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Effects of chronic pesticide and pathogen exposure on honey bee (*Apis mellifera* L.) health at the colony level

Dissertation presented by Richard Odemer

submitted to the Faculty of Agricultural Sciences

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Dedicated to my mother and the memory of my father†

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In order to comply with regulations for a cumulative Ph.D. thesis at the Faculty of Agricultural Sciences, three publications have been included into this work. As these publications have been prepared to fit the regulations of the different peer-reviewed scientific journals, the style, citations and the layout may vary between chapters.

[1] Retschnig G, Williams GR, **Odemer R**, Boltin J, Di Poto C, Mehmann MM, Retschnig P, Winiger P, Rosenkranz P, Neumann P (2015). *Environmental Microbiology* 17:4322–4331. doi: 10.1111/1462-2920.12825

[2] **Odemer R**, Rosenkranz P (2018). *Journal of Apicultural Research* (submitted). doi: 10.1101/293167

[3] **Odemer R**, Nilles L, Linder N, Rosenkranz P (2018). *Ecotoxicology*. doi: 10.1007/s10646-018-1925-5

Abbreviations

ANOVA	Analysis of Variance
Avg	Average
BEE DOC	Bees in Europe and the Decline Of Colonies
CCD	Colony Collapse Disorder
cm	Centimeter
DeBiMo	Deutsches Bienenmonitoring
DWD	Deutscher Wetterdienst
DWV	Deformed Wing Virus
e.g.	*exempli gratia* (for example)
EFSA	European Food and Safety Agency
et al.	*et alii* (and others)
etc.	*et cetera* (and so on)
EU	European Union
FDR	False Discovery Rate
FOAG	Federal Office for Agriculture (Switzerland)
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
GmbH	Gesellschaft mit beschränkter Haftung
h	Hour
HMF	Hydroxymethylfurfural
i.e.	*id est* (that is)
IAPV	Israeli Acute Paralysis Virus
KBV	Kashmir Bee Virus
kg	Kilogram
km	Kilometer
KMN	Kieler Mating Nuc
L	Liter
LAB	Landesanstalt für Bienenkunde
LC-MS	Liquid Chromatography-Mass Spectrometry
LD_{50}	Lethal Dose, 50 %
LOQ	Limit of Quantification
LTD	Limited

m	Meter
mL	Milliliter
mm	Millimeter
N, E	North, East
nAChRs	Nicotinic Acetylcholine Receptors
ng	Nanogram
NOEL	No Effect Level
ppb	Parts Per Billion
qPCR	Quantitative Polymerase Chainreaction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
SD	Standard Deviation
SLU	Sveriges Lantbruksuniversitet
SPE	Solid Phase Extraction
spp.	*speciēs*
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
vs.	Versus
W, L, H	Width, Length, Height
w/v	Weight/Volume
µg	Microgram
µL	Microliter
°C	Degree Celsius

1 Summary

During the last decade the increasing number of honey bee colony losses has become a major concern of beekeepers and scientists world wide. Extensive research and cooperation projects have been established to unravel this phenomenon. Among parasites, pathogens and environmental factors, the use of agrochemicals, most notably the class of neonicotinoid insecticides, are suspected to be a key factor for this collapse. Current approaches not only focus on colony collapse but also on the weakening of honey bees by the exposure to sublethal concentrations of such pesticides.

Recently, the European Food Safety Authority (EFSA) temporarily banned three neonicotinoids including clothianidin, imidacloprid and thiamethoxam, for the use in crops attractive to pollinators. Thiacloprid however, likewise a neonicotinoid insecticide, is still tolerated for agricultural use because it is considered less toxic to bees. Nevertheless, some publications indicate sublethal effects of this agent leading to impairments of the colony.

A general problem for the study of such sublethal effects is that they often are measurable in individual bees without eliciting clear impact at the colony level. In addition, such sublethal effects might only have a consequence in combination with other stressors like pathogens. This thesis presents two new methodical approaches combining the controlled application of stressors to individual bees with an evaluation of the effects under field realistic conditions of free flying colonies. In all approaches, the bees were treated with a combination of different pesticides and/or a combination of pesticides and a pathogen in order to evaluate synergistic interactions. As pathogen, *Nosema ceranae*, a novel intracellular gut parasite introduced from Asia, was used. This parasite is considered to contribute to “CCD”-like symptoms (“colony collapse disorder”), particularly in Spain.

In the first part of this thesis (Retschnig et al., 2015), observation hives at two study sites (Hohenheim in Germany and Bern in Switzerland) were used to clarify possible synergistic effects when honey bees are exposed to pesticides of two different substance classes (the neonicotionoid thiacloprid and the synthetic pyrethroid tau-fluvalinate),

both in combination with an infection of *N. ceranae*. Mortality, flight activity and social behaviour of individually marked and treated worker bees were monitored.

At the Hohenheim site, no impact from any of the treatments could be confirmed except a slightly higher flight activity of the *Nosema* treated bees. At the Bern site however, the pesticide treatments elicited a significant reduction of worker bee lifespan, whereas the *Nosema* infection resulted in higher ratios of motionless periods. Importantly and in contrast to several laboratory studies, in neither of the two sites an interaction among the pesticides and the pathogen could be confirmed. The inconsistency of our results suggests that the effects of both, sublethal application of pesticides and infection with *N. ceranae* were rather weak and that interaction among them may have been overemphasized.

To extend this first approach in small observation colonies, the second part of this thesis (Odemer & Rosenkranz, 2018) focused on performance parameters such as colony development and overwintering success in full sized honey bee colonies, using the same pesticides as in the observation hives. Here, neither the single exposure to thiacloprid or tau-fluvalinate nor their combination had negative effects on the colony performance. However, the chronic application of the acaricide tau-fluvalinate significantly reduced the infestation with *Varroa* mites.

In the third part of this thesis (Odemer et al., 2018), a neonicotinoid (clothianidin) with an extraordinary high toxicity to bees was applied alone and in combination with *N. ceranae* and *N. apis*, the "original" parasite of the European honey bee. A novel approach was developed with individually marked bees that were infected after hatching with a certain number of *Nosema* spores and introduced into mini-hives. In order to simulate worst case field conditions, the pesticide was then applied chronically in sublethal concentrations over the whole lifespan of the bees. Again in contrast to previous laboratory studies, no effect of the clothianidin treatment on mortality or flight activity could be observed. However, the lifespan of *Nosema* infected bees was significantly reduced compared to non-infected bees, but in agreement with the

observation hive experiment, the combination of pesticide and pathogen did not reveal any synergistic effect.

The results of the three experiments of this thesis indicate that (i) individual honey bees are less impaired by neonicotinoids if kept within the social environment of the colony and that (ii) sublethal concentrations of neonicotinoids in the field are not the main driver for colony losses. This should be considered in further honey bee risk assessments. However, these statements refer exclusively to the honey bee colony as a eusocial superorganism that obviously is more resilient to pesticide exposure through mechanisms of “social buffering”. Further research should therefore focus on the question, to what extent pesticides in general and neonicotinoids in particular impair insect biodiversity in rural areas.

2 Zusammenfassung

Synergistische und chronische Effekte von Krankheiten und Pflanzenschutzmitteln auf die Gesundheit von Honigbienen (*Apis mellifera* L.) auf Volksebene

Während des letzten Jahrzehnts ist die zunehmende Zahl an Völkerverlusten zu einem Hauptanliegen der Imker und Wissenschaftler weltweit geworden. Umfangreiche Forschungs- und Kooperationsprojekte wurden eingerichtet, um dieses Phänomen zu untersuchen. Neben Parasiten, Krankheitserregern und Umweltfaktoren wird vermutet, dass der Einsatz von Agrochemikalien, insbesondere die Klasse der Neonicotinoide, ein Schlüsselfaktor für diesen Kollaps ist. Derzeitige Ansätze konzentrieren sich nicht nur auf den Verlust von Völkern, sondern auch auf die Schwächung der Honigbienen durch subletale Konzentrationen solcher Pestizide.

Vor kurzem hat die Europäische Behörde für Lebensmittelsicherheit (EFSA) drei Neonicotinoide einschließlich Clothianidin, Imidacloprid und Thiamethoxam in Beständen die für Bestäuber attraktiv sind, vorübergehend verboten. Thiacloprid, ebenfalls ein Neonicotinoid, bleibt jedoch für die landwirtschaftliche Verwendung frei, da es für Bienen als weniger toxisch angesehen wird. Dennoch weisen einige Publikationen auf subletale Wirkungen dieses Mittels hin, die zu Beeinträchtigungen von Bienenvölkern führen.

Ein generelles Problem bei der Untersuchung solcher subletalen Effekte besteht darin, dass sie oft bei einzelnen Bienen messbar sind, ohne aber dass sie auf der Volksebene eine deutliche Wirkung zeigen. Darüber hinaus könnten solche subletalen Effekte nur in Kombination mit anderen Stressoren wie Pathogenen eine Konsequenz haben. Diese Arbeit stellt zwei neue methodische Ansätze vor, die die kontrollierte Anwendung von Stressoren mit einzelnen Bienen mit einer Bewertung der Effekte unter feldnahen Bedingungen frei fliegender Völker kombinieren. Bei allen Ansätzen wurden die Bienen mit einer Kombination aus verschiedenen Pestiziden und/oder einer Kombination aus Pestiziden und einem Pathogen behandelt, um synergistische Wechselwirkungen zu bewerten. Als Krankheitserreger wurde *Nosema ceranae*, ein neuartiger intrazellulärer Darmparasit aus Asien, verwendet. Es wird angenommen, dass

dieser Parasit insbesondere in Spanien zu "CCD" -artigen Symptomen („Colony Collapse Disorder“) beiträgt.

Im ersten Teil dieser Arbeit (Retschnig et al., 2015) wurden Bienen-Schaukästen an zwei Standorten (Hohenheim in Deutschland und Bern in der Schweiz) verwendet, um mögliche synergistische Effekte zu klären. Dazu wurden Honigbienen Pestiziden aus zwei verschiedenen Substanzklassen ausgesetzt (das Neonicotionoid Thiacloprid und das synthetische Pyrethroid Tau-Fluvalinat), jeweils in Kombination mit einer Infektion von *N. ceranae*. Mortalität, Flugaktivität und soziales Verhalten der individuell markierten und behandelten Arbeiterinnen wurden überwacht.

Mit Ausnahme einer etwas höheren Flugaktivität der mit *Nosema* behandelten Bienen, konnte am Standort Hohenheim keine Auswirkungen durch eine der Pestizid-Behandlungen bestätigt werden. Am Standort Bern führten die Pestizide jedoch zu einer signifikanten Verkürzung der Lebensdauer der Arbeiterinnen, während die *Nosema*-Infektion zu höheren Anteilen bewegungsloser Bienen führte. Im Gegensatz zu diversen Laborstudien konnte an keinem der beiden Standorte eine Wechselwirkung zwischen den Pestiziden und dem *Nosema* Erreger bestätigt werden. Die Unbeständigkeit unserer Ergebnisse deutet darauf hin, dass die Auswirkungen sowohl der subletalen Anwendung von Pestiziden als auch der Infektion mit *N. ceranae* eher schwach waren und dass die Wechselwirkung zwischen ihnen möglicherweise überbewertet wurde.

Um den ersten Ansatz zu erweitern, konzentrierte sich der zweite Teil dieser Arbeit (Odemer & Rosenkranz, 2018) auf Leistungsparameter wie Volksentwicklung und Überwinterungserfolg in Wirtschaftsvölkern, die mit den gleichen Pestiziden wie zuvor die Schaukästen behandelt wurden. Hier hatte weder Thiacloprid oder Tau-Fluvalinat noch deren Kombination negative Auswirkungen auf die genannten Parameter. Die chronische Anwendung des Akarizids Tau-Fluvalinat reduzierte erwartungsgemäß den Befall mit *Varroa*-Milben signifikant.

Im dritten Teil dieser Arbeit (Odemer et al., 2018) wurde ein Neonicotinoid (Clothianidin) mit einer außerordentlich hohen Toxizität für Bienen allein und in Kombination mit *N. ceranae* und *N. apis*, dem "ursprünglichen" Parasiten der

Europäischen Honigbiene, angewendet. Ein neuartiger Ansatz wurde entwickelt bei dem einzeln markierte Bienen nach dem Schlüpfen mit einer bestimmten Anzahl von *Nosema*-Sporen infiziert und in Kieler Begattungskästchen eingesetzt wurden. Um Worst-Case-Feldbedingungen zu simulieren, wurde das Pestizid dann über die gesamte Lebensdauer der Bienen in subletalen Konzentrationen chronisch verfüttert. Auch mit diesem Ansatz konnte im Gegensatz zu früheren Laborstudien keine Wirkung der Clothianidin-Behandlung auf Mortalität oder Flugaktivität beobachtet werden. Allerdings war die Lebensdauer von *Nosema*-infizierten Bienen im Vergleich zu nicht-infizierten Bienen signifikant reduziert. In Übereinstimmung mit unserem Schaukasten-Versuch zeigte die Kombination von Pestizid und Pathogen keinen synergistischen Effekt.

Die Ergebnisse der drei Experimente dieser Arbeit zeigen, dass (i) einzelne Honigbienen durch Neonicotinoide weniger beeinträchtigt werden wenn sie im sozialen Umfeld ihres Volkes gehalten werden und (ii) subletale Konzentrationen von Neonicotinoiden auf dem Feld nicht der Hauptgrund für Völkerverluste sein können. Diese Erkenntnis sollte bei zukünftigen Risikobewertungen von Pflanzenschutzmitteln berücksichtigt werden. Die Aussagen beziehen sich jedoch ausschließlich auf das Bienenvolk als eusozialen Superorganismus, der im Vergleich zur Einzelbiene durch Mechanismen der "sozialen Pufferung" offenbar widerstandsfähiger gegen Pestizid-Exposition ist. Zukünftige Forschung sollte sich daher auf die Frage konzentrieren, inwieweit sich Pestizide im Allgemeinen und Neonicotinoide insbesondere auf die Biodiversität von Insekten und Bestäubern in ländlichen Gebieten auswirken.

3 General Introduction

3.1 The Collapse of Honey Bee Colonies

Due to their outstanding pollination abilities, more than three-quarters of the agricultural crop production benefits from insects (Klein et al., 2007). One-third of this service is owed to the European honey bee (*Apis mellifera* L.) (Spivak et al., 2011). Even though the most essential staple food crops like corn, wheat, rice, soybeans and sorghum are wind-pollinated or self-pollinating, many other produce like fruits, nuts, spices or vegetables rely on cross-pollination from insects. Alfalfa and clover, both important sources of cattle fodder, also depends on insect pollination. As a matter of fact, honey bees play a major role in agricultural dependent economics, providing essential services for both, ecosystem and agriculture. In 2009 Gallai et al. estimated the economic value of pollination on crops worldwide to be 153 billion USD, which represents 9.5 % of the total human food production. Lautenbach et al. (2012) even increased this estimate by a factor of 1.9 largely attributed to purchasing power parities, which was not employed in the former evaluation, corroborating the commercial importance.

In the US, periodic colony losses have been reported since 2006 with average mortality rates of 30 %. However, some beekeepers even had higher losses than that. Similar numbers were reported from Canada (Neumann & Carreck, 2010; vanEngelsdorp & Meixner 2010; Ellis et al., 2010; Lee et al., 2015). In a pan European survey, winter losses were monitored throughout several years resulting in mortality rates ranging from 3.2 to 32.4 % in the winter 2012/13 to 2.4 to 15.4 % in the winter 2013/2014 (Laurent et al., 2016). Within the german bee monitoring project winter losses ranging from 4 to 15 % were detected in the years 2004/08 (Genersch et al., 2010). A particular syndrome in relation to colony losses is the so called CCD (“colony collapse disorder”), first described in the United States. One of the most pregnant indications of CCD is the rapid decrease of foragers resulting in their total absence. Bees from a former healthy colony vanish without any sign of reason. In Europe, however, typical CCD symptoms could not be pinpointed as clearly as in the US and still the causative factors are not unravelled. Although the periodic colony losses – mostly during the overwintering

period – are obviously increasing, the global number of honey bee colonies did not decline (Moritz & Erler, 2016). This is most probably due to the beekeeping management, where colony losses are compensated by splitting hives or making nucleus colonies in spring.

Most research for the reason of increasing colony losses in the past ten years focused on two major drivers assumed to be crucial for the health problems of honey bees around the world: (i) the agricultural use of pesticides, to name especially the group of the highly neurotoxic neonicotionids (Henry et al., 2012; Kessler et al., 2016) and (ii) the invasive parasitic mite *Varroa destructor*, which was introduced to Europe in the late 1970ies where it has spread world-wide ever since (Anderson & Trueman, 2000). In addition, several viruses and other parasites like the microsporidial gut parasites *Nosema spp*. were linked to CCD, too (Evans & Schwarz, 2011; Higes et al., 2008; McMenamin & Genersch, 2015).

Besides that, the ever-growing malnutrition and decline of biodiversity resulting in a lesser variety of pollen supply containing essential amino acids for honey bees have been reported to have a negative impact on honey bee colonies (Naug, 2009). There is a certain necessity of flower diversity to ensure nectar and pollen supply in the right composition (Scofield & Mattila, 2015). Even though the way a honey bee colony functions is rather adjusted to the use of monocultures like oilseed rape, phacelia or other crops like alfalfa or clover, an unbalanced diet or nutrition can be inadequate in value for the proper development of a colony (Decourtye et al., 2011; Huang, 2012). Interestingly, even a natural ground cover may not be more beneficial to a colony than managed farmland, depending on what kind of food source it provides and at which time of the year it flowers and how long it is useful for the honey bees. Municipal areas on the other hand, provide food resources over a relatively long term and can therefore be very beneficial for the colony development (Lecocq et al., 2015). In times when there is no proper food source available and colonies are still rearing large amounts of brood, usually beekeepers supply their colonies with inverted sucrose syrup originated from corn or wheat starch as well as artificial pollen supplements.

In addition to the above mentioned drivers for honey bee health problems or even colony collapses there is to mention, that with the world-wide and local migration of bees for better honey yield or pollination of large scale crops, colonies are exposed to long-distance transports, rough handling, measures for disease prevention, high temperature fluctuations and a drastically reduced access to foraging opportunities can be significant stressors (Simone-Finstrom et al., 2016). It is known that the sugar syrup fed to bees produces over time and with an increased temperature a compound called HMF (Hydroxymethylfurfural). This compound can be toxic to bees, especially when the manufacturer cannot provide an analysis post production, this risk is imminent (LeBlanc et al., 2009; Zirbes et al., 2013). Especially in the US with one of the largest business of pollination in their monocultures, the seasonal dependency on pollination provided from migratory beekeeping is immense. This means that every season colonies are at risk of being introduced to diseases and pests from other colonies arriving from all over the US (Zhu et al., 2014).

During the past research, many other factors were also discussed to have an impact on honey bee health, however are considered to be less important. A few to mention are air pollution (Girling et al., 2013; McFrederick et al., 2008), nanomaterials (Milivojevic et al., 2015), solar radiation (Ferrari, 2014), robbing insects (Core et al., 2012), bee microbiome alteration (Cox-Foster et al., 2007; Mattila et al., 2012), individuality in bee colonies and possible early life stress (Rittschof et al., 2015; Wray et al., 2011) and global warming (Le Conte & Navajas, 2008).

Despite these numerous factors researchers agree that bee pathogens and certain pesticides are the main threats for the weakening of honey bee colonies and the currently increasing numbers of winter losses.

3.2 Pathogens

As mentioned above, the introduction of the parasitic mite *V. destructor* had dramatic impacts on the beekeeping management. Periodic treatments became unavoidable, as otherwise colonies wouldn't have a chance to survive. Still up to date there is no sustainable solution for this problem, yet the principles of *Varroa* population dynamics

are not finally understood (Frey et al., 2013; Lin et al., 2018). Besides the physical damage caused by these mites, they act also as vector for viral diseases. Being lethal for entire colonies, the deformed wing virus (DWV) is among the most serious threats for honey bees transmitted by *V. destructor* (McMenamin & Genersch, 2015). The optimization of existing treatments and the development of new methods for long-term mite control are currently crucial challenges in applied bee research.

Apart from problems caused by a mite, there are several other pathogen threats to honey bees nowadays. Honey bees are impaired by beetles, viruses and many other microorganisms like bacteria, fungi, trypanosomes and amoebae (Cornman et al., 2012). Open to question are the different impacts of these microbes on both, the individual and the colony level. Further, it remains unclear how these pathogens interact amongst each other having a possible significant impact on honey bee health (Singer, 2010).

A monitoring study in the US revealed a high prevalence of two viruses (IAPV and KBV) and two microsporidian species in declining bee colonies in contrast to healthy colonies (Cox-Foster et al., 2007). *Nosema apis* and *Nosema ceranae* are the two species that infect *A. mellifera* and are highly specialized gut parasites. *N. apis* was the first microsporidium to be described (Zander, 1909), exclusively found in the European honey bee (Zander, 1963).

In 1994, when *N. ceranae* was first described by Fries et al. (1996) it was believed to be geographically limited to the distribution of its original host, the Eastern honey bee *Apis cerana*. Approximately ten years later, studies revealed that *N. ceranae* has already spread over Europe, infecting a new host and becoming a new threat to *A. mellifera* (Higes et al., 2006).

Both *Nosema* species are intracellular parasites, injecting a polar filament into the host cells for mass reproduction and subsequent destruction of the gut cell. In a considerable range this can lead to possible dysfunctions in the host including digestive disorders, reduced life span, smaller population size and negative effects on honey production (Fries, 2010; Manzoor, 2013).

Cage studies revealed that *N. ceranae* infection leads to significantly higher mortality when compared to uninfected bees (Higes et al., 2007, Goblirsch et al., 2013). Moreover, a *Nosema* infection may lead to behavioural changes as Kilani (1999) states that foraging activity of bees infected with *N. apis* started at an earlier age due to accelerated aging. Similar effects were found for *N. ceranae* (Goblirsch et al., 2013). Naug & Gibbs (2009) and Mayack & Naug (2009) found higher hunger levels in *N. ceranae* infected bees experienced by the stronger reaction to offered sucrose solution to be possible reasons for this behaviour. Further, it is assumed that these increased nutritional requirements result in higher and more risky flight activity of diseased bees (Dussaubat et al., 2013).

Contrary findings on the overall influence of survivorship and winter losses of diseased colonies were reported. Even though Higes et al. (2008, 2009) are speaking of honey bee colonies collapsing from *N. ceranae* infections in Spain, no such detrimental effects could be noticed in other countries all over the globe (Invernizzi et al., 2009; Gisder et al., 2010; Genersch et al., 2010; Paxton, 2010; Stevanovic et al., 2011). Interestingly there are even contradicting reports from Spain, stating that *Nosemosis* is not correlated to colony collapse at all (Fernández et al., 2012).

To understand pathology and evolutionary epidemiology of honey bee diseases, it is imperative to distinguish between colony level effects and the effects on individual bees (reviewed in Fries, 2010). So, the weakening of a few hundred worker bees might have no measurable effect on the performance of the colony as a highly organized social entity of 20,000 to 40,000 individuals. Recent studies imply that it is impossible to identify a single pathogen solely responsible for colony losses (Genersch, 2010).

3.3 Pesticides

With the introduction of synthetic pesticides into the agricultural production to control weeds, harmful insects and phytopathogenic fungi, concerns raised about negative influences on beneficial insects. Such insecticides (i) might not only control target organisms but also affect honey bees when applied to flowering crops and (ii) herbicides may decrease biodiversity and abundance of forage plants in agricultural

landscapes (Hald, 1999; Albrecht, 2005). Recent discussions about the decline of managed and wild bees have focussed on neonicotionoid pesticides as a possible cause leading to colony collapses (van der Sluijs et al., 2013; Goulson, 2013).

In plant protection, neonicotinoids are meanwhile among the most important agrochemicals worldwide (Elbert et al., 2008) and are mainly used as seed dressings (Sur & Stork, 2003). Seven different neonicotionoids including imidacloprid, thiamethoxam, clothianidin, acetamiprid, thiacloprid, dinotefuran and nitenpyram are commercially in use. They function as neurotoxins by irreversibly binding to nicotinic acetylcholine receptors (nAChRs) of the insect nervous system. Overstimulation of these receptors causes paralysis and death (Matsuda et al., 2001).

A variety of factors have resulted in economic success of these pesticides: on the one hand neonicotinoids are highly selective towards invertebrate organisms, on the other hand they are absorbed systemically by the plant and can be found in all tissues, which makes them effective against a broad range of pests over an extended time period and when applied in small quantities, e.g. as seed dressings (Jeschke & Nauen, 2008). With the exception of exposure to dressing agents (such as, for example dressed maize) from pneumatic seed drills during sowing of dressed seeds (Nuyttens et al., 2013), evaluation of bees' exposure to neonicotinoids in general are considerably lower than levels causing acute mortality (reviewed in Lundin et al., 2015).

Nevertheless, some neonicotinoids have been shown to be highly toxic to bees in very small doses (Iwasa et al., 2004). As a systemic insecticide, they can be translocated into the main sources of food for bees, pollen and nectar and lead to a serious risk of exposure (Cresswell, 2011). Moreover, some compounds only break down gradually and are remaining in the environment (e.g. soil or plants) for months and even years after the application (Goulson, 2013; Hopwood et al., 2016; Krupke et al., 2012; reviewed in Lundin et al., 2015).

A dramatic incident in the German Rhine Valley in 2008 represented a decisive turning point in the discussion on the further use of neonicotinoids and gave rise for great concern in the media and political debates. The sowing of maize with pneumatic drilling

machines caused abrasion of seed dressing (Poncho and Poncho Pro) which was released into the air and environment. It deposited on surrounded blossoms (e.g. rape seed, apple or dandelion). Foraging bees got exposed to the active ingredient clothianidin which resulted in poisoning, death of bees and effects on bee brood. In total, about 12,000 hives were affected (Würfel, 2008).

This incident was one reason for the moratorium to ban the three most toxic neonicotinoids clothianidin, thiamethoxam and imidacloprid as seed dressings for the use on crops that are attractive to bees in the European Union (EU Regulation No 485/2013, 2013) and Switzerland (FOAG, 2013). Coincidental with this moratorium, the authorities called for more field research on the toxicity of these compounds in order to make a final risk assessment on the basis of solid data (Dicks, 2013; Goulson, 2013; Gross, 2013; Carreck, 2017; reviewed in Lundin et al., 2015).

In contrast to these highly toxic agents, thiacloprid and acetamiprid are neonicotinoids considered not harmful to bees (Schmuck et al., 2003). Thiacloprid is therefore commonly used as foliar application and can be sprayed on flowering crops attractive to bees (e.g. oilseed rape). This means honey bees are directly exposed to this agent (Elbert et al., 2007). In 2007 the German Bee Monitoring (DeBiMo) revealed a high prevalence of thiacloprid residues found in bee bread samples (62 positive samples from n=110), but with no negative correlation to colony development or winter losses (Genersch et al., 2010). Under field realistic conditions, other studies also did not show negative effects of thiacloprid on colony health (Schmuck et al., 2003; Retschnig et al., 2015; Siede et al., 2017). However, experiments with individual bees or small groups of bees indicated that navigation is impaired (Fischer et al., 2014) as well as behaviour (Tison et al., 2017) and imunocompetence (Brandt et al., 2017). Studies under laboratory conditions showed even more drastic effects, especially in combination with other stressors like pathogens. They can, for instance, result in a shorter life span of thiacloprid treated worker bees (Vidau et al., 2011; Doublet et al., 2015).

Not only by the use of agricultural pesticides bee health is at risk, but the prevalence of synthetic varroacides and their residues in bee products like beeswax, pollen and honey

increasingly appear to be of huge importance. Residues of the most common varroacides coumaphos (CheckMite) and tau-fluvalinate (Apistan) commercially in use for *Varroa* mite control all over the world are frequently found in bee products nowadays (Cabras et al., 1997; Tsigouri et al., 2004; Mullin et al., 2010; Berry et al., 2013).

Bogdanov et al. (1998) presented data of a tau-fluvalinate accumulation in beeswax with the duration of the treatment, which bears the risk of accumulation from previous treatments contaminating further bee products. On top of that, *V. destructor* has progressively developed resistance against numerous synthetic acaricides in different parts of the world (Milani, 1999; Pettis, 2004; Lodesani & Costa, 2005; Rosenkranz et al. 2010).

Even though broad knowledge on pesticide residues in bee products have been gathered over the last years (Wallner, 1999; Kochansky et al., 2001; Tremolada et al., 2004; Bogdanov, 2006), their consequences for bee health have not yet been identified (Desneux et al., 2007; Martel et al., 2007; Frazier et al., 2008). The impact of chronic exposure to acaricide residues on larvae, pupae and adult bees as well as possible synergistic effects with agrochemicals or pathogens remains unknown.

3.4 Synergistic Effects

Many recent studies conclude, that not the pesticide alone but the interaction between pathogen infections and sublethal exposure to pesticides might weaken honey bees, leading to a steady decline in bee population of the colony. However, there is no common agreement in the scientific community on which the most dominant threats to honey bees are and which combinations of pesticides and pathogens the most detrimental ones for honey bee health are (Jacques et al., 2017; Genersch, 2010; Maini et al., 2010; Ratnieks & Carreck, 2010).

Up to now, most research was focused on the *Varroa* mite (Genersch, 2010; Genersch et al., 2010; Le Conte et al., 2010), *N. ceranae* (Higes et al., 2009; Fries 2010), *Varroa* associated bee viruses (Dainat et al., 2012; Hong et al., 2011, Ai et al., 2012, Noh et al.,

2012) and on pesticides, especially neonicotinoids (Cresswell et al., 2011; Blacquière et al, 2012).

It is beyond question that every single factor mentioned above is able to affect honey bee health at the colony level. If damage thresholds are exceeded entire colonies can be killed either through infections/infestations or through pesticide applications. However, we only have limited knowledge what happens under field-realistic conditions and to what extent interactions of pathogenes and pesticides increase the risk of damages for honey bee colonies. “Field realistic” commonly means that pesticide contamination and pathogen infestations, repectively, are within a sublethal range.

So far, only few experiments included combination effects among pesticides and pathogenes. Some recent studies indicated additive and/or synergistic effects between neonicotinoid pesticides and *N. ceranae* (Alaux et al., 2010; Vidau et al., 2011; Henry et al., 2012; Pettis et al., 2012). Di Prisco et al. (2013) proved increasing virus loads in bees contaminated with the neonicotinoid clothianidin. The possible antagonistic interactions between *N. ceranae* and a honey bee virus however, showed that the situation could become more confusing if more than one pathogen is involved in the interaction process (Costa et al., 2012). These are apparent gaps in our knowledge on the importance of parasite, pathogen and pesticide interactions on honey bee colonies.

A general weakness of the so far published results with such interactions is the nearly exclusive use of individual bees, mostly kept in hoarding cages, for the experiments (Ellis et al., 1997; Suchail et al., 2000; Berry et al., 2013; Doublet et al., 2015). Even the few experiments using free-flying colonies were performed under widely artificial conditions (Henry et al., 2012) or with the use of pesticide concentrations that are considerable higher as known from field conditions (Tison et al., 2016). Therefore, we still have no clear picture whether effects that have been confirmed on the individual bee level will have an impact on the full colony. However, the honey bee “colony” is a functional entity consisting of several thousand cooperating individual bees (“superorganism” concept, Moritz & Southwick, 1992). Indeed, colonies can provide an amazing buffering capacity which may easily mask effects observed at the individual

bee level. For instance, the loss of hundreds of foragers afield for whatever reason(s) may not be noticed even when carefully evaluating the colony population dynamics or colony performance (Straub et al., 2015). Accordingly, the evaluation level of combined harms caused by bee diseases and/or pesticides should be the whole colony. This will allow us to pinpoint thresholds for collapse and interactions of stressors that can cause colony death. Additional standardized methods providing an analysis of combinatory effects at the colony level are therefore urgently required and were a focus of this work.

3.5 Objectives of this Study

The overall objective of this study was to identify interactions between the endoparasite *Nosema spp.*, the miticide tau-fluvalinate and two neonicotinoid pesticides at the level of the honey bee colony. In particular, the problem of the discrepancy between the experiments with individual bees and entire colonies should be overcome by new methodological approaches. Tests on individual bees can be performed under defined and controlled experimental conditions but have the disadvantage that interactions with other bees and buffering effects within the social environment of the colony are not considered. In experiments in full sized colonies, on the other hand, the effects on individual bees within the colony can hardly be measured. The purpose of this work therefore was to investigate sublethal and/or synergistic effects of pesticides and pathogens in individual bees that are kept in free flying colonies.

To study such possible effects, three different approaches with defined exposures to sublethal pesticide doses and pathogens were pursued:

I) Impact of a *N. ceranae* infection under the influence of thiacloprid and tau-fluvalinate exposure on longevity, flight activity and social behaviour of worker bees in free flying observation hives;

II) Effects of a chronic sublethal exposure of the pesticides thiacloprid and tau-fluvalinate on colony development and overwintering of full sized honey bee colonies under field conditions;

III) Effects of a chronic clothianidin exposure in combination with infections of *N. apis* or *N. ceranae* on foraging behaviour and longevity of free flying honey bees kept in specially designed minihives.

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4 Publication 1: RETSCHNIG *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

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Summary

Interactions between pesticides and parasites are believed to be responsible for increased mortality of honey bee (*Apis mellifera*) colonies in the northern hemisphere. Previous efforts have employed experimental approaches using small groups under laboratory conditions to investigate influence of these stressors on honey bee physiology and behaviour, although both the colony level and field conditions play a key role for eusocial honey bees. Here, we challenged honey bee workers under *in vivo* colony conditions with sub-lethal doses of the neonicotinoid thiacloprid, the miticide tau-fluvalinate, and the endoparasite *Nosema ceranae*, to investigate potential effects on longevity and behaviour using observation hives. In contrast to previous laboratory studies our results do not suggest interactions among stressors, but rather lone effects of pesticides and the parasite on mortality and behaviour, respectively. These effects appear to be weak due to different outcomes at the two study sites, thereby suggesting that the role of thiacloprid, tau-fluvalinate, and *N. ceranae* and interactions among them may have been overemphasized. In the future, investigations into the effects of honey bee stressors should prioritize the use of colonies maintained under a variety of environmental conditions in order to obtain more biologically relevant data.

Introduction

All living organisms are exposed to a broad array of environmental stressors, including pests, parasites and contaminants. Mortality represents the strongest and most defined index of effect (i.e. death); however, sublethal impacts affecting behaviour and physiology can also be measured (e.g. Marcogliese and Pietrock, 2011; Pettis et al., 2012; Schneider et al., 2012b). To obtain a thorough understanding of the effects of a particular stressor or combination of stressors, it is therefore crucial to examine multiple potential indices of effect.

The Western honey bee (*Apis mellifera*; hereafter honey bee) is a eusocial insect that can be used to investigate the environmental effects of parasites and pesticides due to its well-described natural history and ease of maintenance in an experimental setting. Additional interest in honey bee health has been stimulated by severe colony mortalities reported recently (Neumann and Carreck, 2010). The widely distributed ectoparasitic mite *Varroa destructor* has been identified as one important driver for colony losses (Genersch et al., 2010; Le Conte et al., 2010; Dietemann et al., 2012); however, it appears that concurrent assaults by multiple other stressors likely have a large influence on colony survival (Potts et al., 2010). While the detrimental consequences of stressor driven mortality are apparent, the dimensions of the impact of sublethal effects on honey bee colonies are often less visible. Sublethal effects can comprise various parameters ranging from anatomical and physiological impairments to more complex processes such as orientation or foraging behaviour (e.g. Desneux et al., 2007). The functioning of the colony superorganism as a unit depends heavily on the social behaviours among the individuals in the hive because the coordination of fundamental tasks in a colony (e.g. brood care, cleaning, foraging, attending etc.) requires the transfer of relevant information among the members of the colony (Moritz and Southwick, 1992). Even though social in-hive behaviours are key for colony functioning, few studies have investigated potential stressor effects on social behaviour, despite data suggesting that stressors can influence other behaviours (e.g. foraging) (Schneider et al., 2012a; Dussaubat et al., 2013a).

The microsporidian *Nosema ceranae* is an obligatory intracellular midgut parasite that host-switched from the Eastern honey bee (*Apis cerana*) to the Western honey bee more than a decade ago (Paxton et al., 2007). It has since developed a nearly ubiquitous distribution worldwide (e.g. Klee et al., 2007; Williams et al., 2008; Giersch et al., 2009; Higes et al., 2009a; Invernizzi et al., 2009; Yoshiyama and Kimura, 2011). Despite numerous investigations of the impact of the parasite, its role in honey bee mortalities is highly debated (Fries, 2010; Higes et al., 2013). Whereas some studies did not detect increased individual bee or colony mortality (e.g. Invernizzi et al., 2009; Genersch et al., 2010; Williams et al., 2011; Dainat et al., 2012; Martin et al., 2013), others have reported lethal effects in the laboratory (Higes et al., 2007) as well as colony deaths (Martín-Hernández et al., 2007; Higes et al., 2008; 2009b). Observed sublethal effects of *N. ceranae* on individuals include host immune suppression (Antúnez et al., 2009), energetic stress (Mayack and Naug, 2009; 2010; Naug and Gibbs, 2009), as well as altered flight behaviour (Kralj and Fuchs, 2010; Dussaubat et al., 2013a) and pheromone production (Dussaubat et al., 2010). Numerous studies have demonstrated that parasites can alter the behaviour of infested honey bees (e.g. Wang and Moflller, 1970; Delfinado-Baker et al., 1992); however, none have investigated if *N. ceranae* affects social behaviour within a colony setting.

Pesticides, acting singly or in combination, can also affect non-target organisms such as solitary bees (Sandrock et al., 2014a), bumble bees (Fauser-Misslin et al., 2014) and honey bees (Bortolotti et al., 2003; Desneux et al., 2007; Aliouane et al., 2009; Wu et al., 2011; Henry et al., 2012; Sandrock et al., 2014b). Doses of pesticides that exceed a certain threshold level (depending on substance and type of exposure) affect the survival of exposed honey bees, while sublethal doses of pesticides can exhibit various effects on individual honey bees, including development, learning performance and orientation (Desneux et al., 2007; Blacquière et al., 2012). While many studies have investigated this kind of pesticide effects on honey bees, similar to *N. ceranae*, little is known about the potential impact of pesticides on honey bee social behaviour at the colony level. The neonicotinoid crop protection insecticide thiacloprid and the pyrethroid tau-fluvalinate are two pesticides widely applied to combat pest insects (Elbert et al., 2008) and

V. destructor (Tsigouri et al., 2001), respectively. Residues of both substances are common in bee hive matrices; thiacloprid in honey (Tanner and Czerwenka, 2011), bee bread (Genersch et al., 2010), nectar and pollen (Pohorecka et al., 2012), and tau-fluvalinate in beeswax and pollen (Chauzat and Faucon, 2007; Mullin et al., 2010). Thiacloprid is of relatively low toxicity to bees (oral LD50 = 17.32 µg bee^{-1}) versus other neonicotinoids, and can act synergistically with *N. ceranae* to kill honey bees in the laboratory (Vidau et al., 2011; Retschnig et al., 2014a). Tau-fluvalinate has an acute contact toxicity of 0.2 g µg bee^{-1}, but was reported to have no lethal effect at daily oral doses of 5 or 10 µg bee^{-1} (Decourtye et al., 2005). However, it was shown to promote honey bee mortality in the presence of the miticide coumaphos (Johnson et al., 2009) as well as influence honey bee locomotion (Teeters et al., 2012). Although combined effects of tau-fluvalinate and any neonicotinoid have not yet been investigated in honey bees, exposure of bumble bees to a similar combination of pesticides (i.e. a neonicotinoid and a pyrethroid) increased worker mortality and impaired foraging behaviour (Gill et al., 2012).

The simultaneous exposure to a combination of parasites and pesticides can lead to interactions between the stressors in the host and can cause increased host mortality or various sublethal effects (Marcogliese and Pietrock, 2011). For example, in honey bees, concurrent exposure to *N. ceranae* and certain neonicotinoid insecticides caused both lethal and sublethal effects (e.g. Alaux et al., 2010; Vidau et al., 2011; Aufauvre et al., 2012; Pettis et al., 2012). In the past, the investigation of specific mechanisms of stressor effects often took place in laboratory studies under standardized conditions (e.g. Alaux et al., 2010; Forsgren and Fries, 2010; Aufauvre et al., 2012), which allowed for the control of potentially interferring factors (Williams et al., 2013). However, it remains unclear to what extent such findings can be extrapolated to honey bees in the field. As demonstrated in previous investigations, the study arena (laboratory versus field) can have a strong influence on the physiological development (Maleszka et al., 2009) as well as measured stressor effects in individual bees, including interactive effects of pesticides on honey bee mortality (Schmuck et al., 2003). Naturally, laboratory studies focus on parameters that can be tested reliably in this particular study

arena, including worker longevity and parasite intensity (e.g. Alaux et al., 2010; Vidau et al., 2011). However, some traits that are crucial for the functioning of the honey bee colony, such as the social in-hive behaviour of the workers, have received too little attention so far. Therefore, the primary objective of this study was to look at potential stressor effects on honey bee worker longevity, the ultimate measure of stress impact, as well as on important behaviours among workers including antennation (communication), grooming (hygiene) and trophallaxis (nutrition), as well as flight activity (nutrition and hygiene) (Moritz and Southwick, 1992). Using observation hives in two locations, we investigated the lethal and sublethal effects of the widely applied pesticides thiacloprid and tau-fluvalinate, as well as the ubiquitous parasite *N. ceranae*, on individual honey bees that faced natural conditions. Experimental individuals were allocated to one of four treatment groups (control, pesticides, *N. ceranae*, and *N. ceranae* and pesticides); pesticide and *N. ceranae* exposure occurred during development and post-emergence, respectively. Due to previous reports of the effects of *N. ceranae* and pesticides on honey bee survival and behaviour (e.g. Alaux et al., 2010; Kralj and Fuchs, 2010; Aufauvre et al., 2012), we expected to observe a similar impact of these stressors and anticipated to find stronger effects on individuals that were exposed to the combination of both *N. ceranae* and pesticides due to potential synergistic interactions (Alaux et al., 2010; Vidau et al., 2011; Aufauvre et al., 2012; Pettis et al., 2012).

Results

Mortality

Location A. Honey bee workers exposed to pesticides during development showed significantly higher mortality than did control individuals during the 14 day trial (Kaplan–Meier, log-rank test with Bonferroni correction, both P*s* = 0.0006, Fig. 1). No such significant difference was observed between control workers and those belonging to the *N. ceranae*-only treatment group (Kaplan–Meier, log-rank test with Bonferroni correction, P = 0.3). Similarly, no significant difference in mortality occurred among the non-control treatment workers (pesticides versus *N. ceranae*, pesticides versus *N. ceranae* and pesticides, and *N. ceranae* versus *N. ceranae* and pesticides, Kaplan–Meier, log-rank test with Bonferroni correction, P = 0.19; 1; 0.23). Mortality, when compared using only data at termination day, was similar to survival analyses that incorporated daily mortality; workers exposed to pesticides showed significantly higher mortality compared with control individuals, and no significant difference was observed among non-control treatment individuals (binary logistic regression with Bonferroni correction, pesticides groups versus control, both P*s* < 0.012, for all other comparisons P*s* > 0.186).

Location B. No significant difference in mortality was observed among treatments when daily deaths (Kaplan–Meier, log-rank test with Bonferroni correction, all *P*s = 1, Fig. 2), or total death number at experiment termination (binary logistic regression with Bonferroni correction, all *P*s = 1) were considered.

Comparison of mortality between locations A and B. In all treatment groups, the workers showed significantly higher mortality in location B compared with location A (log-rank test, all *P*s < 0.001, Table 1, Figs. 1 and 2).

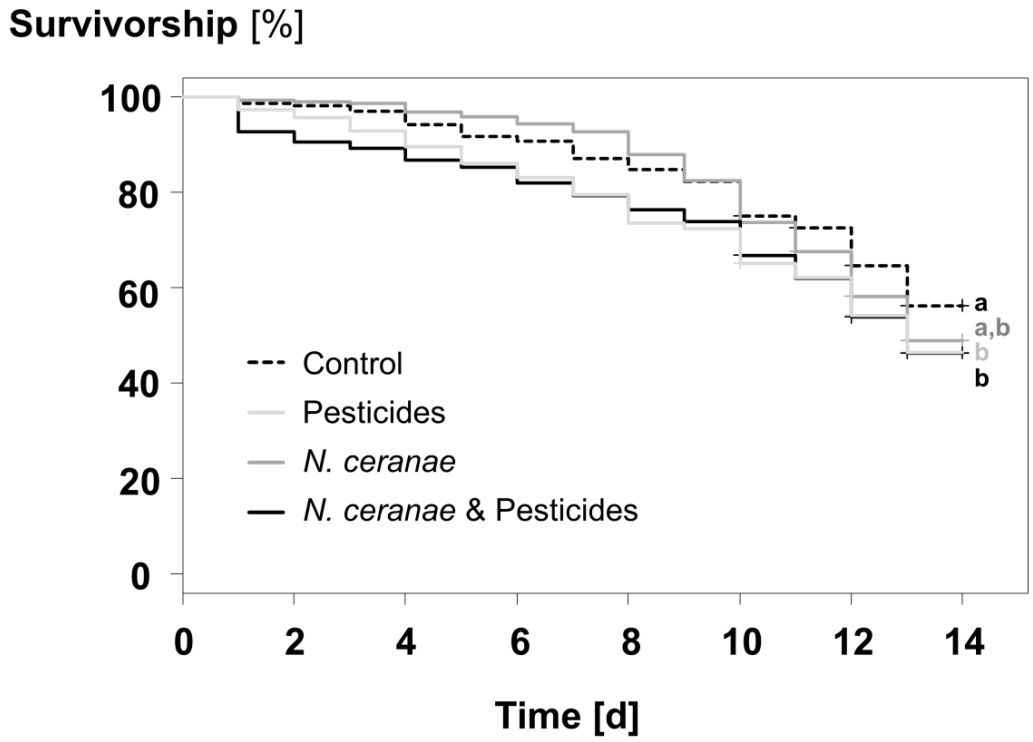

Fig. 1 Kaplan–Meier survival curve of the experimental honey bee (*Apis mellifera*) workers at location A (Switzerland). Workers that were exposed to pesticides (thiacloprid and tau-fluvalinate) during development showed significantly higher mortality than the control group (log-rank test with Bonferroni correction, both P*s* = 0.0006). Significant differences between treatments are marked with different letters (a, b).

Behaviour

Location A – In-hive behaviour. A total of 22147 individual behaviours were observed during 14 days (Fig. 3), with frequency of observations of the three behavioural categories consistent for each treatment: other (including all behaviours except for social behaviours and motionlessness, such as walking, feeding, brood care, cleaning etc.) was observed most (total: 16280 events, 71.41–75.18% events per treatment), followed by motionless (total: 3250 events, 13.09–16.37% events per treatment), and social (antennation total: 1458 events, 6.21–6.94% events per treatment; grooming total: 696 events, 3.0–3.29% events per treatment; trophallaxis total: 463 events, 1.99–2.26% events per treatment).

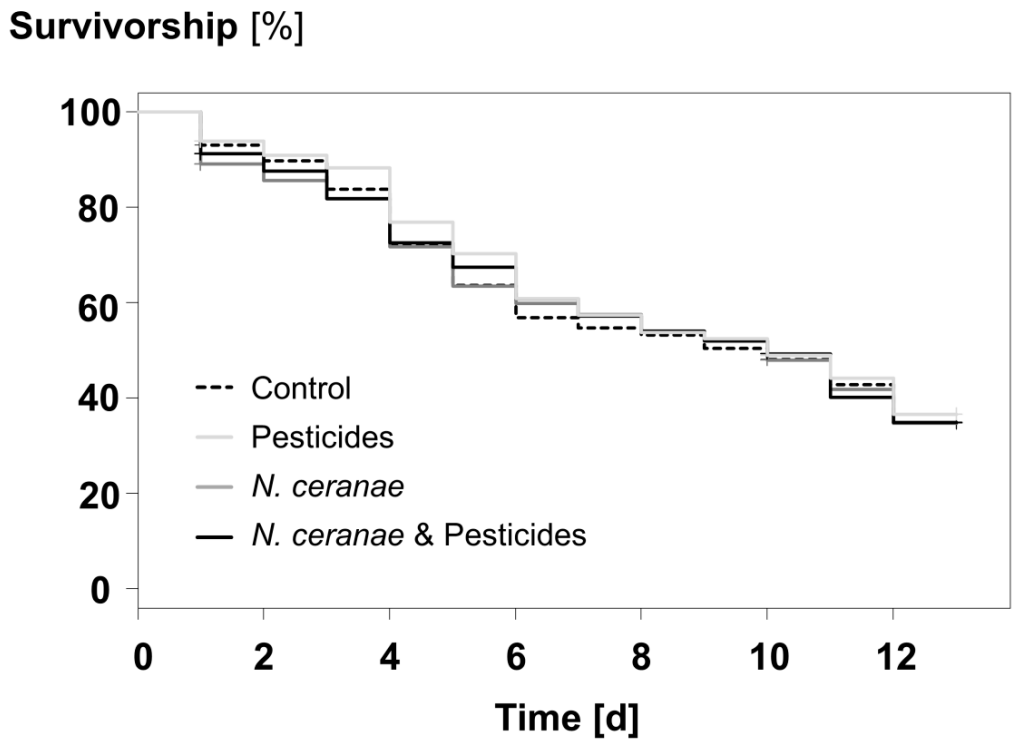

Fig. 2 Kaplan–Meier survival curve of the experimental honey bee workers at location B (Germany). No differences in mortality were observed between the investigated treatment groups (log-rank test with Bonferroni correction, all *P*s =1).

Table 1 Overview and comparison of the stressor impacts on honey bees (*Apis mellifera*) in locations A (Switzerland) and B (Germany).

	Mortality			**Behaviour**	
Treatments	Location A	Location B	A vs. B	Location A	Location B
Control	--	--	higher mortality in B **(*P* <0.001)**	--	--
Pesticides	higher mortality **(*P* < 0.001)**	--	higher mortality in B **(*P* <0.001)**	--	--
N. ceranae	--	--	higher mortality in B **(*P* <0.001)**	higher inactivity **(*P* < 0.001)**	--
***N. ceranae* & Pesticides**	higher mortality **(*P* < 0.001)**	--	higher mortality in B **(*P* <0.001)**	higher inactivity **(*P* < 0.05)**	--

The absence of significant effects is marked as '--' in the table.

For all possible combinations of behaviour comparisons (n = 24), only three showed significant differences (all Ps < 0.05); all others had P-values greater than 0.23 (multinomial logistic regression with false discovery rate (FDR) correction, Fig. 3). Workers inoculated with *N. ceranae* (*N. ceranae*, and *N. ceranae* and Pesticides), regardless of pesticide exposure, were motionless more than control individuals (multinomial logistic regression with FDR correction, both P < 0.016). Additionally, workers exposed to *N. ceranae* only were motionless more than those exposed to pesticides only (multinomial logistic regression with FDR correction, P = 0.0024, Table 2 and Fig. 3).

Location B – Flight activity. There were no significant differences in flight activity, measured as number of flights per minute, among the three treatment groups (pesticides, *N. ceranae* or the combination of both) and the controls [analysis of variance (ANOVA) and Tukey– Kramer test, P < 0.05]. However, the *N. ceranae* treatment group showed

significantly higher flight activity compared with the pesticides treatment group (ANOVA and Tukey–Kramer test, $P < 0.05$).

Table 2 Comparisons of behaviour ratios (reference: category 'other behaviours') in honey bees (*Apis mellifera*) among pairs of treatments.

Compared treatments	**Behaviour (Reference: other behaviours)**	***P-value* with FDR correction**
Control vs. Pesticides	motionless	0.5573
	antennation	0.9698
	grooming	0.9698
	trophallaxis	0.8414
Control vs. *N. ceranae*	motionless	0.0000[(a)]
	antennation	0.2304
	grooming	0.6352
	trophallaxis	0.8238
Control vs. *N. ceranae* & Pesticides	motionless	0.0152[(b)]
	antennation	0.5573
	trophallaxis	0.8238
	grooming	0.9698
Pesticides vs. *N. ceranae* & Pesticides	motionless	0.2619
	antennation	0.5573
	grooming	0.9698
	trophallaxis	0.9698
N. ceranae vs. Pesticides	motionless	0.0024[(c)]
	antennation	0.2304
	grooming	0.7272
	trophallaxis	0.9698
N. ceranae vs. *N. ceranae* & Pesticides	motionless	0.2304
	antennation	0.8238
	grooming	0.6155
	trophallaxis	0.9698

(a) motionless was more frequent in the *N. ceranae* treatment group
(b) motionless was more frequent in the *N. ceranae* & Pesticides treatment group
(c) motionless was more frequent in the *N. ceranae* treatment group

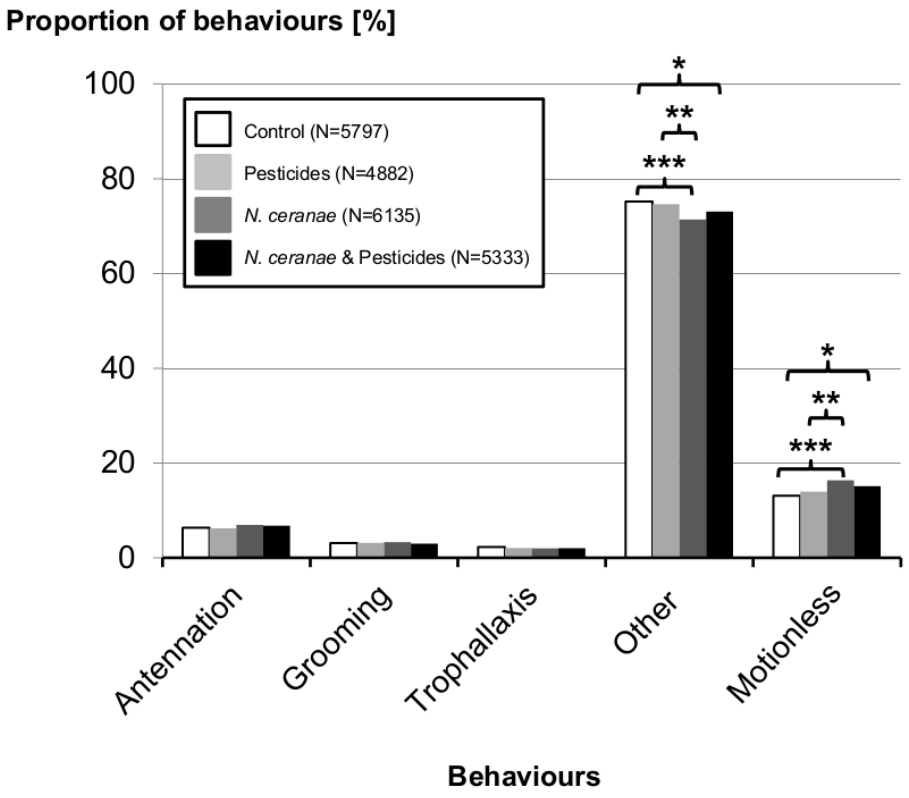

Fig. 3 Frequency of honey bee behaviours in the different treatments at location A (Switzerland). Significant differences among treatments were detected only between the behavioural categories being idle and other behaviours and are indicated with asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Treatment confirmation

Pesticides. Pesticide application to the donor colonies was confirmed by residue analyses of the respective chemical substances in the feeding solutions as well as of different hive matrices. Sucrose feed contained an average level of 611.5 ppb of thiacloprid in the treatment and no detectable thiacloprid residues in the control solutions. In the pesticide-treated colonies, thiacloprid residues were detected in honey (190 ppb), wax (147 ppb) and pollen (68 ppb), whereas tau-fluvalinate was found in wax (8280 ppb) and pollen (105 ppb). In the control colonies, traces of thiacloprid (7.7 ppb in honey, 34.2 ppb in wax and 3.6 ppb in pollen), but not tau-fluvalinate, was detected.

Nosema ceranae

Location A. Workers inoculated with *N. ceranae* showed mean spore amounts of 14.32 × 106 [standard deviation (SD): 5.73 × 106] for the *N. ceranae* only and 14.56 × 106 (SD: 6.31 × 106) for the *N. ceranae* and pesticides treatment group. There was no significant difference between the spore amounts of these two treatment groups (Wilcoxon rank-sum test, $P > 0.05$). Workers that were not inoculated with *N. ceranae* showed median spore amounts (not normally distributed) of 0 spores per bee. However, 21 (35%) and 24 (40%) of the 60 workers analysed from the control and pesticides-only treatment groups were infected with *N. ceranae* at day 14. Mean spore counts in these workers were 8.73 × 106 spores per bee in the control and 10.8 × 106 spores per bee in the pesticides treatment group. Compared with inoculated workers, mean infection level in the non-inoculated individuals was significantly lower (ANOVA and Tukey–Kramer test, $P < 0.001$).

Location B. Mean spore counts of the inoculated workers were 2.93 × 106 (SD: 4.54 × 106) for the *N. ceranae* only and 2.33 × 106 (SD: 3.19 × 106) for the *N. ceranae* and pesticides treatment group. There was no significant difference between spore counts of these treatment groups (Wilcoxon rank-sum test, $P > 0.05$). Workers not inoculated with *N. ceranae* showed mean spore counts of 0.06 × 106 (SD: 0.25 × 106) spores per bee in the control and 0.014 × 106 (SD: 0.07 × 106) in the pesticides treatment group. Five control workers (8.62%) and three (5.45%) individuals of the pesticides-only treatment group were infected with *N. ceranae* at the end of the study with mean spore counts of 0.67 × 106 and 0.26 × 106, respectively. Compared with the *N. ceranae* inoculated workers, the mean infection level in those not fed *N. ceranae* was significantly lower (ANOVA and Tukey–Kramer test, $P < 0.01$).

Discussion

The data consistently revealed for both study locations no evidence of any interactions between parasite and pesticide stressors, as well as no effect of *N. ceranae* on worker mortality. However, overall worker mortality and the effect of pesticide exposure on mortality differed between the two locations. *Nosema ceranae* influenced in-hive activities by increasing frequency of motionless behaviour, but did not show an effect on flight activity.

The field-realistic approach of this study allowed for stressor exposure and collection of mortality and behavioural data under colony conditions. The vast majority of stressor-specific investigations are performed in the laboratory (e.g. Alaux et al., 2010; Forsgren and Fries, 2010; Aufauvre et al., 2012). Although this promotes a relatively controlled environment whereby potentially confounding factors can be more easily excluded (e.g. temperature, humidity, nutrition, etc.) (Williams et al., 2013), results may not always reflect natural conditions because important features to honey bees, like eusociality, are not well represented (e.g. Mattila and Otis, 2006; Maleszka et al., 2009; Retschnig et al., 2014b). Alternatively, incidental exposure of experimental workers to *N. ceranae* and pesticides in colony-level studies is typically much greater than those used for laboratory assays. Similar to Wu and colleagues (2011), traces of pesticide residues were detected in control hives, possibly due to drifting bees or environmental contamination (e.g. Mullin et al., 2010). Likewise, some control workers were infected with *N. ceranae*; this is not surprising as contaminated hive materials are believed to be major sources of *N. ceranae* infection (Higes et al., 2008; Giersch et al., 2009). The mean *N. ceranae* spore amounts of the respective treatment groups were in line with other studies that applied similar methods (e.g. Paxton et al., 2007; Alaux et al., 2010; Pettis et al., 2012).

For both parasite and pesticide stressors, the effects on worker mortality were not consistent at the two study locations. Strong effects on honey bee health are usually highly reproducible, such as the considerable damage due to *V. destructor* parasitism (e.g. Liebig, 2001; Fries et al., 2003; Rosenkranz et al., 2010; Schäfer et al., 2010). Inconsistencies of stressor effects in both locations suggest that they are rather weak.

Regardless, pesticide exposure of immature workers increased mortality at the adult stage in one study location, thereby supporting previous work that showed increased mortality of adults can occur when larvae are exposed to pesticides (Wu et al., 2011; Pettis et al., 2012; Berry et al., 2013).

Sublethal application of either tau-fluvalinate (Berry et al., 2013) or thiacloprid (Siede et al., 2014) on honey bee colonies did not reveal measurable effects on the population dynamics of bees or brood. Here we present the first approach to measure the combined application of these two pesticides at the colony level. In bumblebees, the combination of a neonicotinoid and pyrethroid was demonstrated to increase worker mortality (Gill et al., 2012); our study also observed this effect in honey bees in one location. In contrast to mortality, the data showed no evidence for an impact of the pesticides on the observed behaviours as has been shown in bumblebees (Gill et al., 2012). This could be explained because previous studies that demonstrated sublethal pesticide effects typically applied similar doses of pesticides that have a comparatively higher toxicity, such as clothianidin or imidacloprid (Schneider et al., 2012a; Teeters et al., 2012; Yang et al., 2012).

Nosema ceranae showed no effect on honey bee mortality at both locations. This is in line with a growing number of studies (e.g. Invernizzi et al., 2009; Genersch et al., 2010; Williams et al., 2011; Martin et al., 2013), but contrary to others (e.g. Higes et al., 2007; Martín-Hernández et al., 2007; Higes et al., 2008; Higes et al., 2009b; Williams et al. 2014). This may be explained by variable strains of *N. ceranae* exhibiting a different virulence or differential susceptibility of bees in different geographic regions (Dussaubat et al., 2013b). A further reason for the different outcomes may be that the effect of *N. ceranae* on individual honey bee mortality has so far been tested in laboratory studies only, where the bees might have been influenced by more stressful conditions compared with a natural colony environment (e.g. Retschnig et al., 2014b). Although *N. ceranae* appeared to not influence flight activity at one location, the parasite reduced the overall activity of bees at the other location. This might be explained by the energetic stress caused by *N. ceranae* (Mayack and Naug, 2009; 2010; Naug and Gibbs, 2009).

In contrast to previously reported synergistic effects between neonicotinoid pesticides and *N. ceranae* (Alaux et al., 2010; Vidau et al., 2011; Aufauvre et al., 2012; Pettis et al., 2012), our data provided no such evidence. A potential explanation for this difference may be that previous studies were carried out under laboratory conditions. It is known that influence of stressors may differ depending on test arena (e.g. laboratory versus field) (Schmuck et al., 2003; Mattila and Otis, 2006), which may potentially be a consequence of a higher sensitivity due to the artificial conditions in the laboratory (e.g. Huang et al., 2014; Retschnig et al., 2014b). Experimental workers in the present study lived in a colony environment (i.e. natural hive composition including queen, workers and drones) where they could feed (pollen, bee bread, honey), socially interact and exit the hive.

The mortality of the experimental workers in the two study locations showed clear differences, and the significantly greater worker mortality at one location compared with the other was consistent for all treatments. The workers that remained geographically closer to their donor colonies showed an overall better survival. Although it is difficult to determine mechanisms for these differences due to experimental methods, potential reasons for the higher mortality in the second location may include factors such as genotype–environment interactions (e.g. Costa et al., 2012) or the transportation of the bees in the pupal stage (300 km) (Oldroyd, 2007; Pettis and Delaplane, 2010; Pirk et al., 2014). Such potential impacts should be considered in future studies and closely investigated to improve the investigation of honey bee stressors in natural conditions.

Experimental Procedures

Study set-up

The study was performed in summer 2012 at two locations: location A: Bern, Switzerland; and location B: Stuttgart, Germany. Both locations employed experimental honey bee workers from the same donor colonies located in Bern, Switzerland. Four treatment groups: (i) control, (ii) pesticides (thiacloprid and tau-fluvalinate), (iii) *N. ceranae* and (iv) *N. ceranae* and pesticides were investigated for differences in survivorship (both locations), in-hive behaviour (location A) and flight activity (location B).

At location A, eight local European honey bee colonies (*A. mellifera*) headed by sister queens (hereafter called donor colonies for the experimental workers) were randomly assigned to either the pesticide or the non-pesticide treatment (n = 4 per group). For the pesticide treatments, thiacloprid and tau-fluvalinate were applied for 6 weeks to encompass two complete brood cycles prior to removal of workers for the experiments. Thiacloprid was administered weekly by supplying colonies with 1 kg of 1000 ppb of 98.0% thiacloprid (Dr. Ehrenstorfer GmbH, Germany) sucrose solution (72–73% Hostettler® syrup, Hostettler Spezialzucker AG, Switzerland) using an in-hive feeder; control workers were fed with sucrose solution only. Tau-fluvalinate was applied using two Apistan® strips (Vita [Europe] LTD, UK), each 0.8 g active substance, placed in the lower brood chamber of each colony according to the manufacturer's recommendations. To confirm exposure, the thiacloprid and control solutions, as well as honey, wax and pollen samples were collected and analysed for pesticide residues at the United States Department of Agriculture (USDA) National Science Laboratory, Gastonia, USA, using routine liquid chromatographic procedures (Mullin et al., 2010).

Two weeks prior to the start of data collection, three observation hives were set up using standard approaches (Scheiner et al., 2013) in both locations A and B. Briefly, each observation hive was equipped with a mated egg-laying local queen of the same year and two stacked Zander frames containing ~ 2000 bees: one frame contained brood

in various developmental stages and the other consisted of stored honey and empty cells.

To obtain age cohorts of workers for experiments, queens from the eight donor colonies were caged on an empty brood frame for 48 h. Prior to emergence, brood frames were transferred to the laboratory and maintained in frame holders in the dark at 34.5°C and ≥ 50% relative humidity in an incubator (Williams et al., 2013). For transport to location B, brood frames (1–2 per donor colony) containing age cohorts of workers within capped brood cells (i.e. pupae) were carefully added to the brood chambers of a full-size colony for the ~ 300 km journey by car. Frames were kept under the same conditions as described above upon arrival at the new site.

After emergence, workers (4752 in total) at both locations were randomly assigned to the appropriate treatment group, marked on the thorax using coloured number plates and paints (Marabu Brillant, Gerstaecker, Switzerland) and inoculated with either *N. ceranae* or control suspension using a group feeding approach (Fries et al., 2013). For this, workers were starved for approximately 2 h in disposable plastic cages (20 individuals per cage). For the *N. ceranae* inoculum, fresh spores were obtained from naturally infected foragers that were collected at the hive entrance of local colonies in both sites the day prior. Midguts were carefully extracted from the workers using forceps, crushed in water and then purified by multiple centrifugation runs at 5000 *g* (Fries et al., 2013). Spores were then quantified using light microscopy and a haemocytometer (Cantwell, 1970). Dilution of the suspension using 50% (w/v) sucrose solution yielded a final concentration of 2 000 000 spores per 1.5 ml, whereas the control solution consisted of only freshly prepared 50% (w/v) sucrose solution. Each disposable plastic cage was supplied with either 1.5 ml *N. ceranae* or control inoculum using a 2 ml microcentrifuge tube with a 2 mm diameter hole in bottom tip to allow feeding, thus providing each of the 20 workers per cage with ~ 100 000 spores. Feeding devices were filled with 50% (w/v) sucrose solution when the entire suspension was consumed during frequent checks; after 48 h, all devices were refilled completely. After the inoculation process, a total of 792 workers, 198 individuals per treatment per

observation hive, were sprayed with sucrose solution and carefully inserted into the appropriate observation hive at night.

Location A

Mortality and behaviours of experimental honey bee workers was assessed by examining the observation hives twice daily, between 09h00–12h00 and 14h00–17h00, during 14 consecutive days. Order of observation hive viewing was rotated daily to avoid a potential bias of time. Observed behaviours were allocated to the following categories: (i) social interactions between adults (i.e. antennation, trophallaxis and grooming), (ii) motionless (i.e. individual is not moving) and (iii) other (i.e. performing any task not included in the previous categories, see Scheiner et al., 2013). Social behaviours of experimental workers with two or more other individuals were defined as the following: antennation (contact of the moving antennae), trophallaxis (exchange of food) and grooming (cleaning manipulation using the mouthparts and antennae).

At day 14, all surviving workers were carefully collected using forceps from observation hive frames and immediately frozen at −20°C. To ensure maximum recovery of marked workers, multiple collection attempts occurred during day and night. A subsample of 20 collected workers per treatment group per observation hive was used to determine *N. ceranae* infection levels. This was achieved by homogenizing each individual in a 2 ml Eppendorf tube using a bead mill homogenizer (MM300 Retsch), one metal bead and 1 ml of nuclease-free water. *Nosema ceranae* quantification was performed according previously mentioned techniques.

Location B

Similar to location A, mortality at location B was determined daily by recording all of the marked workers. Flight activity observations occurred between day 7 post-insertion of the marked workers until day 13, when the experiment was terminated. Departing and returning workers were viewed through a 10 cm long transparent plastic tube connecting the colony to the outdoors. Workers surviving to day 13 were collected according to previously discussed methods for location A. Similarly, a subsample of

16–28 workers, depending on number of available bees after collection, per treatment group and observation hive was used to determine *N. ceranae* infection levels. This was achieved by pressing out the midgut content by gently squeezing the abdomen of each individual. The gut suspension was viewed using light microscopy and a haemocytometer according to Cantwell (1970).

Statistics

Differences in survival of experimental workers during the study were tested using Kaplan–Meier survival statistics with the log-rank test (Mantel–Haenszel test) and Bonferroni correction, whereas survival at experiment termination was tested using binary logistic regression using tests that are based on the standard normal z-statistic (Wald statistic). For these analyses, workers collected at the end of the experiment were considered censored, as were those observed but not collected on the final day. Furthermore, workers that disappeared during the experiment were considered dead on the last day they were seen. Differences in survival of the workers between the two locations were analysed using the log-rank test. Comparison of social interactions between adults, motionless and other behaviours among treatments were performed using multinomial logistic regression with *P*-values deduced from Wald statistics using the category 'other behaviours' as a reference. Thus, the ratio of one specific behaviour versus other was compared between two treatment groups for each case. FDR correction was applied to compensate for multiple comparisons (Benjamini and Hochberg, 1995). Flight activity was compared using repeated measures ANOVA. *Nosema ceranae* data were analysed using Kruskal–Wallis one-way ANOVA, because of non-normal data distribution, followed by the Tukey–Kramer multiple comparison tests. All statistical analyses were carried out using the programmes SYSTAT 13 (Systat Software, USA), R (version 3.0.0., The R Foundation for statistical computing platform) and NCSS (version 8, NCSS LLC, USA).

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5 Publication 2: ODEMER & ROSENKRANZ 2018

Chronic exposure to a neonicotinoid pesticide and a synthetic pyrethroid in full-sized honey bee colonies

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Abstract

In the last decade, the use of neonicotinoid insecticides increased significantly in the agricultural landscape and they are meanwhile considered a risk to honey bees. Besides the exposure to pesticides, colonies are treated frequently with various acaricides that beekeepers are forced to use against the parasitic mite *Varroa destructor*. Here we have analyzed the impact of a chronic exposure to sublethal concentrations of the common neonicotinoid thiacloprid (T) and the widely used acaricide τ-fluvalinate (synthetic pyrethroid, F) - applied alone or in combination - to honey bee colonies under field conditions. The population dynamics of bees and brood were assessed in all colonies according to the Liebefeld method. Four groups (T, F, F+T, control) with 8-9 colonies each were analyzed in two independent replications, each lasting from spring/summer until spring of the consecutive year. In late autumn, all colonies were treated with oxalic acid against Varroosis. We could not find a negative impact of the chronic neonicotinoid exposure on the population dynamics or overwintering success of the colonies, irrespective of whether applied alone or in combination with τ-fluvalinate. This is in contrast to some results obtained from individually treated bees under laboratory conditions and confirms again an effective buffering capacity of the honey bee colony as a superorganism. Yet, the underlying mechanisms for this social resilience remain to be fully understood.

Introduction

Neonicotinoid pesticides are among the most used insecticides during the past decades and are dominating the global market for insecticidal seed dressings (Jeschke et al., 2011; Simon-Delso et al., 2015). However, these neonicotinoids are suspected to be a main driver for the decline of honey bees (Hopwood et al., 2016), wild bees (Potts et al., 2010) and even non-target wildlife in general (Goulson, 2013). Recently, the European Food Safety Authority (EFSA) has updated their risk assessment and now considers the three neonicotinoids imidacloprid, clothianidin and thiametoxam to be "a risk for bees" and suggested suitable amendments to the European Commission (EFSA, 2018). These three nitro-substituted compounds have the highest toxicity to bees among the class of neonicotinoids (Iwasa et al., 2004) and have been already banned for the use in flowering crops by the European Union since the year 2014 (EFSA, 2013).

However, other neonicotinoid insecticides with a far lower toxicity to bees - for instance thiacloprid and acetamiprid - are still widely used not only as seed dressings but are even approved as foliar spray in blooming cultures like oilseed rape (Schmuck et al., 2003). This leads to a remarkable high contamination of nectar and pollen and foragers might therefore be continuously exposed to these agents (Genersch et al., 2010; Collison et al., 2016; Rolke et al., 2016; Böhme et al., 2017). There is no doubt about the comparable low acute toxicity of these compounds to bees, however there is a controversial discussion on sublethal and long-term effects. So, it has been shown that thiacloprid can affect the sensitivity of honey bees to the gut parasite *Nosema ceranae* (Vidau et al., 2011; Pettis et al., 2013; Retschnig et al., 2015). More recent publications indicate that sublethal concentrations of thiacloprid alter their social behavior (Forfert and Moritz 2017) and, more importantly, disturb the orientation of foragers (Fischer et al., 2014; Tison et al., 2016, 2017). These studies have been conducted on the level of individual or small groups of bees by performing cage tests or semi-field trials under rather artificial conditions. Therefore, they do not cover important attributes of a social entity, with a more complex perception to its environment. Hence, the transfer of these results to field conditions must be taken with caution. Significantly, the only field study

available so far could not confirm negative effects of thiacloprid at the colony level (Siede et al., 2017).

Another controversial point is the possible interaction of thiacloprid - considered as "non-toxic for bees" - with active compounds of other chemical classes that are applied by beekeepers to control the parasitic mite *Varroa destructor*, requiring multiple annual treatments (Rosenkranz et al., 2010). In an effective and easy to use application, synthetic pyrethroids were, amongst others, introduced to beekeepers (Watkins, 1997) and are besides the formamidine amitraz the most frequently used acaricides in apiculture (Garrido et al., 2016). The exposure of honey bee colonies to a combination of sublethal doses of such pesticides may increase the susceptibility to pathogens and are suspected to contribute to the worldwide health problems of honey bee colonies (Cornman et al., 2013; Matsumoto, 2013; Wu et al., 2012). To study such possible combination effects we have chronically exposed full-sized colonies to the neonicotinoid thiacloprid and the synthetic pyrethroid τ-fluvalinate (Apistan®) in a two-year field study. To our knowledge this is the first study that analyzes the effect of a chronic application of both, a neonicotinoid insecticide and a common acaricide under realistic field conditions at the colony level. An exposure to these two pesticides is very likely under common beekeeping conditions in rural areas. Our crucial endpoints were (i) the overwintering success of treated colonies compared to untreated controls and (ii) the colony population dynamics.

Materials & Methods

Experimental colonies

For each treatment group, five experimental colonies were established in early May of the year 2010. The experiment was repeated with three to four new colonies per group in the year 2011 (Tab. 1). All colonies were set up at our local apiary at the agricultural experimental station Kleinhohenheim, which is an organic farming facility not using any agro chemicals or common pesticides at all. To standardize our experiment, we used artificial swarms made from stock colonies that were screened for low *Varroa* infestation and lack of virus infections prior to the trials. Freshly reared and mated sister queens of the Hohenheim breeding line were provided to each swarm, respectively. After the colonies successfully showed the first open brood stages, we sprayed all of them with a 3.5 % oxalic acid sugar solution for *Varroa* treatment to have a comparable low mite infestation for all experimental groups at the start of the experiment. We used residue free beeswax foundations to minimize the risk of additional contamination through pesticide residues in the wax (Bogdanov et al., 1998; Wallner, 1999). All colonies were set up on one box of 10 Zander frames, which was extended to two boxes when necessary during the summer season.

Tab. 1: List of replications, treatment groups, treatment duration, assessment dates (AD) and no. of colonies (N) at the time of the assessment.

Year	Treatment	Duration [days]	AD 1)	N	AD 2)	N	AD 3)	N	Winter treatment	N	AD 4)	N
2010-2011	Control	56	23. Jul	5	16. Aug	5	8. Oct	5	30. Nov	4	15. Apr	4
	Thiacloprid			5		5		5		3		3
	Fluvalinate			5		5		5		5		5
	Flu + Thia			5		5		5		4		4
2011-2012	Control	62	21. Apr	3	5. Aug	3	13. Oct	3	29. Dec	3	3. Apr	2
	Thiacloprid			4		4		4		4		4
	Fluvalinate			3		3		3		3		3
	Flu + Thia			3		3		3		3		3

Thiacloprid application

For the application of thiacloprid we used the pure substance (98 % purity, Dr. Ehrenstorfer GmbH), which was sonicated in pure water for a stock solution. We aimed to use a field-realistic concentration that was approximately 100-fold lower than the oral LD_{50} for thiacloprid (173.2 mg/kg, Würfel, 2008). We therefore diluted thiacloprid in sucrose syrup (Apiinvert, Südzucker GmbH) in order to receive the respective concentration. The final solution was quantified by an external lab (Eurofins Dr. Specht Laboratorien GmbH, Hamburg, Germany) which confirmed a thiacloprid concentration of 1.6 mg/kg (= 1,600 ppb). This feeding solution was applied to the colonies of the specific treatment groups and control colonies were fed with untreated sucrose syrup. The duration of the treatment in the year 2010 was 56 days (23rd Jul-17th Sep) and in the year 2011 62 days (21st Apr-22nd Jun) during summer season. In this time period we fed 1 kg syrup per week with an internal feeding device, to simulate a chronic exposure. A final amount of 8 kg per colony in 2010 and 9 kg in 2011 was administered in the summer season, respectively. Based on the concentration of 1.6 mg/kg we therefore applied a total amount of 12.8 mg thiacloprid per colony in 8 weeks (2010) and 14.4 mg thiacloprid per colony in 9 weeks (2011) during the summer season, respectively. The treatment was resumed when colonies were fed for overwintering at the end of the season. Every colony was fed with approximately 15 kg of the feeding solution with a total amount of 24.0 mg thiacloprid in each year for winter feeding. After the treatment period in summer, a pooled sample of food (nectar/honey) from the combs was analyzed for residues at Eurofins Dr. Specht Laboratorien GmbH.

τ-fluvalinate application

Apistan® strips (Vita Europe Ltd, Basingstoke, UK) were used for the τ-fluvalinate treatment. As recommended, one strip per box was applied to the τ-fluvalinate treatment groups during the same time of the thiacloprid application. After the treatment period, a pooled sample of beeswax was analyzed for residues at our own lab in Hohenheim. During overwintering, the strips were again inserted to the colonies to resume a chronic treatment.

Assessment of population dynamics

The amount of bees and brood cells (open and sealed) were estimated with the Liebefelder Method (Imdorf et al., 1987), which is a feasible tool that provides accurate and reliable results at the colony level (measuring error +/- 10 %). Care was taken that all colonies were evaluated by the same person on all dates to minimize variation. Colony assessments were usually conducted in the morning before bee flight.

***Varroa* winter treatment**

In order to monitor the level of mite infestation in the colonies and to measure the effectiveness of the τ-fluvalinate treatment, we applied 3.5 % oxalic acid sugar solution to the bees in a brood free stage during late autumn or winter time (30^{th} Nov in 2010 and 29^{th} Dec in 2011). In both years the temperature was below 3 °C for optimal application to a closely spaced bee cluster. Dead mites were counted approximately one week after the treatment with a sticky board, which was inserted at the same day of treatment, respectively.

Statistical analysis

The estimated number of bees and brood cells from both years were checked with a Shapiro-Wilk test for normal distribution ($p>0.05$). Therefore, a one-way ANOVA and a multiple comparison of the means with a post-hoc Bonferroni correction were performed on the four experimental groups, respectively ($\alpha=0.05$).

All tests were performed using WinSTAT (R. Fitch Software, Bad Krozingen).

Results

Overwintering success

In both years, none of the colonies died until the start of wintering in October (Tab. 1). Taken both years together, a total of five of the 33 colonies died over winter. Two of the "Thiacloprid" group (N = 9), one of the "Flu+Thia" group (N = 8), two of the "Control" group (N = 8) and none of the "Fluvalinate" group (N = 8; Tab. 1).

Population dynamics

Experiment 1 (2010 - 2011)

The population of bees and brood cells were estimated four times during the whole season (Tab. 1). The results are shown in Fig. 1a for the number of bees and in Fig. 1b for the number of brood cells. We compared the four treatment groups for each date of the estimates and could not see significant differences (ANOVA) for the number of bees in August 2010 ("AUG"; p=0.254), October 2010 ("OCT"; p=0.473) and April 2011 ("APR"; p=0.388). Likewise, no significant differences of the amount of brood cells were recorded in October 2010 ("OCT"; p=0.590) and April 2011 ("APR"; p=0.128). However, in July the number of bees of the "Control" were significantly lower compared to "Fluvalinate" (p=0.029, ANOVA). The number of brood cells of the "Control" was significantly lower compared to "Thiacloprid" and "Flu+Thia" in July (p=0.012, ANOVA) and compared to "Thiacloprid" in August (p=0.004, ANOVA).

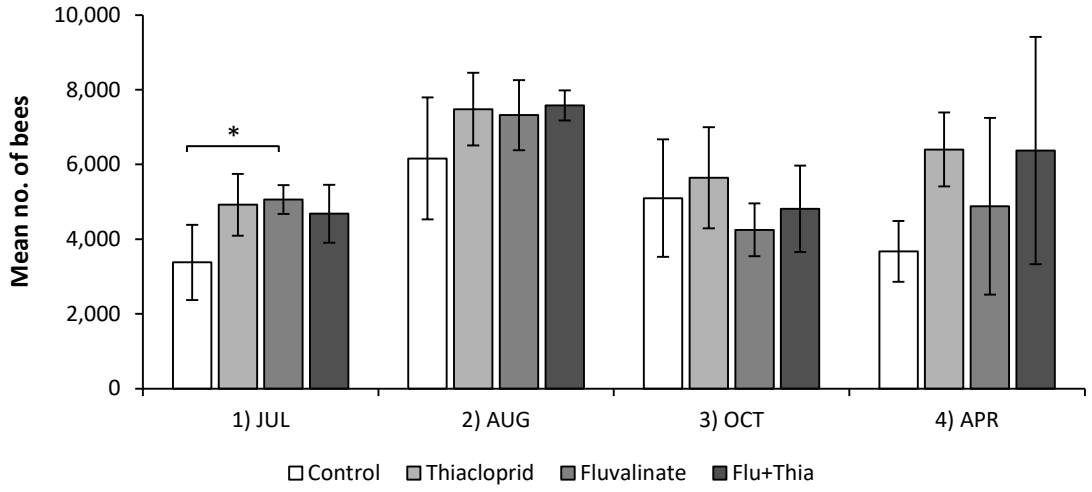

Fig. 1a: Number of bees estimated in the colonies in the year 2010-2011 for the four treatment groups at four different assessments expressed as mean ± standard deviation. * statistically significant ($p<0.05$).

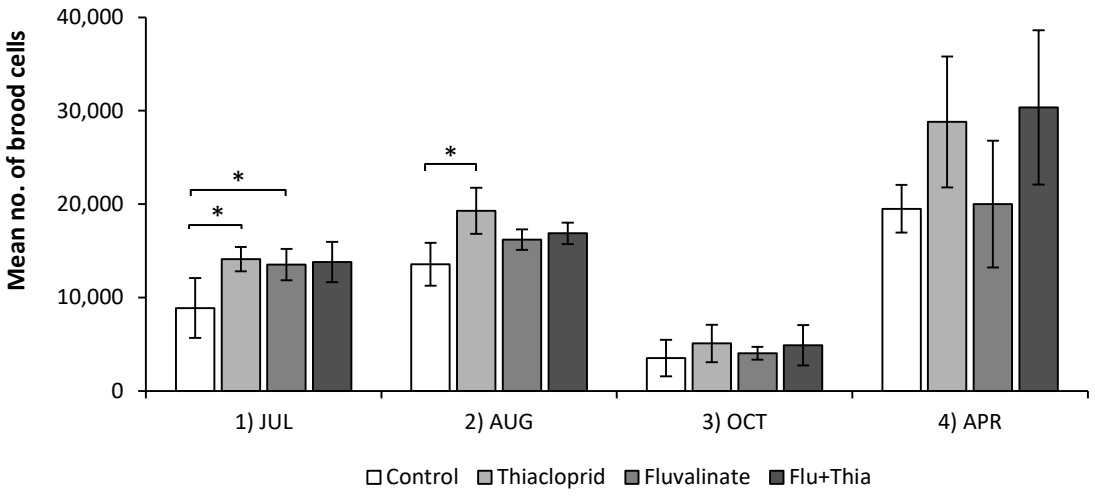

Fig. 1b: Number of brood cells estimated in the colonies in the year 2010-2011 for the four treatment groups at four different assessments expressed as mean ± standard deviation. * statistically significant ($p<0.05$).

Experiment 2 (2011 - 2012)

For the replicate of experiment 1, also four assessments were performed throughout the season. The results are shown in Fig. 2a for bees and in Fig. 2b for brood. We again compared the four groups within each assessment but could not see any significant differences for the number of bees (April 2011 p=0.174; August 2011 p=0.367; October 2011 p=0.664; April 2012 p=0.198) and no significant differences for the number of brood cells in April 2011 (p=0.071), October 2011 (p=0.328) and April 2012 (p=0.176; ANOVA). Solely, in August 2011, the number of brood cells in "Thiacloprid" was significantly lower compared to "Control" and "Fluvalinate" (p=0.017, ANOVA).

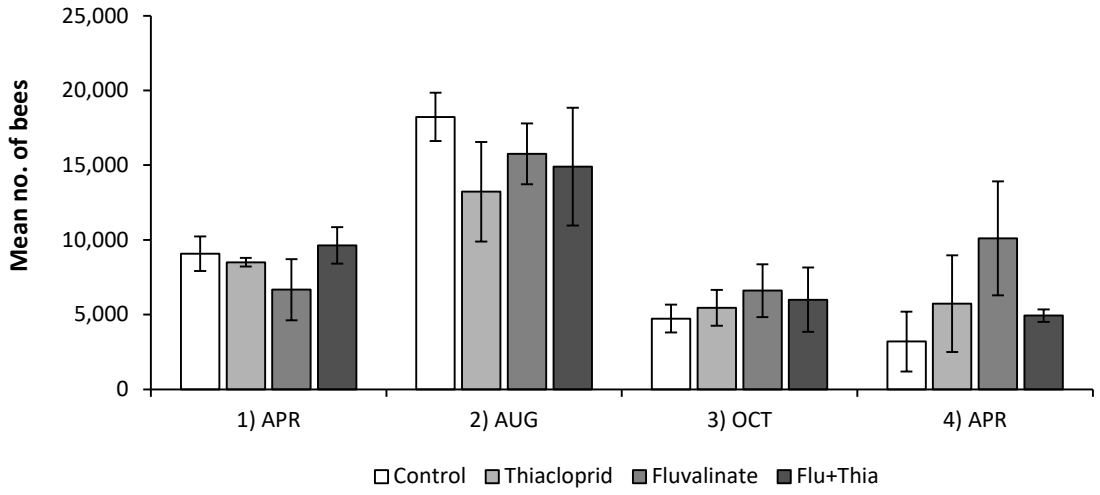

Fig. 2a: Number of bees estimated in the colonies in the year 2011-2012 for the four treatment groups at four different assessments expressed as mean ± standard deviation. We could not see statistically significant differences within the assessments (p>0.05).

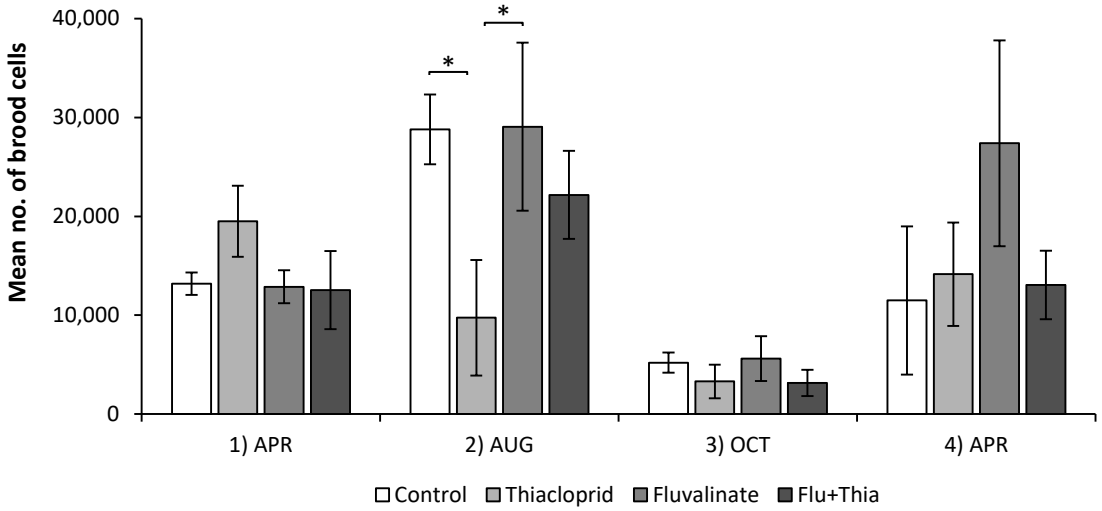

Fig. 2b: Number of brood cells estimated in the colonies in the year 2011-2012 for the four treatment groups at four different assessments expressed as mean ± standard deviation. * statistically significant ($p<0.05$).

Thiacloprid residues

Food from the syrup feeding, which was processed by the bees and stored in honeycombs, was analyzed for thiacloprid residues in both years with QuEChERS method (Limit of Quantification LOQ = 0.01 mg/kg). For the analysis, samples from all colonies and the respective groups per year were pooled. All groups without thiacloprid treatment did not have measurable residues in both years. The pooled samples from the "Thiacloprid" and "Flu+Thia" groups had residues of 0.11 mg/kg and 0.20 mg/kg, respectively, in the year 2010-2011 and 0.29 mg/kg and 0.19 mg/kg, respectively, in the year 2011-2012 (Tab. 2).

τ-fluvalinate residues

Beeswax was analyzed for τ-fluvalinate residues in both years by solid-phase extraction (SPE) and GC-ECD (LOQ = 0.5 mg/kg). For the analysis, samples from all colonies and the respective groups per year were pooled. All groups without τ-fluvalinate treatment did not have measurable residues in both years. Pooled samples from the "Fluvalinate"

and "Flu+Thia" groups had residues of > 100 mg/kg and 16.7 mg/kg, respectively, in the year 2010-2011 and 14.3 mg/kg and 31.6 mg/kg, respectively, in the year 2011-2012 (Tab. 2).

Tab. 2: Thiacloprid residues in pooled food (syrup) samples, which was processed by the bees and stored in the honeycombs from all treatment groups in both years (QuEChERS method, LOQ = 0.01 mg/kg). τ-fluvalinate residues in pooled beeswax samples from all treatment groups in both years (SPE & GC-ECD, LOQ = 0.5 mg/kg).

Year	Treatment	Matrix	Thiacloprid [mg/kg]	Matrix	τ-fluvalinate [mg/kg]
2010-2011	Control	Food	0	Beeswax	0
	Thiacloprid		0.11		0
	Fluvalinate		0		> 100
	Flu + Thia		0.2		16.7
2011-2012	Control	Food	0	Beeswax	0
	Thiacloprid		0.29		0
	Fluvalinate		0		14.3
	Flu + Thia		0.19		31.6
	Feeding Syrup	Syrup	1.6	-	-

Varroa winter treatment

In both years, the winter treatment with oxalic acid killed considerably fewer mites in those groups that have been continuously treated with the acaricide τ-fluvalinate (Fig. 3). In the "Control" and "Thiacloprid" groups between 217 to 409 mites were killed through this winter treatment, on average. In 2010, only one single mite was found in the eight τ-fluvalinate treated colonies! However, in both τ-fluvalinate treated groups the number of mites killed by the winter treatment increased in the second year to an average of 15 mites for the "Fluvalinate" group and 68 mites for the "Flu+Thia" group, respectively.

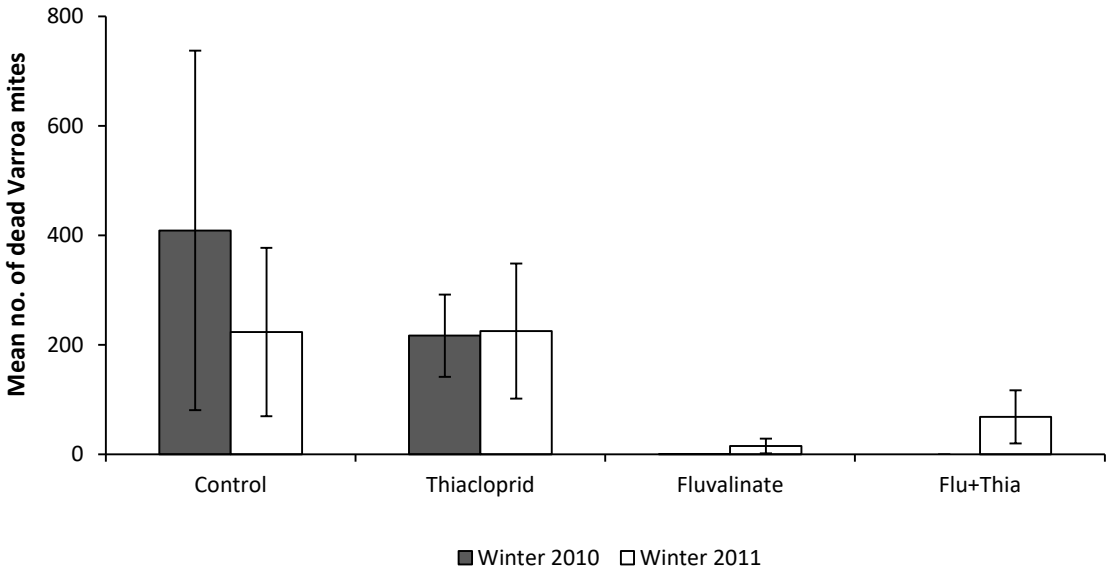

Fig. 3: Graph of the dropped *Varroa* mites approximately one week after oxalic acid treatment during winter time (2010 and 2011) expressed as mean ± standard deviation. In both years a lower number of dead mites could be detected in the τ-fluvalinate treated vs. the untreated groups.

Discussion

We here analyzed the effects of two commonly used pesticides on the population dynamics and the overwintering success of free flying honey bee colonies. The pesticides belong to two different substance classes, one a neonicotinoid insecticide and the other a synthetic pyrethroid widely used as acaricide to combat *Varroa* mites. For both, the insecticide and the acaricide, the applied dosages represent worst case scenarios. Thiacloprid is meanwhile frequently found as residue in pollen and honey, presumably due to the application in flowering oilseed rape and fruit production. Maximum peak concentrations of thiacloprid in bee products such as nectar, honey or pollen range from ~0.05 to 1 mg/kg across the globe (EFSA, 2016; Genersch et al., 2010; Laaniste et al., 2016; Mitchell et al., 2017; Mullin et al., 2012; Pohorecka et al., 2012; Smodis Skerl et al., 2009) but rarely exceed the average level of 0.2 mg/kg (reports of the German Bee Monitoring, see Rosenkranz et al., 2016). It should be mentioned that 0.2 mg/kg is also the maximum value for thiacloprid residues accepted for honey in the EU (EFSA, 2016). The continuous long-term feeding of 1.6 mg/kg thiacloprid to our experimental colonies resulted indeed in residue levels of this magnitude ranging from about 0.1 to 0.3 mg/kg in the stored food. It is interesting to note the significant 8-fold-decrease from the concentration in the original feeding syrup to the honey bee processed syrup stored in the honeycombs. This decrease might be due to a dilution effect, as all colonies could forage and had access to various nectar sources. Furthermore, Iwasa et al. (2004) and Brunet et al. (2005) reported that cyano-substituted neonicotinoids such as thiacloprid and acetamiprid appear to be metabolized more quickly by the honey bee compared to nitro-substituted ones (i.e. imidacloprid, clothianidin). The enzyme that metabolizes thiacloprid very efficiently but lacking impact against imidacloprid was recently identified as a single cytochrome P450, CYP9Q3 (Manjon et al., 2018). As we did not analyze metabolites, this could additionally have contributed to decrease the in-hive concentration of the pesticide by bees processing the syrup.

For τ-fluvalinate, likewise high maximum residue values are reported. Due to their lipophilic property residues are concentrated and accumulated within the beeswax and

can exceed 15 mg/kg (Berry et al., 2013) which is in the range of τ-fluvalinate residues in our experimental colonies after long-term treatment with Apistan® strips. Bogdanov et al. (1998) confirmed an increase of residues with the duration of the strip exposition with a plateau of about 40 to 60 mg/kg after six months whereas other authors found values between 6.6 and 200 mg/kg (Mullin et al., 2010; Adamczyk et al., 2010; Tsigouri et al., 2004).

However, even these residue levels of thiacloprid and τ-fluvalinate are considered to have no acute toxicity to bees or brood (Iwasa et al., 2004; Sanchez-Bayo & Goka, 2014). In our worst case approach we examined whether a long-term exposure to field-realistic peak concentrations of the two pesticides - applied alone or in combination - impairs the development of honey bee colonies under field conditions. In two approaches performed in two consecutive years and using an identical experimental setup we could not detect any negative impact of the treatments on the population of bees and brood and on the overwintering of the colonies. Our moderate overwintering losses of about 15 % (20 % in the first and 8 % in the second winter) are within the range of common winter losses in free flying colonies in Germany and United States (Genersch et al., 2010; Lee et al., 2015) and affected all except the “Fluvalinate” group. Probably, the higher mite load in the untreated groups has contributed to these slightly higher overwintering losses. The mite infestation was quantified in late autumn/winter by an oxalic acid treatment which is known to be highly effective against *Varroa* mites, given that bees are in their winter cluster without brood (Rademacher & Harz, 2006). With the treatment we could also verify that the colonies treated with τ-fluvalinate were sufficiently exposed to this compound during the season, resulting in lower dead mite drops compared to the two groups not treated with τ-fluvalinate. Remarkably, in the winter treatment of the second season our colonies already showed signs of an established τ-fluvalinate resistance in the *Varroa* mite population at our apiary. Such resistance was often reported in the past all over the world (Lodesani et al., 1995; Elzen et al., 1999; Gracia-Salinas et al., 2006; Alissandrakis et al., 2017).

In both years the population of bees and brood was evaluated eight times in a total of 8 - 9 colonies per treatment group. Only in very few cases significant group differences

were recorded. In the first year (2010/2011), the control colonies were slightly weaker at the start of the experiment in spring/summer but revealed no differences any more in the autumn and after-winter evaluations. Although all experimental colonies were established from artificial swarms of approximately the same weight it is not unusual that there are small differences in the first weeks of development in newly established honey bee colonies (Imdorf et al., 2008). In the second year (2011/2012) the "Thiacloprid" group had a significant lower number of brood cells in August, however without differences in the two consecutive assessments and without significant effects on the adult bee population. More importantly, there were no group differences at all in the assessments before and after overwintering, indicating no effects of the pesticide treatment on this crucial colony performance. In a previous study performed in observation hives we could already confirm that behavioral traits like flight activity, antennation, grooming and trophallaxis are not affected by the chronic exposure to high concentrations (1 mg/kg) of thiacloprid (Retschnig et al., 2015). The authors therefore assumed a rather weak impact of the pesticide treatment.

Our results are also in agreement with a three-year study of Siede et al. (2017) who chronically applied two different thiacloprid concentrations (0.2 mg/kg and 2 mg/kg) and could also not confirm any negative impairment on colony health and winter survival. Interestingly, they also found a significant lower amount of brood cells in colonies fed with the high thiacloprid concentration but equally to our results no effect on the colony strength or overwintering was noticed. In contrast to other neonicotinoids (Blacquiere et al., 2012) there has been no prove of acute toxicity of thiacloprid to brood; however, according to our results and those of Siede at al. (2017) this aspect should be considered in future approaches. Berry et al. (2013) could also show for τ-fluvalinate, that exposure to high concentrations in beeswax did not have measurable effects on the amount of brood, amount of honey, foraging rate, time required for marked bees released to return to their hive, percentage of released bees that return to the hive, and colony *Nosema* spore loads. In addition, we here could prove for the first time that a combination of this acaricide with the neonicotinoid insecticide did not have measurable synergistic effects at the colony level.

However, our study is in contrast to many laboratory and semi-field studies providing evidence for negative effects of thiacloprid such as elevated mortality under stress (Doublet et al., 2015) or in combination with pathogens (Vidau et al., 2011), impaired navigation (Fischer et al., 2014), reduced immunocompetence (Brandt et al., 2016), disrupted learning and memory functions (Tison et al., 2017) as well as affected social behavior (Forfert and Moritz 2017; Tison et al., 2016). In most of these studies individual bees were exposed to different concentrations of thiacloprid over a certain time period and subsequently challenged to various physiological tests. The findings were then extrapolated to the colony level without confirmation under field conditions. For example, Tison et al. (2016) found foraging behavior and social communication impaired when applying a concentration of 4.5 mg/kg thiacloprid over one week in a free flying feeder experiment. This exposure corresponds to a 23-fold higher concentration than the maximum value for thiacloprid residues accepted for honey in the EU (0.2 mg/kg; EFSA, 2016). It seems unlikely that honey bees are chronically exposed to such high concentrations under realistic field conditions. Additionally, it makes a difference whether pesticides are applied to individual bees under artificial conditions or to bees within a free flying colony. Obviously, the damage threshold of the honey bee colony as a huge social entity is different from the threshold calculated from the effects on individual bees. This “buffering effect” of the colony has frequently been discussed, however without a final explanation of the underlying mechanisms (Straub et al., 2015; Sponsler & Johnson, 2017). Recently, Odemer at al. (2018) could demonstrate that even the highly bee toxic neonicotinoid clothianidin is significantly less toxic when applied to bees that are kept within the social environment of a colony.

Our results might contribute to the current discussion about the ban of neonicotinoids in agricultural practice which recently led to an assessment of the EFSA considering three neonicotinoids (clothianidin, thiametoxam and imidacloprid) a “risk to bees” (EFSA, 2018). It is an important issue for the agricultural production and for environmental protection, whether neonicotinoids with substantially lower bee toxicity should also be banned. Our results indicate that at least for honey bees the risk is low. It is likely that wild bees or other pollinating insects are more susceptible to thiacloprid as it has been

shown already for bumble bees (Ellis et al., 2017), however more field data on the population level of wild pollinators are necessary for a reliable risk assessment of thiacloprid.

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6 Publication 3: ODEMER *et al.* 2018

Sublethal effects of clothianidin and *Nosema spp.* on the longevity and foraging activity of free flying honey bees

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Abstract

Neonicotinoids alone or in combination with pathogens are considered to be involved in the worldwide weakening of honey bees. We here present a new approach for testing sublethal and/or synergistic effects in free flying colonies. In our experiment individually marked honey bees were kept in free flying mini-hives and chronically exposed to sublethal doses of the neonicotinoid clothianidin. Additional groups of bees were challenged with *Nosema* infections or with combinations of the pesticide and pathogens. Longevity and flight activity of the differentially treated bees were monitored for a period of 18 days. In contrast to previous laboratory studies, no effect of the neonicotinoid treatment on mortality or flight activity could be observed. Although the lifespan of *Nosema* infected bees was significantly reduced compared to non-infected bees a combination of pesticide and pathogen did not reveal any synergistic effect. Our results indicate that individual bees are less impaired by neonicotinoids if kept within the social environment of the colony. The effect of such a "social buffering" should be considered in future risk assessments.

Introduction

The global use of neonicotinoid insecticides has been considered a crucial driver for the decline of insect biodiversity in many parts of the world (Gallai et al., 2009; Potts et al., 2010, Scholer and Krischik, 2014; Stankus, 2014). Neonicotinoids mainly act as specific agonists by binding to acetylcholine receptors (AChR) leading to depolarization and blocking of the synaptic transmission at the postsynaptic membrane of cholinergic synapses. Therefore, they are highly effective in disrupting central nervous system function by overstimulation (Matsuda et al., 2001). In particular bees as the most important pollinator of many agricultural crops (Cresswell et al., 2011; Staveley et al., 2014) have a high risk to come into contact with these neonicotinoids. Due to the systemic property of the neonicotinoids they are often used for seed coating in order to protect the growing plant against herbivores (Elbert et al., 2008). This might result in trace residues of these compounds in pollen/ nectar (Pohorecka et al., 2012) or guttation fluid (Reetz et al., 2011) and therefore, beneficial insects might be exposed to sublethal concentrations. Seed coating is also the preferred application of those neonicotinoid compounds that exhibit an extraordinary high toxicity to bees like imidacloprid, thiametoxam and clothianidin (Iwasa et al., 2004). This high toxicity to bees has been demonstrated in spring 2008 at the Upper Rhine Valley. Here, clothianidin treated corn was sowed with pneumatic drilling machines. The abrasion of the contaminated seed was released into the environment and deposited on surrounding blossoms of orchards and oilseed rape. As a result, 12,000 honey bee hives were heavily damaged (Würfel, 2008).

Besides such obvious impacts through acute poisoning, bees might also come into contact with sublethal concentrations of these neonicotinoids. Traces of the active substances can be translocated into pollen and nectar of the flowering plants (van der Sluijs et al., 2013) or into guttation drops (Girolami et al., 2009; Reetz et al., 2011). Bees might therefore be exposed over longer time periods to sublethal concentrations of neonicotinoids either by foraging in treated crops or later on by consumption of contaminated food storage within the nest which might lead to loss of individual bees (Lu et al., 2014). For individual bees it has been impressively shown that even such

traces of certain neonicotinoids can impair life span (Girolami et al., 2009), memory and orientation (Schneider et al., 2012), foraging efficacy (Henry et al., 2012; Matsumoto, 2013; Karahan et al., 2015), reproductive output (Dussaubat et al., 2016) and immune status (Di Prisco et al., 2013). Additionally, neonicotinoids are supposed to have synergistic effects in combination with honey bee pathogens like honey bee viruses and the intracellular gut parasite *Nosema spp.* (Doublet et al., 2015). Of particular interest in this context is *Nosema ceranae* which is originally a parasite of the Asian honey bee *Apis cerana* and has only recently become invasive in the new host *Apis mellifera* where it is obviously replacing *Nosema apis* in many parts of the world (Paxton et al., 2007; Fries, 2010). There are contradictory statements concerning the impact of *Nosema* infections on colony damages (Chen et al., 2008; Forsgren and Fries, 2010; Gisder et al., 2010; Higes et al., 2013), however several reports confirmed synergistic interactions between *Nosema* infections and neonicotinoids (Alaux et al., 2010; Vidau et al., 2011; Pettis et al., 2012; Doublet et al., 2015).

Consequently, neonicotinoids have been frequently made responsible for periodically high losses of honey bee colonies in Europe and Northern America (Bryden et al., 2013; Lu et al., 2014). Although the absolute number of global honey bee colonies is not decreasing (Moritz & Erler, 2016) the chronic exposure to sublethal concentrations of neonicotinoids together with synergistic interactions are considered a main factor for the weakening of honey bee colonies worldwide (Pettis et al., 2013; Goulson et al., 2015; Sánchez-Bayo et al., 2016).

However, most experiments that confirmed these results have been exclusively performed with individual bees in cage experiments under artificial conditions (Lundin et al., 2015). The few published field studies indicate that the damages of neonicotinoids to honey bees at the colony level are significantly lower than calculated and expected from the results on individual bees (Cutler & Scott-Dupree, 2007; Pilling et al., 2013; Pohorecka et al., 2013; Cutler et al., 2014; Rundlöf et al., 2015). Due to this discrepancy between the individual and colony level more field studies with a chronic application of the pesticides have been required in order to establish a realistic risk

assessment for honey bee colonies that forage in treated crops (EFSA, 2012; Blacquière et al., 2012; Lundin et al., 2015).

General problems for field studies with full sized colonies are the standardization of the colonies and the measurement of weak pesticide effects within the colony. Honey bees can buffer against stressors such as reducing brood production or overcompensating for a particular task allocation. As a superorganism with division of labor and specialization they can afford to overcompensate in response to a particular stress, however only on a group level. Therefore, measuring brood and population dynamics to assess colony health may simply not have enough resolution to detect the harmful effects of stressors such as chronic exposition to pesticides.

We here present a novel approach to combine advantages of laboratory testing in terms of monitoring individual bees over their entire life span with field realistic conditions of free flying honey bee colonies, where treated bees are able to perform age dependent social tasks.

We used newly hatched and individually marked worker bees that were infected or non-infected with *Nosema* spores and put them into small colonies that were chronically fed with either a clothianidin contaminated syrup or a control syrup. With this comprehensive approach we could analyze both, sublethal and synergistic effects of a neonicotinoid and a pathogen on bees. As vitality parameter we used the longevity and the foraging behavior of individual bees. Such approaches are even more important since the ban of three neonicotinoids by the European Union (EFSA, 2013). A final decision whether these pesticides will be available for the agricultural production in future should be taken on the basis of robust field data.

Materials & Methods

Experimental Hive Setup

All hive experiments were performed in a styrofoam mating nuc system ("Kieler mating nuc", KMN) in July and August of the year 2013. Each KMN colony was equipped with four top bars and a strip of a beeswax foundation attached to it (Fig. 1). Every nuc was filled with approximately 800 bees originated from brood frames of two full sized colonies that have been treated against Varroosis and have been proven to be free of *Nosema* spores (Fries et al., 2013). Subsequently, freshly hatched sister queens were introduced to the KMN´s. After one night in a dark and chilled room the KMN colonies were established at a protected apiary of the institute for mating. After a period of five weeks, 12 successfully mated KMN colonies with all stages of brood and freshly built wax combs were used for the following experiments.

Fig. 1 Kieler Mating nuc **(**KMN), equipped with four top bars and stripes of wax foundation and a food container in the back. Outside measurements W 21.5 cm x L 26.0 cm x H 17.0 cm

In front of the hive entrance we installed a special tunnel of lucent plastic material. Thus, the bees had to walk a distance of about 10 cm to enter or leave the hive and marked foraging bees could therefore easily be recorded (Fig. 2).

Fig. 2 Hive entrance with a lucent tunnel device for the observation of flight behavior

Experimental Field Site and Weather Conditions

The KMN hives were set up at the Apicultural State Institute in Stuttgart-Hohenheim (48°42'31.8"N 9°12'38.2"E). Within the closer range of approximately 250 m, no other honey bee colonies were present. In the wider range (> 250 m), other experimental hives as well as observation hives were placed. Main natural food source from local flora mainly was nectar and honeydew from *Tilia spp.*.

The average temperature within the observation period was 22.5 °C with a precipitation of 101.6 L/m². Overall, good weather conditions prevailed to perform the experiment (DWD, 2013).

Clothianidin Treatment

As a metabolite of thiametoxam, clothianidin is a nitro-substituted neonicotinoid of high toxicity to honey bees (Iwasa et al., 2004). The oral LD_{50} was calculated to be 37 µg/kg (37 ppb) or 3.7 ng/bee, respectively with a NOEL of 20 µg/kg (20 ppb) (Würfel 2008).

For the application of clothianidin (Clo) we used the dry compound (99 % purity, Dr. Ehrenstorfer GmbH), which was sonicated in pure water for a stock solution. The amount of stock solution was calculated for a final concentration of 15 µg/kg (or 15 ppb, which was considered to be below an acute toxic concentration (Alkassab and Kirchner, 2016) and diluted in sucrose syrup (Apiinvert, Südzucker GmbH). The same amount of pure water without clothianidin was used for the control treatment.

Treatment groups

Ten of the 12 established KMN colonies were split randomly into two groups of five KMN each. One group received sugar syrup free of any pesticide (Tab. 1) while the other group was chronically fed with 1.12 kg sugar syrup/18 days/KMN containing clothianidin in a concentration of 15 µg/kg, corresponding to a total amount of 16.8 µg clothianidin/18 days/KMN (Tab. 1). The remaining two KMN colonies served as a reserve for potential queen loss. Therefore, bees of each treatment group were allocated to five mini-hives (= replicates).

The effects of clothianidin and/or *Nosema* infection were analyzed in individually marked bees. For this purpose, brood combs from two full sized donor colonies were put into an incubator for 24 hours. Then the freshly hatched bees were mixed and prepared for the experiment. Six groups of 70 freshly hatched bees each were individually labelled with a colored and numbered opalith plate on their thorax. In addition to the individual label per bee we marked the abdomen with a hive specific color (Fig. 3) in order to determine drifting bees that enter “wrong” colonies. Three groups of differently treated bees were added to each KMN colony.

Tab. 1 Setup and color codes of the six different experimental bee groups - 70 of each experimental group split across five mini-hives (KMN), each hosting initially 210 marked bees. Bees from five colonies formed one experimental group of 350 bees.

	Treatment group	Colour code on the thorax	No. of KMN	No. of bees per group per KMN	No. of marked bees per KMN	No. of bees per group in total
Fed with sugar syrup only	***Control (C)***	yellow	5	70	210	350
	N.ceranae (N.cer)	green		70		350
	N.apis (N.apis)	red		70		350
Fed with *Clothianidin* sugar syrup solution	***Clothianidin (Clo)***	light yellow	5	70	210	350
	N.ceranae+Clothianidin (N.cer+Clo)	light green		70		350
	N.apis+Clothianidin (N.apis+Clo)	light red		70		350
Total amount			**10**			**2100**

Fig. 3 Individually labelled honey bees with a group specific colored and numbered opalith plate on the thorax and a hive specific color on the upper side of the abdomen. An amount of 35 bees were put into a stainless steel cage (outside measurements: W 8.5 cm x L 4.5 cm x H 6.5 cm) for mass feeding with either spores of *N. apis*, *N. ceranae* or no spores at all for control

Infection with *Nosema apis* and *Nosema ceranae*

Before the introduction into the KMN the hatched and marked bees were put into a stainless steel cage and fed with sucrose solution (n=35 bees per cage). We used three reversed caps of Eppendorf cups as feeding dish, which were put into each cage and filled with a total amount of 650 µL sucrose solution per cage, corresponding to 18.6 µL solution/bee. Depending on the treatment group, the sucrose solution contained spores of *N. apis*, *N. ceranae* or no spores as a control.

The *Nosema* spores were extracted from the midgut of artificially infected bees, which were previously reared in cages at our institute. Differentiation between *N. ceranae* and *N. apis* species were confirmed via qPCR (Fries et al., 2013). Only freshly extracted spore suspensions were used and purified twice via centrifugation and then diluted in sucrose syrup. The spore count of the solution was performed with a Thoma counting

device to approximately 488,000 spores/650 µL per cage or, on average, 14,000 spores per bee. We waited until the bees consumed all of the food which usually was the case after 24 hours. Subsequently the bees were fed for another 24 hours with pure sucrose solution (without spores) in order to provide enough time that the spores have passed the proventriculus which minimize the risk of cross infections between the different treatment groups.

Analysis of *Nosema* infection

After the observation period ten bees per group and colony were inspected for *Nosema* infection, respectively. Single bees were crushed with 500 mL of pure water each in Bioreba extraction bags. Spores then were counted according to the "Standard methods for *Nosema* research using a light microscope and a Thoma counting chamber (Fries et al., 2013).

Mortality and flight activity

After the artificial *Nosema* infection all marked bees were introduced into the KMN colonies according to Tab. 1. The experiment started 24 hours after the introduction for a period of 18 days. The observation included a daily mortality check, for which all combs including the inside of the hive were photographed for the later on counting of the marked bees on a computer screen. The pictures were taken outside the foraging activity, early in the morning. The overall recovery rate is also shown in Tab. 2.

The flight activity of marked bees of all 10 colonies was analyzed by counting leaving and returning bees at the entrance over a period of 60 minutes per colony and day. Due to the weather conditions flight activity could be recorded at 10 days during the 18 day observation period.

Both, mortality and flight activity were analyzed using individual bees of the 6 treatment groups whereby each treatment group was distributed over five mini-hives.

Fig. 4 Picture of a brood comb from the KMN colonies for the daily mortality assessment

Residue Analysis

Before start of the experiment, a sample of the feeding syrup mixed with clothianidin was collected. Pooled samples of pollen (bee bread) and stored food of the control and clothianidin colonies were collected at the end of the observation period (day 18) out of in-hive storage cells. These samples were analyzed using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive Solid Phase Extraction (SPE) - QuEChERS-method; German version EN 15662:2009 in certified labs (feeding syrup and food: eurofins Dr. Specht Labs Hamburg, LOQ 3 µg/kg; pollen: LUFA Speyer, LOQ 0.3 µg/kg,).

Statistical Analysis

We evaluated the mortality data with a Kaplan-Meier-Survival analysis. Survivorship between control and treatment(s) was compared pairwise and tested for significance with Log-Rank Tests (Cox-Mantel) followed by a Bonferroni correction. Workers which were collected at the end of the experiment were considered censored, equal to those observed but not collected on the last day of the experiment.

Flight activity data were checked with a Shapiro-Wilk test, refusing normal distribution ($p < 0.05$). Therefore, a Kruskal-Wallis-H-Test was performed on the six experimental groups for bees returning to the mini-hives. In case of significant differences, groups then were further tested pairwise using a Mann-Whitney-U-Test with Bonferroni correction ($p = 0.003$).

The different *Nosema* spore counts per group did also not fulfill normal distribution (Shapiro-Wilk test, $p < 0.05$). Therefore a Kruskal-Wallis-H-Test was performed and in case of significant differences, groups then were further tested pairwise using a Mann-Whitney-U-Test with Bonferroni correction ($p = 0.003$). All tests were performed with WinSTAT (R. Fitch Software, Bad Krozingen).

Results

Recovery Rate of Introduced Bees

The recovery rate was calculated by the number of bees that could be rediscovered 24 h after the introduction of 70 particularly treated worker bees per mini-hive. The high recovery rates in all groups ranging from 90.3 to 96.3 % (Tab. 2) indicate that the prior treatment (feeding of clothianidin and *Nosema* spores) did not had an acute negative impact.

Tab. 2 Recovery rates of all treatment groups. "Recovered bees" represent the number of all bees that were identified 24 h after the introduction into the respective mini-hive.

	Introduced bees	Recovered bees*	Recovery rate [%]
Control	70	66.0 ± 3.5	94.3
N. ceranae	70	65.8 ± 2.3	94.0
N. apis	70	65.8 ± 2.6	94.0
Clothianidin	70	63.2 ± 3.5	90.3
N. ceranae + Clo	70	65.0 ± 4.8	92.9
N. apis + Clo	70	67.4 ± 1.9	96.3

*mean of all grouped bees in n=10 KMN colonies at day one of the experiment

Residue Analysis

Samples of feeding syrup, pooled pollen (bee bread) and food from combs of the control and clothianidin colonies were collected at the end of the observation period (day 18) from in-hive storage cells. The intended clothianidin concentration in the feeding syrup could be verified by laboratory analysis. Additionally, we found measurable residues between 2 and 6 µg/kg in stored food and pollen of the clothianidin treated KMN. We could also confirm that the untreated controls were free of clothianidin residues (Tab. 3).

Tab. 3 Residue analysis of control and clothianidin treated feeding syrup prior to observation period. Pooled food and pollen from storage combs of all control and clothianidin treated KMN colonies after 18 days of observation (LC-MS/MS, LOQ: 3 µg/kg for food, 0.3 µg/kg for pollen).

	Control	Clothianidin
Stock solution	-	15 mg/kg
Feeding syrup	0 µg/kg	15 µg/kg
Stored food	0 µg/kg	6 µg/kg
Stored pollen	< 0.3 µg/kg	1.79 µg/kg

Mortality of Worker Bees

The Kaplan-Meyer analysis of the differentially treated bees revealed highly significant differences between the six groups (Log-Rank $p<0.001$) (Fig. 5). A pairwise post hoc analysis with Bonferroni correction of all treatments showed that only the two groups treated with *N. ceranae* ("*N. ceranae"* and "*N. ceranae* + Clo") had a significant higher mortality when compared to the control ($p<0.003$) (Fig. 5). Neither the "*N. apis*" groups nor the clothianidin group had a significant higher mortality compared to the control. Within the untreated control group we analyzed colony-specific effects and did not find significant differences between the 5 mini-hives (Cox regression with pairwise comparison and Bonferroni correction). The results indicate that *N. ceranae* but not clothianidin represented the crucial factor for shortened life span.

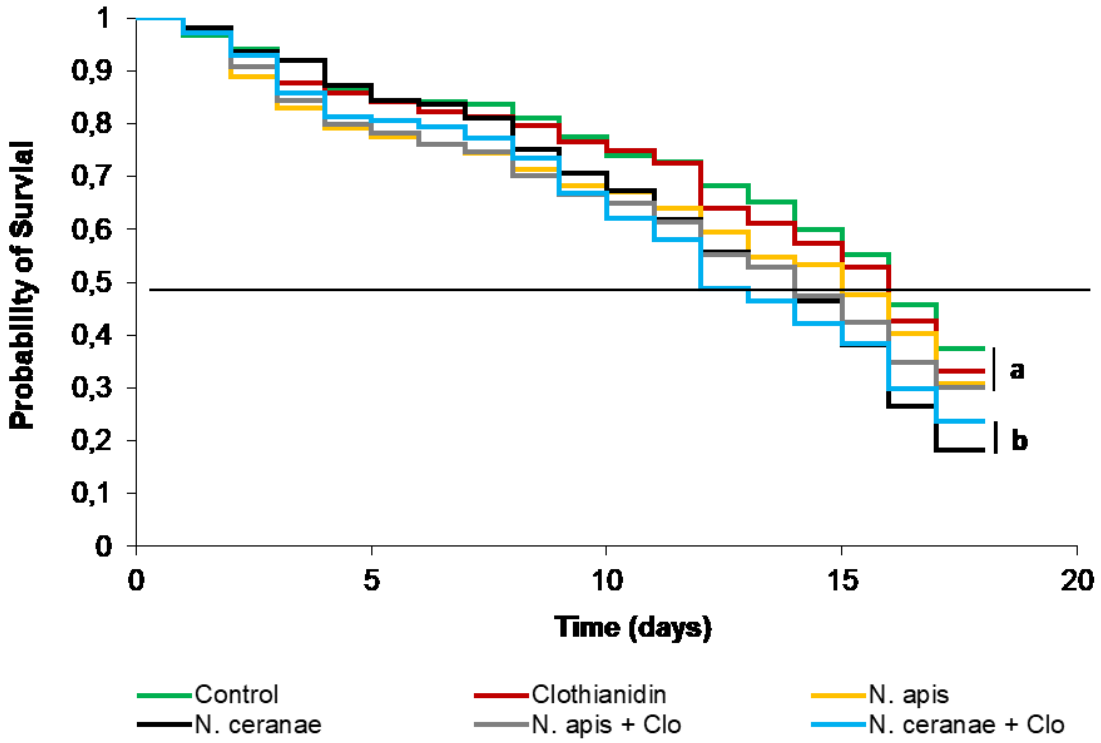

Fig. 5 All six groups were compared with a Kaplan-Meier-Survival analysis. A post-hoc Log-Rank test revealed highly significant differences between those groups (Log-Rank $p<0.001$), therefore we tested groups pairwise. Different letters indicate statistically significantly higher mortality when compared to the control group ($p<0.003$)

Flight Activity

Bees from the "*N. ceranae*" group revealed the highest, and bees from the "clothianidin" group the lowest flight activities (Fig. 6). However only slightly significant differences in the overall flight activity of the six treatment groups (= returning foragers) were found (Kruskal-Wallis-H-Test; $p = 0.04$), but no significant differences were confirmed with a pairwise comparison of the groups (Mann-Whitney-U-Test, $p > 0.003$).

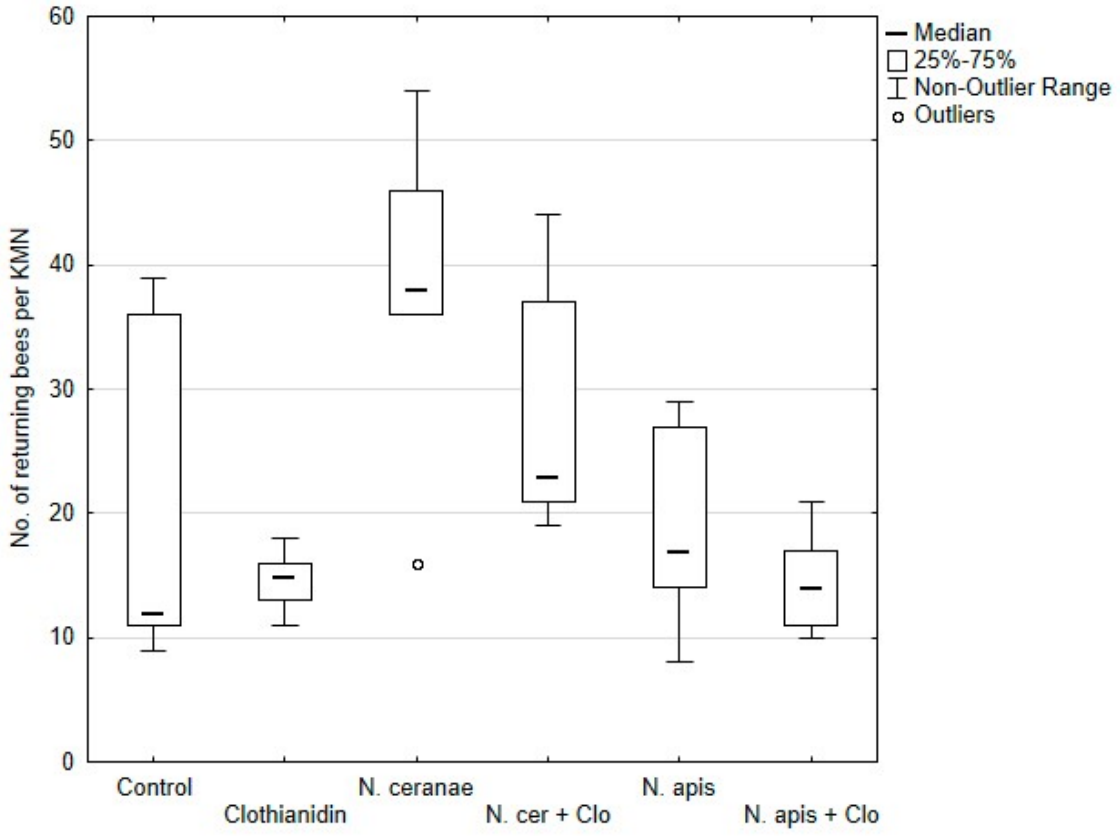

Fig. 6 Box-Whisker-Plot of incoming forager bees of all six treatment groups (n=5 KMN) within the 18 days observation period. *N. ceranae* infected bees revealed the highest and the bees of the control group the lowest flight activities. However, no significant differences were found with a pairwise post-hoc comparison of all groups (Mann-Whitney-U-Test, $p > 0.003$, Bonferroni correction)

Nosema Spore Counts and Infection Ratio

The average numbers of spores per bee from approximately n=50 individuals per treatment group ranged from 925,500 (control) to 7,839,286 ("*N. ceranae* + Clo") after 18 days of incubation (Fig. 7). A Kruskal-Wallis H-Test revealed a highly significant difference between the six groups ($p<0.003$). Bees from both *N. ceranae* groups had the highest amount of spores followed by the two *N. apis* groups. The originally uninfected control and clothianidin treated group also showed slight *Nosema* infections. All *Nosema* treated groups had significantly higher spore counts than the control group (U-Test, $p<0.003$). No differences between clothianidin treated and non-treated groups could be observed, e.g. "clothianidin" vs. "control", "*N. ceranae* + Clo" vs. "*N. ceranae"* and "*N. apis* + Clo" vs. "*N. apis"* (Mann-Whitney-U-Test, $p>0.003$).

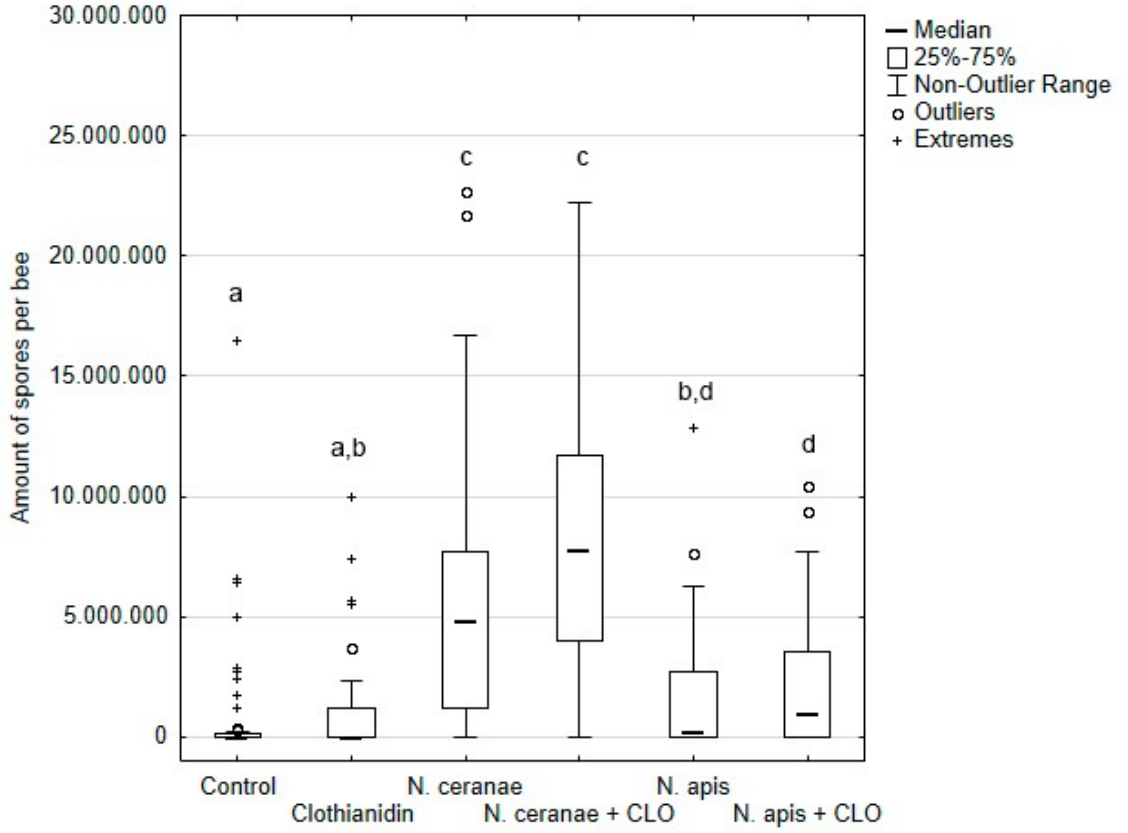

Fig. 7 Box-Whisker-Plot of the amount of spores per bee after 18 days of incubation. Columns with different letters indicate significant differences (Mann-Whitney-U-Test, post-hoc Bonferroni correction, $p < 0.003$)

A successful *Nosema spp.* infection of the respective groups could be validated with the ratio of infected bees (Fig. 8). All intentionally infected bees showed infection rates from 66-93 %, however 28-34 % bees of the non-infected groups showed an infection too but with clearly lower numbers of spores per bee (Fig. 7).

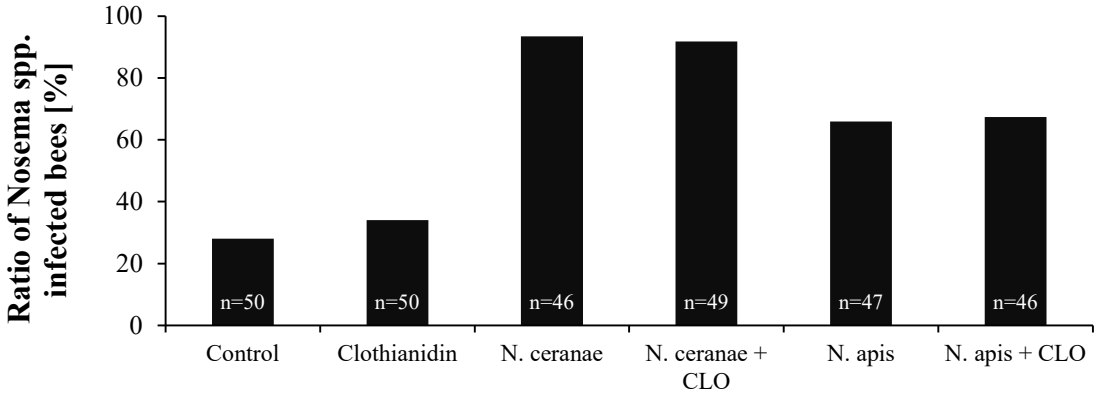

Fig. 8 Ratio of *Nosema spp.* infected bees per group after 18 days of incubation. Both groups originally not infected with *Nosema* spores (control, clothianidin) showed the least rate of infection. Both *N. ceranae* groups were above 90 % and both *N. apis* groups above 66 %

All positive *Nosema* bee samples were analyzed with qPCR to differentiate from the species *N. apis* and *N. ceranae* to determine possible cross infections. Results are shown in Tab. 4. Bees from both *N. ceranae* groups had almost 0 % cross infections, whereas bees from originally not infected groups were nearly entirely infected with *N. ceranae.* In contrast, both *N. apis* groups showed approximately 50/50 cross infection ratios with *N. ceranae.*

Tab. 4: All *N. spp* infected bee samples were analyzed via qPCR for the ratio of both *Nosema* species. Bees from originally not infected groups were almost entirely infected with *N. ceranae*, so were both *N. ceranae* groups. The two *N. apis* groups showed an approximately 50/50 cross infection ratio with *N. ceranae*.

	Total bees (N)	Infected bees (N)	*N. apis* (%)	*N. ceranae* (%)
Control	50	11	0.0	100.0
Clothianidin	50	19	0.3	99.7
N. apis	46	27	46.2	53.8
N. apis + Clo	49	34	50.4	49.6
N. ceranae	47	42	0.0	100.0
N. ceranae + Clo	46	45	2.4	95.4

Discussion

With our new approach we could clearly show that the effects of a chronic exposure of sublethal concentrations of neonicotinoids on honey bees strongly depend on the experimental setup. Obviously, the way of application of the pesticide and the way how the bees are kept during the experiment has a huge impact on the toxicity of the pesticide at the colony level. In many studies, side effects of certain neonicotinoids on individual bees have been described when sublethal concentrations and/or dosages were applied. Among others, learning, memory, orientation and foraging behavior were negatively affected in individual worker bees (Henry et al., 2012; van der Sluijs et al., 2013, Scholer & Krischik, 2014; Fischer et al., 2014; Charreton et al., 2015; Karahan et al., 2015; Tosi et al., 2017) and moreover, the reproductive capacity of queens and drones was significantly reduced (Williams et al., 2015; Kairo et al., 2016; Chaimanee et al., 2016). Furthermore, synergistic effects in combination with honey bee diseases, mainly with viruses (Di Prisco et al., 2013) and *Nosema spp.* infections have been demonstrated (Vidau et al., 2011; Aufauvre et al., 2012; Pettis et al., 2012, 2013; Doublet et al., 2015). However, most of these experiments were performed with single bees that were kept and treated under laboratory conditions, often in cage tests. This was already criticized in a meta-analysis reviewing 268 primary research studies on neonicotionids and bees (Lundin et al., 2015) leading to the demand for more studies that measure effects on the colony level. In contrast to the large number of cage tests the few studies that measured effects on honey bee colony performance in the field could not confirm clear negative effects of neonicotinoids (Blacquière et al., 2012; Pilling et al., 2013; Rundlöf et al., 2015; Henry et al., 2015). A recent large study of Woodcock et al. (2017) in three European countries revealed negative effects on both, wild and managed bees but the effects were not consistent across countries. Another recent study confirms clear negative effects of neonicotinoids on the colony level (Tsvetkov et al., 2017), however after exposure of honey bee colonies to a large cocktail of more than 25 pesticides over a period of several months. To better understand the discrepancy among the various studies we here present an approach that combines the advantage of laboratory tests - i.e. the defined application of certain compound(s) and analysis of

individual bees - with an experimental design where the bees could perform their natural task within the social environment of a bee colony.

For the sublethal treatment we tried to simulate a field realistic worst case exposure (Pecenka and Lundgren, 2015; Rundlöf et al., 2015; Tosi et al., 2017) while staying at the same time below the NOEL of 20 µg/kg clothianidin (Alkassab & Kirchner, 2016; Würfel, 2008). Therefore, we used sugar syrup spiked with clothianidin to a final concentration of 15 µg/kg for the chronic feeding of the test colonies. After each test colony received an amount of more than one kg of this contaminated syrup over a period of 18 days, the analysis of a pooled sample of stored food from all treated colonies confirmed a concentration of 6 µg/kg clothianidin suggesting an approximately 1:1 dilution of the fed syrup with the nectar collected by foraging. This dilution effect may explain why detrimental effects are rather absent in a full colony set-up when compared to lab-testing and may play a crucial role for the "buffering capcity" of a honey bee colony. The control colonies were free of clothianidin residues. Due to our mass feeding approach we cannot exactly determine the pesticide consumption of each individually marked bee. However, since all bees had to use either the syrup or the stored food we can safely assume a chronic intoxication with clothianidin ranging from 6 µg/kg (food) to 15 µg/kg (syrup) over the experimental period of 18 days.

Effect of clothianidin on mortality and flight activity

The median life-span of the untreated control bees was somewhat lower than reported from large free flying colonies but laid in the range of other tests with small experimental units (Retschnig et al., 2015). A chronic feeding with clothianidin, however, did not have any effect on the life span of the bees within the treated colonies. This is in contradiction with experiments on the homing ability of foraging bees that have been treated with clothianidin or thiametoxam (Henry et al., 2012; Tosi et al., 2017). Though, in both studies the concentration of the applied pesticide was two to four times higher than in our experiment which does not correspond to field realistic conditions (Cresswell & Thompson, 2012; Guez, 2013) and is clearly higher than the

concentrations recently measured in the nectar from clothianidin treated fields in Europe (Rundlöf et al., 2015, Henry et al., 2015, Rosenkranz et al., 2013). Furthermore, the bees in the studies of Henry et al. (2012) and Tosi et al. (2017) were fed in the laboratory over a period of several days prior to the homing experiments which might be an additional stress factor. A similar discrepancy between semi-artificial homing experiments and a long-term field study have been recently confirmed for thiacloprid, another commonly used neonicotinoid. While artificially treated bees revealed a clear reduced capacity in navigation and homing behavior (Fischer at al., 2014), a chronic exposure to high concentrations of thiacloprid over three years did not adversely affect the tested honey bee colonies (Siede et al., 2017). Several studies support our finding that sublethal and field realistic concentrations of neonicotinoids does not increase the bee mortality in free flying colonies (Schmuck et al., 2001; Faucon et al., 2005; Cutler & Scott-Dupree, 2007; Pilling et al., 2013; Rundlöf et al., 2015; Woodcock et al., 2017).

It is noticeable that the low mortality of the clothianidin treated bees in our experiment was not a consequence of a reduced flight activity. There were no significant differences between bees from the control group compared to bees from the different treatment groups. This is in accordance with a recent field study (Henry et al., 2015) but again in disagreement with a former study of the same author (Henry et al., 2012). It is further noticeable that both groups infected with *N. ceranae* revealed the highest flight activity which is confirmed by the findings of Dussaubat et al. (2013).

There are several reasons why sublethal concentrations of neonicotinoids might act differently in cage tests, semi-artificial approaches or field tests with entire colonies. Obviously this is not only the consequence of the “buffering capacity” of a honey bee colony as a huge eusocial “superorganism” that is able to quickly compensate for the loss of a certain number of impaired individuals (Henry et al., 2015). Honey bees at the colony level seem to be less impaired and diversely affected than individual bees held under artificial conditions (Straub et al., 2015). This quality however, appears to be reserved to highly eusocial insects only (Ellis et al., 2017). Our results rather indicate that it even makes a difference whether individual bees are exposed to contaminated food within their social environment or whether they are isolated from their social entity

for the application of the pesticide. So far it is unknown how social interaction on the colony level could alter the toxic effects for individuals. According to Sponsler and Johnson (2017), individual- and colony-level effects are linked in a complex and hardly understood way. In addition, the authors make very clear that even studies on the toxicity of pesticides on the colony level require individual-oriented approaches. Our experimental setup fulfills these requirements by applying a defined amount of pesticide and by analyzing individual bees within their social environment.

Nosema infection

The artificial infections with *N. ceranae* were highly successful which is confirmed by the average number of spores per bee ranging from 5.8 to 7.8 million spores for the "*N. ceranae"* and the "*N. ceranae* + Clo" group, respectively. These infection rates match the results of natural infected bees of similar age (Smart & Sheppard, 2012). In contrast, the artificial infection with *N. apis* spores was less successful leading only to infection rates ranging from 1.8 to 2.1 million spores per bee for the "*N. apis"* and the "*N. apis* + Clo" group, respectively. Because we used the same amount of fresh spore material for both *Nosema* species, these differences indicate a slower growth of the *N. apis* infection (Natsopoulou et al., 2015). This is in accordance with studies showing a better growth of *N. ceranae* under higher temperature conditions (Martin-Hernandez et al., 2009; Gisder et al., 2010) and consequently *N. ceranae* is meanwhile the predominant *Nosema* species in Southern Germany (Rosenkranz et al., 2013). Because infected and non-infected bees were kept within the same colony, some cross infection was inevitable. However, due to the low spore load of the non-infected groups a pathogenic effect seems unlikely.

The bees infected with *N. ceranae* showed a significantly reduced lifespan. Also the bees infected with *N. apis* showed a similar but not significant tendency. However, due to the above mentioned lower infection rates the interpretation of pathogenic effects in the *N. apis* groups must be taken with care. This is in agreement with many studies confirming a shorter lifespan in *Nosema* infected bees, primarily caused by an earlier

start of foraging (reviewed in Higes et al., 2013). Accordingly, also in our experiments the two *Nosema* infected experimental groups revealed the highest flight activities.

Although *N. ceranae* had a clear negative impact on the infected bees we could not prove any synergistic or additive effects when *Nosema* infected bees were additionally exposed to chronic clothianidin feeding. This clothianidin feeding did neither shorten the lifespan nor change the flight activity compared to *Nosema* infected bees that received untreated syrup.

At least in terms of an increased mortality we clearly contradict the results of Alaux et al. (2010) and Vidau et al. (2011), who both showed synergistic effects with *N. ceranae* and a neonicotinoid pesticide. However, these studies were conducted in cage experiments under laboratory conditions where bees probably react more sensitive to *Nosema* infections. In addition, *Nosema* strains may vary in infectivity and virulence (Genersch, 2010) and a number of experiments provide evidence that related to the genetic background of the honey bee host, the level of tolerance and resistance to *N. ceranae* can produce a different outcome (Dussaubat et al., 2013; Fontbonne et al., 2013; Huang et al., 2013; Huang et al., 2014). Similar findings of field studies performed in observation hives or full sized colonies assessing synergistic effects between neonicotinoids (thiacloprid, clothinaidin) and *N. ceranae* support and augment our conclusion (Goss, 2014; Retschnig et al., 2015; Rolke et al., 2016). Yet, the results of this experiment cannot certainly exclude synergistic effects between neonicotinoids and parasites of other degrees. Further studies should therefore include a positive control and comprise different concentrations of pesticides and other pathogens like *Varroa* mites or bee viruses (Fries et al., 2011). For such applications, our test system represents a suitable approach.

Conclusion

Our study strongly indicates that in free flying honey bee colonies the effects of sublethal concentrations of neonicotinoids - alone or in combination with a pathogen - on bee mortality are substantial lower compared to *in vitro* experiments with caged

bees. According to our results this “buffering effect” is not a simple replacement of dead worker bees by the huge amount of brood in a full sized colony but rather a lower susceptibility of the individual bee when the pesticide is applied within the well-balanced social community. The physiological mechanisms responsible for this lower susceptibility still need to be clarified.

We could also show that the KMN mini-hives used in our study are suitable for testing effects of pesticide and pathogens on the colony level. As we did not detect synergistic effects in the present approach, further studies have to prove how synergistic interactions are measurable under these colony conditions. The “colony” is the crucial endpoint for a final risk assessment, however typical colony-level performance parameters like population dynamics, honey yields and overwintering rates depend strongly on environmental factors and are difficult to record (Sponsler & Johnson, 2017). As colony level effects are finally the result of the intoxication of individual bees, our approach offers the possibility to measure the impact of pesticide treatments on individual bees in consideration of the complex effects of “social buffering”.

Our results cannot finally answer the question whether certain neonicotinoids should be excluded from the agricultural practice. The great number of studies dealing with the impact of neonicotinoids on honey bees came to varying results and therefore different recommendations concerning the future use of these pesticides. For regulatory authorities and political decision-makers a scientific-based risk assessment is therefore extremely difficult. A better regulation and standardization of the methods that are used for the study of neonicotinoids and honey bees would be an important first step.

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7 General Discussion

Since decades, honey bees were challenged to sublethal doses of pesticides, alone or in combination with other stressors. However, only in recent times this fact has become a major topic for bee research. One reason for this could be the increasing reports regarding continuous or periodic colony losses all over the world. Another likely reason is the increasing use of neonicotinoid insecticides and the global spread of new honey bee parasites such as *Nosema spp*. and *V. destructor*. However, it is a particular challenge to measure the impact of such stressors in honey bees due to the fact that a honey bee colony provides two levels of the phenotype, the individual bee and and the colony as a social superorganism. On the colony level, sublethal effects are often not easily detectable. Therefore, many experiments are performed on individual bees under laboratory conditions. However, such results cannot easily be translated to the state of a huge and free flying colony in the field. Experts therefore agreed that more realistic approaches are needed to measure parameters like longevity, foraging activity and especially social interactions of individual bees within the honey bee "superorganism" (Carreck, 2017). The three publications in this thesis present different methodical approaches for this problem with the aim to quantify the effects of a parasite and different pesticides, applied alone or in combination.

In our first study (Retschnig et al., 2015), we employed observation hives in order to analyse the mortality of treated worker bees, their flight activity and social behaviour. Here the colonies were treated with a combination of two pesticides of different classes (neonicotinoid, pyrethroid) combined with the artificial infection of the widespread gut parasite *N. ceranae.* The study was performed at two different locations in parallel (Germany, Switzerland). In one location, we were able to show a higher mortality in both pesticide treatments but not in the colonies that were exclusively infected with *N. ceranae* (see Publication 1, Fig. 1). In contrast, at the other site we did not observe any differences between the treatments. We therefore conclude that the location – i.e. environmental factors – has a significant influence on the impact of certain stressors. This was recently confirmed in a large field study with full sized colonies demonstrating country-specific effects of pesticides (Woodcock et al., 2017). In our experiment we

have even used the same source of bees for the test colonies in order to standardize our setup in the best possible way. For this, it was necessary to transport source colonies with the respective brood combs from Switzerland to Germany. Obviously, this migration had a sustainable impact on the later on hatching bees. Recently, migratory beekeeping practices were identified to decrease life-span and affect oxidative stress levels in worker bees (Simone-Finstrom et al., 2016), which may here explain differences in both locations. Further, Rueppell et al. (2017) revealed that mortality rate was more affected than social behaviour when honey bee workers were exposed to early life stress, supporting our results.

Other studies have indicated that thiacloprid and tau-fluvalinate alone did not increase worker bee mortality under field conditions (Siede et al., 2017, Berry et al., 2013). In fact, the combination with *N. ceranae* did not reveal any synergistic interactions in our study. This, however, is contrary to many laboratory experiments. For example, Vidau et al. (2011) and Doublet et al. (2015) demonstrated elevated mortality when *N. ceranae* infected bees were exposed to thiacloprid and Forfert and Moritz (2017) found the number of social interactions among caged workers reduced when bees were fed with the pesticide. One explanation for such different findings may be the interpretation of what are field realistic concentrations. The previously stated studies indeed used sublethal thiacloprid levels, but approximately 21- to 1000-fold higher than the maximum value for thiacloprid residues accepted for bee products in the EU (0.2 mg/kg; EFSA, 2016) and 4- to 20-fold higher than the concentration used in our experiment (Tab. 1). Furthermore, the absence of the queen in cage studies can be a major impact in terms of pheromone profiles, colony cohesion and social homeostasis (Botías et al. 2012a; Rangel et al. 2016), representing an additional stressor affecting the experimental outcome.

The inconsistency of our results from the observation hives suggests, that pesticide/pathogen effects were rather weak or remained undetected with the here used methods. As a novelty, we provided evidence that combinatorial stressors which have synergistic effects *in-vitro* do not necessarily translate equally to *in-vivo* conditions.

In the second study (Odemer & Rosenkranz, 2018) we applied the same pesticides from the observation hive experiment but on full sized colonies kept under real beekeeping conditions. In the protocol, we here included colony performance factors such as population dynamics of bees and brood and overwintering success. In addition to the study of Retschnig et al. (2015) we established a worst-case exposure scenario over a long time period. For the first time we could show that neither thiacloprid nor tau-fluvalinate, nor the combination of both as possible synergists had effects on the above mentioned performance parameters. The only significant differences were detected in the number of *Varroa* mites, dropped from treated and untreated colonies during the winter. As expected, tau-fluvalinate colonies were almost mite free in the first year. However, in the second year mite numbers were lower but not significantly different to untreated colonies, demonstrating a strong potential of resistance-building to synthetic acaricides (Milani, 1999). Since more than 20 years now (Watkins, 1997) increasing resistance is largely indicated by the overuse and the associated residues found in bee products world wide (Martel et al., 2007; Lambert et al., 2013; Pohorecka et al., 2017). This development suggests the urgent need to rethink common control strategies for *V. destructor* in terms of alternatives, sustainability, and preservation of natural bee products (Rosenkranz et al., 2010). Moreover, such management practices are suspected of elevating honey bees' sensitivity to insecticide exposure (Rinkevich et al., 2015; Rinkevich et al., 2017).

Other studies support the conclusion that neither thiacloprid nor tau-fluvalinate alone have negative impact on full sized colonies; we could show, in addition, that no synergistic effects of the here used pesticides were measurable (Berry et al., 2013; Faust, 2015; Siede et al., 2017). As one of the few neonicotinoid insecticides classified as not harmful to bees (e.g. category B4: bienenungefährlich in Germany), our results support the findings that cyano-substituted neonicotinoids have a lower acute toxicity to honey bees when compared to nitro-substituted neonicotinoids such as clothianidin or imidacloprid (Iwasa et al., 2004). The authors suggest that with the help of specific enzymes, thiacloprid is quickly metabolized by the bees. The enzyme that metabolizes thiacloprid very efficiently but lacking impact against imidacloprid was recently

identified as a single cytochrome P450, CYP9Q3 (Manjon et al., 2018). Thiacloprid metabolites are not considered toxic, hence, they are representing a step within the honey bee immune system's detoxification process (Berenbaum & Johnson, 2015).

Tab. 1 Thiacloprid residues from different authors found in the field (upper half) and used in experiments (lower half). A field realistic maximum average value was assumed to be 0.2 mg/kg according to the maximum value for thiacloprid residues accepted for bee products in the EU (EFSA, 2016). Therefore a factor was calculated to demonstrate deviations in the different studies.

Matrix	mg/kg	ppb	Publication	Factor	Comment
	173.2	173,200	Würfel, 2008	866	oral-LD_{50}
Bee products	0.2	200	EFSA, 2016	1	Max
Honey	0.002	2	Mitchell et al. 2017	0.01	Avg
	0.047	47		0.2	Max
Honey	0.02	20	Laaniste et al. 2016	0.1	Avg
	0.13	130		1	Max
Nectar	0.2088	209	Pohorecka et al. 2012	1	
Pollen	1.0022	1,002		5	Max
Pollen	0.115	115	Mullin et al. 2010	1	Max
Pollen	0.09	90	Smodis Skerl et al. 2009	0.5	
Beebread	0.009	9	Genersch et al. 2010	0.05	Avg
	0.199	199		1	Max
	0.154	154	Brandt et al. 2016	1	Low
	1.54	1,540		8	High
	0.2	200	Siede et al. 2017	1	Low
	2.0	2,000		10	High
	1.6	1,600	Odemer & Rosenkranz 2018	8	
	1.0	1,000	Retschnig et al. 2015	5	
	144	144,000	Laurino et al. 2011	720	
	5.0	5,000	Doublet et al. 2015	25	
	4.25	4,250	Vidau et al. 2011	21	
	12.5	12,500	Fischer et al. 2014	63	
	4.5	4,500	Tison et al. 2016	23	
	0.02	20	Tison et al. 2017	0.1	Low
	2.0	2,000		10	High
	29.6	29,600	Forfert & Moritz 2017	148	Low
	200	200,000		1000	High

On the contrary, Fischer et al. (2014) performed a catch-and-release experiment, where thiacloprid fed bees showed a significantly reduced homing success when released at a remote site navigating back to the hive (over 50 %). Our data does not match these findings. If homing success would have been affected by thiacloprid exposure on such a large scale, colony development would have indicated a loss of worker bees, respectively. Again, a possible explanation of this effect is most likely due to the use of a 63-fold higher concentration than found under field conditions (Tab. 1).

As we could not detect any effects of thiacloprid in two different experimental setups, we decided to use a neonicotinoid with extremely high toxicity to bees for our last trial ($LD_{50\text{-oral}}$: 3.7 ng/bee clothianidin vs. 17,320 ng/bee thiacloprid; see Würfel, 2008). This compound was crucially involved in the "Rhine Valley incident" in South Germany, where approximately 12,000 colonies were affected by abrasive dust from maize seed dressings (Würfel, 2008). Clothianidin is a nitro-substituted neonicotinoid banned in 2014 for the use in crops attractive to pollinators due to its high toxicity (EFSA, 2013a).

In a novel approach (Odemer et al., 2018) we used Kieler-Mating-Nucs to establish mini-hives, exposing *N. ceranae* and *N. apis* infected honey bees to clothianidin. Once more, we have focused on foraging activity as a performance factor and on longevity of worker bees. Even under field realistic conditions, we did not see adverse effects attributed to the pesticide treatment. However, the lifespan of both *N. ceranae* infected groups was shorter and their flight activity was increased. These findings confirm and corroborate our first study's results (Retschnig et al., 2015), where *N. ceranae* infection caused similar symptoms in one location, but overall no detrimental synergistic pesticide effects occurred. Augmented further by two experiments that found *N. ceranae* placing nutritional stress on individual bees (Mayack & Naug, 2009; Naug & Gibbs, 2009), leading to riskier foraging and greater mortality of forager bees away from the hive (Kuszewska & Woyciechowskski, 2014). Moreover, *N. ceranae* infection significantly accelerates the age polyethism of young bees, causing them to display behaviours typical for older bees (Lecocq et al., 2016). Further studies demonstrated that infected nurse bees significantly outperform controls in odor learning and memory-suggestive of precocious foraging (Gage et al., 2017). Consistent with our previous

results, neither the presence nor the quantity of *N. ceranae* at low, natural levels of infection had any effect on flight distance or duration (Wells et al., 2016).

For *N. apis* however, such effects failed to appear in our experiments. *Nosemosis* caused by *N. apis* is characterized mainly by dysentery, whereas *N. ceranae* is described to cause death of individuals and colonies not preceded by any visible symptoms (reviewed in Genersch, 2010). Additionally, *N. apis* infection is restricted to the midgut epithelium (Fries, 1988), while *N. ceranae* has also been identified in other bee tissues like malpighian tubules and hypopharyngeal glands (Chen et al., 2009, reviewed in Genersch, 2010), representing different consequences for the host. The role of *N. apis* as contributor to honey bee decline remains unclear, as the past research focus was set on *N. ceranae*. With our findings however, a serious threat could not be demonstrated.

Contrary to our data, studies from Spain postulated CCD like symptoms occurring in hives infected with *N. ceranae* along with a high risk of contamination for surrounded apiaries (Higes et al., 2008). As a matter of fact a nearly complete collapse of two professional apiaries was reported (Higes et al., 2009) suggesting that *N. ceranae* is a key factor in colony losses detected over the recent years (Higes et al., 2010). It was stated, that the prevalence of *Nosemosis* in Spain has even reached epidemic levels (Botías et al., 2012b) and *N. ceranae* was found to be the only risk factor strongly associated with colony losses (Meana et al., 2017). None of such strong indications could be validated by our experiments, neither by other authors assessing the global prevalence of the parasite (Chauzat et al., 2007; Chen et al., 2008; Cox-Foster et al., 2007; Fries et al., 2006; Invernizzi et al., 2009; Klee et al., 2007; Paxton et al., 2007; Tapaszti et al., 2009; Williams et al., 2008).

Synergisms define that when taken together, the joint action of agents, i.e. pathogens and/or pesticides, increase each other's effectiveness (Tallarida, 2011). Martin et al. (2013) investigated such possible synergism of the prevalent *N. ceranae* and the deformed wing virus (DWV) in Hawaiian honey bee colonies, which are known to have the highest prevalence of *N. ceranae* in the world. The results showed no correlation between the virus load and spore count and furthermore, no large-scale colony deaths

related to *Nosema* infections at all. In addition, Gisder et al. (2017) monitored hundreds of honey bee colonies in Germany for their *Nosema spp*. prevalence. Within 12-years they could demonstrate that *N. ceranae* infections significantly increased. However, their data revealed no relation between colony mortality and detectable levels of infection, neither for *N. ceranae* nor for *N. apis* (Gisder et al., 2010). This suggests that the drastic symptoms described by Higes et al. (2008) might be a regional problem rather than a global phenomenon (Genersch, 2010).

7.1 Individual Bee Level

In a series of recent reviews, the relationship between pathogens and pesticides were addressed (Collison et al., 2016), pointing the way for future research and how to enhance experimental designs (Benuszak et al., 2017). Even though there are plenty of consistent studies providing evidence for a connection between the exposure to pesticides and the ability of bees to resist or tolerate pathogen infection, only little is known about the mechanisms of such interactions.

To date, many laboratory experiments have found, that sublethal pesticide doses may not only affect social behaviour (Forfert & Moritz, 2017; Tison et al., 2016) and reduce bees' immunocompetence (Brandt et al., 2016), but also impair their navigation (Fischer et al., 2014) and compromise learning and memory functions (Tison et al., 2017) of individual bees. Moreover, both, pesticide residues from agricultural practice, but also from apicultural use can be found in hive environments (Mullin et al., 2010).

The distribution of exposures experienced by individual bees causes a distribution of individual effects, ranging from mild sublethal impairment to death (Fig. 1). More importantly, these individual effects may translate into effects on colony-level functions and should therefore be investigated with regard to such (Sponsler & Johnson, 2017).

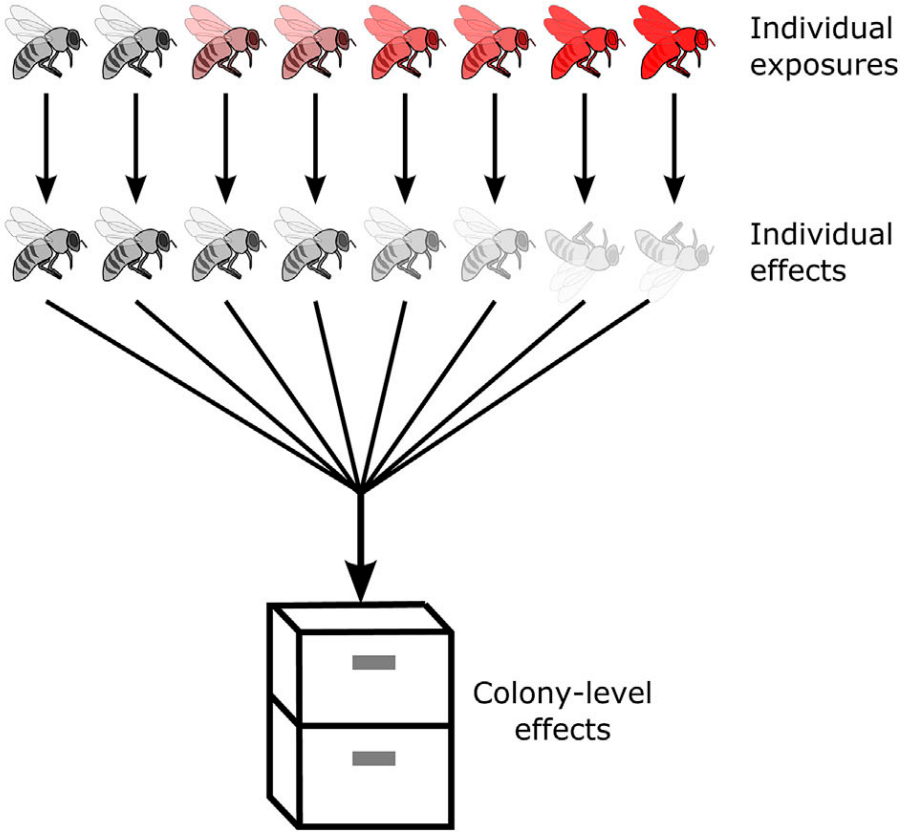

Fig. 1 The distribution of exposures (depicted by red color intensity) experienced by individual bees causes a distribution of individual effects (depicted by opacity), ranging from mild sublethal impairment to death (upside-down bees) after Sponsler & Johnson (2017).

7.2 Colony Level Consequences

To study possible effects of agrochemicals on honey bee health under realistic conditions and in line with the tiered approach in ecotoxicological risk assessment, field studies usually are the first choice (EFSA, 2013b). However, such studies have to face substantial challenges by managing a large number of variables evoked by different biotic and abiotic stressors affecting the superorganism of a colony. In addition, social behavior of bees, such as age-related division of labor, may lead to a misjudgment of pesticide exposures, toxicities and risks for the numerous castes and their specific purpose within the hive environment (Johnson et al., 2010; Wahl & Ulm, 1983; Rortais et al., 2005).

In particular, forager bees are more likely to be exposed to pesticides through contact or oral exposure with contaminated nectar and/or water sources (Fig. 2). Furthermore, such older bees are even more susceptible to these pesticide loads (Krupke et al., 2012; Mullin et al., 2015; Long & Krupke, 2016; Mogren & Lundgren, 2016). As a result, foraging behaviour and cognitive tasks can be affected, leading to decreased brood amounts and food stores, elevated pathogen loads and can ultimately result in greater pesticide sensitivity and disease susceptibility when colonies are under stress (Henry et al., 2012; Alaux et al., 2010; Wahl & Ulm, 1983; Szymas & Jedruszuk, 2003; Gill et al., 2012; Williamson & Wright, 2013).

Unlike foragers, nurse bees are more probably subject of consuming pesticide contaminated pollen. There is the potential risk of undiluted pesticides in pollen, as they convert pollen-derived nutrients into glandular secretions to feed honey bee larvae, the queen and supply drones and other workers (Fig. 2) (Sponsler & Johnson, 2017).

Moreover, nurse bees suffering from secondary infections caused by *V. destructor*, show higher virus titers when feeding on contaminated pollen. In return, these bees are contagious to bee brood and the queen, increasing the risk of transmitting viruses (Rortais et al., 2005; Baily, 1982; Donze & Guerin, 1994; Chen & Siede, 2007).

Pesticide exposure may not only alter honey bees' nursery and breeding habits, they can also affect egg laying, mating behaviour as well as other in-hive tasks that maintain a health-balance within the hive environment. Honey bees' hygienic behaviour, an important trait contributing to the social immunity of the hive, is expressed as defense mechanism identifying and removing diseased brood before pathogens can spread. Recent studies provide evidence for this capacity also to be affected by pesticide exposure, no longer preventing transmission of infectious diseases (Rothenbuhler, 1964; Spivak & Reuter, 1998; Wu-Smart & Spivak, 2016). With the use of chemicals for *Varroa* treatment, beekeepers may inconsiderately promote resistance of mite populations to such drugs, elevating the risk of further intra- and inter colonial pathogen distribution.

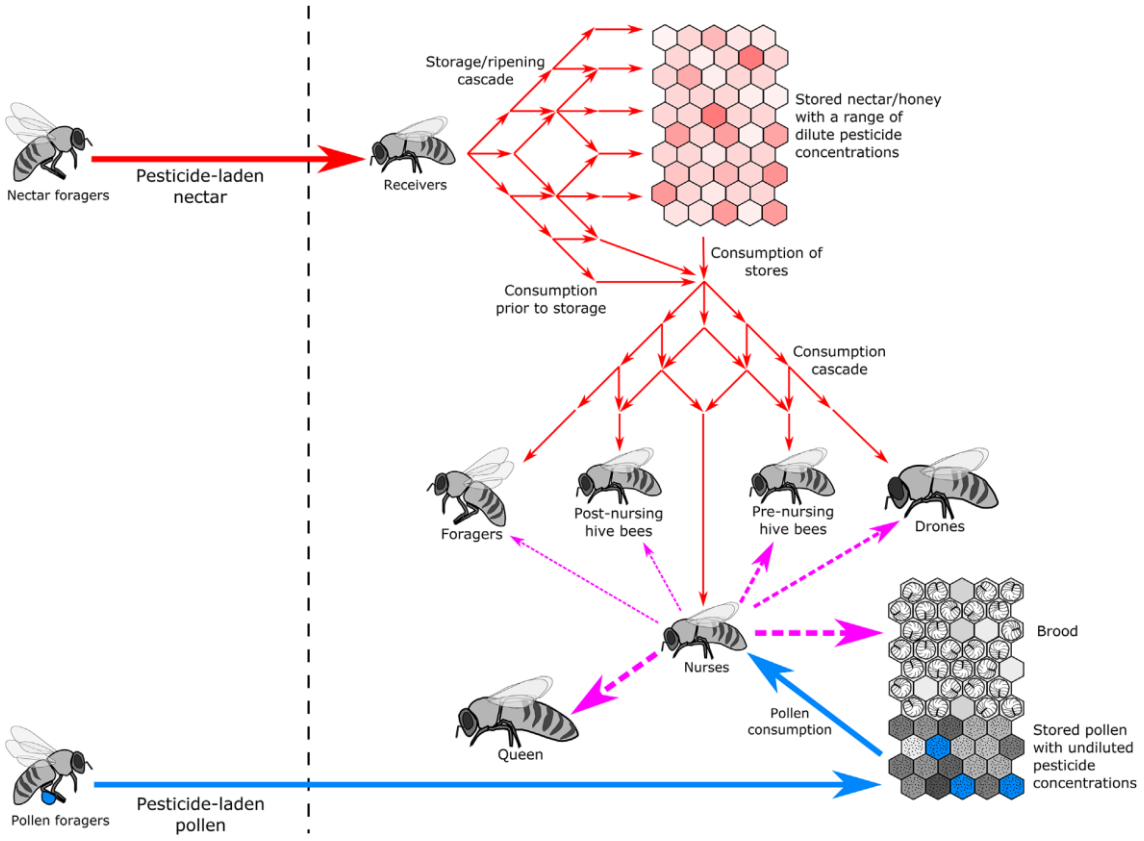

Fig. 2 Transmission pathways of nectar-associated and pollen-associated pesticides. Pesticide-laden nectar (red) undergoes extensive trophallactic transmission prior to consumption, resulting in widespread but dilute ingestion of nectar-associated pesticides. Pesticide-laden pollen (blue) undergoes no mixing or dilution and is consumed almost exclusively by nurse bees, which may, therefore, receive more extreme (higher and lower) pesticide doses than other colony members after Sponsler & Johnson (2017).

7.3 Concluding Remarks and Outlook

As a new and important result, with our studies we were able to show that managed honey bees are evidently more resilient to (pesticide-) stressors at the colony level when compared to individual bees. Interestingly, wild pollinators sharing similar habitats and visiting treated crops do not seem to have such an efficacious defense mechanism (Straub et al., 2015). When bumble bees, solitary bees and other beneficial insects are challenged to equivalent exposure scenarios, effects most often are more fatal and persistent (Gill et al., 2012; Whitehorn et al., 2012; Biddinger et al., 2013; Ellis et al., 2017). In addition, extended agricultural intensification means that pollinators are exposed to larger numbers of pesticides with fewer options for natural food sources

during foraging (Johnson et al., 2010; Mullin et al., 2010; Krupke et al., 2012). Yet, the possible combinatorial effects of pesticide exposure to other species than honey bees have rarely been investigated (Johnson et al., 2009; Pilling & Jepson, 1993; Pilling et al., 1995).

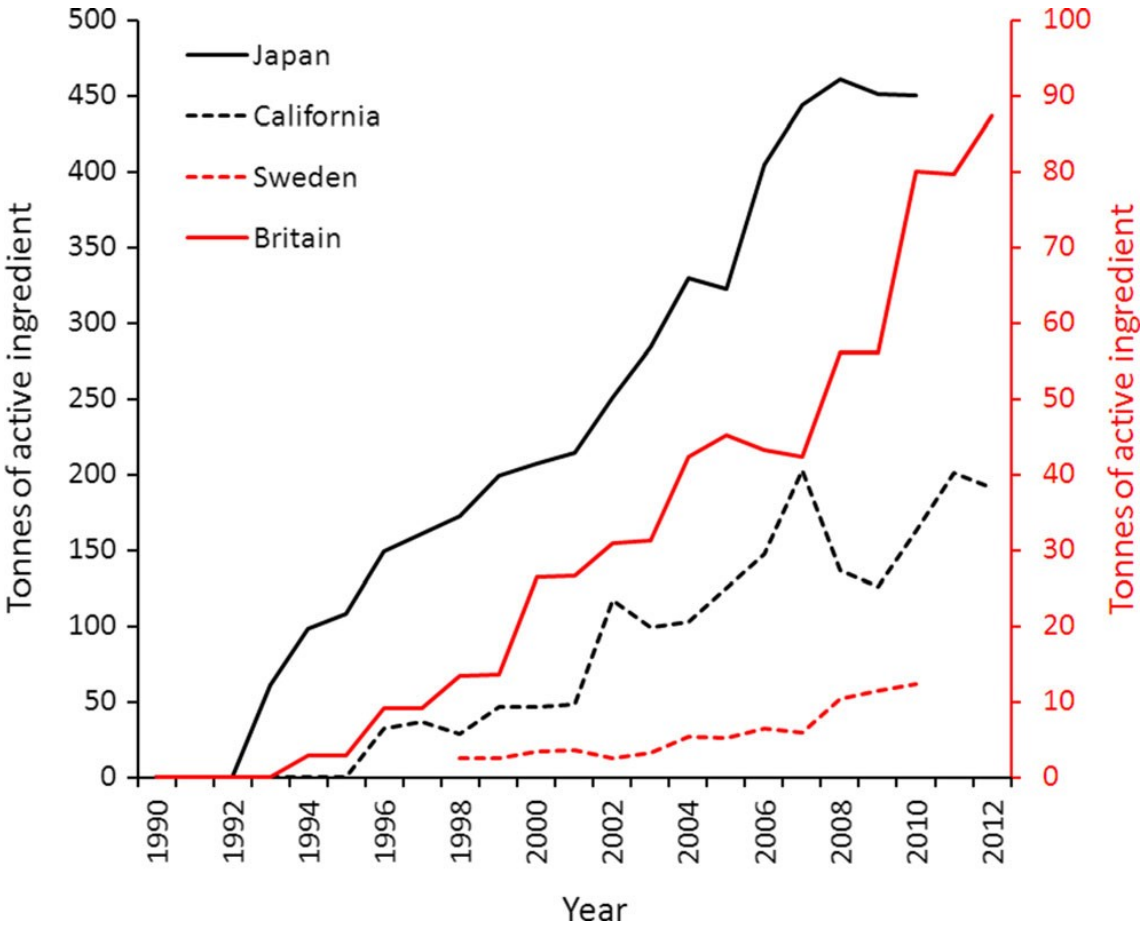

Fig. 2 Trend in the sales (Sweden), domestic shipment (Japan), use (California) and agricultural use (Britain) of all neonicotinoid insecticides and fipronil. All measured in tonnes of active ingredient per year. Note the separate vertical axes for California// Japan, and Britain//Sweden (after Simon-Delso et al., 2015)

Habitat alteration and land use (in particular cropland) are widely considered to be one of the most crucial factors responsible for the dramatic decline of insects in general and pollinators in particular (Klein et al., 2007; Hallmann et al., 2017). Even though the world wide use of neonicotinoid pesticides has substantially increased during the past

three decades (Fig. 2), there is still no clear evidence to what extent these agents have contributed to this trend (reviewed in Godfray et al., 2014). Our results at least suggest that under realistic field conditions and "good agricultural practice" (i.e. correct use of pesticides according to the recommendations) neonicotinoids do not represent a current risk for honey bee colonies. Still, the increasing use of these insecticides is alarming.

Until today, there is a particular uncertainty about the extent that other pollinators are actually exposed to these pesticides. Some may avoid nectar or pollen from treated crops and/or forage on other food sources which might reduce the exposure to the pesticide (Heimbach et al., 2016), making it difficult to give valid statements about the risk they are exposed to. Large scale monitoring studies are necessary to better understand realistic environmental effects on different pollinator species in the current agricultural landscape (Liess et al., 2005), bearing in mind that susceptibility to pesticides might deviate from model species such as the honey bee (Decourtye et al., 2013; Liess et al., 2005). This is important to counteract the decreasing biodiversity in rural areas and can be an essential step forward to a more sustainable use of plant protection products.

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9 Appendix

Eidesstattliche Versicherung gemäß § 8 Absatz 2 der Promotionsordnung der Universität Hohenheim zum Dr. sc. agr.

1. Bei der eingereichten Dissertation zum Thema **„Effects of chronic pesticide and pathogen exposure on honey bee (*Apis mellifera* L.) health at the colony level“** handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Stuttgart, den 10. April 2018

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