Effects of common agrochemicals on the health and function of honey bee colonies

Théotime Colin

BSc, MSc

This thesis is submitted for the degree of Doctor of Philosophy at

Macquarie University

Department of Biological Sciences

Submitted on the 15th of August 2019

Declaration

I certify that this thesis entitled « Effects of common agrochemicals on the health and function of honey bee colonies » has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me. Contributions of co-authors have been appropriately acknowledged, and so has any assistance that I have received in my research.

I certify that all information sources and literature used are indicated in the thesis.

As this research was on invertebrates, the research presented was not subject to approval by the Macquarie University Ethics Review Committee.

Théotime Colin

Contents

List of original publications

Chapter 2: Meikle, W.G., Holst, N., Colin, T., Weiss, M., Carroll, M.J., McFrederick, Q.S., Barron, A.B., 2018. Using within-day hive weight changes to measure environmental effects on honey bee colonies. PLoS One 13, e0197589. https://doi.org/10.1371/journal.pone.0197589

Chapter 3: Colin, T., Bruce, J., Meikle, W.G., Barron, A.B., 2018. The development of honey bee colonies assessed using a new semi-automated brood counting method: Combcount. PLoS One 13, 1–14. https://doi.org/10.1371/journal.pone.0205816

Chapter 4: Colin, T., Meikle, W.G., Paten, A.M., Barron, A.B., 2019. Long-term dynamics of honey bee colonies following exposure to chemical stress. Sci. Total Environ. 677, 660–670. https://doi.org/10.1016/j.scitotenv.2019.04.402

Chapter 5: In review in *Apidologie*, Colin, T.*, Plath, J.A.*, Klein, S.*, Vine, P., Devaud, J.-M., Lihoreau, M., Barron, A.B., Meikle, W.G., n.d. The miticide thymol in combination with trace levels of the neonicotinoid imidacloprid reduces visual learning performance in honey bees (*Apis mellifera*).

Chapter 6: Colin, T.*, Lim, M.Y.*, Quarrell, S.R., Allen, G.R., Barron, A.B., 2019. Effects of thymol on European honey bee hygienic behaviour. Apidologie 50, 141–152. https://doi.org/10.1007/s13592-018-0625-8

Chapter 7: Colin, T., Meikle, W.G., Wu, X., Barron, A.B., 2019c. Traces of a Neonicotinoid Induce Precocious Foraging and Reduce Foraging Performance in Honey Bees. Environ. Sci. Technol. 53, 8252–8261. https://doi.org/10.1021/acs.est.9b02452

* equal contributions

List of contributors

WGM – William G. Meikle; NH – Niels Holst; TC – Théotime Colin; MW – Milagra Weiss; MJC – Mark J. Carrol; QSM – Quinn S. McFrederick; ABB – Andrew B. Barron; JB – Jake Bruce; AMP – Amy M. Paten; MYL – Meng Yong Lim; SRQ – Stephen R. Quarrell; GRA – Geoff R. Allen; JAP – Jenny A. Plath; SK – Simon Klein; PV – Peta Vine; JMD – Jean-Marc Devaud; ML – Mathieu Lihoreau; XW – Xiaobo Wu 吴小波.

	Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7
Conception & design	WGM ; NH	**TC**; JB; WGM; ABB	WGM; ABB; **TC**	ABB; **TC**; SK; JAP; PV; WGM	**TC**; MYL; SRQ; GRA; ABB	**TC**; WGM; ABB
Planning & implementation	WGM; NH; **TC**; ABB	**TC**	WGM; **TC**; ABB	**TC**; PV; SK; JAP	**TC**; MYL; SRQ; GRA; ABB	**TC**; XW
Data collection	WGM; **TC**; MW; MJC; QSM	**TC**; JB	WGM; **TC**; ABB; AMP	**TC**; PV; SK; JAP	**TC**; MYL; SRQ; GRA; ABB	**TC**; XW
Analysis & interpretation	WGM; NH; **TC**	**TC**; JB	**TC**; WGM; ABB; AMP	**TC**; JAP; ABB	**TC**; MYL; SRQ; GRA; ABB	**TC**
Writing the article	WGM; MJC; **TC**; MW; QSM	**TC**; JB; WGM; ABB	**TC**; WGM; ABB	**TC**; JAP; SK; JMD; ML; ABB	**TC**; MYL; SRQ; GRA; ABB	**TC**; WGM; ABB
Overall responsibility	WGM	**TC**	**TC**	ABB; **TC**; SK	**TC**	**TC**

Summary

Pesticides have come under scrutiny in the last two decades for their effects on European honey bees. The effects of the highest doses of insecticides encountered on crops by honey bees are often lethal and well acknowledged. There is, however, much debate surrounding the potential consequences of exposure to smaller amounts of these pesticides for bee colonies. Field studies at these levels are scarce and often report contradictory results across environments. Due to technical constraints, these studies generally lack the precision and temporal resolution necessary to elucidate what causes the effects of a pesticide to vary across environments.

Much attention has been given to the neonicotinoid insecticides, because of early reports of colony failure associated with elevated amounts of these pesticides in dead worker bees. The pesticides used by beekeepers within hives against the parasitic mite *Varroa destructor* have, however, received much less attention. Controlled experiments on the effect of these treatments on whole bee colonies are scarce, even though they are almost ubiquitous in hives.

To lift some of the technical constraints specific to honeybee studies, we developed a method to automatically and continuously measure the daily variations of the weight of hives, such that we can extract from these data the amount of nectar collected daily and indices of foraging intensity. We then developed a new analytical method to quickly measure the area of capped brood and capped honey from photos of hive frames. We applied these methods to a large field study replicated across two continents, where we treated hives with the neonicotinoid imidacloprid and the acaricide thymol, alone and in combination. We found that the effect of imidacloprid on bee colonies varied between environments and that the miticide thymol can have long-term negative effects on bee colonies. We further investigated how these pesticides affect bee colonies by studying the effects of thymol on the hygienic behaviour of honeybees, and the effect of imidacloprid on individual bee foraging performances. These results add to our understanding of bee colony growth and development and how honey bee colony performance is affected by pesticides, and will help improve honeybee health worldwide.

Acknowledgements

1. Aims and overview

Pollinators increase the yields of several important food crops (Gallai *et al.*, 2009; Rollin and Garibaldi, 2019; Woodcock *et al.*, 2019) but beekeepers struggle to supply enough bee colonies to meet the demands for crop pollination (Aizen and Harder, 2009). The main reason for this is the recent increased mortality rate of managed honey bee colonies due to a range of deleterious consequences related to intensive agricultural practices. These practices include; the international trade of bees, which has been responsible for the spread of several bee parasites and pathogens, the rarefaction of wild flowers due to the widespread use of herbicides, and the exposure of pollinating insects to crop insecticides (Potts *et al.*, 2010; Goulson *et al.*, 2015). Despite tremendous scientific and legislative attention, it is still unclear how most agrochemicals affect standard honey bee colonies at the low levels to which bees are commonly and chronically exposed (Meikle *et al.*, 2016; Woodcock *et al.*, 2017; Tihelka, 2018; Osterman *et al.*, 2019). In this thesis, I investigate the mechanisms and effects of common agricultural pesticides used by beekeepers and farmers on the population dynamics of honey bee colonies (*Apis mellifera*).

I first developed two methods to ease the study of fully developed, standard, commercial honey bee colonies (Chapters 2 & 3). Honey bee colonies are complex societies whose operation is dependent on many social interactions, trade-offs and interacting feedback loops (Schmickl and Crailsheim, 2007; Khoury, Myerscough and Barron, 2011; Becher *et al.*, 2014; Perry *et al.*, 2015). Comprehending or measuring the effects of stressors on colony function is complex, costly, and very labour intensive, which has limited the scope of colony-level studies to date (Henry *et al.*, 2015; Meikle and Holst, 2015a). The two methods I developed have

improved our ability to produce detailed accurate and frequent colony-level measurements. I then used these methods to investigate the effects of common agricultural pesticides on the health of honey bee colonies. I measured the long-term population dynamics and performance of bee colonies exposed to these pesticides. To provide a thorough and well-resolved assessment, I then designed a series of short experiments using standard assays to investigate the behavioural and individual mechanisms causal of the effects observed at the colony level.

I chose to focus on the miticide thymol applied by beekeepers within hives and on the neonicotinoid insecticide imidacloprid used by farmers on crops. Thymol is commercially available as a miticide treatment. It is used by beekeepers within hives to kill a widespread parasite of the honeybee (Marchetti, Barbattini and D'Agaru, 1984). The neonicotinoid imidacloprid is one of the most commonly used insecticides, in gardens, orchards and crops such as canola, cotton, maize and sunflowers (Simon-Delso *et al.*, 2015). These two pesticides are likely to be simultaneously encountered by honey bees, so I studied their potential interactions also by exposing some bees to a treatment with both pesticides combined.

2. Development of methodologies for the study of standard bee colonies

Apicultural research is currently limited by the high costs and lack of methods and tools to study the effects of stressors on honey bees at the level of the colony. Most studies of the effects of agrochemicals on bees to date have investigated the effect of single exposures to high doses of pesticides on the behaviours of individual or small groups of honey bees isolated in the laboratory (Cresswell, 2011). A common limitation of these methodologies is that the effects of pesticides on isolated bees often don't linearly translate into consequences for the colony (Henry *et al.*, 2015). This is because low amounts of pesticides have sublethal effects on the behaviour of bees that can be either amplified or buffered through social interactions between members of the colony (Cresswell, 2011; Henry *et al.*, 2015; Lundin *et al.*, 2015; Perry *et al.*, 2015; van Dooremalen *et al.*, 2018). Consequently, it is not simple to extrapolate from studies of individual bees to likely outcomes for a colony. Similarly, synergistic interactions between pesticides and common bee parasites are likely to occur in real conditions, but are often hard to comprehend in lab based studies (O'Neal, Anderson and Wu-Smart, 2018). Despite those limitations, studies of impacts of pesticides on individual bees are most common because they are cheaper and less labour intensive (e.g. see Meikle and Holst, 2015b for a description of colony-level methodologies).

A common solution to avoid the costs of colony-level studies is to use data collected on individual bees to inform mathematical models of honey bee colony function, to predict effects of pesticides on whole bee colonies (Henry *et al.*, 2012; Becher *et al.*, 2014). The validity of the predictions is uncertain due to the lack of empirical data to check predictions from these models (e.g. Khoury, Myerscough and Barron, 2011; Khoury, Barron and Myerscough, 2013; Russell, Barron and Harris, 2013; Barron, 2015; Myerscough *et al.*, 2017). Given the diversity

and rapid development of agrochemicals and agrochemical mixtures (Mullin *et al.*, 2010; Brown *et al.*, 2016), improving models of honey bee colony function could greatly speed up assessments of potential stressors to bee colonies and improve the health of bumblebee and honey bee colonies worldwide. But to do this, more and better quality measures of honey bee colony performance are still needed.

To help resolve these issues, I developed two methods to reduce the costs and labour of colony-level experiments.

I first contributed to the development of a method to automatically estimate the daily foraging effort of bee colonies from continuous measurement of hive, as it changes throughout and between days (Chapter 2, Meikle *et al.*, 2018). This new sensor-driven method helps investigate the effects of pesticides on forager bees, foraging activity and foraging success, and can inform many bee models that include such parameters.

I then developed a free, semi-automated software to assist with measures of capped honey and capped brood areas, commonly used in bee models and in all colony-level studies (Chapter 3). This new tool reduces the time needed to take these measures by 60% in comparison with the most common alternative precise method.

These two methods allowed me to conduct a large field study, to investigated the effects of common pesticides on standard bee colonies.

3. Pesticides of interest

a) Apicultural pesticides

Beekeepers commonly use pesticides inside a bee colony to kill the parasitic mite *Varroa destructor* that has a serious impacts on bee health (Tihelka, 2018). This bee parasite is native to Asia where it was originally only found in colonies of the Asian honey bee *Apis cerana* (Oldroyd, 1999). *V. destructor* is thought to have jumped hosts in the late 1950s and to have colonized *Apis mellifera* colonies across the globe from the late 1970s (Oldroyd, 1999). It is now found wherever *A. mellifera* is present with the exception of Australia and a few small islands. *V. destructor* feeds on fat bodies from bees (Ramsey *et al.*, 2019), disturbing bee larval development and increasing virus load in adult honey bees and their larvae (Rosenkranz, Aumeier and Ziegelmann, 2010).

The pesticides used by beekeepers to control this mite are applied directly within hives. They are commonly referred to as miticides (or as acaricides or varroacides) and correspond to very wide variety of compounds. The term miticide includes organic acids, pyrethroids, monoterpenes and various chemicals with both acaricidal and insecticidal activity such as coumaphos and amitraz (Tihelka, 2018). In-hive pesticides are widely used, and autumn treatment is even mandatory in some countries (Brodschneider, Brus and Danihlík, 2019), but there is surprisingly little knowledge of the cost-benefit ratio of miticide treatments for bee colonies (Tihelka, 2018). Some of them are known or likely to affect the health of honey bees (Tihelka, 2018). There is growing concern that variations in the effects of these treatments on the bees and on the mites could significantly affect bee colony losses (Rosenkranz, Aumeier and Ziegelmann, 2010; Beyer *et al.*, 2018; Tihelka, 2018; Brodschneider, Brus and Danihlík, 2019).

The miticide thymol is a monoterpene pesticide, naturally occurring in thyme, and authorized for use in organic apiculture in Europe. For these reasons (like other synthetic organic pesticides) it is commonly regarded as safe, or at least more natural for the bees by beekeepers (Mullin *et al.*, 2015; Underwood, Traver and López-Uribe, 2019). Thymol may have become popular after several reports of *V. destructor* mites developing resistance to chemical alternatives such as coumaphos, tau-fluvalinate and amitraz (Milani, 1999). There is, however, still not enough data on miticide use to properly compare their popularity, and there are stark differences in miticide use between countries (Brodschneider, Brus and Danihlík, 2019).

b) Crop insecticides

Many crop insecticides have, unsurprisingly, been pointed out for their effects on non-target insects, especially beneficial pollinators. A few commonly applied crop insecticides, are under intense scrutiny for their effects on insects of agricultural importance such as honey bees (Woodcock *et al.*, 2017), various species of bumblebees in the genus *Bombus* (Woodcock *et al.*, 2017) and of solitary bees in the genus *Osmia* (Rundlöf *et al.*, 2015; Woodcock *et al.*, 2017). The effects of the highest doses of common insecticides like fipronil (Decourtye *et al.*, 2011), methoxyfenozide (Meikle *et al.*, 2019) and the pesticides in the neonicotinoid family (Henry *et al.*, 2012; Tsvetkov *et al.*, 2017; Woodcock *et al.*, 2017) on these insects are dramatic, but well-understood (Suchail, Guez and Belzunces, 2001; Hervé Thany, 2010). There is still, however, much debate about the effects of the nearly ubiquitous traces of insecticides in agricultural environments to which pollinators are chronically exposed through dusts, water, pollen and nectar and that sometimes remain in the nectar and pollen stored by bees within their colonies (Suchail, Guez and Belzunces, 2001; Whitehorn *et al.*, 2012; Meikle *et al.*, 2016; Tsvetkov *et al.*, 2017; Woodcock *et al.*, 2017; Osterman *et al.*, 2019). The neonicotinoid insecticide imidacloprid is one of the most commonly applied insecticides in gardens and crops

14

(Simon-Delso *et al.*, 2015). It was used worldwide before its ban in Europe in 2013 on flowering crops and in 2018 for all outdoor use (European Commission, 2018). The bans were implemented amid concerns about its effects on non-target beneficial invertebrates including freshwater crustaceans and pollinating insects (EFSA, 2018).

c) Potential for interactions

Pollinating insects are often exposed simultaneously to more than one pesticide (Mullin *et al.*, 2010). These include fungicides, insecticides and herbicides that they collect in water, nectar and pollen (Schmolke, Kearns and O'Neill, 2018), or are directly exposed to through the dusts and droplets that drift away during crop sowing or spraying (Krupke, Long and Eitzer, 2017; Zhu *et al.*, 2017). In addition, honey bees are commonly treated with antimicrobial drugs and miticides in the hive (Mullin *et al.*, 2010; Johnson *et al.*, 2013). These agrochemicals are almost always found as mixtures in the pollen and honey stores of managed honey bee colonies (Mullin *et al.*, 2010; Mitchell *et al.*, 2017a) and there is growing concern that synergetic interactions or additive effects occur when pesticides are simultaneously encountered by honey bees (Mullin *et al.*, 2010, 2015; Prado *et al.*, 2019).

In honey, neonicotinoids commonly occur in combination with at least one other neonicotinoid (Mitchell *et al.*, 2017a), and residues of several miticides often accumulate together in the wax from hives (Johnson, Pollock and Berenbaum, 2009; Mullin *et al.*, 2010). Neonicotinoids and miticides are likely to co-occur (Mullin *et al.*, 2010; Retschnig *et al.*, 2015; Mitchell *et al.*, 2017b; Calatayud-Vernich *et al.*, 2018).

Individual-based laboratory studies suggest that many pesticides have the potential to act synergistically to reduce bee health (Johnson, Pollock and Berenbaum, 2009; Ciarlo *et al.*, 2012; Johnson *et al.*, 2013; Zhu *et al.*, 2014, 2017; Retschnig *et al.*, 2015; Sgolastra *et al.*, 2017; Raimets *et al.*, 2018; Sgolastra, Arnan, *et al.*, 2018; Sgolastra, Blasioli, *et al.*, 2018; Yao

et al., 2018; Yao, Zhu and Adamczyk, 2018). A recent study following bees throughout their lifespan revealed complex sublethal behavioural effects of certain pesticide blends on worker bees (Prado *et al.*, 2019). But no published work has tried to directly measure the consequences of combined pesticide exposure on standard honey bee colonies (but see Odemer and Rosenkranz, 2018).

To investigate potential interactive effects of common pesticides on honey bee colony health and on individual honey bee learning and short-term memory, I exposed certain bee colonies to a combined imidacloprid and thymol treatment in experiments described in Chapters 4 and 5.

4. Experimental approach

a) Effects on the long term dynamics of whole bee colonies

Using the methods developed in Chapters 2 & 3, I realized a large semi-field colony-level experiment replicated in Australia (where *V. destructor* is absent) and in the USA (where *V. destructor* is present) (Chapter 4). I measured the effects of exposure to two pesticides on food stores and on the short and long-term dynamics of bee populations and food stores. Bee colonies were divided into four groups and treated with the neonicotinoid imidacloprid, the miticide thymol, a combination of both, or left untreated as controls, and followed during nine months in both locations.

b) Identifying individual or group-level mechanisms

Our results showed that the effect of imidacloprid and thymol on bee colonies were long-lasting and strongly dependent on the environment. Based on these results I decided to try to identify the possible mechanisms of action of these pesticides on bee colonies.

Learning and memory are essential to the ability of bees to navigate and forage. A common hypothesis to explain the effect of pesticides on bee colonies is that these functions are particularly vulnerable to stress (Klein *et al.*, 2017). Decreases in the ability of bees to learn could prevent foragers from finding their way home, which could explain important forager losses. Following this hypothesis, we first investigated the effect of the miticides thymol and tau-fluvalinate, of the neonicotinoid imidacloprid and of a combined thymol and imidacloprid treatment on the short-term memory of individual bees. To test this, we measured the ability of bees to avoid a colour previously associated with electric shocks after a short period of time (Chapter 5).

To explain the brood losses in bee colonies exposed to thymol, we then hypothesized that thymol may disrupt hygienic behaviours essential to the health of bee larvae. Thymol is a

17

very volatile aromatic compound that bees often try to remove from the hive or cover with propolis when it is applied to the hives. Such a strong odour is thus likely to affect hygienic behaviours of bees that rely on quite subtle odour discrimination of diseased from healthy larvae. To test this, we measured the efficiency of treated colonies at removing dead brood and bees from the hive (Chapter 6).

To explain the different effects of imidacloprid we observed between replicates in Chapter 4 (colonies were either negatively or positively affected by the treatment depending on the location), we hypothesized that low amounts of imidacloprid may stimulate bees to forage through a process termed hormesis, or may cause bees to begin foraging when younger. To test whether imidacloprid could increase foraging activity or make workers start foraging when younger, we tagged bees with radio-frequency identification transponders and determined the age at which they began foraging and the number of foraging trips they performed (Chapter 7).

5. Main conclusions and perspectives

The following chapters of this thesis are composed of articles that are either published or being reviewed in international scientific journals. Each of them includes a discussion focusing on the interpretation and implications of their own results. I highlight the cohesive nature of the work in Chapter 8 - the General Discussion, and expand on the scope and perspectives that this thesis brings to the field of honey bee research.

In brief, we found that the miticide thymol had a strong negative effect on the bee and brood populations, and that the effects of the neonicotinoid imidacloprid strongly depended on the field site (Chapter 4). Thymol and imidacloprid, when combined, decreased the learning ability or short-term memory of bees (Chapter 5). Thymol increased the removal of dead brood in bee colonies, but may also affect healthy brood or cause its removal (Chapter 6). Imidacloprid caused worker bees to start foraging earlier in life, die younger and perform fewer foraging flights (Chapter 7).

Altogether, these findings show that (1) a thorough reassessment of the benefits of miticide treatments on bees could greatly improve honey bee health, (2) crop insecticides should be studied across a variety of climates before drawing conclusions regarding effects on pollinators, (3) interactions between insecticides and miticides that affect bee behavior can occur, and (4) effects observed at the individual level do not always translate into colony-level effects, and colony-level studies should be preferred when assessing the effects of stressors on honey bees. Along with these conclusions, the six chapters of this thesis provide detailed, well-resolved data that can be used to improve, develop and test models of honey bee colony function.

References

Aizen, M. A. and Harder, L. D. (2009) 'The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination', *Current Biology*. Elsevier Ltd, 19(11), pp. 915–918. doi: 10.1016/j.cub.2009.03.071.

Barron, A. B. (2015) 'Death of the bee hive: understanding the failure of an insect society', *Current Opinion in Insect Science*. Elsevier Inc, 10(Ccd), pp. 45–50. doi: 10.1016/j.cois.2015.04.004.

Becher, M. A. *et al.* (2014) 'BEEHAVE: a systems model of honeybee colony dynamics and foraging to explore multifactorial causes of colony failure', *Journal of Applied Ecology*. Edited by E. Morgan, 51(2), pp. 470–482. doi: 10.1111/1365-2664.12222.

Beyer, M. *et al.* (2018) 'Winter honey bee colony losses, *Varroa destructor* control strategies, and the role of weather conditions: Results from a survey among beekeepers', *Research in Veterinary Science*, 118(November 2017), pp. 52–60. doi: 10.1016/j.rvsc.2018.01.012.

Brodschneider, R., Brus, J. and Danihlík, J. (2019) 'Comparison of apiculture and winter mortality of honey bee colonies (*Apis mellifera*) in Austria and Czechia', *Agriculture, Ecosystems & Environment*. Elsevier, 274(January), pp. 24–32. doi: 10.1016/j.agee.2019.01.002.

Brown, M. J. F. *et al.* (2016) 'A horizon scan of future threats and opportunities for pollinators and pollination', *PeerJ*, 4(3), p. e2249. doi: 10.7717/peerj.2249.

Calatayud-Vernich, P. *et al.* (2018) 'Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure', *Environmental Pollution*, 241, pp. 106–114. doi: 10.1016/j.envpol.2018.05.062.

Ciarlo, T. J. *et al.* (2012) 'Learning impairment in honey bees vaused by agricultural spray adjuvants', *PLoS ONE*. Edited by G. Smagghe, 7(7), p. e40848. doi: 10.1371/journal.pone.0040848.

Colin, T. *et al.* (2018) 'The development of honey bee colonies assessed using a new semi-automated brood counting method: Combcount', *PLoS ONE*, 13(10), pp. 1–14. doi: 10.1371/journal.pone.0205816.

Colin, T. *et al.* (2019) 'Long-term dynamics of honey bee colonies following exposure to

chemical stress', *Science of The Total Environment*. Elsevier B.V., 677, pp. 660–670. doi: 10.1016/j.scitotenv.2019.04.402.

Cresswell, J. E. (2011) 'A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees', *Ecotoxicology*, 20(1), pp. 149–157. doi: 10.1007/s10646-010-0566-0.

Decourtye, A. *et al.* (2011) 'Honeybee tracking with microchips: a new methodology to measure the effects of pesticides', *Ecotoxicology*, 20(2), pp. 429–437. doi: 10.1007/s10646-011-0594-4.

van Dooremalen, C. *et al.* (2018) 'Single and interactive effects of *Varroa destructor*, *Nosema spp.*, and imidacloprid on honey bee colonies *(Apis mellifera)*', *Ecosphere*, 9(8), p. e02378. doi: 10.1002/ecs2.2378.

EFSA (2018) 'Evaluation of the data on clothianidin, imidacloprid and thiamethoxam for the updated risk assessment to bees for seed treatments and granules in the EU', *EFSA Supporting Publications*, 15(2), pp. 1–31. doi: 10.2903/sp.efsa.2018.EN-1378.

European Commission (2018) *Protecting bees: EU set to completely ban outdoor use of pesticides harmful to bees*, *European Commission Press Release Database*. Available at: http://europa.eu/rapid/press-release_MEX-18-3583_en.htm (Accessed: 22 May 2019).

Gallai, N. *et al.* (2009) 'Economic valuation of the vulnerability of world agriculture confronted with pollinator decline', *Ecological Economics*. Elsevier B.V., 68(3), pp. 810–821. doi: 10.1016/j.ecolecon.2008.06.014.

Goulson, D. *et al.* (2015) 'Bee declines driven by combined Stress from parasites, pesticides, and lack of flowers', *Science*, 347(6229). doi: 10.1126/science.1255957.

Henry, M. *et al.* (2012) 'A common pesticide decreases foraging success and survival in honey bees', *Science*, 336(6079), pp. 348–350. doi: 10.1126/science.1215039.

Henry, M. *et al.* (2015) 'Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees', *Proceedings of the Royal Society B: Biological Sciences*, 282(1819), p. 20152110. doi: 10.1098/rspb.2015.2110.

Hervé Thany, S. (2010) *Insect Nicotinic Acetylcholine Receptors*. Edited by S. H. Thany. New York, NY: Springer New York (Advances in Experimental Medicine and Biology). doi: 10.1007/978-1-4419-6445-8.

Johnson, R. M. *et al.* (2013) 'Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*)', *PLoS ONE*. Edited by N. E. Raine, 8(1), p. e54092. doi: 10.1371/journal.pone.0054092.

Johnson, R. M., Pollock, H. S. and Berenbaum, M. R. (2009) 'Synergistic interactions between in-hive miticides in *Apis mellifera*', *Journal of Economic Entomology*. Edited by W. Blenau, 102(2), pp. 474–479. doi: 10.1603/029.102.0202.

Khoury, D. S., Barron, A. B. and Myerscough, M. R. (2013) 'Modelling Food and Population Dynamics in Honey Bee Colonies', *PLoS ONE*. Edited by A. G. Dyer, 8(5), p. e59084. doi: 10.1371/journal.pone.0059084.

Khoury, D. S., Myerscough, M. R. and Barron, A. B. (2011) 'A Quantitative Model of Honey Bee Colony Population Dynamics', *PLoS ONE*. Edited by J. A. R. Marshall, 6(4), p. e18491. doi: 10.1371/journal.pone.0018491.

Klein, S. *et al.* (2017) 'Why Bees Are So Vulnerable to Environmental Stressors', *Trends in Ecology and Evolution*. Elsevier Ltd, 32(4), pp. 268–278. doi: 10.1016/j.tree.2016.12.009.

Krupke, C. H., Long, E. Y. and Eitzer, B. D. (2017) 'Planting of neonicotinoid-treated maize poses risks for honey bees and other non-target organisms over a wide area without consistent crop yield benefit', pp. 1449–1458. doi: 10.1111/1365-2664.12924.

Lundin, O. *et al.* (2015) 'Neonicotinoid insecticides and their impacts on bees: A systematic review of research approaches and identification of knowledge gaps', *PLoS ONE*, 10(8), pp. 1–20. doi: 10.1371/journal.pone.0136928.

Marchetti, S., Barbattini, R. and D'Agaru, M. (1984) 'Comparative effectiveness of treatments used to control *Varroa jacobsoni* Oud.', *Apidologie*, 15(4), pp. 363–378.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLoS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Meikle, W. G. *et al.* (2018) 'Using within-day hive weight changes to measure environmental effects on honey bee colonies', *PLoS ONE*. Edited by A. G. Dyer, 13(5), p. e0197589. doi: 10.1371/journal.pone.0197589.

Meikle, W. G. *et al.* (2019) 'Exposure to sublethal concentrations of methoxyfenozide disrupts honey bee colony activity and thermoregulation', *PLoS ONE*, 14(3), pp. 1–21. doi:

10.1371/journal.pone.0204635.

Meikle, W. G. and Holst, N. (2015) 'Application of continuous monitoring of honeybee colonies', *Apidologie*, 46(1), pp. 10–22. doi: 10.1007/s13592-014-0298-x.

Milani, N. (1999) 'The resistance of *Varroa jacobsoni* Oud. to acaricides', *Apidologie*, 30(2–3), pp. 229–234. doi: 10.1051/apido:19990211.

Mitchell, E. A. D. *et al.* (2017) 'A worldwide survey of neonicotinoids in honey', *Science*, 358(6359), pp. 109–111. doi: 10.1126/science.aan3684.

Mullin, C. A. *et al.* (2010) 'High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health', *PLoS ONE*, 5(3). doi: 10.1371/journal.pone.0009754.

Mullin, C. A. *et al.* (2015) 'The formulation makes the honey bee poison', *Pesticide Biochemistry and Physiology*. Elsevier Inc., 120, pp. 27–35. doi: 10.1016/j.pestbp.2014.12.026.

Myerscough, M. R. *et al.* (2017) 'Why Do Hives Die? Using Mathematics to Solve the Problem of Honey Bee Colony Collapse', in Anderssen, B. et al. (eds) *The Role and Importance of Mathematics in Innovation*. Singapore: Springer Singapore, pp. 35–50.

O'Neal, S. T., Anderson, T. D. and Wu-Smart, J. Y. (2018) 'Interactions between pesticides and pathogen susceptibility in honey bees', *Current Opinion in Insect Science*, 26, pp. 57–62. doi: 10.1016/j.cois.2018.01.006.

Odemer, R. and Rosenkranz, P. (2018) 'Chronic exposure to a neonicotinoid pesticide and a synthetic pyrethroid in full-sized honey bee colonies', *BioRxiv*, p. 293167. doi: 10.1101/293167.

Oldroyd, B. P. (1999) 'Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees', *Trends in Ecology & Evolution*, 14(8), pp. 312–315. doi: 10.1016/S0169-5347(99)01613-4.

Osterman, J. *et al.* (2019) 'Clothianidin seed-treatment has no detectable negative impact on honeybee colonies and their pathogens', *Nature Communications*. Springer US, 10(1), p. 692. doi: 10.1038/s41467-019-08523-4.

Perry, C. J. *et al.* (2015) 'Rapid behavioral maturation accelerates failure of stressed honey

bee colonies', *Proceedings of the National Academy of Sciences*, 112(11), pp. 3427–3432. doi: 10.1073/pnas.1610243113.

Potts, S. G. *et al.* (2010) 'Global pollinator declines: Trends, impacts and drivers', *Trends in Ecology and Evolution*. Elsevier Ltd, 25(6), pp. 345–353. doi: 10.1016/j.tree.2010.01.007.

Prado, A. *et al.* (2019) 'Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees', *Science of the Total Environment*. Elsevier B.V., 650, pp. 1250–1260. doi: 10.1016/j.scitotenv.2018.09.102.

Raimets, R. *et al.* (2018) 'Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees *(Bombus terrestris* L.)', *Pest Management Science*, 74(3), pp. 541–546. doi: 10.1002/ps.4756.

Ramsey, S. D. *et al.* (2019) '*Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph', *Proceedings of the National Academy of Sciences*, 116(5), pp. 1792–1801. doi: 10.1073/pnas.1818371116.

Retschnig, G. *et al.* (2015) 'Effects, but no interactions, of ubiquitous pesticide and parasite stressors on honey bee (*Apis mellifera*) lifespan and behaviour in a colony environment', *Environmental Microbiology*, 17(11), pp. 4322–4331. doi: 10.1111/1462-2920.12825.

Rollin, O. and Garibaldi, L. A. (2019) 'Impacts of honeybee density on crop yield: A meta-analysis', *Journal of Applied Ecology*. Edited by T. Diekötter, 56(5), pp. 1152–1163. doi: 10.1111/1365-2664.13355.

Rosenkranz, P., Aumeier, P. and Ziegelmann, B. (2010) 'Biology and control of *Varroa destructor*', *Journal of Invertebrate Pathology*. Elsevier Inc., 103(SUPPL. 1), pp. S96–S119. doi: 10.1016/j.jip.2009.07.016.

Rundlöf, M. *et al.* (2015) 'Seed coating with a neonicotinoid insecticide negatively affects wild bees', *Nature*, 521(7550), pp. 77–80. doi: 10.1038/nature14420.

Russell, S., Barron, A. B. and Harris, D. (2013) 'Dynamic modelling of honey bee (*Apis mellifera*) colony growth and failure', *Ecological Modelling*. Elsevier B.V., 265, pp. 158–169. doi: 10.1016/j.ecolmodel.2013.06.005.

Schmickl, T. and Crailsheim, K. (2007) 'HoPoMo: A model of honeybee intracolonial population dynamics and resource management', *Ecological Modelling*, 204(1–2), pp. 219–245. doi: 10.1016/j.ecolmodel.2007.01.001.

Schmolke, A., Kearns, B. and O'Neill, B. (2018) 'Plant guttation water as a potential route for pesticide exposure in honey bees: a review of recent literature', *Apidologie*, 49(5), pp. 637–646. doi: 10.1007/s13592-018-0591-1.

Sgolastra, F. *et al.* (2017) 'Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species', *Pest Management Science*, 73(6), pp. 1236–1243. doi: 10.1002/ps.4449.

Sgolastra, F., Arnan, X., *et al.* (2018) 'Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee', *Proceedings of the Royal Society B: Biological Sciences*, 285(1885), p. 20180887. doi: 10.1098/rspb.2018.0887.

Sgolastra, F., Blasioli, S., *et al.* (2018) 'Lethal effects of Cr(III) alone and in combination with propiconazole and clothianidin in honey bees', *Chemosphere*. Elsevier Ltd, 191, pp. 365–372. doi: 10.1016/j.chemosphere.2017.10.068.

Simon-Delso, N. *et al.* (2015) 'Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites', *Environmental Science and Pollution Research*, 22(1), pp. 5–34. doi: 10.1007/s11356-014-3470-y.

Suchail, S., Guez, D. and Belzunces, L. P. (2001) 'Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*', *Environmental Toxicology and Chemistry*, 20(11), p. 2482. doi: 10.1897/1551-5028(2001)020<2482:DBAACT>2.0.CO;2.

Tihelka, E. (2018) 'Effects of synthetic and organic acaricides on honey bee health: A review', *Slovenian Veterinary Research*, 55(3), pp. 114–140. doi: 10.26873/SVR-422-2017.

Tsvetkov, N. *et al.* (2017) 'Chronic exposure to neonicotinoids reduces honey bee health near corn crops', *Science*, 356(6345), pp. 1395–1397. doi: 10.1126/science.aam7470.

Underwood, R. M., Traver, B. E. and López-Uribe, M. M. (2019) 'Beekeeping Management Practices Are Associated with Operation Size and Beekeepers' Philosophy towards in-Hive Chemicals', *Insects*, 10(1), p. 10. doi: 10.3390/insects10010010.

Whitehorn, P. R. *et al.* (2012) 'Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production', *Science*, 336(6079), pp. 351–352. doi: 10.1126/science.1215025.

Woodcock, B. A. *et al.* (2017) 'Country-specific effects of neonicotinoid pesticides on honey

bees and wild bees', *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

Woodcock, B. A. *et al.* (2019) 'Meta-analysis reveals that pollinator functional diversity and abundance enhance crop pollination and yield', *Nature Communications*. Springer US, 10(1), p. 1481. doi: 10.1038/s41467-019-09393-6.

Yao, J. *et al.* (2018) 'Influences of acephate and mixtures with other commonly used pesticides on honey bee (*Apis mellifera*) survival and detoxification enzyme activities', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. Elsevier, 209(January), pp. 9–17. doi: 10.1016/j.cbpc.2018.03.005.

Yao, J., Zhu, Y. C. and Adamczyk, J. (2018) 'Responses of honey bees to lethal and sublethal doses of formulated clothianidin alone and mixtures', *Journal of Economic Entomology*, 111(4), pp. 1517–1525. doi: 10.1093/jee/toy140.

Zhu, W. *et al.* (2014) 'Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0077547.

Zhu, Y. C. *et al.* (2017) 'Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*)', *PLoS ONE*, 12(5), pp. 1–16. doi: 10.1371/journal.pone.0176837.

Chapter 2 Using within-day hive weight changes to measure environmental effects on honey bee colonies

Abstract

Patterns in within-day hive weight data from two independent datasets in Arizona and California were modelled using piecewise regression, and analysed with respect to honey bee colony behaviour and landscape effects. The regression analysis yielded information on the start and finish of a colony's daily activity cycle, hive weight change at night, hive weight loss due to departing foragers and weight gain due to returning foragers. Assumptions about the meaning of the timing and size of the morning weight changes were tested in a third study by delaying the forager departure times from one to three hours using screen entrance gates. A regression of planned vs. observed departure delays showed that the initial hive weight loss around dawn was largely due to foragers. In a similar experiment in Australia, hive weight loss due to departing foragers in the morning was correlated with net bee traffic (difference between the number of departing bees and the number of arriving bees) and from those data the payload of the arriving bees was estimated to be 0.02 g. The piecewise regression approach was then used to analyse a fifth study involving hives with and without access to natural forage. The analysis showed that, during a commercial pollination event, hives with previous access to forage had a significantly higher rate of weight gain as the foragers returned in the afternoon, and, in the weeks after the pollination event, a significantly higher rate of weight loss in the morning, as foragers departed. This combination of continuous weight data and piecewise regression proved effective in detecting treatment differences in foraging activity that other methods failed to detect.

Published version

Introduction

Hive weight data provide information on the interaction of a colony and its environment with little or no disturbance to the colony. Hive weight changes are a function of several factors, including colony food collection and consumption, bee development and disappearance, moisture gain and loss due to nectar inflow, ambient relative humidity, respiration and drinking (Hambleton, 1925), as well as robbing, absconding, and swarming. A central goal of researchers employing hive scales has been to characterize these colony events using weight data combined with improvements in sensor technology and analytical approaches (Meikle and Holst, 2015). The development of automated sensors for continuous monitoring has allowed researchers to quantify colony metrics beyond daily changes in hive weight due to net food storage and population changes. Linking electronic scales to data loggers was first reported in 1990, to show hive abandonment to the tracheal mite *Acarapis woodi* infestation (Buchmann and Thoenes, 1990). Continuous hive weight data have been shown to provide information on weather effects (Hambleton, 1925; Meikle et al., 2006), swarming (Buchmann and Thoenes, 1990; Meikle et al., 2006), differences among honey bee races (Buchmann and Thoenes, 1990), colony growth and consumption (Meikle et al., 2008; W. G. Meikle et al., 2016), hive abandonment (Thoenes and Buchmann, 1992), overwintering (Stalidzans et al., 2017) and the impact of pesticides on bee colonies (William G. Meikle et al., 2016). As equipment costs have

decreased, recent work has focused on integrating hive scales and other sensors into networks (Bayir and Albayrak, 2016; Gil-Lebrero et al., 2016; Meikle and Holst, 2015; Zacepins et al., 2017) that facilitate monitoring more hives at the same time and across different locations. As collecting continuous hive data becomes more efficient and more common, exploiting the data thoroughly and efficiently for information about key colony events and performance has become more important.

A key challenge to extracting information about individual colony events from continuous hive weight data is that the raw hive weight represents the sum effect of several contributing factors, including mass loss as the colony respires, consumes food and water, dries nectar, loses bees, and mass gain as the colony forages for nectar, pollen, water, and propolis. While longer-term weight changes mostly involve gains and losses of honey and pollen stores and the bee population, within-day changes largely involve the departure and return of foragers, influx of nectar, pollen and water, and changes in the moisture content of the food and hive parts due to respiration and ambient relative humidity fluctuations. Analysis of within-day weight changes is likely to reveal factors that can change on short notice, such as the size of the foraging population and the availability of forage. Depending on the magnitude of the effect compared to the precision of the scale, within-day weight changes may reveal colony-level effects that are difficult to measure using periodic hive evaluations. To isolate individual factors, within-day weight changes associated with diurnally-reoccurring activities were examined within highly targeted temporal periods. Using this approach, predictable colony events are detected from daily colony mass fluctuations occurring within specific time periods. Successful modelling of factor-associated mass changes depends largely on selection of appropriate monitoring periods and detection of slope changes by regression modelling.

To test our models, we examined continuous hive weight data from colonies subjected to a major colony stressor routinely encountered by honey bees, namely colony malnutrition. Honey bee colonies experience highly variable forage availability, and therefore, colony nutrition, that impacts colony function and survivorship across seasons and landscapes (Dustmann and von der Ohe, 1988; Kunert and Crailsheim, 1988). Diagnosing colony malnutrition in honey bee colonies can be difficult since such stress is not due to the presence of a particular compound, but rather the absence or imbalance of one or more nutrients. The effects of nutritional stress have been well described at the level of individual bees and colony outcomes. Individual malnourished workers have smaller hypopharyngeal glands, lower protein titers, diminished internal nutrient stores, poorer immune responses, and smaller ovary development than their well-fed counterparts (Alaux et al., 2010; Bitondi and Simões, 1996; Crailsheim, 1990; Sagili and Pankiw, 2007). Functionally, nutritionally-stressed workers mature faster into foragers, live shorter lives, and are less efficient at rearing brood, feeding other adults, foraging, and communicating forage locations (Brodschneider and Crailsheim, 2010; Dustmann and von der Ohe, 1988; Human et al., 2007; Jay, 1964; Mattila and Otis, 2007; Schulz et al., 1998; Scofield and Mattila, 2015; Toth et al., 2005). Colonies are known to collectively respond to poor forage and food store availability through modification of colony-level activities such as worker and reproductive rearing, foraging, colony defence or temperature management, that are critical to overall colony performance (Crailsheim et al., 1992; Maurizio and Hodges, 1950; Meikle et al., 2017; Schmickl et al., 2003; Schmickl and Crailsheim, 2002, 2001; Schmickl and Karsai, 2017; Schulz et al., 2002). Despite the centrality of colony nutrition on colony performance, relatively little is known about the colony-level effects leading up to colony malnutrition. Conventional methods of hive assessments may be too infrequent to adequately capture colony responses to nutritional changes. More frequent checks, more than once a week, of colony functions and performance by conventional periodic

hive evaluations are impractical both in terms of logistics and colony disturbance. Here, we describe how continuous weight data monitoring can be used to assess changes in colony activities associated with nutritional stress.

Materials and methods

a) The CAL 2014 dataset

Eight honey bee hives provided by a commercial beekeeping operation (Hiatt Honey CA LP) were installed in a commercial almond orchard, in four groups of two at regular intervals, on the periphery of a 510 ha irrigated almond orchard near Chowchilla, CA (37° 7'22.25"N, 120°16'21.12"W), in January 2014, as part of an unrelated study on bee foraging (see W. G. Meikle et al., 2016). At the start of the experiment, hives consisted of a single deep Langstroth box (43.65 l capacity) with 8 frames and a division board feeder.

Details about the hive evaluations data are best described in W. G. Meikle et al., 2016. In summary, during a hive evaluation each frame was picked up from the box, gently shaken to dislodge adult bees, then weighed and photographed on both sides using a 16.3-megapixel digital camera (Pentax K-01, Ricoh Imaging Co., Ltd.) and replaced in the hive. The area of sealed brood per frame was estimated from the photographs using ImageJ v. 1.47. The total weight of the adult bee mass was calculated by subtracting the combined weights of hive components (lid, inner cover, box, bottom board, frames, entrance reducer, internal feeder) obtained at the start of the experiment from the total hive weight recorded the midnight prior to the inspection. A second box containing 9 frames was added immediately after the hive evaluation on January 28th. Hives were placed on outdoor electronic scales (TEKFA® model B-2418, Galten, Denmark, max. capacity: 100 kg, precision: ±20g; operating temperature: -30°C to 70°C) and linked to 12-bit data loggers (Hobo® U-12 External Channel datal ogger, Onset Computer Corporation, Bourne, MA, USA) with weight recorded every 15 minutes. Bottom board pollen traps (Sundance bottom mount pollen trap, Brushy Mountain Bee Farm, NC) were placed under each hive at the start of the experiment in January and pollen was collected nearly continuously until March 25th to monitor pollen inflow, after which pollen

traps were removed, standard bottom boards installed, and hives evaluated. Hives were then moved 50 km to Madera, CA (36°56'14.09"N, 119°49'52.90"W) as a single group adjacent to fields planted in almond, grape and citrus, and replaced on the hive scales. Hives were evaluated again on the 27th of May and on the 20th of August. Drought conditions were severe in the area, which impacted available forage.

b) SRER 2014 dataset

Hives monitored in the Santa Rita Experimental Range, AZ (31°46'38.08"N, 110°51'47.39"W) (W. G. Meikle et al., 2016). In April 2013, four honey bee colonies were established from packaged bees of approximately 1.5 kg, with Cordovan Italian queens (C.F. Koehnen & Sons, Glenn, CA). The packages were installed in painted, 10-frame, wooden Langstroth deep boxes fitted with migratory wooden lids (Mann Lake Ltd, Hackensack, MN). The apiary was provided with a permanent water source and hives were spaced 1–3 m apart. The hives were placed on scales linked to data loggers (same models as described above) with weight recorded every 15 minutes. Weight data were collected from the 11th of February to the 3rd of April 2014, and hives were evaluated on the 11th of March and on the 3rd of April 2014.

c) CHBRC 2015 dataset

The objective of this experiment was to prevent the departure of foragers for a known period of time at the start of the active period of the day to observe 1) how the rate of weight change during the night was affected by forager departure times; and 2) the timing of any rate change relative to dawn and to control hives. On the 29th of July 2015 seven honey bee colonies in 10-frame Langstroth deep boxes at the Carl Hayden Bee Research Laboratory, USDA-ARS, Tucson, AZ (32°16'30.17"N, 110°56'28.52"W) were evaluated and placed on hive scales linked to data loggers as described above. The hives were placed under a shelter with a sun screen that protected the hives from both direct sun and precipitation. The hives were established from

packages in April 2013, and had been treated with thymol (Apiguard) prior to start of the experiment to control *V. destructor* mite densities. Data loggers on scales were set to record every minute. Robbing screens (Mann Lake Ltd, cat. no. WW-176) were placed on the entrances of the hives. These screens permit closing the hive entrance without interfering with air exchange.

Colonies were divided into two groups, block 1 with three colonies and block 2 with four colonies. From the 12th to the 28th of August, the entrances of all the hives in one block were closed at 5:30 a.m., before the start of flight activity. Closed entrances were opened after a pre-determined period of time: 1 h, 1.5 h, 2 h, 2.5 h or 3 h (*Supplementary Material 2-1*). Entrances for a given block were closed only on alternate days, and closures were conducted for four to five days per week. Resulting within-day weight data were analysed using piecewise regression, using 100 iterations of regressions with 4 break points, to compare planned and observed delays with respect to data parameters.

d) MU 2017 dataset

The objective of this experiment was to determine the extent to which the slope of the piecewise regression segment associated with morning forager departure is affected by bee movement (arrivals and departures). In November 2016, 16 honey bee colonies established in 10-frame Langstroth deep boxes at Macquarie University, Sydney, Australia (33°46'27.91"S, 151° 6'46.23"E) were placed on hive scales linked to data loggers as described in the sections above. The data loggers were set to record every minute. On the 29th of March and 20th, 21st and 24th of April, hive entrances were recorded on video for one minute per hive on each of four hives. A different set of four hives was chosen each day, so each hive was monitored once. The videos were then analysed frame by frame with the software Cowlog v. 3.0.2 to record all bee departures and arrivals during that minute, which occurred between 8:49 to 8:59 a.m.

depending on the day. Piecewise regression models with four and five break points were fit to the detrended within-day weight data, and the net change in bee number was regressed on slopes of the segments, from 100 iterations of regressions with 5 break points, for that time period during which the observations were made.

e) CAL 2015–16 dataset

In November 2015, 32 honey bee colonies that occupied one to two 10-frame Langstroth deep boxes and had marked queens were identified in apiaries in southern Arizona (see Rothman et al., 2018). The hives were divided into four groups of eight hives. Six hives in each group, for a total of 24 hives, were placed on electronic scales (same scales and data loggers as described in the previous section). All hives were given 250 g of protein patty supplement at the end of November, evaluated to determine adult bee mass in the first week of December, and fed both protein patty supplement and 3 L of 1:1 sugar syrup in mid-December. Hive evaluations were conducted on all hives as described in the previous section. On the 30th December the four groups of eight hives were moved to one of four sites: two sites near Red Rock, AZ (32°33'13.75"N, 111°19'13.76"W), and two sites at the University of Arizona's Maricopa Agricultural Center in Maricopa (33° 5'16.02"N, 111°58'45.19"W). The two hives in each group not on hives scales were fitted with front loader pollen traps (Brushy Mountain, Moravian Falls, NC, USA) to monitor foraging success.

Colonies at the Red Rock sites had access to ample forage provided in a plot of *Brassica rapa* and also foraged on *Encelia farinosa*, *Larrea tridentata*, *Searsia lancea*, and *Erodium spp.*, while colonies at the Maricopa Agricultural Center sites had access to about a third as much forage. Hives were again evaluated on the 26th and 29th of January and on the 2nd and 4th of February all colonies were moved to Blackwell's Corner, CA (35°38'37.28"N, 119°54'56.30"W) where they were placed in an almond grove prior to bloom and fed sugar

syrup. On the 5th of March all colonies were evaluated and then moved to a holding yard at

Keck's Corner, CA (35°40'10.69"N, 120° 6'17.15"W), where they were fed sugar syrup. All

surviving hives were evaluated for a final time on the 4th and 8th of April 2016 and then moved

back to Arizona. As the study proceeded, hives that died were replaced by other hives from the

same treatment groups, replacements were included in statistical analyses.

f) Data analysis

Hive weight data were detrended for each day by subtracting the weight estimate of the

hive between midnight from each subsequent weight value during the day until the last value

just before midnight. This daily dataset had three main parts: the period from midnight until

dawn, when colony's interactions with the environment in terms of weight were limited to

temperature effects and gas exchange (e.g. water vapour), the active period during the day from

the initial departure of bees at dawn until their return at dusk, and the period from dusk to

midnight when the colony was again quiescent. In the CAL 2015–16 field experiment, hive

weight data were also analysed using the method previously described (W. G. Meikle et al.,

2016): data were detrended by subtracting raw data from the hourly 25 hour running average,

sine curves were fit to the resulting data, and the amplitudes of those curves were used as a

response variable (reflecting bee flight activity).

Within-day weight changes were modelled using the "segmented" function in R

(Muggeo, 2008) which fits a segmented line derived from linear or generalized linear model to

a dependent variable. The number of breakpoints can be estimated automatically or through

the use of initial values provided by the user with final determination by the function. Using

the 'segmented' function to automatically determine the number of break points tends to

overestimate the number of break points, so estimation via methods such as visual inspection

is preferred for the final model (Muggeo, 2008). Function outputs include the final break point

values, the slopes and intercepts of the segments, and the overall model fit. An effort was made to use a minimum number of break points to ensure the model did not have an excessive number of arbitrary parameters, but did reflect changes in weight with sufficient precision. Initially models with three break points were fit: break point 1 was the estimated sunrise, which was calculated with respect to day of year, latitude and longitude; break point 2 was the minimum weight value of each day; and break point 3 the estimated sunset. Models with one and two additional break points were also considered, with those initial break point values estimated at regular intervals during the active part of the day. Model output was visually compared to raw data. Our intention was to determine a suitable general model, rather than the optimal model for each day. Parameters for the model with the highest adjusted r^2 value out of 100 iterations were retained.

Results

a) Detrending hive weight data

Detrending data by subtracting hive weight at midnight yielded consistent patterns among hives over time (**Error! Reference source not found.**, **Error! Reference source not found.**). CAL 2014 data showed colony weight loss in mid-winter followed by a nectar flow in March with an ensuing dearth. SRER 2014 data showed colony weight gain during a late winter to early spring nectar flow. While the bee colonies themselves differed in size (**Error! Reference source not found.Error! Reference source not found.**), after detrending the data, the within-day patterns of weight changes were largely consistent over periods of time that corresponded to the nectar flows observed from flower blooms in the vicinity of hives.
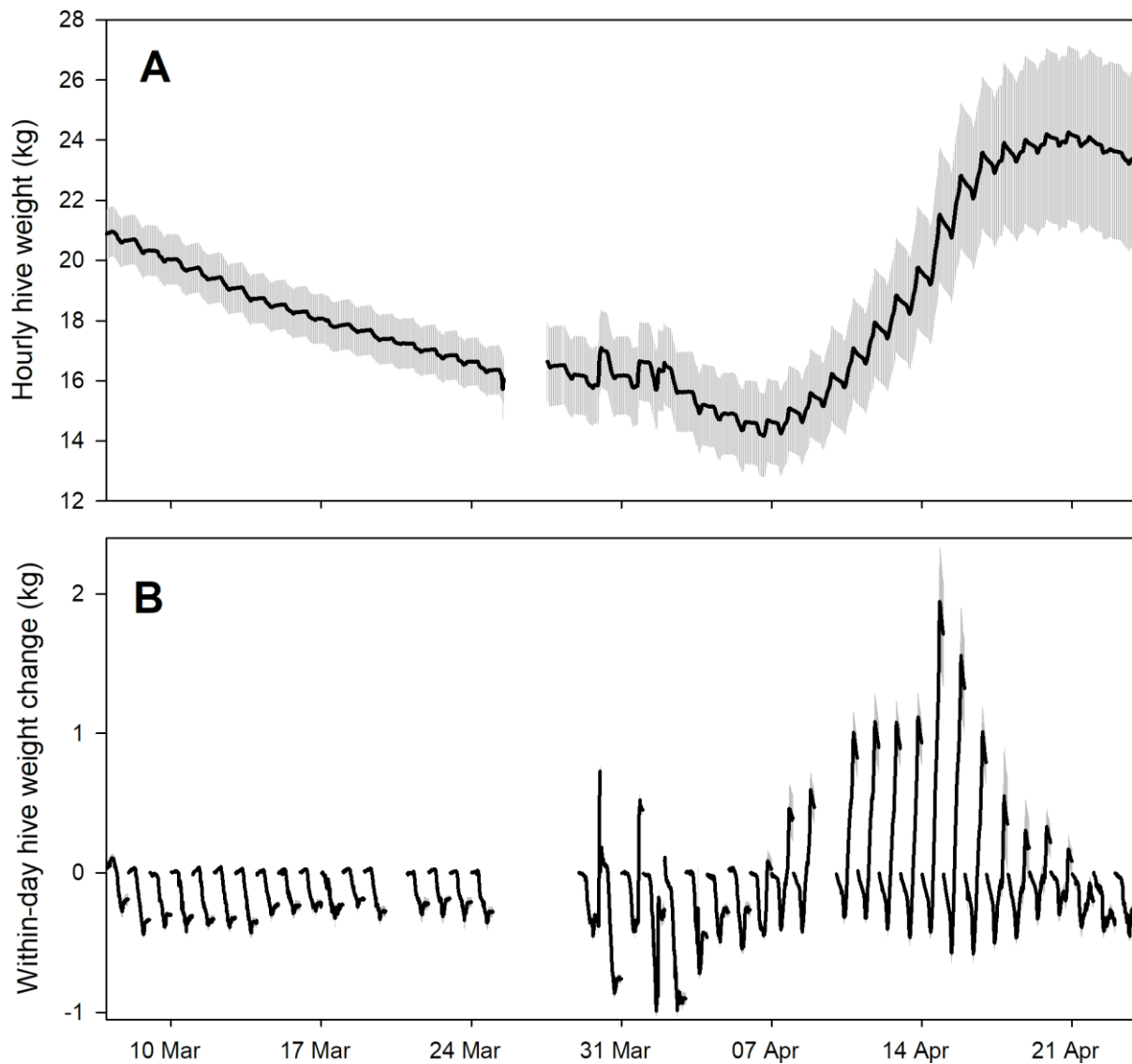
Figure 2-1. **Average (± s.e.) hive weight and within-day weight changes for eight honey bee hives kept near Madera, CA (the CAL 2014 dataset). Data are shown from 7 March to 23 April 2014. A) raw data; B) within-day weight changes.**
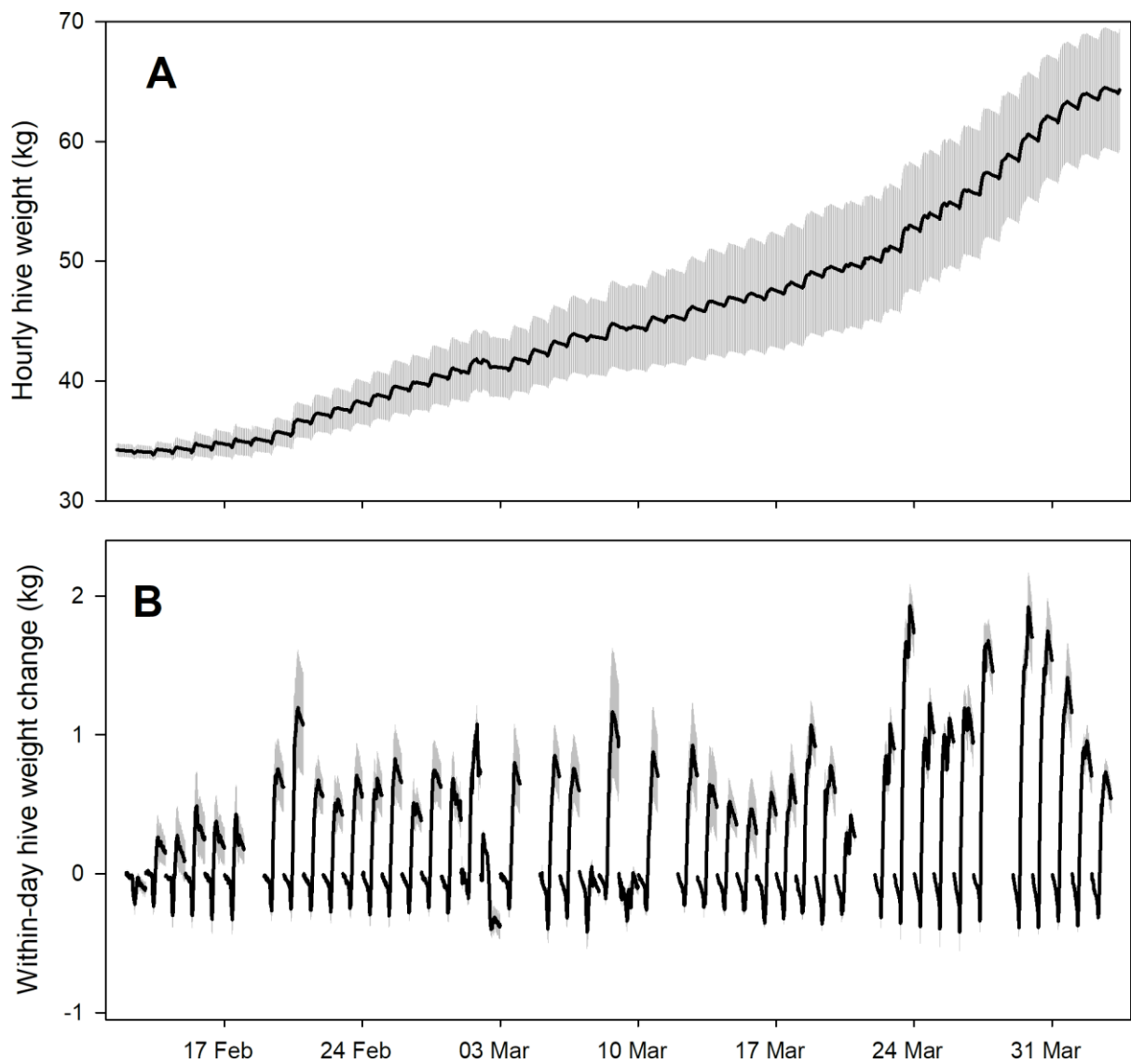
Figure 2-2. **Average (± s.e.) total hive weight and within-day weight changes for 3 honey bee hives kept near Green Valley, AZ (the SRER 2014 dataset). Data are shown from 11 February to 2 April 2014. A) raw data; B) within-day weight changes.**

Table 2-1. **Adult bee masses and capped brood areas (averages ± s.e.) for all sampling occasions of all datasets used in this study. N refers to the number of hives in that treatment group remaining from the original hives on scales at the start of the experiment.**

Data set	Group	N	Date	Adult bee mass (g) average	s.e.	Capped brood area (cm^2) average	s.e.
CAL 2014		8	1/29/2014	2034	±73	-	-
		8	3/25/2014	2010	±154	1773	±62
		8	5/27/2014	3488	±210	2227	±140
		3	8/20/2014	2008	±1000	979	±880
SRER 2014		3	3/11/2014	2606	±284	2946	±244
		3	4/3/2014	4231	±911	4667	±249
CHBRC 2015	Block 1	3	7/29/2015	1494	±492	1343	±352
		3	8/28/2015	1291	±453	917	±275
	Block 2	4	7/29/2015	1443	±153	1438	±148
		4	8/28/2015	1417	±58	1014	±178
CAL 2015–16	MacN	6	12/7/2015	1246	±205	417	±141
		6	1/28/2016	1218	±70	101	±25
		3	3/3/2016	680	±74	479	±11
		3	4/5/2016	854	±418	1115	±711
	MacS	6	12/7/2015	1378	±218	561	±119
		6	1/28/2016	1557	±171	191	±76
		3	3/3/2016	1001	±94	405	±274
		3	4/5/2016	582	±190	928	±838
	RREast	6	12/7/2015	1185	±168	622	±87
		6	1/28/2016	1729	±255	186	±93
		5	3/3/2016	888	±74	501	±150
		5	4/5/2016	763	±234	782	±348
	RRWest	5	12/7/2015	1608	±341	404	±129
		5	1/28/2016	1611	±142	133	±30
		5	3/3/2016	935	±156	854	±224
		5	4/5/2016	996	±195	1409	±243

b) Identifying break points

Most daily activity patterns can be considered as having four to six segments (**Error! Reference source not found.**). Two examples of daily patterns with 3 break points were shown with the following interpretations:

1. Point A: First weight measure at midnight or shortly thereafter;

2. Segment AB: Inactive period in the early morning; hive weight change is likely due to bee respiration and changes in the moisture content of nectar, pollen and wooden hive parts;

3. Point B: Departure of foragers and other bees at the beginning of the active period (usually close to dawn if temperatures permit);

4. Segment BC: Active period usually showing hive weight loss due to greater numbers of departing bees compared to returning bees;

5. Point C: Point at which mass of returning foragers plus nectar and pollen exceeds the mass of departing bees plus weight loss due to drying and respiration in the colony;

6. Segment CD: Active period usually showing hive weight gain due to returning foragers;

7. Point D: Return of bees to the hive around dusk is completed;

8. Segment DE: Inactive period with hive weight change driven mainly by respiration and changes in ambient humidity, usually close to parallel with segment AB;

9. Point E: Last weight measure just before midnight.
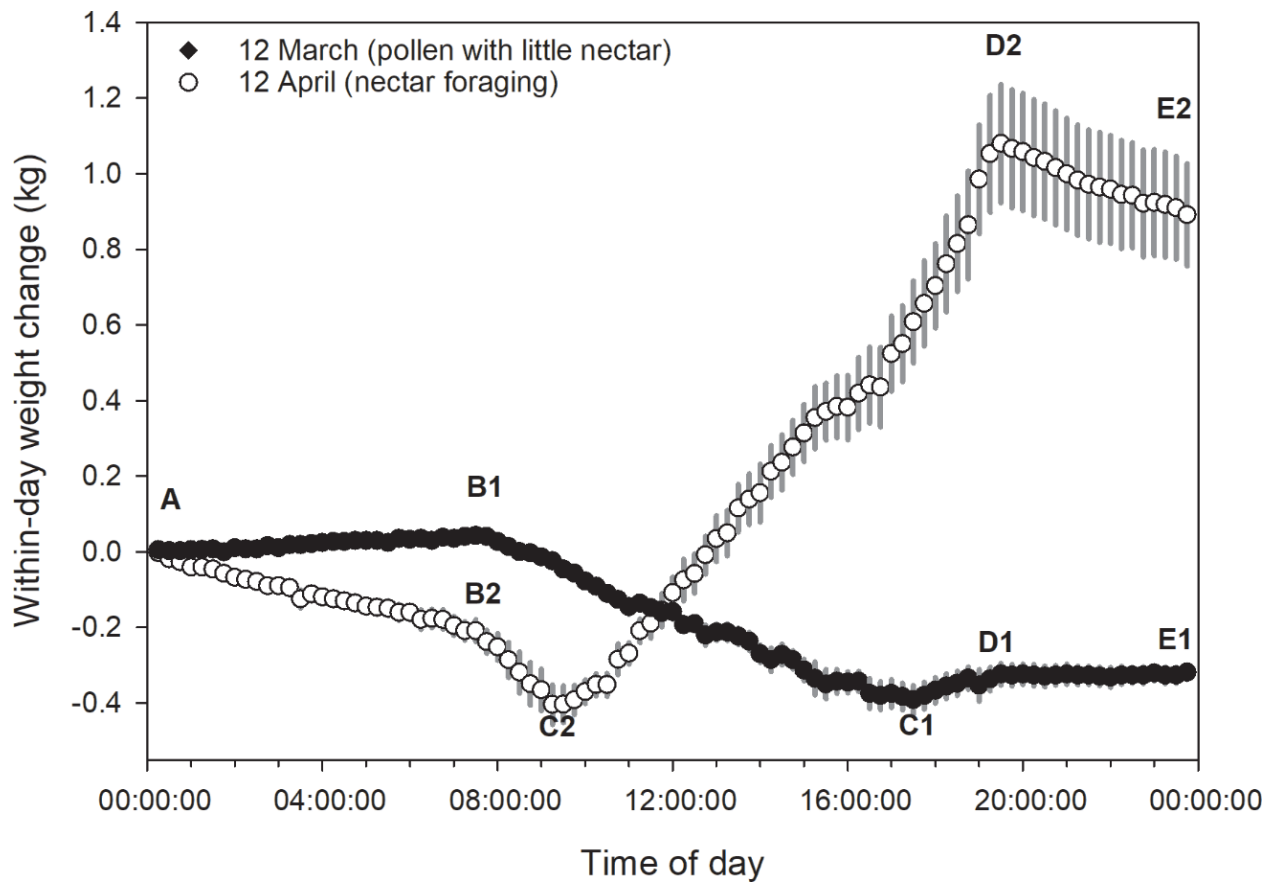
Figure 2-3 **Examples of two within-day weight change patterns obtained from average (±
s.e.) 15-minute weight data from 8 hives kept near Madera, CA (see Figure 1) (the CAL
2014 dataset).**

c) Optimizing the number of model break points

In most locations a hive is active when ambient temperatures permit from about dawn to about dusk. Few if any foragers depart a hive before dawn or return to the hive after dusk. An initial break point before dawn would therefore probably correspond to a weight change caused by something other than initial hive activity, such as rainfall. Similarly, a break point after dusk likely corresponded to an event other than forager return. Considering break points falling between 8 p.m. and 4 a.m. as errors, a model with 3 break points would have had the fewest, followed models with 4 and 5 break points.

The CAL 2014 data were averaged over eight hives and the SRER 2014 data were averaged over 3 hives. Those average values were then modelled using regression lines with 3, 4 and 5 break points. The Hiatt 2014 dataset was divided into a period of hive weight loss with comparatively little nectar (29th of January to 24th of March, which included almond pollination) and a period of citrus nectar flow (29th of March to 26th of May). Five parameters were compared among the different models: the average adjusted r^2 for each fit, the number of days in which any fits were successful, the number of successful fits out of 100 iterations for those days where fits were successful, and number of "errors" for the first and last break points, with a first break error defined as a break before 4 a.m. (i.e. before bees start foraging) and a last break error defined as a break after 8 p.m. (i.e. after bees would have returned to the hive) (Table 2-2).

Table 2-2 **Comparison of piecewise regression model fits using different numbers of break points across different datasets. Shown are average values ± s.e. 1st break point errors were considered initial break points that occurred before 4 a.m. and last break point errors were considered final break points that occurred after 8 p.m.**

Data set	No. break points	Adjusted r^2 (average ± s.e.)		Percentage fits per day (average ± s.e.)		Percentage of days fit	1st break point errors	Last break point errors
CAL 2014	3	0.978	±0.400 a	94.7	±1.6 a	87.3	5	1
29 Jan.-24 Mar.	4	0.984	±0.427 ab	79.4	±3.4 b	89.1	5	2
	5	0.993	±0.103 b	61.9	±3.8 c	74.5	5	2
CAL 2014	3	0.960	±0.500 a	98.2	±0.7 a	93.2	2	11
29 Mar.-26 May	4	0.974	±0.346 b	85.0	±3.3 b	93.2	3	12
	5	0.985	±0.230 b	77.0	±3.1 b	88.1	4	13
SRER 2014	3	0.986	±0.005 a	94.1	±2.2 a	90.0	1	0
12 Feb.-3 Apr.	4	0.993	±0.003 ab	91.0	±2.7 a	90.0	1	1
	5	0.995	±0.002 b	73.7	±2.9 b	90.0	2	0

The regressions with 3 break points had significantly lower adjusted r^2 values, but successfully fit more iterations for days where any 3 break point regressions could be fit than regressions with 5 break points, on average. Regressions with 4 break points had r^2 values equal to or greater than models with 3 break points and fit as many or more daily datasets than models with 5 break points. Hives tended to change weight at rates that were largely constant for sufficient periods of time, often up to several hours, and the average r^2 values of successful fits equalled or exceeded 0.96, which suggested that the piecewise regression approach was appropriate.

Data for April 12, 2014, in the CAL 2014 dataset, as shown in Figure 2-1, were fit with 3, 4 and 5 break point regression models (Figure 2-4).

That particular dataset illustrated another kind of error that would be difficult to correct: the function fails to detect a break point associated with early departure that can be detected through visual inspection. In this case models with 4 and 5 break points detected the departure while the model with 3 break points did not. Models with 4 break points fit to data samples showed an adequate fit (Figure 2-5).
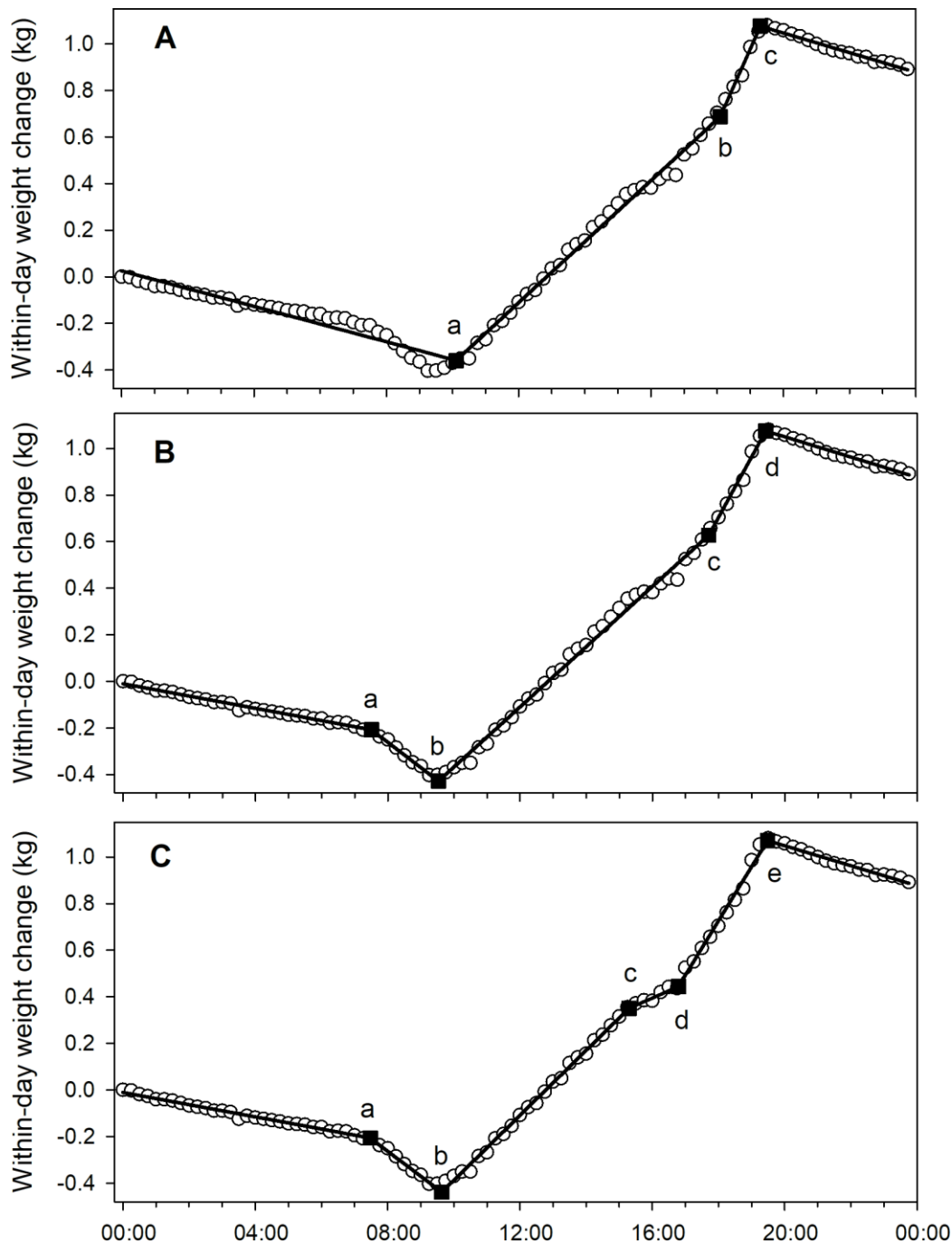
Figure 2-4. **A sample day data subset (April 12, 2014, the same subset as Figure 3; from the CAL 2014 dataset) fit with piecewise regression models with different numbers of break points. A) 3 break points ($r2 = 0.9968$); B) 4 break points ($r2 = 0.9987$); and C) 5 break points ($r2 = 0.9993$). Solid line show regression line, and labelled points a, b, c, d and e (solid squares) show break points 1–5 with a = 1st break point, b = 2nd break point, etc. Empty circles show within-day weight change data.**
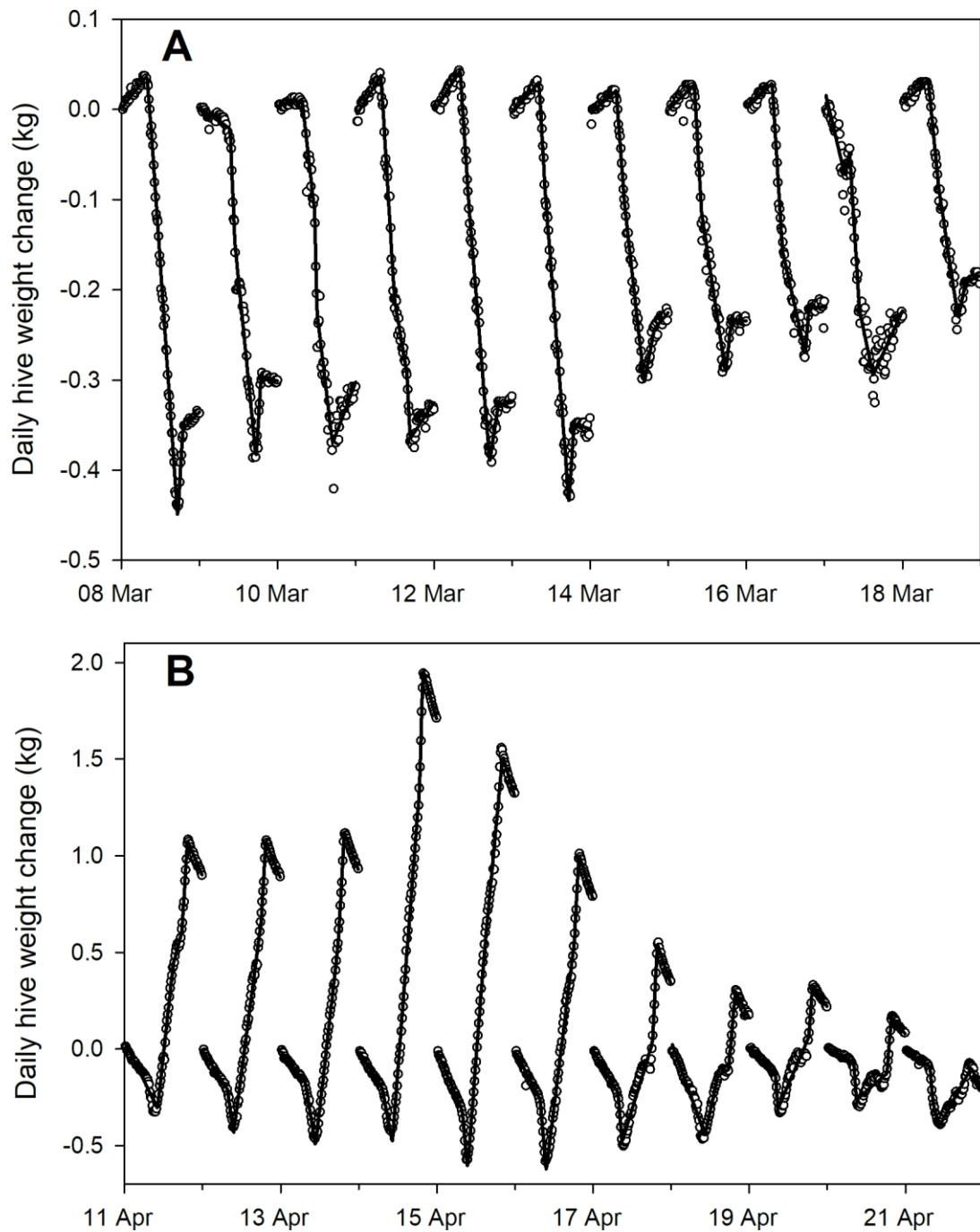
Figure 2-5. **Piecewise regression curves, with 4 break points fit to average within-day hive weight changes from 8 hives kept near Madera, CA (the CAL 2014 dataset). A) Data from 8–18 March 2014 (little or no nectar flow); B) 11–21 April (citrus nectar flow within foraging distance).**

d) Interpreting model parameters

Models with 4 break points were determined to have the best fit and used in subsequent analyses. Models fit to data from the CAL 2014 and SRER 2014 datasets, and the break point and slope values changed from day to day (Figure 2-6, Figure 2-7).

Forager departure and return estimates tended to diverge through the winter and spring, as would be expected as daylight hours and ambient temperatures increased. Hive weight changes during the inactive period at night were influenced by the presence of fresh nectar. A nectar flow was present throughout the SRER 2014 data set but a discrete, short-term citrus nectar flow was observed in the CAL 2014 dataset. Hives tended to lose weight at night during a nectar flow, probably due to moisture loss from drying nectar, but outside of a nectar flow, hives usually maintained or even gained weight, probably because of increasing moisture content of hive wooden parts as the ambient relative humidity rose at night. Meikle et al. (2006) observed that hourly weights of empty hives varied by 50 g or more over 24 hours.
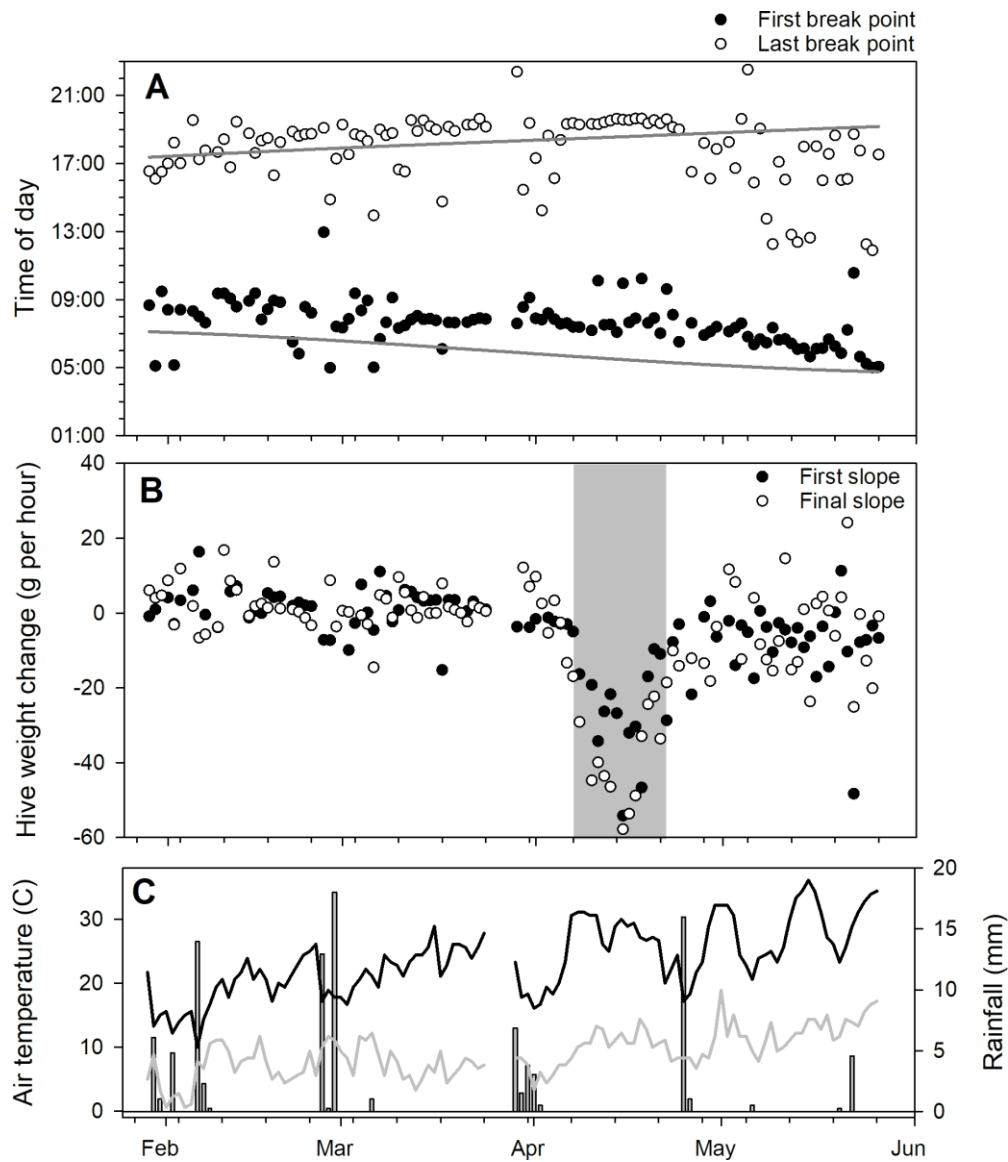
Figure 2-6. **Examination of parameters from 4-break point piecewise regression models fit to average within-day hive weight changes for 8 honey bee hives kept near Madera, CA from 29 Jan. to 26 May 2014 (the CAL 2014 dataset). A) 1st and 4th breakpoints for piecewise regression curves. In cases where the 1st breakpoint occurred before 4AM (i.e. before dawn) the 2nd breakpoint was used. Likewise, if the 4th breakpoint occurred after 8:00PM the 3rd breakpoint was used. Grey lines show local sunrise and sunset. B) 1st and 5th segment slopes for piecewise regression models fitted to average within-day hive weight changes for the same hives. Grey zone indicates a period of nectar flow. C) Rainfall and minimum and maximum temperature data.**
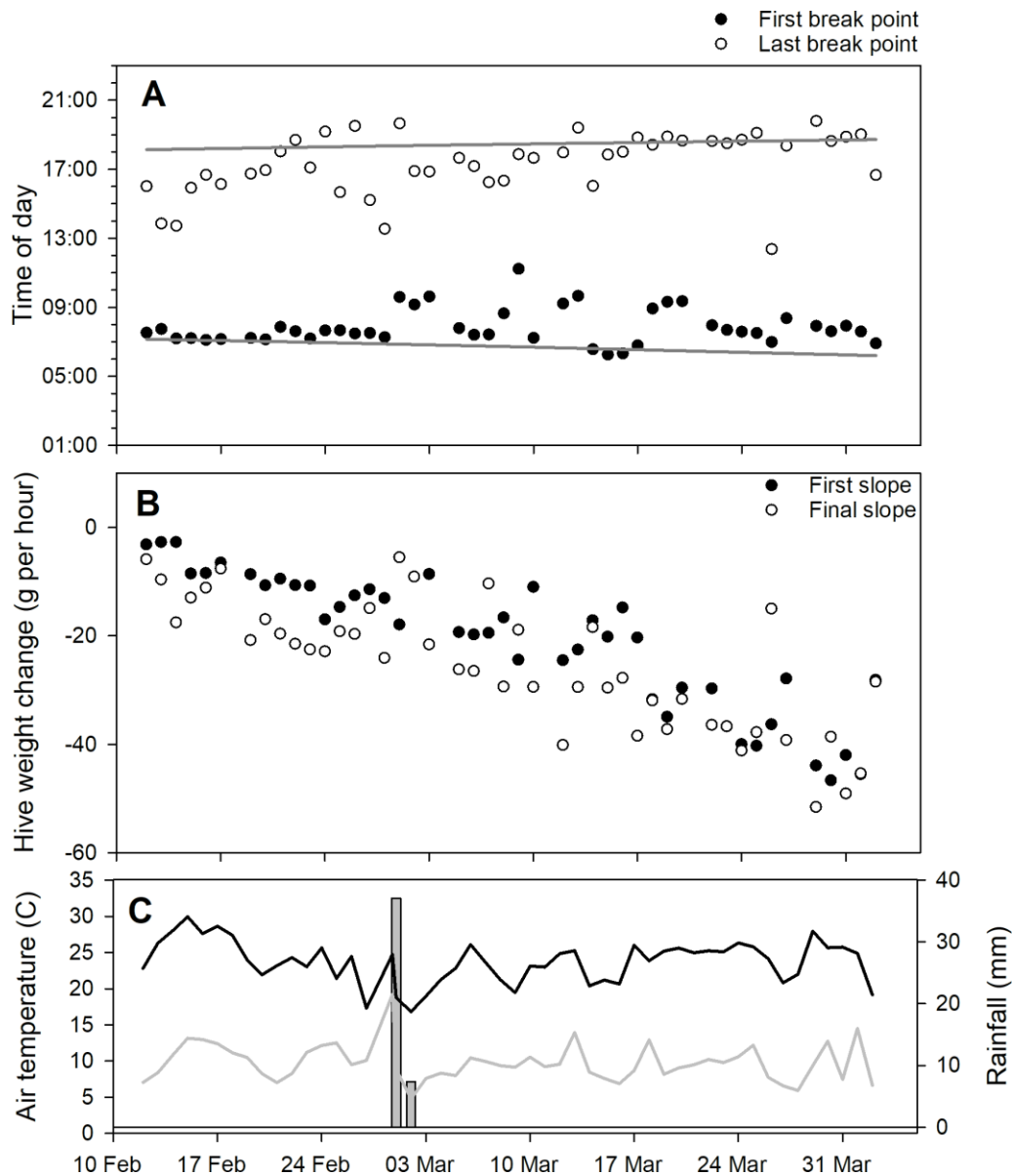
Figure 2-7. **Examination of parameters from 4-break point piecewise regression models fit to average within-day hive weight changes for 3 honey bee hives kept near Green Valley, AZ, from 11 Feb. to 2 April 2014 (the SRER 2014 dataset). A) 1st and 4th breakpoints for piecewise regression curves. In cases where the 1st breakpoint occurred before 4 a.m. (i.e. before dawn) the 2nd breakpoint was used. Likewise, if the 4th breakpoint occurred after 8:00PM the 3rd breakpoint was used. B) 1st and 5th slopes for piecewise regression curves fitted to average within-day hive weight changes for those same hives. C) Rainfall and minimum and maximum temperature data.**

e) Manipulating initial bee departure

Visual examination of the data showed that while the early morning weight curves were generally maintained when the bees were prevented from leaving, some small, additional weight changes were observed, often around the time of the 1st break point for the control colonies (*Supplementary Material 2-2*). The exact cause of those changes was unclear. A comparison of planned vs. observed changes in the first break point showed a significant linear relationship ($F_{1,13} = 73.06$, p<0.001, adj. $r^2 = 0.85$; Shapiro-Wilk normality test and constant variance test both passed) (*Supplementary Material 2-3*). The slope of that line was 0.649; that the slope is not equal to one indicates that there were factors other than bee departure, such as moisture gain or loss due to changing ambient conditions, influencing the break points.

f) Correlating bee traffic with regression slopes

Piecewise regression lines with 5 break points fit data had a higher r^2 (0.987±0.002) and fit the data more often (89.6±4%) (*Supplementary Material 2-5*) than those with 4 break points (0.976±0.005 and 82.5±5%, respectively). The slope of the regression segment fit to weight data collected during the time of the observation was regressed on the net movement of bees (arrivals-departures) at that time (*Supplementary Material 2-4*). Net movement was significantly correlated with hive weight change, as measured by the segment slope ($F_{1,13} = 26.66$, p = 0.0002, adj. $r^2 = 0.65$; Shapiro-Wilk normality test and constant variance test both passed), indicating that bee traffic was a significant component of hive weight change at that time. The absolute value of the regression slope, 0.032 g loss per departing bee, reflects the greater per capita mass of returning foragers, laden with nectar and pollen, compared to departing bees. Assuming all the hive weight change at that point in time was due to bee traffic,

that change can be expressed as: $\Delta W = \dfrac{N_D M_D - N_A M_A}{N_D - N_A}$ where ΔW is the hive weight change

(slope of the regression segment, here 0.032g/min), N_D and N_A are the numbers of departing

and arriving bees, respectively, and M_D and M_A are the masses of departing (unladen) and arriving (laden) bees, respectively. Assuming $M_D = 0.13$ g (Meikle et al., 2008), using data on N_D and N_A, and rearranging the terms, the average mass of an arriving bee, M_A would have been about 0.15 g.

g) Applying within-day analysis to migratory colony nutrition study

The two treatment groups were not significantly different in terms of adult bee mass or brood surface area (see Table 2-1) during any hive evaluations. Continuous hive weight data were divided into three parts: before, during and after almond pollination. Of the 17 colonies in the low-forage treatment just prior to shipping to almond pollination on 28 January, only 8 survived to the last evaluation on 5 April, but of the 19 colonies in the high-forage treatment, 14 were still alive for the final evaluation.

Each hive weight dataset was subjected to a sine curve analysis of 3 days subsets of detrended data (W. G. Meikle et al., 2016) and the resulting curve amplitudes were compared between treatments using repeated measures MANOVA. Amplitude data from every 3rd day were used, to avoid overlap between data subsets. No treatment differences were detected with respect to sine amplitudes for any of three datasets (pre-almond pollination: $P = 0.946$; during almond pollination: $P = 0.274$; post-almond pollination: $P = 0.998$).

Piecewise regression analysis yielded estimates for 10 parameters: 4 break point values, 5 slope values and the adjusted r^2. Because the data were detrended by subtracting the raw data value at midnight, daily datasets were mathematically independent. A repeated measures MANOVA was conducted on these daily parameter values of interest:

1. time of initial forager departure (break point nearest dawn, usually the 1st);

51

2. time of final forager return (break point nearest dusk, usually the 4th);

3. slopes of the 1st segment and 5th segment (associated with weight changes at night when colonies are not foraging);

4. slope of the first segment after initial forager departure, usually the 2nd segment (rate of weight loss largely due to forager departure); and

5. slope of the last segment before dusk, usually the 4th segment (rate of weight gain due forager return).

For statistical analysis, if the 1st break point occurred before 4 a.m., the 2nd break point was used as the time of initial forager departure (with no restrictions placed on that second estimate) and the slope of the 3rd, rather than 2nd, segment was used as the rate of weight loss due to forager departure. Likewise, if the 4th break point occurred after 8 p.m. then the 3rd break point was taken as the time of final forager return (with no restrictions placed on that second estimate).

No differences were observed between treatment groups with respect to any parameter before almond pollination. However, during almond pollination the slope of the last segment before dusk was significantly higher among colonies that had access to forage compared to colonies that did not (Table 2-3, Figure 2-8). The slope of that segment would correspond to the change in hive mass due to returning foragers with nectar and pollen. No other parameters were significantly different between groups during pollination. After almond pollination, only the first segment after dawn, corresponding to weight loss due to forager departure, was significant.
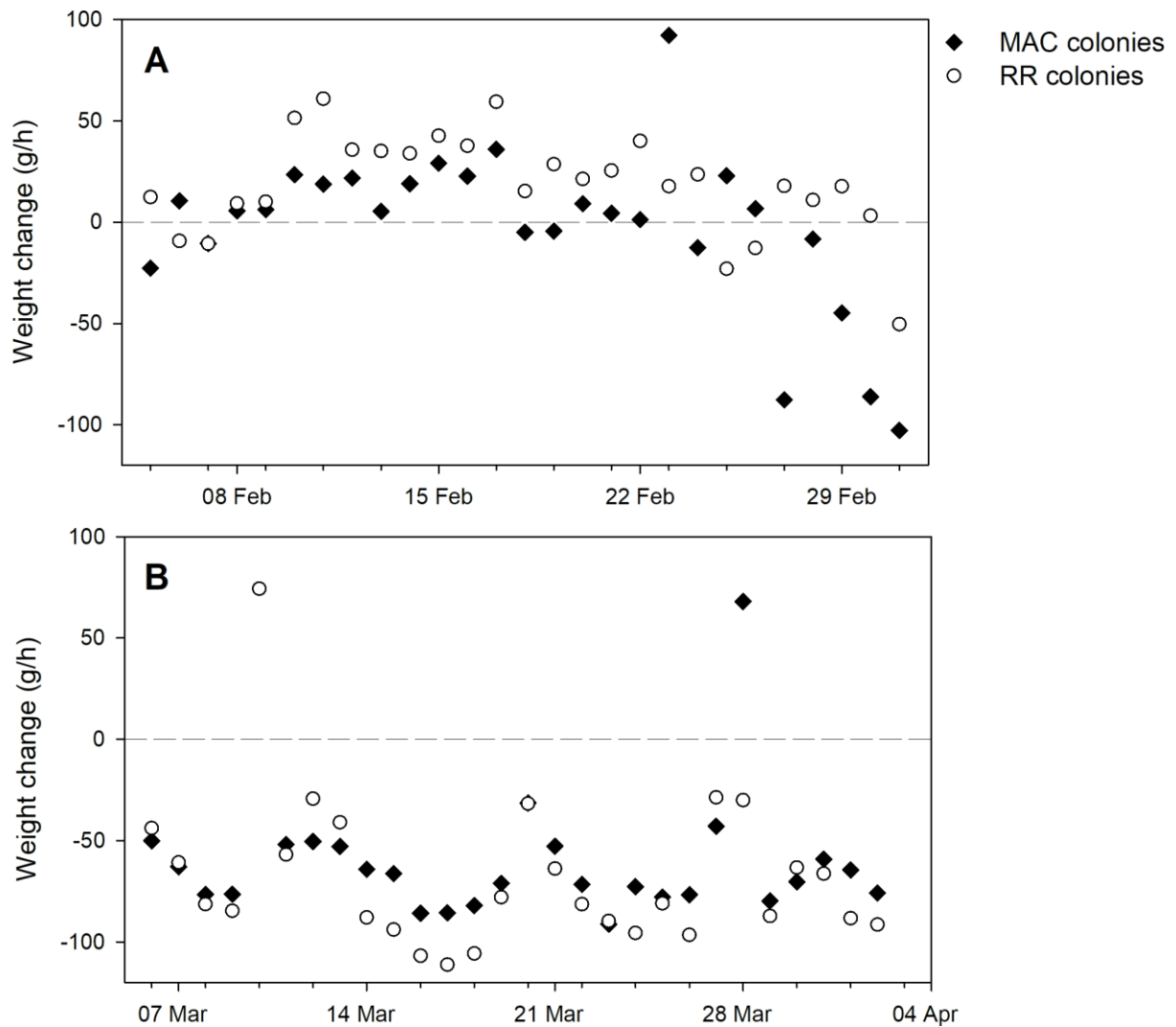
Figure 2-8. **Average slope values of segments from piecewise regression curves fit to within-day weight change data, collected every 15 minutes, obtained from hives placed in proximity to forage (RR) and with little available forage (MAC) prior to exposure to blooming almonds followed by placement in unmanaged fields (the CAL 2015–16 dataset). A) slope of the regression segment just prior to the dusk break point; B) slope of the regression segment just after the dawn break point.**

Table 2-3. **Results of a repeated measures MANOVA conducted on parameters of piecewise regressions fit to continuous hive weight data collected during and after almond pollination in California.**

Dataset	Response variable	Factor	Num DF	Den DF	F Value	Pr > F
CAL 2015–16	4th segment slope	treat	1	105.1	9.99	0.0021
(2 Feb.-3 Mar.)		day	26	278.6	2.70	<0.0001
		day*treat	26	278.6	1.40	0.0987
CAL 2015–16	2nd segment slope	treat	1	168.5	7.06	0.0087
(6 Mar.-4 Apr.)		day	29	458.3	8.29	<0.0001
		day*treat	28	453.9	0.89	0.6341

Discussion

Monitoring the weight of honey bee hives continuously provides information, without colony disturbance, on how the hive interacts with the environment. One of the challenges in the analysis of such data is modelling the data in a way that exploits the information in the dataset while reducing as much as possible the impact of spurious error. Running average weight data and detrended within-day data have been related to colony parameters such as total adult bee mass and foraging activity (Meikle et al., 2008; W. G. Meikle et al., 2016), and the methods they used to detrend and model the data (subtract raw hourly data from the 25 hour running average and fit sine curves to the result) provided robust estimates of bee flight activity. However, information is also lost due to the use of hourly averaging and of 3-days data subsets. In this study raw data were detrended by subtracting the value at midnight, rather than the running average, from each of the values of that day until the next midnight. A piecewise regression model was fit to single-day datasets in the original time scale using an R function (Muggeo, 2008) that is based on a bootstrapping method (Wood, 2001). The user can provide estimated break points, and the function uses a random number generator to provide and test model parameters. The parameters were automatically adjusted until the fit reached a given threshold.

In order to reduce noise in the exploratory phase of model evaluation, daily detrended data were averaged overall 3 to 8 colonies in two independent datasets from different sites in the south-western U.S. The resulting daily patterns were, for the most part, consistent among colonies within site. Because of the high precision and the random number algorithm in the 'segmented' function, parameters for a given fit were rarely identical. One hundred iterations of the function were conducted for each daily dataset and parameters for the best fit line were retained based on the r^2 value. Models with 3, 4 and 5 break points were evaluated and those with 4 break points offered a significantly higher r^2 than those with 3 break points while often fitting more datasets than those with 5 break points. Models with 4 break points offered an alternative break point in the event the 1st or 4th break points were due to chance events unrelated to the study, such as a weather event. The function established break points where the change in the linear slope exceeded a predetermined threshold so providing an excessive number of break points resulted in a failure of model fit. Piecewise regression models in general fit the data well, with r^2 values for regressions with 4 break points on average exceeding 0.97.

Having established that the piecewise regression method fit the data well, two experiments were conducted to test the interpretation of two parameters: the break point and following segment associated with initial forager departure in the morning (usually the first break point and second segment). The first experiment involved blocking the departure of foragers, without blocking gas or temperature exchange, just prior to dawn and keeping them blocked for fixed times. Models fit to the within-day weight data for each hive were used to determine the times of the initial forager departures. The results were consistent and the regression coefficient showed that for every hour the hive was blocked, the forager departure was delayed by about 40 minutes. The lack of a strict correspondence between planned and observed delay was likely due to factors such as the inherent variability in forager departure (particularly outside of a nectar flow) and to hive weight change resulting from moisture loss

55

due to decreasing ambient r.h. after dawn, rather than bee movement. The study did confirm that the dawn break point resulted largely from an increased loss of hive mass due to forager departure. The second experiment involved monitoring bee traffic for one minute in the morning, during the period of time associated with the initial forager departure. Net bee loss (more departures than arrivals) during that minute was significantly related to the slope of the piecewise regression model associated with that time of the morning. The value of that slope was a function of the numbers and masses of arriving (laden) and departing (unladen) bees, and was used to estimate an average payload of arriving bees of about 0.02 g per bee. The better fit of piecewise regression lines with 5 break points to data from Australia compared to 4 break points, which fit Arizona data better, suggested that the optimal number of break points for such analyses may depend on the environment.

The piecewise regression approach was then used to analyse data from an experiment on bee colony nutrition conducted in Arizona and California. Within-day data from hives from two groups, one that had been exposed to abundant forage and one that had been exposed to little forage, were compared with respect to nine regression parameters (4 break points and 5 segment slopes). Neither hive evaluation data nor sine-based models of within-day weight data revealed group differences during any of the time periods (during treatment, during almond pollination, and post pollination), and analyses of bee gut microbiota did not reveal significant differences (Rothman et al., 2018). However, the regression approach did detect a higher rate of weight gain during forager return (usually the 4th segment) during almond pollination, indicating that the well-nourished colonies had greater foraging success even though no significant differences were observed in the mass of foragers. Similarly, during the post almond period, only the forager departure slope was significantly different between groups, indicating that the well-nourished hives likely either produced more foraging bees, or caused increased foraging effort due to factors such as increased demand, even though forage opportunities at

that time and location were poor for all hives (and there was no difference between groups in foraging success, as shown by the lack of a difference in the slopes of segments associated with forager return). In the analysis presented here, no significant differences were detected with respect to break point timing, suggesting that the nutritional state did not affect timing of colony activity.

Within-day weight changes over one to 15 minutes from hives at different locations and years showed consistent patterns that had biological interpretations, that were sensitive to environmental factors such as the presence of a nectar flow, and that could be exploited using piecewise regression. Analyses of within-day data from a field experiment involving colony nutrition detected colony-level treatment differences where other methods, such as visual inspections, did not. In addition, the data were collected without colony disturbance, which can be important, particularly during cold weather. These results demonstrate the potential of continuous monitoring of honey bee hives as a mean of providing easily-interpreted colony-level response variables for longitudinal field experiments.

Conclusions

- The slopes and break points of segmented lines fit to detrended continuous hive weight data using piecewise regression provided information on colony behaviour;

- Piecewise regression lines usually had break points after sunrise, indicating the start of daily colony activity, and before sunset, indicating the end of daily colony activity;

- The interpretation of the break point and following segment associated with the initiation of daily hive activity were confirmed by: 1) manipulating forager departure times by closing the entrance for variable periods of time; and 2) monitoring bee traffic;

- The piecewise regression analysis of detrended within-day weight data were applied to data from fed and starved bee colonies during and after almond pollination in California, and the regression indicated that the fed colonies had greater foraging success during almonds, although the initial rates of mass loss due to forager departures were not different; the opposite was true during a pollen dearth after almond pollination.

Supporting information

Supplementary Material 2-1 **Entrance closing schedule to manipulate the start of flight activity.** https://doi.org/10.1371/journal.pone.0197589.s001

Supplementary Material 2-2 **Sample daily data files from an experiment involving manipulation of time of initial forager departure by blocking the entrance.** Delays were calculated with respect to 5:30AM (about dawn). Black line: average within-day weight change of Block 1 hives (grey shaded area shows s.e.); blue line: average within-day weight change of Block 2 hives (blue shaded area shows s.e.). https://doi.org/10.1371/journal.pone.0197589.s002

Supplementary Material 2-3 **Comparison of planned delay in initial forager departure with observed delay.** Data show 1st break point of Block 1 regression minus 1st break point of Block 2 regression. Regression equation: y = 0.649x-0.225. https://doi.org/10.1371/journal.pone.0197589.s003

Supplementary Material 2-4 **Regression of segment slope, from a piecewise regression fit to within-day hive weight change, on net bee movement (arrivals–departures) over one minute for hives in Sydney, Australia.** Solid line is regression. Regression equation: y = 0.032x-1.1866. https://doi.org/10.1371/journal.pone.0197589.s004

Supplementary Material 2-5 **Figures A to D**. Piecewise regression lines (solid lines) fit to within-day weight changes collected every 5 minutes for 16 hives on 29 March, and 20, 21 and 24 April, 2017, in Sydney, Australia. https://doi.org/10.1371/journal.pone.0197589.s005

Supplementary Material 2-6 **Experimental data.** https://doi.org/10.1371/journal.pone.0197589.s006

Acknowledgments

References

Alaux, C. *et al.* (2010) 'Diet effects on honeybee immunocompetence', *Biology Letters*, 6(4), pp. 562–565. doi: 10.1098/rsbl.2009.0986.

Bayir, R. and Albayrak, A. (2016) 'The monitoring of nectar flow period of honey bees using wireless sensor networks', *International Journal of Distributed Sensor Networks*, 12(11), p. 155014771667800. doi: 10.1177/1550147716678003.

Bitondi, M. M. G. and Simões, Z. L. P. (1996) 'The relationship between level of pollen in the diet, vitellogenin and juvenile hormone titres in Africanized *Apis mellifera* workers', *Journal of Apicultural Research*, 35(1), pp. 27–36. doi: 10.1080/00218839.1996.11100910.

Brodschneider, R. and Crailsheim, K. (2010) 'Nutrition and health in honey bees', *Apidologie*, 41(3), pp. 278–294. doi: 10.1051/apido/2010012.

Buchmann, S. L. and Thoenes, S. C. (1990) 'The electronic scale honey bee colony as a management and research tool', *Bee Science*, 1(1), pp. 40–47.

Crailsheim, K. (1990) 'Protein synthesis in the honeybee (*Apis mellifera* L.) and trophallactic distribution of jelly among imagos in laboratory experiments', *Zoologische Jahrbucher, Allgemeine Zoologie und Physiologie der Tiere*, 94, pp. 303–312.

Crailsheim, K. *et al.* (1992) 'Pollen consumption and utilization in worker honeybees (*Apis mellifera* carnica): Dependence on individual age and function', *Journal of Insect Physiology*, 38(6), pp. 409–419. doi: 10.1016/0022-1910(92)90117-V.

Dustmann, J. and von der Ohe, W. (1988) 'Einfluβ von Kälteeinbrüchen auf die Frühjahrsentwicklung von Bienenvölkern (*Apis mellifera* L.)', *Apidologie*, 19, pp. 245–254.

Gil-Lebrero, S. *et al.* (2016) 'Honey Bee Colonies Remote Monitoring System', *Sensors*, 17(12), p. 55. doi: 10.3390/s17010055.

Hambleton, J. I. (1925) 'Effect of Weather Upon the Change in Weight of a Colony of Bees During the Honey Flow', *Department Bulletin*, 1339(October), pp. 1–50. Available at: http://agris.fao.org/agris-search/search.do?recordID=US201300428684%5Cnpapers2://publication/uuid/975DD5D5-8F26-4464-A1F5-B4567E1324EB.

Human, H. *et al.* (2007) 'Influence of pollen quality on ovarian development in honeybee workers (*Apis mellifera* scutellata)', *Journal of Insect Physiology*, 53(7), pp. 649–655. doi: 10.1016/j.jinsphys.2007.04.002.

Jay, S. C. (1964) 'Starvation studies of larval honey bees', *Canadian Journal of Zoology*, 42(3), pp. 455–462. doi: 10.1139/z64-039.

Kunert, K. and Crailsheim, K. (1988) 'Seasonal Changes in Carbohydrate, Lipid and Protein Content in Emerging Worker Honeybees and their Mortality', *Journal of Apicultural Research*, 27(1), pp. 13–21. doi: 10.1080/00218839.1988.11100775.

Mattila, H. R. and Otis, G. W. (2007) 'Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn', *Ecological Entomology*, 32(5), pp. 496–505. doi: 10.1111/j.1365-2311.2007.00904.x.

Maurizio, A. and Hodges, F. E. D. (1950) 'The Influence of Pollen Feeding and Brood Rearing on the Length of Life and Physiological Condition of the Honeybee Preliminary Report', *Bee World*, 31(2), pp. 9–12. doi: 10.1080/0005772X.1950.11094617.

Meikle, W. G. *et al.* (2006) 'Using balances linked to dataloggers to monitor honey bee colonies', *Journal of Apicultural Research*, 45(1), pp. 39–41. doi: 10.1080/00218839.2006.11101311.

Meikle, W. G. *et al.* (2008) 'Within-day variation in continuous hive weight data as a measure of honey bee colony activity', *Apidologie*, 39(6), pp. 694–707. doi: 10.1051/apido:2008055.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLOS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Meikle, W. G. *et al.* (2017) 'Internal hive temperature as a means of monitoring honey bee colony health in a migratory beekeeping operation before and during winter', *Apidologie*, 48(5), pp. 666–680. doi: 10.1007/s13592-017-0512-8.

Meikle, W. G. and Holst, N. (2015) 'Application of continuous monitoring of honeybee colonies', *Apidologie*, 46(1), pp. 10–22. doi: 10.1007/s13592-014-0298-x.

Meikle, W. G., Weiss, M. and Stilwell, A. R. (2016) 'Monitoring colony phenology using within-day variability in continuous weight and temperature of honey bee hives', *Apidologie*,

47(1), pp. 1–14. doi: 10.1007/s13592-015-0370-1.

Muggeo, V. R. M. (2008) 'segmented: An R Package to Fit Regression Models with Broken-Line Relationships', *R News*, 3(6), pp. 343–4. doi: 10.1159/000323281.

Rothman, J. A. *et al.* (2018) 'Longitudinal Effects of Supplemental Forage on the Honey Bee (*Apis mellifera*) Microbiota and Inter- and Intra-Colony Variability', *Microbial Ecology*. Microbial Ecology, 76(3), pp. 814–824. doi: 10.1007/s00248-018-1151-y.

Sagili, R. R. and Pankiw, T. (2007) 'Effects of protein-constrained brood food on honey bee (*Apis mellifera* L.) pollen foraging and colony growth', *Behavioral Ecology and Sociobiology*, 61(9), pp. 1471–1478. doi: 10.1007/s00265-007-0379-1.

Schmickl, T. *et al.* (2003) 'Collective and individual nursing investment in the queen and in young and old honeybee larvae during foraging and non-foraging periods', *Insectes Sociaux*, 50(2), pp. 174–184. doi: 10.1007/s00040-003-0644-x.

Schmickl, T. and Crailsheim, K. (2001) 'Cannibalism and early capping: Strategy of honeybee colonies in times of experimental pollen shortages', *Journal of Comparative Physiology - A Sensory, Neural, and Behavioral Physiology*, 187(7), pp. 541–547. doi: 10.1007/s003590100226.

Schmickl, T. and Crailsheim, K. (2002) 'How honeybees (*Apis mellifera* L.) change their broodcare behaviour in response to non-foraging conditions and poor pollen conditions', *Behavioral Ecology and Sociobiology*, 51(5), pp. 415–425. doi: 10.1007/s00265-002-0457-3.

Schmickl, T. and Karsai, I. (2017) *Resilience of honeybee colonies via common stomach: A model of self-regulation of foraging*, *PLoS ONE*. doi: 10.1371/journal.pone.0188004.

Schulz, D. J. *et al.* (2002) 'Effects of colony food shortage on social interactions in honey bee colonies', *Insectes Sociaux*, 49(1), pp. 50–55. doi: 10.1007/s00040-002-8279-x.

Schulz, D. J., Huang, Z. Y. and Robinson, G. E. (1998) 'Effects of colony food shortage on behavioral development in honey bees', *Behavioral Ecology and Sociobiology*, 42(5), pp. 295–303. doi: 10.1007/s002650050442.

Scofield, H. N. and Mattila, H. R. (2015) 'Honey bee workers that are pollen stressed as larvae become poor foragers and waggle dancers as adults', *PLoS ONE*, 10(4), pp. 1–19. doi: 10.1371/journal.pone.0121731.

Stalidzans, E. *et al.* (2017) 'Dynamics of Weight Change and Temperature of *Apis mellifera* (Hymenoptera: Apidae) Colonies in a Wintering Building With Controlled Temperature', *Journal of Economic Entomology*, 110(1), p. tow282. doi: 10.1093/jee/tow282.

Thoenes, S. C. and Buchmann, S. L. (1992) 'Colony abandonment by Adult Honey Bees: A behavioural response to high tracheal mite infestation?', *Journal of Apicultural Research*, 31(3–4), pp. 167–168. doi: 10.1080/00218839.1992.11101280.

Toth, A. L. *et al.* (2005) 'Nutritional status influences socially regulated foraging ontogeny in honey bees', *Journal of Experimental Biology*, 208(24), pp. 4641–4649. doi: 10.1242/jeb.01956.

Wood, S. N. (2001) 'Minimizing Model Fitting Objectives That Contain Spurious Local Minima by Bootstrap Restarting', *Biometrics*, 57(1), pp. 240–244. doi: 10.1111/j.0006-341X.2001.00240.x.

Zacepins, A. *et al.* (2017) 'Solution for automated bee colony weight monitoring', *Agronomy Research*, 15(2), pp. 585–593.

Chapter 3 The development of honey bee colonies assessed using a new semi-automated brood counting method: CombCount

Abstract

Precise, objective data on brood and honey levels in honey bee colonies can be obtained through the analysis of hive frame photographs. However, accurate analysis of all the frame photographs from medium to large-scale experiments is time-consuming. This limits the number of hives than can be practically included in honeybee studies. Faster estimation methods exist but they significantly decrease precision and their use requires a larger sample size to maintain statistical power. To resolve this issue, we created 'CombCount' a python program that automatically detects uncapped cells to speed up measurements of capped brood and capped honey on photos of frames. CombCount does not require programming skills, it was designed to facilitate colony-level research in honeybees and to provide a fast, free and accurate alternative to older methods based on visual estimations. Six observers measured the same photos of thirty different frames both with CombCount and by manually outlining the entire capped areas with ImageJ. The results obtained were highly similar between both the observers and the two methods, but measurements with CombCount were 3.2 times faster than with ImageJ (4 and 13 min per side of the frame, respectively) and all observers were faster when using CombCount rather than ImageJ. CombCount was used to measure the proportions of capped brood and capped honey on each frame of 16 hives over a year as they developed from packages to full-size colonies over about 60 days. Our data describe the formation of brood and honey stores during the establishment of a new colony.

Published version

Introduction

The number and intensity of stressors of honeybees has increased considerably in the last decades and may continue to increase in the near future (Brown et al., 2016). It is therefore important to understand the process of development of commercial honey bee colonies, and how that development is impacted by different stressors (EFSA, 2013; Lundin et al., 2015). When assessing colony development and productivity, key parameters of interest include the number of bees, the amount of brood and the mass of honey stored in the hive. Pollen is also a crucial nutrient for honey bees, but pollen stores have a high turnover rate compared to honey, with most pollen being consumed within 96 h of its collection (Carroll et al., 2017). Pollen surface area may thus not reflect pollen influx and use, and is less often estimated.

To date, studies that have focused on brood and honey production have either estimated or manually measured the amount of brood or honey in the hive (e.g. Alburaki et al., 2017, 2015; Bertrand et al., 2015; Floris et al., 2004; Matsumoto, 2013; Meikle et al., 2018; William G. Meikle et al., 2016; Melathopoulos et al., 2000; Odoux et al., 2014; Sandrock et al., 2014; Woodcock et al., 2017). Different methods present different problems. The amount of brood can be estimated using the Liebefelder method (published in German, Imdorf et al., 1987) which is a visual estimation of the surface of the brood of every frame. The variation within datasets and between observers is likely to be higher with this method than with direct counts.

A German publication (Imdorf and Gerig, 1999) stressed that the Liebefelder method requires intensive training before a user can give reliable estimates without placing a grid in front of the frames. A French article (Fresnaye and Lensky, 1961) compared three methods: a visual estimation similar to the Liebefelder method, a measure of the small and large axis of the brood patch to estimate the area of the ellipsis containing the brood, and the use of a planimeter and a grid similar to the one recommended for inexperienced users in the Liebefelder method. They found on average 23.3% of error when estimating the surface of the brood areas visually with up to 78.5% of error per frame, 13.3% of error when calculating the area of an ellipsis and 14.8% of error when measuring the brood with a planimeter and a grid similar to the one recommended for the Liebefelder method. These high error rates can only decrease the power of statistical analyses, forcing researchers to increase their sample size.

The use of photography to measure brood has been considered since 1924 (Fresnaye and Lensky, 1961). Frame photographs can be evaluated with precision and are themselves permanent records of the data, but the technical constraints of film photography made this impractical for a large number of hives. With the development of digital photography, methods have been developed to measure the area covered by capped brood cells on photos either with Photoshop (Emsen, 2006), ArcView (Meikle et al., 2008) or ImageJ (e.g. Delaplane et al., 2013; William G. Meikle et al., 2016; Yoshiyama et al., 2011), although obtaining fast and accurate results is often challenging, especially when the cells are scattered across a frame rather than clustered.

Current methods to accurately quantify capped brood and capped honey are slow, and this may limit sample size (e.g. Bertrand et al., 2015) in colony-level studies in honeybees. Studies involving mature colonies have often been conducted on species that live in small colonies of a few hundred individuals such as bumblebees (e.g. Holland and Bourke, 2015; Woodcock et al., 2017) or certain ants (e.g. Colin et al., 2017; Modlmeier et al., 2012) where

individuals can easily be counted, but they remain uncommon for honeybees because the scale of a bee hive poses real problems for experimental assessment or manipulation (Lundin et al., 2015). Typically, researchers reduce the size of colonies for use in their experiments on honey bee diseases and stressors (e.g. Böhme et al., 2017; Chang et al., 2015; Gill et al., 2012; He et al., 2013; Perry et al., 2015; Wu-Smart and Spivak, 2016), or focus on individual or small groups of bees *in vitro* (e.g. Baines et al., 2017; de Mattos et al., 2017). However, application of results from studies done with small colonies or groups of bees in controlled or laboratory settings to full-size bee colonies in the field may be more complex than simply scaling up due to the increased number and kinds of interactions among members (Fewell, 2003). These concerns have been recently discussed (Böhme et al., 2017; Lundin et al., 2015). Decreasing the time and practical costs of colony-level studies is consequently a better long-term strategy for research on honeybee health than accepting the limitations of studies on individual honeybees.

Here CombCount (an open-source program for Python) was used to facilitate measurement of capped brood and capped honey both in the field when photographing frames and in the lab when analysing them. CombCount can be used on Windows, Macintosh and Linux. CombCount detects uncapped cells but does not automatically differentiate capped honey from capped brood. After capped brood and capped honey areas have been identified visually by the user, wide areas can be quickly drawn around the capped brood and capped honey to measure capped brood and capped honey areas. CombCount can also be configured to fit a wide range of lighting conditions. The time saved, and the accuracy of CombCount compared to ImageJ were measured across six observers on a sample of 30 frames.

CombCount was then used to measure capped brood and capped honey stores to track development of new honey bee colonies from commercial packages of honey bees. A package of bees contains 2kg of workers and a young mated queen, similar to a swarming group. These

are sold commercially and commonly used by bee keepers to establish new commercial colonies. We analysed 1664 photos of frames taken from 16 hives across 8 hive evaluations. Empirical and theoretical studies on the organization of brood, pollen and nectar on the combs have suggested that bees store honey randomly on frames and consume nectar cells closer to the brood first when feeding the larvae (Camazine, 1991; Camazine et al., 1990; DeGrandi-Hoffman and Hagler, 2000; Eyer et al., 2015). Based on observational data on the structure of wild nests from (Seeley and Morse, 1976) and on experimental data from (Johnson and Baker, 2007) showing that bees preferred to store honey on the sides of the hive, on the inner side of the frames and on top of the brood, Johnson (2009) argued that honey deposition was not completely random. However, all these data were obtained from well-established hives. Monitoring how bees fill empty space during the establishment of a new colony can provide information on choices made by the colony as the brood and honey volumes expand. We show that honey is preferentially stored above the frames containing the most brood at the center of the hive, and that bees use the stores that are closer to the center of the brood first when the brood area is expanding.

Methods

   a) Hives and evaluations

In November 2016, 16 hives were established in Sydney, Australia, from commercial packages containing 2kg of bees and a queen, in standard hives of type "Langstroth" containing 7 new frames with printed wax foundation comb and an in-hive feeder frame used to feed syrup to the bees during the establishment of the colonies. Colonies were fed pollen patty containing water, pollen and sugar and syrup for establishment. Colonies were fed an extra 20kg of syrup over six weeks in January and February for an experiment on neonicotinoids. These treatments had no impact on the development of the software. Frame number 7 was located closest to the feeder and consequently had a higher surface of capped honey after feeding. It is unknown whether pesticides can influence patterns of capped brood and capped honey, but no differences in development of brood and food stores were seen between treatment groups. An additional hive box was added on top in January, with no queen excluder, so that queens were left free to choose where they preferred to lay their eggs. Hives were placed on electronic scales and evaluated every four weeks to measure bee number, capped brood and capped honey area using the method described in (William G. Meikle et al., 2016). At each evaluation, frames were taken out of the hives, brushed gently to remove adult bees, placed on a rotating frame holder, and photographed with a 10Mpx Nikon D3000 camera with a Nikon 18-55mm lens and a flash Yongnuo YN-560II equipped with a light diffuser. We used three photos from a similar experiment in Tucson, Arizona, to determine whether CombCount provided similar results in different lightening conditions with a different camera. These photos were obtained with a 16.3 Mpx Pentax K-01 with no artificial light.

b) CombCount implementation details

CombCount was implemented in Python with OpenCV as an image processing dependency. First, every cell of a photo of a frame was identified and labelled as either capped brood, capped honey, uncapped brood, uncapped honey, pollen or empty. Using computer vision techniques to precisely detect and classify all cells is a challenging problem, but uncapped cells exhibit particularly strong visual contrast with the surrounding texture, making them an ideal candidate class for segmentation. We found that the Hough Gradient circle detection algorithm (Yuen et al., 1990) as implemented in OpenCV was reliably able to detect the boundaries of uncapped cells due to their regular and approximately circular shape, and this formed the basis of our quantification method. Using labelled images for validation, we determined that the "luminance" channel of images encoded in CIELAB (a colour space designed such that Euclidean distance approximately preserves human-perceived distance between colours, where the luminance channel corresponds to human-perceived brightness) resulted in the most accurate segmentation of uncapped cells. Although this technique detects only uncapped cells, we can leverage this information to quantify the uncapped area missing from capped regions, using a small amount of human interaction to outline contiguous regions of the different classes of interest. The parameters of the Hough Gradient algorithm include an edge-detector threshold, for which we used the standard value of 200, and a circle-center accumulator threshold, which represents an adjustable sensitivity parameter. We set the circle-center accumulator threshold to an initial value of 20 and allow the human operator to adjust the sensitivity in increments of 2 to achieve the best segmentation results, which helps to account for variation in imaging conditions. The median spacing between the detected circles (median of the set of distances from each circle to its closest neighbour) was used as the radius of the circles to approximate the area occupied by the empty cells. This value is then multiplied by an inflation factor, determined by the operator, to account for variation in cell size and wall

71

thickness between apiaries or experiments. A first phase of tests was conducted on a large sample of frame photos taken in various environmental conditions, with different cameras and lightning conditions, including with and without flash (Figure 3-1).

We encourage researchers to test and configure CombCount on a preliminary sample of their own photos when planning future studies. The parameter "CELL_INFLATE" allows modification of the size of the circles covering the cells, which can be useful if the size of the cells and the thickness of the walls varies across apiaries or experiments. The detection speed can be improved by changing the initial detection threshold with the parameter "THRESHOLD_INC" in case it is constantly too low or too high for a dataset. A full description of the parameters that can be adjusted is given in the supplementary methods.

CombCount is available online under an open-source license that allows copying, changing and redistributing the file. CombCount is accessible on Github under the name "CombCount" (https://github.com/jakebruce/CombCount) where pull requests can be submitted for improvements of the code. The original version of CombCount and instructions on how to launch it are included (see *Supplementary Material 3-7*, *Supplementary Material 3-8*).
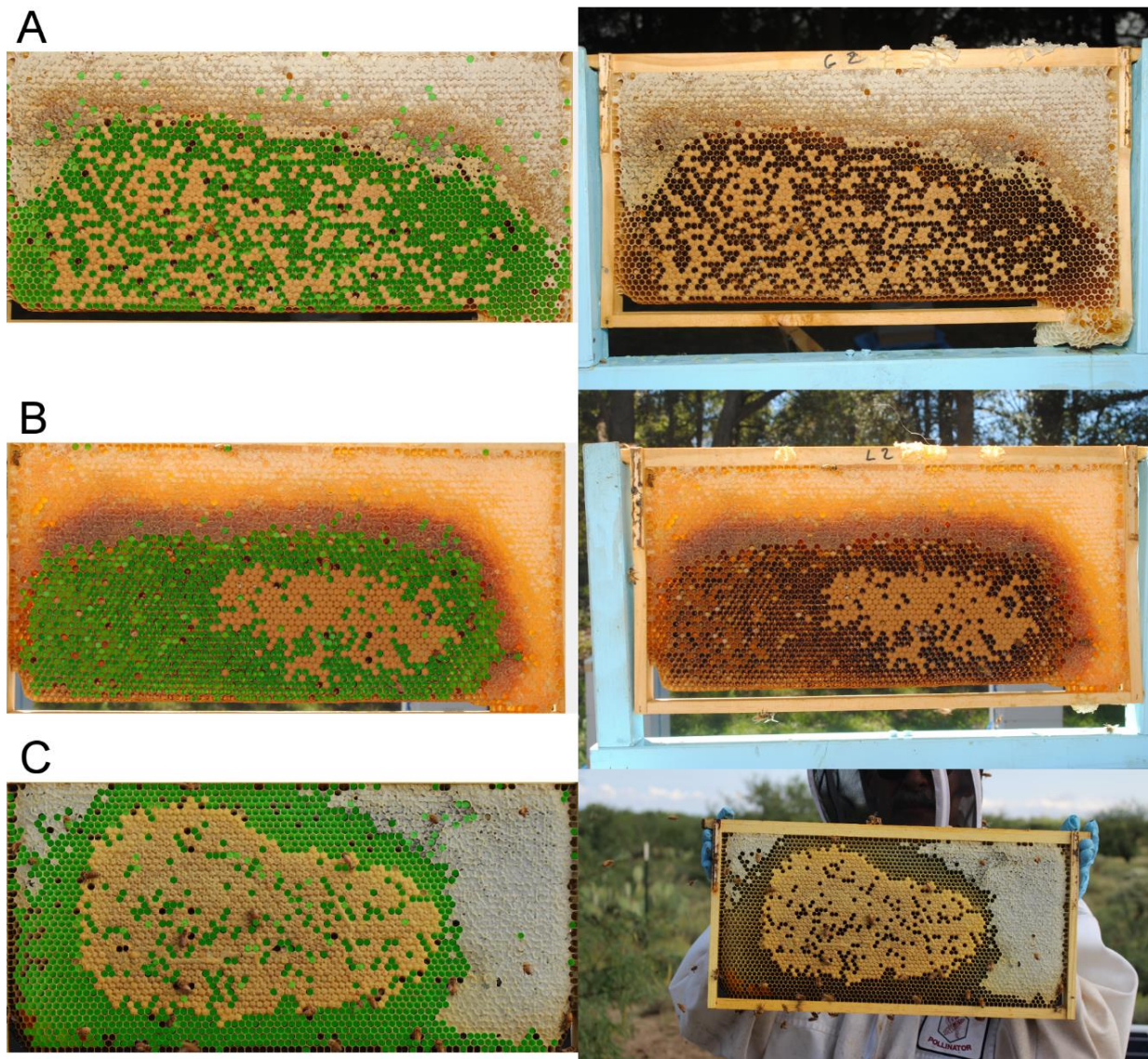
Figure 3-1. **Examples of three frame photos taken in different lightening conditions and their corresponding empty cell detection estimations from CombCount. (A) Photo taken in Sydney, with the 10Mpx Nikon D3000 without flash, (B) Photo taken in the same conditions but with flash, and (C) Photo taken in Arizona, with the 16.3 Mpx Pentax K-01 without flash.**

c) Accuracy of CombCount compared to ImageJ

Thirty photos of frames were randomly selected from a dataset of five hive evaluations on the 16 hives established in Sydney. Photos of frames that did not contain both capped brood and capped honey were removed from the dataset and were replaced with pictures randomly selected from the same dataset (measuring empty frames is irrelevant as it is faster to enter a 0 value). If two sides of the same frame were selected, one of them was replaced, since sides of the same frames tend to be similar. Six different observers measured the 30 selected pictures with both CombCount and ImageJ. Similar amounts of training on both methods were given. Observers were given a short introductory training on how to measure the frames with ImageJ v1.51n and CombCount (with the exception of observer 1 who was already familiar with both methods) until they were confident that they could use both methods. They were asked to record the time to complete all measurements. The detouring tools are remarkably similar in both software applications and great care was given to not influence the users during the introductory training, and to give the same amount of details when explaining both methods. Half of the observers measured the frames with ImageJ first, and the other half with CombCount first. When using CombCount, users were told how to load the photos, how to select the inner corners of the frames, how to change the detection threshold until they were confident that the detection of empty cells could not be improved further, how to select honey and capped brood areas with the selection tool, and where they could access the results. When using ImageJ, users were showed how to load the photos, how to use the "rectangle" tool to measure the inner area of the frame, how to use the "freehand selections" tool to select capped brood and capped honey area, how to subtract empty areas in the middle of large honey or brood patches, and how they could save and access the results. On ImageJ, users were only allowed to use the "freehand selections" tool and did not use the "multi-point" counting tool. Although the counting tool could be more time-efficient for frames with small amounts of

74

capped brood, the number of capped cells was often high, and the counting tool would have been slower than the outlining tool. Further, the counting tool could not have been used for capped honey cells as they were often hard to differentiate from each other. This was also done to avoid imprecision due to the conversion of individual cells into areas.

d) Estimated surface of capped honey and the weight of food stores

The surface of capped honey was measured on 3328 photos corresponding to each side of each frame of 16 hives across 8 evaluations. Evaluations were conducted at least 4 weeks apart. During the two first evaluations, each of the 16 hives was made of a single box containing 7 frames and a feeder. A full depth box was added to each hive after the two first evaluations. During the last six evaluations each hive was made of the same bottom box containing seven frames and a feeder and of a top box containing eight frames. During the hive evaluations, each frame was gently brushed to remove the bees and weighed. We estimated the weight of the stored food alone (including pollen, uncapped honey and capped honey) on each frame by 1) subtracting the weight of the capped brood and the weight of an empty drawn frame from the weight of each frame, and 2) by multiplying the surface of capped honey measured with CombCount by the mass of honey contained in 1 cm^2 of capped honey. Data on the weight of the brood for a given brood surface area, of the weight of an empty drawn frame, and of the weight of honey for a given honey surface were taken from published data (W. G. Meikle et al., 2016). The total mass of honey estimated with both methods was then calculated for each hive and each evaluation.

e) Statistical analyses

All statistical analyses were conducted with R v. 3.5.0 (R Core Team, 2018), the figures were created using the package ggplot2 v. 2.2.1 (Wickham, 2016).

The time each observer took to measure the 30 frames with the two different methods was compared using a paired Wilcoxon test. The area of capped brood, capped honey, and the inner frame area were compared between both methods using three separate ANOVAs, with the area of the parameter measured as a dependant variable and the method, the observers, and their interactions as independent variables. Pearson correlation tests were used to test the correlation between the measurements with the two methods for capped brood and capped honey for each observer. Because the assumption of homoscedasticity was not met, a Spearman correlation test was used to compare the surface of capped honey and the weight of food stores. The relationship between the proportion of honey stored on each frame of the top box and to the proportion of brood on each corresponding frame of the bottom box was analysed with a linear mixed model, hive and evaluation were included as random effects. The proportion of the variance explained by the model was estimated from the method proposed by Xu, 2003. The proportions of honey stored on each frame of the bottom box were compared with an ANOVA, hive and evaluation were included as random effects. Post-hoc contrasts and confidence intervals were estimated using the package emmeans v. 1.2.2 (Lenth et al., 2018).

Results

Measurements were 3.2 times faster with CombCount (on average 4 min for one side of a frame) than with ImageJ (on average 13 min for one side of a frame) (Wilxocon test, V=21, p=0.03125, Figure 3-2). Measurements were correlated for all the observers, both for the capped brood area (for all observers r>0.98 and p<2.2e-16, Figure 3-3A), the honey area (for all observers r>0.96 and p<2.2e-16, Figure 3-3B), and the inner frame area (for all observers r>0.96 and p<2.2e-16, Figure 3-3C).

There was no effect of the method, the observer or of their interactions on the mean of the surface of capped brood (method: Df=1, F=1.309, p=0.253; observer: Df=5, F=0.085, p=0.995; interaction: Df=5, F=0.011, p=1) (Figure 3-4), on the surface of capped honey (method: Df=1, F=0.499, p=0.481; observer: Df=5, F=0.33, p=0.893; interaction: Df=5, F=0.196, p=0.964) (Figure 3-4), and the inner frame area measured with the two methods (method: Df=1, F=1.688, p=0.195; observer: Df=5, F=0.189, p=0.967; interaction: Df=5, F=0.053, p=0.998) (Figure 3-4, *Supplementary Material 3-1*).

The surface of capped honey was correlated with the weight of food stores estimated by subtracting an estimate of weight of brood on the frame, and the known weight of the wooden and wax foundation frame materials from the measured weight of the frame (rho=0.93, p<2e-16) (Figure 3-5).

In the bottom box, there was more capped honey stored in the frames on the sides of the hive than in the center frames, where brood was the most abundant (Figure 3-6, *Supplementary Material 3-3*, *Supplementary Material 3-5*). There was also a significant positive relationship between the proportion of honey stored in the frames of the top box and the proportion of brood in the corresponding frames of the bottom box (estimate=0.50, standard error= 0.05, df=654.78, t=9.02, p<2e-16, 2.5% confidence limit=0.39, 97.5% confidence

limit=0.61, estimated proportion of explained variance = 0.55). Following the addition of the top box, the quantity of honey consistently decreased in the bottom box, and the area of capped brood in the bottom box decreased in the side frames (frames f1 and f7) but increased in the middle frames (frames f3 to f5).
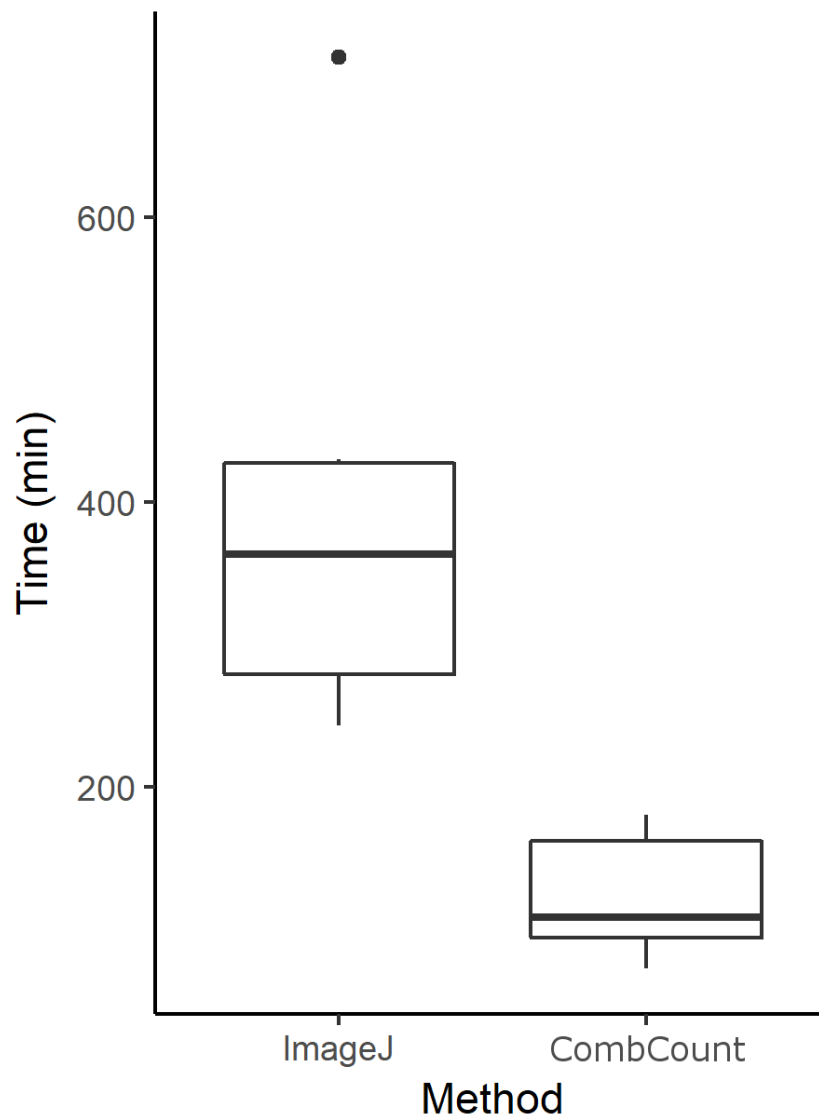
Figure 3-2. **Time taken by the observers to measure 30 frames (minutes). Lower and upper edges of the box represent the 1st and 3rd quartiles respectively, the thick black lines in the boxes represent medians, whiskers extend from minimum to maximum values, one value greater than the 3rd quartile by more than 1.5 times is shown as a dot.**
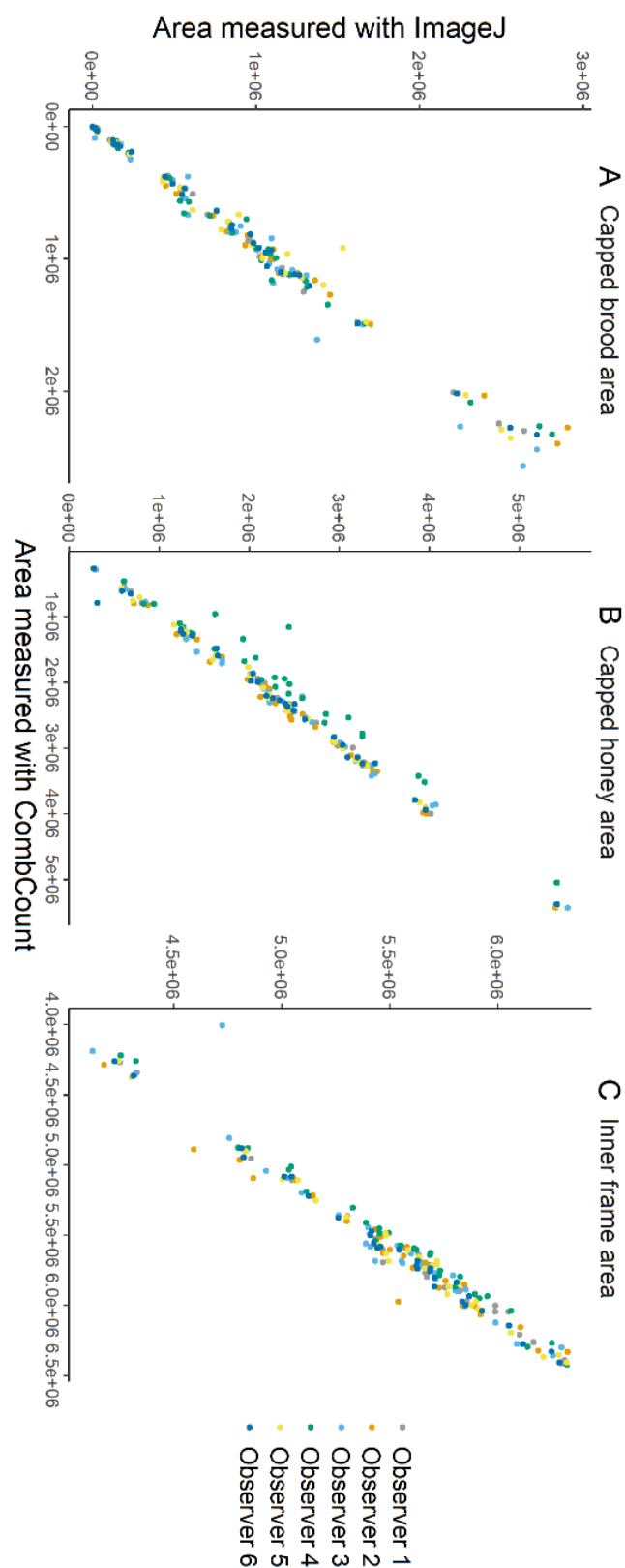
Figure 3-3. **Correlations between the measurements of (A) capped brood, (B) capped honey, and (C) inner frame area with ImageJ and Python from 6 different observers.**
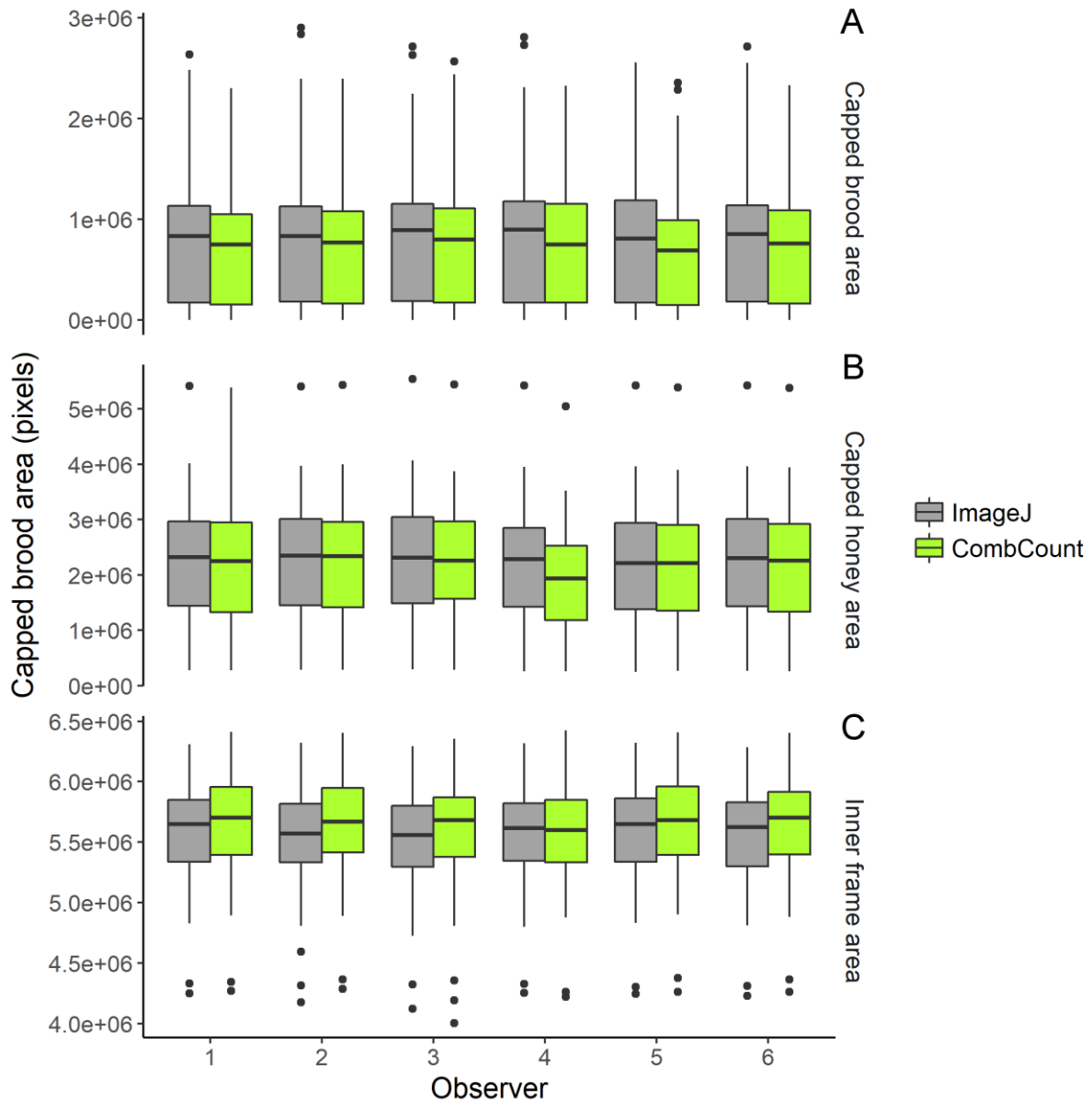
Figure 3-4. **Box and whiskers plots of (A) capped brood area, (B) capped honey area, and (C) inner frame area, with ImageJ and CombCount, by 6 different observers. Measurements with both methods were very similar. Lower and upper edges of the box represent the 1st and 3rd quartiles respectively, black line in the boxes represent medians, whiskers extend from minimum to maximum values, values lesser than the 1st or greater than the 3rd quartile by more than 1.5 times the interquartile range are shown as dots.**
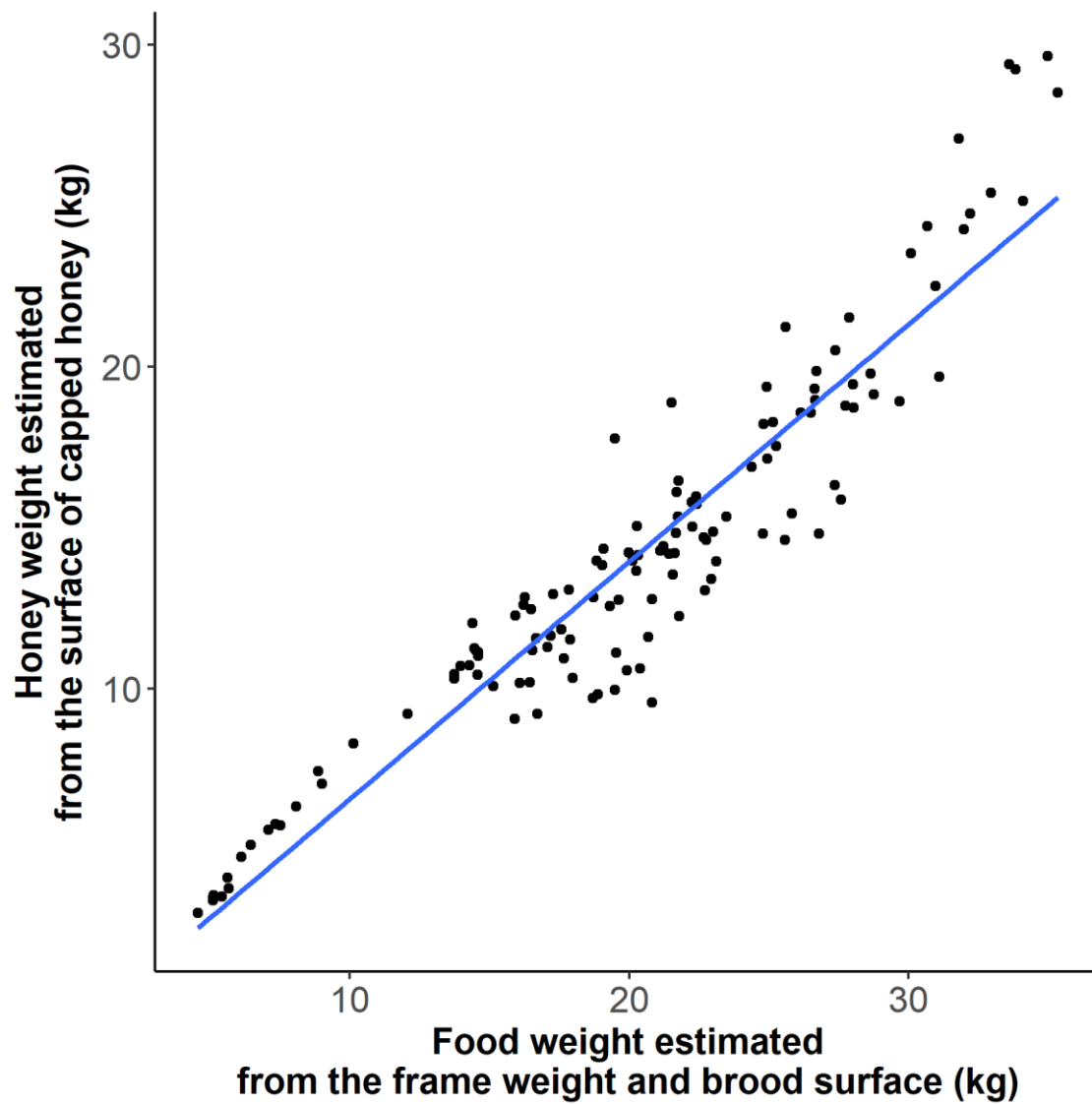
Figure 3-5. **Development of a honey bee colony: estimation of honey weight from the surface of capped honey and by subtracting the estimated weight of brood, wood, and wax from the weight of each frame measured during hive evaluations.**
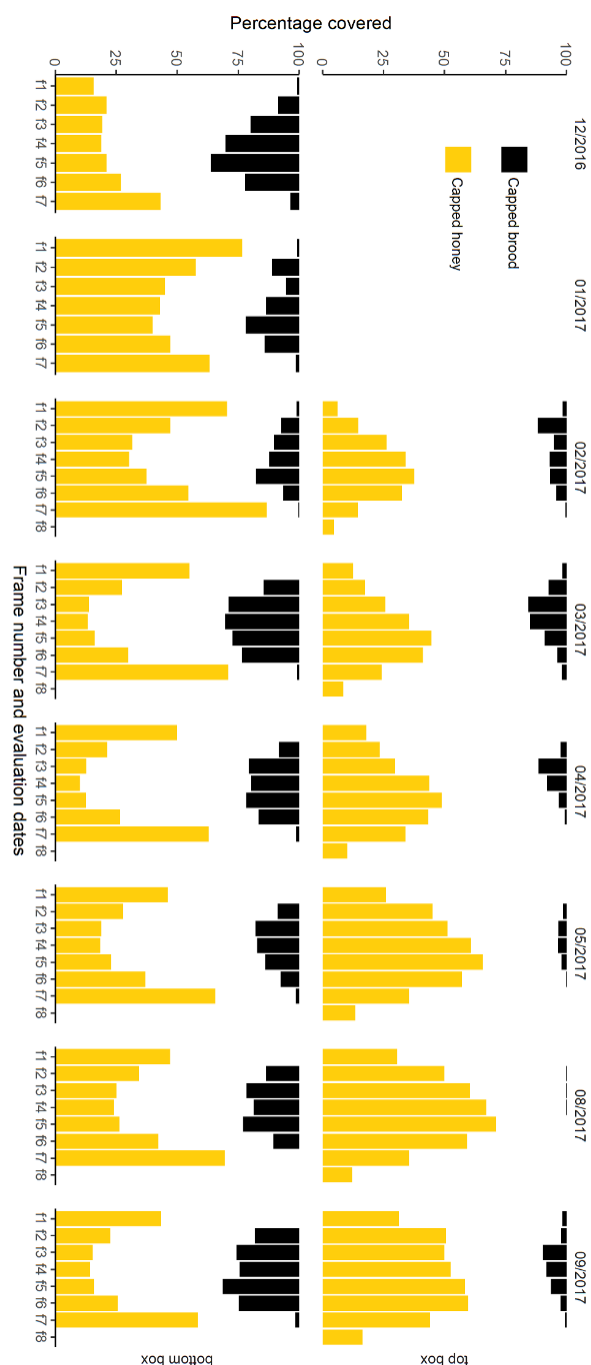
Figure 3-6. **Average value of the amount of capped honey (yellow) and capped brood (grey) for each frame of the 16 hives included in this study at 8 different evaluations. A top box was added in January 2017. The 8th frame of each bottom box was replaced by a frame feeder used during colony establishment and left in the hives. Hives were left undisturbed during Winter (between May 2017 and August 2017). Capped honey and capped brood are represented separately in Supplementary Material 3-5 and Supplementary Material 3-6.**

Discussion

The method described here, CombCount, was successfully used to measure capped brood surface area for a large, long-term colony-level study of colony development from packages in European honeybees. The results obtained were coherent with the literature on self-organized patterns on combs of honeybee colonies, and more detailed than any data previously obtained.

The patterns of capped honey and capped brood that we observed suggest that nectar storage is not random and that bees preferentially store honey in the central frames above the brood, as was previously reported (Johnson and Baker, 2007), and that honey stores decrease on frames where brood is expanding. The first models of self-organized patterns on combs of European honey bee colonies (Camazine et al., 1990; Johnson, 2009) could not predict the patterns observed when the first generation of bees emerges or the vertical patterns of honey storage in natural hives (Johnson and Baker, 2007; Montovan et al., 2013). Parameters such as the use of temperature by the queen to choose optimal brood cells and the difficulty of workers to keep crawling up once they reach the honey zone have been proposed to solve this issue (Johnson, 2009; Johnson and Baker, 2007; Seeley, 1989). Based on the patterns we described, we suggest that further studies should investigate whether bees use variation in temperature to decide where to store honey. A preference for bees to unload nectar in the hottest areas could explain why we found that honey is preferentially stored on top of the brood nest and to a lesser extent on the sides, as heat diffuses and hot air rises from the brood areas to the top of the hive.

In this study we compared the speed and accuracy of measurements of capped honey and capped brood in full-size commercial bee colonies using both ImageJ and CombCount. The measurements of these two parameters with CombCount were similar to those obtained with measures using the standard ImageJ technique, across six different observers (Figure 3-3).

CombCount decreased the time necessary to measure each frame for each observer, with measures taking 69% less time on average (Figure 3-2) although experienced users of the ImageJ method may observe less of a time improvement per photo. With CombCount photos can be taken quickly. They can be analysed if the light conditions allow for a contrast between the empty cells and their borders and as long as the frame is perpendicular to the lens. The ease of this method in terms of equipment needed such as flashes, cameras or tripods allows to keep the costs of photo equipment low, and to work quickly with an open commercial colony.

A commercially-available alternative to CombCount, HoneybeeComplete (WSC Scientific GmbH 2016, n.d.) is available. HoneybeeComplete was not tested in this study and its accuracy and speed have not, as far as we are aware, been published in a refereed journal. According to guidelines provided by its authors, HoneybeeComplete requires high-quality pictures for accurate recognition of brood cells (WSC Scientific GmbH 2016, n.d.). Increasing photo quality may increase camera costs, and increasing the time needed to take such photos may limit the number of colonies that can be studied at the same time. We have not here compared the utility of HoneybeeComplete and CombCount for comb measures.

The surface of capped honey was found to be correlated to the mass of uncapped and capped honey and stored pollen estimated by subtracting the weight of the brood and of an empty drawn frame to the weight of a frame. This indicates that the total food weight can be estimated from the surface of capped honey measured from photographs and confirms the accuracy of CombCount to measure the surface of capped honey.

By reducing the costs and time associated with brood and honey measurements in large apiaries, this tool will allow scientists to increase the number of research projects at the colony level, and to afford a sample size large enough to reduce the risk of false-negative results to an acceptable level.

Acknowledgments

Supporting information

Supplementary Material 3-1 **Inner frame area, capped honey area and capped brood area measured by the different observers with the different methods on 30 pictures of hive frames.** https://doi.org/10.1371/journal.pone.0205816.s001

Supplementary Material 3-2 **S2 Table. Confidence intervals and estimated marginal means for the proportion of honey stored in the frames 1 to 7 of the bottom box.** https://doi.org/10.1371/journal.pone.0205816.s003

Supplementary Material 3-3 **S3 Table.** Percentage of capped brood and capped honey on the frames of the sixteen hives used in this study (Figure 6), and of the honey mass stored in each hive estimated from the photos and from the frame weight (Figure 5). https://doi.org/10.1371/journal.pone.0205816.s008

Supplementary Material 3-4 **S1 Fig. Estimated marginal means (black dot) +/- standard errors (shaded area) of the proportion of honey on each frame.** The degree to which arrows overlap reflects as much as possible the significance of the comparison of the two estimates. https://doi.org/10.1371/journal.pone.0205816.s002

Supplementary Material 3-5 **S2 Fig. Box and whiskers plots of the percentage of each frame covered by capped brood for each of the eight evaluations.** A top box was added in January 2017. The 8th frame of each bottom box was replaced by a frame feeder used during colony establishment and left in the hives. Hives were left undisturbed during Winter (between May 2017 and August 2017). Lower and upper edges of the box represent the 1st and 3rd quartiles respectively, black line in the boxes represent medians, whiskers extend from minimum to maximum values, numbers lesser than the 1st or greater than the 3rd quartile by more than 1.5 times the interquartile range are shown as dots. https://doi.org/10.1371/journal.pone.0205816.s004

Supplementary Material 3-6 **S3 Fig. Box and whiskers plots of the percentage of each frame covered by capped honey for each of the eight evaluations.** A top box was added in January 2017. The 8th frame of each bottom box was replaced by a frame feeder used during colony establishment and left in the hives. Hives were left undisturbed during Winter (between May 2017 and August 2017). Lower and upper edges of the box represent the 1st and 3rd quartiles respectively, black line in the boxes represent medians, whiskers extend from minimum to maximum values, numbers lesser than the 1st or greater than the 3rd quartile by more than 1.5 times the interquartile range are shown as dots. https://doi.org/10.1371/journal.pone.0205816.s005

Supplementary Material 3-7 **S1 Software. Program file written in Python containing the software CombCount.** https://doi.org/10.1371/journal.pone.0205816.s006

Supplementary Material 3-8 **S1 Text. Instructions to launch CombCount and change the default parameters of the software.** https://doi.org/10.1371/journal.pone.0205816.s007

References

Alburaki, M. *et al.* (2015) 'Neonicotinoid-Coated Zea mays Seeds Indirectly Affect Honeybee Performance and Pathogen Susceptibility in Field Trials', *PLoS ONE*. Edited by C. Rodriguez-Saona, 10(5), p. e0125790. doi: 10.1371/journal.pone.0125790.

Alburaki, M. *et al.* (2017) 'Performance of honeybee colonies located in neonicotinoid-treated and untreated cornfields in Quebec', *Journal of Applied Entomology*, 141(1–2), pp. 112–121. doi: 10.1111/jen.12336.

Baines, D. *et al.* (2017) 'Neonicotinoids act like endocrine disrupting chemicals in newly-emerged bees and winter bees', *Scientific Reports*. Springer US, 7(1), p. 10979. doi: 10.1038/s41598-017-10489-6.

Bertrand, B. *et al.* (2015) 'MtDNA COI-COII marker and drone congregation area: An efficient method to establish and monitor honeybee *(Apis mellifera* L.) conservation centres', *Molecular Ecology Resources*, 15(3), pp. 673–683. doi: 10.1111/1755-0998.12339.

Böhme, F. *et al.* (2017) 'Chronic exposure of honeybees, *Apis mellifera* (Hymenoptera: Apidae), to a pesticide mixture in realistic field exposure rates', *Apidologie*, 48(3), pp. 353–363. doi: 10.1007/s13592-016-0479-x.

Brown, M. J. F. *et al.* (2016) 'A horizon scan of future threats and opportunities for pollinators and pollination', *PeerJ*, 4(3), p. e2249. doi: 10.7717/peerj.2249.

Camazine, S. *et al.* (1990) 'A mathematical model of self-organized pattern formation on the combs of honeybee colonies', *Journal of Theoretical Biology*, 147(4), pp. 553–571. doi: 10.1016/S0022-5193(05)80264-4.

Camazine, S. (1991) 'Self-organizing pattern formation on the combs of honey bee colonies', *Behavioral Ecology and Sociobiology*, 28(1), pp. 61–76. doi: 10.1007/BF00172140.

Carroll, M. J. *et al.* (2017) 'Honey bees preferentially consume freshlystored Pollen', *PLoS ONE*, 12(4), pp. 1–21. doi: 10.1371/journal.pone.0175933.

Chang, L., Barron, A. and Cheng, K. (2015) 'Effects of the juvenile hormone analogue methoprene on rate of behavioural development, foraging performance and navigation in honey bees (*Apis mellifera*)', *Journal of Experimental Biology*, 218, pp. 1715–1724. doi: 10.1242/jeb.119198.

Colin, T. *et al.* (2017) 'Decreasing worker size diversity does not affect colony performance during laboratory challenges in the ant Temnothorax nylanderi', *Behavioral Ecology and Sociobiology*. Behavioral Ecology and Sociobiology, 71(6), p. 92. doi: 10.1007/s00265-017-2322-4.

DeGrandi-Hoffman, G. and Hagler, J. (2000) 'The flow of incoming nectar through a honey bee (*Apis mellifera* L.) colony as revealed by a protein marker', *Insectes Sociaux*, 47(4), pp. 302–306. doi: 10.1007/PL00001720.

Delaplane, K. S., Steen, J. Van Der and Guzmán-Novoa, E. (2013) 'Standard methods for estimating strength parameters of *Apis mellifera* colonies', *Journal of Apicultural Research*, 52(1), pp. 1–12. doi: 10.3896/IBRA.1.52.1.03.

EFSA (2013) 'Guidance on the risk assessment of plant protection products on bees *(Apis mellifera*, *Bombus spp.* and solitary bees)', *EFSA Journal*, 11(7), p. 3295. doi: 10.2903/j.efsa.2013.3295.

Emsen, B. (2006) 'Semi-automated measuring capped brood areas of honey bee colonies', *Journal of Animal and Veterinary Advances*, pp. 1229–1232.

Eyer, M. *et al.* (2015) 'No spatial patterns for early nectar storage in honey bee colonies', *Insectes Sociaux*. Springer Basel, 63(1), pp. 51–59. doi: 10.1007/s00040-015-0432-4.

Fewell, J. H. (2003) 'Social Insect Networks', *Science*. Oxford: John Wiley & Sons, 301(5641), pp. 1867–1870. doi: 10.1126/science.1088945.

Floris, I. *et al.* (2004) 'Comparison Between Two Thymol Formulations in the Control of *Varroa destructor*: Effectiveness, Persistence, and Residues', *Journal of Economic Entomology*, 97(2), pp. 187–191. doi: 10.1603/0022-0493-97.2.187.

Fresnaye, J. and Lensky, Y. (1961) 'Méthodes d'appréciation des surfaces de couvain dans les colonies d'abeilles.', *Les Annales de l'Abeille*, 4(4), pp. 369–376.

Gill, R. J., Ramos-Rodriguez, O. and Raine, N. E. (2012) 'Combined pesticide exposure severely affects individual-and colony-level traits in bees', *Nature*. Nature Publishing Group, 491(7422), pp. 105–108. doi: 10.1038/nature11585.

He, X. *et al.* (2013) 'Assessment of flight activity and homing ability in Asian and European honey bee species, *Apis cerana* and *Apis mellifera*, measured with radio frequency tags', *Apidologie*, 44(1), pp. 38–51. doi: 10.1007/s13592-012-0156-7.

Holland, J. G. and Bourke, A. F. G. (2015) 'Colony and individual life-history responses to temperature in a social insect pollinator', *Functional Ecology*, 29(9), pp. 1209–1217. doi: 10.1111/1365-2435.12480.

Imdorf, A. *et al.* (1987) 'Überprüfung der shätzmethode zur ermittlung der brutfläche und der anzahl arbeiterinnen in freifliegenden bienenvölkern.', *Apidologie*, 18(2), pp. 137–146.

Imdorf, A. and Gerig, L. (1999) 'Lehrgang zur Erfassung der Volksstände', *Schweizerisches Zentrum für Bienenforschung*, p. 4.

Johnson, B. R. (2009) 'Pattern formation on the combs of honeybees: increasing fitness by coupling self-organization with templates', *Proceedings of the Royal Society B: Biological Sciences*, 276(1655), pp. 255–261. doi: 10.1098/rspb.2008.0793.

Johnson, B. R. and Baker, N. (2007) 'Adaptive spatial biases in nectar deposition in the nests of honey bees', *Insectes Sociaux*, 54(4), pp. 351–355. doi: 10.1007/s00040-007-0953-6.

Lenth, R. *et al.* (2018) 'Package "emmeans"', *Mran.Microsoft.Com*, 34(1), p. 126. doi: 10.1080/00031305.1980.10483031.

Lundin, O. *et al.* (2015) 'Neonicotinoid insecticides and their impacts on bees: A systematic review of research approaches and identification of knowledge gaps', *PLoS ONE*, 10(8), pp. 1–20. doi: 10.1371/journal.pone.0136928.

Matsumoto, T. (2013) 'Short- and long-term effects of neonicotinoid application in rice fields, on the mortality and colony collapse of honeybees (*Apis mellifera*)', *Journal of Apicultural Science*, 57(2), pp. 21–35. doi: 10.2478/jas-2013-0014.

de Mattos, I. M., Soares, A. E. E. and Tarpy, D. R. (2017) 'Effects of synthetic acaricides on honey bee grooming behavior against the parasitic *Varroa destructor* mite', *Apidologie*, 48(4), pp. 483–494. doi: 10.1007/s13592-017-0491-9.

Meikle, W. G. *et al.* (2008) 'Within-day variation in continuous hive weight data as a measure of honey bee colony activity', *Apidologie*, 39(6), pp. 694–707. doi: 10.1051/apido:2008055.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLoS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Meikle, W. G. *et al.* (2018) 'Using within-day hive weight changes to measure environmental effects on honey bee colonies', *PLoS ONE*. Edited by A. G. Dyer, 13(5), p. e0197589. doi: 10.1371/journal.pone.0197589.

Meikle, W. G., Weiss, M. and Stilwell, A. R. (2016) 'Monitoring colony phenology using within-day variability in continuous weight and temperature of honey bee hives', *Apidologie*, 47(1), pp. 1–14. doi: 10.1007/s13592-015-0370-1.

Melathopoulos, A. P. *et al.* (2000) 'Field Evaluation of Neem and Canola Oil for the Selective Control of the Honey Bee (Hymenoptera: Apidae) Mite Parasites *Varroa jacobsoni* (Acari: Varroidae) and *Acarapis woodi* (Acari: Tarsonemidae)', *Journal of Economic Entomology*, 93(3), pp. 559–567. doi: 10.1603/0022-0493-93.3.559.

Modlmeier, A. P., Liebmann, J. E. and Foitzik, S. (2012) 'Diverse societies are more productive: a lesson from ants', *Proceedings of the Royal Society B: Biological Sciences*, 279(1736), pp. 2142–2150. doi: 10.1098/rspb.2011.2376.

Montovan, K. J. *et al.* (2013) 'Local behavioral rules sustain the cell allocation pattern in the combs of honey bee colonies (*Apis mellifera*)', *Journal of Theoretical Biology*. Elsevier, 336, pp. 75–86. doi: 10.1016/j.jtbi.2013.07.010.

Odoux, J.-F. *et al.* (2014) 'ECOBEE: a tool for long-term honey bee colony monitoring at the landscape scale in West European intensive agroecosystems', *Journal of Apicultural Research*, 53(1), pp. 57–66. doi: 10.3896/IBRA.1.53.1.05.

Perry, C. J. *et al.* (2015) 'Rapid behavioral maturation accelerates failure of stressed honey bee colonies', *Proceedings of the National Academy of Sciences*, 112(11), pp. 3427–3432. doi: 10.1073/pnas.1610243113.

R Core Team, R. (2018) 'R: A language and environment for statistical computing. R Foundation for Statistical Computing'. Vienna, Austria. Available at: https://www.r-project.org/.

Sandrock, C. *et al.* (2014) 'Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure', *PLoS ONE*, 9(8), pp. 1–13. doi: 10.1371/journal.pone.0103592.

Seeley, T. D. (1989) 'Social foraging in honey bees: how nectar foragers assess their colony's nutritional status', *Behavioral Ecology and Sociobiology*, 24(3), pp. 181–199. doi:

10.1007/BF00292101.

Seeley, T. D. and Morse, R. A. (1976) 'The nest of the honey bee (*Apis mellifera* L.)', *Insectes Sociaux*, 23(4), pp. 495–512. doi: 10.1007/BF02223477.

Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. Springer-V. New York: Springer-Verlag New York. Available at: https://ggplot2.tidyverse.org.

Woodcock, B. A. *et al.* (2017) 'Country-specific effects of neonicotinoid pesticides on honey bees and wild bees', *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

WSC Scientific GmbH 2016 (no date) *Software for Evaluation of Honeybee Brood Trials and Colony Assessment*. Available at: http://wsc-regexperts.com/en/honeybees/honeybee-brood-colony-assessment-software.

Wu-Smart, J. and Spivak, M. (2016) 'Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development', *Scientific Reports*. Nature Publishing Group, 6(March), pp. 1–11. doi: 10.1038/srep32108.

Xu, R. (2003) 'Measuring explained variation in linear mixed effects models', *Statistics in Medicine*, 22(22), pp. 3527–3541. doi: 10.1002/sim.1572.

Yoshiyama, M. *et al.* (2011) 'Measuring colony development in honey bees by simple digital image analysis', *Journal of Apicultural Research*, 50(2), pp. 170–172. doi: 10.3896/IBRA.1.50.2.10.

Yuen, H. *et al.* (1990) 'Comparative study of Hough Transform methods for circle finding', *Image and Vision Computing*, 8(1), pp. 71–77. doi: 10.1016/0262-8856(90)90059-E.

Chapter 4 Long-term dynamics of honey bee colonies following exposure to chemical stress

Abstract

Pesticide residues have been linked to reduced bee health and increased honey bee colony failure. Most research to date has investigated the role of pesticides on individual honey bees, and it is still unclear how trace levels of pesticides change colony viability and productivity over seasonal time scales. To address this question we exposed standard bee colonies to chemical stressors known to have negative effects on individual bees, and measured the productivity of bee colonies across a whole year in two environments: near Tucson Arizona and Sydney Australia. We exposed hives to a small concentration of the neonicotinoid imidacloprid and to the acaricide thymol, and measured capped brood, bee and honey production, as well as the temperature and foraging force of the colonies. The effect of imidacloprid on colony dynamics differed between the two environments. In Tucson we recorded a positive effect of imidacloprid treatment on bee and brood numbers. Thymol was associated with short-term negative effects on bee numbers at both locations, and may have affected colony survival at one location. The overall benefits of thymol for the colonies were unclear. We conclude that long-term and colony-level measures of the effects of agrochemicals are needed to properly understand risks to bees.

Published version

Introduction

The effects of sublethal pesticide contaminants on honey bee hive health are now an issue of global concern. This issue is complex because the structure of a honey bee society is such that the consequence of a stressor that impacts bee health does not simply translate to a change in colony function (Henry et al., 2015). Several models have been developed to explore the dynamic relationships between honey bee health and colony function (e.g. Barron, 2015; Becher et al., 2014; Betti et al., 2014; DeGrandi-Hoffman et al., 1989; Khoury et al., 2013, 2011; Myerscough et al., 2017; Perry et al., 2015; Russell et al., 2013). Some of these have become very influential, but none have yet been tested against strong field data. Field studies of agrochemical stressors on bee hives have yielded conflicting results, even within the same study (Kerr, 2017; William G. Meikle et al., 2016; Osterman et al., 2019; Thompson et al., 2019; Woodcock et al., 2017). This may reflect complex interactions between the stressor, colony dynamics and the environment of the hive (Tsvetkov et al., 2017). Further, agrochemical stressors rarely occur in isolation since honey bee colonies are often directly treated with in-hive miticides against the pest *V. destructor* as well as being exposed to insecticides applied to crop plants (Calatayud-Vernich et al., 2018; Mullin et al., 2010). Here we present detailed and long term field data for how bee hives across multiple locations are impacted by trace amounts (5 µg/kg = 5 ppb) of the pesticide imidacloprid, and standard

thymol-based in-hive miticide treatments both separately and in combination. We report multiple measures of colony population and performance over a full year to assess the long-term impacts of stressors on hive performance, and to provide the data needed for validation of models of hive population dynamics.

Colony-level studies of the effects of pesticides remain scarce, consequently models of honey bee colony function rely on limited descriptions of the dynamics of the adult bee and brood populations (e.g. Allen and Jeffree, 1956; Jeffree, 1951; Nolan, 1925 are summarized in Winston (1987) which is cited in Khoury et al., 2011; Russell et al., 2013; Ushitani et al., 2016). The accuracy and precision of these historic measurements were limited by technical constraints (see methods in Allen and Jeffree, 1956; Jeffree, 1951; Nolan, 1925). Some of these constraints have recently been resolved by new sensors and computer programs (Colin et al., 2018; W. G. Meikle et al., 2016) and it is now possible to report frequent or continuous detailed data for individual bee colonies to the level of precision necessary to inform bee colony models (Meikle et al., 2008; Meikle and Holst, 2015). These methods have enabled collection of longitudinal data on the effect of pesticides on whole bee colonies.

Neonicotinoid insecticides are a particular cause for concern. They damage immune function, survival, homing and flight ability of individual honey bees and have been shown to reduce pollen stores and brood production of European honey bee (*A. mellifera*) colonies (Beyer et al., 2018b; Fischer et al., 2014; Forfert et al., 2017; Henry et al., 2012; Lu et al., 2012; Mitchell et al., 2017; Sánchez-Bayo et al., 2016; Sgolastra et al., 2017; Tosi et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017; Wu-Smart and Spivak, 2016). These effects have been reported from lab studies or short-term studies with small experimental colony units. It is not clear how these effects on individuals might translate to changes in function of standard managed colonies of European honey bees in the field (Henry et al., 2015).

Studies of the effects of commonly used neonicotinoid insecticides on European honey bee and bumblebee colonies report negative effects (William G. Meikle et al., 2016; Rundlöf et al., 2015; Samson-Robert et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017), but some have reported positive effects (William G. Meikle et al., 2016; Osterman et al., 2019; Woodcock et al., 2017). This discrepancy remains unexplained. Our understanding of why the effect of neonicotinoids varies so much is limited by the absence of longitudinal data on the duration and magnitude of the effects of pesticides (Osterman et al., 2019).

Interactions between pesticides could explain why the effect of neonicotinoid insecticides on bee colonies varies across environments (Woodcock et al., 2017). In particular, individual honey bees exposed to neonicotinoids are likely to simultaneously encounter miticides. Beekeepers systematically use miticides to kill *V. destructor,* a major pest of honey bees 7 (Mullin et al., 2010). *V. destructor* is found wherever *A, mellifera* is present (apart from Australia, see Cunningham et al., 2002). Miticide treatments and their residues are almost ubiquitous in hives around the world (Beyer et al., 2018a; Bogdanov et al., 1998; Calatayud-Vernich et al., 2018; Mullin et al., 2010; Premrov Bajuk et al., 2017). Miticides themselves have negative effects on honey bees both at the individual and colony level (Dietemann et al., 2012; Kruitwagen et al., 2017; Le Conte et al., 2007; Neumann and Blacquière, 2017; Oddie et al., 2017; Tihelka, 2018). The few studies on the effect of miticides that did not study lethal effects in the short term have reported changes in foraging and hygienic behaviors of individual honey bees and an increase in brood, worker and queen mortality (Colin et al., 2019; de Mattos et al., 2017; Imdorf et al., 1999; Marchetti et al., 1984; Pettis et al., 1991; Prado et al., 2019; Tihelka, 2018; Whittington, 2000; Zhu et al., 2014). Despite their deleterious effects on honey bees, miticides can benefit bee colonies when the advantage provided by the decrease in *V. destructor* population exceeds their negative effects on honey bees. However, the effects of miticides at the colony level have rarely been studied under realistic conditions and over the

long term. Their potential interactions with neonicotinoids have to our knowledge rarely been studied (Johnson et al., 2013; Retschnig et al., 2015; Zhu et al., 2014).

To address these gaps, we recorded over a full year the worker and brood populations, food stores and daily weight dynamics of standard honey bee colonies. Some of these colonies were kept as controls and others were treated with sugar syrup containing 5 µg/kg (= 5 ppb) of the neonicotinoid imidacloprid, or exposed to the commercial formulation Apiguard® of the miticide thymol, or to a combination of thymol and imidacloprid. The 5 µg/kg imidacloprid concentration corresponds to the trace amounts found in bees and hive products, and is less than amounts from pollen and nectar in several crops (Blacquière et al., 2012; Chauzat et al., 2009; Krischik et al., 2007). To investigate the potential interactions of these pesticides with the environment, we replicated this experiment in Sydney, Australia, and in Arizona. These two highly contrasting environments differed notably by the availability of nectar, water and pollen and by the presence (Santa Rita Experimental Range, near Tucson) or absence (Macquarie University in Sydney) of *V. destructor*.

Material and Methods

a) Treatments

At both locations, one milligram of pure analytical-grade imidacloprid (Imidacloprid PESTANAL®, CAS # 138261-41-3) was dissolved in distilled water and added to sucrose syrup to obtain a 5 µg/kg (= 5 ppb) imidacloprid solution as described in William G. Meikle et al. (2016). In brief, the control syrup solution was prepared from sucrose and distilled water at a one for one weight to weight ratio using a large mixing flask on a hot plate and heated to 60°C. The imidacloprid syrup was prepared in the following way: first, a stock solution of imidacloprid was mixed by diluting 1.0 mg of imidacloprid into 100 mL of distilled water using a mixing bar in a glass container placed on a magnetic stirrer for 15 minutes. 1 mL of this solution was then mixed into 199 mL of distilled water and shaken vigorously for 2 minutes. The 200 mL imidacloprid solution was then added to a sucrose solution formulated short of 200 mg of water to achieve a 5 µg/kg imidacloprid sucrose solution of the same sucrose concentration as the control group.

This amount of imidacloprid corresponds to traces of imidacloprid found in bees and hive products (Alburaki et al., 2018; Blacquière et al., 2012; Bonmatin et al., 2005, 2003; Chauzat et al., 2009, 2006; Cresswell, 2011; Krischik et al., 2007). Colonies in the imidacloprid and imidacloprid + thymol treatment groups were fed a total of 20 kg of sugar syrup with 5 µg/kg (ppb) of imidacloprid over 6 weeks. All the syrup was consumed by the bees.

Two weeks after the imidacloprid treatment started, colonies were given one aluminium tray containing 50 g of Apiguard gel (250 g/kg of thymol) that was renewed after two weeks for another two weeks' period (Table 4-1) as per manufacturer's instructions.

Table 4-1. **Treatment dates for thymol and imidacloprid exposure in Tucson and Sydney.**

	Tucson	Sydney
Thymol	1 August to 22 August 2016	3 February to 24 February 2017
Imidacloprid	11 July to 22 August 2016	20 January to 24 February 2017

b) Hives

In April 2016, 28 colonies of European honey bees (*A. mellifera*) were established at a site in the Santa Rita Experimental Range (31°48'5.85"N, 110°53'23.15"W) south of Tucson, AZ, from packages containing 1 kg of bees each. Colonies were stocked with Cordovan-Italian queens (C.F. Koehnen & Sons, Glenn, CA, USA) and housed in painted standard hives referred to as 10-frame wooden "Langstroth" deep boxes fitted with American migratory wooden lids (Mann Lake Ltd, Hackensack, MN, USA). Several frames of drawn comb and foundation comb were placed in each hive. Hives were arranged in groups of four to five. To reduce bee drift among hives, hives were placed around each equipment box (housing the batteries, data loggers and electronics to power hive scales; see below), facing outward in either a North-East, North-West, South-East or South-West direction. Each group was 2 or more meters from neighboring groups. The apiary had a permanent water source and was surrounded by native, unmanaged plants, particularly mesquite (*Prosopis spp.*), creosote (*Larrea spp.*), cactus (mainly *Opuntia spp.*) and wildflowers. No commercial agriculture existed within a 10 km of the apiary. Bees were fed 400 g of pollen patty and 4 L of 50:50 water:sugar syrup on establishment. The colonies did not receive further feeding to avoid creating differences between the study sites and confounding or causing complex interactions between treatments, food supplement and

colony size. The experiment was started on the 13th of June and treatments started on 11th of July 2016. The twenty-two strongest hives were selected and five hives were randomly assigned to the combined thymol and imidacloprid group, six to the thymol group, five to the imidacloprid group and six to the control group. Hive locations were randomized across the apiary.

In November 2016, 20 colonies of European honey bees (*Apis mellifera*) were established on the campus of Macquarie University (33°46'06.6"S 151°06'43.8"E), north of Sydney, NSW, Australia, from packages containing about 1.8 kg of bees. Colonies were stocked with Golden Italian queens (Australian Queen Bee Exporters Pty. Ltd., Blayney, NSW) and housed in painted, standard hives referred to as 8-frame wooden "Langstroth" deep boxes, fitted with Australian migratory lids (vents were closed with tapes for the length of the experiment). Bees were given eight frames with printed wax foundations. Hives were arranged in a grid, separated by at least three meters, and the boxes were painted with different patterns to reduce drift, one month after colony establishment. The sixteen strongest hives were selected and four hives were randomly assigned to each group. Hive locations were randomized across the apiary. The apiary is located in a suburban area within 1km of a national park and surrounded by native plants and gardens, particularly eucalypts and banksias. No commercial agriculture existed within a 10 km of the apiary. Colonies were fed 400 g of pollen patty and 4 L of 50:50 water:sugar syrup on establishment. The experiment started on the 22nd of December 2016 and the treatments on the 20th of January 2017. The sixteen strongest hives were selected and four of each randomly assigned to the combined thymol and imidacloprid, thymol, imidacloprid or control group. Hive locations were randomized across the apiary.

c) Measures

Hives were placed on stainless steel electronic scales (TEKFA® model B-2418 and Avery Weigh-Tronix model BSAO1824-200) (max. capacity 100 kg) connected to a solar power source and to 12- bit dataloggers (Hobo® U-12, Onset Computer Corporation) that were set to record weight every 5 minutes, as described in W. G. Meikle et al. (2016). Prior to placing the hives on the scales, and at the end of the experiment, all scales were calibrated using commercial scale weights. Changes in calibration parameters over time were negligible. The system had an overall precision of approximately ±20 g. Hives were opened every 4 weeks except during the treatment period in Tucson or during winter (Table 4-2). Each frame was taken out, brushed, weighed and both sides photographed.

Capped brood area was measured on each side of the frames using either ImageJ v1.4 or CombCount v1 (Colin et al., 2018), pollen and honey surface were estimated by subtracting the estimated brood weight from the weight of the frame.

Table 4-2. **Dates of hive evaluations. There was no evaluation during winter due to cold temperatures. In Tucson, there was no evaluation during treatment because the low food availability increased the risk of robbing events (bee colonies stealing the food stores of other colonies) that could have caused contaminations between treatment groups.**

Evaluation dates	
Tucson	Sydney
13 June 2016	22 December 2016
11 July 2016 (treatment begins)	19 January 2017 (treatment begins)
No evaluation during feeding to avoid robbing	20 February 2017
8 September 2016	21 March 2017
5 October 2016	13 April 2017
Winter	11 May 2017
16 February 2017	Winter
22 March 2017	10 August 2017
Experiment ended	7 September 2017

On four occasions, *V. destructor* mite falls were measured across all hives in Tucson. Sticky boards covered in a metallic mesh were put on top of the bottom board without opening the hives on the 8th of July 2016 (before thymol treatment), 25th of August 2016 (at the end of the thymol treatment), 28th of November 2016 and 23rd of February 2017, and removed after 3, 4, 3, and 4 days respectively. Total mite falls were divided by the number of days of collection to obtain the average daily mite fall for each hive. *V. destructor* was not detected in Sydney.

One temperature sensor (iButton Thermochron, model DS1922, precision ±0.0625 °C) enclosed in a plastic tissue embedding cassette (Thermo Fisher Scientific, Waltham, MA) was placed at the centre of the brood box at 5 cm below the top bar of the fifth frame and set to record every 30 min. Sensors were replaced every 3 months during hive evaluations when they reached maximum storage capacity. Temperature variation and average temperature were calculated daily.

d) Temperature and rainfall

Temperature data for the Sydney Macquarie Campus site were obtained from the Automatic Weather Station of Department of Environmental Sciences of Macquarie University with a 15-minute interval and averaged per day, daily rainfall data were obtained from the Australian Bureau of Meteorology. Daily temperature and rainfall data for the Tucson Santa Rita Experimental Range site were obtained from the AmeriFlux US-SRM Santa Rita Mesquite station in Tucson (Scott, n.d.).

e) Pesticide detections

In the Tucson experiment, honey samples and wax samples were analysed separately but pooled within treatment groups. Residue analyses were performed by the Laboratory Approval and Testing Division of Agricultural Marketing Service, USDA (LATD), Gastonia, North Carolina USA. A full panel of analyses (192 compounds) was conducted on material collected pre-treatment; thereafter, samples were targeted for thymol and neonicotinoid residues.

In Sydney, 50 bees from each colony were collected on three occasions during the hive evaluations, in February, March and April, by shaking them into a 50 mL plastic tube from one of the inner frames. Certified analytical multi-residue reference standards (LC Multi-Residue Pesticide standard mix #4 and #5), containing organonitrogen compounds, were purchased from Restek (PA, USA). Stock standards for use in calibration and determination of recovery were made up in acetonitrile (ACN) with 1% acetic acid. All solvents used were HPLC grade. Extraction materials magnesium sulfate ($MgSO_4$), Sodium acetate (NaOAc) were purchased from Sigma Aldrich (Canada) and the commercial Q-sep® QuEChERS dSPE mix, containing 1200 mg $MgSO_4$ + 400 mg primary secondary amine (PSA) + 400 mg C18 + 400 mg graphitized carbon black (GCB) from Restek (PA, USA). Samples were extracted using a modified QuEChERS method (Abdel-Ghany et al., 2016; Gbylik-Sikorska et al., 2015). Ten honey bees per hive (average weight in g +/- standard deviation: 1.4 ± 0.15) were homogenised in 4 mL in acetonitrile +1% acetic acid using a tissue lyzer (Qiagen, Germany) at 20 hz for 3 minutes. Homogenised samples were transferred to 10 mL falcon tubes and centrifuged at 4500 rpm for 5 minutes, 2 mL of the supernatant was transferred to a new 10 mL falcon tube containing 800 mg of a 4:1 ratio mix of $MgSO_4$ + NaOAc and shaken and vortexed to ensure all moisture was removed. Samples were then centrifuged at 4500 rpm for 5 minutes and 1 mL of the supernatant was transferred into an Eppendorf tube containing 300 mg of QuEChERs

mix. Samples were vortexed until the solution turned clear, then centrifuged at 16,000 x G for 1 minute. The supernatant was passed through a syringe filter (4 mm, 0.20 µm low protein binding hydrophilic LCR (PTFE) membrane; Millex, Japan) to clean 2 ml LCMS glass vials (Thermo Fisher Scientific, MA, USA). Analytes were separated by HPLC (Agilent 1290 Infinity LC System) with a Hypersil GOLDTM aQ C18 HPLC column (100 x 2.1 mm, 1.9 µm particle size) (Thermo Fisher Scientific, MA, USA), and detected by MS/MS (Agilent 6490 Triple quadrapole equipped with iFunnel) operating in MRM in both positive and negative mode with electrospray source. Pesticide residues (Imidacloprid, Thiamethoxam, Clothianidin, Acetamiprid, Thiacloprid, Dinotefuran and Fipronil) were quantified by comparison to a 7 points calibration curve (0.05, 0.2, 0.5, 1.0, 5.0, 10.0 and 25.0 ng/mL), using retention time and multiple daughter ions.

f) Statistical analyses

Data from the two locations were analyzed separately because of obvious differences in the environments, including the presence/absence of parasites such as *V. destructor* (present in Tucson but absent from Sydney) or small hive beetles (present in Sydney and rare in Tucson).

All statistical analyses were conducted under R v3.4.3 (R Core Team, 2018). We tested the impact of thymol, imidacloprid and of their interaction by building an ANCOVA for each of the dependent variables using the lmer function of the lme4 v1.1-14 package (Bates et al., 2015). We used the individual measures of the number of bees, number of pollen or capped brood cells and food weight on the evaluation days as dependent variables, their respective value at the hive evaluation before the beginning of the treatment as a covariate, and treatment (control, imidacloprid, thymol, imidacloprid + thymol), evaluation number (equivalent to a

time interval of about four weeks) and their interactions as categorical independent variables. Colony mortality between treatment groups was analyzed using a chi-squared test for Tucson only. Because of the limited sample size in our experiment, the absence of statistically significant effects should not be taken for evidence that a particular treatment has no impact on honey bee colonies. Post-hoc contrasts were obtained using the 'emmeans' function of the package emmeans v. 1.3.0 (Lenth et al., 2018) and exact p-values are reported following recommendations from (Moran, 2003).

Mite fall before treatment were analyzed using an ANOVA and then used as a covariate to analyze the differences in daily mite fall between groups after treatment in a separate ANOVA. Daily mite fall were added to the constant 1 (to allow the log transformation of 0 values present in our dataset) before being log transformed to meet the assumptions of normality and homoscedasticity.

The daily weight loss at dawn, correlated to the number of foragers departing the hives in the morning (Holst and Meikle, 2018; Meikle et al., 2018), was automatically measured as the difference in mass between the moment at which foragers started departing, and the hive to lose weight, and the moment where the hive starts to gain weight because of returning foragers. This was done by fitting a linear piecewise regression to the daily weight measurements of each hive using R and the segmented package v. 0.5 – 3.0 (Muggeo, 2008), as described in Holst and Meikle (2018) and Meikle et al. (2018). In some cases, in particular when the weight measures were disturbed by a power outage, beekeeping operations, rainfall, falling branches and wildlife landing or climbing on the equipment, the piecewise regression could not converge and the weight loss at dawn could not be estimated (100 iterations, there was no fit in 14.2% of the cases in Sydney and 19.6% in Tucson). The differences in weight losses at dawn between treatment groups were then analyzed using mixed effect generalized additive models with the package mgcv v. 1.8-26 (Wood, 2017) because of strong seasonal variations in the number of

foragers, possibly caused by heavy rains and nectar flows. For each location, the weight loss

in the morning was used as the dependent variable, the date as a smoothed predictor variable,

the treatment as a categorical variable and the hive as a random effect.

Results

a) Treatments

Samples of wax and honey were collected in Tucson and of bees in Sydney to confirm whether the imidacloprid treatment was successful. In Tucson, no traces of imidacloprid were found in either the wax or the honey samples collected before the treatment period, and all groups had low concentrations of thymol, coumaphos and fluvalinate in the wax (*Supplementary Material 4-1*). After treatment, traces of imidacloprid were found in the honey for the imidacloprid and the imidacloprid + thymol groups only, but not in the wax. Thymol was found in the wax in amounts at least 1587 times larger than in the pretreatment samples for the groups treated with thymol as well as in honey, and only traces of thymol were found in the wax and honey of the control and imidacloprid groups (*Supplementary Material 4-1*). In Sydney, only samples of bees collected inside the hive were analyzed. Traces of imidacloprid were detected during the treatment period in bees from 7 of the 8 colonies exposed to imidacloprid (per bee: mean=0.71 ppb, s.d.=0.51 ppb), and 1 of the 8 colonies that was not fed imidacloprid syrup had a low level of imidacloprid (average per bee: 0.22 ppb, *Supplementary Material 4-2*). This hive was kept in the control group as this detection could have resulted from single worker drift or contamination during the manipulation of the samples. In March, only bees from one of the imidacloprid treated hives still contained trace amounts (0.59 ppb per bee) and the rest were below the level of detection of 0.05 ppb (*Supplementary Material 4-2*). No contamination from other neonicotinoids tested (Acetamiprid, Thiacloprid, Clothianidin, Thiomethoxam, Dinotefuran) or fipronil was detected in any samples (data not shown).

b) General colony dynamics

The dynamics of the brood, bees and food stores were consistent at both locations (Figure 4-1). Increases in brood production in Spring were followed by increases in adult bee population at the beginning of the Summer. In Summer (when the bee population was the largest), the foraging effort, indexed as the weight loss at dawn, was higher and honey stores accumulated faster. In Autumn, there was a reduction of the amount of capped brood in the hives and bee populations slowly declined over winter until the next Spring. Increases in amounts of brood were followed by increases in worker numbers at the next evaluation and food stores increased when the worker population remained high while the number of capped brood cells decreased before winter.

Six of twenty-two hives died in Tucson including four before winter, while the hives in Sydney survived during the entire length of the experiment. These differences in colony mortality after treatment between Tucson and Sydney may have resulted from nectar availability, as colonies in Tucson had on average less than half the mass of honey stored by colonies in Sydney (Figure 4-1). The hives in Sydney stored up to 32 kg honey, whilst most colonies in Tucson stored on average <10 kg honey over the experiment. All six colonies that died in Tucson had low food stores and brood (Figure 4-1) at least a month before they died.
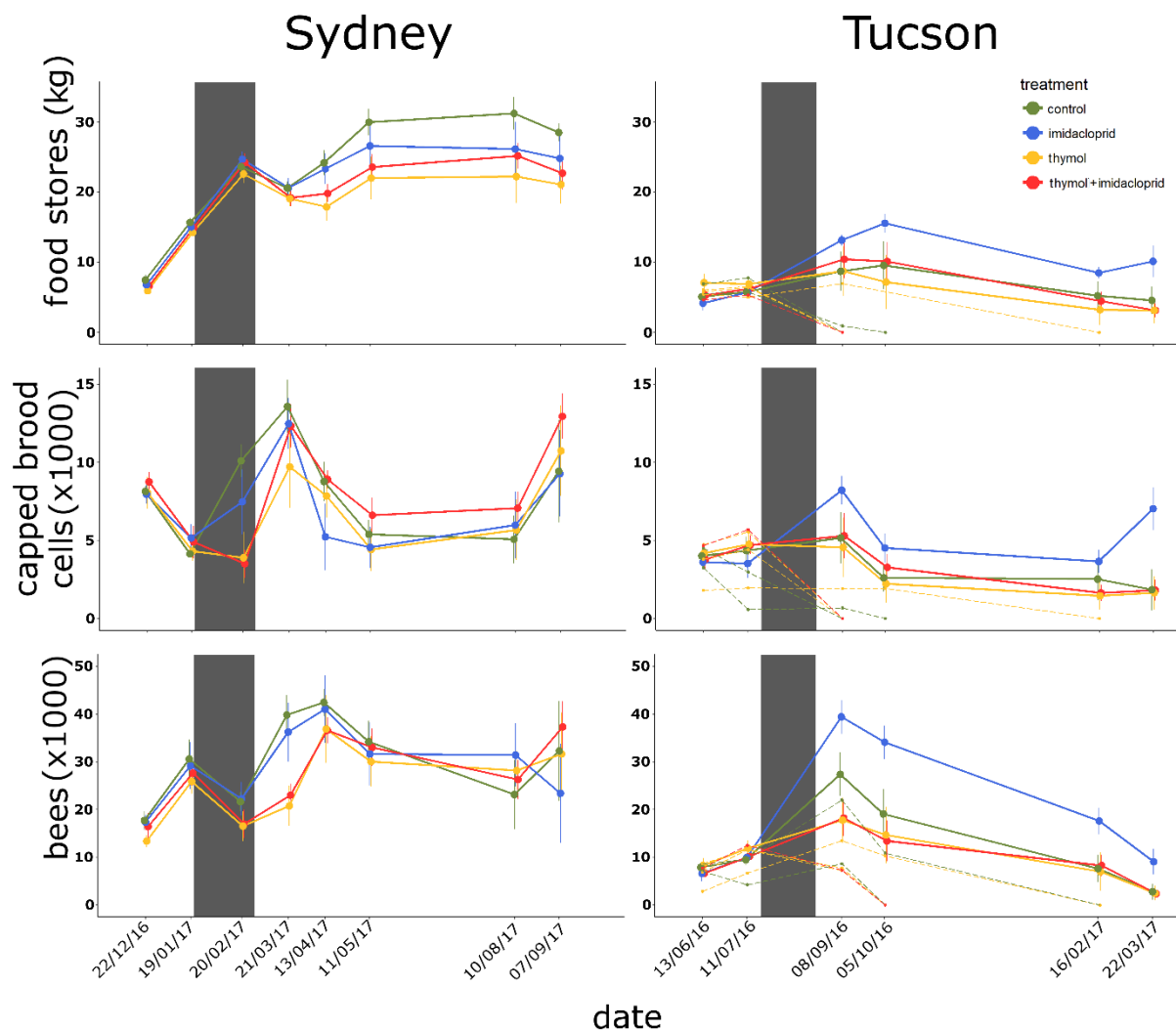
Figure 4-1. **Amounts of brood, food, bees and colony mortality of hives located in Sydney (Australia) and Tucson (USA, Arizona) and treated with thymol, thymol and imidacloprid, imidacloprid or controls. Large dots connected with thick lines represent the number of capped brood cells, bees or the weight of food stores averaged for each group and bars represent the standard deviation. Small dots connected with thin dashed lines represent single colonies that collapsed before the end of the study and that were included as 0 in the statistical analyses. No colonies were lost in Sydney. Evaluations were conducted simultaneously for all groups, but the position of the dots has been slightly altered to ease the reading of the figure. The grey area represents the period of imidacloprid feeding, and/or of exposure to Thymol during the last four weeks. a) Number of capped brood cells over time in Tucson, b) Number of bees over time in Tucson, c) Mass of all stored food (honey and bee bread) over time in Tucson, d) Number of capped brood cells over time in Sydney, e) Number of bees over time in Sydney, f) Mass of all stored food (honey and bee bread) over time in Sydney, g) Number of pollen or bee bread cells over time in Sydney. Detailed pairwise comparisons are given in Supplementary Material 4-3.**

c) Effects of pesticide exposure on bees, brood and food stores

Details of all the pairwise comparisons are reported in Table S3, confidence intervals are shown in Supplementary Material 4-4 for Tucson and Supplementary Material 4-5 or Sydney.

In Tucson, more hives died in the groups treated with thymol (thymol: 3 out of 6; thymol and imidacloprid: 1 out of 5) than in the groups that were not treated with thymol (control: 2 out of 6; imidacloprid: 0 out of 5) groups (Figure 4-1). Although these differences were not significant (Fisher's exact test, p=0.3255), the number of bees was 1.53 times lower in the thymol (mean=17802+/-11530 bees, p=0.0025, df=31, t ratio=3.3) and 1.49 times lower in the combined thymol + imidacloprid groups (mean=18265+/-8477 bees p=0.022, df=32, t ratio=2.4) than in the control group (mean=27383+/-11171 bees) in the first evaluation after treatment (Figure 4-1, Supplementary Material 3-3, Supplementary Material 4-4). This suggests thymol weakened the hives and reduced the amounts of brood and food. Thymol had a similar effect in Sydney where the amount of brood was significantly lower in hives treated with thymol (mean=3893+/-3300 capped brood cells, p=0.02, df=32, t ratio=2.44) and thymol and imidacloprid (mean=3518+/-1814 capped brood cells, p=0.01, df=31, t ratio=2.66) than in the control group (mean=10102+/-2117 capped brood cells) during treatment (Figure 4-1, Supplementary Material 4-3, Supplementary Material 4-5). The number of bees in the hives treated with thymol (mean=20788+/-8501 bees, p=0.039, df=45, t ratio=2.12) and combined thymol and imidacloprid (mean=23044+/-4819, p=0.05, df=46, t ratio=2.02) was subsequently significantly lower in the next evaluation than in the control group (mean=39843+/-8342 bees) (Figure 4-1, Supplementary Material 4-3, Supplementary Material 4-5). and this affected food stores until the end of the experiment (Figure 4-1, Supplementary Material 4-3, Supplementary Material 4-5).

The combined treatment affected the hives similarly to the thymol treatment, suggesting that thymol may have driven the decline in brood, bees and food in this group. Interestingly, the combined treatment group had on average more brood, bees and honey than the thymol group at both locations (

Table 4-3, Supplementary Material 4-3, Supplementary Material 4-4, Supplementary Material 4-5), suggesting an additive effect but no synergistic interactions between the two treatments.

Table 4-3. **Summary of the p-values of the comparisons between treatment groups for the amounts of bees, brood and stored food in the hives at the evaluations in Sydney (A) and near Tucson (B) during and following the exposure to pesticides. Trends (p<0.1) and significant results (p<0.05) are shown in bold.**

A)

	evaluation 3			evaluation 4			evaluation 5			evaluation 6			evaluation 7			evaluation 8		
	brood	bees	honey	brood	bees	honey	brood	bees	honey	brood	bees	honey	brood	bees	honey	brood	bees	honey
thymol	**0.020**	0.838	0.993	0.137	**0.039**	0.895	0.700	0.788	0.133	0.688	0.937	**0.052**	0.840	0.241	**0.028**	0.632	0.689	**0.071**
imidacloprid	0.251	0.813	0.635	0.561	0.728	0.878	0.139	0.962	0.902	0.628	0.839	0.373	0.861	0.199	0.164	0.820	0.281	0.318
Imidacloprid + thymol	**0.012**	0.739	0.715	0.55	**0.049**	0.797	0.94	0.621	0.249	0.736	0.885	**0.083**	0.529	0.457	0.102	0.231	0.319	0.116

B)

	evaluation 3			evaluation 4			evaluation 5			evaluation 6		
	brood	bees	honey	brood	bees	honey	brood	bees	honey	brood	bees	honey
thymol	0.564	**0.002**	0.77	0.673	**0.045**	0.327	0.382	0.236	0.389	0.759	0.265	0.479
imidacloprid	**0.031**	**0.014**	0.213	0.133	**0.002**	**0.098**	0.301	**0.042**	0.354	**0.001**	0.216	0.123
Imidacloprid + thymol	0.946	**0.022**	0.716	0.786	0.121	0.980	0.483	0.886	0.736	0.862	0.698	0.594

In Tucson, the effect of imidacloprid was markedly different. The weight of honey stores and the number of capped brood cells and workers in the imidacloprid group (mean=13141+/-1672kg, mean=8215+/-2012 capped brood cells, mean=39413+/-7949 bees) were 1.51, 1.59 and 1.44 times higher than in the control group (mean=8714+/-6763 kg, mean=5170+/-3995, capped brood cells, mean=27383+/-11171 bees) in the first evaluation after treatment, and these differences persisted until the next spring (Supplementary Material 4-3, Supplementary Material 4-4, Figure 4-1,

Table 4-3). In Sydney, there was no significant effect of the imidacloprid treatment (

Table 4-3, Supplementary Material 4-3, Supplementary Material 4-5), although imidacloprid hives had 1.35 times less brood (mean=7479+/-4134 capped brood cells) than the control group (mean=10102+/-2117 capped brood cells) during treatment (p=0.25, df=30, t ratio=1.17, Figure 4-1). Similar trends were observed for the number of bees shortly after imidacloprid exposure and for honey stores for the rest of the season (Figure 4-1,

Table 4-3). The sample size used in this study may not have allowed us to detect effects of this magnitude (

Table 4-3, Supplementary Material 4-3, Supplementary Material 4-5) .

d) Mite fall

Mite falls were measured in Tucson only, as *V. destructor* is absent from Australia, using sticky boards placed at the bottom of the hive. There were no significant differences in mite falls before treatment (overall: p=0.37, df=3, F=1.11). Hives treated with thymol or thymol + imidacloprid had significantly less mite falls than control hives on the first (average daily mites fall per day +/- sd; control: 1.85+/-0.8; thymol: 0.19+/-0.2, p=0.00025, df=44, t ratio=3.21; thymol+imidacloprid: 0.08+/-0.1, p=0.0003, df=44, t ratio=3.90), second (control: 2.6+/-3.1, thymol: 0.25+/-0.2, p=0.0053, df=44, t ratio=2.93, thymol+imidacloprid: 0.5+/-0.3, p=0.027, df=44, t ratio=2.29) and third (control: 3.38+/=3.4; thymol=0.3+/-0.1, p=0.0026, df= 44, t ratio=3.19; thymol+imidacloprid: 0.56+/-0.4, p=0.0046, df=44, t ratio=2.98) evaluations after treatment, showing that thymol reduced mite populations on the long term. Mite falls were not significantly different between the control and the imidacloprid treatment for any of the evaluations after treatment (evaluation 1: 1.17+/-0.8, p=0.22, df=44, t ratio=1.25; evaluation 2: 1.44+/-0.7, p=0.56, df=44, t ratio=0.6; evaluation 3: 1.2+/-0.4, p=0.08, df=44, t ratio= 1.78, Figure 4-2).
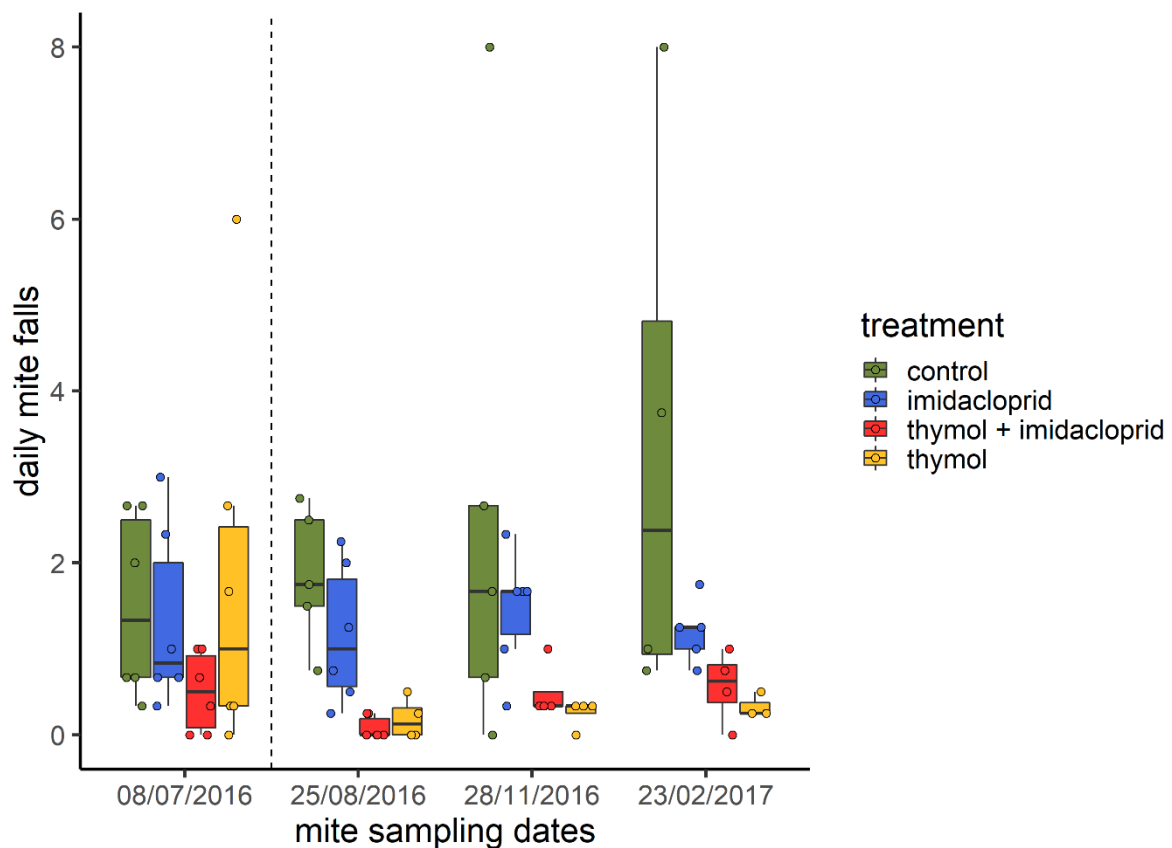
Figure 4-2. **Daily mite falls per hive in Tucson averaged over 4 days. Data on the left side of the dashed line (08/07/2016) were taken before the thymol treatment began. Aluminium trays containing thymol were installed the first week of August for two weeks and then replaced once for another two weeks. On the 25/08/2016, the second thymol trays were removed from the hives. No further treatment was applied. There were no differences in mite falls before treatment. Imidacloprid had no effect on mites falls after treatment. Thymol treated hives had significantly fewer mites falling after treatment.**

e) Temperature regulation

Nest temperature was automatically measured every half an hour using temperature sensors located at the center of the nest just above the brood areas to detect potential effects of pesticides on nest thermoregulation (Figure 4-3, Supplementary Material 4-6) and environmental rainfall and temperature data obtained from nearby weather stations (Figure 4-4). There were no statistically significant effects of the treatments on daily average temperature or daily temperature variations in Sydney (post-hoc contrasts are given in Figure 4-3, Supplementary Material 4-6). In Tucson, significant differences in daily average temperatures and daily temperature variations were observed between the imidacloprid and the thymol group and between the imidacloprid and the combined treatment group during the two weeks following the end of the treatment (Supplementary Material 4-6). These differences seemed to be driven by dying colonies in the thymol and combined treatment groups. After these colonies died and were removed from the assay, differences were no longer statistically significant until the winter. From the beginning of February to the end of March, hives in the imidacloprid group were on average warmer and had lower daily temperature variations than hives in the other groups (Supplementary Material 4-6).
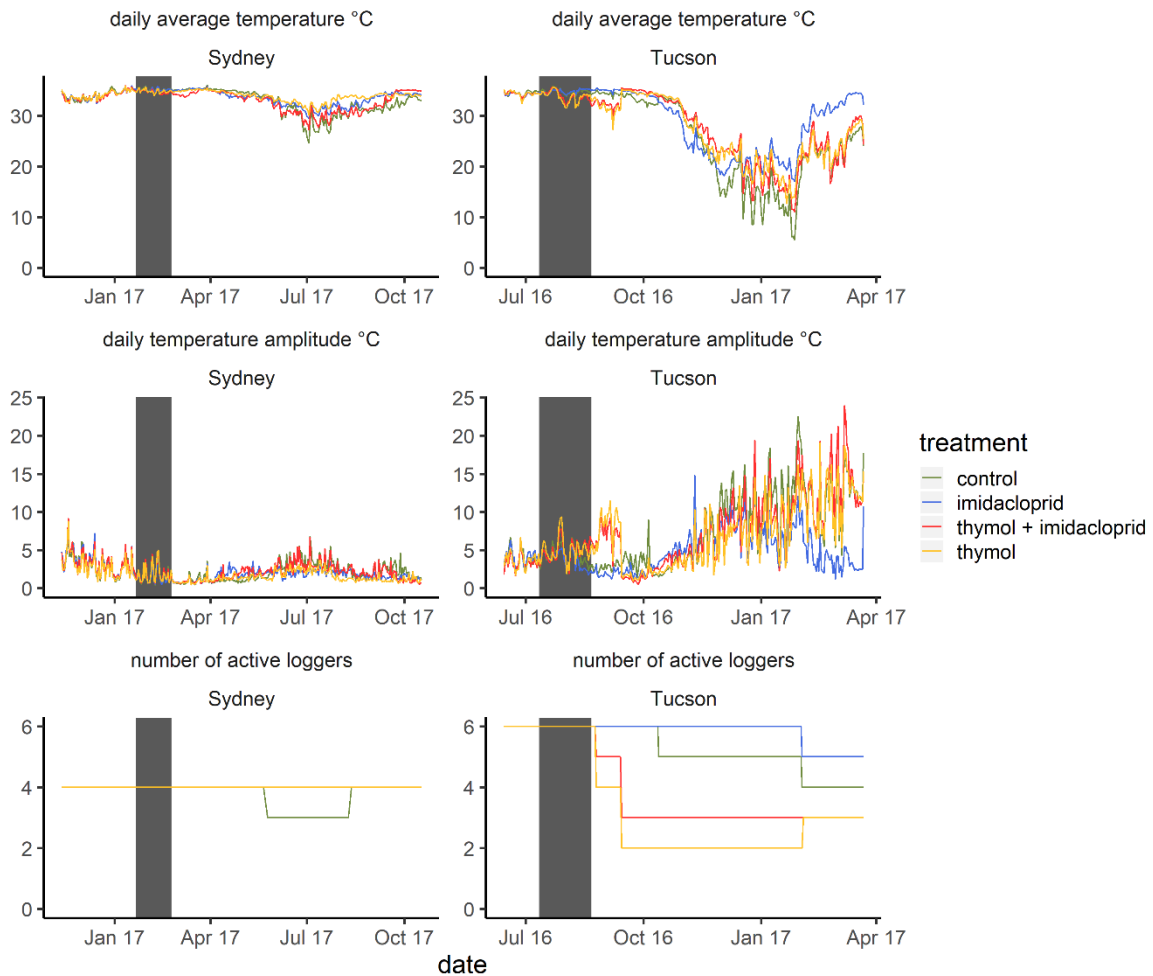
Figure 4-3. **Daily average temperature and temperature fluctuations measured above the brood, between the central frames, inside the hives located in Sydney and Tucson in °C. The two sites had clear differences in in-hive average temperatures during winter, colonies in all the treatment groups in Tucson cooled down to temperatures inferior to 20°C while on average all the hives stayed above 24°C in Sydney. The grey area represents the period of imidacloprid feeding (six weeks), and/or of exposure to thymol (during the last four weeks). There were no clear differences between groups in Sydney but in Tucson the temperature in the hives treated with imidacloprid performed better in late winter and spring. The number of active loggers is shown at the bottom, reductions in the number of loggers were due to logger failures or colony deaths.**
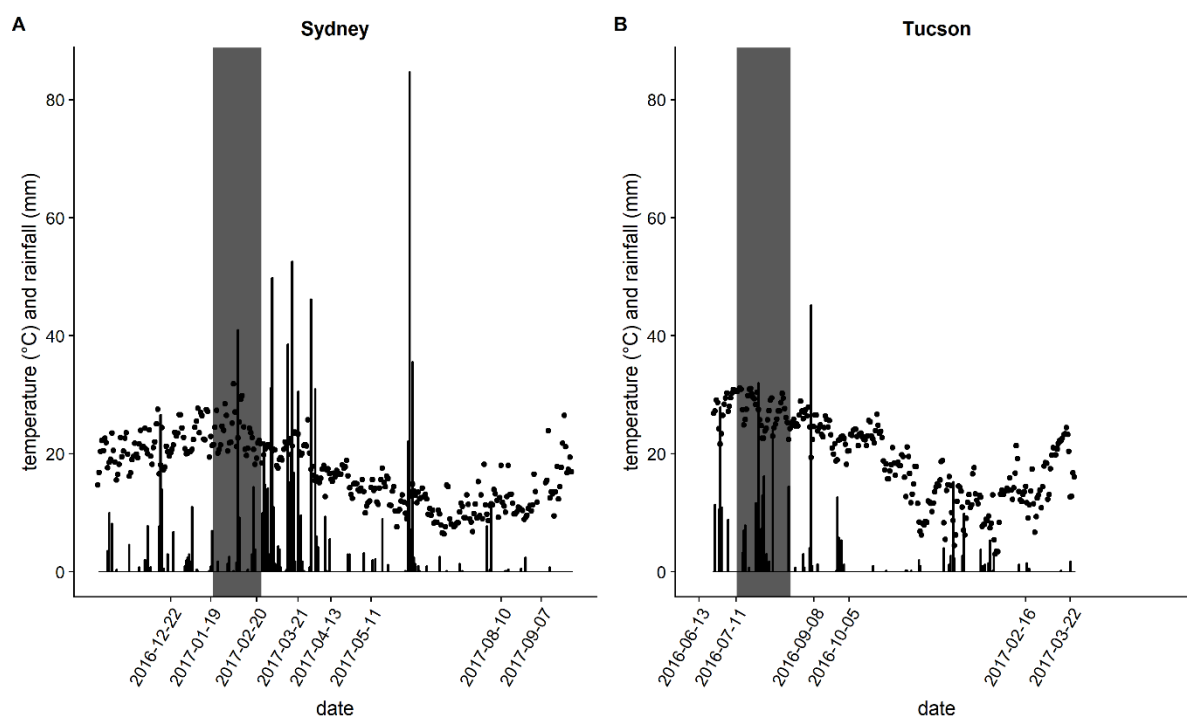
Figure 4-4. **Average daily ambient temperatures and rainfall measured outside the hives from stations close to the Sydney and Tucson apiaries. The grey area represents the period of imidacloprid feeding (six weeks), and/or of exposure to thymol (during the last four weeks). Tucson is drier than Sydney and the daily average temperatures are higher during summer and colder during winter.**

g) Foraging effort

The change in mass at dawn, when more bees are departing than returning from the hive (Holst and Meikle, 2018; Meikle et al., 2018), was monitored throughout both experiments. We found no statistically significant differences in the weight loss at dawn between the control and the treatment groups in Sydney (GAMM; control – imidacloprid, estimate=0.005349, se=0.007548, t=0.709, p=0.479; control – thymol, estimate=0.011594, se=0.007594, t=1.527, p=0.127; control – thymol + imidacloprid, estimate=-0.011202, se=0.007747 , t=-1.446, p=0.148; Fig. 5) but in Tucson, hives in the imidacloprid group lost significantly more weight in the morning than hives in the control group (control – imidacloprid, estimate=0.013996, se=0.005982, t=2.340, p=0.0194) and hives in the thymol and combined treatment group lost significantly less weight in the morning than hives in the control group (control – thymol, estimate=-0.031302, se=0.006382, t=-4.905, p=9.95e-07; control – thymol + imidacloprid, estimate=-0.030014, se=0.006316, t=-4.752, p=2.13e-06; Figure 4-5).
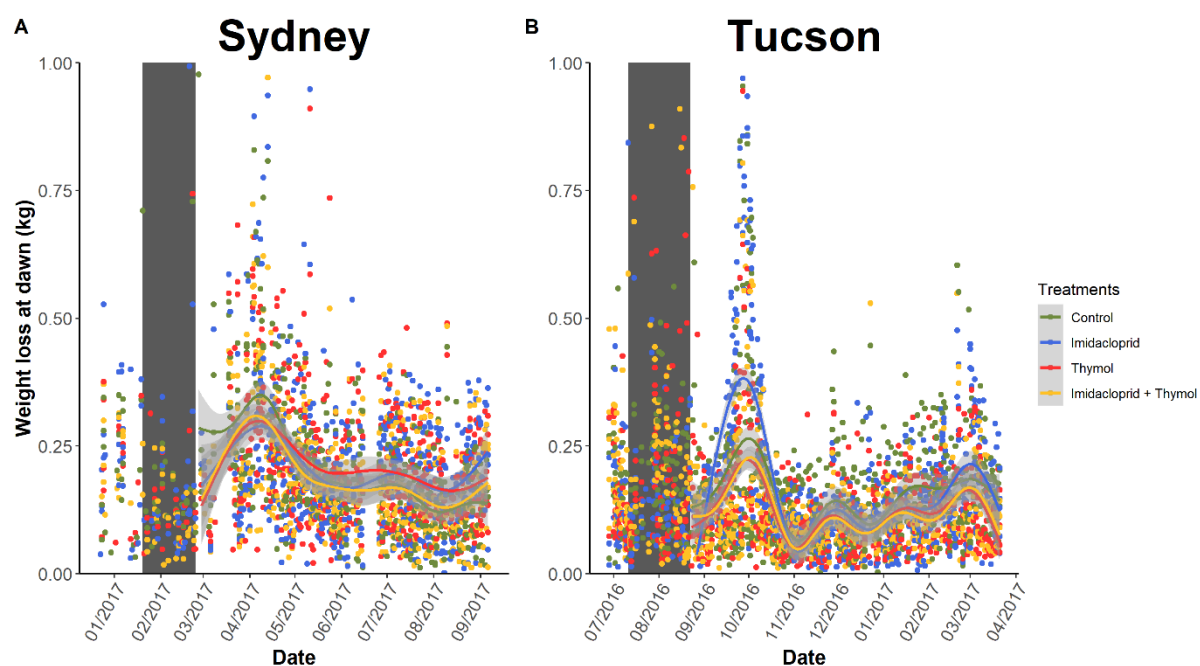
Figure 4-5. **Weight loss at dawn as a measure of the daily foraging force for each hives in the control (green), thymol (yellow), imidacloprid (blue) and combined (red) groups. The grey area represents the period of imidacloprid feeding (six weeks), and/or of exposure to thymol (during the last four weeks). Elevated values indicate periods of intense foraging probably triggered by nectar flows.**

Discussion

Here, we present a detailed dataset of the long-term dynamics of European honey bee colonies. Clear annual cycles in population dynamics were apparent. Changes in brood number were followed by proportional changes in bee numbers within a month. Similarly, increases in the number of bees were followed by increases in food stores within two months. These delays likely reflect the larval developmental time (21 days on average) and the age polyphenism of worker bees, which typically begin foraging when two weeks old as adults (Barron et al., 2002; Barron and Robinson, 2005; Capaldi et al., 2000; Schulz et al., 2002). The effect of stressors on brood should thus be expected to have consequences on bee populations with a delay of about two to three weeks and on honey stores with a delay of at least three to four weeks.

These dynamics differed between the locations studied. Colonies in Sydney accumulated honey stores much more rapidly than the colonies in Tucson in the first three months following their establishment. Interestingly, all the colonies that died during the experiment, six out of thirty-eight studied, had very low food stores and were all located in Tucson. Despite their low food stores, these colonies maintained their brood and bee populations until at least one evaluation before they died. These results suggest that food stress drove the colony losses at Tucson.

The effects of the exposure to the chemical treatments also varied between hive's environments (Figure 4-1, see differences in rainfall and average temperatures in Figure 4-4). In Sydney, the imidacloprid treatment was not found to have any statistically significant effects on the populations of bees and capped brood and only a limited negative impact on the honey stores (Figure 4-1), but this could be due to the limited sample size in our study. However, in Tucson, the imidacloprid treatment was associated with significant increases in brood, honey,

workers, weight losses at dawn and better temperature regulation in Winter (Figure 4-1, Figure 4-3, Figure 4-5).

It is not clear why the effects of imidacloprid differed so much between our two locations. Obvious differences between the two environments were the occurrence of the parasite *V. destructor* and nectar availability. We found no effect of imidacloprid on mite fall indicating that our imidacloprid treatment was not reducing *V. destructor* loads and therefore this is not a likely reason for the positive effect of the imidacloprid treatment at Tucson. Two previously described mechanisms however indicate that neonicotinoids can interact with nectar availability. It has been suggested that honey bees and bumblebees could express biphasic responses to neonicotinoid exposures, a phenomenon termed hormesis (Cutler and Rix, 2015; Potts et al., 2018). Neonicotinoids could stimulate certain biological function of the bees, such has thermogenesis (in bumblebees, Potts et al., 2018), which might explain why the colonies exposed to imidacloprid performed better than control colonies in Tucson. Hormesis is dose-dependent by definition and the amount of neonicotinoids bees are exposed to depends on the concentration in the contaminated nectar from crops, or in our case syrup, and on the ratio of contaminated nectar from crops to uncontaminated nectar from other plants. A positive effect of imidacloprid may thus only be observed at 5 µg/kg when the treatment syrup is not diluted in the nectar of other plants, like in Tucson, but not if the syrup is further diluted among the nectar of wild flowers as it likely was in Sydney.

Stressors often stimulate bee workers to start foraging earlier in their life (Perry et al., 2015; Woyciechowski and Moroń, 2009). An early onset of foraging caused by trace amounts of imidacloprid could have different consequences in terms of brood, bee and honey production depending on the nectar resources in the environment. Specifically, in a food poor environment where bees only raise limited amounts of brood (such as Tucson), colonies could benefit from early foraging because more bees are required to forage than to take care of the brood. In a

food abundant environment where colonies have large food stores and brood amounts (such as Sydney), a colony may receive no benefit from additional foraging since maximal food intake could be limited by space and processing time within the colony. By committing excess resource to foraging the colony could be exposed to increased forager mortality and reduced brood care for no net gain. Our study is not to the first to find neonicotinoids having both positive and negative effects on bee colonies in different environments (see William G. Meikle et al., 2016; Osterman et al., 2019; Woodcock et al., 2017). Clearly the impacts of neonicotinoids on hives are complex, and need further study.

Thymol had a negative impact on the colonies in the short term, but its effect on the long term differed between the two locations (Figure 4-1, Figure 4-3). In Sydney thymol had short-term effects on the brood during treatment (Figure 4-1). As a possible delayed consequence of this effect on brood, bee populations were lower in thymol-treated hives in the first weeks following the treatment (Figure 4-1). Two months after the end of the treatment, as the cohort of workers that grew during treatment was expected to start foraging, honey stores increased more slowly in thymol treated hives than in control hives and remained low until the end of the experiment (Figure 4-1). In Tucson, hives were left undisturbed during the treatment (Supplementary Material 4-7) and the effect of thymol on brood during this period was not measured. In the first month following the end of the treatment bee populations were lower in hives treated with thymol than in the control group (Figure 4-1). Unlike Sydney, there was no long-term effect of the thymol exposure on the amount of honey stored. This could reflect that in Tucson hives benefited from a reduction in parasite load (Figure 4-2) possibly increasing colony-level metabolic efficiency. We note, however, that four out of the six colonies that died in Tucson had been treated with thymol. It was previously suggested that thymol treatments may not always benefit the colonies (Beyer et al., 2018a), possibly by directly affecting or causing the removal of healthy brood (Colin et al., 2019) and our results suggest that a

reevaluation of the benefits of miticide treatments at the level of the colony could benefit the beekeeping industry.

Here, we have reported precise continuous or monthly measurements of the temperature, mite fall, foraging intensity, capped brood cells and worker numbers, and honey mass from thirty-eight hives distributed in four treatment groups and two environments. These data could be used to improve models short and long-term of honey bee colony function. The data available to date on the dynamics of bee colonies were often averaged across a few colonies (Winston, 1987), which has prevented the use of stochastic approaches. To overcome this, we have here provided detailed data for thirty-eight colonies. Similarly, few models have attempted to include abiotic parameters because these data have never been published along with the dynamics of bee colonies. We have reported rainfall and average daily temperatures from two environments. This is a first step to understand how the environment influences the dynamics of honey bee colonies but more studies at the colony level are still needed to answer these questions over the wide range of climates in which the European honey bee is found.

Conclusions

Together, our results suggest that the effects of pesticides and miticides on honey bee colonies can be strongly influenced by the environment through interactions with the function of the colony. Ecotoxicological assessments should be done across a relevant range of environmental conditions before ruling out deleterious effects of pesticides. Colony-level studies are crucial to improve our understanding of the population and food store dynamics of honey bee colonies and help us grasp the long-term effects of stressors on honey bee health.

Acknowledgments

Supplementary Material

Supplementary data to this chapter can be found online at

Supplementary Material 4-1S1 Table. **Concentration of thymol and imidacloprid and detection of other agrochemicals in samples of wax and honey from the hives in the Santa Rita Experimental Range apiary near Tucson.**

Supplementary Material 4-2S2 Table. **Concentration of imidacloprid in samples of bees from the hives located in the Sydney apiary.**

Supplementary Material 4-3S3 Table. **Post-hoc contrasts of the mass of food and amounts of bees and capped brood in hives between treatment groups at both locations.**

Supplementary Material 4-4S4 Figure. **Confidence intervals for the comparisons of the mass of food and amounts of bees and capped brood in hives between treatment groups for the Santa Rita Experimental Range apiary near Tucson.**

Supplementary Material 4-5S5 Figure. **Confidence intervals for the comparisons of the mass of food and amounts of bees and capped brood in hives between treatment groups for the apiary in Sydney.**

Supplementary Material 4-6S6 Table. **Post-hoc contrasts of the temperature amplitudes and average temperatures of the hives at both locations.**

Supplementary Material 4-7S7 Table. **Raw amounts of honey, capped brood and bees measured at each hive evaluation at both locations.**

Supplementary Material 4-8S8 Table. **Raw weight measures of the hives at both locations.**

Supplementary Material 4-9S9 Table. **Mite fall counts in the Santa Rita Experimental Range apiary near Tucson**

Supplementary Material 4-10S10 Table. **Raw temperatures in the hives at both locations.**

Supplementary Material 4-11S11 Table. **Raw temperatures and rainfalls from weather stations close to the study sites.**

References

Abdel-Ghany, M. F. *et al.* (2016) 'Simultaneous determination of eight neonicotinoid insecticide residues and two primary metabolites in cucumbers and soil by liquid chromatography–tandem mass spectrometry coupled with QuEChERS', *Journal of Chromatography B*. Elsevier B.V., 1031, pp. 15–28. doi: 10.1016/j.jchromb.2016.06.020.

Alburaki, M. *et al.* (2018) 'Honey bee survival and pathogen prevalence: from the perspective of landscape and exposure to pesticides', *Insects*, 9(2), pp. 1–15. doi: 10.3390/insects9020065.

Allen, M. D. and Jeffree, E. P. (1956) 'The influence of stored pollen and of colony size on the brood rearing of honeybees', *Annals of Applied Biology*, 44(4), pp. 649–656. doi: 10.1111/j.1744-7348.1956.tb02164.x.

Barron, A. B. (2015) 'Death of the bee hive: understanding the failure of an insect society', *Current Opinion in Insect Science*. Elsevier Inc, 10(Ccd), pp. 45–50. doi: 10.1016/j.cois.2015.04.004.

Barron, A. B. and Robinson, G. E. (2005) 'Selective modulation of task performance by octopamine in honey bee (*Apis mellifera*) division of labour', *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 191(7), pp. 659–668. doi: 10.1007/s00359-005-0619-7.

Barron, A. B., Schulz, D. J. and Robinson, G. E. (2002) 'Octopamine modulates responsiveness to foraging-related stimuli in honey bees (*Apis mellifera*)', *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 188(8), pp. 603–610. doi: 10.1007/s00359-002-0335-5.

Bates, D. *et al.* (2015) 'Fitting Linear Mixed-Effects Models Using lme4', *Journal of Statistical Software*, 67(1). doi: 10.18637/jss.v067.i01.

Becher, M. A. *et al.* (2014) 'BEEHAVE: a systems model of honeybee colony dynamics and foraging to explore multifactorial causes of colony failure', *Journal of Applied Ecology*. Edited by E. Morgan, 51(2), pp. 470–482. doi: 10.1111/1365-2664.12222.

Betti, M. I., Wahl, L. M. and Zamir, M. (2014) 'Effects of Infection on Honey Bee Population Dynamics: A Model', *PLoS ONE*. Edited by O. Rueppell, 9(10), p. e110237. doi:

10.1371/journal.pone.0110237.

Beyer, M., Lenouvel, A., *et al.* (2018) 'Pesticide residue profiles in bee bread and pollen samples and the survival of honeybee colonies—a case study from Luxembourg', *Environmental Science and Pollution Research*. Environmental Science and Pollution Research, 25(32), pp. 32163–32177. doi: 10.1007/s11356-018-3187-4.

Beyer, M., Junk, J., *et al.* (2018) 'Winter honey bee colony losses, *Varroa destructor* control strategies, and the role of weather conditions: Results from a survey among beekeepers', *Research in Veterinary Science*, 118(November 2017), pp. 52–60. doi: 10.1016/j.rvsc.2018.01.012.

Blacquière, T. *et al.* (2012) 'Neonicotinoids in bees: A review on concentrations, side-effects and risk assessment', *Ecotoxicology*, 21(4), pp. 973–992. doi: 10.1007/s10646-012-0863-x.

Bogdanov, S., Imdorf, A. and Kilchenmann, V. (1998) 'Residues in treatment Bogdanov', *Apidologie*, 29(6), pp. 513–524.

Bonmatin, J. M. *et al.* (2003) 'A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants, and in pollens', *Analytical Chemistry*, 75(9), pp. 2027–2033. doi: 10.1021/ac020600b.

Bonmatin, J. M. *et al.* (2005) 'Quantification of Imidacloprid Uptake in Maize Crops', *Journal of Agricultural and Food Chemistry*, 53(13), pp. 5336–5341. doi: 10.1021/jf0479362.

Calatayud-Vernich, P. *et al.* (2018) 'Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure', *Environmental Pollution*, 241, pp. 106–114. doi: 10.1016/j.envpol.2018.05.062.

Capaldi, E. A. *et al.* (2000) 'Ontogeny of orientation flight in the honeybee revealed by harmonic radar', *Nature*, 403(6769), pp. 537–540. doi: 10.1038/35000564.

Chauzat, M.-P. *et al.* (2006) 'A Survey of Pesticide Residues in Pollen Loads Collected by Honey Bees in France', *Journal of Economic Entomology*, 99(2), pp. 253–262. doi: 10.1603/0022-0493-99.2.253.

Chauzat, M.-P. *et al.* (2009) 'Influence of Pesticide Residues on Honey Bee (Hymenoptera: Apidae) Colony Health in France', *Environmental Entomology*, 38(3), pp. 514–523. doi: 10.1603/022.038.0302.

Colin, T. *et al.* (2018) 'The development of honey bee colonies assessed using a new semi-automated brood counting method: Combcount', *PLoS ONE*, 13(10), pp. 1–14. doi: 10.1371/journal.pone.0205816.

Colin, T. *et al.* (2019) 'Effects of thymol on European honey bee hygienic behaviour', *Apidologie*, 50(2), pp. 141–152. doi: 10.1007/s13592-018-0625-8.

Le Conte, Y. *et al.* (2007) 'Honey bee colonies that have survived *Varroa destructor*', *Apidologie*, 38(6), pp. 566–572. doi: 10.1051/apido:2007040.

Cresswell, J. E. (2011) 'A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees', *Ecotoxicology*, 20(1), pp. 149–157. doi: 10.1007/s10646-010-0566-0.

Cunningham, S. A., FitzGibbon, F. and Heard, T. A. (2002) 'The future of pollinators for Australian agriculture', *Australian Journal of Agricultural Research*, 53(8), p. 893. doi: 10.1071/AR01186.

Cutler, G. C. and Rix, R. R. (2015) 'Can poisons stimulate bees? Appreciating the potential of hormesis in bee-pesticide research', *Pest Management Science*, 71(10), pp. 1368–1370. doi: 10.1002/ps.4042.

DeGrandi-Hoffman, G. *et al.* (1989) 'BEEPOP: A honeybee population dynamics simulation model', *Ecological Modelling*, 45(2), pp. 133–150. doi: 10.1016/0304-3800(89)90088-4.

Dietemann, V. *et al.* (2012) '*Varroa destructor* : research avenues towards sustainable control', *Journal of Apicultural Research*, 51(1), pp. 125–132. doi: 10.3896/IBRA.1.51.1.15.

Fischer, J. *et al.* (2014) 'Neonicotinoids interfere with specific components of navigation in honeybees', *PLoS ONE*, 9(3), pp. 1–10. doi: 10.1371/journal.pone.0091364.

Forfert, N. *et al.* (2017) 'Neonicotinoid pesticides can reduce honeybee colony genetic diversity', *PLoS ONE*, 12(10), pp. 1–14. doi: 10.1371/journal.pone.0186109.

Gbylik-Sikorska, M., Sniegocki, T. and Posyniak, A. (2015) 'Determination of neonicotinoid insecticides and their metabolites in honey bee and honey by liquid chromatography tandem mass spectrometry', *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. Elsevier B.V., 990, pp. 132–140. doi: 10.1016/j.jchromb.2015.03.016.

Henry, M. *et al.* (2012) 'A common pesticide decreases foraging success and survival in honey bees', *Science*, 336(6079), pp. 348–350. doi: 10.1126/science.1215039.

Henry, M. *et al.* (2015) 'Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees', *Proceedings of the Royal Society B: Biological Sciences*, 282(1819), p. 20152110. doi: 10.1098/rspb.2015.2110.

Holst, N. and Meikle, W. (2018) 'Breakfast Canyon Discovered in Honeybee Hive Weight Curves', *Insects*, 9(4), p. 176. doi: 10.3390/insects9040176.

Imdorf, A. *et al.* (1999) 'Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies', *Apidologie*, 30(2–3), pp. 209–228. doi: 10.1051/apido:19990210.

Jeffree, E. P. (1951) 'The swarming period in Wiltshire', *Wiltshire Beekprs. Gaz.*, 74, pp. 2–3.

Johnson, R. M. *et al.* (2013) 'Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*)', *PLoS ONE*. Edited by N. E. Raine, 8(1), p. e54092. doi: 10.1371/journal.pone.0054092.

Kerr, J. T. (2017) 'A cocktail of poisons', *Science*, 356(6345), pp. 1331–1332. doi: 10.1126/science.aan6173.

Khoury, D. S., Barron, A. B. and Myerscough, M. R. (2013) 'Modelling Food and Population Dynamics in Honey Bee Colonies', *PLoS ONE*. Edited by A. G. Dyer, 8(5), p. e59084. doi: 10.1371/journal.pone.0059084.

Khoury, D. S., Myerscough, M. R. and Barron, A. B. (2011) 'A Quantitative Model of Honey Bee Colony Population Dynamics', *PLoS ONE*. Edited by J. A. R. Marshall, 6(4), p. e18491. doi: 10.1371/journal.pone.0018491.

Krischik, V. A., Landmark, A. L. and Heimpel, G. E. (2007) 'Soil-Applied Imidacloprid Is Translocated to Nectar and Kills Nectar-Feeding *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae)', *Environmental Entomology*, 36(5), pp. 1238–1245. doi: 10.1603/0046-225x(2007)36[1238:siittn]2.0.co;2.

Kruitwagen, A. *et al.* (2017) 'Naturally selected honey bee *(Apis mellifera)* colonies resistant to *Varroa destructor* do not groom more intensively', *Journal of Apicultural Research*. Taylor & Francis, 56(4), pp. 354–365. doi: 10.1080/00218839.2017.1329797.

Lenth, R. *et al.* (2018) 'Package "emmeans"', *Mran.Microsoft.Com*, 34(1), p. 126. doi: 10.1080/00031305.1980.10483031

Lu, C., Warchol, K. M. and Callahan, R. A. (2012) 'In situ replication of honey bee colony collapse disorder', *Bulletin of Insectology*, 65(1), pp. 99–106.

Marchetti, S., Barbattini, R. and D'Agaru, M. (1984) 'Comparative effectiveness of treatments used to control *Varroa jacobsoni* Oud.', *Apidologie*, 15(4), pp. 363–378.

de Mattos, I. M., Soares, A. E. E. and Tarpy, D. R. (2017) 'Effects of synthetic acaricides on honey bee grooming behavior against the parasitic *Varroa destructor* mite', *Apidologie*, 48(4), pp. 483–494. doi: 10.1007/s13592-017-0491-9.

Meikle, W. G. *et al.* (2008) 'Within-day variation in continuous hive weight data as a measure of honey bee colony activity', *Apidologie*, 39(6), pp. 694–707. doi: 10.1051/apido:2008055.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLOS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Meikle, W. G. *et al.* (2018) 'Using within-day hive weight changes to measure environmental effects on honey bee colonies', *PLOS ONE*. Edited by A. G. Dyer, 13(5), p. e0197589. doi: 10.1371/journal.pone.0197589.

Meikle, W. G. and Holst, N. (2015) 'Application of continuous monitoring of honeybee colonies', *Apidologie*, 46(1), pp. 10–22. doi: 10.1007/s13592-014-0298-x.

Meikle, W. G., Weiss, M. and Stilwell, A. R. (2016) 'Monitoring colony phenology using within-day variability in continuous weight and temperature of honey bee hives', *Apidologie*, 47(1), pp. 1–14. doi: 10.1007/s13592-015-0370-1.

Mitchell, E. A. D. *et al.* (2017) 'A worldwide survey of neonicotinoids in honey', *Science*, 358(6359), pp. 109–111. doi: 10.1126/science.aan3684.

Moran, M. D. (2003) 'Arguments for rejecting the sequential Bonferroni in ecological studies', *Oikos*, 100(2), pp. 403–405. doi: 10.1034/j.1600-0706.2003.12010.x.

Muggeo, V. R. M. (2008) 'segmented: An R Package to Fit Regression Models with Broken-Line Relationships', *R News*, 3(6), pp. 343–4. doi: 10.1159/000323281.

Mullin, C. A. *et al.* (2010) 'High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health', *PLoS ONE*, 5(3). doi: 10.1371/journal.pone.0009754.

Myerscough, M. R. *et al.* (2017) 'Why Do Hives Die? Using Mathematics to Solve the Problem of Honey Bee Colony Collapse', in Anderssen, B. et al. (eds) *The Role and Importance of Mathematics in Innovation*. Singapore: Springer Singapore, pp. 35–50.

Neumann, P. and Blacquière, T. (2017) 'The Darwin cure for apiculture? Natural selection and managed honeybee health', *Evolutionary Applications*, 10(3), pp. 226–230. doi: 10.1111/eva.12448.

Nolan, W. J. (1925) 'The brood-rearing cycle of the honeybee', *Bull. U.S. Dep. Agric*, 1349, pp. 1–56.

Oddie, M. A. Y., Dahle, B. and Neumann, P. (2017) 'Norwegian honey bees surviving *Varroa destructor* mite infestations by means of natural selection', *PeerJ*, 5, p. e3956. doi: 10.7717/peerj.3956.

Osterman, J. *et al.* (2019) 'Clothianidin seed-treatment has no detectable negative impact on honeybee colonies and their pathogens', *Nature Communications*. Springer US, 10(1), p. 692. doi: 10.1038/s41467-019-08523-4.

Perry, C. J. *et al.* (2015) 'Rapid behavioral maturation accelerates failure of stressed honey bee colonies', *Proceedings of the National Academy of Sciences*, 112(11), pp. 3427–3432. doi: 10.1073/pnas.1610243113.

Pettis, J. S. *et al.* (1991) 'Fluvalinate treatment of queen and worker honey bees (*Apis mellifera*) and effects on subsequent mortality, queen acceptance and supersedure', *Apidologie*, 22(1), pp. 1–7. doi: 10.1051/apido:19910101.

Potts, R. *et al.* (2018) 'The effect of dietary neonicotinoid pesticides on non-flight thermogenesis in worker bumble bees (*Bombus terrestris*)', *Journal of Insect Physiology*, 104(May 2017), pp. 33–39. doi: 10.1016/j.jinsphys.2017.11.006.

Prado, A. *et al.* (2019) 'Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees', *Science of the Total Environment*. Elsevier B.V., 650, pp. 1250–1260. doi: 10.1016/j.scitotenv.2018.09.102.

Premrov Bajuk, B. *et al.* (2017) 'Coumaphos residues in honey, bee brood, and beeswax after

Varroa treatment', *Apidologie*, 48(5), pp. 588–598. doi: 10.1007/s13592-017-0501-y.

R Core Team, R. (2018) 'R: A language and environment for statistical computing. R Foundation for Statistical Computing'. Vienna, Austria. Available at: https://www.r-project.org/.

Retschnig, G. *et al.* (2015) 'Effects, but no interactions, of ubiquitous pesticide and parasite stressors on honey bee (*Apis mellifera*) lifespan and behaviour in a colony environment', *Environmental Microbiology*, 17(11), pp. 4322–4331. doi: 10.1111/1462-2920.12825.

Rundlöf, M. *et al.* (2015) 'Seed coating with a neonicotinoid insecticide negatively affects wild bees', *Nature*, 521(7550), pp. 77–80. doi: 10.1038/nature14420.

Russell, S., Barron, A. B. and Harris, D. (2013) 'Dynamic modelling of honey bee (*Apis mellifera*) colony growth and failure', *Ecological Modelling*. Elsevier B.V., 265, pp. 158–169. doi: 10.1016/j.ecolmodel.2013.06.005.

Samson-Robert, O. *et al.* (2017) 'Planting of neonicotinoid-coated corn raises honey bee mortality and sets back colony development', *PeerJ*, 5, p. e3670. doi: 10.7717/peerj.3670.

Sánchez-Bayo, F. *et al.* (2016) 'Are bee diseases linked to pesticides? — A brief review', *Environment International*. Elsevier B.V., 89–90, pp. 7–11. doi: 10.1016/j.envint.2016.01.009.

Schulz, D. J., Barron, A. B. and Robinson, G. E. (2002) 'A role for octopamine in honey bee division of labor', *Brain, Behavior and Evolution*, 60(6), pp. 350–359. doi: 10.1159/000067788.

Scott, R. (no date) *AmeriFlux US-SRM Santa Rita Mesquite*.

Sgolastra, F. *et al.* (2017) 'Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species', *Pest Management Science*, 73(6), pp. 1236–1243. doi: 10.1002/ps.4449.

Thompson, H. *et al.* (2019) 'Thiamethoxam: Long-term effects following honey bee colony-level exposure and implications for risk assessment', *Science of the Total Environment*. Elsevier B.V., 654, pp. 60–71. doi: 10.1016/j.scitotenv.2018.11.003.

Tihelka, E. (2018) 'Effects of synthetic and organic acaricides on honey bee health: A review', *Slovenian Veterinary Research*, 55(3), pp. 114–140. doi: 10.26873/SVR-422-2017.

Tosi, S., Burgio, G. and Nieh, J. C. (2017) 'A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability', *Scientific Reports*. Springer US, 7(1), pp. 1–8. doi: 10.1038/s41598-017-01361-8.

Tsvetkov, N. *et al.* (2017) 'Chronic exposure to neonicotinoids reduces honey bee health near corn crops', *Science*, 356(6345), pp. 1395–1397. doi: 10.1126/science.aam7470.

Ushitani, T. *et al.* (2016) 'Accelerated behavioural development changes fine-scale search behaviour and spatial memory in honey bees (*Apis mellifera* L).', *The Journal of Experimental Biology*, 219, pp. 412–418. doi: 10.1242/jeb.126920.

Whittington, R. (2000) 'Evaluation of the botanical oils neem, thymol, and canola sprayed to control *Varroa jacobsoni* Oud. (Acari: Varroidae) and *Acarapis woodi* (Acari: Tarsonemidae) in colonies of honey bees (*Apis mellifera* L., Hymenoptera: Apidae).', *American Bee Journal*. Philadelphia, PA :, pp. 567–572.

Winston, M. L. (1987) *The biology of the honey bee*. Harvard University press.

Wood, S. N. (2017) *Generalized Additive Models*. Chapman and Hall/CRC. doi: 10.1201/9781315370279.

Woodcock, B. A. *et al.* (2017) 'Country-specific effects of neonicotinoid pesticides on honey bees and wild bees', *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

Woyciechowski, M. and Moroń, D. (2009) 'Life expectancy and onset of foraging in the honeybee (*Apis mellifera*)', *Insectes Sociaux*, 56(2), pp. 193–201. doi: 10.1007/s00040-009-0012-6.

Wu-Smart, J. and Spivak, M. (2016) 'Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development', *Scientific Reports*. Nature Publishing Group, 6(March), pp. 1–11. doi: 10.1038/srep32108.

Zhu, W. *et al.* (2014) 'Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0077547.

Chapter 5 The miticide thymol in combination with the neonicotinoid imidacloprid reduces visual learning performance in European honey bees

Abstract

Despite growing concerns over the impacts of agricultural pesticides on honey bee health, miticides, a group of pesticides used within hives to kill bee parasites, have received little attention. We know very little about how miticides might affect bee cognition, particularly in interaction with other known stressors, such as crop insecticides. Visual learning is essential for foraging bees to find their way to flowers, recognize them, and fly back to the nest. Using a standardized aversive visual conditioning assay, we tested how field exposure to three pesticides affects visual learning in European honey bees (*Apis mellifera*). Our pesticides were two common miticides, thymol in the commercial formulation Apiguard® and tau-fluvalinate in the formulation Apistan® and one neonicotinoid, imidacloprid. We found no significant effect of miticides alone, nor of field-relevant doses of imidacloprid alone, but bees exposed to both thymol and imidacloprid showed reduced performance in the visual learning assay.

Introduction

There is growing concern that agrochemical stressors of honey bees can harm learning and memory, reducing foraging effectiveness and survival (Klein et al., 2017). In agriculturally intensive environments bees are rarely exposed to a single agrochemical in isolation, and we still have very little information on how agrochemical stressors might interact (Benuszak et al., 2017; Calatayud-Vernich et al., 2018; Johnson et al., 2013; Mullin et al., 2010; O'Neal et al., 2018; Prado et al., 2019; Robinson et al., 2017; Sgolastra et al., 2018; Yao et al., 2018a, 2018b; Zhu et al., 2017). Most research has focused on the impacts of pesticides used on crops on honey bees, but honey bees are also frequently exposed to miticides used within hives to control the parasitic mite *V. destructor*.

Here we assessed whether miticides can affect bee visual learning, and whether effects are increased if miticides co-occur with traces of other pesticides. *V. destructor* is considered perhaps the most damaging pest affecting the beekeeping industry (Guzmán-Novoa et al., 2010; Ratnieks and Carreck, 2010). This mite feeds on larvae and adult bees, which causes malformations, sickness and the transmission of many diseases within and between hives (Boecking and Spivak, 1999; Le Conte et al., 2010; Rosenkranz et al., 2010; Wallner and Fries, 2003). *V. destructor* is considered a significant factor driving a global rise in honey bee colony failure rates (Barron, 2015; Ratnieks and Carreck, 2010). In-hive chemical treatments known as miticides or acaricides are commonly used to control *V. destructor*, but the mite has rapidly evolved resistance to many of these miticides, which has reduced the options for control (Berry et al., 2013; Charpentier et al., 2014; Coffey, 2007; Coffey and Breen, 2013; Faucon et al., 1995; Kanga et al., 2010; Marchetti et al., 1984; Milani, 1999, 1995; Smodiš Škerl et al., 2010). In addition, previous research has demonstrated negative effects of two common miticides, coumaphos and tau-fluvalinate, on honey bee survival (Berry et al., 2013), and queen body

weight and longevity (tau-fluvalinate, Haarmann et al., 2009). Tau-fluvalinate (commercialized as Apistan®) is a synthetic pyrethroid (Davies et al., 2007) acting as an arthropod neuron excitoxin which prevents the closure of voltage-gated sodium channels of axonal membranes, leading to prolonged membrane depolarization and thus to paralysis or death (Ray and Fry, 2006). Tau-fluvalinate suppresses neuron excitability in the adult bee brain structures (Zhou et al., 2011) and impairs olfactory memory (Frost et al., 2013). Perhaps because of concerns related to these side effects there has been an increase in alternative miticide treatments such as essential oils and organic acids (Charpentier et al., 2014; Mondet et al., 2011), which are commonly perceived to be safer for bees (Dietemann et al., 2012).

Thymol is a plant allelochemical commonly used for the control of *V. destructor* (Mondet et al., 2011), particularly the commercial formulation Apiguard® in which it is mixed with a slow-release gel. The mode of action of thymol on mites is not well understood, but it is believed that thymol interferes with GABAergic signalling in the central nervous system (Price and Lummis, 2014). GABA (gamma-Aminobutyric acid) is an important neurotransmitter for both mites and insects, and hence there are legitimate concerns that thymol also affects honey bees. For example, Apilife Var®, a commercial formulation of thymol, eucalyptus oil, camphor, and levomenthol, is known to impair olfactory memory and phototactic response in bees shortly after its introduction in the hive (Alayrangues et al., 2016; Bonnafé et al., 2017; Carayon et al., 2014). In this study, we compared the effects of two miticide treatments: the Apiguard® formulation of thymol and the Apistan® formulation of tau-fluvalinate on honey bee visual learning. In intensive agricultural environments bees can be exposed to pesticides used to protect crop plants against pest insect outbreaks, and over time pesticide contaminants accumulate inside the hive (Alburaki et al., 2018; Mullin et al., 2010; Ravoet et al., 2015).

There is now enormous concern regarding the effects of pesticide contaminants on honey bees, especially neonicotinoid pesticides (Fischer et al., 2014; Forfert et al., 2017; Henry et al., 2012; Klein et al., 2017; Lu et al., 2012; Mitchell et al., 2017; Sánchez-Bayo et al., 2016; Sgolastra et al., 2017; Tosi et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017; Wu-Smart and Spivak, 2016). Because bee hives in agricultural environments may be exposed to both miticides and pesticides, here we also explored whether potential deleterious effects of thymol miticides on visual learning could be increased by simultaneous exposure to trace levels of the neonicotinoid insecticide imidacloprid. Learning of colour is vital for bees to distinguish the features of profitable flowers, and to detect natural enemies. To quantify colour learning performance, we used the Automated Performance Index System (APIS) as an aversive visual conditioning assay (Kirkerud et al., 2013). APIS is a small chamber equipped with coloured LEDs to display fields of colour. The floor of the chamber can be electrified to deliver weak electric shocks paired with specific colours. The position of a bee, moving freely within the chamber is continuously recorded by means of infrared sensors. Tracking the bee's position in the chamber indicates the extent to which a bee has learned to avoid a colour field associated with shock. The assay provides a robust and simple measure of rate and degree of visual learning in honey bees. In our first experiment we compared visual learning performance of bees exposed to commercial miticide formulations of tau-fluvalinate and thymol miticides and controls and found no effects of the miticides on aversive learning or memory. In our second experiment we compared visual learning performance of bees exposed to either thymol, a trace level of imidacloprid, or both substances together and controls.

Material and Methods

a) Hive history

Honey bees were of the standard commercial strain available in Australia, and usually referred to as *Apis mellifera* derived from a Golden Italian strain. All bees were sourced from the Macquarie University Research Apiary. Forager bees were collected when leaving the hive, using a hand-held bee vacuum (BioQuip Products, Inc., CA, USA). Bees were captured no longer than 15 minutes prior to testing and stored for a minimum of 10 minutes in the dark, at approximately 24ºC in 50mL Falcon™ conical centrifuge tubes pierced with a pin for ventilation. Since foragers were selected randomly, information about age and foraging experience is unknown, however these factors are expected to be randomized across treatments.

b) The APIS visual learning assay

The APIS assay chamber was developed and built by the University of Konstanz (Kirkerud et al., 2013). The APIS chamber can be illuminated with light fields varying in wavelength and intensity: blue (λ = 465 nm, luminous intensity: 105mcd) or green (λ = 525 nm, Luminous intensity: 119 mcd). It is equipped with an electrifiable grid to deliver pulses of electric shock (10V, 4Hz, 100ms) to the bee, and with infrared sensors to automatically track the bee's movement. APIS specifications and use are fully described in (Plath et al., 2017). In brief: shock and light delivery were automated by software sensitive to the position of the bee in the chamber. Blue light always illuminated the half of the chamber in which the bee was located at light-onset. Prior to each assay, the chamber was cleaned with 70% ethanol solution and left to air dry to remove traces of alarm pheromones, known to impair learning (Urlacher et al., 2010). Bees were placed in the chamber for 10 minutes in the dark to acclimatize. The bee was then subjected to nine training trials, each separated by 30 s of darkness. The chamber

was illuminated for 14 s during each training trial, which comprised a 3 s non-shock period, followed by 11 s of shock pulses delivered whenever a bee was situated in the blue side of the chamber. Shock pulses stopped whenever the bee was located in the green side of the chamber. Following a 30 s delay, the bee was given a learning test of 14 s of illumination without shocks to determine the post-training response to blue and green light fields. As in the training trials, test trials began with blue light illuminating the half of the chamber in which the bee was located. We ran two experiments (see below). Bee colonies were housed in standard hives commonly referred to as eight-frames two-box "Langstroth" hives of a volume of about 76 L.

The sequence of bees selected from treatment hives was randomized and an equal number of bees from all hives were tested each sampling day. Because the position of the bees is measured by infrared sensors with no input from the observer, this assay can be considered blind. Bees moving at an average speed of less than 2 cm/s were excluded from the analyses: since learning is demonstrated by the movement of the bee, at less than this speed it is difficult to demonstrate learning of the association between shocks and colours. The number of bees tested in each treatment group is summarized in Table 5-1. The sequence of bees selected from treatment hives was randomized and an equal number of bees from all hives were tested each sampling day.

Table 5-1. **Number of hives sampled, number of bees tested and number of bees retained for data analysis after exclusion of abnormally slow bees (average speed less than 2 cm/s). All groups included 30 bees or more.**

	Experiment 1			Experiment 2			
Treatment	Control	Tau-fluvalinate	Thymol	Control	Thymol	Imidacloprid	Thymol+ imidacloprid
Number of hives	2	2	2	4	4	4	4
Number of bees	40	39	34	37	38	33	35
Number of bees after exclusion of the slow bees	36	28	31	31	35	30	30

c) Experiment 1: comparing visual learning performance of bees treated with thymol, tau-fluvalinate and controls Bees were sourced from six colonies.

Two hives were treated with thymol using Apiguard® trays containing 25% thymol weight to weight (12.5 g per tray, Vita Europe Ltd, Basingstoke, UK) and two other hives with tau-fluvalinate using Apistan® strips containing 10.3% of tau-fluvalinate weight to weight (824 mg per strip, Vita Europe Ltd, Basingstoke, UK). The final two hives were control colonies. Both miticides were applied following suppliers' instructions. Two Apistan® plastic strips were placed in each of the tau-fluvalinate treated hives, between two brood frames, for six weeks. One Apiguard® gel container was placed on top of the top-box frames of each of the thymol-treated hives for two weeks. After two weeks, the treatment containers were renewed in each of the two hives for another two weeks. Treatments started on the 1st of February 2016.

Weekly hive inspections were performed to check for the presence of a queen and eggs. We started testing the bees six weeks after the beginning of the treatment, on the 14th of March 2016, in order to test foragers that were exposed to the miticides during their development or early adult life. The development of a bee from the egg to eclosion is about 21 days, and typically it takes two weeks for the newly emerged hive bees to become foragers (Winston, 1991). The experiment lasted for nineteen days (from the 14th of March 2016 to the 1st of April 2016).

d) Experiment 2: comparing visual learning performance of bees treated with thymol and/or imidacloprid.

Bees were sourced from sixteen colonies. Four hives were treated with thymol using Apiguard® (Vita Europe Ltd, Basingstoke, UK), four other hives with imidacloprid Pestanal® (Sigma-Aldrich, CAS # 138261-41-3) and four other hives with a combination of Apiguard® and imidacloprid. The final four hives were control colonies. Apiguard® was applied as in Experiment 1. Imidacloprid was fed to the bees in a 50:50 sugar / water (mass for mass) syrup solution containing 5 µg/kg ppb imidacloprid solution as described in Meikle et al. (2016). Treatment was applied over six consecutive weeks using in-hive frame feeders. This corresponds to the trace amounts of imidacloprid that honey bees are commonly exposed to when they forage on treated crops such as canola (Bonmatin et al., 2005, 2003). Treatments started on the 19th of January 2017. We started testing the bees six weeks after the beginning of the treatment, on the 2nd of March 2017. The experiment lasted for 10 days (2nd of March 2017 to 12th of March 2016).

e) Statistical analyses

Analyses were adapted from (Plath et al., 2017). We used a Performance Index (PI) as a metric showing the learning performance of the bees:

$$PI = \frac{t(green) - t(blue)}{t(green) + t(blue)}$$

Where t(green) is the time spent on the green (safe) side of the chamber, and t(blue) is the time spent on the blue (shocked) side of the chamber. The performance index varies between -1 and 1, where the positive values indicate that the bee spent more time in the safe side that on the shocked side. Negative values indicate the opposite. A bee that has learned the association between the blue light and the shocks is expected to run away from the blue side shortly after light-onset and avoid returning to the blue side, and thus have high performance index values. By contrast a bee that has not learned the association is expected to spend equal amounts of time on each side or more time on the blue side, and thus have low performance index values. All analyses were conducted in R v. 3.5.0 (R Core Team, 2018). The performance index and speed of the bees during the test trial were compared using mixed-effect ANOVAs with the function 'lmer' from the lme4 v. 1.1 package (Bates et al., 2015). Pairwise contrasts were performed with the function 'pairs' from the emmeans v. 1.3.0 package (Lenth et al., 2018). Separate ANOVAs were built for each year, with the performance index or the average speed of the bee during the test trial as the quantitative outcome, the treatment as the categorical explanatory variable, and the colony of origin as a random factor. Pairwise comparisons were obtained between the control group and the other treatment groups. Figures were made with ggplot2 v. 3.1.0 (Wickham, 2016) and cowplot v. 0.9.4 (Wilke, 2019).

Results

a) Experiment 1: comparing visual learning performance of bees treated with thymol, tau-fluvalinate and controls.

Bees in all groups were on average able to reach a positive performance index from the second training trial (Figure 5-1A), indicating that they spent less time in the shock associated colour field than in the non-shocked colour field. Their learning performances did not differ. In the test trial, there was no significant difference in performance between the control group (mean=0.43, se=0.08) and either the tau-fluvalinate or the thymol groups (tau-fluvalinate: mean=0.33, se=0.10, p-value=0.58; thymol: mean=0.17, se=0.11, p-value=0.21; Figure 5-1B). There was also no difference in speed of movement in the assay between the control group (mean=4.20 cm/s, se=0.25 cm/s) and the tau-fluvalinate and thymol group (tau-fluvalinate: mean=3.53 cm/s, se=0.30 cm/s, p-value=0.20; thymol: mean=3.87 cm/s, 232 se=0.321 cm/s, p-value=0.48; Figure 5-1C).

Figure 5-1. **Comparing the visual learning performances of bees treated with thymol, tau-fluvalinate and controls. (A) Performance index of the bees during the nine training trials (mean +/- se). Bees were able to obtain a positive performance index from the second training trial. (B) Performance index during the test trial. (C) Individual speed during the test trial.**

b) Experiment 2: comparing visual learning performance of bees treated with thymol and/or imidacloprid.

Bees in all groups were on average able to reach a positive performance index from the third training trial (Figure 5-2A). In the test trial there was no significant difference in performance between the control group (mean=0.44, se=0.09) and the imidacloprid or the thymol groups (imidacloprid: mean=0.16, se=0.11, p-value=0.145; thymol: mean=0.19, se=0.11, p-value=0.188), but bees in the combined thymol and imidacloprid treatment group had an average performance index that was slightly less than zero and significantly lower than the control group (thymol + imidacloprid: mean=-0.09, se=0.15, p-value=0.016; Figure 5-2B). This suggests that visual learning in this group was impaired, both in absolute terms and relative to the performance of the control group. There were no significant differences in the movement speed between the control group (mean=4.41 cm/s, se=0.48 cm/s) and the imidacloprid, thymol, or combined thymol and imidacloprid treatment groups (imidacloprid: mean=4.2 cm/s, se=0.38 cm/s, p-value=0.754; thymol: mean=4.41 cm/s, se=0.37 cm/s, p-value=0.997; thymol + imidacloprid: mean=3.7 cm/s, se=0.49 cm/s, p- value=0.31; Figure 5-2C), providing no evidence that differences in learning performance index were simply due to differences in ability to move in the assay.
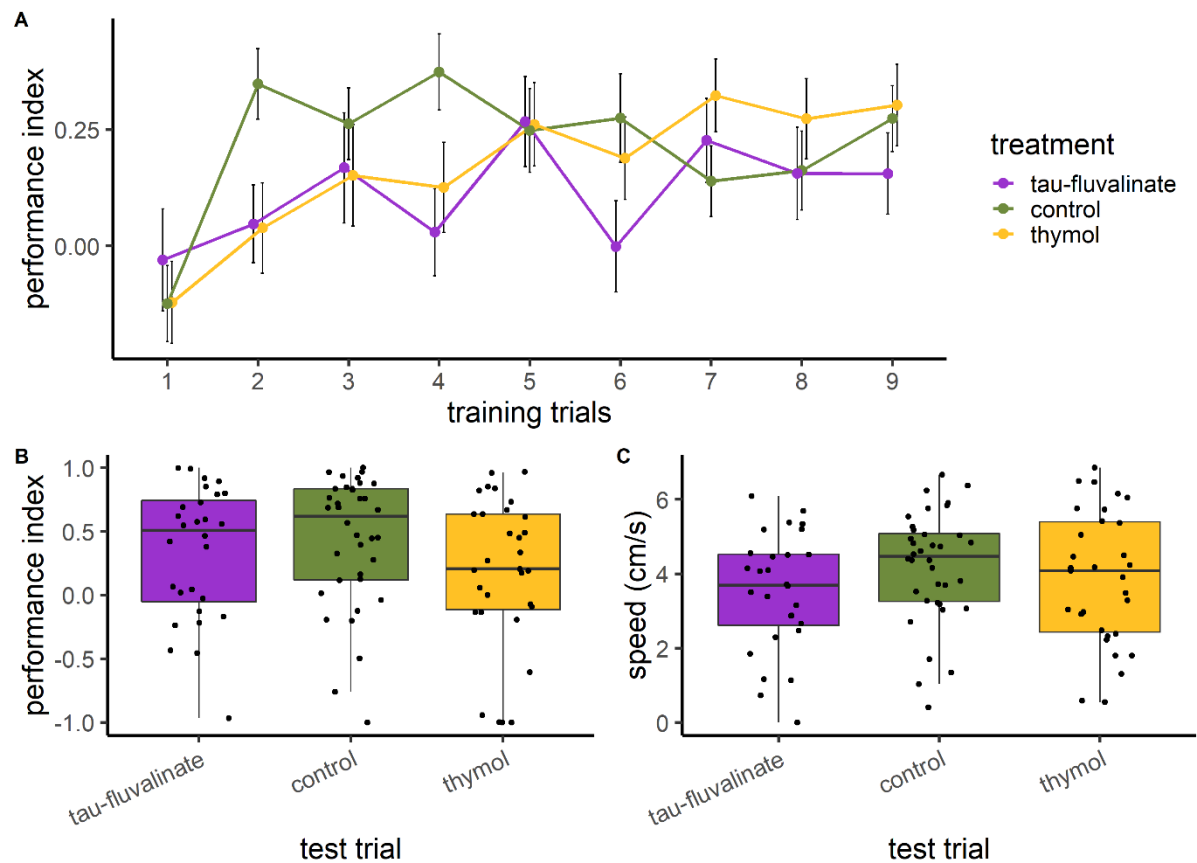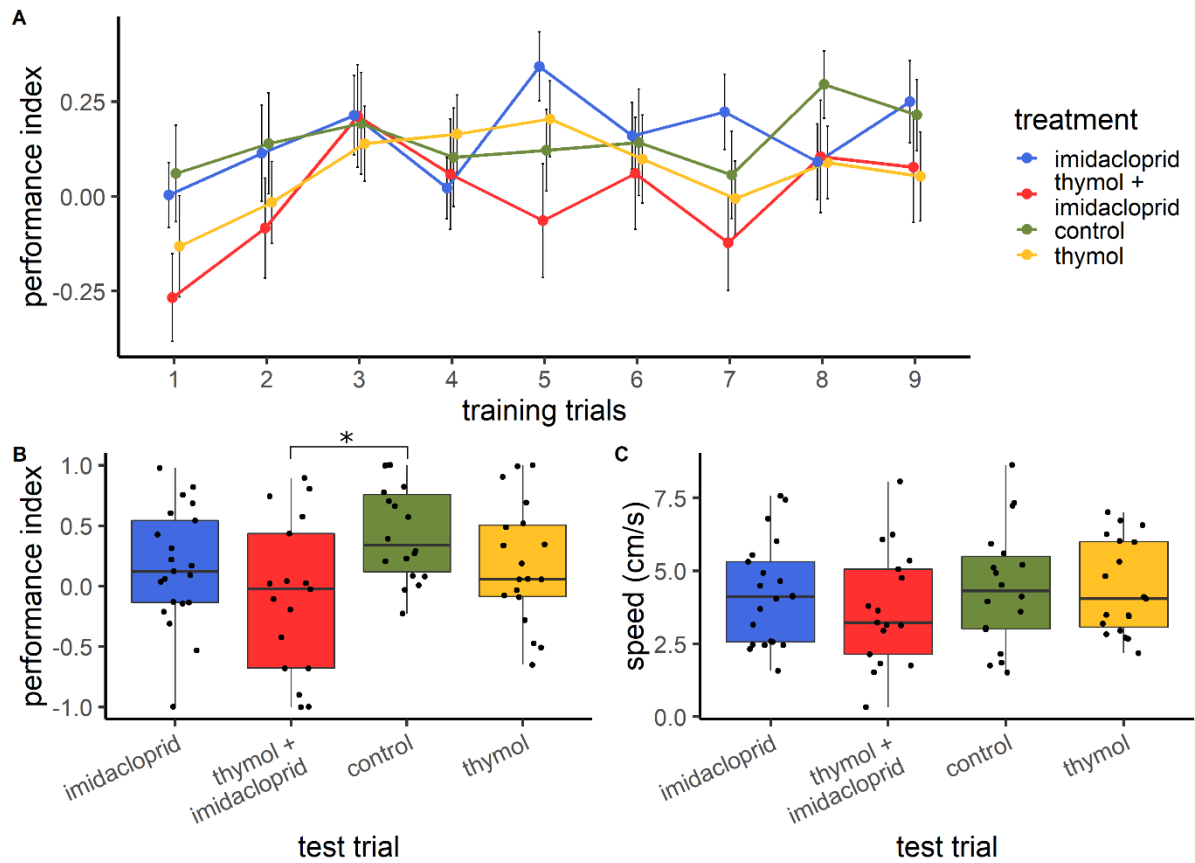
Figure 5-2. **Comparing the visual learning performances of bees treated with thymol and/or imidacloprid. (A) Performance index of the bees during the nine training trials (mean +/- se) Bees were able to obtain a positive performance index from the third training trial. (B) Performance index during the test trial. (C) Individual speed during the test trial.**

Discussion

Here, we investigated if visual learning of honey bees was affected by colony treatments with miticides and pesticides. We did not find any evidence that exposure to either miticide, thymol or tau-fluvalinate, or to the trace amounts of the insecticide imidacloprid during the development and early life of honey bees impairs aversive visual learning when they were applied alone. However, when thymol was applied in combination with imidacloprid, bee workers were not able to associate a specific colour to a weak electrical shock. This interaction provides evidence that accumulating stressors can interact and affect bee behaviours, which could weaken honey bee colonies.

Little is known about the effects of thymol on honey bees and it is unclear why thymol and imidacloprid only significantly affected learning in European honey bees when they were applied together. It has been suggested that thymol acts as an agonist of a GABA receptor in the brains of insects (Price et al. 2014), but imidacloprid mainly binds to neuronal nicotinic acetylcholine receptors (Gauthier 2010). One study however suggests that imidacloprid may be an antagonist of a GABA receptor in insect brains (Taylor-Wells et al. 2015). Therefore, these compounds are not expected to act in the same way and their interactive or additive effects may be complex.

The combined effect of thymol and imidacloprid on honey bees is worrying. Miticides, neonicotinoids and many other types of pesticides are found together in hives located in agricultural landscapes (Tosi et al., 2018), and are known to accumulate in pollen, honey and wax (Boyle and Sheppard, 2017; Mullin et al., 2010). Evidence that accumulating stressors can interact together and affect bee colonies highlights the need for more studies on the effect of combined pesticides exposure on the health and productivity of bee colonies. Miticides in particular are often mixed together in hives (Mullin et al., 2010) and are commercially available

as complex mixtures (e.g. Apilife Var® is a widely used commercial formulation of thymol, eucalyptus oil, camphor, and levomenthol). Successive treatments can also cause miticides to accumulate in bee products (Bogdanov et al., 1998; Mullin et al., 2010).

A previous study found that Apilife Var® had no effect on odour learning using the standard proboscis extension reflex assay, but impaired the olfactory memory of bees for a short period of time after its introduction in the hive (Bonnafé et al., 2017). Apilife Var® also altered phototactic behaviour (Alayrangues et al., 2016; Carayon et al., 2014), which was not required for the bee's response in our setup; this suggests that the treatment has differential effects on response to light and visual learning. The formulation Apiguard® has also been found to change hygienic behaviour (which is associated with olfactory cues) shortly after its introduction in the hive (Chapter 6).

Further research is needed to understand how thymol causes these complex effects on visual learning and olfactory memory and responses. Our research adds to the evidence that chemical miticides are not completely benign for honey bees.

Presently, there has been little research towards the development of non-chemical alternatives for the control of *V. destructor* (Alquisira-Ramírez et al., 2017; Dietemann et al., 2012; Meikle et al., 2012). We argue an integrated approach to *V. destructor* control including mechanical and behavioural control methods, and the selection or hygienic or resistant bee lines to control *V. destructor* would be a productive way forward. It would limit the accumulation of apicultural chemicals in the hives, reduce the risk for potential deleterious interactions with other stressors, and reduce the risk of *V. destructor* evolving resistance to chemical miticides.

Acknowledgements

References

Alayrangues, J. *et al.* (2016) 'Prolonged effects of in-hive monoterpenoids on the honey bee *Apis mellifera*', *Ecotoxicology*. Springer US, 25(5), pp. 856–862. doi: 10.1007/s10646-016-1642-x.

Alburaki, M. *et al.* (2018) 'Honey bee survival and pathogen prevalence: from the perspective of landscape and exposure to pesticides', *Insects*, 9(2), pp. 1–15. doi: 10.3390/insects9020065.

Alquisira-Ramírez, E. V. *et al.* (2017) 'Effects of Bacillus thuringiensis strains virulent to *Varroa destructor* on larvae and adults of *Apis mellifera*', *Ecotoxicology and Environmental Safety*. Elsevier Inc., 142(September 2016), pp. 69–78. doi: 10.1016/j.ecoenv.2017.03.050.

Barron, A. B. (2015) 'Death of the bee hive: understanding the failure of an insect society', *Current Opinion in Insect Science*. Elsevier Inc, 10(Ccd), pp. 45–50. doi: 10.1016/j.cois.2015.04.004.

Bates, D. *et al.* (2015) 'Fitting Linear Mixed-Effects Models Using lme4', *Journal of Statistical Software*, 67(1). doi: 10.18637/jss.v067.i01.

Benuszak, J., Laurent, M. and Chauzat, M. P. (2017) 'The exposure of honey bees (*Apis mellifera*; Hymenoptera: Apidae) to pesticides: Room for improvement in research', *Science of the Total Environment*. Elsevier B.V., 587–588, pp. 423–438. doi: 10.1016/j.scitotenv.2017.02.062.

Berry, J. A. *et al.* (2013) 'Field-Level Sublethal Effects of Approved Bee Hive Chemicals on Honey Bees (*Apis mellifera* L)', *PLoS ONE*, 8(10). doi: 10.1371/journal.pone.0076536.

Boecking, O. and Spivak, M. (1999) 'Behavioral defenses of honey bees against *Varroa jacobsoni* Oud.', *Apidologie*, 30(2–3), pp. 141–158. doi: 10.1051/apido:19990205.

Bogdanov, S., Imdorf, A. and Kilchenmann, V. (1998) 'Residues in wax and honey after Apilife VAR treatment', *Apidologie*, 29(6), pp. 513–524.

Bonmatin, J. M. *et al.* (2003) 'A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants, and in pollens', *Analytical Chemistry*, 75(9), pp. 2027–2033. doi: 10.1021/ac020600b.

Bonmatin, J. M. *et al.* (2005) 'Quantification of Imidacloprid Uptake in Maize Crops',

Journal of Agricultural and Food Chemistry, 53(13), pp. 5336–5341. doi: 10.1021/jf0479362.

Bonnafé, E. *et al.* (2017) 'Monoterpenoid-based preparations in beehives affect learning, memory, and gene expression in the bee brain', *Environmental Toxicology and Chemistry*, 36(2), pp. 337–345. doi: 10.1002/etc.3527.

Boyle, N. K. and Sheppard, W. S. (2017) 'A scientific note on seasonal levels of pesticide residues in honey bee worker tissues', *Apidologie*, 48(1), pp. 128–130. doi: 10.1007/s13592-016-0455-5.

Calatayud-Vernich, P. *et al.* (2018) 'Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure', *Environmental Pollution*, 241, pp. 106–114. doi: 10.1016/j.envpol.2018.05.062.

Carayon, J. L. *et al.* (2014) 'Thymol as an alternative to pesticides: Persistence and effects of Apilife Var on the phototactic behavior of the honeybee *Apis mellifera*', *Environmental Science and Pollution Research*, 21(7), pp. 4934–4939. doi: 10.1007/s11356-013-2143-6.

Charpentier, G. *et al.* (2014) 'Lethal and sub-lethal effects of thymol on honeybee (*Apis mellifera*) larvae reared in vitro', *Pest Management Science*, 70(1), pp. 140–147. doi: 10.1002/ps.3539.

Coffey, M. F. (2007) 'Biotechnical methods in colony management, and the use of Apiguard®and Exomite™ for the control of the varroa mite (*Varroa destructor*) in Irish honey bee (*Apis mellifera*) colonies', *Journal of apicultural research*. Taylor & Francis, 46(4), pp. 213–219.

Coffey, M. F. and Breen, J. (2013) 'Efficacy of Apilife Var® and Thymovar® against *Varroa destructor* as an autumn treatment in a cool climate', *Journal of Apicultural Research*. Taylor & Francis, 52(5), pp. 210–218.

Colin, T. *et al.* (2019) 'Effects of thymol on European honey bee hygienic behaviour', *Apidologie*, 50(2), pp. 141–152. doi: 10.1007/s13592-018-0625-8.

Le Conte, Y., Ellis, M. and Ritter, W. (2010) '*Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses?', *Apidologie*, 41(3), pp. 353–363. doi: 10.1051/apido/2010017.

Davies, T. G. E. *et al.* (2007) 'DDT, pyrethrins, pyrethroids and insect sodium channels',

IUBMB Life, 59(3), pp. 151–162. doi: 10.1080/15216540701352042.

Dietemann, V. *et al.* (2012) '*Varroa destructor* : research avenues towards sustainable control', *Journal of Apicultural Research*, 51(1), pp. 125–132. doi: 10.3896/IBRA.1.51.1.15.

Faucon, J. P., Drajnudel, P. and Fléché, C. (1995) 'Mise en évidence d'une diminution de l'efficacité de l'Apistan ® utilisé contre la varroose de l'abeille (*Apis mellifera* L)', *Apidologie*, 26(4), pp. 291–295. doi: 10.1051/apido:19950403.

Fischer, J. *et al.* (2014) 'Neonicotinoids interfere with specific components of navigation in honeybees', *PLoS ONE*, 9(3), pp. 1–10. doi: 10.1371/journal.pone.0091364.

Forfert, N. *et al.* (2017) 'Neonicotinoid pesticides can reduce honeybee colony genetic diversity', *PLoS ONE*, 12(10), pp. 1–14. doi: 10.1371/journal.pone.0186109.

Frost, E. H., Shutler, D. and Hillier, N. K. (2013) 'Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival.', *The Journal of experimental biology*, 216(Pt 15), pp. 2931–8. doi: 10.1242/jeb.086538.

Guzmán-Novoa, E. *et al.* (2010) '*Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee *(Apis mellifera)* colonies in Ontario, Canada', *Apidologie*, 41(4), pp. 443–450. doi: 10.1051/apido/2009076.

Haarmann, T. *et al.* (2002) 'Effects of Fluvalinate and Coumaphos on Queen Honey Bees (Hymenoptera: Apidae) in Two Commercial Queen Rearing Operations', *Journal of Economic Entomology*, 95(1), pp. 28–35. doi: 10.1603/0022-0493-95.1.28.

Henry, M. *et al.* (2012) 'A common pesticide decreases foraging success and survival in honey bees', *Science*, 336(6079), pp. 348–350. doi: 10.1126/science.1215039.

Johnson, R. M. *et al.* (2013) 'Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*)', *PLoS ONE*. Edited by N. E. Raine, 8(1), p. e54092. doi: 10.1371/journal.pone.0054092.

Kanga, L. H. B. *et al.* (2010) 'Monitoring for Resistance to Organophosphorus and Pyrethroid Insecticides in Varroa Mite Populations', *Journal of Economic Entomology*, 103(5), pp. 1797–1802. doi: 10.1603/ec10064.

Kirkerud, N. H. *et al.* (2013) 'APIS—a novel approach for conditioning honey bees', *Frontiers in Behavioral Neuroscience*, 7(April), pp. 1–19. doi: 10.3389/fnbeh.2013.00029.

Klein, S. *et al.* (2017) 'Why Bees Are So Vulnerable to Environmental Stressors', *Trends in Ecology and Evolution*. Elsevier Ltd, 32(4), pp. 268–278. doi: 10.1016/j.tree.2016.12.009.

Lenth, R. *et al.* (2018) 'Package "emmeans"', *Mran.Microsoft.Com*, 34(1), p. 126. doi: 10.1080/00031305.1980.10483031

Lu, C., Warchol, K. M. and Callahan, R. A. (2012) 'In situ replication of honey bee colony collapse disorder', *Bulletin of Insectology*, 65(1), pp. 99–106.

Marchetti, S., Barbattini, R. and D'Agaru, M. (1984) 'Comparative effectiveness of treatments used to control *Varroa jacobsoni* Oud.', *Apidologie*, 15(4), pp. 363–378.

Meikle, W. G. *et al.* (2012) 'Challenges for developing pathogen-based biopesticides against *Varroa destructor* (Mesostigmata: Varroidae)', *Apidologie*, 43(5), pp. 501–514. doi: 10.1007/s13592-012-0118-0.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLOS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Milani, N. (1995) 'The resistance of *Varroa jacobsoni* Oud to pyrethroids: a laboratory assay', *Apidologie*, 26(5), pp. 415–429. doi: 10.1051/apido:19950507.

Milani, N. (1999) 'The resistance of *Varroa jacobsoni* Oud. to acaricides', *Apidologie*, 30(2–3), pp. 229–234. doi: 10.1051/apido:19990211.

Mitchell, E. A. D. *et al.* (2017) 'A worldwide survey of neonicotinoids in honey', *Science*, 358(6359), pp. 109–111. doi: 10.1126/science.aan3684.

Mondet, F., Goodwin, M. and Mercer, A. (2011) 'Age-related changes in the behavioural response of honeybees to Apiguard®, a thymol-based treatment used to control the mite *Varroa destructor*', *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 197(11), pp. 1055–1062. doi: 10.1007/s00359-011-0666-1.

Mullin, C. A. *et al.* (2010) 'High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health', *PLoS ONE*, 5(3). doi: 10.1371/journal.pone.0009754.

O'Neal, S. T., Anderson, T. D. and Wu-Smart, J. Y. (2018) 'Interactions between pesticides and pathogen susceptibility in honey bees', *Current Opinion in Insect Science*, 26, pp. 57–62.

doi: 10.1016/j.cois.2018.01.006.

Plath, J. A. *et al.* (2017) 'Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay', *Frontiers in Behavioral Neuroscience*, 11(May). doi: 10.3389/fnbeh.2017.00098.

Prado, A. *et al.* (2019) 'Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees', *Science of the Total Environment*. Elsevier B.V., 650, pp. 1250–1260. doi: 10.1016/j.scitotenv.2018.09.102.

Price, K. L. and Lummis, S. C. R. (2014) 'An atypical residue in the pore of *Varroa destructor* GABA-activated RDL receptors affects picrotoxin block and thymol modulation', *Insect Biochemistry and Molecular Biology*. Elsevier Ltd, 55, pp. 19–25. doi: 10.1016/j.ibmb.2014.10.002.

R Core Team, R. (2018) 'R: A language and environment for statistical computing. R Foundation for Statistical Computing'. Vienna, Austria. Available at: https://www.r-project.org/.

Ratnieks, F. L. W. and Carreck, N. L. (2010) 'Clarity on honey bee collapse?', *Science*, 327(5962), pp. 152–153. doi: 10.1126/science.1185563.

Ravoet, J., Reybroeck, W. and De Graaf, D. C. (2015) 'Pesticides for apicultural and/or agricultural application found in Belgian honey bee wax combs', *Bulletin of Environmental Contamination and Toxicology*, 94(5), pp. 543–548. doi: 10.1007/s00128-015-1511-y.

Ray, D. E. and Fry, J. R. (2006) 'A reassessment of the neurotoxicity of pyrethroid insecticides', *Pharmacology & Therapeutics*, 111(1), pp. 174–193. doi: 10.1016/j.pharmthera.2005.10.003.

Robinson, A. *et al.* (2017) 'Comparing bee species responses to chemical mixtures: Common response patterns?', *PLoS ONE*, 12(6), pp. 1–21. doi: 10.1371/journal.pone.0176289.

Rosenkranz, P., Aumeier, P. and Ziegelmann, B. (2010) 'Biology and control of *Varroa destructor*', *Journal of Invertebrate Pathology*. Elsevier Inc., 103(SUPPL. 1), pp. S96–S119. doi: 10.1016/j.jip.2009.07.016.

Sánchez-Bayo, F. *et al.* (2016) 'Are bee diseases linked to pesticides? — A brief review', *Environment International*. Elsevier B.V., 89–90, pp. 7–11. doi: 10.1016/j.envint.2016.01.009.

Sgolastra, F. *et al.* (2017) 'Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species', *Pest Management Science*, 73(6), pp. 1236–1243. doi: 10.1002/ps.4449.

Sgolastra, F. *et al.* (2018) 'Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee', *Proceedings of the Royal Society B: Biological Sciences*, 285(1885), p. 20180887. doi: 10.1098/rspb.2018.0887.

Smodiš Škerl, M. I., Kmecl, V. and Gregorc, A. (2010) 'Exposure to pesticides at sublethal level and their distribution within a honey bee (*Apis mellifera*) colony', *Bulletin of Environmental Contamination and Toxicology*, 85(2), pp. 125–128. doi: 10.1007/s00128-010-0069-y.

Tosi, S. *et al.* (2018) 'A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides', *Science of the Total Environment*. Elsevier B.V., 615(February), pp. 208–218. doi: 10.1016/j.scitotenv.2017.09.226.

Tosi, S., Burgio, G. and Nieh, J. C. (2017) 'A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability', *Scientific Reports*. Springer US, 7(1), pp. 1–8. doi: 10.1038/s41598-017-01361-8.

Tsvetkov, N. *et al.* (2017) 'Chronic exposure to neonicotinoids reduces honey bee health near corn crops', *Science*, 356(6345), pp. 1395–1397. doi: 10.1126/science.aam7470.

Urlacher, E. *et al.* (2010) 'An alarm pheromone modulates appetitive olfactory learning in the honeybee (*Apis mellifera*)', *Frontiers in Behavioral Neuroscience*, 4(August), pp. 1–10. doi: 10.3389/fnbeh.2010.00157.

Wallner, K. and Fries, I. (2003) 'Control of the mite *Varroa destructor* in honey bee colonies', *Pesticide Outlook*. Royal Society of Chemistry, 14(2), pp. 80–84.

Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. Springer-V. New York: Springer-Verlag New York. Available at: https://ggplot2.tidyverse.org.

Wilke, C. O. (2019) 'cowplot: Streamlined Plot Theme and Plot Annotations for "ggplot2"'. Available at: https://cran.r-project.org/package=cowplot.

Woodcock, B. A. *et al.* (2017) 'Country-specific effects of neonicotinoid pesticides on honey bees and wild bees', *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

Wu-Smart, J. and Spivak, M. (2016) 'Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development', *Scientific Reports*. Nature Publishing Group, 6(March), pp. 1–11. doi: 10.1038/srep32108.

Yao, J. *et al.* (2018) 'Influences of acephate and mixtures with other commonly used pesticides on honey bee (*Apis mellifera*) survival and detoxification enzyme activities', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. Elsevier, 209(January), pp. 9–17. doi: 10.1016/j.cbpc.2018.03.005.

Yao, J., Zhu, Y. C. and Adamczyk, J. (2018) 'Responses of honey bees to lethal and sublethal doses of formulated clothianidin alone and mixtures', *Journal of Economic Entomology*, 111(4), pp. 1517–1525. doi: 10.1093/jee/toy140.

Zhou, T. *et al.* (2011) 'Effects of pyrethroids on neuronal excitability of adult honeybees *Apis mellifera*', *Pesticide Biochemistry and Physiology*. Elsevier Inc., 100(1), pp. 35–40. doi: 10.1016/j.pestbp.2011.02.001.

Zhu, Y. C. *et al.* (2017) 'Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*)', *PLoS ONE*, 12(5), pp. 1–16. doi: 10.1371/journal.pone.0176837.

Chapter 6 Effects of thymol on European honey bee hygienic behaviour

Abstract

The parasitic mite *V. destructor* is a major threat to the European honey bee *A. mellifera*. Beekeepers apply the miticide thymol directly within hives to kill this parasitic mite. Thymol is repellent to bees and causes them to ventilate the hive, yet its impact on bee hygienic behaviours that prevent the spread of diseases has never been studied. We measured the efficiency of colonies at removing dead adult bees, uncapping dead pupal cells, and removing dead brood in two miticide-free Australian environments where the mite is absent. Thymol increased the uncapping and removal of dead brood by 24 to 36% after 48h at both locations but had no effect on the removal of dead adult bees. The increased removal of brood could enhance the effect of thymol on *V. destructor*, especially if bees preferentially remove cells infected with the mite.

Published version

Pages 167-192 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Colin, T., Lim, M.Y., Quarrell, S.R. et al. (2019) Effects of thymol on European honey bee hygienic behaviour. *Apidologie*, vol. 50, pp. 141–152. https://doi.org/10.1007/s13592-018-0625-8

Chapter 7 Traces of a neonicotinoid induce precocious foraging and reduces foraging performance in honey bees

Abstract

There is increasing worldwide concern about the impacts of pesticide residues on honey bees and bee colony survival, but how sublethal effects of pesticides on bees might cause colony failure remains highly controversial, with field data giving very mixed results. To explore how trace levels of the neonicotinoid pesticide imidacloprid impacted colony foraging performance we equipped bees with RFID tags that allowed us to track their lifetime flight behaviour. One group of bees was exposed to a trace concentration (5 μg/kg = 5 ppb) of imidacloprid in sugar syrup while in the larval stage. The imidacloprid residues caused bees to start foraging when younger as adults, to perform fewer orientation flights and reduced their lifetime foraging flights by 28%. The magnitude of these effects of a trace imidacloprid concentration delivered only during larval stage highlights the severity of pesticide residues for bee foraging performance. Our data suggest that neonicotinoids could impact colony function by imbalancing the normal age based division of labour in a colony and reducing foraging efficiency. Understanding this mechanism will help the development of interventions to safeguard bee colony health.

Graphical abstract

bees exposed to
imidacloprid in
the larval stage
only, or control

flight behaviour
tracked with Radio
Frequency
Identification tags

imidacloprid exposed
bees start foraging 15%
younger

imidacloprid exposed
bees performed 28%
fewer foraging trips
than controls

bees exposed to
imidacloprid die 1.2
times faster
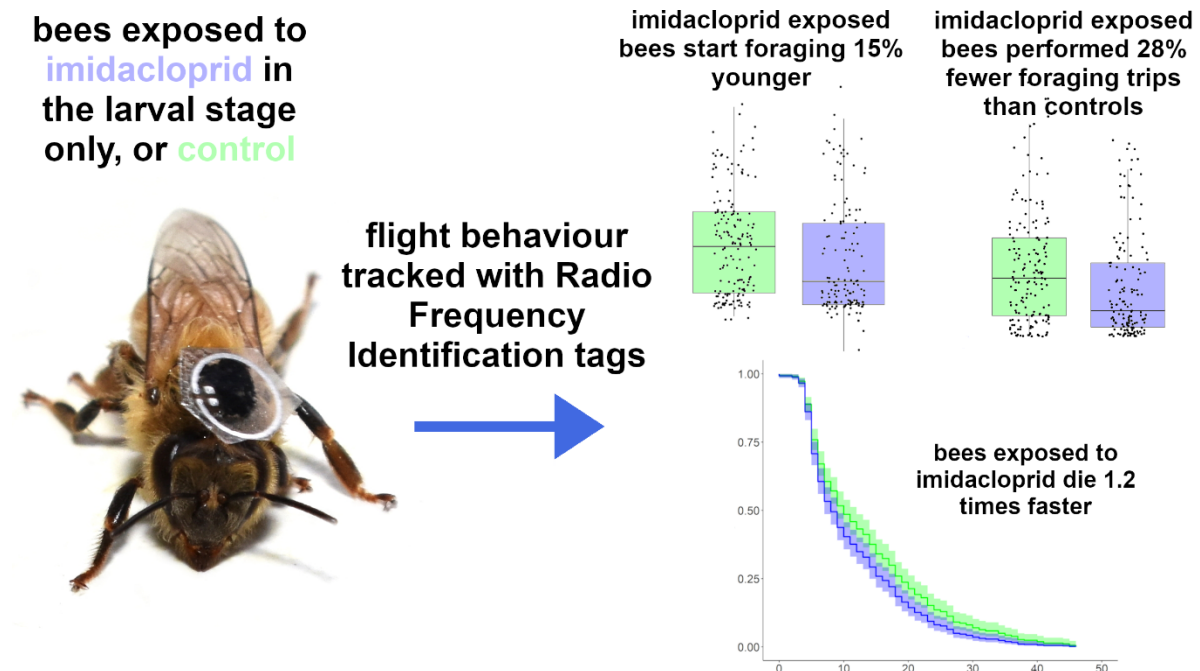
Published version

Colin, T., Meikle, W.G., Wu, X., Barron, A.B., 2019c. Traces of a Neonicotinoid Induce

Precocious Foraging and Reduce Foraging Performance in Honey Bees. Environ. Sci.

Technol. 53, 8252–8261. https://doi.org/10.1021/acs.est.9b02452

Introduction

There is global concern about the impacts of neonicotinoid pesticide residues on honey bee health, and honey bee colony survival (Henry et al., 2015, 2012; Hladik et al., 2018; William G. Meikle et al., 2016; Osterman et al., 2019; Samson-Robert et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017). Many deleterious impacts of neonicotinoid residues on bees have now been reported, and some studies have linked sublethal pesticides exposure to increased colony failure in honey bees and bumblebees (Ellis et al., 2017; Henry et al., 2012; William G. Meikle et al., 2016; Samson-Robert et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017). Different mechanisms have been proposed to explain how pesticide residues cause the rapid failure of a bee colony (Khoury et al., 2011; Nazzi and Pennacchio, 2018; Perry et al., 2015; Sánchez-Bayo and Desneux, 2015; Zanni et al., 2018). The issue is complex because the social dynamics of a bee colony are such that stressors at an individual level do not simply translate to outcomes for a colony (Henry et al., 2015, 2012; Perry et al., 2015). One influential model has argued that a chronic stressor causing a premature death of foragers could cause a dramatic colony failure through the progressive recruitment of younger and younger bees to the foraging force (Perry et al., 2015). Here we examined how traces (5 μg/kg) of the neonicotinoid imidacloprid influenced foraging performance and foraging onset in honey bees, to explore possible mechanisms of pesticide-related colony failure.

In honey bee colonies, foragers are typically the older adult bees, but a loss of foragers is usually compensated by young bees starting foraging earlier in their adult life (Barron et al., 2002; Beshers and Fewell, 2001; Leoncini et al., 2004; Schulz et al., 2002). This is an adaptive response to rapidly restore the colony foraging force, but new theories predict that this mechanism could accelerate colony failure in the face of chronic stress. If workers that start foraging precociously are more sensitive to the stressors and less effective at foraging, they will also die prematurely, ultimately driving a population collapse (Henry et al., 2015; Perry et

195

al., 2015; Tsvetkov et al., 2017). This mechanism for bee colony failure is appealing since it is simple, and precocious foraging is known to be induced by nutritional and pathogen stresses of individual bees (Goblirsch et al., 2013; Schulz et al., 1998; Woyciechowski and Moroń, 2009) and forager losses from the colony (Fahrbach and Robinson, 1996; Perry et al., 2015). We do not yet know, how neonicotinoid contaminants influence the foraging onset in honey bees.

Neonicotinoid pesticides are found in the dust drift during crop sowing (Tapparo et al., 2012; Xue et al., 2015), in the pollen, nectar and guttation drops of many flowering crops (Alburaki et al., 2018; Blacquière et al., 2012; Böhme et al., 2017; Bonmatin et al., 2005, 2003; Chauzat et al., 2009, 2006; Cresswell, 2011; Douglas and Tooker, 2015; Girolami et al., 2009; Hladik et al., 2018; Krischik et al., 2007; Schmolke et al., 2018; Tapparo et al., 2011; Tsvetkov et al., 2017) and nearby wildflowers (Rotheray et al., 2016). The impacts of neonicotinoids on pollinating insects are subject to intensive scrutiny (Henry et al., 2015, 2012; Kerr, 2017; Osterman et al., 2019; Tsvetkov et al., 2017; Woodcock et al., 2017). Four different neonicotinoids, thiametoxam, imidacloprid, clothianidin and thiacloprid have been shown to decrease the homing success of honey bees, and hence the survival of foragers (Fischer et al., 2014; Henry et al., 2012; Tison et al., 2016), probably by affecting the learning and memory functions (T. Colin et al., 2019; Decourtye et al., 2005; Fischer et al., 2014; Gauthier, 2010; Tison et al., 2017; van der Sluijs et al., 2013; Williamson et al., 2013; Yang et al., 2012). Even so, field assessments of the effect of these pesticides on whole bee colonies have often given conflicting results with some studies documenting no effects, or highly variable effects in different environments (Chapter 4; Kerr, 2017; William G. Meikle et al., 2016; Osterman et al., 2019; Woodcock et al., 2017)

Here we assessed in detail how traces of the neonicotinoid imidacloprid influenced lifetime foraging performance of honey bees to give clarity on the mechanisms by which

pesticides could affect bee colonies. To document the effects of imidacloprid on the lifetime forager performance of individual bees we used miniature Radio-Frequency Identification (RFID) transponders, or "tags", fitted on the thorax of bees that were detected at the entrance of the hive as bees come and go (Henry et al., 2015; Perry et al., 2015). A difficulty commonly faced with this method is that young bees perform a series of non-foraging flights known as orientation flights during which they learn how to navigate before they become foragers (Capaldi et al., 2000; Capaldi and Dyer, 1999; Lach et al., 2015; Menzel et al., 2005). This can obscure the point at which a bee transitions to foraging. Previous studies have used the age at the first exit of a bee as a proxy for the age at onset of foraging (Henry et al., 2015), or used a single flight-time threshold to estimate the point at which bees are likely to have transitioned from orienting to foraging (Henry et al., 2015; Perry et al., 2015). Here we developed a new analysis method to estimate from the flight pattern data the likely onset of foraging for each individual.

Using this approach, we studied how larval exposure to a trace level of imidacloprid influenced foraging performance in adult bees. Bees were exposed as larvae by feeding colonies of European honey bees 20 kg of a 5 μg/kg imidacloprid sugar syrup for 6 weeks. Control colonies received sugar syrup only. When the bees emerged as adults they were tagged with RFID transponders and tagged bees from treated and control colonies were introduced together in a full-size bee commercial colony in a standard hive equipped with an RFID reader. We then analysed the flights patterns and history of the bees to measure the number and duration of orientation flights, the age at which individual bees performed their first foraging flight, the number and duration of their foraging flights, and their longevity.

Material and Methods

a) Imidacloprid exposure

To expose colonies to field-relevant concentrations of imidacloprid, we followed a protocol previously published several times (Chapter 4 as Colin et al., 2019; Meikle et al., 2018a; William G. Meikle et al., 2016; Meikle and Weiss, 2017). In brief, a 50:50 sugar to water mix was heated to 60°C to facilitate sugar dissolution and left aside to cool down before it was fed to the colonies in the control group. On the days of treatment, one milligram of pure analytical-grade imidacloprid (Imidacloprid PESTANAL®, CAS # 138261-41-3) was dissolved in 100mL of distilled water using a magnetic mixing bar at ambient temperature (24°C) to obtain a stock solution of imidacloprid at 10ppm. Using a pipette, 0.5mL of the stock solution was then added to 99.5 mL of distilled water and agitated using a magnetic mixing bar. To prepare the syrup, 500 g of sugar was dissolved in 400 mL of distilled water and heated to 60°C and left aside to cool down. Once the syrup is at ambient temperature, the imidacloprid solution was added to obtain a 50:50 sugar to water mix with 5 µg/kg imidacloprid, and fed to the colonies. Colonies were fed a total of 20 kg of control or imidacloprid sugar syrup over 6 weeks, as for previous experiments (Chapter 4 as Colin et al., 2019; William G. Meikle et al., 2016), in a black plastic in-hive frame feeder to keep the syrup in the dark. Bee colonies commonly collect between 1 kg and 2 kg of nectar during nectar flows at the beginning of the summer, so this amount only represents a small fraction of what colonies might gather and process over such time period (Holst and Meikle, 2018; Chapter 2 as Meikle et al., 2018b; W. G. Meikle et al., 2016). All the syrup was consumed by the bees within the 6 weeks. This concentration corresponds to residues of imidacloprid treatments, sometimes referred to as "trace" at concentrations found in plants, bees and hive products (Bonmatin et al., 2005, 2003; Chauzat et al., 2011, 2009; Hladik et al., 2018; Mitchell et al., 2017; Nicholls et al., 2018; Tsvetkov et al., 2017). Imidacloprid is found at an average concentration of 10 µg/kg in

sunflowers (Bonmatin et al., 2003), 2.1 µg/kg in maize pollen (Bonmatin et al., 2005) and on cotton crops its concentration ranges between 1.6 to 64.6 µg/kg in the pollen and between 0 and 1.8 µg/kg in the nectar (Jiang et al., 2018). Chronic exposure to imidacloprid can occur within a hive through consumption of stored contaminated nectar (honey) and pollen (called bee bread). A worldwide survey of neonicotinoids in honey found imidacloprid in more than half the samples analysed, at an average concentration of 0.35 µg/kg with a maximum concentration of 6.3 µg/kg (Mitchell et al., 2017). Imidacloprid was most often found in combination with at least one other neonicotinoid (Mitchell et al., 2017). In France average concentrations of 0.7 µg/kg in honey, 0.9 µg/kg in pollen and 1.2 µg/kg in honey bee workers were found before the 2013 restrictions on outdoor neonicotinoid use (Chauzat et al., 2011, 2009). In a previous experiment where bee colonies were also fed 20 kg of 5 µg/kg imidacloprid syrup over 6 weeks the average concentrations of imidacloprid and imidacloprid metabolites in bees was 0.62 µg/kg (Chapter 4 as Colin et al., 2019). The concentration we use here to feed the colonies lies well within the range of exposure that managed bee colonies are exposed to when collecting pollen and nectar from treated crops.

b) Hives

On the 22nd of November 2017, at the beginning of the Australian summer, six colonies were established outdoors with full access to the outside from packages containing 1.5 kg of bees. The hives were located at Macquarie University (33°46'07.2"S 151°06'46.8"E, New South Wales, Australia). Bees were given wax foundations in standard "deep Langstroth" hives containing seven frames, an in-hive frame feeder and were fed 4 L of sucrose syrup over 3 weeks on establishment. The four colonies with the largest bee population were selected and randomly assigned to the control or imidacloprid group. Hives were kept 4 meters apart and decorated with regular patterns to help minimize worker drift. Entrance reducers were used to avoid robbing (no robbing was observed during the experiment). After three weeks of

treatment, all the frames were full of capped honey stores and a top box was added to allow bees to keep ingesting and storing the syrup.

c) Radio-frequency identification setup

One standard 8-frame single box "deep Langstroth" hive containing a fully established colony was placed indoors at Macquarie University (Sydney, New South Wales, Australia). The entrance was modified to funnel all the bees returning to the hive in one direction and all the departing bees in the other (Supplementary Material 7-7). The tunnels leading in and out of the hive were equipped with four RFID antennae (two on the way in and two on the way out to allow sufficient traffic for a full-size bee colony) connected to an RFID reader (Invengo XC-RF807) previously used in bee experiments (Bordier et al., 2018; Cabirol et al., 2018; Perry et al., 2015; Søvik et al., 2015). Bees had full access to the outside and no access to the inside of the building, were not fed and could only forage outside. The lid of the hive was made of an escape board (a board equipped with a narrow funnel entrance leading the bees to the bottom box) to allow us to add tagged bees without disturbing the hive.

d) Bee eclosions and tagging

To verify reliability, this RFID setup was tested before the beginning of the experiments on a pre-test group of 80 newly emerged bees (less than 10 h old) obtained by placing frames covered in capped brood in an incubator at 34 °C and 37% humidity overnight and tagged with a Radio Frequency Identification tag (RFID; Invengo Technology) glued to their thorax. The tagged bees were introduced into the hive by placing them in a vial above the escape board. The entrance board (Figure S3) was observed two hours a day during 11 days. Bees were observed walking in the right direction at least 93% of the time. A few bees were collected in a box placed under the entrance of the hives and seemed unable to fly possibly due to the presence of glue on their wings. No misdetections of the tags by the antennae were observed.

Between the 13 and the 15th of April 2018, one frame covered in brood was taken each day from each of the four hives treated with imidacloprid or control syrup and placed in the incubator. Bees were obtained, tagged and introduced into the hives as described for the pre-test group. All bees were successfully introduced in the hive.

e) Trip classification

Bees exit the hive to perform various operations at the entrance of the hive and to perform orientation and foraging flights. We excluded all flights of less than a minute from our dataset, as they most likely recorded bees that were sometimes observed fanning under the RFID antennae. Entrances and exits of each tagged bees were collected continuously until the last bee was not seen for 5 consecutive sunny days. To classify flights as orientation or foraging flights, we analysed daily flight patterns of individual bees of an independent dataset. Twenty-nine bees from this pre-test group that performed at least two flights outside the hive were used to develop a way of classifying flights as orientation flights or foraging flights. To do so, the daily flights of these bees were plotted (see Supplementary Material 7-2 for examples) and one observer was asked to indicate when there was a notable change in flight pattern when the number and duration of flights seemed to increase, based on descriptions of the orientation flights from the literature (Capaldi et al., 2000; Degen et al., 2015). The days before the change in flight pattern were classified as days of orientation flights and the days after the change in pattern as days of foraging flights. The daily number of flights, total flight duration, minimum flight duration, maximum flight duration, average flight duration, and the time of first and last flights since sunset were then compared for the putative orientation and foraging periods. The total flight duration and the time of first flight were found to be the most discriminating variables between the two periods (Supplementary Material 7-6, Supplementary Material 7-2, Supplementary Material 7-3). We performed a linear discriminant analysis with the function 'lda' from the Mass v. 7.3-49 package for R (Venables and Ripley, 2002) using daily total

flight duration and time of first flight as discriminant variables and found that days of orientation flights were accurately classified 79.3% of the time and days of foraging flights accurately classified 97.3% of the time when using these two variables alone (Supplementary Material 7-2, Supplementary Material 7-3).

We then used these two variables to estimate the days of first foraging flights for the bees in the experimental dataset. Specifically, we measured for each bee in the control group and the imidacloprid group the daily total flight duration and the time of first flight, and considered that bees started foraging on the first day when they performed their first trip before 5 hours after sunrise and spent more than 20 minutes outside the hive in total (Supplementary Material 7-1).

f) Statistical analyses

All statistical analyses were conducted under R v. 3.4.3. The data obtained were not normally distributed, so a Mann-Whitney test was used to compare the age at onset of foraging. For both orientation and foraging flights, the number of flights, total duration of flights in lifetime and average duration of flights were compared with the function 'wilcox.test' of R base (R Core Team, 2018). A Cox proportional hazards regression model was used to compare the mortality rates of bees in the two treatment groups with the 'coxph' function of the survival v. 2.41-3 package for R (Therneau, 2015) and the survival curves were drawn using the survminer v. 0.4.3 package for R (Kassambara and Kosinski, 2018).

R scripts and data, with additional detail and a polynomial model of the age to number of foraging flights relationship are provided as supplementary information files (Supplementary Material 7-5, Supplementary Material 7-6, Supplementary Material 7-1, Supplementary Material 7-2, Supplementary Material 7-3), results for each colony are explicitly shown in

Supplementary Material 7-4 and information about colony origin is also available in the dataset (Supplementary Material 7-1).

Results

Two hives were fed with 20 kg of control or 5 ppb imidacloprid sugar syrup and two control hives with 20 kg of sugar syrup free of neonicotinoids over six weeks. At the end of the treatment, frames of capped brood from these hives were placed in an incubator at 35°C for 12h overnight. About 295 bees from each hive were equipped with RFID tags at their emergence. All the bees were placed together in a hive with a modified entrance equipped with RFID readers. The exit and entries of individual bees were continuously recorded until the last tagged bee died. 294 bees from two imidacloprid treated hives and 260 bees from two control hives performed at least one trip of more than a minute and were kept for the experiment. The global flight patterns of these bees matched expectations from previous work on bee activity (Holst and Meikle, 2018; Meikle et al., 2018b, 2006; Meikle and Holst, 2015; Prado et al., 2019) i.e. more foragers left the hive in the morning and more returned in the evening (Figure 7-1), orientation flights lasted 2.88 min +/- 0.05 (mean +/- standard error) and foraging flights 21.55 min +/- 0.27, and bees in the control group started foraging 9.24 days +/- 4.46 after emergence.
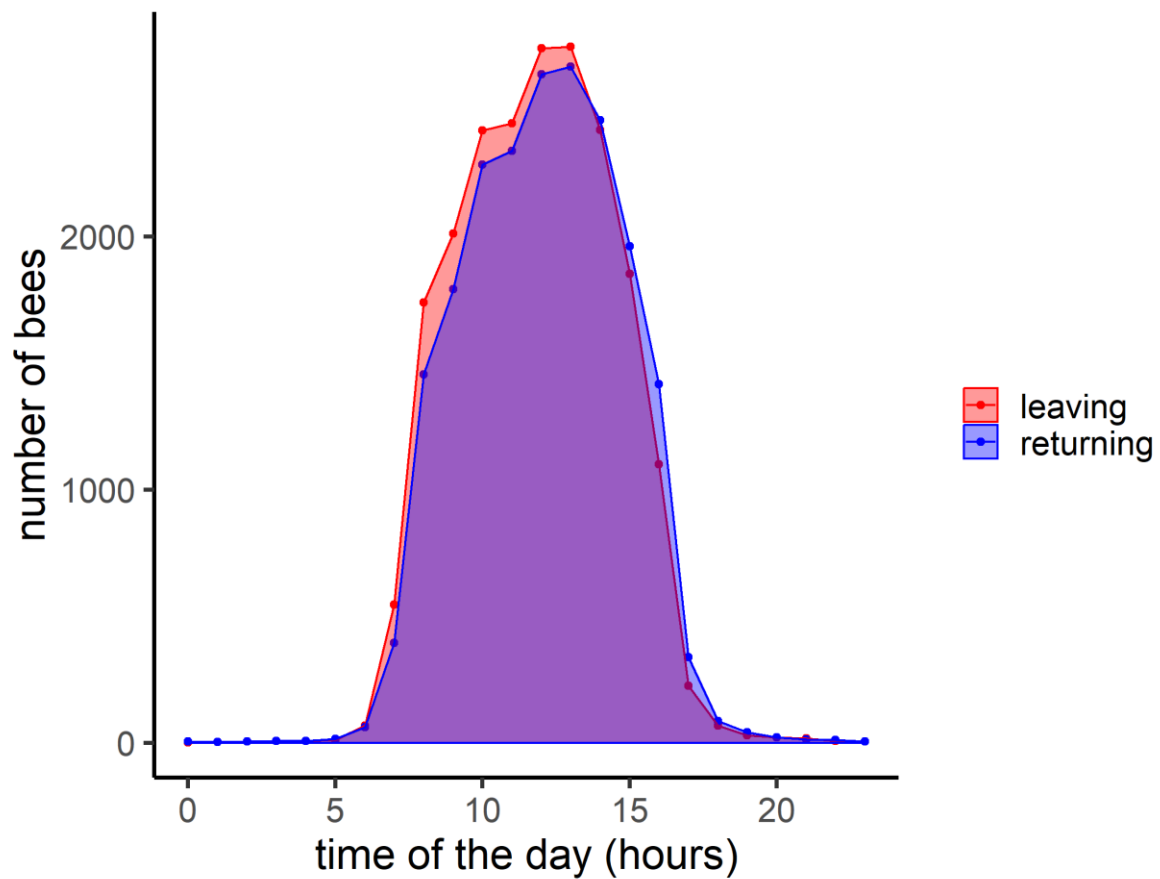
Figure 7-1. **Number of bees departing (transparent red) and returning (transparent blue) to the hive during the day.**

Using a previous, independent dataset, we determined that the total flight duration and the time of first flight were sufficient to identify days of orientation flights 79.3% of the time and days of foraging flights 97.3% of the time (Supplementary Material 7-6, Supplementary Material 7-2, Supplementary Material 7-3). Bees from the imidacloprid experiment were considered to have become foragers on the first day where they started foraging within 5 hours after sunrise and spent more than 20 minutes outside the hive in total. 73.5% of the tagged bees in the control group and 58.7% of those in the imidacloprid group performed foraging flights matching these characteristics.

The bees that were exposed to imidacloprid during their larval stage transitioned from orientation to foraging flights on average 1.38 days younger than bees from the control group (Mann–Whitney test, $W = 13251$, p-value $= 0.001146$) (Figure 7-2) Bees in the imidacloprid group performed on average 2.71 less orientation flights than controls (imidacloprid: 13.26 +/- 1.3 (mean +/- standard error), control: 15.97 +/- 1.01, Mann–Whitney test, $W = 5780.5$, p-value $= 0.0331$, Figure 7-3) and spent on average 8.13 less minutes performing orientation flights in their lifetime than controls (imidacloprid: 34.39 +/- 2.79, control: 42.52 +/- 2.12, Mann–Whitney test, $W = 5933$, p-value $= 0.01226$, Figure 7-4) but the average duration of their orientation flights was not statistically different threshold from those of bees from the control group (imidacloprid: 3.27 +/- 0.28, control: 3.47 +/- 0.19, Mann–Whitney test, $W = 5533$, p-value $= 0.1283$, Figure 7-5).
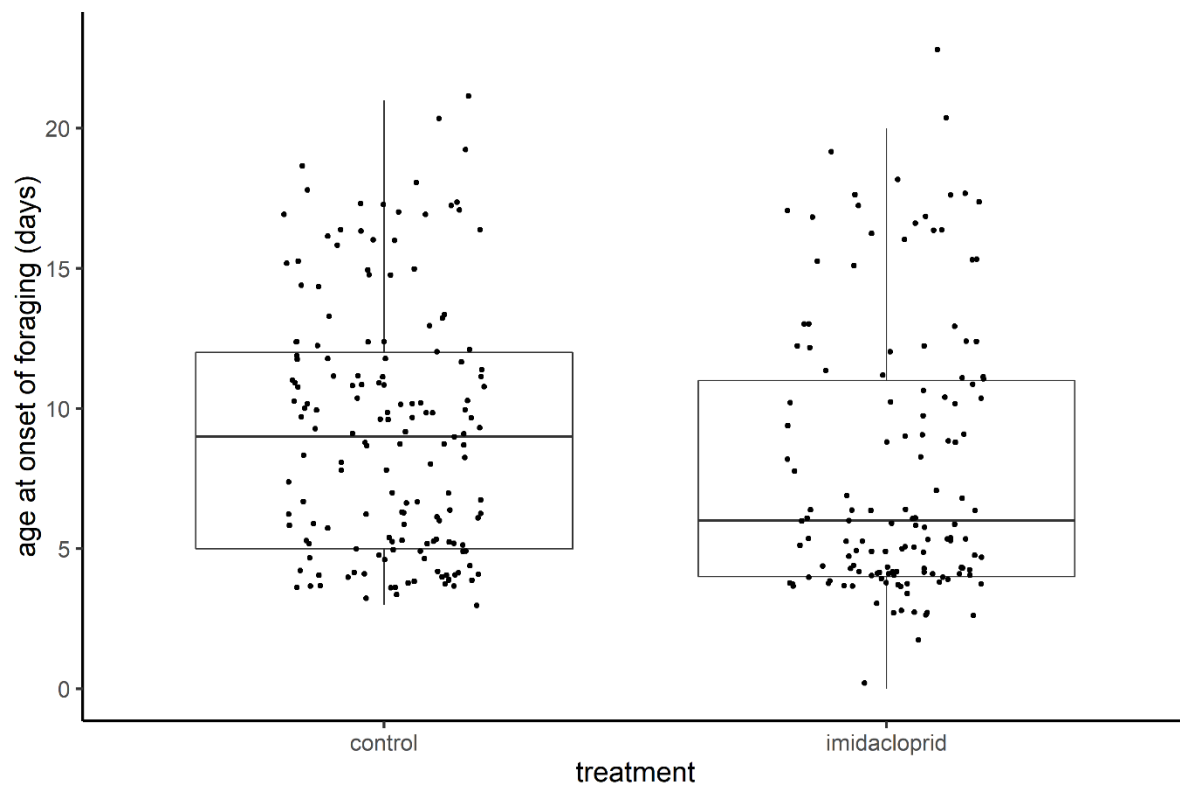
Figure 7-2. **Age at onset of foraging in hives treated with imidacloprid or control syrup. Bees in the imidacloprid group (n=143) started foraging significantly earlier than bees in the control group (n=161).**
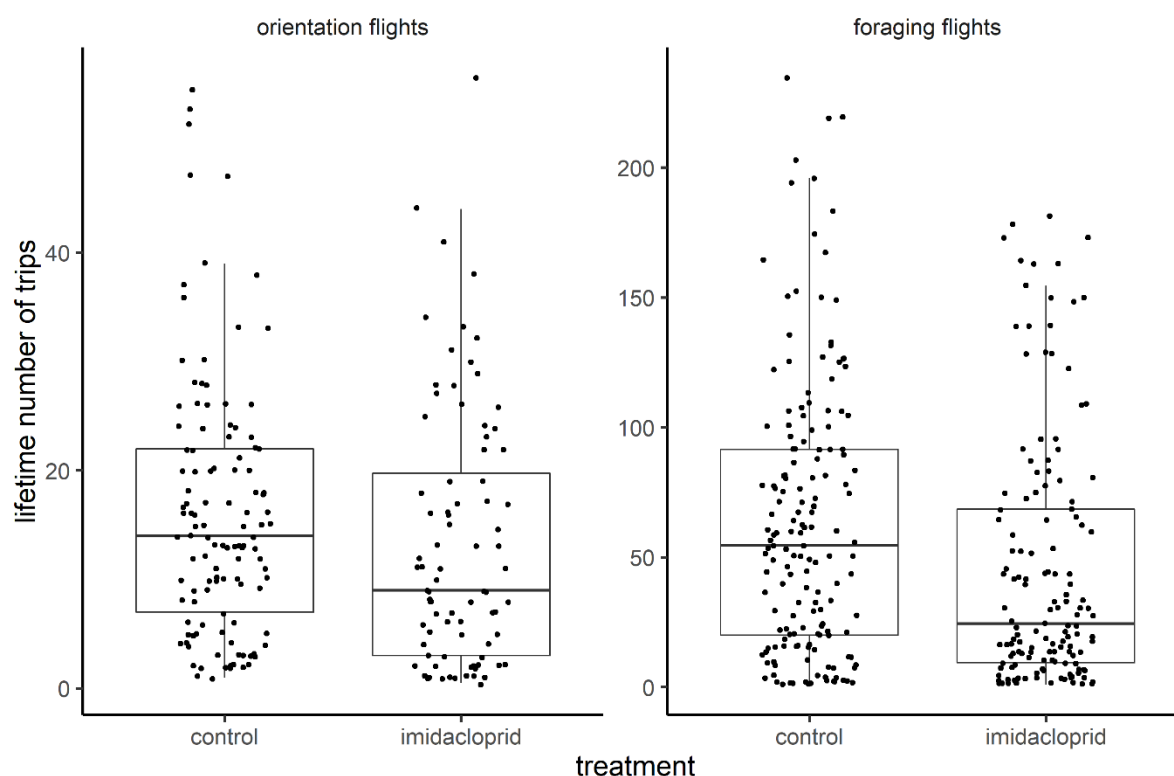
Figure 7-3. **Number of orientation flights performed by bees from the control (n=117) and imidacloprid (n=83) groups and number of foraging flights performed by bees from the control (n=161) and imidacloprid (n=143) groups in their lifetime. Some bees performed no full day of orientation flights and no orientation flights were thus counted for them. Bees exposed to imidacloprid during their larval stage performed significantly fewer orientation and foraging flights during their lifetime. The remainder of the bees died before performing their first foraging flight (n=100 in the control group, n=160 in the imidacloprid group) and are not shown on the figures, these data are available in Supplementary Material 7-1.**
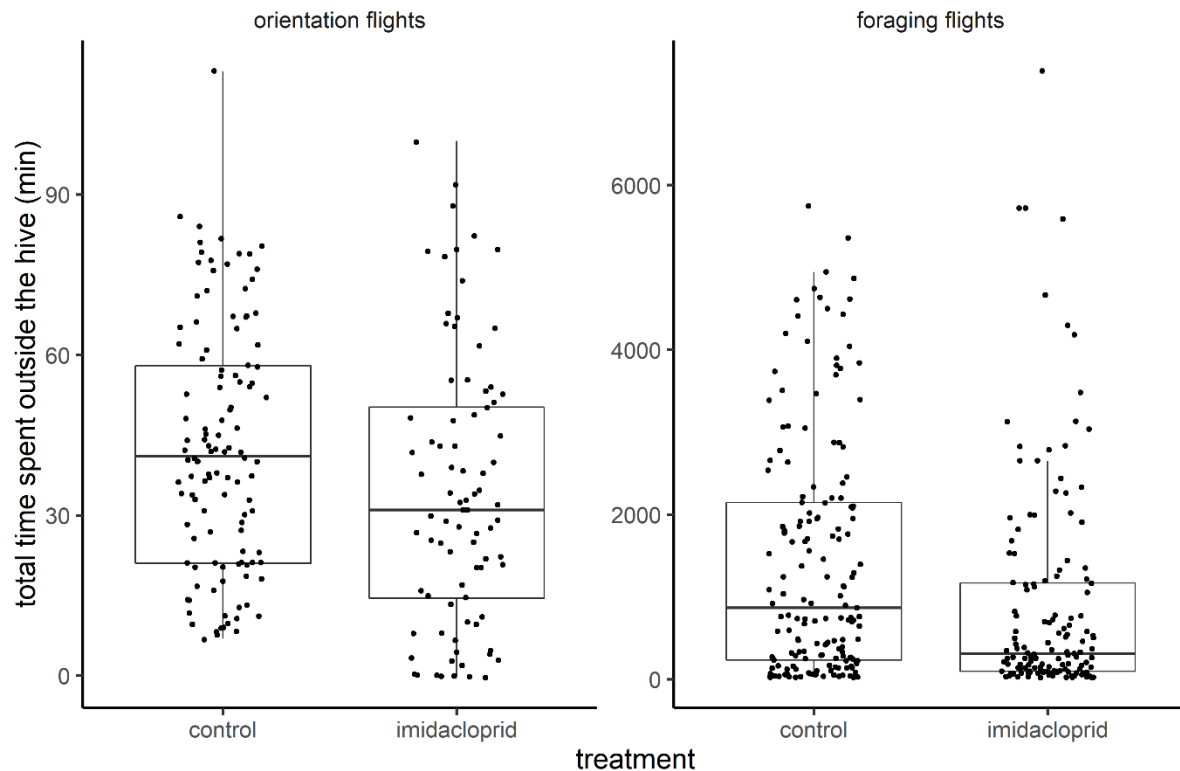
Figure 7-4. **Total time spent outside the hives performing orientation flights by bees from the control (n=117) and imidacloprid (n=83) groups and performing foraging flights by bees from the control (n=161) and imidacloprid (n=143) groups in their lifetime. Some bees performed no full day of orientation flights and no orientation flights were thus counted for them. Bees exposed to imidacloprid during their larval stage spent significantly less time out of the hive during their lifetime for both orientation and foraging flights. The remainder of the bees died before performing their first foraging flight (n=100 in the control group, n=160 in the imidacloprid group) and are not shown on the figures, these data are available in Supplementary Material 7-1.**
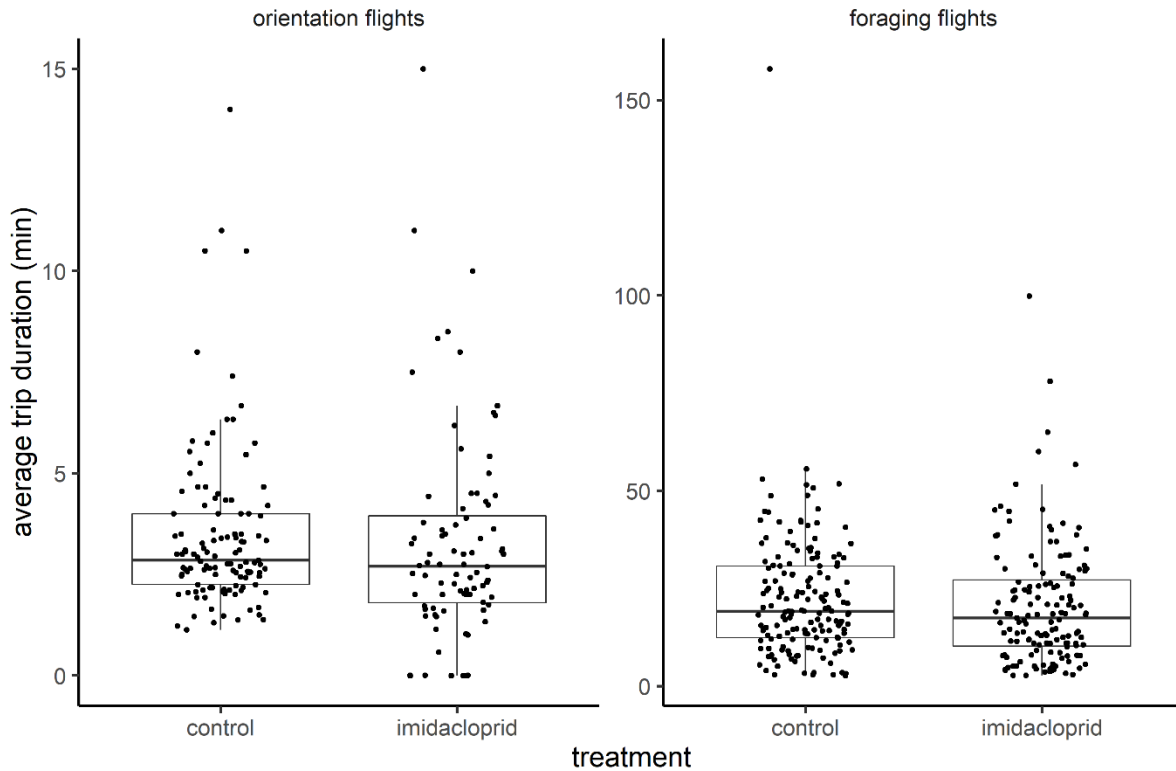
Figure 7-5. **Average trip duration of orientation flights performed by bees from the control (n=117) and imidacloprid (n=83) groups and of foraging flights performed by bees from the control (n=161) and imidacloprid (n=143) groups in their lifetime. Some bees performed no full day of orientation flights and no orientation flights were thus counted for them. There were no significant differences between treatment groups. The remainder of the bees died before performing their first foraging flight (n=100 in the control group, n=160 in the imidacloprid group) and are not shown on the figures, these data are available in Supplementary Material 7-1.**

Bees that started foraging younger performed fewer foraging flights in their lifetime (Figure 7-6), which could explain why bees exposed to imidacloprid during their larval stage performed on average 17.86 less foraging flights than controls (imidacloprid: 45.86 +/- 4.06, control: 63.72 +/- 4.15, Mann–Whitney test, W = 14127, p-value = 0.0006, Figure 7-3) and spent on average 527.33 less minutes foraging in their lifetime than controls (imidacloprid: 910.28 +/- 111.26, control: 1437.61 +/- 114.62, Mann–Whitney test, W = 14438, p-value = 0.0001308, Figure 7-4), and bees in the imidacloprid group tended to perform shorter foraging flights but this was not statistically significant (imidacloprid: 20.53 +/- 1.29, control: 22.68 +/- 1.30, Mann–Whitney test, W = 12826, p-value = 0.08596, Fig 5). Mortality in the imidacloprid treated group was also higher than in the control group (median survival [0.95% confidence interval]: control: 10 [9 - 12], imidacloprid: 8 [7 – 9]; beta coefficient imidacloprid = 0.226, hazard ratio = 1.254, standard error = 0.085, z = 2.814, p = 0.00489) (Figure 7-7).
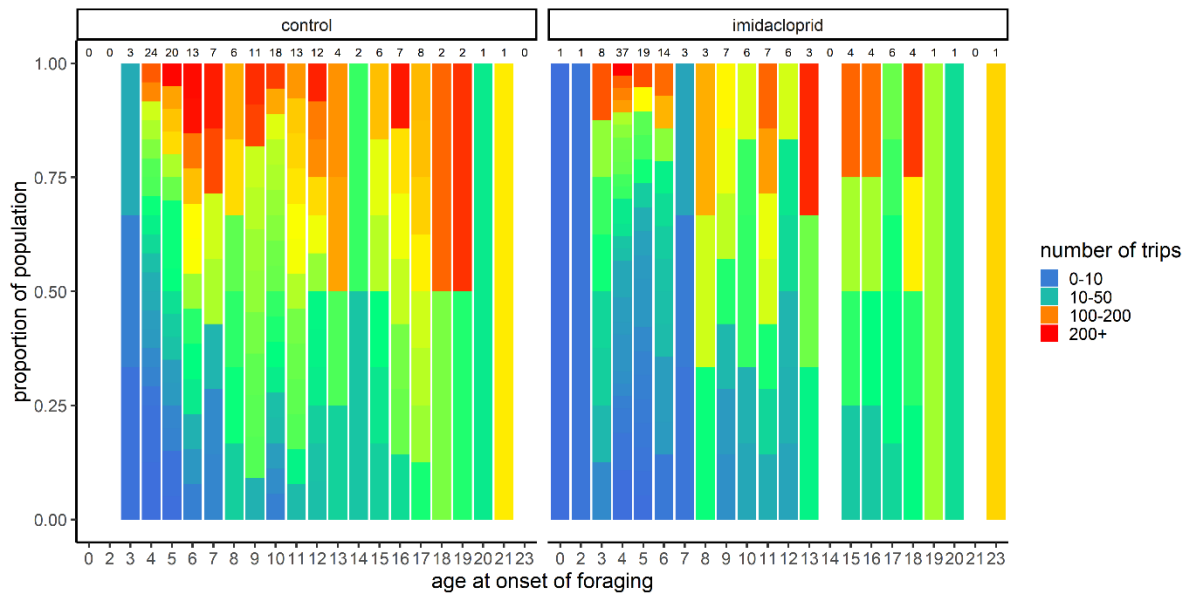
Figure 7-6. **Proportion of the bee population that performed a certain number of foraging flights by the age at which they started foraging. Numbers at the bottom of the bars indicate the age and at the top the sample size for each age. The sample size corresponds to bees that survived the orientation phase and then performed at least one day of foraging flights. Ages at which no bees started foraging are not represented (day 1, day 22).**
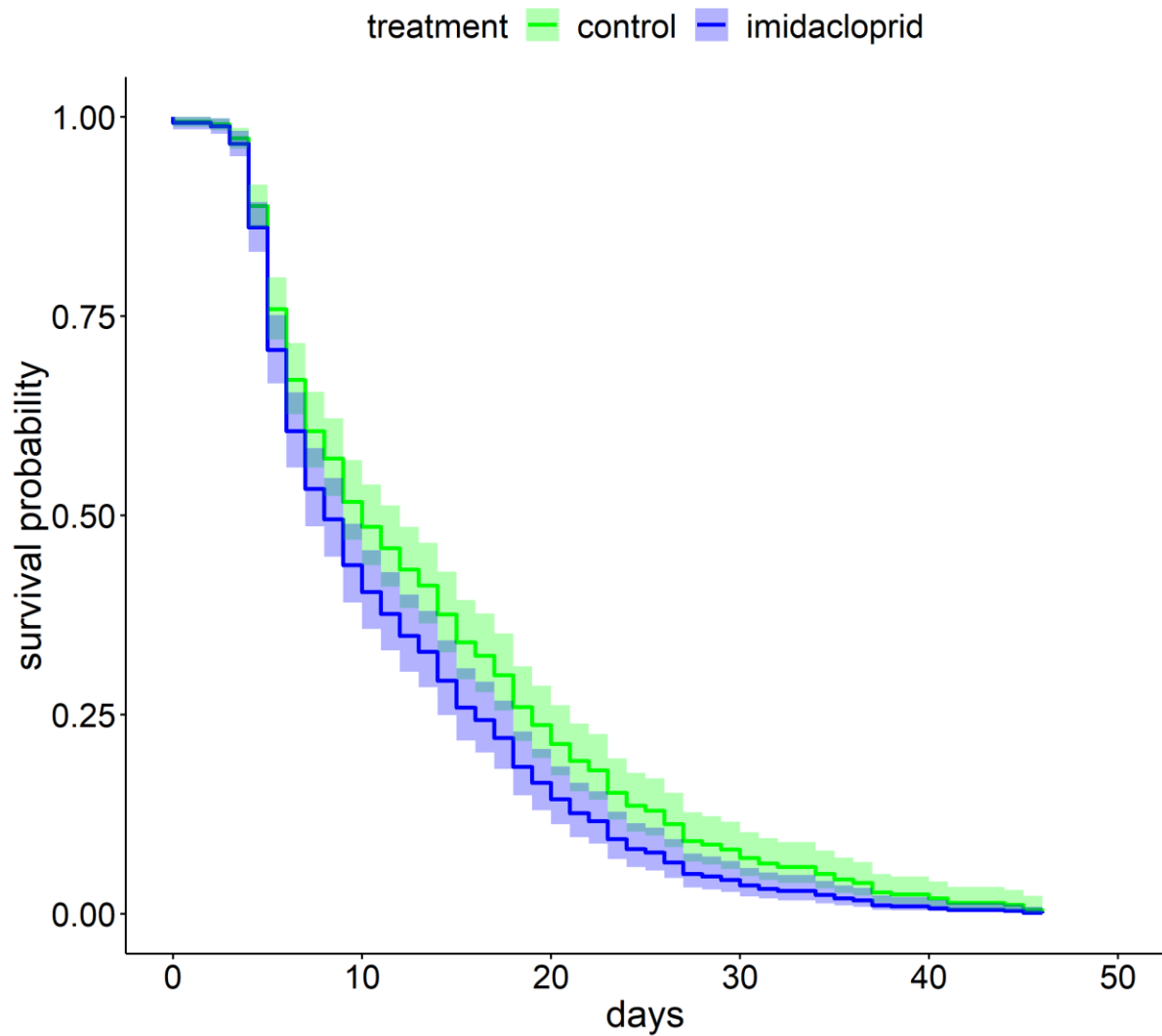
Figure 7-7. **Survival probability of individual honey bees exposed as larvae in the imidacloprid (blue) and control (green) treatments. Adult bees exposed to imidacloprid during their larval stage died significantly faster than bees in the control group.**

Discussion

We found that larval exposure to imidacloprid, at a concentration comparable to the residues found in the nectar of treated crops had dramatic consequences for adult foraging performance. Imidacloprid exposure accelerated foraging onset by 15% from 9.24 days in control bees to 7.8 days on average in imidacloprid exposed bees. In addition, imidacloprid exposed bees performed fewer orientation flights (Figure 7-2, Figure 7-3). Imidacloprid exposed bees performed on average 28% fewer foraging flights than those in the control group (Figure 7-3, Figure 7-6), a reduction similar to that found for the neonicotinoid thiacloprid (Tison et al., 2016). These changes in the age at onset of foraging and foraging performances of bees were similar in magnitude to a drastic manipulation removing the entire population of foragers from a hive (Perry et al., 2015).

Here, we successfully differentiated orientation flights from foraging flights by analysing changes in the daily flight patterns of individual bees (Supplementary Material 7-6, Supplementary Material 7-7, Supplementary Material 7-1, Supplementary Material 7-2, Supplementary Material 7-3). This solves a limitation faced by previous studies, that estimated likely foraging onset based on flight-time thresholds derived from the existing literature (Capaldi et al., 2000; Capaldi and Dyer, 1999; Lach et al., 2015; Perry et al., 2015; Prado et al., 2019; Winston, 1987). We believe this is an important methodological advance since orientation is a crucial phase for all nesting insects that need to navigate back to a stationary nest (Klein et al., 2017). Our analysis method of the RFID data revealed that imidacloprid reduced the number of orientation flights. Since these bees had less time to learn their environment prior to starting foraging this may explain why fewer imidacloprid treated bees successfully transitioned to become foragers (58.7%) than in the control group (73.5%).

An early onset of foraging reduced the life expectancy and foraging efficiency of honey bees. Irrespective of treatment, most of the bees performing foraging flights within their first five days after eclosion as adults performed less than fifteen foraging flights in their lifetime, while others performed more than a hundred (Figure 7-6). Larval imidacloprid exposure significantly accelerated foraging onset (Figure 7-2, Figure 7-6). This suggests that imidacloprid residues, fed to colonies at a concentration of 5 μg/kg, have the potential to cause a rapid failure of bee colonies by imbalancing the age-based division of labour in a colony and progressively reducing the effectiveness of the foraging force by increasing the population of precocious foragers within it (Perry et al., 2015).

It had already been proposed that neonicotinoids could affect the age-based division of labour in bee colonies (Henry et al., 2015, 2012). Our data now show the extent to which imidacloprid treatment accelerates the phase during which bees perform orientation flights, the consequences of this for successful foraging onset, and the reduced performance of foragers. Our data might predict severe consequences for colonies from even trace imidacloprid residues, but we recognise that in field studies such consequences have not always been documented at similar concentrations (Chapter 4 as Colin et al., 2019; William G. Meikle et al., 2016; Osterman et al., 2019; Woodcock et al., 2017). This may be because the social dynamics of a bee hive is complex and nonlinear, and incorporate a capacity to "buffer" stress to a degree, with little measurable consequence to colony performance (Henry et al., 2015). Models and empirical data have emphasized how colony outcomes are influenced by multiple factors, including season, resource base and colony size (Barron, 2015; Becher et al., 2014, 2013; DeGrandi-Hoffman et al., 1989; Khoury et al., 2013, 2011; Meikle et al., 2018b; Myerscough et al., 2017; Perry et al., 2015; Rumkee et al., 2015; Russell et al., 2013; Schmickl and Crailsheim, 2007). Further, we are now beginning to realise how different stressors can interact to have synergistic consequences for colony outcomes (Chapter 5; Hladik et al., 2018; Mullin

et al., 2010; Prado et al., 2019; Sanchez-Bayo and Goka, 2014; Zhu et al., 2014). Even so, better understanding of the processes by which pesticides impact colony function will allow us to improve current models and make better recommendations for colony interventions. From this perspective it is interesting that two non-neonicotinoid pesticide blends that delayed rather than accelerated the age at onset of foraging increased the survival rates of these bees (Prado et al., 2019). Pesticides are often applied as blends on crops and bees are commonly and simultaneously exposed to a variety of fungicide, insecticide and miticide combinations (Mullin et al., 2010; Prado et al., 2019; Woodcock et al., 2017). These findings suggest it may be possible to develop pesticide combinations that are less likely to have negative synergistic or additive effects on bee colonies.

Acknowledgments

Supplementary material

The Supporting Information is available free of charge on the ACS Publications website at

Supplementary Material 7-1 S1 ZIP. **Dataset with flights inferior to a minute removed and R script.**

Supplementary Material 7-2S2 ZIP. **Examples of a typical orientation to foraging flight change patterns.**

Supplementary Material 7-3S3 ZIP. **Dataset and R script for the discriminant analysis, PCA and classification of orientation and foraging flights.**

Supplementary Material 7-4S4 ZIP. **Number, average duration and total time spent on orientation and foraging flights by bees with information about their colonies of origin.**

Supplementary Material 7-5**S1 Figure. Number of flights per age for each forager.**

Supplementary Material 7-6**S2 Figure. Flight characteristics used to classify orientation and foraging flights.**

Supplementary Material 7-7**S3 Figure. Entrance of the hive equipped modified to fit 4 RFID antennae.**

References

Alburaki, M. *et al.* (2018) 'Honey bee survival and pathogen prevalence: from the perspective of landscape and exposure to pesticides', *Insects*, 9(2), pp. 1–15. doi: 10.3390/insects9020065.

Barron, A. B. (2015) 'Death of the bee hive: understanding the failure of an insect society', *Current Opinion in Insect Science*. Elsevier Inc, 10(Ccd), pp. 45–50. doi: 10.1016/j.cois.2015.04.004.

Barron, A. B., Schulz, D. J. and Robinson, G. E. (2002) 'Octopamine modulates responsiveness to foraging-related stimuli in honey bees (*Apis mellifera*)', *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 188(8), pp. 603–610. doi: 10.1007/s00359-002-0335-5.

Becher, M. A. *et al.* (2013) 'Towards a systems approach for understanding honeybee decline: A stocktaking and synthesis of existing models', *Journal of Applied Ecology*, 50(4), pp. 868–880. doi: 10.1111/1365-2664.12112.

Becher, M. A. *et al.* (2014) 'BEEHAVE: a systems model of honeybee colony dynamics and foraging to explore multifactorial causes of colony failure', *Journal of Applied Ecology*. Edited by E. Morgan, 51(2), pp. 470–482. doi: 10.1111/1365-2664.12222.

Beshers, S. N. and Fewell, J. H. (2001) 'Models of division of labor in social insects', *Annual Review of Entomology*, 46, pp. 413–440.

Blacquière, T. *et al.* (2012) 'Neonicotinoids in bees: A review on concentrations, side-effects and risk assessment', *Ecotoxicology*, 21(4), pp. 973–992. doi: 10.1007/s10646-012-0863-x.

Böhme, F. *et al.* (2017) 'Chronic exposure of honeybees, *Apis mellifera* (Hymenoptera: Apidae), to a pesticide mixture in realistic field exposure rates', *Apidologie*, 48(3), pp. 353–363. doi: 10.1007/s13592-016-0479-x.

Bonmatin, J. M. *et al.* (2003) 'A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants, and in pollens', *Analytical Chemistry*, 75(9), pp. 2027–2033. doi: 10.1021/ac020600b.

Bonmatin, J. M. *et al.* (2005) 'Quantification of Imidacloprid Uptake in Maize Crops', *Journal of Agricultural and Food Chemistry*, 53(13), pp. 5336–5341. doi:

10.1021/jf0479362.

Bordier, C. *et al.* (2018) 'Stress decreases pollen foraging performance in honeybees', *The Journal of Experimental Biology*, 221(4), p. jeb171470. doi: 10.1242/jeb.171470.

Cabirol, A. *et al.* (2018) 'Relationship between brain plasticity, learning and foraging performance in honey bees', *PLoS ONE*, 13(4), pp. 1–18. doi: 10.1371/journal.pone.0196749.

Capaldi, E. A. *et al.* (2000) 'Ontogeny of orientation flight in the honeybee revealed by harmonic radar', *Nature*, 403(6769), pp. 537–540. doi: 10.1038/35000564.

Capaldi, E. A. and Dyer, F. C. (1999) 'The role of orientation flights on homing performance in honeybees.', *The Journal of experimental biology*, 202(Pt 12), pp. 1655–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10333511.

Chauzat, M.-P. *et al.* (2006) 'A Survey of Pesticide Residues in Pollen Loads Collected by Honey Bees in France', *Journal of Economic Entomology*, 99(2), pp. 253–262. doi: 10.1603/0022-0493-99.2.253.

Chauzat, M.-P. *et al.* (2009) 'Influence of Pesticide Residues on Honey Bee (Hymenoptera: Apidae) Colony Health in France', *Environmental Entomology*, 38(3), pp. 514–523. doi: 10.1603/022.038.0302.

Chauzat, M.-P. *et al.* (2011) 'An assessment of honeybee colony matrices, *Apis mellifera* (Hymenoptera: Apidae) to monitor pesticide presence in continental France', *Environmental Toxicology and Chemistry*, 30(1), pp. 103–111. doi: 10.1002/etc.361.

Colin, T. *et al.* (2018) 'The development of honey bee colonies assessed using a new semi-automated brood counting method: Combcount', *PLoS ONE*, 13(10), pp. 1–14. doi: 10.1371/journal.pone.0205816.

Colin, T., Lim, M. Y., *et al.* (2019) 'Effects of thymol on European honey bee hygienic behaviour', *Apidologie*, 50(2), pp. 141–152. doi: 10.1007/s13592-018-0625-8.

Colin, T., Meikle, W. G., *et al.* (2019) 'Long-term dynamics of honey bee colonies following exposure to chemical stress', *Science of The Total Environment*. Elsevier B.V., 677, pp. 660–670. doi: 10.1016/j.scitotenv.2019.04.402.

Colin, T. *et al.* (no date) 'The miticide thymol in combination with trace levels of the neonicotinoid imidacloprid reduces visual learning performance in honey bees (*Apis*

mellifera)'.

Cresswell, J. E. (2011) 'A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees', *Ecotoxicology*, 20(1), pp. 149–157. doi: 10.1007/s10646-010-0566-0.

Decourtye, A. *et al.* (2005) 'Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee *Apis mellifera*', *Archives of Environmental Contamination and Toxicology*, 48(2), pp. 242–250. doi: 10.1007/s00244-003-0262-7.

Degen, J. *et al.* (2015) 'Exploratory behaviour of honeybees during orientation flights', *Animal Behaviour*. Elsevier Ltd, 102, pp. 45–57. doi: 10.1016/j.anbehav.2014.12.030.

DeGrandi-Hoffman, G. *et al.* (1989) 'BEEPOP: A honeybee population dynamics simulation model', *Ecological Modelling*, 45(2), pp. 133–150. doi: 10.1016/0304-3800(89)90088-4.

Douglas, M. R. and Tooker, J. F. (2015) 'Large-scale deployment of seed treatments has driven rapid increase in use of neonicotinoid insecticides and preemptive pest management in U.S. Field crops', *Environmental Science and Technology*, 49(8), pp. 5088–5097. doi: 10.1021/es506141g.

Ellis, C. *et al.* (2017) 'The Neonicotinoid Insecticide Thiacloprid Impacts upon Bumblebee Colony Development under Field Conditions', *Environmental Science and Technology*, 51(3), pp. 1727–1732. doi: 10.1021/acs.est.6b04791.

Fahrbach, S. E. and Robinson, G. E. (1996) 'Juvenile Hormone, Behavioral Maturation, and Brain Structure in the Honey Bee', *Developmental Neuroscience*, 18(1–2), pp. 102–114. doi: 10.1159/000111474.

Fischer, J. *et al.* (2014) 'Neonicotinoids interfere with specific components of navigation in honeybees', *PLoS ONE*, 9(3), pp. 1–10. doi: 10.1371/journal.pone.0091364.

Gauthier, M. (2010) 'State of the Art on Insect Nicotinic in Learning and Memory', in *Insect Nicotinic Acetylcholine Receptors*, pp. 98–115.

Girolami, V. *et al.* (2009) 'Translocation of Neonicotinoid Insecticides From Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees', *Journal of Economic Entomology*, 102(5), pp. 1808–1815. doi: 10.1603/029.102.0511.

Goblirsch, M., Huang, Z. Y. and Spivak, M. (2013) 'Physiological and Behavioral Changes

in Honey Bees (*Apis mellifera*) Induced by Nosema ceranae Infection', *PLoS ONE*, 8(3). doi: 10.1371/journal.pone.0058165.

Henry, M. *et al.* (2012) 'A common pesticide decreases foraging success and survival in honey bees', *Science*, 336(6079), pp. 348–350. doi: 10.1126/science.1215039.

Henry, M. *et al.* (2015) 'Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees', *Proceedings of the Royal Society B: Biological Sciences*, 282(1819), p. 20152110. doi: 10.1098/rspb.2015.2110.

Hladik, M. L., Main, A. R. and Goulson, D. (2018) 'Environmental Risks and Challenges Associated with Neonicotinoid Insecticides', *Environmental Science and Technology*, 52(6), pp. 3329–3335. doi: 10.1021/acs.est.7b06388.

Holst, N. and Meikle, W. (2018) 'Breakfast Canyon Discovered in Honeybee Hive Weight Curves', *Insects*, 9(4), p. 176. doi: 10.3390/insects9040176.

Jiang, J. *et al.* (2018) 'Concentrations of imidacloprid and thiamethoxam in pollen, nectar and leaves from seed-dressed cotton crops and their potential risk to honeybees (*Apis mellifera* L.)', *Chemosphere*. Elsevier Ltd, 201, pp. 159–167. doi: 10.1016/j.chemosphere.2018.02.168.

Kassambara, A. and Kosinski, M. (2018) 'survminer: Drawing Survival Curves using "ggplot2".'

Kerr, J. T. (2017) 'A cocktail of poisons', *Science*, 356(6345), pp. 1331–1332. doi: 10.1126/science.aan6173.

Khoury, D. S., Barron, A. B. and Myerscough, M. R. (2013) 'Modelling Food and Population Dynamics in Honey Bee Colonies', *PLoS ONE*. Edited by A. G. Dyer, 8(5), p. e59084. doi: 10.1371/journal.pone.0059084.

Khoury, D. S., Myerscough, M. R. and Barron, A. B. (2011) 'A Quantitative Model of Honey Bee Colony Population Dynamics', *PLoS ONE*. Edited by J. A. R. Marshall, 6(4), p. e18491. doi: 10.1371/journal.pone.0018491.

Klein, S. *et al.* (2017) 'Why Bees Are So Vulnerable to Environmental Stressors', *Trends in Ecology and Evolution*. Elsevier Ltd, 32(4), pp. 268–278. doi: 10.1016/j.tree.2016.12.009.

Krischik, V. A., Landmark, A. L. and Heimpel, G. E. (2007) 'Soil-Applied Imidacloprid Is Translocated to Nectar and Kills Nectar-Feeding *Anagyrus pseudococci* (Girault)

(Hymenoptera: Encyrtidae)', *Environmental Entomology*, 36(5), pp. 1238–1245. doi: 10.1603/0046-225x(2007)36[1238:siittn]2.0.co;2.

Lach, L., Kratz, M. and Baer, B. (2015) 'Parasitized honey bees are less likely to forage and carry less pollen', *Journal of Invertebrate Pathology*. Elsevier Inc., 130, pp. 64–71. doi: 10.1016/j.jip.2015.06.003.

Leoncini, I. *et al.* (2004) 'Worker-worker inhibition of honey bee behavioural development independent of queen and brood', *Insectes Sociaux*, 51, pp. 392–394. doi: 10.1007/s00040-004-0757-x.

Meikle, W. G. *et al.* (2006) 'Using balances linked to dataloggers to monitor honey bee colonies', *Journal of Apicultural Research*, 45(1), pp. 39–41. doi: 10.1080/00218839.2006.11101311.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLoS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Meikle, W. G., Adamczyk, J. J., *et al.* (2018) 'Effects of bee density and sublethal imidacloprid exposure on cluster temperatures of caged honey bees', *Apidologie*, 49(5), pp. 581–593. doi: 10.1007/s13592-018-0585-z.

Meikle, W. G., Holst, N., *et al.* (2018) 'Using within-day hive weight changes to measure environmental effects on honey bee colonies', *PLOS ONE*. Edited by A. G. Dyer, 13(5), p. e0197589. doi: 10.1371/journal.pone.0197589.

Meikle, W. G. and Holst, N. (2015) 'Application of continuous monitoring of honeybee colonies', *Apidologie*, 46(1), pp. 10–22. doi: 10.1007/s13592-014-0298-x.

Meikle, W. G. and Weiss, M. (2017) 'Monitoring Colony-level Effects of Sublethal Pesticide Exposure on Honey Bees', *Journal of Visualized Experiments*, (129), pp. 1–10. doi: 10.3791/56355.

Meikle, W. G., Weiss, M. and Stilwell, A. R. (2016) 'Monitoring colony phenology using within-day variability in continuous weight and temperature of honey bee hives', *Apidologie*, 47(1), pp. 1–14. doi: 10.1007/s13592-015-0370-1.

Menzel, R. *et al.* (2005) 'Honey bees navigate according to a map-like spatial memory', *Proceedings of the National Academy of Sciences*, 102(8), pp. 3040–3045. doi:

10.1073/pnas.0408550102.

Mitchell, E. A. D. *et al.* (2017) 'A worldwide survey of neonicotinoids in honey', *Science*, 358(6359), pp. 109–111. doi: 10.1126/science.aan3684.

Mullin, C. A. *et al.* (2010) 'High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health', *PLoS ONE*, 5(3). doi: 10.1371/journal.pone.0009754.

Myerscough, M. R. *et al.* (2017) 'Why Do Hives Die? Using Mathematics to Solve the Problem of Honey Bee Colony Collapse', in Anderssen, B. et al. (eds) *The Role and Importance of Mathematics in Innovation*. Singapore: Springer Singapore, pp. 35–50.

Nazzi, F. and Pennacchio, F. (2018) 'Honey bee antiviral immune barriers as affected by multiple stress factors: a novel paradigm to interpret colony health decline and collapse', *Viruses*, 10(4), p. 159. doi: 10.3390/v10040159.

Nicholls, E. *et al.* (2018) 'Monitoring Neonicotinoid Exposure for Bees in Rural and Peri-urban Areas of the U.K. during the Transition from Pre- to Post-moratorium', *Environmental Science and Technology*, 52(16), pp. 9391–9402. doi: 10.1021/acs.est.7b06573.

Osterman, J. *et al.* (2019) 'Clothianidin seed-treatment has no detectable negative impact on honeybee colonies and their pathogens', *Nature Communications*. Springer US, 10(1), p. 692. doi: 10.1038/s41467-019-08523-4.

Perry, C. J. *et al.* (2015) 'Rapid behavioral maturation accelerates failure of stressed honey bee colonies', *Proceedings of the National Academy of Sciences*, 112(11), pp. 3427–3432. doi: 10.1073/pnas.1610243113.

Prado, A. *et al.* (2019) 'Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees', *Science of the Total Environment*. Elsevier B.V., 650, pp. 1250–1260. doi: 10.1016/j.scitotenv.2018.09.102.

R Core Team, R. (2018) 'R: A language and environment for statistical computing. R Foundation for Statistical Computing'. Vienna, Austria. Available at: https://www.r-project.org/.

Rotheray, E. L. *et al.* (2016) 'Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops', *Environment International*, 88, pp. 169–178. doi: 10.1016/j.envint.2015.12.011.

Rumkee, J. C. O. *et al.* (2015) 'Predicting Honeybee Colony Failure: Using the BEEHAVE Model to Simulate Colony Responses to Pesticides', *Environmental Science and Technology*, 49(21), pp. 12879–12887. doi: 10.1021/acs.est.5b03593.

Russell, S., Barron, A. B. and Harris, D. (2013) 'Dynamic modelling of honey bee (*Apis mellifera*) colony growth and failure', *Ecological Modelling*. Elsevier B.V., 265, pp. 158–169. doi: 10.1016/j.ecolmodel.2013.06.005.

Samson-Robert, O. *et al.* (2017) 'Planting of neonicotinoid-coated corn raises honey bee mortality and sets back colony development', *PeerJ*, 5, p. e3670. doi: 10.7717/peerj.3670.

Sánchez-Bayo, F. and Desneux, N. (2015) 'Neonicotinoids and the prevalence of parasites and disease in bees', *Bee World*. Taylor & Francis, 92(2), pp. 34–40. doi: 10.1080/0005772X.2015.1118962.

Sanchez-Bayo, F. and Goka, K. (2014) 'Pesticide residues and bees - A risk assessment', *PLoS ONE*, 9(4). doi: 10.1371/journal.pone.0094482.

Schmickl, T. and Crailsheim, K. (2007) 'HoPoMo: A model of honeybee intracolonial population dynamics and resource management', *Ecological Modelling*, 204(1–2), pp. 219–245. doi: 10.1016/j.ecolmodel.2007.01.001.

Schmolke, A., Kearns, B. and O'Neill, B. (2018) 'Plant guttation water as a potential route for pesticide exposure in honey bees: a review of recent literature', *Apidologie*, 49(5), pp. 637–646. doi: 10.1007/s13592-018-0591-1.

Schulz, D. J., Barron, A. B. and Robinson, G. E. (2002) 'A role for octopamine in honey bee division of labor', *Brain, Behavior and Evolution*, 60(6), pp. 350–359. doi: 10.1159/000067788.

Schulz, D. J., Huang, Z. Y. and Robinson, G. E. (1998) 'Effects of colony food shortage on behavioral development in honey bees', *Behavioral Ecology and Sociobiology*, 42(5), pp. 295–303. doi: 10.1007/s002650050442.

van der Sluijs, J. P. *et al.* (2013) 'Neonicotinoids, bee disorders and the sustainability of pollinator services', *Current Opinion in Environmental Sustainability*. Elsevier B.V., 5(3–4), pp. 293–305. doi: 10.1016/j.cosust.2013.05.007.

Søvik, E. *et al.* (2015) 'Negative impact of manganese on honeybee foraging', *Biology Letters*, 11(3), pp. 1–4. doi: 10.1098/rsbl.2014.0989.

Tapparo, A. *et al.* (2011) 'Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds', *Journal of Environmental Monitoring*, 13(6), p. 1564. doi: 10.1039/c1em10085h.

Tapparo, A. *et al.* (2012) 'Assessment of the environmental exposure of honeybees to particulate matter containing neonicotinoid insecticides coming from corn coated seeds', *Environmental Science and Technology*, 46(5), pp. 2592–2599. doi: 10.1021/es2035152.

Therneau, T. M. (2015) 'A Package for Survival Analysis in S'. Available at: https://cran.r-project.org/package=survival.

Tison, L. *et al.* (2016) 'Honey Bees' Behavior Is Impaired by Chronic Exposure to the Neonicotinoid Thiacloprid in the Field', *Environmental Science & Technology*, 50(13), pp. 7218–7227. doi: 10.1021/acs.est.6b02658.

Tison, L. *et al.* (2017) 'Effects of sublethal doses of thiacloprid and its formulation Calypso ® on the learning and memory performance of honey bees', *The Journal of Experimental Biology*, 220(20), pp. 3695–3705. doi: 10.1242/jeb.154518.

Tsvetkov, N. *et al.* (2017) 'Chronic exposure to neonicotinoids reduces honey bee health near corn crops', *Science*, 356(6345), pp. 1395–1397. doi: 10.1126/science.aam7470.

Venables, W. N. and Ripley, B. D. (2002) *Modern Applied Statistics with S.* Fourth Edi. New York: Springer.

Williamson, S. M. *et al.* (2013) 'Exposure to Acetylcholinesterase Inhibitors Alters the Physiology and Motor Function of Honeybees', *Frontiers in Physiology*, 4(February), pp. 1–10. doi: 10.3389/fphys.2013.00013.

Winston, M. L. (1987) *The biology of the honey bee*. Harvard University press.

Woodcock, B. A. *et al.* (2017) 'Country-specific effects of neonicotinoid pesticides on honey bees and wild bees', *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

Woyciechowski, M. and Moroń, D. (2009) 'Life expectancy and onset of foraging in the honeybee (*Apis mellifera*)', *Insectes Sociaux*, 56(2), pp. 193–201. doi: 10.1007/s00040-009-0012-6.

Xue, Y. *et al.* (2015) 'Quantifying Neonicotinoid Insecticide Residues Escaping during Maize Planting with Vacuum Planters', *Environmental Science and Technology*, 49(21), pp.

13003–13011. doi: 10.1021/acs.est.5b03753.

Yang, E. C. *et al.* (2012) 'Impaired Olfactory Associative Behavior of Honeybee Workers Due to Contamination of Imidacloprid in the Larval Stage', *PLoS ONE*, 7(11). doi: 10.1371/journal.pone.0049472.

Zanni, V. *et al.* (2018) 'The reduced brood nursing by mite-infested honey bees depends on their accelerated behavioral maturation', *Journal of Insect Physiology*, 109(June), pp. 47–54. doi: 10.1016/j.jinsphys.2018.06.006.

Zhu, W. *et al.* (2014) 'Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0077547.

Summary

In this thesis, I examined the effect of common agrochemicals on the health of European honey bees. I first developed two methods to ease the study of bee colonies (Chapters 2 and 3), that I later applied to a large-scale field experiment to test the effect of agrochemicals at two locations. Based on results from this study and from the literature, I formulated hypotheses regarding the modes of action of these pesticides that I tested in the last three chapters of the thesis (Figure 8-1, p. 225).

The method in Chapter 2 is a tool that automatically fits piecewise regressions on the daily weight variation data that are obtained from automatic hive scales. This method estimates the daily foraging intensity and net weight gain of colonies automatically, and is useful for large datasets. The method developed in Chapter 3 involves free software that detects empty cells on pictures of frames of comb from hives to speed up measures of capped brood and honey cells. This method effectively reduces the time that was previously needed to precisely measure the amounts of capped brood, honey.

I then used these methods to test, in Chapter 4, the effect of a low concentration of a pesticide commonly applied on several crops (the neonicotinoid imidacloprid) on the growth of bee colonies. We found a small negative, but non-significant, effect on colonies at one location, and a significant positive effect on the brood and bee populations at another location (Figure 4-1, p. 111).

In Chapter 4, we also exposed some hives to the miticide thymol. Thymol targets a parasite of the honey bee, the mite *V. destructor,* a known vector of several diseases thought to be

partially responsible for colony losses (Le Conte et al., 2010). This mite was absent from our field site in Australia but present in Tucson. At both locations, the colonies treated with thymol had fewer bees and less food stores in the three months following the treatment than control colonies. It is interesting that the negative effects of thymol on bee colonies were less pronounced in Tucson (Figure 4-1, p. 111) where *V. destructor* is present, although even in Tucson there were no positive effects on bee colonies to demonstrate a net benefit to using thymol as a miticide in bee colonies. In Tucson, three colonies treated with thymol and two control colonies died during the experiment (Figure 4-1, p. 111), which also suggests that thymol did not prevent colony death despite its effect on *V. destructor* populations (Figure 4-2, p. 117).

In Chapter 4, I also simultaneously exposed some colonies to both pesticides and found no evidence of interactions when these two pesticides were used in combination at these specific doses (the results of the combined treatment group were similar to the results of the thymol group, see Figure 4-1, p. 111).

I then designed a series of short experiments on individuals or groups of bees to test whether common hypotheses from the literature could explain the effects I observed on the bee colonies in Chapter 4.

I tested the effect of imidacloprid, tau-fluvalinate and thymol on the learning or memory abilities of bees in Chapter 5, and only bees simultaneously exposed to imidacloprid and thymol were negatively affected by these treatments (Figure 5-1, p. 148; Figure 5-2, p.150).

I tested the effect of thymol on the removal of freeze-killed brood and bees in Chapter 6. Bees exposed to thymol removed the dead brood more rapidly (Figure 6-2, p. 172), although observations from the literature suggest thymol may kill brood or cause bees to remove healthy brood also (Baggio et al., 2004; Imdorf et al., 1999; Marchetti et al., 1984).

In Chapter 7, I tested whether imidacloprid caused a change in foraging activity or the age at onset of foraging that could be beneficial in certain environments and deleterious in others, and found that bees exposed to imidacloprid as larvae started foraging earlier in life, performed fewer flights in their lifetime, and died younger (Figure 7-2, p. 201; Figure 7-3, p. 202; Figure 7-7, p. 207).

My results highlight how sensitive bees are to low amounts of some of the most common environmental stressors, including those used by beekeepers, and give a clear picture of the complexity of measuring and predicting effects of agrochemicals on standard bee colonies. Taken together, the results from the different chapters show how difficult it is to translate individual-based observations to colony-level effects, and to generalize findings across environments.

Effects of common agrochemicals on the health and function of honey bee colonies

Chapter 2 & Chapter 3	**Development of new methods to ease the study of honey bees at the colony level** **Semi-automation of brood and honey meaurements** **Automation of daily weight changes measurements**

Methods applied to a colony level ecotoxicology experiment

Chapter 4	**Effects of thymol and imidacloprid on honey bees at the colony level** The miticide thymol had a negative effect in the absence and no effect in the presence of *V. destructor* on bee colonies The effects of the neonicotinoid imidacloprid depended on the location. They were negative and limited in Sydney but caused bee colonies to produce more honey and bees in Arizona

How does thymol negativaly affect bee colonies?

How does imidacloprid affect bee colonies? Why does it vary with the environment?

Chapter 6 Hygiene	Chapter 5 Learning and memory	Chapter 7 Ontogeny and performance
Thymol increased hygiene but may have caused the death or removal of healthy brood.	**Thymol** and **imidacloprid** affected the bee's ability to avoid color associated with electric shocks but only when they were combined.	Bees exposed to **imidacloprid** as larvae started foraging earlier in life, performed less trips, died younger. This could be beneficial in environments were food is extremely scarce.

Perspectives

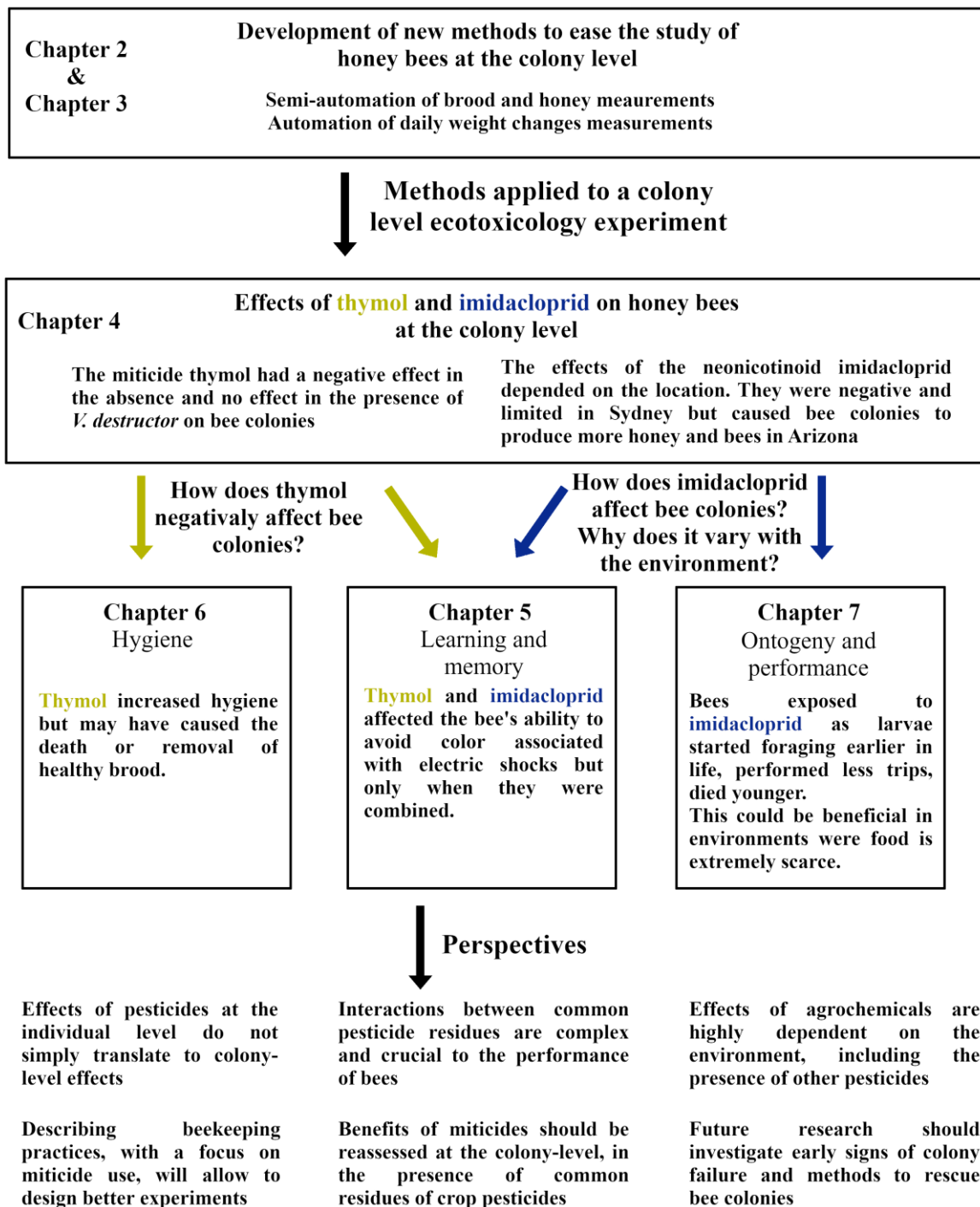|		
Effects of pesticides at the individual level do not simply translate to colony-level effects Describing beekeeping practices, with a focus on miticide use, will allow to design better experiments	Interactions between common pesticide residues are complex and crucial to the performance of bees Benefits of miticides should be reassessed at the colony-level, in the presence of common residues of crop pesticides	Effects of agrochemicals are highly dependent on the environment, including the presence of other pesticides Future research should investigate early signs of colony failure and methods to rescue bee colonies

Figure 8-1 **Main conclusions and summary of the thesis.**

1. Mitigating impacts of pesticide exposures on bees

Avoiding unnecessary or inefficient treatments should thus be a priority to improve the health of pollinators. The benefits of neonicotinoid insecticides and miticides are surprisingly unclear. Several studies have failed to detect a positive effects of neonicotinoid treatments on crop yields (e.g. Adams et al., 2016; Alford and Krupke, 2017; Jordan et al., 2012; Krupke et al., 2017; Wilde et al., 2007) especially given their high cost. The lack of evidence for yield benefits and costs are reviewed and discussed in Goulson, (2013). There are also concerns that miticide treatments may not always benefit bee colonies, because they commonly have negative effects on bees (Tihelka, 2018). In Chapter 4, we have shown that colonies treated with thymol during the summer did not perform better than control colonies in Arizona. Pesticides that do not always have clear benefits for bee colonies or on crop yields are however still commonly applied, possibly because farmers often give great importance to information provided by chemical dealers (Lichtenberg and Zimmerman, 1999). The results presented in this thesis suggest that for cases where these treatments are still applied, beekeepers may be able to mitigate their negative effects or to optimize miticide treatments to maximise their net benefit.

The effects of miticides on bees could be improved by targeting colonies with high mite loads only. In Chapter 4, we tested the effect of thymol on colonies free of *Varroa destructor* in Australia. We showed that thymol temporarily decreased population growth and the accumulation of food stores in the colonies. The difference between the control and the thymol group was less striking in Tucson, where *V. destructor* is present in the colonies (Figure 4-1, p.111). Thymol efficiently reduced the number of mites in the treated colonies in Tucson, which may have compensated for its negative effects on bees without being sufficient to provide clear colony-level benefits. It is possible that in colonies with higher mite loads, the

benefits of the thymol treatment may outweigh its negative effects on bees. The number of mites in the colonies located in Tucson was within the range of what most beekeepers would usually consider low (*Figure 4-2* p.117), but the bee colonies were small and a low number of mite could represent a high parasite load for a small colony.

The effects of miticides seem to vary with the environmental conditions (Beyer et al., 2018) and their negative effects could potentially be mitigated by avoiding their use outside of optimal environmental conditions. In Chapter 4, we applied the thymol treatment during Summer, when it has been suggested to be most efficient (Beyer et al., 2018), but it is also common practice among beekeepers to expose bee colonies to miticides in Autumn. This is probably done by beekeepers because high *V. destructor* loads in Autumn have previously been associated with increased overwintering mortality in the scientific literature (Guzmán-Novoa et al., 2010; Le Conte et al., 2010). Manufacturers of different thymol formulations for the treatment of bee colonies commonly note that the effect of thymol on bee colonies is dependent on the environmental temperature and leads to higher mite death when the temperature exceeds 25°C (Suard, 2009; Vita Bee Health, 2017) but no rigorous tests exist at the colony-level to compare the efficiency of miticides in different environmental conditions.

In Chapter 6, we mentioned observations from the literature suggesting that thymol may cause the removal or death of healthy brood, which could explain the delayed, negative effects on adult bee populations and food stores observed in Chapter 4. Marchetti et al., (1984) noted "When the compound was put near the brood [in a gauze bag], we observed the removal of many larvae and pupae from cells immediately adjacent to the gauze bag." which suggests the negative effects of thymol on brood could be mitigated by increasing the distance between the thymol container and the brood. This may however in turn reduce the efficiency of the thymol treatment because female mites are often located near the larvae onto which they lay their eggs, and the extended exposure to thymol may cause the removal of the brood regardless of its

proximity with the container. It cannot be excluded that part of the efficiency of the thymol treatments against *V. destructor* infestations may be due to increased brood mortality and removal, which would disrupt the varroa's life cycle. The negative effects of thymol on bees may thus be hard to dissociate from its negative effects on *V. destructor*.

The effects of imidacloprid on the amount of brood, bees and honey in Sydney seems to result from effects on brood (Figure 4-1, p.111). This may be because hive bees may start foraging earlier in life after being exposed to imidacloprid (Figure 7-2 p.201) and fewer hive bees may be available to take care of the brood, as I have shown in Chapter 7. These negative effects on the brood seemed to have resulted in a diminution (however not significant) of the bee populations in hives exposed to imidacloprid exposed (Figure 4-1, p.111). This, in turn, could have reduced the growth of the honey stores in the first three months following the end of the treatment. If this is the case, beekeepers could reinforce colonies weakened by exposure to imidacloprid-treated crops by adding brood from colonies located in other environments.

In Tucson, bee colonies exposed to imidacloprid had more bees, brood and food stores after the end of the treatment, but these levels were still below those of the hives located in Sydney. As I have discussed in Chapter 4 and 7, I believe this may be caused by food scarcity. Food scarcity could happen in intensive agricultural landscape once the large-scale blooms are over if herbicides have eliminated other wild flowers from crops and hives start losing weight. This result is however irrelevant to beekeepers who typically do not keep colonies in areas with poor flower resources after crop blooms but migrate them to more propitious areas.

The effects of other insecticides on bee colony function are likely to reflect the wide diversity of chemicals used for crop protection. It is thus difficult to propose general ways to mitigate their effects on honeybees, especially given the scarcity of colony-level studies and the limited knowledge on pesticide interactions.

2. Individual to colony-level effects

a) Colony-level and individual studies

The field of honey bee and bumblebee ecotoxicology is commonly divided between studies conducted on isolated individual bees and studies at the level of the colony. These research practices are best described in Blacquière et al. (2012). Briefly, Individual-based studies usually focus on parameters such as learning, navigation, individual survival, locomotor activity, syrup intake, cell counts, body weight and gene expression or enzyme activity. Bees are usually taken from their hives and isolated in the laboratory in tubes (individual-based studies) or cages (group-based studies) and fed sugar syrup. Colony-level studies usually estimate parameters such as the number of brood cells and adult bees, honey production, regulation of in-hive temperature or hygrometry, daily hive weight changes or activity at the hive's entrance. In colony-level studies, colonies are typically placed in crops treated with pesticides or not treated, or fed sugar and water syrup spiked with the stressors of interest and left to forage freely outside.

Although colony-level effects are the main target of most honey bee ecotoxicology research questions, individual-based studies remain more common than colony-level studies because they are faster, cheaper and less labour-intensive (see discussions of the costs of colony-level studies in Meikle and Holst, 2015; Meikle and Weiss, 2017).

b) Inferring from individual studies to colony-level outcomes

The effects observed on individual bees are difficult to extrapolate. They do not simply linearly translate into colony-level effects, because interactions between members of the colony can buffer or amplify the effects of individual stress for the colony. Colonies may buffer the

234

loss of foragers through the early onset of foraging of young bees that typically stay within the hives during the first two weeks of their lives (Huang and Robinson, 1996; Leoncini et al., 2004). But this recruitment of young foragers may also amplify the effects of stressors if they are sustained, because young bees that start foraging when younger are more sensitive to stress, and may die even earlier than those of the generation they replaced (Perry et al., 2015).

In individual-based studies, bees can also often not freely forage outside the hive. This makes the interpretation and extrapolation of the results from individual based studies particularly difficult given that colonies are most often exposed to complex pesticide blends when they forage on crops (Mullin et al., 2010; Pohorecka et al., 2017). Interactions between pesticides can have complex effects on the longevity and age at onset of foraging of honey bees (Prado et al., 2019).

Other individual-based studies merely investigate the lethal effects of stressors on bees. They traditionally report the concentration of a stressor causing 50% of the bees to die as their main result (LD50). These studies however provide no insights into common sublethal effects known to have dramatic impacts on bee colonies and their results cannot be extrapolated to colonies.

c) Differences between individual and colony-level studies in this thesis

In this thesis, I also observed discrepancies between the results of the colony-level assay from Chapter 4 and from the individual or group-based experiments in Chapters 5 to 7.

In Chapter 4, the bee population in hives treated with thymol dropped in the evaluation following the end of the treatment (Figure 4-1, p. 111). To explain these losses I decided to test the common hypothesis that learning and memory, functions essential to bee workers, were affected by these agrochemicals in Chapter 5 (see Klein et al. (2017) for a review). For this

experiment I used a standardized, aversive visual learning assay, that had previously been developed to answer fundamental questions (Plath et al., 2017). The results from this assay showed a negative effect of the combined exposure to imidacloprid and thymol, but no effect of imidacloprid or thymol alone (Figure 5-1, p. 148; Figure 5-2, p.150). This did not match with our colony-level observations from Chapter 4. At the level of the colony, we found significant effects of the two pesticides alone, and no evidence of a clear interaction between the two pesticides (Figure 4-1, p. 111). The combined treatment group performed very similarly to the thymol group, suggesting that thymol alone was driving the negative effects at the colony-level when bees were exposed simultaneously to both pesticides. Non-speculative interpretation of these contradicting results is limited because it is still unclear how aversive, shock-associated colour learning and memory relates to navigation and more broadly to colony-level effects. Zhang and Nieh, (2015) note that "Aversive learning is important because learning to avoid predators should enhance bee survival." although no reference is mentioned and the authors recognize it is unclear whether olfactory aversive learning is actually linked to survival to predation. Future research should verify whether olfactory or visual learning abilities are linked to the survival of individual bees before the assays are used to evaluate the risks that stressors may affect bee colonies.

In Chapter 4, I also found that thymol caused a reduction in brood and bee populations, and hypothesized this was due to a negative effect of thymol on hygienic behaviours that increased brood mortality. But in Chapter 6, I found that thymol increased the uncapping and removal of dead brood (Figure 6-2, p. 172). Thymol cannot cause negative colony-level effects by simply increasing hygienic behaviours. Observations from the literature however suggest thymol also causes bees to remove healthy, live brood, shortly after its introduction in the hive (Baggio et al., 2004; Imdorf et al., 1999; Marchetti et al., 1984). Thymol may trigger hygienic behaviours

to a level such that bees reject brood from the hive regardless of its condition, or interact with cues that allow bees to perceive the brood as healthy.

In Chapter 4 finally, imidacloprid had a positive effect on the colonies located near Tucson and a limited, non-significant, negative effect on the colonies located in Sydney (Figure 4-1, p. 111). But in Chapter 7, bees exposed to imidacloprid as larvae and equipped with RFID tags showed a drastic reduction in foraging flights (Figure 7-3, p. 202). These observations do not simply fit with my colony-level results. I believe a complex interaction between food availability and the population dynamics of bee colonies can explain these results. An early onset of foraging and increase in mortality could benefit colonies when food is extremely scarce, which was the case in chapter 4 for the colonies located near Tucson. It can also have deleterious effects on the colony when food is abundant, as food processing reaches its maximum capacity and the colony gets no benefits from workers that shift from in-hive tasks to foraging earlier in life. This was likely the case in Sydney were food is abundant (Figure 4-1, p. 111). Increases in forager death could benefit colonies when food is scarce, because bees likely expend energy to forage and come back empty-handed, further accelerating the decline of the hives' food stores.

d) Merits and limitations of individual and colony-level studies

Colony-level studies most often measure colony productivity and the number of workers available to pollinate flowers. They directly address concerns from beekeepers and farmers but offer little insights into the mechanisms that may have caused the changes observed. They often have a low resolution and sample size, which means effects of small amplitudes are hardly detected, and because of their costs they are hardly replicated across landscapes. They do, however, keep bees in more realistic conditions - in their highly social environment - and exposed to other relevant environmental stress. Colony-level measures also leave social

interactions undisturbed so they encompass the potential alterations of the effects of stressors by the social interactions in the colony.

Colony-level studies cannot however detect the effects of stressors that are buffered by social interactions. Stressors that do not cause harm alone to a colony could accumulate or interact with other stressors beyond the colony's buffering capacity. Measuring the extent of the individual effects that are buffered at the colony-level is important. It allows us to determine whether a stressor could affect the capacity of a colony at buffering additional stress. However, few individual-based studies actually investigate interactions between stressors (Benuszak et al., 2017), or try to predict colony-level effects from their results at the individual level (but see Henry et al., 2015, 2012 and Monchanin et al., 2019).

The main limitation of individual-based studies is the difficulty to extrapolate findings. Individual based studies are useful to develop rapid and cheap markers of colony failure (Smart et al., 2016), or to detect negative effects of pesticides that are buffered at the level of the colony (Henry et al., 2015, 2012).

e) Prospects to improve the usefulness of individual-based studies

The multiplication of fast and cheap individual-based studies can bring confusion to policy-makers, when they are not able to detect effects on some of the parameters measured. This is notably the case when it is unclear whether the parameters studied were relevant, or when the sample size is too low to detect any such effects. To ensure honeybee ecotoxicological studies bring more guidance than confusion, I believe it is essential to only resort to individual-based experiments in two cases; (1) when testing clear mechanistic hypotheses; (2) to complement colony-level studies reporting no effects of pesticides, in order to confirm the absence of negative effects that could be merely buffered at the colony-level in the absence of other stressors.

One useful methodology that can bridge individual-based results and colony-level effects is the use of QR codes or RFID transponders to follow the life history of individual bees from their eclosions to their death. Such studies are well-suited to this purpose because they provide detailed data on mortality and foraging efficiency and can still be done over short periods of time. Bees are kept in a relevant environment because they are placed back into a hive after they have been equipped with the RFID transponders or QR codes (Figure 8-2). The data obtained can easily be used to inform models of bee colony population dynamics to predict colony-level effects, because they can easily be extrapolated to match common parameters of these models (Perry et al., 2015). QR code and RFID studies should ideally be complemented by brood survival assays because they cannot detect lethal effects on brood.
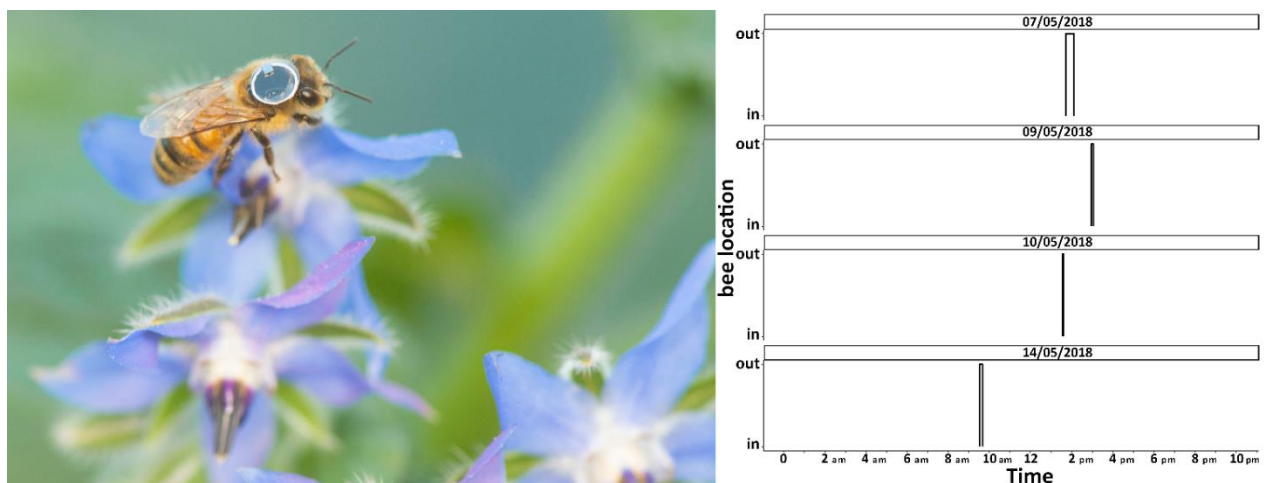

Figure 8-2. **Small RFID tags can be glued on a bees' thorax to detect when it enters or leaves the hive (on the left, a bee carries a small RFID tag of Chinese manufacture). The time and duration of flights can be recorded automatically (right, the orientation flights of a same bee recorded over a week).**

3. Effects of common pesticides on honey bee colonies depend on the environment

How effects of agrochemicals on pollinators generalize across landscapes and climates is a major, recently highlighted issue in the field (Woodcock et al., 2017). The data obtained from the different replicates of my experiments further show how difficult it is to generalize conclusions across locations.

Chapters 4, 5 and 6 were replicated independently in time or location respectively (I took part in one evaluation of the replicate in Chapter 4 to facilitate replicability). The effects of the same pesticides differed between all replicates at least in the timing or duration of the effects, and were opposite in some cases.

In Chapter 4, no colonies died in Sydney while 6 perished in Tucson. Colonies exposed to thymol in Sydney were more negatively affected than those exposed to the same treatment in Tucson, possibly because *V. destructor*, the target of this treatment, is absent from Australia. Hives treated with imidacloprid had lower honey stores than controls in Sydney, but had more honey, brood and bees than controls in Tucson (Figure 4-1, p. 111).

In chapter 5, thymol did not significantly affect the learning abilities or short-term memory of bees unless it was combined with imidacloprid (Figure 5-2, p.150). This suggests that the effects of thymol and imidacloprid on short-term aversive learning or memory depends on the co-occurring pesticide contaminants.

In chapter 6, finally, thymol affected the hygienic behaviour of bees in the same way at both locations, but the effect on brood removal was found only in late treatment in Hobart and only in early treatment in Sydney (Figure 6-2, p. 172).

The methods we used are considered standard in this field (Perry et al., 2015; Plath et al., 2017; Søvik et al., 2015; Spivak and Downey, 1998) and our experiments were well replicated. Small differences in methodologies are difficult to avoid when replicating experiments across continents (for example hives differ slightly in dimensions in Chapter 4), but seem unlikely to cause drastic changes. Rather, interactions with pesticide residues external to the experiments, local presence or outbreaks of parasites and diseases, bee stocks and climate variations are most likely to have caused these differences.

Since the beginning of my PhD in August 2016, more examples of these interactions and of the difficulty to generalize results obtained from honey bee studies have been published. The efficiency of thymol is now known to vary across seasons in Luxembourg (Beyer et al., 2018) and negative effects of several commercial miticides vary between studies (Tihelka, 2018). Complex behavioural effects have been observed in bees exposed to certain pesticide combinations but not in others (Prado et al., 2019) and the effects of neonicotinoids on whole bee colonies vary between states and countries within the same studies (Meikle et al., 2016; Woodcock et al., 2017).

These findings indicate that, in addition to previous recommendation for the study of adverse effects of pesticides on pollinators (EFSA, 2013), future experiments should be replicated under the different relevant climates before ruling out any effects. It also seems essential to keep bees exposed to a representative sample of other pesticides and pollutants that they are susceptible to encounter simultaneously with the pesticide of interest. This can be done by letting them forage freely in landscapes representative of current agricultural practices in terms of pesticide treatments.

4. Future prospects

There is little doubt left that many common crop insecticides and in-hive miticides affect the health of honey bees (Henry et al., 2012; Lundin et al., 2015; Prado et al., 2019; Simon-Delso et al., 2015; Tihelka, 2018; Tsvetkov et al., 2017; Woodcock et al., 2017), but several research questions that could help improve honey bees' health remain unanswered.

One area of central importance is the early detection and prediction of colony failure. Chapter 4 was designed to answer such questions. Frequent hive evaluations and continuous monitoring are useful to detect early warnings and predictors of colony death. In the near future, I will use these data along with unpublished and published datasets to try to identify early signs of colony failure. I will use the data from studies that repeatedly measure the number of brood cells, adults and honey where some colonies have died and other survived (e.g. Meikle et al., 2016; Osterman et al., 2019) to determine if one or a combination of these measures can be used as reliable and simple early predictor of colony failure and productivity. Depending on their availability, environmental data could be included in the analyses to determine environmental conditions that increase the likeliness of colony failure events. To date, such studies have been geographically limited, have focused on overwintering success only, and bee population evaluations were performed shortly before winter. These results are interesting because they show that *V. destructor* levels (Dainat et al., 2012; Smart et al., 2016) and colony-size (Döke et al., 2019) in autumn are predictors of overwintering success, but it can be hard for beekeepers to rescue hives in Autumn as colonies tend to reduce in size and the weather may be too cold for beekeepers to open their hives. Finding early predictors of success, when hives are established or earlier in Summer, would leave more time for beekeepers to rescue their hives through brood transfers or additional treatments against *V. destructor*.

Once early indicators of colony failure have been identified, it will be crucial to focus on methods to prevent the death of bee colonies. Beekeepers already use a wide variety of methods to reinforce "weak" colonies. It is, for example, common practice to add frames of brood, honey or pollen from other colonies, to supplement bees with sugar, pollen or protein-rich mixtures, or to apply additional treatments against *V. destructor*. These practices have been poorly described and their efficiency remains largely untested while their costs are usually high for beekeepers. Common concerns include the spread of diseases through the transfer of equipment or live bees between colonies, the risk of weakening the colony by feeding bees sugar syrup poor in other nutrients or ill-suited to bees, or contaminated protein blends. There is therefore a real risk that some interventions may do more harm than good.

The control of *V. destructor* populations is of major importance for honey bee colony health (Dainat et al., 2012; Smart et al., 2016). The benefits of miticide treatments for bee colonies and honey production are however not well established (Beyer et al., 2018; Dietemann et al., 2012; Tihelka, 2018). All commercially available organic and synthetic acaricide seem to have at least some deleterious effects on honey bees that could undermine their positive action (Tihelka, 2018). Miticides are also likely to interact with other environmental stressors, including insecticides and fungicides (Chapter 5, Johnson et al., 2013; Tihelka, 2018). The effects of miticides can be severe and include increased worker, brood and colony mortality, decreases in bee's learning abilities, effects on the bee's microbiome, or decreases in queen and drone fecundity (Tihelka, 2018). Some miticides also seem to have a limited efficacy against *V. destructor* (Rosenkranz et al., 2010) and mites commonly become resistant to certain treatments (Lodesani and Costa, 2005). Assessments of the effects of miticides on the productivity and survival of bee colonies are mostly lacking (but see Beyer et al., 2018). Despite concerns about the deleterious effects on honey bees research in this field is still limited. This is especially concerning given the continuing development of new commercially

available treatments (e.g. Rademacher et al., 2015). Miticide residues are ubiquitous in hives (Mullin et al., 2010), but research on the effects of miticides on honeybees is increasing much less rapidly than research on crop insecticides (Figure 8-3, data obtained from the Web Of Science database in July 2019). The research and development of non-chemical integrated pest management (IPM) methods for *V. destructor* was previously recommended (Dietemann et al., 2012). This could reduce the risks of side-effects on bees and interactions between miticides and environmental stressors.

Similarly, the treatment methods actually used by beekeepers to treat *V. destructor* are mostly undescribed and many do not seem to simply follow manufacturer's instructions. Most beekeepers seem to combine or repeat treatments with different molecules, treat over a broad range of seasons during the year, and local preferences for certain treatments have been reported (Beyer et al., 2018; Brodschneider et al., 2019). Combined treatments may aim at reducing the emergence of *V. destructor* strains resistant to certain treatments, which is commonly reported for some miticides (Lodesani and Costa, 2005; Milani, 1999). The use of home-made mixtures of organic acaricide is often reported by amateur beekeepers, although undescribed in the scientific literature. Beekeepers seem to resort to home-made treatments when they observe harmful effects of commercial treatments on bee colonies or because they perceive treatments as not efficient or harmful to the environment. Beekeeper's choices in the USA seem to be mostly dependent on their philosophy regarding the use of synthetic treatments and on the number of hives they possess (Underwood et al., 2019). This result could reflect economic choices and differences between the practices of amateurs and commercial beekeepers. Describing these practices is necessary so scientist can test the efficacy of pesticide blends and treatments as they are actually applied by beekeepers.
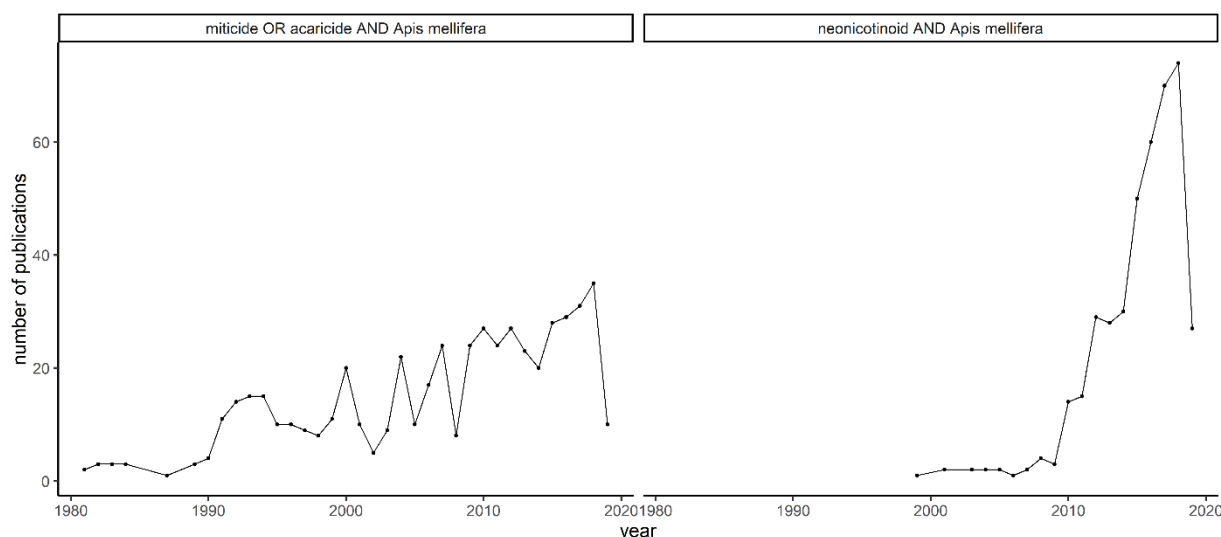
Figure 8-3. **Despite increasing concerns about effects of miticides on honeybees, comparative research is lacking and the field is progressing much slower than those of more recent crop insecticides.**

More colony-level assessments of miticides and crop insecticides are needed. To meet the expectations in terms of the sample size necessary to perform powerful statistical analyses, scientists would benefit from collaborating directly with beekeepers. Such collaborations are difficult to establish but can dramatically increase sample size. Commercial beekeepers can be invited to participate in studies investigating the effects of landscape composition on the development of bee colonies, because they often have several apiaries distributed across different habitats for crop pollination, honey and queen production. It is unlikely commercial beekeepers would allow researchers to experimentally expose their colonies to stressors as this may impact their revenue. These collaborations could also improve how representative studies are of the real hives' condition in commercial apiaries. Most colony-level studies to date have been limited in sample size because of the costs and labour they are associated with. For this

reason, "weak" or "small" colonies are often excluded from the experiment, as they are considered likely to perish independently from the treatments and to add noise to the data (e.g Osterman et al. 2019). These colonies however are part of the normal apiaries and are likely to be part of the colony losses that most recent studies on honey bee health try to investigate. Without including a representative sample of the size and health of bee colonies in apiaries, the negative effects of agrochemicals on bee colonies are likely to be underestimated. Both commercial and amateur beekeepers would however most likely accept studies that compare the effects of miticides, as they usually apply these to their hives once or twice a year. It may however be hard to convince them to keep a control group, because not treating the hives could increase their chances of dying, although a control group may not be necessary if the goal is to determine the most efficient treatment. Another difficulty is to track the beekeeper's interventions on the hives and to prevent interventions that may confound the experiment. Beekeepers may be tempted to rescue weak hives, which could mask the effects of the treatments, and to harvest honey at different periods depending on the treatments to avoid contaminations or if the hives produce different amounts of honey depending on the treatment.

Beekeepers could also provide person power. Many amateur beekeepers are interested in getting involved with bee research. Engaging them to help during experiments could allow scientists to design more relevant experiments, reduce labour costs, and accelerate experiments because beekeepers are already trained to handle bees. Beekeepers would benefit from these collaborations because they would receive additional training and increased access to recent knowledge in the field. These collaborations could also be great opportunities to increase outreach and bring honey bee research closer to its applications, especially if the beekeepers involved are members of beekeeping associations and are likely to disseminate the knowledge acquired.

5. Conclusion

In this thesis, I showed that a crop insecticide and an in-hive miticide have negative effects on honey bees at field relevant concentrations. Imidacloprid can have both negative and positive effects on hives depending on the environment, likely because it changes the ontogeny and foraging performances of worker bees. Thymol has a negative impact on bees' populations, although it can improve the removal of dead brood. Thymol and imidacloprid seem to interact and have adverse effects on the aversive learning or short-term memory of individual bees, but no such interactions were observed at the level of the colony at two different locations.

My results highlight how dependent the effects of field-relevant concentrations of stressors are on environmental conditions, and the necessity to replicate results across relevant landscapes. I argued honey bee ecotoxicology would benefit from more colony-level studies and individual-based studies should be less exploratory and designed to better understand or predict colony-level effects.

Collaborating with beekeepers could ease colony-level studies, because they could increase sample sizes by providing bee colonies or person power. Working along with beekeepers and documenting actual beekeeping practices could greatly improve the relevance of the experiments in terms of the miticide treatments and colonies' initial conditions.

The net benefits of in-hive miticide treatments on bee colonies need to be reassessed, with regards to potential interactions with other stressors and actual beekeeping practices. Non-chemical alternatives for the treatment of *V. destructor* mites could minimize negative effects on bee colonies and the risk of negative interactions with environmental stressors, including crop insecticides and fungicides or miticide residues from previous treatments.

References

Adams, A. *et al.* (2016) 'Efficacy of selected insecticides applied to hybrid rice seed', *Journal of Economic Entomology*, 109(1), pp. 200–206. doi: 10.1093/jee/tov310.

Alford, A. and Krupke, C. H. (2017) 'Translocation of the neonicotinoid seed treatment clothianidin in maize', *PLoS ONE*, 12(3), pp. 1–19. doi: 10.1371/journal.pone.0173836.

Baggio, A. *et al.* (2004) 'Field trials with different thymol-based products for the control of varroosis', *American Bee Journal*, 144(5), pp. 395–400.

Benuszak, J., Laurent, M. and Chauzat, M. P. (2017) 'The exposure of honey bees (*Apis mellifera*; Hymenoptera: Apidae) to pesticides: Room for improvement in research', *Science of the Total Environment*. Elsevier B.V., 587–588, pp. 423–438. doi: 10.1016/j.scitotenv.2017.02.062.

Beyer, M. *et al.* (2018) 'Winter honey bee colony losses, Varroa destructor control strategies, and the role of weather conditions: Results from a survey among beekeepers', *Research in Veterinary Science*, 118(November 2017), pp. 52–60. doi: 10.1016/j.rvsc.2018.01.012.

Blacquière, T. *et al.* (2012) 'Neonicotinoids in bees: A review on concentrations, side-effects and risk assessment', *Ecotoxicology*, 21(4), pp. 973–992. doi: 10.1007/s10646-012-0863-x.

Brodschneider, R., Brus, J. and Danihlík, J. (2019) 'Comparison of apiculture and winter mortality of honey bee colonies (*Apis mellifera*) in Austria and Czechia', *Agriculture, Ecosystems & Environment*. Elsevier, 274(January), pp. 24–32. doi: 10.1016/j.agee.2019.01.002.

Le Conte, Y., Ellis, M. and Ritter, W. (2010) '*Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses?', *Apidologie*, 41(3), pp. 353–363. doi: 10.1051/apido/2010017.

Dainat, B. *et al.* (2012) 'Predictive Markers of Honey Bee Colony Collapse', *PLoS ONE*. Edited by P. V. Aguilar, 7(2), p. e32151. doi: 10.1371/journal.pone.0032151.

Dietemann, V. *et al.* (2012) '*Varroa destructor* : research avenues towards sustainable control', *Journal of Apicultural Research*, 51(1), pp. 125–132. doi: 10.3896/IBRA.1.51.1.15.

Döke, M. A. *et al.* (2019) 'Colony Size, Rather Than Geographic Origin of Stocks, Predicts Overwintering Success in Honey Bees (Hymenoptera: Apidae) in the Northeastern United States', *Journal of Economic Entomology*, 112(2), pp. 525–533. doi: 10.1093/jee/toy377.

EFSA (2013) 'Guidance on the risk assessment of plant protection products on bees (*Apis mellifera, Bombus spp.* and solitary bees)', *EFSA Journal*, 11(7), p. 3295. doi: 10.2903/j.efsa.2013.3295.

Goulson, D. (2013) 'An overview of the environmental risks posed by neonicotinoid insecticides', *Journal of Applied Ecology*, 50(4), pp. 977–987. doi: 10.1111/1365-2664.12111.

Guzmán-Novoa, E. *et al.* (2010) '*Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee (*Apis mellifera*) colonies in Ontario, Canada', *Apidologie*, 41(4), pp. 443–450. doi: 10.1051/apido/2009076.

Henry, M. *et al.* (2012) 'A common pesticide decreases foraging success and survival in honey bees', *Science*, 336(6079), pp. 348–350. doi: 10.1126/science.1215039.

Henry, M. *et al.* (2015) 'Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees', *Proceedings of the Royal Society B: Biological Sciences*, 282(1819), p. 20152110. doi: 10.1098/rspb.2015.2110.

Huang, Z. Y. and Robinson, G. E. (1996) 'Regulation of honey bee division of labor by colony age demography', *Behavioral Ecology and Sociobiology*, 39(3), pp. 147–158. doi:

10.1007/s002650050276.

Imdorf, A. *et al.* (1999) 'Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies', *Apidologie*, 30(2–3), pp. 209–228. doi: 10.1051/apido:19990210.

Johnson, R. M. *et al.* (2013) 'Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*)', *PLoS ONE*. Edited by N. E. Raine, 8(1), p. e54092. doi: 10.1371/journal.pone.0054092.

Jordan, T. A. *et al.* (2012) 'Fall soil sampling method for predicting spring infestation of white grubs (Coleoptera: Scarabaeidae) in corn and the benefits of clothianidin seed treatment in Virginia', *Crop Protection*. Elsevier Ltd, 39, pp. 57–62. doi: 10.1016/j.cropro.2012.04.006.

Klein, S. *et al.* (2017) 'Why Bees Are So Vulnerable to Environmental Stressors', *Trends in Ecology and Evolution*. Elsevier Ltd, 32(4), pp. 268–278. doi: 10.1016/j.tree.2016.12.009.

Krupke, C. H., Long, E. Y. and Eitzer, B. D. (2017) 'Planting of neonicotinoid-treated maize poses risks for honey bees and other non-target organisms over a wide area without consistent crop yield benefit', pp. 1449–1458. doi: 10.1111/1365-2664.12924.

Leoncini, I. *et al.* (2004) 'Worker-worker inhibition of honey bee behavioural development independent of queen and brood', 51, pp. 392–394. doi: 10.1007/s00040-004-0757-x.

Lichtenberg, E. and Zimmerman, R. (1999) 'Information and farmers ' attitudes about pesticides, water quality, and related environmental effects', 73, pp. 227–236.

Lodesani, M. and Costa, C. (2005) 'Limits of chemotherapy in beekeeping: development of resistance and the problem of residues', *Bee World*, 86(4), pp. 102–109. doi: 10.1080/0005772X.2005.11417324.

Lundin, O. *et al.* (2015) 'Neonicotinoid insecticides and their impacts on bees: A systematic review of research approaches and identification of knowledge gaps', *PLoS ONE*, 10(8), pp.

1–20. doi: 10.1371/journal.pone.0136928.

Marchetti, S., Barbattini, R. and D'Agaru, M. (1984) 'Comparative effectiveness of treatments used to control *Varroa jacobsoni* Oud.', *Apidologie*, 15(4), pp. 363–378.

Meikle, W. G. *et al.* (2016) 'Sublethal Effects of Imidacloprid on Honey Bee Colony Growth and Activity at Three Sites in the U.S.', *PLOS ONE*. Edited by G. V. P. Reddy, 11(12), p. e0168603. doi: 10.1371/journal.pone.0168603.

Meikle, W. G. and Holst, N. (2015) 'Application of continuous monitoring of honeybee colonies', *Apidologie*, 46(1), pp. 10–22. doi: 10.1007/s13592-014-0298-x.

Meikle, W. G. and Weiss, M. (2017) 'Monitoring Colony-level Effects of Sublethal Pesticide Exposure on Honey Bees', *Journal of Visualized Experiments*, (129), pp. 1–10. doi: 10.3791/56355.

Milani, N. (1999) 'The resistance of *Varroa jacobsoni* Oud. to acaricides', *Apidologie*, 30(2–3), pp. 229–234. doi: 10.1051/apido:19990211.

Monchanin, C. *et al.* (2019) 'Hazard of a neonicotinoid insecticide on the homing flight of the honeybee depends on climatic conditions and *Varroa* infestation', *Chemosphere*, 224, pp. 360–368. doi: 10.1016/j.chemosphere.2019.02.129.

Mullin, C. A. *et al.* (2010) 'High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health', *PLoS ONE*, 5(3). doi: 10.1371/journal.pone.0009754.

Osterman, J. *et al.* (2019) 'Clothianidin seed-treatment has no detectable negative impact on honeybee colonies and their pathogens', *Nature Communications*. Springer US, 10(1), p. 692. doi: 10.1038/s41467-019-08523-4.

Perry, C. J. *et al.* (2015) 'Rapid behavioral maturation accelerates failure of stressed honey

bee colonies', *Proceedings of the National Academy of Sciences*, 112(11), pp. 3427–3432. doi: 10.1073/pnas.1610243113.

Plath, J. A. *et al.* (2017) 'Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay', *Frontiers in Behavioral Neuroscience*, 11(May). doi: 10.3389/fnbeh.2017.00098.

Pohorecka, K. *et al.* (2017) 'The exposure of honey bees to pesticide residues in the hive environment with regard to winter colony losses', *Journal of Apicultural Science*, 61(1), pp. 105–125. doi: 10.1515/JAS-2017-0013.

Prado, A. *et al.* (2019) 'Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees', *Science of the Total Environment*. Elsevier B.V., 650, pp. 1250–1260. doi: 10.1016/j.scitotenv.2018.09.102.

Rademacher, E., Harz, M. and Schneider, S. (2015) 'The development of HopGuard® as a winter treatment against *Varroa destructor* in colonies of *Apis mellifera*', *Apidologie*, 46(6), pp. 748–759. doi: 10.1007/s13592-015-0363-0.

Rosenkranz, P., Aumeier, P. and Ziegelmann, B. (2010) 'Biology and control of *Varroa destructor*', *Journal of Invertebrate Pathology*. Elsevier Inc., 103(SUPPL. 1), pp. S96–S119. doi: 10.1016/j.jip.2009.07.016.

Simon-Delso, N. *et al.* (2015) 'Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites', *Environmental Science and Pollution Research*, 22(1), pp. 5–34. doi: 10.1007/s11356-014-3470-y.

Smart, M. *et al.* (2016) 'Linking measures of colony and individual honey bee health to survival among apiaries exposed to varying agricultural land use', *PLoS ONE*, 11(3), pp. 10–13. doi: 10.1371/journal.pone.0152685.

Søvik, E. *et al.* (2015) 'Negative impact of manganese on honeybee foraging', *Biology Letters*, 11(3), pp. 1–4. doi: 10.1098/rsbl.2014.0989.

Spivak, M. and Downey, D. L. (1998) 'Field Assays for Hygienic Behavior in Honey Bees (Hymenoptera: Apidae)', *Journal of Economic Entomology*, 91(1), pp. 64–70. doi: 10.1093/jee/91.1.64.

Suard, T. (2009) Thymovar, mode d'emploi, *Andermatt Biovet AG, Switzerland*.

Tihelka, E. (2018) 'Effects of synthetic and organic acaricides on honey bee health: A review', *Slovenian Veterinary Research*, 55(3), pp. 114–140. doi: 10.26873/SVR-422-2017.

Tsvetkov, N. *et al.* (2017) 'Chronic exposure to neonicotinoids reduces honey bee health near corn crops', *Science*, 356(6345), pp. 1395–1397. doi: 10.1126/science.aam7470.

Underwood, R. M., Traver, B. E. and López-Uribe, M. M. (2019) 'Beekeeping Management Practices Are Associated with Operation Size and Beekeepers' Philosophy towards in-Hive Chemicals', *Insects*, 10(1), p. 10. doi: 10.3390/insects10010010.

Vita Bee Health (2017) Apiguard: *Varroa Control*, Vita Europe Limited, London. Available at: https://www.vita-europe.com/beehealth/fr/produits/apiguard/.

Wilde, G. *et al.* (2007) 'Seed Treatment Effects on Early-Season Pests of Corn and on Corn Growth and Yield in the Absence of Insect Pests', *Journal of Agricultural and Urban Entomology*, 24(4), pp. 177–193. doi: 10.3954/1523-5475-24.4.177.

Woodcock, B. A. *et al.* (2017) 'Country-specific effects of neonicotinoid pesticides on honey bees and wild bees', *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

Zhang, E. and Nieh, J. C. (2015) 'The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation', *Journal of Experimental Biology*, 218(20), pp. 3199–3205. doi: 10.1242/jeb.127472.

Appendix: scientific presentations

This presentation was given to the ASSAB in July 2017 in Melbourne.

Does Thymol, a miticide commonly used by beekeepers, impact behaviour and colony dynamic in honeybees?

Théotime Colin

Pesticides are among the main causes of the ongoing honeybee decline. They are believed to interact with each other as well as with stressors such as parasites, pathogens and habitat loss and have been recently placed under increased scrutiny. Most studies seem to focus on pesticides used on crops whereas chemicals used by beekeepers to kill honeybee parasites receive only little attention. Using assays designed to study hygienic behaviour in honeybees, we test whether Thymol, a miticide commonly used by beekeepers to kill parasitic mites, impacts their ability to remove dead workers and brood from the hive.

Using another set of hives, we also investigate whether Thymol impacts colony dynamics and interacts with the insecticide Imidacloprid, widely used on crops. These measurements will allow us to provide specific answers to beekeepers on how they can improve the health of their colonies and will aid our understanding of how pesticides impact honeybee behaviour and colony dynamics.

Long-term monitoring of honey bee colony growth and performance in pesticide studies

Théotime Colin

Many consider agrochemical exposure to be causal of the increase in honey bee colony failure rates, and there is concern about the possibilities of complex synergistic interactions between different agrochemicals. Field data, however, are thus far patchy and often contradictory, and most fails to experimentally address the possibilities of interactions between different chemical stressors. Here we studied the interactions between imidacloprid and thymol on bee colony growth and development on two different continents: North America and Australia. We monitored colony development and performances by measuring the number of bees and the amounts of brood, pollen and honey in hives exposed to thymol, imidacloprid or a combination of both across a full year. The effects of both thymol and imidacloprid were drastically different at both locations. This confirms the crucial role of environmental interactions and synergistic effects in ecotoxicology and highlights the necessity to replicate studies across different environments before generalizing any effects.