C 0-48 hours	-0.166	0.199	-0.832	0.406
21°C 0-72 hours : 27°C 0-72 hours	-0.171	0.234	-0.732	0.465
21°C 0-72 hours : 30°C 0-72 hours	-0.371	0.228	-1.630	0.104
27°C 0-72 hours : 30°C 0-72 hours	-0.200	0.212	-0.943	0.346

Table C18. Experiment 2: Statistical output for the linear mixed model examining walking rate for S10 workers under the no-stimulation scenario, as a function of temperature, time, the interaction between these two categorical variables, and dry mass. Pairwise contrasts were conducted using the Tukey method to compare walking rate at each time point against pre-exposure walking rate for each temperature.

Low Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.079	0.096	0.824	0.843
0 - 48 hours	0.105	0.096	1.092	0.695
0 - 72 hours	-0.022	0.097	-0.231	0.996

Mid Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	-0.151	0.094	-1.610	0.375
0 - 48 hours	0.003	0.094	0.031	1.000
0 - 72 hours	-0.137	0.096	-1.423	0.486

High Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	-0.047	0.085	-0.559	0.944
0 - 48 hours	0.146	0.089	1.642	0.357
0 - 72 hours	0.066	0.090	0.726	0.887

Pooled	Estimate	Std Error	t-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	-0.230	0.134	-1.721	0.086
21°C 0-24 hours : 30°C 0-24 hours	-0.126	0.128	-0.989	0.323
27°C 0-24 hours : 30°C 0-24 hours	0.104	0.126	0.820	0.413
21°C 0-48 hours : 27°C 0-48 hours	-0.102	0.134	-0.764	0.445
21°C 0-48 hours : 30°C 0-48 hours	0.041	0.131	0.314	0.754
27°C 0-48 hours : 30°C 0-48 hours	0.143	0.129	1.112	0.267
21°C 0-72 hours : 27°C 0-72 hours	-0.115	0.136	-0.843	0.400
21°C 0-72 hours : 30°C 0-72 hours	0.088	0.132	0.666	0.506
27°C 0-72 hours : 30°C 0-72 hours	0.203	0.132	1.538	0.125

Table C19. Experiment 2: Statistical output for the linear mixed model examining walking rate for S40 workers under the no-stimulation scenario, as a function of temperature, time, the interaction between these two categorical variables, and dry mass. Pairwise contrasts were conducted using the Tukey method to compare walking rate at each time point against pre-exposure walking rate for each temperature.

Low Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.114	0.087	1.321	0.550
0 - 48 hours	0.141	0.085	1.656	0.349
0 - 72 hours	0.067	0.085	0.797	0.856

Mid Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.282	0.084	3.378	0.005
0 - 48 hours	0.026	0.081	0.317	0.989
0 - 72 hours	0.016	0.085	0.191	0.998

High Temp	Estimate	Std Error	z-value	P-value
0 - 24 hours	0.120	0.082	1.471	0.456
0 - 48 hours	0.083	0.081	1.025	0.735
0 - 72 hours	-0.056	0.087	-0.644	0.918

Pooled	Estimate	Std Error	t-value	P-value
21°C 0-24 hours : 27°C 0-24 hours	0.168	0.120	1.396	0.164
21°C 0-24 hours : 30°C 0-24 hours	0.006	0.119	0.048	0.962
27°C 0-24 hours : 30°C 0-24 hours	-0.162	0.117	-1.389	0.166
21°C 0-48 hours : 27°C 0-48 hours	-0.115	0.118	-0.979	0.328
21°C 0-48 hours : 30°C 0-48 hours	-0.058	0.117	-0.492	0.623
27°C 0-48 hours : 30°C 0-48 hours	0.057	0.115	0.500	0.618
21°C 0-72 hours : 27°C 0-72 hours	-0.051	0.120	-0.426	0.671
21°C 0-72 hours : 30°C 0-72 hours	-0.123	0.121	-1.019	0.309
27°C 0-72 hours : 30°C 0-72 hours	-0.072	0.122	-0.594	0.553

Table C20. Experiment 2: Statistical output for the linear mixed model examining sucrose consumption, as a function of pesticide, temperature, time, as well as pesticide x temperature and pesticide x time interactions. Pairwise contrasts were conducted using the Tukey method to compare sucrose consumption for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.206	-10.411	<0.001
Control - I40	-0.338	-17.086	<0.001
Control - S10	-0.011	-0.571	0.979
Control - S40	-0.018	-0.909	0.893

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.146	-7.370	<0.001
Control - I40	-0.264	-13.342	<0.001
Control - S10	0.007	0.336	0.997
Control - S40	-0.047	-2.373	0.126

High Temp	Estimate	z-value	P-value
Control - I10	-0.142	-7.173	<0.001
Control - I40	-0.185	-9.325	<0.001
Control - S10	-0.001	-0.038	1.000
Control - S40	-0.019	-0.951	0.876

Pooled	Estimate	t-value	P-value
21°C - 27°C	-0.094	-10.799	<0.001
21°C - 30°C	-0.168	-19.294	<0.001
27°C - 30°C	-0.074	-8.495	<0.001
21°C Control - I10 : 27°C Control - I10	0.060	2.259	0.025
21°C Control - I40 : 27°C Control - I40	0.074	2.782	0.006
21°C Control - S10 : 27°C Control - S10	0.018	0.674	0.501
21°C Control - S40 : 27°C Control - S40	-0.029	-1.088	0.278
21°C Control - I10 : 30°C Control - I10	0.064	2.405	0.017
21°C Control - I40 : 30°C Control - I40	0.154	5.767	<0.001
21°C Control - S10 : 30°C Control - S10	0.011	0.396	0.692
21°C Control - S40 : 30°C Control - S40	-0.001	-0.032	0.975
Control - Time : I10 - Time	-0.015	-3.881	<0.001
Control - Time : I40 - Time	-0.019	-5.004	<0.001
Control - Time : S10 - Time	0.003	-0.890	0.374
Control - Time : S40 - Time	0.008	2.196	0.029

Table C21. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining flight distance, as a function of pesticide, temperature, dry mass, mass-specific consumption, and the interaction between pesticide and temperature. Pairwise contrasts were conducted using the Tukey method to compare flight distance for each pesticide treatment against the Control treatment for each temperature, and also to compare all temperatures for Control workers.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.135	-0.540	0.852
Control - S10	-0.145	-0.578	0.832

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.134	-0.526	0.859
Control - S10	0.030	0.117	0.992

High Temp	Estimate	z-value	P-value
Control - I10	-0.762	-3.074	0.006
Control - S10	-0.355	-1.428	0.326

Controls	Estimate	z-value	P-value
21°C - 27°C	-0.236	-0.931	0.621
21°C - 30°C	0.133	0.531	0.856
27°C - 30°C	1.023	0.409	0.912

Pooled	Estimate	t-value	P-value
Control - I10	-0.259	-1.735	0.083
Control - S10	-0.174	-1.170	0.242
Dry Mass	0.017	3.229	0.001
Mass-Specific Consumption	0.490	1.677	0.094
27°C Con - I10 : 21°C Con - I10	-0.269	-0.754	0.452
27°C Con - I10 : 30°C Con - I10	-0.896	-2.522	0.012
21°C Con - I10 : 30°C Con - I10	-0.627	-1.780	0.076
27°C Con - S10 : 21°C Con - S10	-0.175	-0.489	0.625
27°C Con - S10 : 30°C Con - S10	-0.385	-1.080	0.281
21°C Con - S10 : 30°C Con - S10	-0.210	-0.595	0.552

Table C22. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining mean flight velocity over the 0-100m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare mean flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.043	-0.627	0.805
Control - S10	0.066	0.993	0.581

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.039	-0.623	0.808
Control - S10	-0.059	-0.950	0.608

High Temp	Estimate	z-value	P-value
Control - I10	-0.052	-0.917	0.630
Control - S10	-0.033	-0.573	0.835

Pooled	Estimate	t-value	P-value
Control - I10	-0.044	-1.223	0.222
Control - S10	-0.013	-0.359	0.720
Dry Mass	0.005	3.682	<0.001
Mass-Specific Consumption	-0.002	-0.025	0.980

Table C23. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining mean flight velocity over the 100-500m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare mean flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.083	-0.995	0.580
Control - S10	0.077	0.941	0.614

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.065	-0.770	0.721
Control - S10	-0.053	-0.645	0.795

High Temp	Estimate	z-value	P-value
Control - I10	-0.025	-0.312	0.948
Control - S10	-0.009	-0.113	0.993

Pooled	Estimate	t-value	P-value
Control - I10	-0.054	-1.138	0.256
Control - S10	0.004	0.096	0.924
Dry Mass	0.004	2.418	0.016
Mass-Specific Consumption	-0.112	-1.265	0.207

Table C24. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining mean flight velocity over the 500-1000m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare mean flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	0.052	0.495	0.874
Control - S10	0.156	1.494	0.294

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.092	-0.897	0.642
Control - S10	-0.031	-0.309	0.949

High Temp	Estimate	z-value	P-value
Control - I10	0.180	1.630	0.233
Control - S10	-0.035	-0.350	0.935

Pooled	Estimate	t-value	P-value
Control - I10	0.039	0.629	0.530
Control - S10	0.033	0.548	0.585
Dry Mass	0.003	1.237	0.218
Mass-Specific Consumption	-0.131	-1.145	0.254

Table C25. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining mean flight velocity over the 1000-2000m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare mean flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	0.130	1.147	0.485
Control - S10	0.141	1.222	0.440

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.037	-0.295	0.953
Control - S10	-0.036	-0.298	0.952

High Temp	Estimate	z-value	P-value
Control - I10	0.308	1.561	0.263
Control - S10	0.044	0.389	0.920

Pooled	Estimate	t-value	P-value
Control - I10	0.089	1.174	0.243
Control - S10	0.053	0.804	0.423
Dry Mass	-0.002	-0.771	0.442
Mass-Specific Consumption	-0.206	-1.577	0.118

Table C26. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining maximum flight velocity over the 0-100m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare maximum flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.046	-0.857	0.667
Control - S10	0.073	1.412	0.335

Mid Temp	Estimate	z-value	P-value
Control - I10	0.010	0.214	0.975
Control - S10	-0.073	-1.506	0.288

High Temp	Estimate	z-value	P-value
Control - I10	-0.024	-0.531	0.856
Control - S10	0.053	1.178	0.466

Pooled	Estimate	t-value	P-value
Control - I10	-0.016	-0.550	0.583
Control - S10	0.018	0.653	0.514
Dry Mass	0.003	2.885	0.004
Mass-Specific Consumption	0.056	1.017	0.310

Table C27. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining maximum flight velocity over the 100-500m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare maximum flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.108	-1.386	0.348
Control - S10	0.040	0.524	0.860

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.042	-0.531	0.856
Control - S10	-0.108	-1.395	0.344

High Temp	Estimate	z-value	P-value
Control - I10	-0.055	-0.732	0.744
Control - S10	-0.064	-0.874	0.657

Pooled	Estimate	t-value	P-value
Control - I10	-0.068	-1.517	0.131
Control - S10	-0.048	-1.111	0.268
Dry Mass	0.004	2.217	0.028
Mass-Specific Consumption	-0.106	-1.275	0.204

Table C28. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining maximum flight velocity over the 500-1000m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare maximum flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.059	-0.602	0.819
Control - S10	0.005	0.045	0.999

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.149	-1.536	0.274
Control - S10	-0.090	-0.929	0.622

High Temp	Estimate	z-value	P-value
Control - I10	0.097	0.927	0.623
Control - S10	-0.133	-1.392	0.345

Pooled	Estimate	t-value	P-value
Control - I10	-0.051	-0.888	0.376
Control - S10	-0.069	-1.243	0.215
Dry Mass	0.003	1.524	0.129
Mass-Specific Consumption	-0.176	-1.663	0.098

Table C29. Experiment 3: Statistical output for the generalised linear mixed model under a gamma family distribution examining maximum flight velocity over the 1000-2000m flight period, as a function of pesticide, temperature, dry mass, and mass-specific consumption. Pairwise contrasts were conducted using the Tukey method to compare maximum flight velocity for each pesticide treatment against the Control treatment for each temperature.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.058	-0.533	0.855
Control - S10	0.030	0.269	0.961

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.009	-0.072	0.997
Control - S10	-0.067	-0.581	0.830

High Temp	Estimate	z-value	P-value
Control - I10	-0.036	-0.186	0.981
Control - S10	0.094	0.856	0.668

Pooled	Estimate	t-value	P-value
Control - I10	-0.031	-0.419	0.676
Control - S10	0.014	0.224	0.823
Dry Mass	0.002	0.698	0.487
Mass-Specific Consumption	-0.182	-1.453	0.149

Table C30. Experiment 3: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of flight over 100m, as a function of pesticide, temperature, dry mass, mass-specific consumption, the interaction between pesticide and temperature, and the interaction between pesticide and dry mass. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of flight over 100m for each pesticide treatment against the Control treatment for each temperature, and also to compare all temperatures for Control workers.

Low Temp	Estimate	z-value	P-value
Control - I10	0.772	1.820	0.163
Control - S10	0.581	1.379	0.352

Mid Temp	Estimate	z-value	P-value
Control - I10	0.332	0.682	0.774
Control - S10	0.483	1.014	0.568

High Temp	Estimate	z-value	P-value
Control - I10	0.837	1.526	0.279
Control - S10	0.775	1.402	0.340

Controls	Estimate	z-value	P-value
21°C - 27°C	0.553	1.191	0.459
21°C - 30°C	1.252	2.400	0.043
27°C - 30°C	0.699	1.298	0.396

Pooled	Estimate	z-value	P-value
Control - I10	-0.647	-2.211	0.069
Control - S10	-0.613	-2.108	0.088
Dry Mass	0.063	3.250	0.001
Mass-Specific Consumption	0.789	1.483	0.138
27°C Control - I10 : 21°C Control - I10	-0.440	-0.692	0.489
27°C Control - I10 : 30°C Control - I10	-0.505	-0.707	0.480
27°C Control - S10 : 21°C Control - S10	-0.098	-0.156	0.876
27°C Control - S10 : 30°C Control - S10	-0.291	-0.409	0.683
Control - Dry Mass : I10 - Dry Mass	-0.014	-0.555	0.579
Control - Dry Mass : S10 - Dry Mass	-0.029	-1.153	0.249

Table C31. Experiment 3: Statistical output for the generalised linear mixed model under a binomial family distribution examining the likelihood of flight over 1000m, as a function of pesticide, temperature, dry mass, mass-specific consumption, the interaction between pesticide and temperature, and the interaction between pesticide and dry mass. Pairwise contrasts were conducted using the Tukey method to compare the likelihood of flight over 100m for each pesticide treatment against the Control treatment for each temperature, and also to compare all temperatures for Control workers.

Low Temp	Estimate	z-value	P-value
Control - I10	-0.610	-1.469	0.306
Control - S10	-0.621	-1.499	0.291

Mid Temp	Estimate	z-value	P-value
Control - I10	-0.054	-0.131	0.991
Control - S10	-0.116	-0.281	0.957

High Temp	Estimate	z-value	P-value
Control - I10	-1.254	-2.934	0.009
Control - S10	-0.859	-2.092	0.091

Controls	Estimate	z-value	P-value
21°C - 27°C	-0.135	-0.323	0.944
21°C - 30°C	0.193	0.468	0.886
27°C - 30°C	0.328	0.793	0.707

Pooled	Estimate	z-value	P-value
Control - I10	-0.639	-2.639	0.023
Control - S10	0.532	-2.231	0.066
Dry Mass	0.061	3.765	<0.001
Mass-Specific Consumption	0.878	1.862	0.063
27°C Control - I10 : 21°C Control - I10	-0.556	-0.949	0.343
27°C Control - I10 : 30°C Control - I10	-1.200	-2.021	0.043
27°C Control - S10 : 21°C Control - S10	-0.505	-0.862	0.389
27°C Control - S10 : 30°C Control - S10	-0.725	-1.276	0.202
Control - Dry Mass : I10 - Dry Mass	-0.048	-2.218	0.027
Control - Dry Mass : S10 - Dry Mass	-0.046	-2.117	0.034

Appendix D | The effect of combined insecticide and fungicide exposure on bumblebee flight motivation and performance

Please note: This appendix is a summary of Freja Gjerstad's MSc project report.

Freja conducted the majority of experimental work and data analysis presented in this summary, but with significant contribution and close supervision from myself. The summary is written in my own words. For further details, please see the 'Statement of Originality' on page 5.

Introduction & Methods

When considering how pesticides affect bee pollinators, most studies focus on the effect of an individual chemical (Godfray et al., 2014; Lundin et al., 2015; Van der Sluijs et al., 2013). However, isolated pesticide exposure is not a likely scenario in most agricultural landscapes, where farmers commonly use either sequential pesticide sprays, tank mixes, or systemic insecticide seed dressings in combination with spray treatments (Thompson et al., 2014). There is a growing body of evidence demonstrating that bees are commonly subjected to combined pesticide exposure, and come into contact with multiple pesticide classes when foraging in both urban and agricultural landscapes (Botías et al., 2016; David et al., 2016; Hladik et al., 2016; Krupke et al., 2012; Mullin et al., 2010). In spite of this, an often-overlooked issue is that of possible interactive effects between different pesticide classes or active ingredients (Gill et al., 2012; Iwasa et al., 2004; Pilling & Jepson, 1993; Sgolastra et al., 2017). One of the clearest examples of interactive effects between pesticides is the synergism between certain neurotoxic insecticides and azole fungicides, where the observed toxicity to bees of the pesticide mix is vastly greater than would be predicted by the additive toxicities of the individual pesticides (Iwasa et al., 2004; Johnson et al., 2013; Pilling & Jepson, 1993).

In the field it is difficult to pinpoint any additive or synergistic effects of pesticides, or even to determine the impact of a specific chemical, as multiple other factors can influence wild bee success, such as farm management techniques or landscape context (Park et al., 2015; Rundlöf et al., 2015). Controlled lab studies are therefore essential in identifying the risks posed by combined pesticide exposure. However, a key limitation to the current literature is that nearly all published lab studies on this topic to date have looked to use lethal dose (LD₅₀) values as a measure of toxicity (Biddinger et al., 2013; Iwasa et al., 2004; Sgolastra et al., 2017). There is limited data on the relative impacts of

synergists on sublethal endpoints (Desneux et al., 2007; Thompson et al., 2014), but available studies have highlighted the detrimental sub-lethal effects of pesticide chemicals in combination on individual performance and overall colony health (Gill et al., 2012; Tosi & Nieh, 2019; Vandame & Belzunces, 1998).

This study will aim to address the above-mentioned knowledge gaps by investigating the possible sublethal synergistic effects of different pesticide classes on bee flight behaviour and performance. Building on previous research highlighting synergistic interactions between azole fungicides and neonicotinoid insecticides (Iwasa et al., 2004; Sgolastra et al., 2017), the flight mill setup as described in Chapter 3 was used to investigate potential impacts on bumblebee flight motivation, endurance, and speed, from oral exposure to the neonicotinoid insecticide thiacloprid (500 ppb) and the azole fungicide tebuconazole (at both 30 ppb and 3000 ppb), following a fully factorial design so that each chemical concentration was examined both individually and in combination. Thiacloprid was abstained from the EU neonicotinoid ban in 2018, which saw the neonicotinoids imidacloprid, thiamethoxam, and clothianidin prohibited from use outside of closed greenhouses, because it is considered less toxic to non-target insects such as bees (Ellis et al., 2017). Whilst it is now being phased out of use in the UK and across Europe as its licence has not been renewed, up until around 2020 it was one of the most widely used insecticides on pollinator attractive crops such as oilseed rape, and it is still used heavily across many regions worldwide (Garthwaite 2018). The thiacloprid concentration used for this study falls within the range found in crop pollen and nectar as well as in bee collected products in areas where thiacloprid is used in agricultural regimes (David et al., 2016; Ellis et al., 2017; Roszko et al., 2016). Tebuconazole is one of the most used fungicides across the UK and Europe, and the concentrations used in this study represent best (30 ppb) and worst-case (3000 ppb) concentrations found from residue analyses in crop and bee-collected pollen from agricultural areas using this product (David et al., 2016; Garthwaite et al., 2018; Thompson et al., 2014). Concerningly, the toxicity of thiacloprid to honey bees has been found to be enhanced by over 1000-fold when applied in conjunction with azole fungicides such as tebuconazole (Iwasa et al., 2004), and as such it is important we understand how combined exposure to field-realistic concentrations of these chemicals impacts on bee behaviour.

Results & Discussion

Contrary to expectations, no pesticide treatment, either individual or combined, had a significant effect on flight endurance or speed in comparison to control bees ($p > 0.05$ in all cases). However, the tebuconazole 30 ppb individual treatment (GLMM: $z = 2.17$, $p = 0.030$) and thiacloprid

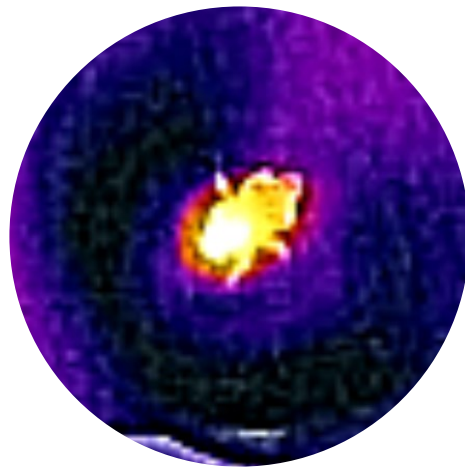
500ppb + tebuconazole 3000 ppb combined treatment (GLMM: $z = 1.99$, $p = 0.047$) had a significant positive effect on the probability of a bee being motivated to fly.

It is interesting that thiacloprid showed no impact on motivation when applied alone but led to increased motivation to initiate flight when applied in conjunction with the high dose of tebuconazole. Azole fungicides function by inhibiting fungal P450 (a class of enzyme) mediated synthesis of ergosterols (organic molecules that are major components of fungal cell membranes), but these fungicides can also inhibit detoxifying P450 activity in bees, such as P450-mediated detoxification of certain insecticides (Johnson et al., 2013; Pilling et al., 1995; Vandame & Belzunces, 1998; Weete et al., 2010). As a consequence, azole fungicides can prevent bees from metabolising and removing these harmful chemicals from their system, and so act synergistically with these insecticides to exacerbate their effects (Sgolastra et al., 2017). As neonicotinoids can stimulate neurons and cause hyperactive motor behaviour (Alkassab & Kirchner, 2018; Tosi & Nieh, 2017), this interaction between thiacloprid and tebuconazole may have led to the increased flight motivation observed in this study. However, the effect size was small, and we observed the same effect on flight motivation in the 30 ppb tebuconazole treatment, which can't be explained by the same mechanistic process. These results may thus be an artefact of the experimental setup, or there may be a different mechanism at play, which future work should aim to address. It would thus be beneficial to test tebuconazole with greater sample sizes over a range of concentrations to examine the concentration response curve for this fungicide.

Conclusion

The finding that thiacloprid had no detectable effect on bee flight performance, either alone or in combination with tebuconazole, suggests that a switch to thiacloprid from the nitroguanidine neonicotinoids (such as imidacloprid and thiamethoxam) in regions that still use them could be a step in the right direction, and are a good sign that thiacloprid and azole fungicides could be safe to use together in agricultural pesticide regimes. However, investigation of further behaviours and functions are needed to support this, and it should be noted that thiacloprid, even at the high 500 ppb concentration tested in this study, may be considerably less effective against some insect pests compared to other neonicotinoids. This study can be added to a growing list of literature that highlights the need for a more holistic approach when assessing the risks of pesticides on bees. Sublethal effects, combined exposure, and the representation of wild pollinators need to be implemented into risk assessments to provide a more complete picture of how the bee community as a whole will respond to realistic and complex pesticide regimes.

Appendix E | The effect of neonicotinoid exposure on bumblebee endothermic ability across a temperature gradient and body mass variation



Connor Lovell

Supervisors: Daniel Kenna & Dr Richard Gill

A thesis submitted in partial fulfilment of the requirements for the degree of
Master of Research at Imperial College London

Submitted March 2020 for the MRes in Ecology, Evolution and Conservation

Please note: This appendix is the submitted MRes project report of Connor Lovell.

Connor undertook the majority of experimental work and conducted the data analysis and write-up, but with significant contribution and close supervision from myself. For further details, please see the 'Statement of Originality' on page 5.

Abstract

Global agriculture has enhanced crop yields, at a cost to wildlife. Neonicotinoids are key contributors to increase crop productivity, however, impacts on key pollinators, such as bumblebees, are reported. Bumblebees are heterothermic and can elevate their thorax temperature above the ambient temperature; an essential process for flight and thermoregulation. Body size influences this warming, with larger bees able to warm faster and to higher temperatures. Neonicotinoids can impair this endothermy and, due to increased energetic demands at lower temperatures, environmental temperature may influence this impairment. Indeed, observations suggest the impact of neonicotinoids on colony-level thermoregulation is magnified at colder night-time temperatures. Despite this, research investigating how environmental temperature and neonicotinoids interact to impair individual-level thermoregulation is minimal, and no previous research accounts for how body size influences the thermal response to neonicotinoids. In this study, I investigated how a neonicotinoid (imidacloprid) affects thorax temperature across a temperature gradient, concurrently testing for any influence of body mass. I found smaller workers held elevated thorax temperatures better over time, with imidacloprid exposure having no significant influence on this relationship. Additionally, imidacloprid lowered thorax temperatures, with lower ambient temperatures increasing this effect, suggesting individual-level thermoregulation is impaired more at colder temperatures by neonicotinoids. These results have important consequences for predicting bumblebee responses to neonicotinoids in a warming world.

Key words: *Bombus terrestris*, thermoregulation, neonicotinoids, environmental temperature, body mass.

Introduction

Insect pollinators are ecologically and economically important (Gallai et al., 2009; Goulson et al., 2008). Worryingly, key pollinators, including bees, are undergoing declines, with consequences to crop yield and wild flower health (Goulson et al., 2008; Soroye et al., 2020). The increasing use of pesticides in agriculture has been implicated as a key driver of these declines (Goulson et al., 2008; Van der Sluijs et al., 2013). Understanding the mechanisms by which these chemicals affect key insect pollinator groups, such as bees, is central to pollinator conservation.

One widely applied class of pesticide, the neonicotinoids, are likely key contributors to declines (Bass et al., 2015; Elbert et al., 2008; Godfray et al., 2014). Their popularity results from both the versatile manner in which they can be applied, and their systemic nature whereby the chemical provides protection for the whole plant (Elbert et al., 2008). Consequently, they are found in the nectar and

pollen of treated flowering plants, through which bees can be exposed (Azpiazu et al., 2019; Rundlöf et al., 2015). Research demonstrates sub-lethal effects of neonicotinoid exposure on bees, such as on foraging and flight ability (Gill et al., 2012; Kenna et al., 2019). Furthermore, Crall *et al* (2018) demonstrated that a neonicotinoid impaired activity levels, nursing, and colony-level thermoregulation significantly more at night, when environmental temperatures were up to 20 °C lower. Many potential mechanisms exist by which neonicotinoids may act to cause these effects, such as by impairing ATP production (Nicodemo et al., 2014), which could impair flight and activity levels.

The mechanisms by which neonicotinoid exposure impairs activity levels and colony-level thermoregulation are unknown. One possibility is that neonicotinoid exposure affects non-flight thermogenesis (NFT) (Potts et al., 2018; Tosi et al., 2016). By shivering flight musculature, an individual bee can generate heat endogenously, raising its thorax temperature above the environment temperature (Esch et al., 1991). This endothermic process, named NFT, is vital for foraging, pre-flight warming, and colony-level thermoregulation (Esch et al., 1991; Heinrich, 1972, 1975). Tosi *et al* (2016) and Potts *et al* (2018) demonstrate that neonicotinoid exposure causes fluctuations in thorax temperature, both over time and as neonicotinoid concentration increases. This effect likely originates from disruptions to signalling in the central nervous system (Barbara et al., 2008; Potts et al., 2018; Tosi et al., 2016). Although Crall *et al* (2018) shows environmental temperature influences the effect of neonicotinoids on colony-level thermoregulation, to my knowledge, no study has investigated this effect on NFT. Understanding the influence of environmental temperature on NFT is crucial in predicting how neonicotinoids may harm bumblebee populations; both throughout the year and with future climate change.

Here, I will study the interaction between environmental temperature and a neonicotinoid (imidacloprid) on NFT. To fully understand how imidacloprid influences NFT, I will additionally investigate whether imidacloprid influences the relationship between NFT and body mass. Research suggests that, both within and between species, there is greater disparity between thorax temperatures and environmental temperatures in larger bees. This contrasts with smaller bees, which typically show greater conformity to their thermal environment, possibly a consequence of increased heat loss due to a larger surface area:volume ratio (Bishop & Armbruster, 1999; Heinrich, 1972). Given that the thermal properties of bees vary depending on size, I expect the influence of neonicotinoids on NFT to also vary depending on size. To my knowledge, this is the first study to investigate how body size influences the thermal response to a pesticide.

I present a series of experiments to further our understanding of how a neonicotinoid (imidacloprid) influences NFT:

1) Before I begin experiments on bee thermoregulation, I will first test the accuracy of the thermal camera to be used for monitoring thorax temperatures. Research has noted accuracy and calibration issues with forward-looking infrared (FLIR) cameras (Malmivirta et al., 2019). I will develop a protocol for identifying whether the thermal camera is adequately calibrated in each thermal image.

2) Secondly, I will test the effect of two experimental feeding strategies on thorax temperature over time, acute (fed once at the start of the experiment) and chronic (fed throughout the experiment). How the feeding strategy influences thorax temperatures will inform the feeding strategy of my main experiment, to ensure changes in thorax temperature are due to imidacloprid and not the feeding strategy. I expect acute feeding to lead to greater drops in thorax temperature.

3) Finally, I will orally expose individual worker bumblebees to imidacloprid, monitoring the ability of exposed workers to maintain an elevated thorax temperature, over time, compared with a control group, across three ambient temperatures. I will also examine how body size mediates the thermal response to imidacloprid. I hypothesise that imidacloprid exposure will cause thorax temperatures to decline compared to control groups, and this will be amplified by lower ambient temperatures. Additionally, I hypothesise body mass will influence the thermal response to imidacloprid.

Methods

Bumblebee husbandry

Bombus terrestris *Audax* colonies were used throughout and stored under red light in a holding controlled environment (CE) room at 25 °C and 60% humidity. Reservoirs with each colony were replaced weekly with a 40% sucrose/water weight/weight nectar substitute (Knopper et al., 2016). Four grams of honeybee-collected pollen was supplied daily to each colony. Supplementary Information 1 (S1) details this husbandry. All colonies underwent this husbandry throughout.

Thermal camera protocol for excluding inadequately calibrated images

Before experiments started, I needed to ensure the thermal camera chosen could provide accurate temperature estimations. A FLIR ONE Pro thermal camera (FLIR Systems) was used, and temperatures were estimated from thermal images using FLIR Tools v.5.13.18031.2002. In November 2019 my investigations into the camera's accuracy demonstrated camera calibration was not always correct, sometimes producing inaccurate thermal images. Details of these experiments are found in S2.

To account for these accuracy issues, and to check if the camera was incorrectly calibrated in thermal images in future experiments, I decided to attach a strip of black electrical tape to the surface which workers were to be placed and monitored on. This was to be measured along with thorax

temperatures in each image. As the tape thermally equilibrated, the temperature of the tape would equal the ambient temperature of the room. The tape had a known emissivity (0.95), which is required when estimating temperatures from thermal imagery. Emissivity is a measure of how effective a surface is at emitting thermal radiation: an emissivity of 0 means no heat is emitted as thermal radiation, and 1 means all heat is emitted as thermal radiation (Li & Wang, 1999). Henceforth, for each image, the camera was deemed inaccurately calibrated if the tape temperature reading strayed from the ambient temperature of the room.

With the tape present in all images throughout future experiments, images were ranked based on how far the tape temperature reading strayed from ambient temperature:

- Rank 1: images where the tape read $\leq 0.1^{\circ}\text{C}$ away from ambient temperature (rounded to one decimal place)
- Rank 2: images 0.2°C away
- Rank 3: images $>0.2^{\circ}\text{C}$ away

Each time a recording was taken in future experiments, Rank 3 images were excluded and Rank 2 images were only used if no Rank 1 images were available. Applying stricter thresholds would exclude more images and reduce sample sizes.

Experimental designs

The following two experiments, on the temporal response of thorax temperatures to feeding strategy and imidacloprid, took place in red light CE rooms at 60% humidity. Experiments were repeated at three test ambient temperatures: 15 °C, 20 °C, and 25 °C. These temperatures were chosen considering the working limits of the CE room and camera; <30 °C and >15 °C respectively.

Colonies were ordered, for each experiment, and workers were chosen equally across available colonies. Instances where this was not possible are discussed in S3.

The camera was clamped and held 0.5m above the monitored workers (Fig.1). At each timepoint, when a thorax temperature recording was taken, multiple images were taken to ensure some were adequately calibrated.

Whenever thermal images were taken, workers were kept unrestrained and individually in holding pots to avoid unnecessary stress potentially influencing thorax temperature, with no barrier between them and the camera (Fig. 1). Consequently, workers infrequently escaped when images were taken. If workers escaped, they were excluded from that timepoint's recording.

Temporal responses of thorax temperature under acute and chronic feeding

This experiment investigated, across November and December 2019, how different feeding strategies influence thorax temperature, and was repeated three times at each of the three test temperatures previously discussed. Two colonies, used to source workers, were delivered five days in advance of the experiment beginning.

Six workers were used each time this experiment was conducted. To reduce the influence of worker mass, as this was not of interest in this experiment, workers were rejected if they fell outside 200 – 300mg and replaced by another worker from the same colony. In the holding room, accepted workers were placed into queen marking tubes (Fig. 2:A) for an initial 10-minute feed. A microscope slide cover

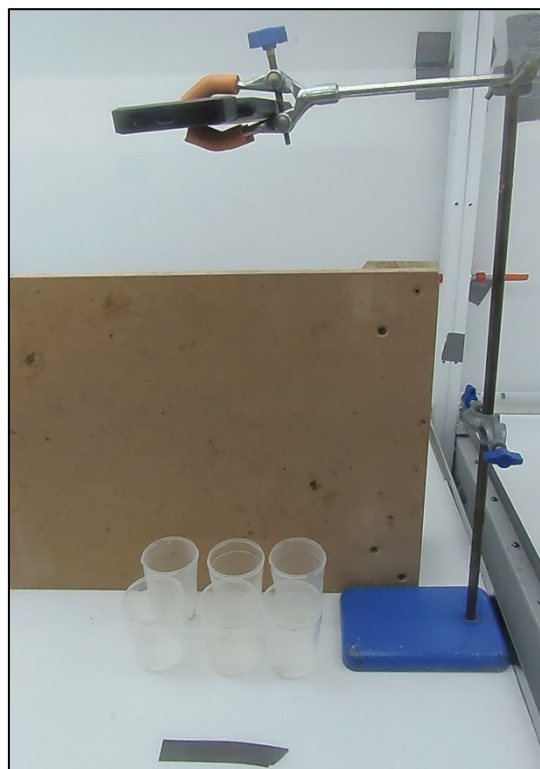
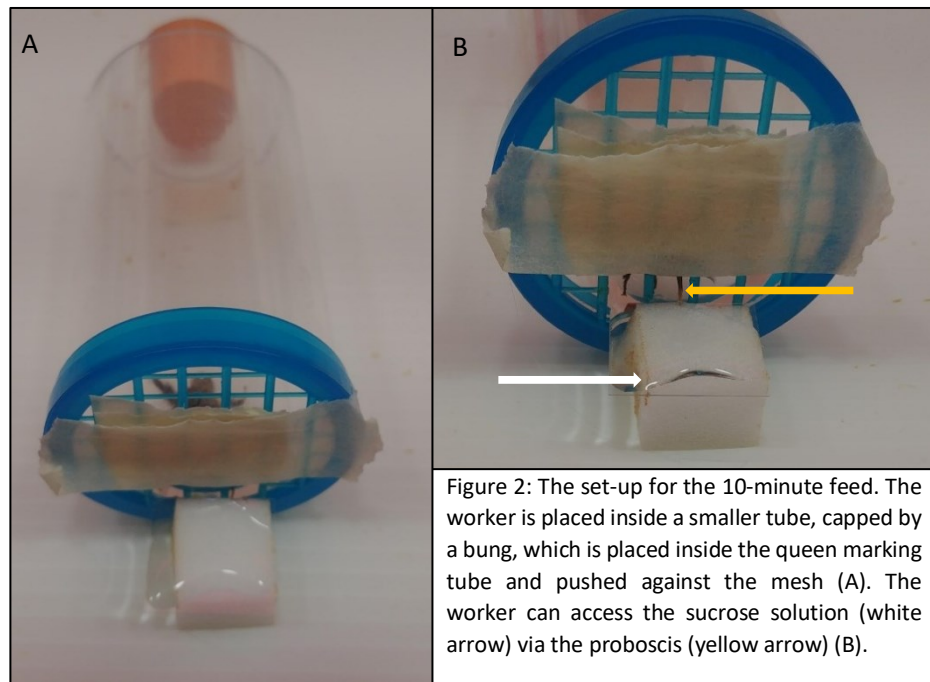


Figure 1: The experimental set-up used to monitor thorax temperatures, photographed under white light for ease of viewing. The camera is held in the clamp 0.5m above the holding pots which workers were to be placed in. This image was angled so pots can be clearly seen.

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with the 40% sucrose solution was placed outside the tube on a sponge. Workers could access this sucrose by extending their proboscis through the mesh (Fig. 2:B), and, if they did, were deemed to have fed. Individuals which did not feed were excluded. Workers were given the opportunity to feed for 10-minutes before the sucrose was removed.



Following this initial feed, the workers were split equally between two feeding classes: ‘acute’ and ‘chronic’. Acute workers were not given access to sucrose throughout the experiment, whilst chronic workers were given *ad libitum* access via the insertion of a sucrose-filled Eppendorf into the bottom of their holding pot. A hole in the Eppendorf allowed workers to access this sucrose. Workers from the same colony were split between feeding strategies as evenly as possible.

Workers were placed into individual holding pots, transferred to a CE room at the test ambient temperature, and arranged alongside each other under the camera (Fig. 1, Fig. 3:A). Eppendorfs were added for chronic workers and all workers were given 20 minutes to settle. Following this, I recorded thorax temperatures at 30-minute intervals for 4 hours, with time = 30 being the end of the 20-minute settling period. All pots were capped with one corrugated fibreboard lid, so only this lid needed to be removed to photograph bees. Whenever thorax temperatures were recorded, the lid was carefully removed, and images were taken of all bees at once (Fig. 3:B), following the thermal camera protocol.

After four hours, workers were marked with a marker pen, ensuring they were not re-used, and returned to colonies. The test temperature was rotated daily through the three test temperatures.

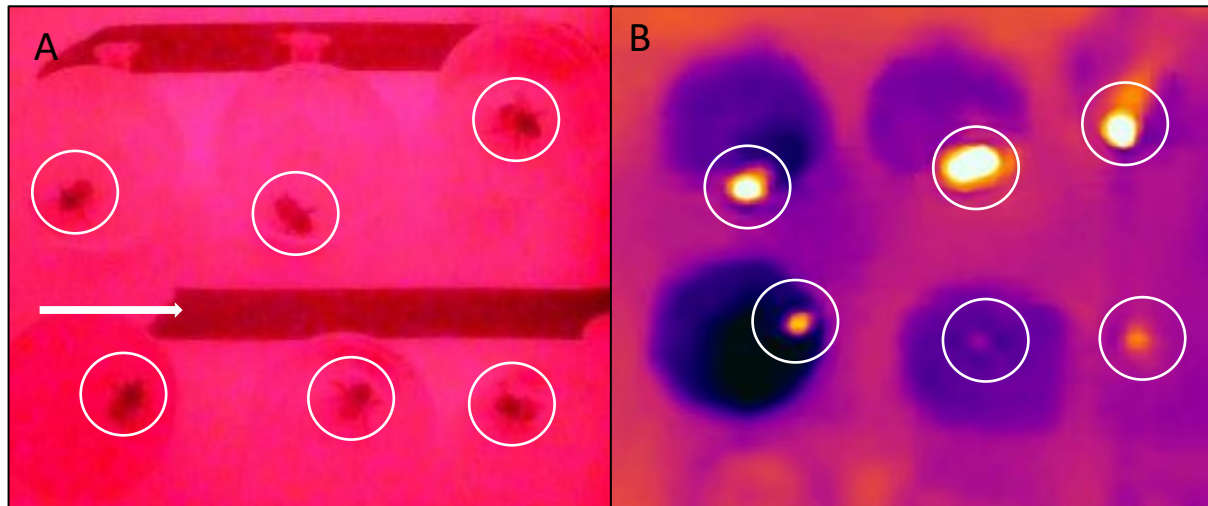


Figure 3: Example images of workers taken by the camera, normal images under red light (A) and thermal images from which thorax temperatures were calculated (B). Note the tape used to ensure thermal images were accurate (white arrow). Workers are circled. Bright white/red indicates hotter temperatures; blue/black indicates cooler (B).

Temporal responses of thorax temperature under pesticide exposure

This experiment investigated, across January and February 2020, response of worker thorax temperature following imidacloprid exposure, and was repeated five times at the three test temperatures. Due to potential time constraints, 15 °C and 25 °C were investigated first, alternated daily, before 20 °C. Five worker donor colonies were delivered five days in advance of this experiment beginning.

Eight workers were used each time this experiment was conducted. Where more or less than eight workers were used is discussed in S4. Each worker was removed from their colony and placed into an individual holding pot. As this experiment was concerned with the effect of worker mass, no masses were rejected. All workers were chronically fed throughout this experiment, following the previously discussed chronic feeding protocol.

The workers were transferred, in their pots, to the CE room at the test ambient temperature. Eppendorfs with either the control or pesticide solution were added, with the workers split equally between these. Control bees were fed the 40% sucrose solution, whilst pesticide exposed bees were fed this spiked with 0.01 mg/l of imidacloprid; a concentration to which wild bumblebees could be exposed (Azpiazu et al., 2019). Details concerning preparation of the imidacloprid solution are found in S5. Research suggests neonicotinoids inhibit feeding (Azpiazu et al., 2019; Laycock et al., 2012), which risked confounding my results. Experiments in January 2020 confirmed imidacloprid did not inhibit feeding at the temperatures and timescales I tested.

Appendix E | The effect of neonicotinoid exposure on bumblebee endothermic ability across a temperature gradient and body mass variation

Upon introduction to the test temperature and solutions, the timer was started. As above, pots were capped with a fibreboard lid, however, separate lids were used for the control and pesticide groups, meaning each group could be photographed individually for ease. Images were taken, as above, after one hour, two hours, then at 30-minute intervals until six hours had elapsed. Six hours was used to ensure any effects of imidacloprid were observed, whilst still being a feasible time period to record over. Additionally, there was no acclimatisation period and, instead, images were taken throughout. Eppendorf test solutions were weighed at the start and each time thorax temperatures were recorded.

After six hours, workers were frozen at -20°C, then dried in 80°C ovens, for 72 hours, and re-weighed, to obtain dry mass.

Estimating thorax temperatures

Within each timepoint, images were excluded following the thermal camera protocol, before thorax temperatures were estimated for workers. Within each image, workers where the thorax view was obscured were excluded. Maximum thorax temperature acted as a proxy for thorax temperature (Belzunces et al., 1996), and emissivity in FLIR Tools was set to 0.97; the estimated emissivity of the cuticle. (Belzunces et al., 1996; Potts et al., 2018; Schmaranzer & Stabentheiner, 1987; Vandame & Belzunces, 1998). To obtain maximum thorax temperatures for each worker in an image, the thorax area was highlighted and FLIR Tools returned the highest pixel temperature. Where multiple acceptable images of the same worker remained at a timepoint, the mean thorax temperature of these images was used. Details regarding a pilot trial investigating how to estimate thorax temperature are found in S6.

Statistical analysis

Statistical analysis was conducted in R studio v1.2.5033 with R v3.6.1. Graphs were produced with ggplot2 v3.2.1 and tables with sjPlot v2.8.3. (Generalized) linear mixed models (GLMM/LMM) were constructed using lme4 v1.1-21. Emmeans v1.4.3.01 calculated post-hoc Tukey pairwise comparisons of LMM means and slopes.

The response variable was always thorax temperature. Fixed effects are described below; if they caused convergence issues it is reported in the results. Three-way interactions between fixed effects were only included if significant. Colony was included as a random factor unless it explained none of the variance. Bee ID nested within time was always included as a random effect to account for temporal pseudo-replication (Crawley, 2013). When GLMM/LMM were used, diagnostic plots were inspected to confirm if assumptions were violated.

Appendix E | The effect of neonicotinoid exposure on bumblebee endothermic ability across a temperature gradient and body mass variation

Mean thorax temperatures (°C) of the raw data over all timepoints, with timepoints representing hour gaps multiplied by two to ensure equal weighting, are reported $\pm$ standard error of the mean. Due to random effects, R^2 values are pseudo- R^2 values and results are reported as significant if $|t| > 2$ or $|Z| > 2$ (Luke, 2017).

Temporal responses of thorax temperature under acute and chronic feeding

Fixed effects were *time*, *ambient temperature*, and *feeding strategy*, alongside associated two-way interactions. Individual workers often showed steep drops in thorax temperature to ambient temperature. Consequently, thorax temperature was modelled using a binomial GLMM; thorax temperature was coded as '1' if it dropped to within 10% of ambient temperature, and '0' otherwise. For example, at 15°C ambient temperature, if thorax temperature fell below 16.5°C at a timepoint it was coded as '1'. A 10% threshold was chosen as a strict limit following visual inspection of histograms displaying thorax temperature at each ambient temperature.

The GLMM only identified whether individual workers were actively thermoregulating. Therefore, the data was also modelled via a LMM with the same fixed effects. The data did not show a clear distribution; however, most values met the assumption of normality.

Temporal responses of thorax temperature under pesticide exposure

Firstly, I wanted to exclude workers which had under-consumed sucrose, as I knew this could influence thorax temperature based on the below results comparing the two feeding strategies. To do this, I constructed histograms at each time point, within each test temperature, visualising the natural log (ln) of the relative sucrose consumption of each worker: $\ln(\text{total sucrose consumption/dry mass})$. These histograms showed the workers consuming the least sucrose were much lower than the rest of the workers. This gave me a cut-off point for each timepoint within each test temperature, below which I excluded workers.

Dry mass was logged throughout so it scaled linearly (Bishop & Armbruster, 1999), then standardized so the intercept represented the thorax temperature of the mean dry mass. The effect of ambient temperature was tested separately from the effect of dry mass, to avoid complicated models with multiple three- and four-way interactions. When testing the influence of ambient temperature, I used *time*, *pesticide*, and *ambient temperature*, plus associated two-way interaction terms, as fixed effects. When testing the influence of dry mass, I used *time*, *pesticide*, and *dry mass*, plus associated two-way interaction terms, alongside *ambient temperature* as a covariate due to its influence on thorax temperature. A LMM and a binomial GLMM were used in the same manner described previously. Again, most values met the assumption of normality. Additionally, when testing the influence of dry

mass, I used a LMM to test for significant three-way interactions when ambient temperatures were considered individually.

Results

In all models, colony explained none of the variance and was removed. Additionally, all three-way interactions between fixed effects were removed due to non-significance and convergence issues. For further details on model selection see S7. For each experiment, the number of samples excluded, and justifications for exclusion, are found in S8.

Temporal responses of thorax temperature under acute and chronic feeding

The final sample size was 362 readings from 47 workers imaged over eight timepoints. Chronic fed workers had a mean thorax temperature of 28.73 ± 0.41 at 15°C , 31.13 ± 0.35 at 20°C , and 32.82 ± 0.24 at 25°C . Acute fed workers had a mean thorax temperature of 18.92 ± 0.73 at 15°C , 25.22 ± 0.68 at 20°C , and 28.25 ± 0.43 at 25°C (Fig. 4).

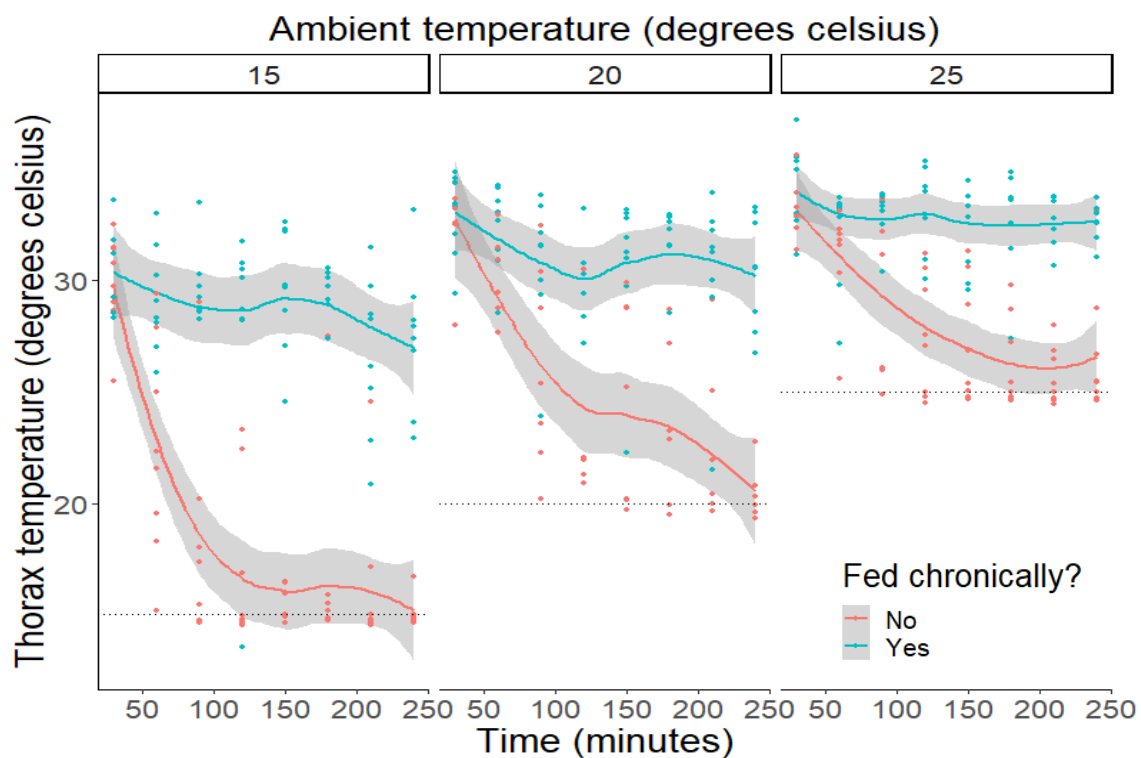


Figure 4: Changes in thorax temperature over time, grouped by ambient temperature and feeding strategy. Lines were constructed via LOESS smoothing. Horizontal dashed lines visualise the ambient temperature for each experiment. 95% confidence intervals are shaded.

When using the binomial GLMM to model the influence of feeding strategy, acute workers were more likely to show a drop of thorax temperature than chronic workers as time progressed (GLMM; $Z = -3.21$). For every minute that passed, the probability of an acute fed worker's thorax temperature dropping increased by 0.41%. Conversely, the probability of a chronically fed workers thorax temperature dropping did not significantly change as time progressed.

Temperature had no significant affect on the probability of a worker's thorax temperature dropping (Fig. 5, Table 1).

Table 1: GLMM output when thorax temperature ~ Feeding strategy*Temperature + Feeding strategy*Time + Temperature*Time. Bold values are significant. 'NA' is present, for conditional R^2 , due the necessity to include random factors explaining no variance to account for pseudo-replication.

Predictors	Drops to ambient temperature		
	Log-Odds	CI	Statistic
Intercept	-3.74	-5.63 – -1.84	-3.86
Time	0.04	0.02 – 0.05	3.94
Chronic - Yes	-0.40	-4.33 – 3.53	-0.20
Temperature - 20	-2.57	-6.08 – 0.94	-1.44
Temperature - 25	0.09	-2.41 – 2.60	0.07
Time * Chronic - Yes	-0.04	-0.07 – -0.02	-3.21
Time * Temperature - 20	0.01	-0.02 – 0.03	0.40
Time * Temperature - 25	-0.00	-0.03 – 0.02	-0.41
Chronic - Yes * Temperature - 20	1.41	-3.03 – 5.85	0.62
Chronic - Yes * Temperature - 25	1.21	-2.45 – 4.88	0.65
Observations	362		
Marginal R^2 / Conditional R^2	0.775 / NA		

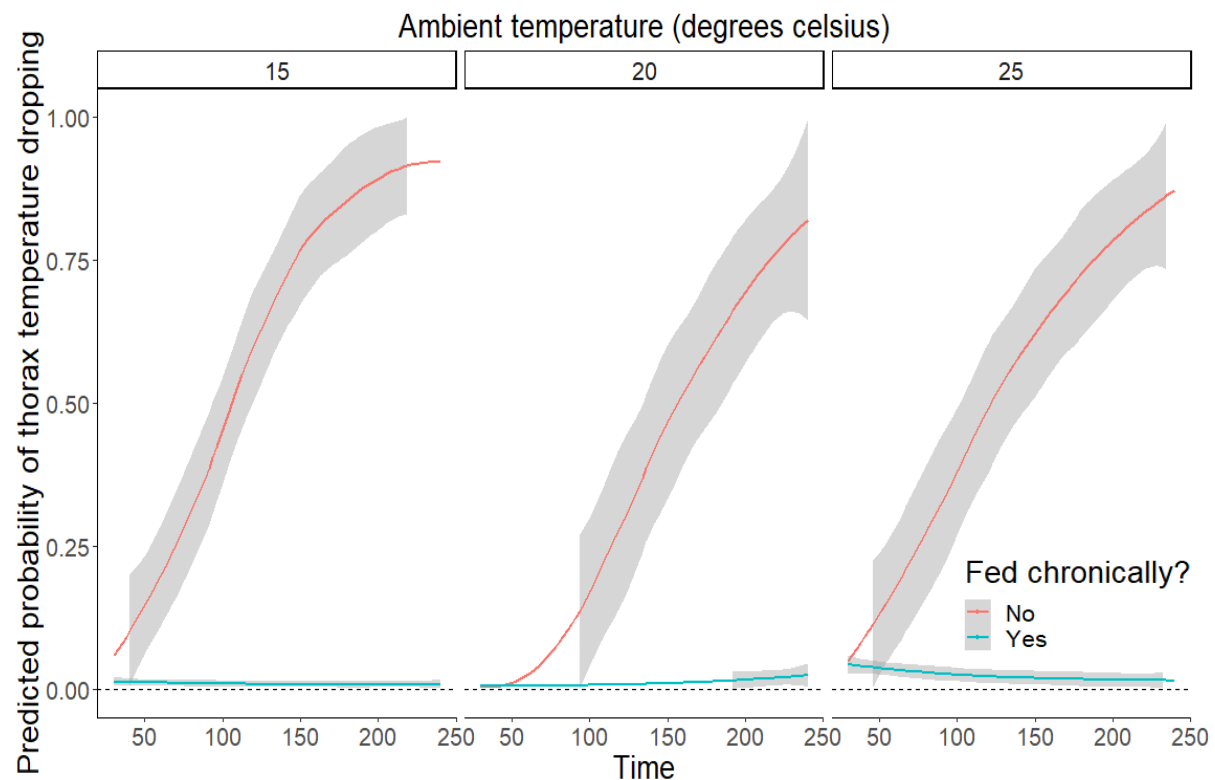


Figure 5: GLMM when thorax temperature $\sim$ Feeding strategy*Temperature + Feeding strategy*Time + Temperature*Time, grouped by feeding strategy and ambient temperature. Lines were constructed via LOESS smoothing. 95% confidence intervals are shaded.

When using the LMM to model the influence of feeding strategy, acute fed workers had significantly lower mean thorax temperatures than chronically fed workers, at all ambient temperatures (LMM; 9.7°C lower at 15°C, $t = -10.69$ | 5.7°C lower at 20°C, $t = -6.05$ | 4.7°C lower at 25°C, $t = -5.05$).

Workers under colder ambient temperatures had lower thorax temperatures. In chronic workers, mean thorax temperature decreased significantly by 2.4°C from 20°C to 15°C (LMM; $t = -2.69$), but there was no significant decrease from 25°C to 20°C (LMM; $t = -1.89$). In acute fed workers, mean thorax temperature decreased significantly by 6.4°C from 20°C to 15°C (LMM; $t = -6.82$), and 2.8°C from 25°C to 20°C (LMM; $t = -3.00$).

Appendix E | The effect of neonicotinoid exposure on bumblebee endothermic ability across a temperature gradient and body mass variation

Chronically fed workers were significantly better at maintaining an elevated thorax temperature over time than acute fed workers under all ambient temperatures (LMM; $t = 8.87$). Per minute, in chronic workers, thorax temperatures fell 0.01°C at 15°C (LMM; $t = 8.87$) but increased by 0.01°C at 25°C (LMM; $t = 3.19$). Conversely, per minute, in acute workers, thorax temperatures fell by 0.05°C at 15°C (LMM; $t = -12.88$) and by 0.03°C at 25°C (LMM; $t = 3.19$). Declines over time in thorax temperature at 20°C , for both feeding classes, were not significantly different from 15°C (LMM; $t = 0.76$) but were significantly steeper than 25°C (LMM; $t = -2.38$, Fig. 6, Table 2).

Table 2: LMM output when thorax temperature $\sim$ Feeding strategy*Temperature + Feeding strategy*Time + Temperature*Time. Bold values are significant. 'NA' is present, for conditional R^2 , due the necessity to include random factors explaining no variance to account for pseudo-replication.

Predictors	Thorax temperature ($^\circ\text{C}$)		
	Estimates	CI	Statistic
Intercept	26.20	24.61 – 27.79	32.33
Time	-0.05	-0.06 – -0.05	-12.88
Chronic - Yes	4.65	2.63 – 6.67	4.51
Temperature - 20	5.90	3.69 – 8.11	5.24
Temperature - 25	7.05	4.89 – 9.21	6.40
Time * Chronic - Yes	0.04	0.03 – 0.05	8.87
Chronic - Yes * Temperature - 20	-3.99	-6.52 – -1.46	-3.09
Chronic - Yes * Temperature - 25	-5.10	-7.59 – -2.61	-4.02
Time * Temperature - 20	0.00	-0.01 – 0.01	0.76
Time * Temperature - 25	0.02	0.01 – 0.03	3.19
Observations	362		
Marginal R^2 / Conditional R^2	0.779 / NA		

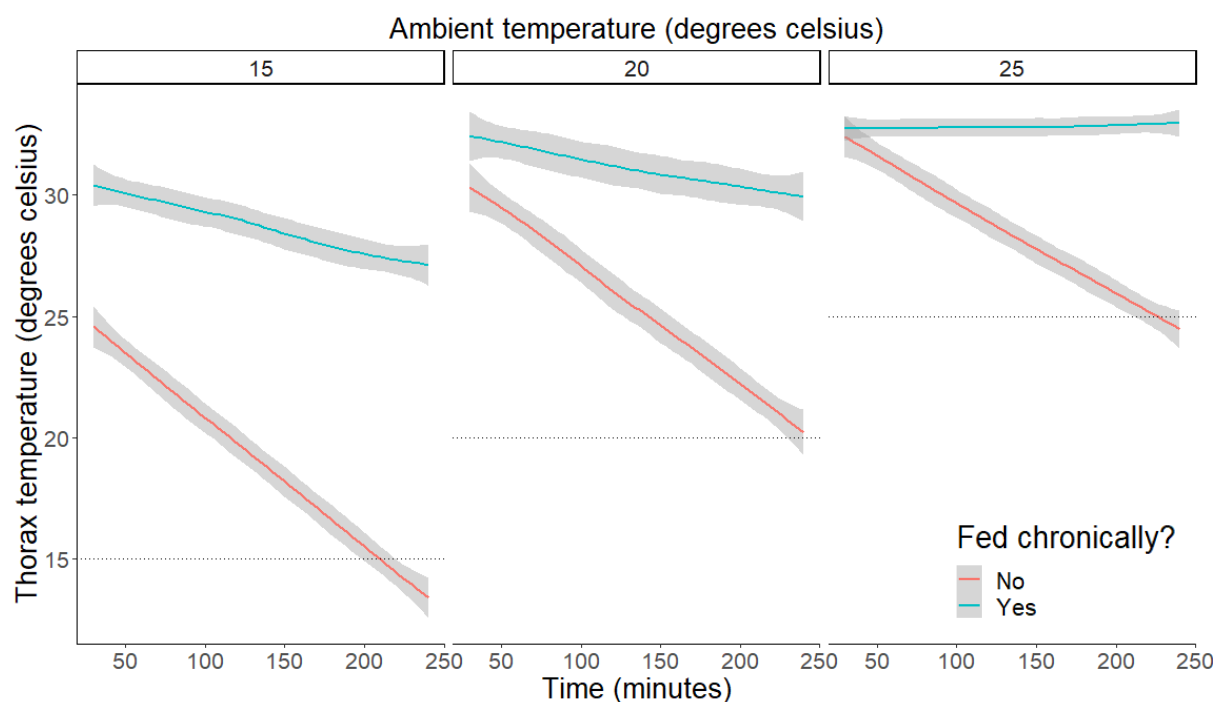


Figure 6: LMM when thorax temperature $\sim$ Feeding strategy*Temperature + Feeding strategy*Time + Temperature*Time, grouped by ambient temperature and feeding strategy. Horizontal dashed lines visualise the ambient temperature for each experiment. 95% confidence intervals are shaded.

Temporal responses of thorax temperature following pesticide exposure

The final sample size was 1129 readings from 127 workers imaged over ten timepoints. For control workers, the mean thorax temperature was 27.94 ± 0.23 at 15°C , 30.71 ± 0.13 at 20°C , and 32.53 ± 0.10 at 25°C . For pesticide-exposed workers, the mean thorax temperature was 25.83 ± 0.37 at 15°C , 29.51 ± 0.21 at 20°C , and 32.38 ± 0.11 at 25°C (Fig. 7).

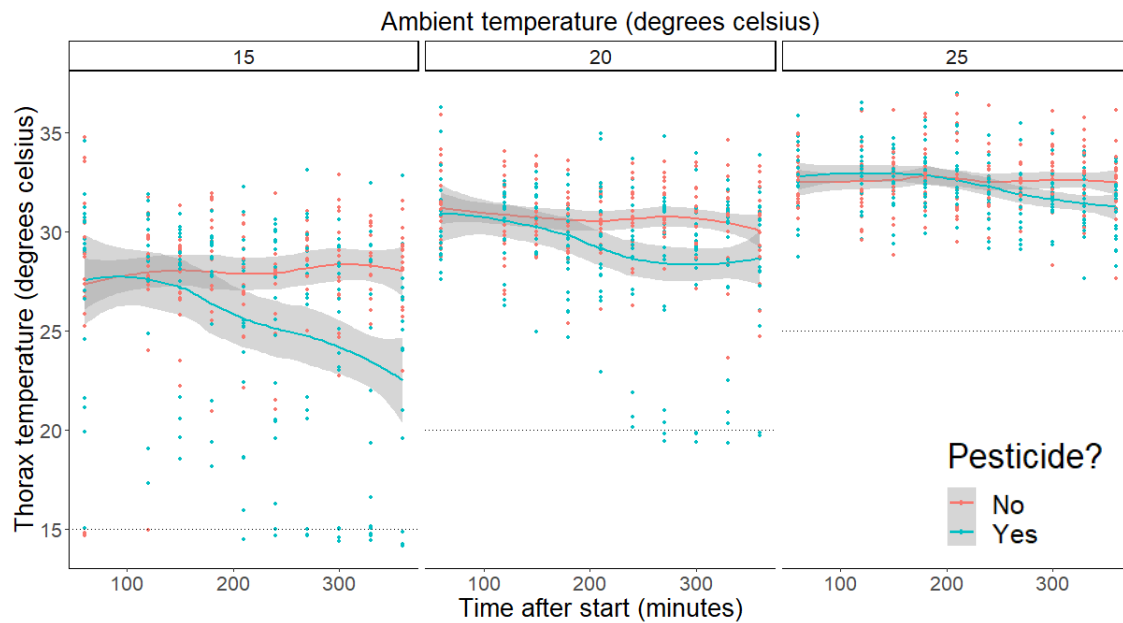


Figure 7: Changes in thorax temperature over time, grouped by ambient temperature and whether workers had been fed a pesticide. Lines were constructed via LOESS smoothing. Horizontal dashed lines visualise the ambient temperature for each experiment. 95% confidence intervals are shaded.

The influence of ambient temperature

When using the binomial GLMM to model the influence of ambient temperature, it did not converge unless ambient temperature was removed. Thus, this model cannot inform whether the drops of thorax temperature to ambient temperature are influenced by ambient temperature.

Both workers without (GLMM; $Z = -1.67$) or with (GLMM; $Z = 1.89$) imidacloprid exposure showed no change in the probability of dropping to ambient temperature over time. For workers exposed to imidacloprid, this probability was greater than 0%, however was still $<0.01\%$ (GLMM; $Z = -2.54$, Table 3).

When using the LMM to model the influence of ambient temperature, workers subjected to lower ambient temperatures held lower mean thorax temperatures. In control workers, mean thorax temperature decreased significantly by 3.0°C from 20°C to 15°C (LMM; $t = -5.17$), and by 1.9°C from 25°C to 20°C (LMM; $t = -3.15$). In imidacloprid workers, mean thorax temperature decreased significantly by 4.0°C from 20°C to 15°C (LMM; $t = -6.84$) and by 2.9°C from 25°C to 20°C (LMM; $t = -4.82$).

Imidacloprid exposure lowered mean thorax temperatures in workers by 2.4°C at 15°C (LMM; $t = 3.96$) and by 0.7°C at 20°C (LMM; $t = 2.43$). Conversely, exposed workers at 25°C held similar mean thorax temperatures to non-exposed workers (LMM; $t = 0.99$).

In both imidacloprid-exposed and control workers, temperature did not significantly affect the rate of thorax temperature decline over time. Imidacloprid-exposed workers showed significant declines in thorax temperature over time compared to control groups (LMM; $t = -3.46$), which showed no significant declines

Table 3: GLMM output when thorax temperature ~ Pesticide*Time. Bold values are significant. Time was rescaled by dividing by 30. 'NA' is present, for conditional R^2 , due the necessity to include random factors explaining no variance to account for pseudo-replication.

Predictors	Drops to ambient temperature		
	Log-Odds	CI	Statistic
Intercept	-2.17	-10.06 – 5.72	-0.54
Pesticide - Yes	-12.82	-22.73 – -2.91	-2.54
Time	-3.18	-6.92 – 0.55	-1.67
Pesticide - Yes * Time	3.64	-0.13 – 7.41	1.89
Observations	1129		
Marginal R^2 / Conditional R^2	0.967 / NA		

Table 4: LMM output when thorax temperature ~ Pesticide*Time + Pesticide*Temperature + Time*Temperature. Bold values are significant. Time was scaled by dividing by 30.

Predictors	Thorax temperature ($^{\circ}\text{C}$)		
	Estimates	CI	Statistic
Intercept	27.86	26.36 – 29.36	36.35
Temperature - 20	3.11	1.19 – 5.03	3.18
Temperature - 25	4.17	2.22 – 6.12	4.20
Pesticide - Yes	0.04	-1.67 – 1.76	0.05
Time	-0.02	-0.21 – 0.17	-0.24
Temperature - 20 * Pesticide - Yes	1.00	-0.62 – 2.62	1.21
Temperature - 25 * Pesticide - Yes	2.00	0.35 – 3.65	2.37
Temperature - 20 * Time	-0.01	-0.24 – 0.22	-0.07
Temperature - 25 * Time	0.10	-0.13 – 0.33	0.83
Pesticide - Yes * Time	-0.33	-0.52 – -0.14	-3.46
Observations	1129		
Marginal R^2 / Conditional R^2	0.417 / 0.733		

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(LMM; $t = -0.24$). Per minute, imidacloprid-exposed worker's thorax temperatures declined by 0.01°C (LMM; $t = -3.46$, Fig. 8, Table 4).

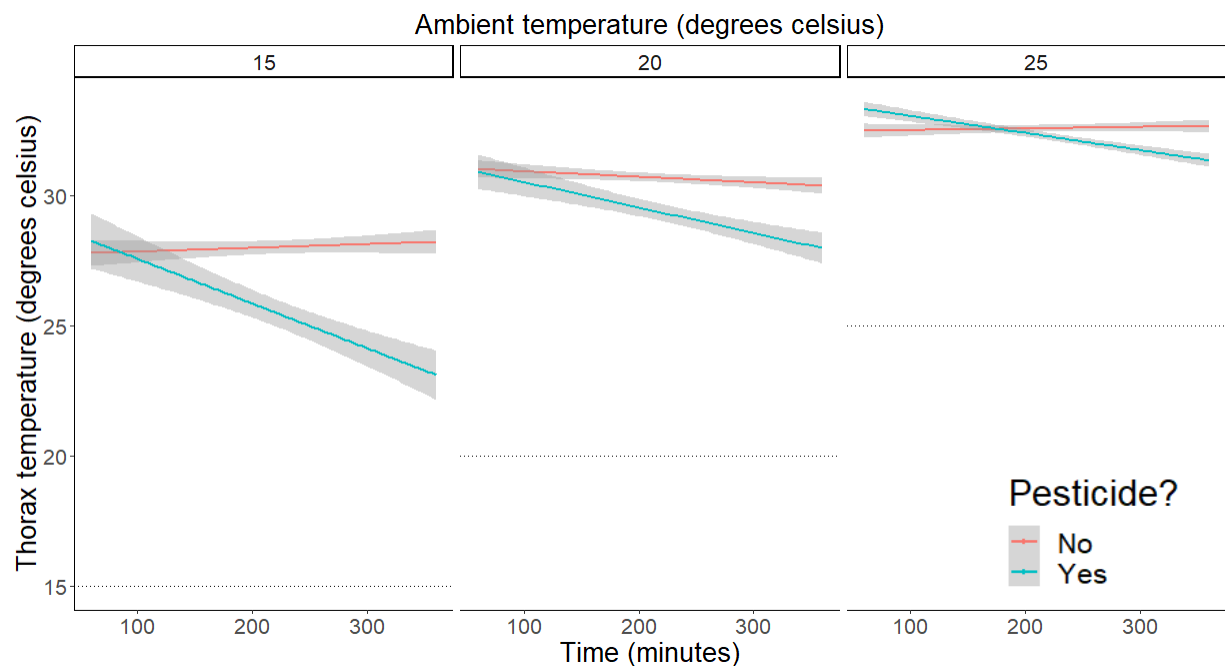


Figure 8: LMM when thorax temperature $\sim$ Pesticide*Time + Pesticide*Temperature + Time*Temperature, grouped by ambient temperature and whether they had been fed a pesticide. Horizontal dashed lines visualise the ambient temperature for each experiment. 95% confidence intervals are shaded.

The influence of dry mass

When using the binomial GLMM to model the influence of dry mass, it only converged when thorax temperature was predicted by time, dry mass, the pesticide, and the interaction between dry mass and the pesticide.

Worker dry mass did not influence whether thorax temperature dropped to ambient temperature; both without (GLMM; $Z = -0.22$) and with (GLMM; $Z = 0.14$) imidacloprid exposure (Table 5).

When using the LMM with ambient temperature as a covariate to model the influence of dry mass, at $t = 0$ in control workers, dry mass had a significant effect on thorax temperature. An increase of one standard deviation in worker mass resulted in an

Table 5: GLMM output when thorax temperature $\sim$ Time + Pesticide*Dry Mass. Bold values are significant. Time was scaled by dividing by 30 and $\ln(\text{dry mass})$ is standardized.

Predictors	Drops to ambient temperature		
	Log-Odds	CI	Statistic
Intercept	-11.35	-17.43 – -5.26	-3.66
Time	-0.14	-0.61 – 0.34	-0.57
Pesticide - Yes	1.83	-2.81 – 6.48	0.77
Dry Mass	-0.45	-4.35 – 3.45	-0.22
Pesticide - Yes * Dry Mass	0.31	-3.98 – 4.59	0.14
Observations	1129		
Marginal R^2 / Conditional R^2	0.006 / 0.984		

Appendix E | The effect of neonicotinoid exposure on bumblebee endothermic ability across a temperature gradient and body mass variation

increase of 2.34 °C in thorax temperature (LMM; $t = 2.21$). This relationship did not differ significantly for imidacloprid-exposed workers (LMM; $t = 0.83$).

Due to the non-significant three-way interaction, this effect of mass on thorax temperature decreased equally in both imidacloprid-exposed and control workers over time (LMM; $t = -2.10$). This relationship was reversed after 255 minutes, where smaller workers held higher thorax temperatures (Fig. 9, Table 6).

When ambient temperatures were considered individually, there was still no significant three-way interactions (LMM; $t = 0.56$ at 15 °C | $t = 0.39$ at 20 °C | $t = -0.75$ at 25 °C).

Table 6: LMM output when thorax temperature $\sim$ Temperature + Pesticide*Time + Pesticide*Dry Mass + Time*Dry Mass. Bold values are significant. Time is scaled by dividing by 30 and $\ln(\text{dry mass})$ is standardized.

Predictors	Thorax temperature (°C)		
	Estimates	CI	Statistic
Intercept	27.05	25.96 – 28.13	48.79
Temperature - 20	3.61	2.78 – 4.43	8.57
Temperature - 25	5.88	5.02 – 6.73	13.44
Time	0.01	-0.11 – 0.14	0.22
Pesticide - Yes	1.33	-0.05 – 2.72	1.89
Dry Mass	0.85	0.10 – 1.60	2.21
Time * Pesticide - Yes	-0.36	-0.54 – -0.17	-3.81
Time * Dry Mass	-0.10	-0.19 – -0.01	-2.10
Pesticide - Yes * Dry Mass	0.29	-0.40 – 0.98	0.83
Observations	1129		
Marginal R^2 / Conditional R^2	0.413 / 0.732		

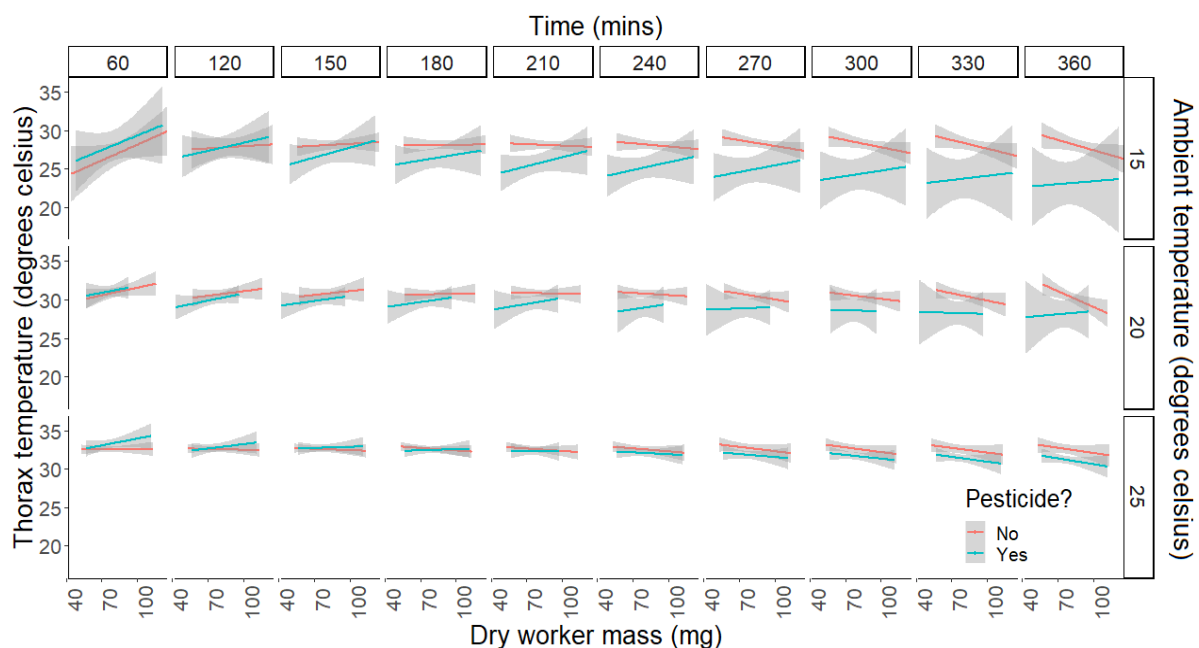


Figure 9: LMM plot, when thorax temperature $\sim$ Temperature + Pesticide*Time + Pesticide*Dry Mass + Time*Dry Mass, grouped by time, ambient temperature, and whether they had been fed a pesticide. 95% confidence intervals are shaded.

Discussion

To my knowledge, this is the first study investigating the interactions of environmental temperature, body mass, and a neonicotinoid on bumblebee NFT. It appears to be the first study demonstrating how different experimental feeding strategies influence thorax temperatures.

Nevertheless, this study had limitations. Workers infrequently escaped during imaging, and subsequent capture and reintroduction to their holding pot likely caused stress, possibly influencing thorax temperature. Ideally, if repeating this experiment, I would test whether a mesh barrier over pots could prevent worker escape whilst still allowing imaging. Additionally, when studying the influence of imidacloprid exposure on thorax temperature, chronic feeding meant workers consumed different quantities of sucrose, potentially influencing thorax temperature. Although I excluded workers who under-consumed sucrose, this was via a crude method. By investigating how the volume of sucrose consumed influences thorax temperature, a more sophisticated method could be developed to eliminate readings influenced by abnormally low sucrose consumption. Alternatively, with more temperature readings, I could have incorporated relative sucrose consumption into models to account for its influence on thorax temperature.

When testing different feeding strategies, acute fed workers (with a single feeding opportunity) were unable to generate endogenous heat, quickly showing drops of thorax temperature until equilibration with ambient temperature. Conversely, chronically fed workers (with unlimited sucrose access) successfully maintained elevated thorax temperatures. Although the mechanism underpinning this is unclear, workers may metabolise their initial energy reserves, with observed drops in thorax temperature due to a type of starvation. Alternatively, this response may be adaptive, and when energy reserves are low, individuals prioritise other physiological processes over thermoregulation. As insect flight muscles are most efficient at high thorax temperatures (Esch, 1976; Heinrich, 1972), and as it appears maladaptive to jeopardise a key process such as flight by reducing thorax temperature, the starvation hypothesis appears more likely. Future research could investigate if workers can rewarm after these drops have occurred; suggesting this effect is not related to starvation.

As bees unable to continuously feed lose their endothermic ability, this has implications in natural settings. Intensification of agriculture can lead to a loss of floral resources and therefore energy-rich nectar (Goulson et al., 2008). If wild bumblebees cannot obtain enough energy when foraging they risk jeopardising endothermy and their potential to warm flight musculature. This may impair foraging ability, an effect subsequently amplified by colder spring temperatures when colonies are more vulnerable (Esch, 1976; Goulson et al., 2008; Heinrich, 1972). Regarding future research into the

effects of neonicotinoids on bee thermoregulation, care should be taken to discern if changes in thorax temperature are caused by the pesticide or feeding strategy. For example, Tosi *et al* (2016) used acute feeding when testing the effect of thiamethoxam on honeybee NFT. My results suggest this feeding strategy may have confounded their results, especially as non-exposed honeybee thorax temperatures also decreased (Tosi *et al.*, 2016).

This study recovered no significant influence of imidacloprid exposure on the relationship between body mass and thorax temperature, although large error sizes may have hindered detection of significance. Regardless, at the start of the experiment, I found larger workers held higher thorax temperatures, as expected (Bishop & Armbruster, 1999; Heinrich, 1972). Intriguingly, this relationship inverted over time until smaller workers thermoregulated to higher temperatures and, as the three-way interaction was consistently non-significant, this meant imidacloprid exposure did not significantly influence this change in relationship over time. To my knowledge, this is the first time this effect of time has been observed. Within foragers, Goulson *et al* (2002) found that smaller workers foraged for greater periods of time. As a raised thorax temperature underpins flight (Esch, 1976; Heinrich, 1972), the findings of Goulson *et al* (2002) infers smaller foragers need to maintain a high thorax temperature for longer time periods compared to larger foragers, a suggestion which my results support. As evidence that body size affects pollen foraging efficiency is not conclusive either way (Goulson *et al.*, 2002), this change in relationship over time may allow smaller foragers to match the pollen foraging efficiency of their larger nestmates. Smaller foragers may also benefit colonies by exploiting different flower types or foraging when predators are common (Gavini *et al.*, 2020; Peat, Tucker, *et al.*, 2005).

Imidacloprid exposure caused significant declines over time in thorax temperature compared to control workers. However, only at 15 °C and 20 °C did imidacloprid exposure lead to a decrease in mean thorax temperature, with 15 °C producing the largest decrease. Interestingly, the rate of decline over time did not differ significantly between temperatures. Although appearing contradictory, this result could be due to a lag time between imidacloprid exposure and the declines of thorax temperature, which appears to increase as temperature decreases (Fig. 7).

Although my findings are consistent with other studies (Potts *et al.*, 2018), Tosi *et al* (2016) reported that, over time, imidacloprid initially stimulates NFT, before oversaturation of receptors disrupts NFT. This disruption manifests as a rise and fall of thorax temperature, which this study did not convincingly observe (Fig. 7), suggesting this may not be important at colder temperatures or when workers continuously feed.

The thermoregulatory impairment caused by imidacloprid was temperature dependent, with lower temperatures causing a greater decrease in thorax temperature in imidacloprid-exposed workers. Crall *et al* (2018) demonstrated a similar effect where colony-level thermoregulation was impaired more by imidacloprid at night, when temperatures were lower. My findings suggest neonicotinoid-driven impairment of NFT increases at colder temperatures, suggesting this contributed to increased impairment of colony-level thermoregulation at night. However, other neonicotinoid-driven effects could act in tandem, such as impairment of ATP production or colony failure to construct an insulating wax canopy (Crall *et al.*, 2018; Nicodemo *et al.*, 2014). A wide range of NFT-dependent behaviours are affected by neonicotinoids, such as flight and foraging (Gill *et al.*, 2012; Heinrich, 1972, 1975; Kenna *et al.*, 2019). Neonicotinoids may impair these behaviours by disrupting NFT; yet more research is needed to test the interaction of lower temperatures and neonicotinoids on the discussed behaviours. As the effect of imidacloprid is more pronounced at colder temperatures, this suggests application of neonicotinoids to crops in spring could lead to amplified impacts on bees. This research could inform agricultural management decisions which reduce the impact of neonicotinoids on bees.

Understanding the implications of the interaction between environmental temperature and neonicotinoids is important in predicting future responses of bees to climate change. Climate change may lead to more frequent extreme weather events; including cold spells (Francis & Vavrus, 2012). Although colonies usually thermoregulate effectively at cold temperatures (Heinrich, 1974a), neonicotinoid exposure could severely impair NFT during these frequent cold spells, with amplified knock-on effects on behaviour and colony-level thermoregulation. Additionally, climate change causes large scale warming (Houghton *et al.*, 2001), and research demonstrates warming leads to increased occupancy by bee species at the colder end of their thermal tolerance range (Soroye *et al.*, 2020). With warming, individuals could hold higher thorax temperatures, something my results and others demonstrate (Heinrich, 1972; Stone & Graham, 1993). Subsequently, warming may improve the efficiency of temperature-mediated behaviours and contribute to occupancy increases. Widespread application of neonicotinoids could inhibit occupancy increases by inhibiting NFT; an effect more severe across lower temperatures, particularly when combined with other effects of warming such as phenological shifts (Memmott *et al.*, 2007).

In summary, this study demonstrated neonicotinoid-driven impacts on NFT can be influenced by environmental temperature. Future research into the impact of neonicotinoids should consider the influence of temperature variation; to better predict how neonicotinoids will impact pollinators both throughout the year and in a warming world.

Supplementary Information

Supplementary Information 1 (S1)

Colonies, supplied by an external company (Agralan Ltd), were delivered in plastic boxes mimicking hives (29 × 22.5 × 13 cm). Each colony started with approximately 50 workers and was visually inspected to ensure a queen was present and colonies were healthy. All colonies were delivered with pollen balls and a sucrose reservoir containing 'biogluc' (a solution of sucrose and antimicrobial compounds). These sources of sucrose and pollen were instantly removed and replaced. Dead bees were removed, and colonies were cleaned weekly. Pollen was stored at -20°C between feeds.

Supplementary Information 2 (S2)

To my knowledge, the chosen thermal camera has not yet been used in thermoregulation studies. Issues have previously been identified with all FLIR technology. FLIR cameras automatically calibrate frequently to ensure that readings remain accurate, however, if the housing changes temperature significantly then this can make the calibration less effective (Malmivirta et al., 2019). Therefore, it was important to develop a protocol to ensure readings were accurate and reliable across different temperatures.

I tested the thermal camera at ambient temperatures at the extremes of my proposed temperature range (15°C and 25°C) in CE rooms. The surface being measured was covered with a strip of black electric tape. This is because the emissivity of black electric tape is known (0.95), and when the camera is correctly calibrated it should read the ambient temperature of the room, when emissivity is accounted for in FLIR Tools. The camera was held in a clamp 0.5m above the tape. Readings were taken of the temperature of the tape every 10 seconds over a period of 10 minutes from camera switch-on, to observe if/how readings changed over time. Temperature readings did converge, but at 25°C this recorded the wrong temperature for the ambient temperature of the room I was in. As a result, I decided to continue using the black electrical tape in images to ensure inadequately calibrated images could be excluded.

Supplementary Information 3 (S3)

When testing the influence of imidacloprid on thorax temperature, workers were initially selected from colony 6 more frequently; colony 6 was much larger and this gave chance for the other four colonies to grow.

Supplementary Information 4 (S4)

I ran eight workers each time (four control, four imidacloprid-exposed). Exceptions to this were trials 1, 2, and 16. Trials 1-2 had 12 tubs per trial (six control, six pesticide). However, this was reduced to eight as it was too difficult to ensure all workers could be photographed adequately. Trial 16 had five tubs to supplement missing pesticide data due to inadequate early thermal photography.

Supplementary Information 5 (S5)

The pesticide solution was prepared from a diluted stock solution of 10,000mg/l stored at -80 °C. During dilution, light exposure was kept to a minimum to prevent breakdown of the active compound (Soliman, 2012), before being separated into aliquots and stored in the dark at -20 °C.

Supplementary Information 6 (S6)

As part of a pilot trial in November 2019, I trialled a different method for estimating thorax temperature. First, I selected the thorax area which a temperature estimate was to be produced from. I then excluded the coolest 50% of pixel temperatures and the hottest 10% of pixels, based on visual inspection of histograms showing pixel temperatures. From the remaining pixels, I calculated the mean. This was trialled in case max thorax temperature resulted in values skewed by abnormally hot pixels. Repeating this for all thorax temperatures in the main trial was too time consuming and not feasible, however, in these pilot trials, max thorax temperature was strongly correlated with this estimate (Spearman's rank; $r = 0.94$, $p < 0.001$). Consequently, max thorax temperature was accepted as a proxy for thorax temperature.

Supplementary Information 7 (S7)

Each model was progressively simplified by the removal of one factor (first colony, followed by the three-way interaction). Each simpler model was compared with the initial, more complex, model to test whether the more complex model was significantly better at explaining the data. After each factor was removed, and if the previous, more complex, model was significantly better at explaining the data, chi-squared < 0.05 (Table 7). The three-way interaction for the GLMM testing the interaction between Pesticide:Mass would not converge, hence being reported as 'N/A'.

Table 7: Chi-squared values from model comparisons. ‘Model type’ and ‘experiment’ identify the exact model being simplified. ‘Factor removed’ shows which fixed factor was removed to simplify the model, with removal if the chi-squared value > 0.05, suggesting the more complex model was not significantly better at describing the data.

Model type	Experiment	Factor removed	Chi-squared
GLMM	Acute vs chronic	Colony	1
GLMM	Acute vs chronic	Three-way interaction	0.85
LMM	Acute vs chronic	Colony	1
LMM	Acute vs chronic	Three-way interaction	0.14
GLMM	Pesticide : Temperature	Colony	0.3
GLMM	Pesticide : Temperature	Three-way interaction	1
LMM	Pesticide : Temperature	Colony	0.29
LMM	Pesticide : Temperature	Three-way interaction	0.17
GLMM	Pesticide : Mass	Colony	0.93
GLMM	Pesticide : Mass	Three-way interaction	N/A
LMM	Pesticide : Mass	Colony	0.42
LMM	Pesticide : Mass	Three-way interaction	0.84

Supplementary Information 8 (S8)

For the temporal response of thorax temperature under acute and chronic feeding, out of a maximum of 54 workers representing 432 thorax readings across treatments and timepoints, seven workers (four acute, three chronic) didn’t initially feed, so were excluded. Following this, 14 temperature readings were then excluded due to either an inadequate thorax view, inadequate camera calibration, or escaped bees. This left 126 recordings at 15°C, 114 recordings at 20°C, and 122 recordings at 25°C.

For the temporal response of thorax temperature to imidacloprid, out of a maximum of 127 workers representing 1270 thorax temperature recordings across treatments and time points, 56 readings were removed due to underfeeding (27 control and 29 pesticide). Following this, 85 readings were then excluded due to either an inadequate thorax view, inadequate camera calibration, or escaped bees. This left 366 recordings at 15°C, 384 recordings at 20°C, and 379 recordings at 25°C.