Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze e Tecnologie Agrarie, Ambientali e Alimentari

Ciclo XXVII

Settore Concorsuale di afferenza: 07/D1

Settore Scientifico disciplinare: AGR/11

SUBLETHAL EFFECTS OF A COMMON NEONICOTINOID PESTICIDE, THIAMETHOXAM, ON HONEY BEES: IMPACT ON LOCOMOTION AND THERMOREGULATION

Presentata da: Simone Tosi

Coordinatore Dottorato

Prof. Giovanni Dinelli

Relatore

Prof. Giovanni Burgio

Correlatori

Prof. James Charles Nieh
Dr. Piotr Medrzycki

Esame finale anno 2015

“If the world can no longer afford the luxury of natural beauty, then it will soon be overcome and destroyed by its own ugliness. I myself feel deeply that the fate of Man, and his dignity, are at stake whenever the earth’s natural splendors are threatened with extinction. (…) Let me tell you this, old friend: in an entirely man-made world, there can be no room for man either.”

Romain Gary
Letter to the Elephant
1967

“La curiosità e il fascino che le api da sempre esercitano in noi possono essere la porta d'accesso al regno della Natura a cui apparteniamo.”

Marina Gallandra
La società delle api:
immagini da un mondo straordinario
2012

SUMMARY

Abstract

Neonicotinoids have been pointed to as a factor responsible for the increased honey bee colony losses in the last decades. Many studies have investigated the effects of the first marketed neonicotinoid, imidacloprid, while fewer have focused on thiamethoxam. One recent study showed that sublethal doses of thiamethoxam lead to colony failure by decreasing forager homing flight success. We thus decided to investigate the mechanism which caused this phenomenon. Our hypothesis was that this effect was caused by impairment of forager locomotion abilities. Therefore we tested the effects of sublethal acute and chronic exposures to thiamethoxam on forager walking (Chapter 2) and flight (Chapter 3) performances. The acute treatment (1.34 ng/bee) affected walking locomotion firstly triggering hyperactivity (30 min post-treatment) and then impairing motor functioning (60 min post-treatment). 2-day continuous exposures to thiamethoxam (32.5, 45 ppb) elicited fewer effects on walking locomotion, however both exposure modes elicited an increased positive phototaxis. Similarly, in flight experiments, the single dose (1.34 ng/bee) elicited hyperactivity shortly after intoxication (increased flight duration and distance), while longer and continuous exposures (32.5, 45 ppb) impaired forager motor functions (decreased flight duration, distance, velocity). It is known that flight muscles temperature needs to be precisely regulated by bees during flight. Therefore, we further hypothesized that the impaired flight performances of neonicotinoid intoxicated bees were caused also by thermoregulation anomalies. We tested the effects that acute thiamethoxam exposures (0.2, 1, 2 ng/bee) elicit on forager thorax temperature (Chapter 4). Foragers treated with high doses exhibited hyperthermia or hypothermia when respectively exposed to high or low environmental temperatures.

In summary, we show that sublethal doses of thiamethoxam affected forager walking and flight locomotion, phototaxis and thermoregulation. We also display the intricate mode of action of thiamethoxam which triggered, at different extents, inverse sublethal effects in relation to time and dose.

CHAPTER 1

General introduction

1.1 The honey bee

The honey bee *Apis mellifera* L., 1758, belongs to the superfamily *Apoidea*, order Hymenoptera. In the world there are over 20,000 species of bees, distributed in nine families. Even if the family *Apidae* includes mainly solitary bees (such as *Osmia* and *Xylocopa* spp.), it is the only one characterized by the presence of species with a strong social structure, like bumblebee (*Bombus* spp.), stingless bees (*Melipona* and *Trigona* spp.) and honey bees (*Apis* spp.). In the genus *Apis*, in addition to the honey bee, there are eight other species, including *Apis cerana*, *Apis dorsata* and *Apis florea* in Southeast Asia. The honey bee was confined to Europe, Asia and Africa until the New World was discovered. It is currently distributed worldwide, except Antarctica and the Arctic region. Due to the extremely vast and different geo-climatic regions in which honey bee are distributed, numerous subspecies evolved, with different morphological, physiological and behavioural characteristics related to adaptations to the area of origin (Ruttner, 1988).

Honey bee colonies can live in natural cavities (e.g. trees) or containers supplied by man (hives). Depending on the climate, a honey bee colony has been reported to vary from a population of about 60,000 thousand bees (Farrar, 1937) to just a few thousand bees in an overwintering colony (Harbo, 1986). The nest is composed by wax honeycombs erected vertically in series, each one formed by cells present on both sides, which are used as containers to either store food (honey or pollen) or nurse the brood. In the latter case, the cell will be built with a different shape or size depending on the caste that will host. In fact, *Apis mellifera* is a holometabolous species characterized by a matriarchal and pluriannual society composed of individuals from three castes, all winged. Reproduction is haplo-diploid: from the fertilized egg is born a diploid female individual, while from an unfertilized the egg is born a haploid male. Each bee colony is composed by one queen bee (the only fertile female), thousands of worker bees (sterile females) and hundreds of drones (fertile males). Each caste is different from the others for the timing and mode of development, morphology, anatomy, tasks performed within the colony and lifespan.

The queen bee is the mother of all the individuals being the only fertile female of the colony. She is characterized by a well-developed abdomen and mates once in life during the "nuptial flight" with multiple different drones. The queen has also the important task, carried out through the use of pheromones, to ensure the cohesion of the colony. The duration of her

life can be up to five years, but as early as the second her efficiency can decline considerably and often she is replaced by a new queen before her death.

Drones are bred with the main purpose to mate with the queen. The individual male is able to live on average 50 days but often, after the period of mating or if the weather/nutritional conditions are not good, most of them will be driven out or killed by the worker bees.

Worker bees constitute the majority of bees within the colony. They are sterile females and attend a much higher variety of tasks, compared to the other two castes. These tasks vary according to age and in fact every worker bee holds in succession each of the various tasks necessary to the society (temporal polyethism). Their first task is to clean cells, then feed and take care of the brood ("nurse bees"), build and repair wax structures, receive and store pollen/nectar, defend the community and finally start foraging ("forager bees") generally after 14 days from emergence (Michener, 1969). However, these indications may be variable in function of colony needs: the division of labour is not so rigid and by necessity it is possible that, for example, young bees turn into foraging and *vice versa* (Schulz et al., 1998). The life span of the worker bee during the active season is of 30 to 45 days, while those born in the fall can survive until the beginning of next active season.

Honey bees communicate to each other with a complex network of chemical signalling (for a comprehensive review see Bortolotti and Costa, 2014). Pheromones have a major role in communication between castes and through individuals of the same caste. The queen mandibular pheromone has the major role to indicate the presence of an active queen and to maintain the cohesion of the colony, mainly preventing any other female to develop the reproductive system (Slessor et al., 2005). Specifically, the queen bee produces a complex blend of odorous stimulus that worker bees transmit through the whole colony via trophallaxis. Other pheromone based communications have been discovered among worker bees and between larvae and nurse bees. Chemical signalling is also used for colony defence purposes and recruitment of worker bees for foraging. The specific information about food sources to forage are communicated between bees through a specific code of movements, called "dances", whose interpretation by von Frisch (1967) gave the most amazing example of the honey bee social complexity and wonder.

1.1.1 The honey bee colony as a superorganism

The honey bee colony is defined as a superorganism, namely a set of individuals who may act in concert to produce phenomena governed by the community (Kelly, 1995; Moritz and Southwick, 1992; Wheeler, 1928). It manifests peculiar collective behaviour and emergent properties which are different from those of individual organisms that constitute the superorganism. It is a complex system composed of a high number of individual organisms which are typically carrying out different tasks within their society (division of tasks). Indeed, as described above, each honey bee colony consists of tens of thousands of individuals, organized in a well-defined division of labour. All the bees within their colony are able to "produce phenomena governed by the community" that are essential to ensure the survival of the family. The system can in fact be preserved only thanks to the different activities carried out by the individuals which, in the absence of the other components of the system, will not survive.

As in humans cellular reproduction does not coincide with the reproduction of the individual, the reproduction of bees does not coincide with the reproduction of their colony. In fact the reproduction of the individual bee is a consequence of the egg deposition and fertilization process of the eggs, while the reproduction process of the bee colony is very different and it is called swarming. This process consists in the separation of a colony into two groups, one of which moves away from the original nest to found another one elsewhere.

1.1.1.1 Thermoregulation

Another phenomenon that only the colony as a community of bees can carry out is the one that allows the thermal homeostasis of the hive. In fact the brood must be maintained at an ideal temperature of 35°C. Variations of less than 2°C from this value, may result in the emergence of bees that are malformed and with neural impairments (Jones et al., 2005; Medrzycki et al., 2010). Therefore, when the ambient temperature deviates from the optimum, worker bees react with specific behaviours aimed at maintaining the optimal temperature for the brood. The bees produce metabolic heat by means of flight muscles, averting the risk of hypothermia of the adult bees and of the brood. Action potentials of thoracic muscles reach the frequency and amplitude that characterize them during the flight, and while maintaining the wings inactive their thoracic temperature can reach temperatures around 40°C (Camazine et al., 1999).

1.1.2 The honey bee and the environment

The fates of the honey bee and the environment are deeply related together, due to the vast influence each one has on the other.

The honey bee is one of the principal pollinator species worldwide. Their broad spectrum efficacy and efficiency as pollinators is fundamental for both managed crops (Roubik, 2002; Southwick and Southwick, 1992; Watanabe, 1994) and wild plants (Burd, 1994; Kearns et al., 1998) success worldwide (Breeze et al., 2014; Klein et al., 2007; Schulp et al., 2014), consequently including honey bees as organisms with both great economical and biodiversity relevance. Indeed, the decline of pollinators can lead to a parallel decline of plant species (Biesmeijer et al., 2006). Numerous yields have shown to decrease consistently in both quality and quantity without honey bees (Southwick and Southwick, 1992). About 70% and 84% of respectively tropical (Roubik et al., 1995) and European (Williams, 1994) crops seem to have at least one variety that depends to some extent upon pollination. Tautz (2008) estimated that worldwide 90% of the fruit trees and 85% of angiosperms pollinated by insects depend mainly on honey bees. According to Accorti and Cerretelli (1991) at least 79% of the Italian agricultural production benefits from insect pollination which leads to an increased agricultural production of over 1.5 billion euros. The economic benefit of honey bees for agriculture has been estimated to be between seven and ten times greater than the value of their honey production (European Parliament, 2011).

However, the modern industrialized agriculture is characterized by the vast use of monocultures and pesticides, which have made the fields a hostile environment to pollinators. Pesticide treatments have reduced the myriads of wild species that served as pollen vectors from flower to flower. The disappearance of many wild pollinators has increased the importance of honey bees as pollinating organisms, overshadowing their most popular role as producers of honey. When wild bees do not visit agricultural fields, managed honey bee colonies are usually the principal solution for farmers to ensure crop pollination. In fact, compared with the management of several wild bee species, honey bees are inexpensive and versatile. For these reasons, honey bees are also used as surrogate organisms for pollinators in risk assessment schemes (Alix and Lewis, 2010; OEPP/EPPO, 2010) and as biological indicators of the environmental well-being (Porrini et al., 1998; Porrini et al., 2002; Smith et al., 2013; Svoboda, 1961).

1.2 Honey bee colony losses

The honey bee population has been declining in the last century (vanEngelsdorp and Meixner, 2010), but in the last decades honey bee colony losses have been even more frequently and extensively reported in numerous parts of the world. Even if beekeepers can duplicate their colonies to overcome the losses (Maini et al., 2010), this implies time, money and environmental costs: beekeeping has become a much more difficult job (Potts et al., 2009).

Honey bee colony losses are likely caused by a multiplicity of factors that, acting individually or in combination, affect colony health (Carreck and Neumann, 2010; Goulson et al., 2015; Maini et al., 2010; Oldroyd, 2007).

The role of each single factor may vary depending on season and environmental circumstances. Among the variety of factors considered the most dangerous for bee health, the principal ones are considered to be pathogens and parasites (Berthoud et al., 2010; Martin et al., 2010; Neumann and Carreck, 2010, Goulson, Lye & Darvill 2008; Cameron et al. 2011; Garibaldi et al. 2011b; Nazzi et al. 2012); loss of genetic diversity (vanEngelsdorp and Meixner, 2010); habitat loss and fragmentation, together with the decreased foraging sites and nutritional value of the pollen (Potts et al., 2010); and at last but not least pesticides (Goulson, 2013; Maini et al., 2010).

Indeed, the widespread use of pesticides for plant protection and their numerous adverse effects on honey bees seem to play a key role in this scenario (Desneux et al., 2007; Farooqui, 2013; Maxim et al., 2013; Sanchez-Bayo, 2014).

1.2.1 Pesticides

The routes of exposure of honey bees to pesticides are numerous, but the principal ones are either through contact or ingestion (Krupke et al., 2012; Rortais et al., 2005; Simon-Delso et al., 2014; Smagghe et al., 2012).

Honey bees can be directly intoxicated by plant protection products via contact while flying during a spray or seed treatment. Considering that spray treatments are influenced by wind drift, the vegetation surrounding the treated field may be contaminated as well, representing an additional source of residual contamination for bees.

In addition, both spray and seed treatments can contaminate honey bee food and be ingested (Bonmatin et al., 2014; Girolami et al., 2009; Krupke et al., 2012; Main et al., 2014;

Morrissey et al., 2015; Rortais et al., 2005; Samson-robert et al., 2014). Nectar, freshly stored pollen and beebread are considered to be the principal sources of in hive contamination for adults and larvae (Krupke et al., 2012). Forager bees that collect contaminated pollen, nectar, honeydew or water can immediately die or, if the doses are not lethal, the media can be transported inside the nest. Subsequently, it is probably accumulated and assumed during the more critical periods, when bees need to consume their food stores. Therefore, sublethal doses of the pesticides may be highly dangerous to the honey bee colony, causing slow but steady depopulation.

The effects elicited by a pesticide depend on the intrinsic properties of the formulation, administered dose and concentration, duration and mode of exposure. Newly developed chemical pesticides have been characterized by an increased toxicity, thus requiring lower quantities to be effective. Systemic insecticides like neonicotinoids represent at the same time the most effective type of plant protection product and one of the most relevant possible threats to honey bees as they can contaminate essential food sources for pollinating insects. Therefore, in this study we focused on this class of pesticides, one of the most popular new generations of agrochemicals used worldwide.

1.2.1.1 Neonicotinoids

The neonicotinoid class of insecticides was first developed and registered in the early 1990s and now represents the largest selling class of seed treatments and approximately one third of the insecticides (data from 2010) on the global market (Elbert et al., 2008; Jeschke et al., 2010; Simon-Delso et al., 2014).

Neonicotinoids act as specific agonists of the insect acetylcholine receptors (Millar and Denholm, 2007; Tomizawa and Casida, 2003) and are very popular in plant protection due to their high toxicity, persistency and systemic property (Maienfisch, 2001; Tomizawa and Casida, 2005).

Their use is versatile and they can be used by various means, such as spray, soil drench applications, trunk injection, bathing and seed coating. Neonicotinoids are used for insect pest management in hundreds of crops in horticulture, agriculture and forestry. In addition, these applications occur across all major agricultural commodities worldwide and on numerous cattle species and companion animals (see Simon-Delso et al., 2014, for a review).

Nevertheless, a variety of non-target organisms, such as pollinators, can also be exposed to these pesticides. Their widespread use combined with their intrinsic properties has

resulted in the routine contamination of wetlands and freshwater resources, agricultural soils and non-target vegetation, estuarine and coastal marine systems, which means that many organisms inhabiting these habitats are being repeatedly and chronically exposed to these insecticides (Simon-Delso et al., 2014).

Among neonicotinoids, in this work we investigated thiamethoxam (Fig. 1.1). It is the second neonicotinoid that was extensively marketed (Elbert et al., 2008; Maienfisch, 2001; Maienfisch et al., 2001; Simon-Delso et al., 2014) and is today one of the most widely used neonicotinoids (Jeschke et al., 2010; Main et al., 2014) although not as extensively studied as imidacloprid, the first neonicotinoid marketed in 1991.

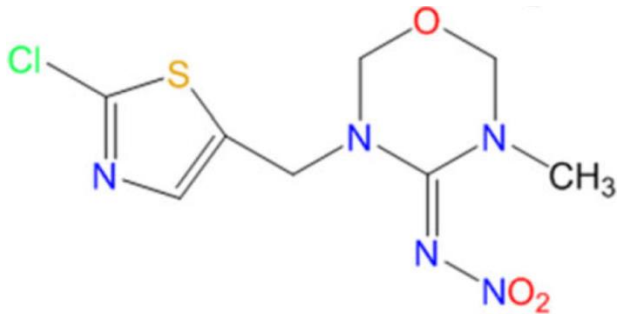

Fig. 1.1. Molecule of the neonicotinoid insecticide thiamethoxam.

Thiamethoxam exhibits exceptional toxicity, persistency and systemic properties (Maienfisch, 2001). If applied to the soil, the product is absorbed by the roots and transported through the xylem throughout the whole plant, showing a remarkable efficacy against insects. As for other neonicotinoids, thiamethoxam is frequently found in various environmental media and in honey bee food sources and nests (generally in the 1–100 ppb range) (ApeNet, 2011; BeeNet, 2013; Bonmatin et al., 2014; Dively and Kamel, 2012; Girolami et al., 2009; Main et al., 2014; Morrissey et al., 2015; Pochi et al., 2012; Samson-robert et al., 2014; Stoner and Eitzer, 2012). Thiamethoxam acts on insects by ingestion and contact blocking the nerve impulses at the level of nicotine receptors. At even very low dose and concentration exposures thiamethoxam triggers sublethal effects on non-target organisms like *Apis* and non-*Apis* bees (see Pisa et al., 2014, for a review).

1.2.1.2 Sublethal effects of neonicotinoids

Sublethal effects are mainly caused by neural impairments which can lead to physiological, behavioural and cognitive alterations (Belzunces et al., 2012).

In the last decades the introduction in the market of the neonicotinoids has revealed the variety and complexity of sub lethal effects that may be elicited on honey bees. As nicotinic acetylcholine receptor agonists, neonicotinoids elicit a variety of sublethal effects (Belzunces et al., 2012; Decourtye and Devillers, 2010; Desneux et al., 2007; EFSA, 2012a; Goulson, 2013; Pisa et al., 2014; Stokstad, 2012; Wu et al., 2011) which can cause dramatic consequences at colony level on highly social organisms such as honey bees. Some of the most relevant sublethal effects that neonicotinoids elicit on honey bees are related to learning and memory (Decourtye et al., 2004a; Decourtye et al., 2004b; Decourtye et al., 2005; Eiri and Nieh, 2012; Gauthier, 2010; Hassani et al., 2005; Hassani et al., 2008; Ping-Li et al., 2013; Yang et al., 2012), communication (Eiri and Nieh, 2012; Kirchner, 1998; Medrzycki et al., 2003), navigation (Fischer et al., 2014; Vandame et al., 1995), homing (Bortolotti et al., 2003; Colin et al., 2004; Henry et al., 2012; Vandame et al., 1994), foraging behaviour (Feltham et al., 2014; Schneider et al., 2012; Scholer and Krischik, 2014; Yang et al., 2008) and locomotion (Lambin et al., 2001; Medrzycki et al., 2003; Williamson et al., 2014).

1.3 Aim of the study

Honey bees are frequently exposed to the commonly used thiamethoxam both in the field and nest, typically at sublethal doses and concentrations. However, even at these low levels of contamination, neonicotinoids commonly elicit sublethal effects on bees. This work wants to focus on some still scarcely investigated sublethal effects that the neonicotinoid thiamethoxam triggers on forager honey bees.

Henry et al. (2012) showed that thiamethoxam administered in sublethal doses impairs the homing flight success of forager bees leading to colony failure. We hypothesized that this effect was caused by an impairment of the locomotion abilities of the bees. Therefore, we tested walking (Chapter 2) and flight (Chapter 3) performances of forager bees after acute (Exp. 1) and after chronic (Exp. 2) exposures to the pesticide.

The same modes of exposure were used in both studies on honey bee locomotion, to allow comparisons of the results. The single dose of thiamethoxam used was specifically based upon Henry et al. (2012) to allow comparisons of our results. A phototaxis arena and several flight mills were used to test respectively forager walking and flight behaviours in controlled environments.

Flight performances depend on flight muscle activity, which in turn depend on regulation of thoracic temperature (Coelho, 1991a; Esch, 1976). Therefore, we decided to investigate the effects of thiamethoxam on forager thermoregulation (Chapter 4).

Thermoregulation is impaired by insecticides like organophosphates (Schmaranzer et al., 1987) and pyrethroids (Belzunces and Vandame, 1998; Belzunces et al., 1996) and by azole fungicides like prochloraz and difenoconazole (Belzunces and Vandame, 1998), that the neonicotinoid chemical class causes on individual bee body temperature and thermoregulation.

Thermoregulation is impaired by insecticides like organophosphates (Schmaranzer et al., 1987) and pyrethroids (Belzunces and Vandame, 1998; Belzunces et al., 1996) and also by azole fungicides like prochloraz and difenoconazole (Belzunces and Vandame, 1998), but there is scarce knowledge on the effects that the neonicotinoid chemical class overall elicit on honey bee thermoregulation. We tested three acute doses of the active ingredient and recorded the thorax temperature of forager bees for two days.

In summary, we carried out three in vitro principal experiments aimed at assessing the sublethal effects thiamethoxam elicits on forager bee locomotion (walking and flight) and thermoregulation.

CHAPTER 2

Sublethal effects of thiamethoxam on honey bee walking locomotion and phototaxis

Abstract

The sublethal effects that the neonicotinoid pesticide thiamethoxam have shown to trigger on bee locomotion are limited, since only few behavioural aspects, doses and concentrations have been studied. We tested the sublethal effects that acute (Exp. 1) and chronic (Exp. 2) exposures to thiamethoxam elicit on forager bees using a phototaxis arena, which allowed investigating hyperactivity, motor functioning and phototaxis features across time restraining environmental variability. The single dose treatment (1.34 ng/bee, based upon Henry et al., 2012) showed to widely impair honey bee locomotion, increasing the hyperactivity of the bees rapidly after treatment (30 min), until an impairment of the motor functioning appeared (60 min). Continuous exposures (32.5, 45 ppb) lead to limited significant effects on the motor functioning, although both modes of exposure to thiamethoxam increased the positive phototaxis. Locomotion is a crucial ability for honey bees, especially because flight proficiency depends on it. Phototaxis is related to the transition of the bees from in-hive to foraging tasks. Therefore, the locomotion and phototaxis alterations elicited by sublethal doses of thiamethoxam can impair foraging activity and consequently colony fitness.

2.1 Introduction

Honey bee colony losses have been extensively reported in recent years (Bacandritsos et al., 2010; Mutinelli et al., 2010; Underwood and vanEngelsdorp, 2007; VanEngelsdorp et al., 2008; vanEngelsdorp et al., 2010) and a multiplicity of factors, acting individually or in combination, is considered to be the cause of this phenomenon (Carreck and Neumann, 2010; Maini et al., 2010; Oldroyd, 2007; Potts et al., 2010). The most critical ones are considered to be pathogens and parasites (Cameron et al., 2011; Nazzi et al., 2012; Potts et al., 2009), habitat loss and fragmentation (Naug, 2009; Potts et al., 2010) and pesticides (Farooqui, 2013; Maini et al., 2010; Maxim et al., 2013; Sanchez-Bayo, 2014; van der Sluijs et al., 2014).

The decline of bees can lead to a parallel decline of plant species (Biesmeijer et al., 2006). In fact, honey bee pollination guarantees the sexual reproduction of a majority of managed crops and wild plants worldwide (Breeze et al., 2014; Klein et al., 2007; Schulp et al., 2014). In particular, managed honey bee hives are often the best solution for farmers to ensure crop pollination when the frequency of wild bee visits to agricultural fields is low. Compared to the management of several wild bee species, honey bees are versatile and cheap. Therefore, together with their key role on biodiversity conservation, honey bees are the most economically valuable pollinators of several crop monocultures (Roubik, 2002; Southwick and Southwick, 1992; Watanabe, 1994). On the other hand, the extensive use of pesticides as plant protection products in agricultural areas lead honey bees to be routinely exposed to a wide variety of pesticides (Chauzat et al., 2006; Dively and Kamel, 2012; Johnson and Pettis, 2014; Johnson et al., 2010; Krupke et al., 2012; Lambert et al., 2013; Smodis Skerl et al., 2009; Stoner and Eitzer, 2012). A specific class of pesticides, the neonicotinoids, have been often linked to bee losses (ApeNet, 2011; Sanchez-Bayo, 2014; van der Sluijs et al., 2013). They have furthermore showed to trigger a variety of both sublethal and synergistic effects, thus leading to critical consequences at low doses and concentrations (Alaux et al., 2010; Beliën et al., 2009; Di Prisco et al., 2013; Glavan, 2013; Pettis et al., 2013; Thompson, 2012; Vidau et al., 2011).

Neonicotinoids are the major group of systemic pesticides worldwide, which was first developed and registered in the early 1990s and now represent the largest selling class of seed treatments and about one third of the insecticides on the global market (Elbert et al., 2008; Jeschke et al., 2010; Simon-Delso et al., 2014). They bind agonistically to the insect nicotinic acetylcholine receptor (nAChR) with high affinity (Millar and Denholm, 2007; Tomizawa and

Casida, 2003). They are widely found in all environmental media such as soil, water and air, including honey bee food and colonies, generally in the 1–100 ppb range (ApeNet, 2011; BeeNet, 2013; Bonmatin et al., 2014; Dively and Kamel, 2012; Girolami et al., 2009; Main et al., 2014; Morrissey et al., 2015; Pochi et al., 2012; Samson-robert et al., 2014; Stoner and Eitzer, 2012). Honey bees exposed to these chemical class exhibited sublethal symptoms such as impaired learning and memory (Decourtye et al., 2004a; Decourtye et al., 2004b; Decourtye et al., 2005; Eiri and Nieh, 2012; Gauthier, 2010; Hassani et al., 2005; Hassani et al., 2008; Ping-Li et al., 2013; Yang et al., 2012), communication (Eiri and Nieh, 2012; Kirchner, 1998), navigation (Fischer et al., 2014; Vandame et al., 1995), homing (Bortolotti et al., 2003; Colin et al., 2004; Henry et al., 2012; Vandame et al., 1994) and foraging behaviour (Feltham et al., 2014; Schneider et al., 2012; Scholer and Krischik, 2014; Yang et al., 2008). Thiamethoxam is today one of the most widely used neonicotinoid (Casida and Durkin, 2013; Jeschke and Nauen, 2008; Jeschke et al., 2010; Main et al., 2014) and exhibits exceptional toxicity, persistency and systemic properties (Maienfisch, 2001). The sublethal effects elicited on bees by thiamethoxam, the second extensively marketed neonicotinoid (Elbert et al., 2008; Maienfisch, 2001; Maienfisch et al., 2001; Simon-Delso et al., 2014), are not as widely investigated as imidacloprid, the first neonicotinoid marketed in 1991.

In this study we focused on thiamethoxam and its effects on forager bee locomotion and phototaxis. Locomotion is an essential behaviour of living organisms and in honey bees plays a central role in colony fitness because bees require use locomotion as they move inside the nest, performing multiple tasks. Indeed, neonicotinoid insecticides elicit an impairment on locomotion behaviour (Aliouane et al., 2008; Lambin et al., 2001; Medrzycki et al., 2003; Williamson et al., 2014) but only few substances like thymol have shown to exhibit effects on phototaxis (Bergougnoux et al., 2012; Carayon et al., 2014). Lambin et al. (2001) observed that topical doses of imidacloprid (1.25, 2.5, 5, 10, 20 ng/bee) influenced the ability of worker bees to move in a PVC box (30 x 30 x 4 cm), similarly to what observed by Medrzycki et al. (2003) at higher concentrations (100-500 ppb). Aliouane et al. (2008) tested the topical and oral effects of acetamiprid (0.1, 0.5, 1 µg/bee) and thiamethoxam (0.1, 0.5, 1 ng/bee) on bee locomotion within an open-field-like apparatus (30 x 30 x 4 cm) and observed increased locomotor activity only after topical applications of acetamiprid. Williamson et al. (2014) studied the effects of various neonicotinoids on locomotion of bees inside petri dishes (15 x

1.5 cm) and observed that those exposed to imidacloprid, thiamethoxam and clothianidin for 24 hours were both more likely to lose postural control and unable to right themselves.

Henry et al. (2012) demonstrated that thiamethoxam reduces forager bee homing flights success and consequently impairs colony survival. We hypothesized that the effect thiamethoxam elicited on flight was related to an impairment of the physical locomotion abilities of bees. We therefore orally administered the bees with the same single dose of thiamethoxam Henry et al. (2012) tested and we focused on locomotion behaviours. We also tested the effects of a chronic exposure of thiamethoxam to replicate a scenario in which forager bees survived a first intoxication and returned to the same contaminated field several times.

2.2 Materials and methods

This study was conducted in 2013 and 2014 at University of California San Diego (UCSD), Division of Biological Sciences (La Jolla, CA, USA).

2.2.1 Honey bee preparation

Returning forager honey bees were collected at the hive entrance of a total of 5 healthy colonies of *Apis mellifera ligustica* Spinola, 1806, located at the UCSD Biological Field Station. After collection, forager bees were placed in plastic cages (11 x 11 x 9 cm) in groups of 10 and maintained in an incubator (30±1°C, 60-80% RH) with sucrose water solution (see Exp. 1 and 2 descriptions below for specific details). After incubation, the locomotion behaviours exhibited inside a phototaxis arena were tested.

2.2.2 Locomotion in the phototaxis arena

The accurate assessments of the locomotion behaviours of the bees were carried out using a phototaxis arena (Fig. 2.1, 30 x 30 x 5 cm) composed of white acrylic walls except for the transparent acrylic front cover that allowed observation. The rear wall was divided in 36 squares of 5 cm side each. The arena had a hole on the bottom side (diameter = 1 cm) that was used to insert the bee. A LED light (280 lumens, 120° illumination angle, 6000K colour temperature) was located at the top pointing to the bottom and was the only source of light. Bees climb inside their nest because their combs are normally vertically oriented, and once the individuals were inserted in the arena they instinctively moved upwards towards the light.

Light intensity was maintained constant at the maximal 280 lumens, since it influences the phototactic behaviour of the bees (Erber et al., 2006). To measure light levels, we used a quantum meter (Apogee model MQ-200, Logan, Utah, USA, spectral range of 410-655 nm). The arena was maintained at 25±1°C and 50-70% RH.

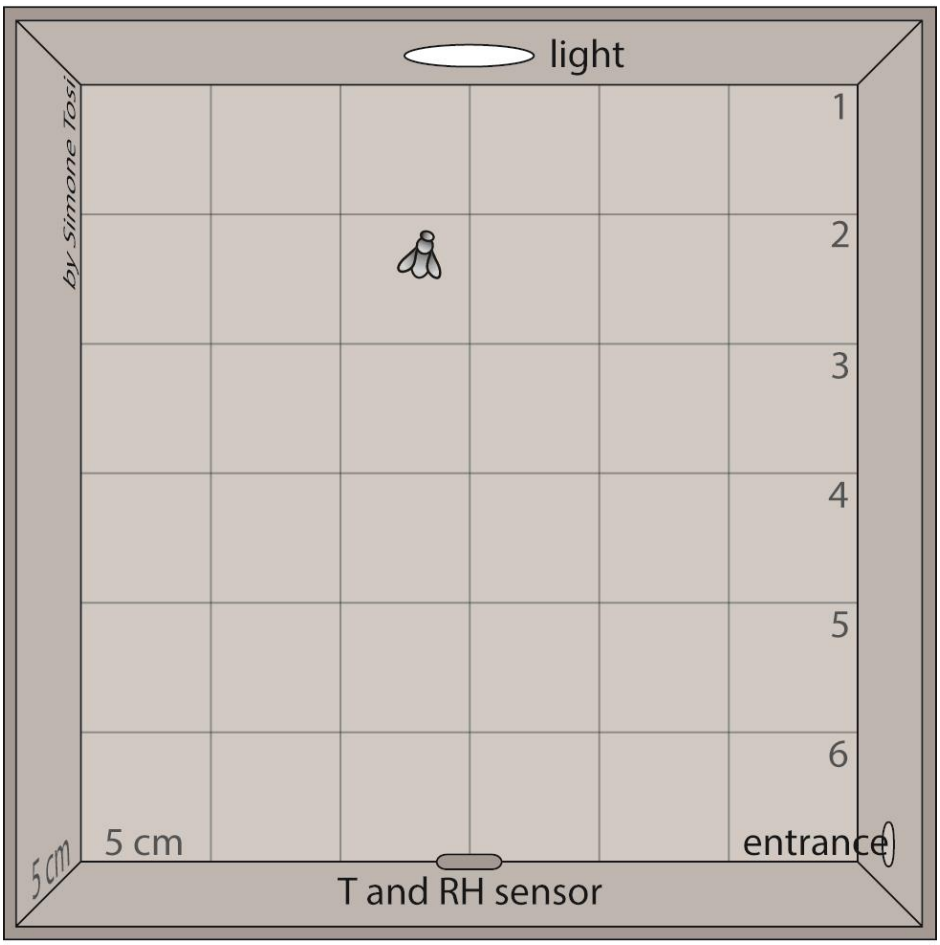

Fig. 2.1. The phototaxis arena used to test walking locomotion and phototaxis behaviour of forager bees.

Forager behaviours related to the positive phototaxis were tested twice, 30 and 60 minutes after treatment. At each assessment time, the movements and behaviours of each bee were recorded for 3 minutes. As a consequence of the bees instinctive positive phototaxis and negative geotaxis behaviour, once the individuals were inserted in the arena they instinctively tended to ascend towards the light and against the force of gravity, similarly to Aliouane et al. (2008) and Lambin et al. (2001). The variables assessed during the test were divided in 3 groups respectively related to the first path to reach the light, hyperactivity and motor functions (Tab. 2.1).

After the recording, each bee was gently caught and removed from the arena, which was then cleaned. In order to test the effects of acute and chronic exposures of thiamethoxam (TMX) on honey bees, two experiments were carried out.

Tab. 2.1. Variables assessed per each bee during the 3 minutes test.

Variable group	Variable assessed (measure unit)	Definition
First path to light *(a)*	Distance covered to light (squares)	Number of squares crossed to reach the light *(b)*
	Time to light (s)	Time spent to reach the light
	Speed to light (squares/s)	Average speed during the first path to reach the light *(c)*
Hyperactivity	Time moving (s)	Time spent moving
	Overall distance covered (squares)	Total number of squares crossed *(b)*
	Overall speed (squares/s)	Average speed during the whole test *(d)*
Motor function	Inability to reach the light (T/F)	The bee does not reach the light source even once
	Inability to ascend (T/F)	The bee tries to climb the phototaxis arena walls but cannot *(e)*
	Abnormal behaviour (T/F)	The bee exhibits a various abnormal behaviour *(f)*
	Falls (n)	Frequency of falls from the arena wall
	Time bottom (s)	Time spent in the bottom row of the arena
	Time top (s)	Time spent in the top row of the arena

(a) Light was considered reached when the bee touched the light source.
(b) A square was considered crossed when head and thorax of the bee fully crossed one of its sides.
(c) Calculated dividing Distance covered to light by Time to light.
(d) Calculated dividing Overall distance covered by total duration of the test (180 s).
(e) True only if exhibited for > 10 s.
(f) E.g. trembling, erratic movements. True only if exhibited for > 10 s

2.2.2.1 Experiment 1: Acute exposure

After collection, forager bees were incubated for 1 h with 0.5 M sucrose solution ad libitum and then starved for 30 minutes. This starving period was necessary to allow the bees to drink completely and in a single feeding event the whole 10 µl of 2 M glucose solution either pure or containing 1.34 ng of thiamethoxam (the latter corresponding to 118 ppb, 134 µg/L and 460 nmol/L). This sublethal dose is 3.7 times lower than the LD_{50} dose of TMX, which is 5 ng/bee (EFSA, 2013a) and does not elicit any significant difference in mortality from control (Henry et al., 2012). This dose was chosen to replicate the one used by Henry et al (2012) in their homing flight experiment. The density of 2 M glucose solution at 20°C and 1 ATM (1.131 kg/L) was used to calculate the concentration.

After treatment, the bees were kept in the incubator (30°C, 60-80% RH) without food. In total, 43 bees were tested.

2.2.2.2 Experiment 2: Chronic exposure

After collection, a total of 36 forager bees were incubated for 2 days with 1.8 M sucrose solution ad libitum containing either 0 or 45 ppb (the latter corresponding to 55 µg/L and 189 nmol/L) of thiamethoxam. The density of 1.8 M sucrose solution at 20°C and 1 ATM (1.230 kg/L, Bubník and Kadlec, 1995) was considered to calculate the concentrations.

The consumption of sucrose solution (and TMX, as appropriate) per cage was evaluated daily, hence the consumption per bee could be calculated indirectly dividing the daily total consumption of sucrose solution per cage by the number of live bees on that day. (EFSA, 2012a) calculated that each forager bee could assume daily doses of thiamethoxam up to 5.89 ng in the worst case scenario. While EFSA considered 5.2 ppb as the highest TMX nectar concentration found in the field (*Phacelia*), Dively and Kamel (2012) reported concentrations of TMX in the nectar that were almost 3 times higher (15 ppb). Therefore, the EFSA calculations could underestimate the dose the bees are exposed to. Nonetheless, it is likely that a wide range of doses and concentrations that can be considered field-relevant over space and time (Pisa et al., 2014; van der Sluijs et al., 2013). Within this context, the daily doses assumed by treated bees in this experiment were always lower than the limit suggested by EFSA (in average, 3.13±0.14 ng of thiamethoxam daily). The doses consumed by bees in this experiment are therefore field-relevant.

2.2.3 Statistical analysis

Generalized Linear Models (GLMs) were used to test the effects of colony and treatment (Exp. 1: *0* or *1.34 ng/bee* of thiamethoxam; Exp. 2: *0* or *45 ppb* of thiamethoxam) at 30 and 60 minutes post-treatment on number of falls, overall speed, time bottom, time moving and time, distance covered and speed to reach the light. This method provided the most appropriate analysis of the data given the trends and the complexity of the interaction with time. Poisson distribution with reciprocal link (for the variable *falls*) or exponential distribution with reciprocal link (for all other variables) were used in the GLMs and their appropriateness was confirmed with Pearson goodness-of-fit analysis. Overdispersion was computed in the GLMs when appropriate (Carruthers et al., 2008).

Nominal logistic analysis were computed to compare the effect of colony and treatment (Exp. 1: *0* or *1.34 ng/bee*; Exp. 2: *0* or *45 ppb*) at 30 and 60 minutes post-treatment on the presence of abnormal behaviours, inability to ascend the arena and inability to reach the light.

In Exp. 2, we used a Spearman correlation to analyse the relationship between of the actual dose of thiamethoxam consumed by the bees during incubation and all other variables.

In Exp. 2, Kruskal-Wallis Rank Sum test was used to assess the effect of treatment on sucrose consumption.

All analysis were computed using JMP v10.0, SAS statistical software.

2.3 Results

2.3.1 Experiment 1: Acute exposure

The summary of the effects elicited by a single dose of thiamethoxam on forager walking locomotion and phototaxis are reported in Tab. 2.2.

Tab. 2.2. Means (SE) of the variables assessed during Exp. 1 in relation to time (30 and 60 minutes post-treatment) and treatment (0 or 1.34 ng/bee of thiamethoxam). Asterisks indicate statistically significant differences between treatments within time assessments (GLMs or Nominal logistics, * p<0.05, ** p<0.01, *** p<0.001).

Variable group	Predicted variable	30 minutes post-treatment				60 minutes post-treatment			
		Control (N=20)	Pesticide (N=21)	L-R χ^2	P value	Control (N=21)	Pesticide (N=21)	L-R χ^2	P value
First path to light	Distance to light (squares)	10.22 (0.97)	10.45 (0.86)	0.01	0.9185	10.35 (0.9)	9.36 (0.58)	1.17	0.1900
	Time to light (s)	45.72 (8.46)	22.35 (4.08)	6.28	0.0122*	29.45 (5.62)	12.71 (1.61)	13.17	0.0003***
	Speed to light (squares/s)	0.36 (0.06)	0.66 (0.07)	8.33	0.0039**	0.49 (0.05)	0.81 (0.06)	14.79	0.0001***
Hyperactivity	Time moving (s)	96.95 (13.78)	148.19 (11.46)	6.46	0.0110*	125.29 (9.56)	135.43 (12.63)	0.28	0.5993
	Overall speed (squares/s)	0.30 (0.05)	0.69 (0.12)	9.92	0.0016**	0.55 (0.10)	0.50 (0.10)	0.37	0.5430
Motor function	Inability to reach light (n)	2	1	0.54	0.4638	1	7	6.21	0.0127*
	Inability to ascend (n)	2	5	1.98	0.1597	3	12	8.78	0.0030**
	Abnormal behavior (n)	2	4	1.3	0.2545	4	11	5.47	0.0193*
	Falls (n)	3.45 (1.11)	7.33 (1.46)	4.13	0.0421*	5.10 (1.28)	8.43 (1.55)	3.92	0.0478*
	Time bottom (s)	44.45 (9.89)	60.00 (9.79)	0.69	0.4000	50.14 (10.06)	99.81 (13.32)	6.62	0.0101*
	Time top (s)	93.20 (11.71)	74.05 (9.71)	1.21	0.2718	88.33 (9.79)	47.43 (11.52)	5.17	0.0229*

2.3.1.1 First path to light

There was no significant effect of treatment on the distance covered to reach the light for the first time (Fig. 2.2A). This result was consistent at 30 and 60 minutes post-treatment.

There was a significant effect of treatment on the time bees needed to reach the light for the first time at both 30 and 60 minutes post-treatment (Fig. 2.2B). In particular, foragers that assumed thiamethoxam reached the light in less time than control bees.

There was a significant effect of treatment on the average speed bees had while reaching the light at both 30 and 60 minutes (Fig. 2.2C). Foragers that ate the pesticide reached the light faster than controls.

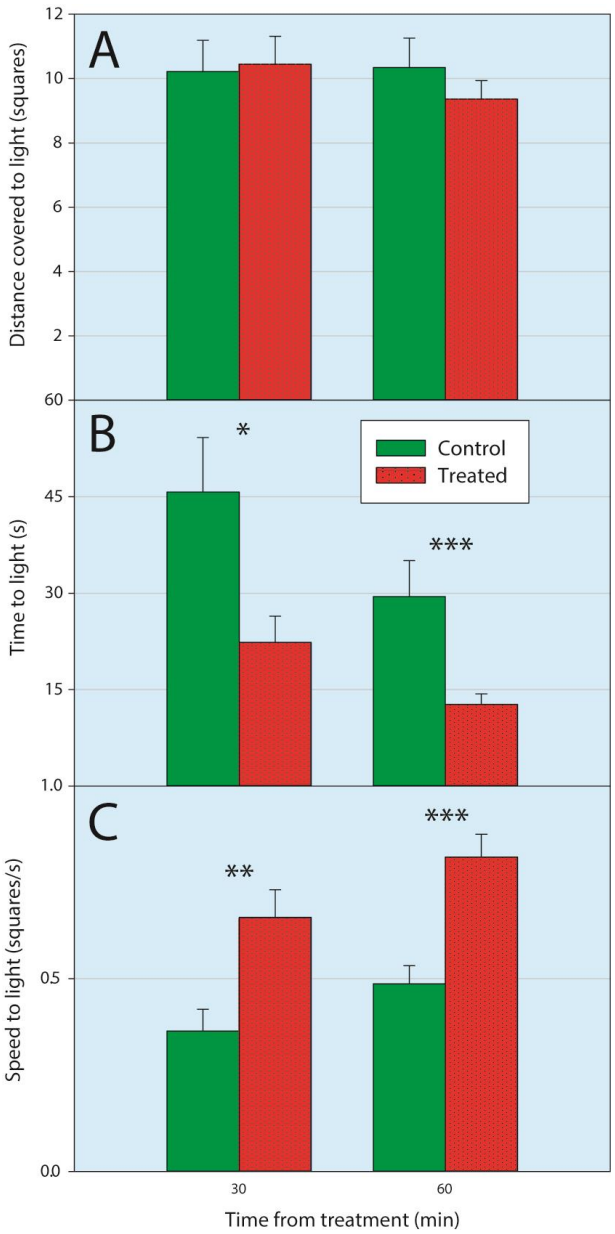

Fig. 2.2. The effects of a single dose (Exp. 1) of thiamethoxam (1.34 ng/bee, red dotted columns) on forager locomotion during their first path to light, 30 (left bars) and 60 (right bars) minutes after treatment. (A) Distance covered, (B) time and (C) speed to reach the light were assessed. Asterisks indicate statistically significant differences between treatments within time assessments (GLMs, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). 30 min: $N_{control} = 20$, $N_{pesticide} = 21$; 60 min: $N_{control} = 21$, $N_{pesticide} = 21$. Error bars are SE.

2.3.1.2 Hyperactivity

There was a significant effect of treatment on the amount of time spent moving only at 30 minutes after treatment. Treated bees spent more time moving compared to controls (Fig. 2.3A). There was no significant effect of *treatment* on the amount of time spent moving at 60 minutes post-treatment.

There was a significant effect of treatment on the overall speed of the bee 30 minutes after treatment. During the 3 minutes evaluation period the treated bees were significantly more fast than untreated ones (Fig. 2.3B).

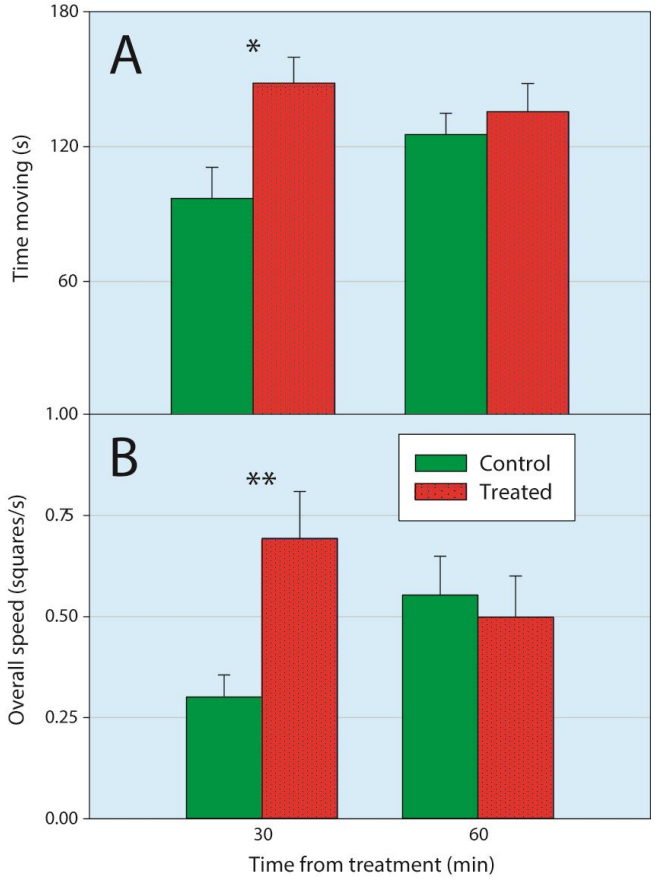

Fig. 2.3. The effects of a single dose (Exp. 1) of thiamethoxam (1.34 ng/bee, red dotted columns) on forager (A) time spent moving and (B) overall speed, 30 (left bars) and 60 (right bars) minutes after treatment. Asterisks indicate statistically significant differences between treatments within time assessments (GLMs, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). 30 min: $N_{control} = 20$, $N_{pesticide} = 21$; 60 min: $N_{control} = 21$, $N_{pesticide} = 21$. Error bars are SE.

2.3.1.3 Motor functions

There was a significant effect of treatment on the number of bees that did not reach the arena light (Fig. 2.4A), were unable to ascend the arena (Fig. 2.4B) and exhibited at least an

abnormal behaviour (Fig. 2.4C), 60 minutes after treatment. Treated bees did not reach the light, showed to be unable to climb the arena and exhibited at least one abnormal behaviour more frequently than controls. No significant effect of treatment was shown at time 30.

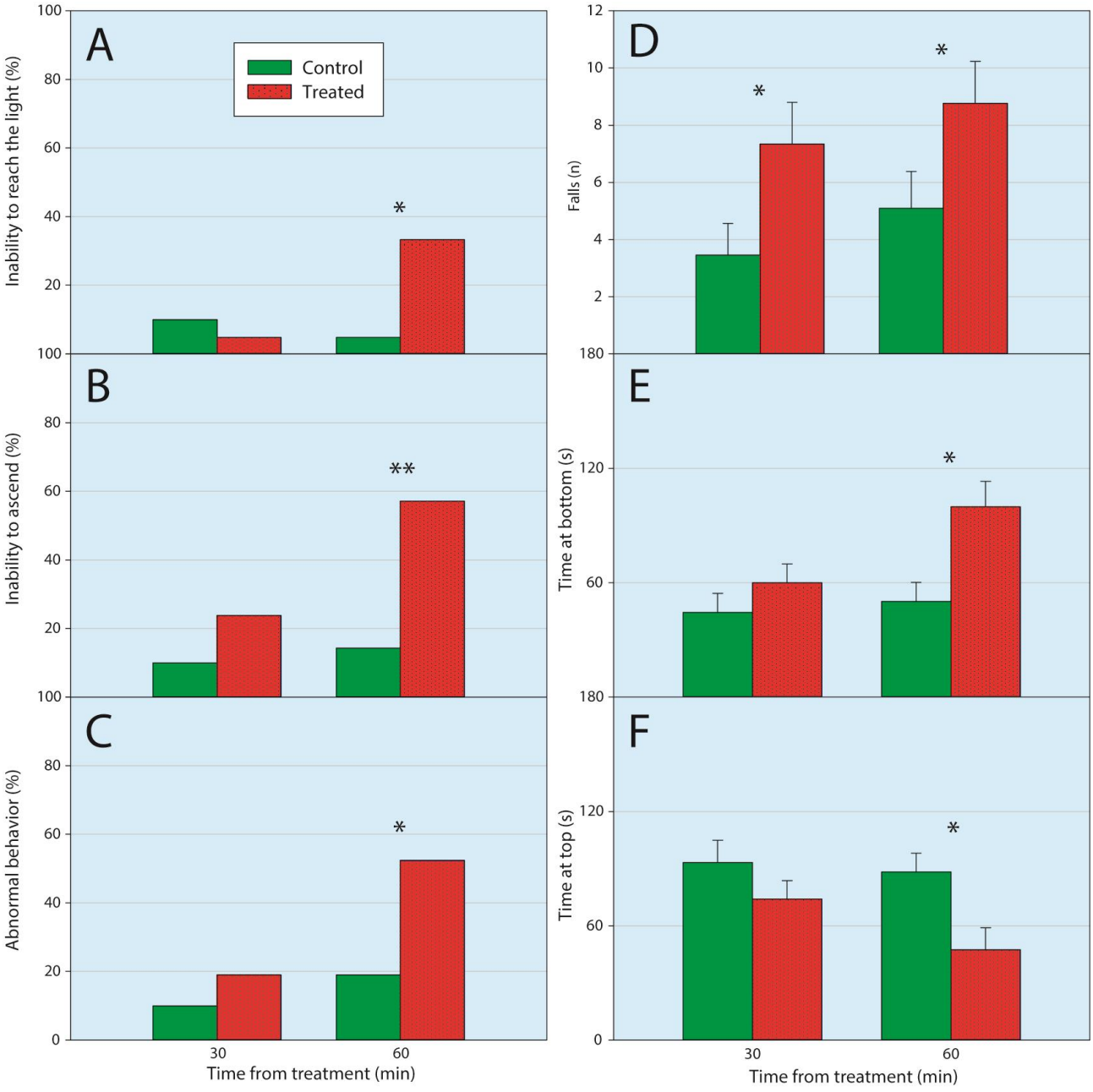

Fig. 2.4. The effects of a single dose (Exp. 1) of thiamethoxam (1.34 ng/bee, red dotted columns) on forager motor functions, 30 (left bars) and 60 (right bars) minutes after treatment. (A) Inability to reach the light, (B) inability to ascend the arena, (C) presence of abnormal behaviours, (D) number of falls per bee, (E) time at bottom and (F) time at top were assessed. Asterisks indicate statistically significant differences between treatments within time assessments (left 3 graphs: Nominal logistics; right 3 graphs: GLMs; * $p<0.05$, ** $p<0.01$, *** $p<0.001$). 30 min: $N_{control} = 20$, $N_{pesticide} = 21$; 60 min: $N_{control} = 21$, $N_{pesticide} = 21$. Error bars are SE.

There was a significant effect of treatment on the number of falls recorded per bee, both at 30 and 60 minutes post-treatment (Fig. 2.4D): treated bees fell more often than control ones.

There was a significant effect of treatment on the amount of time spent at the bottom (Fig. 2.4E) and at the top (Fig. 2.4F) of the phototaxis arena, 60 minutes after treatment. Specifically, treated bees spent less time at the top and more time at the bottom of the arena, compared to controls. There was no significant effect of treatment on the amount of time spent at the bottom or top of the arena 30 minutes post-treatment.

2.3.2 Experiment 2: Chronic exposure

There was no significant effect of *treatment*, *time* and interaction *treatment*time* on the majority of the variables assessed. However, there were significant effects of treatment on the inability to ascend and distance bees covered to reach the light at 60 min post-treatment (Tab. 2.3, Fig. 2.5A-B).

Tab. 2.3. Means (SE) of the variables assessed after 2-day chronic exposure to thiamethoxam (Exp. 2) in relation to time (30 and 60 minutes post-treatment) and treatment (0 or 1.34 ng/bee of thiamethoxam). Asterisks indicate statistically significant differences between treatments within time assessments (GLMs or Nominal logistics, * p<0.05, ** p<0.01, *** p<0.001).

Variable group	Predicted variable	30 minutes post-treatment				60 minutes post-treatment			
		Control (N=21)	Pesticide (N=15)	L-R χ^2	p value	Control (N=21)	Pesticide (N=15)	L-R χ^2	P value
First path to light	Distance to light (squares)	12.37 (2.04)	11.73 (1.65)	0.01	0.9557	15.17 (1.85)	9.83 (0.86)	6.278	0.0122*
	Time to light (s)	34.89 (6.2)	29.73 (10.12)	0.05	0.8302	44.94 (9.66)	27.58 (8.9)	2.25	0.1333
	Speed to light (sq. cross/s)	0.50 (0.08)	0.57 (0.07)	0.08	0.7773	0.48 (0.06)	0.49 (0.06)	0.11	0.7454
Hyperactivity	Time moving (s)	112.29 (10.69)	128.00 (10.12)	0.36	0.5461	121.10 (10.49)	128.67 (13.37)	0.63	0.4289
	Overall speed (sq. Cross/s)	0.35 (0.05)	0.35 (0.06)	0.01	0.9915	0.41 (0.06)	0.36 (0.06)	0.02	0.8812
Motor function	Inability to reach light (n)	2	10	2.39	0.1225	3	9	0.34	0.5593
	Inability to ascend (n)	1	3	1.67	0.1959	0	3	4.39	0.0362*
	Abdnormal behavior (n)	1	0	0.65	0.4216	1	3	1.74	0.1875
	Falls (n)	5.14 (1.01)	7.13 (1.62)	0.29	0.5923	5.62 (1.1)	7.67 (1.37)	1.3	0.2544
	Time bottom (s)	54.52 (9.85)	67.87 (15.04)	0.23	0.6350	65.76 (9.52)	78.13 (15.28)	0.18	0.6756
	Time top (s)	82.19 (9.84)	67.13 (13.16)	0.31	0.5785	62.24 (8.81)	56.40 (10.47)	0.04	0.8479

There was no significant correlation of dose of thiamethoxam assumed and the majority of the variables assessed. However, there was a significant correlation overall

between the dose of thiamethoxam assumed and (i) number of falls and (ii) time to reach the light (Tab. 2.4, Fig. 2.5C-D).

Tab. 2.4. Relationship between the actual dose of thiamethoxam consumed by bees during the 2 days incubation (Exp. 2) and each predicted variable, in relation to time and treatment (Spearman correlation, * p<0.05, ** p<0.01, *** p<0.001). Asterisks indicate statistically significant differences between treatments within time assessments (Spearman correlation, * p<0.05, ** p<0.01, *** p<0.001). $N_{30\ min}$ = 36, $N_{60\ min}$ = 36; $N_{overall}$ = 72.

Variable group	Predicted variable	30 min post-treatment		60 min post-treatment		Overall	
		ρ	p value	ρ	p value	ρ	p value
First path to light	Distance to light (squares)	-0.0098	0.9591	-0.3013	0.1056	-0.1694	0.1958
	Time to light (s)	-0.2210	0.2405	-0.2714	0.1469	-0.2705	0.0366*
	Speed to light (sq. cross/s)	0.3090	0.0966	0.0263	0.8905	0.1550	0.2370
Hyperactivity	Time moving (s)	0.2543	0.1345	0.1554	0.3655	0.2001	0.0920
	Overall speed (sq. Cross/s)	0.0331	0.8479	-0.1122	0.5148	-0.0389	0.7455
Motor function	Falls (n)	0.1875	0.2734	0.2599	0.1259	0.2351	0.0468*
	Time bottom (s)	0.0707	0.6818	0.0406	0.8142	0.0731	0.5419
	Time top (s)	-0.1638	0.3397	-0.0734	0.6706	-0.1219	0.3077

2.3.2.1 First path to light

There was a significant overall effect of treatment on the distance covered by foragers to reach the light (Fig. 2.5A).

There was a significant overall negative correlation between the time foragers spent to reach the light and the average dose of thiamethoxam they consumed (Fig. 2.5C).

2.3.2.2 Motor functions

There was a significant effect of treatment on the inability of the bees to ascend the arena 60 min post-treatment (Fig. 2.5B). Treated bees were more likely unable to climb the walls than control ones.

There was a significant overall positive correlation between the average dose of thiamethoxam consumed by the bees and the number of times they fell (Fig. 2.5D).

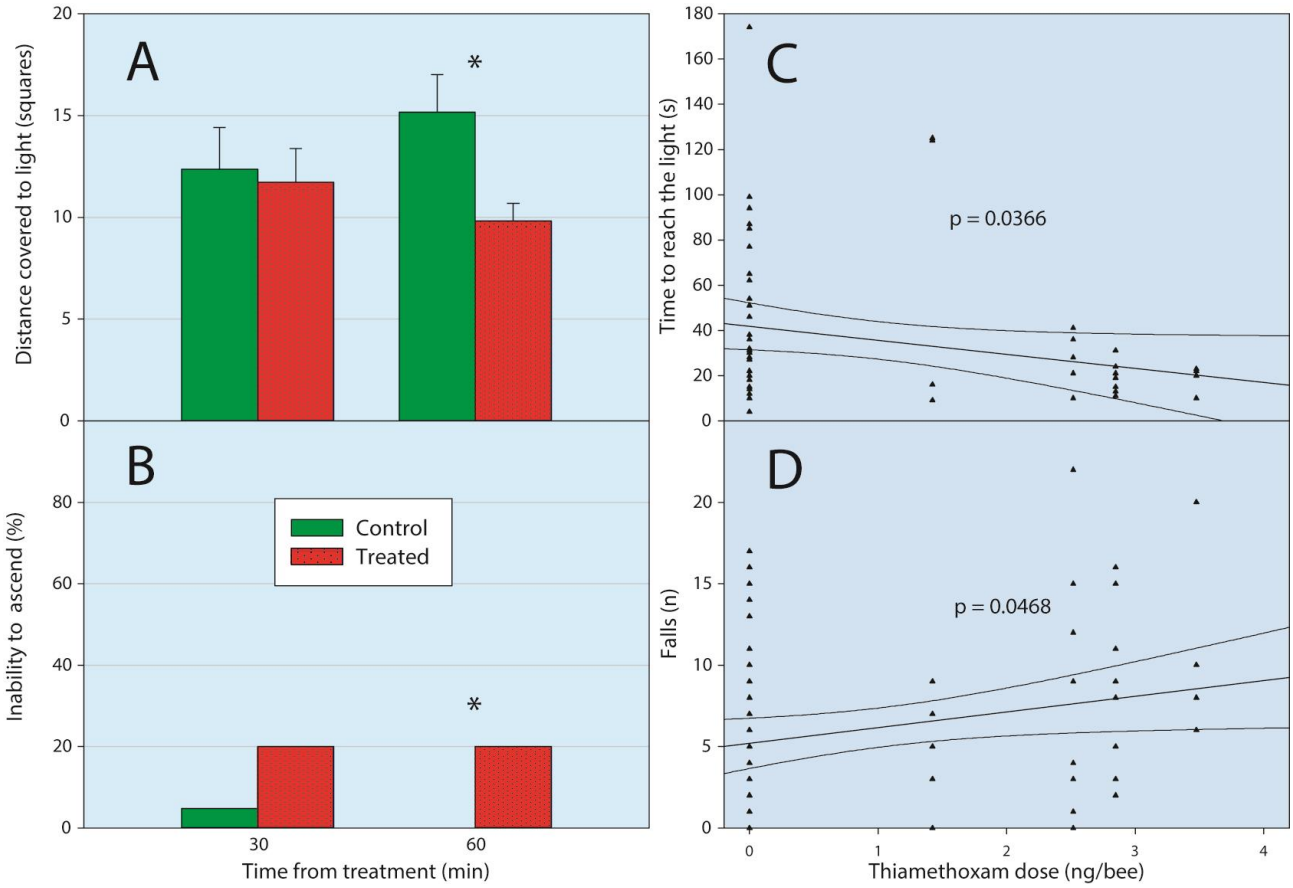

Fig. 2.5. Effect of treatment on forager (A) distance covered to reach the light and (B) inability to ascend, 30 (left bars) and 60 (right bars) min after treatment. Correlation between the actual dose of thiamethoxam consumed by foragers during the 2 days incubation (Exp. 2) and (C) time to reach the light for the first time and (D) number of falls, overall. $N_{control}$ = 21, $N_{pesticide}$ = 15; N_{corr} = 72. In A and B asterisks indicate statistically significant differences between treatments (GLMs, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). In C and D, the 95% confidence intervals are showed (Spearman correlation).

2.4 Discussion

Our study showed that sublethal exposures to thiamethoxam significantly influenced a variety of locomotion behaviours of foragers tested using a phototaxis arena.

Bees treated with a single dose (Exp. 1) were significantly faster and spent more time moving than control bees (Fig. 2.3), suggesting hyperactivity is a symptom of thiamethoxam intoxication (Scheiner et al., 2013). The effects of thiamethoxam on these variables were clearly evident 30 min after treatment but disappeared at 60 min. Such hyperactivity is a typical effect of certain pesticides, like neonicotinoids, in insects (Belzunces et al., 2012). For example, acute exposures to the neonicotinoid imidacloprid (range of tested doses: 0.1-1000 ng/bee) rapidly elicited hyperactivity symptoms that later disappeared (Guez et al., 2003; Medrzycki et al., 2003; Suchail et al., 2001). Hyperactivity was also exhibited by worker bees

after topical application of 100 and 500 ng/bee of the neonicotinoid acetamiprid (i.e. increased distance covered and decreased immobile time), even if those results were not confirmed after oral applications of the same active ingredient (Hassani et al., 2008). Furthermore, shortly after an imidacloprid treatment the German cockroach (*Blattella germanica*) showed hyperresponsiveness and hyperactivity, followed by ataxia (Wen and Scott, 1997).

Foragers fed a single dose of thiamethoxam (Exp. 1) showed an impairment of several motor behaviours (Fig. 2.4). Treated bees, compared to control, fell significantly more often, were more frequently unable to ascend the arena and reach the light. Treated bees also exhibited significantly more abnormal behaviours and spent significantly more time at the bottom and less at the top of the arena. Interestingly, most of thiamethoxam impairment effects on motor functioning were significantly different from control only 60 minutes after treatment. Similarly, Hassani et al. (2008) recorded the locomotion of worker bees 60 minutes after oral and topical applications of thiamethoxam. They recorded the total distance walked, duration of immobility and number of ascents from one level of the arena to a higher one, observing that 1 ng/bee increased the distance walked by the individuals, although the results were non-significant. Their results are consistent with ours that confirm no significant effect of thiamethoxam on these variables at 60 minutes after treatment. Although, differently from this work, they did not plan any assessment 30 minutes post-treatment and at 60 minutes they did not include any motor functioning-related observations. In Exp. 1, our results showed that a single dose of thiamethoxam first lead to hyperactivity and then to a physiological impairment of motor abilities. These differential symptoms elicited by neonicotinoids with time are similar to what Lambin et al. (2001) showed. They noticed opposite effects of acute exposures of imidacloprid on motor activity depending on time. The effects on locomotion behaviour of bees that received a single dose of 2.5 ng/bee were particularly interesting: the bees spent significantly less or more time stationary compared to control respectively at 15 and 60 minutes post-treatment. Lambin et al. (2001) further observed that the increase in time spent immobile (60 min after treatment) was a consequence of the increased trembling, shaking and rolling behaviours, especially at higher doses. Therefore, thiamethoxam, like imidacloprid, impairs the physiological motor abilities of the bees 60 minutes post-treatment.

The adverse effect of thiamethoxam on forager locomotion may have adverse consequences on a variety of other essential bee behaviours, like homing and foraging flights. Specifically, Henry et al. (2012) used the same single oral dose of thiamethoxam that we used

and showed that this sublethal intoxication significantly reduced foragers homing rate and impaired colony survival. Our results, testing the same single sublethal dose they used, provide further insights on their outcome: homing and foraging impairments can be caused by the adverse effect of the neonicotinoid on the physiological motor functioning. Indeed, other neonicotinoid insecticides like imidacloprid elicit an impairment of both locomotion behaviour (Hassani et al., 2008; Lambin et al., 2001; Medrzycki et al., 2003; Williamson et al., 2014) and foraging and homing flight behaviour (Bortolotti et al., 2003; Fischer et al., 2014; Schneider et al., 2012; Yang et al., 2008).

Both acute and chronic exposure modes of thiamethoxam increased the positive phototaxis behaviour of foragers. In fact, forager bees that assumed a single sublethal dose of thiamethoxam reached the light at the top of the arena in significantly less time and shorter distances than controls (Fig. 2.2 and 2.5). Phototactic behaviours of bees depend on their locomotor behaviour (Scheiner et al., 2013), therefore this result may be interpreted as a symptom of hyperactivity at time 30 in Exp. 1, the only case in which a significant effect of treatment on hyperactivity was observed. Interestingly, phototaxis is likely to be related to the transition from in-hive tasks (i.e. a dark environment) to foraging and therefore plays a role on foraging activity and colony fitness (Beliën et al., 2009; Ben-Shahar, 2003; Page et al., 2006; Pankiw, 2003; Tsuruda and Page, 2009).

Unlike the acute treatment (Exp. 1), the chronic one (Exp. 2) showed fewer effects on foragers locomotion, although important significant effects on motor functioning were still observed: treated bees fell and showed inability to climb the arena walls more frequently than control ones (Fig. 2.5). Aliouane et al. (2008) showed no significant effects on bee locomotion after 11-days oral and topical multiple-dose intoxications by thiamethoxam (0.1 and 1 ng/bee), while Williamson et al. (2014) observed that 24-h thiamethoxam treated bees (10 nM) were more likely to lose postural control and unable to right themselves inside petri dishes. Further studies testing various behavioural parameters, concentrations and durations of exposures should be carried out, in fact neonicotinoid sublethal effects can vary widely with time, dose and other experimental methods (Guez et al., 2001; Lambin et al., 2001; Medrzycki et al., 2012; Suchail et al., 2001).

In summary, a single sublethal exposure to thiamethoxam (Exp. 1) first elicited hyperactivity symptoms which then diminished when bees showed motor impairment: a reduced ability to walk and climb. Although a continued exposure to the same neonicotinoid

(Exp. 2) caused limited significant effects on bee motor functioning, both Exp. 1 and 2 showed higher positive phototaxis of treated bees, compared to control. This study provides further evidence on the behavioural effects across time of this neurotoxin. Thiamethoxam effect on locomotion and phototaxis are likely to affect at least foraging behaviour, thus leading to detrimental consequences for individuals and colonies (Decourtye and Devillers, 2010; Henry et al., 2012). The behavioural aspects we assessed should be further investigated, testing different active ingredients, doses and concentrations, eventually on different organisms. In fact, the honey bee is just one of the numerous organisms threatened by pesticide exposure. Recently EFSA (2013b) developed guidance for the evaluation of the effect of chemicals on other pollinators like bumblebees and solitary bees. The phototaxis assay from this study can be adapted for behavioural studies of walking and phototactic locomotion in other pollinators.

CHAPTER 3

Sublethal effects of thiamethoxam on honey bee flight performances

Abstract

The common neurotoxic neonicotinoid pesticide thiamethoxam elicits a variety of sublethal effects on honey bees, including a decreased homing flight success (Henry et al., 2012). However, the effects of neonicotinoids on the physical ability of bees to fly are still uninvestigated. We tested the effects of sublethal acute and chronic exposures to thiamethoxam on the flight performances of forager bees tethered on a flight mill. Rapidly after the single sublethal exposure (1.34 ng/bee, based upon Henry et al., 2012) the treated foragers increased the duration (+71%) and distance (+66%) of their flights. Together with neonicotinoid disorientation effects shown in previous studies the probable consequence of this hyperactivity is that bees abandon the known areas at higher rates decreasing their probability to survive. A continued exposure to the neonicotinoid (32.5 and 45 ppb, for 1 and 2 days) decreased flight duration (1-d: -64%; 2-d: -34%), distance (1-d: -64%; 2-d: -34%), average velocity (only 2-d: -34%) and maximum velocity (only 2-d: -34%) (the percentages showed compare 0 and 45 ppb concentration treatments, the only significantly different treatments). The impaired flight motor abilities observed after longer exposures to the neonicotinoid are likely to cause a decreased foraging proficiency. In summary, the significant effects that thiamethoxam triggers on forager flight performances after sublethal acute and chronic exposures can affect honey bee foraging at individual and colony level.

3.1 Introduction

The honey bee *Apis mellifera* L., 1758, is a global pollinator species for managed crops and wild plants (Breeze et al., 2014; Klein et al., 2007; Schulp et al., 2014). The honey bee is also a bioindicator of environmental health (Devillers and Pham-Delègue, 2002; Svoboda, 1961) because of their high sensitivity to many plant protection products, ability to forage over large distances, variety of environmental media they encounter, body hairs to which environmental contaminants can adhere (Porrini et al., 2002), and typically serves as a surrogate for pollinators in risk assessment schemes (Alix and Lewis, 2010; OEPP/EPPO, 2010). Therefore, increased rates of widespread honey bee losses (Bacandritsos et al., 2010; Mutinelli et al., 2010; Underwood and vanEngelsdorp, 2007; vanEngelsdorp and Meixner, 2010; VanEngelsdorp et al., 2008) have raised concern about ecological stability, wild plant diversity, crop production, food security and human welfare (Chagnon et al., 2014; Potts et al., 2010). Although beekeepers can multiply colonies to overcome some of these losses (Maini et al., 2010), beekeeping is becoming increasingly difficult and expensive (Potts et al., 2009).

Multiple factors contribute to honey bee colony losses (Carreck and Neumann, 2010; Maini et al., 2010; Nazzi and Pennacchio, 2014; Oldroyd, 2007; Potts et al., 2010). Pathogens and parasites present major health challenges (Cameron et al., 2011; Carreck and Neumann, 2010; Potts et al., 2009). Xenobiotics, specifically pesticides used for plant protection, can also harm honey bee health (Desneux et al., 2007; Farooqui, 2013; Maini et al., 2010; Maxim et al., 2013; Sanchez-Bayo, 2014). Honey bees are exposed to a wide variety of pesticides both in the field (Dively and Kamel, 2012; Johnson and Pettis, 2014; Johnson et al., 2010; Krupke et al., 2012; Stoner and Eitzer, 2012) and within the hive (ApeNet, 2011; Chauzat et al., 2006; Lambert et al., 2013; Mullin et al., 2010; Smodis Skerl et al., 2009). Exposure to such multiple compounds in combination with other stressors (e.g. pathologies) can trigger elevated negative effects even when individual chemicals occur at low concentrations (Alaux et al., 2010; Di Prisco et al., 2013; Glavan, 2013; Pettis et al., 2013; Thompson, 2012; Vidau et al., 2011). Attention has focused the neonicotinoids, the most widely used systemic pesticides worldwide (Elbert et al., 2008; Jeschke et al., 2010; Simon-Delso et al., 2014), being also high toxic and persistent (Maienfisch, 2001; Tomizawa and Casida, 2005). They are widely used on many types of crops (Elbert et al., 2008; Jeschke et al., 2010) and have

been implicated in bee losses (ApeNet, 2011; BeeNet, 2013; Maxim and van der Sluijs, 2010; van der Sluijs et al., 2013).

Neonicotinoids and their degradation products are found in media collected by bees such as water (Johnson and Pettis, 2014; Samson-robert et al., 2014; Van Dijk et al., 2013), pollen and nectar (Desneux et al., 2007; Dively and Kamel, 2012; Eggen et al., 2014; Johnson and Pettis, 2014; Main et al., 2014). Generally, these compounds occur at sublethal concentrations, but nonetheless have a wide variety of neural effects (Belzunces et al., 2012; Decourtye and Devillers, 2010; Desneux et al., 2007; EFSA, 2012a; Goulson, 2013; Pisa et al., 2014; Stokstad, 2012; Wu et al., 2011) because they are agonists of insect nicotinic acetylcholine receptors (Millar and Denholm, 2007; Tomizawa and Casida, 2003). For example, neonicotinoids and their degradation products can harm at least bee foraging (Feltham et al., 2014; Schneider et al., 2012; Scholer and Krischik, 2014; Yang et al., 2008), homing (Bortolotti et al., 2003; Colin et al., 2004; Henry et al., 2012; Vandame et al., 1994), locomotion (Aliouane et al., 2008; Hassani et al., 2008; Medrzycki et al., 2003; Williamson et al., 2014), navigation (Fischer et al., 2014; Vandame et al., 1995), learning and memory (Decourtye et al., 2004a; Decourtye et al., 2004b; Decourtye et al., 2005; Eiri and Nieh, 2012; Gauthier, 2010; Hassani et al., 2005; Hassani et al., 2008; Ping-Li et al., 2013; Yang et al., 2012) and communication (Eiri and Nieh, 2012; Kirchner, 1998; Medrzycki et al., 2003).

Neonicotinoid use has been temporarily restricted in Europe (EFSA, 2012b; Gross, 2013) in pre-flowering (e.g. seed treatment), as a consequence of their effects on bees and environment. However, they are still widely used worldwide, thus the sublethal impacts of these compounds deserves further study. The sublethal effects of imidacloprid, the first neonicotinoid inserted in the market in 1991 (Tomizawa and Casida, 2011) are better understood than thiamethoxam, a second generation neonicotinoid that is highly effective and persistent (Maienfisch, 2001). We therefore focused on the effects of thiamethoxam, the second widely commercialized neonicotinoid and one of the most widely used (Elbert et al., 2008; Jeschke et al., 2010; Maienfisch, 2001; Maienfisch et al., 2001; Main et al., 2014; Simon-Delso et al., 2014). Thiamethoxam is found in various environmental media (BeeNet, 2013; Dively and Kamel, 2012; Main et al., 2014; Stoner and Eitzer, 2012) and is also linked to colony losses (ApeNet, 2011; EFSA, 2013a; Tremolada et al., 2010).

Henry et al. (2012) demonstrated that thiamethoxam reduces the return rate of forager bees and consequently impairs colony survival. Subsequently, researchers demonstrated that

sublethal doses of three different neonicotinoids (clothianidin, imidacloprid, and thiacloprid) impaired honey bee navigation (Fischer et al., 2014). Thiamethoxam may similarly impair navigation (Fourrier et al., 2009; Stanley and Raine, 2014), but we hypothesized that it may impair the physical ability of bees to fly. Bees forage by flying, and flight therefore plays an essential role in colony fitness. We tested the effect of acute and chronic doses (based upon Henry et al. 2012 and EFSA, 2012a) and flew tethered bees on flight mill (Jungmann et al., 1989; Scheiner et al., 2013; Smith and Jones, 2012; Sotavalta, 1954) to restrict the effects of environmental variability on flights (Church, 1959; Coelho, 1991a; Scheiner et al., 2013).

3.2 Materials and methods

This study was conducted in 2013 and 2014 at University of California San Diego (UCSD), Division of Biological Sciences (La Jolla, CA, USA).

3.2.1 Honey bee preparation

Returning forager honey bees were collected at the hive entrance of colonies of *Apis mellifera ligustica* Spinola, 1806, located at the UCSD Biological Field Station. A total of 19 colonies were used.

After collection, forager bees were placed into plastic cages (11 x 11 x 9 cm) in groups of 10 and maintained in an incubator at 30±1°C and 60-80% RH. A 1.8 M sucrose water solution was available to the bees for the whole duration of the incubation period.

After incubation, foragers were harnessed. Bees were minimally chilled on ice to reduce their motion. A wire grid (6.5 mm squares) was placed lightly on top of each bee to restrain it during the gluing process. The hairs on the thorax of each forager bee were gently removed using a flat wood toothpick, so a 1 cm-long Teflon tube (AWG22, 0.71 mm inner diameter) could be firmly attached to the thorax using a minimal quantity of contact cement glue (DAP® Weldwood®, Baltimore, Maryland, USA). Correct gluing is the most critical part of this process. Preliminary testing with strengthened cyanoacrylate adhesive or Pattex contact adhesive (Scheiner et al., 2013) showed that the DAP Weldwood provided a superior bond with minimal adhesive between the bees and our plastic tubes.

After harnessing, each bee was individually placed in a cage inside an incubator for 40 minutes at 30±1°C, 60-80% RH, before its flight performances were tested. This allowed the bees to calm down after the harnessing procedure.

3.2.2 The flight mill

We built a modified flight mill (FM, Fig. 3.1) based upon the designs and software provided (Smith and Jones, 2012).

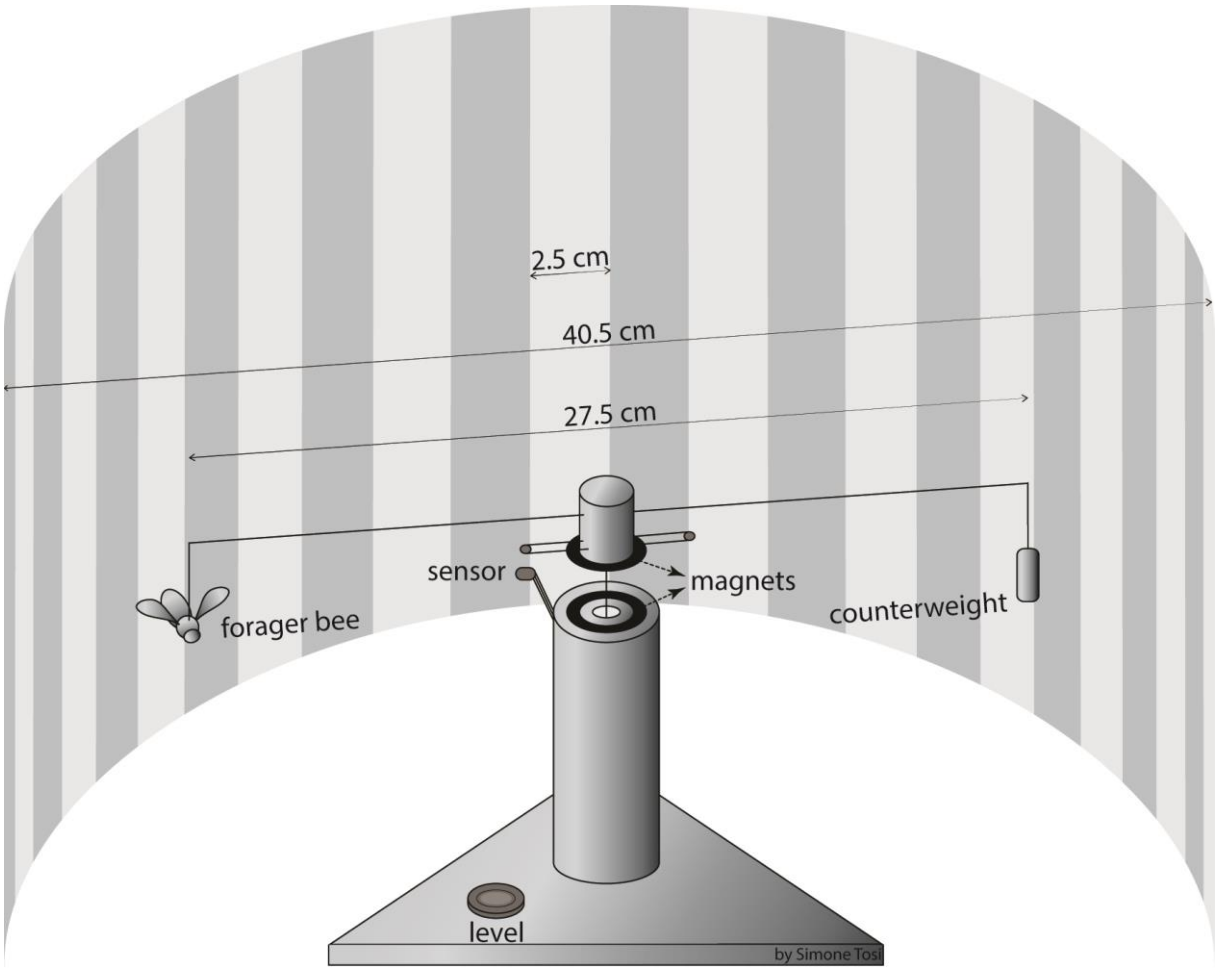

Fig. 3.1. The flight mill used to test the flight performances of tethered forager bees.

The FM allows a tethered bee to fly, using its own power, on a counter-weighted arm (27.5 cm long). This lightweight arm is designed to minimize friction and floats on a magnetic cushion. A fine needle inserted into low-friction Teflon bearing keeps the arm centred and a Hall effect magnetic sensor records each revolution of the arm, transmitting a voltage pulse per rotation that is recorded using LabView software on a desktop PC. The duration of each circuit then allows the calculation of the displacement, velocity, and acceleration per circuit and over the entire flight.

To provide consistent visual feedback, the flight mill is surrounded by 40.5 cm diameter paper cylinder with laser-printed 2.5 cm wide vertical stripes alternating black and

white (100% contrast, 2.5 cm spatial period), yielding a 6.5 cm separation between the bee and cylinder wall. The original mill (Smith and Jones, 2012) was designed to fly small moths. Our primary modification is the use of a fine plastic tube to attach bees to the flight mill arm (see above) and adding a light emitting diode on the flight mill that flashes each time the Hall sensor transmits a pulse. This allows the operator to easily confirm that the sensor is correctly triggered by each pass of the flight arm.

All flight mills were located in the same room maintained at 22±1 °C and 50-70% RH to guarantee the same environmental conditions during the flights. In fact, flight performances can be influenced by various factors like external temperature (Esch, 1976; Esch, 1988; Hrassnigg and Crailsheim, 1999; Woods et al., 2005).

3.2.3 The flight assessment

After harnessing, each bee was maintained 40 minutes in the incubator and then attached to the arm of the FM. When a bee is grabbed by the Teflon tube and lifted from the ground, it instinctively starts flying (i.e. lifts its legs and flaps its wings). A small piece of paper was positioned beneath the forager legs to prevent flying behaviour before the beginning of the test. After the FM arm with the bee attached was inserted on the FM base and thus all the flight parameters could be recorded, the piece of paper was removed from the legs of the bee, which starts to fly. Experience is important to efficiently find the most appropriate flight position of the foragers, which is fundamental to guarantee optimal power transmission from the bee to the flight mill (Scheiner et al., 2013). Duration, distance, average velocity (*avgVel*) and maximum velocity (*maxVel*) of the flight were recorded.

As soon as the flight was concluded, the bee was fed 10 µL of 2 M glucose test solution. Glucose solution was preferred to sucrose to allow a faster recovery of the energy lost, since it is easily metabolized by bees (Brodschneider et al., 2009; Gmeinbauer and Crailsheim, 1993; Hrassnigg et al., 2005). After feeding, the bees were individually placed in cages and maintained in an incubator at 30±1 °C, 60-70% RH, with no food, for 40 minutes. After this period, a second and last flight on the same FM was tested.

Bees that did not fly on the FM were excluded.

Two experiments testing different modes of exposures of thiamethoxam (hereafter also TMX) were carried out.

3.2.3.1 Experiment 1: Acute exposure

After collection, forager bees were incubated for 24 h with 1.8 M sucrose solution ad libitum.

The acute effects of a neonicotinoid pesticide were tested feeding the bees a sublethal dose of thiamethoxam between the two flights. Thus, the flight performances of control and treated bees were recorded both before and after treatment.

Immediately after the first flight was concluded, the bees were administered 10 µL of 2 M glucose solution either pure or containing 1.34 ng of thiamethoxam (the latter corresponding to 118 ppb, 134 µg/L and 459 nmol/L). The density of 2 M glucose solution at 20°C and 1 ATM (1.131 kg/L) was used to calculate the concentration. This sublethal dose is 3.7 times lower than the LD50 dose of thiamethoxam, which is 5 ng/bee (EFSA, 2013a) and does not elicit any significant difference in mortality from control (Henry et al., 2012). This dose was chosen to replicate the one used by Henry et al (2012) in their homing flight experiment, thus allowing comparisons between the results of the two studies.

In total, 42 bees were tested (84 flights).

3.2.3.2 Experiment 2: Chronic exposure

After collection, forager bees were incubated with 1.8 M sucrose solution ad libitum containing either 0, 32.5 or 45.0 ppb of thiamethoxam, corresponding respectively to 0, 40, 55 µg/L and 0, 137, 190 nmol/L. The density of 1.8 M sucrose solution at 20°C and 1 ATM (1.230 kg/L, Bubník and Kadlec, 1995) was considered to calculate the concentrations.

The consumption of sucrose solution per cage was evaluated daily, hence the consumption per bee could be calculated indirectly dividing the daily total consumption of sucrose solution per cage by the number of alive bees in that day. Therefore, the amount of TMX ingested by each bee was determined. EFSA (2012a) calculated that each forager bee could assume in the worst case scenario daily doses of thiamethoxam up to 5.89 ng. While for this evaluation EFSA considered 5.2 ppb as the highest thiamethoxam nectar concentration found in the field (*Phacelia*), Dively and Kamel (2012) reported higher concentrations of thiamethoxam in the nectar (15 ppb), suggesting that EFSA calculations underestimate the dose of active ingredient the bees can be exposed to. Indeed, there is ongoing debate about what doses and concentrations should be considered field-relevant and "it is likely that there is a wide range of these values over space and time" (Pisa et al., 2014; van der Sluijs et al.,

2013). Within this context, the daily doses assumed by the bees in this experiment were always lower than the limit suggested by EFSA (5.89 ng/bee), in both the low and high pesticide treatments. According to the concentration tested, at day 1 the bees consumed respectively 2.68±0.20 and 3.72±0.19 ng of thiamethoxam daily, while at day 2 respectively 2.47±0.07 and 3.46±0.07 ng, in average. As a consequence, the actual doses assumed by the bees in this experiment can be considered field-relevant. In addition, exposure to even higher concentrations of thiamethoxam has been reported (BeeNet, 2013) and since bees can collect guttation droplets exuded from the leaves of corn plants (Girolami et al., 2009).

The bees were divided in two groups, which were tested after either one or two days of incubation. The bees were flown once in the one day trial and twice in the two days trial.

Differently from Exp. 1, all flight performances were evaluated after the pesticide treatment. In total, 223 bees were tested.

3.2.4 Statistical analysis

3.2.4.1 Experiment 1: Acute exposure

Repeated measures ANOVA (JMP v10.0, SAS statistical software) REML algorithm was used to test the effects of colony (random effect), treatment group (bees fed thiamethoxam at *0* or *1.34 ng/bee*), flight period (*before* or *after* treatment) and the interaction treatment group*flight period on *duration*, *distance* and *avgVel* of the two consequent flights of the bees.

A log transformation on distance covered and duration of the flight was applied to normalize the data.

Significant effects were further analysed with contrast post hoc comparisons.

3.2.4.2 Experiment 2: Chronic exposure

Repeated measures ANOVA (JMP v10.0, SAS statistical software) REML algorithm was used to test the effects of colony (random effect), sucrose consumption during incubation, flight number (*first* or *second* flight), treatment (thiamethoxam at *0*, *32.5*, *45 ppb*) and their interactions on d*uration*, *distance*, *avgVel* and *maxVel*. At Day 1, since only one flight was recorded, the flight period effect was not included in the model. A log transformation of distance covered and duration of the flight was applied to normalize the data. Significant

effects were further analysed with Tukey honestly significant difference (HSD) post hoc multiple comparison ($p < 0.05$).

We used a Spearman rank correlation to analyse the relationship between of the actual dose of thiamethoxam consumed by the bees during incubation and their flight *duration*, *distance*, *avgVel* and *maxVel*.

Kruskal-Wallis Rank Sum test was used to assess the effect of treatment on sucrose consumption. Wilcoxon Each Pair tests were computed to further analyse differences among different concentrations.

3.3 Results

Throughout this paper, we report data as means ± SE.

3.3.1 Experiment 1: Acute exposure

The first flight within Exp. 1 recorded the performances of the bees (both *treated* and *control* treatment groups) *before* the actual treatment, while the second flight was recorded *after* the treatment. Thus, only the *after* flight of the *treated* bees was under the influence of thiamethoxam. For this reason, we focus on the interaction *treatment group*flight period*.

3.3.1.1 Flight duration

There was a significant effect of the interaction *treatment group*flight period* on *duration* (Fig. 3.2C, $F_{1,39} = 2.33$, $p = 0.0460$).

As expected, contrast post hoc comparisons showed that *duration* of *control* and *treated* bees were not statistically different *before* they were fed the test solution ($F_{1,68} = 0.0148$, $p = 0.9035$), while *after* the *treated* bees flew longer ($F_{1,73} = 5.61$, $p = 0.0205$) than *control* ones.

Furthermore, *duration* of *control* bees were almost identical *before* and *after* they were fed the test solution ($F_{1,40} = 0.0001$, $p = 0.9919$), while *treated* bees flew significantly more time ($F_{1,37} = 10.08$, $p = 0.0030$) *after* they were fed the pesticide.

Specifically, bees that did eat thiamethoxam flew in average 71% longer than controls in the second flight.

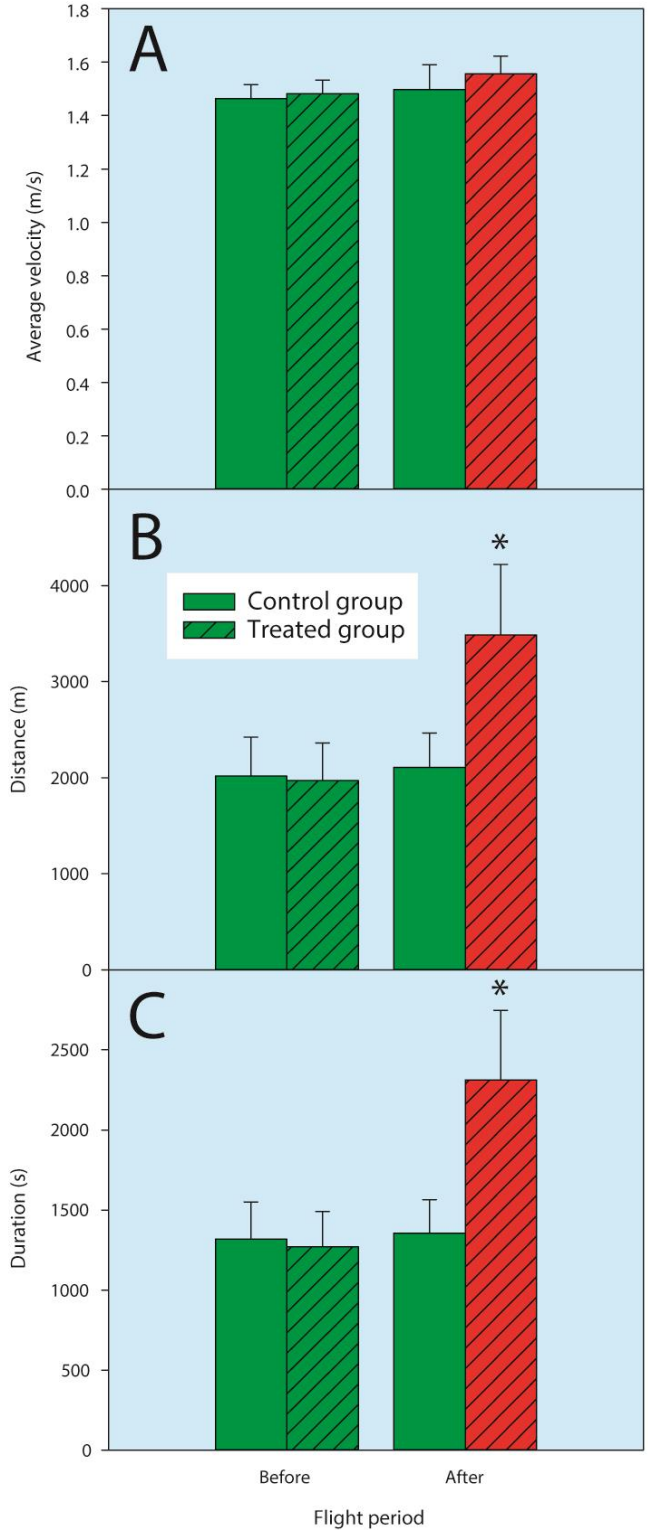

Fig. 3.2. The effect of a single dose (Exp. 1) of thiamethoxam (1.34 ng/bee) on flight (A) average velocity, (B) distance and (C) duration, before (left bars) and after (right bars) treatment. Solid and dashed columns indicate respectively the untreated or treated group of bees. The red column highlights the flight performances of the only bees that assumed thiamethoxam. Asterisks indicate significant differences compared to all other groups (Repeated measures ANOVA $_{REML}$, Contrast, $p < 0.05$). $N_{control} = 18$, $N_{treated} = 24$. Error bars are SE.

3.3.1.2 Flight distance

There was a significant effect of the interaction *treatment group*flight period* on *distance* (Fig. 3.2B, $F_{1,39} = 4.25$, p = 0.0448).

As expected, contrast post hoc comparisons showed that *distance* of *control* and *treated* bees were not statistically different *before* they were fed the test solution ($F_{1,68}$ = 0.0142, p = 0.9055), while *after* the *treated* bees flew farther ($F_{1,73} = 5.65$, p = 0.0200) than *control* ones. Furthermore, *distance* of *control* bees were almost identical *before* and *after* they were fed the test solution ($F_{1,40}$ = 0.0026, p = 0.9594), while *treated* bees flew significantly longer distances ($F_{1,37} = 9.90$, p = 0.0032) *after* they were fed the pesticide.

Specifically, pesticide treated bees flew in average 66% farther than control in the second flight.

3.3.1.3 Flight velocity

There was no significant effect of treatment on flight velocity, either overall or in interaction with the other variables (Fig. 3.2A). *MaxVel* was not calculated.

3.3.2 Experiment 2: Chronic exposure

3.3.2.1 Flight duration

One day trial

There was a highly significant effect of thiamethoxam on *duration* ($F_{2,39} = 5.85$, p = 0.0060). *Post hoc* Tukey HSD_{REML} test showed that the bees fed the high concentration of thiamethoxam (45 ppb) flew significantly shorter time than control and low concentration (32.5 ppb) bees (Fig. 3.3A, $p < 0.05$), while no significant differences were observed between the latter ones.

Spearman correlation confirmed that the duration flown by the bees was significantly related to the actual dose of thiamethoxam they assumed and that *duration* decreased with the increase of pesticide assumed by the bees (Fig. 3.3B, r_s (95) = -0.28, p = 0.0043).

Overall, low and high thiamethoxam concentrations bees flew in average 9 and 64% shorter time compared to control, respectively.

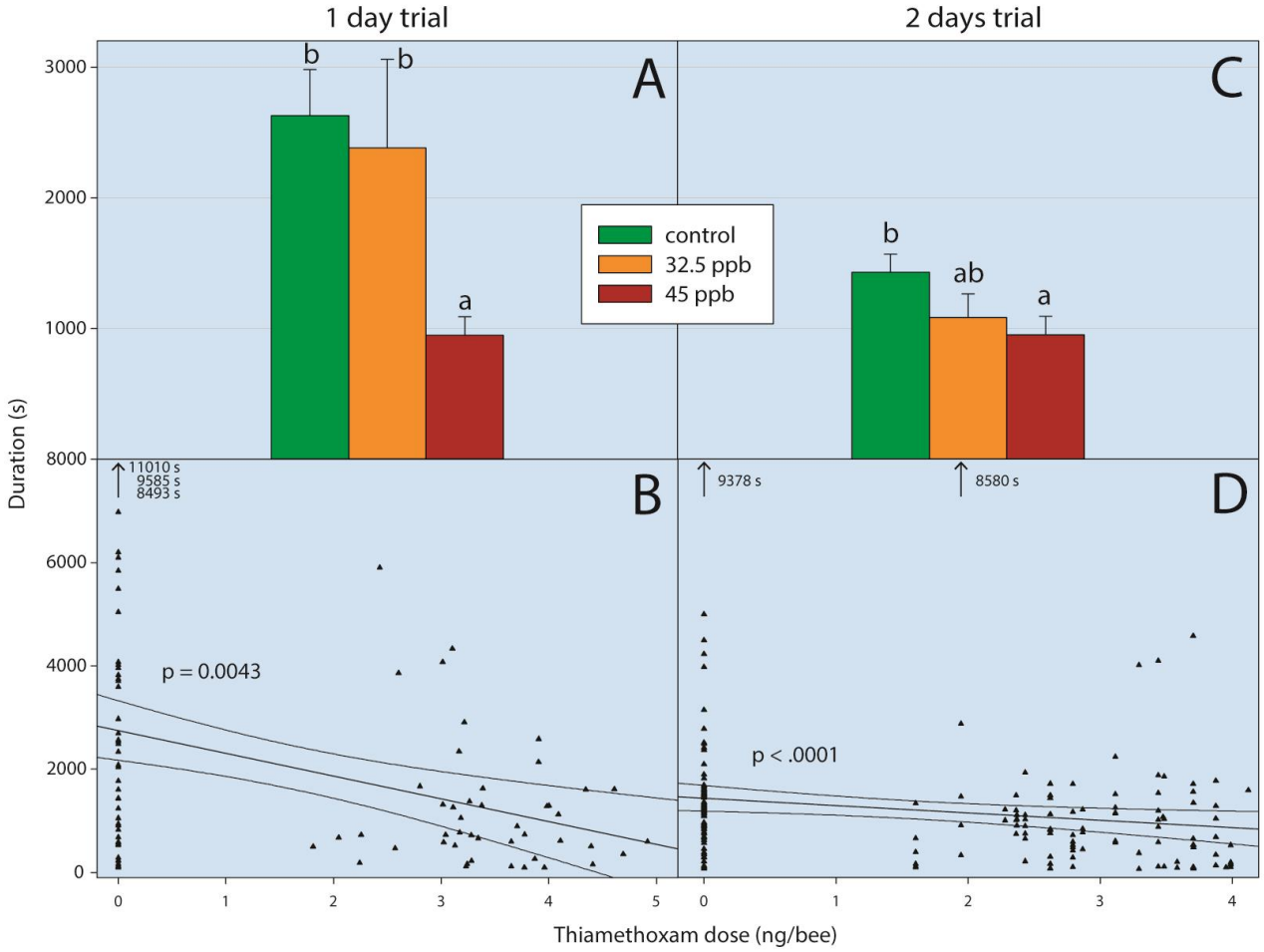

Fig. 3.3. Flight duration of foragers chronically exposed to thiamethoxam in relation to (A, C) the concentration received (0, 32.5 and 45 ppb) and (B, D) the actual dose consumed. The results showed were obtained after (A, B) one and (C, D) two days of exposure. The bees were tested twice in the two days trial, but since there was no statistical difference of neither *flight number* nor interaction *treatment*flight number* the data were pooled. In A and C the effect of treatment was statistically significant overall and different letters indicate significant differences between treatments (Repeated measures ANOVA $_{REML}$, Tukey HSD, $p < 0.05$). In B and D, the 95% confidence intervals are showed (Spearman correlation). One day trial: $N_{control} = 46$, $N_{32.5\ ppb} = 17$, $N_{45\ ppb} = 32$, $N_{corr} = 95$; two days trial: $N_{control} = 85$, $N_{32.5\ ppb} = 47$, $N_{45\ ppb} = 51$, $N_{corr} = 183$. Error bars are SE.

Two days trial

Treatment had a highly significant overall effect on *duration* ($F_{2,76} = 49.437$, $p = 0.0096$). *Post hoc* Tukey HSD_{REML} comparison showed that the high treatment bees flew significantly shorter time than control (Fig. 3.3C, $p < 0.05$), while no significant difference was observed between low and all other concentrations.

There was no significant effect of both flight number overall ($F_{1,72} = 2.09$, $p = 0.1531$) and its interaction with treatment ($F_{2,72} = 0.17$, $p = 0.8446$) on *duration*, suggesting that flight behaviour is consistent between the 2 tested flight.

Spearman correlation confirmed that the *duration* was significantly related to the dose of thiamethoxam they assumed. In fact, with the increase of the dose of thiamethoxam assumed by the bees the duration of the flights decreases (Fig. 3.3D, r_s (183) = -0.29, p < .0001).

Overall, bees that ate low and high concentrations of thiamethoxam flew respectively 24 and 34% shorter compared to control, in average.

3.3.2.2 Flight distance

One day trial

There was a highly significant effect of thiamethoxam on *distance* ($F_{2,57}$ = 5.04, p = 0.0097). In particular, bees fed the high pesticide dose flew significantly shorter distances compared to control (Fig. 3.4A, Tukey HSD_{REML}, $p < 0.05$).

Spearman correlation confirmed that *distance* was significantly related to the actual dose of thiamethoxam they assumed; in particular, *distance* decreased with the increase of pesticide assumed by the bees (Fig. 3.4B, r_s (95) = -0.26, p = 0.0076).

Specifically, bees fed low and high concentrations of thiamethoxam flew in average 14 and 63% shorter than control overall, respectively.

Two days trial

Treatment had a highly significant overall effect on *distance* ($F_{2,78}$ = 69.053 p = 0.0017). Bees that assumed the higher concentration of pesticide flew significantly shorter distances than control (Fig. 3.4C, Tukey HSD_{REML}, $p < 0.05$).

There was no significant effect of flight number ($F_{2,69}$ = 1.15, p = 0.2880) and its interaction with treatment ($F_{2,69}$ = 0.01, p = 0.9962) on *duration*, suggesting that flight behaviour is consistent between the 2 tested flight.

Spearman correlation confirmed that *distance* was significantly related to the dose of thiamethoxam the bees consumed. In fact, with the increase of the dose of thiamethoxam assumed the distance covered during the flights decrease (Fig. 3.4D, r_s (183) = -0.33, p < .0001).

Overall, the bees that ate low and high concentrations of thiamethoxam flew respectively 34 and 39% shorter compared to control, in average.

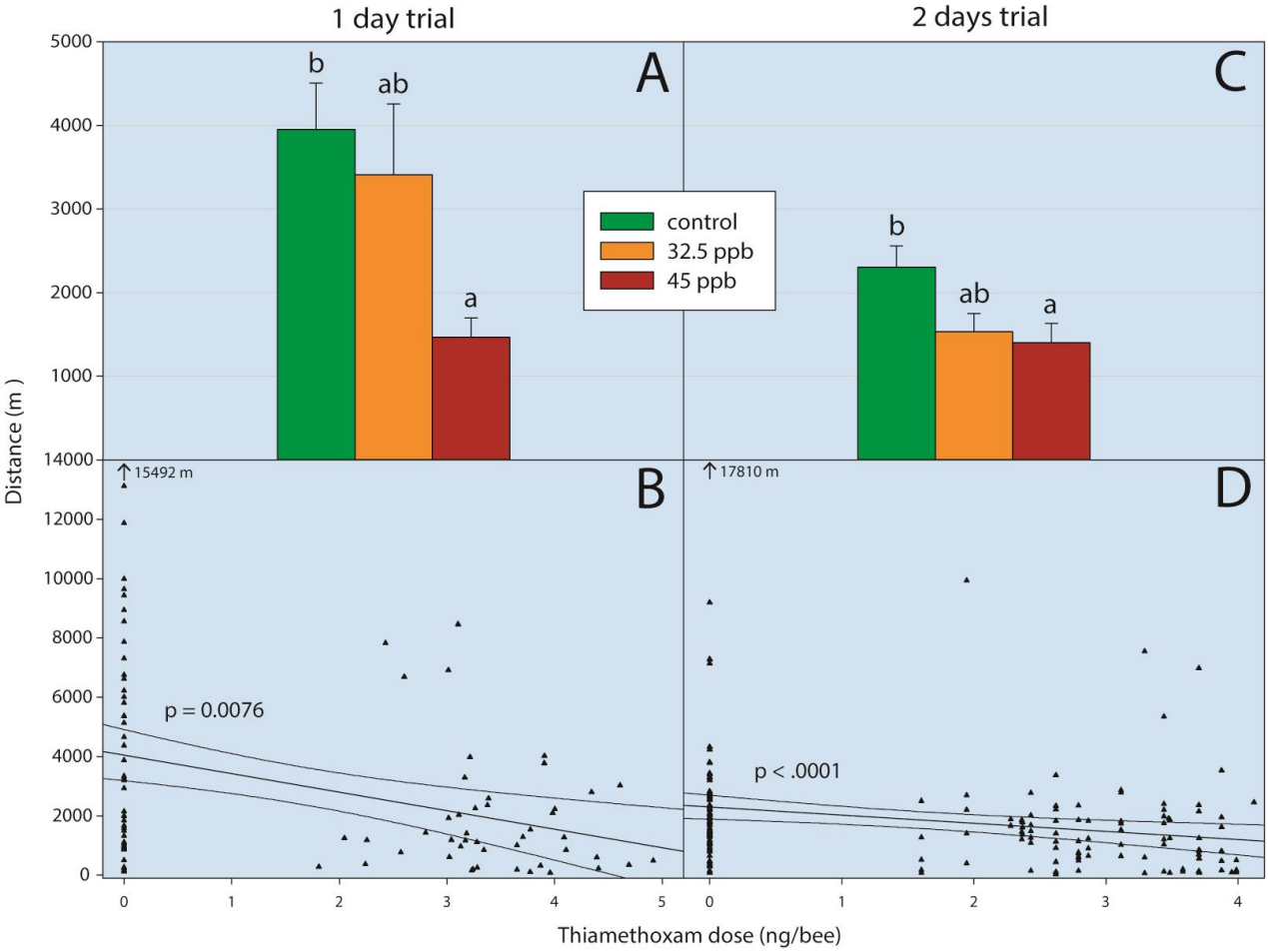

Fig. 3.4. Flight distance of foragers chronically exposed to thiamethoxam in relation to (A, C) the concentration received (0, 32.5 and 45 ppb) and (B, D) the actual dose consumed. The results showed were obtained after (A, B) one and (C, D) two days of exposure. The bees were tested twice in the two days trial, but since there was no statistical difference of neither *flight number* nor interaction *treatment*flight number* the data were pooled. In A and C the effect of treatment was statistically significant overall and different letters indicate significant differences between treatments (Repeated measures ANOVA $_{REML}$, Tukey HSD, $p < 0.05$). In B and D, the 95% confidence intervals are showed (Spearman correlation). One day trial: $N_{control} = 46$, $N_{32.5\ ppb} = 17$, $N_{45\ ppb} = 32$, $N_{corr} = 95$; two days trial: $N_{control} = 85$, $N_{32.5\ ppb} = 47$, $N_{45\ ppb} = 51$, $N_{corr} = 183$. Error bars are SE.

3.3.2.3 Average flight velocity

One day trial

After one day of exposure to the pesticide, there was no significant difference on average and maximum flight velocity ($F_{2,89} = 0.13$, $p = 0.8750$ and $F_{2,89} = 0.37$, $p = 0.6891$, respectively). Also, no significant relationship was found between the velocity of the flight and the dose of pesticide assumed.

For all bees the mean *avgVel* and *maxVel* were 1.46±0.03 m/s (~5.3 km/h) and 1.74±0.04 m/s (~6.3 km/h) overall, respectively.

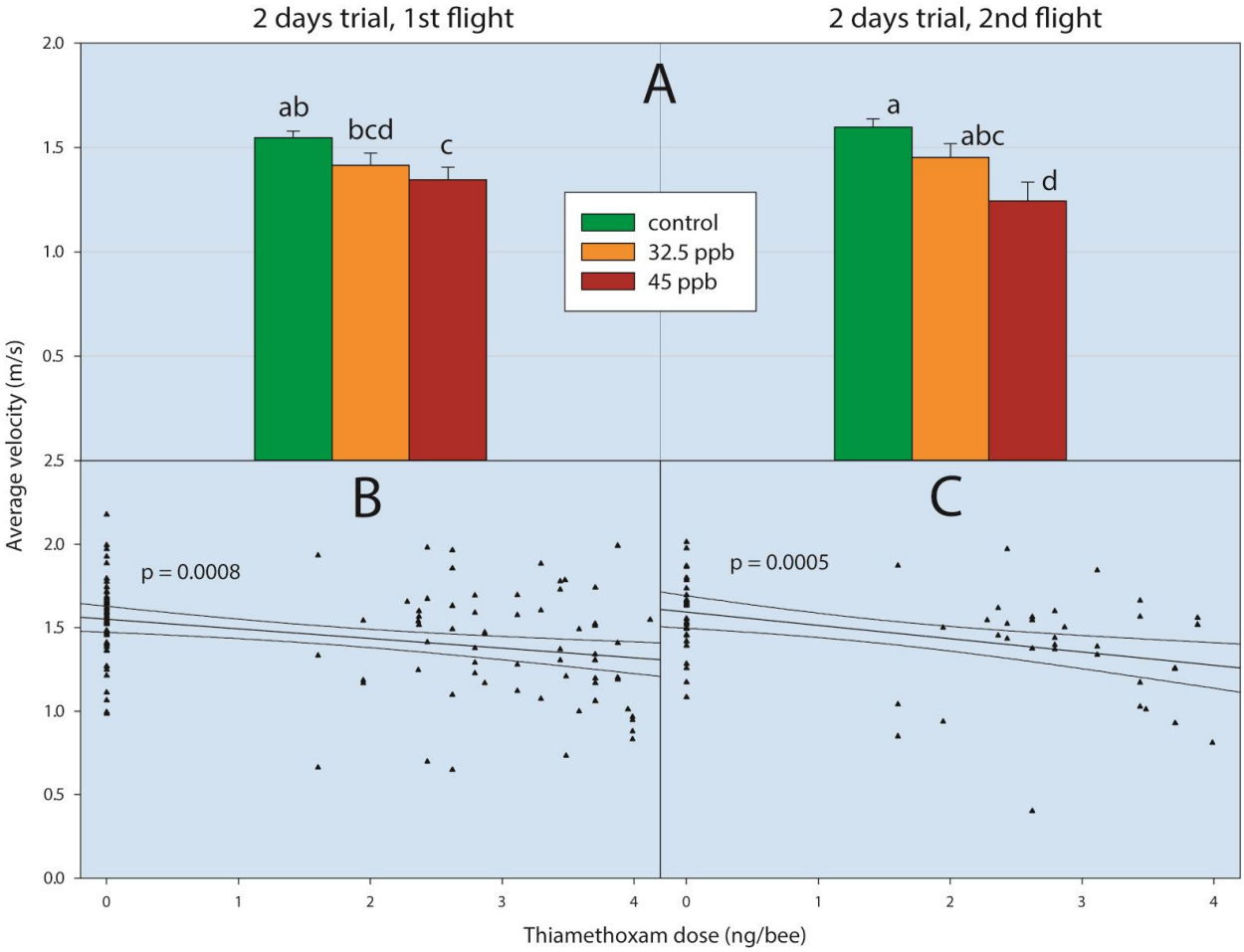

Fig. 3.**5.** Flight average velocity of foragers chronically exposed for two days to thiamethoxam in relation to (A) the concentration received (0, 32.5 and 45 ppb) and (B, C) the actual dose consumed. The results showed were obtained during the (A left bars, B) first and (A right bars, C) second flight tested. In A, there was a significant overall effect of both *treatment* and the interaction *treatment*flight number* (Repeated measures ANOVA $_{REML}$); different letters indicate significant differences (Tukey HSD, $p < 0.05$). In B and C, the 95% confidence intervals are showed (Spearman correlation). One day trial: $N_{control} = 46$, $N_{32.5\ ppb} = 17$, $N_{45\ ppb} = 32$, $N_{corr} = 95$; two days trial: $N_{control} = 85$, $N_{32.5\ ppb} = 47$, $N_{45\ ppb} = 51$, $N_{corr} = 183$. Error bars are SE.

Two days trial

Treatment had a highly significant overall effect on *avgVel* ($F_{2,32} = 72.57$, $p = 0.0025$). In particular, high treatment bees had a significantly lower *avgVel* than control ones (Tukey HSD_{REML}, $p < 0.05$, respectively Fig. 3.5A). There was no significant overall effect of *flight number* ($F_{1,72} = 2.09$, $p = 0.1531$), but there was a highly significant effect of the interaction *treatment*flight number* on *avgVel* (Fig. 3.5A, $F_{2,65} = 56.69$, $p = 0.0054$).

Spearman correlation confirmed that *avgVel* was significantly related to the dose of thiamethoxam they assumed. During the first flight, there was a highly significant negative correlation between the dose of thiamethoxam consumed and *avgVel* (Fig. 3.5B, r_s (117) = -

0.31, p = 0.0008). This relationship was consistent and even more robust in the second flight (Fig. 3.5C, r_s (66) = -0.42, p = 0.0005).

Overall, *avgVel* of bees that assumed low and high concentrations of pesticide was respectively 9 and 16% slowly compared to control, in average.

3.3.2.4 Maximum flight velocity

Two days trial

Treatment had a highly significant overall effect on *maxVel* ($F_{2,30}$ = 74.70, p = 0.0023). In particular, high treatment bees had a significantly lower *maxVel* than control bees (Tukey HSD_{REML}, $p < 0.05$, Fig. 3.6A and 6C).

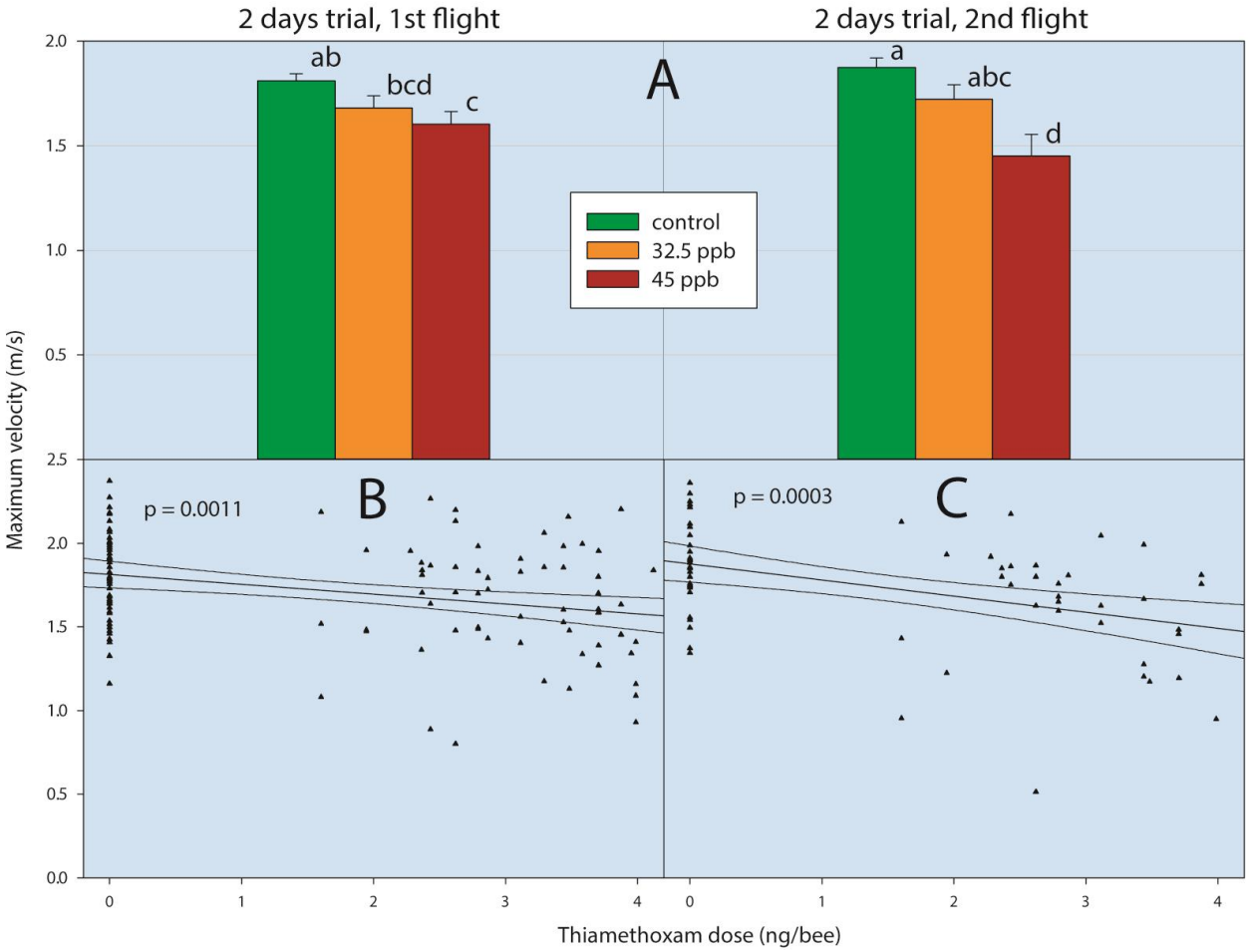

Fig. 3.6. Flight maximum velocity of foragers chronically exposed for two days to thiamethoxam in relation to (A) the concentration received (0, 32.5 and 45 ppb) and (B, C) the actual dose consumed. The results showed were obtained during the (A left bars, B) first and (A right bars, C) second flight tested. In A, there was a significant overall effect of both *treatment* and the interaction *treatment*flight number* (Repeated measures ANOVA REML); different letters indicate significant differences (Tukey HSD, $p < 0.05$). In B and C, the 95% confidence intervals are showed (Spearman correlation). One day trial: $N_{control}$ = 46, $N_{32.5\ ppb}$ = 17, $N_{45\ ppb}$ = 32, N_{corr}= 95; two days trial: $N_{control}$ = 85, $N_{32.5\ ppb}$ = 47, $N_{45\ ppb}$ = 51, N_{corr}= 183. Error bars are SE.

There was no significant overall effect of *flight number* ($F_{1,72}$ = 2.09, p = 0.1531), but there was a highly significant effect of the interaction *treatment*flight number* on *maxVel* (Fig. 3.6A and C, $F_{2,68}$ = 62.66, p = 0.0032).

Spearman correlation confirmed that *maxVel* was significantly related to the dose of thiamethoxam they assumed. During the first flight, there was a highly significant negative correlation between the dose of thiamethoxam consumed and *maxVel* (Fig. 3.6B, r_s (117) = -0.30, p = 0.0011). This relationship between *maxVel* and dose of pesticide assumed was consistent and even more robust in the second flight (Fig. 3.6C, r_s (66)= -0.44, p = 0.0003).

Overall, *maxVel* of bees that assumed low and high concentrations of pesticide was respectively 7 and 15% slowly compared to control, in average.

3.3.2.5 Sucrose solution consumption

There was a highly significant effect of treatment on sucrose solution consumption during the 2 days of incubation (Kruskal-Wallis Rank Sum test, DF = 2, p < .0001). In particular, the bees that were provided low and high pesticide concentrations consumed significantly more sucrose during the 2 days incubation than control bees (Fig. 3.7, Wilcoxon Each Pair comparison, respectively p = 0.0010 and p < .0001), while no significant difference between low and high concentration was found.

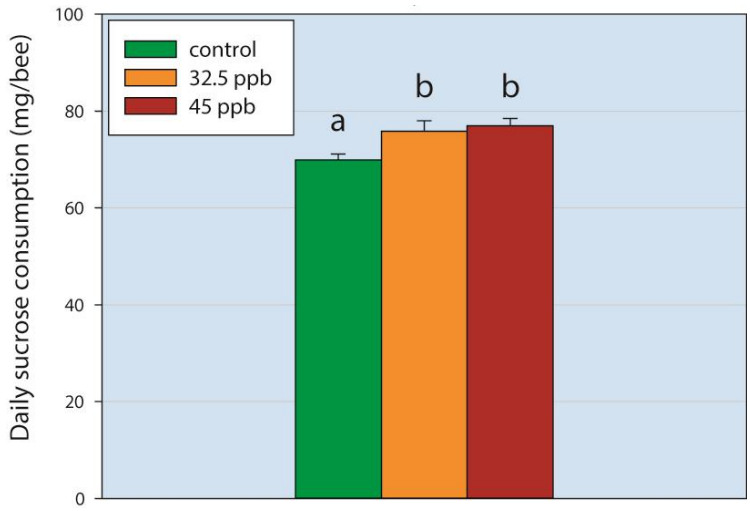

Fig. 3.7. The effect of the 2 days chronic exposure (Exp. 2) of thiamethoxam (0, 32.5 and 45 ppb) on forager 1.8 M sucrose solution consumption (Kruskal-Wallis Rank Sum test, p < 0.05). Different letters indicate statistically significant differences between treatments (Wilcoxon Each Pair test, p < 0.05). $N_{control}$ = 46, $N_{32.5\ ppb}$ = 17, $N_{45\ ppb}$ = 32. Error bars are SE.

The amount of sucrose consumed by the bees during incubation was included in all models to account for its influence on flight performances and resulted non-significant.

3.4 Discussion

Forager honey bee flight performances are influenced by sublethal acute and chronic exposures to thiamethoxam, one of the most common systemic insecticides in the world (Jeschke and Nauen, 2008; Jeschke et al., 2010; Jeschke et al., 2013; Main et al., 2014).

Foragers that assumed a single sublethal dose of thiamethoxam showed a significant rapid increase of both duration and distance flown, compared to control (Exp. 1, Fig. 3.2). Together with the alteration of flight performances, thiamethoxam has also shown disorientation effect (Fischer et al., 2014; Fourrier et al., 2009; Stanley and Raine, 2014), hence bees intoxicated by a sublethal single dose of thiamethoxam fly longer and farther but the probable consequence is that bees abandon the known areas (e.g. their colony or foraging fields), decreasing their probability to successfully return home. The same thiamethoxam dose and exposure mode we tested in Exp. 1 was used by Henry et al. (2012), which in fact showed that forager homing success and consequent colony survival were significantly reduced after the sublethal intoxication.

Forager bees that survived the first single dose intoxication with thiamethoxam can return to forage in the same field, leading to a chronic exposure to the neonicotinoid. Our results show that chronic exposures to thiamethoxam lead to a significant decrease of the duration (Fig. 3.3), distance (Fig. 3.4), average (Fig. 3.5) and maximum velocity (Fig. 3.6) of foragers flights (Exp. 2). These results were consistent when 2 consequent flights were recorded, although more evident after one day of exposure than after two. These chronic exposures to thiamethoxam are likely to impair foraging behaviour: bees would fly closer to their colony, slowly, and for shorter time, reducing their nectar/pollen collection and pollination efficacy. This reduction of bee foraging performances after a neonicotinoid treatment is consistent with previous studies (Schneider et al., 2012; Teeters et al., 2012). The most frequently visited area by foragers is conventionally considered to be about 7 km^2 (Crane, 1984; Porrini et al., 2002), which corresponds to a radius of 1.5 km from the colony. Since thiamethoxam reduces the duration and distance flown by foragers by 34 and 39% respectively, we can hypothesize a reduction of 1/3 of the radius flown around the colony, leading to a 56% reduction of the most frequently visited area. However, particular care

should be taken when results obtained in the laboratory are extrapolated to the field, thus further laboratory and field studies should be carried out to confirm this hypothesis. Certainly, a reduction of the flight area would lead to a reduced (i) pollination service, (ii) nectar and pollen weight gain and (iii) nutritional biodiversity of the collected pollen which is essential to guarantee the development of healthy bees (Black, 2006; Brodschneider and Crailsheim, 2010; Tosi et al., 2013). In addition, our results showed that bees significantly increased their sucrose solution consumption when chronically exposed to thiamethoxam (Fig. 3.7). This may lead to an increased consumption of the food stores and then to even more severe consequences at colony-level. Alteration on mobility can also increase insect vulnerability to predation in the field (Desneux et al., 2007).

Interestingly, chronic and acute exposures to thiamethoxam lead to opposite effects on bee flight behaviour. Neonicotinoid effects on bees are widely sensitive to a variety of factors and thus appear to be highly variable, even in laboratory conditions (Belzunces and Vandame, 1998; Guez et al., 2001; Iwasa et al., 2004; Lambin et al., 2001; Medrzycki et al., 2010; Medrzycki et al., 2013; Nauen et al., 2001; Pisa et al., 2014; Schmuck et al., 2001; Suchail et al., 2001; Tosi et al., 2013). Studies on the interactions between neonicotinoid pesticides and the nicotinic acetylcholine receptors (nAChR) of insects revealed different subunit compositions, locations and desensitization kinetics which unveil the complexity of the effects induced by these insecticides on honey bees (Belzunces et al., 2012; Decourtye and Devillers, 2010; Thany and Gauthier, 2005; Thany et al., 2003). The mode of action of an active ingredient is also influenced by its specific metabolism. Clothianidin is the principal metabolite of thiamethoxam in mammals, insects and plants (Ford and Casida, 2006; Karmakar and Kulshrestha, 2009; Nauen et al., 2003). It is as toxic as thiamethoxam (EFSA, 2013c) and it is used as insecticide itself (Ohkawara et al., 2002). Interestingly, Benzidane et al. (2010) showed that the effect of thiamethoxam on cockroaches locomotor activity is closely associated with the appearance of its metabolite clothianidin. The interaction between the parent compound and its metabolites may even increase the toxic effect (Simon-Delso et al., 2014), which is of particular concern since these two active ingredients are often found together in the environment (Bonmatin et al., 2014). The different mode of action of thiamethoxam metabolites may explain the different effects between exposure methods. While thiamethoxam (a second-generation neonicotinoid, Maienfisch, 2001) is a poor agonist of insects and acts differently to imidacloprid (a first-generation neonicotinoids), its

metabolite clothianidin acts on imidacloprid-sensitive nAChR1 and imidacloprid-insensitive nAChR2 subtypes (Benzidane et al., 2011; Nauen et al., 2003; Simon-Delso et al., 2014; Tan et al., 2007; Thany, 2011). Therefore, the toxicity effect elicited by the metabolites on honey bees may be "completely different" from the parent compound and the different exposure of bees to thiamethoxam and its metabolic derivates across time changes the kinetics and the effects induced (Belzunces et al., 2012). As a consequence, the mode of exposure to an insecticide influences considerably the elicited effects and is often involved in differential effects caused by a given substance (Belzunces et al., 2012). Suchail et al. (2001) showed clear differences between sublethal effects of acute and chronic exposures of a neonicotinoid and its metabolites on bees. They noticed that imidacloprid caused neurotoxicity symptoms such as hyperresponsiveness and hyperactivity rapidly after intoxication, while these symptoms gradually disappeared after several hours, when the bees become hyporesponsive and hypoactive. Comparable symptoms were noticed on *Blattella germanica*: rapidly after treatment the individuals showed hyperresponsiveness and hyperactivity, followed by ataxia (Wen and Scott, 1997). These neurotoxicity symptoms caused by the neonicotinoid imidacloprid are interestingly similar and comparable to those shown by thiamethoxam in this experiment. In the short-term thiamethoxam causes a muscular excitation (e.g. hyperactivity), while in the longer term it produces a muscular depression (e.g. hypoactivity), respectively extending (Exp. 1) and decreasing (Exp. 2) the duration, distance and velocity of the flights (average and maximum velocity are impaired in Exp. 2 only). Similar locomotion hypoactivity symptoms were recorded after a 11-d thiamethoxam chronic exposure by Aliouane et al. (2008).

Hyperactivity was noticed only shortly after intoxication in both acute and chronic experiments. We believe that the hyperactive behaviour and impaired energy metabolism (Decourtye et al., 2004b; Derecka et al., 2013), among other possible sublethal effects elicited just after the intoxication, lead the bees to deplete their nutrients and energy faster than control. As a consequence, we hypothesize that bees tried to compensate these deficits increasing the consumption of sucrose solution (Fig. 3.7), the only food available. However, after both 1 and 2 days of exposure to the pesticide the flight performances of the treated bees were still significantly weaker compared to control. Therefore, the increased sucrose intake was probably not enough to restore the nutritional value lost and/or recover from the

neonicotinoid effect. Further specific studies should be developed to confirm or not this hypothesis.

In summary, our results provide accurate insights into how a neonicotinoid pesticide affects honey bee flight performances. Rapidly after the single sublethal exposure to thiamethoxam the bees increased the duration and distance flown, while a continued and longer exposure to the neonicotinoid decreased their flight duration, distance and velocity. Thiamethoxam elicited clear opposite effects on bee behaviour in relation to its mode of exposure, similarly to what Suchail et al. (2001) noticed studying imidacloprid: our results provide evidence on this tricky mode of action of this second-generation neonicotinoid pesticide.

Flight is a central ability for all pollinators, which are however routinely exposed to pesticides. Therefore, with this study we propose the flight mill - which can be adapted for other pollinators - as a new tool for investigating the effects of pesticides on flight performances. This aspect is particularly important since the actual official guidelines for the risk assessment of plant protection products do not include tests of the effects on flight performances yet.

CHAPTER 4

Sublethal effects of thiamethoxam on honey bee thermoregulation

Abstract

Thiamethoxam is a common neonicotinoid pesticide that, as agonist of the nicotinic acetylcholine receptors, has shown to elicit a variety of sublethal effects on bees. However, information on neonicotinoid effects on bee thermoregulation is lacking. Thermoregulation abilities are essential for honey bees, specifically forager flight success depends on the regulation of the flight muscle temperature. We tested the effects that acute exposures to thiamethoxam (0.2, 1, 2 ng/bee) elicit on forager thorax temperatures exposed to alternating (Exp. 1) or constant (Exp. 2) temperature environments. Thiamethoxam influenced honey bee thorax temperature at all dose tested in different periods after treatment. In Exp. 1 (high and low temperature environment) the high dose elicited hyperthermia 60 and 120 min after intoxication. In Exp. 2 (low temperature environment), contrarily, the high and medium doses lead to hypothermia 60-80 min post-treatment and consistently the following day. Interestingly, thiamethoxam elicited inverse effects on forager thorax temperatures in relation to the environmental temperature and dose the individuals were exposed to. These thermoregulation impairments elicited by thiamethoxam can alter forager flight performances, affecting colony fitness.

4.1 Introduction

Pesticides have been largely used in veterinary medicine and agriculture, however they also affect numerous non-target organism health (Desneux et al., 2007; Schäfer et al., 2012). Recently, attention has focused on the effect of neonicotinoid pesticides on honey bees (for reviews see Godfray et al., 2014 and Pisa et al., 2014). These pesticides are potent agonists of the nicotinic acetylcholine receptors (nAChR) (Iwasa et al., 2004; Matsuda et al., 2001) which are distributed in the central nervous system (Dupuis et al., 2012) disrupting neurotransmission processes (Jones et al., 2006). Therefore, neonicotinoid pesticides affect the numerous processes that involve cholinergic transmission, such as olfaction, learning and memory (Armengaud et al., 2002; Bal et al., 2010; Thany and Gauthier, 2005; Williamson and Wright, 2013). Thiamethoxam is a commonly used second-generation neonicotinoid (Macedo and Castro, 2011; Sirchio and Sutton, 2007) succeeding to the first-generation imidacloprid (Maienfisch et al., 2001; Simon-Delso et al., 2014), however its sublethal effects on bees are not as extensively studied.

Thermoregulation is essential for honey bees both at individual and colony level and is achieved by both physiological and behavioural processes. In fact, bees need to maintain the optimal temperature of both the brood during the whole active season (~35°C) and the winter cluster during the cold periods (Heinrich and Esch, 1994; Jones et al., 2004; Stabentheiner, 2003). Forager bees maintain their thorax above ambient temperature during the whole foraging cycle, therefore flight ability and consequent foraging behaviour depend on their thorax temperature (Esch, 1988; Schmaranzer, 2000; Stabentheiner, 2001). In fact, the thorax contains flight muscles which produce heat when activated (Coelho, 1991b; Kovac et al., 2010). Henry et al. (2012) showed that sublethal doses of thiamethoxam decreased homing flight success leading to colony failure. A possible explanation to this phenomenon is that forager flights were affected by a thermoregulation impairment elicited by thiamethoxam. Thermoregulation is impaired by insecticides like organophosphates (Schmaranzer et al., 1987) and pyrethroids (Belzunces and Vandame, 1998; Belzunces et al., 1996) and also by azole fungicides like prochloraz and difenoconazole (Belzunces and Vandame, 1998), but the effect of neonicotinoids on honey bee thermoregulation has not been investigated yet. Therefore, we studied the acute sublethal effects of the neonicotinoid thiamethoxam on the thorax temperature of individual foragers across time. Two experiments in which the individuals were exposed to different environmental temperatures were carried out.

4.2 Materials and methods

4.2.1 Honey bee preparation

Returning *Apis mellifera scutellata* foragers were collected from four healthy colonies at the experimental farm of University of Pretoria, South Africa, in September 2014. After collection, the bees were immediately brought to the laboratory. Each individual bee was chilled using ice, then inserted into Plexiglas tubes and fixed with a bee wax and rosin mixture on the dorsal part between thorax and abdomen. Thorax of each bee remained free of any material, since its temperature was the principal endpoint evaluated in this study.

Bees were then individually fed 3 µl of 25% sucrose water solution (w/w) and placed in an incubator at 33°C and 50% RH for one hour to help them recover from stressful manipulation.

4.2.2 Administration of the test solutions

After the 1-hour incubation period, the bees were placed at room temperature 22°C and fed 10 µl of 50% sucrose water test solution (w/w). The test solution provided contained either 0 ng/bee (*control*), 0.2 ng/bee (*low*), 1 ng/bee (*medium*) or 2 ng/bee (*high*), corresponding respectively to 0 ppb, 20 ppb, 100 ppb and 200 ppb. The doses consumed by the bees were respectively 25, 5 and 2.5 times lower than the LD_{50} of thiamethoxam on honey bees, which is 5 ng/bee (EFSA, 2013a). Each bee received a randomly assigned treatment and those not consuming the test solution were excluded from the experiment.

4.2.3 Temperature assessment

The thorax temperature of the harnessed bees was recorded using a FLIR SC325 thermal camera (Fig. 4.1). The camera was positioned above the bees for simultaneous recording of the temperature of 80 bees (20 bees per treatment). The thorax temperature of each bee was targeted and defined by a unique identification code through the whole experiment (Fig. 4.2). All temperature recordings started before treatment (time 0) and were carried out in a laboratory room maintained at 22°C. Forager bees that died during the experiment were excluded from the thorax temperature analysis.

Two experiments testing the effect of acute oral exposures of thiamethoxam on honey bee thermoregulation were performed.

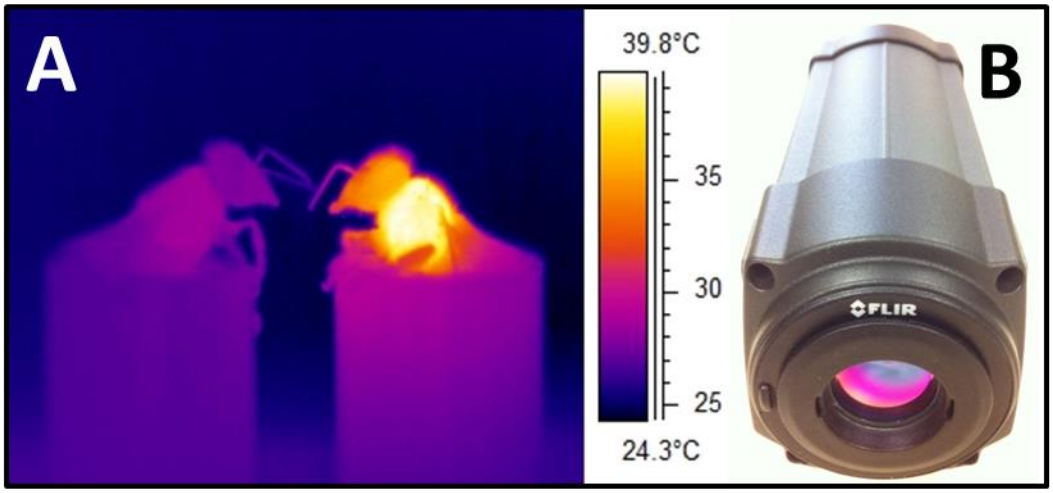

Fig. 4.1. Thermal photo of (A) two harnessed bees inserted and fixed into the Plexiglas tubes. A (B) FLIR SC325 thermo camera was used for all temperature recordings.

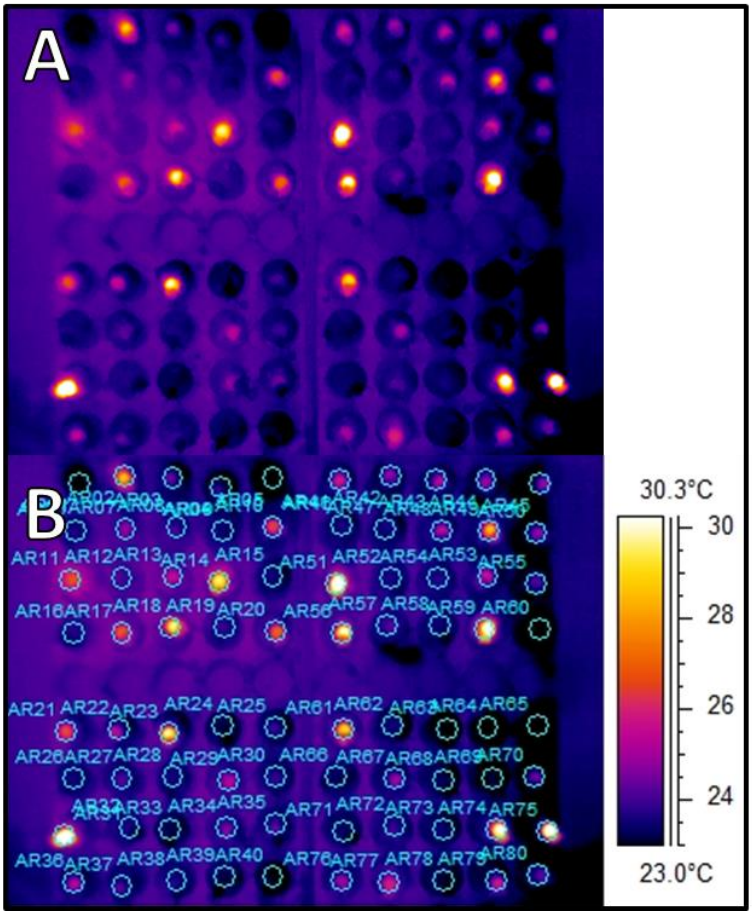

Fig. 4.2. (A, B) Thermal photos of 80 bees recorded from the top during a repetition of the experiment. (B) The thorax of each bee was identified and defined by a specific identification code, thus the temperature of each individual could be assessed.

4.2.3.1 Experiment 1: Alternating temperatures environment

After administration of the test solution, the bees were maintained at 33°C. Exactly one and two hours post treatment, the bees were moved from the incubator to a colder environment at 22°C for 20 minutes, period in which the temperature of their thoraxes were recorded every 10 minutes (time 60, 70, 80, 120, 130, 140).

This experiment was repeated four times.

4.2.3.2 Experiment 2: Constant temperature environment

Day 0 and Day 1

After administration of the test solution, the bees were maintained at 22°C. The first day (Day 0) their thorax temperature was recorded every 30 minutes for a total of four hours (from time 0 to time 240) and at all time assessments recorded in Exp. 1 (time 70, 80, 130, 140). Once the recording at Day 0 was completed, the bees were fed 50% (w/w) sucrose water solution ad libitum and maintained in the incubator at 33°C and 50% RH to guarantee the survival of the bees overnight.

The second day (Day 1), the thorax temperature was recorded for a total of one hour, every 10 minutes in the first half hour and then at 60 minutes (time 1200, 1210, 1220, 1230, 1260).

This experiment was repeated four times.

Cold Shock

After the 1-hour recording on Day 1, a cold shock (CS) was performed. Bees were kept at 4°C for five minutes. Their thorax temperature was assessed every 10 minutes for 30 minutes total (time 0, 10, 20 and 30).

The CS was repeated two times.

4.2.4 Statistical analysis

Kruskal-Wallis Rank-Sum test was used to test the effects of thiamethoxam dose (0, 0.2, 1, 2 ng/bee) on forager bee thorax temperatures overall and at each time assessment. Significant effects were further analysed computing Wilcoxon each pair comparisons among different dose levels. Sequential Bonferroni correction was applied (Sokal and Rohlf., 1995) to correct for 6 pair wise comparisons leading to an adjusted $\alpha = 0.0083$.

ANOVA (REML Algorithm) was used to test the effects of thiamethoxam dose (0, 0.2, 1, 2 ng/bee) and colony (random factor) on honey bee mortality after one day. Mortality percentage data were transformed as shown by Bliss (1938).

All analysis were computed using JMP v10.0, SAS statistical software.

4.3 Results

4.3.1 Experiment 1: Alternating temperatures environment

There was an overall highly significant effect of treatment on the thorax temperatures of the bees (Tab. 4.1 and Fig. 4.3 and 4.4). Wilcoxon each pair comparison showed that the thorax temperature of *high* was significantly higher compared to *control, low* and *medium* overall; in addition, *low* and *medium* had an overall significantly lower thorax temperature than *control* too, while there was no statistical difference between them.

Tab. 4.1. Summary of the results of Exp. 1. The effect of treatment (Kruskal-Wallis Rank-Sum test) and the mean (SE) thorax temperature of the bees in relation to time and dose of thiamethoxam assumed are reported. Asterisks indicate statistically significant differences between doses overall considering each time assessment (Kruskal-Wallis Rank-Sum test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Different letters indicate statistically significant differences between doses within time assessments (Wilcoxon each pair test, $p < 0.0083$ after Bonferroni correction).

Period	Time	N	χ^2	P value	Dose (ng/bee)			
					0	0.2	1	2
Overall		***2156***	*57.64*	*<.0001****	*29.12 (0.15) b*	*28.53 (0.13) a*	*28.54 (0.14) a*	*30.17 (0.18) c*
Before	**0**	**308**	3.25	0.3547	28.83 (0.32)	29 (0.35)	28.99 (0.32)	29.39 (0.34)
	60	**308**	2.56	0.4641	31.19 (0.36)	30.87 (0.32)	30.92 (0.33)	31.86 (0.43)
1 h	**70**	**308**	1.92	0.5894	28.84 (0.42)	28.22 (0.31)	28.21 (0.39)	29.25 (0.51)
	80	**308**	8.92	0.0303*	28.49 (0.42) a	27.97 (0.34) a	27.4 (0.39) a	28.86 (0.48) a
	120	**308**	28.19	<.0001***	31.16 (0.37) bc	29.77 (0.29) a	30.14 (0.3) ab	32.63 (0.45) c
2 h	**130**	**308**	34.40	<.0001***	28.09 (0.38) a	27.35 (0.32) a	27.38 (0.32) a	30.26 (0.45) b
	140	**308**	22.64	<.0001***	27.21 (0.33) a	26.49 (0.29) a	26.75 (0.29) a	28.95 (0.48) b

Furthermore, we tested the effect of treatment on forager thorax temperatures at each time assessment. There was a significant effect of treatment at 120, 130 and 140 minutes post-treatment. Subsequent Wilcoxon each pair comparison showed that 120 min post-treatment *high* had a significantly higher thorax temperature compared to *low* and *medium* and that the thorax temperature of *low* was significantly lower than *control* too. At both 130 and 140 min post-treatment, *high* had a significantly higher temperature than *control*, *low* and *medium*.

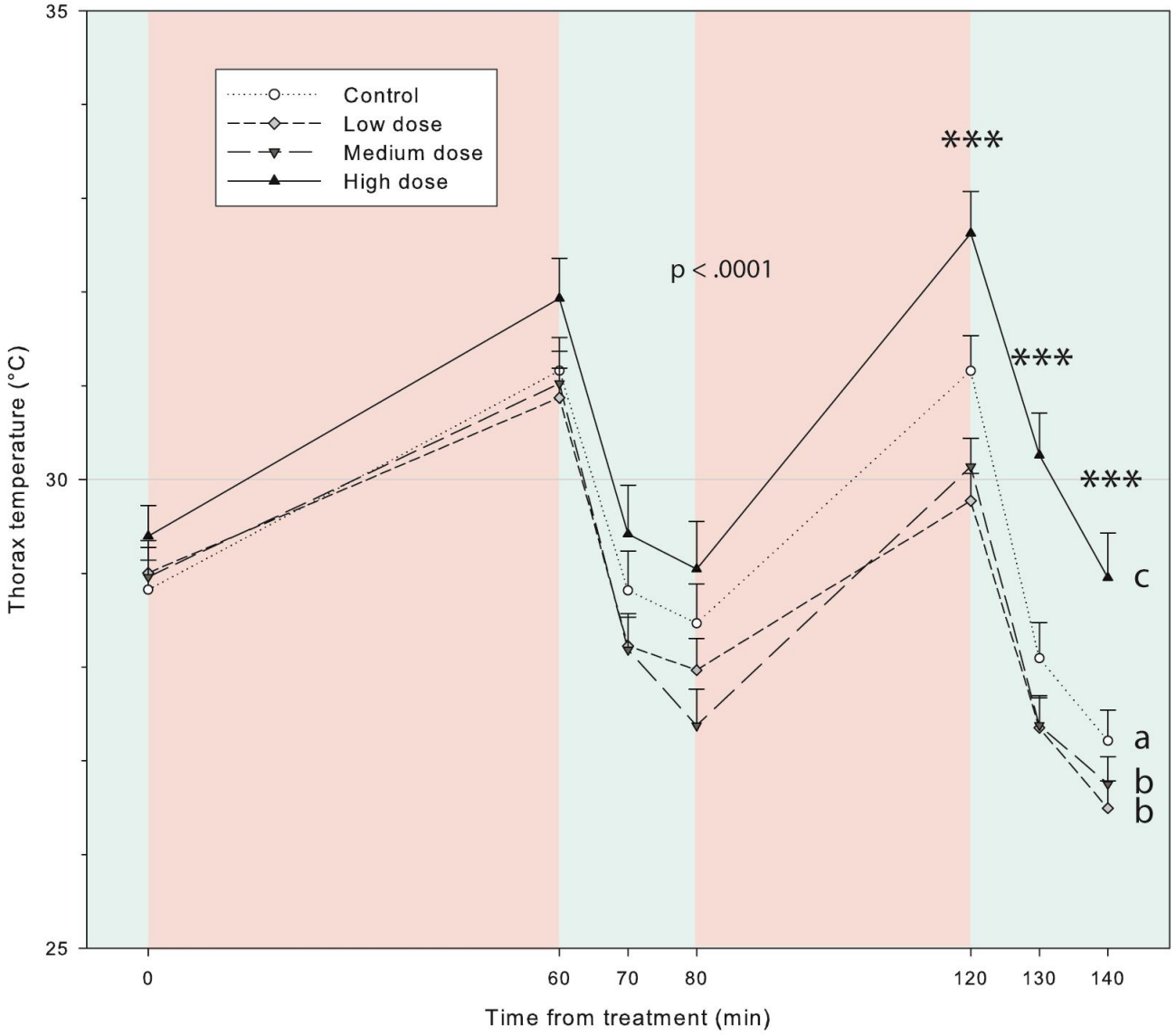

Fig. 4.3. Mean thorax temperatures (±SE) of harnessed forager bees after acute exposures to thiamethoxam (0.2, 1, 2 ng/bee) in Exp. 1. The different colours indicate the different temperature the bees were exposed to (light blue = 22°C, red = 33°C). The effect of treatment was statistically significant overall (Kruskal-Wallis Rank-Sum test). Asterisks indicate statistically significant differences between doses overall within specific time assessments (Kruskal-Wallis Rank-Sum test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Different letters indicate statistically significant differences between doses overall (Wilcoxon each pair test, $p < 0.0083$ after Bonferroni correction). Further post hoc comparisons are reported in Tab. 4.1.

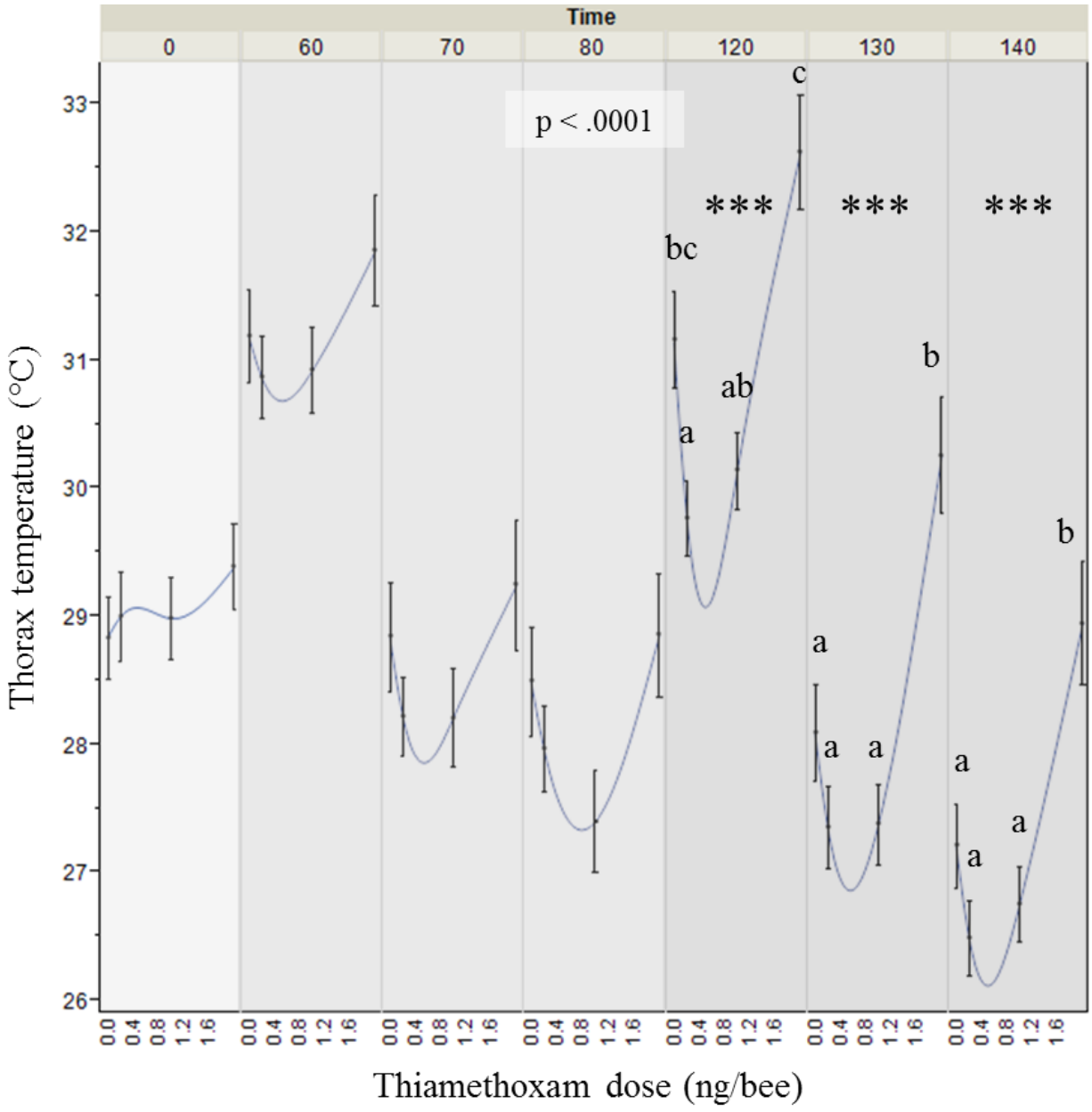

Fig. 4.4. Mean thorax temperatures (±SE) of the harnessed forager bees after acute administrations of thiamethoxam in Exp. 1, in relation to the dose of thiamethoxam assumed (0.2, 1, 2 ng/bee) and the time assessment. The overall effect of the dose of thiamethoxam was statistically significant (Kruskal-Wallis Rank-Sum test). Asterisks indicate statistically significant differences between doses overall within specific time assessments (Kruskal-Wallis Rank-Sum test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Different letters indicate statistically significant differences between doses within specific time assessments (Wilcoxon each pair test, $p < 0.0083$ after Bonferroni correction).

4.3.2 Experiment 2: Constant temperature environment

The results of this experiment are summarized in Tab. 4.2, Fig. 4.5 and 4.6.

Tab. 4.2. Summary of the results of Exp. 2. The effect of treatment (Kruskal-Wallis test) and the mean (SE) thorax temperature of the bees in relation to time and dose of thiamethoxam assumed are reported. Asterisks indicate statistically significant differences between doses overall within specific time assessments (Kruskal-Wallis Rank-Sum test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Different letters indicate statistically significant differences between doses within time assessments (Wilcoxon each pair test, $p < 0.0083$ after Bonferroni correction).

Period	Time	N	χ^2	P value	Dose (ng/bee)			
					0	0.2	1	2
Day 0	***Overall***	*3921*	*18.704*	*0.0003****	*26.22 (0.10) ab*	*26.37 (0.10) b*	*25.95 (0.09) a*	*26.06 (0.11) a*
	0	302	2.1478	0.5423	29.43 (0.32)	29.9 (0.33)	29.56 (0.32)	29.59 (0.37)
	30	302	6.4786	0.0905	27.61 (0.41)	28.12 (0.44)	27.43 (0.38)	26.66 (0.36)
	60	302	14.0782	0.0028**	26.55 (0.37) ab	27.16 (0.38) b	26.23 (0.27) b	25.7 (0.39) a
	70	302	9.2436	0.0262*	26.12 (0.34) ab	26.46 (0.37) b	25.34 (0.25) ab	25.42 (0.37) a
	80	302	9.8616	0.0198*	26.68 (0.42) ab	26.79 (0.37) b	25.96 (0.32) ab	25.67 (0.38) a
	90	302	11.3995	0.0098**	26.71 (0.36) a	26.5 (0.31) a	25.59 (0.27) a	25.81 (0.38) a
	120	302	2.9518	0.7437	26.12 (0.31)	26.08 (0.29)	25.7 (0.28)	25.89 (0.38)
	130	302	1.1722	0.7597	26 (0.32)	25.81 (0.32)	25.61 (0.29)	25.97 (0.42)
	140	302	2.4073	0.4923	25.67 (0.28)	25.79 (0.27)	25.42 (0.28)	26.09 (0.42)
	150	302	1.2387	0.7437	25.47 (0.3)	25.52 (0.31)	25.26 (0.29)	25.59 (0.36)
	180	301	3.2023	0.3615	25.29 (0.31)	24.92 (0.16)	25.13 (0.27)	25.32 (0.27)
	210	301	7.5593	0.0561	24.94 (0.13)	25.25 (0.16)	25.39 (0.28)	25.97 (0.31)
	240	299	4.3933	0.222	24.33 (0.22)	24.47 (0.18)	24.7 (0.27)	25.13 (0.35)
Day 1	***Overall***	*1254*	*59.1467*	*<.0001****	*27.95 (0.22) b*	*28.17 (0.24) b*	*27.87 (0.26) b*	*25.90 (0.18) a*
	1200	251	20.5005	0.0001***	32.1 (0.47) b	32.49 (0.55) b	31.94 (0.5) b	29.4 (0.41) a
	1210	251	20.6177	0.0001***	28.43 (0.37) b	28.36 (0.42) b	28.24 (0.51) b	26.26 (0.27) a
	1220	251	23.5354	<.0001***	26.74 (0.35) b	26.8 (0.39) b	26.76 (0.48) b	24.98 (0.23) a
	1230	251	15.7459	0.0013***	26.75 (0.38) b	27.49 (0.51) b	27.14 (0.56) b	25.05 (0.3) a
	1260	250	22.1019	<.0001***	25.7 (0.43) b	25.68 (0.39) b	25.25 (0.45) b	23.82 (0.27) a
After Cold Shock	***Overall***	*560*	*33.502*	*<.0001****	*25.54 (0.27) b*	*25.76 (0.29) b*	*24.43 (0.15) a*	*24.42 (0.21) a*
	1270	140	7.6936	0.0528	25.9 (0.73)	24.92 (0.4)	24.3 (0.39)	24.02 (0.53)
	1280	140	7.0512	0.0703	27.33 (0.58)	26.49 (0.61)	25.83 (0.23)	25.74 (0.42)
	1290	140	21.6916	<.0001***	24.81 (0.37) b	25.67 (0.6) b	24.06 (0.19) a	24.1 (0.26) a
	1300	140	33.0422	<.0001***	24.13 (0.22) c	25.97 (0.64) b	23.53 (0.21) a	23.81 (0.39) a

4.3.2.1 Day 0 and Day 1

At Day 0, there was an overall highly significant effect of treatment on the thorax temperatures of the bees. Wilcoxon each pair comparison showed that the thorax temperature of *medium* and *high* treatments were lower than *low* overall.

Furthermore, we tested the effect of treatment on forager thorax temperatures at each time assessment during Day 0. There was a significant effect of treatment at 60, 70, 80 and 90

minutes post-treatment. Subsequent Wilcoxon each pair comparison showed that 60 min post-treatment *high* had a significantly lower temperature than *low* and *medium*. At both 70 and 80 min post-treatment, *high* had a significantly lower temperature than *low*.

At Day 1 there was an overall highly significant effect of treatment on the thorax temperature of the bees. Wilcoxon each pair comparison showed that the thorax temperature of the *high* treatment was significantly lower than *control*, *low* and *medium* overall.

In addition, there was a highly significant effect of treatment at all time assessments. Specifically, Wilcoxon each pair comparison showed that *high* had a significantly lower temperature than *control*, *low* and *medium* at all time assessments.

4.3.2.2 Cold Shock

There was an overall highly significant effect of treatment on the thorax temperature of the bees after the CS. Wilcoxon each pair comparison showed that *medium* and *high* thorax temperatures were significantly lower than *control* and *low* overall.

In addition, there was a highly significant effect of treatment at 20 and 30 minutes after the cold shock (respectively time 1290 and 1300). Specifically, Wilcoxon each pair comparison showed that 20 min post-CS *medium* and *high* had a highly significant lower temperature than *control* and *low*. 30 minutes after the CS *low* thorax temperature was significantly higher than *control*, *medium* and *high*; in addition, *control* had a significantly higher thorax temperature than *medium* and *high*.

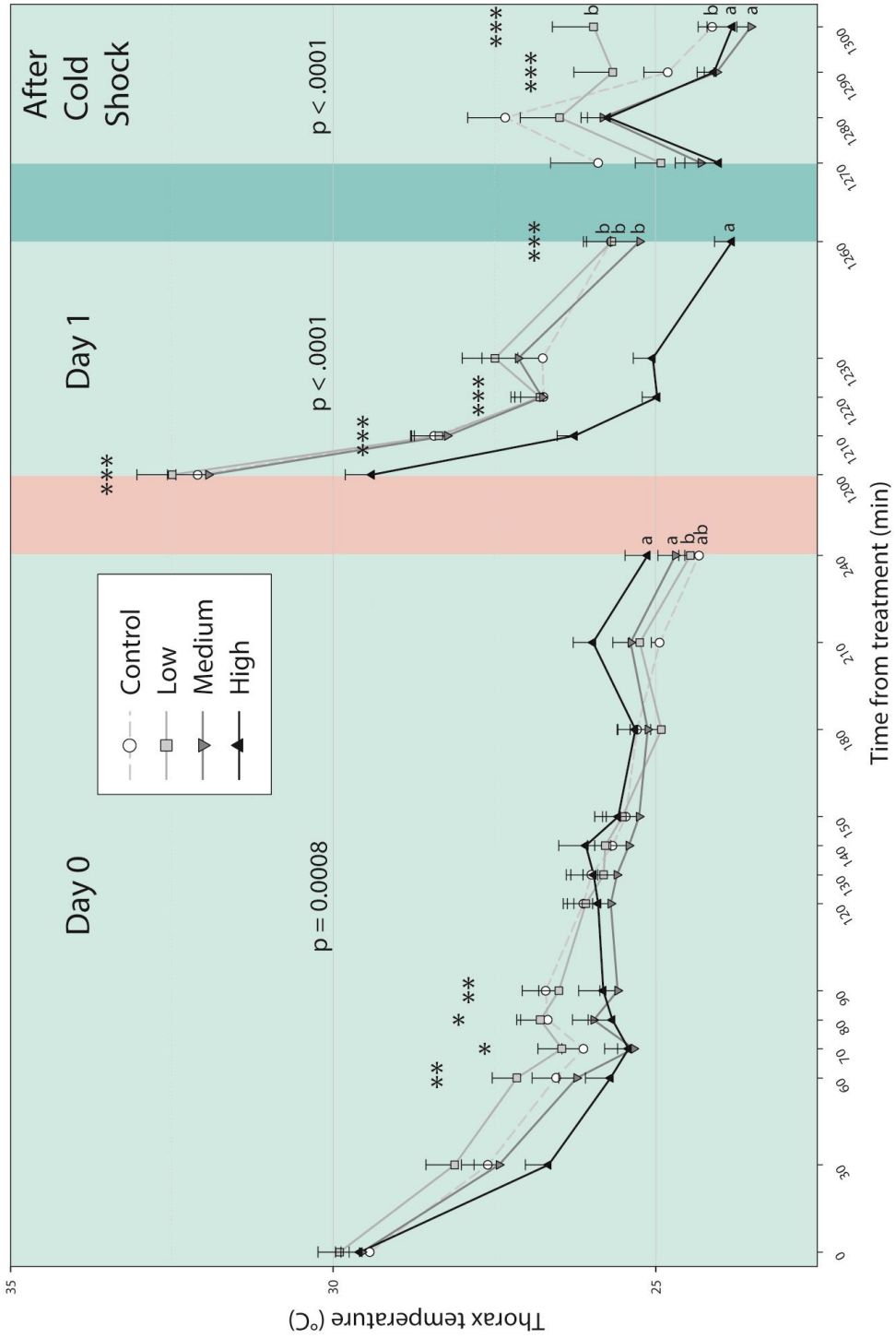

Fig. 4.5. Mean thorax temperatures (±SE) of harnessed forager bees after acute exposures to thiamethoxam (0.2, 1, 2 ng/bee), in Exp. 2. The bees were maintained at 22°C (light blue) when the temperature recordings were carried out. The bees were incubated at 33°C overnight (red). At Day 1, a cold shock was performed (5 min at 4°C, dark blue). The overall effect of dose was statistically significant in each time period (Kruskal-Wallis Rank-Sum test at Day 0, Day 1, After CS). Asterisks indicate statistically significant differences between doses overall within specific time assessments (Kruskal-Wallis Rank-Sum test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Different letters indicate statistically significant differences between doses in each time period overall (Wilcoxon each pair test, $p < 0.0083$ after Bonferroni correction). Further post hoc comparisons are reported in Tab. 4.2.

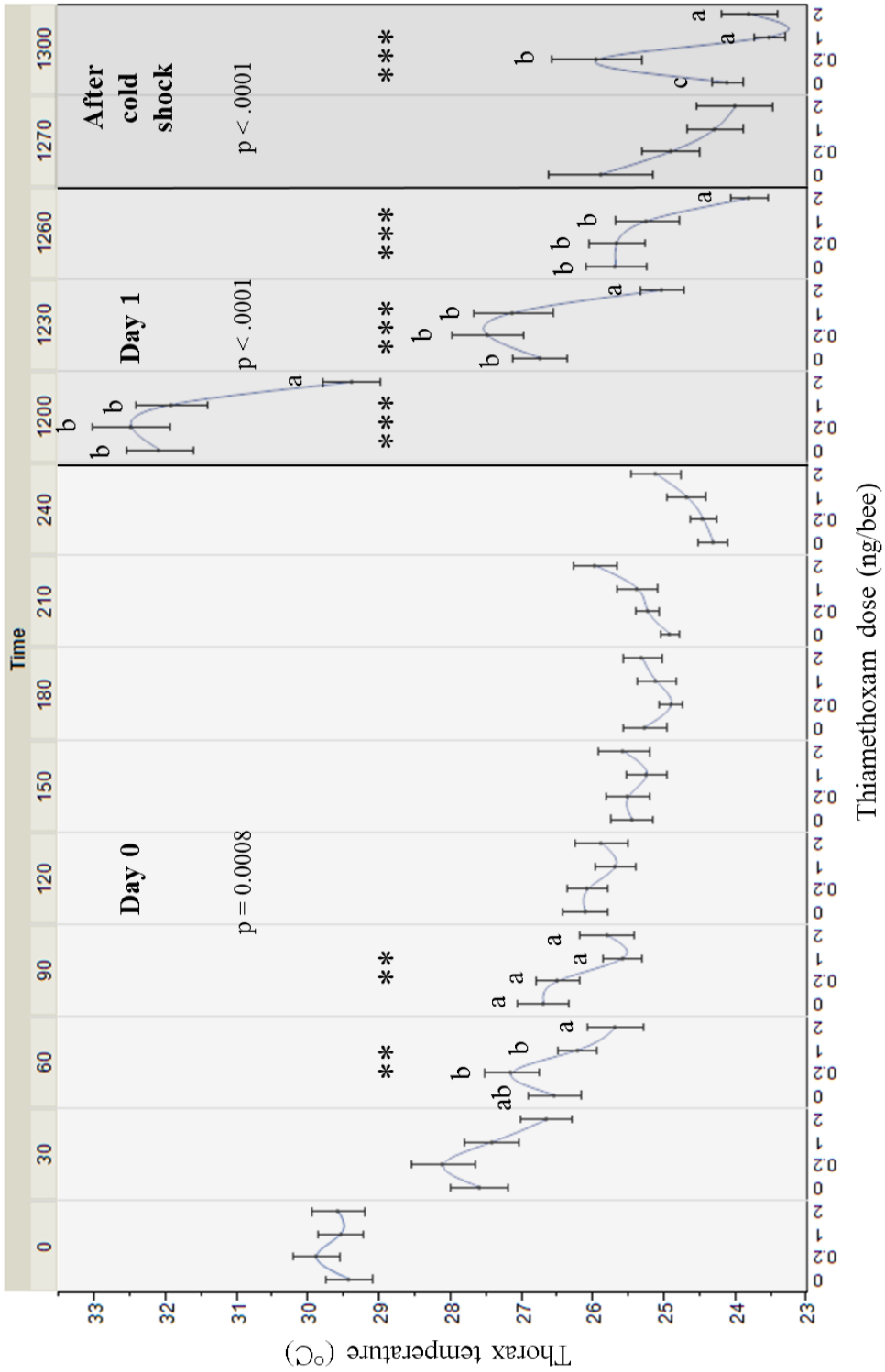

Fig. 4.6. Mean thorax temperatures (±SE) of the harnessed forager bees of Exp. 2 in relation to the dose of thiamethoxam assumed (0.2, 1, 2 ng/bee) and the time assessment. The overall effect of dose was statistically significant in each time period (Kruskal-Wallis Rank-Sum test at Day 0, Day 1, After CS). Asterisks indicate statistically significant differences between doses within time assessments overall (Kruskal-Wallis Rank-Sum test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Different letters indicate statistically significant differences between doses within time assessments (Wilcoxon each pair test, $p < 0.0083$ after Bonferroni correction).

4.3.2.3 Mortality

There was no significant effect of treatment on bee mortality (ANOVA, $F_{3,3} = 2.44$, $p = 0.2419$).

Dead bees were removed before the recording at each time assessment. The temperature data from bees that died during the experiment were excluded.

4.4 Discussion

Acute oral exposures to thiamethoxam significantly influenced the thorax temperature of forager bees exposed at two different temperature environments (Exp. 1 and 2).

When forager bees were maintained at 33°C (optimal temperature environment) and then moved to 22°C 1 and 2 hours after treatment (Exp. 1, Tab. 4.1, Fig. 4.3 and 4.4), the high dose treated bees (2 ng/bee) exhibited higher thorax temperatures than control, low (0.2 ng/bee) and medium (1 ng/bee) doses. The pesticide can have elicited a muscular excitation of the harnessed bees treated with the high dose of thiamethoxam and thus lead to an increased temperature, compared to control. Hyperactivity symptoms were previously observed to appear rapidly after an intoxication with the neonicotinoid imidacloprid on bees (Suchail et al. (2001) and German cockroaches (*Blattella germanica*, Wen and Scott, 1997).

When foragers were constantly maintained at 22°C (Exp. 2), the pesticide treatment significantly influenced their thorax temperature (Tab. 4.2, Fig. 4.5 and 4.6) during the first day of the experiment (Day 0) but especially the day after (Day 1). At Day 0, high dose bees had significantly lower temperatures than low and medium dose ones, 60-80 min post-treatment. The temperature of high dose treated bees started then to increase and became the higher among all treatments at the end of Day 0 (i.e. 4 h post-treatment, n.s.). Consistently during the following day (Day 1), high dose treated bees had significantly lower thorax temperatures than all other treatments, at all time assessments. Completed the Day 1 recording, a cold shock (CS) showed that treated bees (medium and high doses) were less able to produce heat. Contrarily, 20 and 30 minutes post-CS the low dose bees showed a significant increase of temperature while all other treatments showed a decreasing trend. To summarize Exp. 2, thiamethoxam administered at the high dose elicited hypothermia rapidly after intoxication and even more evidently the day after. Similar hypothermia effects were

shown by Belzunces and Vandame (1998) after treatment with deltamethrin (pyrethroid insecticide), prochloraz and difenoconazole (azole fungicides).

The dose-response relationship was non-linear in both Exp. 1 (Fig. 4.4) and 2 (Fig. 4.6). In fact, the effect of lower versus higher doses of thiamethoxam showed opposite effects, compared to control. In Exp. 1 the thorax temperature becomes higher or lower than control if the bees were fed respectively high or low-medium doses of the pesticide. A similar phenomenon, but inverted, is visible in Exp. 2 between 60 and 90 minutes after treatment (Day 0) and after the cold shock (Day 1). The temperature of the bees during these periods was in fact either lower (high and medium doses) or higher (low dose, statistically different from control only after the CS) than control. These opposite results observed between Exp. 1 and 2 may be related to the environmental temperature which the bees were exposed to. In fact, the forager thermoregulation abilities impaired by the high dose of thiamethoxam lead to higher or lower temperatures than control when exposed at respectively high (33°C) or low (22°C) temperatures. The higher temperatures showed by high dose treated bees (consistently in Exp. 1, between 120 and 240 min in Exp. 2) are likely to cause a faster energy depletion which can impede the bees to properly thermoregulate and may thus explain the lower temperatures of bees observed at Day 1 (Exp. 2). Indeed, variability among neonicotinoid effects on bees is interestingly common (Belzunces and Vandame, 1998; Guez et al., 2001; Iwasa et al., 2004; Lambin et al., 2001; Nauen et al., 2001; Suchail et al., 2001) and the interaction between neonicotinoid pesticides and the nicotinic acetylcholine receptors of insects which they target reveal complex kinetics which disclose the intricacy of the effects induced by these neurotoxic insecticides on honey bees (Belzunces et al., 2012; Decourtye and Devillers, 2010; Thany and Gauthier, 2005; Thany et al., 2003). Indeed, the neonicotinoid effects are widely sensitive to a variety of factors (Nauen et al., 2001; Pisa et al., 2014; Schmuck et al., 2001; Tosi et al., 2013), one being temperature (Medrzycki et al., 2010; Medrzycki et al., 2013).

The effect of thiamethoxam on forager thorax temperature can be related to an impairment of their cognitive thermoregulation abilities or physiological functioning. Muscles are activated by bees to produce heat (Esch, 1976) and therefore an impaired muscle activity can consequently affect thermoregulation. Interestingly, imidacloprid and acetamiprid at sublethal doses impair motor functioning (Aliouane et al., 2008; Lambin et al., 2001; Williamson et al., 2014) and thus thiamethoxam may trigger similar effect on motor

functioning. Henry et al. (2012) showed that sublethal doses of thiamethoxam decreased homing flight success leading to colony failure. Flight performances depend on flight muscle activity, which temperature is precisely controlled by bees during flight; our work suggests that one of the reasons leading to impaired flight success of bees exposed to thiamethoxam is thermoregulation.

In summary, thiamethoxam influenced honey bee thorax temperature at all dose tested in different periods after treatment. This neonicotinoid effects also varied in relation to the temperature the bees were exposed to. In Exp. 1, the higher dose tested exhibited hyperthermia 1 and 2 h after the intoxication. Contrarily, in Exp. 2, the higher doses lead to hypothermia rapidly after treatment (60-80 min) and consistently the following day. Interestingly, lower and higher doses elicited opposite effects on thorax temperatures in relation to the environmental temperature they were exposed to, compared to control. Further studies on the effect of pesticides on thorax temperature and thermoregulation should be carried out, addressing specifically the variation of the effect of the pesticide across time, doses and environmental temperatures. Thermoregulation impairment elicited by thiamethoxam can have various negative consequences on bee flight performances decreasing honey bee foraging and homing success, thus affecting overall fitness at both individual and colony level. Therefore, the sublethal effects elicited by thiamethoxam in this study should be considered for the evaluation of its risks on honey bees and other organisms.

CHAPTER 5

Conclusions

In the last decades, honey bee colony losses have been observed at critical rates on a global scale and the explanation of this phenomenon has often been unclear. Attention has focused on the effects that neonicotinoid pesticides trigger on bees. Neonicotinoids are a newly developed class of systemic insecticides that due to their chemical properties and widespread use are found in all environmental media like water, soil and air. They are also found in honey bee food (pollen and nectar) and nest (wax, beebread, honey). At the concentrations found in the field they typically exhibit sublethal effects at behavioural, cognitive or physiological level. The ability to carry out appropriate, precise and specific behaviours is vital for all living organisms and any deviation from normality could lead to detrimental effects at colony level. Since honey bees are one of the most important pollinator species, their behavioural impairments, especially if connected to foraging ability, are a threat for wild plant biodiversity, crop production and environmental health. Accordingly, it is fundamental to investigate and limit what challenges honey bee heath.

We firstly studied the sublethal effects of thiamethoxam on forager walking locomotion using a specifically designed phototaxis arena (Chapter 2). A single sublethal exposure to the neonicotinoid (Exp. 1) rapidly lead to hyperactivity symptoms (increased time spent moving and speed) which then diminished when an impairment of the motor functions appeared (e.g. inability to ascend and reach the light at the top of the arena, increased number of falls and time spent at bottom). The continued exposure to thiamethoxam (Exp. 2) caused only limited significant effects on bee motor functioning. However, thiamethoxam exposures increased the positive phototaxis behaviour of foragers in both experiments. These various behavioural features caused by thiamethoxam may lead to other critical consequences. Indeed, flight depends on motor functioning and its impairment would affect foraging and homing performances, eventually leading to colony failure.

To further investigate the effects showed in Chapter 2, we studied if equal thiamethoxam exposures could affect the actual forager flight performances (Chapter 3). We discovered that a single sublethal exposure to the pesticide (Exp. 1) lead to an increased duration and distance of the flights shortly after intoxication (hyperactivity). On the contrary, a 2-day continued exposure to the neonicotinoid (Exp. 2) elicited a decrease in flight duration, distance and velocity (impaired motor functioning). Thiamethoxam also increased forager sucrose consumption: the effects of the pesticide on forager flight performances could be related to an impaired energy metabolism and inability to balance energy resources. Similarly

to the walking locomotion experiment, the effect of sublethal doses of thiamethoxam on flight behaviour firstly resulted in hyperactivity symptoms followed by impairment of motor functioning at a later stage. Flight is more energy and muscular demanding than walking locomotion. Therefore, this may be the reason of the limited effects observed on motor impairment after the chronic exposure to thiamethoxam in Chapter 2.

In Chapter 4, we showed that acute exposures to thiamethoxam altered the thorax temperature of forager bees. Foragers treated with the higher doses of pesticide, compared to control and lower doses, exhibited hyperthermia or hypothermia when respectively exposed to high or low environmental temperatures just after treatment. We thus conclude that forager thermoregulation is impaired by thiamethoxam and that its effects are particularly evident one day after treatment. The thorax of the bees contains the flight muscles and their temperature is precisely regulated by honey bees during flight. Therefore, the affected thermoregulation abilities triggered by thiamethoxam can consequently impair flight performances, among various other physiological and behavioural aspects.

In summary, we showed that thiamethoxam affected forager motor functioning (Chapter 2) and thermoregulation (Chapter 4), which are likely to play a role in the impaired flight performances evidenced in Chapter 3. This work also provides further evidence on the intricate mode of action of this common neonicotinoid insecticide. Rapidly after intoxication the pesticide triggered hyperactivity which vanished when a motor functioning impairment appeared. All experiments showed, at different extents, inverse effects of thiamethoxam on bees in relation to time and dose assumed. Therefore, further studies testing different doses and concentrations, assessing various behavioural and physiological patterns across time should be carried out to better understand the complex mode of action of thiamethoxam. To conclude, although the interpretation and extrapolation of the subtle effects of thiamethoxam at field-level may be tricky, the effects that this active ingredient elicits at sublethal doses on forager bees are wide and evident. These results are alarming since slight behavioural impairments of the individual honey bee can lead to severe consequences at colony level threatening environment and human wellbeing.

Acknowledgements

We would like to thank Claudio Porrini for his support in the research and all UCSD students that contributed to the work.

We would like to thank Robert Brodschneider for his helpful suggestions on how to attach honey bees to a flight mill. We greatly appreciate the helpful advice of Vincent Jones and Teah Smith on how to build and use their flight mill design. Ryan Hoffman helped to troubleshoot the flight mill software.

We would like to thank Cecilia Costa for her valuable comments on a late version of the thesis.

References

Accorti, M. and Cerretelli, G. (1991). Il valore economico dell'apicoltura. *L'Italia Agric.* **128**.

Alaux, C., Brunet, J.-L. L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L. P. and Le Conte, Y. (2010). Interactions between Nosema microspores and a neonicotinoid weaken honeybees (Apis mellifera). *Env. Microbiol* **12**, 774–782.

Aliouane, Y., El Hassani, A. K., Gary, V., Armengaud, C., Lambin, M., Gauthier, M., Assani, H., Ary, V. I. G., Rmengaud, C. A. A. and Ambin, M. I. L. (2008). Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. *Environ. Toxicol. Chem.* **28**, 113–22.

Alix, A. and Lewis, G. (2010). Guidance for the assessment of risks to bees from the use of plant protection products under the framework of Council Directive 91/414 and Regulation 1107/2009. *EPPO Bull.* **40**, 196–203.

ApeNet (2011). *"Effects of coated maize seed on honeybees" Report based on results obtained from the third year (2011) activity of the APENET project. http://www.reterurale.it/apenet*.

Armengaud, C., Lambin, M. and Gauthier, M. (2002). Effects of imidacloprid on the neural processes of memory in honey bees. *Environ. impact*.

Bacandritsos, N., Granato, A., Budge, G., Papanastasiou, I., Roinioti, E., Caldon, M., Falcaro, C., Gallina, A. and Mutinelli, F. (2010). Sudden deaths and colony population decline in Greek honey bee colonies. *J. Invertebr. Pathol.* **105**, 335–340.

Bal, R., Erdogan, S., Theophilidis, G., Baydas, G. and Naziroglu, M. (2010). Assessing the effects of the neonicotinoid insecticide imidacloprid in the cholinergic synapses of the stellate cells of the mouse cochlear nucleus using whole-cell patch-clamp recording. *Neurotoxicology* **31**, 113–20.

BeeNet (2013). *BEST Bulletin. http://www.reterurale.it/api*.

Beliën, T., Kellers, J., Heylen, K., Keulemans, W., Billen, J., Arckens, L., Huybrechts, R. and Gobin, B. (2009). Effects of sublethal doses of crop protection agents on honey bee (Apis mellifera) global colony vitality and its potential link with aberrant foraging activity. *Commun. Agric. Appl. Biol. Sci.* **74**, 245–53.

Belzunces, L. P. and Vandame, R. (1998). Joint actions of deltamethrin and azole fungicides on honey bee thermoregulation. *Neurosci Lett* **251**, 57–60.

Belzunces, L. P., Vandame, R. and Gu, X. (1996). Modulation of honey bee thermoregulation by adrenergic compounds. *Neuroreport* **7**, 1601–1604.

Belzunces, L. P., Tchamitchian, S. and Brunet, J.-L. (2012). Neural effects of insecticides in the honey bee. *Apidologie* **43**, 348–370.

Ben-Shahar, Y. (2003). cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. *J. Exp. Biol.* **206**, 2507–2515.

Benzidane, Y., Touinsi, S., Motte, E., Jadas-Hécart, A., Communal, P.-Y., Leduc, L. and Thany, S. H. (2010). Effect of thiamethoxam on cockroach locomotor activity is associated with its metabolite clothianidin. *Pest Manag. Sci.* **66**, 1351–9.

Benzidane, Y., Lapied, B. and Thany, S. H. (2011). Neonicotinoid insecticides imidacloprid and clothianidin affect differently neural Kenyon cell death in the cockroach Periplaneta americana. *Pestic. Biochem. Physiol.* **101**, 191–197.

Bergougnoux, M., Treilhou, M. and Armengaud, C. (2012). Exposure to thymol decreased phototactic behaviour in the honeybee (Apis mellifera) in laboratory conditions. *Apidologie* **44**, 82–89.

Biesmeijer, J. C., Roberts, S. P. M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, a P., Potts, S. G., Kleukers, R., Thomas, C. D., et al. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science (80-.).* **313**, 351–354.

Black, J. (2006). *Honeybee nutrition. Review of research and practices*. (ed. Corporation, R. I. R. and D.).

Bliss, C. I. (1938). The Transformation of Percentages for Use in the Analysis of Variance. *Ohio J. Sci.* **38**, 9–12.

Bonmatin, J.-M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E. a D., et al. (2014). Environmental fate and exposure; neonicotinoids and fipronil. *Environ. Sci. Pollut. Res. Int.*

Bortolotti, L. and Costa, C. (2014). Chemical Communication in the Honey Bee Society.

Bortolotti, L., Montanari, R., Marcelino, J., Medrzycki, P., Maini, S. and Porrini, C. (2003). Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. **56**, 63–67.

Breeze, T. D., Vaissière, B. E., Bommarco, R., Petanidou, T., Seraphides, N., Kozák, L., Scheper, J., Biesmeijer, J. C., Kleijn, D., Gyldenkærne, S., et al. (2014). Agricultural policies exacerbate honeybee pollination service supply-demand mismatches across europe. *PLoS One* **9**, e82996.

Brodschneider, R. and Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie* **41**, 278–294.

Brodschneider, R., Riessberger-Gallé, U. and Crailsheim, K. (2009). Flight performance of artificially reared honeybees (Apis mellifera). *Apidologie* **40**, 441–449.

Bubník, Z. and Kadlec, P. (1995). *Sucrose solubility*. (ed. Mathlouthi, M. and Reiser, P.) Boston, MA: Springer US.

Burd, M. (1994). Bateman's principle and plant reproduction: The role of pollen limitation in fruit and seed set. *Bot. Rev.* **60**, 83–139.

Camazine, S., Visscher, P., Finley, J. and Vetter, R. (1999). House-hunting by honey bee swarms: collective decisions and individual behaviors. *Insectes Soc.*

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F. and Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 662–7.

Carayon, J.-L., Téné, N., Bonnafé, E., Alayrangues, J., Hotier, L., Armengaud, C. and Treilhou, M. (2014). Thymol as an alternative to pesticides: persistence and effects of Apilife Var on the phototactic behavior of the honeybee Apis mellifera. *Environ. Sci. Pollut. Res. Int.* **21**, 4934–9.

Carreck, N. L. and Neumann, P. (2010). Honey bee colony losses. *J. Apic. Res.* **49**, 1.

Carruthers, E., Lewis, K., Mccue, T. and Westley, P. (2008). Generalized linear models : model selection , diagnostics , and overdispersion.

Casida, J. E. and Durkin, K. A. (2013). Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **58**, 99–117.

Chagnon, M., Kreutzweiser, D., Mitchell, E. a D., Morrissey, C. a, Noome, D. a and Van der Sluijs, J. P. (2014). Risks of large-scale use of systemic insecticides to ecosystem functioning and services. *Environ. Sci. Pollut. Res. Int.*

Chauzat, M., Faucon, J., Martel, A., Lachaize, J., Cougoule, N. and Aubert, M. (2006). A Survey of Pesticide Residues in Pollen Loads Collected by Honey Bees in France.

Church, N. S. (1959). Heat loss and the body temperatures of flying insects I. Heat loss by evaporation of water. *J. Exp. Biol.* **8**, 171–185.

Coelho, J. (1991a). The effect of thorax temperature on force production during tethered flight in honeybee (Apis mellifera) drones, workers, and queens. *Physiol. Zool.* **64**, 823–835.

Coelho, J. R. (1991b). Heat Transfer and Body Temperature in Honey Bee (Hymenoptera: Apidae) Drones and Workers. *Environ. Entomol.* **20**, 1627–1635.

Colin, M. E., Bonmatin, J. M., Moineau, I., Gaimon, C., Brun, S. and Vermandere, J. P. (2004). A method to quantify and analyze the foraging activity of honey bees: relevance to the sublethal effects induced by systemic insecticides. *Arch Env. Contam Toxicol* **47**, 387–395.

Crane, E. (1984). Bees, honey and pollen as indicators of metals in the environment. *Bee World*.

Decourtye, A. and Devillers, J. (2010). Ecotoxicity of neonicotinoid insecticides to bees. *Adv. Exp. Med. Biol.* **683**, 85–95.

Decourtye, A., Devillers, J., Cluzeau, S. and Charreton, M. (2004a). Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicol. Environ. Saf.* **57**, 410–419.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M. and Pham-Delègue, M.-H. (2004b). Imidacloprid impairs memory and brain metabolism in the honeybee (Apis mellifera L.). *Pestic. Biochem. Physiol.* **78**, 83–92.

Decourtye, A., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S., Pham-Delegue, M. H., Menach, K. Le and Pham-del, M. H. (2005). Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee Apis mellifera. *Arch Env. Contam Toxicol* **48**, 242–250.

Derecka, K., Blythe, M. J., Malla, S., Genereux, D. P., Guffanti, A., Pavan, P., Moles, A., Snart, C., Ryder, T., Ortori, C. A., et al. (2013). Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae. *PLoS One* **8**, e68191.

Desneux, N., Decourtye, A. and Delpuech, J.-M. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* **52**, 81–106.

Devillers, J. and Pham-Delègue, M.-H. (2002). *Bees : Estimating the Environmental Impact of chemicals*. Taylor & Francis.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G. and Pennacchio, F. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 1–6.

Dively, G. P. and Kamel, A. (2012). Insecticide residues in pollen and nectar of a cucurbit crop and their potential exposure to pollinators. *J. Agric. Food Chem.* **60**, 4449–4456.

Dupuis, J., Louis, T., Gauthier, M. and Raymond, V. (2012). Insights from honeybee (Apis mellifera) and fly (Drosophila melanogaster) nicotinic acetylcholine receptors: from genes to behavioral functions. *Neurosci. Biobehav. Rev.* **36**, 1553–64.

EFSA (2012a). Statement on the findings in recent studies investigating sub-lethal effects in bees of some neonicotinoids in consideration of the uses currently authorised in Europe. *EFSA J.* **10**, 2725.

EFSA (2012b). Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (Apis mellifera, Bombus spp. and solitary bees). *EFSA J.*

EFSA (2013a). Conclusion on the peer review of the pesticide risk assessment for bees for the active substance thiamethoxam. *EFSA J.* **11**, 3067.

EFSA (2013b). Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). *EFSA J.* **11**.

EFSA (2013c). Conclusion on the peer review of the pesticide risk assessment for bees for the active substance clothianidin. *EFSA J.* **11**, 3066.

Eggen, T., Odenmarck, S. R. and Loes, A.-K. (2014). *Translocation of imidacloprid from coated rape (Brassica napa) seeds to nectar and pollen*.

Eiri, D. M. and Nieh, J. C. (2012). A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *J Exp Biol* **215**, 2022–2029.

Elbert, A., Haas, M., Springer, B., Thielert, W. and Nauen, R. (2008). Applied aspects of neonicotinoid uses in crop protection. *Pest Manag. Sci.* **1105**, 1099–1105.

Erber, J., Hoormann, J. and Scheiner, R. (2006). Phototactic behaviour correlates with gustatory responsiveness in honey bees (Apis mellifera L.). *Behav. Brain Res.* **174**, 174–80.

Esch, H. (1976). Body temperature and flight performance of honey bees in a servo-mechanically controlled wind tunnel. *J. Comp. Physiol. A* **109**, 265–277.

Esch, H. (1988). The effects of temperature on flight muscle potentials in honeybees and cuculiinid winter moths. *J. Exp. Biol.* **135**, 109–117.

European Parliament (2011). *Opinion of the Committee on the Environment, Public Health and Food Safety on honeybee health and the challenges for the beekeeping sector*.

Farooqui, T. (2013). A potential link among biogenic amines-based pesticides, learning and memory, and colony collapse disorder: a unique hypothesis. *Neurochem. Int.* **62**, 122–36.

Farrar, C. L. (1937). The influence of colony populations on honey production. *J. Agric. Res.* **54**, 945–954.

Feltham, H., Park, K. and Goulson, D. (2014). Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology* **23**, 317–323.

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B. and Menzel, R. (2014). Neonicotinoids interfere with specific components of navigation in honeybees. *PLoS One* **9**, e91364.

Ford, K. A. and Casida, J. E. (2006). Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. *Chem. Res. Toxicol.* **19**, 1549–56.

Fourrier, J., Ruger, C., Devillers, J., Aupinel, P., Gauthier, M. and Decourtye, A. (2009). Sublethal effects of thiamethoxam on the ability of honeybees to orientate in a complex maze. *Proc. Apimondia Congr.* 69280.

Gauthier, M. (2010). State of the art on insect nicotinic acetylcholine receptor function in learning and memory. *Adv. Exp. Med. Biol.* **683**, 97–115.

Girolami, V., Mazzon, L., Squartini, A., Mori, N., Marzaro, M., Di Bernardo, A., Greatti, M., Giorio, C. and Tapparo, A. (2009). Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees. *J. Econ. Entomol.* **102**, 1808–15.

Glavan, G. (2013). The synergy of xenobiotics in honey bee Apis mellifera: mechanisms and effects. *Acta Biol. Slov.* **56**, 11–25.

Gmeinbauer, R. and Crailsheim, K. (1993). Glucose Utilization During Flight of Honeybee(Apis mellifera) Workers, Drones and Queens. *J. Insect Physiol.* **39**, 959–967.

Godfray, H. C. J., Blacquière, T., Field, L. M., Hails, R. S., Petrokofsky, G., Potts, S. G., Raine, N. E., Vanbergen, A. J., Mclean, A. R., B, P. R. S., et al. (2014). A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. R. Soc. B Biol. Sci.* **281**.

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol.* **50**, 977–987.

Goulson, D., Nicholls, E., Botías, C. and Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Sciencexpress* **2010**, 1–16.

Gross, M. (2013). EU ban puts spotlight on complex effects of neonicotinoids. *Curr. Biol.* **23**, R462–R464.

Guez, D., Suchail, S., Gauthier, M., Maleszka, R. and Belzunces, L. P. (2001). Contrasting effects of imidacloprid on habituation in 7- and 8-day-old honeybees (Apis mellifera). *Neurobiol. Learn. Mem.* **76**, 183–91.

Guez, D., Belzunces, L. P. and Maleszka, R. (2003). Effects of imidacloprid metabolites on habituation in honeybees suggest the existence of two subtypes of nicotinic receptors differentially expressed during adult development. *Pharmacol. Biochem. Behav.* **75**, 217–222.

Harbo, J. R. (1986). Effect of population size on brood production, worker survival and honey gain in colonies of honey bees. *J. Apic. Res.* **25**, 22–29.

Hassani, E., Dacher, M., Gauthier, M., Armengaud, C. and El Hassani, A. K. (2005). Effects of sublethal doses of fipronil on the behavior of the honeybee (Apis mellifera). *Pharmacol. Biochem. Behav.* **82**, 30–9.

Hassani, A. El, Dacher, M. and Gary, V. (2008). Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (Apis mellifera). *Arch. Environ. Contam. Toxicol.*

Heinrich, B. and Esch, H. (1994). Thermoregulation in bees. *Am. Sci.* 164–170.

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S. and Decourtye, A. (2012). A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. *Science* **336**, 348–50.

Hrassnigg, N. and Crailsheim, K. (1999). Metabolic rates and metabolic power of honeybees in tethered flight related to temperature and drag (Hymenoptera: Apidae). *Entomol Gener* **24**, 23–30.

Hrassnigg, N., Brodschneider, R., Fleischmann, P. H. and Crailsheim, K. (2005). Unlike nectar foragers, honeybee drones (Apis mellifera) are not able to utilize starch as fuel for flight. *Apidologie* **36**, 547–557.

Iwasa, T., Motoyama, N., Ambrose, J. T. and Roe, R. M. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. *Crop Prot.* **23**, 371–378.

Jeschke, P. and Nauen, R. (2008). Neonicotinoids-from zero to hero in insecticide chemistry. *Pest Manag. Sci.* **64**, 1084–98.

Jeschke, P., Nauen, R., Schindler, M. and Elbert, A. (2010). Overview of the status and global strategy for neonicotinoids. *J Agric Food Chem* **59**, 2897–2908.

Jeschke, P., Nauen, R. and Beck, M. E. (2013). Nicotinic acetylcholine receptor agonists: a milestone for modern crop protection. *Angew. Chem. Int. Ed. Engl.* **52**, 9464–85.

Johnson, J. D. and Pettis, J. S. (2014). A Survey of Imidacloprid Levels in Water Sources Potentially Frequented by Honeybees (Apis mellifera) in the Eastern USA. *water air soil pollut*.

Johnson, R. M., Ellis, M. D., Mullin, C. A. and Frazier, M. (2010). Pesticides and honey bee toxicity – USA. *Apidologie* **41**, 312–331.

Jones, J. C., Myerscough, M. R., Graham, S. and Oldroyd, B. P. (2004). Honey bee nest thermoregulation: diversity promotes stability. *Science* **305**, 402–4.

Jones, J. C., Helliwell, P., Beekman, M., Maleszka, R. and Oldroyd, B. P. (2005). The effects of rearing temperature on developmental stability and learning and memory in the honey bee, Apis mellifera. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **191**, 1121–1129.

Jones, A. K., Raymond-delpech, V., Thany, S. H., Gauthier, M. and Sattelle, D. B. (2006). The nicotinic acetylcholine receptor gene family of the honey bee , Apis mellifera. *Genome Res.* 1422–1430.

Jungmann, R., Rothe, U. and Nachtigall, W. (1989). Flight of the honey bee I. *J. Comp. Physiol. B* **158**, 711–718.

Karmakar, R. and Kulshrestha, G. (2009). Persistence, metabolism and safety evaluation of thiamethoxam in tomato crop. *Pest Manag. Sci.* **65**, 931–7.

Kearns, C. a., Inouye, D. W. and Waser, N. M. (1998). ENDANGERED MUTUALISMS: The Conservation of Plant-Pollinator Interactions. *Annu. Rev. Ecol. Syst.* **29**, 83–112.

Kelly, K. (1995). Out of control: the new biology of machines, social systems and the economic world. In *Out of control*.

Kirchner, W. (1998). The effects of sublethal doses of imidacloprid on the foraging behaviour and orientation ability of honeybees. *Unpubl. Stdy Report, Konstanz*.

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Saul, A., Kremen, C., Tscharntke, T., B, P. R. S., Vaissie, B. E., Cunningham, S. a, et al. (2007). Importance of pollinators in changing landscapes for world crops. *Proc. Biol. Sci.* **274**, 303–13.

Kovac, H., Stabentheiner, A. and Schmaranzer, S. (2010). Thermoregulation of water foraging honeybees--balancing of endothermic activity with radiative heat gain and functional requirements. *J. Insect Physiol.* **56**, 1834–45.

Krupke, C. H., Hunt, G. J., Eitzer, B. D., Andino, G. and Given, K. (2012). Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. *PLoS One* **7**.

Lambert, O., Piroux, M., Puyo, S., Thorin, C., L'Hostis, M., Wiest, L., Buleté, A., Delbac, F. and Pouliquen, H. (2013). Widespread Occurrence of Chemical Residues in Beehive Matrices from Apiaries Located in Different Landscapes of Western France. *PLoS One* **8**, 1–12.

Lambin, M., Armengaud, C., Raymond, S. and Gauthier, M. (2001). Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Arch. Insect Biochem. Physiol.* **48**, 129–34.

Macedo, W. R. and Castro, P. R. de C. e (2011). Thiamethoxam: Molecule moderator of growth, metabolism and production of spring wheat. *Pestic. Biochem. Physiol.* **100**, 299–304.

Maienfisch (2001). Chemistry and biology of thiamethoxam a second generation neonicotinoid. *Pest Manag. Sci.* **57**, 906–913.

Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., Haettenschwiler, J., Sieger, E. and Walti, M. (2001). The discovery of thiamethoxam: A second-generation neonicotinoid. *Pest Manag. Sci.* **57**, 165–176.

Main, A. R., Headley, J. V., Peru, K. M., Michel, N. L., Cessna, A. J. and Morrissey, C. A. (2014). Widespread Use and Frequent Detection of Neonicotinoid Insecticides in Wetlands of Canada's Prairie Pothole Region. *PLoS One* **9**, e92821.

Maini, S., Medrzycki, P. and Porrini, C. (2010). The puzzle of honey bee losses: a brief review. *Bull. Insectology* **63**, 153–160.

Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M. and Sattelle, D. B. (2001). Neonicotinoids: Insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* **22**, 573–580.

Maxim, L. and van der Sluijs, J. P. (2010). Expert explanations of honeybee losses in areas of extensive agriculture in France: Gaucho® compared with other supposed causal factors. *Environ. Res. Lett.* **5**, 014006.

Maxim, L., Sluijs, J. Van Der and van der Sluijs, J. (2013). *Seed-dressing systemic insecticides and honeybees*.

Medrzycki, P., Montanari, R., Bortolotti, L., Sabatini, A. G., Maini, S. and Porrini, C. (2003). Effects of imidacloprid administered in sub-lethal doses on honey bee behaviuor. *Bull. Insectology* **56**, 59–62.

Medrzycki, P., Sgolastra, F., Bortolotti, L., Bogo, G., Tosi, S., Padovani, E., Porrini, C. and Sabatini, A. G. (2010). Influence of brood rearing temperature on honey bee development and susceptibility to poisoning by pesticides Influencia de la temperatura de la cría en el desarrollo de la abeja de la miel y susceptibilidad a la intoxicación por los pesticidas. *J. Apic. Res.* **49**, 52–59.

Medrzycki, P., Sgolastra, F., Bogo, G., Tosi, S. and Venturi, S. (2012). Influence of some experimental conditions on the results of laboratory toxicological tests on honeybees. *Julius-Kühn-Archiv* **437**, 104.

Medrzycki, P., Tosi, S., Bogo, G. and Porrini, C. (2013). Influence of temperature on honey bee susceptibility to pesticides. Proceedings of the American Bee Research Conference, edited by Zachary Huang. *Am. Bee J.* **153**, 306.

Michener, C. D. (1969). Comparative Social Behavior of Bees. *Annu. Rev. Entomol.* **14**, 299–342.

Millar, N. S. and Denholm, I. (2007). Nicotinic acetylcholine receptors: Targets for commercially important insecticides. *Invertebr. Neurosci.* **7**, 53–66.

Moritz, R. F. A. and Southwick, E. E. (1992). Bees as superorganisms: an evolutionary reality.

Morrissey, C. a., Mineau, P., Devries, J. H., Sanchez-Bayo, F., Liess, M., Cavallaro, M. C. and Liber, K. (2015). Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. *Environ. Int.* **74**, 291–303.

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., Vanengelsdorp, D. and Pettis, J. S. (2010). High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS One* **5**, e9754.

Mutinelli, F., Costa, C., Lodesani, M., Baggio, A., Medrzycki, P., Formato, G. and Porrini, C. (2010). Honey bee colony losses in Italy. *J. Apic. Res.* **49**, 119–120.

Nauen, R., Ebbinghaus-Kintscher, U. and Schmuck, R. (2001). Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in Apis mellifera (Hymenoptera: Apidae). *Pest Manag. Sci.* **57**, 577–86.

Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V. L. and Kaussmann, M. (2003). Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pestic. Biochem. Physiol.* **76**, 55–69.

Naug, D. (2009). Nutritional stress due to habitat loss may explain recent honeybee colony collapses. *Biol. Conserv.* **142**, 2369–2372.

Nazzi, F. and Pennacchio, F. (2014). Disentangling multiple interactions in the hive ecosystem. *Trends Parasitol.* **30**, 556–561.

Nazzi, F., Brown, S. P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., Vedova, G. Della, Cattonaro, F., Caprio, E. and Pennacchio, F. (2012). Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog.* **8**.

OEPP/EPPO (2010). Side-effects on honeybees. *EPPO Bull.* **40**, 313–319.

Ohkawara, Y., Akayama, A., Matsuda, K. and Andersch, W. (2002). Clothianidin: a novel broad-spectrum neonicotinoid insecticide. In *The BCPC Conference: Pests and diseases, Volumes 1 and 2. Proceedings of an international conference held at the Brighton Hilton Metropole Hotel, Brighton, UK, 18-21 November 2002.*, pp. 51–58. British Crop Protection Council.

Oldroyd, B. P. (2007). What's killing American honey bees? *PLoS Biol.* **5**, e168.

Page, R. E., Scheiner, R., Erber, J. and Amdam, G. V (2006). The development and evolution of division of labor and foraging specialization in a social insect (Apis mellifera L.). *Curr. Top. Dev. Biol.* **74**, 253–86.

Pankiw, T. (2003). Directional change in a suite of foraging behaviors in tropical and temperate evolved honey bees (Apis mellifera L.). *Behav. Ecol. Sociobiol.* **54**, 458–464.

Pettis, J. S., Lichtenberg, E. M., Andree, M., Stitzinger, J. and Rose, R. (2013). Crop Pollination Exposes Honey Bees to Pesticides Which Alters Their Susceptibility to the Gut Pathogen Nosema ceranae. *PLoS One* **8**.

Ping-Li, D. A. I., Ting, Z., Qiang, W., Yan-yan, W. U., Wen-long, G. and Huai-lei, S. (2013). Effects of Imidacloprid on Learning Performance of Apis mellifera ligustica. *China Acad. J.* **52**, 14–16.

Pisa, L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Downs, C. a, Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M., McField, M., et al. (2014). Effects of neonicotinoids and fipronil on non-target invertebrates. *Environ. Sci. Pollut. Res. Int.*

Pochi, D., Biocca, M., Fanigliulo, R., Pulcini, P. and Conte, E. (2012). Potential exposure of bees, Apis mellifera L., to particulate matter and pesticides derived from seed dressing during maize sowing. *Bull. Environ. Contam. Toxicol.* **89**, 354–61.

Porrini, C., Celli, G. and Radeghieri, P. (1998). Monitoring of pesticides through the use of honeybees as bioindicators of the Emilia-Romagna coastline (1995-1996). *Ann. Chim.* 243–252.

Porrini, C., Ghini, S., Girotti, S., Sabatini, A. G., Gattavecchia, E. and Celli, G. (2002). Use of honey bees as bioindicators of environmental pollution in Italy. In *Honey bees: Estimating the Environmental Impact of Chemicals*.

Potts, S. G., Roberts, S. P. M., Dean, R., Marris, G., Brown, M., Jones, R. and Settele, J. (2009). Declines of managed honey bees and beekeepers in Europe. *J. Apic. Res.* **49**, 15–22.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. and Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* **25**, 345–353.

Rortais, A., Arnold, G., Halm, M.-P. and Touffet-Briens, F. (2005). Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. *Apidologie* **36**, 71–83.

Roubik, D. W. (2002). The value of bees to the coffee harvest. *Nature* **417**, 708.

Roubik, D., Yanega, D., Aluja, M. S., Buchmann, S. and Inouye, D. (1995). On optimal nectar foraging by some tropical bees. *Apidologie*.

Ruttner, F. (1988). *Biogeography and taxonomy of honeybees*. Springer-Verlag.

Samson-robert, O., G, L., M, C. and V, F. (2014). Neonicotinoid-Contaminated Puddles of Water Represent a Risk of Intoxication for Honey Bees. *PLoS One* **9**, 1–17.

Sanchez-Bayo, F. (2014). The trouble with neonicotinoids. *Science (80-.).* **346**, 806–807.

Schäfer, R. B., von der Ohe, P. C., Rasmussen, J., Kefford, B. J., Beketov, M. A., Schulz, R. and Liess, M. (2012). Thresholds for the effects of pesticides on invertebrate communities and leaf breakdown in stream ecosystems. *Environ. Sci. Technol.* **46**, 5134–42.

Scheiner, R., Abramson, C. I., Brodschneider, R., Crailsheim, K., Farina, W. M., Fuchs, S., Grünewald, B., Hahshold, S., Karrer, M., Koeniger, G., et al. (2013). Standard methods for behavioural studies of Apis mellifera. *J. Apic. Res.* **52**, 1–58.

Schmaranzer, S. (2000). Thermoregulation of water collecting honey bees (Apis mellifera). *J Insect Physiol* **46**, 1187–1194.

Schmaranzer, S., Stabentheiner, A. and Heran, H. (1987). Effect of Roxion-S (dimethoate) on the body temperature of the honey bee. *Chem. Biol. Soc. Insects* **241**.

Schmuck, R., Schöning, R., Stork, a and Schramel, O. (2001). Risk posed to honeybees (Apis mellifera L, Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Manag. Sci.* **57**, 225–38.

Schneider, C. W., Tautz, J., Grünewald, B. and Fuchs, S. (2012). RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of Apis mellifera. *PLoS One* **7**, e30023.

Scholer, J. and Krischik, V. (2014). Chronic exposure of imidacloprid and clothianidin reduce queen survival, foraging, and nectar storing in colonies of bombus impatiens. *PLoS One* **9**.

Schulp, C. J. E., Lautenbach, S. and Verburg, P. H. (2014). Quantifying and mapping ecosystem services: Demand and supply of pollination in the European Union. *Ecol. Indic.* **36**, 131–141.

Schulz, D. J., Huang, Z.-Y. and Robinson, G. E. (1998). Effects of colony food shortage on behavioral development in honey bees. *Behav. Ecol. Sociobiol.* **42**, 295–303.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D. W., Giorio, C., Girolami, V., et al. (2014). Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environ. Sci. Pollut. Res.*

Sirchio, K. and Sutton, A. (2007). Syngenta Professional Products Focuses Chemical Technology on New Applications to Enhance the Quality of Life. *Chim. Int. J. Chem.* **61**, 17–22.

Slessor, K. N., Winston, M. L. and Le Conte, Y. (2005). Pheromone communication in the honeybee (Apis mellifera L.). *J. Chem. Ecol.* **31**, 2731–45.

Smagghe, G., Blacquiere, T., van Gestel, C. a M., Mommaerts, V., Blacquière, T., Smagghe, G., van Gestel, C. a M. and Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**, 973–992.

Smith, T. and Jones, V. P. (2012). *The flight mill. http://entomology.tfrec.wsu.edu/VPJ_Lab/Flight-Mil*.

Smith, K. M., Loh, E. H., Rostal, M. K., Zambrana-Torrelio, C. M., Mendiola, L. and Daszak, P. (2013). Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. *Ecohealth* **10**, 434–445.

Smodis Skerl, M. I., Velikonja Bolta, S., Basa Cesnik, H. and Gregorc, A. (2009). Residues of Pesticides in honeybee (Apis mellifera carnica) bee bread and in pollen loads from treated apple orchards. *Bull. Environ. Contam. Toxicol.* **83**, 374–7.

Sokal, R. R. and Rohlf., F. J. (1995). *Biometry: the principles and practice of statistics in biological research*. WH Freman and company: New York.

Sotavalta, O. (1954). On the thoracic temperature of insects in flight. *Ann. ent. fenn.*

Southwick, E. E. and Southwick, L. (1992). Estimating the Economic Value of Honey Bees (Hymenoptera: Apidae) as Agricultural Pollinators in the United States. *J. Econ. Entomol.* **85**, 621–633.

Stabentheiner, A. (2001). Thermoregulation of dancing bees: Thoracic temperature of pollen and nectar foragers in relation to profitability of foraging and colony need. *J. Insect Physiol.* **47**, 385–392.

Stabentheiner, a. (2003). Endothermic heat production in honeybee winter clusters. *J. Exp. Biol.* **206**, 353–358.

Stanley, D. and Raine, N. (2014). Investigating sublethal pesticide effects on bumblebee navigation and foraging. *IUSSI 2014 Proc.* 45.

Stokstad, E. (2012). Field Research on Bees Raises Concern About Low-Dose Pesticides. *Science (80-.).* **335**, 1555–1555.

Stoner, K. a and Eitzer, B. D. (2012). Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (Cucurbita pepo). *PLoS One* **7**, e39114.

Suchail, S., Guez, D. and Belzunces, L. P. (2001). Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in Apis mellifera. *Environ. Toxicol. Chem.* **20**, 2482–6.

Svoboda, J. (1961). "Prumyslové otravy vcel arsenem (Industrial poisoning of bees by arsenic). *Ved. Pr. Vyzk. Ust. Vcelarskeho CSAZV* **2**, 55–60.

Tan, J., Galligan, J. J. and Hollingworth, R. M. (2007). Agonist actions of neonicotinoids on nicotinic acetylcholine receptors expressed by cockroach neurons. *Neurotoxicology* **28**, 829–42.

Tautz, J. (2008). *The Buzz About Bees*.

Teeters, B. S., Johnson, R. M., Ellis, M. D. and Siegfried, B. D. (2012). Using video-tracking to assess sublethal effects of pesticides on honey bees (Apis mellifera L.). *Environ. Toxicol. Chem.* **31**, 1349–54.

Thany, S. H. (2011). Thiamethoxam, a poor agonist of nicotinic acetylcholine receptors expressed on isolated cell bodies, acts as a full agonist at cockroach cercal afferent/giant interneuron synapses. *Neuropharmacology* **60**, 587–592.

Thany, S. H. and Gauthier, M. (2005). Nicotine injected into the antennal lobes induces a rapid modulation of sucrose threshold and improves short-term memory in the honeybee Apis mellifera. *Brain Res.* **1039**, 216–9.

Thany, S. H., Lenaers, G., Crozatier, M., Armengaud, C. and Gauthier, M. (2003). Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, Apis mellifera. *Insect Mol. Biol.* **12**, 255–262.

Thompson, H. M. (2012). *Interaction between pesticides and other factors in effects on bees*.

Tomizawa, M. and Casida, J. E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.* **48**, 339–64.

Tomizawa, M. and Casida, J. E. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.* **45**, 247–268.

Tomizawa, M. and Casida, J. E. (2011). Neonicotinoid insecticides: highlights of a symposium on strategic molecular designs. *J. Agric. Food Chem.* **59**, 2883–6.

Tosi, S., Bergamini, D., Porrini, C. and Medrzycki, P. (2013). Influence of pollen quality on honey bee health. Proceedings of the American Bee Research Conference, edited by Zachary Huang. *Am. Bee J.* **153**, 306–307.

Tremolada, P., Mazzoleni, M., Saliu, F., Colombo, M. and Vighi, M. (2010). Field trial for evaluating the effects on honeybees of corn sown using Cruiser and Celest xl treated seeds. *Bull. Environ. Contam. Toxicol.* **85**, 229–34.

Tsuruda, J. M. and Page, R. E. (2009). The effects of foraging role and genotype on light and sucrose responsiveness in honey bees (Apis mellifera L.). *Behav. Brain Res.* **205**, 132–137.

Underwood, R. M. and vanEngelsdorp, D. (2007). Colony collapse disorder: have we seen this before? *Bee Cult.* **17110**, 1–8.

Van der Sluijs, J. P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.-M. and Belzunces, L. P. (2013). Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr. Opin. Environ. Sustain.* **5**, 293–305.

Van der Sluijs, J. P., Amaral-Rogers, V., Belzunces, L. P., Bijleveld van Lexmond, M. F. I. J., Bonmatin, J.-M., Chagnon, M., Downs, C. a., Furlan, L., Gibbons, D. W., Giorio, C., et al. (2014). Conclusions of the Worldwide Integrated Assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. *Environ. Sci. Pollut. Res.*

Van Dijk, T. C., Van Staalduinen, M. a and Van der Sluijs, J. P. (2013). Macro-invertebrate decline in surface water polluted with imidacloprid. *PLoS One* **8**, e62374.

Vandame, R., Meled, M., Colin, M.-E. and Belzunces, L. P. (1994). alteration homing flight honey bees sublethal dose deltametrin. *Environ. Toxicol. Chem.*

Vandame, R., Meled, M., Colin, M.-E. and Belzunces, L. P. (1995). Alteration of the homing-flight in the honey bee Apis mellifera L. exposed to sublethal dose of deltamethrin. *Environ. Toxicol. Chem.* **14**, 855–860.

vanEngelsdorp, D. and Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J Invertebr Pathol* **103 Suppl**, S80–95.

VanEngelsdorp, D., Hayes Jr., J., Underwood, R. M. and Pettis, J. (2008). A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. *PLoS One* **3**, e4071.

vanEngelsdorp, D., Hayes, J., Underwood, R. and Pettis, J. (2010). A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. *J. Apic. Res.* **49**, 7.

Vidau, C., Diogon, M., Aufauvre, J., Fontbonne, R., Viguès, B., Brunet, J.-L. L., Texier, C., Biron, D. G., Blot, N., El Alaoui, H., et al. (2011). Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by Nosema ceranae. *PLoS One* **6**, e21550.

Von Frisch, K. (1967). *The dance language and orientation of bees*. (ed. Harvard University Press, Cambridge, MA, U.).

Watanabe, M. E. (1994). Pollination worries rise as honey bees decline. *Science* **265**, 1170.

Wen, Z. and Scott, J. G. (1997). Cross-Resistance to Imidacloprid in Strains of German Cockroach (Blattella germanica) and House Fly (Musca domestica). *Pestic. Sci.* **49**, 367–371.

Wheeler, W. M. (1928). *The Social Insects Their Origin And Evolution*. Kegan Paul Trench Trubner And Co Ltd ; London.

Williams, I. H. (1994). The dependence of crop production within the European Union on pollination by honey bees. *Agric. Zool. Rev. (United Kingdom).*

Williamson, S. M. and Wright, G. a (2013). Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *J Exp Biol* **216**, 1799–1807.

Williamson, S. M., Willis, S. J. and Wright, G. a (2014). Exposure to neonicotinoids influences the motor function of adult worker honeybees. *Ecotoxicology* **23**, 1409–18.

Woods, W. a, Heinrich, B. and Stevenson, R. D. (2005). Honeybee flight metabolic rate: does it depend upon air temperature? *J. Exp. Biol.* **208**, 1161–73.

Wu, J. Y., Anelli, C. M. and Sheppard, W. S. (2011). Sub-lethal effects of pesticide residues in brood comb on worker honey bee (Apis mellifera) development and longevity. *PLoS One* **6**, e14720.

Yang, E. C., Chuang, Y. C., Chen, Y. L. and Chang, L. H. (2008). Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **101**, 1743–8.

Yang, E. C., Chang, H. C., Wu, W. Y. and Chen, Y. W. (2012). Impaired Olfactory Associative Behavior of Honeybee Workers Due to Contamination of Imidacloprid in the Larval Stage. *PLoS One* **7**.