# The effects of neonicotinoids and increasing temperatures on the thermoregulation and flight ability of South African honey bees, *Apis mellifera scutellata* (Lepeletier)

by

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Submitted in fulfilment of the requirements for the degree

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# Declaration

I, **Laura Catherine Bester**, declare that the thesis/dissertation, which I hereby submit for the degree **PhD (Entomology)** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date: 09 **January 2023**

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# The effects of neonicotinoids and increasing temperatures on the thermoregulation and flight ability of South African honey bees, *Apis mellifera scutellata* (Lepeletier)

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**Abstract:** Predicted climatic changes throughout the southern African region are likely to affect the distribution of ecological zones, significantly impact agricultural yield, and influence the timing of the flowering season. Climate change has the potential to influence pollinators, especially the honey bee; reducing survival, affecting behaviour and physiology, as well as impacting biodiversity and agriculture. Similarly, agriculturally significant neonicotinoid pesticides have been found to negatively impact many aspects of honey bee physiology and survival. Their widespread usage in Africa and the effects on its pollinators therefore necessitates further investigation. The physiological and behavioural thermoregulatory activities of *Apis mellifera scutellata,* and how they are affected by exposure to three neonicotinoid active ingredients (clothianidin, imidacloprid and thiamethoxam), was investigated. On an individual level, a baseline thermotolerance for *A.m. scutellata* was established ($LT_{50}$=53.77 °C). Exposure to sublethal neonicotinoid doses elicited a 3 °C drop in the $LT_{50}$ of *A.m. scutellata.* Flight efficiency and associated parameters were assessed under three different ambient temperatures, both with and without sublethal neonicotinoid exposure. Using flight parameters as a proxy for flight muscle function and by extension thermoregulatory ability, it was found that the majority of parameters were not significantly impacted in *A.m. scutellate*. However, the honey bees' ability to initiate flight at all was severely affected. Colony level thermoregulatory efficiency was measured using a custom built, in-hive monitoring system that recorded internal hive temperatures at five second intervals over three months. This monitoring was performed both before and after sublethal neonicotinoid exposure. Following neonicotinoid exposure, it was observed that the hives exhibited considerable resilience in terms of maintaining the closely regulated temperatures in the brood frame area. However, the outlying areas within the hive showed more unpredictable fluctuation. These three studies demonstrate that 1) individual LT50 dropped by 3 °C, 2) flight initiation was impaired and 3) colony level thermoregulation efficiency was reduced in non-brood areas, when exposed to sub-lethal neonicotinoid active ingredients clothianidin, imidacloprid and thiamethoxam. More importantly, these results highlight the importance of how the effects may be further exacerbated in the context of both local and global climatic change. These changes include the increased frequency and intensity of extreme weather events. The unique agricultural landscape of South Africa encompasses commercial, small scale and subsistence farming practices that employ varied uses of insecticides, which further contribute to these effects. The results of the studies demonstrate the importance of further research into the effect of these insecticides on honey bees in the unique agricultural context.

**Key words:** honey bees / climate change / neonicotinoids / thermoregulation / flight

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“We have chosen to fill our hives with honey and wax; thus
furnishing mankind with the two noblest of things,
which are sweetness and light.”

~Jonathan Swift (1667-1745)

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# Manuscripts

The literature review in Chapter 1 formed part of the published Academy of Science South Africa report on neonicotinoids in Africa. The data chapters in this thesis have been prepared for publication as separate manuscripts and are structured as such. Data chapters have been prepared as separate manuscripts for publication and subsequently there will be a degree of repetition with regards to methodology. Chapter 2 has been accepted for publication in the journal Apidologie, Chapter 3 has been submitted to the Journal of Apidological Research.

# CHAPTER 1

# General Introduction

## ABSTRACT

Temperature is a pivotal factor affecting biodiversity. An increase in the frequency and intensity of extreme weather events are more commonly experienced nowadays, including heatwaves. This means that predicted changes in temperatures, especially throughout the southern African region, are likely to affect the distribution of ecological zones, significantly impact agricultural yield and influence the timing of the flowering season. Temperature changes in turn have the potential to influence pollinators, such as the honey bee; reducing survival, affecting behaviour and physiology. These influences impact biodiversity and agriculture. Temperature plays an important role in many aspects of honey bee physiology and behaviour and is crucial to their survival. Similarly, agriculturally significant neonicotinoid pesticides have been found to negatively impact many aspects of honey bee physiology and survival. Neonicotinoid usage in Africa was found to be widespread, has been more frequent in use and a chosen method for crop protection to combat pest-resistance to outdated pesticide chemical groups. This is particularly true for Southern Africa. In the context of rising ambient temperatures and increasing incidences of thermal extremes, the effect that

commercially used neonicotinoids may have on honey bee thermoregulatory activities is of great importance.

# GENERAL INTRODUCTION

## *Temperature*

Temperature is a major factor affecting biodiversity (Heller & Zavaleta 2009; Ackerly *et al*. 2010, Dawson *et al*. 2011). Global mean temperatures are predicted to rise by approximately 4 °C by 2100 (Thuiller 2007), along with an increase in both the frequency and intensity of extreme weather events (Meehl *et al*. 2007; Thuiller 2007; Le Conte & Navajas 2008; Westerling & Bryant 2008; Krawchuk *et al*. 2009) including altered rainfall patterns and heatwaves (Schär *et al.* 2004; Luber & McGeehin 2008; Førland *et al.* 2011). Each 1 °C change in global mean temperature has the potential to shift ecological zones on Earth further away from the equator by approximately 160km (Thuiller 2007). Changes in global temperatures and shifting ecological zones may in turn alter the latitudinal and altitudinal distribution of a wide variety of species (Kelly & Goulden 2008; Chen *et al*. 2011) along with the composition and quality of flowering plant communities (Le Conte & Navajas 2008) and timing of seasonal bloom of flowering plants (Fitter & Fitter 2002; Franks *et al.* 2007).

Temperatures in the southern African region are expected to change significantly in the future and many areas are predicted to become warmer and drier and receive comparatively less precipitation (Scholes 2004). Such temperature and precipitation changes are illustrated under the future scenario of continued high emissions (Fig. 1.1). Increasing temperatures and lowered precipitation have the potential to significantly impact agricultural yield, with agricultural yield in South Africa predicted to decrease by 15% to 25% over the period of 2003 to 2080 (Cline 2007). At the global level, changing climatic conditions over the last three decades have already resulted in a global decrease of agricultural yield of around 1-5% per decade (Porter *et al.* 2014). With changing global temperatures comes changing weather patterns, that will in turn be reflected in a number of significant changes, among these are shifts in the timing of the flowering season (Dunnell & Travers 2011; Cleland *et al* 2012) of a number of plant species as well as the changing conditions that pollinators experience due to the nature of their foraging activities (Corbet *et al.* 1993; Hegland *et al.* 2009). These resultant changes will in turn impact their survival and their influence on biodiversity (Fründ *et al.* 2013).

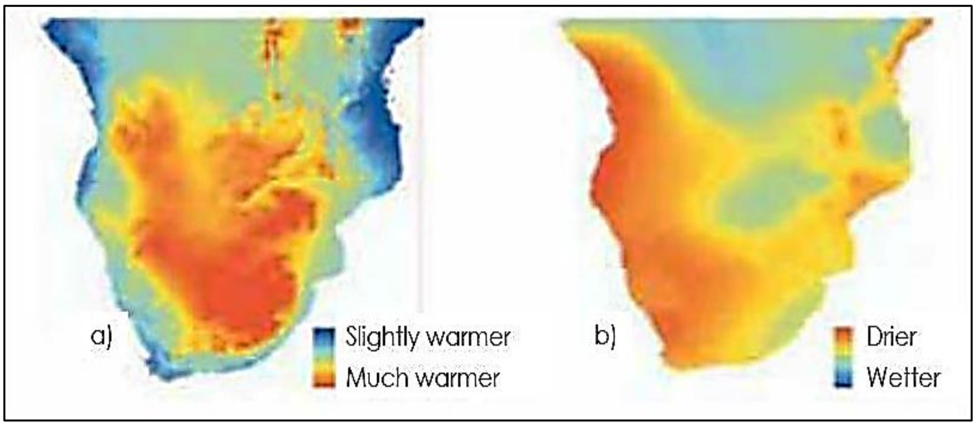

**Figure 1.1:** Projected climate change in southern Africa: HADCM3 climate model projections of change in (a) temperature and (b) precipitation for 2050 relative to mean conditions over the 1961 – 1990 period, under the IPCC SRES A2 (high emissions) scenario (modified after Scholes 2004).

*Pollinators*

Pollinators, specifically honey bees, are an integral part of agricultural production and subsequent global food security (Williams 1994; Gallai *et al*. 2009; Genersch 2010; Eilers *et al*. 2011; Klein *et al*. 2006). Honey bees are of great economic, agricultural and natural importance (McGregor 1976; Watanabe 1994; Roubik 2002; Southwick & Southwick 1992; Klein *et al*. 2006; Potts *et al.* 2006; Moritz & Crewe 2018). While they are by no means the only pollinators of wild and cultivated plants, they are very important for the pollination of a variety of commercial crops including, but not limited to; broad bean (*Vicia faba major*), common bean (*Vicia faba minor*), lima bean (*Phaseolus lunatus*), cucumber (*Cucumis sativus*), okra (*Abelmoschus esculentus*), pumpkin, squash and marrow (*Cucurbita* spp.), apples (*Malus domestica*), avocado (*Persea Americana*), citrus (*Citrus* spp.), cherry (*Prunus avium*), guava (*Psidium guajava*), litchis (*Litchi chinensis*), melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), peach (*Prunus persica*), pear (*Pyrus communis*), plum (*Prunus* spp.), strawberry (*Fragaria* x *ananassa*), cashew nut (*Anacardium occidentale*), macadamia nut (*Macadamia integrifolia*), castor (*Ricinis communis*), coconut (Cocos nucifera), sesame (*Sesamum indicum*), soybean (*Glycine max*), sunflower (*Helianthus annus*), coffee (*Coffea arabica*), and cotton (*Gossypium* spp.) (summarised in Rodger *et al*. 2004). Continued pressure applied by these changing climatic conditions, the ever-increasing global populations and the demand for improved food production and security on the agricultural industry may well necessitate the increased use of various pesticides to guarantee successful yield (Popp *et al.* 2013). The application of pesticides to a variety of agricultural crops is likely to affect not only their target organisms but a variety of important pollinators and other non-target organisms as well (Blacquière *et al*. 2012). Pollinator declines are of great concern worldwide (Gallai *et al.* 2009). These losses have been attributed to a multitude of interacting factors, among them the use of agrochemicals, such as pesticides (Kevan *et al.* 1997; Johnson *et al.* 2010; Potts *et al.* 2010). The majority of available information on the extent of global pollinator losses tends to originate mainly from the United Kingdom, Europe and North America (Ghazoul 2005; Kluser & Peduzzi 2007; Potts *et al.* 2010; Archer *et al.* 2014a; Woodcock *et al.* 2017). Research on pollinator populations in Africa, concerning honey bees in particular, is far less prevalent (Muli *et al*. 2014). Unlike Europe, throughout much of Africa (the other continent of its native distribution) there are still wide-ranging wild honey bee populations (Jaffe *et al.* 2010; Dietemann *et al.* 2009). Accurately quantifying these populations remains challenging and notable inconsistencies in the information surrounding pollinator population declines exists. South Africa has shown consistent signs of decreasing managed populations, with a nationwide survey indicating losses of approximately 29.6% from 2009-2010 and 46.2% decline from 2010 to 2011 (Pirk *et al.* 2014).

Honey bee population density and prevalence differ significantly across continents and across differing geographic and climatic zones. For example, honey bee colony density in wild populations in the harsh conditions of the African dry highland savanna areas in South Africa was still significantly higher than that of Germany, despite intensive beekeeping activities (Moritz *et al*. 2007). Of the ten honey bee species contained in the genus *Apis*, nine of them have their natural distributions concentrated in Asia (Arias & Shepard 2005); the 10th being far more widespread globally. As a species, *Apis mellifera* (Lepeletier). exhibits a wide ranging, natural geographic distribution which spans vast areas of Africa and Europe; from the Cape of Good Hope at the southern tip of Africa, to the oases of the Sahara (Ruttner 1988; Hung *et al.* 2018), to Scandinavia at the northern edge of Europe (Al-Ghamdi *et al*. 2013) and into central Asia. *Apis mellifera* is comprised of over two dozen geographically and morphologically distinct subspecies across its range (Ruttner 1988), with 11 of these subspecies occurring across Africa and Madagascar (Radloff & Hepburn 1998; Engel 1999; Franck *et al.* 2001; Al-Ghamdi *et al.* 2013). Two of these honey bee subspecies are present in South Africa; *A.m. capensis* (the Cape honey bee) and *A.m. scutellata* (the African or Savannah honey bee) (Radloff & Hepburn 1998). The distribution of *A.m. scutellata* spans a wide ranging climatic and biogeographic distribution (Le Conte & Navajas 2008), from the semi-arid desert regions of the Kalahari to the high elevations of the Highveld to the more densely vegetated Lowveld to the warm and humid areas of Durban (Hepburn *et al.* 1998).

In terms of temperature, these honey bees also experience notable environmental variation at colony level, some individuals remaining in constant states of 30 °C-35 °C within the hive (Seeley & Heinrich 1981) while foragers experience a wide range of ambient temperatures throughout the day. Ambient temperatures experienced by foragers include those early in the morning (even in winter) with temperatures averaging 15 °C, and increase right through to far hotter, late afternoon temperatures, especially in summer (Kovac & Schmaranzer 1996; Li *et* *al.* 2016). The wide range of temperatures experienced indicate that the ability to thermoregulate is of vital importance in honey bee physiology. It also plays a crucial role in their maintenance of the hive and all associated functions and processes.

*Thermoregulation*

Thermoregulation in honey bees at both the individual and colony level is an essential component of their physiology and fundamental to their basic functioning and survival. Individual thermoregulation in honey bees is utilised in a number of physiological functions such as dance communication (Stabentheiner & Hagmüller 1991; Stabentheiner *et al*. 1995), aggressive interactions (Stabentheiner *et al*. 2007), and brood care (Basile *et al*. 2008). Dance communication in the hive incorporates thermoregulation, through varying body temperatures depending on the nature of food sources i.e., hotter body temperatures for sweeter-tasting food sources (Stabentheiner & Hagmüller 1991; Stabentheiner *et al*. 1995). Aggressive interactions also result in increased thorax temperatures, with guard bee attacks on humans exhibiting thorax temperatures of 38.6 °C, and up to 46.5 °C during social acts of aggression at the centre of aggression balls (Stabentheiner *et al*. 2007). During prolonged examination of nest mates by guard bees, highly elevated thorax temperatures by the nest mates was observed (Stabentheiner *et al*. 2007). Worker bees utilise their flight muscles during 'shivering' to generate heat and carry out brood warming activities (Basile *et al*. 2008). Individually, honey bees have been shown to be capable of raising their thorax temperatures close to 50 °C for brief periods (Stabentheiner *et al*. 2007). With increases of body temperature, cooling is also an important physiological function. Bees regurgitate water from the crop onto the proboscis as a means of individual evaporative cooling (Esch 1976; Heinrich 1980; Low & Hadley 1985). The determination of a baseline upper threshold of individual thermal tolerance for the *A.m. scutellata* and how that threshold may be affected by neonicotinoids is explored in Chapter Two of this thesis.

At colony level, collective thermoregulation strives to maintain a constant internal climate with stable temperature ((32 °C-36 °C) Seeley & Heinrich 1981; (33 °C-36 °C) Kleinhenz *et al*. 2003; Petz *et al*. 2004; Basile *et al*. 2008; (34.5 °C –35.5 °C) Bujok *et al*. 2002; Kleinhenz *et al*. 2003; Jones *et al*. 2004) and humidity (Ellis *et al.* 2008; Li *et al.* 2016) within the hive, independent of external ambient conditions. One of the main reasons for this is to facilitate optimal brood development. This occurs at an ideal temperature of approximately 35 °C (Jones *et al*. 2005). Constant brood rearing temperature at optimum level promotes speedy development with low mortality and malformation rates (Himmer 1927). Conversely, brood development at sub-optimal temperatures has been investigated (22 °C by Vandame & Belzunces 1998) and although larval development and adult emergence were not found to be significantly affected, the longevity of emerged bees was significantly reduced (Wu *et al.* 2011). Their susceptibility to pesticides also increased (Medrzycki *et al.* 2010). Brain development, cognitive and learning ability as well as foraging has also been found to be affected (Tautz *et al.* 2003; Groh *et al.* 2004; Henry et al. 2012; Ludicke & Nieh 2020). The potential interaction between these factors may further amplify their effects (Fig. 1.2)

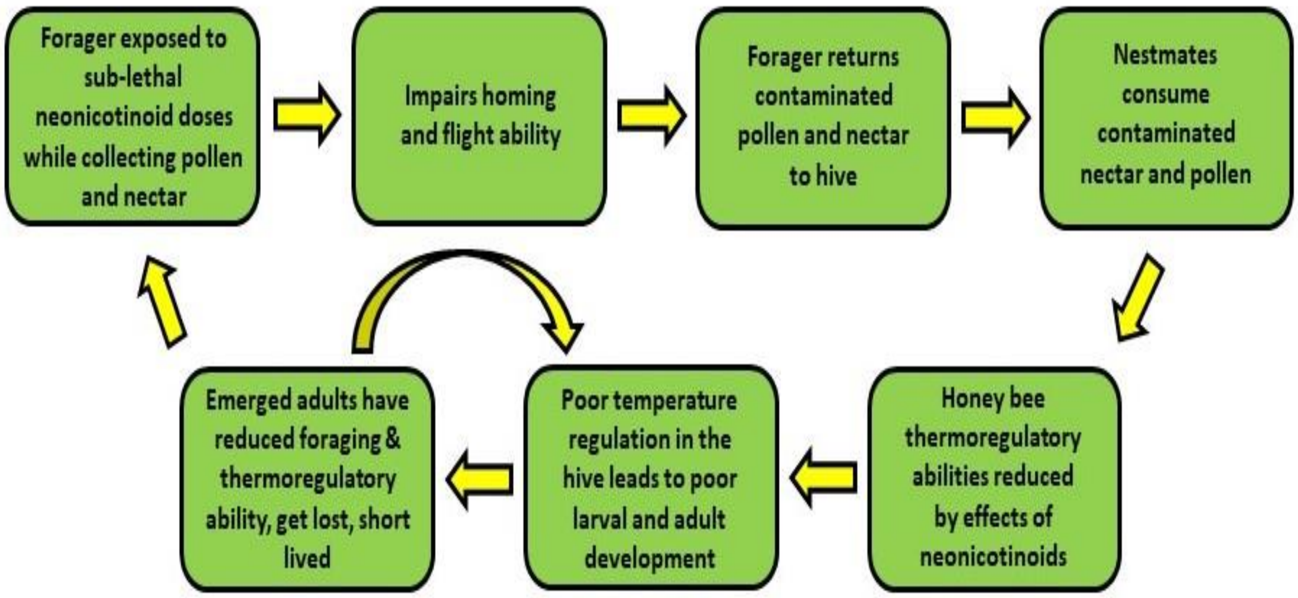

**Figure 1.2**: Positive feedback relationship between sublethal neonicotinoid exposure and reduced thermoregulation efficiency within an *A.m. suctellata* hive.

As a colony, when ambient temperatures begin to fall (overwintering colonies, night time temperatures, etc.) honey bees will increase thoracic temperatures to improve flight muscle function and activate 'shivering' of the flight muscles in the thorax and cluster together. This activity produces the heat required to maintain stable core temperatures at these low ambient temperatures (Esch & Bastian 1968; Owens 1971; Southwick & Heldmaier 1987).

Temperature regulation at elevated environmental temperatures relies mostly on behavioural aspects. The dissipation of internal heat through convection is done using wing fanning, which increases air circulation and colony ventilation of the hive (Hess 1926, Hazelhoff 1941, Southwick & Heldmaier 1987; Southwick & Moritz 1987; Akinwande 2016; Peters *et al.* 2019). Heat is also dissipated through evaporative cooling. Forager bees collect water and deposit droplets on surfaces throughout the hive, both for cooling purposes and to protect the larvae from desiccation (Southwick & Heldmaier 1987; Le Conte & Navajas 2008). Water-foraging bees have proven quite resilient at high temperatures and have been shown to regulate their thorax temperatures at a temperature range as high as 37.0 °C -38.5 °C (Kovac *et al*. 2010). Water foraging and wing fanning, both key activities for cooling, may be affected by the same negative effects that pesticides exerted on homing behaviour, flight capacity and the overall foraging abilities necessary for effective nutritive foraging. These effects occur especially at elevated environmental temperatures. As stated, wing muscle function plays a central role in both individual and colony level thermoregulatory activities at both temperature extremes. Chapter Three of this thesis focuses on the potential effects of pesticides on these activities, using the changes in several features of flight ability as indicators.

*Pesticides*

The use of natural substances, both organic and inorganic, to control organisms considered pests to cultivated plants or animals, dates back to ancient times and continues to present day (Chopra *et al*. 1949; Secoy & Smith 1983; Yang & Tang 1988; Isman 2000; Ranga Rao *et al*. 2007). Pesticides are classified according to the chemical classification of their active ingredients (Buchel 1983; Yadav & Devi 2017), the most notable being organochlorides, organophosphates, carbamates, pyrethrins and pyrethroids, and neonicotinoids. Neonicotinoids are a comparatively young pesticide group, mainly created to combat the increasing resistance of pests to the widely used pyrethroids and carbamates (Corbel *et al.* 2004; Nauen & Jeschke 2011). Continued pesticide use, more particularly neonicotinoid use, has many proven effects on ecosystems and they have the drawback of eventual air, water, soil and organic matter contamination (Bloomfield *et al.* 2006; Kattwinkel *et al.* 2011; Hopwood *et al.*2012; Goulson 2013; Van Dijk *et al*. 2013; Raina-Fulton 2016; Alkassab & Kirchner 2017). They can also leave lingering pesticide residues on fruits and vegetables (Galt 2010; Keikotlhaile *et al.* 2010). Unlike most plant protecting pesticides, neonicotinoids are systemic; accumulating and distributing throughout the entire plant including the pollen and nectar (Elbert *et al.* 2008; Hopwood *et al.* 2012; Fairbrother *et al*. 2014; Codling *et al*. 2018) and can be present at levels known to have both sublethal and lethal effects on terrestrial and aquatic organisms, from beneficial soil microorganisms to invertebrates and vertebrates (Sánchez-Bayo *et al.* 2014; Fernandes *et al.* 2016). The continued use of neonicotinoids will undoubtedly perpetuate these negative effects, impacting both harmful and beneficial insects alike.

Neonicotinoid pesticides constitute around 25% of global insecticide sales (Jeshcke *et al.* 2011; Simon-Delso *et al.* 2015), with imidacloprid, clothianidin and thiamethoxam specifically accounting for around almost 85% of total neonicotinoid sales for use in crop protection in 2012 (Bass *et al*. 2015). In recent years there has been increased pressure to ban the use of these three neonicotinoids due to mounting global concern around their effects on honey bees. Neonicotinoid pesticides are primarily neurotoxins acting as agonists of an insect's nicotinic (acetyl-choline) receptor or nAChR (Iwasa *et al*. 2004). However, insects form a significant part of the pollinator guild and pollinators are a substantial contributor to both natural ecosystems as well as global agricultural as they perform a vital ecosystemic function (Ollerton 2017). The list of beneficial pollinators is long and impressive comprising many invertebrates from multiple orders, including but not limited to; beetles and weevils (Coleoptera), hawkmoths, butterflies and moths (Lepidoptera), flies and midges (Diptera), thrips (Thysanoptera), and wasps, ants, solitary bees, stingless bees, oil-collecting bees, bumble bees, carpenter bees and honey bees (Hymenoptera) (Crane & Walker 1984; McGregor 1976; Free 1996; Steiner & Whitehead 1996; Kevan 1999; Immelman & Eardley 2000; Partap 2003; Rodger *et al*. 2004). The unintentional harm done to this diverse group of pollinators, in our case especially honey bees, has been of great concern for some time (Porrini *et al*. 2003; Ollerton 2017; Raine 2017).

While pesticide residues present in the environment may be below the acute/chronic toxicity levels (Blacquière *et al*. 2012), pesticides are widely utilized in both agriculture and commercial beekeeping practices and have been linked to a variety of sublethal effects on honey bee physiology and behaviour (Mullin *et al*. 2010; Wu *et al*. 2011; Wu *et al*. 2012; Pettis *et al*. 2012). The nature of honey bee foraging potentially exposes them to a multitude of different pesticides, further highlighting the importance of their interacting effects (Vandame & Belzunces 1998; Gill *et al*. 2012). Although field-level pesticide quantities tend to have sublethal effects at individual level (Gill *et al*. 2012), there is the potential for such effects to become amplified, and result in cumulative effects at colony level. This affects colony population performance and productivity (Sandrock *et al.* 2014; Lu *et al.* 2012; Dively *et al.* 2015; Whitehorn *et al.* 2012; Larson *et al.* 2013; Feltham *et al.* 2014; Scholer & Krischik 2014;

Laycock *et al.* 2012; Elston *et al.* 2013; Gill *et al.* 2012). The evidence strongly suggests that the effects of neonicotinoids on honey bees originates from the combined effect of a multitude of factors (summarised in Archer *et al*. 2014b).

Neonicotinoids negatively affect learning and foraging behaviour (Yang *et al*. 2008; Schneider *et al*. 2012; Teeters *et al*. 2012; Frost *et al*. 2013; Williamson & Wright 2013) Olfactory learning is impaired (Han et al 2010; Williamson & Wright 2013; Tan *et al.* 2015), as well as visual learning (Ludicke & Nieh 2020), memory formation (Han *et al.* 2010; Williamson & Wright 2013; Stanley *et al.* 2015), homing behaviour and navigation (Bortolotti *et al*. 2003; Yang *et al.* 2008; Henry *et al.* 2012; Matsumoto 2013; Fischer *et al.* 2014; Henry *et al*. 2014), reduced foraging rates and ultimately colony success (Gill *et al.* 2012; Fauser-Misslin *et al.* 2014).

The ability for individual honey bees to thermoregulate at low temperatures (Vandame & Belzunces 1998), changes in metabolic energy availability for flight muscles, compromised immune-competence (Brandt *et al*. 2016), the suppression of immunity-related genes and increased susceptibility to infection and the deleterious effects of viruses and pathogens (Wu *et al.* 2012; Pettis *et al.* 2012; Doublet *et al* 2015; Sánchez-Bayo *et al*. 2016) have also been demonstrated. There were also effects on dance communication (Kirchner 1999; Eiri & Nieh 2012) and the sensitivity to and consumption of sucrose solutions has also been noted (Kirchner 1999; Schmuck 1999; Eiri & Nieh 2012; Henry *et al.* 2012; Kessler *et al*. 2015; Démares *et al.* 2016). Certain pesticides are thought to cause decoupling of the right and left wing, reducing effectiveness of flight and flight muscle function (Vandame & Belzunces 1998, Vandame *et al*. 1995). The effects of pesticides on flight muscle function, efficiency and coordination (Blanken *et al*. 2015) do not only affect foraging but thermoregulation as well. Pesticides, specifically neonicotinoids, have been shown to have many different effects on individual honey bee thermoregulation. Acute effects of imidacloprid on treated honey bees and German cockroaches included hyperactivity (Suchail *et al*. 2001; Wen & Scott 1997). Mounting evidence suggests that the environmental temperature honey bees are exposed to plays an important role in their responses to pesticide intoxication, most likely influencing their detoxification processes (Tosi *et al*. 2016). One of the neonicotinoids, thiamethoxam, has been shown to affect the thorax temperatures of *A.m. scutellata* foragers as quickly as one hour after exposure for at least 24 hours, possibly longer. This impairment of thermoregulation may be as a result of effects on thoracic/flight muscle function (Tosi *et al*. 2016). Effectiveness of thermoregulation at increasing ambient temperatures may be hindered by pesticides due to the effects they have on flight muscle function, as wing fanning could become less effective. Furthermore, if pesticide exposure is linked to reduced immunity (James & Xu 2012; Di Prisco *et al.* 2013) then the interaction with and accumulative effects of pesticide can result in the reduction of resistance to disease and viruses. This could further weakening their ability to thermoregulate by means of wing fanning (e.g. deformed wing virus), as well as the potential malformations of emerged bees (crumpled, shortened or absent wings) (Atkins & Kellum 1986). Maintaining a constant internal hive environment means that honey bees carry out thermoregulatory activities at the individual and colony level. At low temperatures the interacting effects of deltamethrin and either prochloraz or difenoconazole respectively were shown to significantly intensify the existing individual effects these pesticides have on individual honey bee thermoregulation, resulting in hypothermia in exposed bees (Vandame & Belzunces 1998). Pesticides have been shown to accumulate in brood comb over time (Wu *et al*. 2011), contributing to reduced larval longevity, delayed brood development and increased brood mortality (Wu *et al*. 2011; Zhu *et al*. 2014). Delayed adult emergence and reduced adult longevity (Wu *et al*. 2011), also affects bees of all social castes (Aufauvre *et al*. 2014, Rortais *et al*. 2005). The pesticide-linked delayed development of brood may also provide reproductive advantage to *Varroa destructor,* mites by extending the time period for the mites to infect developing brood (Wu *et al*. 2011). This occurrence increases the potential parasitic load of the colony. Pesticides have also been shown to impact gene expression, subsequently affecting or interrupting important physiological pathways including those responsible for behavioural maturation, detoxification, immunity and nutrition (Schmehl *et al*. 2014). Male (drone) honey bee survival and reproductive success is equally affected by neonicotinoids, and while extremely understudied in comparison to the effects on female honey bees, the lethal and sublethal effects on drones are of major importance for colony health, reproductive success, genetic diversity, etc (Ciereszko *et al.* 2017; Abdelkader *et al.* 2019; Straub *et al.* 2021). A reduction in drone survival, a drastic increase in drifting behaviour to non-maternal colonies, delayed flight activities, reduction in living sperm and affected drone semen quality have been observed (Ciereszko *et al.* 2017; Abdelkader *et al.* 2019; Straub *et al.* 2021). Haploid males are more susceptible to neonicotinoid effects than the diploid females (Friedli *et al.* 2020).

While the research conclusively demonstrates a multitude of negative effects on honey bees, there are also a number of studies with decidedly inconclusive results (reviewed in Scott & Bilsborrow 2019). Prior to 2013, the majority of studies focused on laboratory conditions and arguably did not reliably reflect the prevailing effects and conditions in the field (Cressey 2013). However, following a report from the European Food Safety Authority (EFSA Annual Report 2013) and concerning research and risk assessments, the European Commission instituted a three-year ban in 2013, restricting the use of the three main neonicotinoids (clothianidin, imidacloprid and thiamethoxam) as granules or the seed-coating of bee-attractive flowering crops (EFSA 2013). Blacquière & Steen (2017) found that evaluations of pollinator losses over the subsequent 2-year period following the ban were not sufficient to draw conclusions on whether these were linked to these neonicotinoids, due to the massive variation in colony loss rates (Van der Zee *et al.* 2012). Inconsistent data collection and colony loss included in the contribution to this, along with many other factors. Increased losses experienced since 2000 have been attributed more to honey bee pests, parasites and beekeeping practices than to the neonicotinoids (Blacquière & Steen 2017). Although initially opposed by the UK, the EU ban has since been implemented in the UK. Following further assessment, the UK Department for Environmental, Food and Rural Affairs (Defra) supported further restrictions which would extend to non-flowering crops. In 2018 the EU then instated a total ban on the use of the three major neonicotinoids (Blake 2018).

### *Pesticide uses in Africa*

In contrast to the strongly regulated agricultural landscape of the USA, the United Kingdom and the European Union, the extent of use of these neonicotinoid pesticides on the African continent is not well documented. The effects that neonicotinoids have on pollinators and ecosystems in Africa as a whole cannot be directly inferred from the wide range of research and documented findings from across the EU, UK and North America. This is due to the great differences in ecosystems, ecosystem services and biodiversity between different regions (Moncrieff *et al.* 2015). The far greater prevalence of small scale and subsistence farmers on the African continent (Nagayets 2005), coupled with the rapidly increasing need for improved agricultural yield, widespread illiteracy and limited safety training means that pesticide use in many developing countries remains unregulated and indiscriminate (Kaaya 1994; Ecobichon 2001; Naidoo *et al.* 2010). In many parts of West Africa, for example, market gardening is an important part of the agricultural sector and relies heavily on the use of pesticides (Kouame *et al.* 2013; Agboyi *et al.* 2015).

Of the 54 African countries currently recognised, there is documented use of the most prominent neonicotinoids (clothianidin, imidacloprid, thiamethoxam, acetamiprid and thiacloprid) is available for at least 17 of these countries. The active ingredient Imidacloprid appeared to be the most commonly employed on the continent and the majority of countries employed the same few registered products:

### ***Northern Africa***

*Algeria*: Thiamethoxam based products are widely used in North-eastern Algeria on cereals, tree fruits and vegetable crops (Berghiche *et al*. 2017).

*Egypt*: Products containing acetamiprid, imidacloprid and thiamethoxam were tested against the wood destroying sand termite (*Psammotermes hypostoma*) (Ahmed *et al*. 2015) and as a fava bean seed treatment against aphid damage (Abdu-Allah & Mohamed 2017). Neonicotinoids were also tested against emerging cotton crop pests the cotton mealybug (*Phenacoccus solenopsis*) (El-Zahi *et al*. 2016) and the cotton Leafworm (*Spodoptera littoralis*) (Ahmed 2014). Thiamethoxam and imidacloprid are also recommended as an alternative for treatment against sweet potato whitefly, *Bemisia tabaci* (El-Zahi *et al.* 2017).

*Libya*: Imidacloprid was evaluated for use against the land snail (*Theba pisana*) which causes serious damage to economically important crops in Libya (Mohamed & Radwan 2013).

*Morocco*: Imidacloprid is one of the major neonicotinoids used for the control of aphids in Moroccan citrus groves (Smaili *et al*. 2014).

*Sudan*: Neonicotinoids are among the most commonly used pesticide classes in Sudan (Hammad *et al*. 2017), with thiamethoxam widely used on potato crops against *Aphis gossypii* (Mohamed *et al*. 2014) and on tomatoes against whitefly, *Bemisia tabaci* (Mohamed 2004). Thiamethoxam and imidacloprid are employed as trunk injection treatments in date palm trees to protect against green pit scale insect (*Palmapsis phoenicis*) infestations (Ahmed *et al*. 2010). Imidacloprid residues were detected in exported cantaloup fruit originating from both Khartoum and Gezira state (El Kheir 2004).

*Tunisia*: Neonicotinoid resistance in aphids collected from peach, potato, pepper, tomato and melon crops was detected in the northern region (Charaabi *et al*. 2018). Neonicotinoids are utilized for pest control on cucumber, potato, melon, tomato, pepper and tomato crops throughout this region (Charaabi *et al.* 2015). Acetamiprid and thiacloprid are popular in the citrus industry; mainly protecting against tephritid fruit flies, *Ceratitis capitate* (Harbi *et al*. 2017).

### *Western Africa*

*Benin*: Acetamiprid based pesticides are routinely used to control *Tetranychus evansi* (invasive red spider mite) in southern Benin (Azandémè-Hounmalon *et al.* 2015; Azandémè-Hounmalon *et al.* 2016). Clothianidin based products (Agossa *et al.* 2018a; Agossa *et al.* 2018b) were evaluated as treatment against pyrethroid-resistant *Anopheles gambiae* mosquitoes and malaria. Imidacloprid used extensively in the cotton industry (Zoclanclounon *et al*. 2017)

*Burkina Faso*: Exclusive use of neonicotinoids against white flies on cotton crop has led to widespread resistance (Houndété *et al.* 2010; Gnankiné *et al.* 2013a; Gnankiné *et al.* 2013b; Legg *et al.* 2014). Imidacloprid and acetamiprid residues were among several pesticides detected in water and vegetable samples from areas surrounding lake Loumbila in Burkina Faso (Lehmann *et al.* 2017; Lehmann *et al.* 2018).

*Cameroon*: Imidacloprid is among several pesticides used in vegetable gardening activities in urban, peri-urban and rural areas around Bamenda in Cameroon (Kouame *et al*. 2013). Imidacloprid, acetamiprid and thiamethoxam products were recommended for registration and use on cotton crops in the north of the country (Aboubakary & Mathieu 2008). Imidacloprid, athiametoxam and thiametoxam are used to manage banana and plantain pests including borer weevils (*Cosmopolites sordidus*) (Okolle *et al*. 2009). Acetamiprid is also used against aphids and whiteflies (Silvie *et al*. 2013).

*Cote d'Ivoire*: Around 9.8% of insecticides used in rice cultivation include acetamiprid in conjunction with a pyrethroid (Chouaïbou *et al*. 2016) and vegetable cultivation utilizes mainly pyrethroids with around 30.2% of these insecticides being used in combination with acetamiprid (Chouaïbou *et al*. 2016) in Southern Cote d'Ivoire.

*Ghana*: The Ghana Cocoa Board (COCOBOD) instituted a mass spraying program including the use of Imidacloprid (in Confidor®) and Thiamethoxam (in Actara®) against cocoa mirids in 2001 (Ninsin & Adu-Acheampong 2017).

*Mali*: Anecdotal evidence of acetamiprid has been use reported in Sikasso, Mali (Hamadoun *et al*. 2014). Acetamiprid and imidacloprid are both used on cotton in areas of the Korokoro watershed and Bafinkabougou in Koulikoro, Mali (Maiga *et al*. 2018).

*Nigeria*: Thiamethoxam has been evaluated for use against the cocoa mirid (*Sahlbergella singularis*) in Nigeria (Anikwe *et al*. 2009).

*Togo*: Imidacloprid is popular among market gardening farmers to combat vegetable pests (Agboyi *et al*. 2015; Ahoudi *et al*. 2018).

### *Eastern Africa*

*Eritrea*: Imidacloprid was found to be effective in controlling the citrus pest woolly whitefly and the cottony cushion scale insect (Hussain *et al*. 2017).

*Ethiopia*: Thiamethoxam and imidacloprid were used among farmers surveyed in the Central Eastern part of Ethiopia (Negatu *et al*. 2016).

*Kenya*: Imidacloprid, dinetofuranclothianidin, acetamiprid, thiacloprid and thiamethoxam are approved for use in controlling pests on coffee, French beans, maize, cotton, wheat, forestry nurseries, roses, tobacco and vegetables (PCPB, 1998). Acetamiprid, imidacloprid and thiamethoxam residues were found in pollen and honey samples from Kiambu and Nairobi counties, with thiamethoxam accounting for the highest concentrations in both counties (Mulati 2016). Imidacloprid and thiamethoxam were tested for use as a soil drenching and seed coating treatment against snap pea pests in Mwea, Kenya and were found to be effective against bean fly but not thrips (Otim 2016). This is contrary to the results found by Nyasani *et al*. (2015) for imidacloprid and thrips.

*Uganda*: Pesticide use is relatively unregulated with product identification complicated by the prevalence of mislabelled/counterfeit products (Nalwanga & Ssempebwa 2011). The tobacco and citrus industry rely heavily on neonicotinoids (Srigiriraju *et al*. 2010). Several neonicotinoid residues were found in honey bees and hive products in the three agro-economic zones in Uganda (Mid-Nile, Northern and Eastern) (Srigiriraju *et al*. 2010).

### *Southern Africa*

In South Africa, the varying range of climatic conditions allows for the cultivation of a variety of crops, each susceptible to unique combination of pests, thus requiring unique mixtures of pesticides for best results (Quin et al. 2011). South Africa is one of the four largest importers of pesticides in sub-Saharan Africa (Osibanjo et al. 2002), with over 500 registered pesticides of all classes (Pesticide Action Network (PAN), 2010).

Neonicotinoids are used for pest control on a number of crops in South Africa including barley, canola, citrus, oats, cotton seed, tomatoes, wheat, apples, cucurbits, grapes, maize, sorghum, sunflower seed and peaches (Quinn *et al*. 2011). The Agri-Intel website acts as an agrochemical database comprising of all crop protection products registered for use in South Africa. The site is owned and managed by CropLife SA, a non-profit organization representing the majority of the manufacturers, suppliers and distributors of crop protection products. Not all product registration holders of pesticides submit their products to the Agri-Intel website and registrations fluctuate constantly. The current information on registered neonicotinoids for South Africa includes several key active ingredients (acetamiprid, imidacloprid, thiamethoxam, thiacloprid, clothianidin) under several trade-names. These trade-name products contain either aforementioned active ingredients or combinations thereof (Agri-Intel

2019). Agri-Intel is utilised not only by South Africa, but by several other southern African countries that have registered products for their own local use, summarised here by country:

**Table 1.1**: Summary of neonicotinoid active ingredients registered for use in southern African countries according the Agri-Intel website (Agri-Intel 2019)

| **Country** | **Registered Neonicotinoids** |
|---|---|
| *Angola* | Acetamiprid and imidacloprid based products |
| *Botswana* | Imidacloprid and thiamethoxam based products |
| *Madagascar* | Thiacloprid and imidacloprid based products |
| *Malawi* | Thiacloprid, acetamiprid, and imidacloprid based products |
| *Mauritius* | Imidacloprid based products |
| *Mozambique* | Acetamiprid, imidacloprid and thiamethoxam based products. Imidacloprid was evaluated and recommended for use against leaf miner (*Stomphastis thraustica*) and the leaf beetle (*Aphthona dilutipes*), a major pest of Jatropha in Mozambique, (Cassimo 2011) |
| *Namibia* | Acetamiprid, clothianidin, imidacloprid and thiamethoxam based products |
| *South Africa* | Acetamiprid (13 registered trade names), clothianidin (6 registered trade names), imidacloprid (48 registered trade names), thiacloprid (7 registered trade names), thiamethoxam (16 registered trade names) |
| *Zambia* | Thiacloprid, clothianidin and imidacloprid based products |
| *Zimbabwe* | Thiacloprid, clothianidin and imidacloprid based products (Agri-Intel 2019). Resistance of peach aphid to imidacloprid indicates widespread use of this neonicotinoid in the country (Foster *et al*. 2003) |

There is mounting evidence of the dangers and drawbacks of pesticide use including neonicotinoids and their negative effects on both pollinators (Stokstad 2013) and humans (Zinyemba *et.al* 2018). The majority of this research being conducted in North America and

Europe. While their use remains prevalent as a pest control method worldwide, pesticide use becomes more and more regulated and restricted. This is seen most recently in the banning of the commercial use of the three major neonicotinoids and fipronil in Europe. In many areas of Sub-Saharan Africa pesticide use is increasing (Zinyemba *et.al* 2018). This includes the use of neonicotinoids, often to combat the prevalence of resistance to carbamates and pyrethroids (Corbel *et al.* 2004; Nauen & Jeschke 2011; Charaabi *et al*. 2018). The extent to which widespread pesticide use on the African continent may influence honey bee colony losses requires a great deal more investigation (Pirk *et al.* 2014; Pirk *et al.* 2016). Beekeeping on the African continent remains unique in comparison to the rest of the globe, as only a small proportion of honey bee hives are managed (Johannsmeier 2001). The vast majority of honey bee colonies are wild, estimated at close to 310 million colonies (Dietemann *et al.* 2009). In the context of rising ambient temperatures and increasing incidences of thermal extremes, investigation into the effect that commercially used neonicotinoids may have on honey bee thermoregulatory activities, both managed and wild, is vital.

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## CHAPTER 2

# The influence of sublethal neonicotinoid doses on individual *Apis mellifera scutellata* thermotolerance

## ABSTRACT

Honey bee (*Apis mellifera* L.) thermoregulation plays an integral part in their behaviour and physiology, and has been shown to be vulnerable to the effects of neonicotinoid insecticides. Baseline thermotolerance of 53.8 °C (defined as $LT_{50}$; temperature at which 50% mortality is recorded) was determined for this subspecies. I have evaluated the influence of sublethal dosages of three widely used neonicotinoid insecticides, clothianidin, imidacloprid and thiamethoxam, on the thermotolerance of individual *Apis mellifera scutellata*. Each neonicotinoid group was evaluated through a progression of increasing ambient temperatures. For all three neonicotinoid treatments, *A.m. scutellata* thermotolerance was decreased by more than 3 °C as compared to the baseline data. Such a reduction in honey bee thermotolerance, especially under the increasing frequency and intensity of hot weather events, is cause for concern when considering legislation and use of these neonicotinoids in the South African agricultural and suburban setting.

## INTRODUCTION

The honey bee, *Apis mellifera* L., is of great importance to both agricultural and natural ecosystems. Honey bees play a vital role in our food security (reviewed in Steffan-Dewenter 2005), with the production of approximately one-third of all food crops relying on honey bee pollination (Morse & Calderone 2000). Of equal importance is the contribution of these pollinators have towards biodiversity and ecosystem function (Vanbergen & Insect Pollinators Initiative 2013). Worldwide declines in honey bee numbers and colony health are of particular concern and while the exact causes of colony losses are still unclear, several factors appear to be influential. These factors include climate change (Ruttner 1988; Le Conte & Navajas 2008), anthropogenic activities (Søvik *et al.* 2015), poor beekeeping practices (Gajger *et al.* 2010), habitat loss (Potts *et al.* 2010), monoculture (Kremen *et al.* 2002), introduction and prevalence of parasites (Bowen-Walker *et al.* 1999; Amdam *et al.* 2004), loss of genetic diversity (Meixner *et al.* 2010) and the use of pesticides (Holder *et al.* 2018), particularly neonicotinoid insecticides (Abbo *et al.* 2017; Calvo-Agudo *et al.* 2019).

Neonicotinoid insecticides are classified as systemic insecticides with neurotoxic properties, acting as an agonist on insect nicotinic acetylcholine (nAChR) receptors (Iwasa *et al.* 2004). These receptors provide the majority of excitatory neurotransmissions in the insect central nervous system (Moffat *et al.* 2016). Neonicotinoids have a wide range of target pests and applications (Aliouane *et al.* 2009; Jeschke *et al.* 2011). The use of this class of insecticide has grown steadily worldwide since the 1990s, with various neonicotinoid insecticides registered for use in more than 120 countries, and contributing considerably to global insecticide sales (ASSAf, 2019). Neonicotinoid insecticides constitute around 25% of global insecticide sales, with the three most prominent active ingredients imidacloprid (hereafter IMI), clothianidin (hereafter CLO) and thiamethoxam (hereafter THX) in particular accounting for approximately 85% of total neonicotinoid sales for use in crop protection in 2012 (Bass *et al.* 2015).

The neurotoxic effects of neonicotinoids have been shown to influence a number of aspects of honey bee physiology and behaviour (Pettis et al. 2012) including hyperactivity (Suchail *et al.* 2001), communication of the waggle dance (Kirchner 1999; Schmuck 1999), flight muscle function, efficiency and coordination (Blanken *et al.* 2015), decreased social immune response such as grooming behaviour (Morfin *et al.* 2019), reduced immune-competence and impaired disease resistance (Brandt *et al.* 2016), food collection (Rortais *et al.* 2005), sucrose perception (Démares *et al.* 2016) and honey bee thermoregulation (Tosi *et al.* 2016).

Focusing on honey bee thermoregulation at both the individual and colony level, thermoregulation is a crucial component of basic honey bee function and survival. On a colony level the maintenance of a constant internal hive environment involves multi-level thermoregulatory activities including water evaporation and wing beating/fanning (Belzunces *et al.* 2012).

Individual thermoregulation plays a crucial role in communication, social interaction and foraging activities (Stabentheiner & Hagmüller 1991; Stabentheiner *et al*. 1995; Stabentheiner *et al*. 2007) and involves the tetanic contraction of the flight muscles (thermogenesis) (Belzunces *et al.* 2012). Honey bees have been proven capable of raising thorax temperatures to close to 50 °C for brief periods (Stabentheiner *et al*. 2007). Dance communication in the hive incorporates thermoregulation, for example food sources with a sweeter taste are communicated by hotter body temperatures (Stabentheiner & Hagmüller 1991, Stabentheiner *et al*. 1995). Should honey bee sucrose perception be altered (Démares *et al.* 2016) and thorax temperature and heat generation (Tosi *et al.* 2016) be impacted by neonicotinoid exposure, communication of food sources through the waggle dance is one of many hive activities that may be detrimentally impacted (Tosi *et al.* 2016).

Aggressive interactions also result in increased thorax temperatures; with guard bee attacks on humans exhibiting thorax temperatures of 38.6 °C and temperatures up to 46.5 °C during social act of aggression at the centre of aggression balls (Stabentheiner *et al*. 2007). During prolonged examination of nestmates by guard bees, highly elevated thorax temperatures by the examinees were observed (Stabentheiner *et al*. 2007). Worker bees utilise their flight muscles during 'shivering' to generate heat and carry out brood warming and flight activities (Krogh & Zeuthen 1941; Esch 1960; Goller *et al.* 1991; Bujok *et al.* 2002; Basile *et al*. 2008). Variation and regulation of individual thorax temperatures are influenced by a number of physiological and environmental factors. In *Apis mellifera carnica*, thorax temperature was found to vary strongly at a specific ambient temperature depending on what plant source they had been foraging on, with thorax temperatures differing by up to 4 °C between honey bees foraging on two different plant species under similar conditions, and this is theorised to be due to differences in the motivational state of foragers as well as the quality of food sources (Kovac & Stabentheiner 2011). At a warmer range of ambient temperatures (27 °C-32 °C) the thorax temperature was found to increase almost linearly with ambient temperature (Kovac & Stabentheiner 2011). Honey bees have been found to exhibit high tolerance of heat stress, surviving well at temperatures ranging from 4 °C to 45 °C with some mortalities documented at 50 °C (Koo *et al.* 2015). Existing evidence has demonstrated the effect of neonicotinoids on honey bees at higher temperatures, with the level of the heat shock proteins *hsp70*, *hsp78*, and *hsp90* shown to decrease with exposure to increasing concentrations of imidacloprid (Koo *et al.* 2015).

Individual thermoregulation is therefore not only reliant on the optimal functioning of flight muscles and the action of evaporative cooling, but is also linked to the influence of external ambient temperatures (Bernd 1979; Woods *et al.* 2005). Under conditions of elevated or more frequently fluctuating ambient temperatures and continued exposure to neonicotinoids, even at sublethal levels, the associated negative effects on wing function and efficiency have the potential to not only impair efficient wing fanning activities in the hive, but the individuals' ability to tolerate higher temperatures as well.

The influence of external ambient temperatures on thermoregulation is likely to vary between the diferent honey bee species and numerous subspecies, particularly within the Western honey bee species which has a wide-ranging geographic distribution and a presence on several continents (Suazo *et al.* 1998; Crane 2009). The natural distribution of *A. mellifera* L. extends through much of Africa, Europe and western Asia (Garnery *et al.* 1998; Kotthoff *et al.* 2013; Requier *et al.* 2019). The subspecies existing in temperate and Mediterranean type climates are likely to experience more stress within the lower extremes of their range of temperature tolerance; while those subspecies existing in warmer and drier climates, e.g., areas in their African distribution (Hepburn & Radloff 1998, Pirk 2020), are likely to experience more temperature tolerance stress towards the upper end of their tolerance range. For this reason, investigation into the limits of temperature tolerance of individual subspecies will enable us to better understand how future changes to large scale climate conditions are likely to affect the honey bee species as a whole.

A previous study evaluating the metabolism, critical thermal limit and lethal temperature under conditions of increasing ambient temperature was conducted on two European honey bee subspecies; *Apis mellifera ligustica* (Spinola 1806) and *Apis mellifera carnica* (Pollmann, 1879) in order to ascertain whether any differences between the thermal capacities of the two subspecies populations existed (Kovac *et al.* 2014) (Fig. 2.1). To investigate the lethal temperature aspect of honey bee physiology in particular, this study examined the previously undefined characteristics of lethal thermal limits of African honey bees, i.e., *Apis mellifera scutellata* (Lepeletier 1836). The study was conducted under similar experimental conditions to the European study in order to establish a threshold for the native subspecies and to conduct a subspecies comparison.

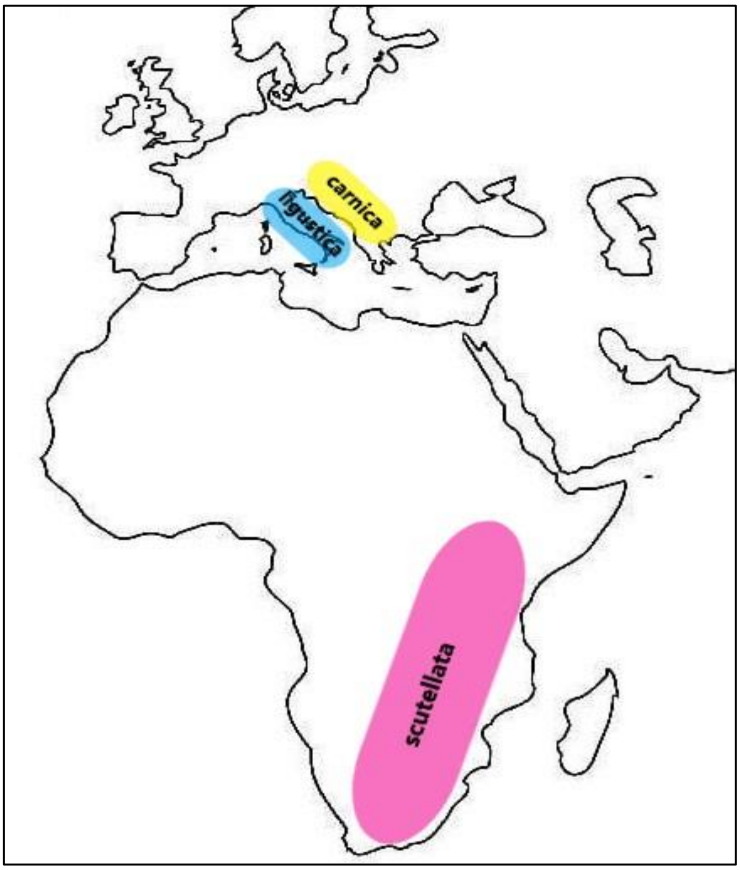

**Figure 2.1**: Geographic distribution of the *Apis mellifera scutellata* (Hepburn & Radloff 1998) utilised in this study and the two European *A. mellifera* subspecies discussed for comparison (according to Franck *et al.* 2000; adapted from Le Conte & Navajas 2008).

The study included the additional effect of three commercially prominent neonicotinoid insecticides on the ability of this honey bee subspecies to thermoregulate at elevated ambient temperatures. The addition was included in order to establish whether these insecticides influence the lethal temperature threshold of this subspecies. The possible implications of the use of neonicotinoid insecticides on the African continent, as well as the rapidly changing global climatic conditions on bee health were discussed.

# METHODS

## *Study Species*

The interacting effects of neonicotinoids and ambient temperature on individual honey bee thermoregulation was conducted on *Apis mellifera scutellata*. This subspecies is naturally distributed across sub-Saharan Africa (Ruttner 1988; Hepburn *et al.* 1998; Pirk 2020), and in South Africa specifically the natural distribution covers the majority of the country, the exception being the Cape region, which is home to the *Apis mellifera capensis* subspecies (Ruttner 1988; Crewe *et al.* 1994). Honey bees were collected from the Social Insect Research Group (SIRG) apiary located at the University of Pretoria's Experimental Farm in Hatfield, Tshwane, Gauteng Province, South Africa from May to August of 2016 and July to December of 2017. At the time of collection, experimental hives were free of obvious signs of disease and deemed large enough to tolerate a continuous removal of small numbers of honey bees. Hives were kept within city limits and not exposed to commercial agriculture agrochemicals.

The study focused on pollen and nectar/water foragers as they are easily detectable at hive entrances, experienced the greatest variation in external temperature and the greatest potential exposure to environmental neonicotinoids while foraging. Not only do foragers consume pollen and nectar potentially contaminated with neonicotinoids, they also become covered in it while foraging (Rortais *et al.* 2005). This puts them at risk of both topical and oral exposure (Rortais *et al.* 2005). Foragers are generally workers over the age of 21 days (Lindauer & Watkin 1953).

## *Baseline Thermal Tolerance*

The baseline thermal tolerance of *A.m. scutellata* was established as per Kovac et al. (2014) methodology with minor modifications. All temperature experiments were conducted using temperature-controlled humidity chambers (HCP108 Memmert® GmbH+ Co.KG), capable of carefully controlling temperature and humidity conditions. The rising temperatures during temperature ramps and the humidity conditions were controlled by the programme Celsius®, specifically designed Memmert® software with which interior chamber conditions could be pre-programmed.

After collection, honey bee foragers were maintained in hoarding cages made of Perspex (120 mm x 95 mm x 80 mm) with sliding panels on both sides, a perforated panel for ventilation on the bottom and two small windows on the front to accommodate the insertion of two 2 mL centrifuge (Eppendorf®) tubes used to administer the diet (Köhler *et al.* 2013) (Fig. 2.2). No comb was provided during the temperature treatment in order to eliminate the influence of the comb's microclimate on thermoregulation. For each trial, cages were kept for 24 hours under controlled conditions i.e., provided with two 2 mL centrifuge (Eppendorf®) tubes standard 50% w/w sugar water solution and maintained at standard hive conditions of 30˚C, 45% relative humidity (RH) (Kovac *et al.* 2014). Feeding tubes were weighed before going into the cage and again after the 24-hour period, ( i.e., before the temperature ramp exposure). One cage per each of the 5 hives, consisting of 30 bees per cage, was evaluated at each of the 11 target temperatures, totalling 55 cages. The target temperature for the first trial was 46˚C, 47˚C for the second, 48˚C for the third and so on, increasing by 1˚C intervals, with the eleventh and final trial terminating at 56˚C. Each set of 5 cages was exposed to a temperature ramp, all starting at an initial temperature of 30˚C and each trial ending at a different end temperature. At the start of each temperature trial the cages were incubated at the initial temperature (30˚C) for 5 minutes and then the temperature was increased gradually by intervals of 0.3 ˚C.min$^{-1}$, terminating at the designated target temperature.

Once the target temperature for the trial was reached the cages were allowed to incubate at the target temperature for a brief stabilisation period of 5 minutes, cooled to the start temperature of 30˚C and incubated at this temperature for a further 8 hours. The total mortality percentage for each cage was recorded directly after the temperature ramp treatment (considered 0 hours), then again at 2 hours, 4 hours, 6 hours and 8 hours following temperature ramp respectively.

The mortality percentages from these 11 trials (150 honey bees per trial, 1650 honey bees in total) gave the baseline $LT_{50}$ ($LT_{50}$ considered the temperature at which a 50% mortality was recorded (Kovac *et al.* 2014)) of *A.m. scutellata*. Based on the baseline temperature, we defined the range of relevant temperatures at which to test the effects of the three individual neonicotinoids via oral exposure, at sublethal concentrations. This temperature range was deemed to be between 52˚C and 56˚C.

**Figure 2.2**: Perspex hoarding cage (120mm x 95mm x 80mm), sliding side panels, perforated base panel and openings for diet tubes (Köhler *et al.* 2013).

### *Neonicotinoid Exposure*

Three commercially utilised neonicotinoid insecticide active ingredients were used in the individual thermoregulation study, namely IMI, THX and CLO. Forager bees were maintained under the same conditions as for the baseline experiment above. Foragers used in the neonicotinoid trials were also provided with two 2 mL centrifuge (Eppendorf®) tubes of sugar solution per cage, both treated with the same sublethal dose of the relevant neonicotinoid (40 µL of 250 nM solution in 2 mL tube of 1:1 w/w sucrose and water, with the final concentration of given neonicotinoid being 5 nM) for a period of 24 hours. Cages with bees exposed to neonicotinoids were exposed to the same temperature ramp methods as described in the baseline experiment. According to standard practice, acetone (hereafter ACE) was used as an organic solvent for the three neonicotinoids to make them soluble in the diet. The proportion of ACE present in each diet, including the control, was lower than 0.05% (Aliouane *et al.* 2009; Démares *et al.* 2016). The neonicotinoid concentration of 5 nM for CLO, IMI and THX respectively was considered comparable to realistic field doses. The period of exposure was sufficient to allow for all honey bees to consume enough treated sugar water to illicit any potential observable effects, while still remaining under the $LD_{50}$ dosage of neonicotinoids (Démares *et al.* 2018; Yao *et al.* 2018).

Each individual neonicotinoid temperature ramp trial consisted of nine experimental cages. Two cages were each treated with IMI, CLO and THX respectively (40 µL of neonicotinoid in 2 x 2 mL tube of 1:1 w/w sucrose and water). Two controls were also included; one cage with the control sugar water solution only (SUC), and one control with sugar water and ACE (40 µL of a dilute ACE solution in 2 mL tube of 1:1 w/w sucrose and water). One cage with the control sucrose solution and no honey bees was included to measure and correct for the amount of evaporation over the 24-hour exposure period. To ascertain the effects of neonicotinoids on this baseline $LT_{50}$ threshold, 5040 honey bees from three hives treated with one of three separate neonicotinoids or two controls, were then evaluated in the same way as the Baseline. Mortality was assessed for all experiments at 0 hours (Fig. 2), 2 hours (Fig. 3), 4 hours (Fig. 4), 6 hours (Fig. 5) and 8 hours (Fig. 6) following temperature ramp exposure. The mortality percentages from these 7 trials (720 honey bees per trial, 5040 honey bees in total) were used to give an indication of the $LT_{50}$ of *A.m. scutellata*, under the influence of three separate neonicotinoids via oral exposure, at sublethal concentrations.

For both the Baseline and Neonicotinoid experiments a honey bee was considered to be alive when it moved, either spontaneously or in response to a gentle stimulus, and was assessed five times at two-hour intervals. Honey bees were provided with sugar syrup and water for the duration of the entire experiment, with the exception of the temperature ramp. This was because the evaporation from the diet, especially at higher temperatures, was found to significantly increase the humidity within the cages and thus the experimental conditions, rendering it impractical.

### *Statistics*

Consumption data was evaluated for normality using a Shapiro-Wilk test (W=0.97; p=0.21). Mean consumption variation among the baseline, three treatments and two control groups were evaluated by an ANOVA and the data met all normality assumptions with a post hoc Tukey HSD test. A linear mixed effects model was used to determine whether consumption (response variable) varied among treatments including the baseline (predictor variable) to account for multiple measurements per hive, with hive being included as the random effect. Models were fit using a maximum-likelihood approach and several plot types were used to assess model fit. The percentage of variance explained by the random effect, i.e. the hive number, was calculated by means of a variance component analysis (Crawley 2007). Models were fitted using the packages *'nlme'* and *'car',* in programme R (Fox & Weisberg 2011, Pinheiro *et al.* 2016, R Core Team 2021).

The recorded mortality percentage was plotted against the experimental target temperature, and the lethal temperature ($LT_{50}$) was ascertained by calculating the best fitted sigmoidal curves. The effects of temperature on honey bee survival across all experiments were also evaluated using Kaplan-Meier survival analysis in Statistica © (version 13.2) and a Gehan's Wilcoxon test used for pairwise comparison was done to ascertain whether treatment affected honey bee survival at each of the various temperatures. An alpha value of 0.05 was used for all stats analysis (Pirk *et al.* 2013).

## RESULTS

A baseline thermal tolerance of was established for the *A.m. scutellata* subspecies (Baseline $LT_{50},\ _{8h}$=53.77 °C; *N*=55 trials). Following the 24-hour exposure period, the consumption of the treated diets (CLO, THX, IMI) and control diets (ACE, SUC) showed little difference between each other. This result factored in the correction for evaporation. Consumption across the different treatments was combined to determine the mean consumption per bee per treatment across all experiments following the standard 24 hours of dietary exposure (Fig. 2.3). Mean consumption did vary but did not show a significant difference across the baseline, three treatments and two controls (ANOVA df = 5; f value = 0.31; p value = 0.9) (Fig. 2.3).

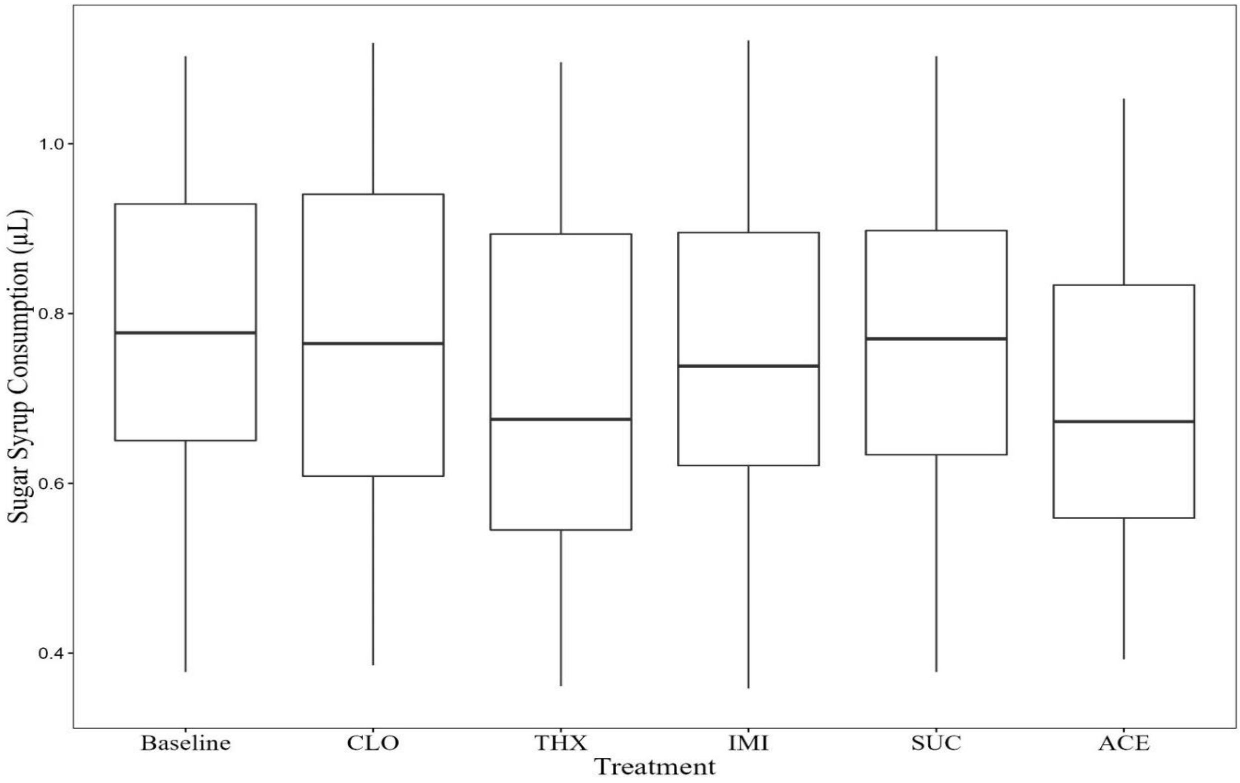

**Figure 2.3:** Mean consumption per A.m. scutellata honey bee (µL) after 24 hours of exposure for the three neonicotinoid treatments; clothianidin (CLO), thiamethoxam (THX), imidacloprid (IMI), and the two controls; acetone (ACE) and sucrose (SUC).

The linear mixed effects model showed there were no differences in consumption between the different treatments ($\mathcal{X}^2 =1.77$; df=5; p value=0.88). Variation among hives explained only 1.85% of the variation in the model, meaning there is no variation amongst the hives. The hive is excluded as a contributing factor for variance in the rest of the analyses.

Honey bee mortality showed a temperature dependence; with increasing mortality with an increase in temperature, as well as a dependence on time across the 8-hour period following the temperature ramp exposure. Mortality percentage data were best fitted with a sigmoidal function ($mortality=a/(1+(x/b)^c)$) and an $LT_{50}$ was determined from these fitted mortality curves. No curves were fitted at zero hours (Fig. 2.4) and two hours (Fig. 2.5) following exposure, but curves were fitted for data collected at four hours (Fig. 2.6), six hours (Fig. 2.7) and eight hours (Fig. 2.8) following exposure.

At the end of the eight hour period, the temperature treatment parameters were *a*=1.0344403E+02 and *b*=5.3879726E+01 and *c*=-7.70101459E+01 for Baseline; *a*=7.3722222E+01 and *b*=4.998623E+01 and *c*=-1.1532962E+03 for CLO; *a*=8.333333E+01 and *b*=4.9994453E+01 and *c*=-1.08302836E+03 for IMI; *a*=7.958333E+01 and *b*=4.9999488E+01 and *c*=-1.0163530E+03 for THX; *a*=7.37777E+01 and *b*=5.0050704E+01 and *c*=-1.0680706E+03 for ACE; and a function could not be fitted for SUC (Fig. 2.8).

The lethal temperature after eight hours, derived from the sigmoidal curves, was not significantly different for the three neonicotinoid treatments (CLO $LT_{50, 8h}$=50.15 °C; IMI $LT_{50, 8h}$=50.05 °C; THX $LT_{50, 8h}$=50.08 °C; *N*=36 trials), slightly higher for both the ACE control (ACE $LT_{50, 8h}$=50.50 °C; *N*=18 trials) and the SUC control (SUC $LT_{50, 8h}$=50.20 °C; *N*=18 trials), and more than 3 °C higher for the Baseline control (Baseline $LT_{50, 8h}$=53.77 °C; *N*=55 trials).

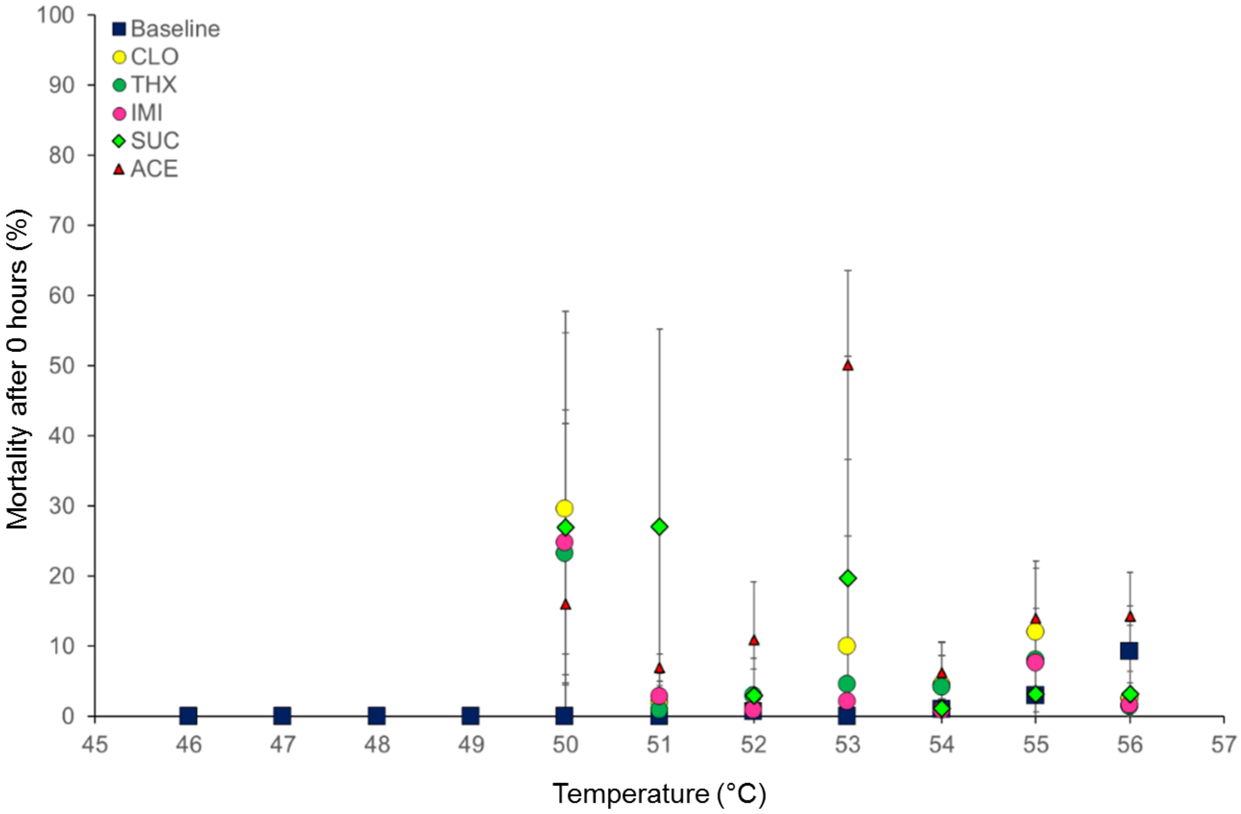

**Figure 2.4:** Mean mortality of the honey bee *Apis melllifera scutellata* recorded at 0 hour following exposure to the designated temperature ramp which terminated at the indicated target temperature (°C), under control (ACE ▲, SUC◆), baseline (Baseline ■) and treated (CLO ●, IMI ●, THX ●) diet conditions. Functions could not be fitted for the 6 treatments. Error bars indicate standard deviation.

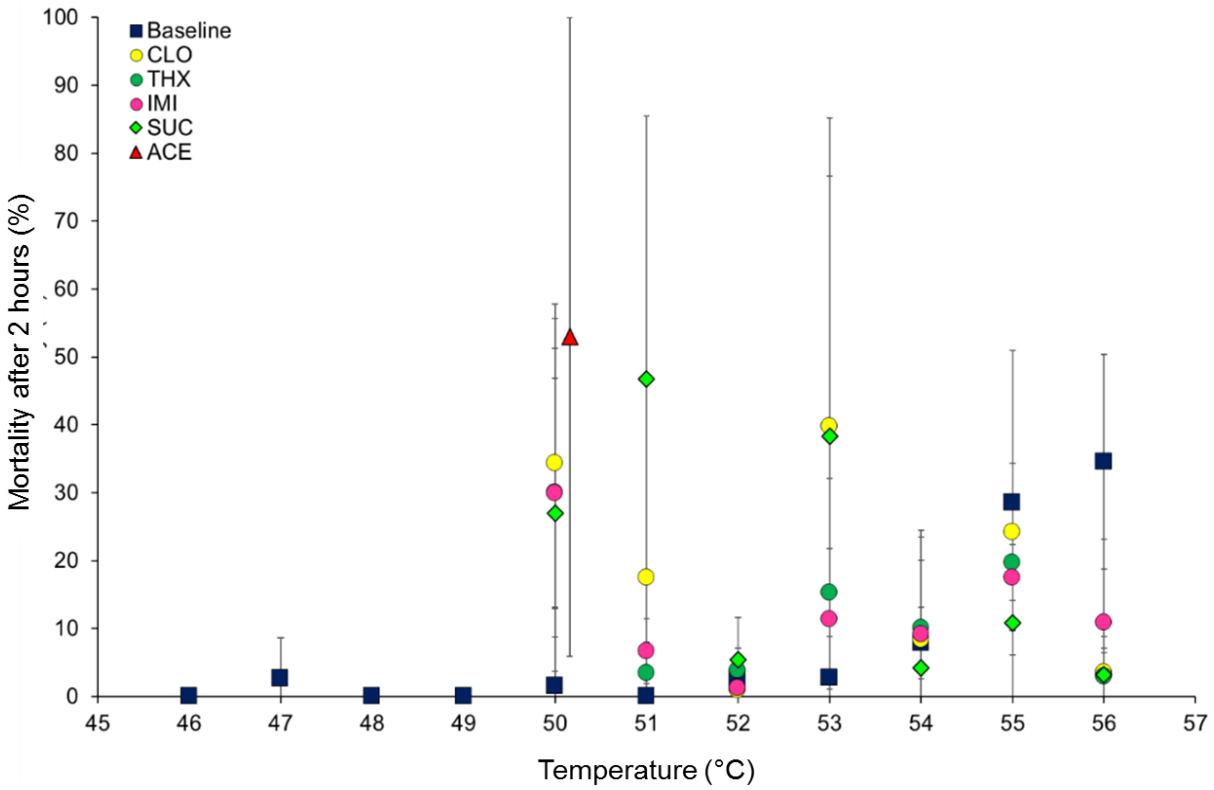

**Figure 2.5:** Mean mortality of the honey bee *A. m. scutellata* recorded at 2 hours following exposure to the designated temperature ramp which terminated at the indicated target temperature (°C), under control (ACE ▲, SUC◆), baseline (Baseline ■) and treated (CLO ● , IMI ●, THX ●) diet conditions. Broken lines indicate lethal temperatures ($LT_{50}$) determined from sigmoidal curves. Functions could not be fitted for the 6 treatments. Error bars indicate standard deviation.

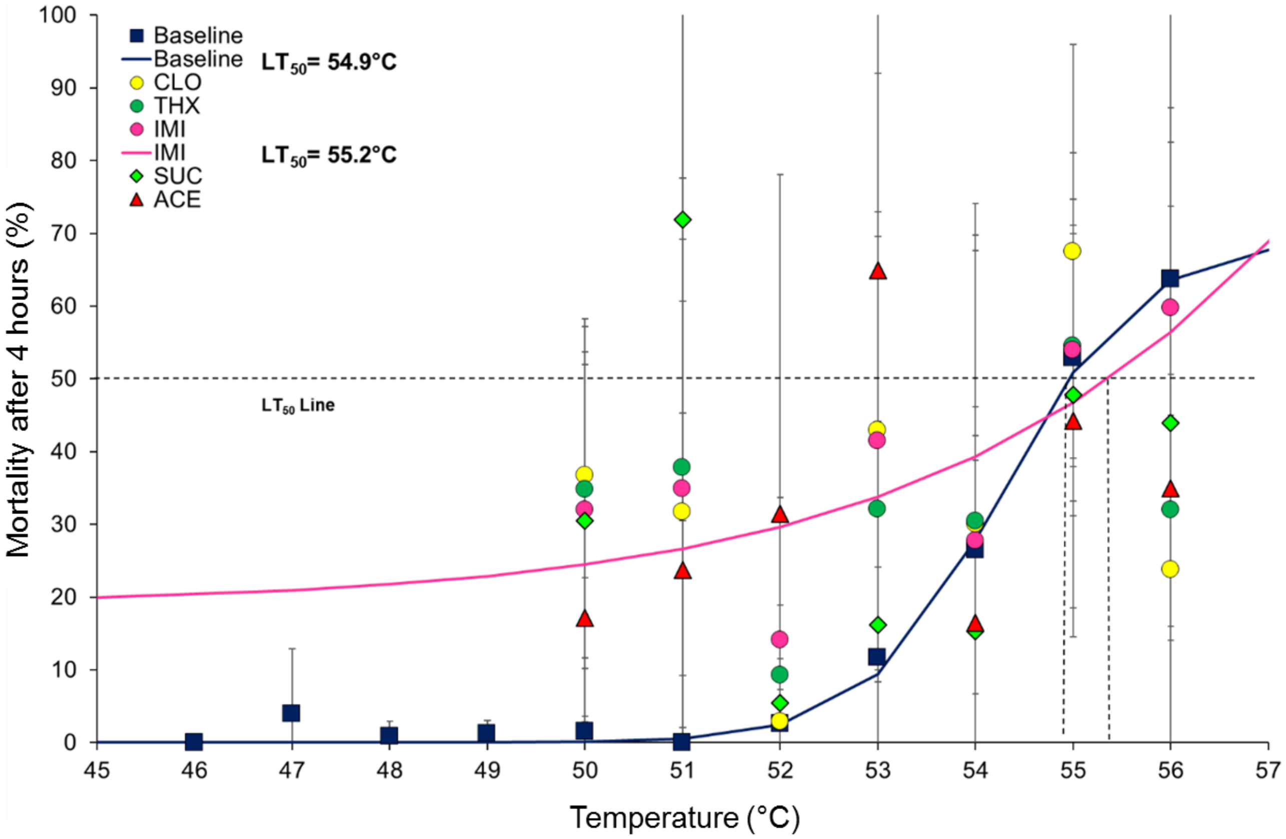

**Figure 2.6:** Mean mortality of the honey bee *Apis melllifera scutellata* recorded at 4 hours following exposure to the designated temperature ramp which terminated at the indicated target temperature (°C), under control (ACE ▲, SUC◆), baseline (Baseline ■) and treated (CLO ●, IMI ●, THX ●) diet conditions. Broken lines indicate lethal temperatures ($LT_{50}$) determined from sigmoidal curves. Curves were best fitted with a sigmoidal function (mortality=a/(1+(x/b)$^{c}$). Parameters for functions: **Baseline (—)**, *a*=7.097023E+01, *b*=5.428830E+01, *c*=-7.515814E+01. **CLO** Function could not be fitted. **IMI (—)**, *a*=9.413888E+01, *b*=5.001369E+01, *c*=-9.733163E+02. **THX** Function could not be fitted. **ACE** Function could not be fitted. **SUC** Function could not be fitted. Error bars indicate standard deviation.

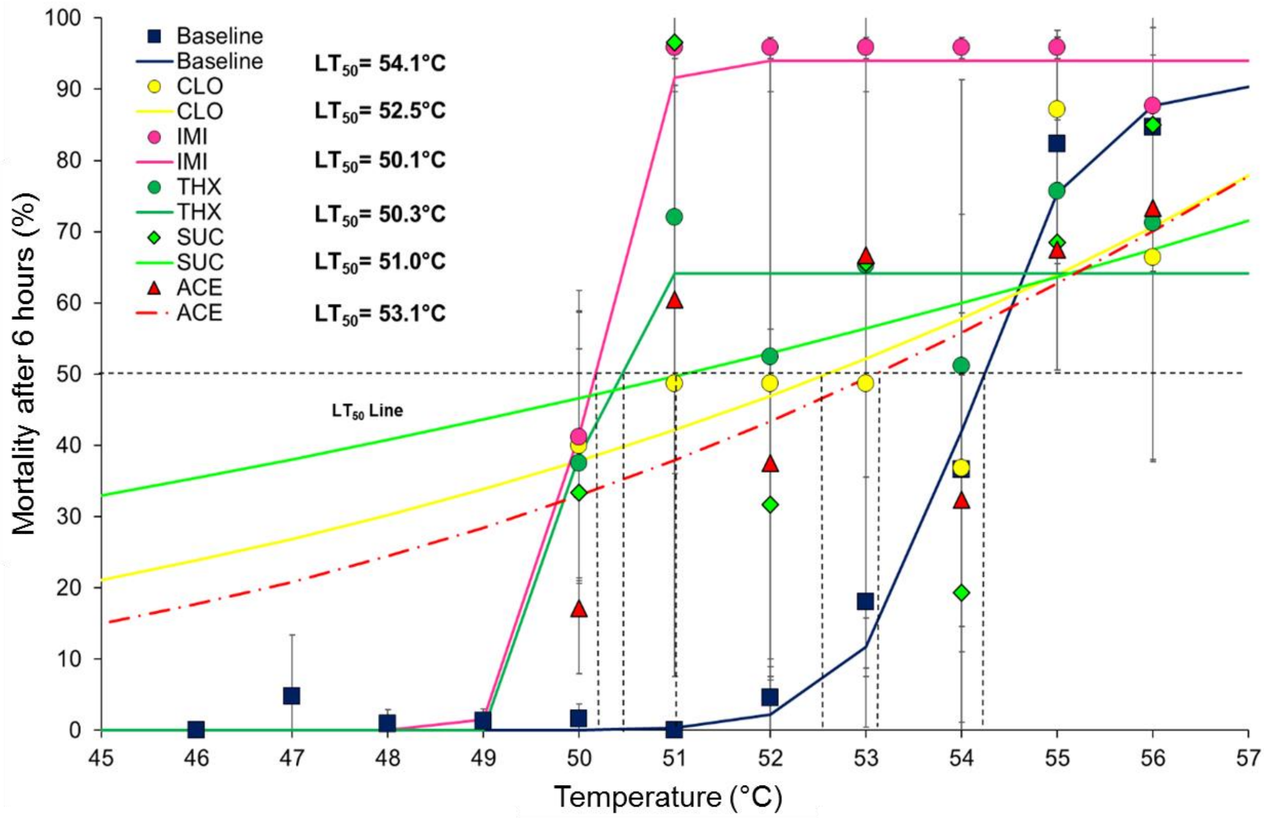

**Figure 2.7:** Mean mortality of the honey bee *Apis melllifera scutellata* recorded at 6 hours following exposure to the designated temperature ramp which terminated at the indicated target temperature (°C), under control (ACE ▲, SUC◆), baseline (Baseline ■) and treated (CLO ●, IMI ●, THX ●) diet conditions. Broken lines indicate lethal temperatures (LT$_{50}$) determined from sigmoidal curves. Curves were best fitted with a sigmoidal function (mortality=a/(1+(x/b)$^{c}$)). Parameters for functions: Baseline **(—)**, *a*=9.228004E+01, *b*=5.408780E+01, *c*=-8.949900E+01. **CLO (—)**, *a*=1.304947E+04, *b*=1.469989E+02, *c*=-5.403435. **IMI (—)**, *a*=9.413888E+01, *b*=5.001369E+01, *c*=-9.733163E+02. **THX (—)**, *a*=6.422222E+01, *b*=4.998610E+01, *c*=-1.065781E+03. **ACE (- - -)**, *a*=1.192658E+02, *b*=5.447768E+01, *c*=-1.1548067E+01. **SUC (—)**, *a*=1.821269E+04, *b*=3.238560E+02, *c*=-3.190418E+00. Error bars indicate standard deviation.

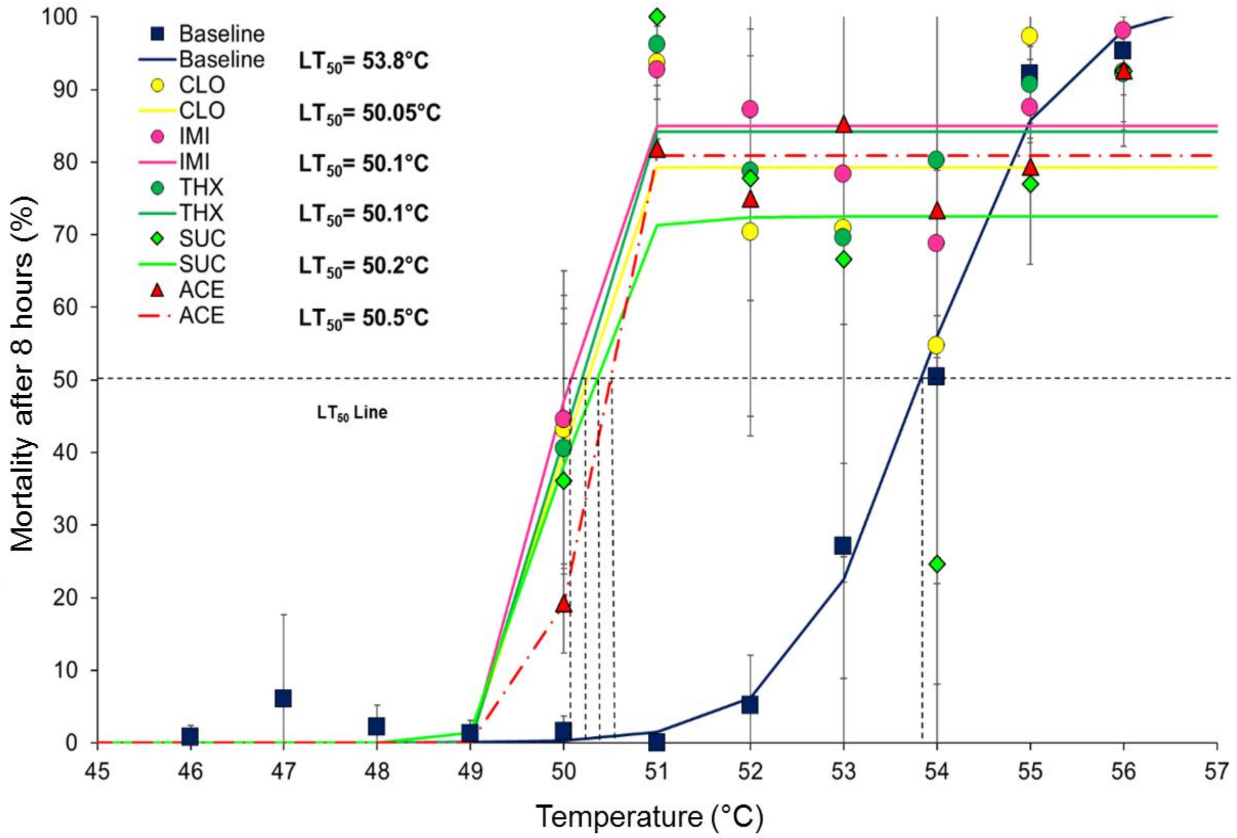

**Figure 2.8:** Mean mortality of the honey bee *Apis melllifera scutellata* recorded at 8 hours following exposure to the designated temperature ramp which terminated at the indicated target temperature (°C), under control (ACE ▲, SUC◆), baseline (Baseline ■) and treated (CLO ●, IMI ●, THX ●) diet conditions. Broken lines indicate lethal temperatures ($LT_{50}$) determined from sigmoidal curves. Curves were best fitted with a sigmoidal function (mortality=a/(1+(x/b)$^c$). Parameters for functions: Baseline **(—)**, *a*=1.0344403E+02, *b*=5.3879726E+01, *c*=-7.70101459E+01. **CLO**, *a*=7.3722222E+01, *b*=4.998623E+01, *c*=-1.1532962E+03. **IMI (—)**, *a*=8.333333E+01, *b*=4.9994453E+01, *c*=-1.08302836E+03. **THX**, *a*=7.958333E+01, *b*=4.9999488E+01, *c*=-1.0163530E+03. **ACE**, *a*=7.37777E+01, *b*=5.0050704E+01, *c*=-1.0680706E+03. **SUC**, a=7.2460823E+01, b=1.3080582E+87, c=4.0139539. Error bars indicate standard deviation.

The mortality rates for the five treatments across the 8-hour period following the temperature ramp exposure was compared to the lowest target temperature (50 °C) (Fig. S2.1) and the highest target temperature (56 °C) (Fig. S2.2). Mortality rate was higher and increased faster over time for all treatments at the higher temperature as opposed to the lower temperature.

The data was processed to produce survival analysis graphs illustrating the survival rates of honey bees. These were recorded at two-hour intervals, over an eight-hour period (0 hours, 2 hours, 4 hours, 6 hours, 8 hours), per treatment (Baseline, CLO, IMI, THX, SUC, ACE) across all seven target temperatures (50 °C to 56 °C) (Fig. S2.4 – Fig. S2.10) as well as a combined graph (Fig. S2.3). These graphs demonstrate the results that come after the bees' exposure to individual temperature ramps. Overall, survival decreased more notably in the treatments than in the Baseline (Fig. S2.3). When separated per temperature, however, survival varied more at lower temperatures and decreased more uniformly at higher temperatures (Fig. S2.4 – Fig. S2.10). Combined survival from all temperatures per treatment was compared for start and end observation time (0 hours vs 8 hours) to ascertain if time was a factor in survival. When comparing only trials from the neonicotinoid experiments, time was not found to be a significant factor (Kaplan-Meier Test $\chi^2$= 7.628; df=4; $p$=0.106). However, when survival data from both the neonicotinoid and baseline experiment data were compared, time was found to be a significant factor influencing honey bee survival (Kaplan-Meier Test $\chi^2$= 326.528; df=5; $p$<0.0001).

Survival data was then evaluated again over the same eight-hour period for the combined five treatments, this time at each individual target temperature separately (Table 2.1). Again, survival decreased over the eight-hour period for all treatments and at all target temperatures, with all seven target temperatures having a significant effect on honey bee survival (Table 2.1).

Survival for the Baseline study was higher than the neonicotinoid treatments at the lower temperatures (50 °C to 53 °C) which lay above the $LT_{50}$ values for the neonicotinoids. Survival for the Baseline study at the higher temperatures (54 °C to 56 °C), which lies above the $LT_{50}$ value for the Baseline data, follows a similar trend to the survival of the neonicotinoid treatments. No significant difference was found between the survival of the Baseline and the respective neonicotinoid treatments under these specific conditions.

**Table 2.1:** Survival analysis statistics for combined neonicotinoid treatment at each target temperature. The relevant survival graph at each temperature is indicated in brackets. Target temperature was found to have a significant (*) effect on honey bee survival.

**Survival Analysis Statistics**

| **Temperature (°C):** | **Chi$^2$:** | **df:** | **p-value:** |
|---|---|---|---|
| 50 (Fig. S2.4) | 82.3508 | 5 | <0.00001* |
| 51 (Fig. S2.5) | 362.996 | 5 | <0.00001* |
| 52 (Fig. S2.6) | 275.436 | 5 | <0.00001* |
| 53 (Fig. S2.7) | 129.427 | 5 | <0.00001* |
| 54 (Fig. S2.8) | 66.3911 | 5 | <0.00001* |
| 55 (Fig. S2.9) | 26.8572 | 5 | <0.00006* |
| 56 (Fig. S2.10) | 91.4639 | 5 | <0.00001* |

Gehan’s Wilcoxon Test was used for pairwise comparison to ascertain whether treatment affected honey bee survival at each of the various temperatures, with treatment differing significantly from Baseline (*p*>0.001) (Table S2.1).

Opportunistic observations throughout the experiment did note more uncoordinated or abnormal behaviour in the neonicotinoid treated bees as opposed to the Baseline or control bees, and while it did not form part of the quantifiable results for the study, it was similar to behaviour noted in other neonicotinoid treated bees (Ludicke & Nieh 2020).

## DISCUSSION

A baseline thermal tolerance of *A.m. scutellata* with an $LT_{50}$ threshold (53.77 °C) was established for this subspecies. This $LT_{50}$ was lowered by several degrees with exposure to sublethal doses of specific neonicotinoids. Mortality rate of these honey bees increased with increasing ambient temperature. While survival analysis also indicated both a decrease in survival over time and a decrease in survival with increasing ambient temperatures, neonicotinoids did not appear to significantly affect survival rates.

Consumption of treated vs. control sucrose solutions over the 24-hour exposure period did not differ significantly across the five treatments, suggesting that no particular diet was preferred or avoided. Previous research has found little or no evidence that honey bees can taste or identify neonicotinoids in food sources (Kessler *et al.* 2015) although they do appear to affect the perception of sucrose (Démares *et al.* 2016).

Despite the decrease of survival in honey bees over time with a concurrent increase in exposure temperature, neonicotinoids did not have a significant effect on mortality under these specific experimental conditions. A diet concentration of 50% w/w was used to ensure comparative consistency with the European study by Kovac *et al.* (2014), however the high quality of diet concentration may play a role in the efficacy of the sublethal neonicotinoid dose. Honey bee forager survival has been found to show minimal impairment under good quality diet conditions (50% sugar solution) but exhibited far more harmful effects under conditions of increased nutritional stress (32.5% and 15% sugar solutions) (Tosi *et al.* 2017). For this study, the mortality data of 1650 individual honey bees across 5 experimental hives over 11 different temperature treatments were used to identify a baseline $LT_{50}$ threshold for our *A.m. scutellata* study population, determined to be 53.77 °C. This is comparatively higher than the previously determined $LT_{50}$ thresholds established for two European subspecies, *A.m. carnica* ($LT_{50}$=50.3 °C) and *A.m. ligustica* ($LT_{50}$=51.7 °C) (Kovac *et al.* 2014). Another study found similar differences between the Eastern (Asian) honey bee *Apis cerana* with an $LT_{50}$=50.7 °C and the Western honey bee *Apis mellifera* with an $LT_{50}$=51.8 °C in Yunnan, China although no specific information about the *A. mellifera* population was provided in the study (Ken *et al.* 2005). For the two European subspecies it was uncertain whether their physiology, their behaviour, or a combination of both was responsible for their respective $LT_{50}$ thresholds as well as the difference in thermal tolerance between the two subspecies This is mainly due to the fact that bees were provided with liquid food throughout the experiment enabling them to employ cooling behaviour (Kovac *et al.* 2014). The honey bees in our South African study were not provided with liquid food during the temperature ramp which may have limited their behavioural cooling ability, suggesting an even stronger resilience at higher temperatures compared to their European counterparts and might indicate a fundamental physiological base for such resilience. The differences in the adaptation of physiological limits to different climatic conditions is one possible explanation for the differences in these value differences between subspecies (Kovac *et al.* 2014).

Other comparative studies on lethal temperature between European subspecies yielded differing results to those of Kovac *et al.* (2014), which were attributed to differences in experimental methodologies. The range of temperatures tested and the differing rates of temperature increase between the studies was the main difference (Abou-Sharaa *et al.* 2012; Kovac *et al.* 2014).

Results from this study are more comparable to the European study as the rate of increase, temperature range and relative humidity parameters were similar (Kovac *et al.* 2014). Differences identified in the LT50 values among the three subspecies (*A.m. carnica, A.m. ligustica* and *A.m. scutellata*) in both the European study and this study may not necessarily be applicable to the respective subspecies as a whole, but rather the specific subspecies' study populations investigated (Kovac *et al.* 2014). This is due to the morphological, behavioural and physiological adaptations to local conditions (Diniz-Filho *et al.* 2000; Alattal & AlGhamdi 2015).

Honey bees from both the European and South African studies were each collected from a single region, for each subspecies, rather than several samples across the entire geographic distribution of each subspecies.

In terms of the European study, samples from *A.m. ligustica* (Italian yellow bee) were collected from Emilia Romagna, Italy which occurs in a warm, temperate climatic region with moderate temperatures (daytime annual average 12.9 °C) and significant rainfall throughout the year (Kovac *et al.* 2014). Samples from *A.m. carnica* (Carniolan honey bee) were collected from Styria, Austria which is a cooler temperate region (daytime annual average 8.3 °C) and significant rainfall throughout the year (Kovac *et al.* 2014). Of the two European subspecies, *A.m. ligustica* occurs in the warmer of the two regions and recorded the higher of the two $LT_{50}$ values suggesting a slightly higher threshold for heat tolerance.

The South African *A.m. scutellata* samples were all collected from the experimental apiary in the city of Tshwane in Gauteng, South Africa. No breeding activities take place at this site and it has been established from wild colonies therefore representing a wider geographical range (Moritz *et al.* 2007). Daytime annual average temperature for this region is 17.8°C with mostly summer rainfall. The *A.m. scutellata* subspecies exhibited a higher $LT_{50}$ value than either of the European subspecies, which could be attributed to the warmer, drier conditions and higher temperature extremes in their native region.

Neonicotinoid trials demonstrated a reduced $LT_{50}$ for all three treatments in comparison to the baseline control $LT_{50}$ data. The CLO, IMI and THX $LT_{50}$ values were more than 3 °C lower than the baseline $LT_{50}$. The SUC and ACE controls from the neonicotinoid trials showed slightly more variation in their mortality and survival trend than the neonicotinoid trials but still echoed similar trends to the Baseline control. This result could be attributed to several factors, such as differences between seasons. Baseline trials were conducted in the autumn and early winter season, whereas neonicotinoid trials were predominantly conducted in the spring and summer seasons. The population demographics of the *Apis mellifera* honey bees cycles in a seasonal manner in response to a colony's needs, which include adapting to the various challenges of winter (Bodenheimer 1937; Seeley & Visscher 1985). The longevity of spring bees (mean lifespan of 30-40 days) and summer bees (mean lifespan of 25-30 days) tends to be notably shorter than that of winter bees (mean lifespan in excess of 100 days, as long as 212-252 days) (Fukuda & Sekiguchi 1966; Mattila *et al.* 2001). This difference in seasonal longevity may be a contributing factor in the more fluctuating mortality of the spring/summer bees in the neonicotinoid control trials as compared to the autumn/winter bees used in the baseline trials.

While notable differences in both longevity and physiological characteristics of summer and winter bees have been well documented in temperate climate zones, there is no documented evidence of these significant differences in winter and summer bees in South Africa. This avenue is therefore an essential topic for future research. The evidence supplied by this study that sublethal doses of three commercial neonicotinoids lower the already established $LT_{50}$ thermal tolerance threshold for *A.m. scutellata* by several degrees adds to the growing body of evidence of the negative effects of neonicotinoids on important pollinators. Further fortification of this subspecies $LT_{50}$ estimate by conducting similar experiments on honey bees is needed, and should include study populations collected from more geographically diverse locations throughout its' natural distribution range.

The lowering of the honey bee $LT_{50}$ as a result of neonicotinoid exposure further substantiates concerns for the long-term survival of honey bees under the current and rapidly changing future climatic conditions on both a local and global scale. The study results coupled with the effects of extreme heat events in several areas around the globe over the last few years, further highlights the increasing environmental pressure that all species face. The African continent is considered particularly vulnerable to the effects of climate change and increasing heatwave frequency and intensity (Russo *et al.* 2016). The probability of heatwaves across the continent are predicted to increase in the near future (Russo *et al.* 2016). Many areas of South Africa have experienced similar heatwaves to those recorded elsewhere, and with record-breaking intensity (Head 2018). Over the last 15 years, the probability of austral summer heatwaves in South Africa have notably increased in comparison to the period 1961-1980 (Lyon 2009). Moving forward, the effects of neonicotinoids on aspects of honey bee thermoregulation should be further quantified, including other areas in the geographical range of this subspecies, as well as evaluating similar aspects of the neighbouring Cape bee subspecies, *A.m. capensis*. This information is crucial when considering future legislation and the use of these neonicotinoids in the South African agricultural and suburban settings.

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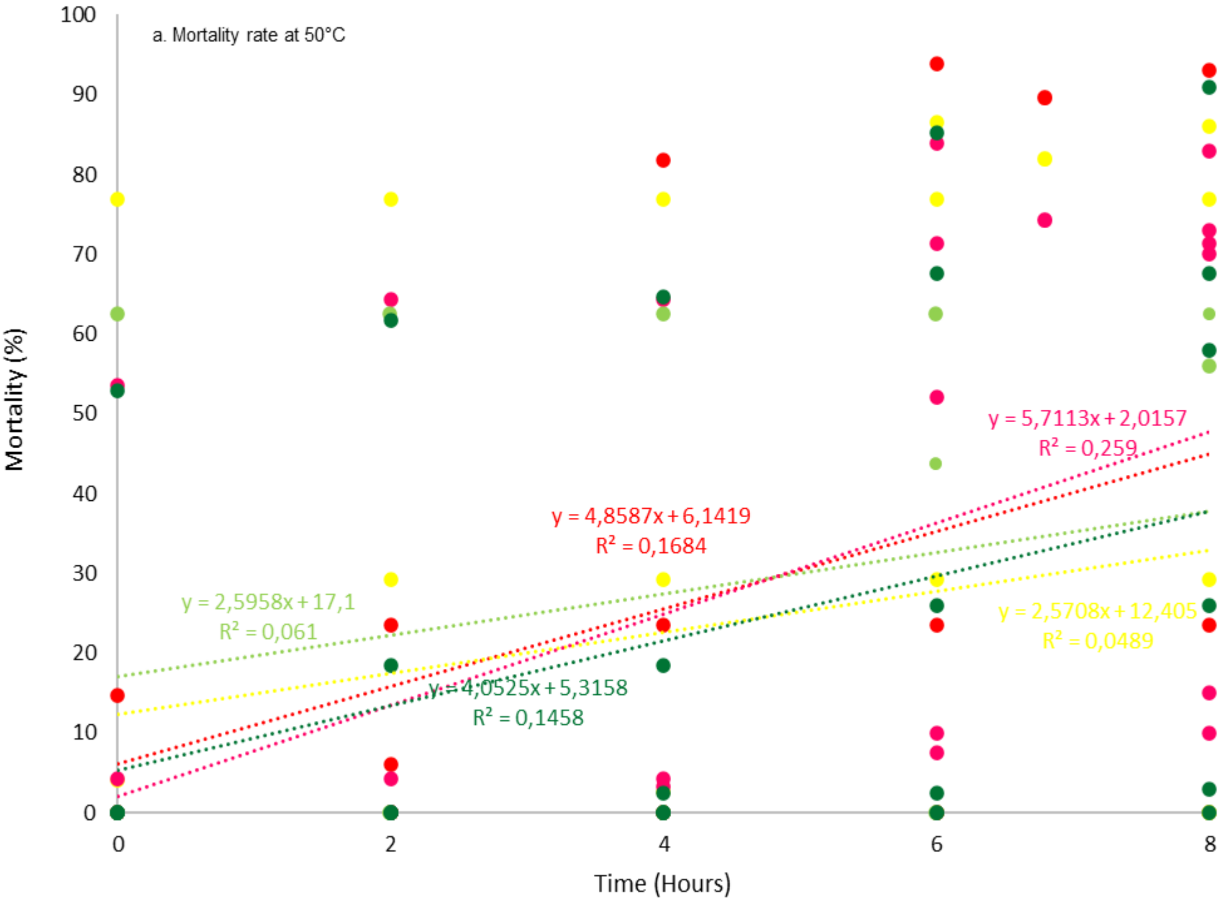
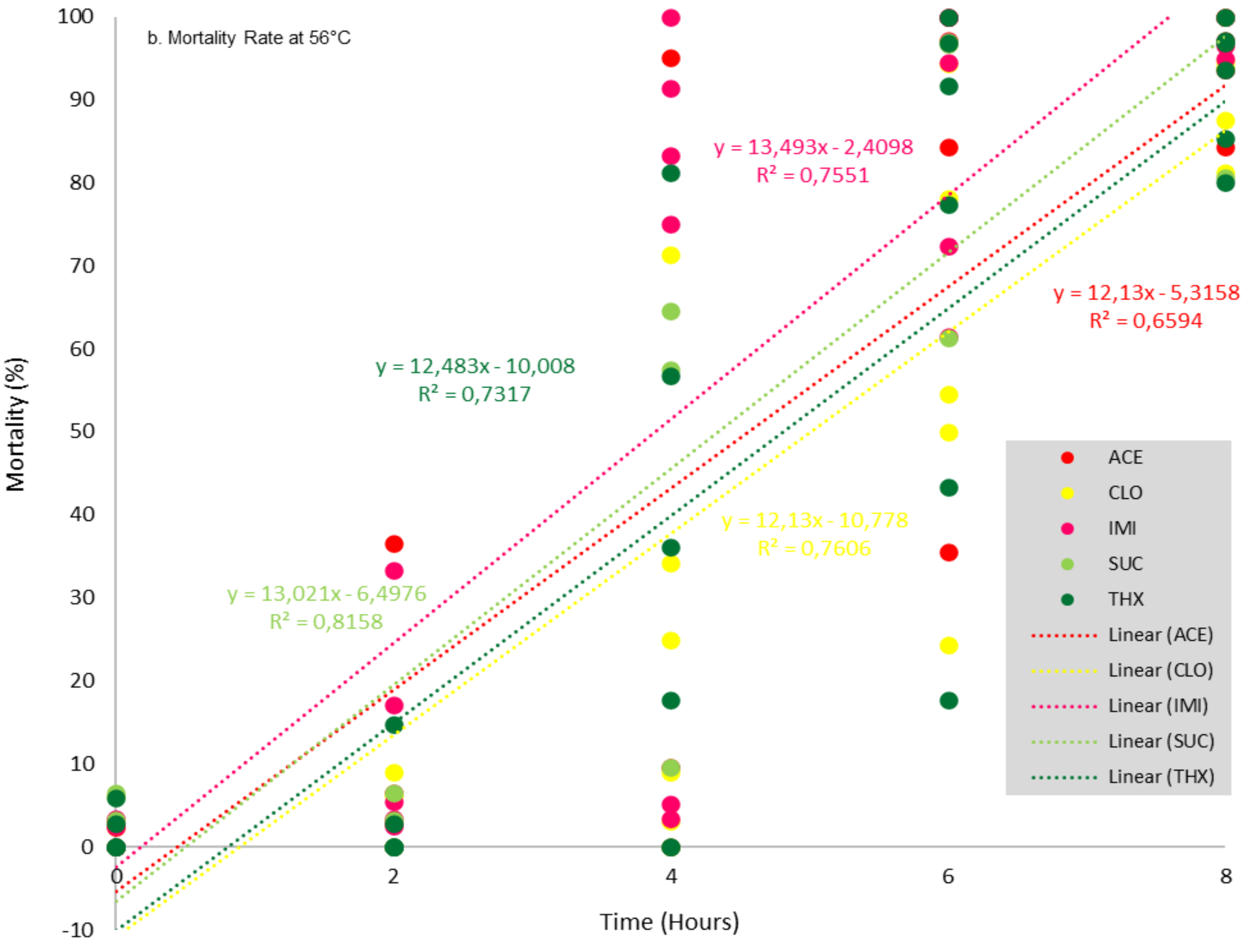
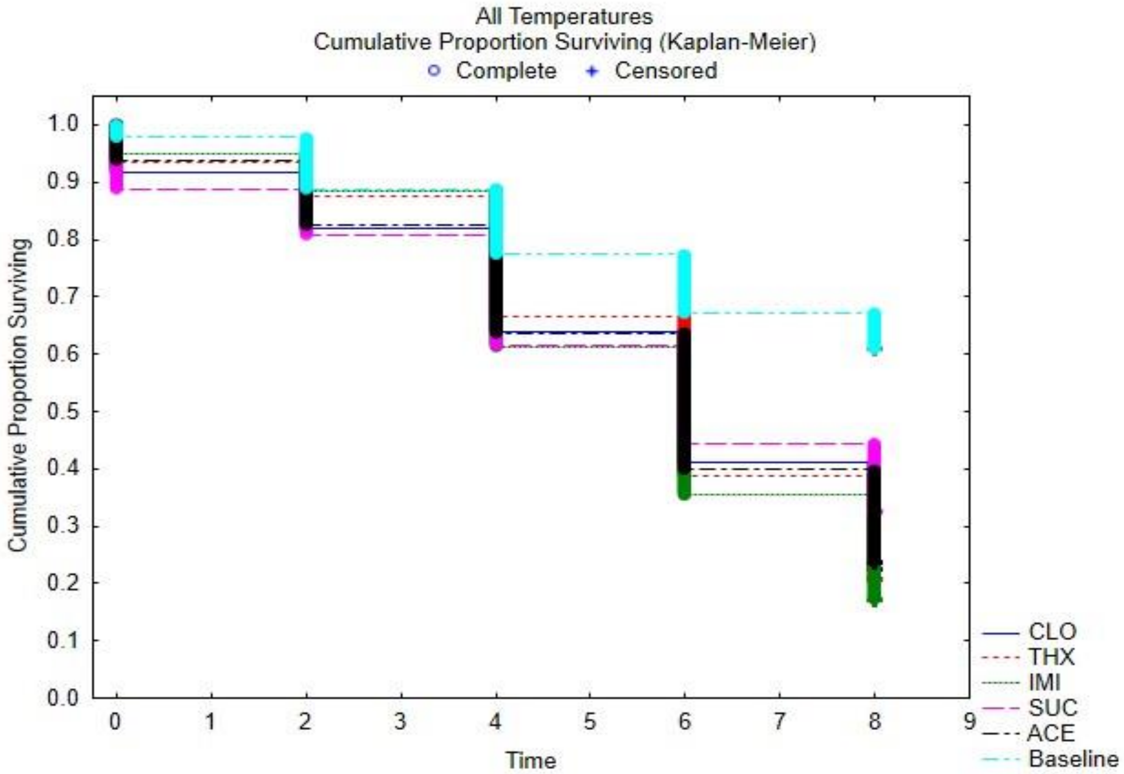
## SUPPLEMENTARY DATA



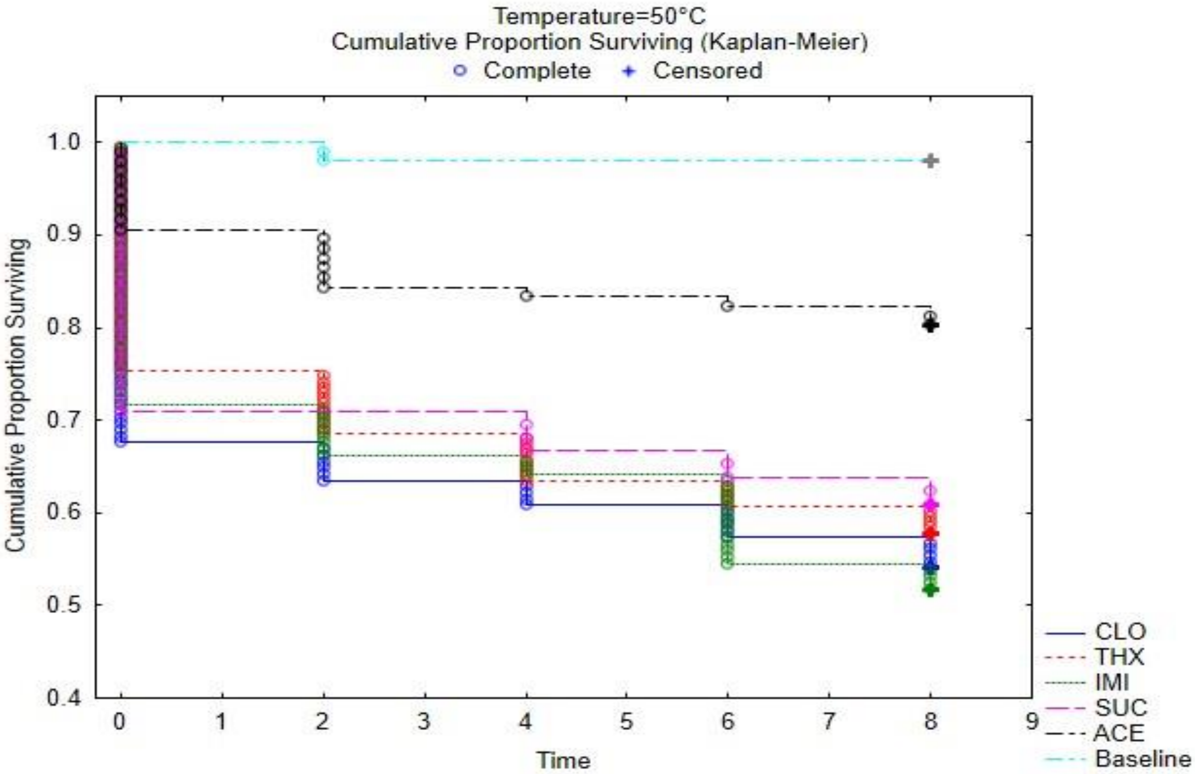
**Supplementary Figure 2.1:** Individual mortalities of *Apis mellifera scutellata* are indicated for the three treatments; clothianidin (CLO), thiamethoxam (THX), imidacloprid (IMI), and the two controls; acetone (ACE) and sucrose (SUC). Mortalities were measured at 2-hour intervals during the 8-hour period. Mortality rates (indicated as the colour corresponding broken lines) for the lowest temperature ramp ending at 50 °C.



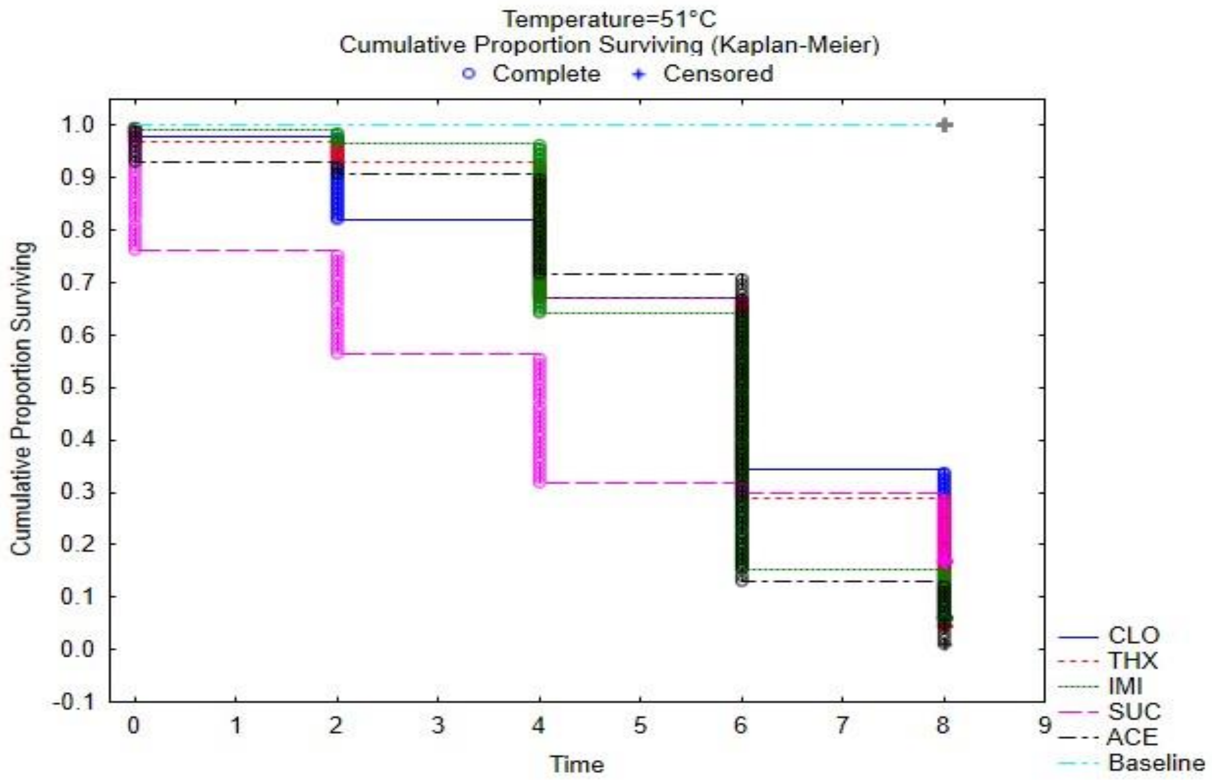
**Supplementary Figure 2.2:** Individual mortalities of *Apis mellifera scutellata* are indicated for the three treatments; clothianidin (CLO), thiamethoxam (THX), imidacloprid (IMI), and the two controls; acetone (ACE) and sucrose (SUC). Mortalities were measured at 2-hour intervals during the 8-hour period. Mortality rates (indicated as the colour corresponding broken lines) for the highest temperature ramp ending at 56 °C.



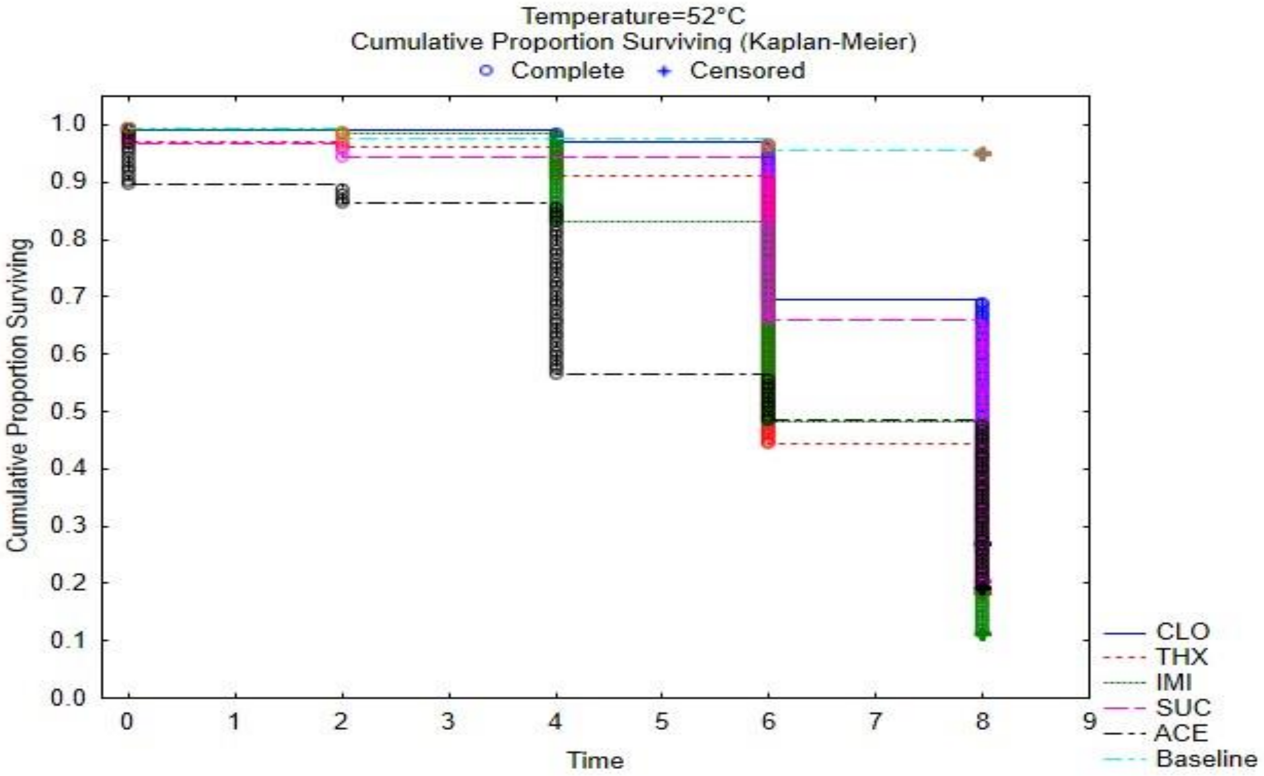
**Supplementary Figure 2.3:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after temperature ramp treatment, combined data for all temperatures per treatment (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



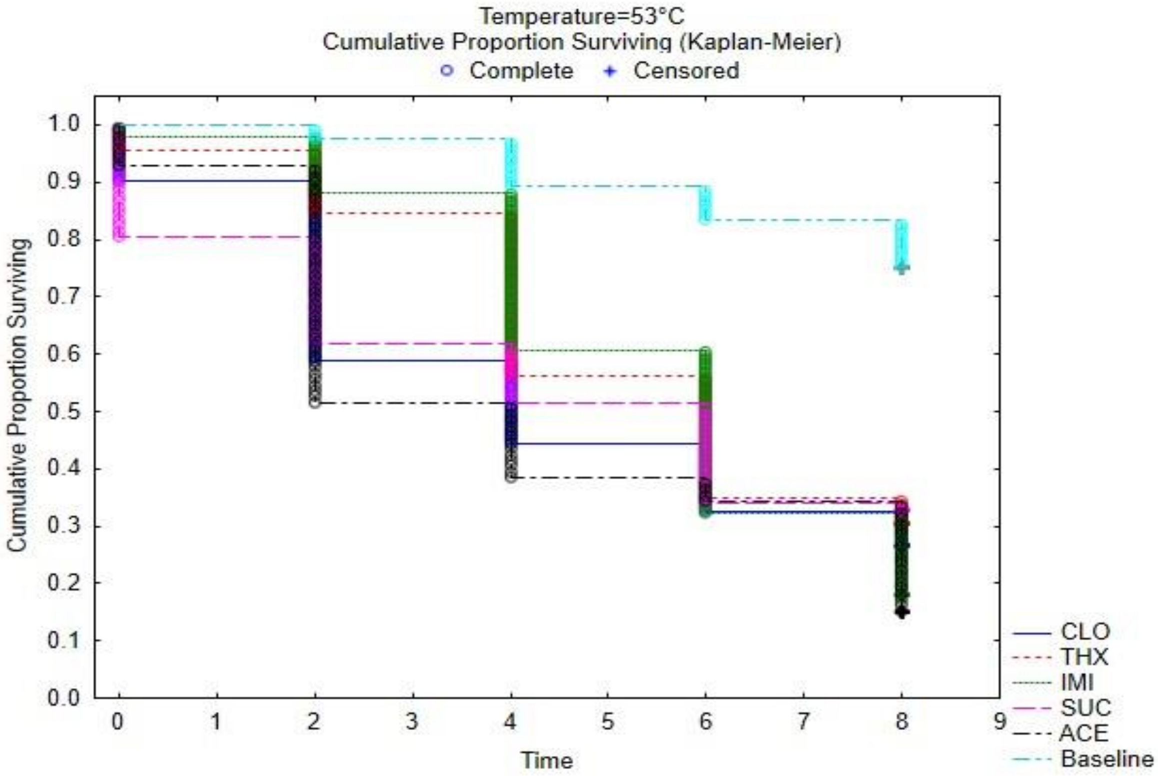
**Supplementary Figure 2.4:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 50 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



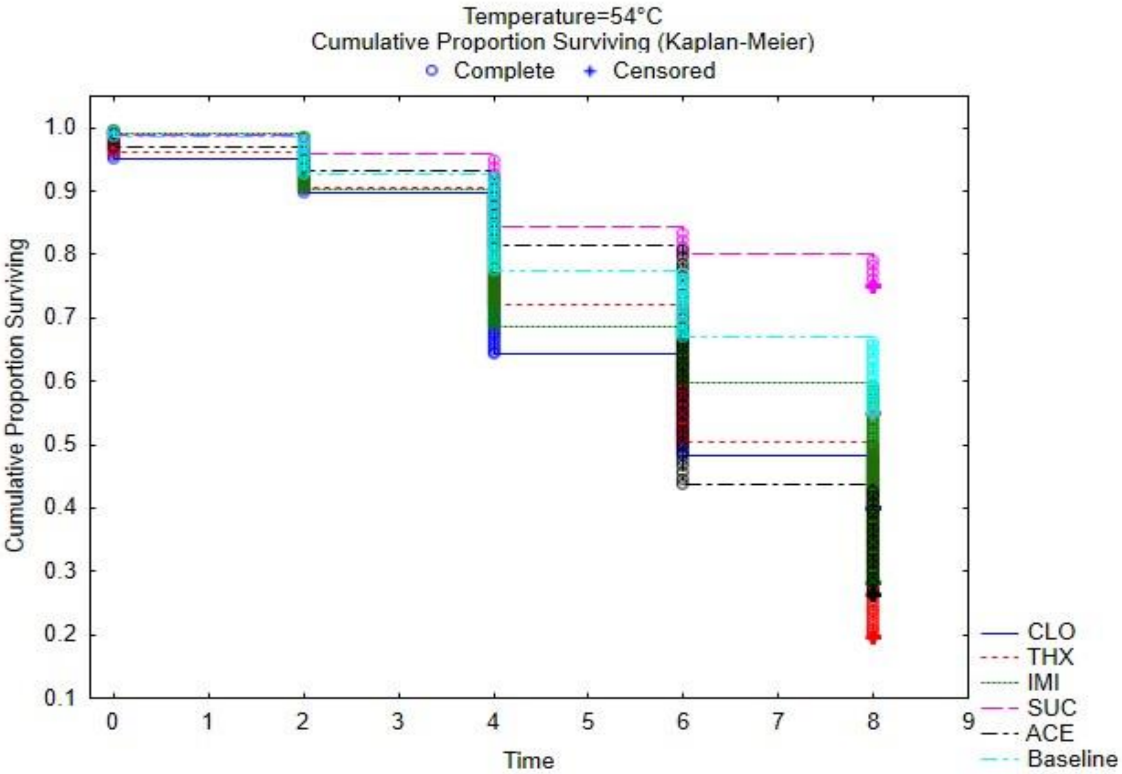
**Supplementary Figure 2.5:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 51 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



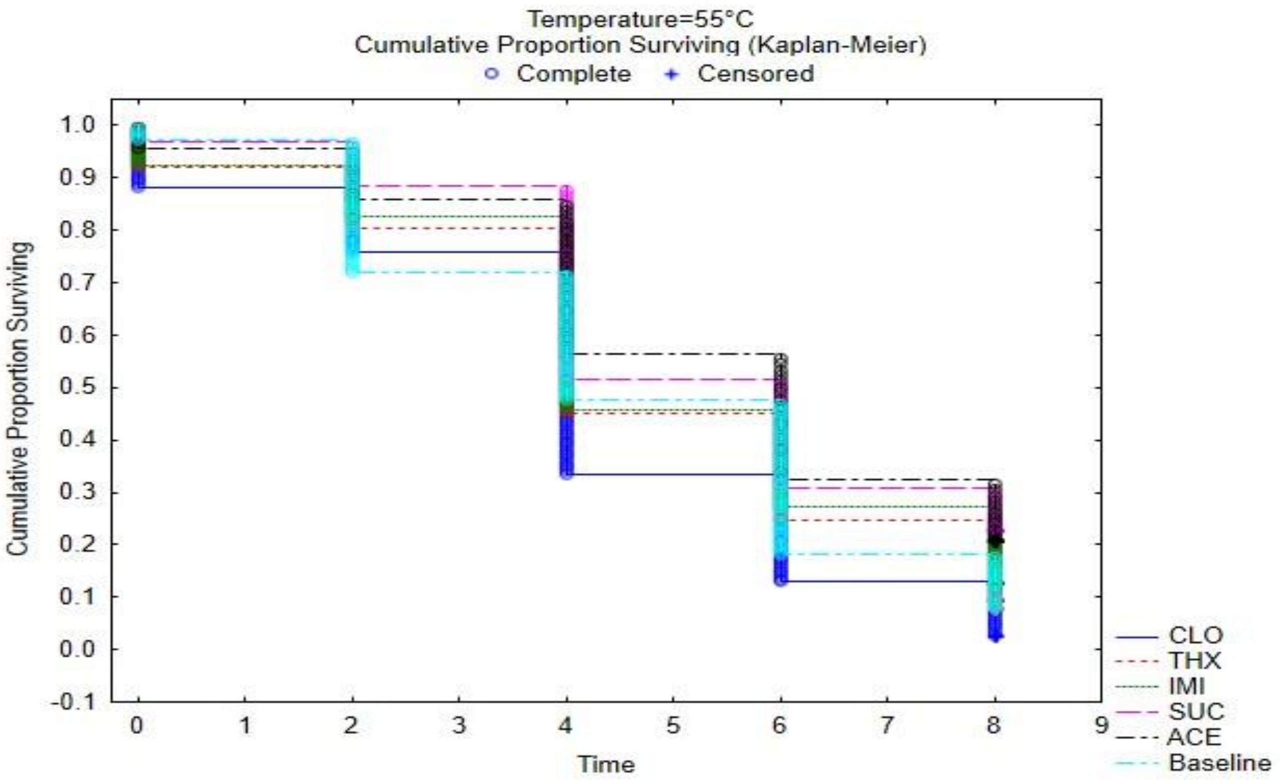
**Supplementary Figure 2.6:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 52 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



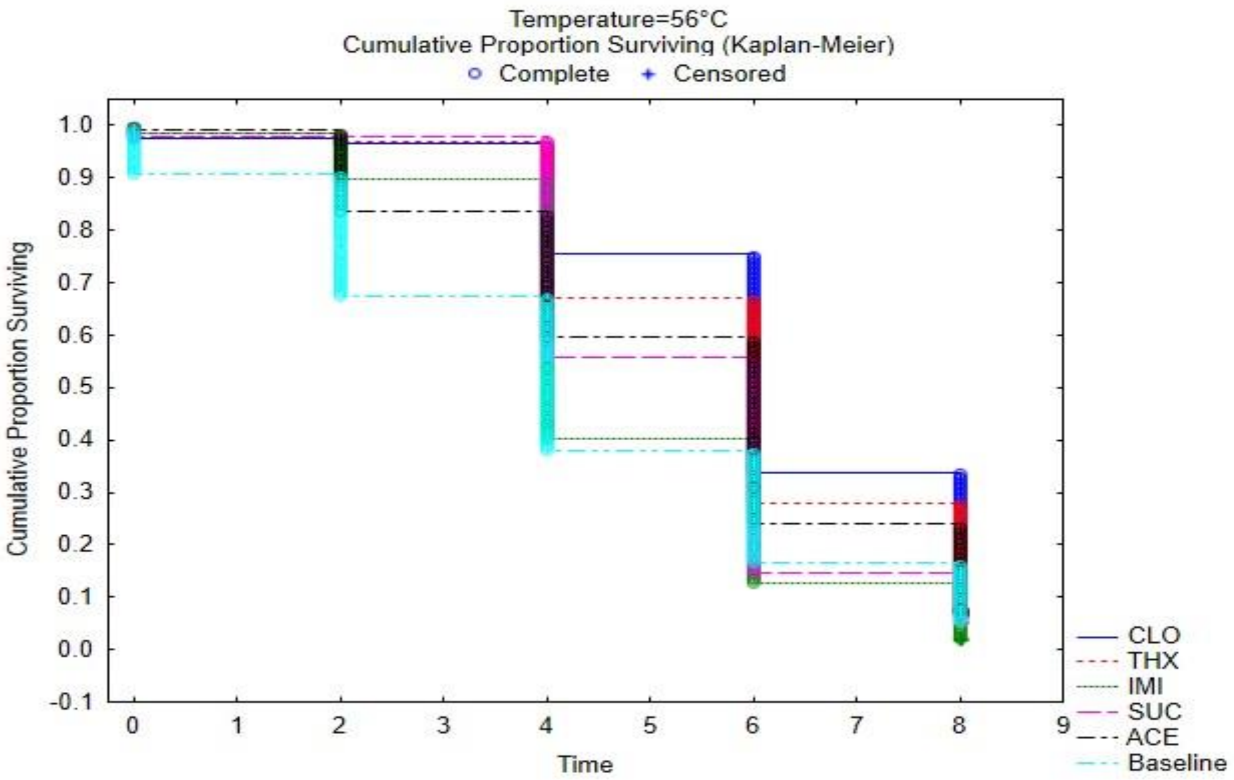
**Supplementary Figure 2.7:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 53 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



**Supplementary Figure 2.8:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 54 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



**Supplementary Figure 2.9:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 55 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.



**Supplementary Figure 2.10:** Survival analysis graph showing the cumulative proportion of surviving *Apis mellifera scutellata* across the 8 hours after the temperature ramp treatment terminating at 56 °C (Kaplan-Meier). Survival rates for honey bees treated with clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX), the acetone (ACE) and sucrose (SUC) controls as well as the Baseline experiment are shown. Survival was recorded at 2-hour intervals.

**Supplementary Table 2.1:** Survival analysis statistics to compare Baseline, the three treatments clothianidin (CLO), imidacloprid (IMI), thiamethoxam (THX), and two controls acetone (ACE), and sucrose (SUC), at each of the seven target temperatures (Temp). Pairwise comparisons were done using the Gehan's Wilcoxon Test to determine whether treatment or temperature influences survival. Test statistic values are indicated in the top right section, and the corresponding *p*-value indicated in the bottom left section.

| **Survival Analysis Statistics** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Temp** | **Baseline** | **CLO** | **IMI** | **THX** | **ACE** | **SUC** |
| **Baseline** | 50 | | 7,719312 | 7,932453 | 7,316304 | 6,493596 | 6,493596 |
| | 51 | | 15,84279 | 16,14244 | 16,09351 | 13,21705 | 13,21705 |
| | 52 | | 11,96384 | 14,34456 | 13,42483 | 11,43902 | 11,43902 |
| | 53 | | 9,144892 | 9,404021 | 8,071751 | 7,268095 | 7,268095 |
| | 54 | | 3,158653 | 3,821306 | 4,888532 | -2,88561 | -2,88561 |
| | 55 | | **1,769400** | -1,19085 | -0,698314 | -2,44702 | -2,44702 |
| | 56 | | -7,06426 | -2,30667 | -6,11184 | -3,68849 | -3,68849 |
| **CLO** | 50 | **0,000001** | | -0,058890 | -0,994403 | -4,33314 | -0,860575 |
| | 51 | **0,000001** | | 1,558709 | 0,1251735 | 1,578066 | 4,037261 |
| | 52 | **0,000001** | | 5,520609 | 4,685847 | 5,076887 | 1,138344 |
| | 53 | **0,000001** | | -3,15863 | -3,25059 | 1,055079 | -0,206061 |
| | 54 | **0,00159** | | 0,1173092 | 1,040331 | -0,088139 | -5,32647 |
| | 55 | 0,07683 | | -3,09031 | -2,59164 | -4,20105 | -4,07356 |
| | 56 | **0,000001** | | 7,259766 | 1,666344 | 2,947474 | 3,732491 |
| **IMI** | 50 | **0,000001** | 0,95304 | | -0,927808 | -4,42674 | -4,42674 |
| | 51 | **0,000001** | 0,11907 | | -1,63405 | -0,305657 | -0,305657 |
| | 52 | **0,000001** | **0,000001** | | -0,816047 | 1,813127 | 1,813127 |
| | 53 | **0,000001** | **0,00159** | | -0,142018 | 3,872698 | 3,872698 |
| | 54 | **0,00013** | 0,90662 | | 1,476613 | 0,4610994 | 0,4610994 |
| | 55 | 0,23371 | **0,00200** | | 0,5259096 | -1,66538 | -1,66538 |
| | 56 | **0,02107** | **0,000001** | | -5,73154 | -2,45575 | -2,45575 |
| **THX** | 50 | **0,000001** | 0,32003 | 0,35351 | | -3,73374 | -3,45596 |
| | 51 | **0,000001** | 0,90039 | 0,10225 | | 1,316785 | 1,43582 |
| | 52 | **0,000001** | **0,000001** | 0,41447 | | 2,446461 | 2,23435 |
| | 53 | **0,000001** | **0,00115** | 0,88707 | | 3,985146 | 3,872698 |
| | 54 | **0,000001** | 0,29819 | 0,13978 | | -0,629328 | -0,629328 |
| | 55 | 0,48498 | **0,00955** | 0,59895 | | -2,09562 | -2,09562 |
| | 56 | **0,000001** | **0,09564** | **0,000001** | | 1,771043 | 1,771043 |
| **ACE** | 50 | **0,000001** | **0,00001** | **0,00001** | **0,00019** | | 2,901582 |
| | 51 | **0,000001** | 0,11455 | 0,75987 | 0,18791 | | 3,271165 |
| | 52 | **0,000001** | **0,000001** | **0,06981** | **0,01443** | | -3,20367 |
| | 53 | **0,000001** | 0,29139 | **0,00011** | **0,00007** | | -0,976003 |
| | 54 | **0,00391** | 0,92977 | 0,64473 | 0,52913 | | -5,69420 |
| | 55 | **0,01440** | **0,00003** | **0,09584** | **0,03612** | | 0,1612038 |
| | 56 | **0,00023** | **0,00320** | **0,01406** | **0,07655** | | 0,2381309 |
| **SUC** | 50 | **0,000001** | 0,38947 | **0,00001** | **0,00001** | **0,00371** | |
| | 51 | **0,000001** | **0,00005** | 0,75987 | 0,75987 | **0,00107** | |
| | 52 | **0,000001** | 0,25498 | **0,06981** | **0,07851** | **0,00136** | |
| | 53 | **0,000001** | 0,83674 | **0,00011** | **0,00011** | 0,32906 | |
| | 54 | **0,00391** | **0,000001** | 0,64473 | 0,68933 | **0,000001** | |
| | 55 | **0,01440** | **0,00005** | **0,09584** | **0,08734** | 0,87193 | |
| | 56 | **0,00023** | **0,00019** | **0,01406** | **0,02066** | 0,81178 | |

# CHAPTER 3

# The influence of sublethal neonicotinoid doses and ambient temperature on individual *Apis mellifera scutellata* flight efficiency

## ABSTRACT

Honey bee (*Apis mellifera*) thermoregulation plays an integral part in their behaviour and physiology and has been shown to be vulnerable to the effects of neonicotinoid insecticides. Flight muscles are a crucial source of physiological heat as well as being vital to behavioural heat regulation, and are negatively affected by neonicotinoid insecticides. In this study, we evaluated the flight efficiency and capacity of *Apis mellifera scutellata* under the influence of both elevated ambient temperatures and sublethal neonicotinoid exposure. The various aspects of flight; distance, speed, and duration, were not notably affected by these factors. However, the honey bees' ability to initiate a successful flight was significantly affected by neonicotinoid exposure. Such a reduction in honey bee flight capacity and flight muscle function in general, especially under the increasing frequency and intensity of hot weather events, is cause for concern when considering legislation and use of these neonicotinoids in the agricultural and suburban setting.

# INTRODUCTION

Among the multitude of negative effects that insecticide use has on both target pests as well as non-target beneficial arthropods such as honey bees, *Apis mellifera*, is an influence on a variety of health and foraging-related factors. Foraging honey bees come into contact with a multitude of pesticides in the environment (Mullin *et al.* 2010; Samson-Robert *et al.* 2014; Woodcock *et al.* 2017; Prado *et al.* 2019) and the residues of these pesticides have been identified in a wide spectrum of food sources for both honey bees and humans alike (Chen *et al.* 2014; Lu *et al.* 2015; Mitchell *et al.* 2017).

Chronic exposure to insecticides impairs honey bee optomotor behaviour and, by extension, their foraging behaviour (Parkinson *et al.* 2022). Of particular concern are the neonicotinoid insecticides and their metabolites, which act as agonists of the nicotinic acetylcholine receptors of insects (Simon-Delso *et al.* 2015). The metabolites of neonicotinoids affect a variety of neurological functions including key learning and foraging behaviours (Schneider *et al*. 2012; Teeters *et al.* 2012; Frost *et al*. 2013; Williamson & Wright 2013) and lowered immune system resistance by suppressing immunity-related genes and increasing the susceptibility to infection by viruses and pathogens (Alaux *et al.* 2010; Vidau *et al.* 2011; Aufauvre *et al.* 2012; Doublet *et al.* 2015; Brandt *et al.* 2016; Sánchez-Bayo *et al*. 2016). Exposure to the neonicotinoid imidacloprid induces rapidly neurotoxic symptoms; including movement coordination difficulty, trembling and tumbling (Suchail *et al.* 2000). Similarly, sublethal doses of the neonicotinoids thiamethoxam, imidacloprid and acetamiprid can impair bee behaviour and motor functions (Aliouane *et al*. 2009; Charreton *et al*. 2015; Lambin *et al*. 2001; Williamson *et al*. 2014). As a result of these affected neurological functions, foraging activities vital for colony survival and reproduction are also negatively impacted (Yang *et al.* 2008; Schneider *et al.* 2012; Gill *et al.* 2014; Scholer & Krischik 2014). Exposure to imidacloprid hampers honey bee pollen collection efficiency, which in turn results in more honey bee workers being recruited for foraging at a younger age (Colin *et al.* 2019) at the expense of sufficient workers available for brood care for example (Gill *et al.* 2014). This may not only result in insufficient colony pollen stores but also in lowered worker production and a further impaired colony work force (Gill *et al.* 2014; Blanken *et al*. 2015).

Neonicotinoid-induced neurological impairment affects foraging through various aspects of flight capacity including flight distance, duration, velocity, and efficiency (Blanken *et al.* 2015; Tosi *et al.* 2017; Ma *et al.* 2019). It can also impair the metabolic energy availability for flight muscles (Nicodemo *et al.* 2014). Imidacloprid exposure reduces flight capacity in honey bees, even more so when acting together with the parasitic mite *Varroa destructor* (Blanken *et al.* 2015).

Flight performance depends on thoracic muscle activity, with the flight muscle temperature precisely controlled by honey bees during flight (Esch 1988; Schmaranzer 2000; Stabentheiner 2001). Apart from the mechanical action of flight, honey bees use their thoracic muscles to produce heat (Esch 1976) and impaired individual thermoregulatory capability could be caused by the effect of pesticide on thoracic muscle activity. *Apis mellifera scutellata* thorax temperature has been shown to be affected by thiamethoxam exposure for at least 24 hours, likely as a result of affected flight muscle function (Tosi *et al*. 2016). Non-flight thermogenesis, found to be negatively affected by dietary neonicotinoids in bumble bees (*Bombus terrestris*) (Potts *et al.* 2018), is crucial for pre-flight warm up (Krogh & Zeuthen 1941; Heinrich 1975; Esch *et al.* 1991).

Environmental temperature is also important for honey bee flight. This includes the efficiency with which they forage at certain temperatures as well as the onset and termination of daily foraging activity (Tan *et al.* 2012). The relatively constant thermal environment within a honey bee hive is advantageous in that it enables workers to commence foraging activities earlier in the day than other stingless or solitary bee species (Heinrich 1981). Flight muscle shivering is employed when elevating the thoracic temperature to suitable levels to facilitate flight; typically being between 36 °C and 38 °C in *Apis mellifera* foragers (Heinrich 1979). Shivering also renders *Apis mellifera* capable of maintaining this elevated $T_{th}$ even at low environmental temperatures (<20 °C) (Heinrich 1979; Dyer & Seeley 1987). However, a negative correlation has been found between foraging and flight activity and elevated ambient temperatures in *A. m. carnica* and *A. m. jemenitica* (Blazyte-Cereskiene *et al*. 2010; Abou-Shaara *et al*. 2013). Foragers are at particular risk of environmental neonicotinoid exposure and the nature of their foraging tasks mean they are also exposed to the widest range of ambient temperatures. Nectar and pollen foragers complete an average of around 10 trips a day (Winston 1987), although there is a great deal of plasticity surrounding foraging capacity, based on various environmental and societal factors (Tenczar *et al.* 2014). These foragers are thus at risk of oral exposure to residues in nectar as well as direct contact exposure with treated plants (Koch & Weisser 1997) through pollen during pollen and nectar foraging (Louveaux 1958; Parker 1981) and the adsorption of contaminated dust particles (Prier *et al.* 2001). They are also at risk of affecting the whole colony with contaminated pollen and nectar (Bos & Masson 1983; Villa *et al.* 2000), a risk which is amplified in the case of systemic insecticides, i.e. neonicotinoids (Waller *et al.* 1984). Water collection is integral to evaporative cooling activities, and the presence of neonicotinoid contaminated surface water (Starner & Goh 2012; Samson-Robert et al 2014; Schaafsma *et al.* 2015; Struger *et al.* 2017) poses an additional risk to water foragers and by extension the colony as a whole (Samson-Robert *et al.* 2014; Simon-Delso *et al.* 2015; Schaafsma *et al.* 2015).

Honey bees are exposed to multiple and overlapping neonicotinoid sources. Existing research has demonstrated that it is important to establish their effects. Previous studies have demonstrated the negative impact of neonicotinoid exposure on honey bee flight (Blanken *et al*. 2015; Tosi *et al.* 2017). In this study, we aimed to further evaluate the effects of exposure of the South African honey bee subspecies *A.m. scutellata* (Lepeletier) to sublethal doses of three commonly used neonicotinoids, namely clothianidin, imidacloprid and thiamethoxam, and their effects on certain aspects of flight ability under varying ambient temperature conditions. This was done by recording tethered flight using flight mills in order to quantify flight success, number of flights, flight distance and flight speed. We predicted that exposure to sublethal doses of neonicotinoids would decrease the tethered flight ability of honey bees and that the effect would be most apparent at lower temperatures, due to potentially reduced capacity for flight muscle shivering.

## METHODS

### *Study species*

The study ran from October 2016 to August 2017, using workers of African honey bees, *A.m. scutellata*, from three healthy experimental colonies housed at the Social Insect Research Group (SIRG) apiary located at the Innovation Africa campus of the University of Pretoria in Hatfield, Tshwane, Gauteng Province, South Africa. Naturally distributed throughout sub-Saharan Africa (Ruttner 1988, Hepburn *et al.* 1998, Pirk 2020). The distribution of this subspecies in South Africa in particular extends over much of the country, with the exception of the Cape region, which is occupied instead by the country's other subspecies, *Apis mellifera capensis* (Ruttner 1988; Crewe *et al.* 1994; Hepburn *et al.* 1998, Pirk 2020).

Pollen and nectar foragers were used as they are easily detectable at hive entrances, are regularly flight active, experience the greatest variation in temperature in the external environment and experience the greatest exposure to pesticides in the environment while foraging (Blanken *et al*. 2015; Tison *et al.* 2016; Tosi *et al.* 2017). Upon inspection, the experimental hives were deemed free of any obvious signs of disease and of a large enough size to withstand the continuous removal of small numbers of honey bees over a prolonged period of time. The forager collection was done on warm days (25 °C-40 °C) when forager traffic was high (no sample collection done on cold/overcast/rainy days as there was minimal forager activity). To ensure that only foragers and not guard bees were collected, the hive entrances were smoked to ensure all bees at the entrance retreated inside and then the entrances (and any other visible openings) were blocked using small sections of foam. After a period of 5-10 minutes, the returning foragers collecting around the inaccessible entrances were collected using aspirators which were lined with foam to eliminate injury.

### *Neonicotinoid exposure*

Once collected from hive entrances, forager bees were transferred to Perspex hoarding cages (120 mm x 95 mm x 80 mm) with sliding panels on both sides, a perforated panel for ventilation on the bottom and two small windows on the front. The two small windows were made to accommodate the insertion of two 2 mL centrifuge (Eppendorf®) tubes used to administer the diet (Köhler *et al.* 2013). Each cage contained 30 bees from the same hive, maintained for no more than two days before using them in flight mill experiments, as the effects of chronic exposure remain the same for this period of time (Blanken *et al.* 2015; Tosi *et al.* 2017). All the tested foragers remained alive throughout the experiment. Foragers used in control (CONT) experiments were provided with two 2 mL microcentrifuge tubes containing only sugar water solution (1:1 w/w sucrose and water) for a period of 24 hours prior to testing (Blanken *et al.* 2015; Tosi *et al.* 2017).

Three commercially available neonicotinoid active ingredients were used in the sublethal exposure treatments, namely thiamethoxam (THX), clothianidin (CLO), and imidacloprid (IMI). As per standard practice, acetone was used as an organic solvent for the neonicotinoids, to make them more soluble in the diet, with the total amount of acetone present per treatment, including the control, amounting to less than 0.05% (El Hassani *et al.* 2008; Aliouane *et al.* 2009; Démares *et al.* 2016). Field-realistic doses and concentrations of neonicotinoids tend to vary widely across space and time (Pisa *et al.* 2015). Neonicotinoid concentrations in this study were considered comparable to realistic field doses and the chronic period of exposure sufficient to allow for all honey bees to consume sufficient treated sugar water to illicit any potential observable effects (Démares *et al.* 2018). Foragers used in the neonicotinoid treated trials were also provided with two 2ml centrifuge (Eppendorf®) tubes, both treated with the same sublethal dose of the relevant neonicotinoid (40 µL in 2 mL tube of 1:1 w/w sucrose and water, final concentration of 5 nM), for a period of 24 hours before being tested. These doses are well below the LD50 = 4-5 ng/bee threshold (Godfray *et al.* 2014; Henry *et al.* 2104; Oliveira *et al.* 2014; Démares *et al.* 2016). Doses were below the LD50 to prevent excessive mortality of the study population.

### *Flight mill methodology*

The flight ability of active foragers was tested using a standard flight mill (Naranjo 1990) (Fig. 3.1). Four flight mills were set up in an incubator (Memmert HCP 108, GmbH+ Co. KG, Schwabach, Germany) and maintained at a constant temperature of 25 °C, 30 °C or 35 °C. As flight is considered unaffected by temperature at 25 °C, this was used as a starting point (Blanken *et al.* 2015). Lighting in the incubator was provided by two standard daylight fluorescent tube lamps and the incubator door was fitted with a transparent observation pane.

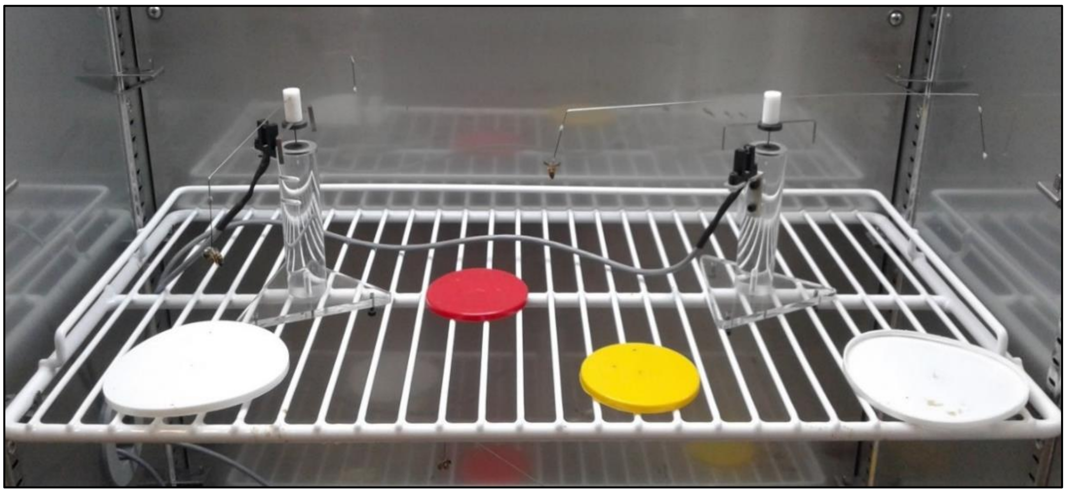


**Figure 3.1:** The flight mill used to evaluate flight capacity of tethered forager *Apis mellifera scutellata* honey bees (red and yellow discs used as environmental enrichment). Foragers were attached to the end of the wire flight mill arm using a small drop of glue placed in the top of their thorax. Tethered bees were then allowed to fly and various flight parameters were recorded by the magnetic contact points.

Flight experiments were performed between 9:00 and 17:00 during the winter, and 8:00 and 18:00 in summer, based on seasonal light and temperature changes and observed activity levels of the hives.

Following the 24-hours, four foragers were removed from the cage at a time and anaesthetised in a cooler box of ice (approximately 5 °C) for 2-3 min (Makumbe *et al.* 2020). Foragers were not completely immobilised, merely cooled until leg and wing movements were minimal and a pin could be applied without any glue interfering with the wings (Blanken *et al.* 2015). Each honey bee was attached to the flight mill by gluing the dorsal side of the thorax to the end of an insect pin using hot melt adhesive (HMA) that had been allowed to cool until safe for application but still adhesive. The end of the pin was bent to provide a 2 mm portion of pin, perpendicular to the main length of the pin, providing sufficient area for attachment while still ensuring that it did not hinder the bee's wing movements. Once attached to the pins, the pins were attached to the end of one arm of a flight mill so that the bee was oriented right way up. In order to ensure the flight mill arm remained exactly level during the experiment and to account for the weight of the honey bee, foragers were weighed prior to the experiment and a counterweight of similar weight (maximum deviation of 2.0 mg; Blanken *et al.* 2015) was attached to an identical pin fitted to the opposing side of the flight mill. Foragers were allowed to rewarm and regain full activity during a 10-minute acclimatisation period at the given temperature before flight mill recording commenced and flight was stimulated. Forager flight activity was recorded on the flight mill for a period of one hour.

The four flight mills each had a diameter of 28 cm and an associated revolution of 87.97 cm. The flight mills were all connected to a specially designed data acquisition system (Vehicle Dynamics Group, 2016) which registered each half rotation and the time taken, recorded with specially designed software (Vehicle Dynamics Group, 2016) (Makumbe *et al*. 2020). Flight mills were spaced within the incubator to minimise any interaction effects between neighbouring test subjects, and to ensure no contact with surrounding walls. Trial runs using various surrounding images and patterns showed little influence on stimulating flight activity in foragers. Small plastic circles of various colours (yellow, green, red and white) distributed on the shelves of the incubator proved to be the most effective visual stimulation (Makumbe *et al.* 2020). In the context of the study, minimum conditions existed that constitute a successful flight and any honey bees measuring below that were discarded and deemed a non-flight. A flight was defined as a period between two breaks, a break being defined as period of three seconds or longer without a revolution. A successful flight was considered as three of more consecutive revolutions.

Baseline flight data for the control and the three neonicotinoid treatments was first determined at a functional ambient temperature of 25 °C in order to eliminate the effects of temperature on flight efficiency (Harrison *et al*. 1996; Brodschneider *et al.* 2009; Blanken *et al.* 2015). Control and neonicotinoid trials were also conducted at 30 °C and 35 °C. The selected temperatures are within the range of temperatures at which honey bee flight is possible, with honey bees able to remain in continuous free flight at high air temperatures up to at least 46 °C (Heinrich 1980).

Bees that failed to exhibit flight during the flight mill test period were designated as non-fliers. The overall percentage of successful flights as well as successful flights per treatment were recorded, and were also used as one of several variables applied to evaluate the influence of neonicotinoids on honey bees.

### *Morphological traits*

Due to the flight capabilities of honey bees also being can also be influenced by a suite of morphometric data, we recorded both wing measurements and bee body weight. Directly following flight tests, honey bee samples were stored at -5 °C for later evaluation of morphometric measurements. Forager wings were carefully removed and wing slides were prepared for each individual honey bee. Each set of wings was mounted in a drop of distilled water on a glass slide and sealed with clear nail varnish before being photographed using a transmission light microscope (Vickers Instrument, York, England) equipped with a Moticam (Motic®, Moticam 5.0 MP, China). Using the photographs, dimensions of each wing were measured using ImageJ image processing and analysis software (version 1.48, US National Institute of Mental Health, Bethesda, Maryland, USA). The measured wing traits were area, length and width (in mm) of the left front (L1), left back (L2), right front (R1), and right back (R2) wings of each bee. The fresh weight of each bee was measured to the nearest 0.001 g (Blanken *et al*. 2015) using an electronic weight balance (Mettler Toledo AG64, Greifensee, Switzerland).

### *Data analyses*

Overall percentage of non-flights vs. successful flights, as well as the percentage of non-flights vs. successful flights, were determined for each treatment and each temperature condition. The flight mill data from successful flights was used to determine various tethered flight parameters including the number of flights, total distance flown and average flight speed. Total distance (m) was calculated using the number of laps and distance covered per lap (calculated using the radius of the flight path covered in one revolution, $c = 2\pi r$). Average speed ($m.s^{-1}$) was calculated using total distance (m) divided by total time flown (s).

In addition to the area, width and length of all four wings, two additional variables were established based on the symmetry of the wings. The area of the left wing was subtracted from the area of the right wing; meaning if wing symmetry was negative, the left wing was bigger, and if it was positive, then the right wing was bigger. A zero value meant the wings were symmetrical. Early exploratory data analysis where each of these wing measurements and bee weight (predictor variables) were plotted against flight success (response variable) showed no differences. Because the wing measurements are all inherently collinear and dependent on each other in some way, we opted to use a principal component regression with a binomial distribution to test whether weight and wing dimensions influenced whether the bee flew or not. A principal component analysis (PCA) was used to include all wing measurement variables and bee weight in order to create a list of orthogonal non-linear principal components performed in R and using base library "*stats*" (R Core Team 2021). Principal components are Eigen values. Only the first 13 principal components were then used in a binomial regression against the predictor variable of whether the bee flew or not; this also done in the R programming language (R Core Team 2021). The first 13 principal components were used because they explained 100% of the deviation in the data. A well performing model with significant principal components is an indication that wing measurements and bee weight would influence a bee's ability or likelihood to fly.

A generalised linear model (GLM) in programme R (R Core Team 2021) was used to test the effects of neonicotinoids and temperature on the flight success, number of flights, flight distance and average flight speed. For flight success, a binomial GLM with a logit-link function was used with the response variable being whether the bee flew (1) or not (0) using all 451 available data points. Only points where the bee flew were used for the remainder of the models (i.e., where the response value was not zero). The number of flights and flight speed followed a gamma distribution, determined using the "fitdistrplus" library (Delignette-Muller & Dutang 2015) in R (R Core Team 2021). Flight distance was severely right-skewed and was subsequently log-transformed after which a Poisson distribution was used in GLM modelling.

Eight (number of flights), four (flight distance) and four (speed) extreme outlier points were not used in these analyses because they all represented anomalous bees who made between 61 and 126 flights; flew further than 2.8 kms; and flew faster than 5.5 m.s$^{-1}$. The excluded outliers recorded extreme values with frenzied flight activity for the full hour of recording. Best models were chosen using chi-squared tests between models and comparing Akaike's information criterion values for small samples sizes (AICc) (Anderson & Burnham 2004). Model fit was assessed using a variety of plots of residuals to make sure the fitted models met the assumptions.

## RESULTS

### *Morphological traits*

The 13 principal components from the PCA explained 100% of the variance in the data (Table 3.1). In the binomial GLM using the principal components as predictor variables only PC 7 was statistically significant, though explaining only 6.8% of the overall data variation (AIC = 130.2, Null deviance = 127.54, *df* = 91; Residual deviance = 102.2, *df* = 78) (Table S3.1). Body mass did not differ significantly across the four treatments at the three different temperatures suggesting it did not influence flight success (Table S3.2). Therefore, the data strongly suggested that whether bees flew or not, it was not coincidentally influenced by the sample of bees we used to investigate the study aims.

### *Flight performance*

Flight mill runs were conducted on a total of 360 foragers across the three ambient temperatures and four dietary treatments. Of those foragers, 169 were designated as non-fliers and 191 were recorded to have performed successful flights. Overall, flight success was 53%. When evaluated per treatment, however, the flight success was far lower in the neonicotinoid treatments than in the control. The binomial GLM determining whether a bee flew or not (n = 451) was only influenced by the treatment (log-ratio $\chi^2$= 53.4, df = 3, P <0.001) and not by temperature (Fig. 3.2). The probabilty of flying was highest if a bee was in the control group (0.72; 95% CI = 0.64,0.78), followed by THX (0.46; 95% CI = 0.37,0.55), then CLO (0.41; 95% CI = 0.32,0.0.51), and lastly the probability of a bee flying was lowest for bees dosed with IMI (0.27; 95% CI = 0.19,0.37) (Fig. 3.2).

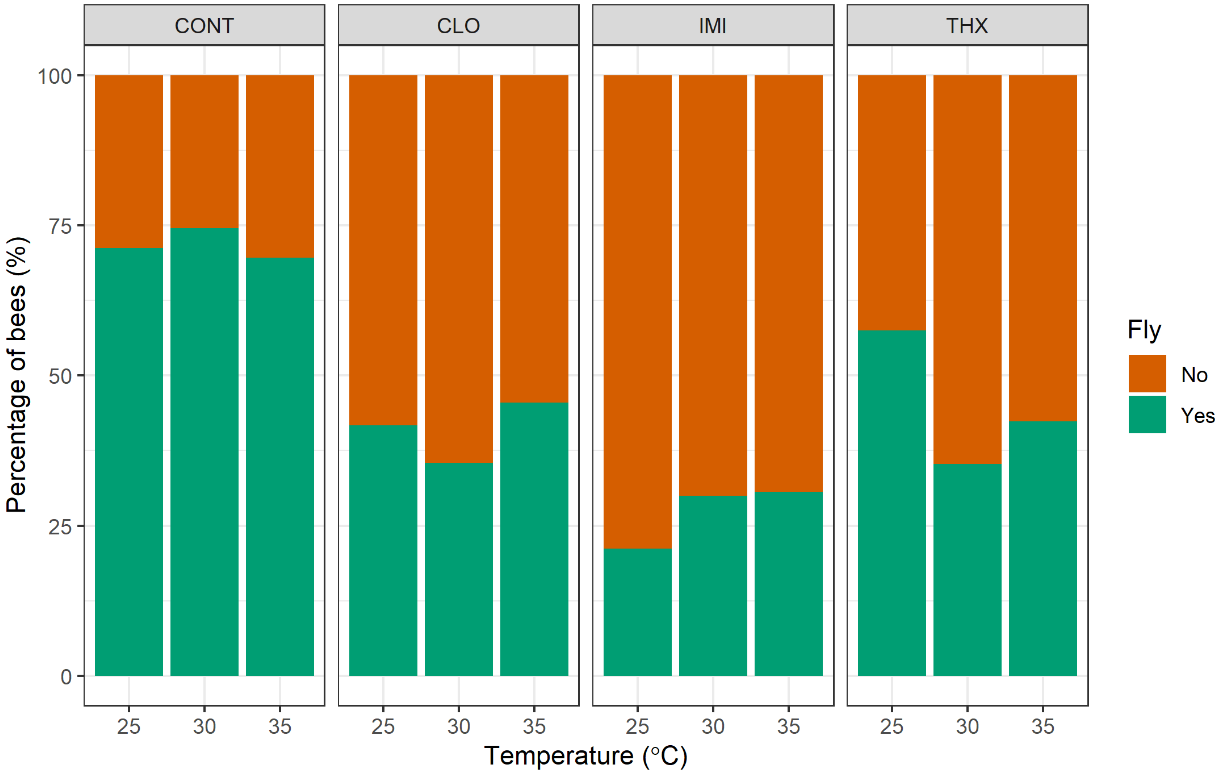


**Figure 3.2:** Summary plot showing the percentage of honey bees used in the experiment that flew (Yes) or not (No) for the control (CONT) and the three neonicotinoid treatments clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (THX), under the three temperature conditions (25 °C; 30 °C, 35 °C).

Superficial examination of the raw data demonstrated that there was a slight increase in the number of flights at 30 °C as compared to 25 °C and 35 °C (Table S3.3). Both THX and IMI showed a decrease in the number of flights with an increase in temperature, while CLO exhibited a noticeable increase of flights at 30 °C as compared to 25 °C and 35 °C. Number of flights under control conditions did not vary significantly between treatments and temperatures. The best model for number of flights included only neonicotinoid treatment as a predictor (AICc = 1378.3; ΔAICc = 0.00; df= 5; weight = 0.538) (Table 3.1).

**Table 3.1:** Generalised linear modelling to test the effects of neonicotinoid treatments (treat), temperature (temp) and their interacting effects (temp:treat) on the number of flights, flight distance and flight speed.

| **Model** | ***df*** | **logLik** | **AICc** | **ΔAICc** | **Weight** |
|---|---|---|---|---|---|
| ***Number of flights*** | | | | | |
| treat | 5 | -683.964 | 1378.3 | 0.00 | 0.538 |
| temp + treat | 6 | -683.679 | 1379.8 | 1.57 | 0.246 |
| intercept | 2 | -688.615 | 1381.3 | 3.03 | 0.118 |
| temp | 3 | -688.452 | 1383.0 | 4.77 | 0.049 |
| temp + treat + temp:treat | 9 | -682.032 | 1383.1 | 4.83 | 0.048 |
| ***Flight distance*** | | | | | |
| intercept | 1 | -342.915 | 678.8 | 0.00 | 0.701 |
| temp | 2 | -342.908 | 689.9 | 2.03 | 0.255 |
| treat | 4 | -342.891 | 694.0 | 6.13 | 0.033 |
| temp + treat | 5 | -342.887 | 696.1 | 8.22 | 0.011 |
| temp + treat + temp:treat | 8 | -342.742 | 702.2 | 14.36 | 0.001 |
| ***Flight speed*** | | | | | |
| temp + treat + temp:treat | 9 | -194.630 | 408.3 | 0.00 | 0.710 |
| intercept | 2 | -204.199 | 412.5 | 4.21 | 0.087 |
| treat | 5 | -201.138 | 412.6 | 4.35 | 0.081 |
| temp | 3 | -203.348 | 412.8 | 4.57 | 0.072 |
| temp + treat | 6 | -200.534 | 413.5 | 5.27 | 0.051 |

The probabilty of flying was highest if a bee was in the control group (0.72; 95% CI = 0.64,0.78), followed by THX (0.46; 95% CI = 0.37,0.55), and CLO (0.41; 95% CI = 0.32,0.0.51), and lastly the probability of a bee flying was lowest for bees dosed with IMI (0.27; 95% CI = 0.19,0.37) (Fig. 3.3).

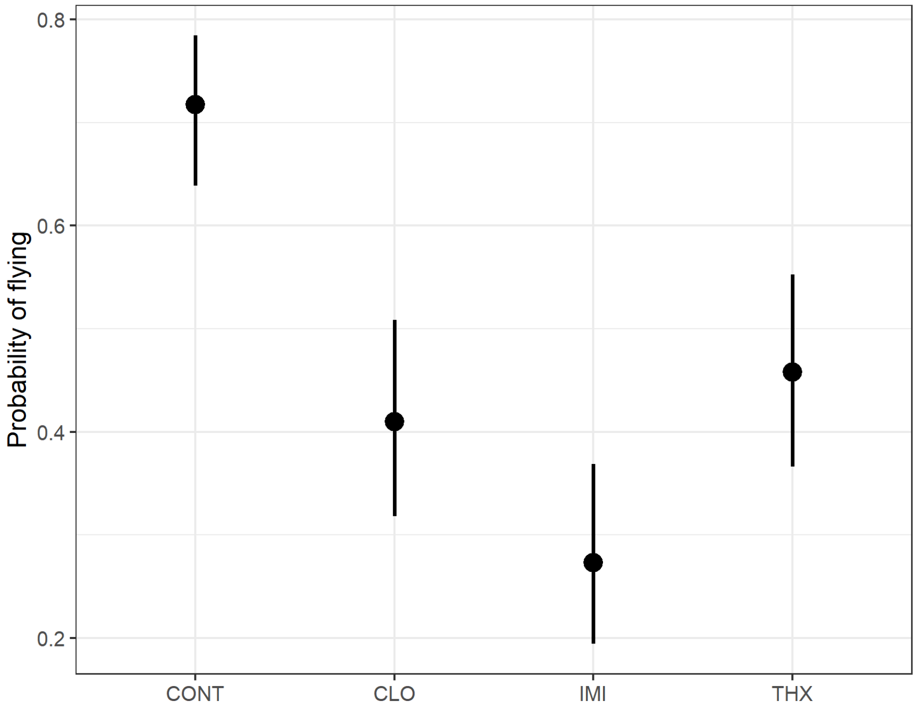


**Figure 3.3:** Model effects of binomial generalised linear model (GLM) showing estimated mean and 95% confidence intervals of the probability of honey bees flying for the control (CONT) and the three neonicotinoid treatments clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX).

The distance flown by the honey bees across the four treatments and three temperatures also varied greatly and no definitive trend was identified (Table S3.3). Similarly, the GLM following a Poisson distribution flight distance was not influenced by treatment, temperature or the interaction term. The null model was better than any of the other models when considering the combinations of predictors (AICc = 678.8; ΔAICc = 0.00, df =1, Weight = 0.0701 vs AICc = 702.2; ΔAICc= 14.36, df = 8, Weight=0.001 for the full model). The second ranked model had a ΔAICc value of 2.03 meaning it was not meaningfully different but not close enough to the null model to be considered a useful model (Anderson & Burnham 2004).

Under control conditions the distance flown differed minimally across the three temperatures. THX and IMI exhibited notably further distances flown at 35 °C and CLO showed similar distances flown across the three temperatures.

The best (lowest ΔAICc value) GLM following a gamma distribution for flight speed was the full model that included temperature, treatment and their interaction term (Table 3.1; AICc = 408.3; ΔAICc = 0.00; df= 9; weight = 0.710), with the second ranked model ΔAICc value increasing by 4.21 indicating a meaningful difference between the two models. Average flight speed was the highest for CLO at 25 °C (2,446 ± 2,028) (when temperature is assumed not influence flight) and highest for CONT at both 30 °C (1,439 ± 1,437) and 35 °C (1,527 ± 1,188) (Table S3.3).

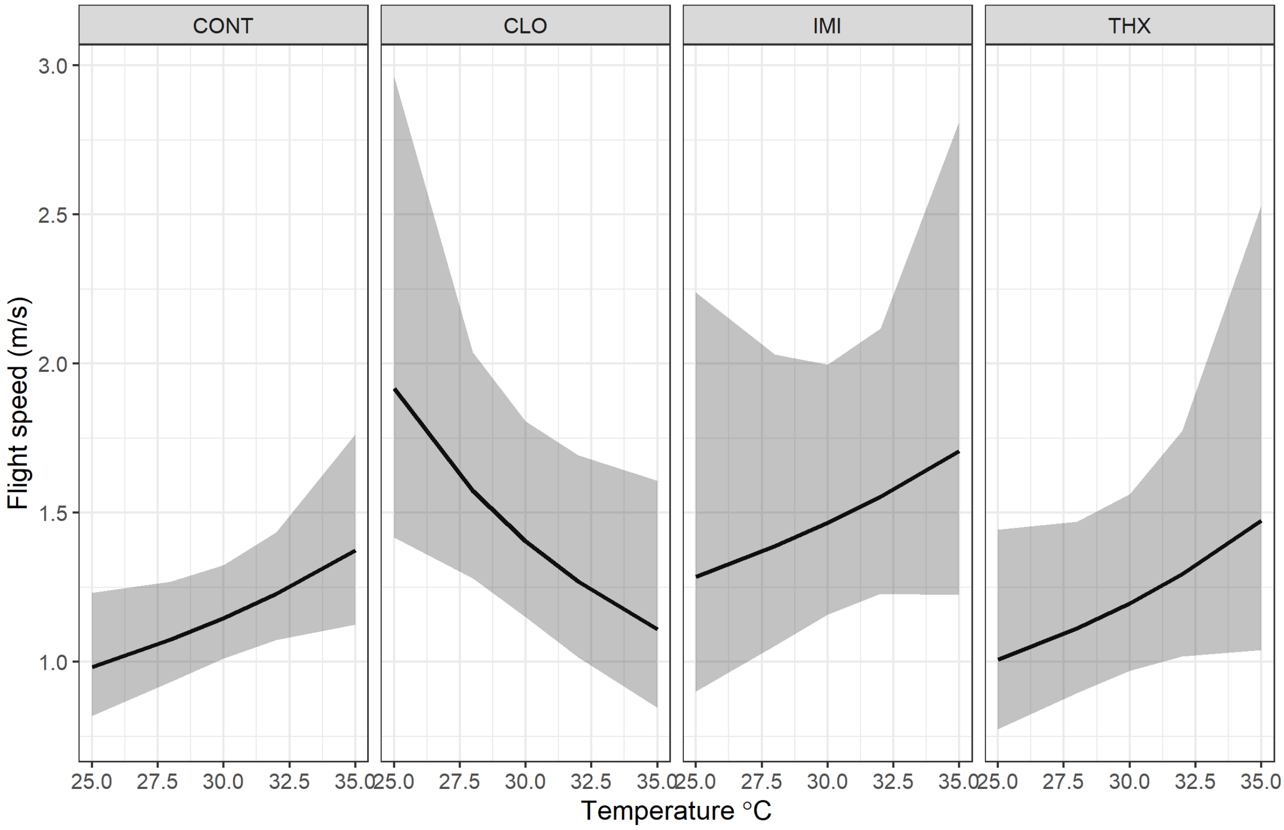


**Figure 3.4:** Model effects of gamma distributed generalised linear model (GLM) showing the estimated mean and 95% confidence intervals of average flight speed (m.s$^{-1}$) for the control (CONT) and the three neonicotinoid treatments clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (THX), under the three temperature conditions (25 °C; 30 °C, 35 °C).

The Chi-squared test, however, indicated that none of the predictors were significant in the full model (Table S3.4). From a biological perspective, the effects plot (Fig. 3.4) indicates that flight speed increased with an increase in ambient temperature and with limited variation. The neonicotinoids IMI and THX showed a similar trend of increasing flight speed with increasing temperature whereas CLO exhibited a decrease (Fig. 3.4).

## DISCUSSION

When exposed to IMI, THX and CLO, under increasing ambient temperature conditions, *A.m. scutellata* exhibited an increased variation in the number of flights, flight duration and flight speed, with a significant difference in flight success among treatments. Similar to Tosi *et al.* (2017), our bees were allowed to feed chronically on treated sucrose solutions for 24 hours but rather than multiple concentrations of one neonicotinoid, we selected three prominent neonicotinoids and administered them at a single, field realistic, sublethal dose to investigate their effects under the three different ambient temperature conditions.

Initial flight success or failure was influenced only by treatment. The probability of a bee flying successfully was far higher in the control, with the three treatments having far lower flight success, IMI having the lowest. Due to far more bees not flying at all under the neonicotinoid treated conditions as compared to the control, the lowered sample size increased the variation across these three treatments and likely contributed to the more varied results. While chronic sublethal THX has been shown to impair flight capacity in honey bees (Tosi *et al.* 2017) after 24 hours, and IMI exacerbates the effect of Varroa infestation on flight capacity (Blanken *et al.* 2015) between 24 hours and 13 weeks, our study demonstrates failure to initiate any flight at all following chronic THX, IMI and CLO after a 24-hour exposure period. This result is consistent with that of Tosi *et al*. (2017) and within the period of exposure noted by Blanken *et al.* (2015). The results in this study were true for all three temperature conditions. Whereas sublethal short-term THX exposure may have elicited an excitatory flight affect, chronic sublethal THX exposure tends to elicit a depressive long-term effect (Tosi *et al.* 2017; Ma *et al.* 2019). One of the neonicotinoids, THX, has already been shown to affect the thorax temperatures (Tosi *et al*. 2016). Thorax temperature in *A.m. scutellata* was affected by THX exposure for at least 24 hours if not longer, likely as a result of changes in thoracic muscle function (Tosi *et al*. 2016).

The number of flights initiated by the bees were influenced by treatment as well as the interaction between treatment and temperature. Under control conditions there was only a slight increase in flight number at 30 °C compared to 25 °C and 35 °C; THX and IMI decreased the flight number as temperature increased; while CLO noticeably increased at 30 °C as compared to 25 °C and 35 °C.Chronic THX exposure significantly decreased honey bee flight duration (−54%) (Tosi *et al.* 2017).

Flight distances were not notably influenced by either temperature or treatment, or interaction term. Descriptive statistics did show more of a variation in the THX and IMI treatments across the three temperature conditions, with far higher flight distances at the highest temperature (35 °C). Tosi *et al.* (2017) noted that chronic THX exposure resulted in a decrease in flight distance (−56%), citing the depressive effect of chronic exposure as a possible explanation. However, the results recorded in this study indicated that the number of flights were not significantly influenced by any of the predictor variables.

Similarly, the sublethal effects of IMI alone were not sufficient to elicit a notable change in the flight parameters in an *Apis mellifera* subspecies in the Netherlands (Blanken *et al.* 2015). The honey bees exhibited reduced flight distances and flight durations only when exposed to field-realistic, chronic sublethal doses of IMI in conjunction with high loads of the *Varroa destructor* mite (Blanken *et al.* 2015). It is possible that as our local populations do not suffer the same higher loads of *Varroa* that they are not influenced by this factor in the same way.

Flight speed was affected by temperature, treatment and their interaction term but the chi-squared test indicated none of the predictors were significant in the full model. While average flight speed may not have been significantly affected by temperature or treatment, the results still have broader biologically relevant implications. Under control conditions, average flight speed increased with an increase in ambient temperature conditions. Ambient conditions of temperatures around 25 °C do not affect flight, as oxygen consumption and metabolic rate are comparatively lower than at other ambient temperatures (Harrison *et al*. 1996; Hrassnigg & Crailshiem 1999; Brodschneider *et al.* 2009; Blanken *et al.* 2015). There was a far greater variation in flight speed under neonicotinoid conditions as opposed to the control. Chronic THX exposure slightly decreased honey bee average velocity (−7%) (Tosi *et al.* 2017), but not significantly. Exposure to neonicotinoids such as THX can induce short-term hyperactivity which can in turn lead to long-term muscular exhaustion and lowered energetic availability (Derecka *et al*. 2013; Tosi *et al*. 2017). This could account for why a greater variation in flight speed was observed among the neonicotinoid treated honey bees. Both IMI and THX chronic exposure has been shown to elicit immediate excitation and hyperactivity followed by decreased activity and responsiveness in bees (Suchail *et al.* 2001; Gill & Raine 2014; Tosi *et al.* 2017), an effect that could explain why these two neonicotinoids had similar progressive effects on flight number, distance and speed across the three temperatures. On the other hand CLO, while still a neonicotinoid acting on nicotine acetylcholine receptors (nAChR), targets nAChR subtypes that differ from those of THX and may have slightly different effects (Simon-Delso *et al.* 2015; Tosi *et al.* 2017). This could account for the contrasting effects of CLO on the flight parameters found in the study. The consumption of the control and treated solutions did not differ significantly in this study. Solution consumption was thoroughly investigated in the previous chapter and due to no changes in the study population and experimental setup parameters,  no notable change was found in this chapter.

Wing dimensions and body weight did not appear to have any effect on whether or not a bee had a successful flight. Only 6.8% of the overall variation in the data was explained by the model. This is most likely an effect of sample size and does not hold any biological significance. While morphometric measurements did not have any bearing on flight success in this study, the difference in results between this study and similar studies on other subspecies, namely *A.m. mellifera* x *A.m. carnica* x *A.m. buckfast,* subspecies hybrid (Blanken *et al.* 2015) and *A.m. ligustica* (Tosi *et al.* 2016) may in part be due to physical differences among these subspecies. For example, the mass-specific metabolism of active, flying *A.m. scutellata* is higher than in honey bees of European subspecies due to their larger thorax-specific capacity and higher flight muscle oxidative capacity. This lead to a difference in flight capacity and metabolism among honey bee subspecies (Harrison & Hall 1993; Hepburn *et al.* 1999; Kovac *et al.* 2014).

Considering that PC 7 only accounted for 6.8% of the overall variation in the data, this is most likely an effect of sample size and does not hold any biological significance

The current study uses flight success and associated flight parameters as an indication of flight muscle function and by extension an indicator of honey bee capacity to thermoregulate both physiologically via thermogenesis and behaviourally via wing fanning and water foraging. Neonicotinoid induced alterations to flight parameters and reduced flight success may not only affect the nectar and pollen foraging capacity of a colony and subsequent nutritional diversity of foraged pollen (Tison *et al.* 2016; Tosi *et al.* 2016), but may also affect the efficacy of both individual and colony-level behavioural and physiological thermoregulation.

The findings of the current study provide valuable information regarding neonicotinoid effects on flight capacity of honey bees in the South African context, particularly in terms of field-applicable results. However, further investigation into the conditions and parameters on actual insecticide treated cropsis required in order to provide mor practical information (Thompson *et al.* 2016).

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## SUPPLEMENTARY DATA

**Supplementary Table 3.1:** Principal components binomial regression analysis of honey bee (*Apis mellifera scutellata*) morphometric data comparison.

| | Estimate | Standard Error | Z value | Pr (>\|z\|) |
|---|---|---|---|---|
| ***(Intercept)*** | 0.0003455 | 0.2434044 | 0.001 | 0.9989 |
| ***PC1*** | 0.0937869 | 0.1149623 | 0.816 | 0.4146 |
| ***PC2*** | 0.1666662 | 0.1722320 | 0.968 | 0.3332 |
| ***PC3*** | -0.2263734 | 0.2030784 | -1.115 | 0.2650 |
| ***PC4*** | -0.0856721 | 0.2323235 | -0.369 | 0.7123 |
| ***PC5*** | 0.2607100 | 0.2377876 | 1.096 | 0.2729 |
| ***PC6*** | 0.0204768 | 0.2578756 | 0.079 | 0.9367 |
| ***PC7*** | -0.5810410 | 0.2831383 | -2.052 | 0.0402 * |
| ***PC8*** | 0.4712969 | 0.3893645 | 1.210 | 0.2261 |
| ***PC9*** | -0.4653991 | 0.4218892 | -1.103 | 0.2700 |
| ***PC10*** | -1.0639088 | 0.5564846 | -1.912 | 0.0559 |
| ***PC11*** | -0.7915564 | 0.5678135 | -1.394 | 0.1633 |
| ***PC12*** | 0.8339161 | 0.6107176 | 1.365 | 0.1721 |
| ***PC13*** | -1.8032980 | 0.7878243 | -2.289 | 0.221 |

*Significance: '***' 0.001; '**' 0.01; '*' 0.05*

**Supplementary Table 3.2:** Mean honey bee (*Apis mellifera scutellata*) body weight (grams) of all bees sampled per treatment at each temperature, for both successful and unsuccessful flights. Honey bees were exposed to either the control (CONT), thiamethoxam (THX), clothianidin (CLO) or imidacloprid (IMI) dosed sucrose diet. Flight mill runs were conducted at 25 °C, 30 °C and 35 °C respectively for each treatment.

| **Temperature** | **Treatment** | **Flight success** | **Mean bee weight (g) (± SD)** | **Sample size (n)** |
|---|---|---|---|---|
| 25 °C | CONT | Yes | 0.0831 ± 0.0211 | 37 |
| | | No | 0.0875 ± 0.0231 | 15 |
| | THX | Yes | 0.0896 ± 0.0123 | 25 |
| | | No | 0.0826 ± 0.0127 | 8 |
| | IMI | Yes | 0.0984 ± 0.0091 | 8 |
| | | No | 0.0851 ± 0.0178 | 12 |
| | CLO | Yes | 0.0807 ± 0.0127 | 14 |
| | | No | 0.0792 ± 0.0160 | 22 |
| 30 °C | CONT | Yes | 0.0765 ± 0.0160 | 35 |
| | | No | 0.0747 ± 0.0167 | 12 |
| | THX | Yes | 0.0847 ± 0.0119 | 12 |
| | | No | 0.0732 ± 0.0139 | 11 |
| | IMI | Yes | 0.0777 ± 0.0192 | 7 |
| | | No | 0.0843 ± 0.0174 | 11 |
| | CLO | Yes | 0.0795 ± 0.0155 | 10 |
| | | No | 0.0894 ± 0.0187 | 16 |
| 35 °C | CONT | Yes | 0.0755 ± 0.0186 | 27 |
| | | No | 0.0649 ± 0.0152 | 9 |
| | THX | Yes | 0.0859 ± 0.0138 | 13 |

| | | | | | |
|---|---|---|---|---|---|
| | No | 0.0867 | ± | 0.0124 | 7 |
| IMI | Yes | 0.0840 | ± | 0.0132 | 11 |
| | No | 0.0772 | ± | 0.0142 | 23 |
| CLO | Yes | 0.0901 | ± | 0.0157 | 16 |
| | No | 0.0796 | ± | 0.0184 | 15 |

**Supplementary Table 3.3:** Average honey bee (*Apis mellifera scutellata*) flight speed (m.s$^{-1}$) and average flight distance (m) of all bees sampled per treatment at each temperature. Honey bees were exposed to either the control (CONT), thiamethoxam (THX), clothianidin (CLO) or imidacloprid (IMI) dosed sucrose diet. Flight mill runs were conducted at 25 °C, 30 °C and 35 °C respectively for each treatment.

| Temperature | Treatment | Average flight speed (m.s$^{-1}$) (±SD) | Average flight distance (m) (±SD) | Average number of flights (±SD) |
|---|---|---|---|---|
| 25 °C | CONT | 0,952 ± 0,494 | 161,772 ± 267,503 | 8,222 ± 8,596 |
| | THX | 1,020 ± 0,432 | 488,126 ± 1325,192 | 16,058 ± 12,831 |
| | IMI | 0,8179 ± 0,226 | 117,602 ± 125,761 | 19 ± 24,952 |
| | CLO | 2,446 ± 2,028 | 417,919 ± 755,143 | 11,125 ± 5,509 |
| 30 °C | CONT | 1,439 ± 1,437 | 271,809 ± 662,421 | 18 ± 23,871 |
| | THX | 1,167 ± 0,513 | 93,824 ± 82,705 | 13,5 ± 5,315 |
| | IMI | 1,015 ± 0,092 | 105,405 ± 77,305 | 9,666 ± 11,556 |
| | CLO | 1,275 ± 0,658 | 223,271 ± 180,424 | 36,3 ± 26,294 |

| | | | | |
|---|---|---|---|---|
| 35 °C | CONT | 1,527 ± 1,188 | 253,931 ± 587,868 | 10,275 ± 12,987 |
| | THX | 1,400 ± 1,263 | 923,140 ± 1564,231 | 9,363 ± 8,326 |
| | IMI | 1,136 ± 0,884 | 2056,725 ± 2052,327 | 3 ± 2 |
| | CLO | 0,978 ± 0,3891 | 216,577 ± 546,294 | 14 ± 15,451 |

**Supplementary Table 3.4:** Chi-squared analysis of the effects of neonicotinoid treatments (treat), temperature (temp) and their interacting effects (temp:treat) for the generalised linear model for honey bee (*Apis mellifera scutellata*) flight speed.

| | **LR Chisq** | **Df** | **Pr(>Chisq)** |
|---|---|---|---|
| ***treat*** | 5.1897 | 3 | 0.15842 |
| ***temp*** | 1.1030 | 1 | 0.29360 |
| ***treat:temp*** | 10.4586 | 3 | 0.15040 |

## CHAPTER 4

# To determine if sublethal pesticide levels affect *Apis mellifera scutellata* colony's ability to maintain a constant internal temperature at elevated ambient temperature

## ABSTRACT

Fluctuations in global climate conditions and increasing frequency and duration of extreme weather events means many organisms, including honey bees, are at risk of having their physiological boundaries more frequently tested. Honey bee sociality allows them to collectively monitor and control their hive's internal environment, largely independent of external conditions. This buffers individuals from stressful conditions. Neonicotinoid insecticide use has many negative effects on various aspects of honey bee behaviour and physiology, most notably that of thermoregulation. In this study, internal hive temperature was used as a proxy measure for internal colony thermoregulation under both controlled and thiamethoxam treated experimental conditions. Internal hive temperature was recorded using a custom built, in-hive monitoring system over the course of three months. Overall, experimental colonies were found to be fairly resilient to external temperature changes. These hives also maintained brood temperatures well following neonicotinoid exposure. However,

outlying areas within the colonies varied more unpredictably following exposure. These outlying areas include pollen stores, honey and nectar frames and unutilized comb.

## INTRODUCTION

Challenging thermal conditions influence the survival and longevity of multiple species (Mołoń *et al.* 2020). With fluctuations in climatic conditions and increasing frequency and duration of extreme weather events, many organisms face the prospect of their physiological boundaries being more frequently tested (Hoffmann *et al.* 2003; Kellermann *et al.* 2009; Chown *et al.* 2010; Fuller *et al.* 2010). Ectothermic organisms which are less able to control their internal body temperature, including insects, are likely to be more affected by changing climatic conditions (Kingsolver *et al.* 2013; Paaijmans *et al.* 2013; Bordier *et al.* 2017). Climate change is a concerning contributor to pollinator decline, including honey bee loss, and a key factor in the loss of synchronization between pollinator activity and flowering (Le Conte & Navajas 2008; Hegland *et al.* 2009; Lever *et al.* 2014). Important in-hive tasks rely on the collective efforts of the colony as well as basic physiological functions, most notably thermoregulation (Tosi *et al.* 2016).

Honey bee sociality allows them to collectively monitor and control the hive’s internal environment, largely independent of external conditions. The social aspect of honey bee existence has the benefit of largely buffering individuals from the impacts of several stressful developmental conditions (Rueppell *et al.* 2017).

Ideal internal hive conditions are of vital importance to ensure optimal colony function, although not all areas of the hive must be as precisely maintained. Precise temperatures are not as important for pollen and honey stores (Fahrenholz *et al.* 1989). Temperature control within the brood comb is more closely maintained, although there is variation here too. Development of larvae, and the survival and longevity of emerged, adult bees is influenced in large part by internal hive temperature and humidity conditions (Himmer 1927; Vandame &

Belzunces 1998; Ellis *et al.* 2008; Wu *et al.* 2011; Li *et al.* 2016). Larvae are less sensitive to temperature fluctuations than pupae (Jones *et al.* 2005). Brood temperature is maintained between approximately 32 °C and 36 °C (32 °-36 °C, Seeley & Heinrich 1981; 33 °C-36 °C, Kleinhenz *et al*. 2003, Petz *et al*. 2004, Basile *et al*. 2008;), with an optimal temperature range of 34.5 °C±1.5 °C (Kronenberg & Heller 1982; Bujok *et al*. 2002; Kleinhenz *et al*. 2003; Jones *et al*. 2004; Jones *et al.* 2005; Tautz 2008). Conditions below 32 °C cause compromised immunity and reduced adult foraging capability (Winston 1987; Tautz *et al*. 2003; Groh *et al.* 2004; Jones *et al.* 2005). Above 36 °C, there is a proliferation of brood mortality, delayed development, and various physical malformations (Fukuda & Sakagami 1968; Winston 1987; Groh *et al.* 2004).

There are many aspects of the internal environmental regulation of honey bee hives (Southwick & Moritz 1987; Jones *et al.* 2004; Bonoan *et al.* 2014). Both individual and colony level thermoregulation factors crucially into basic honey bee functioning. The survival and the maintenance of a constant internal hive environment involves multi-level thermoregulation, both physiological and behavioural. Generating heat through the isometric contraction of thoracic muscles (Heinrich 1980, 1985; Bujok *et al.* 2002; Kleinhenz *et al.* 2003) functions either to raise overall brood comb temperature (Heinrich 1980; Heinrich 1985) or to warm a localized area, i.e., generate heat in an empty brood cell to warm adjacent brood (Bujok *et al.* 2002; Kleinhenz *et al.* 2003).

Decreasing the hive temperatures during hot conditions can be achieved either on a broad scale; through wing fanning or spreading water to stimulate evaporative cooling (Heinrich 1979; Heinrich 1980; Heinrich 1985; Prange 1996; Jones & Oldroyd 2006) or on a smaller scale through heat shielding, whereby bees place themselves between the brood comb and the heat source to act as a shield (Starks & Gilley 1999; Siegel *et al.* 2005; Starks *et al.* 2005).

Choice of hive location is also critical as a sufficiently buffered microclimate can go a long way to ensuring consistent internal hive conditions independent of external fluctuations. Stingless bees, for example, do not make use of water collection and evaporative cooling to modulate hive environment as honey bees do, they rely instead on the insulating properties of the wood and shade of the trees in which they nest (Ramli *et al.* 2017).

The question of how temperature fluctuation affects honey bees is one that should be evaluated at both the singular and collective level. In a study simulating heatwaves under controlled conditions, hybrid European honey bee colonies (*Apis mellifera ligustica* and *Apis mellifera mellifera*) were found to be impressively resilient to the effects of heat waves (Bordier *et al.* 2017). No negative effects at the ind ividual level were observed, the brood environment was well maintained at optimal levels, and pollen and nectar foraging were unaffected (Bordier *et al.* 2017). The number of water foragers however doubled during the simulated heatwave (Bordier *et al.* 2017), suggesting that these honey bees can withstand heat fluctuations with minimal impact.

Termperature resilience could, however, be negatively impacted by insecticide exposure. Insecticide use has been suggested to have a host of negative effects on a wide range of beneficial arthropods over and above their intended target organisms and evidence exists for their negative impacts on insect neurophysiology, biochemistry, development, adult longevity, immunology, fecundity and sex ratios (Desneux *et al.* 2007). For a social insect such as the honey bee, individual effects of sublethal insecticides doses have the potential to have more far-reaching, cumulative effects at the colony level (Rumkee *et al.* 2017). Colony losses worldwide are of increasing concern (Moritz *et al.* 2010; vanEngelsdorp & Meixner 2010). Survey efforts across the globe in recent years have recorded a range of honey bee colony losses (Aston 2010; Brodschneider *et al.* 2010; van der Zee *et al.* 2012; Pirk *et al.* 2014; van der Zee *et al.* 2012; Brodschneider *et al.* 2016; Brodschneider *et al.* 2018). Colony loss is not attributed to any singular force, but a combination of numerous factors (Vanbergen *et al.* 2013) with the role of insecticide use, specifically neonicotinoids, becoming of increasing importance in terms of honey bee colony health (Godfray *et al.* 2014).

Chronic field exposure to thiacloprid, a cyano-substituted neonicotinoid, showed accumulated residues in both foragers as well as other hive members over time. Foraging behaviour, homing success, navigation performance, and social communication were all impaired (Tison *et al.* 2016).

Neonicotinoid exposure is a multifaceted situation. Not only are individual bees exposed to neonicotinoids in the field (Krupke & Long 2015), and susceptible to multiple routes of exposure including oral, inhalation and direct contact, but they are also exposed within the hive as well (Chauzat *et al.* 2006; Smodiš Škerl *et al.* 2009; Mullin *et al.* 2010; Lambert *et al.* 2013; Amulen *et al.* 2017; Rumkee *et al.* 2017). Traces of a multitude of pesticides have been found in pollen (Bonmatin *et al.* 2001; Bonmatin *et al.* 2002; Schmuck *et al.* 2001; Taiwan, Nai *et al.* 2017; Zioga *et al.* 2020), bee bread (Meikle *et al.* 2017), honey (Columbia, López *et al.* 2014; Egypt, Shendy *et al.* 2016; Ghana, Darko *et al.* 2017; Estonia, Karise *et al.* 2017; Pakistan, Farooqi *et al.* 2017), nectar (Lord *et al.* 1968; Schmuck *et al.* 2001; Zioga *et al.* 2020), propolis (Spain and Chile, González-Martin *et al.* 2017) and beeswax (Uganda, Amulen *et al.* 2017; Spain, Calatayud-Vernich *et al.* 2017; Poland, Pohorecka *et al.* 2017).

The resilience of honey bee colonies to fluctuating external conditions therefore has the potential to be altered by the influence of sublethal insecticide exposure. Limited investigation has been done, primarily on European species, and the effects of field-realistic, sublethal insecticide doses are often not taken into account. This study aims to combine these effects to ascertain if there are any effects on colony-level thermoregulation, focusing on the South African honey bee species, *Apis mellifera scutellata*. This subspecies occurs across a large range of environments and altitudes; from the densely vegetated Lowveld, to the high altitudes of the Highveld, and from the semi-arid desert regions of the Kalahari, to humid regions of KwaZulu Natal.

Of the three major neonicotinoids investigated throughout this project in Chapter 2 (Bester *et al.* 2022) and Chapter 3 (Bester *et al.* unpublished), thiamethoxam was identified as the pesticide to focus on in this study. Thiamethoxam is widely used, a known agonist of the nicotinic acetylcholine receptors, and responsible for a range of sublethal effects in honey bees (Maienfisch *et al.* 2001; Tosi *et al.* 2016). Thiamethoxam is utilised as a very successful measure of control against a wide range of commercially relevant agricultural pests, including aphids, jassids, whiteflies, thrips, rice hoppers, Colorado potato beetles, flea beetles, wireworms and several Lepidopteran species (Maienfisch *et al.* 2001). It was developed for both seed treatment as well as foliar and soil applications (Maienfisch *et al.* 2001). It is used to treat crops such as maize, cereals, cotton, sugarbeet, canola and rape seed. While it is not considered toxic to several non-target organisms, it should be considered toxic to bees (Maienfisch *et al.* 2001). Thiamethoxam is considered a second-generation neonicotinoid, first made available in 2000 (Kurwadkar & Evans 2016). Uniquely, one of thiamethoxam's major metabolic by-products is clothianidin (Simon Delso *et al.* 2015). Insects are therefore not only exposed to its primary effects, but after metabolism, they are also exposed to the subsequent effects of another neonicotinoid (Tosi *et al.* 2017). Not only is there an increase in potency and persistence from first generation (e.g., imidacloprid and clothianidin) to second generation neonicotinoids, but solubility of these insecticides in water increases by several orders of magnitude from the first to second generation (e.g., imidacloprid 610mg/L versus thiamethoxam 4100 mg/L) (PPDB 2012). Thiamethoxam intoxication has been shown to reduce foraging success and bring about homing failure (Henry *et al.* 2012) as well has eliciting a depressive long-term effect on various aspects of honey bee flight including distance, duration and velocity (Tosi *et al.* 2017). These impaired aspects of foraging (for both water and food) and increased loss of foragers in the field (Henry *et al.* 2012) contribute to lowered colony health and resource availability, as well as water foraging which is crucial for evaporative cooling and humidity regulation (Southwick & Heldmaier 1987; Le Conte & Navajas 2008). Within 24 hours after thiamethoxam exposure, the overall thorax temperature in *A.m. scutellata* decreases under both high and low ambient temperature conditions (Tosi *et al.* 2016).

The reduced honey bee flying capacity (Tosi *et al.* 2017) suggests reduced neurological and physiological capabilities, lowered flight muscle function and by extension, the potential impairment of other wing muscle related activities. Principal among these flight muscle impairments is heat generation (Tosi *et al.* 2016) and wing assisted hive ventilation (Hess 1926, Hazelhoff 1941, Southwick & Heldmaier 1987; Southwick & Moritz 1987; Akinwande 2016; Peters *et al.* 2019). The interaction between increasing frequency and intensities of hot weather events and wing muscle function could influence the resilience of honey bee colonies to fluctuating external conditions. In particular, when they are exposed to neonicotinoid pesticides. It is therefore important to determine the thermoregulation capabilities of honey bee colonies exposed to both temperature changes and the neonicotinoid pesticides. It is predicted that exposure to noenicotinoid pesticides will negatively affect the colony’s ability to regulate the internal hive temperatures.

## METHODS

The interacting effects of neonicotinoids and ambient temperature on internal colony thermoregulatory capacity was conducted on South African honey bees, *A.m. scutellata*, using 5 colonies at the Social Insect Research Group (SIRG) apiary located at the University of Pretoria’s Experimental Farm in Hatfield, Pretoria, South Africa. The experimental colonies selected for this study were all standard Langstroth ten frame wooden hives (50.48 cm length x 40.64 cm width) with established colonies and none of the hives had additional supers. For the purposes of my investigation, internal colony temperature was used as a proxy measure for internal colony thermoregulation under both control and thiamethoxam treated experimental conditions. Experimental colonies were placed together in the most exposed of the experimental apiary sites with sufficient sunlight during the day. The experimental site received direct sunlight from mid-morning until late afternoon and all three colonies received more direct sunlight on the side facing the afternoon sun.

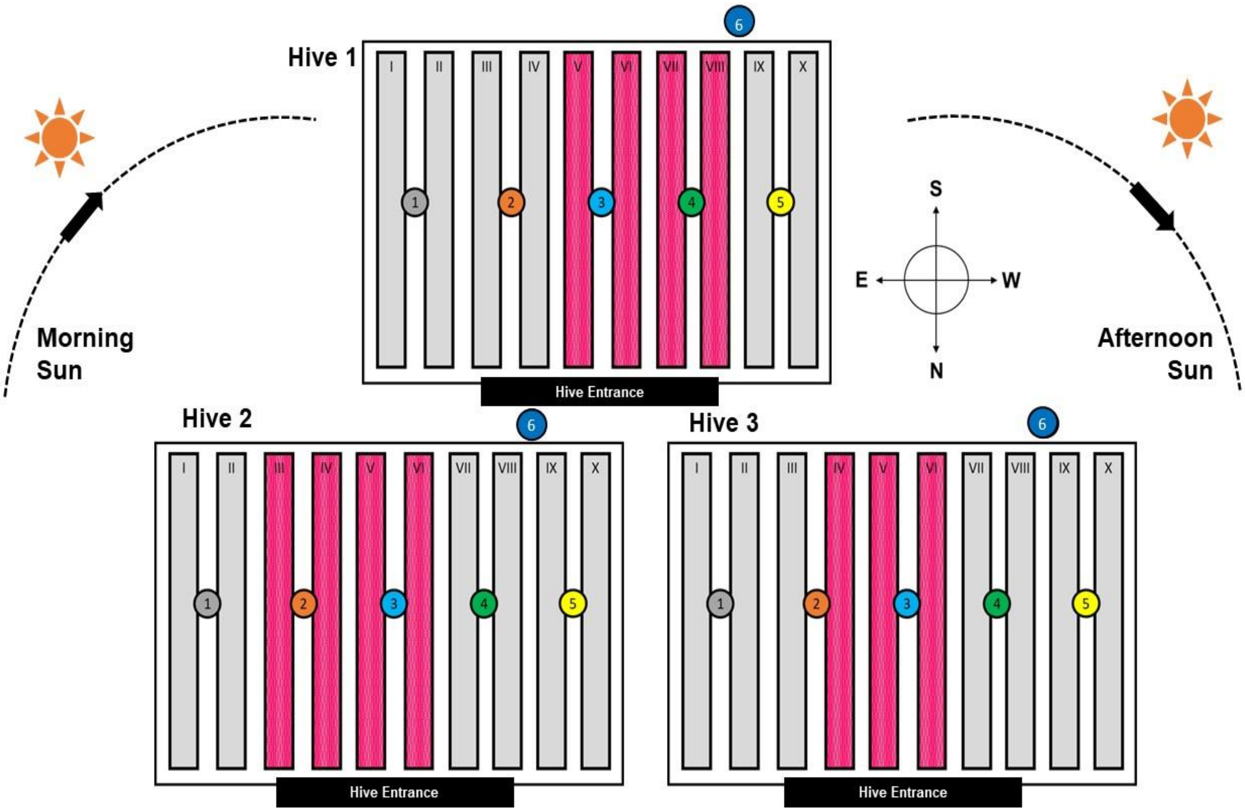

**Figure 4.1:** Internal layout of experimental Hive 1, Hive 2 and Hive 3. Compass denotes direction with West-facing sides of all three hives receiving the majority of the sunlight during the day. Each hive consisted of 10 standard frames, labelled (I) to (X) from left to right in the diagram. Six temperature sensors are indicated, sensor number (1) to (5) distributed across the middle of the hive and sensor (6) on the shaded south side of each hive to record ambient temperature profiles. The orientation of the hive entrances as well as the morning and afternoon sun trajectory are also indicated.

### *Hive 1*

A healthy hive in a sunny area, which experienced slightly dappled shade towards the end of the day. Frames were well populated with both brood and food stores and the hive was a moderate size. Brood frames were located in position V, VI, VII and VIII (Fig. 4.1).

### *Hive 2*

A healthy but slightly smaller hive, comparatively the least populated of the three although still of healthy size, with noticeable space between the frames. Hive two was situated in a spot with slight dappled shade in the morning and more direct sunlight in the afternoon. Brood frames were located in position III, IV, V and VI (Fig. 4.1).

### *Hive 3*

A healthy hive, comparatively the largest of the three. This was the most populated hive with very tightly packed frames and some additional building between the frames and on the hive lid. This hive was in the sunniest position of the three hives, with sun exposure beginning slightly earlier in the morning and terminating slightly earlier in the afternoon compared to the other two hives. Brood frames were located in position IV, V and VI (Fig. 4.1).

### *Temperature Monitoring System*

Internal hive temperature fluctuations were monitored using a custom-designed thermal sensing system. This system was designed and built by Tignique® (quialified mechanical engineer) using industry-grade materials capable of withstanding weather and temperature changes (see Figure 4.2). The system includes six temperature sensors and is powered by an integrated solar panel and battery management system. All data recorded by the device is stored on an SD card inserted into the machine, which is then removed and the data is downloaded and transferrable directly into MS Excel for data analysis. The temperature monitoring system was housed in a water-tight casing and placed inside an empty super on top of the inhabited hive. Instead of using a queen excluder, a thin wooden board was placed between the hive box and the empty super. Five of the temperature sensors were fed through individual holes into the cavity below to record internal hive temperatures (Figure 4.2). The sixth sensor was positioned under the back edge of the hive lid, out of direct sunlight, to record environmental temperature. Temperature readings were taken every five seconds. Temperature sensors were placed between every second frame, across the middle of the hive, equidistant from the top and bottom of the hive. In the event of prolonged rainy or overcast weather that prevented the battery from charging, the battery was connected to an external, portable power bank to allow supplementary charging while remaining in the hive.

The temperature sensors were calibrated to within a 0.25 °C of accuracy (Tignique®). Sensors were re-calibrated before each hive experiment. All six sensors were secured in a single block of aluminium, each in an individual hole. As aluminium is an excellent heat conductor, this setup ensured that all six sensors were exposed to exactly the same ambient temperature alongside a laboratory grade thermometer. Calibrations were done for both low and high extremes with temperatures as low as freezing point and as high as boiling point. A range of moderate temperatures in between these points were also calibrate for (The Cave Pearl Project, 2020).

**Figure 4.2.** Custom-designed and built thermal sensing system (Tignique®). System includes six temperature sensors, with all data recorded in real time and stored on an SD card for download.

### *Pesticide Exposure*

Data collection was done in the form of a cohort within each individual hive. Data for each hive during its control period (2 weeks) and then the THX period (24 hour exposure) thereafter was separated by 3 days. The thermal sensing system remained in place throughout the data collection period. All data collected from one hive by the thermal sensing system was downloaded at the end of the data collection period for that hive. Hives were monitored one at a time, and at the end of the data collection period 3 complete sets of data for control and THX exposure for each of the 3 hives was available for analysis.

A single, outer frame was removed and replaced with an internal feeder frame. For the control, a feeder frame was filled with 2 litres of sugar water solution (1:1 w/w sucrose and water). For the experiment, the 2 litres of sugar solution were dosed with a sublethal dose of THX to produce a solution with a final concentration of 5 nM. These doses are well below the LD50 = 4-5 ng/bee threshold (Godfray *et al.* 2014; Henry *et al.* 2104; Oliveira *et al.* 2014; Démares *et al.* 2016).

The colony was allowed to consume the sugar water in the feeding frame overnight. The feeding frame was removed the following day and replaced with the original frame. Initial trials indicated that temperatures in the vicinity of the feeding frame did not change between the replacement of a normal frame with the sugar water filled feeding frame and therefore would not influence temperature readings by the thermal sensor. Changes would only be due to pesticide introduction, if any.

### *Statistical Analysis*

Given the many confounding factors (differing colony densities over time, time of day, and season) that influence the results of the hive thermoregulation, we opted for a simple descriptive statistical approach to compare control and experimental time-series data.

For each of the three hives and each of the six sensors, the control and experiment temperature time-series data were aligned for the number of days since the start of the experiment and the time of the day. The first day of the experiment was designated 0 days since start of the experiment, increasing by 1 every day for the duration of the recordings. Time of day in reality was aligned to the closest similar time of the day in the recording, ensuring that aligned actual and recorded times differed by less than 10 seconds.

For some of the experiments and controls, some of the readings towards the end of the recording period were inadvertently dropped, but this was done for two reasons: 1) to ensure that if the introduction of the temperature sensors affected hive behaviour and subsequent temperatures, that it would be the same for both (experiment and control), and 2) to ensure that the time of day for both sets of data to be compared lined up with external ambient temperature fluctuations and to ensure daily behavioural cycles of the colony were comparable.

Once these were lined up, the temperature difference between control and experiment was calculated for each hive and each sensor ($T_{difference} = T_{control} – T_{experiment}$). This meant that a positive temperature difference indicated temperatures recorded during the control were higher, and a negative temperature difference indicated that temperature readings recorded during the experiment were higher. These new temperature differences were plotted and the mean and standard deviation calculated per hive and per sensor. Biologically, only temperature differences larger than 2 °C-3 °C were considered to have a meaningful impact on the hive (Seeley & Heinrich 1981; Kronenberg & Heller 1982; Bujok *et al*. 2002; Kleinhenz *et al*. 2003; Jones *et al*. 2004; Petz *et al*. 2004; Jones *et al.* 2005; Basile *et al*. 2008; Tautz 2008).

## RESULTS

***Hive 1*** - Temperatures were fairly well regulated across time, with more tightly regulated temperatures recorded by sensor 3, 4 and 5, located within or in close proximity to the brood frames (V, VI, VII, VIII). Temperatures recorded by sensor 1 and 2 were slightly less tightly controlled and followed the external temperature fluctuations, but all still within the ±35 °C range (Fig. 4.2). Temperature regulation across all five hive sensors slowly began to fluctuate more noticeably across the week following the THX exposure. The hive continued to maintain temperatures in the brood area (sensors 3 and 4) most reliably, still within the ±35 °C range (Fig. 4.2). In the area outside the brood area, temperatures oscillated more with daily fluctuations (sensor 1, 2 and 5), dropping down to the ±30 °C range (sensor 1 and 2) with some fluctuations dropping to ±25 °C (sensor 5) (Fig. 4.2).

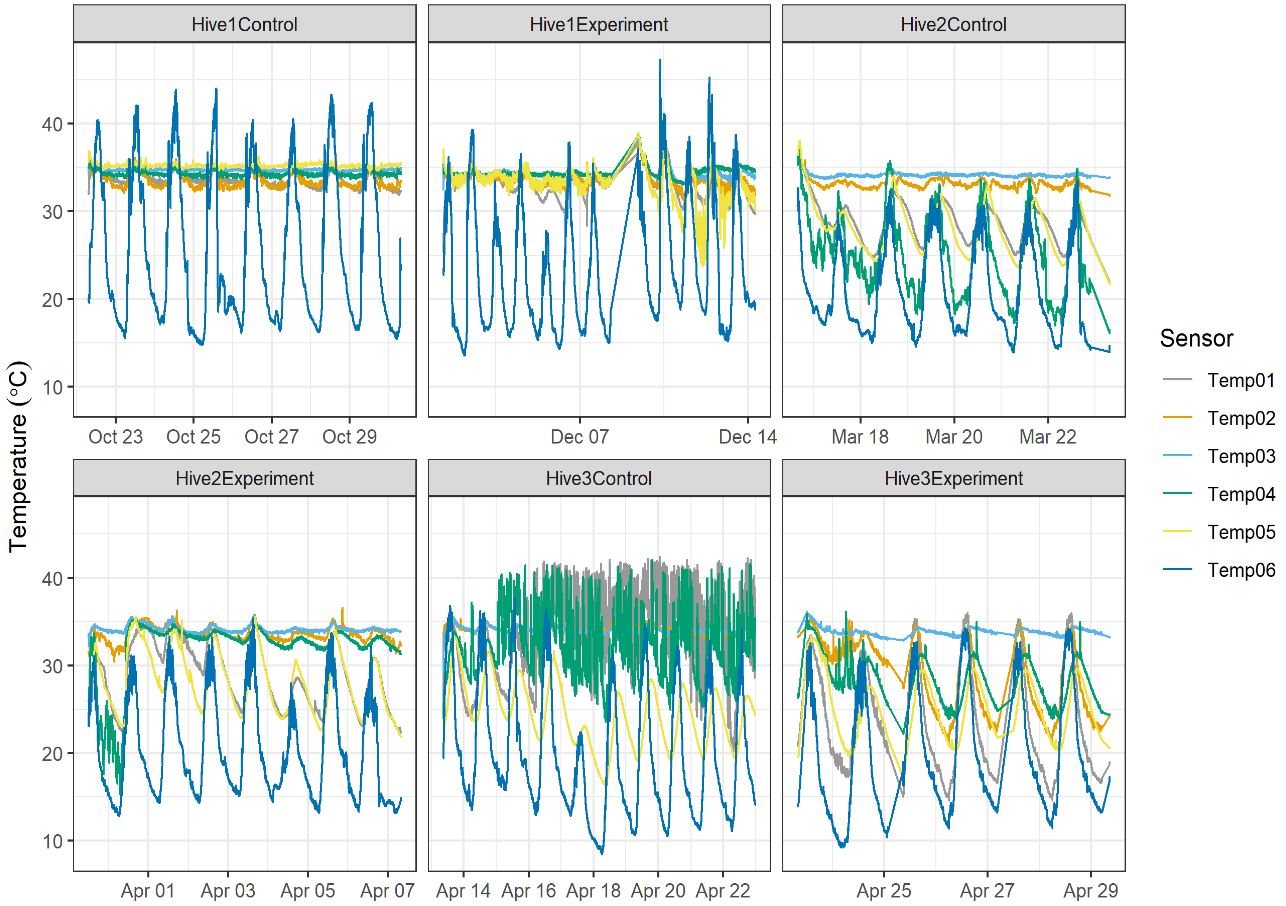

**Figure 4.3:** Time series plots indicating the six temperature sensor readings for each of the three experimental hives (Hive 1, Hive 2 and Hive 3) over time. The first five temperature sensor profiles in each graph (Temp01 – Temp05) indicate in-hive temperature readings and the sixth sensor profile indicates external ambient temperature (Temp06).

When comparing mean temperatures (recorded for both control and treatment conditions) for each of the five in-hive temperature sensors, they exhibit similar temperature readings with noticeable differences exhibited following the THX treatment (Fig. 4.3). When evaluating temperature differences between control and experiment, the majority of the sensors exhibited a positive difference, indicating a drop in temperatures from the control to the experiment (Fig. 4.4, Fig. 4.5). The temperature differences were minor and well below the 3 °C threshold of biological significance (Fig. 4.5).

***Hive 2*** - Under control conditions, temperatures were fairly well regulated across time in the brood area (III, IV, V, VI), with more tightly regulated temperatures recorded by sensor 2 and 3 within the ±35 °C range (Fig. 4.2). Temperatures recorded by sensor 1 and 5 fluctuated noticeably more and followed the external temperature fluctuation trend, dropping down to the ±30 °C range, with sensor 4 dropping to ±25 °C.

Following the THX exposure, temperature regulation across all five hive sensors began to fluctuate more noticeably across as the week progressed. The hive continued to maintain temperatures in the brood and adjacent area (sensors 2, 3 and 4) most reliably, still within the ±35 °C range but oscillating more in line with external temperature fluctuation trends. In the area outside the brood area, the temperatures fluctuated more noticeably along with daily temperature fluctuations (sensor 1 and 5), dropping down to the ±30 °C range (sensor 1 and 2).

When comparing mean temperatures (recorded for both control and treatment conditions) for each of the five in-hive temperature sensors, the brood area remains tightly controlled with a slight increase in variation following treatment (Fig. 4.3). Temperatures were under 30 °C in the outermost areas of either side of the hive, also with an increase in the variation following the treatment (Fig. 4.3). Sensor 4 exhibited a noticeable increase in the average temperature following THX treatment (Fig. 4.3). When evaluating temperature differences between control and experiment, the majority of the sensors exhibited a negative difference, indicating a rise in temperatures from the control to the experiment (Fig. 4.4, Fig. 4.5). The temperature differences were minor in the brood frame area (Temp02 and Temp03) but more noticeable in the other sensors (Temp01, Temp04, Temp05) which met the 3 °C threshold of biological significance (Fig. 4.5).

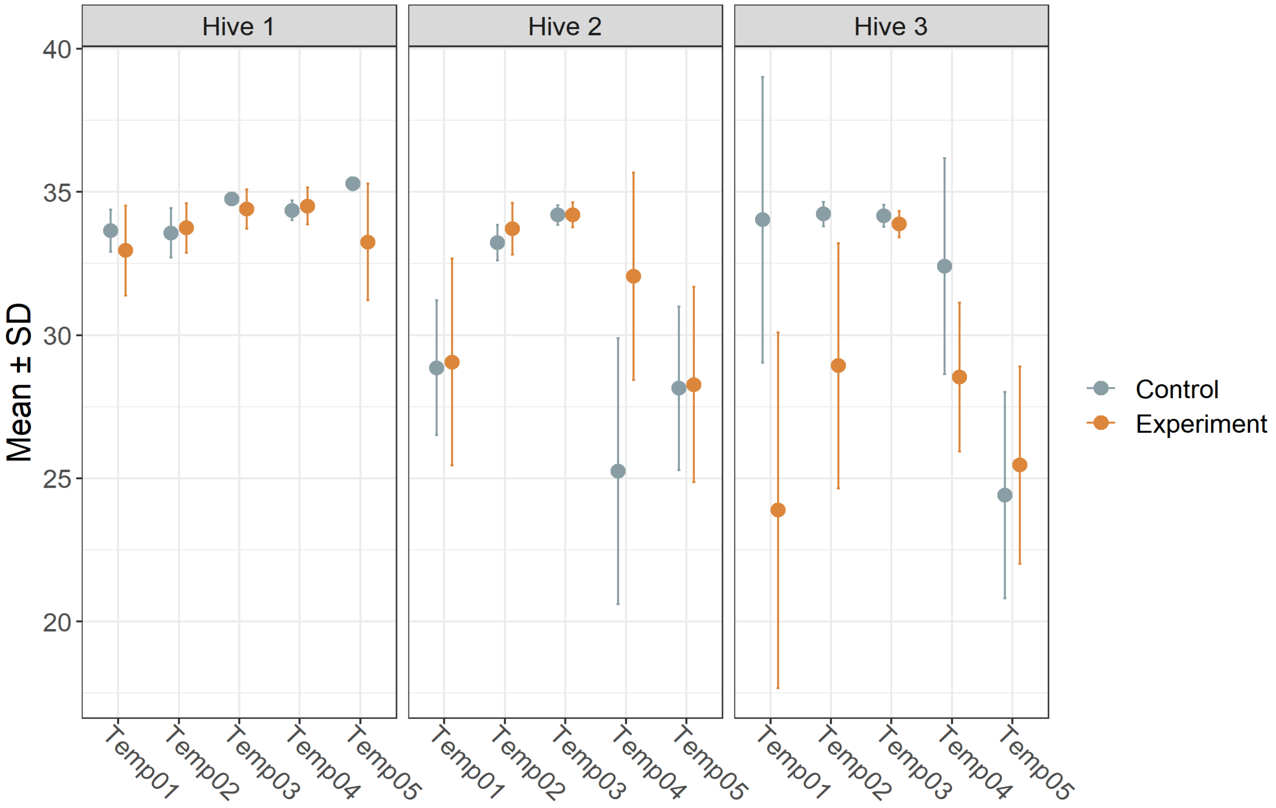

**Figure 4.4:** Box plot indicating the mean and standard deviation of temperatures recorded by each of the five in-hive sensors (Temp01 – Temp05) for each of the three experimental hives (Hive 1, Hive 2 and Hive 3). The sensors located in the brood area were Temp03 and Temp04 (Hive 1), Temp02 and Temp03 (Hive 2), and Temp02 and Temp03 (Hive 3) respectively.

***Hive 3*** - Under control conditions, temperatures were fairly well regulated across time in the brood area (IV, V, VI), with more tightly regulated temperatures recorded by sensor 2 and 3 within the ±35 °C range. Temperatures recorded by sensor 1 and 4 fluctuated noticeably and far more frequently during the day, independent of the daily external temperature fluctuation trend, fluctuating between ±25 °C and ±40 °C range. Temperatures recorded by sensor 5 were poorly regulated and followed the external ambient temperature fluctuation trend.

Following the THX exposure, temperature regulation within the ±35 °C range was recorded only by sensor 3 in the centre of the brood area (Fig. 4.2). On either side of the brood area temperatures fluctuated more noticeably (sensors 2 and 4) with the most fluctuating temperature profiles recorded by the outermost sensors (sensors 1 and 5), dropping down as low as the ±15 °C range.

When comparing mean temperatures recorded for both control and treatment conditions for each of the five in-hive temperature sensors, the brood area remains tightly controlled, with noticeable variation in other parts of the hive (Fig. 4.3). For four of the five sensors, average temperatures dropped following treatment (Fig. 4.3). When evaluating temperature differences between control and experiment, the majority of the sensors exhibited a positive difference, indicating an overall drop in temperatures from the control to the experiment (Fig. 4.4, Fig. 4.5).

The temperature differences were the greatest of the three hives, with a small change recorded by the sensor central to the brood frame (Temp03) but far greater changes in the other sensors (Temp01, Temp02, Temp04, Temp05) which met the 3 °C threshold of biological significance (Fig. 4.5).

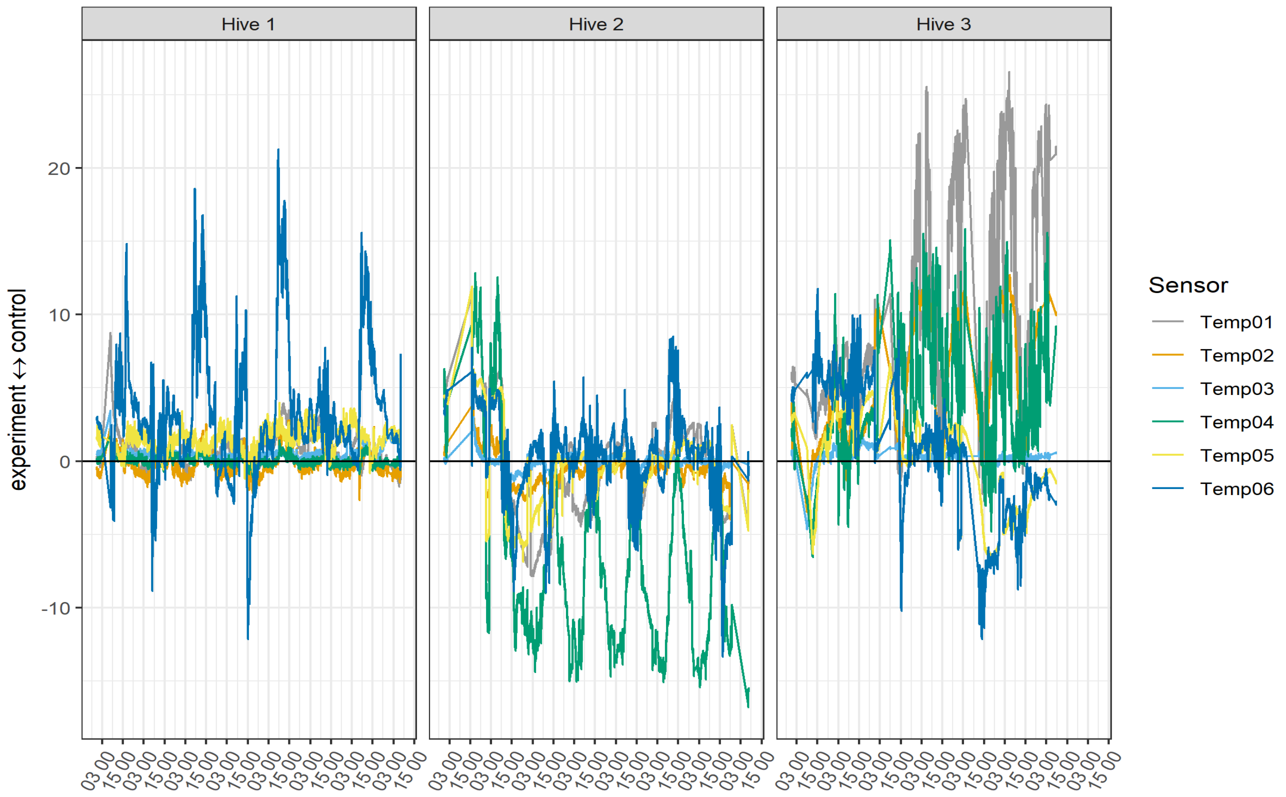

**Figure 4.5:** Temperature differences* between the control and experiment were calculated for each hive (Hive 1, Hive 2 and Hive 3) and each sensor (Temp01 – Temp06). Positive temperature differences indicate that control temperatures were higher; negative temperature differences indicate that experiment temperatures were higher.

*($T_{difference} = T_{control} - T_{experiment}$)

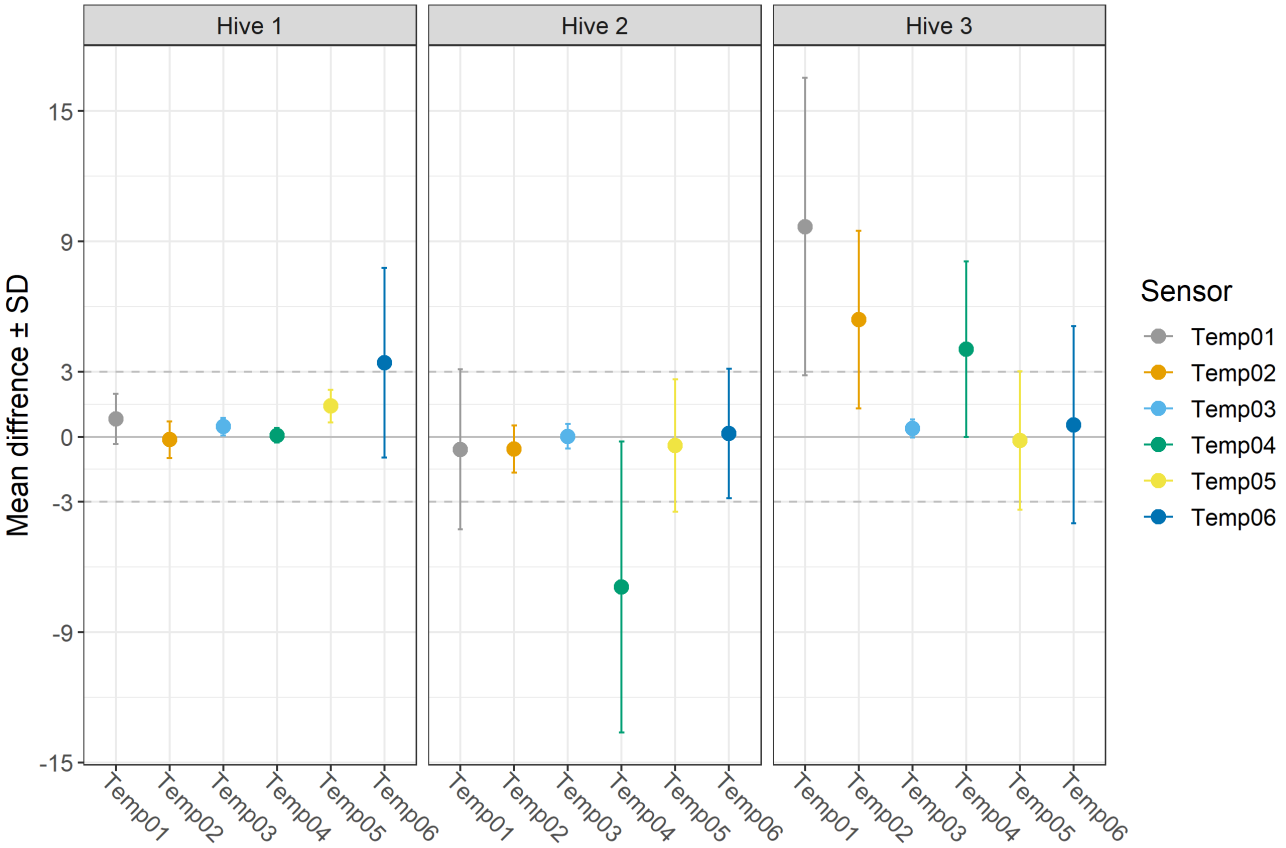

**Figure 4.6:** Temperature differences between the control and experiment calculated for each hive (Hive 1, Hive 2 and Hive 3) and each sensor (Temp01 – Temp06). These new temperature differences were plotted and the mean temperature difference and standard deviation were calculated per sensor (Temp01 - Temp06) for each of the three hives (Hive 1, Hive 2 and Hive 3). *($T_{difference} = T_{control} – T_{experiment}$)

## DISCUSSION

The ability of *A.m. scutellata* to maintain a constant internal thermal hive environment was affected by chronic exposure to sublethal THX doses. The three hives included in the study were as similar as possible with respect to geographic location, sun and shade exposure, standard Langstroth hive design, no addition of a super, etc. Internally, the brood frame location did differ slightly from hive to hive, but the position of the in-hive sensors was standardized. While there were minor differences between the hives, making use of the same three hives throughout the study ensured that other factors (concentration and quality of available food sources, distance to the food source, localized microclimate conditions, etc.) that all play a role in influencing individual body temperature, and by extension have an effect on overall internal hive temperature, were standardized as far as possible.

The size of the colony appeared to have the most effect on how the colony responded to THX exposure. While all colonies maintained the temperature within their respective brood areas quite consistently, the temperature fluctuations in the outer areas of the hive fluctuated more following THX exposure in each case. The smallest of the three colonies exhibited increased internal hive temperatures following THX exposure, possibly due to the fact that the smaller number of bees available to regulate and dissipate heat (Jones *et al.* 2006). This led to a lowered ability to tolerate ambient heat on an individual and colony level, and heat regulating activities were further concentrated in the brood area leading to less temperature regulation and dissipation in the outer hive regions.

The largest and most densely populated of the three hives exhibited elevated internal temperatures in the outer hive areas prior to exposure to THX. This suggests that perhaps the densely populated interior of the hive inhibited sufficient heat regulation and dissipation under control conditions (Hess 1926, Hazelhoff 1941, Southwick & Heldmaier 1987; Southwick & Moritz 1987; Akinwande 2016; Peters *et al.* 2019). The brood area remained relatively well controlled before and after exposure, but the outer areas exhibited a noticeable drop in temperature under treated experimental conditions. This could be attributed to a number of factors, including the loss of a small percentage of the colony upon initial consumption of treated sugar water. The reduction in honey bee numbers could have facilitated improved ventilation and heat dissipation within the hive (Bonoan *et al.* 2014). The THX exposure may also have inhibited the honey bee's ability to generate and regulate heat effectively and as a result, less heat created in the hive meant a lowered internal hive temperature, especially outside the brood area. The third and most 'average' of the three experimental hives exhibited optimal brood conditions both before and after THX exposure, with slightly increased fluctuations in outer hive temperatures following treatment. As brood temperature is ideally maintained within the general range of approximately 32 °C to 36 °C (32 °C-36 °C, Seeley & Heinrich 1981; 33 °C-36 °C, Kleinhenz *et al*. 2003, Petz *et al*. 2004, Basile *et al*. 2008;), but ideally within the narrow, optimal temperature range of 34.5 °C±1.5 °C (Kronenberg & Heller 1982; Bujok *et al*. 2002; Kleinhenz *et al*. 2003; Jones *et al*. 2004; Jones *et al.* 2005; Tautz 2008), all three hives remained resilient in the face of sublethal THX exposure with respect to brood temperature regulation. This meant that despite THX exposure, colonies did well to prevent brood temperatures from falling below 32 °C (which can cause compromised immunity and reduced adult foraging capability (Winston 1987; Tautz *et al.* 2003; Groh *et al.* 2004; Jones *et al.* 2005)) as well as prevent brood temperatures from rising above 36 °C (conditions which facilitate the proliferation of brood mortality, delayed development, and physical malformations (Fukuda & Sakagami 1968; Winston 1987; Groh *et al.* 2004)).

Hybrid European honey bee colonies (*A.m. ligustica* and *A.m. mellifera*) exposed to simulated heatwaves under controlled conditions (although without the added factor of neonicotinoid exposure), exhibited similar resilience and minimal impact (Bordier *et al.* 2017).

The buffering effect of the internal microclimate meant that both individual and colony-level function remained unaffected, and the brood area as well as honey and pollen stores were well maintained (Bordier *et al.* 2017).

Current climatic conditions in many areas of the world have exceeded previous records in the last few years, further highlighting the impending threat of increased frequency and intensity of extreme weather events, particularly heat waves. In 2022, much of Europe experienced unprecedented maximum temperatures with many areas surpassing the highest recorded temperatures to date. One particularly concerning example is the future temperature predictions for the UK (originally forecast for 2050 if action was not taken to mitigate climate change) that have already been experienced this year, nearly 30 years ahead of schedule.

This study's findings suggest that hives are mostly resilient to the effects of elevated environmental temperatures, with the exposure to a neonicotinoid having limited influence on in-hive thermoregulation. However, with maximum temperatures continuing to climb in many areas, evaluating *A.m. scutellata* colonies under more extreme environmental conditions would be a prudent future extension to these current findings.

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# CHAPTER 5

# General conclusion

## SYNTHESIS

The importance of temperature as a crucial component in biodiversity, ecosystem and physiological function of organisms is well studied. Continued climate change and the increased frequency and intensity of extreme weather events (including changes in rainfall patterns, heat waves, etc.) continue to influence various aspects of thermoregulation in a variety of organisms. Changes to the seasonal timing of flowering plants as a result of changing temperatures are likely to impact pollinators. Honey bee physiology and behaviour centres crucially around temperature, both in terms of their individual and collective behaviour, as well as their physiology, biology and development.

Insecticides have proved important in the development of large-scale agriculture to increase crop yield and quality. While insecticides, specifically neonicotinoids, are highly effective against target organisms, they may influence various aspects of non-target organisms as well. Commercially utilized neonicotinoids, specifically clothianidin, imidacloprid and thiamethoxam have demonstrated an array of non-lethal effects on honey bee behaviour and physiology. Several aspects of *Apis mellifera scutellata* physiology were used to build on the existing evidence of sublethal neonicotinoid insecticide exposure on this southern African honey bee subspecies.

Honey bees, by no means the only pollinators of a number of agricultural crops and wild plants, are still important and responsible for the pollination of a very wide range of plants that we greatly rely on for food, clothing and livestock feed. Managed colonies are under threat worldwide and recently recorded hive losses, especially in South Africa, are quite concerning. Pollinator losses have negative ecological and economic effects, and do not only impact agricultural production and food security, but also wild plant diversity and by extension, healthy ecosystem function, which in turn affects human welfare. The reasons for global honey bee losses are complex and often interacting. Although parasites, diseases and loss of genetic diversity are important contributors to honey bee loss, environmental stressors, particularly insecticide use and climate change, are equally important.

The state of insecticide use on a global scale is well documented in some areas and very poorly investigated in others. Insecticide use, particularly use of the newer neonicotinoid family, has been well established in Europe and North America and has allowed for the development and implementation of legislation to ensure safe and responsible agricultural practices. However, the stark contrast between the agricultural landscape in these regions as opposed to that of Africa makes the extrapolation of, or the direct comparison to these laws impractical. The African agricultural landscape differs substantially from that of Europe and North America, with Africa relying heavily on subsistence and small-scale farming practices as opposed to the almost exclusively commercial setup in the Northern Hemisphere. I compiled an extensive literature review of all available research done on neonicotinoid use on the continent (Chapter 1), the bulk of which was included in a comprehensive evaluation of the impacts of neonicotinoids on agriculture and biodiversity in Africa by the Academy of Science South Africa (ASSAf 2019). This further highlighted the need for a continent and region-specific evaluation of the influence of neonicotinoids on resident pollinators.

Individual thermal tolerance and flight efficiency and colony-level thermal regulation were all used as a means of examining *A.m. scutellata* thermoregulation and its interacting effects with elevated ambient temperature and insecticide exposure.

Individual thermal tolerance was reduced following chronic exposure to the three neonicotinoids (Chapter 2). Not only was a baseline thermal tolerance with an $LT_{50}$ threshold of 53.77 °C established for this subspecies, but a reduced $LT_{50}$ for all three treatments of more than 3 °C lower than the baseline $LT_{50}$ was observed. The evidence that sublethal doses of three commercial neonicotinoids lower the already established $LT_{50}$ thermal tolerance threshold for *A.m. scutellata* by several degrees adds to the growing evidence of the negative effects these substances have on important pollinators. The substances also impact the bees' ability to tolerate changing ambient temperatures as well as perform vital behavioural and physiological tasks.

Various aspects of flight efficiency were not significantly impacted by exposure to the three neonicotinoids, however the honey bees' ability to initiate flight was noticeably affected. The majority of honey bees exposed to the insecticide treatments were unable to initiate flight at all (Chapter 3). Using flight success and associated flight parameters as a proxy for flight muscle function, my study highlighted potential neonicotinoid induced alterations to honey bee thermoregulation; both physiologically via thermogenesis and behaviourally via wing fanning and water foraging.

With respect to the in-hive thermoregulation, no significant differences were observed in the three hives between the control and the treatment temperature readings. However, there were differences in the way the hives responded to insecticide treatment based on the size of the colony (Chapter 4).

In general, we can conclude from this that individual thermoregulation in honey bees is more susceptible to sublethal neonicotinoid exposure, whereas the effects on the hive are less noticeable, most likely mitigated by the microclimate created by the colony and the colony's collective plasticity (Chapter 2, Chapter 3, Chapter 4).

The same experimental hives were used to explore individual thermal tolerance (Chapter 2), flight efficiency (Chapter 3) and colony thermoregulation (Chapter 4). The same experimental hives were used throughout the study, for all of the experiments, and while there were undoubtedly internal changes to the colony over time, we kept as many of the variables between repetitions and experiments as consistent as possible. Foraging quality of the surrounding environment, water, weather conditions, beekeeping activities, agricultural practices and the size, location and orientation of the hives were constant and comparable across all experiments. Chapter 4 raises some important points regarding the individual differences between hives in terms of their internal structure and the population size of said colony.

While not all these results may have been statistically significant, there are definite biological implications. A reduced individual honey bee thermal tolerance of 3 °C, a drastically reduced ability to initiate flight, and an altered thermoregulatory efficiency at colony level is biologically significant. These changes may translate to; a lowered tolerance to more frequent and intense heat waves, reduced pollen and nectar foraging capacity, the subsequent loss of nutritional diversity of the foraged pollen, the impaired ability to ventilate the hive through wing fanning, reduced capacity for water foraging and subsequent evaporative cooling, affected thoracic muscle heat generation capability and any number of other individual and colony level behavioural and physiological thermoregulatory activities. When considering these effects in conjunction with a South African agricultural landscape that encompasses small-scale and subsistence farming that utilises insecticides in a less regulated manner, the likelihood of honey bee exposure to these neonicotinoids becomes of even greater concern.

## FUTURE RESEARCH

Results of this research demonstrate the importance of both laboratory and field-based investigations and their complimentary contribution to improving our understanding of the interacting effects on colony loss. While the results of all three studies looking into the individual and colony-level effects of commercial neonicotinoids on thermoregulation yielded important results, local and global climate conditions and predictions continue to change. Recorded maximum temperatures during extreme heat events were higher in 2022 in many parts of the world than any of the preceding years of the study. The results of individual and colony-level experiments serve as a strong base from which future studies under more extreme weather conditions would yield valuable information on how the *A.m. scutellata* honey bee subspecies will continue to adapt and be affected by accelerating changes to global and local climatic conditions, especially in the context of an ever-changing South African agricultural landscape. Future research could be conducted over a longer study period, looking at all seasons, on multiple sites and across the extensive *A.m. scutellata* distribution range. Additionally, future investigations can be conducted on the efficacy of current honey bee thermoregulatory mechanisms in response to future extreme weather conditions over an extended period.

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The End

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