Effects of neonicotinoid exposure on the strength of honey bee (*Apis mellifera*) colonies

by

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Abstract

A growing body of research continues to document and describe the unintended effects of insecticides on non-target organisms, such as insect predators, parasitoids, and pollinators. Specifically, different classes of insecticides, including neonicotinoids, have been documented to negatively influence honey bee, *Apis mellifera* L., health. Despite a large body of research on the effects of exposure to individual honey bees, few studies have explored effects on colony-level performance. Consequently, I sought to reduce this knowledge gap by performing colony-level experiments that examined the effects of neonicotinoids on the quantities of workers and food stores, and how colony genetic diversity may buffer such effects. Overall, results highlighted the negative effects of the neonicotinoid on all colony parameters investigated in one study, but that neither neonicotinoid exposure or genetic diversity, as managed for my experiment, had little influence on the second study. This work sheds light on the effects of field-realistic concentrations of this widely used class of insecticides on an important non-target organism.

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Statement of purpose

Honey bees, *Apis mellifera* L. (Hymenoptera: Apidae) have experienced recent severe colony loss due to multiple factors acting singly or in combination (Havard et al., 2020; Vanengelsdorp & Meixner, 2010). Neonicotinoid insecticides are routinely used on flowering crops visited by insect pollinators, and are believed to negatively impact individual honey bees. Here, I sought to evaluate the effects of two neonicotinoids on honey bee colonies, and determine the influence of genetic diversity of colonies to managed possible negative effects.

1.1 Honey bees

Honey bees, *Apis mellifera* L., provide vital pollination services to crops and wild plants that are necessary for the maintenance of biodiversity and food security through agricultural productivity (Gallai et al., 2009; Garibaldi et al., 2011; Klein et al., 2007). The ease of maintenance and transportation of honey bees to pollinator-dependent crops has earned them being called the single most valuable animal pollinators to agriculture. Beyond agriculture, the honey bee is also a highly valued resource around the world. For example, they are prized for their honey and wax production (Vanengelsdorp & Meixner, 2010). Of the insect pollinators, honey bees exhibit a eusocial level of social organization and are one of the most efficient pollinators owing to this sociality and biology which allow for the management of very large colonies (Degrandi-Hoffman et al., 2019). Like other eusocial organisms, honey bees exhibit overlapping generations, cooperative brood care, and reproductive division of labor (Nowak et al., 2010; Wilson & Holldobler, 2005). In the case of honey bees, three different types of individuals - the queen, worker, and drone - perform intertwined roles and live together (Hossam & Abou-Shaara, 2015).

The main role of the queen, which is a type of female honey bee, in the colony is reproduction and colony functioning (Leimar et al., 2012), including successful mating flights that can initiate molecular, physiological, and behavioral changes (Niño et al., 2013). The developing queens are provided with copious amounts of high-quality food known as “royal jelly” from the hypopharyngeal and mandibular glands of nursing workers (Milone et al., 2021). The queen is fully developed in 16 days, and they are the only perennial member of the colony, able to live several years, even though are commonly replaced annually by beekeepers (Sammataro and Avitabile, 1998). The queen's life span is linked to both quantity and quality of stored sperm, which is used to fertilize eggs she deposits, resulting in daughters of mixed paternity because of their polyandrous mating system (Ostroverkhova et al., 2016). As queens emerge from their brood cell as young adults, they embark upon polyandrous mating flights to look for drones (male honey bees) at drone congregation areas (DCAs). The queen can mate with an average of 12 drones upon reaching DCA. Subsequently, the healthy queen returns to the colony to oviposit female eggs of mixed paternity until stored sperm is depleted (Niño et al., 2013). With their polyandrous mating behavior, the genetic diversity of the colony's future progeny is increased; this has been shown to improve disease resistance (Ding et al., 2017; Ostroverkhova et al., 2016). The ability of a queen to mate with multiple drones is therefore imperative for the fitness of the colony (Forfert et al., 2017). It is known that as the queen begins to fail or lay fewer eggs, the workers may replace her (Sammataro and Avitabile, 2011). Recent investigations have identified failing honey bee queen health to be a primary cause of colony mortality (Genersch et al., 2010; Pettis et al., 2016; Vanengelsdorp et al., 2008).

The workers are another type of female honey bee, like the queen, and are fully developed in 21 days. Unlike the queen that is primarily responsible for laying eggs and producing

pheromones, workers construct the nest because of the presence of wax glands on the underside of their abdomens. They also perform many other tasks, such as foraging for pollen and nectar because of the pollen basket on their hind legs (Hossam & Abou-Shaara, 2015). Nurse bees are young workers that are not yet mature enough to go forage. They care for the adult queen and the developing brood by transferring proteinaceous secretions from their food glands (Winston, 1987). During the foraging season, workers will transition to act as foragers, moving out of the colony in search of propolis, water, pollen, and nectar (Calderone, 1998; Robinson, 1992; Seeley, 1995).

Drones are male honey bees and are fully developed in 24 days. Like the queen, they undergo morphological and physiological changes in their reproductive organs with age (Koeniger et al., 2014; Winston, 1987). They are present during the active period as they seek to mate with virgin queens at DCAs (Langowska & Zduniak, 2020), typically in spring and summer (Rangel & Fisher, 2019). Drones die after mating with the queen. In addition to fulfilling reproductive roles, drones contribute to some thermoregulation of brood in the hive (Harrison, 1987).

1.2 Honey bees colony mortality

Between 2019 – 2020, U.S. beekeepers lost 43.7% of their colonies (Bruckner et al., 2020) reportedly double what beekeepers considered normal historically (Kulhanek et al., 2017). Recent studies, such as by (Tosi et al., 2018) (2018) and (Straub et al., 2016), noted that honey bee health is compromised by complex multiple interactions. To date, it is believed that insecticides, introduced parasites, genetic background, climate change, and anthropogenic land-use practices all play important roles (Neumann & Carreck, 2010; Sandrock, Tanadini, Tanadini, et al., 2014; Williams et al., 2010). Honey bees have experienced severe colony loss due to multiple factors,

both biotic and abiotic, acting singly or in combination (Havard et al., 2020; Vanengelsdorp & Meixner, 2010). It is reported that within the last decade, colonies have experienced severe annual mortalities in the northern hemisphere (Hristov et al., 2020).

The environment is frequently mentioned as a potential cause of mortality of honey bee colonies, with food resources and climatic factors cited (Conte and Navajas, 2008; Hristov et al., 2020). Honey bee workers, for example, consume a variety of pollens to meet the majority of their requirements for protein and lipids (Corby-Harris et al., 2018). However, combined exposure to multiple insecticides can have synergistic adverse effects on honey bees as they feed on contaminated food resources (Gill et al., 2012; Iwasa et al., 2004; Sandrock, Tanadini, Tanadini, et al., 2014; Tosi et al., 2017). Researchers have described the effects of classes of insecticides like organophosphates, neonicotioids, organochlorines, and pyrethroids on honey bees (Li and Rangel, 2018; Sandrock, Tanadini, Tanadini, et al., 2014; Tosi et al., 2017). In particular, neonicotinoid insecticides, a ubiquitously employed class of agricultural insecticide (Blacquière et al., 2012; Goulson, 2013), have been implicated in honey bee colony mortality (Maxim & Sluijs, 2010), as foragers are exposed to many different agricultural chemicals while visiting flowers of treated crops (Mullin et al., 2010).

1.3 Neonicotinoid insecticides

Neonicotinoids are systemic insecticides commonly used on flowering crops visited by pollinators (Williamson et al., 2014). Neonicotinoids were developed in the 1980s (Goulson, 2013). Since their discovery in the late 1980s, they have become the most widely used class of insecticides worldwide, with vast applications ranging from crop protection to invertebrate pest control (Simon-Delso et al., 2015). These insecticides are synthetic compounds, structurally similar to nicotine, and target insect nicotinic acetylcholine receptors (nAChRs) (Millar &

Denholm, 2007). The increased preference for the usage of neonicotinoid usages such as imidacloprid, clothianidin, thiamethoxam, and thiacloprid is in part due to the their lower toxicity to vertebrates. This is because, compared to invertebrates, vertebrates have significantly fewer nAChRs (Simon-Delso et al., 2015). Although neonicotinoid insecticides are applied to control pests in a variety of crops, their residues in plants and plant parts pose a challenge to bees once exposed (Blacquière et al., 2012). Neonicotinoids, as a result of their extensive use and their physicochemical properties, are found in environmental compartments as soil, water, and air (Simon-Delso et al., 2015). Consequently, plant roots can pick up neonicotinoid residues and translocate them up to the plant shoot such as the leaf, flower, or fruiting bodies. Thus, a visit by a pollinator such as the honey bee can lead to unintended exposure (Colwell et al., 2017). The binding of neonicotinoids to an insect's nAChR induces continuous excitation of the neuronal membranes which can lead to paralysis, energy exhaustion, and memory loss (Simon-Delso et al., 2015; Tasman et al., 2021).

1.4 Impacts of neonicotinoid insecticides on honey bees

At the individual bee level, neonicotinoids can induce acute mortality, as well as a range of physiological (Renzi et al., 2016), mechanical (Williamson et al., 2014), and behavioral (Wright et al., 2015) responses. In addition, field-realistic concentrations of neonicotinoids induce several sublethal effects such as learning abilities, memory, and respiratory functions (Decourtye et al., 2003; Hatjina et al., 2013) on honey bees, thereby impairing the performance of the entire colony (Cutler et al., 2014; Osterman et al., 2019; Sandrock, Tanadini, Pettis, et al., 2014). For example, sub-lethal neonicotinoid exposure could have significant consequences for the honey bee colony through compromising the queens' cognitive abilities or immune system, thus reducing performance at the colony-level (Sandrock, Tanadini, Tanadini, et al., 2014). Studies have also

shown that honey bees exposed to neonicotinoids exhibit slower rates of learning and poor memory formation (Wright et al., 2015). Colony-level impacts of neonicotinoids on honey bees, however, have been investigated by researchers (Sandrock, Tanadini, Tanadini, et al., 2014; Tsvetkov et al., 2017; Zee et al., 2015). For example, Sandrock *et al*. (2014) reported significant unfavorable short and long-term impacts on colony performance and queen fate, and they thus suggest that neonicotinoids may contribute to colony weakening.

Thiamethoxam, along with its metabolite clothianidin, are relatively moderately toxic neonicotinoids, represent model neonicotinoids in many studies (Tasman et al., 2021), and are widely used against multiple insect pests. Their usage having been scrutinized by regulatory agencies in the United States (EPA, 2016) and the European Union (Blacquière et al., 2012; Bonmatin et al., 2015; *Guidance on Risk Assessment on Bees*, 2013). For example, a recent study investigating the effects of clothianidin on honey bee colonies demonstrated an increase in mortality of individual larvae and a decrease in the production of jelly in the individual worker caste (Schott et al., 2021).

1.5 Mitigating of neonicotinoid insecticides on honey bees

The need to continually protect agricultural plants from other insect pests while catering to the world's increasing human population size means that neonicotinoids and other insecticides will continue to be used in agroecosystems. Yet, concerted efforts must be made to mitigate exposure of neonicotinoids to unintended and useful insects such as the honey bee. The first of many steps is to limit exposure. This would require the need to strictly follow label instructions (Johnson, 2015). Keywords to look for include the level of toxicity of the insecticide on the pesticide label. Where and when possible, highly toxic insecticides and extended residual insecticides should not

be used. Similarly, pesticides should not be applied to the blooming part plants routinely visited by honey bees (Krupke et al., 2012). Second, the coordination of crop timing and pesticide application with dates of apiary arrival and departure can help reduce honey bee exposure (Frazier et al., 2015). Understandably, this approach would require synchrony between crop growers and beekeepers. Thirdly, in the advent of exposure to insecticide, honey bee colonies should be effectively managed to allow them to buffer against effects. The ability of honey bees to buffer against insecticide exposure is not new. For example, honey bee colonies were observed to compensate for exposure to clothianidin-treated seeds by decreasing royal jelly composition and increasing brood production (Schott et al., 2021).

But these approaches do not have to be always reactionary. A pro-active approach could be to use good food and nutrition in the development of honey bees to improve immunocompetence and detoxification. (Alaux et al., 2010) noted that protein feeding improved immunocompetence in honey bees. Reducing interactions with stressors such as *Varroa destructor* can boost the fitness and biological parameters of honey bees (Straub et al., 2019). These approaches could help improve the genetic diversity and super-organism resilience in honey bees (Straub et al., 2015).

1.6 Polyandry in honey bees

The habit of a honey bee queen mating with diverse drones is known as polyandry (Delaplane et al., 2015), and it has been noted to confer significant adaptive advantages that in turn positively affects colony productivity (Tarpy et al., 2013). For example, research has shown that promiscuous honey bee queens increased colony productivity by conquering the ability of workers to rear worker-derived males and concentrate on raising the queen progeny (Heather et al., 2012).

Given that the highest known mating frequencies in the social Hymenoptera are found in honey bees (Tarpy & Seeley, 2006), the degree of a queen's polyandry is positively associated with measures of colony fitness (Delaplane et al., 2015). Honey bee queens mate with unusually high numbers of males to produce a workforce with high diversity of worker genotypes within the colony (Tarpy et al., 2013).

Polyandry is achieved in different ways. Honey bee queens naturally embark on series of polyandrous mating flights to mate with different males at DCAs (Tarpy et al., 2013). Polyandry is also achieved through intra- and inter-specific brood transfer in a managed system (Tan et al., 2009). By way of artificial insemination and in contrast with taking advantage of natural selection to manage honeybee health, breeders choose drones of the right age, which have not made it to DCAs yet, and may therefore not have full reproductive potential (Neumann & Blacquière, 2017), for queen insemination (Delaplane et al., 2015).

1.7 Significances of polyandry

Honey bee susceptibility to insecticides may also be mitigated through polyandry. Multiple matings by queens may be significant for adaptation to disease as it results in genetically diverse subfamilies of workers with different alleles for disease resistance within a colony (Tarpy & Seeley, 2006). Previously, authors have hypothesized that genetic diversity may increase the behavioral diversity of worker force, which may enable colonies to extract resources more efficiently and that genetic diversity may reduce the prevalence of parasites and pathogens within the colony (Oldroyd et al., 1991, 1991; Tarpy, 2003). Polyandry ensures that the queen has enough sperm for her egg-laying lifetime (Tarpy et al., 2013). For example, hyper polyandrous queens have been observed to confer colony-level benefit that includes suppression of varroa mites populations (Delaplane et al., 2015). This observation illustrates the possible advantages of

polyandry on biotic effects. Interestingly, how polyandry affects non-biotic effects such as neonicotinoid insecticides remains unknown.

Thesis objective

The overall objective of my thesis research was to evaluate and describe the detrimental effects of thiamethoxam and its metabolite, clothianidin, on honey-bee colony strength by which means population of adult bees and brood. I did this by performing two colony-level studies experiment. First, I investigated the effects of field-realistic concentrations of thiamethoxam and clothianidin on honey bee population and food stores using 17 honey bee hives. Then, I investigated the effects of neonicotinoid exposure and colony genetic diversity on honey bee colony population, food stores using 24 honey bee hives.

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Chapter 2

Effects of neonicotinoid exposure on honey bee colony strength

2.1 Abstract

A growing body of research suggests unintended effects of insecticides on non-target organisms such as predators, parasitoids, and pollinators. Although different classes of insecticides have been documented to negatively influence honey bee (*Apis mellifera*) health, the effects of neonicotinoids on colony-level performance have not been fully explored. To help reduce this knowledge gap, I performed a colony-level experiment that examined the effects of neonicotinoids on quantities of worker adults, worker capped brood, honey, and pollen. Apart from worker capped brood, I found that all colony parameters were negatively affected by neonicotinoid exposure. This work sheds light on the effects of field-realistic concentrations of this widely used class of insecticides on an important non-target organism.

2.2 Introduction

The honey bee, *Apis mellifera* L., is an important pollinator for many crops. In the United States alone, the economic implications of its pollinating prowess are estimated to be around $18 billion (Calderone, 2012). Increased colony mortality in recent years has attracted interest among researchers and regulators (Fairbrother et al., 2014; Neumann & Carreck, 2010). Recently, several biotic and abiotic factors are believed to contribute to increased colony mortality, including the mite *Varroa destructor*, multiple viruses, weather, and insecticides (Decourtye et al., 2010; Neumann & Carreck, 2010; Sandrock et al., 2014).

Neonicotinoid insecticides, discovered in the late 1980s, have become the world's most widely used class of insecticides (Simon-Delso et al., 2015); they can bind to acetylcholine (AChE) receptors to interfere with the excitatory action of the neuron and act (Moffat et al., 2016). Given

their systemic properties, they are frequently applied to the exterior surfaces of seeds, after which they translocate through the plant tissues and become widespread throughout the plant, including pollen and nectar (Bromilow et al., 1990). Honey bees rely on these plant products for their primary sources of carbohydrates (Haydak, 1970) and protein (Corby-Harris et al., 2018; Standifer, 1967), respectively. Studies have also demonstrated exposure of honey bees to neonicotinoids via contaminated honey bee food (Girolami et al., 2009; Samson-Robert et al., 2014; Tapparo et al., 2011).

Several laboratory and field investigations involving the effect of neonicotinoids on individual honey bees have reported that field-realistic concentrations of neonicotinoids can elicit sub-lethal effects on learning ability, memory, and respiratory function (Decourtye et al., 2003; Hatjina et al., 2013) on individual honey bees. Additionally, Thiamethoxam, and it's metabolite clothianidin, are two commonly used neonicotinoids, and are relatively toxic to bees. For example, Monchanin et al. (2019) described the cost of sublethal exposure of forager honey bees to thiamethoxam to include homing flight failure, a vital normal function of the colony and the ecosystem service it provides. It is believed that these individual-level effects can ultimately lead to broader effects on the colony (Sandrock et al., 2014; Tosi et al., 2017). Thus, it is pertinent to explore ways to mitigate biotic and abiotic interactions with honey bees. Straub et al. (2015) noted that such mitigations could help improve honey bees' resilience and entrain eusociality in honey bees.

The objective of this study was to determine the effects of two neonicotinoids - thiamethoxam and clothianidin - on honey bee colonies. Based on the results of Sandrock et al., (2014), which were a similarly performed experiment but performed in Europe, I expected to observe a negative effect of the neonicotinoids on honey bee colonies.

2.3 Materials and Methods

2.3.1 Experimental set-up

Seven-teen honey bee packages were established in May 2019 in Auburn, Alabama on deep Langstroth hive equipment; each was headed by a laying sister queen and contained 1.36 kg of workers. Colonies were obtained from the Blue Ridge Honey Company (Lakemont, GA). The experiment included two treatment groups – Control and Neonicotinoid. Six were randomly assigned to the control treatment group, whereas, 11 were randomly assigned to the neonicotinoid treatment group.

2.3.2 Neonicotinoid exposure

Colony exposure to each treatment group was achieved through feeding with experimental pollen paste (60% pollen, 30% powdered sugar, 10% organic honey) (Figure 1) (Sandrock et al., 2014; Williams et al., 2015). Additionally, the neonicotinoid colonies received pollen paste that contained two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin; both Sigma Aldrich, St. Louis, MS) (Straub et al., 2016; Williams et al., 2015). Each colony was equipped with an entrance pollen trap during the entire experimental period to limit forager-collected corbicular pollen brought back to the hive from the surrounding environment to promote pollen paste feeding. Pollen paste was provided for 42 days to ensure colonies were exposed for at least two complete brood cycles (Friedli et al., 2020; Sandrock et al., 2014; Straub et al., 2016). These concentrations and exposure duration were chosen as they represent filed relevant concentrations reported to cause a range of lethal and sublethal effects in honey bees (Wood and Goulson, 2017).

Figure 1. Application of an in-hive pollen patty to a honey bee (*Apis mellifera*) colony. Pollen patties fed to colonies belonging to the neonicotinoid treatment group contained 4.5 ppb thiamethoxam and 1.5 ppb clothianidin.

2.3.3 Assessing honey bee populations and food stores

Colony strength assessments were conducted using the Liebefelder method of visual estimation (Delaplane et al., 2013). Detailed colony assessments, including estimates of worker adults, worker immatures (i.e. capped brood), and food stores (honey and pollen cells) were performed to assess the potential impact of the treatment on colony performance and productivity. Assessments were performed three times – at day 0, immediately before application of experimental pollen patty, on day 70, after one complete worker brood cycle of exposure, and at day 158, one complete worker brood cycle after final exposure of experimental pollen paste. These assessment dates were chosen based on the brood cycle. Two observers visually estimated the

surface area of a comb covered by worker adults, capped broods – by assuming all capped brood contain pupae – honey, and pollen (Delaplane *et al*., 2013). At each assessment, the worker adults, worker capped brood, food, and honey cells were first estimated visually based on percent coverage of each Langstroth deep frame side. The number of worker adults was calculated assuming 1,215 individuals can occupy each frame side; and the number of workers capped brood, honey, and pollen cells was calculated assuming 3.8 cells / cm^2 (Delaplane *et al*., 2013).

2.3.4 Statistical analyses

The R statistical software was used to perform all statistical analyses and to produce boxplots. For the analysis, we used generalized linear mixed models (GLMMs) to analyze data with repeated measures to explain the nested structure. To investigate the impact of neonicotinoids (thiamethoxam and clothianidin) on honey bee colony performance over time, we examined all critical endpoint measurements in the framework of mixed effect models. Colonies were treated as random effects, while treatment groups (control and neonicotinoids) and assessment points (days 0, 70, and 158) as fixed effects.

2.4 Results

2.4.1 Worker adults

As expected, at day 0 there was not a significant difference in the population of worker adults between neonicotinoid-exposed and control colonies before treatment application (Fig. 1) ($P = 0.89$ and F-value= 0.02). In contrast, the number of worker adults on day 70 ($P = 0.07$ and F-value = 3.86) and 158 ($P = 0.07$ and F-value= 3.76) was observed to be significantly higher for the control group compared to the neonicotinoid-exposed colonies (Fig. 2).

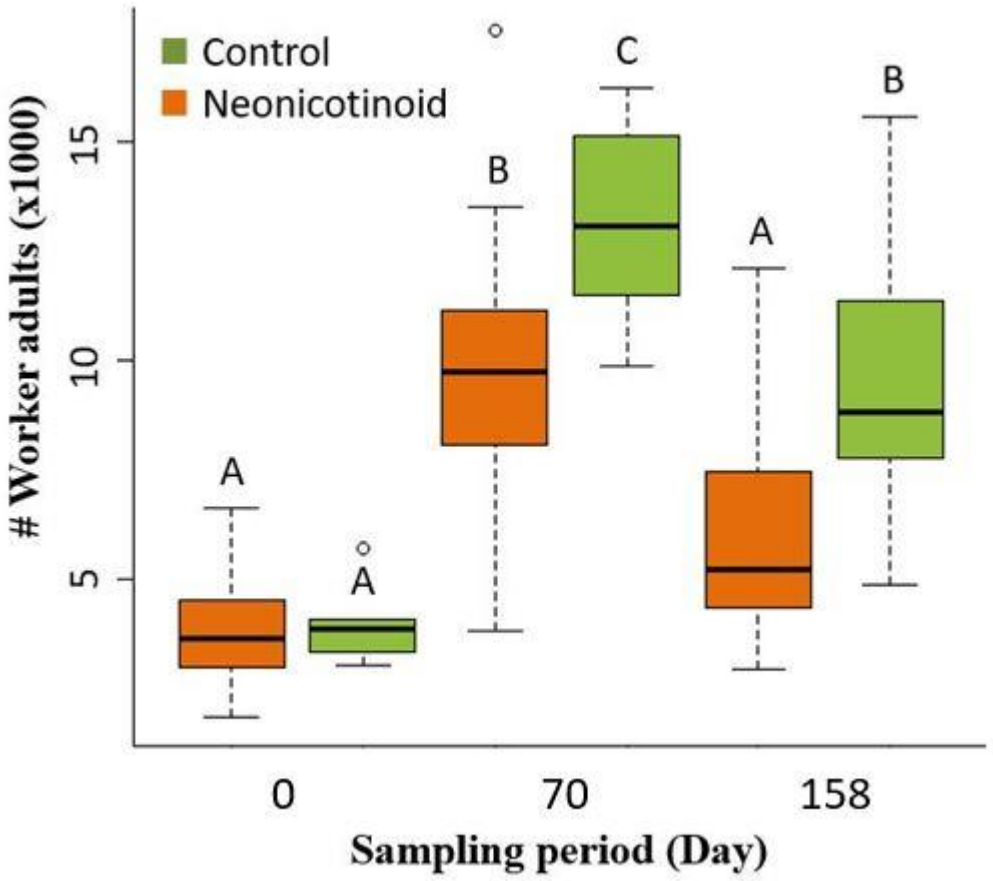

Fig 2. The abundance of *Apis mellifera* worker adults at sampling days 0, 70, and 158 between treatment groups. A significant difference (logistic regression, $p \leq 0.05$) between treatment groups are indicated by different letters. All boxplots show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), and outliers (black dots).

2.4.2 Worker capped brood

Pre-exposure to neonicotinoids at day 0, similar to worker adults, did not yield any significant difference (P = 0.1009 and F-value= 3.055) in the number of workers capped brood (Fig. 3) between the treatment groups. On day 70, however, the number of workers capped brood in the control group was significantly higher than those in the neonicotinoid-exposed group (P = 0.04304 and F-value= 4.885), whereas, at day 158 no significant difference was observed between the control and the neonicotinoid-exposed colonies (P = 0.2222 and F-value= 0.02867).

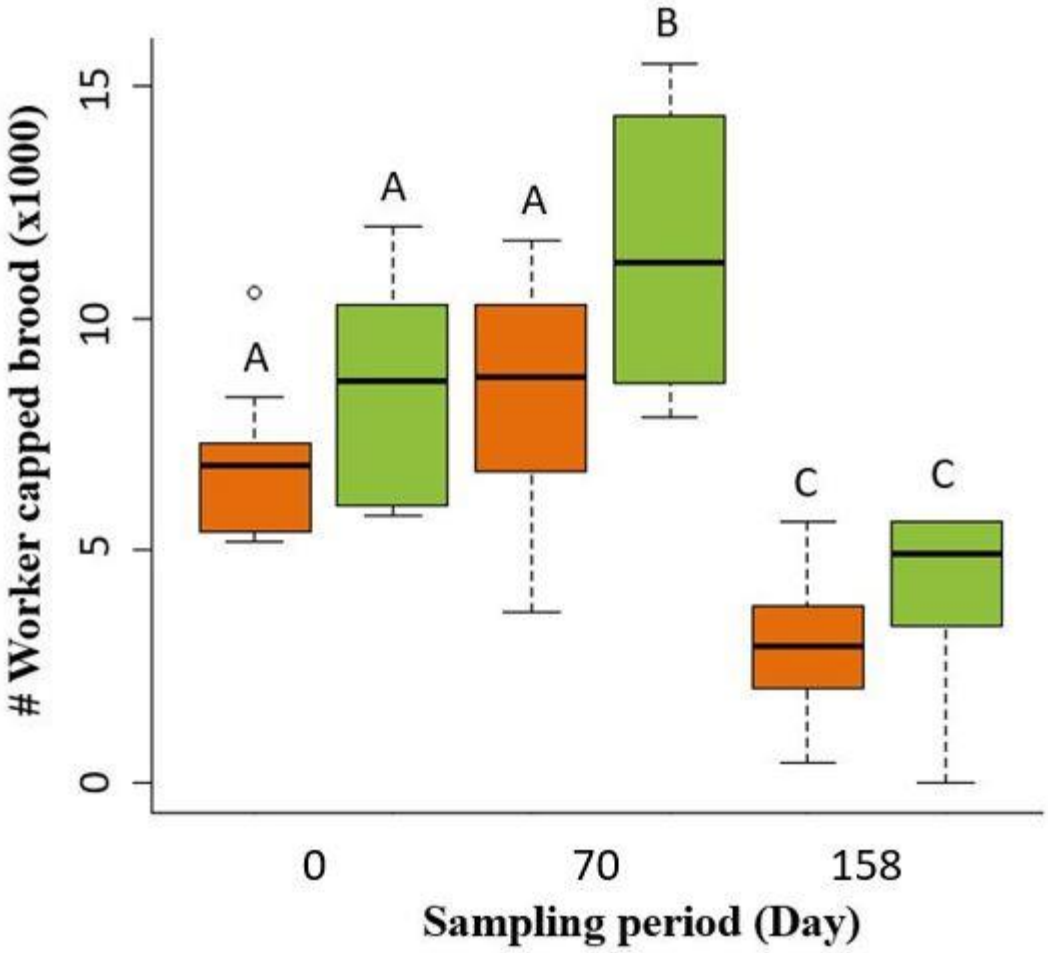

Fig 3. The abundance of *Apis mellifera* worker capped brood at sampling days 0, 70, and 158 between treatment groups. A significant difference (logistic regression, $p \leq 0.05$) between treatment groups are indicated by different letters. All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from the box), and outliers (black dots).

2.4.3 Honey cells

There was no significant difference between the number of honey cells in the neonicotinoid-exposed group compared with the control on day 0 (P = 0.4824 and F-value= 0.5188) (Fig. 4). Exposure to neonicotinoids yielded a significant reduction in the number of honey cells at both day 70 (P = 0.01566 and F-value= 7.424) and day 158 (P = 0.007627 and F-value= 9.485), when compared with the controls.

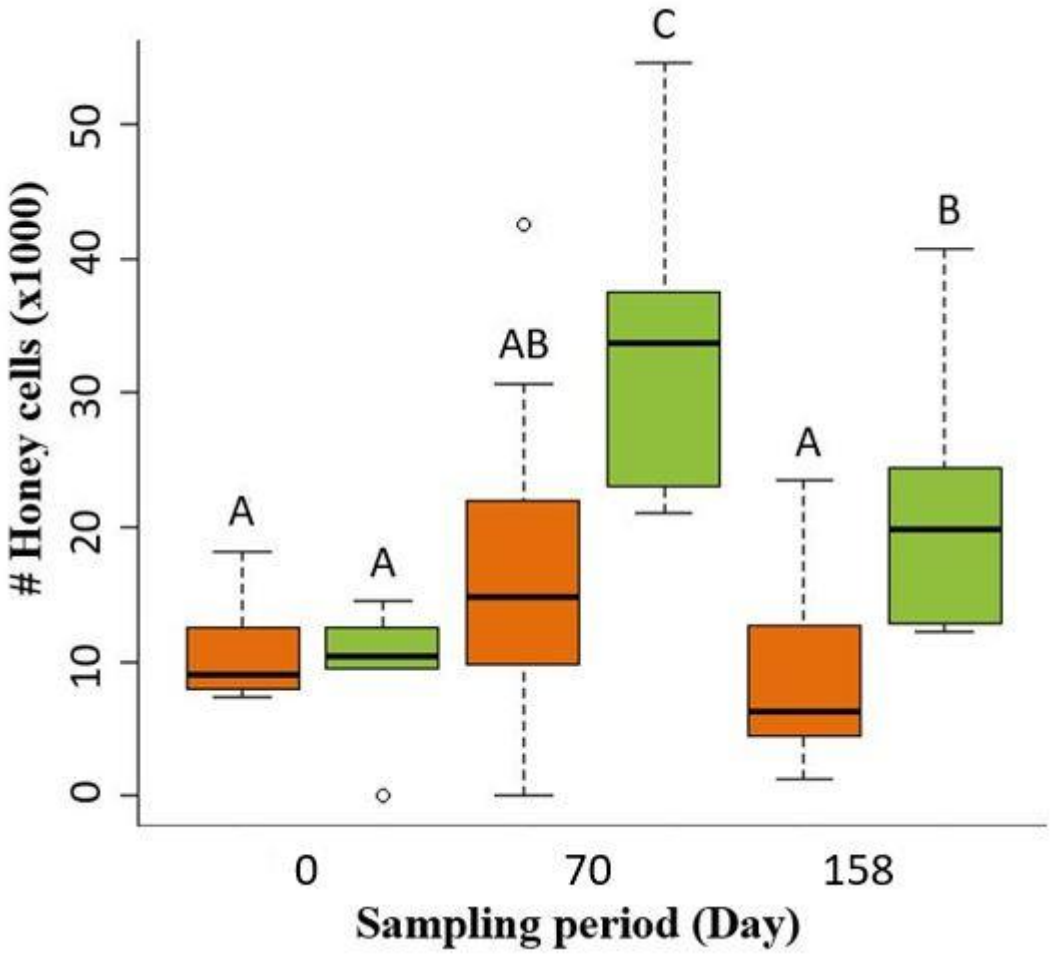

Fig 4. The abundance of *Apis mellifera* honey cells at sampling days 0, 70, and 158 between treatment groups A significant difference (logistic regression, $p \leq 0.05$) between treatment groups are indicated by different letters. All boxplots show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), and outliers (black dots).

2.4.4 Pollen cells

Pre-exposure (day 0) for pollen cells as well revealed no significant difference between neonicotinoid-exposed group and control group (P = 0.1636 F-value = 2.145) (Fig. 5). Similarly, there was no observed difference in the number of pollen cells between the control group and the neonicotinoid-exposed group on day 70. However, on day 158 a higher number of pollen cells were observed in the control group compared to the neonicotinoid-exposed colonies, but this difference was not significant (P = 0.3239 and F-value = 1.04).

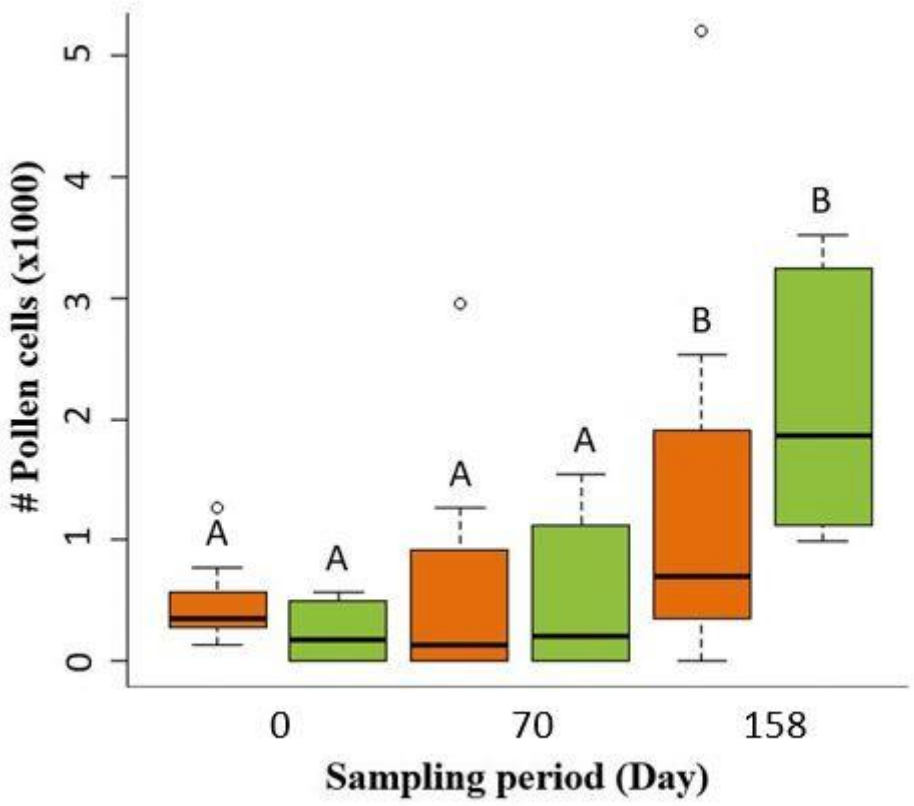

Fig 5. The abundance of *Apis mellifera* pollen cells at sampling days 0, 70, and 158 between treatment groups. A significant difference (logistic regression, $p \leq 0.05$) between treatment groups are indicated by different letters. All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from the box), and outliers (black dots).

2.5 Discussion

Insecticides are reported to sub-lethally affect honey bees (Corby-Harris et al., 2018; Fairbrother et al., 2014; Wu et al., 2012), with varied health consequences (Desneux et al., 2007; Iqbal et al., 2019; Tosi et al., 2017) that can reduce their performance and productivity (Sandrock et al., 2014). Here we considered the extent to which neonicotinoid may impact honey bees at the colony-level. Our experimental findings revealed that neonicotinoids had a strong negative effect on worker adult and stored honey cell numbers, had a marginal negative effect on worker capped brood numbers, but had no effect on stored pollen.

For worker adults, there was a significant reduction in the population of the neonicotinoid-exposed group after approximately one and three brood cycles compared to the controls. This finding is in line with other publications that studied the sublethal effects of the neonicotinoid on honey bees. Field realistic concentrations of neonicotinoid, indeed, have shown several sublethal effects as impairment of learning abilities, memory, and respiratory functions (Decourtye et al., 2003; Hatjina et al., 2013). These could have affected the foraging efficiency of the neonicotinoid-exposed group, as a result of compromised navigation and forage ability (Yang et al., 2008), hence a decline in the performance of the entire colony (Sandrock et al., 2013; Tosi et al., 2017).

Although the number of worker capped brood in the neonicotinoid-exposed colonies was significantly lower than the control group on day 70, the difference between them was not significant by the end of the trial. Possibly, long-term larvae survivorship in the control group was dependent on an additional factor. Over time, the waning of pesticides is a feasible phenomenon (Straub et al., 2019). This may be why a difference in honey cells reported in this study.

Furthermore, a significant decline in the number of honey cells was recorded for the

neonicotinoid-exposed group compared with the control group, both after a brood cycle and three brood cycles. This could be a result of direct consequence of a reduction in the population of worker adults, as a general reduction in foraging efficiency is observed to have a comparative reduction with honey production (Sandrock et al., 2014). Higher forager losses from homing flight failure (Monchanin et al., 2019) associated with changes in foraging structure (Sandrock et al., 2014).

Even though pollen was consumed similarly in both treatment groups after a brood cycle, a higher number of pollen cells remained in the control colonies compared with the neonicotinoid-exposed colonies on day 158. The more demand for pollen by the neonicotinoid-exposed group after three brood cycles is an indication that more pollen was required in-hive by nurse bees, larvae, and queen to keep the colony productive (Bourke & Chan, 1999; Tarpy & Olivarez, 2014).

In summary, I found that capped broods and eggs were affected by thiamethoxam and clothianidin. However, worker adults, eggs, and food storages were not affected. Our findings are important because it gives insight on the probable effects of thiamethoxam use in agricultural and indicates on a need to call for action between honey bee stakeholders (i.e., apiaries) and farmers.

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Chapter 3

Effects of neonicotinoid exposure and brood mixing on honey bee (*Apis mellifera*) colony strength

Abstract

Multiple studies have reported the adaptive benefits of a honey bee (*Apis mellifera*) colony possessing genetically diverse workers, especially in light of widespread observations of colony mortality. Despite the pollinator's immense economic importance, there is no information about how genetic diversity among workers in a colony may affect honeybee susceptibility to abiotic stressors like neonicotinoids. To investigate this, I executed a colony level experiment with four treatment groups: 1. High genetic diversity (via one round of inter-colony brood exchange) & No neonicotinoid exposure, 2, High genetic diversity (via one round inter-colony brood exchange) & Neonicotinoid exposure, 3. Normal genetic diversity & No neonicotinoid exposure, and 4) Normal genetic diversity & Neonicotinoid exposure. At pre-determined periods (days 21, 42, and 180), I assessed colony worker populations and food stores. Colonies did not differ among treatment groups for any measured variable, with the exception that a significantly higher number of eggs were recorded in the high genetic diversity group that was not exposed to neonicotinoids during the second assessment period. Notably, I did not find a negative effect of neonicotinoid exposure on colonies, regardless of genetic diversity status. Future work should investigate if multiple rounds of inter-colony exchange of brood frames can be a tool used by beekeepers to promote colony populations and food stores, and resilience against environmental stressors.

3.1 Introduction

Multiple factors, in various combinations, are believed to contribute to increased honey bee colony mortality observed in the northern hemisphere during the last decade (Blacquière et al.,

2012; Fairbrother et al., 2014; Genersch et al., 2010). By and large, modern agricultural practices consisting of large monocultures and simplified ecosystems tend to promote insect pest populations (Gurr *et al*., 2003; Winston, 1987). As a result, management schemes often employ acute toxins to ensure high-quality crops for both human and animal consumption (Oerke and Dehne, 2004). However, advances in fundamental research and environmental risk assessment of agricultural chemicals reveal a general lack of knowledge of the sub-lethal effects of pesticides on non-target species such as predators, parasitoids, and pollinators that provide vital ecosystem services (Desneux et al., 2007).

Neonicotinoids are believed to negatively influence honey bee health because the timing of their widespread adoption by agricultural practitioners coincided with increased colony mortality (Simon-Delso et al., 2015). In recent decades, neonicotinoids have become the most ubiquitously used class of agricultural pesticides, globally (Blacquiere *et al*., 2012; Goulson, 2013). Due to their solubility in water, neonicotinoids have systemic properties that result in broad protection against a range of insect pests (Matsuda *et al*., 2001; Tomizawa and Casida, 2005), but also promote expose pollinators such as honey bees to residues during foraging (Bonmatin *et al*., 2014).

Interestingly, honey bee queen polyandry contributes to the genetic diversity of colonies and may confer colony-level benefits against biotic stressors (Tarpy and Seeley, 2006). Honey bee reproduction relies on intimate connections among all bee morphs, including the two female castes (queens and workers) and the males (drones) (Koeniger *et al*., 2014; Laidlaw and Page, 1997; Winston, 1987). Successful mating by the queen induces significant molecular, physiological, and behavioral changes. This promote the queen to reside in the colony to oviposit female eggs of mixed paternity until stored sperm is depleted (Nino *et al*., 2013; Winston, 1987). As sexually matured drones as well perform physiologically and behaviorally demanding flights to drone

congregated areas (DCA) to mate (Koeniger *et al*., 2014; Laidlaw and Page, 1997; Winston, 1987). Usually, a queen mates with 12-40 males (Neumann et al., 1999).

Despite the benefits of increased intracolonial worker genetic diversity to biotic stressors (Delaplane *et al*., 2015), there are currently no data on how it may interact with abiotic stressors, like neonicotinoids (Seeley and Tarpy, 2007; Deplane *et al*., 2015). Data from the most harmonized experimental designs, however, suggest that thiamethoxam and clothianidin have wide-ranging negative consequences on queens (Chaimanee *et al*., 2016; Sandrock *et al*., 2014; Williams *et al*., 2015), drones (Straub *et al*., 2016), and workers (Bruckner, 2017). Beekeepers can promote genetic diversity in colonies through instrumental insemination and inter-colony exchange of brood frames, although the latter procedure results in a brief flush of genetic diversity, followed by a graduate decline to normalcy as workers die.

The objective of my work was to investigate how genetic diversity and neonicotinoids interact to affect honey bee colony strength. Based on a previous study that demonstrated increased intracolonial worker genetic diversity mitigated effects of an important biotic stressor (Delaplane et al., 2015), I predicted that higher genetic diversity of worker-nestmates would mitigate effects against abiotic stressors like neonicotinoid insecticides.

3.2 Materials and Methods.

3.2.1 Experiment set-up

The work was conducted during the summer of 2019 in Auburn, Alabama at a research apiary (32.62676692843044, -85.54428795558492) maintained by Auburn University. Twenty-four established honey bee (*Apis mellifera*) colonies containing sister queens were used for the experiment; bees were housed in Langstroth deep equipment. The number of adults and brood (i.e.,

immatures), were quantified visually using the Liebefeld assessment method (Delaplane et al., 2013). They were subsequently equalized down to 3 brood frames, the greatest common denominator for all colonies, that contained all stages of brood.

3.2.2 Inter-colony brood exchange

Each colony was randomly allocated to high colony genetic diversity (via inter-colony brood exchange) (B+) or normal colony-level genetic diversity (no inter-colony brood exchange) (B-) treatments. For the former, colonies were grouped into two groups of three. Within each triplet group, each colony served as donor and recipient of one brood frame to and from the other two colonies, respectively. As a result, each B+ colony possessed one original brood frame and two introduced brood frames from two separate colonies. This temporarily increased the genetic diversity of the B+ colonies (Tarpy et al., 2013). In contrast, colonies belonging to B- did not exchange brood frames with other colonies. In total, 12 colonies of each B+ and B-.

3.2.3 Insecticide exposure

Half of the B+ and B- colonies were allocated randomly to either a neonicotinoid (N+) or control (N-) treatments. Colony exposure to each treatment occurred by feeding with experimental pollen paste (60% pollen, 30% powdered sugar, 10% organic honey) according to an established method (Sandrock et al., 2014b; Williams et al., 2015). The neonicotinoid colonies (N+; 6 colonies from each B+ and B-) received pollen patty that contained field-realistic concentrations of two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin; both Sigma Aldrich, St. Louis, MS) (David et al., 2016; Friedli et al., 2020; Tosi et al., 2018). Control colonies (N-; 6 colonies from each B+ and B-) received pollen patties that did not include neonicotinoids. Pollen patties were supplied *ad libitum* every two days for or 42 days (two brood cycles). Each colony was

equipped with an entrance pollen trap during the entire experimental period to limit forager-collected corbicular pollen brought back to the hive from the surrounding environment; this was to promote pollen paste feeding (Goulson, 2013).

In summary, there were six colonies replicates for each of the four treatment groups: 1) normal intra-colony genetic diversity (no brood mixing) and no neonicotinoid exposure (control, B-/N-), 2) high intra-colony genetic diversity (via brood mixing), and no neonicotinoid exposure (brood mixing only, B+/N-), 3) normal intra-colony genetic diversity (no brood mixing) and neonicotinoid exposure (neonicotinoids only, B-/N+), and 4) high intra-colony genetic diversity (via brood mixing) and neonicotinoid exposure (both treatments, B+/N+).

3.2.4. Measuring honey bee populations and food stores

Colony strength, measured as numbers of workers (adult and brood) and food stores (honey and pollen) were conducted using the Liebefeld method of visual estimation (Delaplane et al., 2013). Worker adults and food store assessments were performed simultaneously with the brood assessment. They were conducted on days 0, 21, 42, and 180. These days were chosen due to the brood cycle. Two observers visually estimated percent coverage area the surface area of a comb covered by worker adults, worker brood (worker egg cells, worker larvae cells, worker capped brood), and food stores (honey and pollen cells). The number of worker adults was calculated assuming 1,215 individuals occupy each frame side. Similarly, the numbers of brood and food stores were calculated assuming 3.8 cells / cm^2 per frame side (Delaplane et al., 2013).

3.2.5. Statistical analyses

The R (Version 3.5.0., 05.30.2019) statistical software was used to perform all statistical analyses and to produce boxplots. To investigate the impact of polyandry status and neonicotinoid

exposure (thiamethoxam and clothianidin) on honey bee colony performance over time, a variety of critical endpoints were examined using mixed-effect models. Endpoint measures included the number of worker adults, worker brood (i.e. capped brood, larvae cells and egg cells), and food stores (honey and pollen cells). Polyandry status, neonicotinoid exposure, and assessment periods were included as fixed effects, whereas colonies were added as random effects. Differences between treatment groups (i.e. controls, brood mixing only, neonicotinoid only, and both treatments) were compared using a repeated-measures ANOVA (mma) (Maxwell and Delaney, 2004) followed by a Bonferroni post-hoc test (bht) using the 'emmeans' package.

3.3 Results

3.3.1 Worker adults

Worker adult population was not affected by inter-colony brood exchange status, neonicotinoid exposure, or assessment period (all $p>0.05$)(Fig. 6).

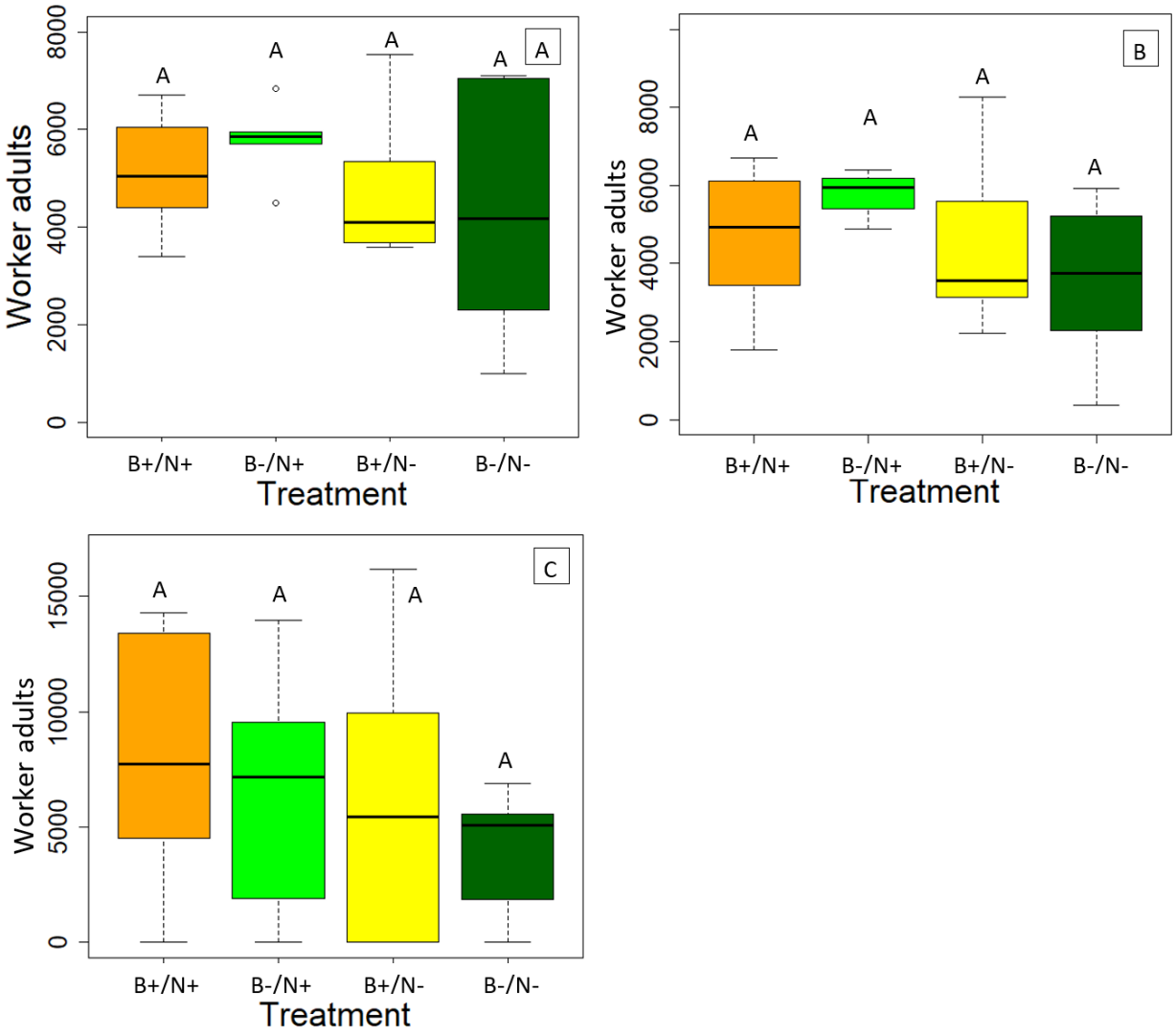

Fig 6 (A-C): The abundance of *Apis mellifera* worker adults at day 21 (A), 42 (B), and 180 (C) among treatment groups. There is a significant difference among treatment groups at $p \leq 0.05$. Boxplots, as applicable, show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), data collection order (letter in square box) and outliers (black dots). B+/N+: brood mixing and neonicotinoid exposure, B+/N-: brood mixing and no neonicotinoid exposure, B-/N+: no brood mixing and neonicotinoid exposure, B-/N-: no brood mixing and no neonicotinoid exposure.

3.3.2 Worker brood

Overall, neither pesticide exposure nor brood exchange status affected worker brood variables (i.e. capped brood, larvae cells, and egg cells) (all $p>0.05$). There was a significant effect of the assessment period on capped worker brood ($p<0.001$), but not on larvae and egg cells (both

p>0.05)(Fig.7). Similarly, the mean number of worker larvae cells was not different between treatment groups at any assessment period (all p>0.05) (Fig. 8). Mean number of worker egg cells did not differ among treatment groups at day 0 (F-statistics = 1.456, P-value = 0.257; Fig. 9a), day 21 (F-statistic = 1.415, p-value = 0.268, Fig. 9b) and day 180 (F-statistic = 0.279, p-value = 0.840, Fig. 9c). However, there was a significant difference at day 42 (F-statistic = 3.283, p-value = 0.042, Fig. 9d) when the brood mixing only group (B+/N-) had significantly more worker egg cells (N=1951) compared to the other treatment groups (all p<0.05). Numerically, the brood mixing group also had the highest mean number of worker egg cells on day 0, 21, and 180 (N= 1811, 3428, and 2856, respectively.). The least number of egg cells was found in colonies that received both treatments (B+/N+) on day 0, 21 and 42 (N=1108, 2341, and 920), whereas, control (B-/N-) and neonicotinoid only (B-/N+) treatment groups had the least amount on day 180 (both N= 2118).

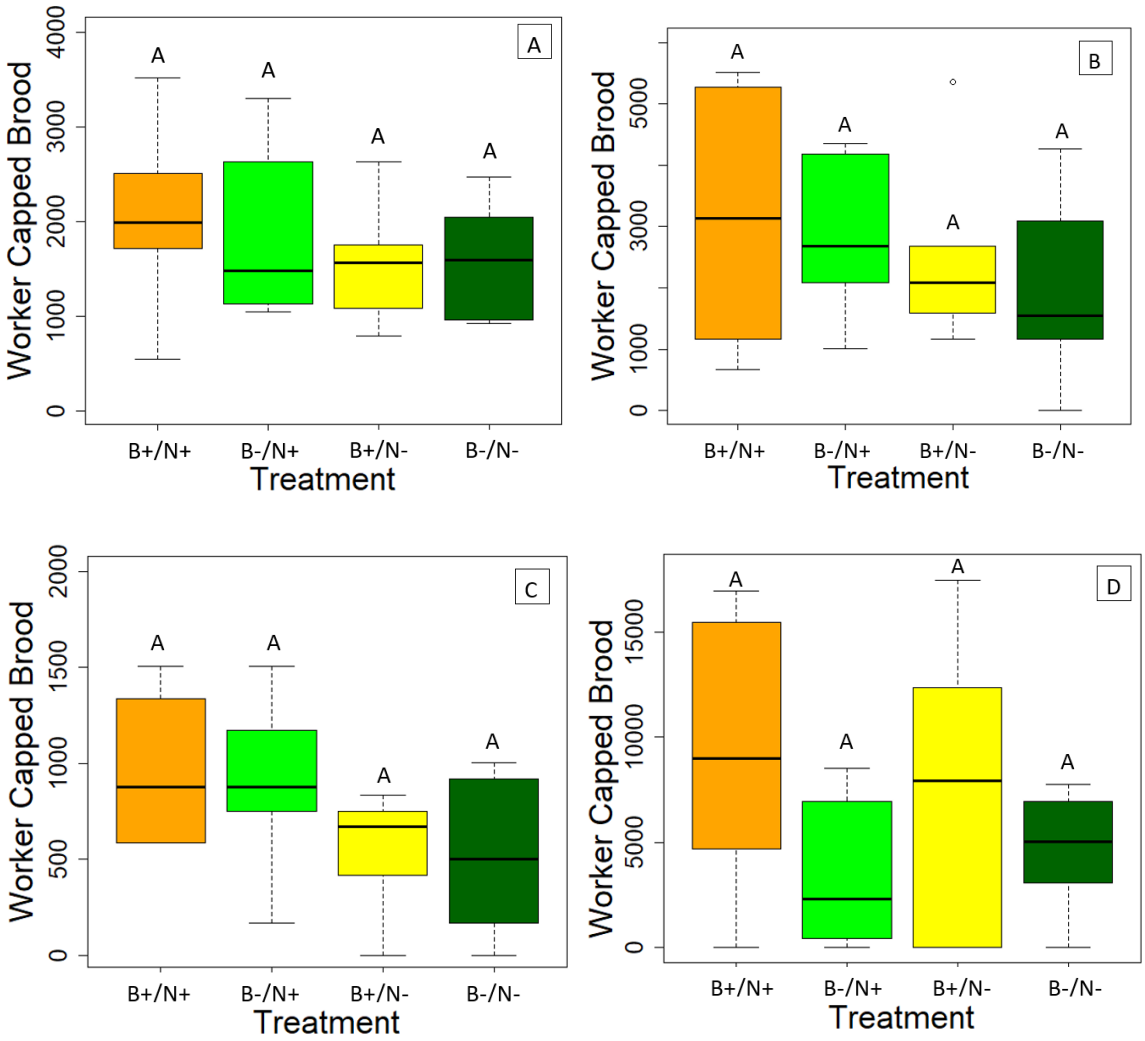

Fig 7 (A-D): The abundance of *Apis mellifera* worker capped brood at day 0(A), 21(B), 42(C), and 180 (D) among treatment groups. There is a significant difference among treatment groups at $p \leq 0.05$. Boxplots, as applicable, show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from the box), data collection order (letter in square box) and outliers (black dots). B+/N+: brood mixing and neonicotinoid exposure, B+/N-: brood mixing and no neonicotinoid exposure, B-/N+: no brood mixing and neonicotinoid exposure, B-/N-: no brood mixing and no neonicotinoid exposure.

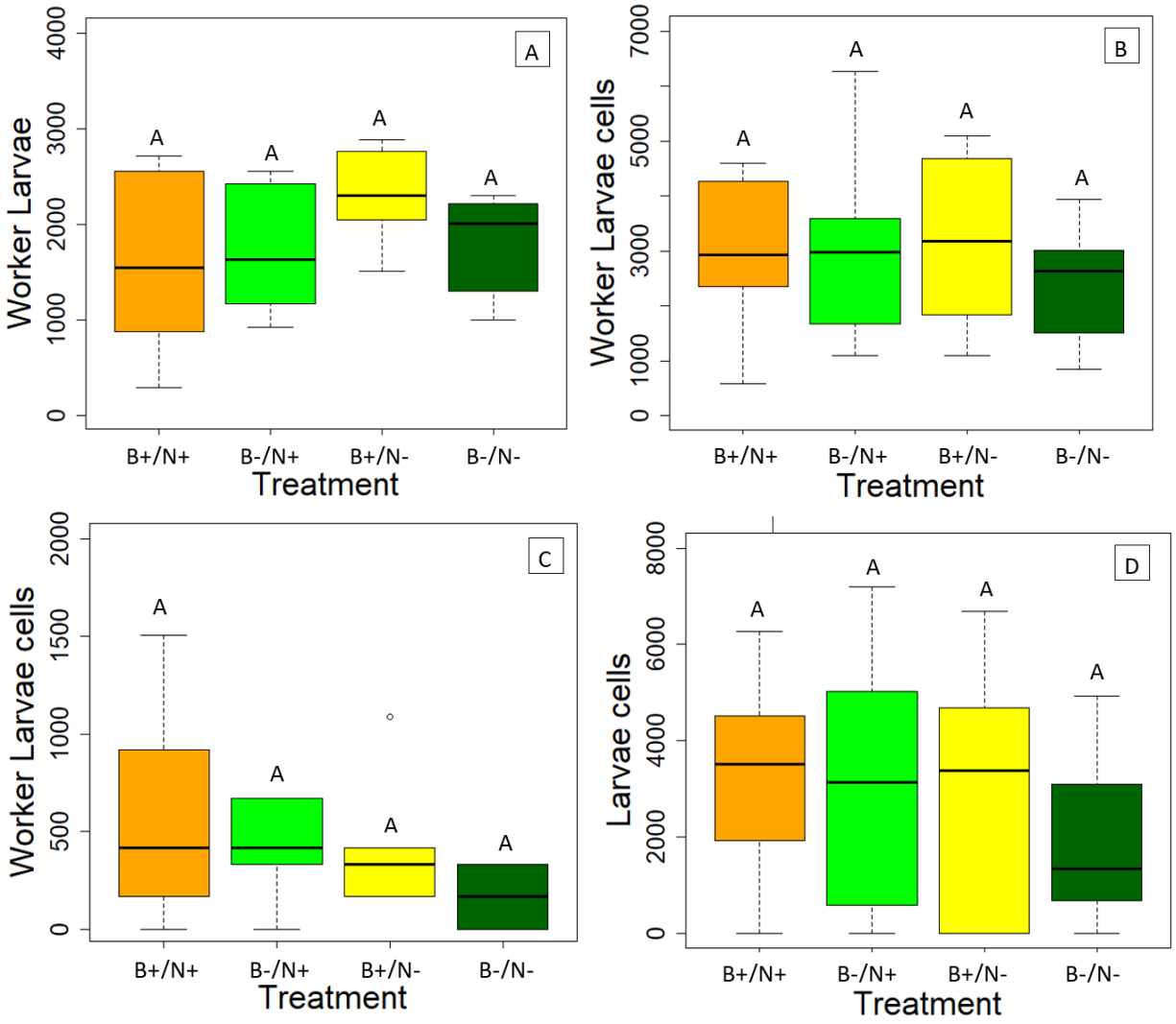

Fig 8 (A-D): The abundance of *Apis mellifera* worker larvae cells at day 0(A), 21(B), 42(C), and 180(D) among treatment groups. There is a significant difference among treatment groups at $p \leq 0.05$. Boxplots, as applicable, show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), and outliers (black dots). B+/N+: brood mixing and neonicotinoid exposure, B+/N-: brood mixing and no neonicotinoid exposure, B-/N+: no brood mixing and neonicotinoid exposure, B-/N-: no brood mixing and no neonicotinoid exposure.

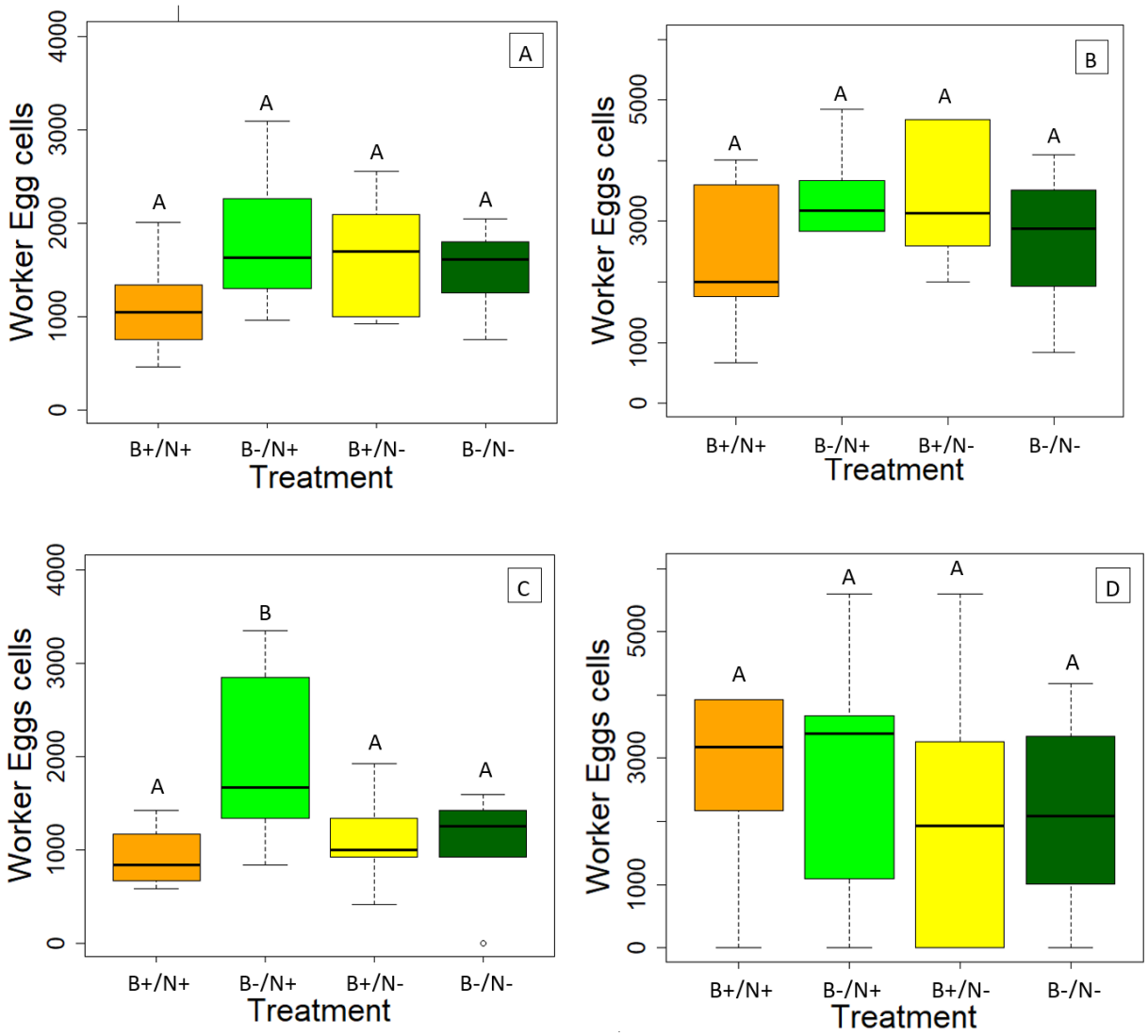

Fig 9 (A-D): The abundance of *Apis mellifera* worker egg cells at day 0(A), 21(B), 42(C), and 180(D) among treatment groups. There is a significant difference among treatment groups at $p \leq 0.05$. Boxplots, as applicable, show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), and outliers (black dots). B+/N+: brood mixing and neonicotinoid exposure, B+/N-: brood mixing and no neonicotinoid exposure, B-/N+: no brood mixing and neonicotinoid exposure, B-/N-: no brood mixing and no neonicotinoid exposure.

3.3.3 Food stores

Neither polyandry status nor neonicotinoid exposure had an effect on the mean number of pollen and honey cells (all $p>0.05$). Both food store variables were significantly affected by assessment period however (both $p<0.001$). The mean number of pollen cells did not differ between treatment groups at any assessment period (all $p>0.05$) (Fig. 10). Similarly, the number of honey cells across the treatment groups was not significantly different at any assessment period (all $p>0.05$)(Fig. 11).

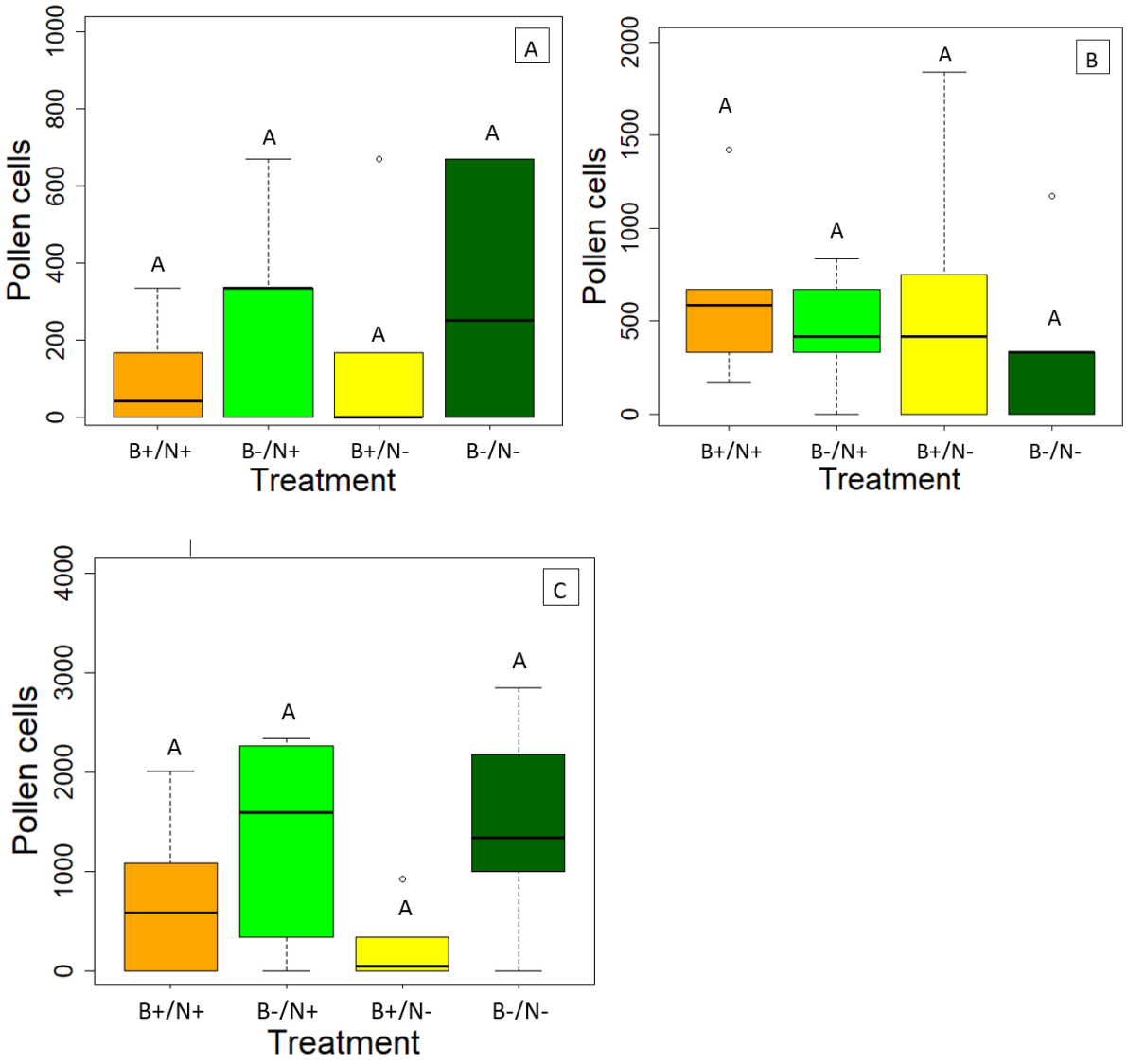

Fig 10 (A-C): The abundance of *Apis mellifera* pollen cells at day 21(A), 42(B), and 180(C) among treatment groups. There is a significant difference among treatment groups at $p \leq 0.05$. Boxplots, as applicable, show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), and outliers (black dots). B+/N+: brood mixing and neonicotinoid exposure, B+/N-: brood mixing and no neonicotinoid exposure, B-/N+: no brood mixing and neonicotinoid exposure, B-/N-: no brood mixing and no neonicotinoid exposure.

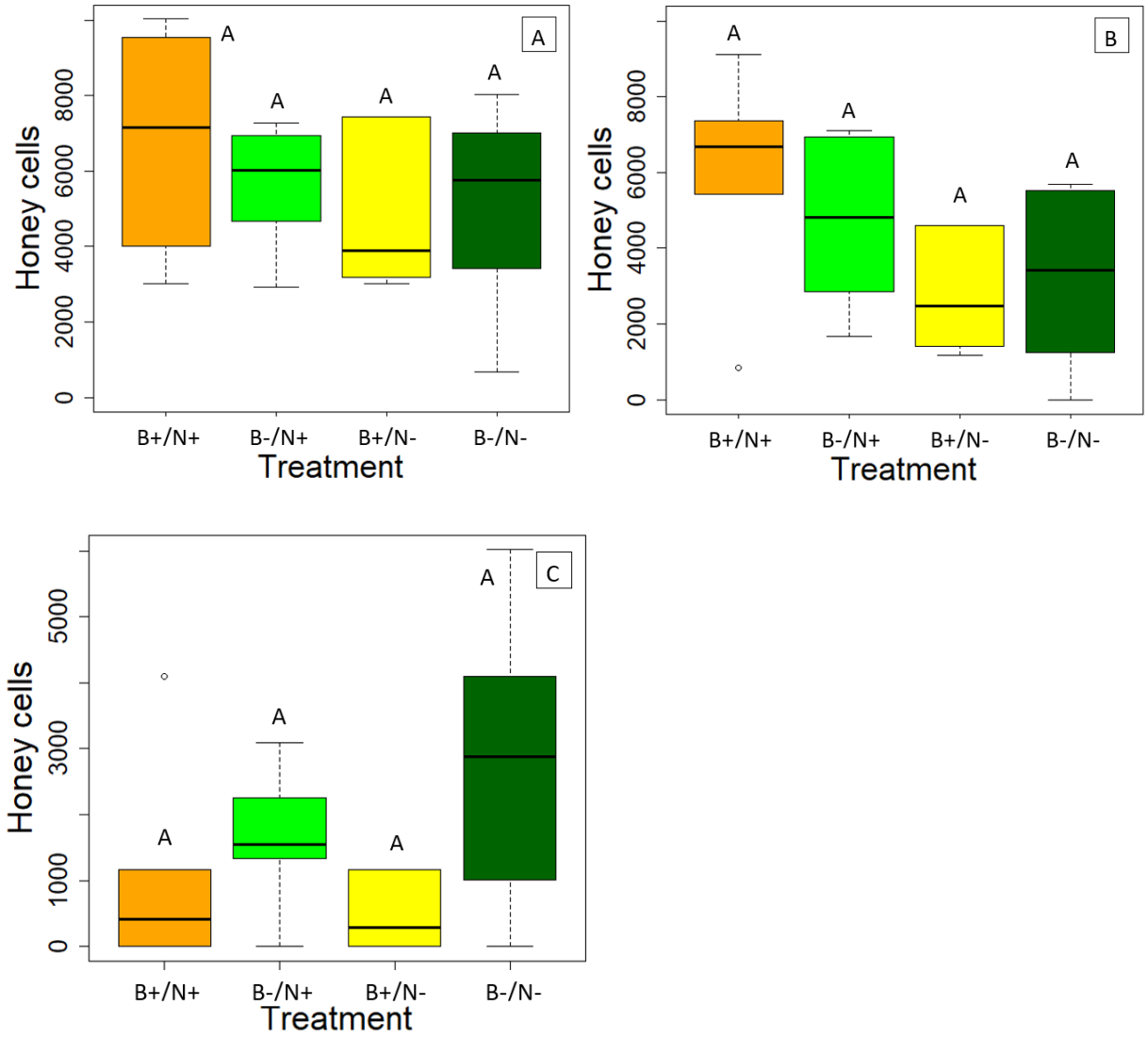

Fig 11 (A-C): The abundance of *Apis mellifera* honey cells at day 21(A), 42(B), and 180(C) among treatment groups. There is a significant difference among treatment groups at $p \leq 0.05$. Boxplots, as applicable, show the inter-quartile range (box), the median (black line within box), date range (horizontal black lines from the box), and outliers (black dots). B+/N+: brood mixing and neonicotinoid exposure, B+/N-: brood mixing and no neonicotinoid exposure, B-/N+: no brood mixing and neonicotinoid exposure, B-/N-: no brood mixing and no neonicotinoid exposure.

3.4 Discussion

The significance of honey bees to agriculture and biodiversity because of the pollination services they provide justifies the need to fully investigate how multifactorial environmental interactions affect their health (Alaux et al., 2010; Sandrock et al., 2014). One such important abiotic stressor is the commonly used neonicotinoid class of insecticides (Alaux et al., 2010; Sandrock et al., 2014; Simon-Delso et al., 2015). Here for the first time, we assessed the combined effects of neonicotinoid-exposure and colony genetic diversity in a mixed model framework to investigate the potential mitigating effects of honey bee brood mixing against neonicotinoid insecticides. Worker egg cells in the brood mixing only treatment group were significantly different from the other treatment groups at day 42; all other endpoint measurements suggest little to no effect of both neonicotinoid exposure and polyandry status on the colonies.

Polyandry is significant for the evolution of genetically diverse subfamilies of workers with distinct alleles within a colony (Tarpy and Seelay, 2006). Although Delaplane et al. (2015) reported that field studies have shown a positive relationship between the queen's expression of polyandry and measures of colony's fitness as population growth and amongst other observed factors, no effect of inter-colony brood exchange was recorded in most of our findings across the measurement variables. For example, there was no significant variation for populations of worker adults across the treatment groups irrespective of the sampling dates (days 21, 42, and 180), even though most populations resided in the B+/N+ and B+/N- relative to the B-/N+ and B-/N- treatment groups. Our study demonstrated that chronic exposure (30 days) of brood mixed and non-brood mixed colonies during the summer season to neonicotinoids treatment did not affect the wintering (180 days) ability of the honey bees, as no significant difference was recorded among the treatment groups.

Similarly, the brood did not yield any differences, except for the worker egg cell on day 42. A significantly greater number of worker egg cells recorded in the B+/N- treatment group at day 42. It could be ascribed to the resultant efforts of queens of the colonies, about the amount of polyandrous mating, consequently resulting in prolonged storage of sperm in the queens' spermatheca (Delaplane et al., 2015; Tarpy et al., 2013). Previously, on day 21, the B+/N+ treatment group had recorded the lowest quantities of worker egg cells. Also, there was more population of egg cells in the B+/N+ treatment group compared to other treatment groups at day 180, even though the variation was not significant. To further put these findings into perspective, Sandrock *et al*. (2014) maintained that reproductive success is vital for inferring long-term population-level consequences. According to Delaplane *et al*. (2015), the constraint limiting higher mating numbers in nature associated with the queen ranges from sexually-transmitted disease to hazards associated with repeated mating flights, thereby making a rare allele of key importance.

Despite the notion that a general reduction in foraging efficiency of worker adults is also indicative of a reduction in food stores (Sandrock et al., 2014), it followed that non-significantly lesser quantity of food stores were distributed between B-/N+ and B-/N- relative to the B-/N+ and B-/N- throughout the sampling dates.

The majority of our findings were statistically non-significant. Perhaps this is because of the relatively brief benefits inter-colony brood exchanged offered, as workers obtained from other colonies only live approximately 3 weeks post-emergence during summer months (Free and Spencer-Booth, 1959). As Tarpy and Seeley (2006) earlier reported that honey bee intra-colony diversity confers colony-level defense against stressor and particularly for colonies whose queens are highly polyandrous, Delaplane *et al*. (2015) reported that instrumental insemination could be employed in improving genetic diversity against honeybee health challenges that could reduce

colony strength in a managed system. By adjusting number of queen matings, rather than simply exchanging brood frames, relatively prolonged benefits from higher genetic diversity are likely Furthermore, Tarpy et al. (2013) reported that colonies of lower genetic diversity are more likely to experience colony death than those with greater diversity. Non-significant occurrences in our study may be attributed to the low levels of genetic diversity. Additionally, since reproduction is an important process to the furtherance of the colony, a loss of brood may be more detrimental for the colony than the loss of older bees (Decourtye and Devillers, 2010). It may also be important to consider the highest level of polyandry essential for mitigating neonicotinoids, as Delaplane *et al*., (2015) reported its significance concerning achieving colony-level benefit.

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