Potential Effects of Biotic and Abiotic Stressors on the Reproductive Health of Honey Bees

by

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Abstract

Apis mellifera Linnaeus honey bees are the most economically important pollinator species in the United States, yet colonies consistently experience high losses as a result of interacting biotic and abiotic stressors. My three-year monitoring effort, performed in collaboration with the Bee Informed Partnership to document and better understand national honey bee colony losses, revealed that high losses continue and vary according to region, season, and year. It also revealed that losses are tightly associated to beekeeping operation size, most likely because of differences in management philosophy concerning the ubiquitous mite *Varroa destructor* Anderson and Trueman, which deprives parasitized bees from essential nutrients and challenges their immune systems. As an important abiotic stressor, neonicotinoid insecticides can elicit significant negative effects on honey bees; however, the nature of these effects during simultaneous pressure alongside *V. destructor* is not well known. My experiments suggest that *V. destructor* and neonicotinoids can act synergistically to reduce worker and drone survival, but not affect other features such as hypopharyngeal gland size in workers and sperm quality in drones. To investigate possible practical mitigation actions by beekeepers against neonicotinoids, I found that artificially increased colony genetic diversity through inter-colony brood mixing had a positive effect on worker survival, but no effect on worker hypopharyngeal gland size Overall, my dissertation provides novel evidence for interactions between two common honey bee stressors, highlights the need for in-depth studies to understand how individual-level effects translate to the colony-level, and demonstrates that honey bee colonies in the United States continue to experience high losses.

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Chapter 1

Dissertation introduction

In addition to being an important sentinel species for environmental change, the honey bee (*Apis mellifera* Linnaeus) is the most economically important insect pollinator species for agriculture in the United States (Aizen and Harder 2009). Recent data show that acreage of agricultural crops depending on animal pollination increased rapidly over the last 50 years, which raises questions concerning if appropriate ecosystem services can be provided adequately through the current pollination capacity (Aizen and Harder 2009). This question is especially important considering recent global reports of high honey bee colony losses, including from the United States (Pirk et al. 2015, Lee et al. 2015b, Kulhanek et al. 2017, Brodschneider et al. 2018, Gray et al. 2019).

To better understand the nature of honey bee colony losses, monitoring groups have formed in various countries to document regional losses to compile long-term, multi-year data crucial for providing both spatial and temporal context (vanEngelsdorp et al. 2007, Zee et al. 2013, Lee et al. 2015a). For example, the Apiary Inspectors of America conducted a nationwide colony loss survey in 2007 as a response to inexplicable colony losses, now known as Colony Collapse Disorder (CCD), across the United States (vanEngelsdorp et al. 2007). Subsequently, the Bee Informed Partnership, an American non-profit organization, was founded and has continued efforts to collect colony loss data on national and state levels for more than a decade (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012, Spleen et al. 2013, Steinhauer et al. 2014, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017). Historically, the annual survey focused exclusively on colony mortality during winter, a time when colonies experience death because of

unfavorable environmental conditions, and most beekeepers are limited in their management options and report the highest losses (Seeley and Visscher 1985). Upon additional reports of losses during the active brood-rearing season (Currie and Gatien 2006), the survey expanded to also include the summer period (Steinhauer et al. 2014). Based on this large data set, it appears that colony losses vary across regions and years in the United States, and that size of beekeeping operation matters (USDA NASS 2020). The majority of beekeepers in the United States are considered small-scale, managing less than 50 colonies, not relying on honey bees as their primary source of income. These beekeepers keep bees for environmental stewardship or enjoyment (Underwood et al. 2019). Although large-scale operations, managing more than 50 colonies, represent the smallest fraction of beekeepers, they are responsible for most of the national honey bee stock (USDA NASS 2020). They rely on strong honey bee colonies to generate income by selling honey or honey bees, or by providing pollination services to important agricultural crops (Calderone 2012). To achieve these goals, they employ different management approaches than small-scale operations (Underwood et al. 2019, Goodrich 2020).

Although actively managed by beekeepers, honey bees are still highly dependent on their environment, which also harbors a variety of biotic and abiotic risk factors (Mills et al. 1993, Gómez-Ramírez et al. 2014, Steinhauer et al. 2018). Proposed abiotic risk factors include including extreme weather events like wildfires and floods, and environmental contaminants such as fungicides and insecticides (Potts et al. 2010, Johnson et al. 2013). Likewise, there is an array of important biotic risk factors, like limited floral resources, the bacterium *Paenibacillus larvae* White, newly arrived pests such as the African *Aethina tumida* Murray small hive beetle, and entomopathogenic mites like the *Varroa destructor* Anderson and Trueman mite (Neumann and

Elzen 2004, Boecking and Genersch 2008, Alaux et al. 2010, Genersch 2010). Combined, these risk factors can affect the health of individual honey bees, as well as the colony.

As the sole reproductive female, the queen is arguably the most important individual in a colony (Winston 1991), directly contributing to colony strength by producing offspring and colony cohesion by releasing pheromones (Walsh et al. 2020). As in other eusocial insect species (Arnqvist and Nilsson 2000), honey bee queens are polyandrous. They embark on a few nuptial flights early in their adult life to mate with approximately 12 male honey bees, called drones, which will provide her with a life-long supply of sperm (Schlüns et al. 2005, Koeniger and Koeniger 2007). Since the queen rarely leaves the nest after the mating flights, the acquired sperm is stored in a special organ called the spermatheca (Winston 1991). Exhibiting haplodiploid sex-determination, stored sperm is subsequently used to fertilize eggs that will develop into diploid females, whereas haploid males develop from eggs not fertilized (Winston 1991, Aamidor et al. 2018). The number of drone mating partners of a queen is positively correlated with enhanced colony fitness and productivity (Reed and Frankham 2003, Mattila and Seeley 2007), at least partly via increased colony resilience to biotic and abiotic risk factors due to increased genetic diversity (Palmer and Oldroyd 2003, Schmickl and Crailsheim 2004, Delaplane et al. 2015).

Workers are responsible for colony maintenance and exhibit age-dependent division of labor (Winston 1991, Johnson 2010, Nowak et al. 2010). For example, workers 8-12 days old play a nursing role (Winston 1991, Deseyn and Billen 2005) and, using special glands located in the anterior part of their heads called hypopharyngeal glands (HPGs), produce brood food to nourish developing individuals, including queen larvae (Knecht and Kaatz 1990, Crailsheim and Hrassnigg 1998). The future queen will be fed with a very specific proteinaceous substance produced by nurse HPGs called royal jelly that is vital to queen development (Haydak 1961,

Albert et al. 2014). HPGs are not only involved in brood food production, but they also contribute specific sterilizing enzymes that are added to brood food to help preserve and protect it against several larval diseases (Yang and Cox-Foster 2005). Furthermore, nurse HPGs contribute to the nourishment of other adult members of the colony, including the drones (Crailsheim 1991).

Although generally overlooked in the past, drones provide indirect benefits to colony maintenance and health by inseminating future queens (Pettis et al. 2016, Rangel and Fisher 2019). As sperm from various drones is collected before being mixed and stored in the queens' spermatheca, it contributes greatly to a genetically diverse worker population that ultimately confers many benefits to the colony, like improved resilience to disease (Tarpy and Seeley 2006, Delaplane et al. 2015). However, as haploid individuals, drones are expected to be more susceptible to environmental stressors due to the lack of allelic variation at important immune and possibly detoxification genes (O'Donnell and Beshers 2004, Retschnig et al. 2014, Rautiala et al. 2019, Friedli et al. 2020). This is important given their contribution to colony fitness and considering that a very important biotic risk factor, the *V. destructor* mite, parasitizes drones more frequently (Rosenkranz et al. 2010).

The ectoparasitic mite *V. destructor* is considered the major biotic threat to honey bees (Boecking and Genersch 2008, Rosenkranz et al. 2010). Despite its importance for the beekeeping industry (Steinhauer et al. 2021), many gaps in knowledge exist concerning how it interacts with honey bees. For example, it was recently reported to primarily feed on the fat body of its host (Ramsey et al. 2019), rather than its hemolymph as previously thought (Rosenkranz et al. 2010). The fat body is a crucial tissue for the immune system of honey bees, including the recognition of foreign particles (Arrese and Soulages 2010). This is essential given that *V. destructor* is a vector for several viruses including the Deformed Wing Virus (DWV) and Acute Paralysis Virus (APV)

complex (Martin 2001). *Varroa destructor* depends entirely on its host for nutrition as well as successful dispersal and reproduction (Kuenen and Calderone 1997, Traynor et al. 2020). During the dispersal stage, adult female *V. destructor* mites will use adult honey bees to travel within and between colonies while feeding on them (Seeley and Smith 2015, Ramsey et al. 2019); the reproductive stage of the lifecycle of *V. destructor* occurs once a mature female mite enters a suitable honey bee brood cell using olfactory cues (Trouiller et al. 1992, Nazzi et al. 2004). The foundress mite will submerge in larval brood food to avoid detection and removal by workers before cell capping (Ifantidis 1983, Rosenkranz et al. 2010). Generally, *V. destructor* foundress mites will initiate oviposition upon brood cell capping, and produce one male and one or two female offspring (Traynor et al. 2020). The entire mite family will subsequently feed on the fat body of the developing bee until it undergoes metamorphosis and emerges as an adult from the brood cell (Rosenkranz et al. 2010, Ramsey et al. 2019). *Varroa destructor* infestation during development can have serious consequences for adults, such as reduced body weight and lifespan (Kovac and Crailsheim 1988, Bowen-Walker and Gunn 2001). Additionally, *V. destructor* can negatively affect the reproductive quality of drones and nursing capacity by workers (Schneider and Drescher 1987, Duay et al. 2002, Yousef et al. 2014). Thus, *V. destructor* infestation can directly affect individual worker and drone health, which can lead to down-stream, indirect negative effects on the queen and ultimately the colony (Martin 2001, Boecking and Genersch 2008). Beekeepers can mitigate damage by *V. destructor* by employing a variety of management practices, including in-hive acaricides (Haber et al. 2019, Steinhauer et al. 2021). However, there is evidence that commonly used acaricides can negatively affect honey bees, especially by reducing the reproductive health of queens and drones (Johnson et al. 2013), possibly because of high residue levels persisting within the colony (Mullin et al. 2010).

Neonicotinoid insecticides have garnered much attention in the last decade because of their widespread use and relatively well-documented effects on non-target organisms, including honey bees (Simon-Delso et al. 2015, Rundlöf et al. 2015, Ardestani 2020). Neonicotinoids account for the majority of sales in the global insecticide market (Jeschke and Nauen 2008). Their systemic properties allow widespread application as a crop seed-coating, which reduces risks to farmers while providing maximal protection to plants through translocation to all tissues (Alford and Krupke 2017). However, because of their systemic properties, residues of neonicotinoids and their metabolites can be detected in nectar and pollen, both collected by forager bees and used by the colony as a source of carbohydrate and protein, respectively (Bonmatin et al. 2007, Botías et al. 2015). Neonicotinoids can have lethal effects in honey bees (Suchail 2000, Iwasa et al. 2004), but these results are mainly derived from laboratory experiments employing acute and chronic exposures (Suchail et al. 2001, Laurino et al. 2011, Abbo et al. 2017). In contrast, most experiments employing field-relevant concentrations of neonicotinoids do not report lethal effects (Cresswell 2011). However, there is ample evidence of sub-lethal effects on honey bees exposed to field-realistic concentrations of neonicotinoids, including impaired learning ability and homing behavior (Schneider et al. 2012, Blacquière et al. 2012, Simon-Delso et al. 2015, Muth et al. 2019). Moreover, sub-lethal concentrations of neonicotinoids can induce physiological changes in ovaries of queens (Williams et al. 2015), sperm in drones (Straub et al. 2016), and HPGs in workers (Hatjina et al. 2013).

Despite the ubiquitous nature of both *V. destructor* and neonicotinoids, and their known adverse effects on honey bee health, little is known about potential interactions between these two risk factors (Blanken et al. 2015, Abbo et al. 2017, Straub et al. 2019). Generally, interaction outcomes can range from synergistic to antagonistic, when the effect of simultaneous exposure to

both factors is greater or lesser than the sum of individual effects, respectively (Maher et al. 2019). They can also be negative or positive, depending on the directionality (Piggott et al. 2015) of the factor effects.

For my dissertation, I investigated potential interaction effects of *V. destructor* and neonicotinoids on lethal and sub-lethal measures in individual honey bees, specifically workers and drones, by assessing their effects on mortality and anatomy, respectively. Additionally, I employed a practical beekeeping approach to examine the effects of artificially increased intra-colony genetic diversity on the ability of a honey bee colony to mitigate the effects of neonicotinoids. To acknowledge the important contributions of my collaborators, I used the plural possessive 'we' in each chapter that communicated a scientific investigation. Ultimately, those bodies of work included in this dissertation are my responsibility.

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Chapter 2

Elevated losses of managed *Apis mellifera* honey bee colonies in the United States vary seasonally and according to beekeeping operation size.

Results of the Bee Informed Partnership's National Colony Loss Survey – 2017-18, 2018-19, and 2019-2020.

Abstract

For more than a decade, beekeepers in the United States and around the world have experienced consistently high losses of managed honey bee (*Apis mellifera* Linnaeus) colonies. Long-term, multi-year monitoring efforts are crucial to provide a temporal and spatial context to these losses, and shed light on how beekeeper management affects this economically important species. The Bee Informed Partnership has conducted a national honey bee colony loss survey in the United States for more than a decade. Here we present survey results from the last three years − 2017-18, 2018-19, and 2019-20. Each year, colony losses were estimated for three periods – Summer, Winter, and Annual – and compared between three operation types based on size − Backyard, Sideline, and Commercial Beekeepers. Our results revealed that beekeepers during 2018-19 experienced the highest national Total Winter colony loss (37.7%) since the start of winter colony loss data collection in 2007-08. This was followed by the highest ever documented national Total Summer colony loss (32.1%) during the 2019-20 survey year. Consistent with reported surveys, Backyard Beekeepers usually experienced higher losses in Winter compared to any other operation type, whereas Commercial Beekeepers experienced higher losses during the Summer. Overall, our results highlight the variability of colony losses according to season, year, and operation type in the United States.

Introduction

Reports of managed *Apis mellifera* Linnaeus (hereafter honey bee) colony losses received public attention as early as 1905, when an infamous, unexplainable honey bee epidemic raged on the Isle of Wright in the United Kingdom (Bailey 1964). More recently, a mysterious phenomenon now known as Colony Collapse Disorder (CCD) resulted in large-scale colony losses in the United States starting in 2006 (vanEngelsdorp et al. 2007). CCD has since been attributed to only a small fraction of lost colonies in the United States (Ellis et al. 2010, vanEngelsdorp et al. 2010), which like many countries has experienced consistently high losses for more than a decade (Neumann and Carreck 2010, Steinhauer et al. 2014b, Seitz et al. 2015, Gray et al. 2019).

Given their status as sentinel species because of their close connection to the environment (Bromenshenk et al. 1985, Mills et al. 1993, García-Fernández et al. 2020), honey bees are presented with a plethora of biotic and abiotic risk factors (Mills et al. 1993, Gómez-Ramírez et al. 2014, Rortais et al. 2017, Steinhauer et al. 2018). Biotic risk factors of honey bees include availability of floral resources in natural and agricultural regions, historic fungi like the introduced *Nosema ceranae* Fries microsporidian, newly arrived pests such as the African *Aethina tumida* Murray small hive beetle, and introduced macro-parasites like the *Varroa destructor* Anderson and Trueman mite (Neumann and Elzen 2004, Paxton et al. 2007, Boecking and Genersch 2008). Similarly, diverse abiotic risk factors exist, including extreme weather events like drought or floods, and environmental contaminants such as fungicides and insecticides that are used in a variety of landscapes (Alaux et al. 2010, Potts et al. 2010, Thompson et al. 2014, Goulson et al. 2015, Lundin et al. 2015, Nicholls et al. 2018). Although these risk factors are

proposed by scientists, they largely conform with beekeeper-perceived causes of colony loss (Pirk et al. 2015, Kulhanek et al. 2017, Gray et al. 2019).

The modern-day honey bee relies on human handlers to provide appropriate colony-level requirements, ideally via scientifically-derived Best Management Practices that are tailored to the specific needs of a beekeeper while prioritizing colony health (Project Apis m. 2020). Some beekeepers maintain a handful of colonies in the area that they reside, often for honey production, environmental stewardship, or leisure (Underwood et al. 2019). Other beekeepers manage thousands of colonies as a business, strategically moving them across the country to provide migratory pollination services to a variety of economically important crops like almonds, blueberries, and carrot (Calderone 2012, vanEngelsdorp et al. 2013b), or in search of high-intensity floral nectar flows for honey production (Whynott 1991).

The impacts of different beekeeping practices on colony health are starting to become apparent, and seem to be largely driven by a management approach that is closely related to the number to colonies managed (Underwood et al. 2019). For example, beekeepers caring for large numbers of colonies were more likely to employ in-hive chemicals to combat the *V. destructor* mite; this possibly reduces risk of colony mortality in winter (Haber et al. 2019). Large-scale beekeepers are also more likely to take their colonies to California almond orchards to provide pollination services (Morse and Calderone 2000), thereby increasing their exposure to a variety of agricultural land use practices or inputs, like insecticides and fungicides, that could severely affect colony health and fitness (Pettis et al. 2013, Zhu et al. 2014b, Smart et al. 2016, Chessler et al. 2018, Alger et al. 2018, Wade et al. 2019). Despite this, evidence suggests that seemingly risky activities like providing pollination services to high intensity agriculture, or moving colonies thousands of miles, are relatively less important than other risk factors, such as *V. destructor,*

experienced by most managed honey bee colonies around the country (Lee et al. 2015b, Kulhanek et al. 2017, Haber et al. 2019, Steinhauer et al. 2021). Possibly because of this, small-scale beekeepers managing fewer than 50 colonies consistently lose a higher proportion of their colonies than larger operations, especially during winter (Guzmán-Novoa et al. 2010, Dainat et al. 2012, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017).

Despite persistent high colony losses reported by beekeepers in the United States (Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017), the total number of managed honey bee colonies has increased from 2.39 million in 2006 to 2.67 million in 2020 (USDA NASS 2007, 2020a). Beekeepers are able to compensate for colony losses by splitting surviving colonies − a common beekeeping practice to divide a colony into two or more parts − by purchasing new colonies from another beekeeper, or by installing captured swarms (Delaplane 2010, 2015). Therefore, experienced losses do not necessarily translate into a decrease of the national colony stock (vanEngelsdorp and Meixner 2010b, Steinhauer et al. 2021). Active replacement of colonies is incentivized by high almond pollination fees and relatively stable honey prices (Moritz and Erler 2016, USDA NASS 2017, 2020b, Goodrich 2020), however the rapid intensification of pollinator dependent crop acreage across the United States places tremendous strain on the beekeeping industry (Aizen and Harder 2009). Beekeepers frequently cite increased resources required to maintain colony numbers as a major threat to the long-term sustainability of the industry (Somerville 2003). Therefore, long-term multi-year colony loss monitoring is crucial to provide the temporal and spatial context of honey bee colony health, and to contribute to the scientific understanding of managed colony losses (Lindenmayer et al. 2012).

In 2007, the Apiary Inspectors of America launched its first national colony loss survey in response to CCD (vanEngelsdorp et al. 2007). Subsequently, an American non-profit organization

called the Bee Informed Partnership (BIP) was founded and then administered the annual survey (vanEngelsdorp et al. 2008, 2017, Steinhauer et al. 2014a, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017). Initially only colony losses during wintering (Brodschneider et al. 2010, Ellis et al. 2010, Zee et al. 2014, Pirk et al. 2015, Laurent et al. 2016), which is historically the period of the year when beekeepers reported highest losses (Seeley and Visscher 1985), were recorded. Because of additional reports that large-scale beekeepers were losing colonies during the active honey bee brood-rearing season, the national survey expanded to include the summer period starting in 2012 (Currie and Gatien 2006, Steinhauer et al. 2014a). Colony loss appears to vary across years and regions; however, in depth studies are lacking. The national ten-year average of total colony loss during winter is 28%, which is higher than the 17% that beekeepers deem to be an acceptable level of loss during this period (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012, Spleen et al. 2013, Steinhauer et al. 2014b, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017). This suggests that beekeepers continue to experience losses greater than historic levels prior to this long-term annual survey.

Here we present the results of the Bee Informed Partnership's national managed honey bee colony loss survey conducted during the last three years − 2017-18, 2018-19 and 2019-20. The primary objective of this work was to continue the standardized reporting of managed honey bee colony losses in the United States, especially in the context of common beekeeping practices and experiences such as location and size of operation, as well as key pollination events and other associated activities. Secondarily, we investigated the seasonal relationship between colony loss and beekeeping operation size. Based on previous American and international colony loss reports (Seitz et al. 2015, Kulhanek et al. 2017, Brodschneider et al. 2018, Gray et al. 2019), we expected to observe higher than historic colony losses, and that smaller beekeeping operations would

continue to experience higher losses compared to large ones, especially in winter. This is likely because of differences in beekeeping management philosophy concerning important risk factors like *V. destructor* (Underwood et al. 2019).

Material and Methods

Full survey design

An online survey was created using Select Survey (https://10.selectsurvey.net) to collect information about managed *Apis mellifera* honey bee colony losses across the United States beginning 1 April for each of three survey years – 2017-18, 2018-19, and 2019-20. The present work was closely aligned to previously published national colony loss surveys of the Bee Informed Partnership (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012, Spleen et al. 2013, Steinhauer et al. 2014b, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017). Prior to the most recent survey year (2019-20), respondents had to complete the survey questionnaire in one sitting, or had to start over. Therefore, respondents created a login to the online platform that allowed for responses to be revisited prior to submission in the survey year 2019-20. The survey consisted of three sections - the "Loss Survey", the "Management Survey", and additional questions concerning socioeconomics. This work focused on the "Loss Survey".

Loss survey design

Comparable to previous surveys, three colony loss periods were defined as 1 April 201X – 1 October 201X (Summer), 1 October 201X – 1 April 201Y (Winter), and 1 April 201X – 1 April 201Y (Annual), whereby "X" and "Y" represent successive years. Respondents were asked quantitative questions about the number of colonies in their operation at the start of each loss

period, as well as experienced increases by splitting existing colonies or purchasing new ones, and decreases in colony numbers by combining or selling existing colonies between loss periods. Like previous years, respondents were also asked to provide the location of their operation at the state-level, and to answer two subjective questions about "perceived cause of loss" and "acceptable annual loss rate". To address the former, respondents could choose from a selection of pre-defined "Causes of Winter Loss" (COWLs), but also freely write any subjective reasons not included in the pre-defined list. This list included common previously reported risk factors by beekeepers and scientists. The selection was adjusted in the 2018-19 survey year with the primary motive to remove "Colony Collapse Disorder" (CCD) from the pre-selected list. CCD inappropriately served as an umbrella term, and may wrongfully be selected to define inexplainable colony losses (Williams et al. 2010), because it has very specific characteristics that are often overlooked by beekeepers (Cox-Foster et al. 2007). Prior to 2018-19, the list included 12 COWLs; from then on, the list contained 14 COWLs to reduce ambiguity among selection items and to streamline answers. In the survey year 2018-19, we also added a list of 14 pre-defined "Causes of Summer Loss" (COSLs) for the first time to assess whether beekeeper-perceived causes of loss differ based on season. Finally, respondents were asked to specify what percentage of Winter colony loss they deem acceptable in their operation. This purpose of this question was to identify if beekeepers have habituated to consistently high losses.

All submitted data were cleaned by removing invalid responses (e.g. managed colony numbers exceeding 100000, negative colony numbers), and duplicated entries. Only colonies residing within the 50 U.S. states, the federal District of Columbia, and the five permanently inhabited self-governing territories, were included. Three data subsets corresponding to the Summer, Winter, and Annual loss periods were created. Each included beekeeper respondents

that maintained at least one colony at the start of the given loss period. This allowed for the inclusion of beekeepers that did not manage one or more colonies throughout an entire year. We differentiated between three different operation types based on the number of colonies managed on 1 October of each year. Respondents were categorized by operation type: "Backyard Beekeeper" managed 1-50 colonies, "Sideline Beekeeper" managed 51-500, and "Commercial Beekeeper" managed 501 or more colonies (Steinhauer et al. 2014b, Kulhanek et al. 2017). Finally, respondents were further subdivided based on two beekeeping activities: if they moved colonies across state lines (i.e. "Migratory Beekeepers") or if they sent colonies to California for almond pollination. For both activities, we compared Winter colony losses to non-migratory beekeepers (i.e. "Stationary Beekeepers") and operations that were not involved in almond pollination, respectively.

<u>Survey distribution</u>

The survey represents a non-random subsample of beekeepers in the United States, and is convenience-based because respondents provided their answers voluntarily upon personal invitation, advertisement, or word of mouth (Zee et al. 2013). The survey was advertised electronically to beekeepers that voluntarily provided their e-mail address to the Bee Informed Partnership in previous colony loss surveys, or in the USDA Animal Plant Health Inspection Service National Honey Bee Disease Survey. Recipients were encouraged to forward the survey link to other beekeepers to increase response rate. The survey was also promoted by two national beekeeping organizations (American Beekeeping Federation and American Honey Producer's Association), a beekeeping supply company (Brushy Mountain Bee Farm), two national beekeeping journals (American Bee Journal and Bee Culture), and two subscriber email lists (Catch the Buzz and ABFAlert). Furthermore, we asked the Apiary Inspectors of America, state

extension apiculturists, beekeeping industry leaders, and a number of regional beekeeping clubs to promote the survey among their stakeholders and members. Traditionally, Backyard Beekeepers account for the majority of online survey responses and also represent the majority of beekeepers in the country (USDA NASS 2018, 2019, 2020a). To collect more responses from Commercial Beekeepers that manage the majority of colonies in the United States, staff from the Bee Informed Partnership conducted telephone interviews with beekeepers participating in Technology Transfer activities of the organization. Furthermore, approximately 600 paper-versions of the survey were distributed each year to beekeepers participating in Bee Informed Partnership projects or the USDA APHIS honey bee health survey program.

The online survey went live from 1-30 April each survey year, whereas paper versions were mailed to beekeepers during the last week of March (Appendix Figure 14, Figure 15 & Figure 16); those returned by 30 May of each respective year were included in the study.

Statistical analyses

All calculations and statistical tests were performed using the statistical program R (R version 3.6.2), using a significance level of $\alpha = 0.05$. According to vanEngelsdorp et al. (2013), weighted ("Total") and unweighted ("Average") colony losses were calculated for the three fixed time periods (Summer, Winter, and Annual) using the R code presented in Steinhauer et al. (2014). For Total Loss, every colony was counted individually without considering operation size which is more representative of respondents with larger operations like Commercial Beekeepers since they manage the majority of colonies in the United States (USDA NASS 2018, 2019, 2020a). Average Loss, on the other hand, weighs each operation as one unit, which facilitates the comparison between operation types. It therefore is more representative of smaller operations like Backyard Beekeepers since they generally represent the majority of participants (Steinhauer et al. 2014b,

Seitz et al. 2015, Kulhanek et al. 2017). Ninety-five percent confidence intervals were calculated by employing a bootstrap sampling distribution (Frost 2020). Using the Kruskal-Wallis rank sum test, we identified differences in colony loss between operation type (Backyard, Sideline, Commercial), migratory status, and California almond pollination status. For multiple comparisons, a Kruskal-Wallis test was followed by Tukey's honestly significant difference (HSD) test. State colony losses were reported by including colonies of multi-state beekeepers in each state in which they reported having colonies (USDA NASS 2018). To maintain respondents' anonymity, losses for states with five or fewer respondent(s) were not reported.

Results

Loss Survey responses

Loss Survey response rate declined each year, with 5599 nationally validated responses 2017-18 and 3773 in 2019-20. The number of respondents per operation type was variable among both type and year (Table 1). Since most U.S. beekeeping operations are small, most responses consistently came from Backyard Beekeepers (>95%, 3-year cumulative N=12779). However, most colonies (3-year cumulative N=631222) were managed by Commercial Beekeepers which comprised the smallest fraction of the total number of respondents (<1.5%, 3-year cumulative N=152). The number of total managed colonies by respondents on October 1 was highest for the 2018-19 survey year, when responses by Commercial Beekeepers was highest (Figure 1, Table 1). Colonies managed by respondents represented 6.3, 11.1, and 8.7 % of the total number of estimated honey producing colonies in the United States in 2017, 2018, and 2019, respectively (USDA NASS 2018, 2019, 2020a).

Table 1: Responses to a national managed *Apis mellifera* honey bee colony loss survey performed for the United States (U.S.) during years 2017-18, 2018-19 and 2019-20. For each year the analytical dataset of valid United States respondents was used to create three seasonal subsets used to calculate loss – Summer (1 April 201X– 1 October 201X), Winter (1 October 201X – 1 April 201Y) and Annual (1 April 201X– 1 April 201Y), whereby "X" and "Y" represent successive years. Number of colonies managed by survey respondents on 1 October of each year.

			Valid respondents –seasonal subsets			
Survey Period	**Valid U.S. respondents (N)**	**Operation Type**	**Summer (N)**	**Winter (N)**	**Annual (N)**	**Colonies managed 1 Oct (N)**
2017-18	5,599	All	4,074	4,864	3,889	179,285
		Backyard	3,908	4,674	3,730	30,505
		Sideline	127	152	125	21,265
		Commercial	39	38	34	127,515
2018-19	5,510	All	3,818	4,631	3,652	318,932
		Backyard	3,668	4,465	3,513	28,028
		Sideline	91	108	87	14,951
		Commercial	57	58	52	275,953
2019-20	3,773	All	2,834	3,301	2,887	262,044
		Backyard	2,712	3,169	2,574	21,986
		Sideline	81	91	78	12,304
		Commercial	41	41	36	227,754

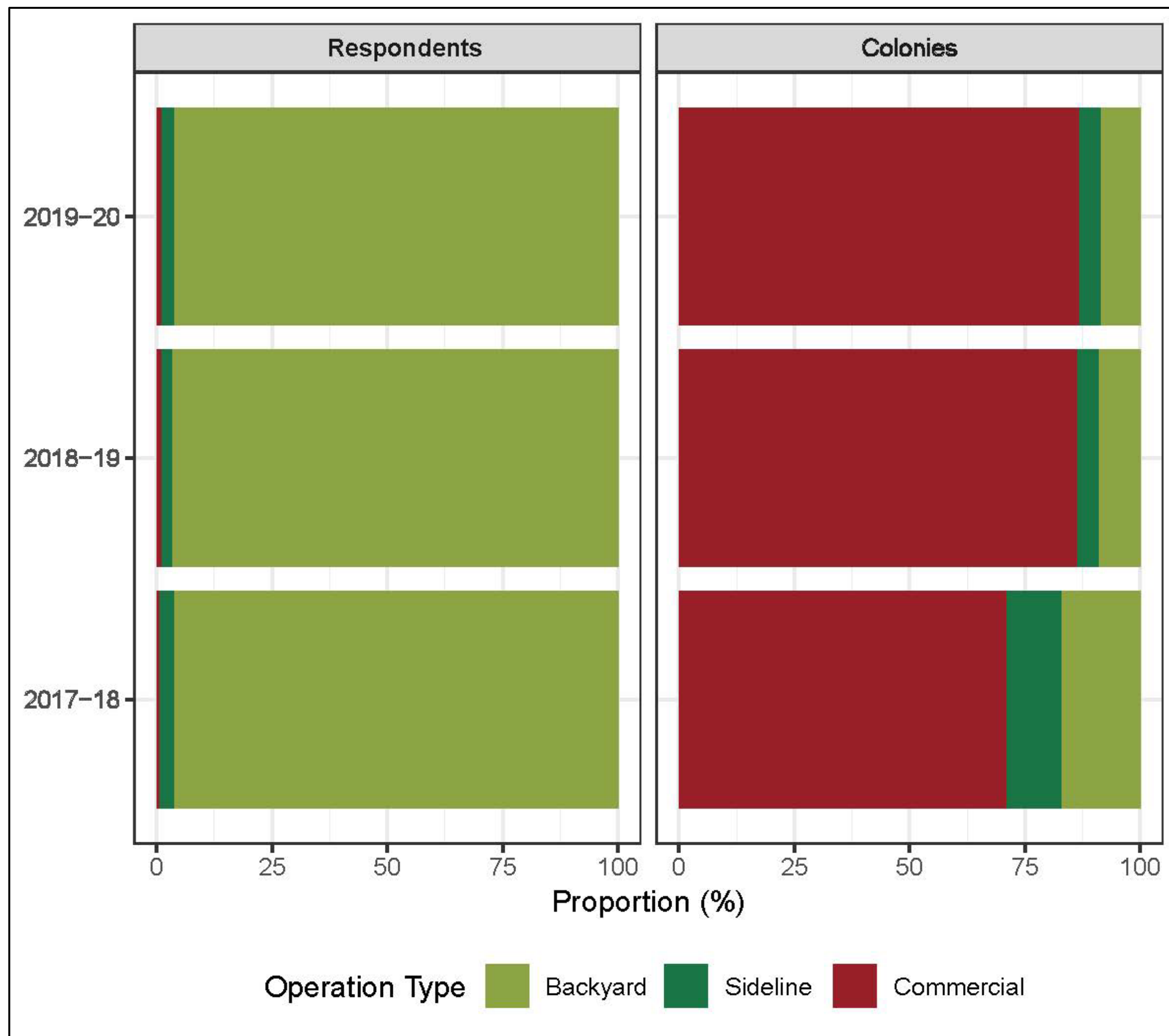

Figure 1: Composition of valid respondents to a national managed *Apis mellifera* honey bee colony loss survey performed for the United States during years 2017-18, 2018-19 and 2019-20 according to operation type and number of colonies managed on October 1 for each survey year. The total number of survey respondents and managed colonies on October 1 were treated as 100% for each survey year. Illustrated here are the composition of survey respondents according to operation type – Backyard, Sideline, and Commercial beekeeper, managing 1-50, 51-500, and 501 or more colonies, respectively. The proportion of colonies managed by each operation type is also included.

National Total and Average colony loss estimates

For the three survey years, the national estimate for Total colony loss during Summer was highest in 2019-20 (32.1%) and lowest in 2017-18 (17.9%) . For Winter, the highest national Total colony loss was 37.0% in 2018-19; it was lowest in 2019-20 (22.9%) . National Total Annual loss ranged between 40.4% in 2018-19 and 44.0% in 2019-20 (Table 2, Figure 2).

Among all three survey years, the national estimate for Average colony loss during Summer was highest in 2019-20 (21.5%) and lowest in 2018-19 (14.6%) . For Winter, the highest national Average colony loss was 50.4% in 2017-18, and lowest in 2019-20 with 35.7% . Annually, the national Average colony loss ranged between 46.1% in 2019-20 and 57.3% in 2017-18 (Table 2).

Table 2: A summary of the managed *Apis mellifera* honey bee colony losses in the United States during three survey years – 2017-18, 2018-19 and 2019-20. For each survey year and loss period (Summer [1 April 201X – 1 October 201X], Winter [1 October 201X – 1 April 201Y], and Annual [1 April 201X – 1 April 201Y], whereby “X” and “Y” represent successive years), we listed: Number of valid respondents (n), the number of collectively managed colonies (N) at the start, the middle and the end of each survey year (i.e. April 1 201X (start), October 1 201X, April 1 201Y (end)), the total number of colony increases (+) and decreases (-) between key dates, as well as national Total and Average loss (%) [95% CI]. Net change is defined as increasing operation size (+) by splitting and purchasing colonies, and reducing operation size (-) by selling or giving away colonies. Colonies that died or combined between key dates are excluded from the net change calculation.

			Total colonies (N) alive on:						
Survey Year	**Season**	**n**	**April 1 (start)**	**Net change**	**October 1**	**Net change**	**April 1 (end)**	**Total loss (% [95%CI])**	**Average loss (% [95%CI])**
	Summer	4,074	157,803	(+) 57,736	177,459	-	-	17.89 [14.1-22.4]	18.55 [17.8-19.4]
2017-18	Winter	4,864	-	-	179,285	(+) 30,818	145,146	31.10 [26.2-36.2]	50.36 [49.3-51.4]
	Annual	3,889	136,406	(+) 56,312		(+) 28,333	130,986	41.14 [35.5-47.5]	57.34 [56.2-58.4]
	Summer	3,818	264,170	(+) 65,398	261,488			20.7 [20.1-21.1]	14.6 [13.9-15.3]
2018-19	Winter	4,631			318,969	(+) 47,670	228,236	37.7 [36.9-38.6]	43.8 [42.7-44.8]
	Annual	3,652	248,069	(+) 65,790		(+) 47,670	212,894	40.9 [40.3-41.5]	49.4 [48.3-50.5]
	Summer	2,843	215,904	(+) 36,627	255,064	-	-	32.07 [20.2-45.5]	21.49 [20.5-22.5]
2019-20	Winter	3,301			262,033	(+) 5,658	218,510	22.85 [17.4-29.3]	35.70 [34.5-36.9]
	Annual	2,887	202,290	(+) 36,526		(+) 5,509	203,092	43.95 [31.7-55.8]	46.06 [44.8-47.3]

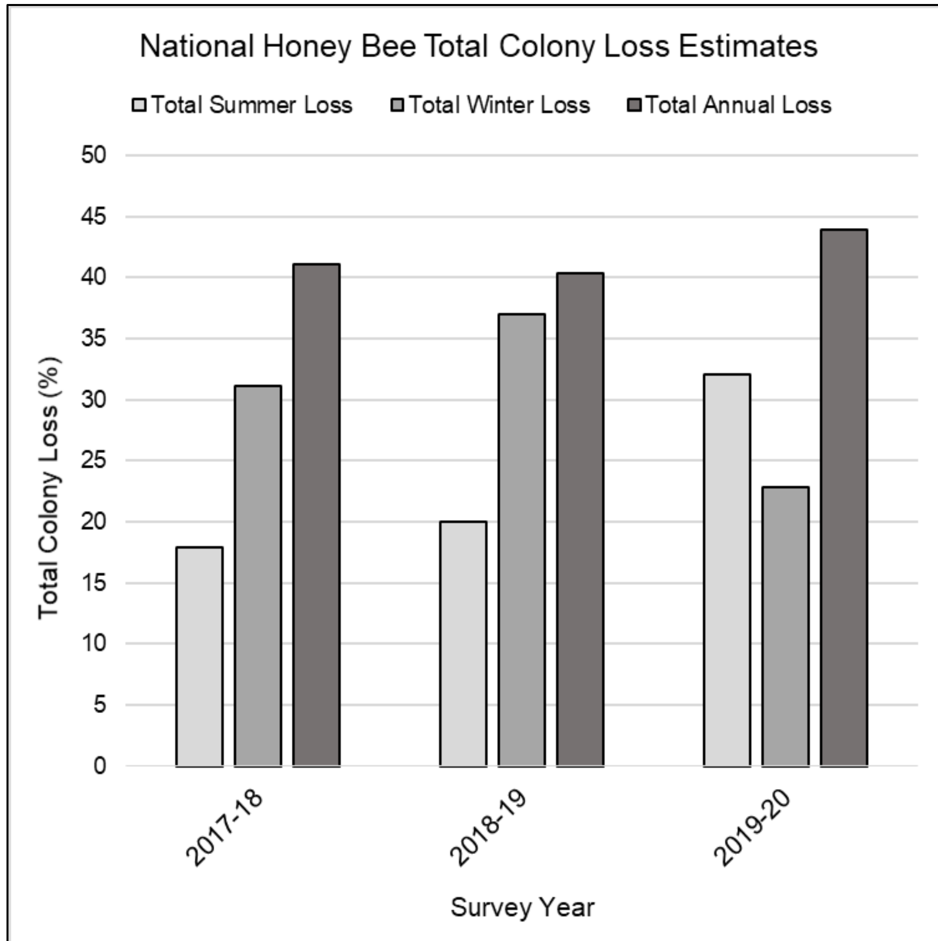

Figure 2: Managed *Apis mellifera* honey bee colony Total Loss estimates per season for three survey years in the United States – 2017-18, 2018-19 and 2019-20. The bar graphs indicate the national total loss estimates [%] for the Summer (1 April 201X– 1 October201X), Winter (1 October 201X – 1 April 201Y) and Annual (1 April 201X– 1 April 201Y) loss periods, whereby "X" and "Y" represent successive years.

State-specific Total and Average colony loss

The number of state-specific respondents was highly variable with respect to state and loss period (Appendix 1, Figure 17 & Figure 18, Table 24, Table 25 & Table 26). In 2017-18, Total colony loss estimates during Winter ranged from 16.9% in Arkansas (N=42) to 62.9% in Tennessee (N=80). In contrast, Average colony loss estimates during Winter were lowest in Oklahoma (N=32, 23.7%) and highest in Wisconsin (N=156, 73.5%). In 2018-19, Total colony loss estimates during Winter were lowest in South Carolina (N=41, 12.8%) and highest in Nevada (N=15, 64.0%). Average colony loss estimates during Winter were between 21.6% in Florida (N=37) and 65.2% in Illinois (N=108). Finally, in 2019-20, Total colony loss estimates during Winter were between 10.2% in Nevada (N=10) and 49.1% in Utah (N=70), whereas Average colony loss estimates during Winter ranged from 19.3% in Texas (N=96) to 55.8% in Minnesota (N=63) (Figure 3).

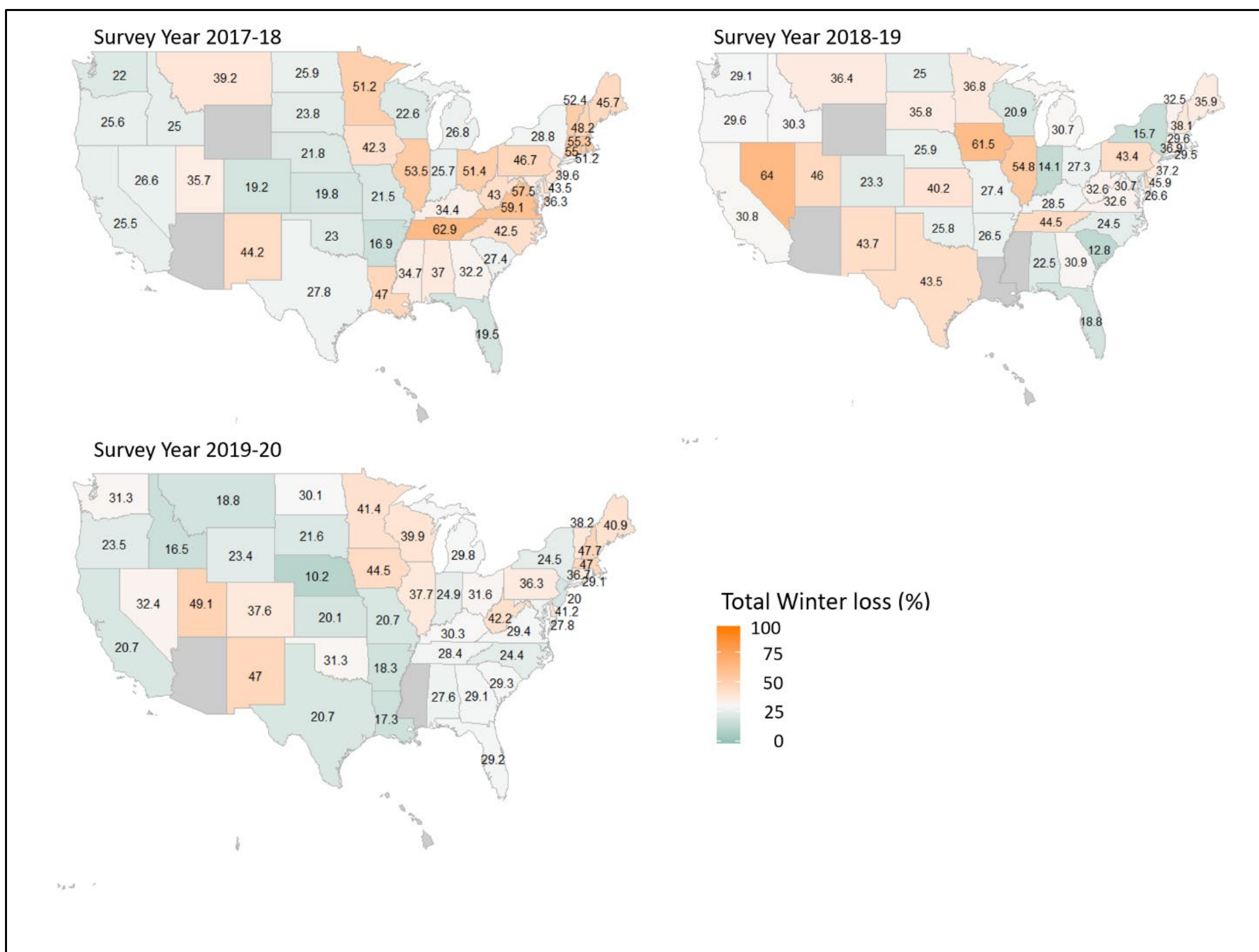

Figure 3: Managed *Apis mellifera* honey bee colony Total Loss estimates during Winter per state for three survey years in the United States – 2017-18, 2018-19 and 2019-20. Maps represent state-specific Total Winter loss estimates [%] (Winter: 1 October 201X – 1 April 201Y, whereby "X" and "Y" represent successive years). State-specific estimates, written within or near each state boundary, were only calculated if there were more than ten respondents.

National Total and Average colony loss estimates by operation type and activity

For all three loss periods per year, Total and Average colony losses were estimated by operation type (Backyard, Sideline, Commercial). Generally, Commercial Beekeepers experienced higher Average colony loss in Summer compared to Backyard and Sideliner Beekeepers in all three survey years ($p<0.05$, Table 3, Figure 4). In contrast, during the Winter and Annual loss periods, Backyard Beekeepers lost more colonies than Sideliner and Commercial Beekeepers, although significant statistical differences between groups were not observed for the survey year 2019-20 (Table 3, Figure 4).

Table 3: A summary of managed *Apis mellifera* honey bee colony losses in the United States by operation type during three survey years – 2017-18, 2018-19 and 2019-20. Each year includes three seasonal loss periods: Summer (1 April 201X– 1 October201X), Winter (1 October 201X – 1 April 201Y) and Annual (1 April 201X– 1 April 201Y), whereby "X" and "Y" represent successive years. Total and Average colony loss estimates are listed for respondents (N) belonging to one of three operation types: Backyard (managing 1-50 colonies), Sideliner (managing 51-500 colonies), and Commercial (managing 501 or more colonies) beekeepers. For each survey year, Average colony loss estimates within one particular loss period (e.g. Summer) were compared between the three operation types. Different significance letters identify statistically significant differences in Average colony loss within a given loss period of the associated survey year.

Survey Year	Season	Operation type	N	Total loss (% [95%CI])	Average Loss (% [95%CI])	χ2, p	Significance letters
2017-18	Summer	Backyard	3,908	21.2 [19.7-22.9]	18.7 [17.9-19.5]	15.94, <0.01	a
		Sideline	127	22.4 [16.8-26.7]	14.7 [12.0-17.6]		a
		Commercial	39	16.6 [11.7-22.3]	22.8 [18.2-27.7]		b
	Winter	Backyard	4,674	46.0 [44.4-47.3]	51.0 [49.8-52.0]	22.20, <0.01	a
		Sideline	15	38.8 [33.3-44.7]	37.4 [32.9-41.4]		b
		Commercial	38	26.4 [20.0-33.0]	31.6 [24.2-39.2]		c
	Annual	Backyard	3,730	56.1 [54.7-57.4]	57.9 [56.8-58.9]	24.57, <0.01	a
		Sideline	125	51.1 [45.1-57.0]	45.9 [41.5-50.2]		b
		Commercial	34	36.0 [29.2-43.7]	43.8 [36.4-50.7]		b
2018-19	Summer	Backyard	3,669	15.4 [14.4-16.6]	14.5 [13.8-15.3]	46.07, <0.01	a
		Sideline	91	25.8 [17.9-35.3]	14.1 [10.6-17.9]		b
		Commercial	57	20.1 [15.4-25.1]	20.8 [17.2-24.9]		c
	Winter	Backyard	4,466	39.8 [38.5-41.2]	44.2 [43.1-45.4]	9.31, 0.01	a
		Sideline	108	34.7 [28.4-41.1]	33.3 [28.5-37.8]		ab
		Commercial	58	37.3 [23.9-52.7]	26.2 [20.8-31.6]		b
	Annual	Backyard	3,514	47.6 [46.2-49.0]	49.8 [48.6-50.9]	10.67, <0.01	a
		Sideline	87	45.6 [38.2-52.7]	41.4 [36.2-46.9]		ab
		Commercial	52	39.4 [34.3-45.3]	36.4 [31.9-41.3]		b

Table 3 (continued): A summary of managed *Apis mellifera* honey bee colony losses in the United States by operation type during three survey years – 2017-18, 2018-19 and 2019-20. Each year includes three seasonal loss periods: Summer (1 April 201X– 1 October201X), Winter (1 October 201X – 1 April 201Y) and Annual (1 April 201X– 1 April 201Y), whereby "X" and "Y" represent successive years. Total and Average colony loss estimates are listed for respondents (N) belonging to one of three operation types: Backyard (managing 1-50 colonies), Sideliner (managing 51-500 colonies), and Commercial (managing 501 or more colonies) beekeepers. For each survey year, Average colony loss estimates within one particular loss period (e.g. Summer) were compared between the three operation types. Different significance letters identify statistically significant differences in Average colony loss within a given loss period of the associated survey year.

Survey Year	Season	Operation type	N	Total loss (% [95%CI])	Average Loss (% [95%CI])	χ2, p	Significance letters
2019-20	Summer	Backyard	2,712	23.7 [22.3-25.2]	21.5 [20.5-22.5]	8.96, 0.01	a
		Sideline	81	23.9 [17.9-30.3]	17.5 [14.3-20.9]		a
		Commercial	41	32.9 [18.9-48.4]	28.2 [21.3-35.4]		b
	Winter	Backyard	3,169	32.9 [31.4-34.3]	36.0 [34.8-37.3]	0.005, 0.99	a
		Sideline	91	31.6 [25.3-38.6]	28.9 [24.2-33.7]		a
		Commercial	41	21.4 [14.9-28.6]	28.9 [22.6-35.9]		a
	Annual	Backyard	2,574	46.1 [38.9-51.0]	46.3 [45.0-47.6]	2.86, 0.24	a
		Sideline	78	45.1 [38.9-51.0]	38.8 [34.0-43.8]		a
		Commercial	36	43.6 [30.6-56.9]	46.2 [39.4-53.0]		a

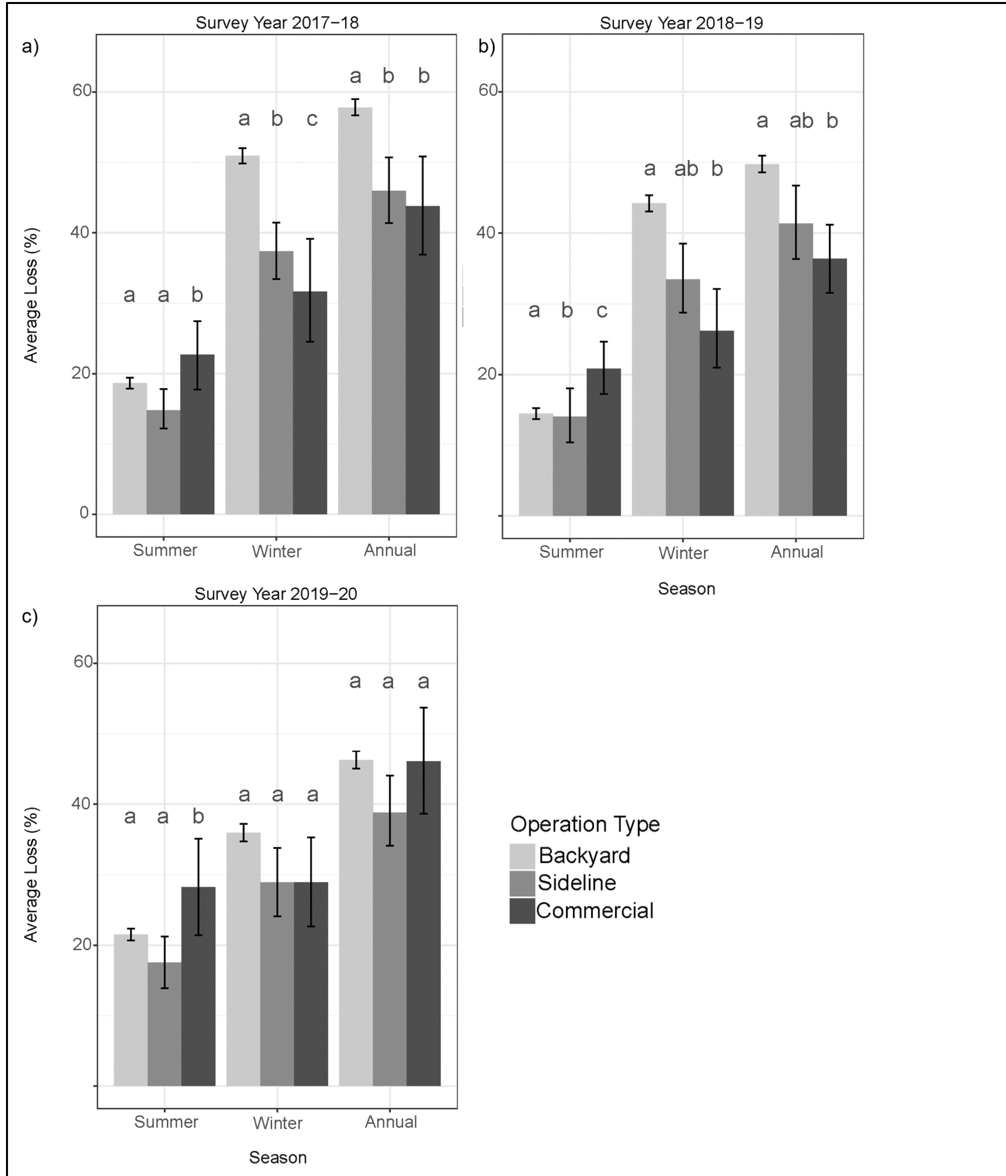

Figure 4: Managed *Apis mellifera* honey bee colony Average colony loss estimates in the United States by operation type during three survey years – 2017-18, 2018-19 and 2019-20. Each year contains three seasonal loss periods : Summer (1 April 201X– 1 October201X), Winter (1 October 201X – 1 April 201Y) and Annual (1 April 201X– 1 April 201Y), whereby "X" and "Y" represent successive years. Bar graphs illustrate Average colony loss estimates for three operation types: Backyard (managing 1-50 colonies), Sideliner (managing 51-500 colonies), and Commercial (managing 501 or more colonies) beekeepers. Comparisons between loss estimate comparisons were analyzed within one particular loss period (e.g. Summer) resulting in significance letters above bars. Different significance letters identify statistically significant differences in Average colony loss within a given loss period of the associated survey year.

Average Winter colony loss estimates did not differ according to migratory status in any of the three survey years ($p \geq 0.05$, Table 4). Similarly, Average Winter colony loss estimates were not different for operations that sent colonies to California for almond pollination compared to ones that did not ($p \geq 0.05$, Table 4).

Table 4: A summary of managed *Apis mellifera* honey bee colony losses in the United States during Winter according to key activity for three survey years – 2017-18, 2018-19 and 2019-20. Average colony loss estimates are listed according to one of four groups based on two key activities. N represents number of respondents. Average colony loss estimates were compared between Migratory and Stationary Beekeepers, as well as differences between beekeepers sending colonies to California for almond pollination (Yes almonds) or not (No almonds). Different significance letters identify statistically significant differences in Average colony loss between groups within a particular survey year.

Survey Year	Operation type	N	Average Loss (% [95%CI])	$\chi 2$, p	Significance letters
2017-18	Stationary	116	40.7 [35.6-45.8]	3.79, 0.05	a
	Migratory	66	31.2 [25.3-37.2]		a
2018-19	Stationary	87	34.03 [28.3-39.8]	2.14, 0.14	a
	Migratory	65	26.6 [21.7-31.5]		a
2019-20	Stationary	87	27.75 [22.9-32.6]	1.59, 0.21	a
	Migratory	43	31.81 [25.1-38.5]		a
2017-18	No almonds	120	40.2 [35.1-45.3]	3.61, 0.06	a
	Yes almonds	51	29.3 [23.7-34.9]		a
2018-19	No almonds	88	33.6 [28.0-39.3]	1.29, 0.26	a
	Yes almonds	55	27.7 [22.1-33.4]		a
2019-20	No almonds	84	28.59 [23.6-33.5]	0.39, 0.53	a
	Yes almonds	46	30.01 [23.5-36.6]		a

Self-reported acceptable winter colony loss

The three-year average acceptable winter loss was 21.31%. However, Average Colony Loss during Winter was greater than this for more than half of respondents (Table 5).

Table 5: Summary of self-reported acceptable Winter colony loss of managed *Apis mellifera* honey bee colonies in the United States. In each considered survey period, more than 90% of all valid winter loss respondents (VWL) indicated the percentage of operational loss they would deem acceptable during winter. The majority of these respondents experienced greater colony losses than the national average acceptable loss.

Survey Period	**Total respondents (N)**	**Average acceptable loss (% [95%CI])**	**Respondents with greater than average acceptable loss (N (% total respondents))**
2017-18	4643	20.6 [19.9-21.1]	3371 (73%)
2018-19	4340	22.3 [21.7-22.9]	2864 (66%)
2019-20	3238	21.1 [20.4-21.7]	1807 (56%)

Self-reported Cause of Winter Loss

Causes of Winter Loss (COWLs) varied according to operation type and survey year, except for “Varroa”, which was the most frequently selected option under all circumstances (Figure 5 & Figure 6, Table 6). After “Varroa”, Backyard Beekeepers most frequently selected “Weather”, “Starvation”, “Weak in Fall”, and “Don’t know” as COWL across all survey years. In contrast, “Queen Failure”, “Starvation”, and “Weak in Fall” belonged to the most frequently selected COWLs for Sideliner Beekeepers, whereas Commercial Beekeepers mostly selected “Queen Failure”, “Pesticides”, and “Pollen Deprivation” (Table 6).

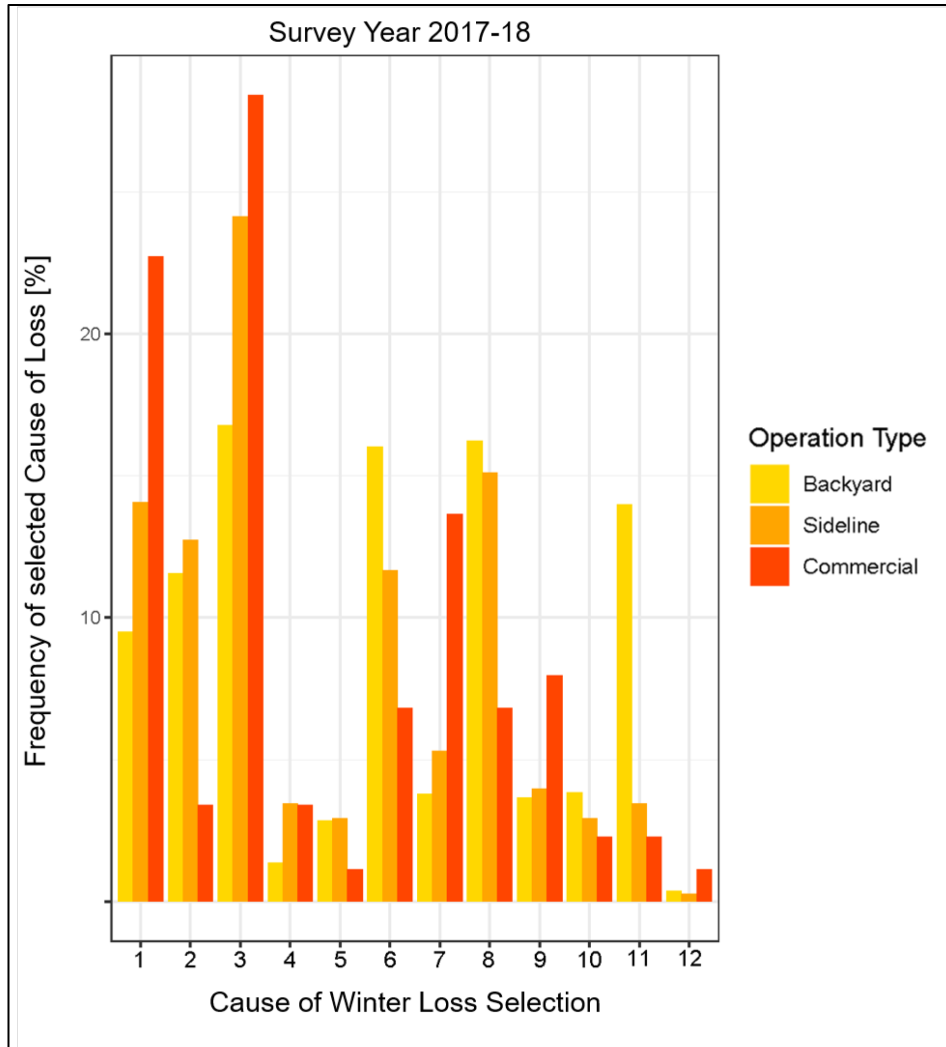

Figure 5: Frequency of beekeeper perceived causes of managed *Apis mellifera* honey bee Winter colony losses in the United States by operation type for the survey year 2017-18. Respondents were able to select from a list of 11 Causes of Winter colony loss: 1) Queen Failure, 2) Starvation, 3) *Varroa destructor*, 4) Nosema, 5) Small Hive Beetle, 6) Weather, 7) Pesticide, 8) Weak in Fall, 9) Colony Collapse Disorder, 10) Disaster, and 11) Don't know. In addition, they could provide beekeeper defined causes as open entry which correlates with 12) "Other cause". Differently colored bars indicate the frequency of each selected Cause of Winter loss from three operation types: Backyard (managing 1-50 colonies, yellow), Sideliner (managing 51-500 colonies, orange), and Commercial (managing 501 or more colonies, red) beekeepers.

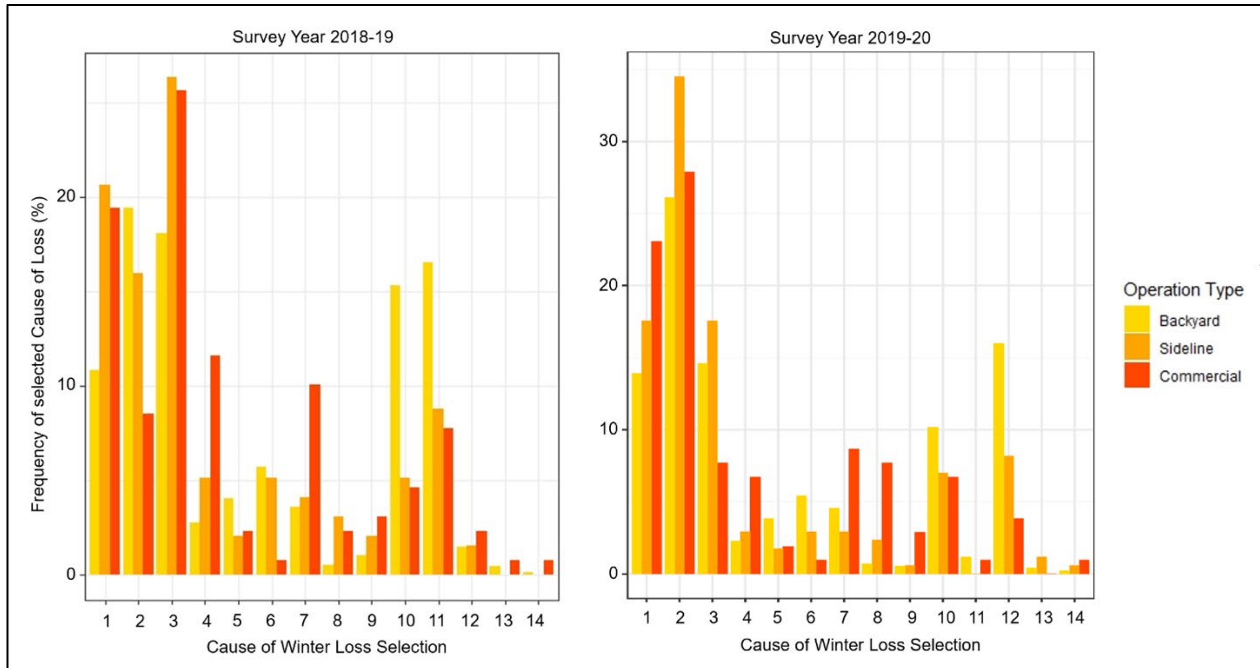

Figure 6: Frequency of beekeeper perceived causes of managed *Apis mellifera* honey bee Winter colony losses in the United States by operation type for the survey years 2018 -19 and 2019-20. Respondents were able to select from a list of 13 predefined Causes of Winter colony loss: 1) Queen Failure, 2) Starvation, 3) *Varroa destructor*, 4) Pollen deprivation, 5) Scavenger, 6) Equipment Failure, 7) Pesticide, 8) Brood disease, 9) Disaster, 10) Don't know, 11) Weather, 12) Predators, and 13) Mismanagement. In addition, they could provide beekeeper defined causes as open entry which correlates with 14) "Other cause". Differently colored bars indicate the frequency of each selected Cause of Winter loss from three operation types: Backyard (managing 1-50 colonies, yellow), Sideliner (managing 51-500 colonies, orange), and Commercial (managing 501 or more colonies, red) beekeepers.

Table 6: Self-reported Cause of Winter Loss for managed *Apis mellifera* honey bee colonies in the United States during the survey periods 2017-18, 2018-19 and 2019-20. Respondents selected items from a list of potential "Causes of Winter Loss". The total number of respondents that selected a cause were further categorized into number of selections per operation type (Backyard, Sideliner, and Commercial Beekeeper).

	Winter 2017-18 N				Winter 2018-19 N				Winter 2019-20 N			
Cause of Loss	Total	Backyard	Sideline	Commercial	Total	Backyard	Sideline	Commercial	Total	Backyard	Sideline	Commercial
Varroa	1319	1193	93	33	951	867	51	33	989	894	64	31
Weak in Fall	1218	1152	58	8	-			-	-	-	-	-
Weather	1168	1116	46	6	827	800	17	10	404	383	14	7
Don't know	1031	1016	13	2	745	729	10	6	591	571	14	6
Starvation	866	814	48	4	979	937	31	11	571	531	32	8
Queen failure	776	697	54	25	587	523	39	25	563	501	35	27
Pesticides	317	282	21	14	193	172	8	13	181	166	6	9
Disaster	305	290	11	4	57	49	4	4	26	22	1	3
CCD	267	243	15	9	-	-	-	-	-	-	-	-
SHB	240	227	11	2	-	-	-	-	-	-	-	-
Scavengers	130	129	1	0	201	194	4	3	152	146	4	2
Nosema	111	93	14	4	-		-	-	-	-	-	-
Equipment failure	103	-	-	-	287	276	10	1	201	195	5	1
Management	34	-	-	-	24	23	0	1	22	20	2	0
Brood disease	22	-	-	-	34	25	6	3	36	24	4	8
Predators	21	-	-	-	79	73	3	3	45	43	0	2
Other	19	17	2	0	14	13	0	1	11	9	1	1
Pollen	-	-	-	-	159	134	10	15	107	93	7	7

Self-reported Cause of Summer Loss

"Queen Failure" was the most frequently selected Cause of Summer Loss (COSLs) for all operation types and survey years, followed by "Varroa" (Figure 7, Table 7). The third most frequently chosen COSL in 2018-19 and 2019-20 was "Don't know" for Backyard Beekeepers and "Pesticides" for Commercial Beekeepers. Sideliner Beekeepers' third most frequently selected COSL was different between survey years, and included "Pollen Deprivation", and "Pesticides" in 2018-19 and 2019-20, respectively (Table 7).

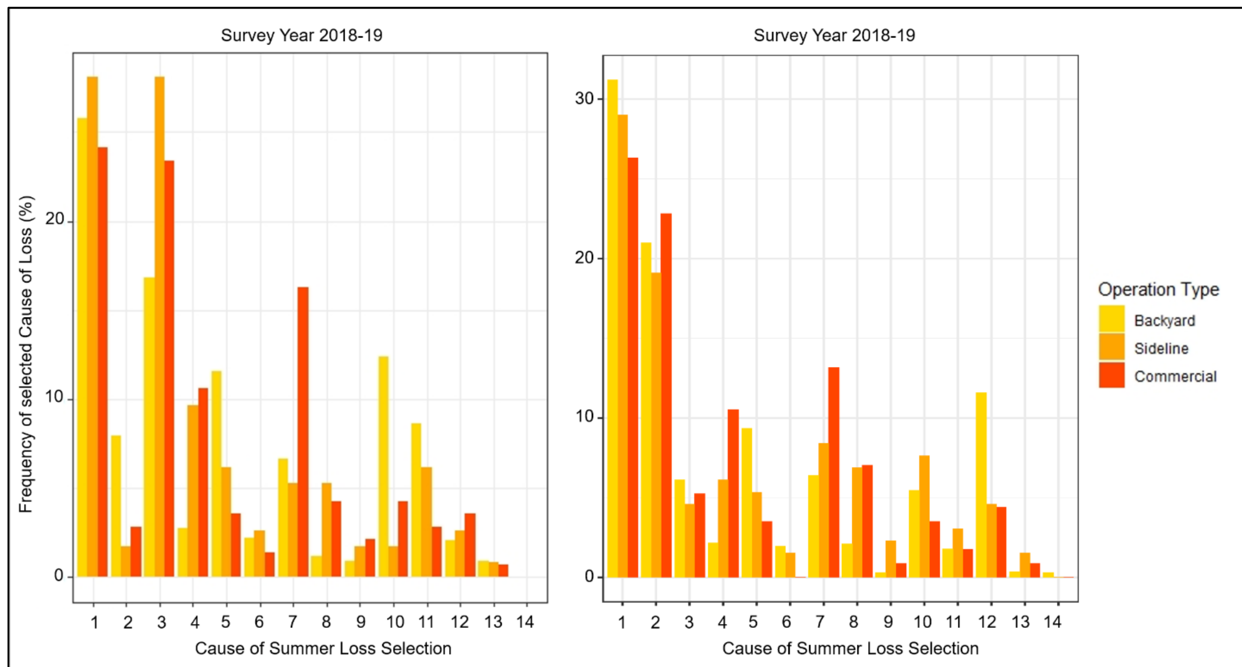

Figure 7: Frequency of beekeeper perceived causes of managed *Apis mellifera* honey bee Summer colony losses in the United States by operation type for the survey years 2018 -19 and 2019-20. Respondents were able to select from a list of 13 predefined Causes of Summer colony loss: 1) Queen Failure, 2) Summer starvation, 3) Varroa destructor, 4) Pollen deprivation, 5) Scavenger, 6) Equipment Failure, 7) Pesticide, 8) Brood disease, 9) Disaster, 10) Don't know, 11) Weather, 12) Predators, and 13) Mismanagement. In addition, they could provide beekeeper defined causes as open entry which correlates with 14) "Other cause". Differently colored bars indicate the frequency of each selected Cause of Summer loss from three operation types: Backyard (managing 1-50 colonies, yellow), Sideliner (managing 51-500 colonies, orange), and Commercial (managing 501 or more colonies, red) beekeepers.

Table 7: Self-reported Cause of Summer Loss for managed *Apis mellifera* honey bee colonies in the United States during the survey years 2018-19 and 2019-20. Respondents either selected items from a list of 14 potential "Causes of Summer Loss" and/or added beekeeper-defined causes as open entry. The total number of respondents that selected a particular cause were further categorized into number of selections per operation type (Backyard, Sideliner and Commercial Beekeeper).

	Summer 2018 (N)				Summer 2019 (N)			
Cause of loss	Total	Backyard	Sideline	Commercial	Total	Backyard	Sideline	Commercial
Queen failure	495	429	32	34	688	602	49	37
Varroa	345	280	32	33	467	403	33	31
Don't Know	214	206	2	6	267	254	8	5
Scavengers	204	192	7	5	186	173	9	4
Weather	155	144	7	4	133	118	11	4
Pesticides	140	111	6	23	152	124	11	17
Starvation	138	132	2	4	127	114	7	6
Pollen	72	46	11	15	65	43	9	13
Predator	43	35	3	5	41	35	4	2
Equipment Failure	42	37	3	2	36	34	2	0
Brood Disease	32	20	6	6	61	41	10	10
Disaster	20	15	2	3	11	7	3	1
Mismanagement	17	15	1	1	14	11	2	1
Other	1	1	0	0	5	5	0	0

Discussion

Long-term monitoring of managed *Apis mellifera* Linnaeus honey bee colony losses in the United States and elsewhere is critical given the recent decline in health of this economically and societally important species (Brodschneider et al. 2010, Zee et al. 2013, Pirk et al. 2015, Lee et al. 2015a, Kulhanek et al. 2017). It provides spatial and temporal context for honey bee health, and promotes understanding and potential mitigation of these losses (Lindenmayer et al. 2012, Lee et al. 2015a). Consistent with similar surveys performed in preceding years (Steinhauer et al. 2014b, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017), we observed that managed honey bee colony loss during the last three winters (2017-18, 2018-19, 2019-20) (Total Winter colony loss; TWCL) in the United States remained higher than historically reported (Neumann and Carreck 2010), at 31, 38, and 23%, respectively. Furthermore, we observed that backyard beekeeping operations managing fewer than 50 colonies experienced the highest colony losses among all beekeeping operation types during winter for two of the three surveyed years, whereas commercial operations managing more than 500 colonies experienced the highest colony losses among all operation types during all three summers. This work re-affirms the continued need for sustainable, beekeeper-specific Best Management Practices to improve the health of managed honey bees in the United States.

National TWCL fluctuated around 30% for the first 7 years of the Bee Informed Partnership's standardized surveys (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012, Steinhauer et al. 2014b), with the exception of Spleen et al. (2013) that observed 23% TWCL. National TWCL trended downward in the four years preceding this work (Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017, Steinhauer et al. in prep), suggesting that improvements to honey bee health were occurring. But, elevated national TWCL observed during 2017-18 (31%)

and 2018-19 (38%), the latter of which represented the highest level observed since the survey began in 2007- 08, highlights the dynamic nature of colony losses in winter throughout the country and this can only be observed through long-term monitoring. Our results also highlight a general trend of relatively constant high winter colony losses. When compared to historical losses of 10-15% (Neumann and Carreck 2010), these high losses appear set to remain. When averaged across the three reported survey years, beekeepers believed 21% colony loss during winter was acceptable. This is 4% above the ten-year average acceptable loss percentage of 17% (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012, Spleen et al. 2013, Steinhauer et al. 2014b, Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017), and may indicate habituation by beekeepers to elevated losses. Historically, honey bee colony loss surveys have focused exclusively on wintering mortality (vanEngelsdorp et al. 2007, 2008, Neumann and Carreck 2010, vanEngelsdorp and Meixner 2010b) because winter is considered to be a relatively inactive season due to climatic conditions (i.e. cold temperatures and shortened daylight hours) in most regions of the country. Winter losses were highly variable among regions and states, but there was a trend for lower winter losses in areas with more mild climates such as the southeast or west coast. Winter is also a high risk period, as many potential intervention actions to prevent colony losses, like queen replacement, are limited if not impossible at that time (Seeley and Visscher 1985).

Despite beekeepers traditionally experiencing higher colony losses in winter compared to summer, the latter is still a period of substantial losses, particularly by large-scale beekeepers (Lee et al. 2015b, Kulhanek et al. 2017). Since the first documentation of national Total Summer colony loss (TSCL) in 2012 (Steinhauer et al. 2014b), estimates have fluctuated around 20% (Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017). National TSCL estimates for 2017-18

(17. 9%) and 2018-19 (20.7%) fit this trend; however, at 32.1%, 2019-20 marked the worst on record. Interestingly, we documented record high national TWCL and TSCL in consecutive years, 2018-19 and 2019-20, respectively, which might indicate a spill-over effect. Beekeepers that lost high numbers of colonies during winter compensated for their losses in early spring by installing newly obtained or recently split pre-existing colonies; both bear high risks (Delaplane 2010, 2015, Steinhauer et al. 2021). Although summer affords beekeepers the flexibility to perform a range of management options (Project Apis m. 2020), self-reported causes of loss through queen failure and the *Varroa destructor* Anderson and Trueman mite by all beekeeper operation types suggest areas for future research and extension efforts to mitigate loss during this period.

Number of colonies managed is closely connected to the current goals of beekeepers, and strongly shapes their philosophies of management (Underwood et al. 2019). This likely helps to explain seasonal difference in colony losses experienced by the different types of beekeepers. Similar to previous surveys here and abroad (Kulhanek et al. 2017, Gray et al. 2019, USDA NASS 2020a), Backyard beekeepers experienced higher losses during winter compared to Commercial beekeepers, with exception of Winter 2019-20. Many Commercial beekeepers rely on their colonies for income generation, and need strong colonies at the beginning of the brood rearing seasons to ensure they can provide adequate pollination services in spring (Underwood et al. 2019). To accomplish this, those beekeepers are more likely to use chemical controls against the important biotic risk factor *V. destructor* (Haber et al. 2019). These management actions likely reduce the risk of winter mortality resulting from *V. destructor* pressure, which is reflected in our results (Steinhauer et al. 2021). Interestingly, *V. destructor* was selected as the main beekeeper-perceived cause of winter loss across all three survey years, regardless of operation

type. This suggests that even Backyard beekeepers are aware of the risk of *V. destructor*, but that their reluctance to use synthetic acaricides may affect the effectiveness of their management practices (Underwood et al. 2019). Conversely, Commercial beekeepers lost significantly more colonies during summer compared to all other beekeeper operation types. Although they diligently manage their colonies in fall to ensure strong colonies in the spring (Underwood et al. 2019), in-hive use of chemicals to manage *V. destructor* may have consequential secondary effects. Losses during the brood-rearing season are concerning, and may be related to what beekeepers perceived to be the most likely cause for summer losses − queen failure. A number of studies have demonstrated that residues persisting from commonly used acaricides for *V. destructor* control can negatively affect the reproductive health and behavior of queens, as well as their potential mates (Rangel and Tarpy 2015, Rangel and Fisher 2019). Although large-scale operations commonly pursue risky beekeeping activities like providing migratory pollination services to important agricultural crops including almonds (Whynott 1991, Morse and Calderone 2000), our results support previous work that suggests these activities do not significantly impair honey bee health (Lee et al. 2015b, Seitz et al. 2015, Kulhanek et al. 2017). Experiments are needed to truly tease apart how other management activities, especially in consideration of *V. destructor*, impact colony mortality.

Managed honey bee colonies in the United States are continuously threatened by simultaneous pressures from multiple, possibly interacting risk factors (Potts et al. 2010, Smith et al. 2013, Steinhauer et al. 2018). Our results highlight not only the variability in colony losses among regions, seasons and years, but also by beekeeping operation type. Considering the importance of managed honey bee colonies across the globe, our long-term efforts, as well as

those by others, provide crucial baseline information and valuable insight into relationships between honey bees and their beekeepers.

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Chapter 3

Effects of *Varroa destructor* mites and neonicotinoid insecticides on *Apis mellifera* honey bee worker survival and hypopharyngeal glands

Abstract

Nursing workers are crucial to honey bee (*Apis mellifera* Linnaeus) colony functioning. Their hypopharyngeal glands (HPGs) produce brood food that nourishes developing larvae and contributes to social immunity. The ubiquitous ectoparasitic mite *Varroa destructor* Anderson and Trueman and commonly employed neonicotinoid insecticides can cause lethal and sub-lethal effects in honey bees. Although concurrent exposure to both stressors is likely, little is known about how the two may interact to affect honey bees. Using nursing worker survival and HPG quality as a proxy for honey bee health, we performed a fully crossed experimental design to assess the effects of simultaneous neonicotinoid exposure and *V. destructor* infestation. Known age cohorts of workers were obtained from 20 colonies − half the colonies received patties containing field-realistic concentrations of two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin), whereas the other half received patties without neonicotinoids. Workers from each colony were artificially emerged, assessed for *V. destructor* infestation, and allocated to treatment groups of no dietary neonicotinoid with and without *V. destructor* added, and dietary neonicotinoid with and without *V. destructor* added. Workers were kept in laboratory cages for 10 days before being decapitated for HPG examination at the typical age of nursing. Individually, neonicotinoids and *V. destructor* significantly reduced worker survival and HPG development. Contrary to our expectations and similar studies, our results suggested an antagonistic interaction between the two stressors possibly explained by differences in route and

timing of exposure to stressors, or physiological pathways affected. This work highlights the interactive nature of important biotic and abiotic stressors of the economically and ecologically important honey bee.

Introduction

Eusocial hymenopteran insects like the honey bee (*Apis mellifera* Linnaeus) exhibit a dynamic temporal polyethism, with workers performing different tasks according to age (Winston 1991, Johnson 2010). Changes in task responsibilities are accompanied by an adjustment of anatomy and physiology which can be plastic depending on the needs of the colony (Winston 1991, Crailsheim and Hrassnigg 1998). For example are the hypopharyngeal glands (HPGs), paired glands coiled within a worker's head and composed of secretory subunits called acini (Snodgrass 1956). HPGs are largest and fully functional in adult workers aged 8 to 12 days (Knecht and Kaatz 1990, Smodiš Škerl and Gregorc 2015), when workers perform nursing duties, such as the oral delivery of glandular secretions that contribute to brood food, the substance used to nourish developing honey bee larvae (Winston 1991, Crailsheim 1991, Crailsheim et al. 1992). Once workers begin to fly, usually starting around 22 days, their HPGs cease to produce brood food and acini simultaneously atrophy (Knecht and Kaatz 1990, Ohashi et al. 1999, Deseyn and Billen 2005, Seeley 2009). HPGs also play a role in social immunity by secreting glucose oxidase to sterilize brood food; this supposedly prevents larval diseases and provides trans-generational immunity (Yang and Cox-Foster 2005, Harwood et al. 2019). Factors that might impair HPG development or function could have negative down-stream effects on other individuals in the honey bee colony that rely on its glandular secretions, both during development and adulthood (Crailsheim 1991). Ultimately, this might affect the entire colony if enough individuals are compromised (Khoury et al. 2011, Henry et al. 2012, Sponsler and Johnson 2017).

Understanding the consequences of HPG dysfunction is especially important in the light of high honey bee colony losses in recent years (Potts et al. 2010, Kulhanek et al. 2017, Bruckner et

al. 2019). Given the close connection of honey bee colonies to the environment, multiple biotic and abiotic risk factors could directly or indirectly impair HPG function posing a hazard to the colony (Steinhauer et al. 2018). Biotic risk factors include fungi, bacteria, viruses, and parasitic mites (Genersch 2010, McMenamin and Genersch 2015, Steinhauer et al. 2018). In contrast, xenobiotics like weather, heavy metals, fungicides, and insecticides are examples of abiotic risk factors (Mao et al. 2009, Johnson et al. 2010, 2013, Niu et al. 2011, Sgolastra et al. 2018). Recently, there is consensus that many honey bee colony losses can be attributed to interactions among risk factors but in-depth studies are scarce (Potts et al. 2010).

Arguably the most important biotic threat to apiculture is the ectoparasitic mite *Varroa destructor* Anderson and Trueman (Rosenkranz et al. 2010, Le Conte et al. 2010, Guzmán-Novoa et al. 2010, Steinhauer et al. 2018). Feeding by mites on the developing honey bee host results in reduced body weight and shortened life span for the host, as well as impaired development of anatomical structures such as HPGs (Schneider and Drescher 1987, Donzé and Guerin 1994, Bowen-Walker and Gunn 2001, Gregorc et al. 2012a, Yousef et al. 2014). Historically described as hemophageous, researchers recently discovered that *V. destructor* mainly feeds on the fat body of honey bees, which serves as nutrient storage and is essential for protein synthesis, hormone regulation, pesticide detoxification, food storage, and the innate immune response (Arrese and Soulages 2010, Rosenkranz et al. 2010, Gätschenberger et al. 2013, Ramsey et al. 2019). Despite its importance to the beekeeping industry, research on potential effects of *V. destructor* on tissue, glands, or internal organs is limited. HPGs are one of the few anatomical structures that have been investigated, and were shown to be smaller in workers parasitized by the mite (Schneider and Drescher 1987, Yousef et al. 2014).

Similarly, insecticides, specifically neonicotinoids can also have sub-lethal effects on honey bees exposed to field-realistic concentrations of neonicotinoids (Williams et al. 2015, Straub et al. 2016). Neonicotinoids such as thiamethoxam are primarily applied as a seed-coating treatment to manage insect pests of plants, and belong to the most widely used agricultural insecticides in the world (Jeschke et al. 2011, Wieben 2020). Due to their systemic properties, neonicotinoids are translocated throughout the entire plant including nectar and pollen, which poses a route of exposure to non-target organisms like honey bees (Goulson 2013). Therefore, not only can foraging workers be exposed to contaminated nectar and pollen, but so too can the entire colony population via contaminated stored food causing sub-lethal effects (Sanchez-Bayo and Goka 2014). For example, exposure to field-realistic concentrations of neonicotinoids resulted in smaller HPGs of nursing workers in both the laboratory and field (Heylen et al. 2011, Hatjina et al. 2013, Smet et al. 2017).

Although both *V. destructor* and neonicotinoids alone can negatively affect honey bee health (Boecking and Genersch 2008, Lundin et al. 2015), and simultaneous exposure to these ubiquitous stressors is likely (Mitchell et al. 2017), only few experiments have studied their potential interactive effects on honey bees (Blanken et al. 2015, Morfin et al. 2019). For example, interaction effects of *V. destructor* and neonicotinoids on honey bee survival were documented by Straub et al. (2019) and Morfin et al. (2020), whereas, Siede et al. (2018) did not find any interaction effects.

To address this knowledge gap, we used a fully crossed experimental design to evaluate potential effects of *V. destructor* and neonicotinoids on worker honey bee survival and HPG development. Based on previous work (Morfin et al. 2019, 2020, Straub et al. 2019), we expected to observe an

interaction with larger negative effects on survival and HPG development when workers were exposed to both stressors.

Methods

In May 2018, twenty honey bee (*Apis mellifera carnica*) packages (Blue Ridge Honey Company, Lakemont, GA, USA), each headed by a laying sister queen and 1.5 kg workers, were established in Auburn, AL, USA. Three weeks later, the colonies were randomly assigned to either a control or neonicotinoid treatment group.

Neonicotinoid Exposure

Insecticide treatments were provided *ad libitum* to all colonies via pollen patties (60% corbicular pollen, 30% powdered sugar, 10% organic honey) using an established method (Sandrock et al. 2014b, Straub et al. 2019). Corbicular pollen was sourced from honey bee colonies residing in a low intensity agricultural region of Colorado and contained no detectable levels of agricultural chemicals (Appendix 2, Figure 19). Similar to our previous studies, colonies belonging to the neonicotinoid treatment group (N+) received pollen patties spiked with field-relevant concentrations of two neonicotinoids − thiamethoxam and clothianidin (4.5 ppb and 1.5 ppb, respectively, both Sigma Aldrich) (Pilling et al. 2013, Williams et al. 2015, Straub et al. 2016). Pollen patties fed to control colonies (N-) were not spiked (Appendix 3). To promote in-hive patty consumption, each colony was equipped with a Sundance pollen trap (Rossman Apiaries, LLC., Moultrie, GA, USA) to prevent an influx of natural pollen (Sandrock et al. 2014b, Williams et al. 2015). Like previous studies using a similar experimental exposure regime (Forfert et al. 2015, Williams et al. 2015, Straub et al. 2016, 2019, Friedli et al. 2020),

pollen patties were provided for 49 days to cover two entire brood cycles and to mimic a realistic exposure period encountered by foraging honey bees (Goulson 2013).

Source of experimental workers and *Varroa destructor* mite infestation

Forty-two days after initial feeding, the queen of each colony was caged onto an empty brood frame to obtain known age cohorts of experimental workers (Williams et al. 2013). Similar to Dietemann et al. (2013), experimental brood frames were transferred to the laboratory just after cell capping so ~30 randomly selected capped worker cells per frame could be artificially infested with *V. destructor*. In brief, a small incision was made along the margin of each brood cell before a single mature female mite was introduced using a fine-tip brush then, the cell was immediately re-sealed. *V. destructor* females used for infestations were collected during their phoretic stage and were sourced from highly parasitized non-experimental colonies. Artificially infested frames were maintained in the incubator (34.5 °C, 75% RH, DR-41NL, Percival Scientific, Inc., Perry, IW) until ~24 hours prior to adult worker emergence (Dietemann et al. 2013). Then, for each frame in all experimental colonies (N+ and N-), sixty workers were artificially emerged. Of these, 30 that were artificially infested with *V. destructor* (V+) and 30 that were not (V-). Upon removal of the worker, each cell was assessed for the presence of a female mite and her offspring to ensure appropriate treatment group allocation (Straub et al. 2019). Teneral body mass of each worker was measured before the individual was added to a hoarding cage (Williams et al. 2013), then grouped according to treatment: 1) control colony and *V. destructor* absent (control, N-/V-), 2) control colony and *V. destructor* present (*V. destructor* only, N-/V+), 3) neonicotinoid colony and *V. destructor* absent (neonicotinoid only, N+/V-), and 4) neonicotinoid colony and *V. destructor* present (both stressors, N+/V+). Ultimately, each cage contained 10 workers that originated from the same colony.

Cage mortality and hypopharyngeal glands

Hoarding cages were maintained in the incubator (30 °C, 60% RH) and equipped with a syringe containing sucrose solution (50% w/v) and a pollen feeder (60% corbicular pollen, 40% powdered sugar) provided *ad libitum* to promote proper development of physiological structures (Brodschneider and Crailsheim 2010, Williams et al. 2013). Dead individuals were removed daily until 10 days post emergence, the typical age of nursing (Knecht and Kaatz 1990). At this time, all survivors were decapitated and their heads were preserved in 2% paraformaldehyde PBS buffer at 4 °C (Lanier and Warner 1981) until the hypopharyngeal glands (HPGs) could be removed. Each gland was then added to 0.1 M PBS buffer, stained with Bradford solution (Coomassie Brilliant Blue G-250, both Sigma Aldrich), then slide-mounted (Hartfelder et al. 2013) and photographed under a light microscope with 5.5x magnification (Leica, DM2500, Morrisville, USA). The diameters of 20 arbitrarily selected acini per gland per individual were measured using computer ImageJ software (https://imagej.net/) according to Hatjina et al. (2013).

Statistical analyses

All statistical analyses were performed in R (version 4.0.2., 11/2/20) using a significance level of α=0.05. Survival curves (Kaplan-Meier plots) were produced using the "survival" package, and the "survminer" package allowed for pairwise comparisons between treatment group specific cumulative survival with a Bonferroni correction.

Longevity data were not normally distributed. Therefore, a generalized linear mixed effect model was built to assess effects of neonicotinoid exposure and *V. destructor* infestation. Since individual workers represented experimental units, colony and worker identification numbers

were included as random effects to account for clustering effects. Multiple pairwise comparisons between treatment groups (N-/V-, N-/V+, N+/V-, and N+/V+) were performed using the "emmeans" package while employing a Bonferroni correction to account for multiple comparisons.

Measurements of HPG acini width were normally distributed, and effects of neonicotinoid exposure and *V. destructor* infestation were assessed using a linear mixed effect model. Cage and worker identification numbers were included as random effects since individual acini diameters represent the experimental unit. Multiple pairwise comparisons between the aforementioned treatment groups were performed using a pairwise Student t test with a Bonferroni correction to account for multiple comparisons.

Potential interactions between neonicotinoid exposure and *V. destructor* infestation were identified based on an additive framework (Folt et al. 1999), whereby an interaction is synergistic or antagonistic if the observed combined stressor effects are greater or smaller than the observed individual stressor effect, respectively. The percent difference in treatment groups compared to controls was calculated using mean longevity [d] for survival and median acini width [μm] for the HPGs.

Results

Cage mortality

Both neonicotinoid exposure as well as *V. destructor* infestation significantly reduced worker longevity ($p=0.008$ and $p<0.0001$, respectively) (Table 8). Longevity of control workers (N-/V-) was greatest (9.4±2.1 days, mean±standard deviation (s.d.)) compared to any other

treatment group (all p<0.05). Neonicotinoid only workers (N+/V-) lived significantly longer (8.7±3.0 days, mean±s.d.) than *V. destructor* only workers (N-/V+, 7.3±4.0 days, mean±s.d., p<0.001), and the combination of both stressors (N+/V+, 7.2±3.9 days, mean±s.d., p<0.001). There was no difference in mean longevity between the two *V. destructor* infested treatment groups (N-/V+ and N+/V+, p>0.05) (Table 9). The combined stressors reduced worker longevity by 23% compared to controls, which was smaller than the sum of the individual stressors – a 8% and 23% reduction for neonicotinoid only and *V. destructor* only workers, respectively, compared to controls. This suggests an antagonistic interaction between the two stressors.

Similarly, survival until the end of the assay was greatest in control workers (N-/V-, 88±0.02%, cumulative survival (CS)%±standard error (s.e.)) compared to the other treatment groups (all p≤0.01). Survival of neonicotinoid only workers (N+/V-, 77±0.03%, CS%±s.e.,) was greater than workers from the treatment groups *V. destructor* only (N-/V+, 63±0.04% CS%±s.e., p=0.02) and both stressors (N+/V+, 58±0.05%, CS%±s.e., p<0.01) (Figure 8). There was no difference in survival between treatment groups infested with *V. destructor* (p=0.7).

Table 8: Generalized linear mixed effect model summary for honey bee (*Apis mellifera*) worker longevity [days]. Potential effects on worker longevity in cages were assessed for two explanatory variables: 1) Treatment (control versus neonicotinoid (N)) and 2) *Varroa destructor* infestation (no versus yes (V)). Neonicotinoids included in the experiment were thiamethoxam and clothianidin. *V. destructor* infestation was represented by artificial infestation of mites to brood cells. Estimates of each variable represent the (negative) effect on longevity.

Model: glmer(Longevity~Treatment+Varroa+(1|Worker_ID), family=poisson)				
Variable	**Estimate**	**Std. Error**	**z-value**	**p-value**
TreatmentN	-0.03	0.03	-0.63	0.008**
VarroaV	-0.21	0.05	-3.48	<0.0001***

Table 9: **Summary table for honey bee (*Apis mellifera*) worker longevity [days] per treatment group.** Treatment groups consisted of workers reared in either control (N-) or neonicotinoid (N+) colonies, of which half each were artificially infested with *Varroa destructor* mites (present, V+) and half were not (absent, V-). Upon emergence, workers were maintained in hoarding cages based on treatment group assignment. Dead workers were removed daily until the end of the assay, 10 days post emergence. Acronym definitions: Sample size (N), Standard Deviation (Std. Dev.), Minimum (Min.), Perc. (Percentiles) and Maximum (Max). Different significance letters indicate significant differences between treatment groups (p<0.05).

Variable	Treatment	Code	N	Mean	St. Dev	Min.	0.05 Perc.	0.25 Perc.	Median	0.75 Perc	0.95 Perc.	Max.	Significance letter
Longevity [days]	Control - *V. destructor* absent	N-/V-	332	9.39	2.06	0	3	10	10	10	10	10	a
	Neonicotinoid – *V. destructor* absent	N+/V-	266	8.65	2.95	0	0	10	10	10	10	10	b
	Control – *V. destructor* present	N-/V+	123	7.27	4.03	0	0	3.5	10	10	10	10	c
	Neonicotinoid – *V. destructor* present	N+/V+	112	7.23	3.87	0	0	3	10	10	10	10	c

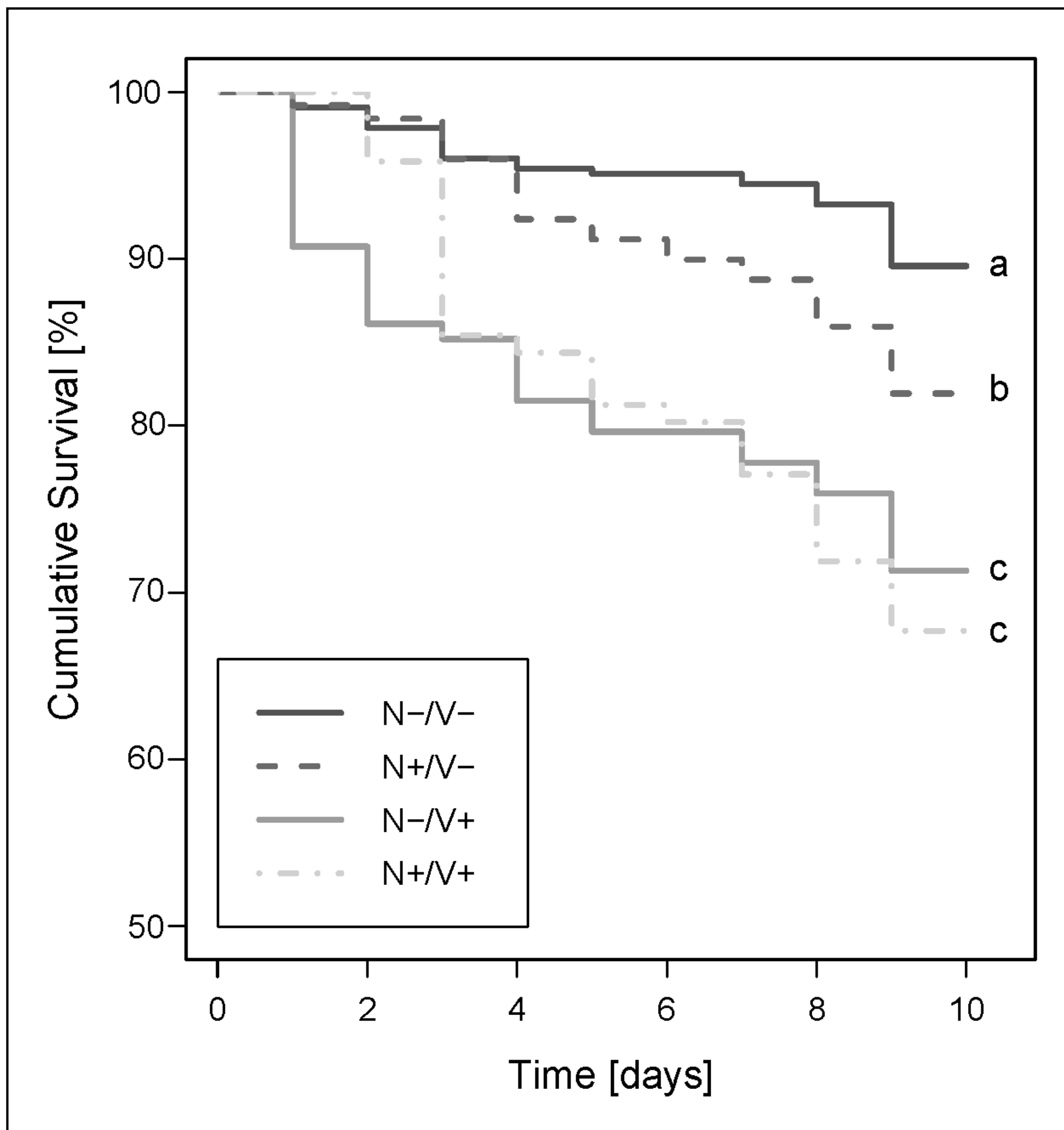

Figure 8: Cage survival for experimental honey bee (*Apis mellifera*) workers post emergence. Survival curves represent cumulative survival [%] of workers. Survival of workers reared in the absence of neonicotinoid exposure and *Varroa destructor* infestation (control, N-/V-, N= 326 workers) was higher compared to any other treatment group ($p<0.05$), followed by workers reared under neonicotinoid exposure only (N+/V-, neonicotinoid only, N=249 workers) Workers from control colonies that were infested with *V. destructor* (*V. destructor* only, N-/V+, N=108 workers) and workers exposed to a combination of neonicotinoids and *V. destructor* (combined stressors, N+/V+, N=96 workers) showed the lowest cumulative survival ($p<0.0001$). Different letters indicate statistically significant differences ($p \leq 0.05$).

Hypopharyngeal gland size

Both neonicotinoid exposure and *V. destructor* infestation had significant negative effects on hypopharyngeal gland (HPG) acini width (p=0.04 and p<0.0001, respectively) (Table 10). Control worker HPG acini width (P-/V-, 75.8±21.0 µm, mean±standard deviation (s.d.)) was larger compared to neonicotinoid only workers (N+/V-, 68.3±20.8µm, mean±s.d.), workers exposed to both stressors (N+/V+, 59.1±16.3 µm, mean±s.d.), and *V. destructor* only workers (N-/V+, 56.2±12.4µm, mean±s.d.) (all p<0.0001). HPG acini width of the two treatment groups infested with *V. destructor* (N-/V+ and N+/V+) did not differ from each other (p=0.14), but were smaller compared to neonicotinoid only workers (N+/V-, p<0.001) (Figure 9, Table 11). The combined stressors reduced HPG acini width by 32% compared to controls, which was smaller than the sum of individual stressor effects – a 13% and 26% acini width reduction for neonicotinoid only and *V. destructor* only workers, respectively compared to controls. Therefore, this suggests an antagonistic interaction between the two stressors.

Table 10: Linear mixed effect model summary for potential stressor effects on honey bee (*Apis mellifera*) hypopharyngeal gland (HPG) acini width. Acini width [µm] was used as a proxy for HPGs size. Two explanatory variables were assessed for potential effects: 1) Treatment (control versus pesticide (N)) and 2) *Varroa destructor* infestation (V).Estimates of each variable represent the (negative) effect on acini width.

Model: lme(Acini_Width~Treatment+Varroa, random=~1|Colony/Worker_ID)				
Variable	**Estimate**	**Std. Error**	**t-value**	**p-value**
TreatmentN	-9.26	4.02	-2.3	0.035*
VarroaV	-19.18	4.09	-4.69	<0.0001***

Table 11: Summary table of honey bee (*Apis mellifera*) hypopharyngeal gland (HPG) acini width per treatment group. Acini width [µm] was used as proxy for hypopharyngeal gland size. Treatment groups consisted of workers reared in either control (N-) or pesticide (N+) colonies of which half were artificially infested with *Varroa destructor* mites (present, V+) and half were not (absent, V-). Acronym definitions: Sample size (N), Standard Deviation (Std. Dev.), Minimum (Min.), Perc. (Percentiles) and Maximum (Max). Different significance letters indicate significant differences between treatment groups ($p<0.05$).

Variable	Treatment	Code	N	Mean	Std. Dev.	Min.	0.05 Perc.	0.25 Perc.	Median	0.75 Perc.	0.95 Perc.	Max	Significance letter
Acini width [µm]	Control - *V.destructor* absent	N-/V-	1140	75.83	20.95	25.14	43.84	61.09	73.58	88.9	112.41	165.86	a
	Pesticide - *V.destructor* absent	N+/V-	760	68.3	20.76	21.76	38.8	53.09	65.75	80.02	109.14	148.24	b
	Control - *V. destructor* present	N-/V+	460	56.24	12.41	26.05	36.13	47.14	55.8	64.57	105.32	277.23	c
	Pesticide - *V.destructor* present	N+/V+	500	59.11	16.29	22.36	35.19	47.87	58.3	67.97	90.4	107.99	c

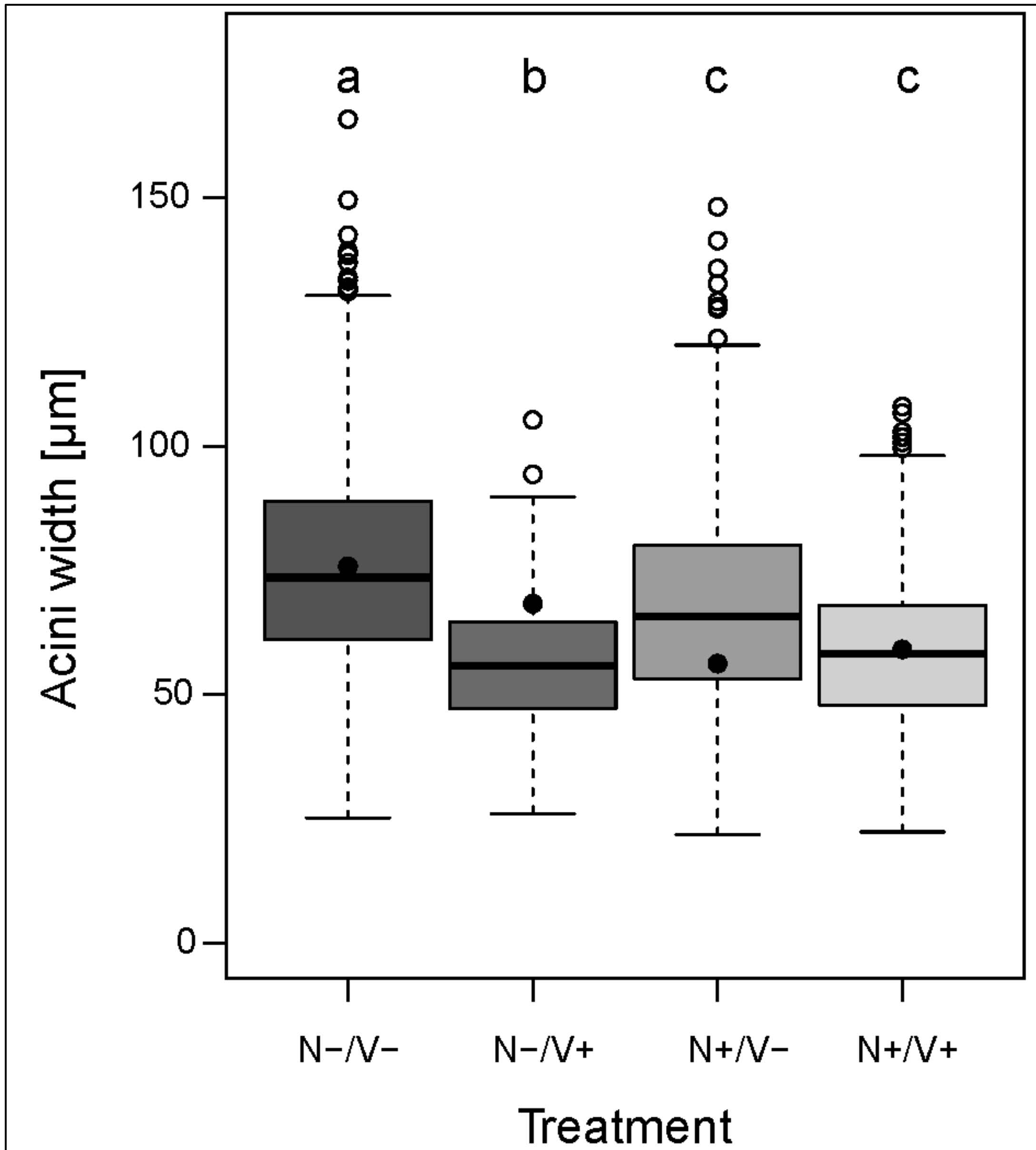

Figure 9: Differences in experimental worker honey bee (*Apis mellifera*) hypopharyngeal gland (HPG) acini width among treatment groups. Compared to any other treatment group, HPG acini width was largest in workers reared in the absence of neonicotinoid exposure and *Varroa destructor* infestation (control, N-/V-, N=1140 acini) ($p<0.0001$), followed by *V. destructor* absent workers exposed to neonicotinoids (N+/V-, N=760 acini). The smallest HPG acini widths were found in workers reared in both control (N-/V+, N=460 acini) and neonicotinoid (N+/V+, N=500 acini) colonies that were infested with *V. destructor* mites, regardless of neonicotinoid exposure ($p=0.12$). Boxplots show the inter-quartile range (box), the median (black line within box), data range (vertical black lines from box), means (black dots) and outliers (open circles). Different letters above boxplots indicate statistically significant differences ($p \leq 0.05$).

Discussion

Honey bees (*Apis mellifera* Linnaeus) are frequently and concurrently exposed to multiple stressors that might affect colony functioning (Alaux et al. 2010, Burgher-MacLellan et al. 2010, Shutler et al. 2014, Colwell et al. 2017). Yet relatively little knowledge exists about how multiple exposure scenario may affect honey bees, especially concerning important known stressors like insecticides and parasitic mites. Given the high colony losses experienced across the northern hemisphere (Lee et al. 2015b, Kulhanek et al. 2017, Brodschneider et al. 2018, Bruckner et al. 2019, Gray et al. 2019), this is urgently needed. For the first time, we assessed the potential lethal and sub-lethal effects of neonicotinoids and *Varroa destructor* Anderson and Trueman, alone and in combination, on worker honey bees by assessing survival and hypopharyngeal glands (HPGs), respectively. Under our experimental conditions, our results suggest that an antagonistic interaction can occur between neonicotinoids and *V. destructor* for both lethal and sub-lethal effects on honey bees. Furthermore, our results suggest that *V. destructor* has stronger negative lethal and sub-lethal effects on worker honey bees than neonicotinoid insecticides.

Recent efforts have highlighted the effects of concurrent multiple stressors on honey bees, including the possibility of interactions, whereby combined observed effects do not equal the sum of all individual stressor effects (Maher et al. 2019). Contrary to recently published data produced from work that employed a similar experimental design (Straub et al. 2019), we observed an antagonistic effect of neonicotinoids and *V. destructor* on worker survival. We also observed a similar antagonistic effect on worker HPG size, which further suggests that an antagonistic interaction can occur between neonicotinoids and *V. destructor* under certain field-relevant conditions. This antagonistic interaction can possibly be explained by the unique

physiological pathways that are affected by the two stressors. For example, honey bee larvae exposed to neonicotinoids increased transcription of defense genes against parasites and pathogens (Gregorc et al. 2012a). Given that neonicotinoids have been shown to interact synergistically with other biotic stressors under different experimental designs, such as the fungus *Nosema ceranae* (Aufauvre et al. 2012) and even *V. destructor* (Blanken et al. 2015, Morfin et al. 2020), we expected to observe a similar stressor interaction. Reasons for disparity among studies can possibly be explained by route and timing of exposure, as well as measured response variables. For example, some experiments administered neonicotinoids via sugar syrup and not pollen. For adult bees, this might greatly influence insecticide exposure, as exposure via sugar syrup ingestion can result in increased uptake by several magnitudes (Azpiazu et al. 2019). This likely increases the concentration of neonicotinoids delivered to developing larvae via brood food, and could possibly alter potential stressor interactions (Wittmann and D 1982, Davis and Shuel 1988). Furthermore, we exposed developing individuals to both neonicotinoids and *V. destructor*, whereas others like Morfin et al. (2020) exposed newly emerged adults to both stressors. It is well known that honey bees exhibit age-related susceptibility to many stressors, including insecticides (Smirle and Winston 2011). While Straub et al. (2019) assessed effects on worker survival using a similar experimental design as in our study, it was performed on a different continent at a different time of the year and using a different type of western honey bee. Differences in susceptibility to insecticides and *V. destructor* can occur among types of honey bees (Martin and Medina-Medina 2004, Laurino et al. 2013, Rinkevich et al. 2015). This difference can also be driven by seasonal timing of experiments (Straub et al. 2019). Additionally, we focused on effects of neonicotinoids and *V. destructor* on an anatomical structure −HPGs − which are important during early worker adulthood (Deseyn and Billen

2005); factors influencing HPG development are likely to be different than those responsible for gene expression of neural related genes or flight activity, which were measured by Blanken et al. (2015) and Morfin et al. (2020).

Similar to previous studies (Schneider and Drescher 1987, Bowen-Walker and Gunn 2001, Hatjina et al. 2013, Retschnig et al. 2015), we found that neonicotinoids and *V. destructor* negatively affected worker honey bee survival and HPG size, even during single exposure scenarios. Our survival results conform to the general knowledge that *V. destructor* is a devastating parasite of honey bees as infestation results in depressed immunity and physical deformities (Schneider and Drescher 1987, Yang and Cox-Foster 2005, Jong et al. 2015, Di Prisco et al. 2016). Together, these effects are expected to increase susceptibility of honey bees to other stressors (Martin 2001). Our HPG results for exposure to neonicotinoids or *V. destructor* alone revealed negative effects of the two stressors on workers, and potentially the entire colony. Since HPG size is correlated with gland activity (Knecht and Kaatz 1990, Crailsheim and Hrassnigg 1998), nursing workers with small glands likely produce deficient brood food and sterilizing enzymes (Kubo et al. 1996), and may even shift from protein synthesis to carbohydrate metabolism (Simpson et al. 1968, Ohashi et al. 1999). This could result in inadequately fed brood that could subsequently affect their adult performance (Cruz-Landim and Hadek 1969, Knecht and Kaatz 1990, Hatjina et al. 2013). Additionally, reduction in HPG size could lead to precocious foraging behavior, thereby limiting the number of nurses in the colony (Jaycox et al. 1974). Ultimately, this could impair social immunity, brood rearing capacity, and possibly jeopardize colony survival (Brodschneider and Crailsheim 2010, Khoury et al. 2011).

Interestingly, our results suggest that *V. destructor* has a more pronounced effect than neonicotinoids on both survival and HPGs of worker honey bees. Routes and timing of exposure,

as well as affected physiological pathways, are known to influence the effects of a particular stressor (Holmstrup et al. 2010, Morfin et al. 2020). For example, *V. destructor* feeds on the fat body of developing workers during the pupal stage (Rosenkranz et al. 2010, Ramsey et al. 2019), a vital time when HPG development takes place (Klose et al. 2017). The fat body is the primary site of nutrient storage of lipids and proteins, which are used by developing workers during morphogenesis of HPGs and final metamorphosis into an adult (Arrese and Soulages 2010, Martins et al. 2010, Omar et al. 2017). Furthermore, the fat body plays an important role for the immune system of workers (Arrese and Soulages 2010). Therefore, direct feeding on this important organ, as well as the timing of feeding and affected physiological pathways, likely contribute to the observed negative effects of *V. destructor* on workers.

Developing workers were indirectly exposed to neonicotinoids during the larval stage by nurses that previously ingested contaminated food resources (Zhu et al. 2014a). The concentration of neonicotinoids can decrease the time between the pollen is collected from flowers to when it is fed to brood (Böhme et al. 2018), suggesting that possible negative effects on worker development could be reduced. Early stage honey bee larvae also contain fewer receptors for neonicotinoids compared to pupal and adult stages (Yang et al. 2012), therefore potentially diminishing observed effects. This may compensate for the reduced quantity of detoxification enzymes in individual honey bees compared to non-social insects, as well as the inability of larvae to avoid exposure due to their brood cell confinement (Berenbaum and Johnson 2015). However, honey bee larvae are known to upregulate immune genes involved in energy and xenobiotic metabolism when exposed to low levels of neonicotinoids (Derecka et al. 2013, Morfin et al. 2020). This could affect resource allocation during development, and perhaps even adult survival (Derecka et al. 2013).

In summary, our results confirm that both neonicotinoid exposure and *V. destructor* infestation during development can induce lethal and sub-lethal effects on nursing worker honey bees; however, an antagonistic interaction between these two stressors during simultaneous exposure is possible. Antagonistic stressor interactions are rarely documented in ecological literature (Holmstrup et al. 2010), possibly because synergistic interactions are considered more severe given their potential to exacerbate negative effects on organisms (Maher et al. 2019). Regardless, our results suggest that antagonistic stressor interactions between neonicotinoids and *V. destructor* can elicit considerable negative effects on worker honey bees. Therefore, the directionality of interactions outcomes should not be overlooked when investigating concurrent stressors. More knowledge is needed regarding how interaction effects observed at the individual bee level translate into potential colony level effects, especially given that the latter is the fundamental biological unit for the honey bee.

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Chapter 4

Effects of *Varroa destructor* mites and neonicotinoid insecticides on *Apis mellifera* honey bee drone survival and sperm quality

Abstract

Honey bee (*Apis mellifera* Linnaeus) colony losses are caused by biotic and abiotic stressors acting singly or in combination. The most important biotic threat to the beekeeping industry is the parasitic mite *Varroa destructor* Anderson and Trueman. In addition, neonicotinoid insecticides used widely for pest management represent important abiotic stressors. Honey bees may be simultaneously exposed to both *V. destructor* and neonicotinoids, their potential interaction effects are severely understudied though, especially with respect to reproductive castes. A fully crossed experimental design was used to assess the effects of simultaneous neonicotinoid exposure and *V. destructor* infestation on drones. Known age cohorts were obtained from 10 colonies − half the colonies received patties containing field-relevant concentrations of two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin), whereas the other half received patties without. Drones from each colony were artificially emerged, assessed for *V. destructor* infestation, and allocated to appropriate treatment groups of no neonicotinoid with and without *V. destructor* added, and dietary neonicotinoid with and without *V. destructor* added. Drones were kept in laboratory cages until sexual maturity, and then assessed for sperm quality traits. Individually, neonicotinoids and *V. destructor* can significantly reduce drone survival, and there was evidence for a synergistic interaction between the two stressors under simultaneous exposure. Contrary to our expectations, neonicotinoids and *V. destructor*, alone or in combination, had no effect on drone sperm quality traits. Nonetheless,

a reduction in drone survival at time of maturity could severely affect honey bee populations since drones are crucial to colony health through their contribution to mating.

Introduction

Honey bees (*Apis mellifera* Linnaeus) are a eusocial species that exhibits haplodiploid sex determination where unfertilized eggs develop into haploid males called drones and fertilized eggs develop into diploid females (worker or queen), depending on her caste allocation (Hamilton 1964a, Herrick and Seger 1999, Goudie and Oldroyd 2018). The haploid susceptibility hypothesis suggests that drones, due to a lack in allelic variation, might exhibit reduced resilience to environmental factors like parasitism and toxins (Hamilton 1964a, O'Donnell and Beshers 2004, Retschnig et al. 2014, Friedli et al. 2020). This is problematic as drones contribute indirectly to colony fitness by inseminating future queens of other colonies (Rangel and Fisher 2019). Polyandrous queens acquire sperm from an average of 12 drones (Winston 1991) and sperm collected by queens during the first few days of adulthood serve as a life-long supply to fertilize eggs that subsequently develop into female offspring (Tarpy and Nielsen 2002, Koeniger and Koeniger 2007). Therefore, the availability of high quality drones within the flight range of a young queen is crucial for her colony (Koeniger and Koeniger 2007). Indeed, colony performance is significantly improved when headed by queens inseminated by many drones (Tarpy 2003, Delaplane et al. 2015). This may be explained by higher genetic variation within the colony, which confers benefits such as more specialized division of labor and increased resilience to biotic stressors (Page et al. 1995, Beshers and Fewell 2001, Delaplane et al. 2015).

Various biotic and abiotic stressors, acting in concert rather than individually, have been suggested to cause these elevated colony losses (Steinhauer et al. 2018). Proposed biotic stressors include poor habitat, bacteria, viruses, and parasites, whereas heavy metals, fungicides, and insecticides are considered important abiotic stressors (Mao et al. 2009, Genersch 2010, Johnson et al. 2013, McMenamin and Genersch 2015, Sgolastra et al. 2018, Traynor et al. 2020).

It is currently believed that many honey bee colony losses can be attributed to interactions among concurrent stressors (Potts et al. 2010).

The ectoparasitic mite *Varroa destructor* Anderson and Trueman is considered to be the most important biotic threat to honey bees (Rosenkranz et al. 2010, Le Conte et al. 2010, Guzmán-Novoa et al. 2010). *Apis mellifera* honey bees lack effective defense mechanisms and experience parasitism of both worker and drone brood (Rosenkranz et al. 2010). However, mature female *V. destructor* mites prefer drone brood over worker brood (Fuchs 1992, Rosenkranz et al. 2010). Infestations of *V. destructor* negatively affects drone mating efficiency through increased mortality, reduced flight activity, and low sperm quality (Collins and Pettis 2001, Bubalo et al. 2005, Straub et al. 2019).

Neonicotinoids are a class of systemic insecticides that are among the most widely applied insecticides in the world (Simon-Delso et al. 2015). Therefore, neonicotinoids can pose a risk to non-target organisms such as honey bees that forage on contaminated food resources (Bonmatin et al. 2007). The risk of exposure extends from the individual forager to the entire colony when contaminated resources are shared with other adults and developing individuals (Sanchez-Bayo and Goka 2014). Field-relevant concentrations of neonicotinoids can have sub-lethal effects on honey bees, eliciting behavioral, physiological, and anatomical changes (Lundin et al. 2015, Williams et al. 2015). For example, nursing workers have smaller food glands, queens store less sperm, and drones produce low quality sperm after neonicotinoid exposure which can ultimately affect colony reproductive potential and overall performance (Hatjina et al. 2013, Williams et al. 2015, Straub et al. 2016).

Simultaneous exposure of honey bees to *V. destructor* and neonicotinoids is likely as both are relatively ubiquitous stressors in the environment (Little et al. 2015, Wilfert et al. 2016, Colwell

et al. 2017, Mitchell et al. 2017). Despite this, little is known about the potential interaction effects of these stressors. The available studies on worker bees have yielded conflicting results, ranging from no interaction to synergism (Straub et al. 2016, Siede et al. 2018, Morfin et al. 2020). No studies thus far have examined drones, and only a few studies have looked at possible negative effects of *V. destructor* and neonicotinoids individually on drone health (Bubalo et al. 2005, Straub et al. 2016). Given the importance of drones to colony performance and overall population health, investigations into the effects of important honey bee stressors on them is urgently needed.

Therefore, we assessed for the first time the effects of simultaneous exposure to neonicotinoid insecticides and the *V. destructor* mite on honey bee drone survival and sperm quality. Based on previous investigations using workers (Straub et al. 2016, Morfin et al. 2020), and considering the haploid susceptibility hypothesis, we expected that both stressors when presented alone would have strong negative effects on drones. Furthermore, we expected that *V. destructor* parasitized drones could not buffer against other environmental stressors such as exposure to neonicotinoids (O'Donnell and Beshers 2004, Blackmon et al. 2015, Maher et al. 2019). Therefore, we expected to observe a synergistic negative effect on drones during simultaneous exposure of both studied stressors.

Methods

On 18 March 2020, ten honey bee (*Apis mellifera ligustica*) packages (Rossman Apiaries LLC. Moultrie, GA, USA) , each headed by a laying sister queen and 1.5 kg workers, were

installed in Auburn AL, USA. Two weeks later, they were randomly assigned to either a control or neonicotinoid treatment.

Neonicotinoid Exposure

Treatments were administered *ad libitum* via pollen patties (60% corbicular pollen, 30% powdered sugar, 10% organic honey) following an established protocol (Sandrock et al. 2014a, Williams et al. 2015, Straub et al. 2016). Honey bee colonies residing in a low intensity agricultural region of Colorado sourced the corbicular pollen; subsequent analysis detected no traceable levels of agricultural chemicals (Appendix 2, Figure 20). As in previous experiments (Pilling et al. 2013, Williams et al. 2015, Straub et al. 2016), colonies allocated to the neonicotinoid treatment (N+) received pollen patties spiked with field-relevant concentrations of two neonicotinoids, thiamethoxam and clothianidin (4.5 ppb and 1.5 ppb, both Sigma Aldrich). Control treatments (N-) colonies were fed non-spiked pollen patties (Appendix 3). Prior to feeding, each colony was equipped with a Sundance pollen trap (Rossman Apiaries, LLC., Moultrie, GA, USA) to promote in-hive patty consumption and prevent influx of local pollen (Sandrock et al. 2014a, Williams et al. 2015). Following a previously employed feeding regime (Forfert et al. 2015, Williams et al. 2015, Straub et al. 2019), pollen patties were provided for 49 days to cover two entire brood cycles, and to mimic a realistic exposure period encountered by foraging honey bees (Goulson 2013).

Source of experimental drones and *Varroa destructor* mite infestation

Forty-two days post initial feeding, the queen of each colony was caged on a drone brood frame and left for 48 hours to obtain known age cohorts of drones (Williams et al. 2013). Approximately 21 days later, when drones were expected to emerge (Winston 1991), we

artificially emerged drones from capped brood cells on each previously caged frame to assess them for *V. destructor* status; drones were subsequently allocated to either "*V. destructor* absent" (V-) or "*V. destructor* present" (V+) treatments. Individuals were added to a laboratory hoarding cage (Williams et al. 2013), then grouped according to one of four treatment groups: 1) control colony and *V. destructor* absent (control, N-/V-), 2) control colony and *V. destructor* present (*V. destructor* only, N-/V+), 3) neonicotinoid colony and *V. destructor* absent (neonicotinoid only, N+/V-), and 4) neonicotinoid colony and *V. destructor* present (both stressors, N+/V+). Each cage confined up to 10 drones, as well as up to 20 workers, from the same colony; the latter were included to provide caretaking duties (Ruttner 1966, Straub et al. 2016).

Longevity and sperm quality

Each hoarding cage was kept in an incubator (30°C and 60% RH, DR-41NL, Percival Scientific, Inc., Perry, IW) and equipped with a syringe containing sucrose solution (50% w/v) to feed workers, an in-cage feeder containing sucrose solution (50% w/v) to promote autonomous drone feeding (Mindt 1960, Williams et al. 2013), and a pollen feeder (60% corbicular pollen obtained from Colorado, 40% powdered sugar) to promote proper development and maturation of male reproductive organs (Mindt 1960, Hrassnigg and Crailsheim 2005). All food resources were provided *ad libitum*.

Dead drones were removed from each cage daily, until 14 days post emergence when all surviving individuals were expected to be sexual mature (Rhodes et al. 2011). These individuals were sacrificed for subsequent sperm quality assessments. To prevent sperm migration from seminal vesicles to the bulb, reproductive organs were dissected from living drones (Mazeed and Mohanny 2010). In brief, each abdomen was detached using dissection scissors, then pinned onto a wax plate before removing ventral sternites so that the testes, mucus glands, and seminal

vesicles could be removed using forceps. For each individual, all structures were then placed in a 1.5 ml Eppendorf tube containing 500 µl Kiev buffer and crushed to make a diluted Sperm Stock Solution (SSS) (Carreck et al. 2013).

Sperm quality traits were assessed following Straub et al. (2016). To assess sperm viability, immediately following dissection 50 µl SSS was transferred to a new 1.5 ml Eppendorf tube containing 50 µl Kiev buffer. For this, we added 2 µl Propidium Iodide (PI) solution (1 mg ml^{-1}) and 2 µl Hoechst 33342 (0.5 mg ml^{-}1) (both Sigma-Aldrich) to each sample before incubating the tubes for 20 min at room temperature in complete darkness. The suspension was then gently mixed before 10 µl of the solution was mounted on a microscope slide, covered with a glass slip, and then examined using a fluorescent microscope (400x magnification, Leica, DM2500 LED, Morrisville, NC, USA) outfitted with filter cubes for UV excitation. The quantity of living and dead sperm was counted in 10 arbitrarily chosen visual fields from which the average viability was calculated. To calculate total sperm quantity, 20 µl SSS were transferred to another 1.5 ml Eppendorf tube containing 80 µl Kiev buffer (1:5 dilution). Then, 10 µl of the diluted solution was transferred into a Neubauer counting chamber so that sperm could be quantified under light microscopy (Leica, DM2500 LED). Total sperm quantity (in 500 µl SSS) was determined by multiplying the average number of sperm counted in two Neubauer counting chambers by the dilution factor (1:5) by the volume used for Neubauer counting chamber (10 µl) by the SSS volume (500 µl) (Straub et al. 2016). Lastly, living sperm quantity was calculated by multiplying sperm viability by total sperm quantity.

Statistics

All statistical analyses were performed in R (version 4.0.2., 11/2/20) using a significance level of α=0.05. Survival curves (Kaplan-Meier plots) were produced using the "survival" package, and the "survminer" package allowed for pairwise comparisons between treatment group specific cumulative survival with a Bonferroni correction.

Since drone longevity data were not normally distributed, a generalized linear mixed effect model (glm) was fitted to assess effects of neonicotinoid exposure and *V. destructor* infestation (i.e. fixed factors). Likewise, total sperm quantity and living sperm quantity were not normally distributed; fixed factor effects were analyzed by fitting a glm model that included cage identification number as random factor to account for potential clustering effects. Multiple pairwise comparisons between treatment groups (N-/V-, N-/V+, N+/V-, and N+/V+) were performed using the "emmeans" package and a Bonferroni correction for multiple testing.

In contrast, a linear mixed effect model (lm) was fitted to assess effects of fixed factor on sperm viability data [%] , cage identification number was included as a random factor to account for potential clustering effects (Table 5). The "emmeans" package and a Bonferroni correction for multiple testing was used to perform multiple pairwise comparisons between treatment groups.

To identify potential interactions between neonicotinoid exposure and *V. destructor* infestation, an additive effects framework was employed (Folt et al. 1999). Interactions were considered synergistic or antagonistic if the effect of the combined stressor treatment group (N+/V+) was greater or smaller than the sum resulting from individual stressors (N-/V+ and N+/V-) (Hay 1996). To assess this, the percent difference in treatment groups compared to the

controls (N-/V-) were calculated using mean survival [d], total sperm quantity [#], living sperm quantity [#], and sperm viability [%].

Results

Longevity

Both neonicotinoid exposure and *Varroa destructor* infestation significantly reduced drone longevity (p=0.006 and p=0.05, respectively) (Table 12). Longevity of control drones was greatest (N-/V-, 10.0±5.4 days, mean±standard deviation (s.d.), N=247) compared to any other treatment group (all p≤0.001). Longevity of drone exposed only to neonicotinoids (N+/V-, 8.2±5.6 days, mean±s.d., N=367) was not different from drones exposed only to *V. destructor* (N-/V+, 8.7±5.1 days, mean±s.d., N=91) (p>0.05). Longevity of drones exposed to both stressors was the shortest (N+/V+, 5.1±5.6 days, mean±s.d., N=85) (all p<0.001) (Table 13). Longevity of drones exposed to both stressors (N+/V+) was reduced by 48% compared to controls. This was greater than the sum of individual stressor effects – 18% and 16% reduction in longevity when compared to controls for neonicotinoid only (N+/V-) and *V. destructor* only (N-/V+) drones, respectively. This suggests a synergistic interaction between the two stressors.

Similarly, survival until the end of the assay was greatest in control drones (N-/V-, 44.5±0.03, cumulative survival (CS)%±standard error (s.e.)) compared to the other treatment groups (all p≤0.01). Survival of neonicotinoid only workers (N+/V-, 37.1±0.03%, CS%±s.e.,) was not different from *V. destructor* only workers (N-/V+, 31.9±0.05% CS%±s.e.) (p=0.97). Survival was lowest for drones exposed to both stressors (N+/V+, 20±0.04%, CS%±s.e.) compared to any other treatment group (all p<0.001) (Figure 10).

Table 12: Generalized linear mixed effect model summary for honey bee (Apis mellifera) drone longevity [days]. Potential effects on drone longevity kept in laboratory cages were assessed for two explanatory variables: 1) Treatment (control versus neonicotinoid (N)) and 2) *Varroa destructor* infestation (no versus yes (V)). Neonicotinoids included in the experiment were thiamethoxam and clothianidin. Estimates of each variable represent the effect on longevity.

Model: glmer(Longevity~Treatment+Varroa+(1|Cage_ID), family=Gamma)				
Variable	**Estimate**	**Std. Error**	**t-value**	**p-value**
TreatmentN	0.08	0.03	2.76	0.006**
VarroaV	0.07	0.04	1.96	0.05*

Table 13: Summary table for honey bee (*Apis mellifera*) drone longevity [days] per treatment group. Treatment groups consisted of drones reared in either control (N-) or neonicotinoid (N+) colonies; from each of these colonies, we obtained drones that were either infested with *Varroa destructor* mites (present, V+) or not (absent, V-). Upon emergence, drones were maintained in hoarding cages based on treatment group assignment. Daily drone mortality was recorded until the end of the assay, 14 days post emergence. Acronym definitions: Sample size (N), Standard Deviation (Std. Dev.), Minimum (Min.), Perc. (Percentiles) and Maximum (Max). Different letters indicate significant differences between treatment groups ($p<0.05$).

Variable	Treatment	Code	N	Mean	St. Dev	Min.	0.05 Perc.	0.25 Perc.	Median	0.75 Perc	0.95 Perc.	Max.	Significance letter
Longevity [days]	Control - *V. destructor* absent	N-/V-	247	9.97	5.39	1	2	5	13	14	14	14	a
	Neonicotinoid – *V. destructor* absent	N+/V-	367	8.19	5.62	1	1.25	2	9	14	14	14	b
	Control – *V. destructor* present	N-/V+	91	8.73	5.09	1	2	5	8	14	14	14	b
	Neonicotinoid – *V. destructor* present	N+/V+	85	5.14	5.57	1	1	1	2	11	13.25	14	c

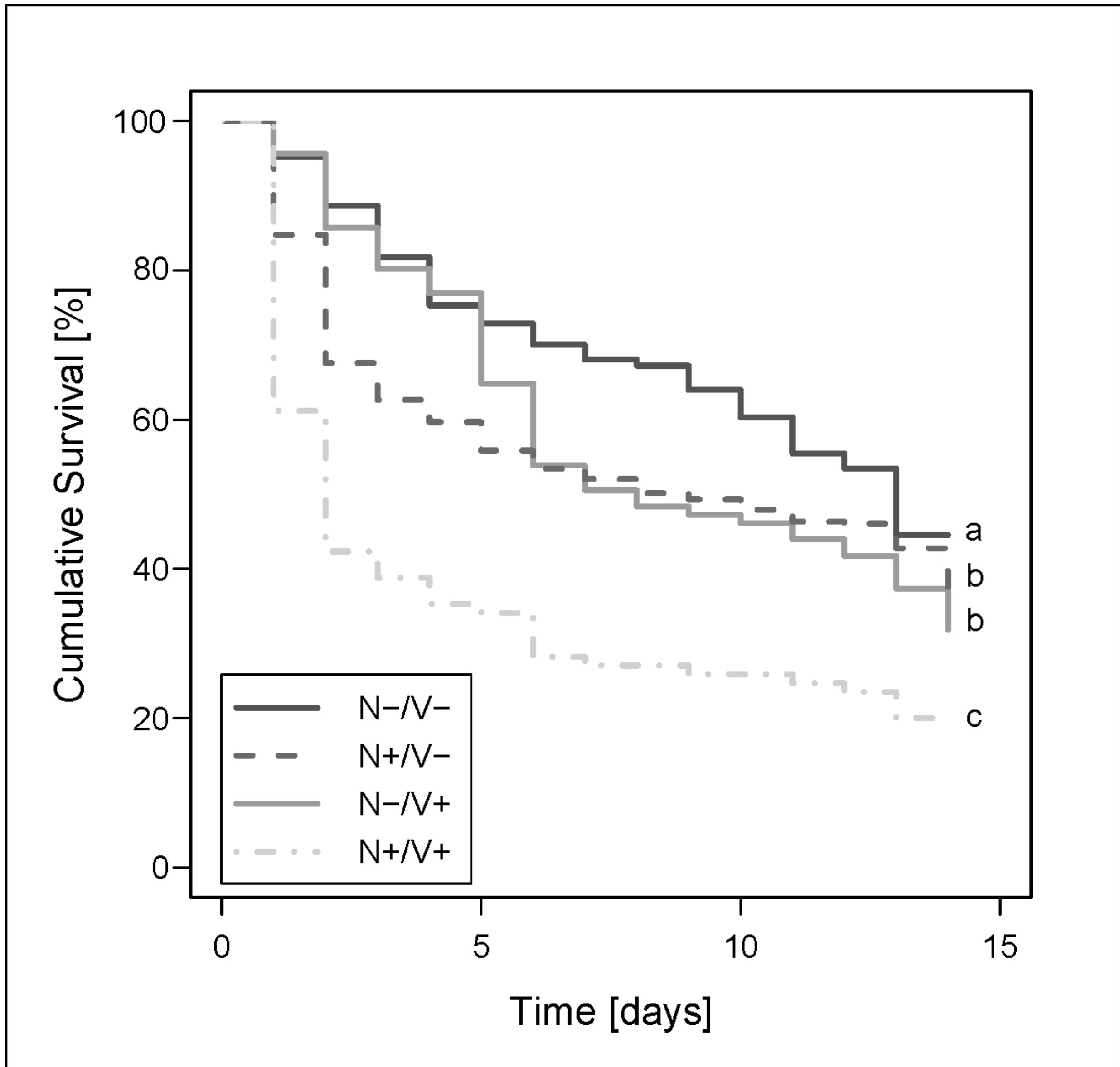

Figure 10: Cage survival for experimental honey bee (*Apis mellifera*) drones post emergence. Survival curves represent cumulative survival [%] of drones. Survival of drones reared in the absence of neonicotinoid exposure and *Varroa destructor* infestation (control, N-/V-, N=247) was higher compared to any other treatment group (p<0.05). There was no difference in survival between drones only infested with *V. destructor* (*V. destructor* only, N-/V+, N=91) and drones only exposed to neonicotinoids (neonicotinoid only, N+/V-, N=367) (p=0.97). The lowest survival was observed in drones exposed to both neonicotinoids and *V. destructor* (both stressors, N+/V+, N=85) compared to any other treatment group (all p<0.001). Different letters indicate statistically significant differences (p<0.05).

Sperm quality

Exposure to neonicotinoids or *V. destructor* infestation did not have a significant effect on any sperm quality trait – sperm viability [%], total number of sperm [#] and total living sperm quantity [#], respectively (all p>0.05, Table 14, Table 15 and Table 16). There was no difference between treatment groups for any measured sperm quality trait (all p>0.05, Figure 11).

Table 14: Generalized linear mixed effect model summary for potential stressor effects on total sperm quantity for honey bee (*Apis mellifera*) drones. Two explanatory variables were assessed for potential effects on total sperm quantity [# million]: 1) Treatment (control versus pesticide (N)) and 2) *Varroa destructor* infestation (V). Cage identification number was included as a random effect. The data were analyzed using a negative binomial regression. Estimates of each variable represent the effect on total sperm quantity.

Model: glmer(TotalSpermQuantity~Treatment+Varroa + (1|Cage), family = neg. bin.				
Variable	**Estimate**	**Std. Error**	**z-value**	**p-value**
TreatmentN	0.09	0.24	0.38	0.71
VarroaV	0.13	0.40	0.33	0.75

Table 15: Linear mixed effect model summary for potential stressor effects on sperm viability for honey bee (*Apis mellifera*) drones. Two explanatory variables were assessed for potential effects on sperm viability [%]: 1) Treatment (control versus pesticide (N)) and 2) *Varroa destructor* infestation (V). Cage identification number was included as random effect. Estimates of each variable represent the effect on sperm viability.

Model: lme(SpermViability~Treatment+Varroa, random=~1|Cage)				
Variable	**Estimate**	**Std. Error**	**t-value**	**p-value**
TreatmentN	-4.67	2.63	-1.77	0.08
VarroaV	-0.56	4.61	-0.12	0.90

Table 16: Generalized linear mixed effect model summary for potential stressor effects on living sperm quantity for honey bee (*Apis mellifera*) drones. Two explanatory variables were assessed for potential effects on living sperm quantity [# million]: 1) Treatment (control versus pesticide (N)) and 2) *Varroa destructor* infestation (V). Cage identification number was included as random effect. The data were analyzed using a negative binomial regression. Estimates of each variable represent the effect on living sperm quantity.

Model: glm(LivingSpermQuantity~Treatment+Varroa +(1|Cage), family = neg. bin.				
Variable	**Estimate**	**Std. Error**	**z-value**	**p-value**
TreatmentN	0.21	0.17	1.22	0.22
VarroaV	-0.24	0.32	-0.75	0.45

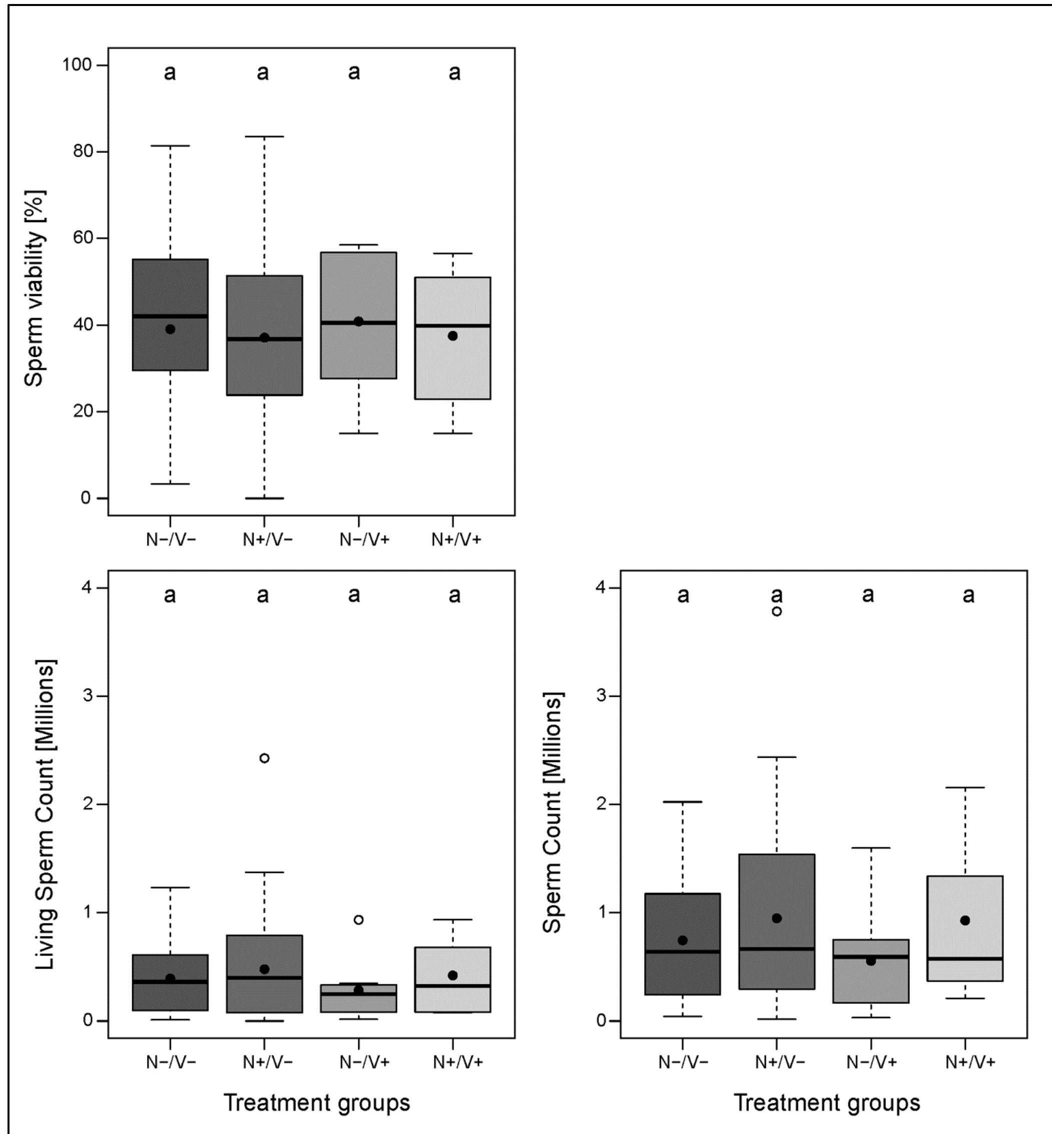

Figure 11: Differences in drone (male) honey bee (*Apis mellifera*) sperm quality among treatment groups. The sperm quality traits were compared between four treatment groups: Drones reared in absence of neonicotinoid exposure and *Varroa destructor* infestation (control, N-/V-), drones reared under neonicotinoid exposure that were free from *V. destructor* (neonic only, N+/V-), drones reared in absence of neonicotinoid exposure but were infested with by *V. destructor* (*V. destructor* only, N-/V+) and drones reared under neonicotinoid exposure and *V. destructor* infestation (combined stressors, N+/V+). Boxplots illustrate three sperm quality traits: sperm viability (a) − $N_{(N-/V-)}$ = 79, $N_{(N+/V-)}$ = 120, $N_{(N-/V+)}$ = 13, $N_{(N+/V+)}$ = 6; total sperm quantity (b) − $N_{(N-/V-)}$ = 74, $N_{(N+/V-)}$ = 104, $N_{(N-/V+)}$ = 10, $N_{(N+/V+)}$ = 5; and, living sperm quantity (c) − $N_{(N-/V-)}$ = 66, $N_{(N+/V-)}$ = 93, $N_{(N-/V+)}$ = 8, $N_{(N+/V+)}$ = 5). There were no statistically significant differences in any of the sperm traits between treatment groups ($p>0.05$). Boxplots show the inter-quartile range (box), the median (black line within box), data range (vertical black lines from box), means (black dots) and outliers (open circles). Different letters above boxplots indicate statistically significant differences ($p < 0.05$).

Total sperm quantity in control drones (N-/V-, N=74) was 0.75 ± 0.72 million (mean ± standard deviation (s.d.)). For drones exposed to neonicotinoids only (N+/V-, N=104) and *V. destructor* only (N-/V+, N=10), total sperm quantity was 0.95 ± 1.32 million and 0.55 ± 0.55 million (mean± s.d.), respectively. Total sperm quantity for drones exposed to both stressors (N+/V+, N=5) was 0.93 ± 0.75 million (mean± s.d.) (Table 17).

Table 17: Summary table of total sperm quantity of honey bee (*Apis mellifera*) drones. Total sperm quantity [# million] was calculated for four treatment groups consisting of drones reared in either control (N-) or pesticide (N+) colonies; from each of these colonies, we obtained drones that were either infested with *Varroa destructor* mites (present, V+) or not (absent, V-). Acronym definitions: Sample size (N), Standard Deviation (Std. Dev.), Minimum (Min.), Perc. (Percentiles) and Maximum (Max). Different significance letters indicate significant differences between treatment groups ($p<0.05$).

Variable	Treatment	Code	N	Mean	Std. Dev.	Min.	0.05 Perc.	0.25 Perc.	Median	0.75 Perc.	0.95 Perc.	Max	Significance letter
Total sperm quantity [# million]	Control - *V. destructor* absent	N-/V-	74	0.75	0.72	0.04	0.01	0.24	0.64	1.17	1.77	2.02	a
	Pesticide - *V. destructor* absent	N+/V-	104	0.95	1.32	0.02	0.00	0.30	0.67	1.53	3.11	3.78	a
	Control - *V. destructor* present	N-/V+	10	0.55	0.55	0.03	0.00	0.21	0.59	0.72	1.33	1.56	a
	Pesticide - *V. destructor* present	N+/V+	5	0.93	0.75	0.21	0.06	0.37	0.57	1.33	1.91	2.15	a

Sperm viability in control drones (N-/V-, N=79) was 39.1 ± 27.9% (mean±standard deviation (s.d.)), 37.1 ± 27.5% (mean±s.d.) in drones exposed to neonicotinoid only (N+/V-, N=120), and 40. 9 ± 20.9% (mean±s.d.) in drones exposed to *V. destructor* only (N-/V+, N=13). It was 37.5 ± 15.1% (mean±s.d.) in drones exposed to both stressors (N+/V+, N=6) (Table 18).

Table 18: Summary table of sperm viability of honey bee (*Apis mellifera*) drones. Sperm viability [%] was calculated for four treatment groups consisting of drones reared in either control (N-) or pesticide (N+) colonies; from each of these colonies, we obtained drones that were either infested with *Varroa destructor* mites (present, V+) or not (absent, V-). Acronym definitions: Sample size (N), Standard Deviation (Std. Dev.), Minimum (Min.), Perc. (Percentiles) and Maximum (Max). Different significance letters indicate significant differences between treatment groups ($p<0.05$).

Variable	Treatment	Code	N	Mean	Std. Dev.	Min.	0.05 Perc.	0.25 Perc.	Median	0.75 Perc.	0.95 Perc.	Max	Significance letter
Sperm Viability [%]	Control - *V. destructor* absent	N-/V-	79	39.08	27.90	0	6.34	25.37	40.08	53.60	76.02	83.50	a
	Pesticide - *V. destructor* absent	N+/V-	120	37.11	27.53	0	3.30	24.94	36.76	51.30	73.84	83.50	a
	Control - *V. destructor* present	N-/V+	13	40.85	20.94	15	5.90	27.62	40.53	56.74	57.97	58.50	a
	Pesticide - *V. destructor* present	N+/V+	6	37.52	15.07	15	17.82	26.27	39.83	49.10	54.69	56.55	a

Living sperm quantity in control drones (N-/V-, N=66) was 0.39 ± 0.44 million (mean±standard deviation (s.d.)), and 0.48±0.86 million and 0.28 ± 0.32 million (mean±s.d.) in drones exposed to neonicotinoids only (N+/V-, N=93) and *V. destructor* only (N-/V+, N=8), respectively. It was 0.42 ± 0.35 million (mean±s.d.) in drones exposed to both stressors (Table 19).

Table 19: Summary table of living sperm quantity of honey bee (*Apis mellifera*) drones. Living sperm quantity [# million] was calculated for four treatment groups consisting of drones reared in either control (N-) or pesticide (N+) colonies; from each of these colonies, we obtained drones that were either infested with *Varroa destructor* mites (present, V+) or not (absent, V-). Acronym definitions: Sample size (N), Standard Deviation (Std. Dev.), Minimum (Min.), Perc. (Percentiles) and Maximum (Max). Different significance letters indicate significant differences between treatment groups ($p<0.05$).

Variable	Treatment	Code	N	Mean	Std. Dev.	Min.	0.05 Perc.	0.25 Perc.	Median	0.75 Perc.	0.95 Perc.	Max	Significance letter
Living Sperm Quantity [# million]	Control - *V. destructor* absent	N-/V-	66	0.39	0.44	0.01	0.00	0.10	0.36	0.61	1.04	1.23	a
	Pesticide - *V. destructor* absent	N+/V-	93	0.48	0.86	0	0	0.08	0.40	0.79	1.94	2.43	a
	Control - *V. destructor* present	N-/V+	8	0.28	0.32	0.02	0	0.09	0.25	0.33	0.75	0.93	a
	Pesticide - *V. destructor* present	N+/V+	5	0.42	0.35	0.08	0.02	0.08	0.32	0.68	0.86	0.94	a

Discussion

Honey bees (*Apis mellifera* Linnaeus) are frequently exposed to multiple concurrent stressors that likely play an important role in the health of both individuals and colonies (Little et al. 2015, Retschnig et al. 2015, Kulhanek et al. 2017, Steinhauer et al. 2018, Gray et al. 2019, Straub et al. 2019). Despite this, only a few studies have documented how the interactions among stressors such as neonicotinoid insecticides and *Varroa destructor* Anderson and Trueman mites may affect honey bees (Alaux et al. 2010, Burgher-MacLellan et al. 2010, Shutler et al. 2014, Colwell et al. 2017). For the first time, we investigated the potential lethal and sub-lethal effects of neonicotinoids and *V. destructor*, alone and in combination, on drone honey bees by assessing survival and sperm quality. We found that neonicotinoids and *V. destructor* can act synergistically to severely impact the survival of this important type of honey bee.

The possible effects of concurrent exposure to multiple stressors on honey bees are of great interest, especially given the threat of detrimental interactions, whereby the effect of multiple concurrent stressors are far worse than the sum of the individual stressor effects (Maher et al. 2019). As expected, we observed a synergistic effect of neonicotinoids and *V. destructor* on drone survival supporting the haploid susceptibility hypothesis (O'Donnell and Beshers 2004, Blackmon et al. 2015). A similar experiment performed on workers yielded no interaction effect between stressors on survival (Straub et al. 2019). This interaction was observed for drones but not workers perhaps because of their hemizygosity at loci involved in immunity that diminishes allelic diversity (Blackmon et al. 2015). Regardless, even diploid workers exhibiting heterozygosity can be negatively affected by interacting stressors despite their predicted lower susceptibility (Alaux et al. 2010, Blanken et al. 2015, Straub et al. 2019, Morfin et al. 2020). Both neonicotinoids and *V. destructor* can impair the immune response of honey bees

(Claudianos et al. 2006, Prisco et al. 2013, Brandt et al. 2016), which might explain the observed synergistic effect.

In contrast, neonicotinoids and *V. destructor* did not have any effect on sperm quality. This is opposite to a similar study by Straub et al. (2016) that showed neonicotinoids can negatively affect sperm viability and number of living sperm, but not total sperm number. However, these experiments were conducted on a different continent using a different type of honey bee which may influence the susceptibility to insecticides and *V. destructor* (Martin and Medina-Medina 2004, Laurino et al. 2013, Rinkevich et al. 2015). Given that spermatogenesis begins during the larval stage and terminates at pupation (Snodgrass 1956, Yániz et al. 2020), it is not surprising that limited effects of neonicotinoids were observed in our study because drones were exposed to the insecticides during the larval stage, a fraction of the total spermatogenesis process. Therefore, upregulated immune genes (Derecka et al. 2013, Morfin et al. 2020), coupled with the limited availability of receptors for neonicotinoids in young larvae (Yang et al. 2012) may have been sufficient to prevent insecticide damage. The discrepancies between studies highlight the importance of factors such as study location and honey bee genetics can potentially have effects on experimental outcomes.

Similar to exposure to neonicotinoids only, no effect of *V. destructor* on sperm quality was observed. This corresponds to Collins and Pettis (2001a) and (Rinderer et al. 1999), but not Duay et al. (2002) and Bubalo et al. (2005). The latter two studies maintained newly emerged drones in a colony setting compared to our controlled laboratory one, possibly subjecting drones to unexpected temperature fluctuations known to impact sperm quantity (Bieńkowska et al. 2011). Furthermore, those studies examined sperm in older, flying individuals that are known to experience sperm senescence (Rueppell et al. 2005, Reyes et al. 2019). Together, these

differences in testing arena and experimental design again highlight the difficulties that exist when comparing observations among multiple studies, and demonstrate the importance of standardized experiments performed in multiple laboratories when investigating the effects of agricultural chemicals on the environment (Medrzycki et al. 2013, van der Sluijs et al. 2015).

Similar to previous studies (Collins and Pettis 2001, Straub et al. 2016), exposure to neonicotinoids or *V. destructor* negatively affected drone survival, although the latter stressor was much more severe. This can likely be explained by the low level of neonicotinoid exposure drones experienced, as Zhu et al. (2014) and Böhme et al. (2018) recently demonstrated that residues in brood food are severely diluted. It is more likely that the negative effects observed on drone survival were the result of compromised worker nurses, as previous investigations demonstrated that neonicotinoid exposure under a similar experimental setting severely affected their food glands (Hatjina et al. 2013). Conversely, experimental drones were directly exposed to parasitism by foundress *V. destructor* mites and their offspring. Numerous studies have shown this results in reduced body weight and survival of workers (Bowen-Walker and Gunn 2001, Yang and Cox-Foster 2007). Surprisingly, few studies have investigated the effects of *V. destructor* on drone survival, especially given that *V. destructor* prefers drone brood cells over worker ones for their reproductive stage (Fuchs 1992, Collins and Pettis 2001). The strong negative effect of *V. destructor* observed here could be further explained by the extended length drone brood persist as pupae compared to workers (Winston 1991). This allows *V. destructor* to produce twice as many offspring that subsequently feed on the developing drone (Rosenkranz et al. 2010), potentially resulting in additional damage (Ellis and Nalen 2019).

Despite elevated losses experienced by honey bees in many regions of the world (Lee et al. 2015b, Kulhanek et al. 2017, Brodschneider et al. 2018, Gray et al. 2019), relatively little

information exists concerning the effects of important honey bee stressors on drone honey bees. Our results confirm that both neonicotinoid exposure and *V. destructor* infestation can induce severe lethal effects on this important type of honey bee. Furthermore, our observation of a synergistic interaction between the two stressors demonstrates the importance of using realistic exposure scenarios for honey bees, and supports the haploid susceptibility hypothesis that haploid drones are less resilient to environmental stressors compared to diploid workers (O'Donnell and Beshers 2004). Although we observed no difference in sperm quality in drones surviving to sexual maturity, colony-level impacts of fewer individuals capable of mating should be investigated, especially given the importance of drones to queen mating and overall colony resilience to biotic stressors like *V. destructor* (Delaplane et al. 2015).

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Chapter 5

The potential for increased genetic diversity in a *Apis mellifera* honey bee colony to mitigate abiotic stressor effects

Abstract

Intra-colonial genetic diversity of the *Apis mellifera* Linnaeus honey bee superorganism is largely driven by the polyandrous reproductive strategy of the queen. Recent evidence suggests that hyper-polyandrous queens, mated with an above-average number of drones, enhance colony fitness. This is likely because increased intra-colonial genetic diversity can confer improved regulation of worker housekeeping tasks and resistance to biological stressors like the *Varroa destructor* Anderson and Trueman mite. However, little information exists about if the same mitigating benefits are observed for abiotic stressors like neonicotinoids insecticides. Using worker survival and hypopharyngeal gland (HPG) development as a proxy for colony health, we performed a fully crossed experimental design using 24 honey bee colonies to assess the potential of genetic diversity to mitigate effects of neonicotinoids. For 12 experimental colonies, intra-colonial genetic diversity was artificially increased through inter-colony brood frame exchange (Hyper-polyandry), whereas the other 12 did not exchange frames (Normal polyandry). Colonies were further sub-divided according to neonicotinoid exposure, whereby half of each polyandry level received pollen patties spiked with field-realistic concentrations of two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin) (Yes Neonicotinoids); the other half received patties not spiked (No neonicotinoids). Ultimately, four treatment groups were distinguished:

1) Normal polyandry / No neonicotinoids, 2) Normal polyandry / Yes neonicotinoids,

3)Hyper-polyandry / No neonicotinoids and 4) Hyper-polyandry / Yes neonicotinoids. From each treatment group, we obtained known age cohorts of workers reared during the predicted maximum of intra-colonial genetic diversity, and kept them in laboratory cages until the typical age of nursing when they were decapitated for HPG assessments. Our results suggest that even temporarily increased intra-colonial genetic diversity has the potential to mitigate lethal effects of neonicotinoids. This highlights the benefits of genetic diversity on resilience to an important group of environmental stressors, and demonstrates the possibility of employing a practical management method – inter-colony brood frame exchange – to improve colony health.

Introduction

Despite active management of honey bee (*Apis mellifera* Linnaeus) colonies by beekeepers, many ecological and environmental risk factors can severely impact colony health (Tarpy et al. 2013). Colony health is a function of its components (i.e. individuals), their contribution (i.e. division of labor), and their communication (mostly chemically) to the unit (Hölldobler and Wilson 2009). The honey bee queen is a central component of a colony, especially her health and fitness, via her role as the primary reproductive female and source of cohesion (Bortolotti and Costa 2014).

Like the other type of female in the colony − workers − queens develop from a fertilized egg, but are exclusively nourished with a rich proteinaceous substance called royal jelly that is produced by the hypopharyngeal glands (HPGs) of nursing workers (Haydak 1943, Crailsheim and Hrassnigg 1998). The different composition of worker brood food and royal jelly is likely an adaptation to the shorter development time from egg to adult (16 days) in queens compared to workers (21 days) (Haydak 1943). Within 7 to 14 days upon emergence as a young adult, a queen usually performs up to five nuptial flights (Roberts 1944), collecting and storing sperm from an average of 12 drones (Woyke 1964, Winston 1991). Only about 5% of the acquired sperm is ultimately stored, therefore it is crucial that a queen mates with many high quality drones for the sperm to last her lifetime (Winston 1991, Schlüns et al. 2005).

Although this example of polyandrous mating behavior contradicts the inclusive fitness theory (Hamilton 1964b, Crozier et al. 1996), and can bear risks for queens during mating (Ruttner 1956, Moritz and Southwick 2012), several benefits can result. Polyandry has been shown to diversify worker genotypes in a colony (Page et al. 1995), and thereby confer improved resilience to abiotic stressors like temperature fluctuations (Jones et al. 2004), and biotic

stressors like the bacterium *Paenibacillus larvae* White and the ectoparasitic mite *Varroa destructor* Anderson and Trueman (Sherman et al. 1988, Palmer and Oldroyd 2003, Delaplane et al. 2015). Given the importance of healthy queens to honey bee colonies, reports of poor queen health are concerning, and intersect both fundamental and applied science (vanEngelsdorp and Meixner 2010b, Steinhauer et al. 2014b, Kulhanek et al. 2017, Bruckner et al. 2019).

In recent years, beekeepers in the northern hemisphere have experienced consistently high colony losses (Lee et al. 2015b, Kulhanek et al. 2017, Brodschneider et al. 2018, Gray et al. 2019). Given their interaction with the environment, honey bees encounter a variety of biotic and abiotic stressors (Steinhauer et al. 2018). Biotic stressors include forage availability, mites, and bacteria, whereas weather, heavy metals, and insecticides are important abiotic stressors (Boecking and Genersch 2008, Mao et al. 2009, Genersch 2010, Sponsler and Johnson 2017). Colony losses have been attributed to the interaction of many concurrent stressors rather than a single one (Potts et al. 2010, Steinhauer et al. 2018).

Neonicotinoid insecticides have become one of the most widely used active ingredients in the world (Simon-Delso et al. 2015). Despite the low uptake, residues can also be detected in nectar and pollen of treated plants, which can pose a route of exposure to non-target organisms such as honey bees that are foraging on contaminated food sources (Bonmatin et al. 2007). Ultimately entire colonies can be affected as collected resources are shared with both adults and immatures (Crailsheim 1991, Sanchez-Bayo and Goka 2014). Field-relevant concentrations of neonicotinoids can have sub-lethal effects on honey bees, resulting in behavioral, physiological, and anatomical changes in workers (Schneider et al. 2012, Hatjina et al. 2013, Lundin et al. 2015, Friedli et al. 2020). As a superorganism, honey bee colonies can buffer against reasonable

loss of workers as long as the reproductive individuals − the drones and the queen − are maintained (Scharf et al. 2012).

Beekeepers can implement activities that promote queen reproductive health, and ultimately the health and fitness of an entire colony. Key activities include proper management of *V. destructor*, the major biotic threat to honey bees, as it negatively affects HPG development and drone flight performance which could result in inadequately fed and mated queens, respectively (Duay et al. 2002, Bubalo et al. 2005, Yousef et al. 2014). Preferably, intensive agricultural landscapes should be avoided since various agrochemicals including neonicotinoid insecticides can negatively affect HPG development (Hatjina et al. 2013), and reduce the reproductive potential of both queens and drones, possibly resulting in decreased intra-colony genetic diversity (Williams et al. 2015, Straub et al. 2016). However, beekeepers have options to artificially increase genetic diversity. This can occur involuntarily through admixture because of open mated queens (Harpur et al. 2012), or voluntarily through instrumental insemination of queens (Cobey 2007) or inter-colony exchange of frames containing brood from different patrilines (Brodschneider et al. 2012). Instrumental insemination of queens ensures that the spermatheca is filled with a highly diverse sperm mixture (Harbo 1985, Cobey 2007), and also allows for the production of 'hyper-polyandrous' queens which receive sperm collected from far more drones than they would contact through natural mating (Withrow and Tarpy 2018). Similar to natural polyandry, it can increase colony resilience to biotic and abiotic stressors like pathogens and temperature, respectively (Sherman et al. 1988, Page et al. 1995, Jones et al. 2004, Cobey 2007, Delaplane et al. 2015). However, it is not known if differences in colony-level genetic diversity can influence the observed negative effects of neonicotinoids. Despite the seemingly broad benefits to increased genetic diversity in a colony, instrumental insemination is

labor intensive, and requires costly equipment and highly specialized expertise that limits accessibility for many beekeepers. In contrast, inter-colony brood frame exchange is a practice that many beekeepers possessing more than one colony can relatively easily perform. We employed a fully-crossed experimental design to investigate if artificially induced hyper-polyandry can mitigate sub-lethal effects of two model neonicotinoids, thiamethoxam and clothianidin (Williams et al. 2015, Straub et al. 2016, 2019, Friedli et al. 2020). Hyper-polyandry was mimicked by exchanging brood frames between colonies under the assumption that this will temporarily increase intra-colony genetic diversity once individuals have emerged from these frames. The ability of hyper-polyandry to mitigate the effects of neonicotinoids were assessed on young workers called nurses that were reared under temporarily increased genetic diversity by measuring their longevity and hypopharyngeal glands, a model organ used in many toxicological studies (Heylen et al. 2011, Hatjina et al. 2013, Renzi et al. 2016). Since both measures have been shown to be affected by neonicotinoids (Hatjina et al. 2013), we predicted reduced negative effects in hyper-polyandrous colonies.

Methods

In August 2019, 24 queenright honey bee (*Apis mellifera ligustica*) colonies (Rossman Apiaries LLC., Moultrie, GA, USA) in Auburn, AL, USA, each occupying two Langstroth brood chambers, were visually assessed using the Liebefeld estimation method to quantify colony strength parameters (Delaplane et al. 2013). Brood cover estimates of eggs, larvae, and pupae were used to equalize colonies to three frames of similarly aged brood. Excess frames were removed from the study. Additionally, brood cover composition estimates were used to predict

the time of maximum abundance of specific worker age groups in the colony (e.g. nurses) (Bartlett et al. *unpublished data)*.

Polyandry status

Equalized colonies were randomly assigned to either a hyper-polyandry (HP+, N=12) or normal polyandry (HP-, N=12) treatment level. Hyper-polyandry was artificially induced by exchanging brood frames between selected colonies at the beginning of the experiment. For that, colonies belonging to the HP+ were grouped into triplets. Within these triplets, each colony served as donor and recipient of one brood frame to and from the other two colonies, respectively. Therefore, after the exchange each HP+ colony possessed one original brood frame and two introduced brood frames from two separate colonies. In contrast, colonies belonging to HP- did not exchange brood frames with other colonies. Then, half of HP+ and HP- colonies were randomly allocated either a control or neonicotinoid treatment level.

Neonicotinoid Exposure

Following an established method, all colonies were provided pollen patties *ad libitum* (60% corbicular pollen, 30% powdered sugar, 10% organic honey) (Sandrock et al. 2014b, Williams et al. 2015, Straub et al. 2019). The corbicular pollen component was sourced from honey bee colonies residing in a low intensity agricultural region of Colorado. Residue analyses performed according to Mullin et al. (2010) revealed that the pollen did not contain agricultural chemical compounds above the level of detection (Appendix 2, Figure 20). Pollen patties fed to colonies in the neonicotinoid treatment group (N+; 6 colonies from each HP+ and HP-) were spiked with field-relevant concentrations of two neonicotinoids − thiamethoxam and clothianidin (4.5 ppb and 1.5 ppb, respectively, both Sigma Aldrich) (Pilling et al. 2013, Williams et al. 2015, Straub

et al. 2016). Control colonies (N-; 6 colonies from each HP+ and HP-) received pollen patties that were not spiked (Appendix 3). To prevent influx of local, natural pollen and promote in-hive patty consumption, each colony was equipped with a Sundance pollen trap (Rossman Apiaries, LLC., Moultrie, GA, USA) (Sandrock et al. 2014b, Williams et al. 2015, Straub et al. 2016). Pollen patties were provided for 30 days to cover at least one entire brood cycle, and to mimic a realistic exposure period encountered by foraging honey bees (Goulson 2013).

Source of experimental workers

The queen of each colony was caged on an empty brood frame 10 days after the Big Mix to obtain the largest possible number of workers of a known age cohort that could raise subsequently examined workers. In other words, workers examined from HP+ colonies were raised by nursing workers that were expected to possess high genetic diversity because of previous inter-colony frame exchange. Twenty-four hours prior to adult emergence of these workers, experimental brood frames housing them were transferred to the laboratory and maintained in the incubator (34.5 °C, 75% RH; DR-41NL, Percival Scientific, Inc. Perry, IW) (Williams et al. 2013). From each frame of every colony, up to 120 newly emerged workers were individually weighed before being added to a hoarding cage (Williams et al. 2013), grouped according to their previous HP+/HP- and N+/N- experiences: 1) normal polyandry colony and no neonicotinoid exposure (control, HP-/N-), 2) hyper-polyandry colony and no neonicotinoid exposure (hyper-polyandry only, HP+/N-), 3) normal polyandry colony and neonicotinoid exposure (neonicotinoids only, HP-/N+), and 4) hyper-polyandry colony and neonicotinoid exposure (both treatments, HP+/N+). Ultimately, we obtained up to five cages per colony; each cage contained up to 25 workers.

Cage mortality and hypopharyngeal glands

Hoarding cages were maintained in the incubator (30°C, 60% RH, Percival Scientific, Inc. Perry, IW). Workers had *ad libitum* access to both sucrose solution (syringe feeder, 50% w/v) and pollen (Eppendorf tube feeder, 60% corbicular pollen from Colorado, 40% powdered sugar) to promote proper development of physiological structures (Brodschneider and Crailsheim 2010, Williams et al. 2013). Deceased individuals were removed daily, and 10 days post emergence a subset of experimental workers (i.e. 2 workers per cage) was euthanized by decapitation. Their heads were preserved in 2% paraformaldehyde PBS buffer at 4°C (Lanier and Warner 1981) until hypopharyngeal glands (HPGs) were removed. Each gland was then added to 0.1M PBS buffer and stained with Bradford solution (Coomassie Brilliant Blue G-250, both Sigma Aldrich) before being slide-mounted (Hartfelder et al. 2013) and photographed under a light microscope with 5.5x magnification (Leica, DM2500, Morrisville, NC, USA). Diameters of 20 acini per gland per worker were measured using the computer software ImageJ following Hatjina et al. (2013).

Statistical analyses

All statistical analyses were performed in R (version 4.0.2., 11/2/20) using a significance level of $\alpha=0.05$. The “survival” and “survminer” packages were used to produce survival curves (Kaplan-Meier plots) and to perform multiple pairwise comparisons with a Bonferroni correction between treatment group specific curves, respectively.

Longevity data were not normally distributed. Therefore, a generalized linear mixed effect model (glm) with a Gamma distribution was fitted to assess effects of polyandry status and neonicotinoid exposure (i.e. fixed factors). Individual workers represented the experimental units

and cage identification number was included as a random factor to account for clustering effects. HPG acini width data were normally distributed. Therefore, a linear mixed effect model was fitted to assess effects of polyandry status and neonicotinoid exposure. Individual acini represented experimental units, and colony was included as a random effect. For both longevity and HPG acini width, multiple pairwise comparisons between treatment groups (HP-/N-, HP-/N+, HP+/N-, and HP+/N+) were performed using the "emmeans" package that included a Bonferroni correction.

Employing an additive framework, potential interactions between polyandry status and neonicotinoid exposure were identified (Folt et al. 1999). Interactions were considered synergistic or antagonistic if the effect of the treatment group experiencing both treatments (i.e. HP+/N+) was greater or smaller than the sum of the individual treatments effects (i.e. HP+/N- and HP-/N+), respectively (Hay 1996). This was calculated as the percent difference in median survival [%] and HPG acini width [μm] of the three treatment groups compared to controls (HP-/N-).

Results

Cage mortality

Both polyandry status and neonicotinoid exposure had a significant effect on worker longevity (both $p<0.001$) (Table 20). Longevity was lowest in control workers (HP-/N-, 3.36 ± 3.98 days, mean±standard deviation (s.d.), N=216) compared to any other treatment group (all $p<0.001$). Longevity for hyper-polyandry only workers (HP+/N-) was 5.52±5.48 days (mean±s.d, N=273), 6.31±5.18 (mean±s.d, N=166) for neonicotinoid only workers (HP-/N+),

and 8.37±4.27 days (mean±s.d, N=91) for workers from colonies that experienced both treatments (HP+/N+). None of these three treatment groups were different from each other (all p>0.05) (Table 21).

Table 20: Generalized linear mixed effect model summary for potential stressors on honey bee (*Apis mellifera*) worker longevity. Potential effects on worker longevity in cages were assessed for two explanatory variables: 1) Polyandry status (normal versus hyper- (HP)) and 2) Neonicotinoids (present (N) versus absent). Cage identification number was included as random effect. Estimates of each variable represent the effect on longevity.

Model: glmer (Longevity~PolyandryStatus+Neonicotinoids+(1|Cage_ID), family=Gamma)				
Variable	**Estimate**	**Std.Error**	**t-value**	**p-value**
PolyandryStatus(HP)	0.06	0.01	-6.35	<0.001
Neonicotinoids(N)	-0.08	0.01	5.12	<0.001

Table 21: Summary table of honey bee (*Apis mellifera*) worker longevity per treatment group. Workers were reared in colonies assigned to either a normal (HP-) or hyper-polyandry (HP+) treatment. Treatments were then further sub-divided into two groups exposed to neonicotinoids (N+) or not (N-). Upon emergence workers were maintained in laboratory hoarding cages based on treatment group assignment (Code). Worker mortality was recorded daily until the end of the assay (day 15). Acronym definitions: Sample Size (N), Standard Deviation (S.D.), Minimum (Min.), Percentiles (0.05, 0.25, 0.75 and 0.95) and Maximum (Max.). Different letters indicate significant differences between treatment groups (p<0.05).

Variable	**Treatment**	**Code**	**N**	**Mean**	**S.D.**	**Min.**	**0.05**	**0.25**	**Median**	**0.75**	**0.95**	**Max.**	**Significance letter**
Longevity [days]	Polyandry - Control	HP-/N-	216	3.36	3.98	1	1	1	1	4	14	15	a
	Polyandry-Neonicotinoids	HP-/N+	166	6.31	5.18	1	1	1	5	11.75	15	15	b
	Hyper-polyandry - Control	HP+/N-	273	5.52	5.48	1	1	1	2	10	15	15	b
	Hyper-polyandry - Neonicotinoids	HP+/N+	91	8.37	4.27	1	2	4	9	10	15	15	b

Similarly, both hyper-polyandry and neonicotinoid exposure had a significant positive effect on survival (both $p<0.001$). Median survival was highest in workers from colonies that received both treatments (HP+/N+, 60±26.7-66.7%, median survival±95% Confidence Interval (CI)) compared to the other treatments (all $p<0.05$). Hyper-polyandry only workers (HP+/N-) did not differ from neonicotinoid only workers (HP-/N+) in survival (13.33±6.7-66.7% and 33.33±6.7-78.3%, median survival±95% CI, respectively) ($p=0.92$). Control workers (HP- /N-) had the lowest survival compared to any other treatment group (6.7±6.7-26.7%, median survival±95% CI) (all $p<0.001$) (Figure 12). There was a 796% increase in median survival for drones that experienced both treatments (HP+/N+) compared to controls (HP-/N-). This was greater than the sum of individual treatments – 393% and 94% for neonicotinoid only (HP-/N+) and hyper-polyandry only (HP+/N-) workers compared to controls, respectively. This suggests a positive synergistic interaction between the two treatments.

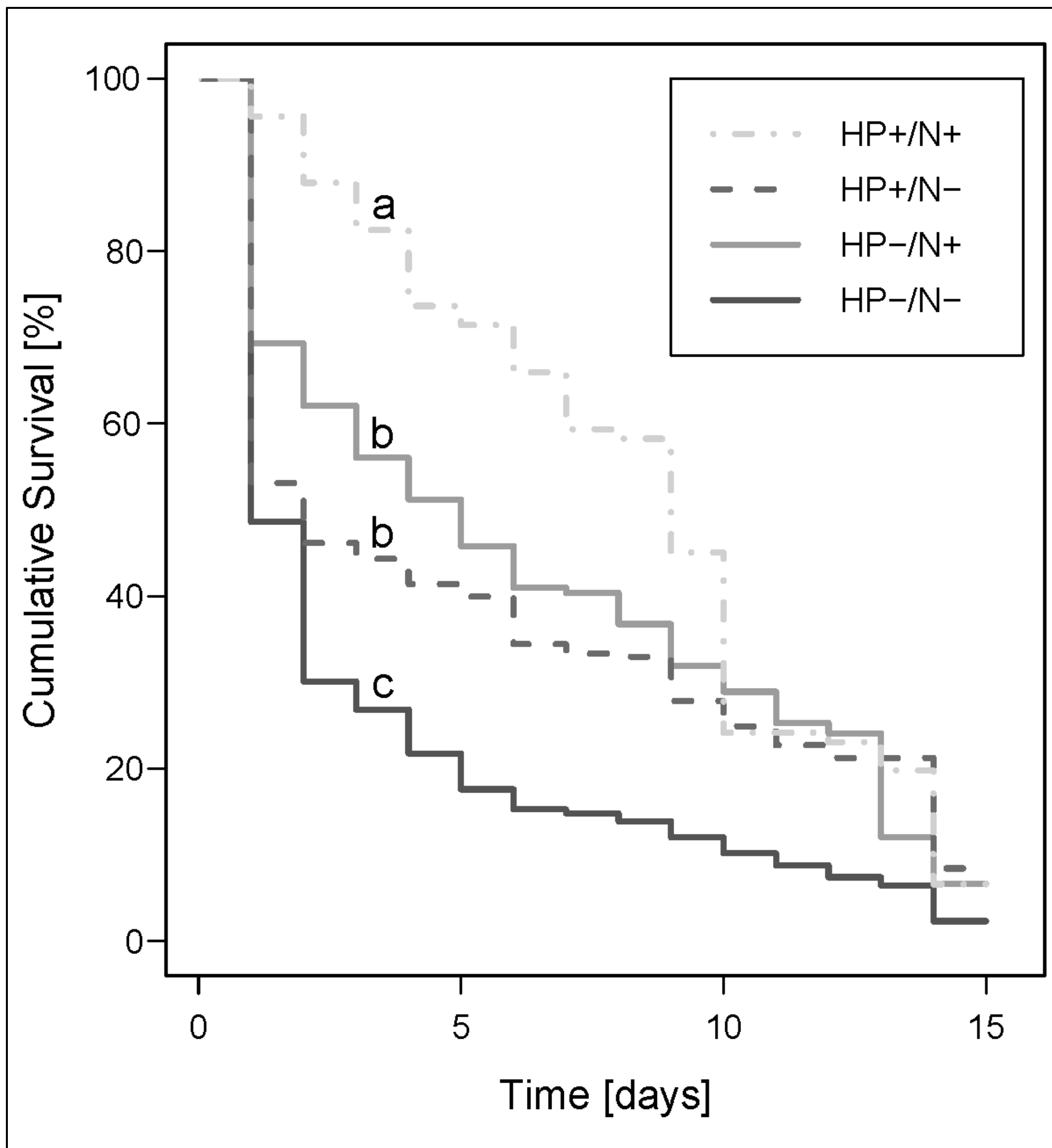

Figure 12: Cage survival of honey bee (*Apis mellifera*) workers post emergence. Survival curves represent cumulative survival [%] of workers. Survival of workers reared in hyper-polyandrous colonies exposed to neonicotinoids (both treatment, HP+/N+, N= 91 workers) was higher compared to any other treatment group (all $p<0.05$). Survival of workers reared in hyper-polyandrous group in the absence of neonicotinoids (hyper-polyandrous only, HP+/N-, N=273 workers) was not different from survival of workers reared in normal polyandrous colonies in the presence of neonicotinoids (neonicotinoid only, HP-/N+, N=166). Workers from normal polyandrous colonies in the absence of neonicotinoids (control, HP-/N-) had the lowest survival (all $p<0.001$). Different letters indicate statistically significant differences ($p < 0.05$).

Hypopharyngeal gland size

Neither hyper-polyandry nor neonicotinoid exposure had a significant effect on hypopharyngeal gland (HPG) acini width (both p>0.05) (Table 22). Control workers (HP-/N-) had the smallest acini width (92.87±44.77µm, mean±standard deviation (s.d.), N=1000 acini). Acini width for neonicotinoid-only workers (HP-/N+) was 101.21±46.83µm (mean±s.d., N=1020 acini) and 102.85±24.92µm (mean±s.d., N=1080 acini) for hyper-polyandry only workers (HP+/N-). Workers from colonies that received both treatments (HP+/N+) had the largest acini width at 105.28±19.22µm (mean±s.d., N=1000 acini) (Table 23, Figure 13). However, these differences in size among treatment groups were not statistically significant (all p>0.05) which suggests that there is no interaction effect of polyandry status and neonicotinoids on HPG acini width.

Table 22: Linear mixed effect model summary for potential stressors in honey bee (*Apis mellifera*) hypopharyngeal gland (HPG) acini width. Acini width [µm] served as a proxy for HPG activity. Two explanatory variables were assessed for potential effects: 1) Polyandry status (normal versus hyper- (HP)) and 2) Neonicotinoids (absent versus present (N)). Colony identification number was included as random effect. Estimates of each variable represent the effect on acini width.

Model: lme(Acini width ~ Treatment+Neonicotinoids, random=~1|Colony_ID)					
Variable	**Estimates**	**Std.Error**	**DF**	**t-value**	**p-value**
Treatment (HP)	-7.77	4.95	17	-1.57	0.13
Neonicotinoids (N)	5.82	4.98	17	1.17	0.26

Table 23: Summary table of honey bee (*Apis mellifera*) hypopharyngeal gland (HPG) acini width per treatment group. Acini width [µm] was used as proxy for hypopharyngeal gland activity. Treatment groups (Code) consisted of workers reared in either normal (HP-) or hyper-polyandry (HP+) colonies. Both groups of colonies (HP- and HP+) were further sub-divided into neonicotinoid absent (N-) or neonicotinoid present (N+). Acronym definitions: Sample size (N), Standard Deviation (S.D.), Minimum (Min.), Percentiles (0.05, 0.25, 0.75 and 0.95) and Maximum (Max). Different letters indicate significant differences between treatment groups ($p<0.05$)

Variable	Treatment	Code	N	Mean	S.D.	Min.	0.05	0.25	Median	0.75	0.95	Max.	Significance letter
Acini width [µm]	Polyandry - Control	HP-/N-	1000	92.87	44.77	30.14	41.34	74.94	96.78	110.85	152.80	166.78	a
	Polyandry - Neonicotinoids	HP-/N+	1020	101.21	46.83	33.82	46.97	86.40	103.15	117.21	163.18	178.50	a
	Hyper-polyandry - Control	HP+/N-	1080	102.85	50.22	37.84	55.48	88.41	104.82	118.89	174.15	192.57	a
	Hyper-polyandry - Neonicotinoids	HP+/N+	1000	105.28	38.38	59.28	67.49	92.1	104.49	116.88	161.34	176.16	a

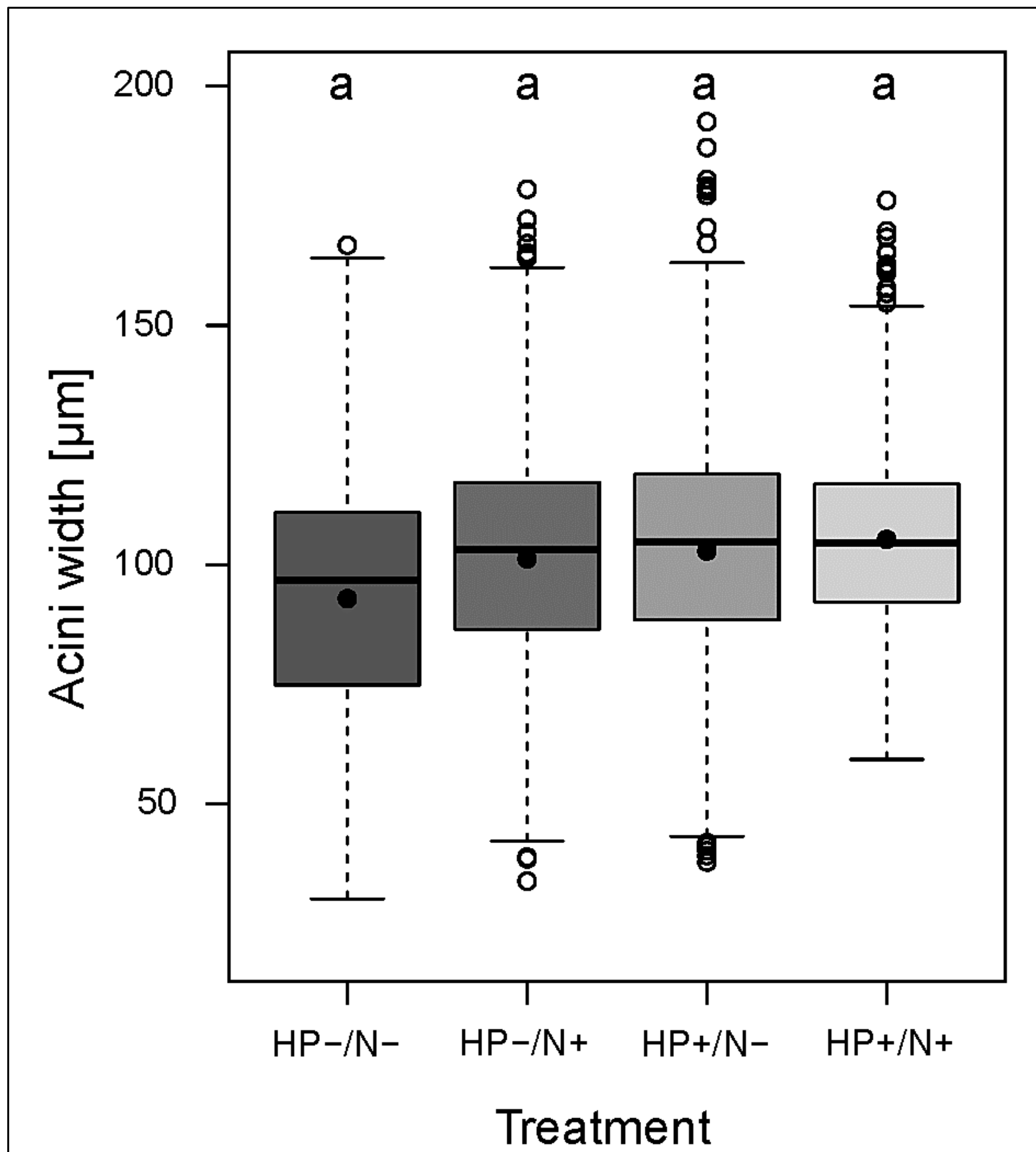

Figure 13: Differences in worker honey bee (*Apis mellifera*) hypopharyngeal gland (HPG) acini width among treatment groups. There was no difference in HPG acini width among treatment groups, regardless of if workers were reared in hyper-polyandrous (HP+) or normal polyandrous (HP-) colonies or exposed to neonicotinoids (N+) or not (N-). Numerically, acini were largest in workers raised in hyper-polyandrous colonies that were exposed to neonicotinoids (HP+/N+, N=1000 acini) and smallest in workers reared in normal polyandrous colonies not exposed to neonicotinoids (HP-/N-, N=1000 acini). Boxplots show the inter-quartile range (box), the median (black line within box), data range (vertical black lines from box), means (black dots) and outliers (open circles). Different letters above boxplots indicate statistically significant differences ($p<0.05$).

Discussion

Polyandrous queens confer many benefits to a honey bee (*Apis mellifera* Linnaeus) colony by producing genetically diverse worker sub-families (Robinson and Page 1989, Reed and Frankham 2003) that result in more efficient division of labor (Mattila and Seeley 2010, Saar et al. 2018) and improved colony resilience to biotic and abiotic stressors (Seeley and Tarpy 2007, Oldroyd and Fewell 2007, Delaplane et al. 2015). For the first time, we assessed the potential of artificially increased intra-colony genetic variation to mitigate known lethal and sub-lethal effects of neonicotinoid exposure by measuring worker survival, longevity, and hypopharyngeal gland (HPG) size (Hatjina et al. 2013, Abbo et al. 2017). Our results suggest that increased intra-colonial genetic variation improved worker survivorship, but not HPGs size, in workers simultaneously exposed to neonicotinoid insecticides. Surprisingly, we also observed a positive effect of neonicotinoid exposure on worker survival, but again no effect on HPG size. Together, this resulted in a synergistic positive interaction between hyper-polyandry and neonicotinoids on worker survival.

The relationship between genetic diversity and fitness benefits is well documented at various levels of biological organization, including social Hymenoptera (Robinson 1992, Cole 1999, Mattila and Seeley 2007). As social organisms with polyandrous queens, honey bees have evolved to exploit these benefits (Page, and Metcalf 1982). More recently, queens have been documented to acquire sperm from far more than the average of 12 drones (Delaplane et al. 2015), resulting in even greater fitness benefits to colonies headed by such “hyper-polyandrous” queens. For example, highly diverse patrilines in a colony can exhibit improved efficiency of worker task performance, ultimately benefitting a colony when important tasks like nursing or thermoregulation are emphasized (Jones et al. 2004, Oldroyd and Fewell 2007, Mattila and

Seeley 2010). This could explain the positive effects of increased genetic diversity on worker survival since both nursing and temperature in the brood nest are crucial for successful brood rearing (Schmickl and Crailsheim 2004, Mattila and Seeley 2007).

Interestingly, our results also suggested a positive effect of exposure to field-relevant concentrations of neonicotinoids. This observation could be a result of hormesis, a biphasic dose-response phenomenon whereby low concentrations of a stressor can stimulate biological processes while high concentrations are inhibitory (Calabrese 2005). Despite great efforts in honey bee toxicology, the potential occurrence of hormesis has been greatly overlooked (Cutler and Rix 2015). Given that experimental workers were indirectly exposed to neonicotinoids via nurses that previously ingested contaminated food (Zhu et al. 2014a), and that concentrations of insecticides steadily decrease from collected pollen to brood food (Böhme et al. 2018), it is possible that developing individuals were exposed to concentrations that had a stimulatory effect on biological processes involved in survival. Despite employing the same neonicotinoids and route of exposure (in-hive pollen patties), a previous study documented no effect or a reduction in worker survival (Straub et al. 2019) upon neonicotinoid exposure. However, this study exposed colonies for two brood cycles, which could result in neonicotinoid accumulation in nurse bees and subsequent exposure of developing workers to concentrations that might have reached an inhibitory level (Calabrese 2005). Exposure only lasted for one brood cycle under our experimental conditions, which might not have been enough time for substantial exposure of neonicotinoids by nurses. Therefore assessed workers may have been exposed to concentrations that were stimulatory, resulting in improved survival (Decourtye and Devillers 2010, Rondeau et al. 2014).

Our results suggest a positive synergistic interaction of increased genetic diversity and neonicotinoid exposure on worker survival. Interestingly, synergistic interactions – when the effect of two simultaneously occurring factors is greater than the sum of each individual one − are mostly discussed in the context of stressors in biological sciences (Folt et al. 1999, Dunne 2010). This is likely due to the potential of exacerbating negative effects on individuals and the associated uncertainty in predicted ecological change (Darling and Côté 2008, Maher et al. 2019). Piggott et al. (2015) addressed this bias and the importance of directionality − positive or negative – of individual factors by highlighting that interactions between potential protective and risk factors (e.g. hyper-polyandry and neonicotinoid exposure) can indeed result in a positive outcome. In our case, both factors worked in a positive direction for worker survival. Improved genetic diversity, potentially via increased care taking, and neonicotinoid exposure, potentially via hormesis, can possibly explain the positive synergy observed (Piggott et al. 2015).

In contrast, HPG development was not affected by neonicotinoid exposure or increased genetic diversity, alone or in combination. To date, most studies have measured benefits of increased genetic diversity on the colony rather than on the individuals (Mattila and Seeley 2007, Oldroyd and Fewell 2007, Delaplane et al. 2015). Simone-Finstrom et al. (2016) found that individual larvae reared in genetically diverse colonies did not differ in immune response when challenged with *Paenibacillus larvae* White, the causative agent of American Foulbrood, but showed increased specificity in recognizing this disease agent. Thus, there might not be a direct positive effect of increased genetic diversity on the process of HPG development itself, but rather higher specificity in worker nursing performance later. Given that HPGs are involved in royal jelly production that are crucial to queen nourishment, potential delayed positive effects on nursing performance should be further investigated.

According to Forbes (2000), the lack of a positive effect of neonicotinoid exposure on HPG development, could be due to temporary re-allocation of resources typical for hormesis. Under conditions that favor hormesis, an organism likely allocates fewer resources to one biological process such as HPG development in favor of stimulating other, possibly more important processes that may indirectly benefit survival. Furthermore, the probability of a hormesis dose-response greatly depends on the measured variable (Calabrese 2005). Thus, although our results for worker survival suggest hormesis, the response of HPG development is not necessarily expected to be identical.

In summary, our results suggest a positive effect of genetic diversity on honey bee health, even when artificially induced for a brief period of time. Furthermore, our findings highlight the importance of different dose-response scenarios that should be considered in honey bee toxicology. Although neonicotinoids evidently cause lethal and sub-lethal effects, we observed that low doses may have positive effects. Although rarely discussed, our results also suggest that positive synergistic interactions can occur between protective and risk factors, if they have the same directionality under particular circumstances. Future experiments should investigate if extending the period for increased genetic diversity through repeated brood frame exchange may expand benefits to other fitness traits such as HPG development, and more importantly, how this could translate into colony-level effects.

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Chapter 6

Dissertation Conclusions

My dissertation investigated the current status of managed *Apis mellifera* Linnaeus honey bee colony mortality in the United States, how important stressors affect the survival and anatomy of different honey bee types, and a possible management action that beekeepers can employ to mitigate effects of an important stressor.

Monitoring efforts were conducted in collaboration with the Bee Informed Partnership (BIP) to document national honey bee colony losses during the last three years – 2017-18, 2018-19, and 2019-20. These data were the first to be collected in the second decade of the annual BIP national honey bee colony loss survey (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012, Spleen et al. 2013, Steinhauer et al. 2014, Lee et al. 2015, Seitz et al. 2015, Kulhanek et al. 2017), and contribute to a long-term multi-year data collection effort that provides valuable insights into the variability of colony losses according to region, season, year, and beekeeper operation size. During these three years, beekeepers continued to experience higher winter losses than historically recorded, including a national record high during Winter 2018-19. This was followed by a record high national colony loss during Summer 2019. Following an established trend, our results suggest that small-scale beekeepers with fewer than 50 colonies experience higher losses during winter, whereas large-scale beekeepers managing more than 500 colonies suffer more losses during summer. This observation could be linked to differences in management philosophies between the two operation sizes, especially when considering impacts of the economically important ectoparasitic mite *Varroa destructor* Anderson and Trueman (Underwood et al. 2019).

Varroa destructor is considered the major threat to the beekeeping industry (Rosenkranz et al. 2010). My work confirms that infestation can significantly reduce survival in both workers and drones. Moreover, I found that drone survival can be severely reduced during simultaneous exposure to *V. destructor* and neonicotinoids via a synergistic interaction between the two stressors. This is a novel insight into potential stressor interactions for male honey bees, and supports the haploid susceptibility hypothesis since diploid worker survival was not affected synergistically (Retschnig et al. 2014, Straub et al. 2019, Friedli et al. 2020).

Beekeepers can employ a range of tactics to mitigate the effects of honey bee stressors, including the promotion of genetic diversity in their colonies (Bienefeld 2016, Project Apis m. 2020, Steinhauer et al. 2021). Temporary artificial increased genetic diversity as a result of brood frame exchange did not mitigate the negative effects of neonicotinoids on worker hypopharyngeal glands, but it did improve worker survival via a synergistic interaction. This may have occurred because of the positive effect of neonicotinoid exposure on worker survival, possibly because of hormesis, whereby low concentrations of a stressor can stimulate beneficial biological processes (Calabrese 2005, Cutler and Rix 2015).

In summary, my dissertation highlights the continual challenges faced by beekeepers in the United States, and suggests that experimental condition, type of honey bee investigated, and specific variable measured, are important to the outcomes of investigations. This holds true for both single and multiple stressor exposure scenarios. This highlights the potential limitations of using very targeted bee health studies that employ honey bee workers and measure a select few endpoints to understand broad effects of stressors on beneficial insects.

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Appendix 1 – Chapter 2 – Supplementary material

1* In what state(s)/territory did you keep your colonies between April 2017 and April 2018? *(Select all that apply)*

☐ Alabama	☐ Georgia	☐ Maryland	☐ New Jersey	☐ Rhode Island	☐ Vermont
☐ Alaska	☐ Hawaii	☐ Massachusetts	☐ New Mexico	☐ South Carolina	☐ Virginia
☐ Arizona	☐ Idaho	☐ Michigan	☐ New York	☐ South Dakota	☐ Washington
☐ Arkansas	☐ Illinois	☐ Minnesota	☐ North Carolina	☐ Tennessee	☐ West Virginia
☐ California	☐ Indiana	☐ Mississippi	☐ North Dakota	☐ Texas	☐ Wisconsin
☐ Colorado	☐ Iowa	☐ Missouri	☐ Ohio	☐ Utah	☐ Wyoming
☐ Connecticut	☐ Kansas	☐ Montana	☐ Oklahoma	☐ Other, please specify:	
☐ District of Columbia	☐ Kentucky	☐ Nebraska	☐ Oregon		
☐ Delaware	☐ Louisiana	☐ Nevada	☐ Pennsylvania		
☐ Florida	☐ Maine	☐ New Hampshire	☐ Puerto Rico		

*For the purpose of this survey: a "**colony**" is a **queen right** unit of bees that includes full size colonies and queen right nucs (do NOT include mating nucs); "**Living**" means alive on that date, independent of future prospects; "**Increases**" include successfully hived swarms and/or feral colonies.*

2* How many living colonies did you have last spring on **April 1, 2017**?

3* How many colonies, splits and/or increases did you make or buy between April 1, 2017 and October 1, 2017?

4* How many colonies, splits and/or increases did you sell or give away between April 1, 2017 and October 1, 2017?

5* Did you purposefully combine colonies between April 1, 2017 and October 1, 2017?
❍ No ❍ Yes If yes: # colonies that were combined: # colonies that resulted from combination:

6* How many living colonies did you have last fall on **October 1, 2017**?

7* How many colonies, splits and/or increases did you make or buy between October 1, 2017 and April 1, 2018?

8* How many colonies, splits and/or increases did you sell or give away between October 1, 2017 and April 1, 2018?

9* Did you purposefully combine colonies between October 1, 2017 and April 1, 2018?
❍ No ❍ Yes If yes: # colonies that were combined: # colonies that resulted from combination:

10* How many total living colonies (overwinter surviving colonies plus purchase or splits) did you have on **April 1, 2018**?

11 What was the largest number of living colonies you owned between April 1, 2017 and April 1, 2018?

12 What was the smallest number of living colonies you owned between April 1, 2017 and April 1, 2018?

13 Of the colonies in question 2, how many of those specific colonies were **still** alive on October 1, 2017?

14 Of the colonies in question 6, how many of those specific colonies were **still** alive on April 1, 2018?

15 What is the average size of your apiary or apiaries? *(How many colonies share the same location, on average)*

16 In how many different apiaries do you keep your bees? (if stationary)

17* What **percentage** of loss, over the last winter, would you consider acceptable? *Between 0-100%*

18 Was your winter loss this year higher or lower than last year? *(choose one)*
❍ Higher ❍ Same ❍ Lower ❍ I'm unsure ❍ I did not keep bees last year

19 What **percentage** of the colonies died over the winter (between October 1, 2017 and April 1, 2018) without dead bees in the hive or apiary? *Between 0-100%* ☐ None

20 In your opinion, what factors were the most prominent cause(s) of colony death in your operation between October 1, 2017 and April 1, 2018? *(select all that apply)*

☐ I did not experience winter loss	☐ Queen failure	☐ Starvation	☐ Colony Collapse Disorder (CCD)
☐ Varroa mites	☐ Nosema disease	☐ Small Hive Beetle	☐ Natural disaster and the like (eg. flood, bear…)
☐ Poor wintering conditions	☐ Weak in the fall	☐ Pesticides	☐ I don't know

☐ Other, please specify:

Between April 1, 2017 and April 1, 2018…

21* Did you move any of your colonies **across state lines**? ❍ Yes ❍ No *(skip Q 22)*

22 Approximately what **percentage** of your operation moved **across state lines** at least once between April 1, 2017 and April 1, 2018? *Between 0-100%*

23 What **percentage** of your hives did you send to or move into California almond orchards for pollination in 2018? *Between 0-100%* ☐ None

24 Please indicate in which states/territory you kept bees for the months listed *(mark relevant months with an " x ")*. Also list the number of living colonies you had **in each state** on December 31, 2017 *(note in the last column)*.

State	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sept	Oct	Nov	Dec	Living colonies on 12/31/2017
-													
-													
-													
-													
-													

25 In what zip code is your operation based?

26 In what zip code do you live most of the year?

27 Did you take part in any of the following monitoring efforts? *(select all that apply)*

☐ APHIS National Honey Bee Survey [1] — NHBS sampling code

☐ Bee Informed Tech Transfer Teams [1] — BIP Tech Team participant code

☐ Bee Informed Sentinel Apiary (real time disease monitoring) [1] — Sentinel Apiary participant code

☐ None of the above ☐ I prefer not to say ☐ I'm unsure

[1] *If you participated in any of the BIP sampling programs, or the APHIS NHBS survey, would you be willing to provide your participant code (above) and/or contact information so we can link your survey responses with your bee health measures?*

Figure 14: **A national managed *Apis mellifera* honey bee colony loss survey performed for the United States (U.S.) in 2017-18 – Paper version.** This paper version of the survey was distributed to specific respondents. Questions included in this paper version were also accessible via an online survey that was live between 1 April 2018– 30 April 2018.

1* In what state(s)/territory did you keep your colonies between April 1, 2018 and April 1, 2019? *(Select all that apply)*

☐ Alabama	☐ Georgia	☐ Maryland	☐ New Jersey	☐ Rhode Island	☐ Vermont
☐ Alaska	☐ Hawaii	☐ Massachusetts	☐ New Mexico	☐ South Carolina	☐ Virginia
☐ Arizona	☐ Idaho	☐ Michigan	☐ New York	☐ South Dakota	☐ Washington
☐ Arkansas	☐ Illinois	☐ Minnesota	☐ North Carolina	☐ Tennessee	☐ West Virginia
☐ California	☐ Indiana	☐ Mississippi	☐ North Dakota	☐ Texas	☐ Wisconsin
☐ Colorado	☐ Iowa	☐ Missouri	☐ Ohio	☐ Utah	☐ Wyoming
☐ Connecticut	☐ Kansas	☐ Montana	☐ Oklahoma	☐ Other, please specify:	
☐ District of Columbia	☐ Kentucky	☐ Nebraska	☐ Oregon		
☐ Delaware	☐ Louisiana	☐ Nevada	☐ Pennsylvania		
☐ Florida	☐ Maine	☐ New Hampshire	☐ Puerto Rico		

*For the purpose of this survey, a "**colony**" is a **queen right** unit of bees that includes full size colonies, queen right nucs and packages once installed (do NOT include mating nucs); "**Living**" means alive on that date, independent of future prospects; "**Increases**" include successfully hived swarms and/or feral colonies.*

2* How many living colonies did you have last spring on April 1, 2018?

3 If known, how many of those specific colonies were still alive on October 1, 2018?

4* How many **colonies, splits and/or increases** did you make or obtain between April 1, 2018 and October 1, 2018?

5 If known, how many of those **colonies, splits and/or increases** were still alive on October 1, 2018?

6* How many **colonies, splits and/or increases** did you sell or give away between April 1, 2018 and October 1, 2018?

7* Did you purposefully combine colonies between April 1, 2018 and October 1, 2018?
❍ No ❍ Yes If yes: # colonies that were combined: # colonies that resulted from combination:

8* How many living colonies did you have last fall on October 1, 2018?

9 If known, how many of those specific colonies were still alive on April 1, 2019?

10* How many **colonies, splits and/or increases** did you make or obtain between October 1, 2018 and April 1, 2019?

11 If known, how many of those specific **colonies, splits and/or increases** were still alive on April 1, 2019?

12* How many **colonies, splits and/or increases** did you sell or give away between October 1, 2018 and April 1, 2019?

13* Did you purposefully combine colonies between October 1, 2018 and April 1, 2019?
❍ No ❍ Yes If yes: # colonies that were combined: # colonies that resulted from combination:

14* How many living colonies did you have this spring on April 1, 2019?

15 What percentage of your colonies established or made the previous spring and summer (between April 1, 2018 and October 1, 2018) were still alive on April 1, 2019? *Between 0-100%*
*(In other words, what % of your colonies **successfully overwintered for the first time**.)*

16* What **percentage** of loss, over the last winter, would you consider acceptable? *Between 0-100%*

17 Of your colonies that died between October 1, 2018 and April 1, 2019, what **percentage** experienced the following symptoms: *Between 0-100%*
Lack of dead workers around hives AND rapid loss of adult worker force, but presence of brood and laying queen AND delayed invasion of hive pests/robbing

18 In your opinion, what factors were the most prominent **cause(s) of colony death** in your operation **in Summer** (between April 1, 2018 and October 1, 2018)? *(select all that apply)*
☐ I did not experience summer loss ☐ Natural disaster (e.g. hurricane, flood) ☐ Pesticides and Apicultural treatments
☐ Adverse weather (e.g. drought) ☐ Nutritional stress (pollen deprivation) ☐ Predators (e.g. bears)
☐ Brood diseases (e.g. EFB, AFB) ☐ Starvation (honey/nectar/sugar water) ☐ Scavenger Pests (e.g. Small Hive Beetle, Wax moth)
☐ Queen issues ☐ Equipment failure (e.g. moisture, ventilation) ☐ Varroa mites and associated viruses
☐ I don't know ☐ Other, please specify:

19 In your opinion, what factors were the most prominent **cause(s) of colony death** in your operation **in Winter** (between October 1, 2018 and April 1, 2019)? *(select all that apply)*
☐ I did not experience winter loss ☐ Natural disaster (e.g. hurricane, flood) ☐ Pesticides and Apicultural treatments
☐ Adverse weather (e.g. cold snap) ☐ Nutritional stress (pollen deprivation) ☐ Predators (e.g. bears)
☐ Brood diseases (e.g. EFB, AFB) ☐ Starvation (honey/nectar/sugar water) ☐ Scavenger Pests (e.g. Small Hive Beetle, Wax moth)
☐ Queen issues ☐ Equipment failure (e.g. moisture, ventilation) ☐ Varroa mites and associated viruses
☐ I don't know ☐ Other, please specify:

Between April 1, 2018 and April 1, 2019…

20 Please provide the zip code of your **primary apiary**.

21* Did you move any of your colonies **across state lines**? ❍ Yes *(skip Q 25 -27)* ❍ No *(skip Q 22-23)*

22 Approximately what **percentage** of your operation moved **across state lines** at least once between April 1, 2018 and April 1, 2019? *Between 0-100%*

23 What **percentage** of your hives did you send to or move into California almond orchards for pollination in 2019? *Between 0-100%* ☐ None

24 For each month last year, please provide the number of colonies you maintained in each states/territory.

State	1 Apr '18	1 May '18	1 Jun '18	1 July '18	1 Aug '18	1 Sept '18	1 Oct '18	1 Nov '18	1 Dec '18	1 Jan '19	1 Feb '19	1 Mar '19
-												
-												
-												
-												
-												

25 What is the average size of your apiary or apiaries? *(On average how many colonies share the same location)*

26 In how many different apiaries did you keep your bees?

27 If you maintained stationary apiaries, please consider to include their GPS coordinates.
(Disclaimer: This information is confidential and will not be published. GPS data is valuable to estimate apiary densities in different regions.)
N W
N W
N W
N W

Figure 15: A national managed *Apis mellifera* honey bee colony loss survey performed for the United States (U.S.) in 2018-19 – Paper version. This paper version of the survey was distributed to specific respondents. Questions included in this paper version were also accessible via an online survey that was live between 1 April 2019– 30 April 2019.

1* In what state(s)/territory did you keep your colonies between April 1, 2019 and April 1, 2020?
(Please specify ALL relevant states, including states visited for pollination, or if you manage apiaries in multiple states.)

☐ Alabama
☐ Alaska
☐ Arizona
☐ Arkansas
☐ California
☐ Colorado
☐ Connecticut
☐ District of Columbia
☐ Delaware
☐ Florida
☐ Georgia
☐ Hawaii
☐ Idaho
☐ Illinois
☐ Indiana
☐ Iowa
☐ Kansas
☐ Kentucky
☐ Louisiana
☐ Maine
☐ Maryland
☐ Massachusetts
☐ Michigan
☐ Minnesota
☐ Mississippi
☐ Missouri
☐ Montana
☐ Nebraska
☐ Nevada
☐ New Hampshire
☐ New Jersey
☐ New Mexico
☐ New York
☐ North Carolina
☐ North Dakota
☐ Ohio
☐ Oklahoma
☐ Oregon
☐ Pennsylvania
☐ Puerto Rico
☐ Rhode Island
☐ South Carolina
☐ South Dakota
☐ Tennessee
☐ Texas
☐ Utah
☐ Other, please specify:
☐ Vermont
☐ Virginia
☐ Washington
☐ West Virginia
☐ Wisconsin
☐ Wyoming

*For the purpose of this survey, a "**colony**" is defined as any unit of bees housed in a hive. That is, a full-sized colony, a nuc, a recently created split, a newly installed package or swarm, BUT NOT a mating nuc. It must be headed by a mated queen, or at least contain young brood, a queen cell, or a virgin queen. In other words, it should not be hopelessly queenless.*

2* Enter the **number of colonies**, regardless of future survival prospects or queen status, you had on the given date.
1 April 2019 | 1 October 2019 | 1 April 2020

3* How many colonies did you **obtain** from outside your operation? *(Please include both those that did AND did not establish in your operation after your initial installation).*
Summer (1 Apr 2019 – 1 Oct 2019) | Winter (1 Oct 2019 – 1 Apr 2020)

4* How much **net increase** in colonies did you make from **splitting** your own colonies? *(Please include both those that did AND did not establish. Also include packages and splits that were eventually sold).*
Summer (1 Apr 2019 – 1 Oct 2019) | Winter (1 Oct 2019 – 1 Apr 2020)

Example of net increase by splitting:
*- You might break down an entire colony while making splits. If you break down 1 colony into 3 smaller colonies (e.g. nucs), this represents a **net increase** of 2 colonies.*
*- You might take extra bees and brood from strong colonies. Two strong colonies results in one new colony AND the two original colonies. This represents a **net increase** of 1 colony.*

5* Of your **newly set up** colonies, how many **did not establish** within the first month after installation (e.g. queen was not accepted, swarm absconded, etc.)
Consider both those you obtained from outside your operation and colonies you made by splitting, but do not include parent colonies.
Summer (1 Apr 2019 – 1 Oct 2019) | Winter (1 Oct 2019 – 1 Apr 2020)

6* How many colonies did you **sell** or **give** away?
***Remember**, according to our definition of a colony, this includes full-sized colonies, as well as newly made splits/nucs and packages.*
Summer (1 Apr 2019 – 1 Oct 2019) | Winter (1 Oct 2019 – 1 Apr 2020)

7* How much colonies did you **net decrease** from combining your own colonies?
Summer (1 Apr 2019 – 1 Oct 2019) | Winter (1 Oct 2019 – 1 Apr 2020)

Example of net decrease by combining: You might combine 2 weak colonies into one. This represents a net decrease of 1 colony.

8* What **percentage** of loss, over the last **winter**, would you consider **acceptable**? *Between 0-100%*

9 Of your colonies that died during **winter** (between October 1, 2019 and April 1, 2020), what **percentage** experienced the following symptoms:
Few dead workers on the bottom board and around the hive, AND rapid loss of adult worker force, but presence of brood and laying queen, AND delayed invasion of hive pests/robbing *Between 0-100%*

10 In your opinion, what factors were the most important **cause(s) of colony death** in your operation **in Summer** (between April 1, 2019 and October 1, 2019)? *(select all that apply)*

☐ I did not experience summer loss
☐ Adverse weather (e.g. drought)
☐ Brood diseases (e.g. EFB, AFB)
☐ Queen issues
☐ I don't know
☐ Natural disaster (e.g. hurricane, flood)
☐ Nutritional stress (pollen deprivation)
☐ Starvation (honey/nectar/sugar water)
☐ Equipment failure (e.g. moisture, ventilation)
☐ Pesticides and treatments
☐ Predators (e.g. bears)
☐ Scavenger Pests (e.g. Small Hive Beetle, Wax moth)
☐ Varroa mites and associated viruses
☐ Other, please specify:

11 In your opinion, what factors were the most important **cause(s) of colony death** in your operation **in Winter** (between October 1, 2019 and April 1, 2020)? *(select all that apply)*

☐ I did not experience winter loss
☐ Adverse weather (e.g. cold snap)
☐ Brood diseases (e.g. EFB, AFB)
☐ Queen issues
☐ I don't know
☐ Natural disaster (e.g. hurricane, flood)
☐ Nutritional stress (pollen deprivation)
☐ Starvation (honey/nectar/sugar water)
☐ Equipment failure (e.g. moisture, ventilation)
☐ Pesticides and treatments
☐ Predators (e.g. bears)
☐ Scavenger Pests (e.g. Small Hive Beetle, Wax moth)
☐ Varroa mites and associated viruses
☐ Other, please specify:

Between April 1, 2019 and April 1, 2020...

12 Please provide the zip code(s) of your **primary apiary/base of operation**.
(You can provide more than one if you have multiple bases of operation)

13 At the time of 2020 bloom, what **percentage** of your operation's colonies were used to **pollinate California almonds**? *Between 0-100%* ☐ None

14* Did you **move** any of your colonies **between states**? *Do not include colonies you obtained from out of state before your installed them.* ❍ **Yes** *(skip Q 16-17)* ❍ **No** *(skip Q 15)*

15 Approximately what **percentage** of your operation moved **across state lines** at least once between April 1, 2019 and April 1, 2020? *Between 0-100%*

16 What is the **average size** of your bee yards?
(On average how many colonies share the same location. Do not include mating yards or pollination drops.)

17 In how **many** different bee yards did you keep your bees?
(Do not include mating yards or pollination drops.)

18 Please enter the estimated **number of colonies** you had on the first of each month in each state/territory.

State	1 Apr '19	1 May '19	1 Jun '19	1 July '19	1 Aug '19	1 Sept '19	1 Oct '19	1 Nov '19	1 Dec '19	1 Jan '20	1 Feb '20	1 Mar '20
-												
-												
-												
-												
-												

Figure 16: A national managed *Apis mellifera* honey bee colony loss survey performed for the United States (U.S.) in 2019-20 – Paper version. This paper version of the survey was distributed to specific respondents. Questions included in this paper version were also accessible via an online survey that was live between 1 April 2020 – 30 April 2020.

Table 24*: A summary of managed Apis mellifera* honey bee Summer colony losses in the United States by states during three survey years – 2017-18, 2018-19 and 2019-20. Total and Average loss (%) are listed for all 50 states, the federal District of Columbia, and five permanently inhabited self-governing territories for the Summer period (1 April 201X - 1 October 201X). Estimates are not reported for states with less than 10 respondents to protect their privacy.

	Survey Year 2017-18			Survey Year 2018-19			Survey Year 2019-20		
State	N	Total Loss [95% CI]	Average Loss [95% CI]	N	Total Loss [95% CI]	Average Loss [95% CI]	N	Total Loss [95% CI]	Average Loss [95% CI]
AL	57	15.2 [7.5-27.6]	17.6 [11.7-23.9]	68	18.4 [10.6-26.9]	16.7 [11-22.4]	72	31.5 [25.3-40.7]	33.1 [26.8-39.9]
AK	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
AZ	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
AR	35	37.9 [23.9-53.9]	29.4 [20.3-39.9]	44	21.8 [15.4-27.9]	20.4 [14.5-27.2]	29	35.5 [14.1-46.1]	15.2 [9-22.4]
CA	120	16.3 [10.8-24.1]	21.1 [17-25.2]	142	20.5 [15.7-25.8]	20.6 [17.1-24.5]	109	31.7 [18.7-47.3]	26.8 [22.5-31.3]
CO	87	15.7 [10.4-27.3]	22.4 [16.4-29.4]	107	17.4 [10.9-28.1]	13.7 [9.1-18.6]	63	23.3 [17.1-29]	20.5 [14.6-26.3]
CT	36	21.7 [8.7-38.2]	21.9 [12.2-33.1]	20	9.1 [2.3-18.5]	10.9 [3.7-18.7]	32	18.6 [11-27.2]	18.8 [12.5-26.1]
DC	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
DE	20	19.9 [10.4-24.5]	18.9 [8.8-31.6]	13	15.8 [3.9-38.1]	18.3 [5.1-35]	12	30.3 [14.2-50.6]	31.7 [16.9-48.5]
FL	61	33.1 [14-48.5]	25.2 [17.4-32.5]	41	34.5 [16.7-48.1]	16.2 [8.7-24.3]	27	58.3 [4.6-94.2]	30.7 [20-42.2]
GA	142	20.8 [15.2-29.9]	24.1 [19.9-28.4]	92	35.2 [21.3-51.3]	20.9 [15.6-26.7]	93	30.5 [15.2-44.1]	29.8 [24.1-35.5]
HI	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
ID	27	18.7 [9.4-33.7]	10.8 [4.8-18.9]	26	25.6 [14.3-39.5]	24.6 [15.7-35.8]	23	18.5 [10.1-33.9]	21 [44196]
IL	104	17 [12.6-21.6]	19 [14.7-23.3]	82	9.1 [4.8-15.1]	11.2 [7.3-15.9]	40	16.6 [9.4-24.6]	18.5 [12.4-25.4]
IN	117	14 [11.6-19.9]	12.4 [9.4-15.9]	99	32.3 [6.4-51.4]	14.2 [10.6-18.3]	69	8.6 [2.4-20.4]	21 [15.1-27.2]
IA	85	31.5 [10.8-50.9]	17 [12.4-22.4]	69	18.2 [7.1-28.6]	11.1 [7.4-15.1]	35	37.4 [11.7-55.3]	24.4 [16.2-32.1]
KS	25	32.5 [7.8-41.3]	9.6 [3.6-15.9]	29	34.7 [15.4-47.7]	20.8 [12.5-29.8]	26	16.9 [10.2-22.6]	19 [10.9-27.2]
KY	99	32.3 [19.2-40.4]	20.6 [15.5-25.8]	87	10.9 [7.2-15.2]	13.2 [9.7-17.3]	67	24.6 [15.2-36]	20.1 [14.6-25.9]
<10	14	16.7 [12-22.8]	17.9 [7-30.5]	<10	- [---]	- [---]	<10	- [---]	- [---]
ME	59	10.7 [5.6-16.8]	12.7 [7.2-18.6]	49	9.7 [6.3-13.4]	10 [5.2-14.7]	48	19.5 [8.5-33.5]	16.7 [10.7-23.2]
MD	123	24.1 [19-26.8]	20.4 [15.7-24.8]	170	15.4 [12.9-18.7]	16.5 [12.9-20.4]	141	18.7 [16.3-23.6]	21.7 [17.9-25.8]
MA	87	32.8 [11.7-55.2]	16.1 [11.3-21.4]	71	13.3 [8.6-18.6]	12.5 [8.6-17.2]	51	13.4 [8.8-21.3]	15 [9.2-20.5]
MI	203	19.5 [8.3-36.4]	16.5 [13.4-19.9]	154	38.3 [22.3-49.8]	11 [8.5-13.8]	141	22.3 [6.3-39.7]	23.9 [20.1-28.1]
MN	75	28.2 [17.4-31.4]	17.2 [13.2-21.5]	81	24.6 [14.1-43.8]	17.4 [12-23.6]	43	31.8 [22.2-55.4]	29.1 [21.6-36.4]
MS	13	30.7 [17.4-37.4]	22.6 [10.9-33.8]	<10	- [---]	- [---]	<10	- [---]	- [---]
MO	71	15.1 [10.1-21.1]	15.2 [10.8-20]	81	15 [10.9-18.8]	13.2 [9-17.4]	75	17.1 [9.9-26.2]	16.4 [11.5-21.8]
MT	19	17.6 [10.4-43.9]	26.1 [14.5-40.3]	19	9.1 [4-16.6]	10.1 [3.9-17.8]	20	20.2 [4.7-38]	10.4 [5.1-16.4]
NE	13	24.8 [5.1-64.2]	25.2 [12.8-38.4]	13	13.3 [10-26.6]	17.3 [8.4-27.1]	<10	- [---]	- [---]
NV	13	28.9 [4.1-50.6]	13.8 [1.4-29.8]	15	17.8 [10.5-24.2]	13.2 [5-21.8]	12	33 [17.7-50]	29.4 [17-42.4]
NH	40	9 [3.3-19]	12.5 [7-18.1]	29	13.9 [6.1-23.9]	15.1 [8.1-23.1]	31	14.2 [9.7-23.2]	18.4 [44190]
NJ	70	15.9 [9.5-23.6]	16.4 [10.7-22.4]	54	7.4 [2.7-14.9]	10.3 [6.1-15.3]	52	13.5 [7.5-22.3]	17.2 [12.2-22.2]
NM	19	45.7 [7.6-71.8]	22.2 [8.1-37.8]	14	11.4 [4.2-22]	15.2 [5.1-25.6]	12	40.9 [25.8-56.1]	42.4 [25-59.6]
NY	126	20.5 [12.3-24.7]	18 [14.2-22.2]	105	22.9 [13-30.9]	9.8 [6.7-13.3]	75	12.8 [7.3-22.2]	22.7 [17.1-28.7]
NC	237	19.6 [15.3-23.9]	20.2 [17-23.7]	190	16.3 [12.6-20.2]	16.8 [13.5-20.4]	133	22.1 [16.3-28.2]	22 [18-26.7]
ND	13	13.5 [6.2-35.1]	23.6 [11.4-38.1]	20	21.2 [14.4-29.1]	24.7 [16.9-34.1]	13	45.4 [23.6-63.2]	37 [25-48.6]
OH	179	17.7 [12-25.4]	19 [15.3-22.8]	144	10.9 [7.3-14.9]	13.6 [10.5-16.8]	128	15.4 [11.3-24.8]	18.6 [14.7-22.9]
OK	32	32.6 [17.2-47.6]	22.9 [13.5-34.3]	65	13.9 [9-25.5]	15.8 [11.2-21.1]	32	21.4 [13.2-32.7]	25.1 [14.7-36.5]
OR	92	16.2 [12.3-17.4]	10.4 [7.2-14]	103	14.1 [10.2-17.9]	15.4 [11.2-19.9]	71	28.1 [8.6-37.6]	18.6 [13-24.9]
PA	468	15.3 [11.3-20.6]	14.9 [12.9-16.9]	499	13 [7.9-20.3]	11.6 [9.7-13.4]	479	15.8 [12.8-18.7]	18.7 [16.6-20.8]
PR	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
RI	15	21.2 [6.9-35.4]	14.6 [5.8-24.4]	14	12.4 [3.4-25.8]	14.3 [3.3-29.9]	<10	- [---]	- [---]
SC	67	20.2 [15.4-26.3]	18 [13-23.9]	38	35.3 [10.4-53.7]	14.2 [7.8-21.1]	43	18.9 [11.8-35.4]	30.5 [21.3-40.4]
SD	15	7.1 [3.5-16.7]	17.9 [7.9-31.9]	12	13.5 [5.8-24.9]	15.5 [5.9-27.7]	14	22.4 [8.4-42.9]	26.8 [15.4-38.4]
TN	85	28.4 [16.7-41.6]	32 [25.7-38.8]	61	12 [7.6-16.9]	11.5 [7.4-16.5]	42	22.6 [13.6-35.3]	18.6 [11.8-25.8]
TX	95	13.8 [7.7-22.3]	18.5 [14.1-23.2]	139	20.7 [16.5-25.3]	18 [14.4-22.4]	97	43.6 [23.5-61.9]	20.9 [16.1-26.1]
UT	133	16 [11.4-20.4]	17.9 [13.3-22.4]	102	17.5 [12.6-23.9]	18.9 [13.8-24.8]	58	39.1 [9.5-56.3]	26.2 [19.2-33.7]
VT	46	10.1 [4.6-18.9]	20.3 [12.1-28.7]	36	10.2 [6.4-12.8]	7.3 [3.4-12]	24	20.9 [6.8-26.5]	13 [5.8-20.9]
VA	484	20.1 [16.4-24.7]	21.1 [18.8-23.6]	523	15.9 [12.4-20.4]	15.8 [13.8-17.8]	236	23.3 [16.7-30.1]	20.8 [17.9-24]
WA	100	8.1 [3.5-16.1]	18.1 [13.2-23.2]	115	19.6 [5-39.1]	18.6 [14.4-23.3]	55	27 [10.9-40.8]	23.1 [16.9-29.4]
WV	48	15.7 [8.6-20.2]	13 [8.3-18.4]	35	15.1 [7.2-25]	15.4 [9.3-23.2]	19	65.4 [10.8-94.4]	21.3 [12.5-32.6]
WI	118	14.5 [9.9-25.9]	18.5 [15-22.5]	91	37.6 [18.2-49.2]	15 [11-19.1]	57	39.3 [15.6-54.2]	25.3 [19.3-32.5]
WY	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
Other	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
Multi State	105	15.9 [10.8-22.2]	18.4 [15.1-21.6]	117	21.5 [16.7-26.3]	19.2 [16.2-22.5]	78	37.6 [24.5-52.2]	26 [21-31.1]

Table 25: A summary of managed *Apis mellifera* honey bee Winter colony losses in the United States by states during three survey years – 2017-18, 2018-19 and 2019-20. Total and Average loss (%) estimates are listed for all 50 states, the federal District of Columbia, and five permanently inhabited self-governing territories for the Winter period (1 October 201X – 1 April 201Y, whereby “X” and “Y” represent successive years). Estimates are not reported for states with less than 10 respondents to protect their privacy.

	Survey Year 2017-18			Survey Year 2018-19			Survey Year 2019-20		
State	N	Total Loss [95% CI]	Average Loss [95% CI]	N	Total Loss [95% CI]	Average Loss [95% CI]	N	Total Loss [95% CI]	Average Loss [95% CI]
AL	65	37 [20-54.6]	30.4 [22.8-37.9]	75	22.5 [16.2-30.7]	24.8 [17.5-31.9]	68	27.6 [17.6-35.6]	25.4 [19.6-31.7]
AK	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
AZ	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
AR	42	16.9 [5.7-28.7]	24.6 [15.9-33.1]	47	26.5 [18.2-37]	34 [24.4-44.6]	29	18.3 [13.8-31.1]	23.9 [14.3-35]
CA	136	25.5 [19.6-32.5]	32.1 [27.3-37]	144	30.8 [21.8-40]	35.6 [30.6-40.7]	115	20.7 [14.7-28]	32.4 [27-37.8]
CO	114	19.2 [7.1-46.5]	42.4 [35-49.8]	132	23.3 [10.6-47.6]	46.5 [40-53.6]	78	37.6 [29-46.3]	34.7 [27.1-43.1]
CT	49	55 [39.6-70.8]	56.4 [45.6-66.6]	36	36.9 [23.5-52.5]	41.2 [28.3-54]	37	36.7 [18.8-59.4]	41.3 [28.8-54.3]
DC	10	57.5 [33.3-81.8]	64.4 [38.3-90]	11	30.7 [15-38.8]	26.1 [14.1-38.4]	<10	- [---]	- [---]
DE	26	43.5 [35.3-67.5]	66.3 [53.9-78.2]	15	45.9 [25.7-56.9]	39.4 [20.4-56.6]	14	41.2 [17-64.8]	32 [15.1-50.7]
FL	57	19.5 [9.7-35.5]	23.9 [17.5-30.5]	37	18.8 [4.1-36.9]	21.6 [12.8-31]	26	29.2 [18.7-46.7]	30.5 [19.6-42.1]
GA	150	32.2 [20.7-51.4]	45.7 [39.7-52.2]	90	30.9 [14.6-44.7]	38.8 [31.6-45.8]	82	29.1 [13.3-61.5]	26.5 [20.1-33.5]
HI	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
ID	34	25 [17.6-37.6]	42.7 [29.7-55.6]	32	30.3 [9-59.1]	47 [33.9-61]	29	16.5 [6.7-35.4]	27.1 [15.5-39.8]
IL	120	53.5 [44.8-62]	52.4 [45.9-59]	108	54.8 [46.9-62.9]	65.2 [57.9-72]	55	37.7 [20.8-54.1]	39.9 [30.6-50.1]
IN	139	25.7 [16.9-41.7]	46.9 [40.7-53.3]	122	14.1 [8.9-40]	39.2 [32.7-45.7]	70	24.9 [19.4-27.6]	30.8 [23.9-38.4]
IA	91	42.3 [32.7-54.1]	54.4 [47.4-61.9]	83	61.5 [53.1-67.5]	62.4 [55.1-69.7]	40	44.5 [26.7-58.5]	32.7 [24.1-41.6]
KS	29	19.8 [9.8-33.9]	32.6 [22.2-44]	30	40.2 [34.1-54.9]	46.7 [34.9-59.1]	24	20.1 [14.6-26.4]	23.6 [14.5-33.1]
KY	112	34.4 [26.4-43.5]	35.7 [29.4-41.7]	95	28.5 [19.8-39.3]	37.5 [30.9-44.7]	81	30.3 [23.8-37.4]	32.3 [25.2-39.6]
LA	13	47 [15-65.2]	25.9 [12.3-40.2]	<10	- [---]	- [---]	12	17.3 [7.6-39.1]	35.7 [16.4-56]
ME	78	45.7 [32.7-61.4]	53.1 [44.6-61.4]	71	35.9 [28.1-45]	46.3 [37.7-55.3]	65	40.9 [16.8-62.5]	26.6 [18.5-35.4]
MD	152	36.3 [27-51.8]	60.5 [54.5-66.2]	193	26.6 [21.9-34.3]	37.2 [32.1-42]	162	27.8 [23.6-34.8]	39.1 [33.6-44.8]
MA	100	55.3 [36.1-68.3]	58.2 [50.8-65.2]	100	29.6 [21.5-43.2]	42.7 [35.4-50.1]	70	47 [23.3-66.9]	38.6 [30.8-46.5]
MI	284	26.8 [19.1-42.2]	64.7 [60.4-68.9]	223	30.7 [12.8-54]	43.8 [38.9-48.7]	203	29.8 [15.9-53.3]	42.4 [37.1-47.4]
MN	101	51.2 [39.4-70.3]	68.1 [60.9-75.1]	108	36.8 [18.7-50.7]	60.3 [54-67.2]	63	41.4 [37.5-54.2]	55.8 [46.4-64.8]
MS	12	34.7 [25.4-37.9]	24.4 [17-31.2]	<10	- [---]	- [---]	<10	- [---]	- [---]
MO	85	21.5 [17.1-28.7]	23.8 [18.3-29.9]	91	27.4 [20.9-35.3]	26 [19.5-33]	90	20.7 [16.1-25.7]	23.4 [18-29.5]
MT	22	39.2 [24.8-57.4]	53.6 [38.4-69.3]	26	36.4 [4-85.1]	32.8 [17.9-49.3]	20	18.8 [13.9-37.1]	22.2 [11.2-35.2]
NE	14	21.8 [13.2-48.1]	35.6 [22.3-50.9]	17	25.9 [13.1-65]	62 [43.4-79.6]	10	10.2 [6.5-28.8]	35.9 [17.3-56.8]
NV	16	26.6 [16.3-39.4]	34 [17.9-52.5]	15	64 [46.1-75.7]	53.9 [34.2-72.4]	12	32.4 [10-58]	23.8 [6.6-42.9]
NH	52	48.2 [29.8-57.2]	44.2 [33.8-55.1]	43	38.1 [24.3-51.7]	37.4 [26.9-47.8]	43	47.7 [21.1-70]	29.1 [19.3-40.3]
NJ	77	39.6 [26.7-53.3]	51.7 [43.3-60.3]	66	37.2 [25-53]	51.4 [42.5-59.9]	56	20 [10.2-34.4]	28.3 [20.4-37.1]
NM	18	44.2 [33.6-57.7]	48.4 [36.2-61.4]	20	43.7 [28.4-64.7]	53.3 [34.6-71.5]	17	47 [27.5-69.1]	41.5 [24.5-58.1]
NY	154	28.8 [24-36.9]	46.7 [41.1-52.2]	134	15.7 [6.8-28.4]	42.9 [36.6-49.6]	101	24.5 [14.6-42.6]	42.1 [35.4-48.5]
NC	258	42.5 [34.9-49.7]	50.7 [46.5-55.1]	201	24.5 [18.5-31]	34.2 [29.3-39.1]	129	24.4 [19-30.7]	32.2 [26.7-37.6]
ND	13	25.9 [18.5-37.2]	52.6 [36.3-69.8]	19	25 [12.9-36.9]	31.7 [20.7-45]	14	30.1 [21.1-40.4]	42.5 [30.5-56.5]
OH	211	51.4 [40.8-63.6]	51.7 [46.4-56.7]	186	27.3 [22.4-32.4]	34.4 [29.6-39.7]	140	31.6 [26.1-43]	37.7 [32-43.8]
OK	32	23 [13.4-31.4]	23.7 [15-33.5]	72	25.8 [21.9-37.5]	28.4 [21.3-35.7]	32	31.3 [8.8-41.8]	22.6 [13.9-32]
OR	122	25.6 [1.8-46.1]	35.8 [29.9-42.3]	112	29.6 [13.8-44.7]	50.5 [43.4-57.1]	83	23.5 [9-36.7]	37.5 [29.5-46]
PA	607	46.7 [41.6-52.3]	55.2 [52.2-58.2]	674	43.4 [37.2-49.8]	47.1 [44.3-50]	556	36.3 [30.2-42.6]	42.7 [39.5-45.9]
PR	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
RI	19	51.2 [36.6-65.9]	57 [39.8-74.2]	16	29.5 [13.1-48.2]	26.5 [11.2-43.7]	12	29.1 [9-59.6]	39.9 [17.5-63.3]
SC	64	27.4 [21-41.5]	43.1 [34.3-52]	41	12.8 [10.8-21.8]	25.1 [15.2-35.2]	43	29.3 [15-37.2]	25.9 [17.4-35.7]
SD	14	23.8 [16.3-34.9]	57.8 [42.5-74.1]	14	35.8 [13.7-51.7]	34.8 [22.1-48.6]	15	21.6 [9.1-36.1]	31.2 [14.7-51]
TN	80	62.9 [42.5-80.9]	46.4 [38.5-54.4]	68	44.5 [33.1-53.2]	38.7 [30.8-47]	44	28.4 [15.2-39.9]	35.7 [24.1-47.6]
TX	101	27.8 [17.1-44]	26.9 [21.3-33.1]	140	43.5 [19.2-69.1]	25.1 [20.4-30]	96	20.7 [11-33.4]	19.3 [14.7-25.1]
UT	163	35.7 [28.7-43.5]	43.3 [36.4-50.1]	116	46 [21.4-69.7]	62.6 [55.8-70]	70	49.1 [13.2-80.4]	30.5 [21.9-40.2]
VT	57	52.4 [41-60.9]	64.4 [55-74.1]	49	32.5 [10.8-65.7]	59.5 [50.1-68.4]	35	38.2 [17.8-67.9]	35.5 [23.9-48.7]
VA	551	59.1 [53.3-64.9]	59 [55.9-62.2]	621	32.6 [28.6-36.6]	43.1 [40.2-46.2]	269	29.4 [25.4-33.7]	35.2 [31.2-39.2]
WA	134	22 [4.2-40]	36.7 [30.4-43.3]	133	29.1 [11.6-39.3]	59.2 [52.7-65.4]	73	31.3 [23.8-44.5]	42 [33.3-51]
WV	60	43 [34.7-56.1]	48.1 [39.5-56.6]	41	32.6 [22.5-44.3]	48.9 [37-60.5]	20	42.2 [31.9-52]	39.6 [26.5-53.1]
WI	156	22.6 [16.5-35.7]	73.5 [68.5-78]	121	20.9 [9.8-41.5]	52.3 [45.9-59]	88	39.9 [8.5-70]	41.3 [33.8-48.1]
WY	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	23.4 [7.9-40.3]	31.6 [11.9-54]
Other	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
Multi State	117	24.8 [19.1-30.6]	40.1 [35-45.1]	120	30.4 [22-40.1]	32.1 [27.5-36.9]	86	24.1 [17.1-31.3]	33.6 [28.3-39.1]

Table 26: A summary of managed *Apis mellifera* honey bee Annual colony losses in the United States by states during three survey years – 2017-18, 2018-19 and 2019-20. Total and Average loss (%) are listed for all 50 states, the federal District of Columbia, and five permanently inhabited self-governing territories for the Annual period (1 April 201X – 1 April 201Y, whereby "X" and "Y" represent successive years). Estimates are not reported for states with less than 10 respondents to protect their privacy.

	Survey Year 2017-18			Survey Year 2018-19			Survey 2019-20		
State	N	Total Loss [95 % CI]	Average Loss [95% CI]	N	Total Loss [95 % CI]	Average Loss [95% CI]	N	Total Loss [95 % CI]	Average Loss [95% CI]
AL	52	45.4 [31.5-60.3]	39.7 [31.4-48.2]	61	32.9 [26.9-37]	30.3 [22.8-37.6]	61	46.4 [39.4-51.1]	44.5 [38.1-51.1]
AK	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
AZ	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
AR	32	47.1 [36.3-56.7]	42.2 [32.1-52.8]	43	40.3 [30.8-52.3]	44.6 [34-55]	27	46.4 [28.9-54]	37.4 [27.9-47.7]
CA	111	34.8 [27.5-43.7]	41.5 [36.9-46.4]	130	39.7 [34.4-45.4]	45.1 [40-49.8]	100	41.8 [28.7-56.5]	46.1 [40.3-51.5]
CO	81	30.6 [17.3-58.1]	54.3 [46.6-62.5]	101	34.7 [20.1-58.1]	51 [43.4-58.7]	61	51.7 [41.4-61.2]	47.6 [38.8-55.6]
CT	33	72.3 [60.7-83.3]	64.9 [53.4-75.5]	20	40 [23.3-61.3]	47.1 [30-64.5]	32	56.5 [40.3-72.6]	50.7 [39.4-62.8]
DC	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
DE	19	50 [36.6-66.7]	66 [51.4-79.8]	13	53.3 [41.1-65.4]	54.2 [38.9-69.6]	11	50.5 [32.2-67.7]	49.8 [33.1-65.1]
FL	52	40.8 [25.4-60.5]	38.7 [31.3-45.9]	32	39.7 [27-49.1]	32.9 [23.6-42.7]	23	40.3 [31.7-48.5]	41.7 [29.9-54.1]
GA	132	43.9 [30.6-64.6]	55.2 [49.5-60.6]	81	50.3 [42.3-57.6]	48.3 [41.4-55.2]	75	52.2 [28.8-80.9]	41.5 [35.6-47.8]
HI	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
ID	26	35.1 [26.6-43.8]	41.4 [28.4-55.6]	24	35.4 [21.1-47.7]	52.3 [38.8-65.2]	22	28.1 [16.1-51.6]	42.7 [28.8-56.7]
IL	100	58.6 [50.7-66.8]	60.4 [53.9-66.9]	80	57.9 [48.6-67.5]	63.2 [55.3-70.7]	39	40.1 [29.7-52.7]	46.9 [36-57.8]
IN	115	33 [24.3-50.8]	52.4 [46.4-58.6]	97	37.6 [25.6-60]	45.9 [40-52.2]	65	31.4 [27.6-37.9]	42.8 [35.6-49.5]
IA	82	59.4 [50.5-63.8]	59.6 [52.4-66.5]	68	67.3 [55.1-75.3]	61.8 [53.8-69.4]	34	62.6 [34.4-81.2]	47.5 [38.1-56.8]
KS	25	41.5 [28.2-50.6]	38.6 [27.4-50.4]	28	56.1 [47.7-68.7]	54.7 [41.5-67]	23	34.6 [26.3-43]	34.1 [23.4-46.6]
KY	96	52.1 [39.1-60.2]	45.9 [39.5-52.2]	85	40.2 [31.5-49.4]	45.6 [38.5-53.3]	67	46.9 [39-56.4]	45.5 [38.2-53]
LA	11	55.3 [23.4-72.8]	35.2 [18.8-49.8]	<10	- [---]	- [---]	<10	- [---]	- [---]
ME	57	56.1 [41-71.4]	53.8 [45.4-63.1]	49	42.6 [34.1-51.6]	48.6 [38.8-57.3]	46	36.7 [22.2-47.7]	35.9 [27-44.8]
MD	121	51.6 [45.5-64.2]	64.2 [58.4-70.3]	162	35.3 [32.2-42.1]	44.2 [38.9-49.3]	136	39.5 [35.7-45.8]	47.3 [42.1-52.7]
MA	83	61 [42.3-73.7]	56.8 [49.9-63.4]	69	46.1 [39-54.3]	48.7 [40.8-57]	51	36.6 [28.3-49.3]	44.4 [35.6-53.4]
MI	198	37.7 [28.2-57.7]	68.1 [63.5-72.4]	153	45.1 [39.4-54.1]	48.9 [42.7-54.6]	135	53.3 [30.8-80.6]	56.5 [50.7-62.4]
MN	74	60.3 [48.3-77.8]	72.2 [65.1-79.3]	76	48 [29.7-59.5]	64.7 [57.3-72.8]	40	55 [53-64.8]	64.1 [53.9-73]
MS	12	47.4 [33.5-54.2]	35.3 [24.4-46]	<10	- [---]	- [---]	<10	- [---]	- [---]
MO	70	34 [27.3-41.2]	35 [28-41.7]	79	35.9 [29-43.2]	34 [27-41.4]	72	32 [26.2-38.5]	33.4 [27.4-39.9]
MT	17	61.6 [43.7-77.4]	56.9 [40.3-73.1]	19	16.8 [11.3-18.4]	39.2 [24.2-57.6]	19	24.1 [18-47.8]	28.2 [16.2-41]
NE	13	37.8 [17.6-75.1]	48 [31.6-64.1]	13	35.9 [22.6-76]	64.6 [48.5-80.5]	<10	- [---]	- [---]
NV	12	43.6 [22.8-69.6]	42.1 [19.7-64.8]	15	70.1 [55.1-79.9]	57.8 [37.8-76.4]	12	53.4 [33-72.6]	44.9 [27.6-61.6]
NH	40	52.8 [37.5-60.4]	50.3 [38.9-61.2]	29	45 [28-62.4]	51.3 [39.4-62.1]	31	37.7 [29.6-51.8]	40.3 [29.9-51.1]
NJ	68	46.3 [34.8-59.1]	58 [49.9-65.9]	54	41.2 [26.7-58.4]	55.3 [46.5-64.1]	51	30.5 [18.4-48.6]	38.8 [31.2-47]
NM	17	64.9 [41-83.8]	52.9 [41.1-66.1]	14	54.3 [35.1-80.7]	68 [49.5-86.8]	11	58.5 [42.4-75]	55 [35.5-74.1]
NY	121	40.7 [36-46.7]	53 [47.6-58.8]	103	30.2 [25.2-37.2]	47.9 [40.8-55]	72	33.4 [20.7-54.9]	50.3 [42.9-58]
NC	226	55.1 [50.8-59.9]	60.6 [56.9-64.4]	176	33.4 [27.1-40.3]	42.1 [37.2-46.9]	115	38.5 [31.3-45.9]	41.5 [35.8-46.8]
ND	12	32.6 [22.9-45.4]	60.3 [43.4-76.5]	18	38.1 [29-47.7]	41 [31.8-51.2]	13	59.9 [45.3-70.6]	60.1 [50.6-69.7]
OH	169	59.4 [49.2-70.3]	55.9 [50.7-60.9]	142	32.8 [26.8-39.5]	38.1 [32.7-43.6]	125	42 [34.6-55.9]	46.4 [40.3-52]
OK	26	40.9 [20.6-58.7]	34.5 [22.9-46.6]	62	35.6 [29.4-52.1]	37.7 [30.4-45]	27	42.7 [19.8-54.2]	31 [22.4-40.1]
OR	92	35.8 [12.1-55.3]	43.1 [36-50.8]	97	40.2 [23.2-53.8]	55.6 [48.7-62.4]	67	41.3 [36.7-46.1]	46.2 [37.3-55]
PA	456	53.2 [47.9-58.7]	59.9 [56.7-63.2]	486	46.8 [41.5-52.6]	52.1 [48.8-55.2]	468	44.5 [39-49.7]	50.9 [47.8-54]
PR	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
RI	14	61.9 [42.9-76.8]	67.6 [50.9-82.9]	13	33 [16.7-51]	28 [12.7-43.8]	<10	- [---]	- [---]
SC	61	40.2 [34.9-53]	51.7 [43-60.6]	35	32.9 [19.5-41.9]	35.9 [24.9-47.4]	40	40.9 [28.2-46.7]	42.5 [33.2-51.6]
SD	13	29 [18.9-52.9]	62.8 [48.3-77.9]	12	43 [27-59.3]	42.5 [27.2-57.7]	14	37.9 [20-61.9]	44.1 [27.2-60.4]
TN	74	72.9 [54.3-86.4]	59.2 [51.9-66.2]	60	48.9 [36.3-59.5]	42.4 [33.9-50.3]	41	40.8 [26.8-51.7]	43.5 [32.4-54.5]
TX	90	35.2 [22.7-53.9]	37.9 [31.7-44.2]	127	42 [31.3-52.1]	34.1 [29.1-38.8]	90	54.5 [31.4-69.9]	32.8 [27-38.8]
UT	127	44.9 [38.2-52.3]	53.7 [47.2-60.1]	94	53.7 [30.6-77.6]	65.3 [58-72.7]	54	65.2 [21.5-91.9]	44.3 [35-52.9]
VT	44	57 [48.4-65.1]	68.3 [59-77]	36	40.8 [19.5-73.3]	66.5 [55.9-76.6]	24	35.8 [23-41.3]	37.6 [24.5-51.9]
VA	460	65.3 [60.4-70]	68.1 [65.1-70.8]	495	41.4 [36.9-46.1]	49.8 [46.5-52.8]	226	44.3 [37.8-50.8]	45.6 [41.7-49.6]
WA	96	24.9 [12.7-43.9]	48.2 [41.8-54.8]	113	44.1 [37.8-50.8]	66.5 [60.2-72.4]	52	46 [29.2-62.8]	57.7 [48.3-66.6]
WV	48	54.8 [48.2-65.4]	56.7 [48.6-65]	35	41 [28.8-54.5]	51.8 [39.2-63.9]	18	51.1 [41.7-59.3]	50.8 [37.7-63.1]
WI	116	34.6 [26.9-71.7]	78.6 [74.3-82.7]	90	42.2 [30.4-54]	56.8 [50.3-63.4]	55	52.9 [16.4-83.3]	53.7 [45.7-61.2]
WY	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
Other	<10	- [---]	- [---]	<10	- [---]	- [---]	<10	- [---]	- [---]
Multi State	100	34.1 [27.6-41.5]	47.2 [42-52.2]	107	39.5 [33.6-45.3]	39.5 [34.7-44.1]	73	49.6 [35.9-61.9]	47.2 [41.4-53.4]

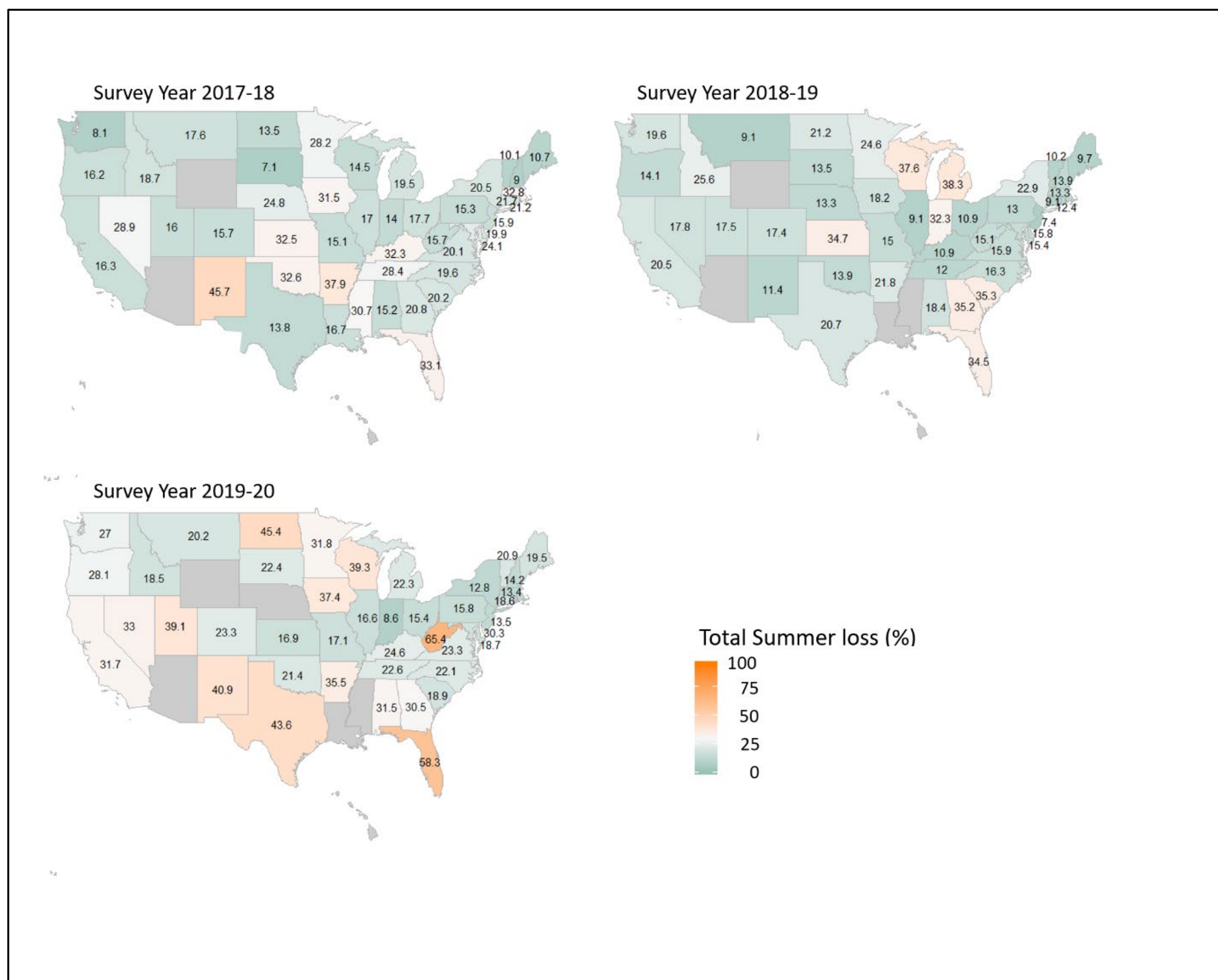


Figure 17: Managed *Apis mellifera* honey bee colony Total Loss estimates during Summer per state for three survey years in the United States – 2017-18, 2018-19 and 2019-20. Maps represent state-specific Total Summer loss estimates [%] (Summer: 1 April 201X – 1 October 201X). State-specific estimates, written within or near each state boundary, were only calculated if there were more than ten respondents.

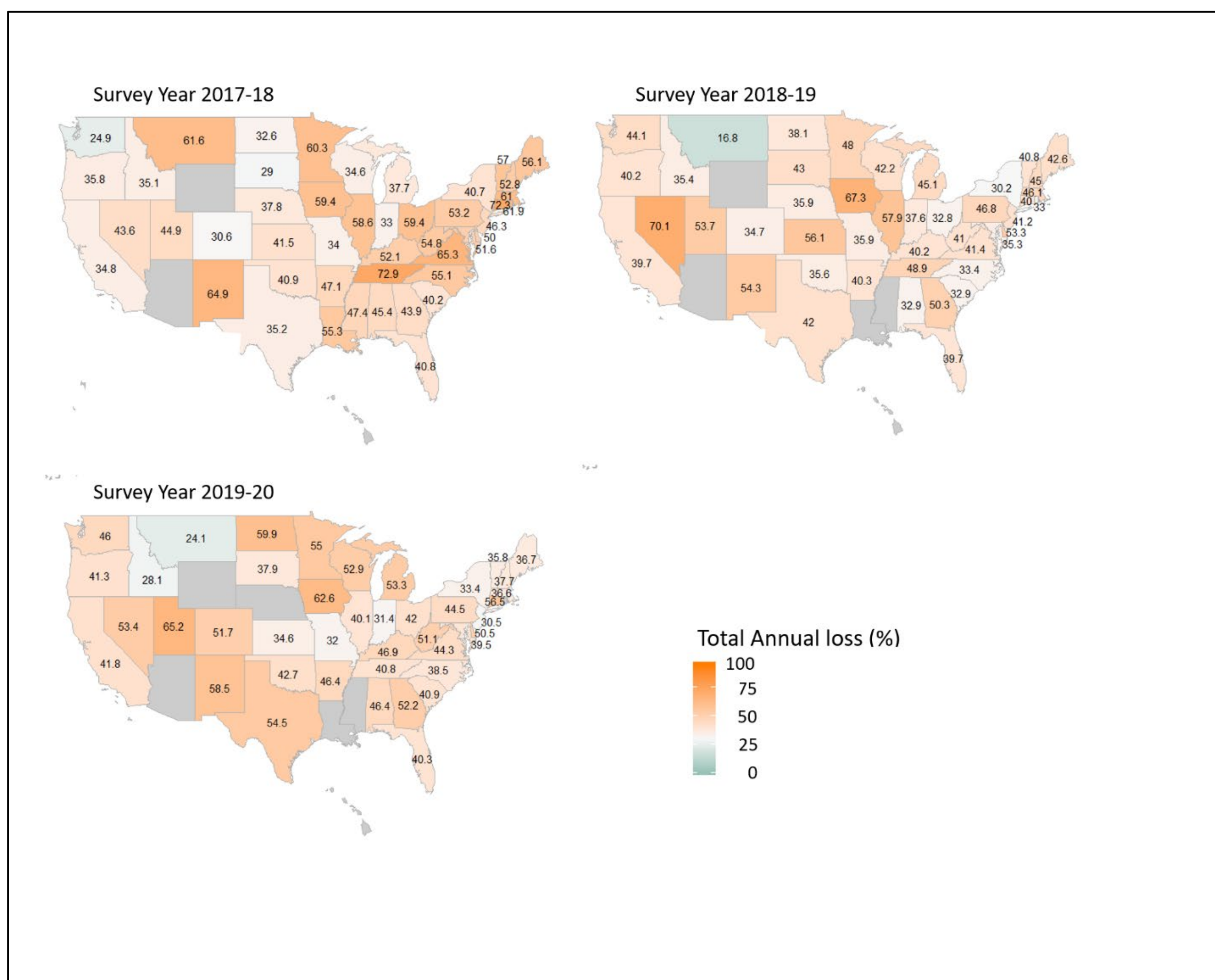
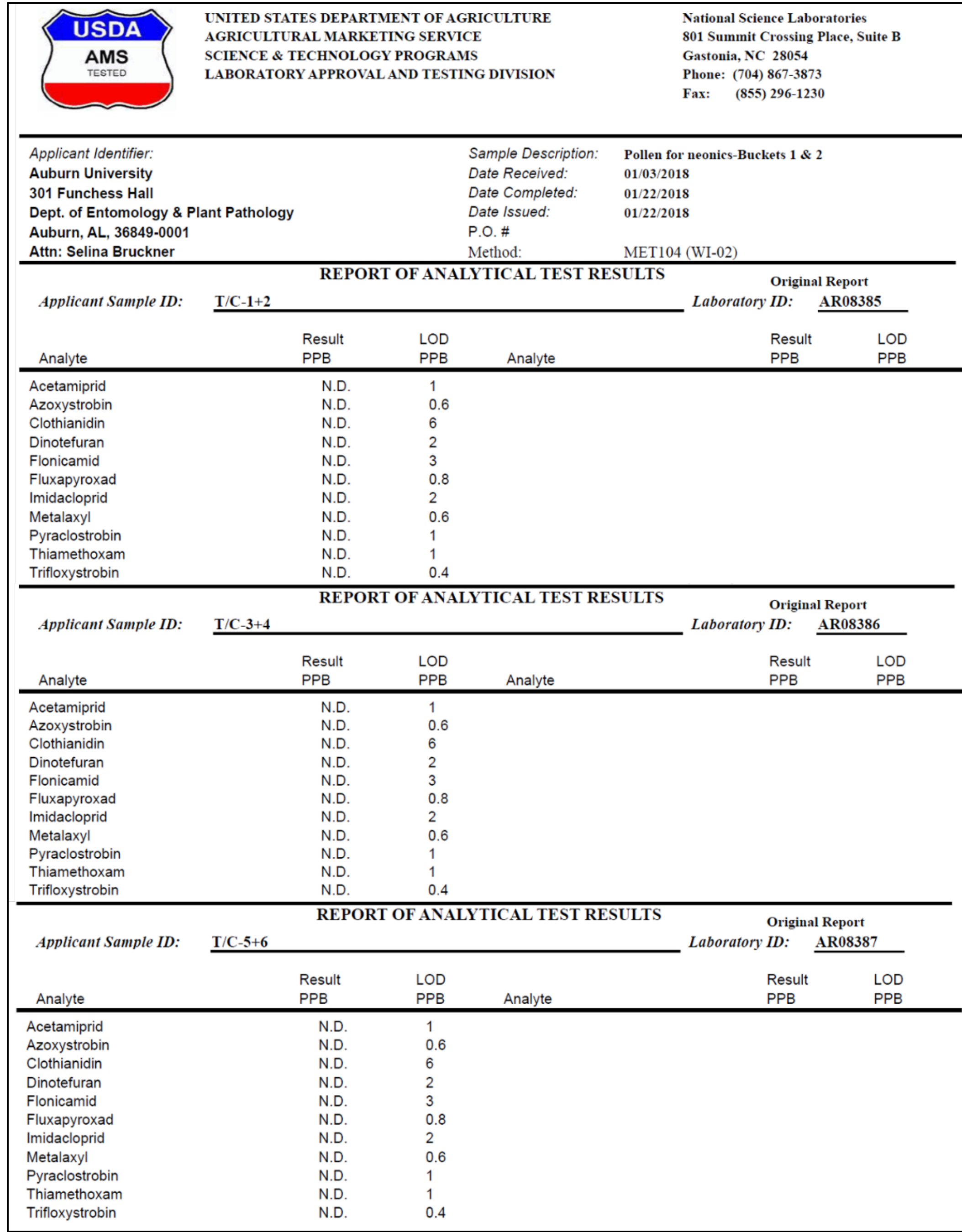


Figure 18: Managed *Apis mellifera* honey bee colony Total Loss estimates during Annual per state for three survey years in the United States – 2017-18, 2018-19 and 2019-20. Maps represent state-specific Total Annual loss estimates [%] (Annual: 1 April 201X – 1 April 201Y, whereby "X" and "Y" represented successive years). State-specific estimates, written within or near each state boundary, were only calculated if there were more than ten respondents.

Appendix 2 – Neonicotinoid residue analyses in corbicular pollen



USDA AMS TESTED

UNITED STATES DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
SCIENCE & TECHNOLOGY PROGRAMS
LABORATORY APPROVAL AND TESTING DIVISION

National Science Laboratories
801 Summit Crossing Place, Suite B
Gastonia, NC 28054
Phone: (704) 867-3873
Fax: (855) 296-1230

Applicant Identifier:
Auburn University
301 Funchess Hall
Dept. of Entomology & Plant Pathology
Auburn, AL, 36849-0001
Attn: Selina Bruckner

Sample Description: **Pollen for neonics-Buckets 1 & 2**
Date Received: **01/03/2018**
Date Completed: **01/22/2018**
Date Issued: **01/22/2018**
P.O. #
Method: MET104 (WI-02)

REPORT OF ANALYTICAL TEST RESULTS

Original Report

Applicant Sample ID: **T/C-1+2** ***Laboratory ID:*** **AR08385**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Fluxapyroxad	N.D.	0.8			
Imidacloprid	N.D.	2			
Metalaxyl	N.D.	0.6			
Pyraclostrobin	N.D.	1			
Thiamethoxam	N.D.	1			
Trifloxystrobin	N.D.	0.4			

REPORT OF ANALYTICAL TEST RESULTS

Original Report

Applicant Sample ID: **T/C-3+4** ***Laboratory ID:*** **AR08386**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Fluxapyroxad	N.D.	0.8			
Imidacloprid	N.D.	2			
Metalaxyl	N.D.	0.6			
Pyraclostrobin	N.D.	1			
Thiamethoxam	N.D.	1			
Trifloxystrobin	N.D.	0.4			

REPORT OF ANALYTICAL TEST RESULTS

Original Report

Applicant Sample ID: **T/C-5+6** ***Laboratory ID:*** **AR08387**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Fluxapyroxad	N.D.	0.8			
Imidacloprid	N.D.	2			
Metalaxyl	N.D.	0.6			
Pyraclostrobin	N.D.	1			
Thiamethoxam	N.D.	1			
Trifloxystrobin	N.D.	0.4			

Figure 19: Analyses of neonicotinoid concentration in corbicular pollen (2018). Six batches of corbicular pollen were tested in 2018 for the presence of detectable levels of neonicotinoid insecticides (T/C1+2, T/C3+4, T/C5+6, T/C7+8, T/C9+10, and T/C11+12). The pollen was sourced from *Apis mellifera* Linnaeus honey bee colonies residing in a low intensity agricultural region of Colorado and used to make pollen patties for subsequent treatment exposure. Analyses confirmed that the pollen did not contain detectable levels of neonicotinoids (N.D.).

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Phone: (704) 867-3873
Fax: (855) 296-1230

Applicant Identifier:
Auburn University
301 Funchess Hall
Dept. of Entomology & Plant Pathology
Auburn, AL, 36849-0001
Attn: Selina Bruckner

Sample Description: Pollen for neonics-Buckets 7 & 8
Date Received: 01/03/2018
Date Completed: 01/22/2018
Date Issued: 01/22/2018
P.O. #
Method: MET104 (WI-02)

REPORT OF ANALYTICAL TEST RESULTS

Original Report

Applicant Sample ID: T/C-7+8 ***Laboratory ID:*** AR08388

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Fluxapyroxad	N.D.	0.8			
Imidacloprid	N.D.	2			
Metalaxyl	N.D.	0.6			
Pyraclostrobin	N.D.	1			
Thiamethoxam	N.D.	1			
Trifloxystrobin	N.D.	0.4			

REPORT OF ANALYTICAL TEST RESULTS

Original Report

Applicant Sample ID: T/C-9+10 ***Laboratory ID:*** AR08389

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Fluxapyroxad	N.D.	0.8			
Imidacloprid	N.D.	2			
Metalaxyl	N.D.	0.6			
Pyraclostrobin	N.D.	1			
Thiamethoxam	N.D.	1			
Trifloxystrobin	N.D.	0.4			

REPORT OF ANALYTICAL TEST RESULTS

Original Report

Applicant Sample ID: T/C-11+12 ***Laboratory ID:*** AR08390

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Fluxapyroxad	N.D.	0.8			
Imidacloprid	N.D.	2			
Metalaxyl	N.D.	0.6			
Pyraclostrobin	N.D.	1			
Thiamethoxam	N.D.	1			
Trifloxystrobin	N.D.	0.4			

Figure 19 (continued): Analyses of neonicotinoid concentration in corbicular pollen (2018). Six batches of corbicular pollen were tested in 2018 for the presence of detectable levels of neonicotinoid insecticides (T/C1+2, T/C3+4, T/C5+6, T/C7+8, T/C9+10, and T/C11+12). The pollen was sourced from *Apis mellifera* Linnaeus honey bee colonies residing in a low intensity agricultural region of Colorado and used to make pollen patties for subsequent treatment exposure. Analyses confirmed that the pollen did not contain detectable levels of neonicotinoids (N.D.).

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National Science Laboratories
801 Summit Crossing Place, Suite B
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Phone: (704) 867-3873
Fax: (855) 296-1230

Applicant Identifier:
Auburn University
301 Funchess Hall
Dept. of Entomology & Plant Pathology
Auburn, AL, 36849-0001
Attention: Selina Bruckner

Sample Description: **Crude Wildflower Pollen (Neonic Testing)**
Date Received: 01/24/2019
Date Completed: 03/14/2019
Date Issued: 03/14/2019
P.O. #
Method: MET104 (WI-02)

REPORT OF ANALYTICAL TEST RESULTS

Original Report

***Applicant Sample ID:* ZWFB 1-10** — ***Laboratory ID:* AS10542**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Imidacloprid	N.D.	2			
Thiamethoxam	N.D.	1			

***Applicant Sample ID:* ZWFB 11-19** — ***Laboratory ID:* AS10543**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Imidacloprid	N.D.	2			
Thiamethoxam	N.D.	1			

***Applicant Sample ID:* ZVB 1-10** — ***Laboratory ID:* AS10544**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Imidacloprid	N.D.	2			
Thiamethoxam	N.D.	1			

***Applicant Sample ID:* ZVB 11-19** — ***Laboratory ID:* AS10545**

Analyte	Result PPB	LOD PPB	Analyte	Result PPB	LOD PPB
Acetamiprid	N.D.	1			
Azoxystrobin	N.D.	0.6			
Clothianidin	N.D.	6			
Dinotefuran	N.D.	2			
Flonicamid	N.D.	3			
Imidacloprid	N.D.	2			
Thiamethoxam	N.D.	1			

Figure 20: Analyses of neonicotinoid concentration in corbicular pollen (2019). Four batches of corbicular pollen were tested in 2019 for the presence of detectable levels of neonicotinoid insecticides (ZWFB1-10, ZWFB11-19, ZVB1-10 and ZVB 11-19). The pollen was sourced from *Apis mellifera* honey bee colonies residing in a low intensity agricultural region of Colorado and used to make pollen patties for subsequent treatment exposure. Analyses confirmed that the pollen did not contain detectable levels of neonicotinodis (N.D.).

Appendix 3 –Pollen Patties: Protocol for Field Season 2018, 2019 and 2020

Technical grade materials

37924-100MG-R Thiamethoxam

33589-100MG Clothianidin PESTANAL

Required patties: 100 g X 55 days X 16 colonies = 88 kg

Round up to 100 kg pollen patties → 50 kg per treatment group

Note: Adjust calculations based on number of colonies per treatment group

Composition per treatment

	Parts (%)	Requirement netto (kg)	+ 7.5% losses (kg)	Total requirements (kg)
Pollen	60%	30	2.25	**32.25**
Honey	10%	5	0.375	**5.375**
Powder sugar	30%	15	1.125	**16.125**
Total	100%	50		**53.75**
H_20	Ca.0.576%			**288 ml**

Concentration for toxics required (for 53.75 kg pesticide treatment in total):

- Thiametoxam: 4.5 µg / kg (4.5 ppb) : 4.5 µg / kg * 53.75 kg = **241.875 µg in total**
- Clothianidin: 1.5 µg / kg (1.5 ppb) : 1.5 µg / kg * 53.75 kg = **80.625 µg in total**

Step 1: Stock pesticide solution (in MilliQ-Water + Aceton)

Thiametoxam: Exactly **45.2 mg** adissolved into **50.0 ml** water (1 hour with sonication at 50°C water bath). Final concentration thiamethoxam: **0.904 mg / ml** = **904 ug / ml**; stored in a glass vial at 4°C.

Clothianidin: Exactly **69.4 mg** dissolved into **3.75 ml** acetone + **47** ml water (1 hour with sonication at 50°C water bath). Final concentration clothianidin: **1.367** mg/ml **= 1376 µg / ml**; stored in a glass vial at 4°C.

Step 2: Dilute Stock Solution to 10 ug / g

- Thiamethoxam: 1.0 mg / 0.904 mg/ml = **1.1062 ml = (1000 µg/ml solution)**

  1.1062 ml (1000 ug/ml solution) + 100 ml H_2O = **10 µg/ml**

- Clothianidin: 1.0 mg / 1.367 mg/ml = **0.7315 ml = (1000 µg/ml solution)**

  0.7315 ml (1000 ug/ml solution) + 100 ml H_2O = **10 µg/ml**

Step 3: Volume of Pesticide Solution (10 µg/ml) required per Treatment

5.375 kg Honey required per Treatment

For the pesticide:

- **24.19** ml thiametoxam 10 µg/ml (final concentration 4.5 ppb)
- **8.07** ml clothianidin 10 µg/ml (final concentration 1.5 ppb)

For the control:

- **24.19** ml **water**
- **8.07** ml (water + diluted **acetone**)

Do 3 batches of pollen patties per treatment group

	(kg)	Partition to 3 (kg)
Pollen	**32.25**	10.75
Honey	**5.375**	1.79
Powder Sugar	**16.125**	5.375
Total	**53.75**	17.915 per batch