

Horizons in bee science

Edited by

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Horizons in bee science

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Editorial: Horizons in bee science

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KEYWORDS

global warming, bees, honey bee (*Apis mellifera* L.), bumble bee, pollination, *Varroa destructor*, microbiota, chromosomes

Editorial on the Research Topic

Horizons in bee science

Bees are vital pollinators facing unprecedented pressure from human intervention, including climate change, habitat loss, and introduced parasites (IPBES, 2016; Potts et al., 2010). Recent research is highlighted in 11 publications in the Research Topic “Horizons in Bee Science”. Collectively, they emphasize the complex interactions between environmental stressors and bee biology, based on information concerning both social and solitary species. The global importance of these contributions is exemplified by the ~55,000 views and downloads to date (July 2025). Below, we examine the key themes explored in these 11 publications and their implications for advancing our understanding and supporting the well-being of bees.

Bees currently face numerous challenges. Global warming (and resulting thermal stress) negatively impacts both social and solitary species (Acosta et al.; Kevan et al.; Le Conte and Navajas, 2008; Polidori et al.; Soroye et al., 2020; Vilchez-Russell and Rafferty). Honey bees (*Apis mellifera*), the world’s most economically important pollinator, are particularly vulnerable to a suite of threats: most notably the parasitic mite *Varroa destructor* and its associated viruses (Francis et al., 2013; Lamas and Evans; Lester; Morfin et al.; Traynor et al., 2020). Studies about bee biology, genetics, interactions between species, and ways to improve their efficiency as an agricultural input (Owen; Anderson and Copeland; Oddie and Dahle; Farina et al.) complement the contributions to this series of articles.

Climate change and bees

Climate-induced heat stress is a growing threat to bee diversity. Solitary bees appear to be particularly vulnerable due to their inability to thermoregulate their nests (Vilchez-Russell and Rafferty). Studies have shown that heat stress reduces survival and adult emergence rates, and alters body size, development time, sex ratios, and diapause in solitary bees. Increasing temperatures can lead to a lack of synchrony between bee emergence and floral resource availability. There is thus a need for habitat management to provide thermal refugia, along with integrated conservation planning that considers climate variability, and species-specific responses and resilience mechanisms.

Acosta et al. propose a methodological framework to identify priority zones for monitoring wild bee species and their response to climate change, using a Brazilian stingless bee, *Melipona fasciculata*, in a case study. They identified three main types of habitat zones for this species using their new methodology: Loss zones – will become unsuitable; Gain zones – will become suitable; and Persistent zones – remain suitable and are best for long-term monitoring. Their approach offers an option for achieving better spatial precision for conservation measures for bees.

Urban Heat Island Effect (UHI) refers to elevated temperatures in urban areas due to human infrastructure such as buildings and roads, particularly affecting ectothermic organisms such as bees (Polidori et al.; Camilo et al., 2025). Temperature increases are generally known to affect bee physiology, reduce foraging efficiency and reproductive success. Not all bees respond in the same way to warming. Specialists and thermosensitive species are at risk of local extinction. UHI can shift flowering times and resource availability in urban areas, leading to mismatches between bees and plants. More comprehensive, multi-scalar research combining urban ecology, thermal biology, and pollination science is needed for informing urban biodiversity planning.

Bumble bee populations are declining globally, with climate warming identified as a key driver (Kevan et al.; Soroye et al., 2020). Bumble bee colonies maintain a remarkably consistent brood rearing temperature across species and biogeographic regions, suggesting limited evolutionary plasticity. They are more susceptible to heat stress than to cold. We know that nest placement and structure significantly affect nest temperatures, but few data exist on thermal gradients and insulation properties in bumble bee nests. There is a need to understand how bumble bee physiology, ecology, and nest architecture affect responses to climate change.

The Varroa mite and its impact on *Apis mellifera*

There is a devastating interplay of honey bees, Varroa mites, and viruses, which is a major cause of colony losses worldwide (Francis et al., 2013; Lamas and Evans). Common beekeeping practices (e.g., splitting colonies, transporting hives) inadvertently facilitate virus and mite dispersal. While miticides help control mites, they do not eliminate circulating viruses, and mite resistance to acaricides is a growing problem. There is a need to understand how management strategies affect bee-mite-virus dynamics. Effective bee health management requires tackling the synergistic threat posed by mites and viruses together, rather than separately.

Varroa infestation reduces honey bee lifespan and exacerbates viral infections by suppressing bee immune responses and increasing virus titers (Morfin et al.). Infestations can lead to colony collapse, if unmanaged. Resistance traits (e.g., Varroa Sensitive Hygiene) have potential for selective breeding programs to promote resistance. Integrative approaches combining genomics, ecology, and behavior could be combined into a multifaceted solution. The complex impact of *Varroa destructor* on honey bee health underscores the need for sustainable, science-based management strategies to mitigate its effects.

Overuse and reliance on acaricides have led to the evolution of resistance in Varroa populations (Lester). Coordinated control management is needed to prevent resistance development. Possible strategies to manage resistance include rotating acaricides with different modes of action and applying treatments only when thresholds are exceeded. Improved monitoring of resistance alleles for resistance patterns is essential. Beekeepers, researchers, and regulators are encouraged to work collaboratively to preserve the efficacy of current treatments.

Advances in bee science and their use in crop pollination

There is much chromosome diversity in bees (superfamily Apoidea), with a wide range of haploid chromosome numbers ($n=3-28$). Owen showed that chromosome structure and number varies widely among bee species, but a lack of data for many bees makes it difficult to determine relationships among species and how they evolved. Methods for preserving and analyzing chromosomes have improved, including C-banding and FISH techniques, helping advance our understanding of chromosomal rearrangement and evolution. Modern genomic tools can be used to explore adaptive significance and phylogenetic patterns in chromosomal variation (Beye et al., 2006; Christmas et al., 2019; Zayed, 2009).

The microbiota of bees contributes to preservation of stored pollen and honey, inhibition of pathogens, and colony-level disease resistance. Anderson and Copeland conducted a meta-analysis of 35 studies of the microbiota of honey bee hives, finding that bees have a distinct and relatively stable hive microbiota, dominated by aerobic or aerotolerant bacteria, shared across aerobic niches including larva, worker and queen mouthparts, worker crops and hypopharyngeal glands, queen crops and midguts, bee bread, royal jelly, and honey. This study points to the need for more research exploring how hive management and climate affect the hive microbiome (Engel et al., 2012), which also should be considered in colony health diagnostics.

Honey bees can rapidly abandon a crop and switch to alternative flowering species depending on nectar and pollen quality (Levin and Anderson, 1970; Wojcik et al., 2018). Farina et al. showed that bees can be trained to associate floral scents with food rewards. Once trained, they visit target crops more frequently, potentially enhancing pollination precision. Continued improvement of automated within-hive conditioning has the potential to make honey bees more effective agents of pollination services. Additional research and adjustments are needed, however, to determine if this system is viable for specific crops.

Conflicts and common ground: unified strategies for pollinator protection

Concern about competition between wild and managed pollinators sometimes leads to conflicts between beekeepers and conservationists (Oddie and Dahle; Prendergast et al.). However, given that honey

bees and wild pollinators face common threats, beekeepers can be early detectors of environmental stressors. Fortunately, enlightened beekeepers have increasingly become allies in the advocacy for pollinator-friendly practices. They help promote landscape-scale solutions, such as creating pollinator corridors or pesticide-free zones, and promote floral diversity. Aligned interests between managed and wild pollinators open the door for unified conservation strategies, with beekeepers potentially playing a key role in pollinator protection. This is especially relevant considering that superior fruit set and quality are obtained when wild and managed bees visit the same flowers, showing additive or synergistic effects (Brittain et al., 2013).

Conclusions

A common theme in the contributions to “*Horizons in bee science*” has been to identify pathways for improving conditions, both managed and natural, for these vital pollinators. The next challenges involve prioritizing and implementing these improvements. A coordinated international effort, incorporating changes in management practices, is needed to address the adverse conditions currently confronting bees.

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Targeted crop pollination by training honey bees: advances and perspectives

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As pollinator-dependent crops continue to expand globally, management strategies are needed to meet the current demand for pollination services. Improving the efficiency of pollinators depends on knowledge about crop plant biology as well as pollinator behavior. In this sense, we will review the scope and challenges of implementing a targeted pollination strategy based on the behavioral individual and social plasticity of the honey bee *Apis mellifera*. Here we summarize current knowledge on the bees' ability to perceive, learn and generalize floral odors, the bias of their foraging preferences after in-hive experiences and the transfer of food source information within the social context of the colony, all aspects that impact on foraging decisions and can be used to direct pollinators to target crops. We focused on describing how key olfactory cues that mimic crop floral scents are acquired in the hive and propagate among colony mates to guide foraging to specific crops. Knowledge gaps, including volatiles variability between flowers of the same or different crop varieties, alternative managed pollinators, and potential impact on food industry are discussed.

KEYWORDS

floral volatiles, learning, mimic odors, honey bee, *Apis mellifera*, pollinator-dependent crops, foraging behavior

Introduction

In the last 70 years, the agricultural area devoted to pollinator-dependent crops has increased monotonically (Aizen et al., 2019). Animal pollination, mostly bee pollination, directly affects the yield of 87 of 115 leading single crops (Klein et al., 2007). Given the central place that pollination services have achieved in agriculture, it is necessary to improve the efficiency of pollinators, in particular of those managed by humans, like the honey bee. The challenge of improving pollination services depends on several factors, including knowledge about plant biology and pollination requisites, landscape features, environmental conditions, as well as the pollinator needs (McGregor, 1976; Free, 1993; Delaplane and Mayer, 2000; Abrol, 2012). In particular, improving pollination by managed

honey bees requires monitoring the colonies introduced into the crop to assess levels of foraging activity and the resources collected before, during and after the blooming period. This knowledge allows the design of a pollinator management strategy to define the number, placement, and timing of colony introduction to obtain high yields. In addition, healthy and populous colonies are essential to ensure the success of pollination service (McGregor, 1976; Free, 1993; Delaplane and Mayer, 2000; Abrol, 2012).

The crop requirements and the management of its pollinators are covered within the topic known as “managed pollination” or “directed pollination” (Delaplane and Mayer, 2000; Vásquez Romero et al., 2011; Rosa et al., 2018). However, this area does not consider aspects of the individual and social behavior of honey bees, the most commonly managed pollinator worldwide, which directly impacts the pollination services provided. For instance, honey bees exhibit behaviors that advertise and recruit nestmates to the most profitable food sources. In this sense, honey bees have the ability to communicate spatial information about profitable sites through the waggle dance (a figure-of-eight maneuver on the vertical wax combs), and to transfer food-related information, such as scents or tastes, through mouth-to-mouth trophallactic food exchanges among nestmates (von Frisch, 1967; Farina et al., 2005). So far, honey bee plastic behavioral responses to new conditions required by crop pollination management, either by moving hives between environments that offer different floral availability, and/or after the sudden onset of a massive and dominant blooming, are seldom considered for pollination services. Furthermore, honey bees’ orientation and navigation abilities, as well as their capacity to learn floral-related cues, were often neglected. Within the behavioral sciences, these aspects are covered by cognitive ecology (Dukas, 1998), which considers how animals obtain and process information from their environments, and how they relate and use such information to make decisions according to their perception and learning abilities (Healy and Braithwaite, 2000).

Honey bees can visit a wide range of flower types as long as the resources offered are profitable (Visser and Seeley, 1982; Steffan-Dewenter and Kuhn, 2003). Regardless of their generalist foraging strategy, honey bees exhibit fidelity to a single plant species within the same foraging bout (von Frisch, 1967). Such behavior is known as flower constancy (Free, 1963; von Frisch, 1967) and it implies that experiences with flowers that offer sufficient reward (pollen and/or nectar) encourage bees to keep collecting on the same floral type (Menzel and Erber, 1978; Chittka et al., 1999). It may vary according to the quantity and quality between the food sources (Wells and Wells, 1986). Thus, floral constancy together with the ability to communicate food-related information (location, profitability and chemosensory cues) within the nest (von Frisch, 1967; Farina et al., 2005), make the honey bee an efficient pollinator throughout a broad spectrum of agricultural settings (McGregor, 1976; Free, 1993).

The first attempts to improve food production in agricultural landscapes considering the plastic behavior of the honey bee were reported in the famine time before and during the World War II by different research groups from Germany and the ex-Soviet Union. In that time, different procedures to direct pollinators to target crops were based on the seminal study of von Frisch (1923),

showing that recruited forager bees were prone to visit flowers of the same species previously exploited by scouting colony mates. von Frisch observed that the efficiency of the recruitment to a target feeding site depended on the distance to the hive and on the presence of floral odors, which were either emitted by parts of flowers attached to a feeder or diluted in the food. von Frisch noted that the offering of scented food enabled a better recruitment, likely because the chemical properties of the odors were better maintained until the liquid food was shared via trophallaxis with the colony mates. Pioneering practices to promote bee responses to target crops were then based on soaking fragrant flowers in sugar water, which produced a scented solution expected to bias foraging towards the target flowers (Smaragdova, 1933; Gubin, 1936; Gubin, 1938; von Frisch, 1943; von Frisch, 1947). The use of in-hive scented-food stimulation showed increases both in the number of bees that visited the crop (Gubin, 1938; Sorokin, 1938; Komarov, 1939) and in seed yields (Sorokin, 1938; von Frisch, 1947). von Frisch also tested the offering of scented sugar solution outside the hive (i.e., in the crop surroundings; von Frisch, 1943; von Frisch, 1947) and proved that this procedure was effective in increasing both the colony activity level, and the amount of honey produced (von Frisch, 1943), likely as it promotes the display of dances. However, a study of Free (1958) in apple and red clover crops using either the offering of scented sugar solution outside or inside the hive, or the combination of both, showed no evidence of increases in crop yields. Later, Free (1969) tested the extent to which the odor of nectar stored in combs affected foraging preferences in a double-choice test. Although the results were highly variable, Free was able to detect a brief biased response to the odor present in the honeycomb.

Despite their relative success in guiding bees to target crops, procedures that soak fragrant flowers in sugar water have several disadvantages, such as the poor stability of the odor extracted from the flowers and the cost involved in obtaining large quantities of flowers to achieve a stimulus sufficiently intense to modify bee responses. Furthermore, the cutting and crushing of flowers for the syrup preparation may promote the release of unwanted volatiles, related to tissue damage or wilting, being a strong source of variation among results of pioneering studies. For this reason, it is relevant to integrate aspects related to floral odors and honey bee social behavior as part of a targeted pollination strategy. With this in mind, the objective of this review is to summarize some pertinent elements related to individual and social honey bee learning of floral scents that affect foraging responses, which are potentially applicable for guiding bees to target crops to enhance pollination services. Floral volatiles of specific crops, honey bee odor perception, social foraging, and the procedures in the field will also be discussed as necessary components within the targeted pollination framework (Figure 1).

Floral scent and its recognition by honey bees

Floral bouquets are complex mixtures of volatile organic compounds (VOCs) which are directly involved in plant-

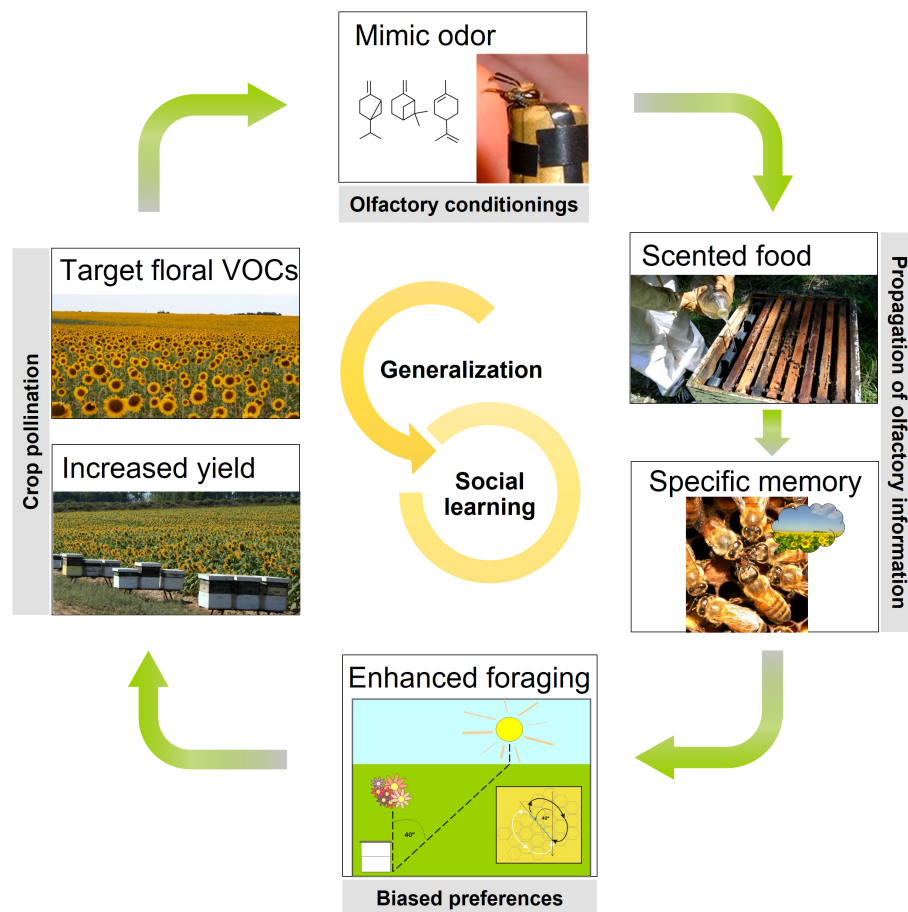


FIGURE 1

The targeted pollination framework integrates aspects related to floral odors and honey bee social behavior. Floral volatile organic compounds (VOCs) of specific crops are collected and identified to determine a set of potential odor mixtures that mimic the bouquet of the target crop flower. The odorant mimic which honey bees broadly generalize to the natural floral bouquet, but which is also the less discriminating, is selected as the mimic odor to be evaluated in the field. The circulation of sugar syrup scented with the mimic odor inside the colony establishes specific olfactory memories among nestmates. The propagation and persistence of the food-related information at the colony level releases recruiting mechanisms and foraging toward the target crop, consequently improving pollination services (adapted from Farina et al., 2020). Reproduced with permission from Farina and co-workers, Current Biology; published by Cell Press, 2020 (CC-BY 4.0).

pollinator interactions (Knudsen et al., 1993; Raguso, 2008; Pichersky and Dudareva, 2020). Volatile emissions can be altered by several factors, such as cultivar, time of day and pollination status (Rodriguez-Saona et al., 2011; Twidle et al., 2017). Depending on the identity and concentration of the VOCs emitted by the different plant species, diverse specific pollinator groups are attracted (Dobson, 2006). In particular, the olfactory cues that honey bees use to perceive specific flowers has been investigated for different crops, such as oilseed rape (Wadhams et al., 1994), kiwifruit (Twidle et al., 2015), pear (Su et al., 2022) and other Brassicaceae species (Kobayashi et al., 2012), among others. In such studies, honey bee odor detection was assessed by means of electro-antennography (EAG) where the antennal response towards the different floral bouquets is measured. It is well known that, although plants emit large amounts of VOCs, honey bees detect a small subset of these compounds or key odorants (Reinhard et al., 2010; Mas et al., 2020). Moreover, not all odors detected by the peripheral olfactory system are behaviorally meaningful, and most must be learned before they can influence behavior (Riffell et al., 2009b; Menzel, 2012).

Honey bee learning abilities and tools to train individual bees to complex floral scents

Many odors, which initially are neutral to honey bee foragers, may become good predictors of food sources after being learned (von Frisch, 1967; Lindauer, 1970; Gould, 1984; Menzel, 2012). Learning allows individuals to flexibly respond to a changing environment (Menzel, 1999), with varying availability of food sources during the season (Núñez, 1977; Vogel, 1983), being extremely important in species with generalist habits. In this way, honey bees as well other pollinators are able to associate floral cues, such as odors and colors, with the rewards (nectar, pollen) that the source provides (Gould, 1984; Chittka and Thomson, 2001). If bees repeat cue-reward experiences, these associations turn into memories that influence foraging behaviors, by biasing flight orientation (Chaffiol et al., 2005; Nery et al., 2021), landing (Arenas et al., 2007; Arenas et al., 2008), and/or extension of the proboscis (Grüter et al., 2006; Arenas and

Farina, 2012). The latter is an innate reflex response that occurs when a bee's antennae contact the nectar of a flower, leading to an immediate ingestion of the food. In the laboratory, the proboscis extension response (PER) can be evoked by touching the antennae of restrained bees with an enough concentrated sucrose solution (Kuwabara, 1957; Takeda, 1961). Moreover, bees can be trained to associate an odor with a sucrose reward, by means of an olfactory conditioning protocol (Takeda, 1961; Bitterman et al., 1983). Prior to conditioning, bees do not usually respond to the conditioned stimulus (CS), but after successive paired presentations between the odor and a sucrose reward, the previously neutral stimulus now takes control over the proboscis reflex.

Within the PER paradigm, it is possible to train bees to learn that an odor predicts an oncoming reward in the absence of other alternative stimuli, in the so-called absolute conditioning (Giurfa, 2007; Giurfa and Sandoz, 2012). This protocol allows us to test the extent to which the conditioned response can be generalized to different but equivalent stimuli. The phenomenon of generalization is widespread among animal kingdom (Shepard, 1987; Ghirlanda and Enquist, 2003), and it is essential for the foraging behavior of the bees since it enables foragers to respond to different floral odors if they are perceived as similar (Pham-Delegue et al., 1989; Guerrieri et al., 2005). Alternatively, bees trained in a differential conditioning learn not only the characteristics of a reinforced stimulus (rewarded conditioned stimulus, henceforth: CS+), but also those of a nonreinforced one (non-rewarded conditioned stimulus, henceforth: CS-) (Bitterman et al., 1983; Giurfa, 2007; Giurfa and Sandoz, 2012). This protocol allows us to evaluate bees' abilities to discriminate between both conditioned stimuli. Combined, the two types of conditionings enable investigating how bees learn, generalize and discriminate odorant mixtures.

Previous studies about insect behavior demonstrated that plant-pollinator interactions can be mediated by a few key odorants (Riffell et al., 2009a; Riffell et al., 2009b; Reinhard et al., 2010; Mas et al., 2020). Using the moth *Manduca sexta* as a study model, it was observed that food source attraction and innate foraging behavior could be elicited by a few of the volatile compounds that conform the natural flower bouquet (Riffell et al., 2009a; Riffell et al., 2009b). The evidence that a few key volatiles are sufficient to account for a complex odor mixture is not limited to innate behaviors but extends to learned responses as well. In honey bees, response to mixtures composed of a few selected key odorants (some of only 3 pure compounds) were sufficient to elicit levels of PER comparable to those evoked by the complete olfactory mixtures of 14 odorants to which the bees were initially conditioned (Reinhard et al., 2010). The key odorant processing of floral scents may be adaptive to maintain stimulus identity in a constantly changing environment while it gives us the possibility for using simple mixtures to mimic the complex floral scent of a species of interest to manipulate odor-mediated responses of pollinators. In fact, it has been recently shown that the conditioning of synthetic mixtures with 3 or 4 constituents could be enough to successfully generalize the natural floral scent of agriculturally important species, such as sunflower, pear, apple, and almond (see Figure 2A as example), which in turn resulted in a bias of the bees' foraging behavior towards the target crop (Farina et al., 2020; Farina et al., 2022; Farina et al., 2023).

Apart from evaluating the degree of generalization of specific odor mixtures designed to mimic the scent of crop flowers, Farina and coworkers (2020; 2022; 2023) assessed to what extent bees could discriminate them from the respective natural floral scents. Differential PER conditionings using the mimic odor and the natural floral scents both as CS+ and CS- revealed that bees could only discriminate between the stimuli when the natural blend was presented as CS+ and each mimic (either for the sunflower, pear, or apple flower) as CS-. Interestingly, bees failed to distinguish between stimuli if the mimics acted as CS+ and the natural blend as CS-, indicating that discrimination was not symmetric (see Figures 2B, C as example). Such asymmetry between a small subset of key odors that make up a behaviorally effective mixture and the natural floral bouquet denotes the complexity of insect olfactory perception (Sandoz et al., 2001) and suggests that a mimic odor could be more effective in biasing foraging behavior if learned while bees remain naive to crop flower's olfactory cues than in experienced individuals.

Considering that memories decay in time (Menzel, 1999), some studies focused on the effect of the addition of nonsugar nectar compounds on honey bee olfactory associative learning with the aim to establish more stable long-term memories (Wright et al., 2013; Marchi et al., 2021). On the one hand, the alkaloid caffeine triggers long-term memory in honey bee (Wright et al., 2013), meanwhile the essential amino acid arginine participates in the synthesis of nitric oxide, and therefore promotes protein synthesis during long-term memory formation (Müller, 1996; Müller, 1997). A recent related study showed a positive effect of the combination of caffeine and arginine in the reward, increasing bees' learning performance and long-term memory formation (Marchi et al., 2021). Thus, the joint administration of nonsugar nectar compounds with synthetic mimic odors could further enhance the persistence of olfactory memories and therefore, the efficacy of a procedure that aims to modify bee's preferences based on experience.

Social foraging strategies

A honey bee colony can rapidly adjust its foraging behavior and guide its workforce toward the most rewarding flowers in the surrounding environment (Seeley, 1995). Within the hive, social interactions among nestmates facilitate the propagation of food-related information, allowing not only experienced foragers to access information about other available sources, but also new recruits to locate profitable foraging sites via the waggle dance (von Frisch, 1967). There is a consensus that olfactory cues of the discovered resource play an important role in orientation at short distances (von Frisch, 1923; Sherman and Visscher, 2002). On the other hand, further studies support the idea that olfactory cues alone are not sufficient to recruit nestmates (Gould, 1984; Riley et al., 2005) and that orientation of foragers fails if olfactory stimuli from the food source to which they have been trained are relocated beyond 200 meters (Menzel and Greggers, 2013). However, odorants can assist recruits to reach the target if they are learned in the colony and within a recruiting context (Farina et al., 2005; Díaz et al., 2007).

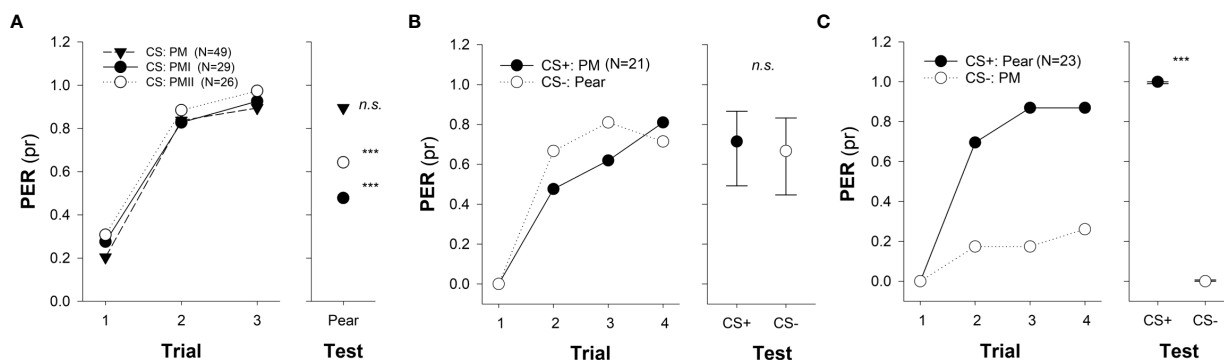


FIGURE 2

Odor generalization and discrimination of memories from pear mimic odors to natural floral scents. (A) Odor generalization was tested towards the single unrewarded presentation of the pear natural odor (right panel) after one of the pear mimics (PM, PMI or PMII) was used as conditioned stimulus (CS) during an absolute three-trials classical conditioning of the proboscis extension reflex (PER; left panel). Asterisks indicate significant differences between responses obtained at the third conditioning trial and the test (***, $p < 0.001$). No significant difference (n.s.) indicates that bees could successfully generalize PM to the pear natural scent (test). (B, C) Discrimination was evaluated towards the single presentation of the pear natural odor and the pear mimic (PM) at the test (right panel) after a four-pair-of-trials differential PER conditioning (left panel), for which both odors were used as rewarded (CS+) and non-rewarded stimulus (CS-). (B) Pear natural odor (floral natural scent) was used as CS+ and the pear mimic (PM) as CS-. No difference (n.s.) at test indicates that bees could not discriminate between PM and the unrewarded pear natural scent. (C) Pear natural odor (natural floral scent) was used as CS+ and PM as CS-. Asterisks indicate significant differences between tested odors (***, $p < 0.001$). The experimental subjects were all foraging bees and had no previous access to any pear tree. Numbers between brackets indicate sample size. Circles indicate the probability of PER (GLMM predicted data) and bars (in test) show the 95% confidence intervals (adapted from Farina et al., 2022). Reproduced with permission from Farina and co-workers, Scientific Reports; published by Nature Portfolio, 2022 (CC-BY 4.0).

Beside the transmission of spatial information, the dance increases the attention and activity of bees in the vicinity, attracting them to the dancer (von Frisch, 1967; Grüter and Farina, 2009; Balbuena et al., 2012a; Ai and Farina, 2023). Then, more, and highly motivated bees around the dancer can learn the floral odor molecules attached to its body (Mo Mauro et al., 2018). Dancing bees often briefly interrupt the dance and offer food samples to surrounding bees (von Frisch, 1967; Díaz et al., 2007). These oral interactions (i.e. trophalaxis) can be very brief, but just long enough to act as a reward in olfactory learning (Díaz et al., 2007; Farina et al., 2007; Farina and Grüter 2009; Grüter and Farina, 2009). For scented nectars, trophallactic interactions enable the establishment of memories from odors diluted in the food that is being shared, an effective mechanism when scouts forage from distant sources while the odors attached to their body fade during the trip back to the hive. For pollen foragers, cues associated with pollen loads carried on the hind legs of dancers may also be perceived and learned by other foragers (Díaz et al., 2007; Nery et al., 2020) giving selectivity to recruitment (Arenas et al., 2021).

Memorization of olfactory cues within the nest, albeit outside the dancing context, could also assist recruits locate the feeding site (Balbuena et al., 2012b). Olfactory cues could also be learned from scented nectars that are unloaded to the food processor bees (Grüter et al., 2006; Grüter et al., 2009). The food odors learned inside the nest can be retained by colony mates for up to 10–11 days suggesting that olfactory experiences occurring within the colony can propagate to many individuals (Grüter et al., 2009). Moreover, circulation of scented sugar solution biases foraging preferences towards the learned odor, a response that is extended until four days after removing the scented-food stores and the combs where the syrup could have been stored (Arenas et al., 2007; Arenas et al.,

2008). It is not trivial to mention that when the odor is not offered in the food but presented as a volatile that aromatizes the nest environment (Arenas et al., 2008), an avoidance rather than an improvement of the landing response towards the exposed odor is observed. These results suggest that the presentation of odors, unpaired with the reward, triggers cognitive processes other than associative learning, which prevents the nectar foragers to visit sources scented with the exposed odor. In summary, although other sensory modalities (e.g. visual) may be much more effective for long-distance flights during searching resources (Chittka and Menzel, 1992; Dyer et al., 2011; Menzel and Greggers, 2013), the use of floral odors via olfactory memories are crucial in the search for food sources when combined with other social interactions occurring in the nest.

Targeted pollination procedures

Given that olfactory information transfer can also occur when odors are directly provided inside the nest (Arenas et al., 2007; Arenas et al., 2008), the offering of scented food can be used as a standardized procedure to establish specific long-term memories among foragers being part of a targeted pollination strategy. A common practice of beekeepers is feeding colonies with sugar syrup at certain times of the year, for instance during dearth periods of nectar (Geslin et al., 2017a; Sammataro and de Guzman, 2018; FAO et al., 2021). Furthermore, feeding colonies with syrup in the fall can ensure survival through winter and the provision of sugar syrup inside the hive stimulates brood rearing and thereby promotes foraging for pollen (Goodwin, 1997; Sammataro and de Guzman, 2018). Sugar syrup can be offered by means of in-hive feeders of

different types, such as division board or top hive feeders. Such supplemental feeding practice is suitable for olfactory conditioning of colonies providing pollination services (Farina et al., 2020; Estravis-Barcala et al., 2021; Farina et al., 2022; Farina et al., 2023). Scented food can be obtained by diluting a small volume of the mimic odor (50 µL) per liter of sucrose solution (50% weight/weight, henceforth: w/w). Scented syrup can be offered using in-hive feeders or even poured over the top of the central frames of the hives (for 1,000–1,500 mL or 500 mL of scented solution, respectively).

Some important aspects to be considered in honey bee management for pollination services are colony size (Goodwin, 1997; Geslin et al., 2017a; Ovinge and Hoover, 2018; Chabert et al., 2021) and the timing of colony introduction into the plots (Free, 1959; Free et al., 1960; Al-Tikrity et al., 1972; Moeller, 1973; Sammartaro and de Guzman, 2018). If colonies are introduced into the agricultural setting long before blooming, bees could forage on other attractive non-target flowers and may ignore the crop when it blooms. On the other hand, if colonies are settled when the crop is already in full bloom, they may not be able to learn the cues related to the crop flowers before blooming ends and may not have enough time to learn the landmarks needed to orient themselves. The timing of stimulation is critical as well. Feeding should be done at the beginning of the blooming period to guarantee bees an early access to relevant olfactory information which will assist them in finding the target flowers in a novel environment. It is advisable to perform the stimulation of colonies when the target crop is 10–40% in bloom. A single stimulation event should suffice to guide bees to the target crop. But it should be considered that the number of events may vary with the specific requirements of the crops and the weather conditions.

The targeted pollination strategy has the advantage of being specific to the crop, and usually requires only one application (Farina et al., 2020; Farina et al., 2022; Farina et al., 2023). This method facilitates the propagation of food related information among nestmates, which can be retrieved several days later. These studies demonstrate that specific olfactory memories established within the honey bee colony result in faster foraging that increases crop production, showing the advantages of a targeted pollination approach to enhance pollination services in commercial crops (see [Supplementary Table S1](#)).

It should be mentioned that there is a study testing the method of osmoguiding bees with a maceration and cooking of crop flowers, which failed to promote visits to the target pollen (Higuera-Higuera et al., 2023). So far, these results are inconclusive, as some of the assays need more controls to be confirmed.

Alternative methods to improve pollination services

In addition to the targeted pollination strategy, alternative methods to improve pollination services involve the use of non-crop-specific attractants derived from plant natural extracts or pheromonal compounds, with varying degrees of success (see studies reviewed in Delaplane and Mayer, 2000; Abrol, 2012 and

illustrative examples in [Supplementary Table S1](#)). Among the first group, the spraying of crop flowers with an essential oil extracted from *Lavandula hybrida* leaves or with olive pomace extract showed ambiguous results in bee visits but had a positive effect on yield (Meroi Arcerito et al., 2021; Monasterio et al., 2023). Among the latter, the evaluation of commercial attractants based on pheromonal compounds (e.g. Bee Scent, Bee-Here, Pollinus and Polynate) reported mixed results. While some authors documented increases in bee visits (Higo et al., 1995) and seed yields (Przybylska et al., 2021), other studies showed no improvement of either the number of bees on the target crop or fruit set (Schultheis et al., 1994; Ellis and Delaplane, 2009; Williamson et al., 2018). These methods might be limited due to the mode of application. As not only flowers but whole plants were sprayed with the attractant (or attractant dispensers were attached to the branches), bees will not necessarily reach the target flowers and associate nectar and pollen resources with the attractant through learning, reducing the chances of a successful pollination (Knauer and Schiestl, 2015). Also, even when some pheromone-based compounds (Nasonov gland or queen mandibular pheromones) might generate an initial innate response, the repeated exposure to the attractant without a floral reward could result in the losing of the stimulus meaningfulness. This process known as habituation is well documented in bees (Scheiner, 2004).

Another group of attractants is commercialized as food lures (e.g. BeeLure, Beeline and Bee-Q) containing protein, sugars, fats, minerals and/or vitamins. They have been widely tested in several crops with limited success (for example, Rajotte and Fell, 1982; Schultheis et al., 1994; Jayaramappa et al., 2011; Dorjay et al., 2022; Jailyang et al., 2022). These studies do not fall within the scope of this review, as our aim was to focus on cognitive and behavioral aspects of the bee-crop interaction, discarding those attractants which consider bee nutritional matters.

Measurements in the field

After feeding colonies with scented sugar solution, the effect of the stimulation can be measured both from the bee perspective, on foraging-related activities, and from the crop perspective, on yield (see [Supplementary Table S1](#)). To do that, it is relevant to consider the placement of hives within the field, to avoid overlapping treatments (scented and unscented food) and to ensure bees a similar availability of flowers and, therefore, of resources. Although honey bees can forage over vast areas around the nest, up to 10 km or more if food is scarce (Jay, 1986; Beekman and Ratnieks, 2000), they prefer to forage within 1–2 km from their colonies (Seeley, 1995; Aras et al., 1996; Vaissière et al., 2011). Ideally, the groups of treated hives should be at least 2 km apart, which can be difficult to achieve in agricultural settings. Particularly, it has been observed in various crops that the number of foraging bees decreases as distance from the hives increases (Noetzel, 1968; Gary et al., 1976; Johannsmeier et al., 1997; Johannsmeier and Mostert, 2001; Hagler et al., 2011; Cunningham and Le Feuvre, 2013; Chabert et al., 2022), and that

honey bees forage at an average distance of 80–1,663 m, and up to more than 6 km, from their colonies in adjacent fields depending on the crop (Gary et al., 1972; Gary et al., 1973; Gary et al., 1975; Gary et al., 1976; Gary et al., 1978; Hagler et al., 2011). Future research should further investigate bee foraging distances in different agricultural scenarios.

Assessment of bee foraging-related activities

Display of waggle dances: To test whether the offering of scented food positively biases bee foraging choice toward the target crop, it is possible to decode waggle dances and reveal the location of their foraging sites (von Frisch, 1967; Visscher and Seeley, 1982). Therefore, waggle dances can be used as indicators to determine the spatial and seasonal ecology of honey bees in both rural and urban landscapes (Couvillon et al., 2014; Balfour and Ratnieks, 2017; Danner et al., 2017; Bänisch et al., 2020a). To that end, two-frame observation hives (each with about 4,000 workers and a mated queen) can be settled in the field. Honey bee dances can be video recorded during the experimental period. The recording times should be equally distributed to morning and afternoon hours to account for pollen and nectar availability of different plant species throughout the day. Based on the angles and the duration of the waggle runs, the observer can then identify dances recruiting toward a given target and perform a daily dance map. Finally, the percentage of dances advertising locations within the target crop at different moments of the experiment can be calculated and the time elapsed since the onset of dances can be measured (Figure 3; Farina et al., 2020).

Hive entrance activity: To evaluate whether the circulation of scented food inside the hives alters foraging activity, the number of incoming bees can be assessed since a large part of these bees is expected to return from foraging sites (Fewell and Winston, 1996; Díaz et al., 2013), while another part is expected to be learning foragers operating orientation flights (Capaldi et al., 2000; Degen et al., 2015). When successful foragers return to the hive and display dances, the activation or reactivation of unemployed foragers is promoted, as well as, in a minor proportion, of those nestmates ready to initiate foraging tasks (Lindauer, 1954; Seeley, 1986; Seeley, 1995; Thom et al., 2007). Incoming foragers at the entrance of the hive can be counted for a short period (1 min) at the same time on consecutive days (Delaplane et al., 2013a; Farina et al., 2020; Estravis-Barcala et al., 2021; Farina et al., 2022; Farina et al., 2023; see Figures 4A, 5A as examples). Ideally, 3 to 5 independent measurements should be done before feeding the colonies to control for environmental conditions, pre-existing colony differences and behavioral inertia (Rodet and Henry, 2014). Then, using a Before-After-Control-Impact (BACI) design is a robust approach to analyze the effect of feeding colonies with scented food on the hive entrance activity by testing the interaction between the periods 'before'/'after treatment' and the treatments 'control'/'scented food' (Christie et al., 2019; Christie et al., 2020; Wauchope et al., 2021).

Pollen collection: The effect of the stimulation with scented food on pollen collection can be assessed by measuring the abundance and weight of corbicular pollen loads. The measurement of these

variables is particularly pertinent when honey bees actively exploit this resource in the target crop (Hoover and Ovinge, 2018; Bänisch et al., 2020b), but see below the *Case studies* section for more discussion. Pollen loads from returning foragers can be collected using conventional pollen traps (frontal-entrance trap), consisting of a wooden structure with a removable metal mesh inside (Delaplane et al., 2013a). The traps should be placed at the hive at the same time on consecutive days, depending on the timing of the crop pollen availability. Ideally, 3 to 5 independent measurements should be done before feeding the colonies as mentioned before. Pollen pellets can be identified as coming either from the target crop or from other competing floral sources based on their color, by comparison with pellets obtained from bees captured foraging on the crop. Finally, the number and weight of the target pollen loads can then be determined (Farina et al., 2020; Farina et al., 2022).

Another way to assess honey bee pollen collection is to quantify the pollen reserves by estimating the amount of stored pollen inside the colony. For this purpose, colonies are thoroughly inspected by sequentially removing frames and recording the area occupied by cells containing pollen on both sides of each frame (Delaplane et al., 2013b; Farina et al., 2023). This measurement must be done before stimulation and at a defined time later, to evince any difference in pollen foraging. The interval of time can be set according to the blooming period of the target crop.

Crop foraging activity: To evaluate whether the offering of scented food affects honey bee foraging intensity on the target crop, densities of foragers visiting the target flowers in the surroundings of the colonies can be assessed. Ideally, foragers can be assessed in all the field, until 2 km away from the colonies (Vaissière et al., 2011). Forager density can be measured by scan sampling on a fixed number of open flowers or inflorescences along a row in herbaceous crops, or in focal trees in orchards (Vaissière et al., 2011; Farina et al., 2020; Estravis-Barcala et al., 2021; Farina et al., 2023; see Figures 5B, C, 4B, respectively, as examples). These measurements must be repeated at the same time (during peak hours of foraging) on consecutive days. As mentioned above for the other variables, ideally, 3 to 5 independent measurements should be done before feeding the colonies as for previous variables. It should be kept in mind that to estimate the floral resources available to honey bees in the field, the recording of flower density or phenology of the crop should be done at the same time as assessing the bee density (Vaissière et al., 2011; Estravis-Barcala et al., 2021).

Assessment of crop yield

Although many factors not related to the pollination level during flowering can interfere with the crop production variables, it is possible to evaluate the contribution of honey bee pollination on yield (Vaissière et al., 2011), as long as the crop yield potential is properly controlled with hand pollination treatments (Chabert et al., 2022). Fruit set (the proportion of flowers that develop into mature fruits) is usually correlated to crop yield and it can be strongly affected by pollinator visitation in a wide variety of crop systems (Garibaldi et al., 2016; Reilly et al., 2020). Thus, the effect of the offering of scented food on the target crop yield can be evaluated by means of the fruit set and/or seed set depending on the crop

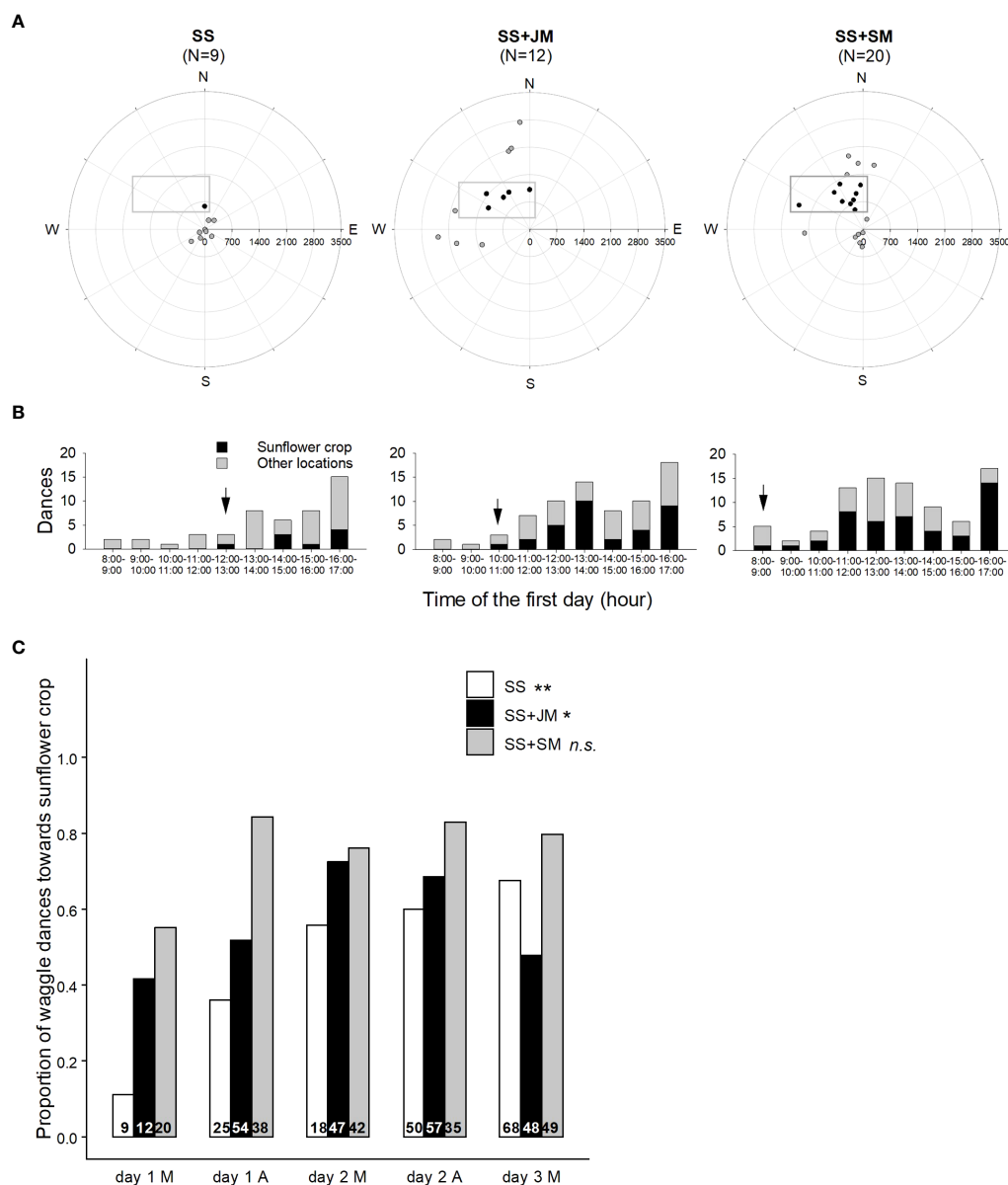


FIGURE 3

Effect of sunflower mimic odor on the display of waggle dances. **(A)** Colonies located in two-combs observation hives were fed with unscented sucrose solution (SS), sucrose solution scented with jasmine mimic (SS+JM), or sucrose solution scented with sunflower mimic (SS+SM) in a distant apiary, 2 days before the onset of the stimulation. Food scented with JM, the mimic of a flower that is not available in the surroundings, was offered as control for the unspecific effect of an odor in the solution compared to the specific mimic odor (SM). Radial maps show the foraging locations (circles) decoded by the waggle dances on the first morning of the experiment. Hives (centers) were settled 600 m SE from the sunflower plot (grey rectangles). Dances were categorized according to the location they were indicating, i.e., inside (black circles) or outside (grey circles) of the sunflower plot. Numbers between brackets indicate the number of dances observed. The decoded waggle dances revealed the location of their foraging sites which showed that the offering of SM-scented food positively biased bees' foraging choice toward the sunflower crop. **(B)** Distribution of waggle dances indicating the sunflower plot (black bars) or other locations (gray bars), displayed on the first day. Black arrow indicates the first dance pointing at the sunflower plot. Dances recruiting toward the target plot occurred earlier in the colony fed SS+SM. During the first morning of the experiment, more than half of the dances in this colony recruited toward the sunflower, and it increased during the afternoon **(C)** Distribution of waggle dances advertising resources within the sunflower plot in each colony during the mornings (M) and afternoons (A) from 1 to 3 days after moving the colonies. Display of recruiting dances in observation hives were affected by the colony treatment (Fisher's exact two-sided test). Asterisks indicate significant differences throughout the experimental period for each treatment (**, $p < 0.01$; *, $p < 0.05$; n.s., non-significant). Differences in proportions of dancers were noticeable during the first day of the experiment, especially during the afternoon up to 84%, a value that was much higher than those exhibited by the other hives (day 1 A). Differences between colonies fed SS+SM and SS+JM persisted during the rest of the experiment. As SS-treated hive showed an increase in the proportion of dances for the sunflower plot by the end of the experiment, previously observed differences with colonies fed SS+SM were attenuated. Numbers inside bars indicate the number of dances observed. (Adapted from Farina et al., 2020). Reproduced with permission from Farina and co-workers, Current Biology; published by Cell Press, 2020 (CC-BY 4.0).

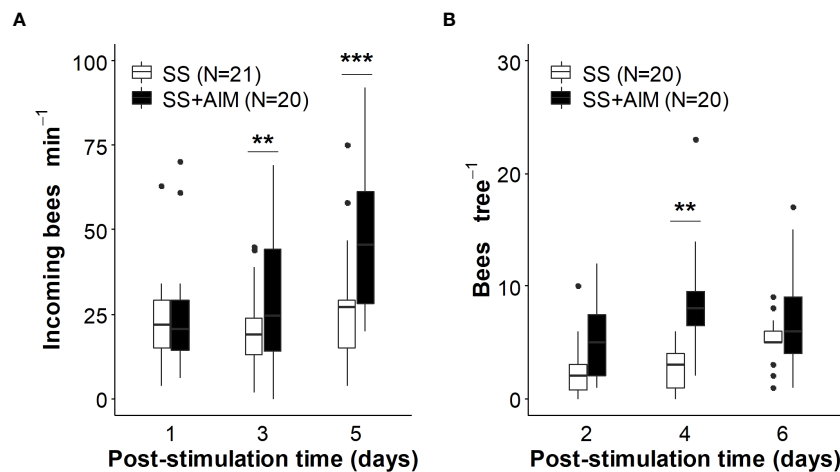


FIGURE 4

Effect of almond mimic odor on bees' foraging related activity. Colonies were stimulated with unscented sucrose solution (SS) or almond mimic scented sucrose solution (SS+AIM). (A) The number of incoming bees per minute was monitored up-to 5 days post-stimulation. (B) The density of bees foraging on almond flowers was quantified in trees within 40 m of the treated beehives up-to 6 days post-stimulation. Boxplots (observed data) show the median and interquartile range (IQR), with whiskers showing the maximum value within 1.5 IQR, and individual points mark showing values outside this range. Asterisks indicate significant differences between treatments (**, $p < 0.01$, ***, $p < 0.001$, GLMM predicted data). Numbers between brackets indicate sample size (adapted from Farina et al., 2023). Reproduced with permission from Farina and co-workers, Apidologie; published by Springer, 2023 (CC-BY 4.0).

(Estravis-Barcala et al., 2021; Farina et al., 2022). Another way to assess crop yield is by quantifying fruit production (number of fruits and fruit mass) at plant/tree level (Sáez et al., 2020; Estravis-Barcala et al., 2021; Farina et al., 2022). At this small scale, fruit production (number and weight of fruits) should be estimated ideally in specimens in all the field, until 2 km away from the colonies (Vaissière et al., 2011). At larger scales, yield is usually reported by the producers as total fruit weight per unit area (Farina et al., 2020; Farina et al., 2022; see Figure 6 as examples).

Case studies

In the last decades, several management methods were developed in an attempt to improve honey bee pollination of crops with ambiguous results (Goodwin, 1997; Delaplane and Mayer, 2000). Recently, the use of mimic odors based on crop floral volatiles has proven to be successful in guiding honey bees toward a target crop, which in turn positively affected foraging activity in systems highly dependent on pollinators, such as sunflower for hybrid seed production, apple, pear and almond, and consequently, increased yields (Farina et al., 2020; Farina et al., 2022; Farina et al., 2023). Although the mentioned species are mass-flowering crops, offering plentiful floral resources for bees, they differ in the type of plantation. While sunflower and almond are usually grown on large-scale monoculture fields and orchards (Farina et al., 2020; Estravis-Barcala et al., 2021; Farina et al., 2023), apple and pear trees are cultivated at a much smaller scale (< 10 ha) and sometime coexist within the same orchard (Díaz et al., 2013; Quinet et al., 2016).

Pollen versus nectar collection

From the bee perspective, olfactory learning of the mimic scents translated into higher levels of foraging activity both at the hive entrance and on the target crop (for sunflower hybrids seed production: Farina et al., 2020; for almond trees: Farina et al., 2023). In the case of apple and pear crops, olfactory memories established within the hive differentially affected bee foraging activity according to the floral resources mainly exploited by bees on these two crops (nectar in apple flowers and pollen in pear flowers; Díaz et al., 2013; Quinet et al., 2016). While the circulation of scented food with the apple mimic promoted a higher number of incoming foragers at the hive (associated with greater activity of nectar foragers), the offering of pear mimic-scented sugar solution did not increase the hive entrance activity, but positively affected pollen collection (Farina et al., 2022). Treatment of colonies with scented foods is expected to increase nectar foraging in the target crop of interest, but not especially to increase pollen foraging. This reasoning is because the odors of the crop flowers are supplied (contingent) with the sugar reward, and not with a pollen reward (Nery et al., 2020; Moreno and Arenas, 2023). However, differences in pollen and nectar foraging patterns in pear and apple tree plantations suggests that the information acquired from sugar syrup can be adjusted and updated based on the availability of resources in the field (Arenas and Kohlmaier, 2019) and thus, be functional to improve pollen collection. Although administration of a scented sucrose solution may activate mainly nectar foragers, a percentage of these bees would have the ability to change their preferred resource by switching to pollen collection (Arenas and Kohlmaier, 2019). This transition is favored especially if the nectar

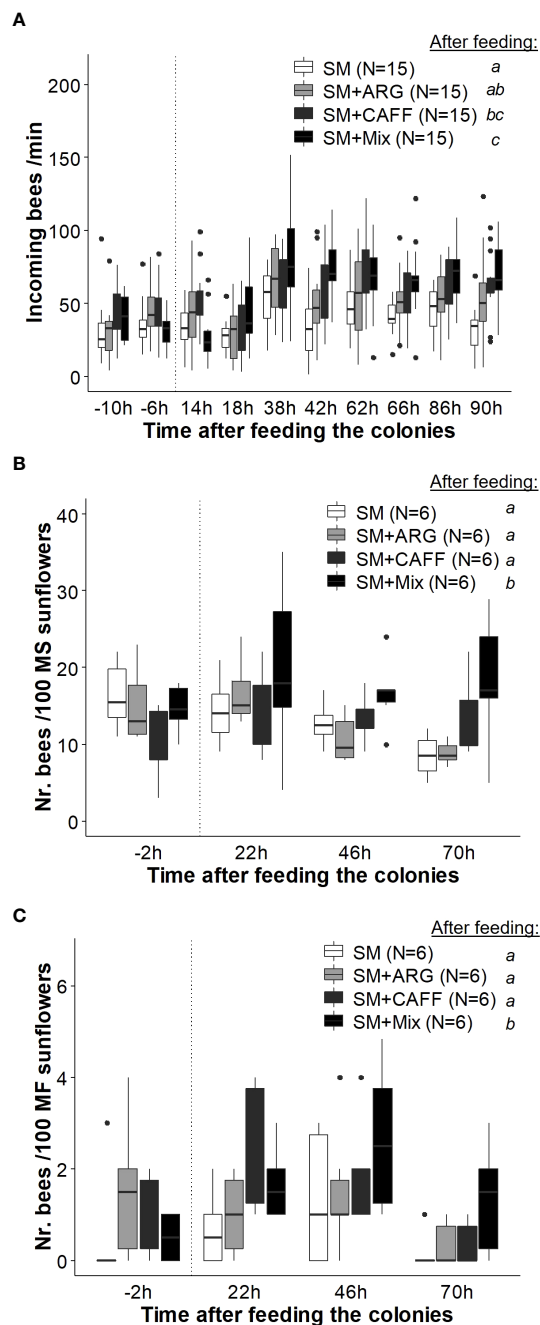


FIGURE 5

Effect of the sunflower mimic (SM) combined with nectar's nonsugar compounds on honey bee foraging. Colonies providing pollination services in a field of sunflower hybrid seed production were fed: SM-scented food (as control), and SM-scented food supplemented with either caffeine (SM+CAFF), L-arginine (SM+ARG), or a mixture of both compounds (SM+Mix). **(A)** Rate of incoming bees before (–10, –6 h) and after the offering of the treatments (up to 90 h). **(B)** Honey bee density on male sterile (MS) sunflower heads in the surroundings of the treated colonies. **(C)** Honey bees' density on male fertile (MF) sunflower inflorescences in the surroundings of treated colonies. Boxplots show the median and interquartile range (IQR), with whiskers showing the maximum value within 1.5 IQR, and individual points mark values outside this range. The vertical dotted line indicates the administration of the treatments. Different letters indicate significant differences ($p < 0.05$) for each treatment after feeding the colonies as assessed with *post hoc* comparisons. Numbers between brackets indicate sample size (adapted from Estravis-Barcala et al., 2021). Reproduced with permission from Estravis-Barcala and co-workers, Scientific Reports; published by Nature Portfolio, 2021 (CC-BY 4.0).

sources visited exhibit a lower productivity than the expected based on foragers' in-hive experience. Considering that the odors learned predicted a very productive source (i.e., an *ad libitum* feeder offering a 50% sucrose solution) and that the nectar productivity of pear flowers is relatively low (estimated nectar sugar concentrations: 6.8

$\pm 0.26\%$ w/w, Díaz et al., 2013; ~10–15% in average depending on the cultivar; Quinet et al., 2016), we speculate that some foragers, initially motivated to collect nectar, may end up collecting pollen. From the early discovery and collection of pollen from pear flowers, which is indeed very productive in terms of pollen reward, the

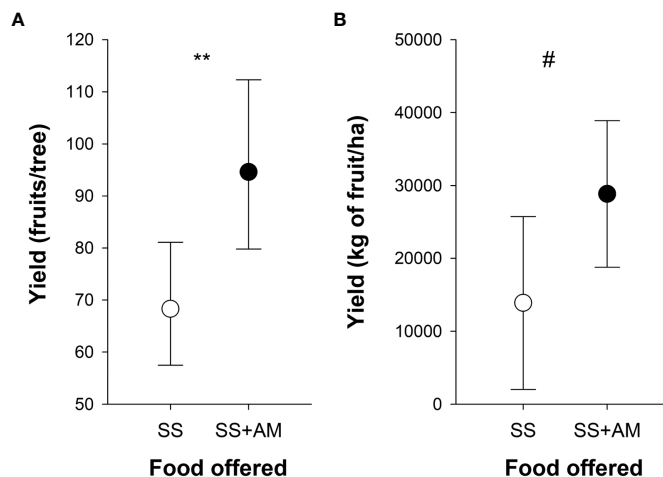


FIGURE 6

Effect of the apple mimic odor on fruit yield and on plantation yield. (A) Fruit yield was calculated as the counts of fruits (fruit set) per tree in two apple plots, where 30 trees were surveyed. Colonies that provided for each apple plot were fed with apple mimic-scented sucrose solution (SS+AM) or with unscented sucrose solution (SS). (B) Crop yield was obtained either from 11 apple plots provided with 130 colonies in total that had been fed with apple mimic-scented sucrose solution (SS+AM) or from 11 apple plots provided with 139 colonies that had been fed with unscented sucrose solution (SS). Asterisks indicate significant differences between the treatments (**, $p < 0.01$; #, $p = 0.06$). Symbols indicate the mean values (GLMM predicted data) and bars show the 95% confidence intervals (adapted from Farina et al., 2022). Reproduced with permission from Farina and co-workers, Scientific Reports; published by Nature Portfolio, 2022 (CC-BY 4.0).

propagation of pollen-related cues (Díaz et al., 2007; Arenas et al., 2021) and information of pollen sources might be guaranteed through the behavioral pathways already described for nectar sources. To this end, higher amounts of pear pollen were collected per foraging bout in the mimic-scented sucrose solution (SS + PM)-treated colony than the control one (Farina et al., 2022). Similarly, almond flowers are also productive in terms of pollen with moderate productivity in nectar values (estimated nectar sugar concentration, $16.7 \pm 1.1\%$ w/w; Farina et al., 2023). In this regard, higher areas of pollen reserves were found in colonies fed almond mimic-scented sucrose solution (SS + AlM) than in control colonies (Farina et al., 2023).

Yield measurements

From the crop perspective, the offering of scented food increased yield significantly in different sunflower cultivars (i.e., kg of seeds per hectare; Farina et al., 2020), and a higher number of fruits per tree was measured both in pear and apple trees (Farina et al., 2022; see Figure 6A as example). It is worth mentioning that the observed increase in the yield of different apple cultivars at a larger scale (kg of fruits per hectare) resulted in no significant increase (see Figure 6B), suggesting that there may be variation in the extent to which bees generalize the mimic odor to the natural scent of diverse apple varieties. This was not the case for the different lines in sunflower hybrid seed production, where the same formulation was effective in guiding foragers in plots dominated by different hybrid lines (Farina et al., 2020).

Effect of additional compounds

Finally, effect of the joint administration of a mimic odor and non-sugar nectar compounds in liquid food was studied in a sunflower field (Estravis-Barcala et al., 2021). Feeding colonies with scented syrup supplemented with both caffeine and arginine resulted in higher foraging activity both at the hive entrance and on the target crop (Figure 5), as well as in increased yields (in terms of seed set and seed mass) compared to the individual effect of the mimic-scented food. Thus, it is suggested that nonsugar compounds, which act as memory enhancers (Marchi et al., 2021), could improve olfactory learning of the mimic odor and its effect on crop pollination.

Conclusions and perspectives

The growing global demand for pollination services (Aizen et al., 2019) leads to propose new strategies in honey bee management to improve its efficiency in agroecosystems. The implementation of a targeted pollination strategy mediated by honey bee plastic responses integrates aspects related to floral odors and honey bee social behavior, including communication processes. Within this framework, the results so far obtained suggest that conditioning bees to simple synthetic odorant mixtures which mimic specific flowers could enable the establishment of in-hive odor memories that bias bees to the target crop and potentially increase yields (Farina et al., 2020; Farina et al., 2022; Farina et al., 2023). From the growers' perspective, this method might decrease the honey bee stocking

rate by increasing the pollination activity of the honey bee colonies and therefore to save on input costs. While at the same it can help to decrease the detrimental effects of managing too many honey bees at the same location on wild flora and entomofauna (Geslin et al., 2017b; Morales et al., 2017; Russo et al., 2021). Nevertheless, it is worth remarking that there are knowledge gaps to be further investigated. The use of volatile mixtures as odor mimic could be challenging for crops that involve different varieties and this will require a thorough understanding of cultivar-specific floral bouquets (Rodríguez-Saona et al., 2011; Twidle et al., 2017). Additionally, specific crops might present certain characteristics detrimental to pollination by honey bees (i.e., brief blooming period, restrictive floral morphology, lack of nectar as reward). Also, future works are necessary to determine how long the effect of the treatment of feeding colonies with sugar syrup scented with mimic odors lasts. At the same time, it remains to be assessed the extent to which this procedure can be implemented with alternative managed bees for those crops where honey bees are less efficient pollinators. The honey bee is not the most efficient pollinator in many cases (Ne'eman et al., 2010) due to quite limited single visit pollen depositions (Földesi et al., 2021; Page et al., 2021), because they are not especially effective to transfer cross-pollen on cultivars requiring cross-pollination, especially when wild entomofauna is absent (Garibaldi et al., 2013), or they forage on a large area around their nest, resulting in a high probability to be diverted to other competing bloom (Jay, 1986; Quinet et al., 2016; Osterman et al., 2021a). This is of particular interest since many native bees (e.g., bumble bees and solitary bees) are currently reared for agricultural purposes (Osterman et al., 2021b). Lastly, although this procedure has great potential for positive impacts on food industry, research on a proper packaging to maintain the chemical stability of the mixture will also be needed to determine its economic viability. The economic impact of an efficient and sustainable entomophilous pollination procedure for the most high-market valuable crops could improve yields in quantitative and qualitative terms in a global context of increasing demand of pollination services.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

National Scientific and Technical Research Council of Argentina (CONICET) has the intellectual property AR082846B1 and Pat. 20110102441 on the commercial use of the sunflower and apple formulations to improve honey bee pollination efficiency, in which WF and AA are coinventors. CONICET and the University of Buenos Aires (UBA) have filed the patent application PCT/IB2018/055550 on the commercial use of the pear formulation to improve honey bee pollination efficiency, in which WF is coinventor. CONICET and UBA have filed the patent application PCT/IB2018/055549 on the commercial use of the almond formulation to improve honeybee pollination efficiency, in which WF, FP, and MCEB are coinventors. WF is coinventor and shareholder of ToBEE S.A., the licensee of these technologies.

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frbee.2023.1253157/full#supplementary-material>

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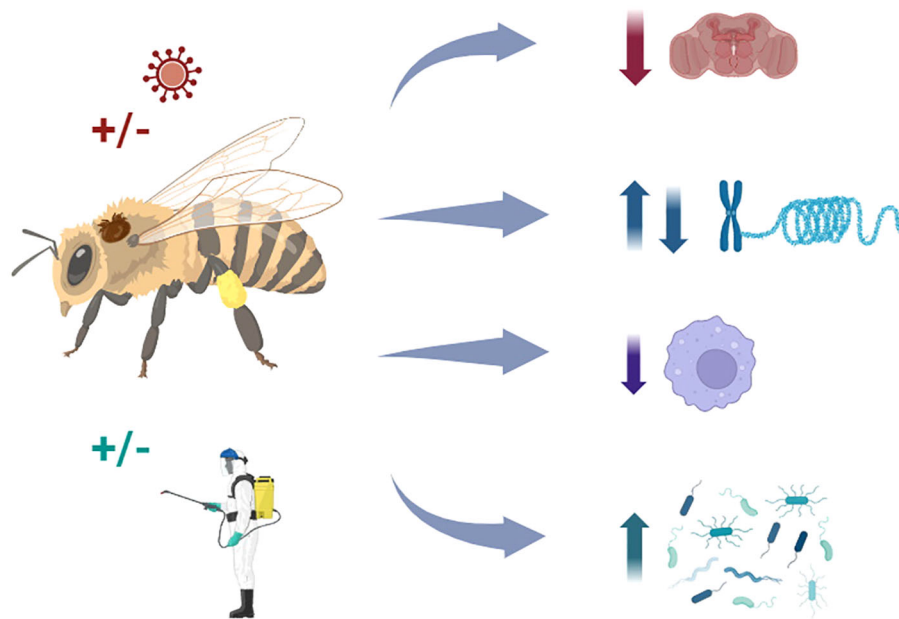
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Varroa destructor is considered one of the greatest threats to the health of the honey bee, *A. mellifera*. In recent years, there has been a considerable increase in the number of studies on the mite and its interaction with the bee at a cellular and molecular level. However, these studies have also revealed just how complex the interaction is. A significant factor in the virulence of *V. destructor* is the proteins secreted in its saliva, but only a fraction of these have yet been examined. These proteins can negatively affect the bee's immune system and promote viruses associated with mite parasitism. Initially, studies on parasitized bees concentrated on immune-related genes, but as more genes of the bees have been examined, it is clear that many other aspects of the bee are affected, such as metabolism and neural functioning. Some of those could be responsible for the detrimental changes in certain behaviors of parasitized bees, which compromises the health of the entire colony. Several viruses are associated with *V. destructor* parasitism, but it remains difficult to distinguish the effects of the viruses from those of the mite. Reduced immunity in parasitized bees also opens the possibility of secondary microbial infections, adding complexity to the mite-bee interaction. Further complicating studies is the impact of other factors, like agrochemicals, which can alter how *V. destructor* parasitism affects bee immunity, metabolism, and neural functioning. In addition, differences due to age and sex of the bee being parasitized is a factor that needs to be considered in all studies. While much has been learned in recent years about this complex interaction, the number of unanswered questions only increases.

KEYWORDS

Varroa destructor, varroosis, virus, honey bees, gene expression, behavior, immunity



GRAPHICAL ABSTRACT

The mechanisms by which *Varroa destructor* affects honey bee health are not trivial; they are associated with complex interactions with other stressors, including pathogens and agrochemicals, which may impact behaviors, gene expression, and immune responses, compromising the survival and performance of honey bee colonies.

1 Introduction

Among the seven to 11 species of *Apis*, the most important economically managed pollinator worldwide is the western honey bee, *A. mellifera*, followed much less by the Asian honey bee, *A. cerana*, which is primarily found in East Asia (Caron and Connor, 2013). Insect pollination is estimated to increase global food production by 15–30%, which can be up to 6,700% compared to self-pollination (Verma and Partap, 1993; Papa et al., 2022). In the USA, honey bee pollination has a value of approx. 11 billion USD (Khalifa et al., 2021) and approx. €153 billion worldwide (Gallai et al., 2009). Pollination increases plant yield and quality (Papa et al., 2022). Vegetables and fruits are the leading types of crops utilizing insect pollination, followed by edible oil crops, stimulants, nuts, and spices (Gallai et al., 2009). Also, honey bees are essential for pollinating wild plants, helping to maintain biodiversity (Papa et al., 2022).

Starting in the winter of 2006–2007, there has been a notable increase in *A. mellifera* colony losses, particularly in the USA and Europe (vanEngelsdorp et al., 2010). An overwintering loss of colonies of approx. 10% was considered normal, but in the winter of 2006–2007, losses in the USA reached 32% (vanEngelsdorp et al., 2009). Additionally, researchers from other parts of the world, including the Middle East and parts of Asia, reported high rates of colony losses for the same period, in some cases reaching more than 50% (Neumann and Carreck, 2010). More recent losses of colonies have been reported from Spain (36%), Mexico (27.65%), Slovenia (28.9%) (Medina-Flores et al., 2021; Gray et al., 2023), and the USA

(51%) (Bruckner et al., 2023a). These high rates of colony losses have been described as Colony Collapse Disorder (CCD) (vanEngelsdorp et al., 2009). With CCD, most worker bees in a colony disappear and leave behind a queen, plenty of food and a few nurse bees to care for the remaining immature bees and the queen (<https://www.epa.gov/pollinator-protection/colony-collapse-disorder>). CCD is likely due to multiple factors, including arthropod parasites, such as *Varroa destructor* (Genersch, 2010; Guzman-Novoa et al., 2010), *Acarapis woodi* (Oldroyd, 2007), and *Aethina tumida* (Morawetz et al., 2019), and pathogens, such as RNA viruses, like *Deformed wing virus* (DWV) and *Acute bee paralysis virus* (ABPV), microsporidians, such as *Vairimorpha* (*Nosema*) *ceranae*, and bacteria, such as *Mellisococcus plutonius* and *Paenibacillus larvae* (Genersch, 2010; Hristov et al., 2020). In addition, queen failure (Genersch, 2010), weak colonies in autumn (Genersch, 2010; Guzman-Novoa et al., 2010), synthetic acaricides (vanEngelsdorp et al., 2009), deforestation and habitat loss, agrochemicals, and climate change have been reported as contributing factors (Oldroyd, 2007; Khalifa et al., 2021). Among the causes of CCD, *V. destructor* is often cited as the most important. For example, in the winter of 2008–2009 in Canada, *V. destructor* was associated with >85% of colony deaths (Guzman-Novoa et al., 2010) between 2012 and 2015 in the Netherlands, 83% of colonies not treated for *V. destructor* died (van Dooremalen and van Langevelde, 2021), in the winter of 2015–2016 in Austria, losses were 54.6% when *V. destructor* levels were at 30% (Morawetz et al., 2019).

Originally, *Varroa jacobsoni* was described as a brood parasite of *A. mellifera* that had switched host from *A. cerana* (Oldroyd,

1999). However, *V. jacobsoni* was a complex of at least two species, *V. jacobsoni* and a new species, *V. destructor* (Anderson and Trueman, 2000). After switching hosts, *V. destructor* spread relatively rapidly, reaching Europe by the early 1970s, and worldwide by approx. 2000 (Traynor et al., 2020). One reason for its rapid spread is that *A. mellifera* has lower individual behavioral defenses against the mite than its original host, *A. cerana*. These defenses include grooming, hygienic behavior, varroa sensitive hygiene (uncapping and removal of infested brood), entombing of infested brood, and inhibition of mite fertility (Rath, 1999; Traynor et al., 2020; Grindrod and Martin, 2023).

2 *Varroa destructor* genetic variants

Both *V. jacobsoni* and *V. destructor* can be divided into several variants. Based on the sequence of the mitochondrial DNA cytochrome oxidase subunit I (COI/CO-I/COXI), there were 12 haplotypes of *V. jacobsoni* and four of *V. destructor* in *A. cerana*. In contrast, there were two haplotypes (Korea and Japan-Thailand) of *V. destructor* in *A. mellifera* (Anderson and Trueman, 2000). The Korea haplotype is the most virulent and common worldwide, while the less virulent Japan-Thailand haplotype is limited to Japan, Thailand, French Guyana, Chile, and Brazil (Traynor et al., 2020). However, more haplotypes or sub-haplotypes have been found by analyzing additional mitochondrial sequences. By examining sequence variation in cytochrome B (CytB) in addition to COXI, Lin et al. (2021) identified two variants for the Korea haplotype in China with *A. mellifera*, and Gajić et al. (2019), found five variants of the Korea haplotype in Serbia with *A. mellifera*. By examining sequence variation in cytochrome c oxidase subunit III (COX3), ATP synthase subunit 6 (ATP6), CytB, and COXI, Navajas et al. (2010) found three variants of the Korea haplotype and four variants of the Japan-Thailand haplotype, both in northeast Asia with *A. mellifera*. However, with that same set of genes, Ogiwara et al. (2020) did not find any variation within the Korea haplotype in Japan.

The amount of sequence differences between the genes used to define *V. destructor* haplotypes can vary considerably. Navajas et al. (2010) failed to detect any single nucleotide polymorphisms (SNPs) in CytB within the Korea haplotype of *V. destructor* parasitizing *A. mellifera* in Asia, and all the variation was detected with COXI. For the Korea haplotype in Turkey, the genetic distance between *V. destructor* samples was greatest for COX3 (0.55%), followed by CytB (0.06%), and none for ATP6 (0.00%) (Koç et al., 2021). For *V. destructor* in Serbia, COXI and CytB revealed SNPs in 51.8 and 16.3% of the samples, respectively (Gajić et al., 2019). For the Korea haplotype in Japan, Ogiwara et al. (2020) found no variation in ATP6, COXIII, and CytB among 15 apiaries. Thus, it appears that COXI and COXIII are generally better for distinguishing haplotypes/sub-haplotypes. However, an examination of *V. destructor* from Argentina with COXI, NADH dehydrogenase subunit 4 (ND4), subunit 4 L (ND4L), and subunit 5 (ND5) showed that the ND4 was best as it could reveal two Korea sub-haplotypes (Muntaabski et al., 2020). Thus, there may be other

sequences that can provide better differentiation of *V. destructor* haplotypes.

Another approach to determining variation among *V. destructor* is to examine polymorphisms in microsatellites. Analysis of the *V. destructor* transcriptome revealed 27,775 potential microsatellite loci, and among 60 randomly selected microsatellite loci, six were confirmed to be polymorphic in *V. destructor* from five locations in China (Duan et al., 2020). Thus, there are many possible microsatellites in *V. destructor*, but only a fraction may show polymorphisms. For samples of the Korea haplotype from France, Chile, USA, and Philippines, only two of 20 microsatellites revealed variability (Solignac et al., 2005). For samples from Madagascar, three of 11 microsatellites showed polymorphisms, one with two alleles, another with three, and the last one with four alleles (Rasolofoarivao et al., 2017). For samples from China, five of six microsatellites were polymorphic, and four showed polymorphisms with nine to 16 alleles within two Korea sub-haplotypes (Lin et al., 2021). Greater success can be obtained if *V. destructor* microsatellites are chosen that were previously shown to be polymorphic. Dynes et al. (2016) used 10 microsatellites showing polymorphisms in earlier papers and found all showed polymorphism at the apiary and colony level in the USA. The same approach was used to select six microsatellites, all of which were highly polymorphic for Philippine and Vietnam samples belonging to the Korea haplotype (Beaurepaire et al., 2015). However, Strapazzon et al. (2009) used some of the same microsatellites as Dynes et al. (2016) and Beaurepaire et al. (2015), and none of the four chosen showed polymorphisms within the Korea or Japan haplotypes in two locations in Brazil.

Genetic variability of *V. destructor* has been compared in a variety of ways, mostly by sequencing mitochondrial genes or microsatellite length polymorphisms. Ultimately, the best approach is full genome sequence comparisons. The genome of *V. destructor* is approx. 565 Mbp, like many other Acari (Cornman et al., 2010). As sequencing technology continues to advance, it should become more feasible to compare complete *V. destructor* genomes (Hasegawa et al., 2021). Even a limited comparison between complete genomes of samples could be useful in identifying better markers, such as sequences with the highest number of SNPs or microsatellites with the most significant length variation. As more markers are developed to distinguish sub-haplotypes, like those within the Korea haplotype, it will also be important to compare their virulence to better understand how that varies within a haplotype. Also, one factor that has not been considered much in these studies is sampling time. It appears that mite population can shift considerably over time. The number of alleles varied over a brood season in Germany when examined with seven microsatellites (Beaurepaire et al., 2015).

3 *Varroa destructor* life cycle and virulence

The life cycle of *V. destructor* can be divided into two phases, the dispersal or phoretic phase and the reproductive phase. In the

phoretic phase, *V. destructor* females adhere and parasitize adult bees, which enable them to be transported to worker or drone brood cells where they reproduce, and to spread between colonies on parasitized robber or drifting bees (Boecking and Genersch, 2008). The reproductive phase involves *V. destructor* females entering cells of bee larvae before they are capped (5-day-old instar bee larvae). Once inside the cell, the female mite hides underneath the larva's food until the cell is capped (De Jong et al., 1982). Drone cells appear to be preferred as they were invaded 11.6 times more frequently than worker cells (Boot et al., 1995). During the molt of the bee prepupa (48 h after cell capping), the mother mite, also known as the foundress mite, prepares a feeding site for her daughters on the bee pupa by puncturing the prepupa's cuticle at the sternite of the second abdominal segment of drone pupae or in the mesothorax of worker pupae. Following penetration by the mite's toothed chelicerae and serrated-edged corniculi to form a channel in the bee's cuticle, a pharyngeal pump is used to feed on the bee's internal fluids at 4.5 cycles per second with each feeding event lasting approx. 10 seconds separated by approx. 2 minutes (Li et al., 2019). Bee fluid is extracted from the feeding site by the foundress mite and her progeny throughout the bee's pupal phase (Kanbar and Engels, 2003). Also, the foundress mite establishes a fecal accumulation site on the wall of the cell, where the mating of her offspring also takes place (Donzé and Guerin, 1994).

Approximately 60 to 70 h after the cell is capped, the foundress lays her first egg, which is unfertilized and develops into a haploid male (Rehm and Ritter, 1989). After this, the foundress lays 2 to 5 fertilized eggs at about 30 h intervals between them, and these eggs become females. The developmental period of *V. destructor* from egg to adult is 5.8 days for females and 6.6 days for males. Mating starts approximately 230–280 h post cell capping with female mites reaching sexual maturity 10 to 20 h after the males (Donzé and Guerin, 1994). Males fertilize a sister or sexually mature female mite from a different foundress mite, and the female stores the semen in her spermatheca. A female has to mate at least four times to obtain sufficient spermatozoa to achieve 1.6 to 1.7 reproductive cycles (Ifantidis, 1983). A male can fertilize an average of 3.75 females in a worker cell but 7.5 females in a drone cell since the metamorphosis period is longer in drone cells (Donzé et al., 1996). When workers or drones hatch from their cells, *V. destructor* female progeny leave the cells attached to their hosts. In contrast, progeny males remain in the cell where they starve to death since their mouthparts (chelicerae) are modified to perform sperm transportation into the female receptacle (oviduct II) and thus are unable to feed (Alberti and Hänel, 1986).

A key element of the virulence of *V. destructor* to bees is its saliva. Typically, a damaged cuticle and epidermis of an insect will heal preventing loss of hemolymph, but puncture wounds caused by *V. destructor* remain open (Kanbar and Engels, 2003). There were at least 15 different proteins detected by SDS-PAGE and electroblotting in the saliva of *V. destructor*, and when incubated with hemocytes of the caterpillar, *Lacanobia oleracea*, the saliva damaged the hemocytes and suppressed their ability to extend pseudopods and form aggregates, which are needed for wound healing (Richards et al., 2011). However, a much greater number (356 proteins) were detected in the mite saliva by SDS-PAGE, gel

sectioning and nano-liquid chromatography coupled to mass spectrometry (Zhang and Han, 2019). Saliva was toxic to the larvae of *A. cerana* workers, and some of the proteins in it were potential virulence factors, such as lysophospholipase for membrane destabilization, antimicrobial factors, such as lysozyme for bacterial cell wall degradation, nutrient utilization factors, such as dipeptidyl peptidase III for ingesting host erythrocytes, antioxidant/oxidation–reduction factors, such as thioredoxin peroxidase for hydrogen peroxide breakdown, and detoxification factors, such as sulfotransferase for xenobiotic elimination. Another protein in the mite's saliva is a chitinase (Vd-CHIsal) related to chitinases of parasitic arthropods (Becchimanzi et al., 2020). The gene encoding it was highly expressed in mite salivary glands and silencing it reduced mite survival. It may be essential for hydrolysis of the bee's chitin keeping the wound site open and anti-microbial activity attacking the cell wall of any opportunistic bacteria near the wound site. Enzyme activities of cholinesterases, carboxylesterases and phosphatases were also detected in the secretion products of *V. destructor*, presumably in the saliva, that could enhance virulence by causing cell lysis and degrading bee tissues (Dmitryjuk et al., 2014). Perhaps the clearest example of a saliva protein being involved in mite virulence is Varroa toxic protein, which is lethal to *A. cerana* worker larvae and pupae, but not *A. mellifera* worker larvae and pupae. Also it was not toxic to *A. cerana* worker adults and drones (Zhang and Han, 2018). Considering the significant number of proteins, in the saliva, it will be important to conduct more studies involving gene silencing to assess their importance.

Much of the literature about factors secreted by *V. destructor* is related to honey bee-associated viruses. In *V. destructor*, peptides were detected from Varroa destructor Macula-like virus (VdMLV), DWV and ABPV (Erban et al., 2015). Lack of detection of non-structural proteins compared with high amounts of structural proteins suggested that the viruses did not replicate in the mite but more likely accumulated in the mite gut from hemolymph feeding. Santillan-Galicia et al. (2008) also did not find that DWV replicated in *V. destructor* since there was no specific antibody binding to DWV in any mite tissues, and DWV was only detected in the mite's midgut lumen in structures resembling fecal pellets. However, the variant DWV-B was able to infect the mite's intestinal epithelium and salivary glands, while variant DWV-A could not infect the mite (Gisder and Genersch, 2021). DWV was detected among the mite's saliva proteins, but other viruses, ABPV, Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), Israeli acute paralysis virus (IAPV) and Sacbrood virus (SBV), were not (Zhang and Han, 2019). However, another study reported SBV and Kashmir bee virus (KBV) in mite saliva (Shen et al., 2005). Therefore, at least DWV-B, and perhaps other viruses, can be transmitted in mite saliva.

In addition to transmission in saliva, DWV in bees can be affected by *V. destructor* parasitism. Mite parasitized brood can have overt DWV infections with deformed wings, general paralysis, discoloration, and bloated abdomen compared to covert DWV infections where none of those symptoms appear (Martin and Brettell, 2019). In workers, DWV was found in the thorax and abdomen, but not the head with covert infections, whereas the virus was spread throughout the body in overt infections at elevated levels

(Yue and Genersch, 2005). The saliva of *V. destructor* may be a factor in triggering overt infections as it stimulated the appearance of deformed wings in *A. mellifera* adults in the presence of DWV (Zhang and Han, 2019). Mite parasitism suppressed bee immunity based on reduced expression of genes for antimicrobial peptides and immunity-related enzymes allowing for increased DWV replication (Yang and Cox-Foster, 2005). One explanation for this could be Varroa toxic protein in the saliva, which was not toxic to *A. mellifera*, but increased DWV levels and the subsequent development of deformed-wing symptoms in adults (Zhang and Han, 2018). Another explanation is that *V. destructor* suppresses the immune systems by feeding on the fat body of the bee mechanically removing a tissue that is responsible for immune responses (Galbraith et al., 2015; Ramsey et al., 2019). Mite reproduction was also increased by DWV infection as the virus adversely impacted the bee's immune responses by interfering with NF- κ B signaling (Di Prisco et al., 2016). However, proteome changes in the bee during interactions with *V. destructor* and DWV showed that they were mostly due to the mite rather than the virus (Erban et al., 2019). The effects of the mite and DWV were both cooperative and antagonistic. Opposite effects included the mite and DWV activating and suppressing NF- κ B signaling, respectively, while cooperative effects included both the mite and DWV increasing p53-induced apoptosis, hyperactivation of the JAK/STAT pathway and disruption of p53-BCL-6 feedback. Another way that *V. destructor* could affect viruses in bees is through hemolymph removal during feeding as loss of increasing volumes of hemolymph from bees increased DWV densities by destabilizing viral immune control (Annoscia et al., 2019). Hemolymph removal favors the extraction of antiviral molecules, triggering greater viral replication. While some of the studies do conflict, it is clear that the relationship between the virulence of the mite and honey bee-associated viruses is more driven by the mite and that possibly both mite saliva proteins and physical removal of hemolymph are involved.

4 Individual honey bee damage by mite parasitism

The above review of the life cycle and virulence of *V. destructor* shows that it is highly damaging to bees both directly and indirectly. Effects on individual bees include decreased body weight, lifespan, water content, immune gene expression, neural processes, learning and behaviors, such as flying and orientation (Noël et al., 2020). One of the most common measurements in studies of the effects of the mite on individual bees is the bee's weight, and thus can be compared between multiple studies. For newly emerged bees without deformed wing symptoms, Bowen-Walker and Gunn (2001) found that non-parasitized bees weighed 5% more than parasitized bees inoculated with one mite per capped brood, but this increased to 7% more than bees with three mites per capped brood. For newly emerged bees with deformed wing symptoms, the impact was greater with 12% more weight for non-parasitized bees than those with one mite per capped brood, and 17% more than those with three mites per capped brood. Similar results were obtained by

Yang and Cox-Foster (2007) where newly emerged non-parasitized bees weighed 5 and 8% more than those with one or three mites per capped brood, respectively, without deformed wing symptoms, and 11, 21 and 27% more with one, three or 10 mites per capped brood with deformed wing symptoms. Annoscia et al. (2012) results without deformed wing symptoms were similar with non-parasitized newly emerged bees weighing 8 and 12% more than those with one or three mites per capped brood, respectively. However, with deformed wing symptoms, the weight of non-parasitized newly emerged bees was 4% and 13% higher than those with one or three mites per capped brood, respectively, indicating that deformed wing symptoms did not greatly affect weight reductions. Other studies have only examined parasitized bee weight without deformed wing symptoms. These include van Dooremalen et al. (2013) with non-parasitized bees at seven days of post emergence having 5% more weight than those with one or two mites per capped brood; Strauss et al. (2016) with non-parasitized bees at emergence having 7 and 15% more weight than those with one or with two to three mites per capped brood, respectively; Morfin et al. (2020a) with non-parasitized bees at emergence having 8% more weight than those with one mite per capped brood; and Yang et al. (2021) with non-parasitized bees at emergence having 17% greater weight than those with two mites per capped brood. A summary of studies of the effect of *V. destructor* parasitism of brood on weight of the emerged adult indicates that weight loss always increased with more mites per capped brood, which was relatively consistent, mostly with non-parasitized bees being 5–8% heavier than those with one mite and 15–20% than those with two to three mites. However, more studies are needed to compare the effect of DWV infections on weight loss, particularly by quantifying the amount of DWV. In contrast to brood parasitism, only Morfin et al. (2020b) examined weight loss where mites were placed on adults, showing that non-parasitized bees weighed 7% more than those with one mite per adult bee at 21 days of parasitism with no deformed wing symptoms.

Another factor commonly measured for the effect of *V. destructor* on individual bees is bee survival, and thus can be compared between studies. The median lifespan of bees without deformed wing symptoms after emergence was 13.6 d and 8.9 d with one or two mites per brood, respectively, compared to 27.6 d for non-parasitized bees (De Jong and De Jong, 1983). The time of 75% cumulative dead after emergence with one mite per capped brood was 18.5 d without deformed wing symptoms and 1.1 d with deformed wing symptoms compared to 20.7 d for non-parasitized bees (Yang and Cox-Foster, 2007). The average lifespan of emerged bees with one and three mites per capped brood was 16 or 8.5 d, respectively, without deformed wing symptoms, and 7.5 or 4 d, respectively, with deformed wing symptoms compared to 19 d for non-parasitized bees (Annoscia et al., 2012). The average lifespan of emerged bees with two mites per capped brood was 8.5 d without deformed wing symptoms compared to 14.4 d for non-parasitized bees (Reyes-Quintana et al., 2019). Similar to studies on weight, there has been little examination of the effects of mite parasitism on adult bee lifespans. Morfin et al. (2020b) did not calculate the average lifespan but showed that 20% of adult bees with one mite per bee survived by 21 days compared to 83% for non-parasitized

adult bees. All these studies show that mite parasitism of brood reduced the lifespan of the bee with much greater effects with DWV symptoms, although the effect on adults appears much more limited. Also, the degree of impact of *V. destructor* parasitism on bee lifespan is much greater than that on weight.

In the above studies, weight reductions due to mite parasitism was hypothesized to be due to hemolymph loss (Bowen-Walker and Gunn, 2001; Annoscia et al., 2012; Morfin et al., 2020a), fat body consumption (Morfin et al., 2020a), DWV symptoms (Bowen-Walker and Gunn, 2001; Morfin et al., 2020a), reduced bee water content (Annoscia et al., 2012; Strauss et al., 2016), and reduced bee protein content (van Dooremalen et al., 2013). Reduced lifespan due to mite parasitism were proposed to be due to piercing of cuticle and membranes (Morfin et al., 2020a), suppression of bee immunity (Yang and Cox-Foster, 2007; Reyes-Quintana et al., 2019; Morfin et al., 2020b), up-regulation of genes associated with cardiac pathology, and down-regulation of genes associated with glycolysis/glucogenesis (Morfin et al., 2020b), and DWV symptoms resulting in bees being unable to feed themselves (Yang and Cox-Foster, 2007; Annoscia et al., 2012; Reyes-Quintana et al., 2019).

5 Impacts of *V. destructor* on haemocytes and cellular immunity

Hemolymph is the circulating fluid of insects, composed mostly of water (approx. 20–50% of total water in an insect), but it also contains a variety of chemicals, such as inorganic compounds, low molecular weight organic compounds and proteins, as well as circulating cells called hemocytes (Kanost, 2009). All of these are important for the proper functioning of an insect's metabolism, such as ions to help maintain pH, trehalose as an energy source, glycerol, and sorbitol to lower the freezing point for cold damage protection, and proteins to maintain osmotic pressure, transport lipids, provide food storage, and contribute to the immune response. Also, hemolymph transports nutrients from the digestive system, and removes cellular wastes (Caron and Connor, 2013). The physical loss of hemolymph during *V. destructor* parasitism could negatively affect any of these functions to varying extents. For example, body water content of bees was negatively correlated with the degree of mite parasitism (Bowen-Walker and Gunn, 2001), and total hemolymph basic proteins decreased 34% with one to three mites and 56% with four to six mites per larva (Glinski and Jarosz, 1984).

Insect hemocytes are divided into plasmatocytes, granulocytes and lamellocytes based on their morphology/presumed function with plasmatocytes (a pleiomorphic hemocyte) being the most abundant (Ling and Yu, 2006; Marmaras and Lampropoulou, 2009). Plasmatocytes, granulocytes and lamellocytes are part of the immune response involved in phagocytosis of microbes, nodule formation where multiple hemocytes aggregate to trap microbes, encapsulation where hemocytes attach to the surface of a larger parasite and form a multilayered capsule killing the parasite. In addition, plasmatocytes aggregate to seal wounds preventing hemolymph loss, and granulocytes synthesize an extracellular

matrix that covers tissues exposed to the hemolymph (Strand, 2008). Newly emerged bees with *V. destructor* had up to 90% reduction in the number of normal hemocytes compared to the non-parasitized control, but by 8 to 25 days post-emergence, there were no significance differences from the non-parasitized control (Amdam et al., 2004). Another study of newly emerged bees with *V. destructor* showed approx. 25% reduced hemocyte concentration when exposed to one mite per capped pupa (Morfin et al., 2020a). For nurse bees from colonies parasitized with *V. destructor*, there was an approx. 33% decrease in hemocyte concentration (Belaïd and Doumandji, 2010). For adult worker bees in cages with *V. destructor*, there was an approx. 50% reduction in total hemocyte concentrations by 12 hours of parasitism, although the numbers recovered by 24 and 48 hours (Koleoglu et al., 2018). For drones taken from infested colonies, hemocyte concentrations were decreased by 47%, 13% and 65% for infested larvae, pupae, and adults, respectively (Salem et al., 2006). While all these studies show that parasitism reduces hemocytes in bees, there is a considerable range in the level of reductions, and evidence that the reductions can be transient.

Reductions in hemocyte concentrations by *V. destructor* parasitism as well as down-regulated immune gene expression (Yang and Cox-Foster, 2005; Erban et al., 2019) may help explain the ability of pathogens or potential pathogens to invade the hemolymph of mite parasitized bees. The pathogen, *V. ceranae*, is normally restricted to the bee's digestive system, but its DNA was found in 68% of the hemolymph samples of bees parasitized by *V. destructor* (Glavinić et al., 2014). *Serratia marcescens* was found in the hemolymph of more than 90% of dying worker bees in winter hives and about half of the *V. destructor* in those colonies (Burritt et al., 2016). The bacterium was not generally found on the surface or digestive tract of dying bees, and it was not found in healthy bees. Sequencing the genome showed that this was a novel strain with unique genes related to those of certain bacterial insect pathogens, and there was evidence that the bacterium could be reducing hemocyte populations and permeabilizing some bee organ membranes. Varroa toxic protein could be involved as injection of it into *A. mellifera* larvae resulted in the isolation of *Stenotrophomonas maltophilia* and *Staphylococcus aureus*, while bacterial colonies were not detected from the larval hemolymph with injection only (Balakrishnan et al., 2021). The authors suggested that those species of bacteria were present in the hemolymph of healthy larvae at undetectable levels and Varroa toxic protein stimulated their growth. While none of these studies have shown that the microbes detected are causing diseases in the hemolymph, they do indicate that microbes that normally would be controlled by the bee's cellular immunity now have the opportunity to proliferate and spread in the hemolymph during *V. destructor* parasitism.

6 Impacts of *V. destructor* on honey bee tissues

While it is generally assumed that mite uptake of hemolymph was related to consumption of hemocytes as their numbers declined with parasitism, there is evidence that fat body cells are fed upon.

The mite preferred to feed on the ventral rather than the dorsal region of the bee's metasoma providing access to the fat body, and degraded fat body cells were found directly underneath the intersegmental membrane at the feeding site, likely a result of extra-oral digestion by the mite (Ramsey et al., 2018; Ramsey et al., 2019). Marking hemolymph and fat body in honey bees with different fluorescent stains revealed that the guts of mites contained the stain fluorescence of the fat body rather than the hemolymph (Ramsey et al., 2019). Also, mites feeding on the fat body lived longer and produced more eggs than those feeding on the hemolymph. All of this indicated that fat body cells were being fed upon rather than hemolymph following extra-oral digestion by the mite creating dissolved semisolid fat body tissue in the bee. Ramsey et al. (2019) proposed that the diverse symptoms of *V. destructor* parasitism would be consistent with the loss of fat body tissue during feeding as the bee's fat body is important for immunity, detoxification, nutrient storage, and other functions.

There are other bee organs affected by mite feeding, such as the hypopharyngeal gland. The size of the hypopharyngeal gland in parasitized nurse bees was reduced with fewer vacuoles suggesting decreased secretion by the gland, which could be detrimental to brood development and queen nutrition (Pinto et al., 2011). Similarly, newly emerged bees with mite parasitism of the brood showed reduced hypopharyngeal gland size, which could result in bees with less secretion of royal jelly and brood food as well as less secretion of antimicrobial enzymes reducing immunity (Bruckner et al., 2023b). Pinto et al. (2011) proposed that reduced hypopharyngeal gland size was due to direct parasitism and viral infections. Other evidence that DWV may be involved was the smaller hypopharyngeal glands of bees with deformed wings that also had few small vacuoles but many eosinophilic granules, suggesting increased production of serous secretion, more typical of foragers (Power et al., 2021). Power et al. (2021) proposed that this was an impact of DWV on the gland in both symptomatic as well as asymptomatic bees.

Other organs affected by mite parasitism are the mandibular glands and smaller reservoir glands (Zakaria and Abd El-Wahab, 2004), antennal sense organs (Abd El-Wahab et al., 2006), and flight muscles and mid-gut (Power et al., 2021). Mandibular and smaller reservoir glands were smaller in newly emerged bees with mite parasitism and more severe effects were observed with deformed bees, which could be related to inadequate protein levels during development (Zakaria and Abd El-Wahab, 2004). Mandibular glands are needed to produce pheromones for communication among colony members, and so reduced function would negatively affect the entire colony (Zakaria and Abd El-Wahab, 2004). Mite parasitized bees also have fewer sensilla trichodea and smaller antennal flagellum (Abd El-Wahab et al., 2006). Such a reduction in the antennal sense organs could adversely affect the bee's behavior with a reduced sense of touch, smell and taste. In addition, mite parasitized bees showed an incomplete development of flight muscles and an inflammation of the midgut and hemocele (Power et al., 2021). There were increased numbers of inflammatory cells (plasmacytes and granulocytes) and the accumulation of melanin between the midgut villi and the hemocele. These changes were observed in symptomatic and asymptomatic bees infected with

DWV. While ABPV is typically associated with DWV in bees and was also found in all samples with altered morphology, it was believed to be less likely responsible as ABPV does not trigger an immune response in the bee. The inflammation of the midgut could be related to reduced immunity against microbial infections, consistent with the finding of *N. ceranae* in the hemolymph samples of parasitized bees (Glavinić et al., 2014).

Finally, the brain of the bee appears to be negatively affected with *V. destructor* parasitism. The size of the total brain and specific brain regions were reduced by approx. 13% in workers that developed from mite parasitized brood (Lucas et al., 2006). Mite parasitism also dysregulated many brain metabolites, including fatty acids, amino acids, carboxylic acid, and phospholipids, with the greatest changes in linoleic acid, propanoate, glycine, serine, and threonine metabolism (Wu et al., 2017). The result would be reduced brain function negatively affecting the processing of sensory inputs as well as outputs like bee behaviors, which would adversely impact both individuals and colonies. Other evidence for reduced brain function is altered gene expression of parasitized capped brood related to decreased dopamine production and suppression of the prevention of neural degeneration, which could result in greater neuronal apoptosis during aging causing cognitive impairment (Navajas et al., 2008). Mite parasitism of adult bees also down-regulated expression of several neural genes indicating disruption of synaptic function and reduced ability to counteract neurodegeneration (Morfin et al., 2020b; Morfin et al., 2020c). These negative changes in the bee brain during mite parasitism may contribute to the behavioral changes in parasitized bees reviewed below.

7 Impacts of *V. destructor* on immune gene expression

One of the highest impacts of *V. destructor* on individual bees is the effect on the humoral immune system. Activation of humoral immunity relies on changes in protein levels regulated by intracellular signaling pathways (Morfin et al., 2021). These signaling pathways include Toll, Immune deficiency (Imd), Janus kinase-signal transducer and activator of transcription (JAK/STAT), and c-Jun N-terminal kinase (JNK). The activation of any of these pathways culminates in the synthesis of antimicrobial compounds, mainly antimicrobial peptides (AMP), defensive enzymes, and complement-like proteins (Morfin et al., 2021).

Several studies have found differential immune gene expression in developing and adult honey bees with mite parasitism, either showing or not showing symptoms of viral infection. For example, Yang and Cox-Foster (2005) found differences in gene expression between bees with no signs of wing deformity and no mites, mite-parasitized bees with normal wing development, and mite-parasitized bees with deformed wings due to DWV infection. When these bees were challenged with *Escherichia coli*, immunosuppression was detected by the downregulation of *hymenoptaecin*, which was not observed in non-parasitized control bees (Supplementary Table 1). Navajas et al. (2008) compared bee gene expression from colonies that were

presumably susceptible or resistant to *V. destructor* and reported differences in transcriptomic profiles as a response to *V. destructor* parasitism during the pupal stage. Of the differentially expressed genes that were identified, 15 genes were upregulated and 17 were downregulated. The differentially expressed genes were linked to biological pathways related to embryonic development (perhaps explaining wing deformity), cell metabolism, and immune responses. Another study also found that *V. destructor* parasitism affected the expression of bee immune-related genes, including the downregulation of *defensin* and *spatzle* in parasitized honey bee pupae (Khongphinitbunjong et al., 2015). Defensin and hymenoptaecin are AMPs synthesized after the activation of the Toll pathway by the protein *spatzle* (Rolff and Reynolds, 2009), which suggests that all the proteins regulated by the Toll pathway are suppressed by the mite during bee development. However, in adult bees, Abbo et al. (2017) found an up-regulatory effect of mite parasitism on the humoral immunity genes, *defensin* and *hymenoptaecin*, and the cellular immunity gene, *eater*, indicating that the effects may be quite different between parasitism of developing and adult bees. They also reported a positive correlation between the expression of *defensin* and *hymenoptaecin* with levels of varroa mite parasitism. Barroso-Arévalo et al. (2019) found a negative correlation between mite loads and the downregulation of the defense gene *defensin* and the development gene *dorsal*, both related to the Toll pathway, but a positive correlation with the up-regulation of the humoral immunity regulator gene, *relish*. However, Gregory et al. (2005) found that pupae parasitized with one to four mites had lower expression levels of the immunity genes, *abaecin* and *defensin*, compared to non-parasitized pupae or pupae parasitized with five to six mites, suggesting a non-linear expression pattern with respect to the number of mites parasitizing the bees. However, not all studies have reported immunosuppression during parasitism. Kuster et al. (2014) found immunostimulatory effects of varroa parasitism in developing bees based on the expression of 10 immune related genes (including *defensin-2* and *hymenoptaecin*) at 24, 72, 120, 192, and 240-hours post cell capping (hpc). However, the upregulation of immune-related genes only occurred at some hpc and with ≥ 3 mites per cell, indicating an inconsistent effect of *V. destructor* on immune responses in developing bees. Their study found that experimental wounding also increased the expression of immune genes, suggesting that the mechanical injury caused by the mite's feeding is related to changes in immune gene expression. Likewise, Koleoglu et al. (2017) found that the effect of *V. destructor* parasitism and the injection of a saline buffer had similar effects, but in this case resulting in downregulation of the immune-related genes *hymenoptaecin* and *defensin*, in developing and adult honey bees, suggesting that the effects of the mite on immune responses could be, at least in part, related to the wounds caused by the mite or the needle used to inject the buffer. In contrast to all these studies, Aronstein et al. (2012) found no significant effects of the mite on the expression of the immune genes *defensin-1*, *abaecin*, and *hymenoptaecin* studied in parasitized bees at different stages of development.

In addition to cellular immune responses mediated by hemocytes that circulate in the hemolymph of bees, there are a

number of genes related to cellular immunity (Morfin et al., 2021). Besides a reduction in hemocyte numbers, Koleoglu et al. (2018) found that the mite caused a downregulation of *AmPPO*, a prophenoloxidase gene whose expression was directly correlated with hemocyte count. Morfin et al. (2020a) found that a reduction of uncharacterized hemocytes in newly emerged bees parasitized with *V. destructor* during their pupal stage occurred along with 21 upregulated and 45 downregulated differentially expressed genes, that included biological pathways linked to leucocyte transendothelial migration and phagosomes, linking the effect of *V. destructor* on cellular immune responses. Furthermore, Zaobidna et al. (2015) found mostly down-regulatory effects of *V. destructor* parasitism on the expression of *proPO*, and a reduced enzymatic activity of prophenoloxidase in most of the developmental stages of parasitized honey bee workers and drones. The cleavage of prophenoloxidase by serine proteases leads to activation of phenoloxidase, which in turn allows the production of melanin. Melanin and the reactive oxygen and nitrogen species formed during its synthesis are toxic to parasites, bacteria, fungi, and viruses (Morfin et al., 2021). Thus, not only are hemocyte numbers reduced in response to *V. destructor* parasitism, gene expression and enzyme activities related to them are also suppressed. However, it is not yet clear if such reductions are simply a result of fewer hemocytes or whether haemocytes are not functioning normally. Studies on the effects of *V. destructor* on hemocytes need to identify gene expression in the different types of hemocytes as well.

Although it is evident that *V. destructor* alters gene expression in honey bees, there are clearly inconsistencies among the above studies in the way *V. destructor* dysregulates the expression of immune related genes. Differences between the studies could be related to the experimental setup, developing versus adult bees, or the levels of other stressors, like viruses. For example, Zaobidna et al. (2017) observed effects on 14 genes related to the Toll pathway in parasitized workers and drones during different developmental stages, finding significantly increased expression of 10 of the 14 genes, including *defensin-1* and *defensin-2*, in larvae, with a slower increase in drones. However, expression was silenced in later life stages showing that the impact of mite parasitism on bee gene expression was sex and life-stage specific.

8 Honey bee transcriptomic responses to *V. destructor*

The effect of mite parasitism on honey bee gene expression in most of the studies above have been limited to three to 14 genes, but transcriptomic studies have allowed the examination of a much broader range of genes shedding light on the complex effects of *V. destructor* on honey bee health. For example, Doublet et al. (2017) identified 88 upregulated genes and 79 downregulated genes in bees parasitized by *V. destructor*, and a gene ontology (GO) analysis identified nutrient reservoir activity linked to the down-regulated genes. Differentially expressed immune related genes identified by Doublet et al. (2017) included *iap2*, *rel*, *tube*, and *defensin-2*, which are part of the Imd and Toll immunity pathways. The involvement

of the aforementioned genes in anti-bacterial and anti-fungal mechanisms has been well defined, but most recently their activation in response to viral infections has also been described (McMenamin et al., 2018). However, Doublet et al. (2017) failed to identify transcripts related to the antiviral defense mechanism, RNAi, and as discussed by the authors, this was possibly due to the transient nature of the transcripts and the technical inability of RNA sequencing to detect them. However, they found that *vitellogenin* (*Vg*) was down-regulated and *malvolio* (*Mvl*) was up-regulated. These genes are involved in behavioral division of labor, indicating a potential effect of *V. destructor* on behavioral social responses (Alaux et al., 2012; Salmela and Sundström, 2018). Similarly, Amdam et al. (2012) showed lower titres of *Vg* protein in parasitized worker bees infested during their pupal stage that could be affecting the onset of foraging behavior in parasitized bees. Zanni et al. (2017) identified 1333 differentially expressed genes in honey bees highly parasitized by *V. destructor* in field and laboratory experiments, and apart from observing higher DWV levels in parasitized bees, they identified dysregulated genes linked to stress responses, immune responses, nervous system function, metabolism, and behavioral maturation. Dysregulated expression of immune genes included up-regulation of *PGRP-2* and *hymenoptaecin* and down-regulation of *UNC93*, and up-regulation of the neural genes for nicotinic acetylcholine receptors (*nAChRa9* and *nAChRb2*), indicating a possible impact of the mite on both immunity and behavioral immune responses (discussed in section 11). Using a combined Omics approach (transcriptomics, proteomic, metabolomic, and functional analysis), Kunc et al. (2023) showed an activation of immune responses and sphingolipid metabolism in parasitized 10-day old worker bees, but an inhibition of olfactory recognition and oxidative stress. Their metabolome analysis indicated a decrease in nutrients and energy stores in parasitized bees, which agrees with the mite's feeding behavior of consuming primarily fat body but also hemolymph (Annoscia et al., 2019; Ramsey et al., 2019). The up-regulated immune response genes by *V. destructor* identified by Kunc et al. (2023) included *apidaecin 1*, *abaecin*, *hymenoptaecin*, *defensin 1*, *RISC-loading complex subunit TARBP2*, *dicer*, *argonaute-2*, *peptidoglycan recognition protein S2*, and *beta-1,3 glucan binding protein*. However, no down-regulated genes linked to immune response. Down-regulated genes were involved in the maintenance of structural integrity and cell development and included *cell wall integrity* and *stress response component 1*, *collagen alpha-2(IV) chain like*, *tubulin alpha chain*, and the detoxification genes *cytochrome P450 6A1* and *cytochrome P450 9e2*. Other down-regulated genes were related to metabolic processes including *pancreatic triacylglycerol lipase-like*, *glucose dehydrogenase*, and *major royal jelly protein 6*, and sensory recognition, such as *carotenoid isomeroxygenase* and *chemosensory protein 6*. Thus, a quite broad range of impacts were observed. These studies showed the complex effects of *V. destructor* on different biological pathways (immune and metabolic pathways) that could be interconnected.

Although *V. destructor* has been considered the main culprit of honey bee colony mortality in the last three decades, most of the conclusions regarding the effect of the mite on honey bee immune

responses are based on the expression of a few genes used as molecular markers (Supplementary Table 1). However, this has started to change as more studies have examined the effect of the mite on the immunity and other biological pathways using high throughput transcriptomic, proteomic, and metabolomic studies (Duay et al., 2002; Navajas et al., 2008; Zanni et al., 2017; Morfin et al., 2019; Morfin et al., 2020a; Morfin et al., 2020b; Kunc et al., 2023). Nevertheless, it is apparent that the response of bees differs based on sex and age (Zaobidna et al., 2017), and many of the studies so far conducted have focused on worker bees, but little is known about the effect of *V. destructor* parasitism on immune mechanisms of queens and drones (Supplementary Table 1). Moreover, studies on relationships between *V. destructor* parasitism, energetic cost of immune responses, and consequences to other metabolic pathways (like neural processes and reproduction) are warranted. The crosstalk between immune and metabolic pathways, known as immunometabolism, is a new area of research in other animal models (i.e. *Mus musculus*) (Lercher et al., 2020), and needs to be further explored in bees. Lastly, there are difficulties in determining the sole effect of *V. destructor* as there are many factors, like viruses and altered neural and metabolic processes, that could be interacting with the effects of varroa mite parasitism. Addressing the sole effects of the mite as well as the synergistic effects from the interaction of the parasite with different stressors (biotic and abiotic) and their influence on different biological processes is not trivial (discussed in section 5). Hopefully the use of high throughput molecular techniques and studies on the description and quantification of biomolecules and their role on different biological pathways and networks will help understand the impact of *V. destructor* on honey bee health at the molecular level, as well as to help develop therapeutic strategies for varroosis control.

9 Interaction of *V. destructor* with biotic stressors

In addition to the host-parasite interaction between *V. destructor* and *A. mellifera*, there is also the interaction between the mite and viruses. Several studies have found an increase in viral levels (i.e. DWV and IAPV) in honey bees from *V. destructor*-parasitized colonies, and also an increase in bee mortality in parasitized and infected bees of those colonies, compared to colonies not parasitized by the mite (Dainat et al., 2012; Francis et al., 2013). Zhu et al. (2022) found that colonies that were treated with the synthetic acaricide Apivar® (containing the active ingredient amitraz) not only had lower mite loads and lower DWV levels compared to untreated colonies, but they also showed higher tolerance (lower mortality) to insecticides such as imidacloprid, cyhalothrin and oxamyl, indicating that *V. destructor* parasitism may affect tolerance to insecticide exposure. Several studies support the notion that viruses associated with *V. destructor* have detrimental effects on honey bee health, including immune responses. For example, a negative correlation between DWV levels and the cellular immune responses of melanization and encapsulation has been documented in honey bee brood artificially

parasitized with *V. destructor* (Di Prisco et al., 2016). This correlation resulted from a down-regulatory effect of DWV on *Amel102*, a gene involved in cellular immunity, which is regulated by the transcription factor NB-kB of the Toll signaling pathway. Di Prisco et al. (2016) also proposed that DWV increased *V. destructor* fitness because the proportion of reproducing mites increased as DWV levels in the pupae increased, up to a threshold of 10^8 DWV genome copies per bee. Conversely, higher DWV levels seemed to negatively impact *V. destructor* reproduction. Furthermore, mites showed twice the fertility rate (40%) when they parasitized larvae that later emerged with evident signs of wing deformity, compared to mites that parasitized asymptomatic bees (22% fertility rate), suggesting that DWV infections might favor *V. destructor* fitness. However, confirmation of the levels of DWV in the bees was lacking, and thus, further studies are needed to confirm the claims made by the authors of the study.

Three known variants of DWV that infect honey bees (DWV-A, DWV-B, and DWV-C) are mechanically vectored by *V. destructor*, but apparently only DWV-B can infect mite tissues (intestinal epithelium and salivary glands), which turns the parasite into a biological vector and a parasite of *V. destructor* (Gisder and Genersch, 2021). However, very few studies have been conducted on the relationship between DWV variants and *V. destructor*, and if the interaction with the vector (*V. destructor*) is affecting the mutation rate of DWV (Regoes et al., 2013). It is possible that variants of DWV will eventually appear that are primarily pathogens of the mite rather than the bee. Variants of *Varroa destructor virus* (VDV; Iflaviridae), including VDV-2, VDV-3, and VDV-5, seem to selectively infect mites but not bees (Levin et al., 2016; Herrero et al., 2019; Chen et al., 2021). Most of these studies have used RNA extraction and sequencing, which is a technique able to identify the presence of the virus but is not capable of determining if the virus is replicating and damaging tissues of either host. However, there are not many techniques available to confirm the infectivity of viruses in *V. destructor* and *A. mellifera*, as there are limited or no commercial cell lines. Identifying negative RNA strands, immunoassays, or *in situ* hybridization could be the first steps to study virus infectivity (Shen et al., 2005; Yue and Genersch, 2005; Shah et al., 2009). Moreover, the interactions between the different viruses may be a confounding factor. For example, Gregorc et al. (2012) found higher and lower DWV and BQCV transcripts, respectively, in mite parasitized bees, indicating a possible competition between viruses, which should be further explored. Moreover, there are less well studied viruses associated with honey bee and/or mite tissues, such as *Bee Macula-like virus* (BeeMLV), and De Miranda et al. (2015) suggested that mites are likely a biological vector of this virus based on the identification of sub-genomic RNA, which allowed the detection of actively replicating virus in bee and mite tissues. Other less studied viruses have been identified in *V. destructor* and bees using high throughput RNA sequencing, like *Moku virus* and *Bee macula virus*, and the implications of these viruses on *V. destructor* and bee health are unknown (Mordecai et al., 2016; Morfin et al., 2022).

Varroa destructor seems to interact with pathogens other than viruses, including the microsporidians *Vairimorpha* (*Nosema*) spp., as it appears that honey bee colonies parasitized by the mite tend to

have higher *Vairimorpha* (*Nosema*) spp. loads (Bermejo and Fernández, 1997; Mariani et al., 2012; Little et al., 2015). Additionally, van Dooremalen et al. (2018) found that colonies parasitized by *V. destructor* that were exposed to imidacloprid, showed higher *Vairimorpha* (*Nosema*) spp. levels, but they found no interactions between the stressors on colony size or colony survival. Contrary to these findings, Ostermann (2003) found no effect of *V. destructor* on *Vairimorpha* (*Nosema*) *apis* levels, nor an effect of the mite and formic acid treatment on the prevalence of chalkbrood disease (*Ascosphaera apis*) in their experimental colonies.

In recent years, there has been an increasing interest in understanding how the gut microbiome impacts bee health, as evidence suggests that it plays an important role in bee metabolism, immunity, and development (Raymann and Moran, 2018). Parasitized bees showed a reduction in the relative abundance of *Bartonella apis* and *Lactobacillus apis*, and an increase of *Lactobacillus helsingborgensis*, *Lactobacillus mellis*, and *Commensalibacter intestini*, in whole bodies compared to non-parasitized bees (Hubert et al., 2017). The effect of *V. destructor* on the bee gut was greater for bacteria than for fungi, such as *V. apis* and *V. ceranae*, or trypanosomes, such as *Lotmaria passim*, which is interesting because *Vairimorpha* (*Nosema*) spp. and *L. passim* infect the epithelial cells of the bees' gut, and thus, a more pronounced effect on the microbiome would be expected from these pathogens than from *V. destructor*. Marche et al. (2019) also found that the abundance of bacterial community in whole bee bodies was altered by mite parasitism, with increased relative abundance of Gammaproteobacteria, Betaproteobacteria, *Lactobacillus* spp., *Bifidobacterium* spp., and *Brevibacillus laterosporus*.

There are a few reports on the interaction of *V. destructor* parasitism and multicellular organisms attacking bees. For example, a decrease in mite levels in apiaries infested with the small hive beetle (SHB), *Aethina tumida*, was reported, but no effects resulting from the interaction between *V. destructor* and SHB were observed on parameters associated to honey bee health like adult bee population, body mass, brood density, or colony weight (Delaplane et al., 2010). More studies are needed to confirm the nature of the interaction between *V. destructor* and SHB, and the possible consequences on honey bee health.

10 Interaction of *V. destructor* with abiotic stressors

Abiotic stressors also interact with *V. destructor* parasitism, impacting honey bee health. Most studies have focused on analyzing the effect of the mite and insecticide exposure on different aspects of bee health, behavior, and neural gene expression. For behavior, negative effects on flight capacity (i.e. bees flying shorter distances) and homing success of forager bees from colonies exposed to neonicotinoid insecticides (i.e. imidacloprid or thiamethoxam) and parasitized by *V. destructor* have been reported (Blanken et al., 2015; Monchanin et al., 2019). Detrimental effects on memory retention and in the expression of neural related genes, like *neurexin* (*AmNrx-1*), in honey bees parasitized by *V. destructor* and exposed to sublethal doses of

neonicotinoid insecticides, like imidacloprid and clothianidin, have been described, which could be responsible for the altered behaviors (Morfin et al., 2020b; Morfin et al., 2020c; Schwartz et al., 2021). The complex formed by pre and postsynaptic proteins regulated by neural genes such as neurexin and neuroligin is essential for neurotransmission and linked to learning processes and memory retention in bees (Biswas et al., 2010). Therefore, these effects of insecticide exposure and *V. destructor* parasitism affect the performance of behaviors that are essential for colony survival, such as foraging behavior and navigation. An effect of sublethal doses of clothianidin and *V. destructor* parasitism on the proportion of bees performing intense self-grooming and an effect of both stressors on genes linked to neurological dysfunction was reported by Morfin et al. (2019).

Exposure to imidacloprid and thiamethoxam plus *V. destructor* parasitism also dysregulated immune related genes in bees at different developmental stages, like a down-regulation of *abaecin* and *defensin-1* in white-eyed pupa, and an up-regulation of *PPOact* and *spaetzle* in brown-eyed pupae (Tesovnik et al., 2017; Tesovnik et al., 2019). The mechanism by which neonicotinoid insecticides interact with *V. destructor* seems complex. Annoscia et al. (2020) found that bees exposed to sublethal doses of clothianidin had lower expression levels of immune related genes (*Amel102* and *dorsal 1*), higher DWV levels, and lower mite fertility (reproducing mites/total mites). Although the immunosuppressive effect of clothianidin could be exacerbating the effects of the other two biotic stressors (DWV and *V. destructor*), the nature of the interactions needs to be investigated.

The combined effects of *V. destructor* parasitism and neonicotinoid insecticide exposure may reduce the body mass of newly emerged bees as well as their subsequent longevity (Straub et al., 2019), and this could be related to the detrimental effect of the parasite and insecticide on hypopharyngeal gland size in workers, and on drone body mass at emergence (Bruckner et al., 2021; Bruckner et al., 2023b). Other studies have found that the interaction between *V. destructor* and neonicotinoid insecticides impact bee metabolism by dysregulating genes linked to the biosynthesis of secondary metabolites and amino acids, as well as genes involved in fat digestion and absorption (Morfin et al., 2020a; Morfin et al., 2020b).

Most of the studies conducted so far have analyzed the effect of *V. destructor* in combination with other stressors on worker bees, but it would be interesting to know more about the synergistic impact of these stressors on the reproductive casts and the possible consequences to colony health and fitness. Additionally, the effect of *V. destructor* in combination with stressors other than insecticides used in agriculture that honey bees are exposed to, like a variety of agrochemicals, such as herbicides, fungicides, and acaricides (including the ones used to treat varroosis), low or high temperatures, drought, etc, warrant investigation to find out if their interaction with the mite impacts honey bee health and colony fitness.

11 Honey bee behavioral responses to *V. destructor* parasitism

Relatively little is known about how *V. destructor* affects bee behavior. The best documented behaviors of honey bees in response

to *V. destructor* parasitism are hygienic and grooming behavior, but other behaviors such as foraging and defensive behavior may also be affected by the mite.

Parasitism by *V. destructor* affects honey bee foraging behavior in different ways. Foragers infested by the mite perform longer foraging trips and many do not return to their hives at all. Parasitized bees also show impaired homing and orientation ability at the hive entrance (Kralj and Fuchs, 2006), which may limit the ability of colonies to collect resources. The fact that bees take long foraging trips or do not return to the hive could be an adaptive behavior of the insects to reduce parasitism load in the hive by leaving the hive or could be due to neurological damage caused by the mite, affecting recognition and responsiveness to environmental stimuli. This last hypothesis was tested by Kralj et al. (2007), who found that bees infested with *V. destructor* significantly decreased their responses to a reward stimulus (sucrose syrup) when a habituation test was conducted by repeatedly presenting the stimulus to the bees. The parasitized bees also showed a lower response to a scent stimulus than non-parasitized bees, suggesting that mites may interfere with neural transmission that enables learning and memory retention. Morfin et al. (2020c) also showed a detrimental impact of *V. destructor* on the ability of honey bees to respond to foraging-related stimuli, and possibly other behaviors that require cognitive processing. Studies have also found a down regulatory effect of *V. destructor* on neural related genes, like *AmNrx-1*, *neuroligin* (*Am-Nlg-1*) and *acetylcholinesterase* (*AmAChE-2*) (Morfin et al., 2020b; Morfin et al., 2020c). Likewise, Duay et al. (2002) found that *V. destructor*-parasitized drones showed decreased flight performance and lower ability to mate with queens compared to non-parasitized drones. Neural disorders because of *V. destructor* parasitism could also be due to infections caused by viruses transmitted by the mite. For example, DWV and IAPV affect the expression of neural genes and impair the homing ability and learning processes in honey bees (Iqbal and Mueller, 2007; Li et al., 2013). Therefore, the combination of mite parasitism and viral infections could be detrimental to the foraging activity and fitness of honey bee colonies.

Varroa destructor parasitism and reproduction in honey bee brood stimulates hygienic behavior by adult bees. Hygienic worker bees detect and uncap comb cells containing larvae infected with bacteria or fungi, as well as parasitized with *V. destructor* (Guzman-Novoa and Morfin, 2019). A parasitized larva may be removed from its cell (Boecking and Spivak, 1999; Arathi et al., 2000) or the cell may be posteriorly recapped (Hawkins and Martin, 2021), all of which interrupts the mite's life cycle. It is well established that hygienic behavior is a heritable trait (Lapidge et al., 2002; Unger and Guzman-Novoa, 2010) and that some honey bee strains are more hygienic than others (Spivak and Reuter, 1998), particularly those expressing the varroa sensitive hygiene (VSH) trait, that allows them to identify cells infested with reproducing mites (Harbo and Harris, 2009). Worker bees that are hygienic can detect *V. destructor*-parasitized larvae by perceiving with their antennae chemical odorants from the brood such as hydrocarbons and oleic acid (Nazzi et al., 2004; Mondet et al., 2015; McAfee et al., 2018) that elicit hygienic responses in the bees. Some of these

compounds have also been identified from *V. destructor* and DWV (Wagoner et al., 2019), which again, shows that the combination of both pathogens can influence several honey bee behaviors.

Grooming behavior of adult honey bees may also be triggered by *V. destructor* infestations. Parasitized workers use their legs and mandibles to remove mites from their bodies, sometimes biting and injuring them (Boecking and Spivak, 1999). The presence of *V. destructor* as well as the wounds inflicted by the mite while feeding irritate the bees, which elicits grooming responses (Morfin et al., 2019) that are governed by neural processes (Hamiduzzaman et al., 2017; Morfin et al., 2023). Grooming behavior is a heritable trait (Arechavaleta-Velasco et al., 2012; Morfin et al., 2020d), and the expression of this behavior varies among different genotypes and strains of honey bees (Arechavaleta-Velasco and Guzmán-Novoa, 2001; Rinderer et al., 2001; Guzmán-Novoa et al., 2012; Bakk and Wilde, 2015; Invernizzi et al., 2015). Interestingly, younger daughter mites elicit stronger grooming responses by honey bees than older foundress mites (Kirrane et al., 2012). Perhaps these responses are related with the greater reproductive potential of younger mites or with different chemical signals produced by the mites.

Varroa destructor may also influence defensive behaviors of honey bees. De la Mora et al. (2021) measured the stinging response threshold of *V. destructor*-parasitized and non-parasitized worker bees by exposing them to a constant electric stimulus while measuring the time that the bees took to sting a leather patch. Parasitized bees stung significantly faster than non-parasitized bees. The authors concluded that the irritation caused by the parasite affected neural processes that made the bees more sensitive and prone to sting. The implications of these results are that colonies infested with the mite may be better adapted to protect their nests from predators, which would be beneficial for their ecological success, but they would also be more difficult to manage by beekeepers for production purposes.

While it is clear that *V. destructor* affects different honey bee behaviors, these could result in higher or lower mite infestation rates. More research, particularly related to breeding and selection for different behaviors are needed. In addition to better understanding the effects of the mite on bee behaviors, such studies could have practical outcomes for improved health and fitness of honey bee colonies.

12 Conclusions

Undoubtedly, *V. destructor* parasitism is one of the main impacts currently affecting *A. mellifera* worldwide. Although advancements have been made to understand the development of *V. destructor* parasitism, there is insufficient knowledge on the effects of the parasite on different immune responses in honey bees and the consequences (or interactions) on biological processes, like metabolic pathways. However, recent studies using more comprehensive methods to investigate the effect of *V. destructor* on molecular processes, like the use of 'omic' tools, can help understand this interaction. Nevertheless, they need to be applied

to bees of different developmental stages and sexes rather than concentrating mostly on newly emerged bees parasitized as brood. They would also be useful to combine with studies showing behavioral changes. Complicating all studies of *V. destructor* parasitism is its interaction in different ways with many biotic and abiotic factors. Such interactions are not trivial, especially those with viruses, as separating the effects of viral diseases (e.g. DWV) and *V. destructor* is challenging. While research on the effect of *V. destructor* on immune pathways, cellular immunity, and metabolic pathways may appear to have limited practical outcomes, it will hopefully lead to the development of therapies and bees that could better fight *V. destructor*, thus reducing economic losses, which is urgently needed.

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Supplementary material

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Warming up through buildings and roads: what we know and should know about the urban heat island effect on bees

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Urbanization leads to cities having higher temperatures than surrounding non-urban areas [this is known as the urban heat island (UHI) effect]. Very little is known about the impacts of the UHI effect on bees, despite the importance of temperature on many aspects of bees' life suggesting that these may be not negligible. In this study, we aimed to highlight how the UHI effect could impact relevant functional traits of bees in cities, proposing several *ad hoc* hypotheses for traits that have thus far been investigated only in few studies or not at all, based on what we know from non-urban studies. The UHI effect was shown to influence bee body size, and generally tended to reduce the body size of bees in cities. Urban temperature may also affect bees' wing morphology, and thus their overall flight morphology parameters. Individuals may be more brightly colored in cities. Bee ommatidial size and the number of antennal thermoreceptors they have may be smaller and fewer, respectively, in cities than in non-urban areas. As expected, because urban bees face a higher risk of desiccation, higher proportions of alkanes and longer main-carbon chain lengths are expected in their cuticular hydrocarbon (CHC) profiles. Stress biomarkers can also occur at greater concentrations in bees in cities and specific bacteria in the bee gut may occur at lower abundances. Warm urban temperatures may impact the life cycle of pathogens by reducing their proliferation. Aggression levels may be increased, and eusocial species may present more worker phases per year due to the UHI effect. All of these proposed impacts could be likely more visible in solitary and primitively eusocial bee species, which are those suspected to have a more limited dispersal ability. Comparative studies would help in the proper testing of these hypotheses.

KEYWORDS

urbanization, temperature, morphology, cuticular hydrocarbons, stress markers, symbionts, behavior

1 Introduction

Urbanization, defined as the expansion of cities, represents one of the main drivers of land-use change since the 21st century and is predicted to increase in the future (Kalnay and Cai, 2003). Approximately 3% of the earth's surface is covered by urban land, and more than 50% of the global population lives in cities, across an urban surface that has expanded by 9,687 km² per year in the last few decades (Grimm et al., 2008; Liu et al., 2014; Liu et al., 2020). More than 95% of the net increase in the global population will be in cities, and individual cities are rapidly growing, creating new megacities (i.e., those containing > 10 million people) (Grimm et al., 2008). Hence, the impact of urbanization on earth is currently, and will continue to be, enormous. This seems to be especially true for megacities in the southern hemisphere, which are already characterized by having higher greenhouse gas emissions than those in the northern hemisphere (Venter et al., 2021). In addition to the reduction of green areas and fragmentation of the remaining green patches, urbanization is well known to produce a non-negligible rise in temperature within cities compared with in the surrounding non-urban areas. Such climatic alteration—which elevate city temperatures approximately 2°C–4°C (sometimes even more)—is called the “urban heat island effect” (hereafter, the UHI effect) (Deilami et al., 2018) and is due to both the impervious (i.e., concrete) surface acting as a heat sink (Cheela et al., 2021) and the loss of tree cover (Rakoto et al., 2021). We note again that such increases in temperature in the urban matrix are predicted to be more severe in the future for cities in the Global South (Huang et al., 2019). Given the extent of this temperature shift, it is thus not surprising that in many studies, the UHI effect was associated with variations in the abundance, diversity, phenology, and physiology of both plants and animals.

Due to the important ecosystem service provided, bees (Hymenoptera: Apoidea)—the most important group of pollinators in cities (Lowenstein et al., 2015)—are increasingly receiving attention in the context of urbanization, though mostly from a community ecology point of view (e.g., Biella et al., 2022; Ferrari and Polidori, 2022; Geppert et al., 2023). Indeed, recent literature reviews clearly highlight the large amount of diversity and abundance data for urban bees accumulated in the last 20 years (e.g., Winfree et al., 2011; Wenzel et al., 2020; Brant et al., 2022; Ferrari and Polidori, 2022). On the other hand, there has been much less research published on the effect of urbanization—and in particular on the UHI effect—on bee functional traits (Buchholz and Egerer, 2020), spanning morphology, physiology, and behavior, among other traits (Brant et al., 2022). All we know regarding the UHI effect on bees comes from studies on a very limited number of species and traits (e.g., Hamblin et al., 2017; Burdine and McCluney, 2019; Zeballos et al., 2022). However, since temperature is a key factor in the life of bees (Atkinson, 1994), it is expected that the UHI effect will impact bees in cities, and a range of their traits, in a variety of ways.

From this perspective, we aimed to highlight how the UHI effect could impact the essential functional traits of bees in cities. We present the few available, previously published studies on the topic, and then propose *ad hoc* hypotheses for traits not yet investigated,

based on what we know from non-urban studies on bees and on other organisms (Figure 1).

2 External morphology of bees and the UHI effect

2.1 Body size

Body size is a crucial functional trait in bees, as it is positively correlated with dispersal abilities, fecundity, foraging efficiency, and other fitness-related traits (e.g., Araújo et al., 2004; Bosch and Vicens, 2006; Benjamin et al., 2014). It is possible that this is the main reason why body size is by far the most investigated functional trait of wild bees in relation to the UHI effect (Buchholz et al., 2020). In such studies, the general hypothesis is that increasing temperatures due to the UHI effect should induce shrinkage in the body size of bees [Bergman's rule, Bergmann (1847)]. This could occur through two different mechanisms: either higher temperatures may accelerate the larval development of the bees (Howe, 1967), or it could be an adaptation to hotter urban landscapes, in accordance with Bergmann's rule. The evidence supporting the body size-shrinking hypothesis in urban areas has been obtained at the intraspecific level in different species and countries: in Europe on different *Bombus* species (Apidae) (Theodorou et al., 2021; Tommasi et al., 2022), and in central America on two *Euglossa* species (Apidae) (Garlin et al., 2022). The variation in intertegular distance (proxy of body size) reached up to 5% in an approximate gradient of 10°C (*Bombus terrestris*, Theodorou et al., 2021). However, some studies found no variation in body size, for example in an *Anthophora* (Apidae) in Europe (Banaszak-Cibicka et al., 2018); or found larger bees (i.e., the opposite trend) in more urbanized landscapes such as in north America in a *Bombus* species (Austin et al., 2022). The data on body size variation at a community levels support this hypothesis also in time and space. For example, the species richness of large bees (measuring > 15 mm) was higher—and the species richness of medium-sized species (measuring 8 mm–15 mm) lower—in larger (hotter) cities (Ferrari and Polidori, 2022). In addition, Herrera et al. (2023) found a reduction in mean body size in a community of solitary wild bee species across time. This somewhat contrasting evidence might be due to the tested species. In fact, most of the studies have been performed on single or congeneric species (usually *Bombus*), with only a few studies conducted on solitary species. In addition, such contrasting patterns may arise as a result of particular trade-offs between body size and other traits, so that the expected effects might not be always seen. For example, limited food availability either leads to smaller individuals or the production of more males (the cheaper sex in bees), preserving female body size (Torchio and Tepedino, 1980). Furthermore, it is still largely unclear if body size reduction in urban environments is due to the effects of temperature on larval development or on genetic processes (i.e., plasticity vs. adaptation). In at least one study on bumblebees, body size clines were observed across the urban gradient, despite a lack of population genetic structure (Austin et al.,

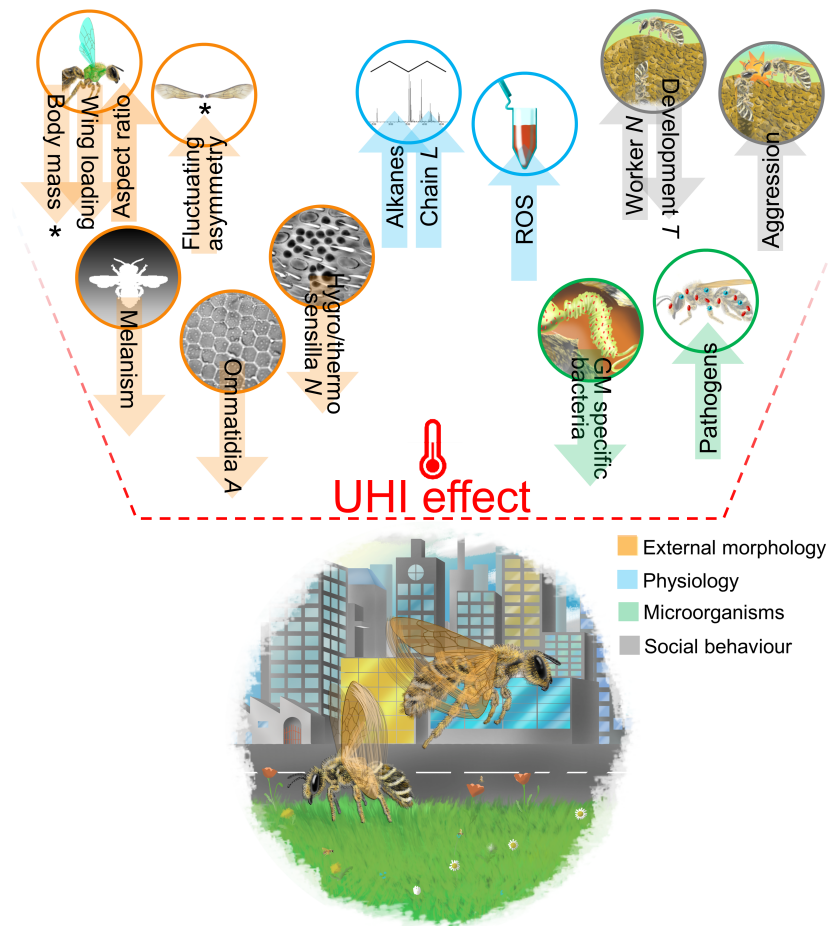


FIGURE 1

Schematic graphical representation of the proposed impacts of the UHI effect on bee functional traits. An asterisk marks the traits for which an effect of the UHI was detected in at least one urban study on bees. All the other proposed impacts are hypothesized following either urban studies on other animal groups or non-urban studies on bees and other insects. L, length; A, area; N, number; T, time; ROS, reactive oxygen species; GM, gut microbiome. An up-directed arrow identifies a positive effect of the UHI on the trait, whereas a down-directed arrow identifies a negative effect of the UHI on the trait. For more details on the different hypotheses, please see the text.

2022). Nonetheless, analyses including both physiological assessment and the detection of possible genetic signatures in urban vs. non-urban populations would be highly welcome. In addition, studies would be easily directed to abundant species of certain bee genera such as *Andrena* (Andrenidae), *Bombus* (Apidae), *Halictus*, and *Lasioglossum* (Halictidae), or *Osmia* and *Megachile* (Megachilidae) in Europe. Finally, one should consider that variations in bee body size may be differently driven according to the actual size of the bee species and its thermal tolerance. This highlights the need to further investigate this phenomenon, considering as many bee genera, sizes, and cities as possible.

2.2 Wing morphometry and flight morphology

Morphological variation driven by temperature shifts may also occur in wings. Wings are known to respond to environmental

changes (Hayes et al., 2019) through subtle variations in their bilateral symmetry (Møller and Swaddle, 1997). This fluctuating asymmetry (FA) is commonly considered to reflect developmental instability and it is increasingly used as an indicator of environmental stress (Benítez et al., 2020). It is therefore hypothesized that increasing temperature due to the UHI effect may increase the stress during the development of the larvae, and hence the wing FA. The existing studies, however, yield contrasting results. For example, Tommasi et al. (2022) found increasing FA in hotter urban areas in only one of the two *Bombus* species investigated, whereas Banaszak-Cibicka et al. (2018) found no variation in wing asymmetry along an urbanization gradient in an *Anthophora* species. We note again that this contrasting evidence may be the result of the investigated species. As different species have different thermal tolerance limits, the UHI effect may act as a high or low stress factor. This highlights a general lack of knowledge about what these tolerance limits are, which prevents us from knowing, or even estimating, how close species are to this threshold and thus how stressed they might be.

Allen's hypothesis states that higher temperatures favor longer and less-thick appendages and meets some confirmation in several insect species (e.g., Shelomi and Zeuss, 2017). This may in turn affect two parameters used as proxy of the flight abilities, which are based on wing morphology. Wing loading (WL) is the ratio between the body weight and wing area; lower values minimize the energetic cost of flight and improve flight maneuverability (Marden, 1987). The aspect ratio (AR) is the relative front-to-back width of the wing relative to its length (i.e., narrow vs. broad); higher values (i.e., longer and narrower wings) are associated with better flight maneuverability (Danforth, 1989). Bee wing morphology has rarely been investigated in urban contexts, and we are aware of no studies that have considered WL and AR. Beasley et al. (2019) studied wing size in a solitary bee along an impervious surface gradient (i.e., a proxy of urbanization, but not necessarily for the UHI effect) without finding any significant differences. Examples from other temperature gradients might be useful in guiding future urban-based studies. For example, Lozier et al. (2021) found reduced wing loading in colder environments in a *Bombus* species along an altitudinal gradient. If this trend applies in urban contexts, then bees would have a higher WL in cities, negatively affecting them. On the other hand, if Allen's rule applies to urban bees, then AR would be increased in cities, favoring flight ability. Since WL and AR allometrically scale with body size in bees (Marden, 1987; Danforth, 1989), future studies should focus on conducting comparative analyses of flight morphology in species with different body sizes.

2.3 Melanism

Melanins (eumelanin and pheomelanin) confer darker colors, such as black, red, or brown, to animals, including bees (Polidori et al., 2017; Popadić and Tsitlakidou, 2021). The melanism variations can be explained by two main rules: the Gloger's rule (Gloger, 1833) and the thermal melanism hypothesis (e.g., Lusis, 1961; Clusella-Trullas et al., 2008). The first rule states that animals tend to be darker in habitats with more intense solar ultraviolet (UV) radiation (since darker surfaces are more protected from UV radiation) and/or humid environments (since darker surfaces may be more protected against degrading bacteria) (Delhey, 2019). The second hypothesis states that colder habitats select for melanic individuals since they can absorb more light and become hot faster than lighter individuals. Thus, darker individuals may have an advantage over lighter ones (Forsman, 2011).

The color variation associated with the UHI effect has never been investigated in bees. In the wasp *Vespa vulgaris* (Vespidae), there are differences in the frequency of color morphs in different climatic conditions (Badejo et al., 2018) and between urban and rural areas (Badejo et al., 2020), thus suggesting that the increased temperatures in urban spots can impact the pigmentation of the wasps. Other investigations have been carried out along wide geographical gradients. For example, more melanic species of *Polistes* inhabit colder latitudes and altitudes more than the brighter species, thus supporting the thermal melanism hypothesis (de Souza et al., 2017;

de Souza et al., 2020). Similarly, honeybees are darker in colder climates (Ruttner, 1988). Laboratory experiments also support the thermal melanism hypothesis (Pereboom and Biesmeijer, 2003). In that single study, carried out on stingless bees (Apidae: Meliponini), paler bees warmed up less rapidly and had lower temperature excesses than darker bees, both in interspecific and intraspecific comparisons. Therefore, new studies should aim to test for the two alternative hypotheses on melanism variation in urban environments, considering bee species with different colorations and body size. Furthermore, attention should be given to which type of pigment is responsible for the coloration, as eumelanins (which confer black color) and pheomelanins (which confer yellow to red colors) are both detected in bees (Polidori et al., 2017), and are predicted to increase (the former) or decrease (the latter) with humidity (Delhey, 2019).

2.4 Sensory system

The bee sensory system processes both internal and external stimuli, through signal transfer from sensory receptors to the brain. The main receptors are the light/visual detectors, the mechanoreceptors, the chemoreceptors, and the temperature receptors (Wyatt, 2014; Souto et al., 2022). Eyes, ocelli, and antennae are the main structures bearing one or more types of these receptors.

Concerning visual system, no studies were carried out in urban habitats. The limited evidence suggests that insects respond to higher developmental temperatures by reducing cell size, because of the high demand for oxygen in such conditions. Kierat et al. (2017) manipulated the thermal conditions inside the nests of a solitary *Osmia* species (Megachilidae) and found that ommatidial size, a proxy for general cell size in insects (Blanckenhorn and Llaurens, 2005), decreases in response to a higher temperature. Hence, the UHI effect may lead to bees with decreased ommatidial size, compared with non-urban populations. Smaller ommatidia, in turn, may impact bee movements in flight, though not necessarily negatively. Indeed, the interaction between ommatidia size and number either increases light capture (larger but fewer ommatidia) or image resolution (smaller but more ommatidia) (Land, 1997). However, the link between ommatidial size and flight behavior is not well understood, and we need new studies to properly test for this hypothesis.

Concerning antennal system, bees possess a wide range of sensillar types with a variety of functions (Chapman, 2013). Although density and morphology of the sensilla was shown to be correlated in bees with diet (Polidori et al., 2020), kleptoparasitic lifestyle (Wcislo, 1995), and sociality (Wittwer et al., 2017), none addressed the UHI effect on the antennal sensory system of bees. In a study on the socially polymorphic sweat bee *Halictus rubicundus* (Halictidae), a species whose social behavior depends on climate (Field et al., 2010), it was found that bees from a mid- and northern latitude possess more of hygro/thermoreceptive sensilla than bees from the southern areas (Boulton and Field, 2022). Hence, future studies should test if bees possess lower numbers or densities of

hygro/thermoreceptive sensilla in urban environments, compared with rural or natural ones.

3 Physiology of bees and the UHI effect

3.1 Cuticular hydrocarbon profiles

The primary and ancestral function of the cuticular hydrocarbon (CHC) layer on bees' cuticle is to provide waterproofing and, hence, desiccation resistance (Blomquist and Bagnères, 2010). The CHC profile comprises a mixture of saturated compounds (i.e., *n*-alkanes, methyl-branched alkanes) and unsaturated hydrocarbons (i.e., alkenes). The former are especially important when facing temperature increases and desiccation risk, as they aggregate more tightly than unsaturated hydrocarbons (Gibbs and Pomonis, 1995). Furthermore, a CHC profile with a higher mean chain length provides a better resistance to increased temperatures (Menzel et al., 2017).

Although we know that CHC profile characteristics vary according to these expectations at both inter- and intraspecific level, available studies in insects have focused on large geographical scales (latitudinal or altitudinal) (Menzel et al., 2017; Rajpurohit et al., 2017; Mayr et al., 2021). In bees, studies are still rare. In one study on alpine *Bombus*, Maihoff et al. (2023) showed that workers translocated to a warm region tended to possess longer hydrocarbon chains than bumblebees translocated to cool regions. In addition, Mayr et al. (2021) found that *Lasioglossum* species (Halictidae) at higher elevations along Mount Kilimanjaro (Tanzania) possess higher proportions of alkanes. No evidence is available for similar trends along urbanization gradients. Because it was shown that CHC profiles can alter plastically in the short term under changing climatic conditions (Menzel et al., 2018), we can hypothesize that the UHI effect may shift CHC profiles in bees, not only as a long-term adaptation, but also as a rapid acclimation. Interestingly, since CHC profiles are greatly involved in chemical communication (Blomquist and Bagnères, 2010), such a shift may potentially affect behavioral interactions, though new studies are necessary to properly test for this hypothesis.

3.2 Molecular biomarkers of stress

Reactive oxygen species (ROS) are normal byproducts of aerobic metabolism, which can increase disproportionately under environmental stresses. Heat stress is triggered by temperatures that exceed the optimum growth conditions and it is a problem under the current global warming (Medhaug et al., 2017). Heat stress generates ROS, such as hydroxyl radicals, hydrogen peroxide (H₂O₂), and superoxide anions, which if they occur at high levels can lead to damage to DNA, proteins, and lipids, and an increase in age-related diseases and aging, and, ultimately, to cell death (Slimen et al., 2014). However, studies on the deleterious effects of heat

stress on bees are still scarce. In honeybees, heat stress can impact growth and development of larval and pupal stages, and their ability to learn and forage as adults. In addition, it can promote senescence and death (Tautz et al., 2003; Abou-Shaara, 2015; Abou-Shaara et al., 2017). It could be expected that ROS are overproduced in the warmer urban conditions, negatively affecting bees; therefore, this is a new analysis that should be performed through both laboratory assays and physiological investigations on field-collected individuals.

4 Microorganisms associated with bees and the UHI effect

4.1 Symbionts

The symbionts in the gut of bees [i.e., the gut microbiome (GM)] can be vulnerable to increasing temperatures, and responses can be species specific. An analysis on honeybees and bumblebees showed that the GM of the latter tend to be less heat tolerant than that of honeybees (Hammer et al., 2021). Such a greater effect of the UHI on the GM in primitively eusocial species, such as bumblebees, and solitary species, could be expected since the bacterial community in the honeybee is notably very simple and conserved (Kwong and Moran 2016), compared with that of other bees. In fact, the GM of solitary species is known to be more dependent on the environment (Keller et al., 2021). Some bee symbionts can survive at temperatures up to 52°C, whereas others are not able to grow below a certain thermal limit (29°C). On the other hand, functionally important bacteria, such as *Apilactobacillus*, are overrepresented in areas with lower annual temperatures (Nguyen and Rehan, 2022). Moreover, in honeybees there is a seasonal dominance of the non-core bacteria *Bartonella*, which is associated to dietary shifts caused by low temperatures in winter (Li et al., 2022a). In Ceratina (Apidae) from different climatic areas across Australia a variation in microbiome composition was observed (McFrederick and Rehan, 2019). *Lasioglossum* bees also change the gut microbial communities along an elevation gradient, this is very likely due to the constraints in the availability of food resources in the areas with harsher temperatures (Mayr et al., 2021). To date, no studies have considered the effects of UHI on the GM of bees. In the future, it could be tested if relevant bacteria may occur at a lower, non-optimal abundance at the higher urban temperatures, thus in turn negatively affecting bees.

4.2 Pathogens

The disturbance of the host–parasite equilibrium can produce unpredictable consequences for bee communities (Goulson et al., 2015; Meeus et al., 2018; Piot et al., 2022). Temperature is believed to have species-specific effects on both hosts and pathogens, thus influencing ecological interactions (Wojda, 2017; Meeus et al., 2018). Despite several researchers attempting to explore the

impact of urbanization on bee epidemiology (Cohen et al., 2022; Ivers et al., 2022; Tommasi et al., 2023), to our knowledge, none of them have evaluated the UHI effect on host–pathogen dynamics in bees.

However, insights on the potential impacts come from non-urban studies. In a *Bombus* species (Apidae), *Crithidia bombi* infection intensity was found to decrease with an increasing temperature (Palmer-Young et al., 2019), and it seems that fungal or viral infections ameliorate with relatively high temperatures in honeybees (Martín-Hernández et al., 2009; Dalmon et al., 2019). At the same time high temperatures reduce the viability of pathogens, especially viruses, present on flowers. Given that flowers are known to act as a platform for pathogen dispersal between bees (Alger et al., 2019) warmer temperatures may reduce their transmission (Piot et al., 2022). Furthermore, high temperatures may also shape the host–parasite equilibrium, thus in turn altering the susceptibility of hosts toward infections. In *Megachile rotundata* (Megachilidae), for example, higher temperatures promote the expression of immune response genes, suggesting a positive effect of warmer temperatures due to the improved efficiency in activating infection prevention systems (Xu and James, 2012). Similarly, the expression of antimicrobial peptide genes was found to increase in response to increasing temperature in *Apis mellifera* and *Apis cerana* (Li et al., 2022b).

Some of this evidence appears to support a positive effect of the UHI effect on bees, especially in terms of a reduction of pathogen transmission and susceptibility of hosts toward infections. However, exposure to elevated temperatures may simultaneously cause irreversible damage and increased mortality to the host (Dalmon et al., 2019; Vanderplanck et al., 2019; Walters et al., 2022) making the disadvantages posed by the UHI effect greater than the advantages. Species ecological traits may mitigate the exposure of individuals to stressors. For example, social bees that can thermoregulate the hive may be less affected (Palmer-Young et al., 2019; Vanderplanck et al., 2019). Essentially what we know comes from eusocial Apidae, so it is necessary to include solitary bee species in new studies. They can harbor the same pathogens but show different symptoms compared with eusocial bees (Ravoet et al., 2014). Also interesting to perform in the future would be the analysis of infections in social vs. solitary species. Indeed, the thermally more constant conditions in the nests of eusocial bees may lead to the adaptation of pathogens to a limited temperature range, compared with the nests of solitary bees.

5 Social behavior of bees and the UHI effect

Several studies reported an association between the frequency of social vs. solitary species and urbanization level. However, these results seem inconsistent across studies (Buchholz et al., 2020), as some studies report a greater abundance of solitary bee species in

cities, whereas others indicate a higher abundance of social species (reviewed in Ferrari and Polidori, 2022).

At the intraspecific level, the possible shifts in social behavior of bees along an urbanization gradient is completely unknown. Weissel et al. (2006), in a study carried out at a German University campus, showed that higher soil temperatures shortened the duration of the pauses between worker phases in the ground-nesting, eusocial *Lasioglossum malachurum* (Halictidae). Therefore, the annual nesting cycle of this bee species was also shortened. The acceleration of larval development may be at the base of such phenomenon, and it may reflect a gradient in social behavior of eusocial halictid bees along an urbanization gradient. For example, a quicker cycle may lead to a higher number of worker phases produced by the queens, potentially increasing colony success. At larger gradients (e.g., across latitudes), social behavior of some bees seems also to be affected by temperature variations. For example, using field transplant experiments, it was shown that across a south–north cline in the UK, *Halictus rubicundus* (Halictidae) foundresses shift the initiation of provisioning of the first brood, hence changing size and maturation time of their offspring. Most importantly, such plasticity leads to a switch between two strikingly different social phenotypes, with northern populations being solitary and the southern ones being eusocial (Field et al., 2010). Furthermore, Schürch et al. (2016) demonstrated that worker numbers in this sweat bee species should increase throughout Great Britain under predicted climate change scenarios, and that sociality should appear in the northern areas where, at the moment, only solitary nests occur. Though this is a quite extreme situation due to the great social plasticity of this species, it may be expected that, in general, for primitively eusocial bees the timing of colony development and the number of brood phases produced may also show some variations across urbanization clines. Therefore, new studies should aim to collect detailed data on the colony composition along urbanization gradients. Furthermore, it should be tested if the UHI effect can negatively affect cognitive skills—and, consequently, foraging behavior—in bees. Indeed, bumblebees tested at 32°C (a condition often met during heatwaves in cities) were significantly worse at forming an association between a colored light and a sucrose reward, compared with individuals tested at 25°C (Gérard et al., 2022).

6 Conclusions

In this article we have highlighted barely or not yet-tested hypotheses on the impacts of the UHI effect on several relevant functional traits in bees. It remains open the question on which bee taxa may be more affected by the UHI effect. It is likely that dispersal ability has an important role in this sense. Indeed, populations of species with limited dispersal ability may be affected for long times in urban habitats. Dispersal ability is not well studied in most bee species, but a strong philopatric tendency while nesting (e.g.,

Yanega, 1990) and a foraging distance rarely exceeding 1 km from the nest (e.g., Greenleaf et al., 2007) suggest that the proposed impacts could be likely more visible in solitary and primitively eusocial bee species (especially small species, which have a smaller foraging distance compared with large species), rather than in honeybees. On the other hand, among solitary species, oligolectic species may be more affected as they may not successfully forage their nests if their few used plant species suffer a reduction or a temporal mismatch with bees due to the UHI effect. In addition, bees can be also lethally affected if ambient temperature increases above certain levels. Such lethal effects may be more likely to appear in very hot cities during the increasingly more common heatwaves. For example, at an air temperature of 35°C, the thoracic temperature of a flying bumblebee almost reaches 45°C, that is, close to the lethal limit (Heinrich, 2004).

Although we have focused on direct effects, it is important to note that warmer urban conditions can also indirectly affect bees. For example, elevated temperatures alter flower resource phenology, diversity, and abundance with direct consequences for the bee diet (Moss and Evans, 2022; Sexton et al., 2023). This may in turn potentially decrease bees' tolerance toward pathogens (Dolezal and Toth, 2018; Meeus et al., 2018), affect their GM composition (Voulgari-Kokota et al., 2019), and increase aggression in intraspecific contexts, as seen in other animals (Parker and Nilon, 2008; Davies and Sewall, 2016; de Tranaltes et al., 2022). Furthermore, in the warmer urban conditions, pollen foraging may compromise bees to an unsafe body temperature, since it was shown, at least in a *Bombus* species, that bees carrying pollen have a thorax temperature approximately 1°C –2°C hotter than those without pollen (Naumchik and Youngsteadt, 2023).

In order to test for these hypotheses and fill the many gaps in our knowledge on this topic, it would be desirable to (1) enlarge the taxonomic diversity of species considered in such studies, especially trying to span different life cycles, biological features, and morphology (e.g., solitary vs. social, kleptoparasites vs. non-parasites, ground-nesting vs. aerial nesting, oligolectic vs. polylectic, small sized vs. large sized), as well as from different bee lineages. For example, since solitary bees cannot regulate their internal nest temperature as eusocial ones (Jones and Oldroyd, 2006), it may be expected that nest settlement by solitary species might be more dependent on local temperature conditions. This, in turn, could lead to some future research directions on the behavioral plasticity of nest settlement as a function of external temperature. (2) Carry out genetic studies to ascertain if these morphological, physiological, and behavioral traits can show outcomes in urban habitats because of adaptation processes or plastic responses to higher temperatures. (3) Consider different cities that vary in their characteristics (e.g., human population size, temperature difference between cities and their surrounding areas). In addition, not only absolute temperature, but also temperature fluctuations may affect the discussed bee traits. For example, temperature daily and seasonal variations could be stronger in cities, with large nocturnal and seasonal variability (Cao et al., 2021), and such fluctuations were also seen to affect bees (e.g., Kierat et al., 2017). Hence, also this climatic parameter should be taken into

account in future investigations. Since bee species considerably vary in their biology among and within lineages, it would also be important to apply phylogenetic corrections to comparative analyses.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

CP: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. AF: Conceptualization, Writing – original draft, Writing – review & editing. FR: Writing – original draft, Writing – review & editing. NT: Funding acquisition, Writing – original draft, Writing – review & editing. EN: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author CP declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Integrated resistance management for acaricide use on *Varroa destructor*

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The global beekeeping industry faces an escalating challenge in the form of *Varroa destructor*. Synthetic chemicals serve as a cornerstone for varroa management, although they face a major challenge in the form of acaricide resistance. Here, I examine acaricide resistance in varroa under the framework of Insecticide Resistance Management (IRM). I assess the potential of diverse IRM strategies, such as pesticide rotation and mixtures, refuge utilization, synergists and the integration of non-persistent chemicals. The peculiar life history of varroa, characterized by its incestuous breeding system, challenges conventional IRM strategies. There is little published evidence that pesticide rotation is beneficial for resistance management in varroa, with several studies showing resistance is maintained despite rotation. Fitness costs associated with pesticide resistance are often an essential component for IRM strategies, but there are no current data from varroa demonstrating such specific fitness costs (e.g., a reduced relative oviposition rate) associated with resistance. The single published experimental study directly examining relative fitness found that here was little or no reproductive fitness cost associated with pyrethroid resistance. More work is needed on fitness effects of the key acaricides, which would better guide the use of rotation and refuge strategies. A key prospect for future work that has been identified through simulation modeling is offered by pesticide mixtures and the role of synergists to elevate acaricide efficacy. Additional tools for varroa IRM include 'soft' acaricides, including oxalic acid, and biopesticides such as dsRNA. In light of the widespread prevalence of acaricide resistance and an increasing varroa problem, there is an urgent need for nuanced, data-driven varroa IRM strategies.

KEYWORDS

honey bee (*Apis mellifera*) health, pesticide resistance, resistance fitness costs, acaricide rotation and mixtures, synergists, refuges, novel acaricide development

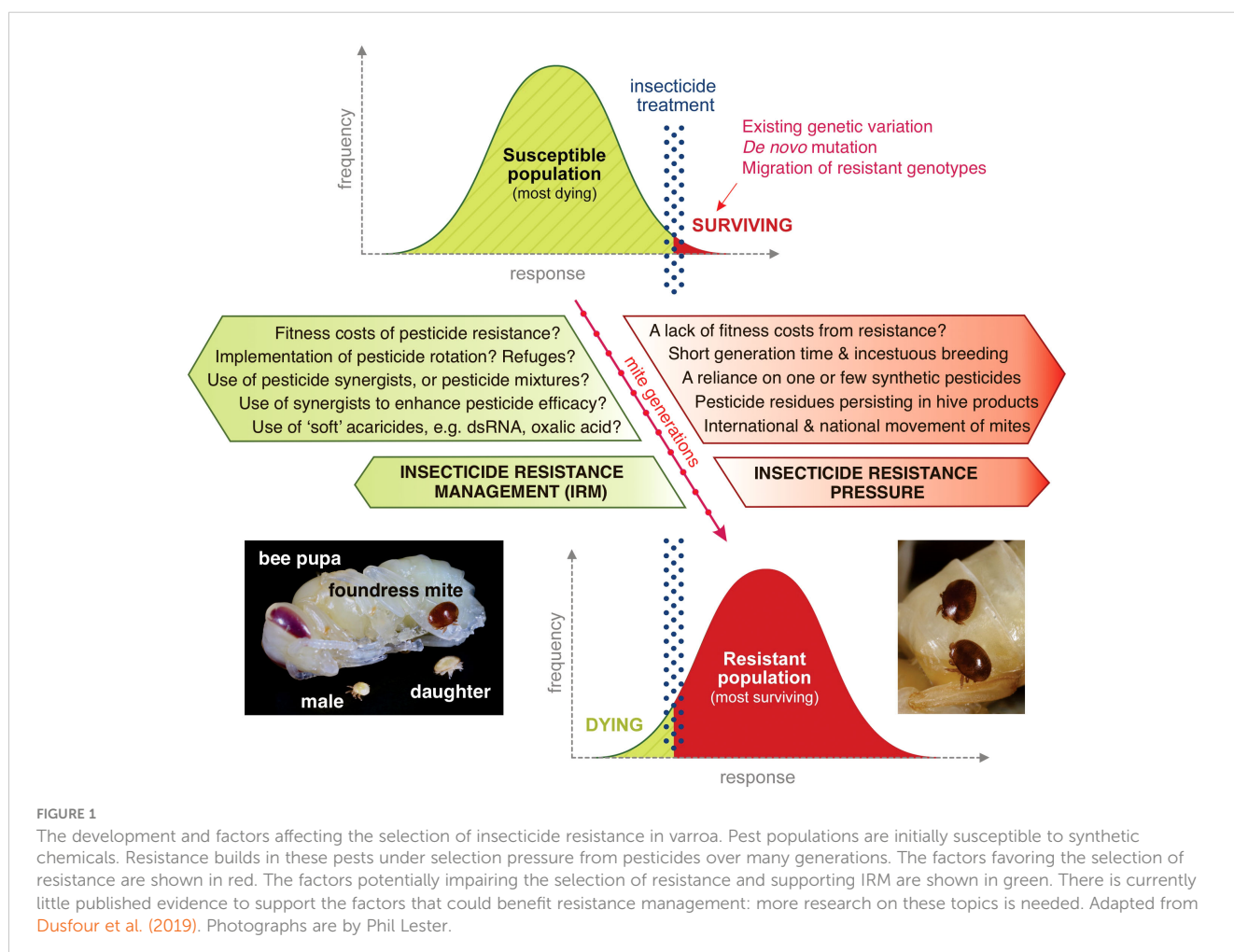
1 Introduction

Varroa destructor, along with the associated Deformed wing virus (DWV) and other viruses associated with the mite, are key pests that are resulting in an ongoing decline in honey bee health around the globe (Rosenkranz et al., 2010; Traynor et al., 2020; Jack and Ellis, 2021; De Jong and Lester, 2023). *Varroa* was first reported on *Apis mellifera* outside its natural distribution area of Southeast Asia in 1949, spreading quickly to reach Europe in the 1970s, and then rapidly to North America, South America, Africa, and the Asia Pacific region (Nazzi and Le Conte, 2016). In 2022 it was first detected in Australia, which has been the last major landmass previously free of the parasite and DWV (Chapman et al., 2023). In many of these countries including New Zealand, for example, the combined effects of this parasite and virus have been consistently identified as the leading cause of overwintering honey bee hive mortality (Stahlmann-Brown et al., 2022; McGruddy et al., 2023). This parasite now fits the description of a ‘superpest’, or a species that has the capacity to invade and adapt to environments around the globe, causing significant damage, and evading control strategies (Whitfield and Rotenberg, 2023).

Throughout its global distribution, synthetic chemicals are and will likely continue to be an essential tool for beekeepers in managing varroa. Currently, only a small number of these

acaricides are widely used for varroa control: pyrethroids (such as tau-fluvalinate and flumethrin), the organophosphate coumaphos, and the formamidine amitraz. Their ease of use and historical efficacy have led to a significant global reliance on these products (Mitton et al., 2022). A recent survey indicated that 23% of beekeepers in New Zealand used only amitraz or flumethrin, while another 31% used these two pesticides in rotation (Stahlmann-Brown et al., 2022). Similarly, in the UK, 18% of beekeepers depended solely on amitraz, and 20% utilized a rotation or mixed-method approach to mite treatments (Valentine and Martin, 2023). High concentrations of these chemicals or their metabolites are commonly found in bees, honey, wax, and other components of the hive (Mullin et al., 2010; Kiljanek et al., 2017; Vegh et al., 2023).

Pesticide resistance can be defined as a relative decrease in the susceptibility of a pest population to a particular pesticide (Figure 1). Due to the demonstrated ability of many other mite species to rapidly develop resistance to pesticides, it was considered inevitable that varroa would also become resistant (Martin, 2004). Resistance to the pyrethroid tau-fluvalinate was first observed in 1991 and spread quickly (Lodesani et al., 1995). For instance, in the UK, it was initially discovered in 2001 but had already spread over many hundreds of kilometres by 2004 (Martin, 2004). Similarly, resistance was first observed for amitraz in 1991, flumethrin in



1995, and coumaphos in 2001, and has since become globally widespread (Mitton et al., 2022). The development of resistance has historically been classified as mechanisms of decreased exposure (pharmacokinetic) such as through behavioral modification, increased metabolism, sequestration, and excretion, or mechanisms of decreased sensitivity (pharmacodynamic) that include target-site insensitivity (Van Leeuwen and Dermauw, 2016). Pyrethroid resistance in varroa has commonly been associated with amino acid changes in the voltage gated sodium channel (VGSC), although esterase activity has also been speculated to play a role (Mitton et al., 2022). Amitraz resistance has been correlated with the mutations in the receptor for the neuromodulator octopamine (Rinkevich et al., 2023). Coumaphos resistance has been linked to varroa down-regulating the expression of a P450 monooxygenase enzyme on the Greek island of Andros (Vlogiannitis et al., 2021b). It is likely that varroa have developed a diversity of resistance mechanisms that are only beginning to emerge, as mites have an array of gene families that can confer resistance and which are not well studied (Van Leeuwen and Dermauw, 2016).

The primary objectives of Insecticide Resistance Management (IRM) are to prevent the evolution or emergence of resistance in susceptible pest populations, slow down the development of resistance, or reverse it to a level that allows for the effective and efficient use of existing or new insecticides, all while minimizing adverse environmental impacts (Denholm and Rowland, 1992; Dusfour et al., 2019). IRM has been widely recognized as essential for maintaining effective pest control across various systems, including insect vectors of malaria and arboviruses (WHO, 2012; Mnzava et al., 2015; Dusfour et al., 2019). Georghiou (1994) categorized IRM approaches into three main groups: management through multiple attack, management through saturation, and management through moderation. Management through multiple attack involves the application of multi-directional selection pressures, often achieved by using pesticide mixtures or rotating unrelated insecticides. Management through saturation seeks to eliminate the selective advantage of resistant individuals, which can be accomplished by increasing insecticide uptake through attractants or by suppressing detoxification enzymes with synergists. Management through moderation focuses on reducing selection pressure while supplementing control with non-chemical measures (Georghiou, 1994).

To ensure that synthetic chemicals remain a viable tool for varroa control in beekeeping, the global honey bee industry must align its management and research efforts with the objectives of IRM. Our goals should include maintaining or extending the effectiveness of acaricides while minimizing their environmental and non-target impacts. Additionally, we should strive to integrate acaricides more effectively into holistic varroa control strategies. This article aims to examine varroa mite management via acaricides within the framework of IRM. I explore the key factors that could influence IRM in the context of varroa control, assess the utility of foundational IRM components like pesticide rotation, and identify the research needs required to enhance future varroa IRM. Below, I address these issues using the IRM framework proposed by Georghiou (1994).

2 Varroa management by multiple attack

Management by multiple attack involves the application of multi-directional selection pressures, including by the use of pesticide mixtures or rotations of unrelated insecticides that have different modes of action (Georghiou, 1994). This strategy operates on the premise that control can be achieved through the influence of multiple, independent stressors, which may encompass the use of insecticides. Each stressor imposes a selection pressure below the threshold required for the development and sustenance of resistance. It is an approach that can include the application of chemicals in rotations or in mixtures.

The rotation of acaricides with different modes of action has widely been considered an essential strategy for preventing or delaying resistance development in varroa (Jack and Ellis, 2021; Mitton et al., 2022; Morfin et al., 2022; Warner et al., 2024). In a simulation analysis, Sudo et al. (2018) illustrated how rotation represents the optimal strategy for IRM in many scenarios, particularly when pesticide efficacy is high in the presence of pre-mating selection and dispersal. Pesticide rotation, therefore, remains an indispensable tool for managing many pest species. However, the analysis by Sudo et al. (2018) also identified scenarios in which no known pesticide-use strategy could delay resistance development. An absence of conditions such as pre-mating dispersal and existing high levels of pesticide resistance rendered resistance management strategies unavailable for certain pest species, including the mite *Tetranychus urticae* (Sudo et al., 2018). Indeed, pesticide rotation has been shown to be ineffective for resistance management in this spider mite (Overmeer et al., 1975). Varroa shares several life-history attributes that theoretically limit the efficacy of pesticide rotation, and the literature contains several examples where rotation failed as a resistance management strategy. Maggi et al. (2011) documented coumaphos resistance in apiaries in Uruguay despite strict acaricide rotation. Subsequently, coumaphos resistance persisted in these apiaries even after 9 years of non-use (Mitton et al., 2018). Similarly, in Spanish apiaries, coumaphos resistance endured for at least 5 years in the absence of this pesticides use (Higes et al., 2020). It is possible that resistance was maintained in the absence of coumaphos. It is also possible that resistance was maintained due to selection pressure via coumaphos residues from contaminated beeswax or other hive matrices. Or alternatively, immigration of resistant mites may have contributed to this outcome. Genetic and dispersal data would be needed to discriminate between mechanisms of mite drift or genetic factors for resistance maintenance.

Despite simulation modeling approaches that have indicated resistance is predicted to be maintained in varroa even in the absence of pesticide use, there are some observations that pesticide resistance can be reduced when the use of a synthetic chemical has been halted. Milani (1999) cite unpublished observations that the proportion of varroa resistant to fluvalinate showed a slow decrease after the use of this pesticide was halted. In published work from this Italian region, they describe the decline of pyrethroid resistance to take many generations (Milani and Della Vedova, 2002). It was predicted that pesticide rotation could be

useful but only if the pyrethroid was used once every 4–6 years and only then if it was promptly followed by an alternative treatment to eliminate as many remaining pyrethroid-resistant mites (Milani and Della Vedova, 2002). Of note from this work was that the authors considered toxicological parameters such the LD₅₀ to be of limited use for resistance management because of co-occurring combinations of susceptible and resistant mites (Milani and Della Vedova, 2002). To my knowledge, there is only one other publication reporting an instance of varroa undergoing a reversion from a resistant to a susceptible population through pesticide rotation management. Elzen & Westervelt (2004) describe a ‘modest’ reversion over a relatively short 10-month period after discontinuing the use of fluvalinate. While it is possible that susceptible mite immigration contributed to these results, the authors considered it unlikely (Elzen and Westervelt, 2004).

Much more common in the scientific literature are reports of rotation being ineffective and which question the usefulness of this strategy for resistance management (Alissandrakis et al., 2017; Mitton et al., 2018; Higes et al., 2020).

For a pest population to revert from pesticide-resistant to susceptible genotypes, there needs to be a significant evolutionary fitness cost (Kliot and Ghanim, 2012). There is, however, limited experimental evidence specifically demonstrating any relative fitness effects for varroa. Relative fitness estimates need to compare both the survival and reproductive performance of susceptible and resistant genotypes (Orr, 2009). Martin et al. (2002) is the only published study that I am aware of to examine egg-laying and daughter mite production in a pyrethroid-resistant and susceptible population of mites. They found little or no reproductive fitness cost associated with pyrethroid resistance in varroa from Texas, which was concluded to be likely if the point mutations associated with resistance cause little or no metabolic cost to the mite (Martin et al., 2002).

An absence of relative fitness cost in resistant individuals would not be unique. Other pest species, including a blowfly and a flour beetle, have developed heritable modifiers that appear to have eliminated fitness costs and have resulted in resistance traits becoming fixed in pest populations (Kliot and Ghanim, 2012). Some authors have suggested point mutations in varroa can induce major fitness costs, though have not measured any life history parameters (González-Cabrera et al., 2018). Another example was a report of no disadvantage or only a small but consistent reduction in the fitness of pyrethroid-resistant mites in Italy (Martin, 2004), although the authors of the cited Italian study did not directly measure reproduction or fitness parameters (Milani and Della Vedova, 2002). A more comprehensive understanding of the selection pressure and fitness effects exerted by different pesticides on varroa is needed.

It is clear that pesticide rotation does not always work for resistance management in varroa. A more nuanced analysis is needed for this management approach (Rosenkranz et al., 2010; Mitton et al., 2021; Mitton et al., 2022). The “mainly non-professional structure of the beekeepers’ community” in many countries or regions has implied rotation may have only ever been a short-term solution for resistance management

(Rosenkranz et al., 2010). Nevertheless, the few reports of resistance reversion and decline offer some hope for ongoing varroa management through the rotation of synthetic chemicals. These observations should be a top priority for further investigation: can we transform these exceptions into the norm? Have resistant genes genuinely disappeared from the population, or do resistance reservoirs persist? What are the fitness implications of various mutations? How quickly will resistance re-emerge when synthetic chemicals are reintroduced into a rotation schedule, whether through reservoirs or re-invasion? It is crucial to disentangle the impacts of resistance through the two potential mechanisms of genetic maintenance within populations and reinvasion. What is the ideal pesticide rotation schedule to maintain pesticide efficacy? A rotation schedule that involves using a pesticide only once every 4–6 years (Milani and Della Vedova, 2002) probably isn’t practical for a beekeepers toolbox. Other researchers have reached the same conclusion: there is an urgent need for a more sophisticated assessment of rotation as a resistance management tool, and how it might be best integrated varroa control approaches (Mitton et al., 2021).

A further complicating aspect to achieve rotation is that relatively high concentrations of acaricides or their metabolites persist in honey, wax, and other components of the hive (Mullin et al., 2010; Kiljanek et al., 2017; Vegh et al., 2023). Beekeepers often recycle and reuse wax and hive components from hives for many years. Varroa may thus be being exposed to low concentrations of different acaricides even when under rotation. This accumulation and management of miticides in beeswax is considered to be a widespread problem of increasing importance for resistance management (Le Conte et al., 2010).

An approach that has received little attention in the context of varroa IRM is the use of pesticide mixtures. Simulations, however, suggest that mixtures can be extremely beneficial. Helps et al. (2020) developed a model to explore the factors influencing optimal insecticide resistance management strategies. Their model incorporated variables such as fitness effects on the pest, the rotation of different insecticides, and the use of refuges. Their findings revealed that the primary determinant of the optimal strategy was the reproductive strategy of the pest. For pests with a sexual reproductive mode, employing a pesticide mixture emerged as the nearly always optimal strategy for IRM. Interestingly, a reduced application dose mixture often outperformed the label dose mixture, and regardless of the pests reproductive mode, a reduced dose mixture frequently represented the optimal strategy (Helps et al., 2020). There is reason to expect that mixtures might be beneficial for varroa IRM. For example, coumaphos resistance on the Greek the island of Andros has been linked to varroa down-regulating the activation of a P450 monooxygenase enzyme (Vlogiannitis et al., 2021b). Perhaps this resistance could be negated or exploited by using an alternative or a mixture of pesticides. Vlogiannitis et al. (2021b) discuss their results in regard to IRM, citing other instances of how the gene expression in the P450 pathway of other pests confers resistance to one pesticide but can increase the susceptibility to chemicals. Such negative cross-resistance between different insecticide or pro-insecticides caused by differential gene regulation in resistant

insects could be exploited for varroa IRM via a push–pull strategy (Vlogiannitis et al., 2021b).

In summary, regarding varroa IRM through multiple attack:

- There is currently no empirical evidence indicating that pesticide resistance imposes a specific relative fitness cost on varroa. There is a pressing need for pesticide-specific, laboratory-based assessments of fitness costs associated with resistance. Such assessments would provide valuable insights into the implementation of various aspects of varroa IRM. It is possible that fitness costs may arise for some pesticides or specific genetic mutations linked to resistance but not for others.
- Pesticide rotation has been frequently suggested a key component of IRM for varroa, although there is limited current evidence that rotation is or could be effective. A simulation model suggests rotation is likely to be ineffective for varroa IRM (but would not be deleterious). There is an urgent need for a nuanced analysis of rotation and how it might be best integrated into varroa IRM strategies.
- Pesticide mixtures are projected to hold potential value for IRM based on simulation models, yet experimental investigations in this area are lacking and warrant further research before implementation can be considered.

3 Varroa management by saturation

Management by saturation requires an elimination of the selective advantage of resistant individuals, perhaps by increasing insecticide uptake through attractants or by suppressing of detoxication enzymes via synergists (Georghiou, 1994).

Semiochemical attractants or lures are used as a component of IRM in a range of pest management systems. The ‘lure and kill’ approach has been proposed as an effective method for pest control and even potential eradication (El-Sayed et al., 2006). These treatments of lure and kill can be further combined with additional pheromone disruption practices (Suckling et al., 2016). Varroa produce or respond to a wide variety of semiochemicals that influence their behavior and physiology (Plettner et al., 2016). Some semiochemicals can deter and repel varroa (Pernal et al., 2005), arrest their movement (Calderone and Lin, 2001), alter their host-selection behavior (Eliash et al., 2014), or even induce gravid adult females to reabsorb eggs (Frey et al., 2013). Despite these observations, no commercially available attractants that lure or attract varroa currently exist. Research in this area is ongoing, with one report of an experimental attractant capable of causing 35–50% of mites to disengage from bees (Suszkiw, 2009). An efficient attractant that could enhance varroa exposure to pesticides would be of significant value for IRM, as would non-pesticide control options such as mating disruption. In addition to ‘lure and kill’ approaches being used directly on the mites, an additional or alternative approach would be to investigate ways to selectively attract varroa-infested bees to acaricide treatments.

There is potential for the suppression of detoxification enzymes through synergists, which could in some situations enhance or sustain the effectiveness of synthetic chemicals in varroa IRM. For example, in pyrethroid-resistant mosquito populations the benefits of using a synergistic pesticide (synergist piperonyl butoxide, or PBO) are thought to be via the inhibition of the cytochrome P450 enzymes that catalyze pyrethroid detoxification (Farnahm, 1998). The degree of pyrethroid enhancement via the PBO synergist was found to be mosquito species-dependent and dependent on the level of pyrethroid resistance in the population (Churcher et al., 2016). PBO was similarly shown to restore pyrethroid efficacy in resistant populations of whitefly and cotton bollworm (Young et al., 2006). Similarly, to chemical synergists, dsRNA can silence P450 genes that confer resistance to pests against plant defenses (Mao et al., 2007). dsRNA can also knock-down host immune responses and augment the virulence of pathogens (Wang et al., 2021; Felden et al., 2023). The synergistic use of biopesticides has also been observed to substantially increase pyrethroid efficacy (Nishimatsu and Jackson, 1998). An important caveat in the use of synergists for varroa, however, is that synergists may only be of benefit in situations where resistance arises from metabolic mechanisms. Alternatively, if the major mechanism is the target site modification, as with the majority of known resistance cases for varroa to date (Mitton et al., 2022), it is unlikely that there will be significant benefits from the use of such compounds.

In summary, regarding varroa IRM by saturation:

- Varroa respond to an array of semiochemicals, which could be useful for IRM if mites could be attracted to pesticide exposure as in ‘lure and kill’ methods. Semiochemical use and development for varroa has been challenging and further research is needed in this area.
- The suppression of detoxication enzymes via synergists is also an under-studied area in varroa IRM, although may be of limited benefit for varroa IRM if the key resistant mechanisms are target site modifications. Results from other IRM systems demonstrate that enhanced efficacy and even the restoration of pesticide efficacy can be achieved by the use of synergists.
- The elimination of the selective advantage of resistant varroa might be achieved in some instances through the use of a wide variety of synergists including other pesticides, biopesticides including dsRNA.

4 Varroa IRM by moderation

Management by moderation acknowledges the value of pesticide-susceptible genes and individuals as a valuable resource. It entails reducing the selection pressure and complementing control efforts with non-chemical measures (Georghiou, 1994). These management actions may involve decreasing insecticide application rates, reducing the frequency of application, using non-persistent chemicals, or preserving susceptible genes and individuals through the utilization of untreated refuge populations.

Lowering or eliminating the selection pressure for a particular pesticide is a crucial component for successful IRM in many pest species. Low rates of application might be useful for IRM with many pests that are yet to develop resistance. Pesticide resistance in varroa, however, has developed and is already widespread (Mitton et al., 2022).

Could lowering rates or infrequent application of pesticides successfully cause a reversion of varroa populations to become dominated by susceptible individuals? The inbreeding behavior of varroa makes resistance development and management especially challengingly and very different from many other pests. This mite displays arrhenotokous parthenogenesis (Reams and Rangel, 2022). In hives with low density mite infestations bee pupal cells are typically occupied by single foundress mites. Inbreeding necessarily then results, with the first offspring being a male from an unfertilised egg. This male then mates with subsequent sister female offspring that develop from fertilized egg. Outbreeding between lineages only occurs in scenarios of high mite abundance when multiple foundress mites inhabit a single bee pupal cell. Low to moderate mite densities thus limit the opportunity for selection and outbreeding and genes for pesticide resistance to be lost from populations. It is the number of mites per brood cell in hives that will determine the genetic populations structure of varroa, with simulations demonstrating that inbreeding and low mite abundance will enhance the fixation of mite with homozygous pesticide resistance alleles (Beaurepaire et al., 2017). Resistant allele homozygosity in varroa does appear to enhance physiological pesticide resistance (Mitton et al., 2021; Vlogiannitis et al., 2021a).

Compounding this inbreeding outcome, low mite densities limit the propensity of varroa to move between hives. In low abundance, mites appear likely to remain within the bee colony by an ability to selectively choose nurse bees and quickly move to new cells for reproduction (Cervo et al., 2014). It is only when mites attain higher densities that they are more frequently found on older foraging bees that have a higher likelihood of movement outside the hive and between hives.

What implications does the varroa life history and inbreeding have for a beekeeper in an area where varroa have developed resistance to synthetic chemicals? These mites (and their genes) have been moved around between beekeeping operations due to management practices such as receiving packaged bees that may already have pesticide resistance (Strange et al., 2008). Natural mite movement behaviors can also contribute to the spread of resistance genotypes, including via bee drifting (Kulhanek et al., 2021), robbing (Peck and Seeley, 2019), or even by mites moving between bees while foraging on flowers (Peck et al., 2016). A beekeeper who acquires resistant mites but successfully maintains low varroa infestations, possibly even without relying on synthetic chemical pesticides and instead employing varroa-resistant bee strains and oxalic acid treatments, might inadvertently facilitate the persistence of resistance to synthetic chemicals. By sustaining low mite densities, this diligent beekeeper essentially perpetuates inbreeding and limits opportunities for outbreeding. Genetic drift alone cannot be counted on to eliminate genetic variation, and the inbreeding mating behavior of varroa will likely result in numerous inbred mite lineages, each harbouring different alleles in a

homozygous state (Beaurepaire et al., 2017). Even if all beekeepers in the region cease using a particular pesticide for several years, those who successfully maintain low mite numbers during this period are essentially preserving reservoirs of resistance. Thus, management actions that involve decreasing insecticide application rates, reducing the frequency of application, or even using non-persistent chemicals may not contribute toward IRM in varroa.

The use of 'refuges' can be beneficial for the management other pests including with other mite species (Lester et al., 1998). However, refuges may offer limited utility in the context of varroa management. For varroa IRM, a requirement for refuges would be to maintain populations of homozygous, susceptible females. These susceptible females would need to migrate from refuges to cohabit bee pupal cells with resistant mites to enable the potential for crossbreeding between lineages. As noted previously, for cohabitation or co-occurrence to occur would generally require high mite densities. Deleterious fitness effects are also assumed as necessary for the selection of and reversion to susceptible genotypes. It is possible that beekeepers who use non-chemical means of controlling varroa could develop such refuge populations of acaricide-susceptible varroa. However, it is hard to be optimistic regarding the use of refuges for varroa IRM given the previously discussed, frequent long-term maintenance of resistance in varroa after the cessation of pesticide use. Further, the national and international movement of bees could negate any benefits of refuges. Beekeeping operations frequently entail the movement of bee colonies within and between regions, countries, and even across continents. Packages or nucleus colonies of bees, and even truckloads of entire hives, inadvertently carrying varroa mites and associated viruses, are transported within countries and globally (Martin, 2004; Strange et al., 2008; Budge et al., 2020). The high rate of varroa movement within and between countries via bee packages or the transport of entire hives makes resistance development in one region a global problem. Any benefits for resistance management that beekeepers may accrue through the use of refuges could be negated through hive and mite movement.

Additional, new synthetic chemical acaricides are being investigated including carbamates (Jack et al., 2022) and lithium salts including lithium formate (Ziegelmann et al., 2018; Sevin et al., 2022). Non-synthetic acaricide measures may be a key method to reducing varroa selection pressure and complementing control efforts. The 'soft' acaricides that include oxalic acid, thymol, and formic acid have become widespread, with oxalic acid often seen as most effective of the soft acaricides in terms of mite control and honey yield (Qadir et al., 2021). In 2020/2021 the most common acaricide treatment method in the UK was oxalic acid, followed by thymol and amitraz (Valentine and Martin, 2023). There has been no reported evidence of resistance to oxalic acid treatments. An additional emerging technology is gene silencing or dsRNA based biopesticide treatments, which can have be directly lethal to varroa (Garbian et al., 2012) or be used in the silencing of genes in resistance pathways (Vlogiannitis et al., 2021b). Honey bee bacterial symbionts have recently been produced to express dsRNA for both DWV and varroa control (Leonard et al., 2020). Another bee-safe approach might be through selective acaricides

targeting the varroa mite-specific neuropeptides and signaling systems (Jindal et al., 2022). These biopesticides and soft acaricides offer an opportunity for enhancing varroa IRM, as they could reduce acaricide selection pressure and complement synthetic pesticide control efforts (Georghiou, 1994).

In summary, regarding varroa IRM through moderation:

- Pesticide-susceptible genes and individuals represent a valuable resource. Currently, it remains unclear how IRM practices can be effectively employed to increase the frequency of susceptible genes and individuals within a population.
- Due to varroa's inbreeding system, resistance is likely to persist in populations even after discontinuing the use of a particular pesticide. This is especially true in scenarios where mite populations are kept at low levels. Consequently, IRM strategies like infrequently applying different acaricides with diverse modes of action are expected to have limited efficacy in reversing resistance in varroa populations.
- Resistance is already widespread in varroa, likely implying that in IRM management actions such as lowering insecticide application rates having limited value. The value of refuges could be via supplying homozygous, susceptible females that could reduce or dilute resistance. However, the value of refuges is largely unknown for varroa IRM.
- The adoption of non-persistent soft acaricides like oxalic acid, as well as the introduction of new acaricides and emerging treatments such as biopesticides including varroa-specific double-stranded RNA (dsRNA), presents opportunities for IRM. The integration of these alternatives into varroa IRM strategies should be encouraged as well as resistance monitoring in mite populations to understand their benefit.

5 Conclusions

The global plan for managing insecticide resistance in malaria vectors was formulated by the World Health Organization Global Malaria Program (WHO, 2012). This plan has been recognized as one of the few comprehensive documents addressing the management of a worldwide insecticide resistance crisis. It places a strong emphasis on five interconnected "pillars" for a global strategy, namely: (i) devising and implementing insecticide resistance management strategies, (ii) ensuring ongoing resistance monitoring, (iii) developing novel control tools, (iv) addressing knowledge gaps, and (v) establishing essential enabling mechanisms, which encompass advocacy and the allocation of financial resources (WHO, 2012; Sternberg and Thomas, 2018;

Dusfour et al., 2019). My assessment is that IRM is far less developed for varroa than it is for malaria vectors or than for other pests including lepidopteran species in horticulture (Walker et al., 2017). Varroa IRM needs a global strategy that could be modelled using the malaria vector approach.

Varroa management encompasses three fundamental, broad beekeeping practices: employing resistant bee strains, implementing apitechnical measures such as brood removal, and utilizing chemical control methods (Bubnič et al., 2021). Chemical control methods are likely to remain as one of these key pillars for the foreseeable future, and thus IRM should be a priority. Research is required across nearly all the facets of varroa IRM, including the evaluation of mixtures, synergists, assessing fitness costs associated with resistance, and even in exploring the advantages of acaricide rotation. Among the numerous avenues and approaches proposed, the examination of the effectiveness and advantages of pesticide mixtures and synergists stands out as particularly promising (Helps et al., 2020), as well as the continuing development and use of oxalic acid, new chemicals including lithium salts, and biopesticides such as dsRNA. New developments and long-term analyses in varroa IRM are desperately needed in order to help stem colony losses due to varroa infestations around the globe.

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A methodological approach to identify priority zones for monitoring and assessment of wild bee species under climate change

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Climate change is affecting wild populations worldwide, and assessing the impacts on these populations is essential for effective conservation planning. The integration of advanced analytical techniques holds promise in furnishing detailed, spatially explicit information on climate change impacts on wild populations, providing fine-grained metrics on current environmental quality levels and trends of changes induced by estimated climate change scenarios. Here, we propose a framework that integrates three advanced approaches aiming to designate the most representative zones for long-term monitoring, considering different scenarios of climate change: Species Distribution Modeling (SDM), Geospatial Principal Component Analysis (GPCA) and Generalized Procrustes Analysis (GPA). We tested our framework with a climatically sensible Neotropical stingless bee species as study case, *Melipona (Melikerria) fasciculata* Smith, 1854. We used the SDM to determine the climatically persistent suitable areas for species, i.e. areas where the climate is suitable for species today and in all future scenarios considered. By using a GPCA as a zoning approach, we sliced the persistent suitable area into belts based on the variability of extremes and averages of meaningful climate variables. Subsequently, we measured, analyzed, and described the climatic variability and trends (toward future changes) in each belt by applying GPA approach. Our results showed that the framework adds significant analytical advantages for priority area selection for population monitoring. Most importantly, it allows a robust discrimination of areas where climate change will exert greater-to-lower impacts on the species. We showed that our results provide superior geospatial design, qualification, and

quantification of climate change effects than currently used SDM-only approaches. These improvements increase assertiveness and precision in determining priority areas, reflecting in better decision-making for conservation and restoration.

KEYWORDS

methodological framework, species distribution modeling, conservation, decision making, priority zones, climate change, stingless bee, *Melipona fasciculata*

1 Introduction

Climate change and biodiversity loss are two major environmental challenges facing the planet. Studies have shown that the current climate crisis is already affecting the physiologies, genomes, distributions, interactions, and behaviors of many wild species, including bees (Kerr et al., 2015; Hegland et al., 2021; Barbosa et al., 2022; Schonfeldt et al., 2022; Sgolastra et al., 2022). These changes are expected to worsen in the coming decades, with significant implications for species survival and ecosystem functioning (IPCC-AR6, 2021). To address the urgent need for effective conservation strategies in the face of climate change crisis, new integrative approaches are required (Foden et al., 2018).

Species Distribution Modeling is an effective approach used to evaluate both the present status and future scenarios of potential changes in climate suitability for species, especially in the context of climate change. This analytical method can play a crucial role in aiding decision-making processes for biodiversity restoration and conservation (Kearney et al., 2010; Ferraz et al., 2012; Guisan et al., 2013; Angelieri et al., 2016; Sofaer et al., 2019). Climate suitable areas represent sites that are most adequate for the occurrence of species in different climate scenarios. Combined with Geographic Information System, they also can provide straightforward and intuitive graphical representations of possible trends of change for species and areas, facilitating the visualization and interpretation of the effects of climate change in temporal and spatial dimensions (Kearney et al., 2010; Ferraz et al., 2012; Guisan et al., 2013; Angelieri et al., 2016; Sofaer et al., 2019). SDM relies on the relationship between environmental variables and empirical data on the target-species occurrence. The most relevant ranges and composition of environmental factors in explaining species occurrence are quantified and mapped, generating a baseline scenario (Diniz-Filho et al., 2009; Espindola and Plischoff, 2018; Hao et al., 2019; Huang et al., 2022; Marshall et al., 2023). Subsequently, the characteristics detected in the baseline scenario are then projected on a new set of variables with predicted variations for future climate change scenarios (Hulme, 2005; Ludena and Yoon, 2015; Li et al., 2018). This projection enables the identification of areas that will suffer different changes in the

climate suitability for the species. It is plausible to expect that greater changes will impose greater climate pressure on populations, and these areas can be defined as priorities for actions aimed at mitigating the impacts of climate change. Equally important are the currently climatically suitable areas that will suffer milder impacts in the future, as they are essential for the conservation of the species, safeguarding the species for future. Therefore, areas that are suitable for species today and will continue to be suitable in the future are targeted for this approach. Here, we call this zone as Persistent Suitable Extent (PSE), which are contiguous regions that will consistently offer a suitable climate for species survival from the present into the future, and can function as potential sanctuaries, since adverse effects of climate change in populations are less intense, potentially allowing the maintenance of the populations. Therefore, the PSE are of great interest for long-term monitoring and status assessments, as well as, priority zones for land cover restoration and conservation planning (Hulme, 2005; Ludena and Yoon, 2015; Li et al., 2018).

The Generalized Procrustes Analysis (GPA) is a multivariate analysis used for comparing the concordance (and discordance) among a set of matrices (Gower, 1975; Dijksterhuis and Gower, 1991; Goodall, 1991; Ren et al., 2016; Cheng et al., 2021). For example, it has been frequently used for studies on morphological shapes (Rohlf, 1998; Nicola et al., 2003; Mitteroecker and Gunz, 2009; Viscosi and Cardini, 2011; Klingenberg, 2016; dos Santos et al., 2019). But it can also be employed as sensory analysis (Risvik et al., 1994; Mauricio et al., 2016; Ser, 2019; Rodríguez-Noriega et al., 2021), ecology (Lisboa et al., 2014; Camiz et al., 2017) and genetics (Bramardi et al., 2005; Xiong et al., 2008a). GPA is obtained after Procrustes transformation, defined as translation, rotation, reflection, and isotropic scaling (Gower, 1975; Ten Berge, 1977; Dijksterhuis and Gower, 1991; Goodall, 1991; Risvik et al., 1994). In short, translation means that all individual configurations are displaced to the center of the projective mapping, while they are rotated and reflected for an optimal agreement with one another. Yet, isotropic scaling is concerned to shrinking/stretching of individual configurations in a way that they are, as much as possible, alike to one another while relative distances among objects are preserved (Gower, 1975; Ten Berge, 1977; Dijksterhuis

and Gower, 1991; Goodall, 1991; Risvik et al., 1994). Thus, GPA has two relevant attributes: it is based on a consensus configuration that is projected in a multidimensional space and it allows calculating the level of within-variance of each assessed item. Therefore, according to environmental matrices extracted from climate variables into the persistent suitability area delimited by the suitability models, GPA inform the level and direction of potential convergences among scenarios. In addition, since the persistent suitability area of the target organism was sliced in a set of inner regions by a Geospatial Principal Component Analysis (GPCA) zoning approach (please do not confuse with Generalized PCA; see details in Material and Methods), the GPA analysis is able to indicate inner regions where all combined variables are prone to a greater within-variation, indicating where populations may face a higher climatic pressure. Conversely, regions within climatic suitability would exhibit lower within-variation, indicating places under lower climatic disturbance, therefore, populations under lower pressure. Such sites are considered as priority areas for long-term ecological, genetic or morphological studies, but also contributes to decision-making on the prioritization of areas for assessments, monitoring, restoration and conservation planning, as these locations can function as potential wildlife sanctuaries for many species in the future, and help guarantee the essential conditions for the existence or reproduction of resident and migratory fauna.

We relied on a native Brazilian stingless bee species to test our framework, the stingless bee *Melipona (Melikerria) fasciculata* Smith, 1854. Bees play the major pollinator role, providing several ecological, economic and social benefits, with significant impact in nature conservation, in agriculture and human societies (Klein et al., 2007; Potts et al., 2010; Ollerton et al., 2011; Chaplin-Kramer et al., 2014; Potts et al., 2016; Hristov et al., 2020; Klein et al., 2020; Patel et al., 2020). The stingless bees are the most diverse group of social bees and are found in the tropical and subtropical regions of the world, comprising more than 605 described species (Moure et al., 2022; Engel et al., 2023; Roubik, 2023). Several stingless bee species are traditionally managed by human populations around the world having not only economic but cultural importance (Cortopassi-Laurino et al., 2006; Quezada-Euán et al., 2018; Hill et al., 2019). *Melipona fasciculata* is a prominent stingless bee species inhabiting natural areas within Amazon biome (northern Brazil), managed for honey production by people living therein (Jaffé et al., 2015; Venturieri et al., 2015; Farfan et al., 2022), playing a key role as pollinator of native plants in the forest and in cultivated plants requiring buzz-pollination (Maués and Couturier, 2002; Nunes-Silva et al., 2013; Giannini et al., 2015; Gostinski et al., 2018). Their foraging flight and homing capability has been estimated to cover large distances as 10 km (Nunes-Silva et al., 2020). *M. fasciculata* is one of the most studied and reared stingless bees in North Brazil (Venturieri et al., 2017).

Here, we propose a methodological framework that integrates three advanced analytical approaches to determine the most representative sampling areas for monitoring aimed at assessing ecological and molecular long-term effects of climate change on populations. Our framework aims to: (A) identify the persistent suitable extent for the species under climate change scenarios; (B) discriminate inner zones

based on climatic variability inside the persistent suitable extent by applying GPCA analysis as a zoning approach and; (C) determine different levels of spatial and temporal climatic variability in each inner zone by applying GPA. We used a native stingless species to test our framework, but it can subsidize other studies aiming to prioritizing areas for planning actions and policies for restoration and environmental conservation to protect other species.

2 Materials and methods

2.1 Species occurrence data acquisition, database reliability assessment and filtering

We collected empirical data for *M. fasciculata* occurrence from the following databases: (a) speciesLink biodiversity database (<http://splink.org.br/>); (b) Global Biodiversity Information Facility biodiversity database (GBIF, 2023; DOI: 10.15468/dl.gvxe6y); and a database resulted from recent fieldwork (Almeida, 2022). After an initial screening, we excluded records containing inaccurate coordinates (e.g. lacking numbers and signals), dubious positions (e.g. records over water/ocean) and repeated records with the same coordinates for the target species (database available in Supplementary Material 1).

2.2 Climate variables and climate change scenarios

The recent recommendations of IPCC report (IPCC-AR6, 2021) consolidated the Coupled Model Intercomparison Project (CMIP6) for as Global Circulation Models (GCM), which enhanced the accuracy of climate effect estimates, with substantial changes in emission trends and spatial distributions (Hausfather, 2019; McBride et al., 2021). Updated climatic variables, which have a direct effect on terrestrial species, suggest that climate suitability models for species must be either renewed or newly proposed to support accurate decisions (McBride et al., 2021; Schramek, 2021).

We retrieved three sets of digital layers representing 19 bioclimatic variables and altitude from Worldclim (Fick and Hijmans, 2017). These bioclimatic variables geographically express averages, seasonality, and extremes of precipitation and temperature, and combinations thereof. The first set is historical variables, representing interpolated empiric climatic conditions of the period 1970–2000. The other two sets are the most recent (CMIP6) Global Circulation Models (GCM) projections generated for future climate scenarios released by the Japanese Agency for Marine-Earth Science and Technology (GCM named MIROC6) according to the SSP585 scenarios. Each set represents the climate estimated for two upcoming periods, from 2021 to 2040 and from 2041 to 2060. Although they are represented as periods, these layers reflect the outcome of each scenario to be achieved at the end of each period. For practical purposes, the scenario periods will be referred to as the last year of each respective period: 2040 and 2060. Since *M. fasciculata* occurs only in the Brazilian territory, all the environmental variables were cropped for the Brazilian administrative extent (IBGE, 2022), and

were used at the same geographical extension (Brazil) and high resolution (2.5 arc minutes).

Among the Shared Socioeconomic Pathways (SSP), the 5-8.5 is one of the most intensive global greenhouse gas emission trajectories estimated by the AR6 (IPCC-AR6, 2021). Although it is not possible to guarantee the consolidation of any scenario as the most likely climate of the future, this seems to be the most appropriate scenario for this study. We argue that more intense effects improves the approach's discriminative capacity by detecting regions whose species will be under lower and higher climatic pressure. Additionally, future projections aiming to prioritize regions for monitoring and to define conservation areas must observe the worst possible scenario, so that the actions planned to safeguard species will be efficient in the future consolidation of any possible scenarios, including the pessimistic one. The set of 19 bioclimatic variables (baseline period from 1970 to 2000) was tested aiming to exclude variables with highest correlation between each other, which could compromise the predictive quality of the models from regression algorithms due to effect of multicollinearity. For this purpose, we applied the Variance Inflation Factor Analysis (*vifcor* function) from R package's *usdm* (Naimi et al., 2014). Thereafter, we used the selected set of variables for the modeling procedure (see [Supplementary Material 2](#)).

2.3 Data analysis

2.3.1 SDM for baseline and future climate scenarios

To map the geographical shape of species climatic suitability for the species in the baseline conditions (1970–2000), we used a habitat suitability approach (Hirzel and Le Lay, 2008) by means of the SDM tool *Biomod2* package version 3.5.1 (Thuiller et al., 2021) in R platform (R Core Team, 2021). We selected five *Biomod2* algorithms: GLM – Generalized Linear Model (package *glm*: Hastie and Pregibon, 1992); GBM – Generalized Boosting Model (package *gbm*: Greenwell et al., 2020); GAM – Generalized Additive Model (package *mgcv*: Hastie and Tibshirani, 1990); RF – Random Forest (package *randomForest*: Breiman, 2001); MAXENT – Maximum Entropy (package *maxent*: Phillips et al., 2021). We chose these algorithms based on the results of Aguirre-Gutiérrez et al. (2013) and Acosta et al. (2016), who showed high performance for similar applications. We followed the parameterization of the algorithm as recommended by the authors (Guisan and Thuiller, 2005; Phillips et al., 2006; Thuiller et al., 2009; Thuiller et al., 2021).

In addition to the species presences, the modeling approach also requires data on absences. However, this type of data is rarely available, and for the targeted species, there is no confirmed

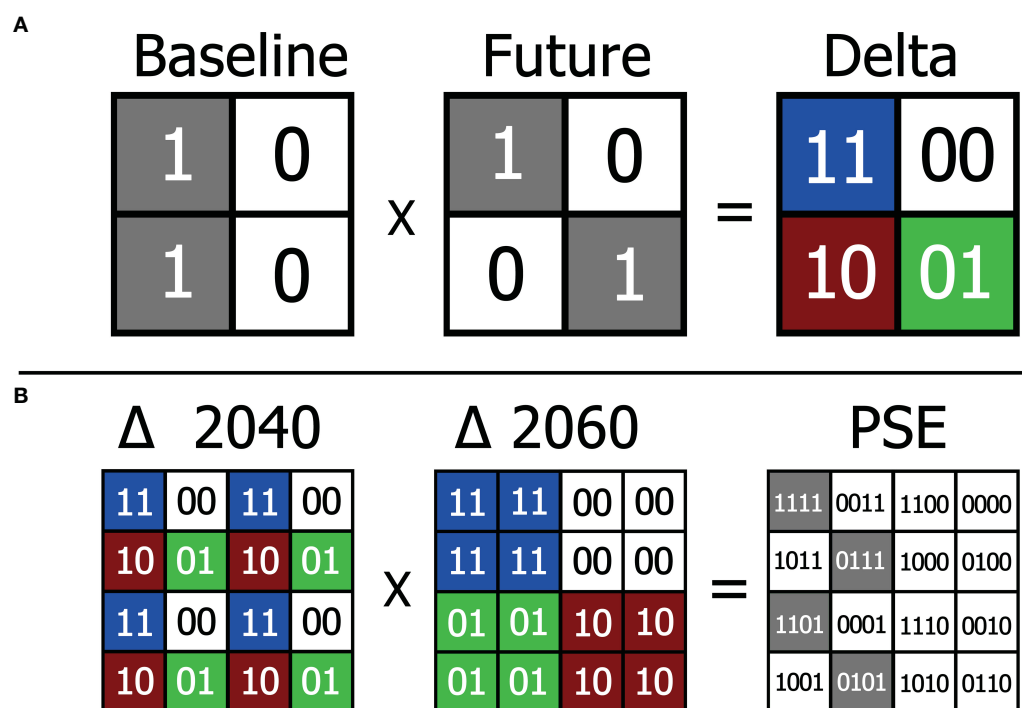


FIGURE 1

The upper part of the figure (section A) shows raster fragments of four cells from baseline and future EFMs, presenting values = 1 for areas suitable for the species and = 0 for areas unsuitable. Overlapping the baseline with the future fragment (e.g. 2040) we obtained the Delta Model, therefore the sum of the binary values generate four possible values: 11 for locations that will remain climatically suitable; 00 for locations that will remain unsuitable; 01 for unsuitable locations that will gain suitability and; 10 for locations that will lose suitability. At the bottom (section B) we have an example with 16 cells showing all possibilities of crossing between Deltas (2040 x 2060), obtaining a single combined result. For our study, only cells with suitability in both future scenarios (green and blue) were considered safe for long-term maintenance of the species, hence the extension we called persistent suitable zone (PSE; gray cells).

geographic statement of absences. To fulfill this demand, five pseudo-absences datasets with 10 times the number of presences (based in [Chefaoui and Lobo, 2008](#); for better predictive performance in similar modeling circumstances) were randomly generated outside the predefined geographic exclusion zones (20 km buffer radius) around the presences. These zones represent twice the distance species individuals could travel from their colonies throughout their lifetime ([Nunes-Silva et al., 2020](#)), and thus no pseudo-absences were assigned within them.

At each round of modeling, the set of presence points was randomly divided into 80% dedicated to model training, and 20% to evaluate the predictive quality by true skill statistics (TSS; [Allouche et al., 2006](#)). This random selection was replaced at each modeling run (RUN), in the same way that the pseudo absence datasets were also randomly replaced per round (PA). We then generated 125 models for *M. fasciculata* in the baseline climate scenario. This amount results from the combination of five algorithms, five randomly generated pseudo-absence datasets, and five random partitioning of the species presence datasets ($5 \times 5 \times 5 = 125$).

In the modeling framework, we only maintained the models with best predictive performance per species, which were evaluated by TSSs > 0.8 (evaluation data and selection in [Supplementary Material 3](#)). These selected models were used to build a baseline ensemble forecast model (BEFM) based on the Committee Averaging method. This method was defined as a conversion of probabilistic predictions from single models into binary predictions using a threshold maximizing specificity and sensitivity, and then averaging them ([Wisz et al., 2008](#); [Hao et al., 2019](#); [Thuiller et al., 2021](#)). The same set of mathematical models used to generate the BEFM, were also projected for future scenarios using Biomod2 projection function. Subsequently, each ensemble forecast model (baseline, 2040 and 2060) were reclassified as follows: zones showing suitability values $\geq 75\%$ were converted into 1. The remaining areas, with < 0.75%, were converted into zero, meaning unsuitable for the species. The reason for classifying the continuous models into binary values is to geographically circumscribe four types of areas: A) currently climatically suitable area and that will remain suitable in the future ([Figure 1A](#); Delta blue cell=11); B) currently climatically suitable area that will become unsuitable in the future ([Figure 1A](#); Delta red cell=10); C) currently unsuitable area that will become climatically suitable in the future ([Figure 1A](#); Delta green cell=01) and; D) areas always unsuitable to the species ([Figure 1A](#); Delta white cell=00). For this, we used a geospatial overlay strategy of binary models to detect these four areas (see [Figure 1A](#)). After generating the delta models, which compare the baseline and each future scenario individually, we compared the both deltas generated by concatenating side-by-side binary values ([Figure 1B](#)). Only cells matching suitability in both future scenarios were determined as Persistent Suitable Extent (PSE; [Figure 1B](#)). Although areas showing loss of suitability may be of interest for wildlife monitoring, rescue and management, for our approach (oriented to long-term ecological and genetic studies) these areas were not targeted. Likewise, zones that maintained or gained suitability from the baseline to a single future scenario, but not in both futures, were also not considered.

2.3.2 Partitioning of persistent suitable extent using GPCA as a zoning approach

For better spatial discrimination of the zone with variable intensity (higher to lower) of climate change within the environmental range still suitable for the occurrence of the species today and in the both future scenarios (PSE), we divided it into 10 inner belts based on the current variability of climatic conditions. For that, we used Principal Component Analysis as an approach for the geographical zoning. Since our approach is a geographic space-oriented PCA, we refer to this analysis as Geospatial PCA; however, we emphasize that we are not dealing with Generalized PCA, but Geospatial PCA (GPCA). This approach has demonstrated to be very accessible, as it is present at almost all free geographic information system and programming platforms, and ideal for our purposes, as it can reduce the dimensionality of large data sets, increasing interpretability while minimizing the loss of information. This technique assesses multiple variables simultaneously and creates other variables, either reducing or nullifying the level of correlation among them and successively maximizing the variance, which is an aspect of great interest for this study ([Gavioli et al., 2016](#); [Jolliffe and Cadima, 2016](#)).

This zoning approach aims to show climate variability along the extension of the persistent suitability area, aggregating into belts the climatically most similar areas in terms of climatic variation based on the thermal and precipitation extremes and averages. Thus, we will maximize the variability convergence within each belt and, simultaneously, maximize the variability divergence between each belt. Therefore, the climatic variability within belt 1 will be quite different from that found in belt 10, and although belt 1 differs from belt 2, it does so to a lesser level than in belt 10. This 10-zone gradient allows us to look at persistent suitability areas as ten climatically particular zones, and to compare them we applied the GPA.

As axes for the GPCA, we used a set of six variables in current condition from Worldclim ([Fick and Hijmans, 2017](#)), the ones that better represent the centrality and extremes of the thermal and rainfall amplitudes into the persistent suitability areas. They are Annual Mean Temperature (Bio 1); Maximum Temperature of Warmest Month (Bio 5); Minimum Temperature of Coldest Month (Bio 6); Annual Precipitation (Bio 12); Precipitation of Wettest Month (Bio 13); Precipitation of Driest Month (Bio 14).

Here, to determine the belts, we exclusively selected the eigenvalues from the first PCA axis (into GPCA), which often explain more than 50% of the total variance. The range of eigenvalues variation was normalized from 0 to 1, and subsequently, the value was partitioned into deciles, so that these ten ranges could represent spatial clippings that we call the GPCA Zones as PCAZ. Subsequently, we plotted and enumerated these PCAZ (from 1 to 10) for posterior identification. Therefore, the PCAZ 1 will always be the most divergent in variance with respect to the opposite extreme, the PCAZ 10, which does not necessarily imply linearity. The relevant climatic differences among PCAZ were later depicted in the GPA. We performed the Geospatial PCA (GPCA) approach by using the R software with the function *rasterPCA* from package RStoolbox v. 0.3.0 ([Leutner, 2022](#)). After the GPCA zoning, the climatic values from all respective six variables projected for both future scenarios (2040 and 2060) were also extracted to be further used in the Generalized Procrustean Analysis.

2.3.3 Concordance and variability of bioclimatic variables and zones based on Generalized Procrustes Analysis

We performed the Generalized Procrustean Analyses (GPA; Gower, 1975; Dijksterhuis and Gower, 1991) to assess the positioning, displacement (trajectories) and variability of bioclimatic factors (above mentioned), relating and comparing the three scenarios (baseline, 2040 and 2060), considering exclusively and individually the ten geospatially compartmentalized PCAZ into the persistent suitability areas.

GPA is particularly relevant for investigating the concordance among a set of matrices having as a baseline a consensus configuration projected in a multidimensional space, and it allows calculating the level of within-variance of each assessed item (Gower, 1975; Dijksterhuis and Gower, 1991). GPA was performed with the 'GPA' function in the package *FactoMineR* (Lê et al., 2008). The tolerance level for solution convergence was set to 100^{-10} and the number of iterations was set to 199. Then, we applied a permutation test with 99 permutations by means of the "GPA.test" function of the package *RVAideMemoire* (Hervé, 2020) to test whether both the bioclimatic variables and the zones were

not positioned randomly in the GPA multidimensional spaces (Wakeling et al., 1992; Xiong et al., 2008a; Xiong et al., 2008b). Such a permutation test provides an observed R_c value that can be interpreted as the proportion of the original variance explained by the consensus configuration significantly higher than 95% of the results that are obtained when permuting the data (Xiong et al., 2008b).

We used two output metrics of the GPA, the bioclimatic similarity between future periods with the baseline scenario, and the size of the residuals (within-variance) of the bioclimatic variables and PCAZ. In the first case, similarity was extracted from the 'Residual variance' (RV) coefficients resulting from GPA outputs (Lê et al., 2008). RV coefficient varies from 0 to 1, values close to 1 would indicate scenarios more similar each other, while values closer to 0 would indicate higher discrepancy between scenarios. GPA output also generates the size of residual for each item investigated by minimizing the residual sum of squares between points and its underlying centroids corresponding n dimensions (Lisboa et al., 2014). Thus, the higher the residuals, the more the elements differ in terms of the consensus configuration. In fact, this suggests a poor fit and, consequently, a

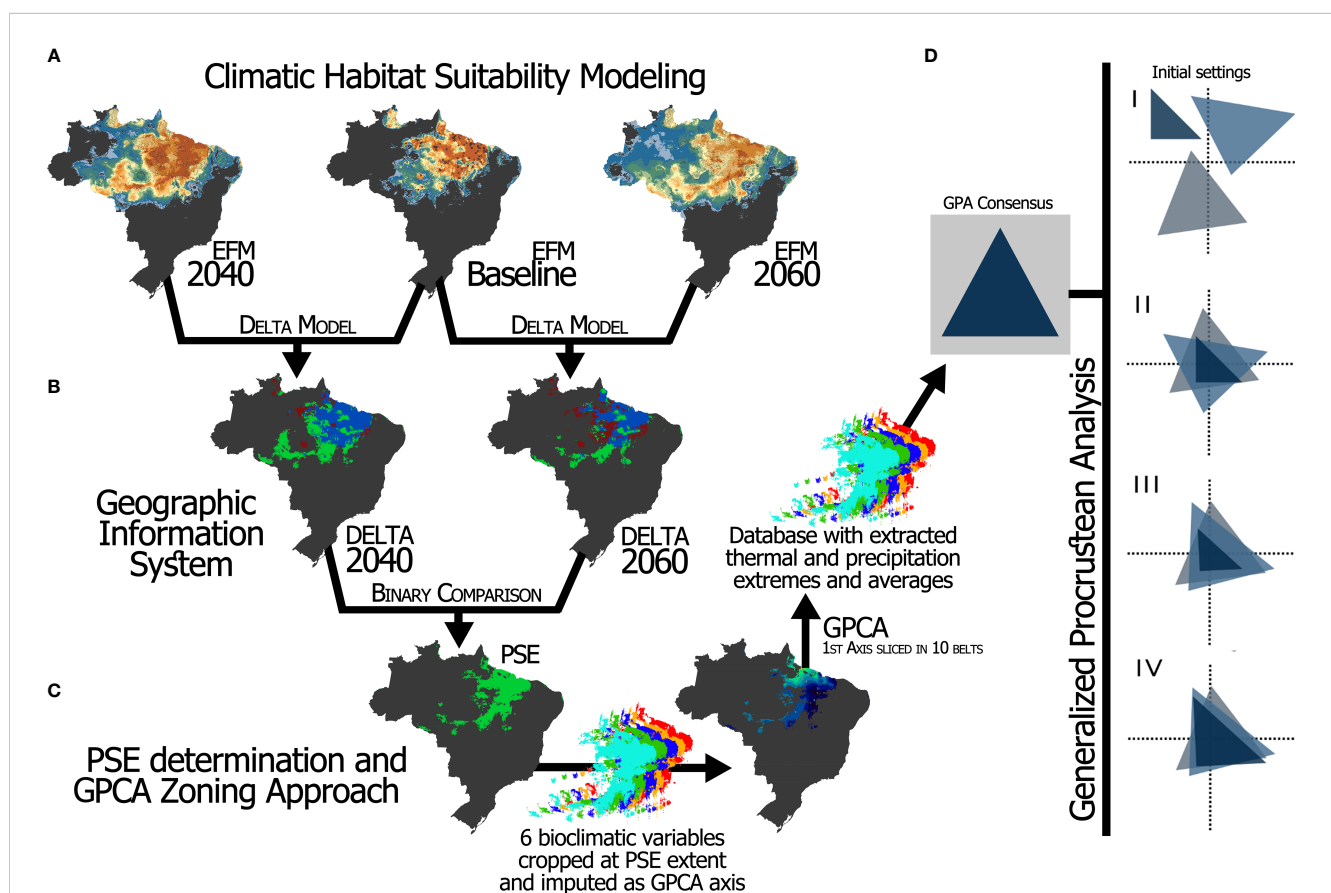


FIGURE 2

Main steps showing environmental layer extraction, how datasets from different period are overlapped and how Procrustes superimposition analysis is conducted. (A) Environmental modeling (above) resulting in habitat/climate suitability. (B) Extraction of datasets containing all variables obtained from modeling in (A). (C) Determination of the spatial extent of the PSE zone and application of the GPCA approach using the values of the bioclimatic layers cropped in this respective area as axis. (D) Procrustes superimposition showing a triangle configuration as a model: (I) original configurations, (II) being translated to superimpose their centroids (III) rotating and reflecting their corresponding vertices (tips) to the same position, (IV) and scaling them to maximize their coincidence by minimizing the sum of the squared deviations.

higher underlying variance that can be interpreted as having higher within-discrepancies (Lisboa et al., 2014). As result, we labeled the bioclimatic variables and/or zones as more or less congruent according to how concordant (small or negligible residuals) or discordant (greater residuals) they were following the GPA. That is, one might expect a higher ecological pressure on the populations of *M. fasciculata* if there are large discrepancies among all bioclimatic variables within the zones, which may expose the analyzed species to an increased climatic pressure. The Figure 2 presents the main steps of the framework, so that the methodological structure can be visually understood.

3 Results

3.1 Species distribution models and GPCA zoning of persistent suitable extent

We obtained 134 unique records of *Melipona fasciculata* to run species distribution models after the data filtering (Database available at [Supplementary Material 1](#)). Among the 19 variables initially considered, 13 were maintained after collinearity evaluation (see Sup. Mat. 2). Out of the 125 computed models, 33 showed high predictive quality (TSS>0.8) and were kept for the ensemble forecasting. Among the algorithms, Random Forest had the best performance, contributing 12 models, then GAM with 7, Maxent and GBM with 6 each, and GLM with only 2 models. (see Sup. Mat. 3 and 4). Some areas of uncertainty regarding suitability prediction were detected in South American countries. However, in the focal area of the study, comprising the administrative extension of Brazil, only a few islands with the lowest level of uncertainty were detected in the northeast (access [Supplementary Material 6](#) for details).

The ensembles were projected on maps (Figure 3) showing the variation of climate suitability for the species according to the three scenarios: baseline and respective projections for both future periods 2021–2040 (referred as 2040) and 2041–2060 (referred as 2060).

After the binary classification, the differences in suitability between baseline and future scenarios can be identified in the delta maps (Future minus Baseline EFM; Figure 4). We geospatially compared the delta maps and kept only the common areas that will remain (from baseline) suitable in all future periods. Thus, the blue areas of both maps (Figure 4) that overlap in space, as well as the gain zones in 2040 that remain suitable until 2060, were cropped, combined and mapped (Figure 5), composing the persistent suitable extent (PSE) displayed in Figure 5. The colored bands on this extent display the PCAZ, whose zones range in colors from dark blue (PCAZ zone 1) to yellow (PCAZ zone 10). Our PCAZ showed a pattern of undulating belts, cutting the PSE into ten irradiating zones from southeast to northwest. The map shows some smaller and spatially more restricted belts (PCAZ zone 9 and 10), and others quite extensive (PCAZ zone 4 and 3), and still others fragmented and with isolated islands (PCAZ zone 1 and 7). GPA applied to this layer enabled to calculate the intensities and trends of climate change from baseline to future per PCAZ.

3.2 Similarity among scenarios based on the variability of bioclimatic variables

We observed a significant projection of the bioclimatic variables through the multidimensional space of GPA (Permutation test; $R_c = 99.79$, p -value < 0.001; Figures 6A, B). The GPA model had an excellent fit (94.7%; Table 1), suggesting the occurrence of a common structure underlying how bioclimatic variables were projected among the different scenarios. Furthermore, the first two Procrustes dimensions accounted for 71.88% of the variation (dimension 1: 58.46%; dimension 2: 13.42%; respectively X and Y axis for Baseline, 2040 and 2060 frames in Figure 6C). This suggests that the positioning of these variables was different from the centroid and other individual configurations indicating that each bioclimatic variable could be properly distinguished according to its behavior in the multidimensional space. Our analysis reveals that

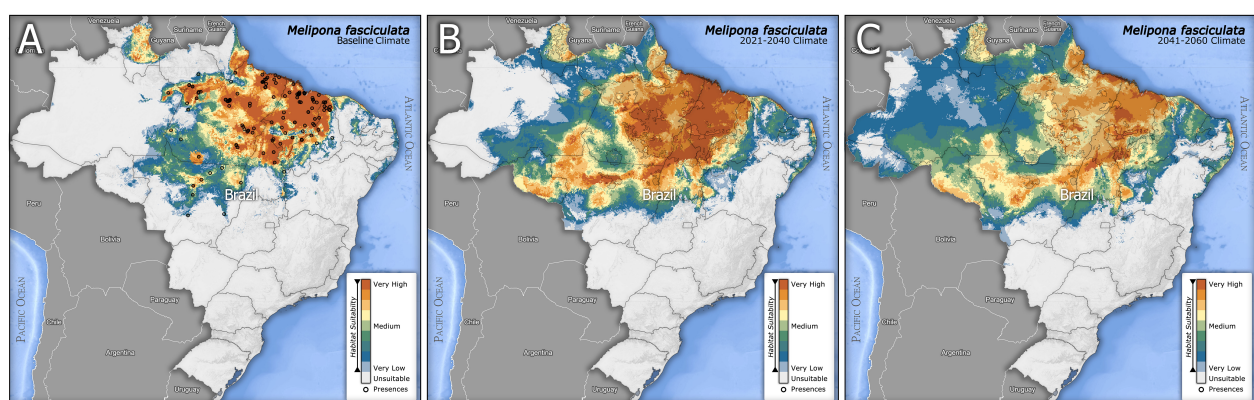


FIGURE 3

Ensemble models showing the climatic suitability at continuous values for *Melipona fasciculata* in Baseline (A), Future 2021–2040 (B) and Future 2041–2060 scenarios (C). The black dots in (A) represent the presence records of *M. fasciculata*. The hatched polygon over (B, C) (better visualized in the high-resolution figures) represent the extent of the baseline climatic suitability in a binary classification (Suitability ≥ 0.75 to 1 reclassified to Bin=1), aiming at an easier comparison between baseline and future scenarios.

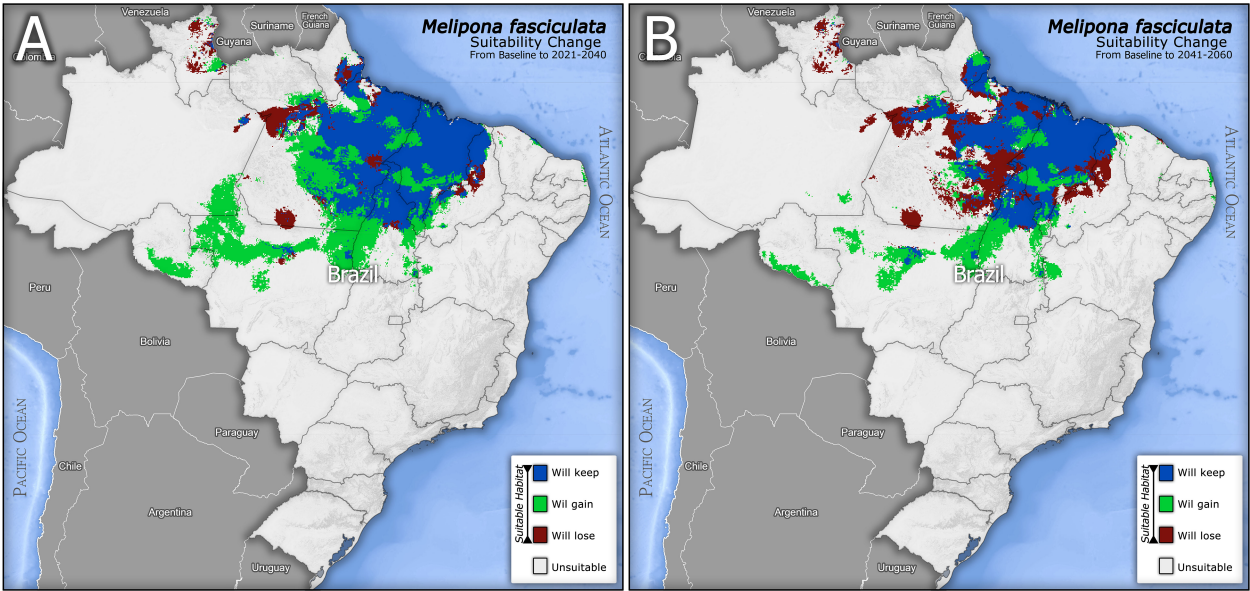


FIGURE 4
Differences between baseline and future climate suitability projections for *Melipona fasciculata* for the two future predictions: 2021-2040 (A) and 2041-2060 (B). The white and light tones represent the unsuitable areas for the species in Brazil; red represents previously suitable areas that will be lost; green represents climate suitable gained areas; and blue represents the areas that are currently climate suitable and will be kept in future scenarios.

nearly all bioclimatic variables were relevant with respect to the amount of explained variation for the consensus configuration (Table 1). Overall, our findings indicate that climatic similarity between both Baseline and 2040 scenarios will achieve 78% (Table 1 and 2040 frame in Figure 6B) within the next two decades, while the

similarity between the baseline and 2060 scenarios is assumed to be more discordant from baseline with 64% (Table 1 and 2060 frame in Figure 6B).

The main variables contributing to this climate disparity appear to be Bio1 (annual mean temperature) and Bio12 (annual

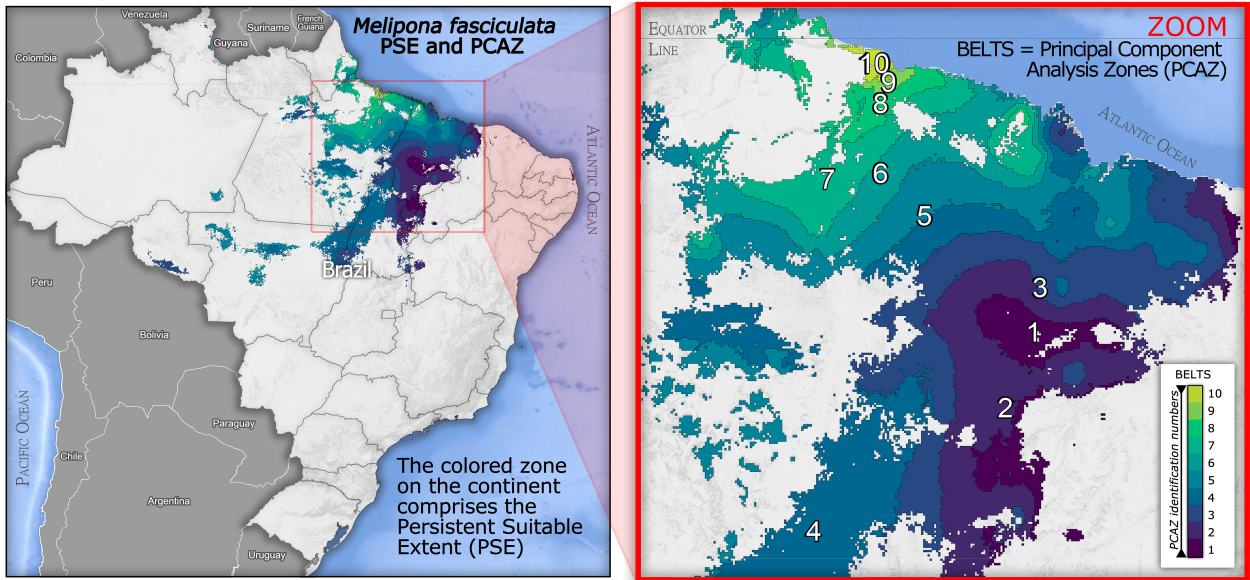
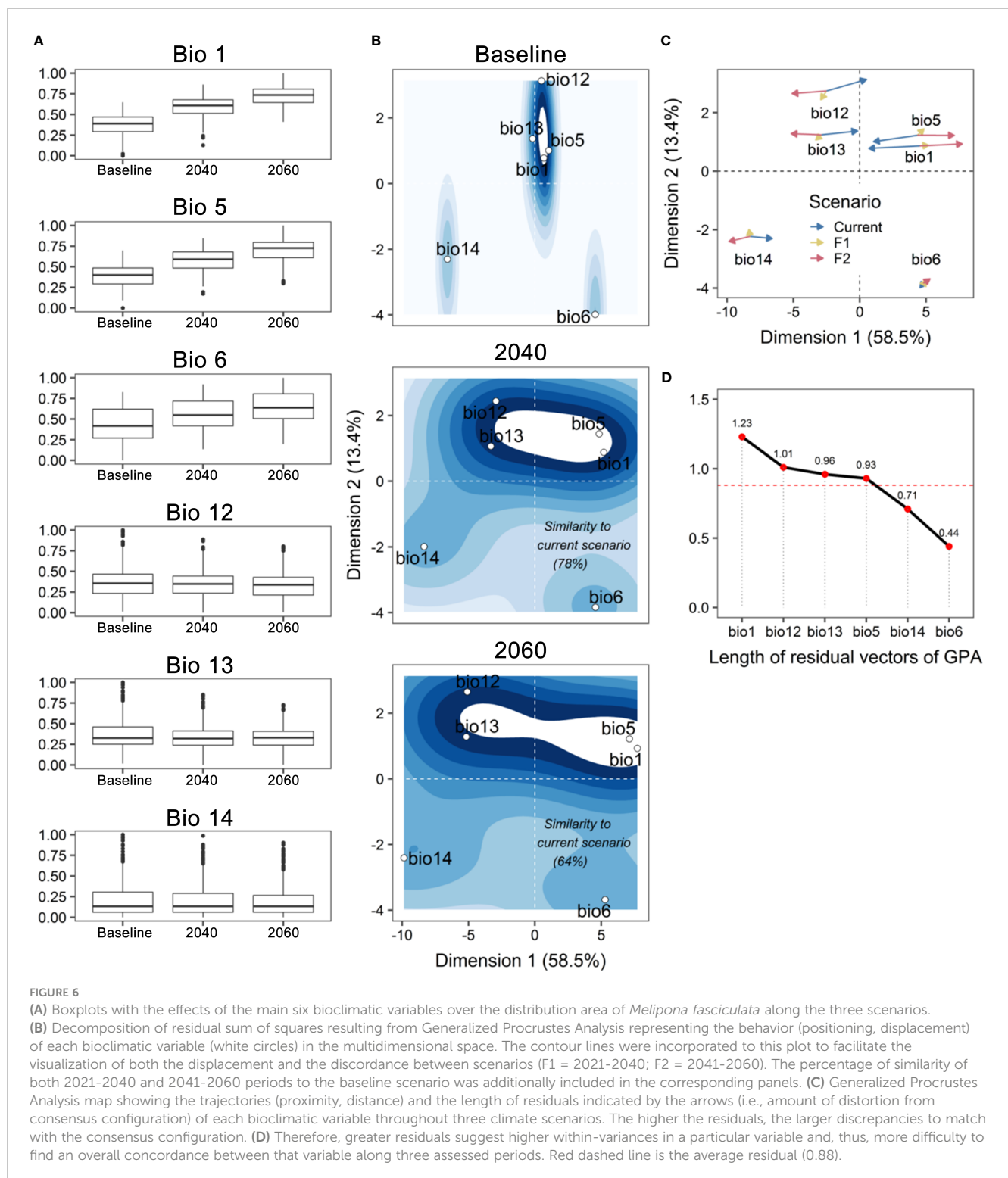


FIGURE 5
The total extent of the colored area in Brazil represents the Persistent Suitable Extent (PSE) for *Melipona fasciculata* considering all scenarios. The map also shows the ten PCAZ belts (ranging from green to blue) that spatially express the climatic variability over the area, whose variation was analyzed in detail through GPA.



precipitation), with the highest residual vector being observed in Bio1 (1.23; Figure 6D) followed by Bio12 (1.01; Figure 6D) among the six bioclimatic variables (Table 1). This means that it has a greater effect size, i.e., an elevated intra-variance resulting in a low concordance among three evaluated scenarios. Thus, it somehow suggests that Bio1 will likely have a more pervasive shift over the distribution area of *M. fasciculata* in the next decades. The Bio12

closely followed Bio1 in terms of the observed residual variance, having the second most discrepant residual vector (1.01). These results demonstrate that both variables are expected to behave with more uncertainty toward the next decades. Consequently, a rise in annual mean temperatures together with a decrease in annual precipitation might affect more drastically the distribution of *M. fasciculata* in the coming decades.

TABLE 1 Procrustes Analysis of Variance (PANOVA) summarizing the total variability in the bioclimatic variables and the pairwise similarity (in percentage) among periods according to combined variance of all six bioclimatic variables examined.

	Consensus	Residuals	Total variance
Annual Mean Temperature (Bio1)	13.2	1.23	14.4
Max Temperature of Warmest Month (Bio5)	12.4	0.93	13.3
Min Temperature of Coldest Month (Bio6)	16.8	0.44	17.2
Annual Precipitation (Bio12)	11.7	1.00	12.7
Precipitation of Wettest Month (Bio13)	11.0	0.96	11.9
Precipitation of Driest Month (Bio14)	29.7	0.71	30.5
Sum	94.7	5.27	100
RV – % of similarity	Baseline	2021-2040	2041-2060
Baseline	1		
2021-2040	0.78	1	
2041-2060	0.64	0.98	1

3.3 Candidate zones for further investigation

As we plot the scaled values of bioclimatic zones, we can overall observe a similar pattern in its within-variance. However, the PCAZ 9 and 10 seem to behave slightly different (Figures 7A, B; Table 2), being GPA robust enough to corroborate this. As such, there was a significant effect of the PCAZ along multidimensional space of GPA (Permutation test; $R_c = 99.93$, $p\text{-value} < 0.001$), with a near perfect fitting of the GPA model (99.9%). This indicates a shared zoning structure being projected along the different scenarios. Nevertheless, the first two Procrustes dimensions accounted for only 30.15% of the observed variation (dimension 1: 17.2%; dimension 2: 12.9%; Table 2, Figure 7B). This low explanatory value represented by the position of the climatic zones (located next to the center of configuration) reveals that adjacent zones were more like each other than expected (Figure 7A). As such, its behavior along the multidimensional space becomes harder to discriminate. However, at least three geographical regions emerge as good candidates for future ecological and molecular investigations, namely the geographical regions covered by PCAZ 9 and 10, in order to contrast their populations with each other, as well as from them to the populations in the remaining aggregated PCAZs, especially by comparing PCAZ 9 and 10 with those located in the more distant PCAZ, 1 to 4 (Table 2, Figure 7A). These three regions were positioned far from each other (Figure 7B) with higher positive values in the first two dimensions analyzed. Furthermore, they presented larger residues that can be observed by their values in Table 1 and the size of the arrows (Figures 7B, C). Although the

small overall climate variability between the 10 PCAZ, especially when comparing adjacent PCAZ (Figure 7D), the process of discriminating the variable climate effects in the persistent suitability area (PSE) was efficient in supporting long-term sampling prioritizations.

4 Discussion

Our spatially explicitly results indicate important changes in habitat suitability for the species *Melipona fasciculata*, highlighting zones of gain, persistence, and loss of suitability. These findings provide support to the determination of conservation planning and population management actions to safeguard genetic variability of this species in the face of climate change. The areas of loss can guide monitoring actions and potential population translocation strategies from regions severely impacted by climate change. The areas of suitability persistence will be essential for the conservation of local populations and, depending on prior ecological assessments, may receive managed populations from areas of loss. Conversely, for the areas of suitability gain, it cannot be assured that they will effectively act as climatic refuges, as the timeframe of the results may be too short for a natural dispersal process on a broad geographical scale. However, they may also be of interest for assisted management, but this requires a very cautious prior assessment to avoid impacts on the species that already inhabit the sites, particularly other bees in terms of negative interactions, which could induce competition for resources with the risk of species exclusion and/or spread/spillover of diseases between specimens and species.

Previous studies already focused in the impact of climate change on the distribution of Brazilian stingless bees (Meliponini) (e.g., Giannini et al., 2012), showing detrimental losses of suitable habitat in the future. Other works highlighted the relationships with plants used by the bees (Giannini et al., 2013), and crop production (Giannini et al., 2017). Studies conducted in a preserved area of the eastern Amazon, in the Carajás National Forest, however, highlighted the extent of the problem, examining the impact of climate change on 216 bee species collected in the area: 85% of them would not find survival conditions in this forest by 2050-2070. Other work presented data on the climatic impacts on *Melipona* bee species used in Brazilian meliponiculture (Lima and Marchioro, 2021). This work highlights the future distribution areas for each species, with gains and losses depending on the species considered. In Colombia, ten Meliponini species were analyzed, considering the genera *Melipona*, *Paratrigona*, *Plebeia*, *Frieseomelitta*, and *Scaura* (Gonzalez et al., 2021). For most of them, the evidence indicated habitat loss in the coming years, with habitat gains for only two species. Also in the dry areas of Brazil, other analyses estimated the impact of climate change for *Plebeia flavocincta* (Maia et al., 2020), a small stingless bees largely used for meliponiculture.

By using our methodological framework, we were able to reveal that changes of climate variability are concordant throughout three scenarios. In short, while the three temperature variables tend to increase, the three precipitation variables seem to decrease. By integrating these results with the Procrustes analysis, it is possible to observe an expansion in the potential distribution area considering the future scenarios. This tendency was likely due to

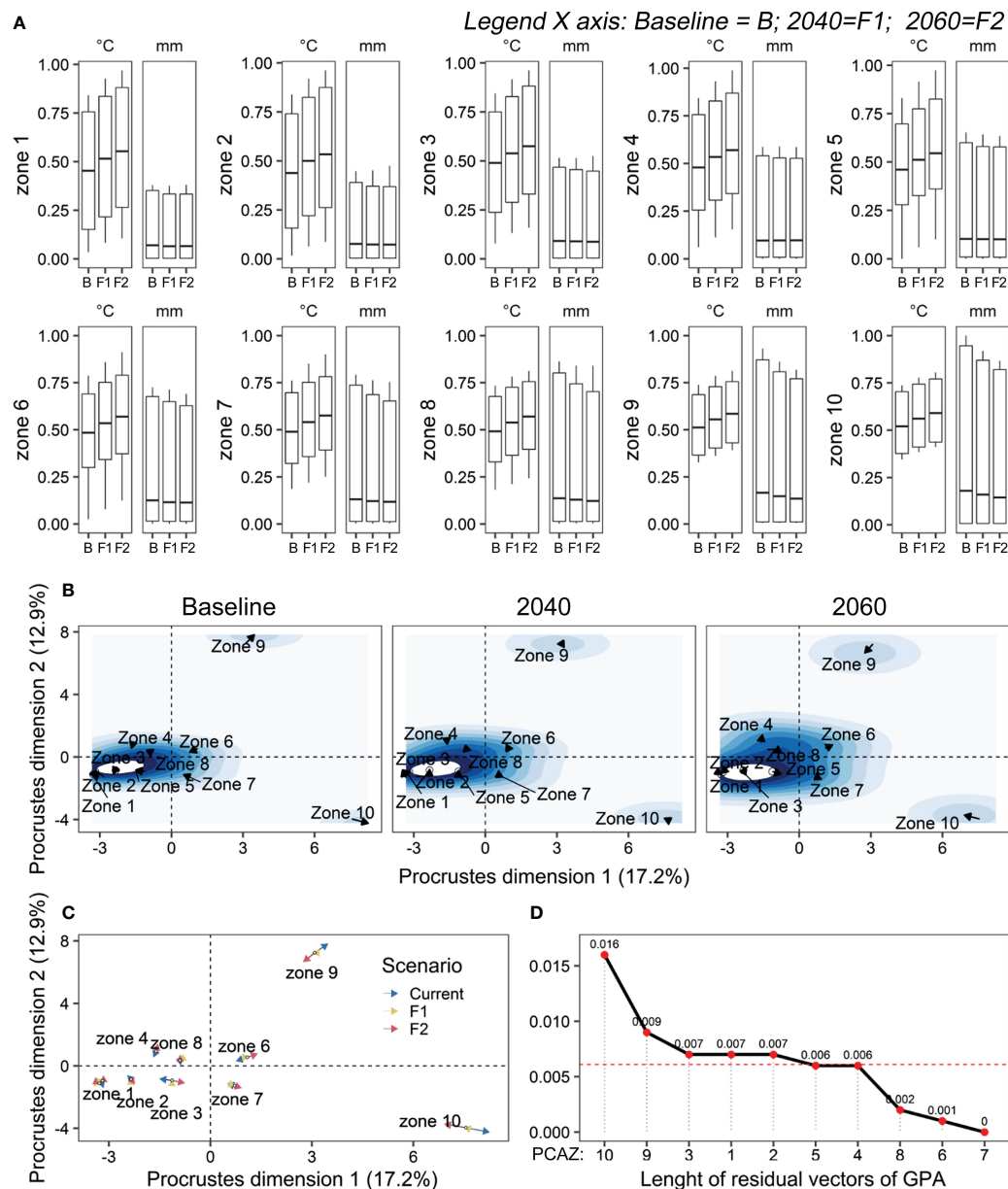


FIGURE 7

(A) Variation of temperature (°C) and precipitation (mm) along three periods (C = baseline; F1 = 2021–2040; F2 = 2041–2060) over 10 zones (PCAZ) extracted from GPCA in the distribution area of *Melipona fasciculata*. (B) Decomposition of residual sum of squares resulting from Generalized Procrustes Analysis representing the behavior (positioning, displacement) of each zone (black arrows) in the multidimensional space. The contour lines were incorporated to this plot to facilitate the visualization of proximity and distance and how discordant were each scenario from each other. (C) Generalized Procrustes Analysis map showing the trajectories (proximity, distance) and length of residuals were indicated by the arrows (i.e., amount of distortion from consensus configuration) of each zone throughout the three climate scenarios. The higher the residuals, the larger discrepancies to the consensus configuration. (D) Therefore, greater residuals suggest higher within-variances in a particular zone and, thus, more difficulty to find an overall concordance between that zone along three assessed periods. Red dashed line is the average residual (0.0061).

the position of all variables that progressively (from baseline to 2040 and 2060) move away from consensus configuration over the multidimensional space of Procrustes. These displacements of variables probably cause the observed disparity between 2040 and 2060 scenarios to the baseline climate. As a result, it should be expected a significant climate inconsistency within the geographic range of *M. fasciculata* in the next two decades baseline with an expected climate incompatibility of up to 22%. In fact, such

mismatching might be still higher, achieving 36% of incompatibility within the next four decades.

The size of residuals was calculated by evaluating the difference between the location of the items of each configuration and the position of the item in the consensus configuration being, therefore, a good predictor of within-group variance (Gower, 1975; Ten Berge, 1977; Dijkstra and Gower, 1991; Goodall, 1991; Risvik et al., 1994). Here, the variance of each environmental variable provides a

TABLE 2 Procrustes Analysis of Variance (PANOVA) summarizing the total variability in the PCAZ after Generalized Procrustes Analysis transformations per configuration and the pairwise similarity (in percentage) among periods according to combined variance of all ten bioclimatic zones evaluated (zones according [Figure 5](#)).

PCAZ	Consensus	Residuals	Total variance
Zone 1	8.9	0.007	8.9
Zone 2	9.1	0.007	9.1
Zone 3	8.9	0.007	8.9
Zone 4	8.9	0.006	9.0
Zone 5	9.7	0.006	9.7
Zone 6	9.1	0.001	9.1
Zone 7	9.3	0.000	9.3
Zone 8	10.3	0.002	10.3
Zone 9	11.8	0.009	11.8
Zone 10	13.8	0.016	13.8
sum	99.9	0.061	100
RV – % of similarity	Baseline	2021-2040	2041-2060
Baseline	1		
2021-2040	0.99	1	
2041-2060	0.99	0.99	1

measure of deviation from the average scenario or GPCA Zones (referred as PCAZ). Consequently, it is possible to select the main zones according to the size of their residuals, which means that they likely have a larger effect on the different climatic scenarios.

The 10 zones partitioned within the persistent suitability area for *M. fasciculata* among the three scenarios presented varying levels of deviations from the consensus configuration, with PCAZ 9 and 10 standing out as better candidates for further surveying and/or monitoring. Both zones are located further northwest of the suitable habitat for *M. fasciculata*. Nevertheless, despite being adjacent to each other, zone 10 had the highest variance, with nearly twice the variance observed in zone 9. The comparison of these two PCAZ with the others, especially with the more distant ones (1-4), also proves to be an important prioritization to evaluate the differential climate effect between populations in the long term. Even though these three geographic regions (covering 9, 10 and 1-4 PCAZ) are within the appropriate climatic limits for the species until 2060, climate change will clearly generate effects of different intensities on their populations, which will possibly respond differently.

The final maps provided by our framework are easy to interpret, which allows estimating which changes might be expected with respect to the geographic range of target organisms over time assuming different climate change scenarios. Nevertheless, a more robust comparison among scenarios may be restrained by the lack of available data. Here we show that the Procrustes analysis was

successful in accurately describing and quantifying the concordance among the evaluated scenarios and zones.

The use of more recent climate suitability models integrated with the Procrustes analysis was able to map and accurately describe the level of change among scenarios on the geographic distribution area of the studied species, highlighting distortions among bioclimatic variables of interest and pointing out areas of interest for decision-makers. Our framework also identified the environmental variables with higher relative importance among scenarios according to the size of their residuals. Accordingly, some zones along the distribution area of the target species can be prioritized for permanent monitoring of impacts on the species due to the expected higher change. Furthermore, some other zones, in which the climate change will be likely less pronounced, can be selected to act as a potential climate refuge. These areas could be prioritized to their conservation to sustain sanctuaries where the target species might continue to thrive.

Global conventions and reports continually issue warnings to assess and conserve biodiversity in the face of climate change. However, for such demands to be effectively met, the scientific community needs to develop operationalization strategies and orientation in more intuitive and simplified results to effectively convey the message to a wider audience. Research whose outcomes are presented in a complex way, requiring extensive scientific experience to be understood and interpreted, make it difficult to access fundamental information for decision-making at crucial moments, when scientific knowledge must be guided to support immediate action.

Analysis that generates spatially explicit results, especially presented through maps, have been an efficient support to decision making. With such support, they can point out where, when, and how they should act aiming reliable conservation and restoration plans. Besides these maps, the measure that quantifies percentages of change among baseline and future scenarios and the level of variance (degree of deviation) among variables of interest, may reinforce the evidence being sustained along studies of species distribution modeling and climate change modeling.

The combined use of Species Distribution Modeling (SDM) and Generalized Procrustes Analysis (GPA) provided a comprehensive approach for pollinator biomonitoring programs, such as bees, in the face of contemporary environmental challenges like climate change and biodiversity loss. SDM is essential for assessing the current and future climatic suitability of species, identifying critical areas that require conservation and restoration interventions. Combining SDM with GIS (Geographic Information System) offers clear visual representations of species distribution changes under different climate scenarios. In contrast, GPA allows for the assessment of concordance between climate scenarios and the identification of zones with significant variations. The integration of these analytical approaches deepens our understanding of climate change impacts on pollinators, prioritizes areas for conservation, sustainable use and research, and facilitates scientific communication, making information accessible for conservation decisions and nature-based solutions. We also suggest the need to identify areas for continuous monitoring of bee populations, following the considerations of [Hoban et al. \(2023\)](#)

for biodiversity in general, and our proposed methodology fits this goal.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

AA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Resources, Visualization. CdS: Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. VI-F: Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. RO: Methodology, Visualization, Writing – review & editing, Writing – original draft. TG: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frbee.2024.1329844/full#supplementary-material>

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One for all and all for one: a review on the commonality of risk to honeybees and wild pollinators and the benefits of beekeepers in conservation

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Pollinator declines across the globe are centrally driven by a synergistic interaction between intensive land use, pesticides, and climate change. Competition between managed and wild pollinators has been a growing topic of research, however the ensuing social conflict builds antagonism between beekeepers and conservationists, two parties that have an interest in protecting natural diversity for pollinators. The threats perpetuating this potential for competition are as real for managed bees as wild species and uniting both groups, wherever possible, can create long lasting and meaningful change in current agricultural practices. This review examines the most recent literature on pollinator competition and the common threats that drive it. It also delves into the social elements of beekeeping and examines the potential for beekeepers to contribute to the protection of natural habitats. Beekeepers have a genuine interest to preserve natural space and with their charismatic species, dutiful observations, and innovative techniques, they can be valuable assets in filling knowledge gaps and generating public interest. Pollinator strategies in the future should include beekeepers as key stakeholders if their impacts are to be improved.

KEYWORDS

beekeepers, competition, conservation, honeybees, sustainability, wild pollinators

1 Introduction

Insect pollination is a vital service to both natural ecosystems and humans. At least 30% of the most nutritionally valuable crop yields produced for human consumption require insect pollinators (Klein et al., 2007; Eilers et al., 2011; Garibaldi et al., 2013; Rollin and Garibaldi, 2019). The dependence of human crops on pollination is increasing over time (Aizen et al., 2009) and plant-pollinator networks build a crucial base for the effectiveness of this ecosystem service (Kremen et al., 2004; Dainese et al., 2019). Managed pollinators,

mostly managed honeybees (*Apis mellifera*), are tightly linked to this service in many human-mediated landscapes, now both in their native and non-native ranges (Rollin and Garibaldi, 2019). It has been well documented that pollinators are in global decline and in nearly all cases, the central driver is the intensification of land management (Kevan and Viana, 2003; Potts et al., 2010a; Burkle et al., 2013; Durant and Otto, 2019; Seibold et al., 2019; Dicks et al., 2021).

With increasingly limited natural resources, conflict between managed and wild pollinators becomes inevitable. Recently, there has been a research focus on identifying and assessing the potential for competition between wild pollinators managed bees (Mallinger et al., 2017; Wojcik et al., 2018). Likely this has come, at least partially, as a pushback against the misguided concept that honeybee conservation is a functional equivalent to species-level biodiversity conservation (Wilson et al., 2017; Geldmann and González-Varo, 2018). In their non-native ranges, managed species like honeybees kept at high densities, or large populations of escaped, feral bees can have severe impacts on local wildlife.

Though the risks of competition are evident, the social implications of this divisive stance splits two parties with a vested interest in protecting the same thing, namely, healthy environments for pollinators. The beekeeping industry has grown increasingly vulnerable in many areas due to its reliance on private and public land permissions. Shifting land use and conservation policy has created a more exclusionary environment which beekeepers must now navigate (Durant, 2019). As a result, many beekeepers have turned from small scale wild honey operations to large scale industrial honey or pollination services, that perpetuate unsustainable farming practices and make little room for wild space (Maderson, 2023b). Often, beekeeping cannot break away from agricultural landscapes, which are more exposed to threats that contribute to both pollinator decline and competition, making their presence and their plight more visible (Seibold et al., 2019).

This review explores the most current research on the underlying causes of competition and outlines the commonality between the threats that face both wild pollinators and kept honeybees in the Anthropocene. It also investigates the social elements that define the relationship between these threats and stakeholders and explores the role beekeepers have played as well as their future potential in insect conservation. The data presented here details the essential practicality of pooling the efforts of both beekeepers and conservation groups to improve conditions collectively for all pollinators in human-mediated landscapes.

2 Competition: when does it happen and why?

When species experience niche overlap, there is potential for competition. Exploitative competition decreases the fitness of at least one competitor group due to reduced access to a finite resource (Elton, 1946; Schoener, 1983; Abrams, 2022). The common belief is that a highly social, generalist pollinator like the honeybee, in high enough densities, can reduce the available nectar or pollen for wild pollinator species. This may often remove specialized resources for

an already declining group of insects (Thomson, 2004; Cane and Tepedino, 2017; Wojcik et al., 2018; Rasmussen et al., 2021), or shift plant-pollinator interactions to affect the habitat as a whole (Valido et al., 2019). Honeybees also have the potential to spread pests and disease, and their large, tight-knit colony lifestyles creates opportunity for zoonotic shifts, which could lead to massive outbreaks in wild pollinator communities (Otterstatter and Thomson, 2008; Fürst et al., 2014; Goulson and Hughes, 2015; Mallinger et al., 2017). By human hands, honeybees have been moved to many places where they are not native. High domestic hive densities like those in the Americas (Geslin et al., 2017), as well as released, feral populations like those in Australasia (Prendergast, 2023; Pyke et al., 2023), have the potential to magnify the above-stated issues because of the sheer number of colonies present where they have never been before. Due to the increasing disappearance of key habitats, the ever-shifting use of harmful chemicals, climate change and invasive species, the effect of managed pollinators certainly has the potential to become damaging to local pollinator communities under the right circumstances (Brown and Paxton, 2009; Goulson et al., 2015; Brown et al., 2016; Baldock, 2020; Herrera, 2020; MacInnis et al., 2023).

Much of the current research on pollinator competition extrapolates potential risk from field observations but provides very little direct experimental evidence: The studies actively testing for impacts of honeybee-wild pollinator competition on fitness are surprisingly few, and relegated to handful of species, mostly bumblebees. Two recent reviews (Mallinger et al., 2017; Wojcik et al., 2018) investigated the number of papers on pollinator competition and pathogen spillover. In the field of competition, the first review examined 81 papers and found 19 that met their criteria for “direct impacts” on fitness, presenting experimental evidence and not only observational fluctuations in species abundance and richness based on proximity to honeybee hives or hive density. Of those 19, 10 found evidence of exploitative competition and 9 found no direct evidence. The second review found 38 out of 72 papers reporting negative effects of honeybees on wild pollinator foraging. Of the 27 papers investigating viral transmission, only 2 documented active transmission of viruses from honeybees to wild bees. An updated review (Iwasaki and Hogendoorn, 2022) found that the body of literature is increasing rapidly, growing by 47% since Mallinger et al. (2017), and the reporting of negative effects of competition has increased by about 13%, indicating a disproportionately slow growth of negative findings to the overall growth of the literature body. In short, there is little evidence that shows that the presence of honeybees has direct, negative impact on wild pollinator fitness, and likely, competition does not create easily measurable impacts in every instance of shared land.

Even with moderate rates of positive evidence, competition is inarguably a risk, and every case of honeybee presence must be considered as having the potential to impact wild pollinators negatively. There is much to be said for the precautionary principle in cases where honeybee competition with wild pollinators seems likely (Pyke, 1999). However, shifts in social perspective and the corresponding calls for policy change have been highly focused on mitigating the effects by restricting beekeeper access to often much-needed resources as a blanket

strategy in all cases (Durant, 2019; Matsuzawa and Kohsaka, 2021). Efforts to mitigate the effects of competition may be more successful overall, if the focus was less on the damage from honeybees and more on the conditions under which that damage could occur. The largest underlying threats facing wild pollinators are very much the same threats facing honeybees. In this light, a growing body of research is being produced outlining the tight-knit similarities between the needs, problems, and solutions for all pollinators. Acknowledging beekeepers as fundamental stakeholders in the health of the natural environment plays well on two stages: scientifically beekeepers can offer much in their constant monitoring of a species that lives and thrives on natural biodiversity, and socially, beekeepers, their bees and their industry are charismatic bannermen for campaigns to involve the public in environmental challenges. If meaningful change is to occur for pollinators, all active players must be unified.

3 Common threats (and solutions)

3.1 Habitat loss

Habitat destruction is the most significant cause of biodiversity decline (Caro et al., 2022). Recent maps indicate an area of untouched “wild” land at just 25% across the globe (Allan et al., 2017). Additionally, species made vulnerable by the removal of needed habitat are much more susceptible to other threats like climate change and invasive species (Ganuza et al., 2022). Habitat loss is driven by the changing of land from a natural state to a state that provides food and other resources for human use (Tilman et al., 2017). Agricultural landscapes have historically been interlaced with natural and semi-natural habitats that were suitable for a large diversity of pollinators, but with the rapid increase in human population and general wealth, land is being handed to urbanization, farming practices are becoming more intense and this removes practical habitats that house many species (Shi et al., 2021). It is well-known that wild pollinators can only thrive if a suitable diversity of flowering plants is present in their environment. The issue lies in the area of land that is needed to maintain these natural landscapes. Stakeholders often see a loss of opportunity in natural landscapes, where they could instead be managed as more cropland (Kleijn et al., 2015; Montoya et al., 2020), and if pollinators are required, they can be purchased and fed with supplements (Noordyke and Ellis, 2021). The demand of pollination in today’s landscapes in many areas however, is rising faster than the increase in honeybee colonies (Potts et al., 2010b). Wild pollinators can augment the performance of managed bees and sometimes surpass it (Garibaldi et al., 2013; Monasterolo et al., 2022), and there is now clear evidence that restoring natural diversity in intensely-managed landscapes may be required for honeybees as well. Natural and semi-natural habitats can improve nutritional intake and provide diverse resources during times of food scarcity in predominantly monofloral environments.

3.1.1 Nutrition and stressor resistance

A large body of literature illustrates the link between poor nutritional intake and honeybee susceptibility to other external

stressors (Naug, 2009). Poor nutrition can lead to higher instances of disease (DeGrandi-Hoffman and Chen, 2015; Branchiccela et al., 2019; Dolezal et al., 2019) and a greater susceptibility to environmental toxins (Tosi et al., 2017). Numerous studies now link the loss of natural and semi-natural habitats to poor nutritional health in honeybees: A US study found a strong correlation between the decrease in rangeland (grazed natural grasslands) and honeybee colony losses. States with the highest areas of natural land cover had a higher honey production per hive. This tells us that lands with more diverse resources improved overall colony survival, likely by providing a higher diversity and volume of pollen and nectar (Naug, 2009). Similar patterns were found recently in Canada (Richardson et al., 2023), and more diverse pollen collection, a key for good nutrition, was also linked to natural landscapes in Great Britain (Woodcock et al., 2022), France (Odoux et al., 2012) and Papua New Guinea (Cannizzaro et al., 2022). A wide variety of pollen can even work synergistically to improve honeybee health (Donkersley et al., 2017). Looking at the impacts on individual bees, a higher natural diversity can reduce microbial imbalances (Gorrochategui-Ortega et al., 2022) and improve the production of vitellogenin (Alaux et al., 2017), a protein that has been linked to better toxin processing (Barascou et al., 2021) and is crucial for winter survival in temperate climates (Amdam et al., 2005).

Access to a variety of different pollens plays a large role in many aspects of honeybee health (Di Pasquale et al., 2016), and not all roles are entirely understood, therefore replicating the needed diversity artificially through food substitutes may not serve as a good long term strategy.

In addition to maintaining preexisting diversity, restoring natural diversity in agricultural landscapes can increase the volume of food available: An experiment performed by Zhang et al. (2023) examining prairie strips in an agricultural landscape found that honeybees collected 50% more pollen and colonies were 24% larger at the end of season monitoring. Many of the resources in the strips were left uncollected, meaning there was the potential capacity to provide food for other species. This study offers direct evidence that replacing some natural diversity in highly managed agricultural landscapes can work to reduce nutritional stress in honeybees and possibly reduce competition with wild pollinators.

3.1.2 Temporal availability

Floral diversity in natural habitats can increase the availability of resources like pollen and nectar on a temporal scale too (Mallinger et al., 2016). A study in Western France examining the composition of collected pollen over time revealed that honeybees collected up to 40% of their pollen from weed species growing between the desired crop flowerings (Requier et al., 2015). Honeybees do not often use a high level of diversity at any given point in a season, but the types of resources collected in diverse environments changes significantly over time (Jones et al., 2022). Temporal shifts in floral availability are just as present in tropical climates as temperate (Souza et al., 2018), so consideration of temporal diversity in landscape management planning is as important as area coverage. Limiting this availability exposes honeybee colonies to greater risk of malnutrition or starvation and could increase competition at key points in a season.

3.1.3 Strategies for maximizing floral diversity in agricultural landscapes

There are many strategies being implemented to improve conditions for pollinators, including diverse cropping (planting more than one crop in an area: [Martínez-Núñez et al., 2022](#)), flower strips (narrow lengths of planted flowers in or around cropland: [Scheper et al., 2015](#)) and lower crop seed densities ([Sidemo-Holm et al., 2021](#)). However, it is natural floral diversity that stands out as the most effective resource for wild pollinator and honeybee health:

Studies have shown that though flower strips and semi-natural habitats provide similar resources, the pollinator diversity supported by semi-natural habitats is often superior ([Morandin et al., 2007](#); [Hevia et al., 2021](#); [Hadrava et al., 2022](#)). This means that natural and semi-natural habitats provide better resources for rare species. Choosing seed mixtures can be a complex affair when considering the effects they must have, and often, natural mixtures provide the best nutrition for pollinating species ([Haaland et al., 2011](#)). A combination then, of natural, semi-natural and floral strip habitats might offer the best spread of strategies to accommodate a variety of landscape assemblages.

Though the benefit of natural diversity in farming landscapes is generally accepted, there is a large gap in knowledge from an economic and social perspective on the direct benefits of these strategies to the farmers who produce crops ([Uyttenbroeck et al., 2016](#)). It is not known if these strategies produce enough incentive alone to employ them in all cases, and in many cases, subsidies are required to encourage their use. The fastest solution may currently involve interested stakeholders like beekeepers and conservation groups working together to make natural diversity a requirement in landscape planning and not only an option, as it often is ([Durant, 2019](#); [Pe'er et al., 2022](#)).

3.2 Agrochemicals

Agrochemicals are essentially all chemicals used in agriculture, from fertilizers to pesticides that target a number of taxa and can have severe detrimental effects. Most current agrochemical application practices for the globalized agricultural sector are unsustainable ([Weltin et al., 2018](#)). Often these chemicals affect species and areas outside the intended and cause toxin buildup in the environment. This endangers organisms on all trophic levels and can eventually damage ecosystem services, food production and subsequently human health ([Singh et al., 2018](#)). The effects of many agrochemicals on pollinators are no different. Depending on the method and timing of application, these chemicals have the capacity to cause mass deaths and serious sublethal conditions for honeybees and wild pollinators alike ([Woodcock et al., 2017](#); [Holder et al., 2018](#); [Fikadu, 2020](#)).

Honeybees are often chosen as a model organism for assessing the toxicity levels of pesticides, however honeybees, due to their eusocial, large colony-nesting strategies are often more resistant to the effects, and not the best proxies for assessing the threats to wild pollinators ([Franklin and Raine, 2019](#)). Even other eusocial bees, like neotropical stingless bees, can suffer stronger effects than those

measured in managed honeybees. Two studies found a much more profound effect of a combination of pesticides and fungicides on one species of stingless bee (*Partamona helleri*) than the tested *A. mellifera* ([Tomé et al., 2017](#); [Almeida et al., 2021](#)). So, if a chemical is found to affect honeybees in a serious way, it may well affect many wild pollinator species more profoundly.

Methods of assessing the toxicity of agrochemicals often fall very short of the entire system of effect they can have on species in the field. Until recently, focus for assessment had generally been on the LD50: the concentration of the chemical that caused a 50% mortality rate in tested subjects ([Trevan and Dale, 1927](#)). This would be realistic if there were no other stressors posing challenges to pollinator health, but pesticides can affect learning and memory in foraging ([Henry et al., 2012](#)), reduce reproductive success ([Sandrock et al., 2014](#); [Woodcock et al., 2017](#)), alter parasite loads ([Evans et al., 2018](#); [Schwartz et al., 2021](#)), lower immunity ([Pettis et al., 2013](#); [Sánchez-Bayo et al., 2016](#)), and cause changes to food webs and species assemblages in the ecosystems that pollinators require to sustain themselves ([Tooker and Pearsons, 2021](#)). In short, with all other challenges, both natural and man-made, pesticide effects can become synergistic with other threats to make “sublethal” effects very lethal indeed ([Goulson et al., 2015](#); [Siviter et al., 2021a](#)).

3.2.1 Lack of knowledge in policymaking

Methods for successfully predicting pesticide exposure and the (not so) sub-lethal effects are still under development ([Barmaz et al., 2010](#); [Siviter et al., 2021b](#)). Currently, many regulatory bodies are relying on the published results of independent studies and the hope that large-scale decision-makers will take the data into account when reworking policies. As of yet, there have been very few steps taken to include any species other than honeybees in most assessments ([Siviter et al., 2021b](#)).

Current EFSA guidelines for the risk assessment of plant protection products (PPPs) only have clear sublethal effect thresholds for honeybees, thresholds for wild pollinators remain ‘undefined’, due to insufficient data ([Authority \(EFSA\) et al., 2023](#)). Today, studies on the agrochemical effects on honeybees still dominate the literature at about 80% ([Vanbergen, 2021](#); [Dirilgen et al., 2023](#)), and the majority of studies on other insects are focused on bumblebees, mason bees and leaf-cutter bees ([Dirilgen et al., 2023](#)). There are large gaps in the knowledge on the synergistic effects of pesticides and by this, policy makers may excuse non-committal opinions in favor of continued, intense agricultural production.

3.2.2 Strategies for minimizing the effects of agrochemicals on non-target systems

The growing global human population is increasing the demand on our agricultural systems ([Noel et al., 2016](#)), and land users often see the call for a reduction of agrochemicals as a threat to their productivity ([Young et al., 2022](#); [Argüelles and March, 2023](#)). However, food production cannot persist outside the framework of stable ecosystems ([Dudley and Alexander, 2017](#); [Kopittke et al., 2019](#)), and some reduction in the intensity of management to make way for that healthy framework may be necessary for the production to continue indefinitely.

Interestingly, pollinators themselves, both domestic and wild may provide an incentive to reduce the use of chemical pest control. One study found that by reducing pesticide use in oilseed rape fields, the subsequent increase in pollinator abundance raised crop yield to the point where it negated the cost of product loss from pest species, and cut production costs by reducing the volume of the purchased pest control chemicals (Catarino et al., 2019). Similar evidence was found when measuring wild pollinator abundance in watermelon fields in relation to a reduction in pesticide applications (Pecenka et al., 2021), increasing yield via improved pollination services beyond the crop loss from pests.

Knowing the unsustainability of current agrochemical applications in most countries and given the evidence that providing for pollinators can nullify the crop loss from pest species, governments must put a high value on farmers, crops and pollinators (both domestic and wild) and work to consider strategies beneficial to all groups.

Beekeepers have a stake in making the landscape more hospitable for pollinators, and beyond the use of their bees, their power of advocacy can be a formidable tool. As an example of policy change regarding agrochemicals in Europe, the three neonicotinoids Clothianidin, Thiamethoxam and Imidacloprid, which were where three of the most widely used pesticides at the time, were banned for outdoor use (EC, 2013), partly as a result of lobbying by beekeepers (Demortain, 2021). Beekeepers have a very keen awareness of how their bees fair in the environments they navigate, and honeybee potential as an ecological monitor, through the attentive beekeepers, may be a strong resource to provide some missing data for policymakers (Cunningham et al., 2022), keeping in mind the effects of these chemicals are likely more severe for any pollinator that is not a honeybee (Thompson and Pamminger, 2019).

Honeybees are often used as indicator species to measure the effects of agrochemicals like pesticides, however they are often not good representatives for the other insect species present in the systems. The increasing pressure on food production systems is pressing for a higher-level of chemical inputs, but this in turn, decreases the stability of the land processes required to grow food successfully. Now we are discovering that there may be alternatives to more intensive land management and using natural solutions, like reducing pesticides to encourage pollinators may prove just as profitable. Accounting for a trade-off between crop productivity and ecological sustainability, involving indirect stakeholders like beekeepers, and pushing for more research to close knowledge gaps should bring us closer to building an agricultural system that can coexist with natural diversity and continue safely into the future.

3.3 Climate change

Climate change is likely one of the most daunting yet seemingly vague threats facing the world. It is difficult to attribute real time events to a force that cannot be seen or measured except over long periods of time, however recent data have now illuminated the effects quite clearly.

Climate change is impacting global temperatures (Hansen et al., 2006; Seneviratne et al., 2006; Sun et al., 2014) and this is causing more frequent and more violent extreme weather events like droughts, floods, wildfires and storms (National Academies of Sciences, Engineering, and Medicine, 2016; Stott, 2016). The effects of these events and increasing temperatures pose problems for most life on earth, and makes current human problems, like taxing an already stressed food production system, harder (Brás et al., 2021).

3.3.1 The varying effects of climate change on pollinators

The effects of climate change on honeybees (both managed and feral) and on other wild and native bees will likely be similar. Extreme weather, for example, can limit forage, and in areas where habitat quality is already reduced (intensely managed landscapes) this could have serious impacts for food collection and nesting habitat (Goulson et al., 2015). Changing weather and temperature patterns can alter local assemblages and shift home ranges, hinder flowering phenology, and make way for invasive species (Parmesan, 2007; Schweiger et al., 2010; Duchenne et al., 2020). Wildlife, and to an extent honeybees, recover from extreme events by being recolonized from surrounding populations that were not affected (Macarthur and Wilson, 1967; Venturini et al., 2017), but reducing habitat patch size and increasing distance between those patches affects recolonization potential (Parmesan et al., 2000). This means there must be suitable habitat within reachable distance with which to support populations that will recolonize after a drastic disturbance (Vasiliev and Greenwood, 2021) created by climate change. Recolonization might be less problematic for domestic pollinators, that can be repopulated by human means, but decreasing the amount of suitable environment makes even honeybees weaker and more susceptible to other threats (Naug, 2009; Potts et al., 2010b). Data on the direct impacts of climate change on pollinators are scant (Decourtye et al., 2019). Many papers detailing potential effects rely on laboratory studies or theoretical modelling which, though insightful, is not a substitute for tangible evidence (Forrest, 2017; Giannini et al., 2017; Hannah et al., 2017).

Despite this lack of concrete knowledge, there are strategies providing necessary data. The effects of climate change, though difficult to predict, are very measurable in their impacts.

3.3.2 Strategies for mitigating the effects of climate change for pollinators

Because it is so hard to measure, and studies that capture discernible effects must be long term and include many samples, there simply is not a large amount of conclusive field data detailing climate change effects on pollinators. The problem then, is one of time and work force. Beekeepers often make excellent watch dogs for the effects of extreme weather. For example, professional beekeepers in Italy retained good records and offered insight into the changes of nectar amount and type in tandem with the weather from year to year (Vercelli et al., 2021). These data might be useful as a proxy for floral abundances of significant resources for pollinators in the

environment and could be valuable data to inform policy makers on climate change mitigation strategies. The fact is, much of a beekeeper's data is empirical, they are highly motivated, and with a small amount of training, many can provide high quality data for long term monitoring projects (Maderson and Wynne-Jones, 2016; Gratzer and Brodschneider, 2021).

To directly mitigate the impacts of climate change, restoring natural habitat can be a viable option. A recent global study concluded that insect biodiversity benefitted directly from more natural habitat in the area, reducing the synergistic impact of climate change and landscape degradation, though the amount of natural habitat needed was great (Outhwaite et al., 2022). A less intense strategy for agriculture could permit a large amount of natural diversity to persist interlaced with needed cropland, and indeed, multiple studies have found that smaller, more diverse crop spaces increase biodiversity and landscape connectivity with minimal losses to productivity by area (Dudley and Alexander, 2017; Tscharnkte et al., 2021).

On a local scale, native plants can be more drought resistant than monoculture crops, and a high enough diversity makes sure there are more floral resources that can tolerate varying conditions. Honeybees in Iowa, when given access to natural resources during times of drought switched from their main source of pollen at the time (clover, *Trifolium* spp), which was much less abundant, to a small variety of natural prairie species and the amount of pollen collected was statistically comparable to other years (Zhang et al., 2022), effectively mitigating a climate-induced forage dearth.

To bring it together: Direct measures to mitigate the effects of climate change, apart from reducing greenhouse gases, lie most prominently in doing what we can to restore natural habitat and reduce the intensity of management in affected landscapes, while also protecting the natural diversity that remains. More data on the effects of climate change would help drive decisions, and beekeepers might offer the efficient and long-term information collection that could help obtain it. Beekeepers have a keen understanding of the importance of natural diversity, and they can provide a force for conservation that is both insightful and passionate. Ultimately, beekeepers have much to offer in the battle for biodiversity.

4 Beekeepers in conservation

Beekeepers have a vested interest in protecting resources for their bees, and this ultimately includes resources for wild pollinators as well. There has recently been significant dialogue on whether or not honeybees fall under the category of “pollinators that need conserving” (Geldmann and González-Varo, 2018; Kleijn et al., 2018; Saunders et al., 2018; Alaux et al., 2019). Arguably, they are managed in most cases, their survival is aided, their numbers bolstered beyond natural limits, or, they are non-native to where they occur. In addition, many projects promoting beekeeping have been pedaled as efforts to increase biodiversity, but this claim is false (Colla and MacIvor, 2017). Outside of wild honeybee colonies in their natural ranges, the conservation of honeybees does very little to aid other species directly.

In terms of practical solutions for habitat loss, the discourse on whether honeybees should be the targets of conservation is irrelevant. Resources required by honeybees and wild pollinators overlap enough that the commonality can be exploited, as ultimately it is not the species that should be the focus in many cases, but the habitats they need to live.

Negatively targeting beekeepers in the effort to preserve wild pollinators alienates an industry that has the most to gain by aligning with them. The repercussions of being restricted from forage for the sake of conservation may be effective in some cases, and short term, but the longstanding, widespread consequences might be that beekeepers seek a sustainable profit in other areas, like industrial scale commercial pollination, and end up supporting a practice even more hostile to the preservation of natural space (Maderson, 2023b). The effects of competition, even those highlighted in the vying for policy attention, can be effectively mitigated in many cases if efforts are combined by these two passionate sides to preserve natural ecosystems in our changing landscapes. Resources must be considered common between both parties and equally protected by both. Wild pollinator conservation groups and the beekeeping industry have unique resources to lend to this cause, and they complement each other in ways that could be synergistic in solving the common problems outlined at length in previous sections.

4.1 Lessons from developing countries: value creation for intact natural habitat

Compared to the West, the story of pollinator conservation in Africa and Southeast Asia includes beekeeping, it being adopted to generate a sustainable income for those who would otherwise depend on trades that are damaging to natural landscapes (Kassa Degu and Regasa Megerssa, 2020; Harianja et al., 2023). Many countries like Ethiopia, Kenya, Tanzania Uganda, India, Indonesia, and Nepal have created conservation programs that center around managing honey-producing bees. The strategy is to generate value in intact natural landscapes and restore disturbed habitats by promoting an industry that can harvest resources with minimal impact on ecosystem function (Wagner et al., 2019; Bareke et al., 2022). As a result, the people who practice beekeeping have gained economic benefits (Kadigi et al., 2021) and are more aware of factors affecting the health of their forests and surrounding land. Some projects have found that beekeepers are active drivers in restoring and protecting local diversity (Sialuk, 2014). Though restricted resources and difficulties in developing appropriate training programs hinder success, observers are optimistic about the potential of this strategy to eventually safeguard natural space (Wagner et al., 2019; Ghode, 2022).

The circumstances surrounding rural communities in developing countries obviously differ from many issues present in places like Europe and North America, however working to create economic value in intact natural landscapes offers an additional level of protection and a new cohort of people ready to defend them. Beekeeping raises conservation awareness wherever it has been measured, and beekeepers have a lot to give in the push for better pollinator conservation (Maderson and Wynne-Jones, 2016).

4.2 Beekeeper drive and public motivation

Applying a monetary value to natural diversity is a solid conservation strategy, but beekeepers' understanding of its real value goes beyond money. A study done in Massachusetts found that beekeepers are more aware of conservation issues than the general public, and more willing to engage actively in pollinator conservation beyond their own bees (DiDonato and Gareau, 2022). Another study found that some beekeepers can be more willing to work for and pay for the conservation of wild pollinators (Penn et al., 2019). These findings are understandable when considering the day-to-day of a beekeeper and their livestock. Beekeepers, especially commercial beekeepers, live the threats to biodiversity every day, because they are often the same threats that affect their own livelihoods. This would not apply to all beekeepers, however, the awareness and incentives are present enough as to consider the beekeeping community as a valuable resource in the endeavor of preserving natural landscapes.

One of the most common reasons given by people starting a hobby beekeeping business is to aid in conserving natural diversity (Duarte Alonso et al., 2021). Beekeeping is used to raise environmental awareness, promote local identity (regional honey) and reignite an interest in traditional and low-impact farming practices (Kohsaka et al., 2017; Cho and Lee, 2018). Though there are apparent misunderstandings of the real impact of honeybees on natural diversity, the willingness to be part of the solution is irrefutably present (Lorenz and Stark, 2015; Duarte Alonso et al., 2021). So, hobby beekeepers want to help, and potentially focusing on environmental education for this particular group of stakeholders may be an easy method to engage them in impactful solutions.

Another possible way beekeepers might influence changes in agricultural strategies is by indirect contact, engaging with crop-based farmers that rely on pollination services for productivity. Studies investigating farmer perception on pollinator declines and supporting management strategies found that knowledge of the threats and the willingness to enact change was related to on-farm experiences and age (Bloom et al., 2021) rather than their use of managed pollinators, and was also linked to their level of knowledge on the subject (Osterman et al., 2021). Still, no studies were found on the perception of crop farmers on the topic of pollinator conservation and their level of engagement with beekeepers, so the possibility exists that beekeepers may well be able to interact as intermediaries between farmers and wild pollinator conservation strategies, providing education to crop farmers by simple interaction.

Ultimately, be it commercial beekeeper, hobby beekeeper or non-beekeeper, there is a great deal of human love for the honeybee both in and outside of beekeeping circles; they have been consistent and valuable partners for millennia (Prendergast et al., 2021). We have many reasons to look on honeybees favorably. They are pollinating allies that make us food, provide sweet treats, and draw us in with their complex social behavior that is easily related to our own societies: Honeybees work together, they care for their young and they dance.

When looking at media representation, honeybees receive much more attention than wild pollinators (Smith and Saunders, 2016;

van Vierssen Trip et al., 2020). This has been seen by conservationists as part of the problem as honeybees are the least threatened pollinator globally (Iwasaki and Hogendoorn, 2021), but it can also be seen as part of the solution.

A flagship species is defined as a charismatic species that draws the attention and sympathy of the public to raise awareness and action for a specific cause (Jepson and Barua, 2015). In many countries it is part of our cultural upbringing to be aware of honeybees and what they do. The awareness of the plight of the pollinators is growing around the world (Hall and Martins, 2020), and within that, our practical and emotional connections to the honeybee creates a drive that galvanizes many people to take action (Schönfelder and Bogner, 2017). A fantastic example of this was presented during the debate which ended in a Europe-wide ban of the three damaging neonicotinoid pesticides. The public cry "Save the bees" is still well-known to this day (Demortain, 2021). There is a pitfall to be avoided here however, and strategies must be careful to use honeybees to draw attention but build the focus of conservation around needed habitats and not the honeybees themselves (Basset and Lamarre, 2019). If properly harnessed with structured outreach and education (elements that honeybees already contribute to), our collective love for honeybees could be one of the central public drivers to sway policy in favor of more sustainable practices and protect natural habitats for all pollinators.

4.3 For science: practical contributions of beekeepers and their bees

Both beekeepers and their bees have a large potential to contribute practically to conservation projects. Honeybees may not be the most sensitive bioindicators, nesting in large numbers and using a suite of effective eusocial behaviors to reduce stressors at the individual level (Franklin and Raine, 2019), however they are abundant, easily managed, respond predictably to their environment, their data can be standardized across large areas, and they come with their own passionate people who are already collecting their data (Quigley et al., 2019; Cunningham et al., 2022). Honeybees will not be able to provide all data needed for informed decision-making on pollinator conservation, but in situations where large amounts of similar data are needed on a multi-regional scale, they may be one of the best options for scientists to use.

This is an example of how beekeepers can contribute to scientific knowledge. Scientific knowledge is one of the most reliable types of knowledge, using strict, repeatable methods and numerical quantification to form conclusions. However, it is expensive, time-consuming, and very limited due to restricted funding. If practical solutions are to be found for issues like the growing decline of insect pollinators, other types of knowledge must be included and taken seriously, knowledges like the practical and experiential knowledges of people who keep bees for a living. Oftentimes issue is taken with incorporating the knowledge of laymen because it is not considered as well-collected or based in concrete scientific understanding and therefore, is not as valuable (Maderson, 2023b, 2023a). However traditional and lay knowledges have the benefit of direct, long term experience with the systems under study, a different lens that can add

much-needed insight to solving on-the-ground problems (Maderson and Wynne-Jones, 2016).

A hard truth is that scientists often do not obtain sufficient funding to complete the tasks that would supply all the information needed for solid policymaking. Citizen science is defined as a collection of volunteers that participate in data collection for scientific studies (Cohn, 2008). It requires an interest in the subject and a consistent time commitment from the participants. Today we have an extensive list of tools to train and employ large numbers of dedicated volunteers in science monitoring projects. The fact that almost everyone on the planet now carries a smartphone, effectively a small computer, has made the idea of citizen science all the more practical. Combine these tools with the expertise, passion, and keen observational skills of a beekeeper, and you are likely to get data that rival the fastidious detail found in the scientific community.

Several studies have now been published using the data collected by beekeepers on their honeybees: some to assess pollen availability and the use of forage plants by honeybees over the active season (Brodschneider et al., 2019), others to examine the field levels of pesticides (Woodcock et al., 2022) or heavy metals (Shaw et al., 2023). These studies identified seasonal declines in pollen diversity, successfully linked foliar insecticides to an increase in disease, and monitored levels of several heavy metals present in an environment.

In addition to participating in data collection, beekeepers have been shown to contribute to the design of novel data collection tools. They optimized methods to align with their capabilities, identified pitfalls and streamlined the collection plans when set to the task of improving technologies (Phillips et al., 2013). Beekeepers are natural innovators and are often willing to lend their expertise to improving projects when invited.

Citizen science offers an address to the problem of resources for scientific studies, and involving beekeepers in science is, in itself, a form of outreach and education. One of the central issues around pollinator declines is a lack of understanding of the core elements of the issue, both by the public and some beekeepers. Involving these key stakeholders in scientific solutions and data collection can provide the education missing in many of the stakeholder groups that have been historically excluded from direct scientific findings (Vohland et al., 2021).

In the end too, creating strategies around people is usually the most long-lasting form of conservation, as it creates value for the communities using these systems, includes them as stakeholders in solutions and ensures that effects can be intergenerational, building local culture around sustainable principles and allowing an internalization of core practices.

5 Conclusion

In the end, honeybees do not contribute directly to improving the environment for wild pollinators, and in some cases, can be detrimental, but this does not mean that beekeepers are, by default, antagonists to conservation. Managed honeybees, like wild pollinators, stand to lose a considerable amount of stability with

the reduction of natural and semi-natural habitats, and without sufficient natural diversity in the landscape, competition between the two species groups is inevitable. Competition dynamics have the potential to cause both great harm to natural systems and threaten to restrict the land beekeepers depend on for their livelihoods. The effects of land use intensification can combine with other threats like climate change and competition to exacerbate conditions and magnify problems, and it is only in the direct mitigation of these larger threats that broadscale solutions can be found. The literature body is growing however, knowledge is still lacking in key areas and this allows policymakers to sidestep meaningful action.

Beekeepers have a good deal to offer in needed data collection and the development of strategies to mitigate habitat loss. For example, they are the bannermen that generate public interest for pollinators, and the honeybees they keep are charismatic flagships. Beekeepers are present on the ground for many direct changes and can offer pinpointed local information as well as largescale, long-term data that can aid in policymaking. With precise education, training and guidance, beekeepers can harness their interest and passion and the weight of their industry to afford better protections for natural lands and push for more sustainable agriculture.

It would be a powerful combination to arm beekeepers with the factual knowledge of conservation and channel their endeavors into a collective effort to protect all pollinators. The beekeeping industry is one that closely aligns with the goals of sustainable agriculture due to their livestock's dependency on natural diversity for good health. Honeybees and beekeepers, in the context of human-mediated landscapes could be said to have a symbiotic relationship with wild pollinator groups in the context of their mutual need for wild space and the drive of the domestic bee industry to create a sustainable future for itself. A collaborative, unified effort between beekeepers and wild pollinator advocates will provide a louder voice when pushing for policy improvements regarding the preservation of natural habitats. Both groups standing together may serve as a needed example on how our agricultural systems in their entirety could benefit from taking the needs of the natural environment into account.

There must be a push to understand the underlying causes of competition, and education for both beekeepers and other farmers will be needed to deepen knowledge and understanding of the issues surrounding it. Like the Three Musketeers, beekeepers and wild pollinator conservationists must rally together and unite their efforts. Involving beekeepers in decision-making, acknowledging them as key stakeholders and keepers of valuable knowledge, and promoting their potential to be part of the solution is the only way to create long-lasting and self-perpetuating change directly in the environments that must be conserved, both for wild pollinators and for our bees.

Author contributions

MO: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. BD: Writing – review & editing.

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Thermodynamics, thermal performance and climate change: temperature regimes for bumblebee (*Bombus* spp.) colonies as examples of superorganisms

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Evidence is widespread that many species of *Bombus* are in population and biogeographical decline in response to adverse effects of global climate warming. The complex interactions of the mechanisms at the root of the declines are poorly understood. Among the numerous factors, we posit that heat stress in the nests could play a key role in the decline of bumblebee species. The similarity of the optimum temperature range in incubating nests is remarkable, about 28–32 °C regardless of species from the cold High Arctic to tropical environments indicates that the optimal temperature for rearing of brood in *Bombus* spp. is a characteristic common to bumblebees (perhaps a synapomorphy) and with limited evolutionary plasticity. We do note that higher brood rearing temperature for the boreal and Arctic species that have been tested is stressfully high when compared with that for *B. terrestris*. The Thermal Neutral Zone (TNZ), temperatures over which metabolic expenditure is minimal to maintain uniform nest temperatures, has not been studied in *Bombus* and may differ between species and biogeographic conditions. That heat stress is more serious than chilling is illustrated by the Thermal Performance Curve Relationship (TPC) (also sometimes considered as a Thermal Tolerance Relationship). The TPC indicates that development and activity increase more slowly as conditions become warmer until reaching a plateau of the range of temperatures over which rates of activity do not change markedly. After that, activity rates decline rapidly, and death ensues. The TPC has not been studied in eusocial bees except *Apis dorsata* but may differ between species and biogeographic conditions. The importance of the TPC and the TNZ indicates that environmental temperatures in and around bumblebee nests (which have been rarely studied especially in the contexts of nest architecture and substrate thermal characteristics) are factors central to understanding the adverse effects of heat stress and climatic warming on bumblebee populations, health, and biogeographical decline.

KEYWORDS

thermoregulation, Thermal Neutral Zone, thermal performance, thermal tolerance, nest architecture, incubation, nest insulation, diet

1 Introduction

Bumblebees are eusocial, large and conspicuous insects. More than 250 species are described throughout the world (Williams, 1998), but in some respects they are remarkably uniform in form and behaviour. Their colonies can be considered individually to be single “superorganisms” (i.e., a group of synergetically interacting organisms of the same species) because the entire colony, rather than the individual resident bees, is the reproductive unit subject to Darwinian selection. An important feature of the “superorganism” concept is the ability of the whole colony to maintain its temperature within a range over which metabolic rates are minimal and activity can be maintained. That range is the Thermal Neutral Zone (TNZ) (Figure 1) and was elucidated by Moritz and Southwick (1992) for the western honeybee (*Apis mellifera*). Thermal stress has been indicated to result in workers of smaller size and poorer condition through accelerated development and more energy devoted to respiration during development. Similarly, the reproductives, especially the gynes, may mature at a smaller size with concomitant adverse effects on overwintering success, spring founding of new colonies, fewer eggs in the ovaries, and overall reduced colony strength (see Vanderplanck et al., 2019).

Bumblebees are recognised as important pollinators from the High Arctic to the tropics and are exploited in commercial agriculture

(Cameron and Sadd, 2019). They form a monophyletic genus, *Bombus*, that includes the subgenus of socially parasitic *Psithyrus*, within the Family Apidea (Hymenoptera) (Cameron et al., 2007). Aspects of the bionomics of several species of bumblebees have contributed to their commercial value in pollination of crops (Velthuis and Van Doorn, 2006), assessing natural problems in pollination and associated issues in conservation (IPBES, 2016; Potts et al., 2016).

Bumblebees are widely considered to be in decline globally, with climate change (especially warming) being a major contributor (Vannote and Sweeney, 1980; Williams, 1986; Colla and Packer, 2008; Cameron et al., 2011; Bommarco et al., 2012; Rasmont and Iserbyt, 2012; Sunday et al., 2014; Kerr et al., 2015; Martins et al., 2015; Rasmont et al., 2015; Arbetman et al., 2017; Ogilvie et al., 2017; Woodard, 2017; Koch et al., 2018; Sirois-Delisle and Kerr, 2018; Françoso et al., 2019; Naeem et al., 2019; Vray et al., 2019; da Silva Krechmer and Marchioro, 2020; Jacquemin et al., 2020; Rollin et al., 2020; Soroye et al., 2020; Marshall et al., 2021; Martínez-López et al., 2021; Oyen et al., 2021). In the few examples when the diversity and abundance of bumblebees have been assessed by a standard method over several years, wide interannual fluctuations have not been explained (Turnock et al., 1997). The conclusions from most studies rely on inferences, circumstantial evidence, and correlations without invoking experimentally elucidated root physiological causes, or suggesting a suite of interacting ecological, behavioural to physiological causalities.

We suggest that assessment of the effects of stress at the superorganism level (i.e., colony) would help explain their plight under climate change. Conditions within nesting habitats are a rather neglected but probably important factor under conditions of heat stress, such as during heat waves (Vanderplanck et al., 2019; Martinet et al., 2021; Gradišek et al., 2023), in constraining and adversely affecting the health and survival of bumblebees as the effects of global warming become increasingly severe. The effects of stress may be well measured by whole colony respirometry which seems to have been rarely undertaken. The stress of parasitic tracheal mites adversely affects the respiratory rates of whole colonies of honeybees (*Apis mellifera*) and helped understand winter mortality (Skinner, 2000). Respirometry of honeybees in clusters held at differing temperatures has helped understand superorganism effects in colony thermoregulation (Moritz and Southwick, 1992; Stabentheiner et al., 2021).

The aim of this report is to stress the importance of nest thermoregulation and brood incubation, and their apparent consistency throughout the genus, as being highly sensitive to disruption, especially under heat stress. Then, by returning to classic understandings of thermal performance, we point out that more and comparative studies on this common character could explain the recorded sensitivities of various *Bombus* spp. to the effects of climate change.

Coupled with the remarkable uniformity in the nest incubation temperatures across the genus (Table 1), other studies on nesting ecology are needed, such as the ambient heat load (soil temperature), nature of the substrate, architecture of the nests, thermal buffering properties of the substrate, considerations of aspect to insolation, shading, and other environmental factors.

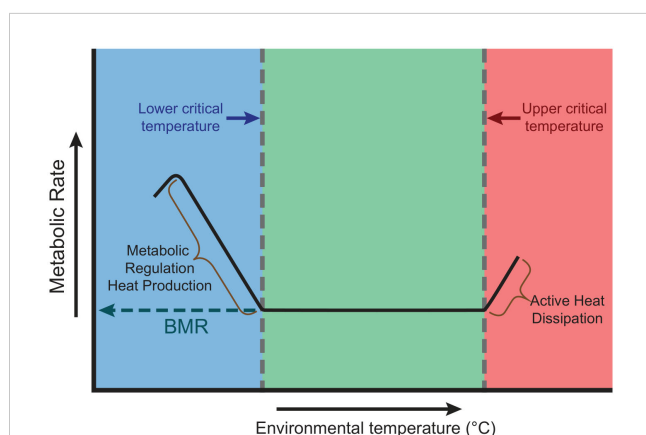


FIGURE 1

Generalised relationship between rate of performance (i.e. activity) and environmental temperature in animals, notably in those with capacity to thermoregulate as in the nests of eusocial insects (from Moritz and Southwick, 1992). As conditions become colder (in blue), thermoregulation requires more and more metabolic energy (e.g., through vibration of thoracic muscles) until cold torpor and then death set in (BMR). The Thermal Neutral Zone (TNZ) (in green) represents temperature conditions under which the metabolic rate required to thermoregulate remains minimal. If heat stress occurs (in red), the metabolic cost of thermoregulation for cooling increases (e.g., by fanning, evaporation, mass defecation, etc.) until thermoregulation becomes impossible and death ensues. In general, the slopes of metabolic and thermal stress are longer and shallower for warming than for cooling (i.e. overheating is more metabolically stressful than chilling) (see Figure 2). The TNZ has not been studied in *Bombus* and may differ between species and biogeographic conditions.

The extent of behavioural plasticity is probably greater than noted in published studies but may be constrained in the face of other risks, such as flooding, predation and potential for disease.

2 Uniformity of nest incubation temperatures

Seeley and Heinrich (1981), Heinrich (1993), and Jones and Oldroyd (2007) review what is known about temperature regulation in the nests of various social insects. It is widely known that honeybees [*Apis* spp. (Hymenoptera: Apidae)] control the environmental conditions in their colonies (Huber, 1814; Milum, 1929; Seeley and Heinrich, 1981; Moritz and Southwick, 1992; Sudarsan et al., 2012; Stabentheiner et al., 2021), but the extent and mechanisms by which other eusocial insects, such as ants (Roeder et al., 2021; Nascimento et al., 2022) and bumblebees [*Bombus* spp. (Hymenoptera: Apidae)] do so is less understood (Goulson, 2010).

Newport (1837) presented probably the first detailed accounts of the temperatures generated for the incubation of the brood by bumblebees, as later pointed out by Hoffer (1882) as being similar to birds incubating their eggs and chicks. The informative books by Heinrich (1979, 1993, 2004) summarise the scientific information on both cooling and heating in terms of temperature control in individual bumblebees and of the nest.

Table 1 presents information on the species of *Bombus* for which nest incubation and body temperature data are published. From the first publication by Newport (1837), it has become increasingly accepted that the general optimum temperature within the brood of various species bumblebee is much the same, at about 28–32 °C, regardless of species. That narrow temperature range applies when the colony is well developed, but the brood nest may be much cooler when a lone queen needs to leave her nest to forage at the early phase of nest establishment. In small, queenless, colonies of *B. terrestris* thermoregulation is also tightly controlled, notably under conditions of cold stress. More recent research (e.g., Vogt, 1986; Weidenmüller et al., 2002; Weidenmüller, 2004; Gardner et al., 2007; Vanderplanck et al., 2019) has stressed behavioural responses, especially fanning, in response to high temperatures, cooling, and preventing the overheating of brood in developed colonies.

The temperature regime at the periphery of the cluster and within the cavity occupied by the nest varies depending on the ambient temperature outside the cavity or domicile in which the colony lives. The ambient temperature immediately outside the cavity is, of course, similarly influenced but the surrounding materials (e.g., wood, straw, animal nest residues, soil and so on) and their thermal properties, especially conductance (see below).

Our review of the published literature over the past 180 years indicates that bumblebees of all species so far studied maintain constant brood nest temperatures of 30–35 °C at the core of the colony (Table 1). This range seems to be wider at lower temperatures than for honeybees, however comparative studies have not been made. As with honeybees, the narrow temperature range of incubation and thermoregulation seems not to have been studied in bumblebees in relation to brood survival. It can be

TABLE 1 Generalised brood temperatures of various species of *Bombus* arranged primarily in chronological order of studies made.

Species	Brood temperatures	Reference
<i>Bombus terrestris</i>	22.5–34.5 °C	Newport, 1837
	30–34 °C	Linhard, 1912
	29–35 °C	Hasselrot, 1960
	31.3–33.4 °C	Weidenmüller et al., 2002
	31 °C	Livesley et al., 2019
	28–34 °C	Wynants et al., 2021
<i>Bombus pascuorum</i> (as <i>B. agrorum</i>)	29–32.5 °C	Himmer, 1933
<i>Bombus hypnorum</i>	30 °C	Nielsen, 1938
<i>Bombus borealis</i>	30.5 °C	Fye and Medler, 1954
<i>Bombus rufocinctus</i>	29.5 °C	
<i>Bombus fervidus</i>	29.5–32.2 °C	
<i>Bombus lapidarius</i>	20–35 °C	Hasselrot, 1960
<i>Bombus terrestris</i>	29–35 °C	
Also <i>B. hypnorum</i> , <i>B. lucorum</i> , <i>B. so[a]ro[e]nsis</i> , <i>B. pratorum</i>		
<i>Bombus lapidarius</i>	27–33 °C	Wojtowski, 1963
Also <i>B. pascuorum</i> , <i>B. derhamellus</i> , <i>B. terrestris</i> , <i>B. silvarum</i>		
<i>Bombus polaris</i>	25–35 °C (despite the cold ground surrounding the nest)	Richards, 1973
<i>Bombus vosnesenskii</i>	24–34 °C	Heinrich, 1974
<i>Bombus impatiens</i> , <i>B. terrestris</i> , <i>B. ruderatus</i>	29–32 °C	Pomeroy and Plowright, 1980
<i>Bombus agrorum</i> (= <i>B. pascuorum</i>), <i>B. hortorum</i> , <i>B. saroeeensis</i> , <i>B. subbaicalensis</i> , <i>B. distinguendus</i>	27–34 °C (29–31 °C)	Eskov and Dolgov, 1986
<i>Bombus impatiens</i> and <i>Bombus affinis</i>	30–32 °C	Vogt, 1986
<i>Bombus lapidarius</i>	27 – (29–33) °C	Schultze-Motel, 1991
<i>Bombus huntii</i>	28–32 °C (implied as optimum)	Gardner et al., 2007
<i>Bombus atratus</i>	28 (by implication)	Vega et al., 2011
<i>Bombus hypnorum</i> , <i>B. hortorum</i> , <i>B. argillaceus</i> , <i>B. pascuorum</i> , <i>B. humilis</i> , and <i>B. silvarum</i>	ca. 31 °C for all species, especially at night	Gradišek et al., 2023

assumed that bumblebee brood would not survive at temperatures above 36 °C, and at temperatures at about 30–32 °C the brood survives and develops normally, prolonged chilling can be assumed to be detrimental.

3 Thermal performance & tolerance

In general, temperature affects rates of biological activity (A), from enzymatic reactions, bacterial population growth rates, various metabolic functions, organ development and maturation, to organismal growth and development. Together and in combination temperatures and rates of biological activity (also metabolic rates) constitute the Thermal Performance Curve (TPC) (Schulte et al., 2011; Rezende and Bozinovic, 2019) (Figure 2), sometimes referred to as the Thermal Tolerance Relationship (TTR). TPC is highly diverse across life forms, from organisms that thrive at near freezing temperatures to extreme thermophiles. Nevertheless, for all life forms, TPC may be modelled as the product of two functions, an exponentially increasing rate of the forward reaction to the optimum and a steeper exponential decay (till death) presumably resulting from enzyme denaturation, membrane dysfunction, deceleration of mitochondrial activity as temperatures increase beyond the optimum, and perhaps effects on DNA-methylation. The optimum, where the change in activity remains nil (homeostatic) as temperature increases ($dA/dT = 0$ for TTR) may be extended by biochemical plasticity, acclimation, thermoregulatory behaviour, and evolutionary adaptation.

In classical ecology it is generally recognised that the ranges of most terrestrial organisms, especially animals, can be explained ecophysiologically by temperature and moisture (Uvarov, 1931; Andrewartha and Birch, 1954; Lutterschmidt and Hutchison, 1997; Kinsolver and Umbanhowe, 2018). In particular, the

relationship between temperatures for development and survival of all organisms shares the common feature of being negatively skewed (i.e., the TPC, which indicates that once the optimal temperature for development, activity, or survival is exceeded, the decline is steep with death ensuing after a few more degrees of heat) (bacteria: Rezende and Bozinovic, 2019; plants: Rezende and Bozinovic, 2019; fungi: Rezende and Bozinovic, 2019; insects: Uvarov, 1931; Wigglesworth, 1972; Kinsolver and Umbanhowe, 2018; Rezende and Bozinovic, 2019; vertebrates: Lutterschmidt and Hutchison, 1997; Rezende and Bozinovic, 2019).

Surprisingly, it seems that few experimental studies have been made to elucidate the thermal performance and tolerances (TT, see discussion below) of bees and their brood together (but see Vogt, 1986; Vanderplanck et al., 2019). It may be assumed that brood temperatures lower than optimal are more tolerable and less damaging over a greater range than temperatures above optimal. That is because temperatures lower than the optimum just slows down metabolism but going higher causes irreversible damage. In general, one may assume that heat stress may be lethal even if slightly elevated above optimum. Himmer (1932) suggested that the vital upper limit for temperature of honeybee brood (*Apis mellifera*) is 36 °C and Mardan and Kevan (2002) showed that for *Apis dorsata* brood does not survive when incubated at 36 °C or above. For adult bumblebees it is known that this curve may have different positions on the temperature continuum, with some species able to function at lower or higher temperatures and some species that can acclimate in response to ambient and local conditions (Oyen et al., 2016; Oyen and Dillon, 2018; Oyen et al., 2021; Gonzalez et al., 2022).

Food is the source of energy for thermoregulation and colony maintenance and can be influenced by climate change (e.g., Miller-Struttmann et al., 2015). Vanderplanck et al. (2019) studied the interactive effects of heat stress reduction of dietary resource quality, and colony size in bumblebee colonies. Using 117 colonies of *B. terrestris*, they applied a fully cross-treatment experiment to test the effects of three dietary quality levels under three levels of heat stress with two colony sizes. Both nutritional and heat stress reduced colony development and caused lower investment in offspring production. Small colonies were much more sensitive to heat and nutritional stresses than were large ones, possibly because a higher proportion of workers are needed to maintain social homeostasis in the former. From a nutritional viewpoint, the effects of heat stress were far less pronounced for small colonies which received relatively good diets than for those that received inferior diets.

4 Bumblebee nest architecture and soil temperature profiles

We suggest that the thermal environments in which bumblebee nest (mostly underground, some in cavities, and few on the surface of the ground) are a neglected area in bumblebee ecology. We have not found any reports that link experimentally the various factors involved.

Bumblebee nests take on many forms but are generally enclosed. The most well-known form is ovoid enclosed in a

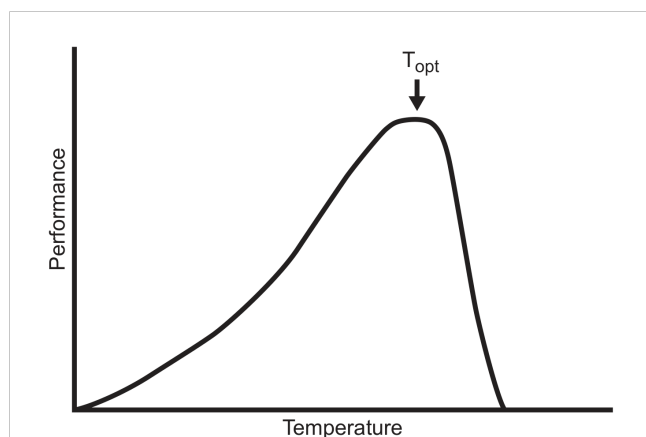


FIGURE 2

Generalised relationship between temperature and activity (Thermal Performance Curve; TPC) showing the more pronounced impact of heat stress (steep decline in activity after optimal temperatures are exceeded) than of chilling (left-hand tail). The position and breadth of the curve differs between organisms, being transposed to the right for thermophiles and to the left for organisms adapted to cooler conditions. The optimal temperature range, at which activity can be the greatest, may be broader or narrower depending on adaptation and/or acclimation. For poikilotherms the relationship is generally broader, especially at cooler temperatures, but for homeotherms the relationship is tight across the range of tolerable temperatures as is well-known for incubation and successful development of birds' eggs. The general form of the relationship is recognised by biologists who have studied temperatures and activity/development from bacteria to mammals (see Rezende and Bozinovic, 2019).

protective cavity. Those bumblebees using vacated rodent nests may be as much as 20 cm beneath the soil surface (Free and Butler, 1959; Goulson 2010; Martinet et al., 2022). The nest itself includes the central complex of brood cells within an involucre of waxy material adhering to an outer layer of insulation. That insulation may be a centimetre or so thick. Outside the cavity is the substrate in which the nest is enclosed is soil, wood (as in cavity nesters), or rarely vegetation (living and/or dead) for surface nesters (Figure 3) (for examples, see Free and Butler, 1959; Sakagami et al., 1967; Hines et al., 2007; Goulson, 2010; De Meulemeester et al., 2011; Martinet et al., 2022).

From a thermodynamic viewpoint, nest temperatures are influenced by thermoregulation by the colony (metabolic or behavioural) (Figure 1) (Vogt, 1986). The buffering of the temperature within the colony must be influenced by the insulation afforded by the involucre and insulation; that is, the capacity of the materials together to resist the flow of conductive heat (R- or RSI values and U-units) (see Figure 3). The involucre of the nest is likely to have high insulational value with RSI at about 20 ([https://en.wikipedia.org/wiki/R-value_\(insulation\)](https://en.wikipedia.org/wiki/R-value_(insulation))) based on data for sheep fleece, straw, etc. Outside the involucre, the soil or wood substrate likely has a much lower RSI at less than half that of the involucre but its conductivity presumably varies widely depending on water content, compaction, and composition. We know of no measurements of RSI values for materials that make up bumblebee nests.

The actual temperature profile from the outside of the nest's involucre to the surface presumably and typically would show the highest heat loads at the surface, especially if insulated with temperatures exceeding lethal temperatures for many organisms. Under shaded, night-time and rainy conditions the surface temperature of the soil is known to be close to that of the ambient air (Geiger et al., 2003; Holmes et al., 2008). We know of no records in which the soil temperature profile over bumblebee nests is given but Linhard (1912) provided data on ambient

temperatures associated with wooden-cavity nests (bird nesting boxes) inhabited by *B. hypnorum*.

5 Discussion

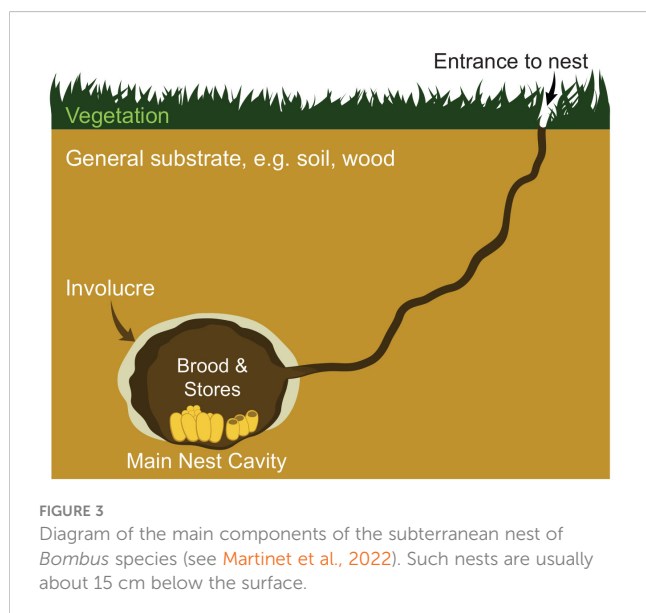
This essay brings to readers' attention that the issue of climate change and ensuing range reductions in bumblebees is complex. We hope that our readers recognise that we have attempted to provide a vista of the "Horizon" by presenting this "Frontier" for contemporary "Bee Science".

Climate change and especially increases in global temperatures seem to affect bumblebees in various and interrelated ways. Anatomical adaptations have been recorded and include changes in size, body part dimensions (notably mouthparts) and possibly pilosity (Miller-Struttmann et al., 2015; but see de Keyser et al., 2016 and, more recently, Gérard et al., 2018, 2021, 2022a, 2022b). Other adaptive morphological and physiological changes in bee species have been proposed for bees living in urban heat islands (Polidori et al., 2023). Flight activity is influenced by temperature, insolation, and other weather conditions in nest-seeking queens, foraging workers, and the next generation of sexuals. Thermoregulation in nests and incubation of brood are in themselves thermodynamically and behaviourally complex and presumably part of species and colony differences in nest site choice, nest design and construction, and use of materials. The nest differences must also reflect strategies and adaptations for overcoming periods of adversity (e.g., heat, cold, damp, dearth of resources and even hibernation/diapause/metamorphosis).

Excessive heat is clearly a major issue for perhaps most species of bumblebees and it is the factor considered in most detail for the effects of climatic warming. Most contemporary research has focussed on standard meteorological data and coincidental or circumstantial changes in the ranges of various species on bumblebees. Experimental explanations of cause and effect need more detailed and integrated studies with special attention paid to colonies as "superorganisms" (Moritz and Southwick, 1992).

Thermal performance and tolerance (TT) studies in perhaps all organisms show that heat is more hazardous than cold [see Figure 2 (TPC) with its negatively skewed shape]. After a certain ambient temperature is reached, the effects of increasing heat are more pronounced and deadly than are the effects of cooling. The relationship between temperature and larval death has been studied in only a few species of social insects and not in bumblebees. The maximum for the level of activity with temperature can be presumed to reflect a preferred range and the temperature at which social insects, including bumblebees, would try to keep their nests. That range lends to the general relationship (Figures 1, 2) but the variability in that feature seems not to have been assessed in insects, neither physiologically nor in the broader contexts of evolutionary ecology and behaviour. We suggest that such studies, specific and comparative, could elucidate explanatory aspects to climate change and biogeographical range shifts as well as providing insights into possible variability in the TNZ (Figure 1).

The remarkable similarity of nest temperatures recorded from numerous species of bumblebee (Table 1) needs to be seriously



considered as a possible limitation on their abilities to withstand not just cool conditions but heat as well. The idea of the TNZ (Figure 1) provides insights as to the possible nature of ecological and evolutionary constraints. Few species have been studied for nesting TNZ. We suggest that various species differ in that respect, with cool-climate species possibly having broader TNZ than others from subtropical and tropic environments. Tropical and subtropical bumblebees may have narrow TNZs reflecting that thermoregulation for cooling is more energetically expensive than in temperate zone species. Thus, the latter with their abilities to live over a broad range of temperatures may represent greater variability in potential survival under relatively limited hot and cold conditions. That aspect of comparative energetics and thermoregulation remains unexplored but may suggest reasons for some changes in bumblebee ranges. The apparent and approximate inverse relationship between TPC (Figure 2) and TNZ (Figure 1) reflects mostly the general shape of the TPC curve with its extended tail at the cold end of the temperature range coupled with the preferred temperature range as maintained by thermoregulatory processes (TNZ, Figure 1).

Thermoregulatory behaviours are various. Air exchange is the most commonly considered reaction to temperature changes in the nests of various bees. The thermodynamics and biophysics of colony clustering within the nest have been elucidated by Sudarsan et al. (2012) for western honeybees (*A. mellifera*) and those principles likely apply to other cavity nesting social insects. Fanning to expel excess heat from the nest is well known in honeybees and bumblebees. That behaviour can be coupled with the evaporation of water (the swamp cooler effect) within the nesting cavity (e.g. hive) or on the surface of open nests, such as in *A. dorsata* which indulge in gobetting and mass defecatory flights related to cooling the colony under conditions of heat stress (Mardan and Kevan, 1989). Those behaviours seem not to have been recorded in bumblebees but may be important.

The placement, construction, and architecture, of nests of social bees and wasps have been described to greater or lesser extents for several species but not especially so for thermoregulatory considerations. Subterranean nesting is well-known in bumblebees. They use various substrates and materials, but nests are usually only a few centimetres below the surface (Figure 3). Less seems to be known about the nesting structures of bumblebees that nest in above ground cavities (e.g., hollow trees or bird-nesting boxes). Some may nest above ground in available cavities (even discarded household furniture) or, as the tropical Amazonian *B. transversalis* which even forages for nest-building thatch materials along its established walking trails (Cameron and Whitfield, 1996). Human beings have domesticated a few species so that they inhabit artificial domiciles (Velthuis and Van Doorn, 2006). Interspecific and intraspecific comparative studies on the nature of the nests and their locations, together with considerations on thermoregulation, may elucidate factors that pertain to physiological ecology and biogeographic limitations. A major problem confronting bumblebee studies is finding nests in the wild. For locating nests of subterranean species, perhaps non-invasive studies by ground-

penetrating radar (GPR) could be useful. Sherrod et al. (2019) record nesting burrows of the ground-nesting, solitary bee, *Colletes inaequalis* at about 0.5 m depth. We know of no published studies using that technique for bumblebees.

We suggest that studies using flow-through respirometry (Skinner, 2000; Stabentheiner et al., 2021) of whole colonies whereby the temperature of the atmosphere, its saturation deficit, and possibly partial pressures of gases can be manipulated would provide more comprehensive and comparative ecophysiological understandings of the TNZ and TPC. Such studies could also involve measurements of the insulation properties of materials from which nests are constructed and of their surrounding matrix. We know of no measurements of heat loss through bee nesting materials. Skowron and Kern (1980) used basic principles of thermodynamics and heat flux to measure the insulation afforded by birds' nests. Their approach could be followed with modifications possible by modernisation of instrumentation. We suggest that measuring heat flow (i.e., thermal resistance or conductance) across bee nest walls may be instructive and can be done when the temperatures inside and outside are known so as to calculate the temperature difference from inside to the outside of the nest. Then, by measuring the area or volume of the nesting material or matrix being considered and the amount of time taken for the temperature difference to stabilise the insulational properties of the nest can be measured (R-value or U-value for heat transmittance). We are not aware of any such measurements for materials comprising bumblebee nests, either artificial or natural. Calculation could be adjusted to account for the thickness and nature of the wall materials. Readers are referred to Çengel and Afshin (2015) and Tritt (2004) for principles and methods.

By linking the various ecophysiological and behavioural factors, with environmental concerns and constraints on evolutionary plasticity through the superorganism approach to bumblebee nesting, several novel approaches suggest themselves for understanding how climate change asserts its influence on bumblebees' population dynamics, changing biogeography, and possibly conservation. We suggest that the same principles should be applied to the effects of climate change on other eusocial and other insects (e.g., ants, termites, other Hymenoptera, community gall-formers, etc.).

Author contributions

PK: Writing – original draft, Writing – review & editing. PR: Writing – original draft, Writing – review & editing. BM: Writing – original draft, Writing – review & editing.

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Conflict of interest

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Effects of heat shocks, heat waves, and sustained warming on solitary bees

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Along with higher average temperatures, global climate change is expected to lead to more frequent and intense extreme heat events, and these different types of warming are likely to differ in their effects on bees. Although solitary bees comprise >75% of bee species, and despite their ecological and economic value as pollinators, a literature search revealed that only 8% of studies on bee responses to warming involve solitary bees. Here we review studies that have addressed how solitary bees are affected by three main types of warming that vary in magnitude and duration: heat shocks, heat waves, and sustained warming. We focus on direct physiological and behavioral effects of warming on solitary bees, rather than the underlying mechanisms. We find that heat shocks have received little attention in solitary bees both in terms of number of studies and relative to social bees, and all of those studies examine the effects of heat shocks on a single genus, *Megachile*. This work has shown that heat-shocked eggs, larvae, and pupae tend to upregulate heat shock protein genes, while heat shock at the adult stage can increase mortality in male bees, potentially altering population sex ratios. We find that solitary bee responses to heat waves have received even less study, but the few studies suggest that these events can increase larval mortality and slow development time, and that bees may not be able to physiologically acclimate to heat wave conditions by increasing their critical thermal maxima. Finally, sustained warming, which has been relatively well-studied in solitary bees, can speed development rate, reduce body mass, increase mortality, and alter foraging behavior. Our review reveals knowledge gaps in the effects of heat shocks and heat waves on solitary bees and, more broadly, in the responses of unmanaged solitary bees to warming. To improve our ability to anticipate the consequences of climate change for these critical pollinators, we encourage research on solitary bee thermal responses that examines short-term, extreme warming and incorporates greater ecological realism and complexity.

KEYWORDS

climate change, foraging, nesting, pollination, pollinators, thermal ecology

1 Introduction

Climate change represents a major threat to global biodiversity, with effects spanning levels of biological organization from genes to biomes (Scheffers et al., 2016). In particular, rapidly increasing temperatures will challenge the limits of acclimatization and adaptation to novel environmental conditions (Sears and Angilletta, 2011). With regions experiencing simultaneous increases in mean average temperatures and in the magnitude of temperature fluctuations (Lewis and King, 2017; Chen et al., 2018; Arnell et al., 2019), it is crucial to understand how both sustained warming and shorter-term episodes of extreme warming will affect species, their interactions, and the ecosystem services and functions they provide.

Among the ecological systems threatened by warming, plant-pollinator interaction networks are critical for crop production (Klein et al., 2007; Eilers et al., 2011) and the maintenance of biodiversity in natural communities (Wei et al., 2021). Globally, 85% of crops are pollinated by animals, and cultivation of pollinator-dependent crops is increasing (Klein et al., 2007). Additionally, almost 90% of flowering plants rely on animals for pollination, and these plants provide resources for many other species (Ollerton et al., 2011). Thus, it is important to understand how warming, spanning various magnitudes and durations, will affect pollinators and thus pollination services.

Bees are ecologically valuable pollinators (Pardee et al., 2022) and are facing various environmental stressors thought to be responsible for widespread population declines, including warming associated with climate change (Goulson et al., 2015). Given bees are largely ectothermic, and many aspects of their life histories are known to be sensitive to temperature, climate warming is likely to have strong direct effects on their physiology, phenology, development, and behavior (Hegland et al., 2009; Scaven and Rafferty, 2013). Indeed, recent studies have documented multifaceted effects of warming on bees (Hayes and López-Martínez, 2021; Zaragoza-Trello et al., 2021; Melone et al., 2024). However, variation in the magnitude and duration of warming under study make it challenging to synthesize these effects. Furthermore, as we demonstrate herein, most research has focused on social bee responses to warming, leaving much to be understood about the effects of warming on the solitary bees that comprise more than 75% of all bee species (Danforth et al., 2019).

Here, we review the direct physiological and behavioral effects of climate warming on solitary bees, focusing on three main types of warming: heat shocks, heat waves, and sustained warming. Because exact, quantitative definitions of these different types of warming are not well-resolved (e.g., IPCC, 2018; Barriopedro et al., 2023), we use these categorical terms to identify and group studies on warming and solitary bees. In general terms, heat shocks refer to extreme, short-term spikes in temperature, whereas heat waves are mid-magnitude and medium-duration events. Finally, sustained warming reflects lower-magnitude, longer-term increases in global mean temperatures associated with climate change.

By organizing empirical studies according to these three different magnitudes and durations of warming, we can discern whether they have differing effects on solitary bees. Whereas heat

shock may cause acute stress and immediate mortality if temperatures exceed critical thermal maxima, heat waves may cause chronic physiological stress as individuals attempt to acclimate (e.g., Hayes and López-Martínez, 2021; Gonzalez et al., 2024). The effects of sustained warming may be more subtle and cumulative, expressed in terms of altered sex ratio, size, phenology, behavior, and lifespan (e.g., Slominski and Burkle, 2019; de Manincor et al., 2023). Although small-scale spatial heterogeneity in temperature is likely to shape bee responses to warming (Sears et al., 2011), we focus on temporal aspects of warming while acknowledging that bees can encounter many different microclimates as the sun rises and heats up a nesting site, during flight and as they forage, and inside flowers they visit (Herrera, 1995).

We first present the results of a literature survey to quantify how many studies have considered social vs. solitary bee responses to warming. We use this survey to draw attention to the paucity of work on the responses of solitary bees to heat shocks and heat waves, in particular. We conclude by suggesting directions for future research to improve our understanding of the impacts of warming on solitary bees.

2 Literature survey

We conducted literature searches to determine how many studies on bee responses to warming have involved solitary bees. We also used these searches to identify and synthesize studies on solitary bee responses to heat shocks, heat waves, and sustained warming. All topic searches were conducted in the Web of Science database, included “article,” “early access,” and “review article” document types, and spanned all years. Aside from the terms of interest, the parameters for each search were identical.

We first conducted a search for studies on all three types of warming and social bees, using a topic search for (warming OR warmed OR heating OR heated OR “heat shock*” OR heatshock* OR “heat wave*” OR heatwave*) AND (“social bee*” OR honeybee* OR “honey bee*” OR bumblebee* OR “bumble bee*” OR “stingless bee*”). This search yielded 1,013 results. We then repeated this search but replaced the social bee search terms with solitary bee search terms, using the topic search string (warming OR warmed OR heating OR heated OR “heat shock*” OR heatshock* OR “heat wave*” OR heatwave*) AND (“solitary bee*” OR “nonsocial bee*” OR “non-social bee*”), which returned 86 results.

We next constructed search strings that focused on either heat shocks or heat waves and either social or solitary bees. First, focusing on heat shocks, a topic search for (“heat shock*” OR heatshock*) AND (“social bee*” OR honeybee* OR “honey bee*” OR bumblebee* OR “bumble bee*” OR “stingless bee*”) gave 139 results that dealt with social bees. In contrast, the same topic search on solitary bees using the string (“heat shock*” OR heatshock*) AND (“solitary bee*” OR “nonsocial bee*” OR “non-social bee*”) produced 10 results. Second, focusing on heat waves, a topic search for (“heat wave*” OR heatwave*) AND (“social bee*” OR honeybee* OR “honey bee*” OR bumblebee* OR “bumble bee*” OR “stingless bee*”) yielded 37 studies on social bees. Repeating the same search

for solitary bees with the string (“heat wave*” OR heatwave*) AND (“solitary bee*” OR “nonsocial bee*” OR “non-social bee*”) returned 2 results.

In summary, of the 1,099 studies that were detected in our literature search on warming and bees, only 7.8% (86) considered solitary bees in their titles, abstracts, or keywords (Figure 1). Narrowing to heat shocks and heat waves, we found that solitary bees were similarly underrepresented. Only 6.7% of the studies on heat shocks and 5.1% of the studies on heat waves considered solitary bees, amounting to 10 and 2 studies, respectively (Figure 1). Because many terms are used to describe lower-magnitude, longer-term warming, we were not able to isolate studies on bee responses to sustained warming specifically, but of the 1,099 studies identified in our first two broad searches on all types of warming, 911 did not consider heat shocks or heat waves, meaning that 82.9% of studies dealt with less-extreme warming. Thus, the vast majority of studies on bee responses to warming do not address heat shocks or heat waves.

In the sections that follow, we summarize the main findings of the studies identified in our literature search that pertain to solitary bee responses to heat shock, heat waves, and sustained warming. As this is a mini-review rather than a systematic review, our goal is to highlight key findings across different types of warming, rather than to exhaustively review all studies.

3 Heat shocks

Of the studies identified by our literature survey, all those that directly applied heat shock focused on species of *Megachile* (Megachilidae), examining the effects of heat shock at various developmental stages. In *M. rotundata*, work has involved heat shock at the prepupal, pupal, and adult stages, with documented consequences for gene expression and life history traits. In *M. apicalis*, the effects of heat shock were similarly documented at the genetic level and in terms of survival across developmental stages. We first summarize the findings for *M. rotundata* before turning to those for *M. apicalis*.

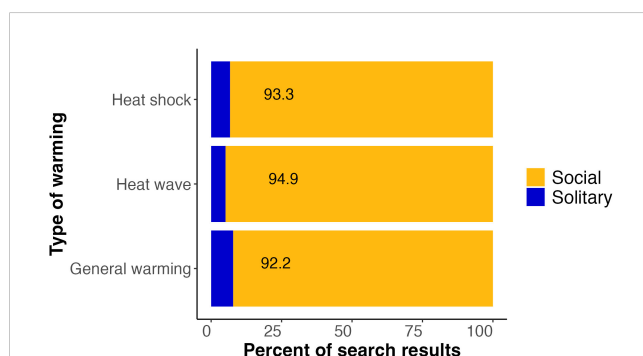


FIGURE 1

Bar plot showing the percent of literature search results for three different categories of warming (heat shock, heat wave, and general warming) that included terms related to social (orange) or solitary (blue) bees in the titles, abstracts, or author keywords. Across the three types of warming, 5.1–7.8% of the studies identified by these searches involved solitary bees. Detailed information on search terms is provided in the text.

A study focused on *M. rotundata* pupae examined the expression of heat shock protein genes following a 1-hour heat shock at 40°C in the lab (Yocum et al., 2005). Transcript expression screens indicated that certain heat shock proteins were strongly upregulated in heat-shocked pupae post-diapause (Yocum et al., 2005). Relatedly, possible differential upregulation of heat shock protein genes was hypothesized to underlie differences in adult *M. rotundata* survival following a 1-hour heat shock at 50°C in the lab (Hayes and López-Martínez, 2021). Timing of the heat shock was an important predictor of starvation survival, with bees shocked in the morning surviving longer than bees shocked in the afternoon, possibly because preparatory stress responses follow a circadian rhythm. Life stage was also an important predictor of response to heat shock. Overall, prepupae and pupae tolerated heat shock, whereas adults had reduced activity, shorter lifespans, and elevated risk of mortality. Indeed, for adult male bees the mortality rate was 80% during the 3 weeks following the heat shock treatment, whereas none of the control bees died in that time. In contrast, adult female bees that were heat-shocked showed no difference in mortality rates compared to control bees. The reasons underlying this difference in male and female mortality rates are not well-understood in *M. rotundata*, but the greater sensitivity of males to heat shock could translate into altered sex ratios. Similarly, a slightly less severe 1-hour heat shock at 45°C delayed adult male emergence but did not delay female emergence. Together, these findings point to life stage-specific and sex-specific heat shock effects in *M. rotundata*, with adult males exposed to afternoon heat likely being the most vulnerable (Hayes and López-Martínez, 2021).

In *M. apicalis*, nest site preferences, stress responses, and offspring survival rates were studied in the context of heat shock in a field setting (Hranitz et al., 2009). Artificial nesting cavities were placed in the field and oriented such that they were either exposed to direct sunlight (south-facing) or shaded (north-facing), with maximum temperatures within nests approaching 50°C during the hottest 2–3 hours of the day in the exposed treatment. Females preferentially nested in the shaded cavities, where the rate of nest failure was lower (8.9% vs. 23.2%). Indeed, the exposed cavities induced stress responses in the form of elevated heat shock protein concentrations across offspring developmental stages, especially eggs. Although offspring survival did not differ between exposed and shaded nests, a greater proportion of offspring died for unexplained reasons in the exposed cavities, while a greater proportion of larvae died due to parasitoids in the shaded cavities. Thus, heat shock can induce physiological stress responses in offspring within semi-natural nesting cavities and can likely result in similar levels of mortality as those imposed by parasitoids in *M. apicalis* (Hranitz et al., 2009).

4 Heat waves

To the best of our knowledge, the effects of heat waves have been studied in only two taxa of solitary bees, *Osmia* (Megachilidae) and *Xylocopa* (Apidae). In *Osmia*, work has primarily examined how heat waves affect development of larvae and critical thermal

maxima of adults, whereas in *Xylocopa*, work has assessed shifts in critical thermal maxima of adults in response to heat waves. We summarize the findings for *Osmia* first, then *Xylocopa*.

Using *Osmia lignaria*, a lab study subjected eggs and larvae to four different heat wave treatments, with maximum temperatures of either 31 or 37°C, lasting 4 or 7 days (Melone et al., 2024). Although heat wave duration did not affect mortality, eggs and larvae exposed to heat waves of 37°C had higher mortality than those exposed to heat waves of 31°C, which in turn did not differ in mortality rates compared to larvae maintained at the control temperature (25°C). Similarly, larval development time was slowed by 37°C heat waves in comparison to the control treatment, whereas 31°C heat waves did not affect development time. Finally, for bees that completed larval development and spun cocoons, heat wave treatment did not translate into any effects on adult emergence probability. Thus, magnitude rather than duration of warming was key to explaining the larval responses of this cavity-nesting species to heat waves, indicating that even shorter-term episodes of temperatures that exceed the optimum temperature range by as little as 6°C can cause mortality of immature life history stages (Melone et al., 2024).

Although not identified in our literature search, likely because the term “extreme heating event” is used in place of heat wave, another lab study examined heat wave responses in adult females of both *O. lignaria* and *O. cornifrons* (Porrás et al., 2023). Specifically, critical thermal maxima were measured after bees were warmed to approximately 30°C and held at that temperature for approximately 1 day. In both species, the critical thermal maxima of female bees previously subjected to the heat wave treatment were reduced by several degrees on average, suggesting that exposure to heat waves can erode thermal tolerances in these species, exacerbating their vulnerability to near-future warming events (Porrás et al., 2023).

A larger lab study that exposed six bee species to heat waves included two species of solitary bees, *Xylocopa violacea* and *X. olivieri* (Gonzalez et al., 2024). Although both *Xylocopa* species had higher critical thermal maxima than the other, smaller species in the study, neither maxima increased when adult bees were exposed to 32°C for 2 days. Thus, bees did not acclimate by this important metric when warmed to a moderate temperature that, although more than 10°C below the critical thermal maxima of either species, represents a 5°C increase above mean summer temperatures for these wild-caught bees. These findings indicate critical thermal maxima do not respond plastically to heat wave conditions in these solitary species, suggesting that similar warming events will force bees to rely on behavioral mechanisms to avoid overheating (Gonzalez et al., 2024).

5 Sustained warming

Experimental sustained warming of solitary bees has been implemented in several studies, especially those focusing on agriculturally important bee genera, such as *Osmia* and *Megachile*. Because these studies encompass a wide array of lower-magnitude, longer-term warming treatments, rather than detailing the methods of each study, we outline some of the key findings, largely organized by species.

In a field experiment focused on *O. ribifloris*, nest boxes were painted black or white to promote passive warming or cooling, respectively (CaraDonna et al., 2018). Bees that developed within the warmed nests emerged with lower body mass and fat content, likely due to elevated metabolism, and experienced 30–73% mortality, whereas bees that developed in the cooler nests experienced <4% mortality. Warmed bees also emerged later, which could reduce synchrony with floral resources. Ultimately, such high mortality rates could lead to extirpation of populations from the warmest parts of the distribution (CaraDonna et al., 2018).

In *O. bicornis* (synonym *O. rufa*), offspring survival decreased by about 30% in experimentally warmed nests in the field primarily due to egg mortality, and females preferentially selected nest sites with more moderate temperatures. For adults that successfully completed development within warmed nests, sustained warming led to weight loss in both sexes (Ostap-Chec et al., 2021). Another study that imposed sustained warming on *O. bicornis* nests in the field found warming (in combination with low flower abundance) was associated with reduced female body size but increased male body size, as well as a male-biased sex ratio that likely reflects greater female investment in the offspring sex that requires fewer resources. Although the reasons for larger male size with warming are not understood, larger males may have reduced mating success, and possible consequences of smaller female size include reduced reproductive output (Zaragoza-Trello et al., 2021). In a lab setting, the duration of almost all developmental phases (except prepupal) decreased with elevated temperatures in *O. bicornis*, as did adult survival rates (Giejdasz and Fliszkiewicz, 2016). Additionally, rearing *O. bicornis* at constant high temperatures vs. naturally fluctuating temperatures in the lab resulted in high mortality of adults (up to 100%), likely because sustained warming elevated their metabolic rates and exhausted their energy reserves (Radmacher and Strohm, 2011).

A lab study that exposed *O. lignaria* to elevated temperatures throughout development (from egg to adult) found that development time was shortened by 50% under the warmest conditions (Bosch and Kemp, 2000). In a follow-up study that imposed constant warmer temperatures only after cocoon completion, development time was similarly generally reduced, and surprisingly longevity of adult females increased slightly under the warmest treatment (Kemp and Bosch, 2005). A lab study focused on experimentally elevated overwintering temperatures for *O. lignaria* documented early emergence, increased mortality, greater fat body depletion, and decreased longevity in response to warming (Bosch and Kemp, 2003). Finally, in a greenhouse study sustained warming of adult *O. lignaria* during the nesting season resulted in bees spending less time foraging, making fewer, shorter visits to flowers, and being more generalized in their foraging behavior (de Manincor et al., 2023).

Several species of *Osmia* and *Megachile* were subjected to temperatures projected under continued climate change in a lab study exploring the effects of changes in fall duration and spring onset in overwintering solitary bees (Slominski and Burkle, 2019). Pre-emergence weight loss was associated with temperature treatment in bees of both genera but tended to increase with warming in *Osmia* while decreasing with warming in *Megachile*. Post-emergence lifespan was reduced by 1–2 days in male and by 1.5 days in female *Osmia* due to sustained warming, but this effect depended

on the timing of spring onset and the duration of cooler temperatures in the fall. In contrast, *Megachile* post-emergence lifespan was longer in the warm temperature treatments. The disparate findings for these two genera may relate to a key difference in their life histories; whereas *Megachile* overwinter as prepupae, *Osmia* overwinter as adults, and therefore may be likely to exhaust their energy reserves before emerging (Slominski and Burkle, 2019).

Altogether, these findings suggest sustained warming will have varied, largely negative effects on these solitary bee taxa. At the same time, study- and species-specific responses point to the need for meta-analytic and trait-based syntheses of solitary bee responses to increases in global mean temperatures.

6 Discussion

In grouping studies by the magnitude and duration of warming, our review indicates that a warmer climate with more frequent and intense intervals of heat will likely negatively affect solitary bees physiologically and behaviorally (Figure 2). First, we found that the effects of heat shocks on solitary bees have been studied solely in Megachilid bees and cause upregulation of heat shock protein genes in immature stages and increased mortality in adult male bees (Yocum et al., 2005; Hranitz et al., 2009; Hayes and López-Martínez, 2021). Second, we found few studies on solitary bee responses to heat waves, but the limited evidence available indicates these events cause higher mortality and slower development in larvae (Melone et al., 2024). Further, not only may solitary bees fail to acclimate by increasing their critical thermal maxima but also their thermal tolerances may be reduced by heat waves (Gonzalez et al., 2024; Porras et al., 2023). Third, our survey revealed that of the three types of warming we considered, sustained warming has been relatively well-studied in solitary bees across developmental stages. Multiple studies found that sustained warming can accelerate development and result in lower body mass, likely as fat bodies are depleted,

ultimately elevating mortality (Bosch and Kemp, 2003; Radmacher and Strohm, 2011; CaraDonna et al., 2018). Changes to solitary bee foraging behavior are also likely, with direct consequences for pollination success (de Manincor et al., 2023). Altogether, the available empirical data on these different types of warming indicate negative fitness consequences that span life history stages.

Our review points to several gaps in our understanding of how continued climate warming will affect solitary bees. First, we identified a need to broaden the assessment of heat shock to other solitary taxa beyond the two species of *Megachile* that, to the best of our knowledge, represent the only studies to date. A second area where more research is needed is in understanding the effects of heat waves on solitary bees, particularly in a field setting. Heat waves have increased in frequency and intensity in most land areas over the past several decades and are projected to continue to intensify with greater levels of global warming (IPCC, 2023). The few lab-based studies we have identified here suggest that heat waves may have disastrous effects on solitary bees, potentially causing high levels of larval mortality (Melone et al., 2024).

Our review also suggests a pattern of studying the effects of heat shocks, heat waves, and warming in general on social, colony-forming bees, which are unlikely to reflect the responses of solitary bees. For example, although the nests of some social bees such as *Bombus* species (Apidae) may be more vulnerable to warming than previously realized (Kevan et al., 2024), many social bees (e.g., *Apis* species [Apidae]) rely on workers to behaviorally moderate nest temperatures (Jones et al., 2004). However, behavioral thermoregulation of nests does not occur in solitary bees, potentially leaving their offspring especially defenseless to extreme warming.

Lastly, many of the studies we reviewed involved bees used in commercial pollination, likely due to the relative ease of rearing and experimentally manipulating managed bees. Thus, although we acknowledge the value of using agriculturally important pollinators for climate change research, we have much to discover about the effects of warming on truly wild solitary bees.

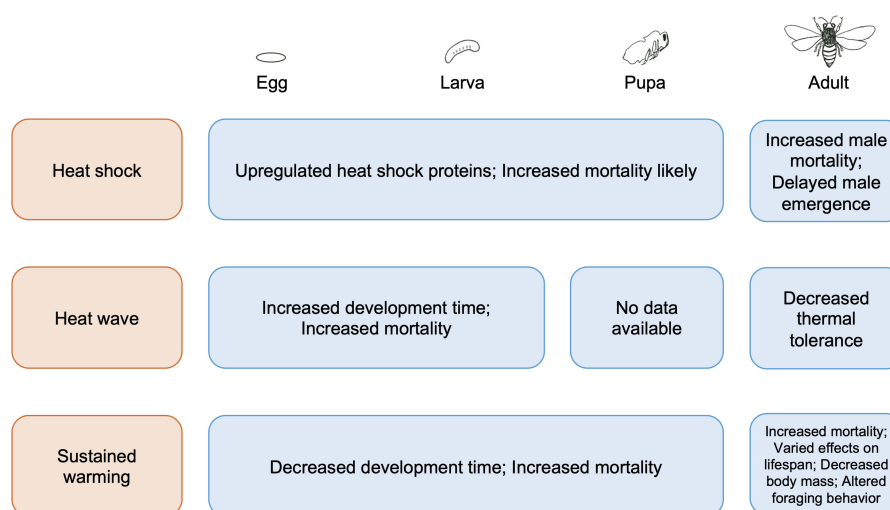


FIGURE 2

Summary of the direct effects of heat shocks, heat waves, and sustained warming on solitary bees at various life stages (egg, larva, pupa, and adult).

Taking a longer-term view from observational field studies that encompass multiple generations, there is evidence that traits such as body size and nesting habit will interact with climate warming to influence bee population dynamics and community composition. For example, trait-based studies indicate that small-bodied bee species may increase in abundance under warmer temperatures (Pardee et al., 2022; Maihoff et al., 2023). In contrast, large-bodied, comb-building cavity-nesting bees are expected to decline in relative abundance under long-term climate warming (Pardee et al., 2022). Finally, a trait-based analysis conducted in the context of urban environments that can act as heat islands suggests that both solitary and cavity-nesting bees are likely to decline under future warming (Hamblin et al., 2017). These longer-term projections, together with the effects of the types of warming we reviewed, point to major perturbations to wild bee ecology and suggest key functional roles may be lost from bee communities with climate change.

In conclusion, there is a pressing need to improve our understanding of how solitary bees, particularly unmanaged solitary bees, will be affected by warming of various magnitudes and durations. An important step will be to incorporate greater complexity and realism in our study of bee thermal ecology, a goal that can help to reveal the emergent effects of warming on bees as parts of ecological communities. As we approach a future in which bees are faced with heat shocks, heat waves, and sustained warming, concurrently with drought and other environmental stressors, this basic information is critical to informing conservation of wild solitary bees and the valuable pollination services they provide.

Author contributions

KV-R: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. NR: Conceptualization,

Investigation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The honey bee “hive” microbiota: meta-analysis reveals a native and aerobic microbiota prevalent throughout the social resource niche

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The microbiome of the honey bee worker hindgut has been explored thoroughly with culturing and next-generation sequencing revealing both composition and function. However, less effort has been devoted to the aerobic social niches associated with the hive environment and colony process. We performed a meta-analysis of 3,800+ publicly available 16S rRNA gene sequence libraries examining the hypothesis of a native aerobic microbiota associated with social interaction and colony resources. We selected high-throughput studies to represent tissue-specific samples, including nine distinct aerobic niches throughout the colony and hive, defined by social nutrient processing. These included queen and worker gut tissues, foregut, midgut, ileum, rectum, mouthparts, worker social glands, developing larvae, and secreted and stored nutrition. We found that the aerobic mouthparts, foregut and midgut niches of queens and workers share a significant portion of their microbiome with that of larval rearing and nutrient secretion and storage, defining the microbiota of the social resource niche. Characterized by species dominance and rapid growth, the social resource microbiota functions primarily in disease prevention at both the individual and colony level and may also function in social communication and gut microbiome resilience. Defining the microbiota of social function contributes to a systems-level understanding of host–microbial interactions in the honey bee.

KEYWORDS

aerobic bacteria, social microbiome, nest microbiome, larvae, queen, disease resistance

Introduction

The honey bee (*Apis mellifera*) is a colonial insect species domesticated worldwide for honey production and pollination services (Gallant et al., 2014; Aslan et al., 2016). The colony and associated built structure consist of a reproductive queen, thousands of cooperative sterile workers, developing larvae, stored food, and a highly predictable

hindgut microbiome that populates worker bees (Kwong and Moran, 2016). The honey bee colony has been described as a superorganism because complex social communication and behavioral interactions between individuals result in emergent group properties that benefit the colony as a whole (Fewell, 2003). Herein, we consider the ecology of the holobiont, a unit of selection that includes the genomes of the host and its associated microbiome (Bordenstein and Theis, 2015; Moran and Sloan, 2015). More specifically, we define and discuss the aerobic microbiome associated with healthy social (group) hygiene that occurs on, within, and throughout colony and hive environment of the honey bee host. Strongly allied with the processing and sharing of information and nutrition, the collection of aerobic or microaerophilic niches including stored and secreted nutrition, and host anatomical features we refer to herein as the social resource niche (SRN). While this broad niche space includes “nest materials” like honey and stored pollen, taxonomic similarity across studies suggests that the SRN may extend to the larval gut and the mouthparts, glands, foreguts, and mid-guts of both queens and workers (Figure 1).

Honey bee life history is amenable to the co-evolution of strict host–microbial relationships. The founding worker population (reproductive swarm) presents a broad and continuous microbial niche for holobiont evolution (Winston, 1987; Engel et al., 2012; Rothman et al., 2018). The honey bee colony shows perpetual worker production, continuous colony fission, and continuously overlapping adult worker generations (Seeley, 1989). The worker bees of the present moment have had continuous and intimate physical contact with their ancestral lineage, as it stretches back through the ages. The depth and continuity of the surviving swarm and lack of a reproductive bottleneck have thus facilitated intimate co-evolution of the total microbiome with the host organism. Here, we suggest that similar to other complex social groups, microbes that contribute to the informational and hygienic function of the nest and colony environment are transmitted across generations with the founding host (Breed et al., 1988; Haeder et al., 2009; Goldstein and Klassen, 2020). With this idea in mind, we review data from 35 NCBI bio-projects to better define the microbiota inhabiting the SRN (Anderson et al., 2013; Vojvodic et al., 2013; Maes et al., 2016; Anderson and Ricigliano, 2017; Anderson et al.,

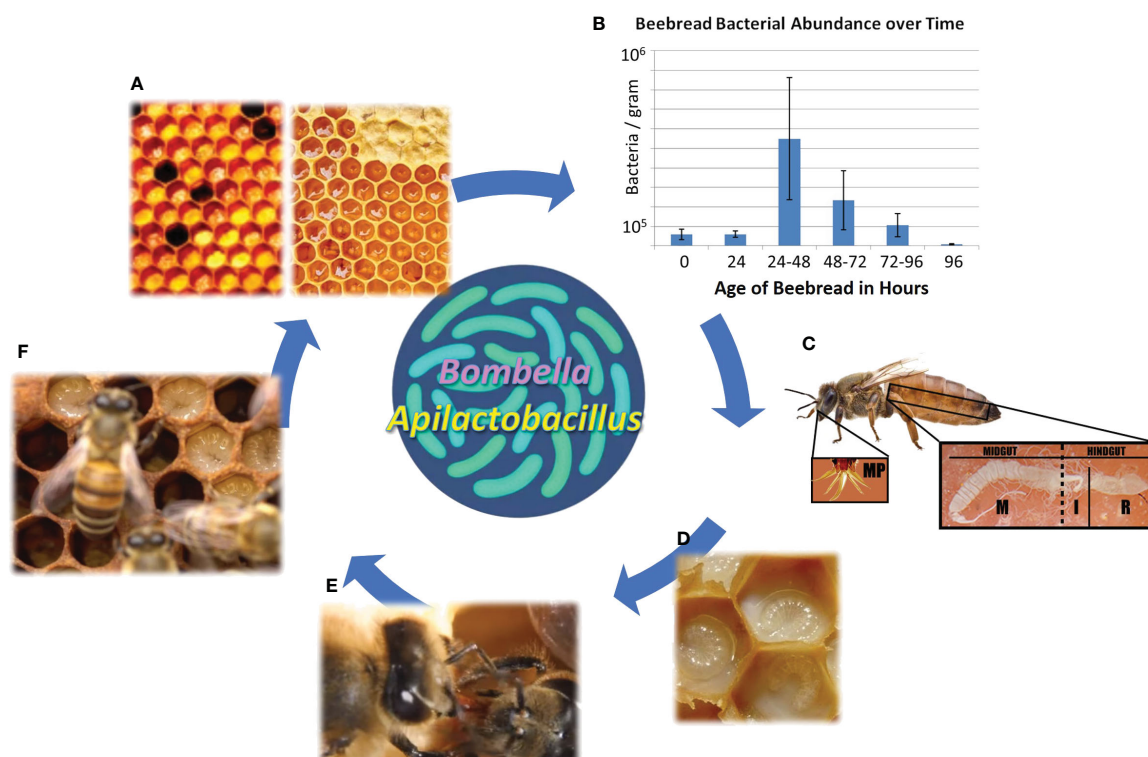


FIGURE 1

The social resource niche includes the anatomical features and bioactive substances associated with sharing behavior and colony hygiene.

(A) Collected nectar is processed into honey, while collected pollen is processed into beebread, both of which are consumed by young adult worker bees as their gut microbiome self-assembles. (B) The storage of beebread fuels a predictable burst of native microbial growth, including sugar tolerant yeasts, that peaks at 24–48 h post-collection before declining precipitously. This is the preferred age at which stored pollen is consumed by nurse bees, and the process aids in the hygienic filtering of bacteria and yeasts vectored from the pollination or floral environment. (C) New adult worker bees become nurse bees, and from a modified head gland (HPG), secrete a highly nutritious and antimicrobial jelly that sustains queen egg-laying and larval development. The queens mouthparts and midgut are often saturated with royal jelly. (D) The highly nutritious jelly contains antimicrobial peptides and other microbial deterrents and can be customized to some degree to meet the immediate social needs of the colony. (E) Social sharing results in the colony-level distribution of antimicrobial substances and associated microbes. (F) The substances and associated microbes provide pathogen protection for developing larvae.

2022). We predict that the social resource microbiota (SRM) is similar across studies and locations and shared throughout the SRN in accordance with colony function. As a null hypothesis, non-native (environmental) microbes vectored from the local pollination environment may typify the SRN.

The SRN is deeply antimicrobial and influenced by colony-level factors like worker activity level and behavioral role. The mechanical processing associated with nutrient storage, honey production, and brood rearing are layered with raw nutritional resources (Anderson et al., 2013). Three substances produced by the honey bee dominate the SRN and are integral to microbial health: honey, jelly, and propolis. All three are uniquely antimicrobial, function throughout distinct but overlapping niche space, and display specialized relationships with native hive microbes (Vojvodic et al., 2013; Corby-Harris et al., 2014b; Dalenberg et al., 2020; Anderson and Maes, 2022). In general, fructophilic lactic acid bacteria (*Apilactobacillus*) specialize on honey and *Bombella* species on jelly (Endo et al., 2012; Vojvodic et al., 2013). Produced from collected tree resin, propolis is mixed with host secreted wax and distributed throughout the colony. Propolis promotes microbiome and immune health and provides a form of socialized medicine (Simone et al., 2009; Evans and Spivak, 2010; Dalenberg et al., 2020). Active in both individual and group level immunity (Evans and Spivak, 2010; Anderson and Ricigliano, 2017; Zheng et al., 2018), these three substances and their associated co-evolved microbiotas strongly mitigate microbial growth throughout the SRN. When the activity of worker bees (the colony) is removed from the built structure, resident fungal and bacterial opportunists consume the hive environment. It appears that many of the behavioral processes, substances, and microbes associated with the SRN actively maintain colony hygiene, mitigating disease, and opportunism (Figure 1).

The SRM aids in the rapid sterilization of collected pollen or “beebread” (Figure 1), a process dominated by *Bombella*, *Apilactobacillus*, and sugar-tolerant yeasts (Anderson et al., 2014; Anderson and Mott, 2023). Pollen foraging introduces a spectrum of environmental bacteria to the hive environment, but the subsequent treatment of collected pollen and nectar within the hive promotes survival of the SRN microbiota relative to introduced microbes (Anderson et al., 2014; Anderson and Mott, 2023). Half honey by weight, beebread coopts most of its antimicrobial properties from honey (Nicolson, 2011). During the conversion of collected pollen into beebread, the SRM grows fast, producing an extreme acidic environment at the oxygen interface, much like the production of silage in agriculture (Anderson et al., 2014). *Apilactobacillus* can grow rapidly with exposure to oxygen and can also metabolize p-coumaric acid, a biologically vital monomer abundant in pollen cell walls (Mao et al., 2013; Endo et al., 2018). The SRM peaks in size in 1–2-day-old pollen stores, concurrent with significantly increased beebread consumption by newly emerged worker bees (Anderson et al., 2014; Carroll et al., 2017). Beebread is consumed quickly by the worker bee population to avoid negative host effects associated with long-term pollen storage like increased mortality, delayed development, gut dysbiosis, and increased disease susceptibility (Anderson et al., 2014; Maes et al., 2016; Roessink and van der Steen, 2021). Following the digestion of

beebread in the midguts of newly emerged worker bees, hypopharyngeal glands in the head synthesize royal jelly as food for developing larvae and the queen (Feng et al., 2009; Harwood et al., 2021). The HPG and its secreted jelly can contain tailored cocktails of pro-oxidants, antioxidants, and antimicrobial peptides that interface constantly with the SRM and the social information network (Buttstedt et al., 2013; Vojvodic et al., 2015; Anderson and Maes, 2022).

While populations of aerobic bacteria are common in the worker mouthparts, foregut, and midgut, the rectum houses generally anaerobic fermentative metabolism, and the ileum represents a transition from microaerophilic to anaerobic metabolism (Engel et al., 2012; Zheng et al., 2017). However, the hindgut microbiota alters in accord with gut physiology, often supporting aerobic growth in the rectum (Anderson and Ricigliano, 2017; Callegari et al., 2021). In this contribution, we perform a niche-specific meta-analysis of the total honey bee microbiota. We include oxygenated and nutrient-rich niches throughout the SRN including tissue-specific sequencing of aerobic to anaerobic gut niches of workers and queens, mouthparts, foreguts, hypopharyngeal (social) glands and their secretions (royal jelly), developing larvae, beebread, and honey. Using >3,800 libraries from publicly available datasets, we curate, distill, and standardize taxonomy of the total microbiome, testing the hypothesis of a consistent microbiota shared throughout the SRN.

Methods

We selected a broad list of microbiome studies to represent variation of the total honey bee microbiome and emphasize aerobic niches. We normalized these datasets by retrieving the raw data sets then processing them via the same bioinformatics pipeline. The selected studies differ by method (primer sets and extraction protocols) and biological variation: physiological subsets of honey bee life history capturing environments within the gut, colony, and hive, including aerobic, microaerophilic, and anaerobic niches. As our primary goal, we test the hypothesis of a social resource microbiota, using a subset of libraries that have explored a variety of aerobic niches. Supplementary Table S1 describes some of the features (primers, niche, and location) of the 35 NCBI bio-projects included in this meta-analysis (Anderson et al., 2014; Corby-Harris et al., 2014a; Kapheim et al., 2015; Jia et al., 2016; Hubert et al., 2017; Anderson et al., 2018; Jones et al., 2018; Motta et al., 2018; Rothman et al., 2019; Subotic et al., 2019; Taylor et al., 2019; Daisley et al., 2020; Dalenberg et al., 2020; Kešnerová et al., 2020; Sopko et al., 2020; Vernier et al., 2020; Alberoni et al., 2021; Callegari et al., 2021; Damico et al., 2021; Maes et al., 2021; Wang et al., 2021; Anderson and Maes, 2022; Anderson et al., 2022; Liberti et al., 2022; Copeland et al., 2022a, 2022b; Anderson et al., 2023).

Selected studies used 454-amplicon sequencing or Illumina high-throughput sequencing to target 16S rRNA genes producing paired-end reads. Read libraries were processed using mothur v.1.44.3 (Schloss et al., 2009). Paired-end reads were merged and quality filtered using the make.contigs command in mothur v.1.44.3

(Schloss et al., 2009). The command `fastq.info` was used to create a fasta and quality file. Next, `rim.seqs` was used to remove sequencing barcodes. The `merge.files` command was used to combine fasta, count_tables, and group files. The command “`screen.seqs`” was used to filter sequences with >1 ambiguous bases and a maximum homopolymer length of eight bases.

To assign sequences across different hypervariable regions of the 16S rRNA gene, we employed a closed-reference OTU workflow using the BEEexact database (Daisley and Reid, 2021). BEEexact allows for species-level taxonomic resolution for honey bee-associated bacteria compared to traditional databases like GreenGenes and Silva (Daisley and Reid, 2021). Chimeras were removed and sequences were clustered into OTUs at 97% similarity using VSEARCH (Rognes et al., 2016). The “`merge.otus`” command in `mothur` (Schloss et al., 2009) was used to generate species and genus-level OTUs for further analysis.

Many of the sequenced environments or tissues included in this meta-analysis reflect high exposure to oxygen and highly concentrated sources of nutrition, including early instar larvae, queen and worker mouthparts, foreguts, and midguts. These niches can vary greatly in microbial load (10^4 – 10^8 gene copies per tissue) but average low microbial biomass relative to the worker hindgut (10^6 – 10^9 gene copies per tissue). To determine the likelihood of native aerobic microbiota, we applied a threshold of 1% relative abundance and 70% prevalence across all libraries. The threshold of 70% was chosen to account for the high degree of variability (gut and hive niches) in our dataset, allowing us to capture more biologically relevant signal. Because some of our samples were from low-abundance DNA environments (Salter et al., 2014), we curated sequences to identify and remove sources of contamination. We classified potential contaminants and sparse OTUs based on known culturing results, curated data collections, and documented contaminants specific to reagents or laboratories (Salter et al., 2014; Anderson et al., 2023).

The community composition of samples by niche was assessed with ANOSIM (Bray–Curtis dissimilarity) implemented in `mothur` (Clarke, 1993). We used a PERMANOVA test to estimate beta diversity variation associated with niche using the ADONIS function from the `vegan` package performed with 1,000 permutations. We ran a linear discriminant analysis (LDA) of the top 10 OTUs to test the hypothesis of a SRN microbiota shared between larvae, workers, queens, and the hive environment. We used Circos plots (Krzywinski et al., 2009) to display the bacterial relationships by niche.

Results

We analyzed high-throughput 16S rRNA gene sequence archives from 35 NCBI bio-projects representing honey bee alimentary tract tissues, reproductive caste, larval development, secretory glands, and nutritional resources. Overall, VSEARCH assigned 5,773,026 of 7,118,258 (81.10%) of unique sequences and 128M of the 141M sequence reads (90.47%) were at least 97% similar to a representative full-length sequence in the BEEexact database (Daisley and Reid, 2021). Sequences that failed to match

97% identity to any reference sequence are examined in a different publication. Our species and genus-level OTU tables provided 1,972 and 669 OTUs, respectively. OTUs occasionally clustered into “x_bxid####”, where x is the first initial of the taxonomic level and the #s are unique identifiers distinguishing group members at each taxonomic rank. These are placeholder names in the BEEexact database given to sequences with <98.7% identity to type strain representatives (Supplementary Table S1).

Associated with high-throughput metagenomics studies investigating low-abundance DNA environments, we identified a number of contaminant OTUs consistent with previous identification from amplicon libraries investigating early instar larvae (Anderson et al., 2023). Our results linking particular OTUs to contamination is further reinforced by previous results sequencing low-abundance queen gut environments and blank controls (Anderson et al., 2018). As determined by previous criteria, we designated the following OTUs as the top 8 contaminants: *Ralstonia*, *Caulobacter*, *Bradyrhizobium*, *Pelomonas*, Cyanobacteria, *Lysinibacillus*, *Shigella*, and *Nevskia*, and more generally, Chitinophagaceae, Comamonadaceae, Caulobacteraceae, Burkholderiaceae, and Bradyrhizobiaceae. We note that many OTUs confirmed by culturing can also present the character of a contaminant, and the rare biosphere of honey bees remains to be confirmed. Although most prevalent in low-abundance DNA environments, contaminant sequences are found at lower abundance throughout the data set in association with deep sequencing efforts of the worker gut.

A total of 10 genera met a minimum threshold of 70% prevalence with at least 1% relative abundance: *Lactobacillus*, *Gilliamella*, *Snodgrassella*, *Bombilactobacillus*, *Frischella*, *Bifidobacterium*, *Bombella*, *Apilactobacillus*, *Commensalibacter*, and *Bartonella* (Figure 2). We found that the SRM is comprised of two major genera, *Bombella* and *Apilactobacillus*, and various species of *Lactobacillus* shared across aerobic niches including larvae, worker and queen mouthparts, worker crops, worker hypopharyngeal glands, queen crops and midguts, beebread, royal jelly, and honey. Based on linear discriminant analysis and the resulting feature space, we found a strong taxonomic overlap of nine distinct niches, driven primarily by the frequency and abundance of three major bacterial genera (Figure 3). These niches are dominated by *Apilactobacillus*, *Bombella*, and *Lactobacillus*. *Bombella* and *Apilactobacillus* account for >40% of reads throughout the SRN (Figure 4). *Fructobacillus* accounted for approximately 1% of the reads in this study but did not meet our 70% prevalence criteria. *Fructobacillus* clustered with *Apilactobacillus* and *Bombella* by study, and it was most prevalent and abundant in larvae, worker mouthparts, and the anterior queen gut.

We found significant separation of microbiomes by niche with both ANOSIM (R, 0.145, $p = 0.001$) and ADONIS (F15 = 28.9, $p = 0.001$). Linear discriminant analysis reveals an overlap of various bacterial species shared by aerobic niches including larvae, worker mouthparts, worker crops, worker hypopharyngeal glands, queen mouthparts, queen midguts, beebread, royal jelly, and honey. The SRN microbiota is dominated by a few major bacterial species: *Bombella apis*, *Bombella intestini*, *Bombella* spp. (bxid5328),

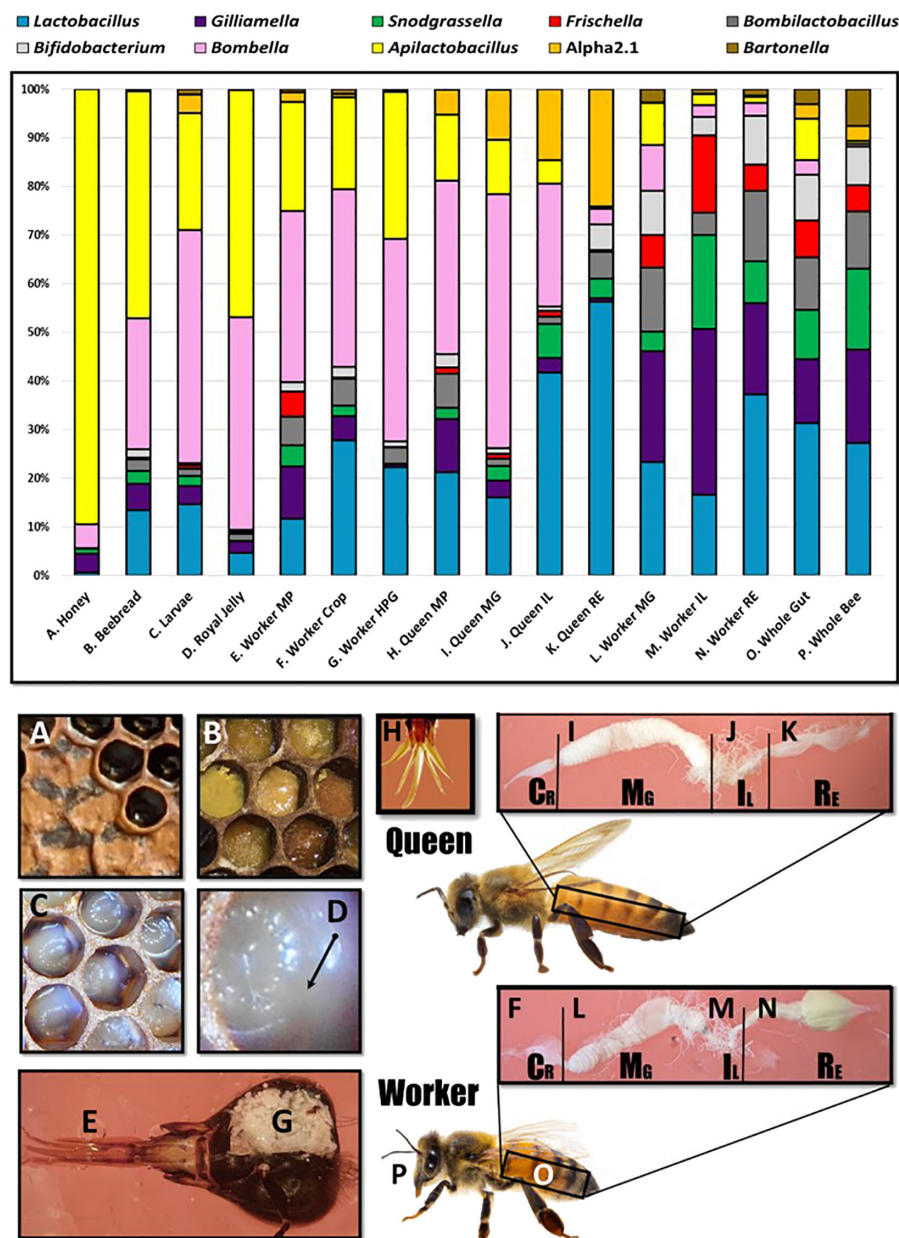


FIGURE 2

Microbiota of the social resource niche displayed as relative abundance in the upper panel. The niche is saturated with processed and shared nutrition: honey (A), beebread (B), and royal jelly (D). Larvae (C) are fed these substances in varying amounts throughout development. The niche also includes anatomical features associated with producing, processing, consuming, or sharing nutrition including the mouthparts (E), crops (F), and hypopharyngeal (social) glands (G) of workers. The mouthpart microbiota of queens (H) and workers is similar, but beginning at the midgut (I, L), the microbiota diverges significantly in membership and structure by caste. Queens (H–K) contain more Acetobacteraceae, both *Bombella* and *Commensalibacter*, while workers (L–P) are typified by the presence/abundance of *Snodgrassella*, *Gilliamella*, and *Frischella*.

Apilactobacillus kunkeei, *Apilactobacillus apinorum*, *Apilactobacillus* spp. (bxid5570), *Fructobacillus fructosus*, and *Fructobacillus* spp. (bxid5666).

Discussion

This meta-analysis united >3,800 raw sequence read libraries to normalize bioinformatic methods and evaluate niche specificity by taxonomic group. Our results highlight a native aerophilic, acid-

resistant and osmotolerant microbiota shared across a number of socially interconnected niches, referred to herein as the social resource niche, SRN (Endo and Salminen, 2013; Vojvodic et al., 2013; Anderson et al., 2014; Corby-Harris et al., 2014b). Results suggest that the social resource microbiota (SRM) is continuously transmitted to the new budding colony and nesting location in the glands, mouthparts, and guts of workers (Figure 2). Correspondingly, the entire core hindgut microbiota of workers occurs with prevalence and abundance throughout the SRN. Prevalent in the literature, a competing hypothesis had long

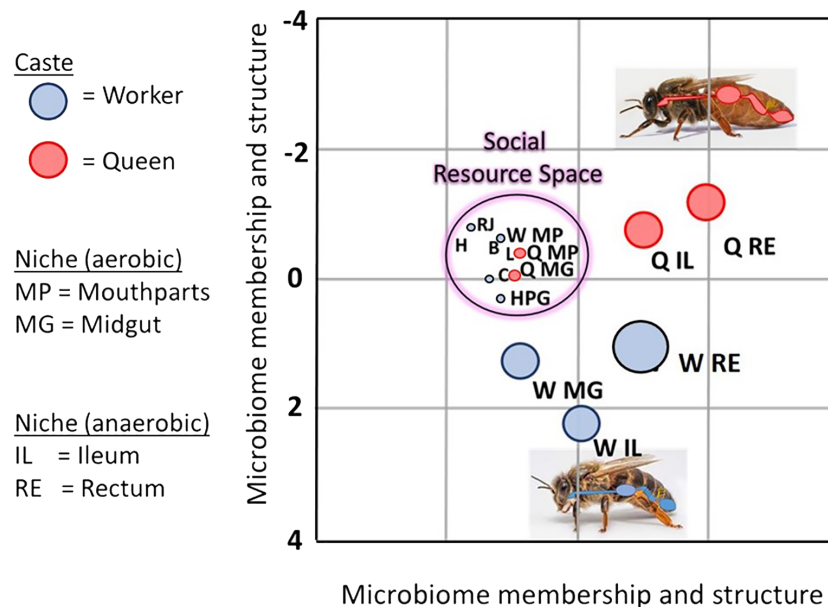


FIGURE 3

Linear discriminant analysis LDA depicts an overlap of various bacterial species shared by aerobic niches including larvae (L), worker mouthparts (W MP), worker crops (C), worker hypopharyngeal glands (HPG), queen mouthparts (Q MP), queen midguts (Q MG), beebread (B), royal jelly (RJ), and honey (H). Dominated by a few major bacterial species, the social resource niche supports the microbiota of the “hygienesphere” because the associated substances, activities, and microbiota are the demonstrated antagonists of the pathosphere.

speculated that these intimate colony niches were dominated by “environmental microbes” introduced from the local foraging environment (Endo et al., 2012; Anderson et al., 2013; Vojvodic et al., 2013; Corby-Harris et al., 2014b; Djukic et al., 2015; Kwong and Moran, 2016). While microbes often originate from the local foraging environment, relatively few microbes have evolved to endure the active hive and colony environment of *Apis mellifera*. The SRM is closely related to bacteria that populate flowers and solitary bees (McFrederick et al., 2012; Vojvodic et al., 2013; Anderson and Ricigliano, 2017; McFrederick et al., 2017), but has evolved to prosper in the antimicrobial environments of royal jelly, propolis, and honey (Kwakman et al., 2010; Dalenberg et al., 2020; Harwood et al., 2021).

Much like the fermentative hindgut microbiome of workers, the SRM provides a living layer of protection that thwarts the growth and establishment of undesirable microbes, both native and introduced. Simply by surviving on the fringe of the colony activity, within the niche preferred by the pathosphere, the SRN microbiome plays a protective role similar to that of human skin or nasal pharyngeal microbiome. The dynamic SRN connects the behavioral and metabolic activities of a colony and maintains continuous contact with the nutrition, substances, activities, and microbiota that typify colony health and growth. The SRN contains molecular information associated with group nutritional state and microbial threats that inform key behaviors of social immunity and more general colony process (Evans and Spivak, 2010; Spivak et al., 2019). Below, we discuss the SRN and SRM in the context of colony hygiene and social life history. Although many core hindgut OTUs (e.g., *Lactobacillus*) occur with frequency and abundance

throughout the SRN, we limit our discussion to the highly aerobic and fast-growing genera *Apilactobacillus* and *Bombella*.

Based on linear discriminant analysis and the resulting feature space, we found a strong taxonomic overlap of nine distinct niches, driven primarily by the prevalence and abundance of two major bacterial genera, *Bombella* and *Apilactobacillus* (Figure 3). Throughout this broad and interconnected niche, the lack of moisture, abundance of oxygen, and the combination of host and microbial products result in a somewhat continuous layer of antimicrobial activity that accompanies the colony processes of nutrition processing, queen maintenance, and larval development (Anderson et al., 2014; Anderson and Ricigliano, 2017; Anderson and Maes, 2022; Anderson and Mott, 2023). Anatomically, this niche includes the surface rich and versatile mouthparts and foreguts of queens and workers, queen midguts, worker head glands that produce social secretions, and developing larvae (Figure 2). The niche also includes stored, processed, and secreted nutrition. More generally, the collective worker behaviors and physiology associated with colony process nurture a broad and interconnected niche conducive to the growth of beneficial microbes and inhibitory towards the growth of non-native or pathogenic microbes (Figure 2).

The SRM is the demonstrated antagonist of the honey bee pathosphere (Schwarz et al., 2015) and non-native microbes that populate flowers. *A. kunkelii*, prevalent in the crop and on the mouthparts, inhibits the growth of 60 different transient flower microorganisms based on *in vitro* growth experiments (Vásquez et al., 2012). *Apilactobacillus* also inhibits the major honey bee pathogens *Nosema*, American foulbrood and European foulbrood

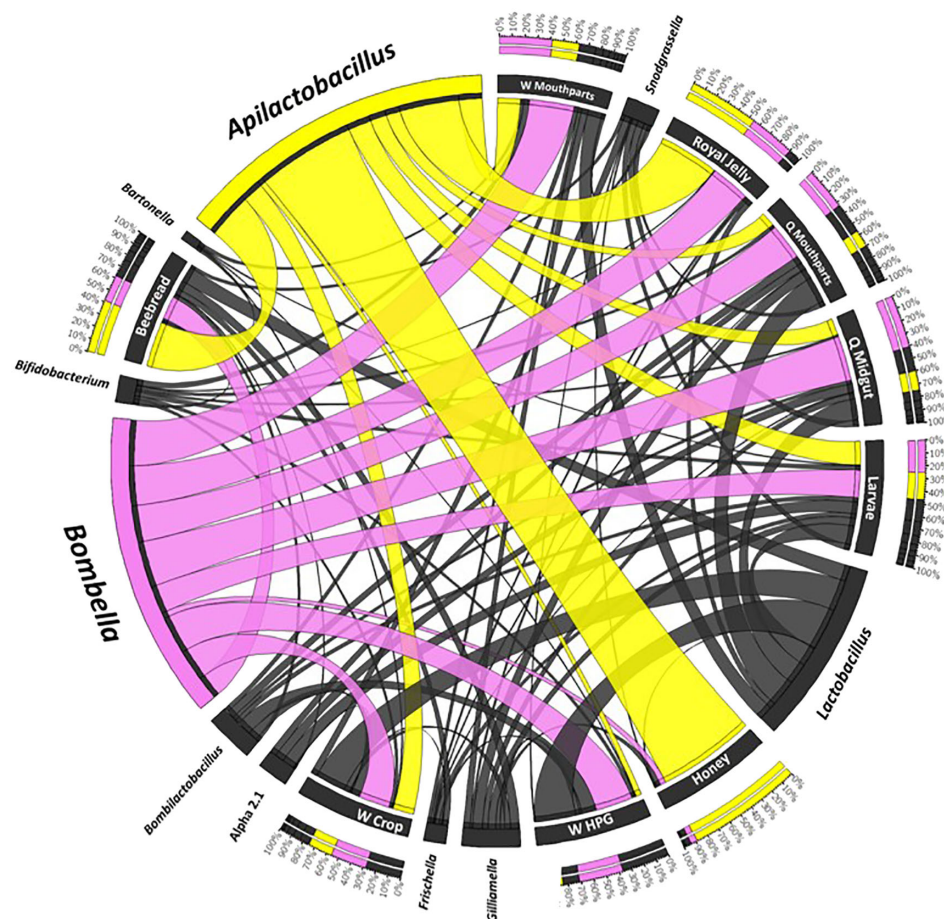


FIGURE 4

A Circos plot of the social resource niche and genera found therein. Plots were generated from a CSS-normalized OTU table with all singletons removed. Each genus is assigned a specific color, and its abundance is directly proportional to the width of each ribbon connecting bacterial taxa to its respective niche. The outer ring represents the cumulative percent of 16S sequences assigned to a given genus from each sample, while the inner circle represents the number of 16S rRNA sequences assigned to a given taxa in a given sample.

(Forsgren et al., 2010; Vásquez et al., 2012; Arredondo et al., 2018; Zendo et al., 2020; Miller et al., 2021). More specifically, *A. kunkleei* produces Kunkecin, a bacteriocin with specificity for European foulbrood (*M. plutonius*), a widespread and destructive pathogen (Zendo et al., 2020). *Bombella* (*Parasaccharibacter*) treatment at the colony level is associated with significantly lower *Varimorpha* (*Nosema*) counts in workers experimentally fed 10,000 *Nosema* spores (Corby-Harris et al., 2016) and often proliferates in the worker midgut, where *Nosema* and sugar-tolerant yeast find their reproductive niche (Corby-Harris et al., 2016). The abundance of *Bombella* shows significant negative associations with general fungal abundance throughout the worker gut (Anderson and Maes, 2022; Anderson et al., 2022). *Bombella* inhibits the growth of a major fungal pathogen *Aspergillus flavus*, based on *in vitro* inhibition assays. This phenotype was confirmed with *in vivo* larval rearing; bee brood supplemented with *Bombella* were significantly less likely to be infected by *A. flavus*. Comparative genome analysis of *Bombella* suggests that fungal inhibition occurs via the secretion of secondary metabolites (Miller et al., 2021).

The worker mouthparts and foregut work together to generate pathogen protection or “social immunity”, including continuous trophallaxis between worker adults, feeding the larvae and queen and nectar dehydration (Nicolson, 2009; Anderson et al., 2013; Dalenberg et al., 2020). The foregut (crop) is simply an expandable bag in the anterior worker gut used to process, hold, and distribute liquid resources, while the mouthparts are rich with surface area and capable of unfolding, reconfiguring, biting, sucking, and lapping. In a process known as “bubbling”, the mouthparts and the foregut swap nectar loads to dehydrate collected nectar (Nicolson, 2009), a highly oxidative and water-removing process that significantly inhibits non-native microbial growth and selects for the growth of native beneficial species (Corby-Harris et al., 2014a). This antimicrobial effect is transmitted throughout the colony by the continuous sharing of liquid food (Crailsheim, 1998). Our results confirm that only *Bombella* and *Apilactobacillus* are primary to the crop niche (Corby-Harris et al., 2014a). While species of *Lactobacillus*, *Bombilactobacillus*, and *Bifidobacterium* are often sampled from the crop and the SRN,

these taxonomic groups show much stronger fidelity for the oxygen depleted hindgut (Moran et al., 2012; Vásquez et al., 2012; Corby-Harris et al., 2014a). Similarly, the worker mouthparts were also dominated by *Apilactobacillus* and *Bombella* and, to a lesser extent, *Fructobacillus fructosus*, another aerobe demonstrated to enhance the growth of other beneficial bacteria throughout the system (Rokop et al., 2015; Dalenberg et al., 2020).

Ubiquitous in the worker crop and on the mouthparts, the nutritional resources jelly and honey (often mixed) are associated with distinct but overlapping microbiomes (Vojvodic et al., 2013; Corby-Harris et al., 2014b; Maeno et al., 2016; Anderson and Maes, 2022). Worker brood rearing and feeding activities are reduced during periods of colony stress and disturbance, and the quality of jelly provided by workers may affect the protective powers of the SRN/SRM. The occurrence and abundance of native aerobes throughout the data set suggest that honey, royal jelly, and propolis promote rapid aerobic growth by providing nutrition, decreasing competition, and reducing the cost of using oxygen (Simone-Finstrom and Spivak, 2010). In one study, *Bo apis*, *A. kunkeei*, and *F. fructosus* were all significantly enriched on the mouthparts in colonies with increased propolis collection and deposition (Dalenberg et al., 2020). These same bacteria can also flourish in honey, royal jelly, larvae, and queens (Endo and Salminen, 2013; Vojvodic et al., 2013; Anderson et al., 2023). Although not considered by this study, similar niche-related factors including worker behavior likely mitigate native populations of sugar-tolerant yeast (Detry et al., 2020).

Honey represents one of the most extreme antibiotic environments known to science (Kwakman et al., 2010). Both *Apilactobacillus kunkeei* and *Fructobacillus fructosus* are specialized to exploit honey. First recognized by Endo, these genera are obligately fructophilic, preferring D-fructose as a carbon source abundant in honey (Neveling et al., 2012; Endo et al., 2018). Oxygen tolerance and utilization is a primary attribute of the SRM, as O₂ is required for rapid growth. *A. kunkeei* grows exceedingly fast under aerobic lab conditions with a doubling time of approximately 1 h, a trait attributed to a suite of large mystery (unannotated) genes that flank the origin of replication (Tamarit et al., 2015), genes that likely enhance survival in honey. Microbes can survive briefly on the periphery of honey, whereas pure honey quickly kills or inactivates all microbial growth due to severe osmotic conditions and acidic pH, a byproduct of honey bee salivary enzymes and microbial fermentation occurring at the hygroscopic and oxygen-rich surface. Fully processed honey (dehydrated to >82% sugars) is sealed with beeswax, a substance impervious to water and atmospheric gasses.

Abundance measures of *Apilactobacillus* and *Bombella* are positively correlated in many studies suggesting resource partitioning. The two major SRN bacteria are specialized to exploit different sugar monomers abundant through the system; *Bombella* prefers glucose. Highly osmotolerant, gluconic-acid producing strains of *Bombella* continue growth at 40%–50% sugar concentrations and pH 3, demonstrating their tolerance for honey-rich environments (Ruiz-Argueso and Rodriguez-Navarro, 1975). *Bo. apis* has lost alternative oxidative pathways and harvests energy

almost exclusively from glucose using oxygen as an electron receptor (Bonilla-Rosso and Engel, 2018). Both *Apilactobacillus* and *Bombella* are core to the queen's gut microbiota (Tarpay et al., 2015; Anderson et al., 2018; Copeland et al., 2022a), and *Bombella* often dominates the queen mouthparts, foregut, ileum, and midgut. The queen's gut supports a magnitude less gut bacteria than workers, suggesting a relatively less hospitable microbial environment (Anderson et al., 2018; Copeland et al., 2022b). This may result from continuous royal jelly exposure and constitutive expression of vitellogenin and other antimicrobial molecules throughout the queen's system (Salmela and Sundström, 2017; Harwood et al., 2019). In the similar environment of larval guts, *Bo. apis* is the first bacterium to populate larvae based on culture-dependent and culture-independent data, and *Bo. apis* and/or *A. kunkeei* dominate later instars (Vojvodic et al., 2013; Floyd et al., 2020; Anderson et al., 2023).

Genome analysis of *Bombella* indicates intimate host-microbial evolution. Niche dominance of *Bo. apis* is facilitated by host-produced glucose oxidase, an enzyme converting glucose into gluconic acid, producing H₂O₂ as a byproduct (Ohashi et al., 1999). *Bo. apis* can then further oxidize gluconic acid, fueling its metabolism (Bonilla-Rosso and Engel, 2018; Smith and Newton, 2020). Genome evidence also suggests that *Bo. apis* has evolved to quickly divert the readily available energy stores in honey or jelly to alleviate osmoregulatory and oxidative stress (Smith and Newton, 2020). As a fast growing aerobe, *Bo. apis* possesses all the conventional mechanisms of oxidative stress management, including superoxide dismutase, catalase, peroxidase, and the suite of enzymes involved in glutathione cycling. Between the inner and outer membrane of *Bo. apis* cells are extensive networks of periplasmic glucans, providing resistance to acids, enzymes, reactive oxygen species (e.g., H₂O₂), and rapid changes in osmolarity encountered in food stores and in the queen and larval gut. Not found in its closest relatives, *Bo. apis* possesses Aquaporin Z, a highly stable transmembrane protein channel that facilitates rapid osmoregulation and resists denaturing due to heat or extremes of pH found throughout the SRN. Consistent with other recent findings (Bonilla-Rosso and Engel, 2018; Smith and Newton, 2020; Härer et al., 2023), our analysis of 16S rRNA sequence variation suggests at least four major *Bombella* species with fidelity for distinct niche space including a novel species of *Bombella* that populates the midgut and ileum of queens (Figure 4).

Although their ecology is poorly known, highly osmotolerant and native species of yeast have likely influenced the evolution of the SRN, SRM, and the hindgut microbiota (Gilliam, 1979; Tauber et al., 2019; Detry et al., 2020; Anderson and Mott, 2023). In the human gut, fungi regulate host physiological processes and assembly of the co-residing gut bacterial microbiome (Nash et al., 2017). In honey bees, a general survey throughout the gut using universal fungal primers shows strong abundance relationships of fungi with species of hindgut bacteria (Maes et al., 2021; Anderson and Maes, 2022; Anderson et al., 2022). Based on detailed microscopy, it appears that the vast majority of fungi found throughout the SRN are native osmotolerant yeasts that can attain high numbers where oxygen is readily available (Detry

et al., 2020; Anderson and Mott, 2023). Consistently, *Apilactobacillus* and *Bombella* show strong negative associations with yeast abundance in the worker gut; fungal load decreases concurrent with increasing bacterial load, attaining greatest values in the midgut and lowest values in the hindgut (Anderson and Maes, 2022; Anderson et al., 2022). However, these yeasts appear unwelcome in some aerobic environments rich in royal jelly including larvae and the queen gut. Queen guts do not tolerate a high fungal load, showing significantly lower fungal load relative to worker guts (Maes et al., 2021; Copeland et al., 2022a). Yeast blooms in times of bacterial gut dysbiosis (Anderson et al., 2022), and *Bombella* is a demonstrated fungal antagonist, inhibiting the growth of both yeasts and molds ubiquitous throughout the SRN (Corby-Harris et al., 2014b; Anderson et al., 2018; Dalenberg et al., 2020; Miller et al., 2021). *Apilactobacillus* also inhibits yeast growth (Vásquez et al., 2012; Bisson et al., 2017).

This exploration and review of the SRN uncovered a variety of Enterobacteriaceae with consistent taxonomy across studies, known to participate in gut dysbiosis of workers and invade the hemolymph (Gilliam and Valentine, 1974; Burritt et al., 2016; Raymann et al., 2019; Anderson and Maes, 2022). We suggest that the SRM acts to supplement the function of a compromised worker gut microbiome and discourage the establishment of opportunistic/pathogenic microbes. Following a disturbance, *Apilactobacillus* and *Bombella* often replace core hindgut bacteria (Anderson et al., 2022). Typically abundant in the worker mouthparts, foregut, and midgut, the SRM may act in gut microbiome resilience and generally suppress the growth of pathogenic microbes throughout the gut (Corby-Harris et al., 2014b, 2016; Anderson and Ricigliano, 2017; Anderson and Maes, 2022). With a shift in gut physiology, the speed with which these two fast growing and obligate aerobes can dominate available resources and niche space may be important for pathogen protection. Similar to core hindgut bacteria, *Bombella* and *Apilactobacillus* expel short-chain fatty acids as a final product of oxidative metabolism. Their presence in guts is tolerated by *S. alvi*, a core gut bacterium and obligate aerobe with a complete TCA cycle capable of assimilating short-chain fatty acids (Kwong and Moran, 2016). Beyond this, the SRM frequently co-occurs in the worker midgut with *Gilliamella*, native yeast, and *Varimorpha* (Nosema). Based on relationships within and among studies, various gut microbiome dynamics are associated with the development of Nosema disease and viral infections in both queens and workers (Ptaszyńska et al., 2016; Tauber et al., 2019; Anderson et al., 2022; Copeland et al., 2024). Understanding the dynamics of the SRM in the context of gut microbiome resilience presents a new perspective on honey bee health.

Conclusion

Research on the honey bee microbiota has focused primarily on six core genera that comprise the worker hindgut microbiome. To entertain the holobiont perspective (Bordenstein and Theis, 2015;

Moran and Sloan, 2015), we performed a meta-analysis that included aerobic microbiomes associated with colony maintenance and social interaction, examining both anterior and posterior gut environments by reproductive caste. We identified two genera and a collection of species that are shared among intimate social niches, describing an aerobic “surface-rich” ecosystem maintained by continuous social processing of colony resources. The osmotolerant and acidophilic microbes that evolved to endure the social resource niche have come to play functional roles in disease ecology either by suppressing deleterious microbial growth or through their participation in perturbed hindgut enterotypes. Based on the results presented here and those of others, it is easy to speculate that this native aerobic microbiota contributes many functions at the group level including social communication, preserving stored nutrition, preventing disease and opportunism, and perhaps even gut microbiome resilience.

Author contributions

KA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DC: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frbee.2024.1410331/full#supplementary-material>

SUPPLEMENTARY TABLE 1

Reports the list of NCBI bio-projects and associated publications used for this meta-analysis, and bacterial (OTU) prevalence and abundance by niche.

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Deadly triangle: honey bees, mites, and viruses

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Honey bees are managed by humans on all continents except Antarctica, leading to an exceptional database of colony growth and survival. Honey bee colony losses in the United States are approximately 50% annually, and losses in other countries range from 10% to 60%. These losses reflect chemical, climatic, and nutritional stresses alongside immense pressure from diverse parasites and pathogens. The combination of RNA viruses and parasitic mites that vector these viruses plays a primary role in colony losses. Here, we discuss virus infection with and without mite vectors, bee defenses, colony vulnerabilities, and the roles of managed beekeeping in mitigating and aggravating the impacts of *Varroa* mites and viral disease.

KEYWORDS

pollination, vector biology, host-parasite, pathology, virus

1 Introduction

As a concentrated resource, honey bee colonies are beset by diverse parasites and pathogens (Boncristiani et al., 2020). Among these, honey bee viruses are ancestral and are maintained thanks to horizontal and vertical transmission routes inherent to crowded social settings. Deformed wing virus (DWV) in the family Iflaviridae is a common virus in honey bee colonies. DWV was named for developmental pathologies that can occur when levels are high during the formation and differentiation of imaginal wing discs (de Miranda and Genersch, 2010). Honey bees have evolved defenses that mitigate the effects of viral transmission and fitness costs, including immune responses at the individual level (Steinmann et al., 2015) and social traits such as hygienic removal of diseased nestmates (Wagoner et al., 2019) and behavioral task shifts that minimize contact between bees most prone to high infection and susceptible nestmates (Geffre et al., 2020). These defenses and the entire system were greatly destabilized when the western honey bee, *Apis mellifera*, was first parasitized by the ectoparasitic mite *Varroa destructor* roughly one century ago. At this moment, the disease system transitioned from communicable infection transmitted host-to-host to a complex vector-borne system where pathogens move freely among mites and bees. With help from the beekeeping trade, *Varroa* mites have steadily moved across the globe, catching up with *A. mellifera* in virtually every part of this host's current range (Chapman et al., 2023). The arrival and proliferation of *Varroa* in honey bee populations almost inevitably leads to wide-scale colony losses and pathologies (Steinhauer et al., 2018).

These losses reflect, in large part, the effective vectoring of DWV among bees by *Varroa* (Bowen-Walker et al., 1999), a known factor in colony mortality (e.g., Dainat et al., 2012). The spread of *Varroa* as a novel vector has both increased DWV prevalence worldwide and helped shape the evolution of different DWV variants (Martin et al., 2012; Wilfert et al., 2016; Grindrod et al., 2021; Hasegawa et al., 2023; Doublet et al., 2024). Here, we contrast the direct impacts of *Varroa* mites while feeding on their hosts with the far more damaging role these mites play as vectors of disease. We will focus on the DWV group since this species is most prevalent and impactful, resulting in abundant insightful research.

2 Individual disease

As an intracellular pathogen, DWV is, by definition, a disease of individuals. Virus replication depends on host organelles and proteins, and viral impacts happen at the individual level. While wing pathologies are rare, honey bees emerging with overt DWV infection show high viral loads in the brain and nervous tissues (Yue and Genersch, 2005), while individual bees with covert infections have been shown to have memory deficits (Tang et al., 2021), reduced brood rearing (Zanni et al., 2018), and poor foraging success (Benaets et al., 2017). Hosts also respond as individuals to viral infection, mounting immune responses that likely play some role in mitigating disease impacts (Brutscher et al., 2017). Similarly, *Varroa* parasitism happens at the individual level, with mites exerting a physiological cost on developing bees by damaging and consuming tissues just below the bee cuticle (Ramsey et al., 2019), and by consuming hemolymph of both developing and adult bees (Han et al., 2024). As in viral responses, parasitized bees can mount deterrents to mite feeding, including melanization of feeding sites, although these deterrents themselves seem to be weakened by mite saliva (Richards et al., 2011) and/or microbial allies (Kanbar and Engels, 2005). DWV might itself be one such ally since DWV presence could, at least conditionally, affect the abilities of bees to mount cellular defenses at mite feeding sites (Gregory et al., 2005; Yang and Cox-Foster, 2005; Fang et al., 2022). Interestingly, mites with high levels of DWV kill their adult bee hosts quickly, while similar feedings by mites with low viral loads impart a low relative risk of death. The latter bees can develop infections and share viruses while remaining asymptomatic (Lamas et al., 2023), putting additional colony members at risk.

3 Colony dynamics and communicable infection

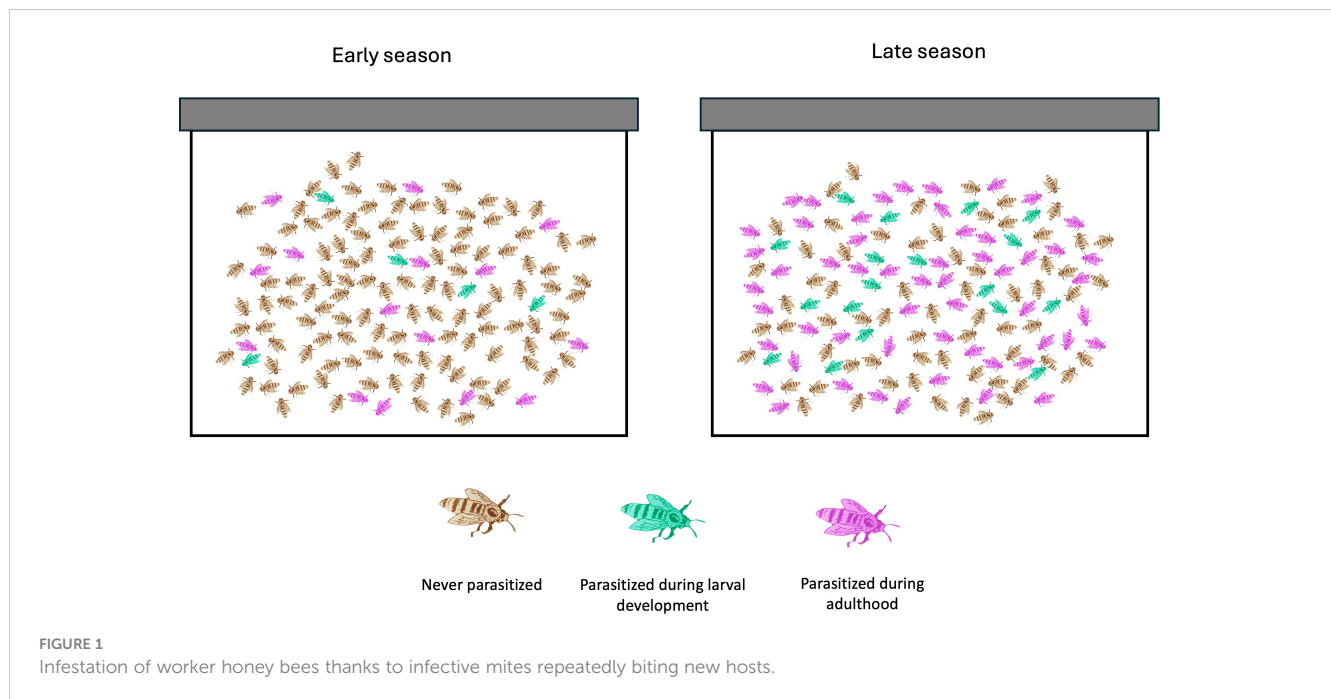
Viral transmission between bees is complex, involving both host-host and vector-borne transmission. DWV is transmitted between bee hosts vertically and horizontally and is very much driven by the social and reproductive systems of honey bee colonies. DWV is transmitted vertically by queens to their offspring (Amiri et al., 2018), potentially infecting hundreds of individual colony members daily. The source of these transmitted viruses can be the queen herself or her infected mates (Chen et al., 2006; Yue et al.,

2007). Horizontal transmission occurs via behaviors ranging from food sharing (Lamas et al., 2024b) to cannibalism of infected nestmates (Posada-Florez et al., 2021). Finally, the shared enclosed colony structure of honey bees can itself be a reservoir of viruses and other pathogens (Schittny et al., 2020). All of these transmission routes impact honey bees, but current research suggests that their impacts on colony health pale in the face of vector-driven transmission by parasitic *Varroa* mites.

Varroa parasitism has historically been studied as a phenomenon of developing bees, with great attention paid to the invasion of brood cells and parasitism of late-instar larvae and pupae. In fact, while mites reproduce only on developing bees, the majority of host contacts involve adult bees. Between reproductive bouts, *Varroa* are mobile, actively switching from one adult host to another in order to feed (Figure 1). Thus, the mite population in a colony is always significantly lower than the number of parasitized bees (Lamas et al., 2023). *Varroa* parasitism is not constant, seasonally shifting across age and sex cohorts at the colony level (Lamas, 2022). *Varroa* form highly aggregated distributions on adult male (drone) bees early in the season. Co-infestation on bee hosts is an efficient strategy for pathogen transfer between vectors, allowing naive mites to become infectious vectors (Lamas et al., 2024b).

While drones are arguably a preferred food source, the more numerous worker bees are better candidates for virus maintenance within the colony. Maintenance hosts are important for pathogen persistence, and such worker bees have been shown to harbor high levels of DWV infection while remaining asymptomatic and interactive with naive nestmates. Worker bees are excellent candidates as maintenance hosts as they share nutrients, and hence potentially viruses, with every life stage in the colony, from larvae to adult workers, drones, and queens. Some worker hosts are especially susceptible to virus infection, later acting as highly infectious agents in their colony. These so-called super-spreaders have a disproportionate impact on infection, and although they appear as outliers, they are regular features of epidemics (Stein, 2011). Infectious hosts increase the risk of infection to susceptible nestmates and the naive *Varroa* that feed upon them (Lamas et al., 2024b). In laboratory assays, adult cannibals of infectious pupae subsequently infect their nestmates and the mites that feed on these newly infectious bees also become infectious (Posada-Florez et al., 2021; Lamas et al., 2024b). Superspreaders may also present themselves as vectors. In other vector-borne systems, the vector biting rate is a key parameter in disease spread (Garrett-Jones and Shidrawi, 1969). A small number of vectors will have a disproportionate effect on disease transmission dynamics, as the most frequent host switchers are more likely to acquire and then transmit a pathogen (Cooper et al., 2019). *Varroa* exhibit similar features as they rapidly move from one adult bee to another in order to feed. On average, mites switch hosts once every two and a half days, but a smaller number of mites switch at much higher frequencies, parasitizing far more hosts than their slower switching conspecifics (Lamas et al., 2023). Unlike most disease systems, this triad between mites, bees, and viruses leads to an intense combination of both vector-borne and social transmission.

Virus dynamics continue at the level of apiaries, which have stocking densities ranging from one to hundreds of adjacent



colonies. Within apiaries, bees tend to drift into adjacent colonies, bringing their mites and/or viruses. Intriguingly, virus-infected bees seem to be more readily accepted as drifters into adjacent colonies, following changes in cuticular hydrocarbons that mask their 'alien' chemical profiles (Geffre et al., 2020). This is perhaps reflective of a pathogen manipulation of its host. Even more potently, diseased colonies in a state of weakness can be robbed by healthier colonies for honey and pollen, at which point raiding colonies readily acquire mites and/or viruses (Peck and Seeley, 2019). Bees living in collapsing colonies also regularly disperse and seek entry into distant colonies (Kulhanek et al., 2021).

4 Seasonal variation: the drone-to-worker shift

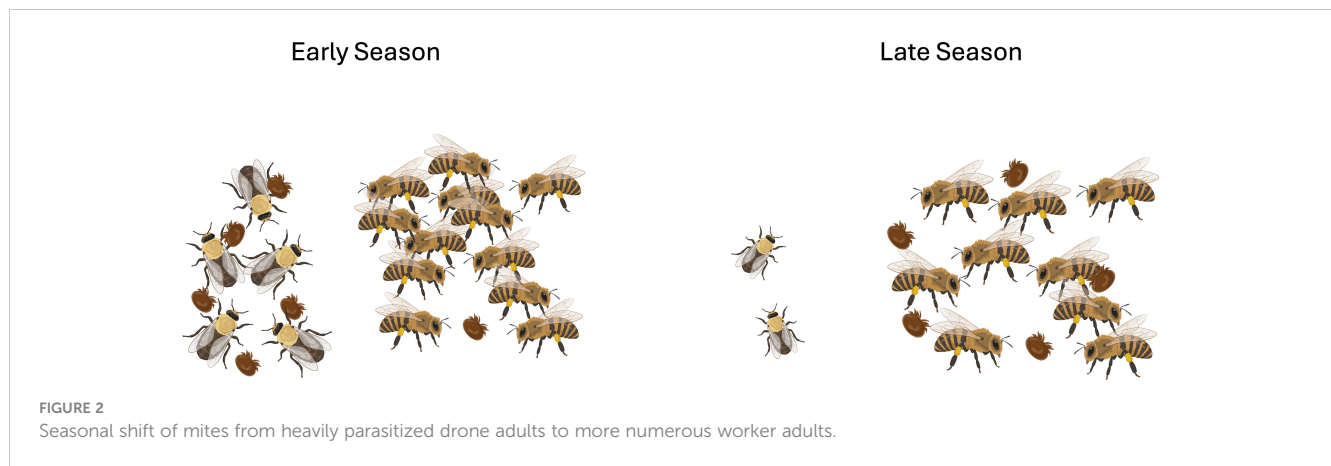
Varroa are highly attracted to drones when they are plentiful during the spring mating season. In fact, when drones are prevalent inside a colony, the absolute parasite burden is high on drones and low on workers. While drones attract this parasitism within their colony, they are unlikely candidates to infect their worker nestmates with viruses since they do not engage in colony maintenance tasks and receive but do not provide food donations during trophallactic events. Infected drones nonetheless pose a risk to the community of colonies surrounding them through their mating.

Honey bee colonies reproduce by fissioning in the spring and early summer, expelling the existing queen with more than half of the workforce while raising replacement queens who will mate on the wing with multiple drones. Drone production peaks in spring in preparation for these fission events. Given the great attractiveness of developing and adult drones to mites, drones are hotbeds of viral infection and hence excellent candidates for vertical transmission of DWV during mating (Amiri et al., 2016). Queens that harbor

infectious levels of DWV in their ovaries lay eggs coated with high levels of DWV, potentially abetting the long-term persistence of the virus through numerous rounds of egg production (Amiri et al., 2018). Spring mite parasitism focused on drone bees drives vertical (venereal) transmission through queen mating to her offspring. These infections impact subsequent colonies, but not their birth colonies.

When drones become seasonally less abundant, mites originally on drones inevitably shift to the worker bee population, spreading disease to the critical workforce (Figure 2). While quantification of this shift is needed across apiaries, regions, and climates, movement of mites from heavily infested drones to workers likely has an impact on worker fitness and both bee-bee and mite-bee spread of viruses. Biting on worker bees becomes the defining feature of late-season infestation, when the absolute parasite burden significantly increases on the worker population. Invasions into worker brood significantly increase, as does parasitism of young worker bees. Due to the highly mobile nature of mites, upwards of 60% of worker bees can be parasitized in a little over a week. Given this dynamic, it is not surprising that DWV levels in worker bees and pupae are low early in the season when mite feedings predominate on drones and appreciate greatly in the late summer and fall when they are more frequent targets and when mite populations increase (Tentcheva et al., 2004; Francis et al., 2013; Diao et al., 2019). This increase in viral pressure is tied to increasing colony losses in fall and winter (Dainat et al., 2012).

Vertical transmission pathways likely aid the long-term persistence of covert viral infections in honey bee populations (Chen et al., 2006; Peck and Seeley, 2019; Kulhanek et al., 2021). Beekeepers who allow individual colonies to develop heavy infestation in their dense apiaries may be benefiting mites and viruses, as there will always be new, healthy host colonies to accept them after their original host colony collapses.



5 Beekeeper interventions and the deadly triangle

Beekeepers are part of this complex pathogen-vector-host system, in that the interjection of husbandry and business decisions can have much influence over the persistence and dispersal of pathogens and vectors (Figure 3). Routine reproduction of colonies through the artificial splitting of colonies by beekeepers has enabled beekeepers to maintain a relatively consistent population of honey bee colonies in the United States despite high annual losses. Despite this consistency, the relationship between beekeepers and this system is anything but stable, and many beekeeping operations have high losses annually (Lamas et al., 2024a). At times, business decisions intrinsic to beekeeping

are at odds with biosecurity and disease prevention. First, beekeepers keep tens if not hundreds of colonies in close contact in working apiaries. Beekeepers also routinely split colonies to recover from losses, promoting intra-operation dispersal of DWV and *Varroa*. At a larger scale, the sale of live colonies between operations promotes inter-operational dispersal at a continental scale. Infected and infectious colonies sold into new operations can bring pathogens into naive, susceptible populations. From the perspective of pathogens and parasites, which need to disperse in order to acquire new hosts, these management strategies and sales provide a crucial lending hand on behalf of mites and viruses.

Recent suggestions that managing beehives in ways more consistent with natural colonies and populations (Seeley, 2017) have been taken to heart by some beekeepers as a strategy for

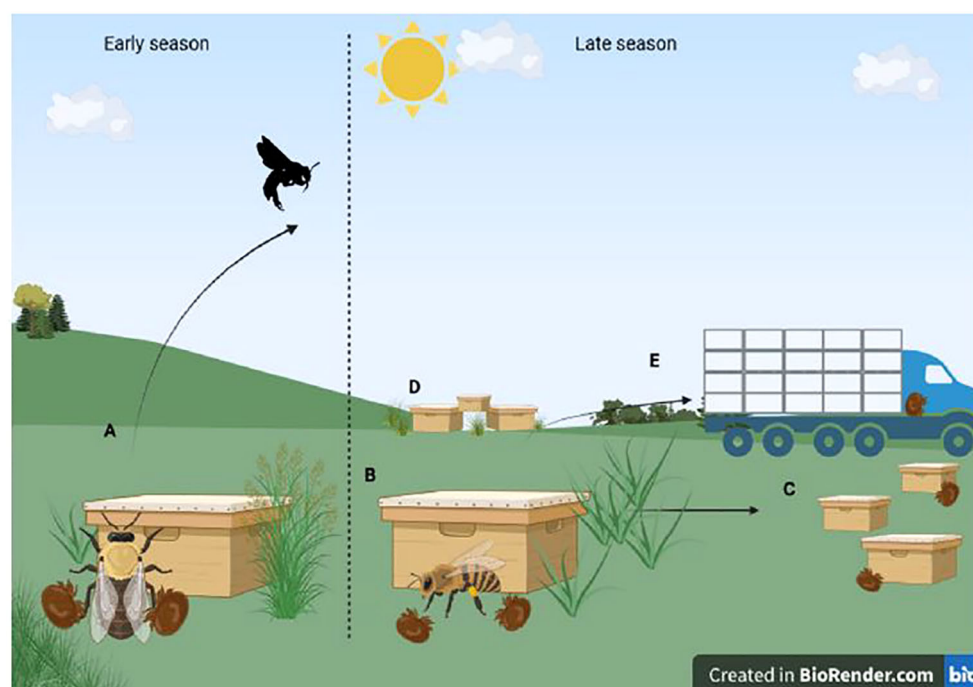


FIGURE 3

Multiple routes of movement by mites and vectors. (A) Drone infection and subsequent vertical transmission, (B) worker infection within hives, (C) Drift of workers between hives, (D) Diseased colonies as a local source of mites, (E) movement of diseased hives long distances by beekeepers. Source: Created with BioRender.com.

disease control. Nevertheless, beekeepers in much of the world deploy chemical miticides to reduce vectoring and direct damage by mites, with treatments that have evolved for over 50 years. Heavy reliance on miticides has led to repetitive and rotating treatments, especially when, inevitably, mites evolve resistance (Haber et al., 2019). IPM treatment methods, including regular mite counts, can economize treatments and prolong the lifetimes of specific chemicals. Even when beekeepers successfully control *Varroa* populations, they can be left with circulating viruses among colony and apiary members (Locke et al., 2017). Given difficulties in monitoring both viruses and individual symptomatic bees, the first indication of high viral loads might be “dwindled” colonies that fail to grow or whose remaining bees are unable to thermoregulate or regenerate their lost adult bee population. When this occurs, beekeepers are left with economically non-viable units (Lamas et al., 2024a). More urgently, effective antiviral drugs could help release honey bees from some of the costs of persistent mite infection.

6 Hot topics and unknowns

This review highlights disease models for this complex pathogen-vector-host triad in light of ecological and economic importance. Many questions remain open in light of this triad and the protection of honey bee health. A few of the more pressing questions are below:

- Which current beekeeping strategies exacerbate parasites and viruses in colonies? For example, beekeepers split colonies for sale or to replace lost colonies, but splitting will inevitably aid in the artificial dispersal of mites and viruses. Which new practices would allow the movement of bees and re-use of equipment while reducing the risk of infection?
- What are the carrying capacities of landscapes with respect to unhealthy disease pressures?
- *Varroa* is a biological vector of some but not all DWV strains. How does this impact the triangle?
- More generally, roughly half of the known honey bee viruses seem to be vectored by *Varroa*; why are these distinct from other bee viruses?
- Continued work is needed to understand colony components that harbor and cause infection to new bees.

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How infectious are hive substrates? And brood food given to larvae?

- What are the roles of environmental stressors such as poor nutrition, sublethal pesticide exposures, and co-infection in increasing honey bee susceptibility to viruses?
- How can beekeepers reduce viral impacts with prevention, medication, or recovery?

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Chromosome evolution in bees

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Of the about 1850 species of Hymenoptera for which chromosome counts are known, only just over 200 of these are bees (Apoidea). Haploid numbers (n) range from 3–28, which probably does represent the true range of chromosome numbers in this superfamily. The modal number is 17, with another peak at $n=9$, representing a clade of meliponid bees which has been well studied. Although much is known about the chromosomes of bees there is still much to learn about overall trends in haploid number and chromosome organization. We are still lacking this information for many important families of bees. The only andrenid bee karyotyped, *Andrena togashii* has the low n of 3, so we certainly need to know which other species in this family have low chromosome numbers to see if this is an exception and to further test the Minimum Interaction Theory (MIT) of Imai and colleagues which predicts the evolutionary increase in chromosome number. In general, an overall increase from low numbers ($n=3-8$) to the higher numbers found in the Apidae, Colletidae, Halictidae, and Megachilidae (modal numbers 17, 16, 16, 16, respectively) does appear to be followed. However, within groups this is not always the case; the Meliponid clade with $n=9$ being an example. The potential adaptive value of chromosome number *per se* is of great interest. I propose a hypothesis to account for the high ($n=25$) chromosome number found in the social parasitic bumble bee subgenus *Psithyrus*. More sophisticated techniques beyond chromosome counting and karyotyping using C-banding, will yield much more detailed information about chromosomal rearrangements as shown by the work on the neotropical meliponid bees by the Brazilian cytogeneticists, and when these are applied to other taxa of bees will undoubtedly reveal features of great interest. Genomic approaches are starting to identify chromosomal rearrangements such as inversions and this holds much potential to explore their adaptive significance.

KEYWORDS

Hymenoptera, bees, Apidae, chromosomes, karyotypes, inversions

1 Introduction

There is a vast amount known about the chromosomes of animals (White, 1977), and the study of chromosomes has been central to the discipline of genetics since its early days. Similarly, chromosome rearrangements within species are of evolutionary significance, and have also been of importance for revealing phylogenetic patterns among different species (White, 1977; Wellenreuther and Bernatchez, 2018). Indeed, interest in the former has also now undergone resurgence in the era of genomics (Wellenreuther and Bernatchez, 2018). It is a striking fact that species, even of one taxonomic group, can vary dramatically in

chromosome number; ants (see below) are a good example of this. However, almost paradoxically, although chromosome numbers can vary by almost an order of magnitude [for example the *Lycaenoidea* butterflies have a range of haploid chromosome number from 10 to a high of 223 (White, 1977)] there is no obvious visible effect on the phenotypes; all the butterflies look like butterflies in gross morphology. In some ways this is not really surprising as differences in chromosome number just represent different ways of packaging the total genetic material – the genome. Nevertheless, it would be naïve to think that there is no importance to this variation, and at the very least there are likely to be limits to the effectiveness of meiosis and mitosis with very large numbers of chromosomes. The events of chromosome replication and meiosis are obviously critical as well known from classical genetics. Even a mutation at a single base (not even a base pair) can be transmitted to the offspring as a half-chromatid mutation and can potentially result in a very large phenotypic effect such as phenotypic mosaics and even gynandromorphs in the Hymenoptera (Owen, 2023). This is the apparent paradox; large scale changes in chromosome number may have no obvious phenotypic effect on an organism but a single base change can have a drastic effect. Chromosomal variation simply cannot be without significance and thus still needs to be understood. As Imai et al. (2001) remark, "...karyotypes might be important as an isolating mechanism in speciation and have their own evolutionary trends independent of genetic evolution (King, 1993)". However the exact role of chromosomes and chromosomal changes in speciation remains unclear and is still subject to considerable debate (King, 1993; Coyne and Orr, 2004).

In this review I will summarize some of what is known about chromosome variation in bees, and discuss some possible causes and adaptive consequences of this variation. I will start by putting bee chromosome numbers in the context of chromosome variation the Hymenoptera as a whole.

2 Hymenopteran chromosomes

The most obvious, and dramatic, difference in chromosome number in the Hymenoptera is that between males and females

(Figure 1) which arises because of the genetic system of haplodiploidy, whereby females arise from fertilized eggs and males from unfertilized eggs. Thus, males have just one haploid (n) set of chromosomes while the females have the full diploid complement of $2n$. This represents the successive breaking of constraints that allows development to proceed by mitosis from the unfertilized egg (Gallis and van Alphen, 2020). In many species of Hymenoptera there is a genic system of sex-determination underlying this; the single-locus complementary sex-determination (sl-CSD) proposed by Whiting (1933) to explain sex-determination in *Habrobracon*. Sex is determined a single locus with multiple alleles $x_1, x_2, x_3, \dots, x_k$ (where k varies from 12–20 in many natural populations); any heterozygote (e.g. x_1x_3) is female, haploids are normal males while homozygotes are diploid males. In any finite population diploid males are expected to occur regularly at frequency of 5–10% (Owen and Packer, 1994). Diploid males are inviable in some species, while in others are viable and fertile (Garofalo and Kerr, 1975). Karyotypes have been obtained of diploid males for a number of species of bees, for example honeybees and bumble bees (Hoshiba, 1984b; Hoshiba et al., 1995). That these dramatic ploidy differences between males, females and diploid males results in perfectly viable adults suggests that the Hymenoptera may be able to tolerate changes in chromosome number rather well. Also, somatic polyploidy is well known and widespread in the Hymenoptera (Crozier, 1975) but will not be discussed in this review.

2.1 Chromosome nomenclature

The conventional system of classifying and naming chromosomes follows that of Levan et al. (1964) which is based on the ratio of length between the long and short arms. Thus, we have metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) chromosomes, and this is widely used for most plant and animal taxa. In contrast a rather more detailed, and complex system of naming chromosomes has been used by Imai and coworkers and has been applied particularly to the chromosomes of the Hymenoptera (Hoshiba and Imai, 1993; Imai, 1978). It is worth going into this in some detail as it forms the basis of the

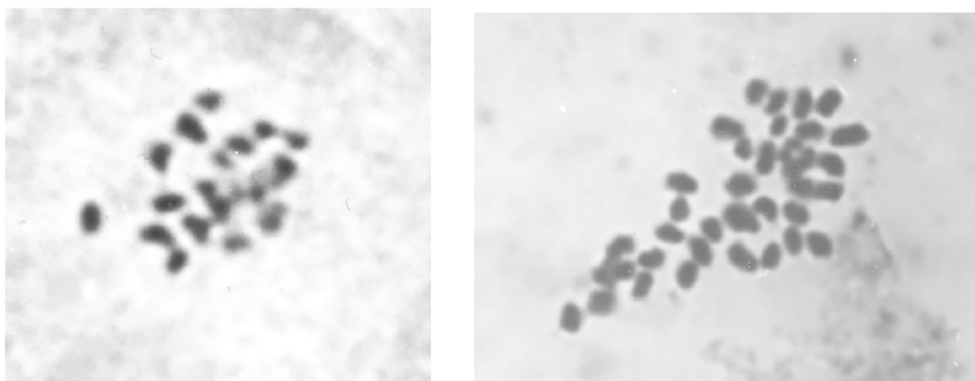


FIGURE 1
Bombus (Thoracobombus) fervidus male (left) and female (right) with haploid number $N = 18$, and diploid number $2N = 36$ respectively.

minimum interaction theory to account for chromosomal evolution in the Hymenoptera (Imai et al., 1986; Hoshiba and Imai, 1993). This theory is interesting and important because it attempts an adaptive explanation for changes in chromosome number and of the amount and distribution of heterochromatin observed.

The system of chromosome morphology, called the TAM system by Imai (1978, 1991) classifies chromosomes into three basic categories, telocentric (T) acrocentric (A) and metacentric ($\bar{M}$) chromosomes (Hoshiba and Imai, 1993). The A and M chromosomes are defined by two features; (i) chromosome arms are euchromatic or heterochromatic, and (ii) ratio of the width (i.e., length) of short (W_S) and long arms (W_L) as follows:

A, heterochromatic short arm, euchromatic long arm, and $W_S < W_L$

M, both arms are euchromatic, and $W_S = W_L$

Modified types of the A chromosomes are A^c , A^M (pseudocentric), and A^h which are defined as

A^c , both arms are euchromatic, and $W_S < W_L$

A^M , either short or long arms heterochromatic and $W_S = W_L$

A^h , both arms are heterochromatic, and $W_S < W_L$

Heterochromatic regions are located by the C-banding technique (see below), and C-band(s) located at the pericentromeric, interstitial or, terminal regions of chromosome arms are represented as A^C , A^i , A^c , M^C , M^i , M^t , etc. The terms “A group” and “M group” chromosomes are used by Hoshiba and Imai (1993), and include respectively A, A^M , A^{Mc} , A^{Mc} , etc., and M, M^c , M^i , etc. Some examples of the A and M group chromosomes with C-banding found in bees and wasps show in Figure 2, which is modified from Hoshiba and Imai (1993). It is the cyclic transition between these types of chromosomes that is the essence of the minimum interaction theory (Imai, 1978). Imai (1978) proposed that centric fission of an M chromosome $\rightarrow$ T + T (two telocentric chromosomes), this is followed by tandem growth of heterochromatin, thus $T \rightarrow A$, and finally pericentric inversion reverting $A \rightarrow M$ (Imai, 1978). Thus, the overall trend is the evolution of higher chromosome numbers and numbers of chromosome arms within lineages (Imai, 1978). Telocentric chromosomes although rare in nature, do occur (Marks, 1957; White, 1977); for example, are common in some birds (Takagi and Sasaki, 1974). Telocentric chromosomes (or telosomes) are thought to be unstable as Koo et al. (2015) state “they arise through misdivision of centromeres in normal chromosomes, and their cytological stability depends on the structure of their

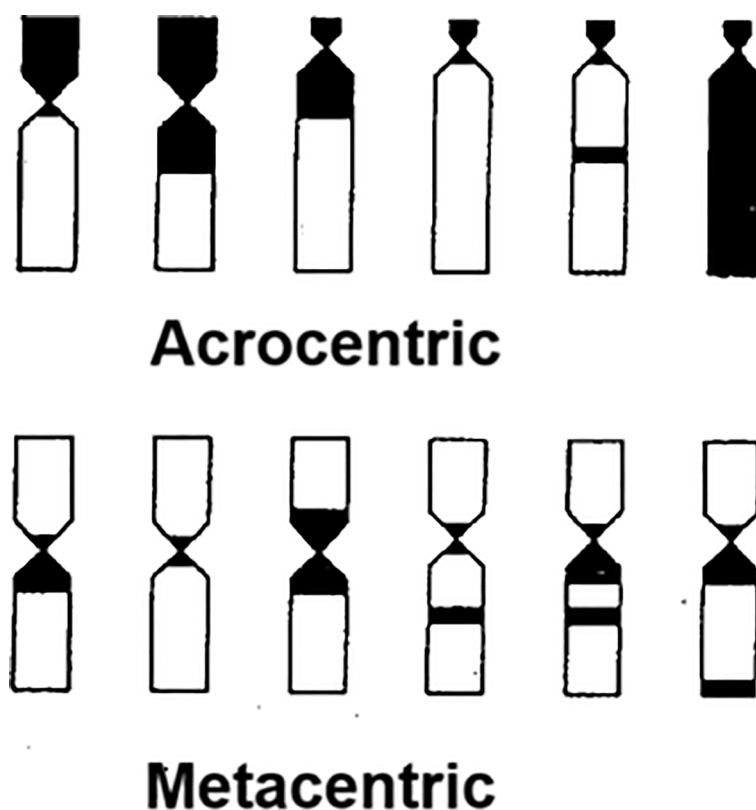


FIGURE 2

A selection of some of the main “A” group and “ $\bar{M}$ ” group chromosomes found in bees and wasps showing C banding. Modified and simplified from Hoshiba and Imai (1993).

kinetochores. The instability of telosomes may be attributed to the relative centromere size and the degree of completeness of their kinetochore". Thus, in their analysis of chromosomes evolution in the Hymenoptera Hoshiba and Imai (1993) treat the T chromosome as only a theoretical construct and it is used only for theoretical discussions of chromosome evolution, and is regarded as a member of A chromosome group (Hoshiba and Imai, 1993). In this system the T chromosomes transform into acrocentrics through increase in heterochromatin ($T \rightarrow A$, see above).

2.2 Theories of chromosomal evolution

From the study of the diversity of mammalian chromosome numbers three different mechanisms or hypotheses emerged to account for this karyotype evolution (Imai and Crozier, 1980; Menezes et al., 2014). The hypotheses are general enough to be applicable to any group of animals:

1. The fusion hypothesis (White, 1977) assumes that chromosome numbers have decreased by centric fusion from an ancestral high number of acrocentric chromosomes.
2. The fission hypothesis is the opposite, assuming ancestral chromosome numbers to be low and subsequently increasing by centric fissions and pericentric inversions (Todd, 1970; Imai and Crozier, 1980). Centric fission of chromosomes was long thought to be unlikely given the nature of the centromere (Imai et al., 2001). However, more recent molecular genetics has revised this idea, (Imai et al., 2001) and there is now direct evidence of fission in hymenopteran chromosomes (Rousselet et al., 2000).
3. The modal hypothesis (Matthey, 1973) assumes that ancestral chromosome numbers are intermediate and subsequently have increased or decreased in lineages through fission and fusion.

As an alternative to all of the above, as already mentioned, Imai et al. (1986) have proposed a modified form of the fission hypothesis, which is:

4. The minimum interaction theory (MIT) to account for chromosomal evolution in ants, bees and wasps (Imai et al., 1986; Hoshiba and Imai, 1993). As succinctly put by Hoshiba and Imai (1993) under the minimum interaction hypothesis "...chromosome evolution proceeds toward minimizing chromosomal interactions which induce deleterious chromosomal mutations such as reciprocal translocation, and predicts that increasing chromosome number by centric fission is one of the adaptive solutions".

Here I am following Imai et al. (2001) by describing "chromosome evolution" as a general term covering three distinct concepts: (i) morphological alteration of individual chromosomes, (ii) evolution of individual karyotypes, and (iii) evolution of mass-karyotypes, which refers to evolution of karyotype at the generic, familial or ordinal level Imai et al. (2001).

Keeping these hypotheses in mind, I will briefly summarize some trends in chromosomal evolution as seen in some of the other major groups of Hymenoptera, and then turn to the bees in detail.

2.3 Hymenopteran chromosomes: variation in number

Chromosome counts for 1846 species of Hymenoptera are given in Table 1. Chromosome numbers vary widely in the Hymenoptera, from an $n = 1$ to a high of $n = 60$ (both ants)! and their numbers, karyotypes and evolution have been studied intensively in many groups and have been reviewed elsewhere (e.g. Crozier, 1975; Gokhman, 2023; Lorite and Palomeque, 2010; Ross et al., 2015). However, in spite of this variation, haploid numbers of the vast majority of Hymenoptera lie within the range typical for animals as a whole which range from 6-20, and which presumably represents the limits of the spindle apparatus to function with either large or small numbers of chromosomes (White, 1977). Interestingly Hymenoptera do exhibit some of the lowest chromosome numbers in the animal kingdom. The lowest possible chromosome number, $n = 1$, in the Australian ant *Myrmeca croslandi* (Crossland and Crozier, 1986) is rivaled only by one species of nematode (White, 1977), nonetheless the low haploid number of three ($n=3$) is found in some bees, parasitoids and ants (Table 1). On the other hand, the highest n of 60 in the South American ant *Dinoponera lucida* (Mariano et al., 2008) is much less than the highest chromosome numbers recorded in animals, the record being an $n = 217-223$ in the butterfly *Lysandra atlantica* (White, 1977).

Polyploidy (of the germ line, as distinct from somatic polyploidy) has been suggested as a cause of major trends in hymenopteran chromosomal evolution (reviewed by Crozier, 1975) and in certain groups (for example bees, Kerr and Silveira, 1972). However, is little evidence for this except in some specific cases and other types of chromosomal rearrangements can account for the observed karyotypic evolution in the Hymenoptera (Crozier, 1975). An interesting exception is the gall wasp *Diplolepis eglanteriae* (Sanderson, 1988). Three other species in this genus have an $n=18$, whereas as *D. eglanteriae* has $n=27$, and triploidy is strikingly seen by the presence of three distinctively large chromosomes (Figure 3, in Sanderson, 1988). Similarly, Naito and Inomata (2006) identified a triploid thelytokous sawfly, *Pachyprotasis youngiae*. Thirty chromosomes were seen at mitotic metaphase and the karyotype clearly consists of three sets of $n = 10$ chromosomes (Figure 4, Naito and Inomata, 2006). Given that most other Japanese species of *Pachyprotasis* have a haploid number of 10, triploidy of *P. youngiae* is strongly implied (Naito and Inomata, 2006).

In terms of total DNA content, it is worth noting that there is no obvious relationship between C-value and chromosome number in the Hymenoptera (Ardila-Garcia et al., 2010). This is the so-called C-value paradox (Thomas, 1971), for which there appears to be no one satisfactory explanation (Lakhotia, 2023). The haploid genome

TABLE 1 A summary of chromosome numbers in the Hymenoptera. Haploid numbers (*n*) for 1846 species of Hymenoptera are given.

Taxon		Number of Species	Minimum	Maximum	Mode(s)
Superfamily	Family				
Symphyta		357			
Pamphilioidea		10	11	35	—
Tenthredoidea		339	5	22	10
	Argidae	9	8	13	8
	Cimbicidae	4	8	16	8
	Diprionidae	39	6	15	7
	Tenthredinidae	287	5	22	10
Cephoidea	Cepidae	3	9	26	20 (median)
Sircoidea	Siricidae	5	8	18	8
Apocrita		1489			
Parasitica		398			
Ceraphronoidea		1	9	9	—
Chalcoidea		147	3	12	5
Cynipoidea		27	9	12	10
Evanoidea		2	14	16	—
Ichneumonoidea	Braconidae	63	3	23	6, 10, 17
	Ichneumonidae	155	8	17	11
Proctotrupoidea	Diapriidae	2	8	10	9 (median)
Platygastroidea	Scelionidae	1	—	—	10
Aculeata		1091			
Apoidea		212	3	28	17
	Crabonidae	5	3	5	3
Anthophila	<i>Andrenidae</i>	1	3	3	3
	<i>Apidae</i>	178	8	26	17
	<i>Colletidae</i>	5	8	28	16
	<i>Halictidae</i>	14	6	20	16
	<i>Megachilidae</i>	8	16	16	16
Chrysidoidea		3	10	19	14 (median)
Vespoidea		873	1	60	10
	Formicidae	791	1	60	10
	Pompilidae	4	14	15	15
	Vespidae	49	5	34	25
	Sphecidae	19	4	24	14
	Eumeninae	21	4	18	6

Thus review focusses on the bees the Anthophila (superfamily Apoidea) with the families discussed shown in italics. The numbers in bold indicate the total number of species in that taxon karyotyped, or the minimum and maximum haploid chromosome numbers counted.

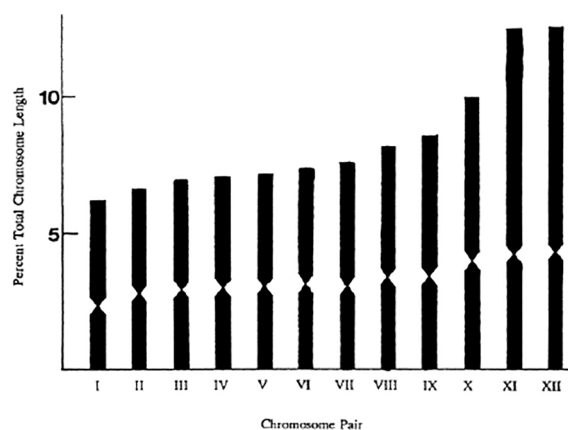


FIGURE 3

Ideogram of *B. (Pyrobombus) perplexus*. Mean percent chromosome length based on a sample of 14 nuclei, with a mean haploid total complement length of 24.1 μ m. From: Owen et al. (1995).

sizes (picograms, pg) for 131 species of Hymenoptera range from 0.1 pg in a braconid wasp to 1.14 pg in the yellow mud dauber *Sceliphron caementarium* (Ardila-Garcia et al., 2010). Ardila-Garcia et al. (2010) give additional genome size data for 89 species of Hymenoptera, which have a mean genome size of 0.38 pg $\pm$ 0.20 SD. For the 18 species of bees (Anthophila) they examined, taking the data from their Table 1, gives a mean of 0.57 pg $\pm$ 0.18 SD. The values ranged from 0.24 pg in *Apis mellifera* to 0.90 pg in the

halicted *Augochloropsis metallica*. Thus, there appears to be no clear connection between chromosome number and genome size in the Hymenoptera as a whole, or the bees in particular.

2.4 Symphyta

Only a small fraction (about 360/7170 $\approx$ 5%, Table 1) of the described species in this suborder have been studied cytogenetically and for many families chromosome numbers are unknown (Westendorff, 2006). Likewise modal chromosome numbers cannot be confidently given for all families which have been studied (Westendorff, 2006). However, some families, such as the large family Tenthredinidae are relatively well studied (Westendorff et al., 1999; Kuznetsova et al., 2001), but few species have been karyotyped in the Argidae, Cimbicidae and Cephidae (Westendorff and Taeger, 2002). Overall, haploid (n) chromosome numbers in the Symphyta vary from 5–35 (Table 1) (Westendorff, 2006), with some genera showing great variation both in number and morphology of chromosomes. For example, *Cephalcia* (Pamphiliidae) n varies from 23–35, and chromosome morphology is also highly diverse with metacentrics predominating in some species and acrocentrics in others (Westendorff, 2006). Nevertheless, given the phylogeny of the suborder (Vilhelmsen, 2006) and its position as a basal group of the Hymenoptera, it is reasonable to conclude that low n values of 7–8 is ancestral to the group, and as Hoshiba and Imai (1993) and Gokhman and Quicke (1995) suggest the ancestral chromosome number of the Hymenoptera and also the Apocrita was low; such as $n = 8$ or less. Thus, in the Symphyta and Apocrita chromosome evolution has, to a large extent, proceeded by fission, a major tenet of the minimum interaction theory of Imai et al. (1986). Interestingly there is direct evidence of chromosomal fission in sawflies as Rousselet et al. (2000) suggested that the origin of *Neodiprion abietis* with $n = 8$ was due to fission from a karyotype with $n = 7$ (typical for the Diprionidae), and that the break point of the chromosome fission was located close to an rRNA gene cluster.

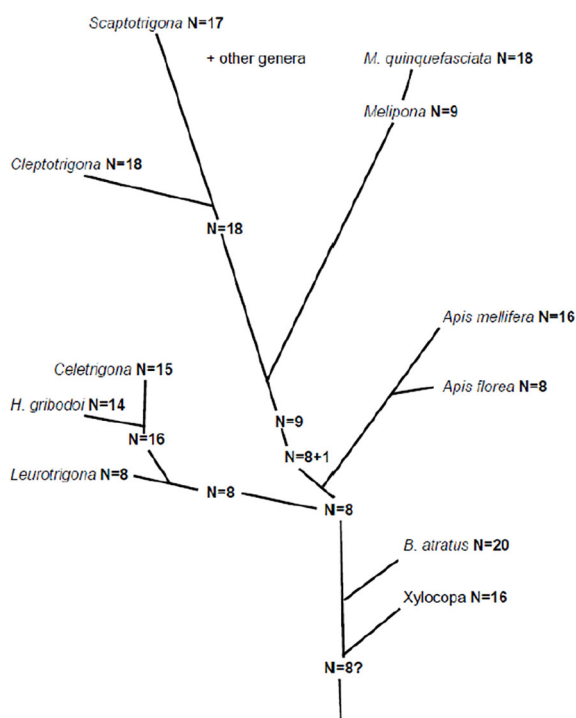


FIGURE 4

The polyploid hypothesis of Kerr and Silveira (1972).

2.5 Parasitica

Gokhman (2006) reviewed the trends in chromosome evolution of parasitic Hymenoptera. Haploid chromosome numbers of parasitic wasps (Parasitica + Chrysoidea) for the approximately 400 species examined range from 3–23 (Gokhman, 2006, 2009, 2023) (Table 1). He considers chromosome numbers (n) of 14–17 with a symmetrical karyotype to be ancestral. A symmetrical karyotype is one containing metacentric chromosomes of a similar size, in contrast to an asymmetrical karyotype which has predominantly acrocentric and telocentric chromosomes (intrachromosomal asymmetry) and highly variable chromosome sizes (interchromosomal asymmetry) (White, 1977; Peruzzi and Eroğlu, 2013).

The two major trends have been independent reduction in chromosome number in the major lineages and increasing asymmetry of the karyotype. Reductions to $n \leq 10$ –11 occurred in some groups of the Ichneumonoidea, and independently in the common ancestor of the Proctotrupoidea, Ceraphronoidea, Cynipoidea and Chalcidoidea (Gokhman, 2006).

2.6 Vespoidea: Formicidae

Ants (Formicidae) are the most variable chromosomally of all Hymenoptera (Table 1), and a great deal is known about their karyotypic evolution, as reviewed by Lorite and Palomeque (2010). Robertsonian centric fusions and fissions, and also inversions and translocations appear to be the most important mechanisms for karyotypic evolution in ants whereas polyploidy and aneuploidy probably have a minor role (Lorite and Palomeque, 2010). For example, Santos et al. (2012) found large numbers of acrocentric chromosomes in four closely related *Dinoponera* ants, *D. australis* ($n = 57$), *D. gigantea*, ($n = 41$), *D. lucida*, ($n = 59/60$), *D. quadricipes* ($n = 46$) suggesting that these arose from centric fissions of metacentric chromosomes. Imai et al. (1986) have proposed the minimum-interaction theory (MIT) to account for ant chromosomal evolution, and under which chromosome numbers tend to increase, but obviously to an upper limit (Lorite and Palomeque, 2010; Cardoso and Cristiano, 2021).

2.7 Vespidae and Sphecidae

The haploid chromosome numbers of the wasps (Vespidae) vary widely from 5–34 (Table 1). Hoshiba and Imai (1993) concluded that the pattern of chromosome evolution in both of these groups conformed with the MIT starting with an ancestral low n of around three, and then proceeding in a characteristic zig-zag pattern through the karyograph and evolving high numbers. However, a more recent study by Menezes et al. (2014) on the Epiponini (swarm-founding Polistine wasps) concluded that in this group a high chromosome number of $n = 33$ was ancestral and that a gradual reduction in chromosome number had occurred. For example, chromosome numbers had decreased to an $n = 16$ in

Polybia, and even within this genus a similar reduction in n had occurred within each subgenus (Menezes et al., 2014). These trends contradict the MIT of Imai et al. (1986) (Menezes et al., 2021). Menezes et al. (2014) used the computer program chromEvol v1.3 of Mayrose et al. (2010) to evaluate the direction and type of chromosome change. The program uses a probabilistic approach to estimate the most likely chromosome changes (polyploidy, gain or loss of chromosomes by fission or fusion) that have occurred when a set of chromosomes is mapped onto a known phylogeny (Mayrose et al., 2010). Menezes et al. (2014) found that a process of constant gain and loss, and no duplication (polyploidy) gave the best fit to the data and indicated the ancestral n of 33.

Twenty one species from 10 genera of the subfamily Eumeninae have been karyotyped with n values ranging from 5–18 (Tavares and Teixeira, 2021). Tavares and Teixeira (2021, 2022, 2023) have examined in detail chromosomal evolution in various solitary wasps. Notable among these is the variation they observed in *Ancistrocerus flavomarginatus* (Tavares and Teixeira, 2023). In the karyotype they found two larger chromosome pairs, which were almost entirely heterochromatic, and many subtelo-centric chromosomes with heterochromatic short arms. They concluded that the latter resulted from chromosomal fissions (Tavares and Teixeira, 2023).

3 Chromosome numbers and karyotypes in bees

Here I will examine in some detail trends in evolution of chromosome numbers in the major lineages of bees, which are a monophyletic group – the Anthophila (Sann et al., 2018) – of the superfamily Apoidea (Table 1). Also included in the Apoidea is the Crabronidae which are a polyphyletic group of wasps (Sann et al., 2018) which will not be considered here.

I will first briefly describe some of the advances in cytological techniques that have led to an improved understanding of bee chromosomes. Conceptually it may seem that counting the chromosome number of a species is one of the easiest tasks. However, what may not be realized is that definite chromosome counts of even some of the most common and well-known species on Earth, such as *Homo sapiens*, and some *Apis* species have only been relatively recently been determined! It wasn't until 1956 that the chromosome number of humans was definitely established as 46 by Tjio and Levan (1956). This was possible by using the squash technique rather than the old method of sectioning of testicular tissue embedded in paraffin (O'Connor, 2008).

Petrunkewitsch (1910) (quoted in Milne, 1986) in 1901 correctly determined the chromosome number of *A. mellifera* when he observed 32 very small chromosomes in oogonia (Milne, 1986). This was subsequently confirmed with better techniques and differences in chromosome size were observed (Sanderson and Hall, 1948). However, it wasn't until 1977 that Fahrenhorst was able to lay to rest “the contradictory reports in the existing literature” regarding chromosome numbers in the other *Apis* species (Fahrenhorst, 1977) and state unequivocally (in the English

abstract) “The haploid chromosome number is 16 and this was found uniformly in *Apis mellifera*, *A. cerana*, *A. dorsata* and *A. florea*. As such the reported number of 8 haploid chromosomes in the last two species mentioned above should be rejected. It is doubtless, that in all the species of *Apis*, the diploid set of female germline cells consists of 32 chromosomes, as this was established for *A. mellifera*” (Fahrenhorst, 1977).

3.1 Cytological techniques

As with humans (O'Connor, 2008), sectioning of tissue embedded in paraffin was previously used for hymenopteran chromosomes counts; for example, an incorrect (Owen et al., 1995) $n = 12$ for *Bombus fervidus* was obtained by Whelden (1954). Although reliable chromosome counts have been obtained with sectioning (e.g. Deodikar et al., 1959), generally this is an unreliable technique (Crozier, 1975). Squash methods using fresh brain, testis, or ovary tissue yield much more better chromosome preparations (Figure 5) and have been successfully used to count bee chromosomes (e.g. Owen, 1983; Owen et al., 1995). Following dissection and fixation, the tissue can be stained using either the Feulgen technique or aceto-carmin method (Darlington and LaCour, 1976; Owen, 1983; Owen et al., 1995). Slides are prepared by teasing out a small piece of tissue into a drop of 50% acetic acid and then squashing under a cover slip (Figures 1, 5). Although the results are acceptable, a simpler air-drying technique that yields superior preparations was developed by Imai et al. (1977) and is widely used (Figure 6). In brief, the tissue to be examined is, after treatment in a colchicine-hypotonic and Fixative (I) solution, macerated on a slide under a dissecting microscope to separate out single cells and clumps of cells, then Fixative II solution is added which is then drained off the slide (Imai et al., 1977). The slide is left to dry for one day (Barcia, 2007) and then stained with Giemsa solution, and after brief washing the slide is again left to dry; it is now permanent and needs no

coverslip (Imai et al., 1977). Furthermore, it also gives C-bands spontaneously (Imai et al., 1977). C-bands occur where Giemsa stains intensely, and identify regions of constitutive heterochromatin on the chromosome; however, it is not clear whether this results from DNA denaturation-renaturation (Gokhman, 2009; Kumar et al., 2021) or staining of heterochromatin-specific proteins (Gokhman, 2009). C-banding has been widely used on hymenopteran chromosomes, particularly by Imai and coworkers on bees and wasps (Hoshiba, 1984a, b; Hoshiba and Imai, 1993; Imai, 1991). The classic Giemsa stain, perfected by Gustav Giemsa in 1904, and developed originally to identify malarial plasmodia in the blood, was successful due to its stability and because of its ability to stain chromatin deeply (Fleischer, 2004; Barcia, 2007).

There are numerous other staining techniques in addition to C-banding that give chromosome bands (e.g. Q, G, R, T) or identify certain regions on the chromosome (Kumar et al., 2021), one of these is NOR banding. Nucleolus organizer regions (NOR) of metaphase chromosomes can be visualized by silver staining techniques, either Ag-As or Ag (Kumar et al., 2021). Maffei et al. (2001) localized NORs in the bees *Euglossa* sp., *Melipona marginate*, *Plebia* sp., and the parasitic wasp *Mellitobia australica*, with AgNOR. Similarly, Rocha et al. (2002) used NOR banding on ten Brazilian species of *Melipona*. Similarly, Beye and Moritz (1993) used DNA probes specific to *Drosophila melanogaster*, coding for 28S and 18S rRNA, and found that these hybridized *in situ* to distinct regions of two chromosomes of the honeybee, identifying NORs.

More recently the development of single-stranded DNA probes that anneal to complementary DNA (Bishop, 2010) has allowed very specific regions of chromosomes to be identified. Of these Fluorescence *In Situ* Hybridization (FISH) has been used extensively for the Hymenoptera (Gokhman, 2009), with ribosomal DNA and microsatellite-satellite sequences commonly mapped (Cunha et al., 2023). For example, Cunha et al. (2023) used probes for four microsatellites to help identify possible Robertson fusion events in Neotropical meliponid bees (see below).

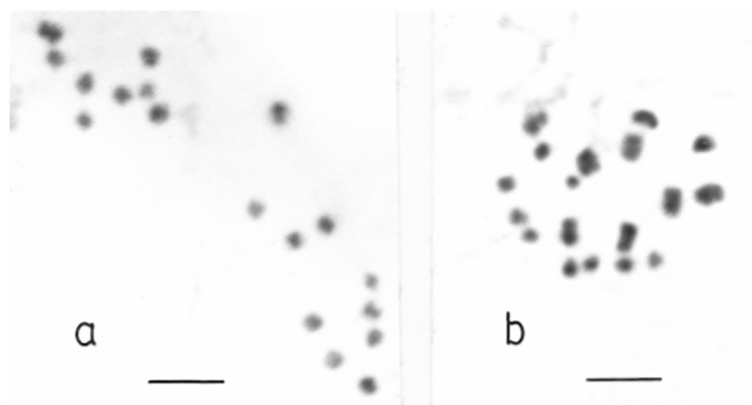


FIGURE 5

Squash preparations of (a) *Bombus* (*Pyrobombus*) *ephippiatus* (male brain tissue), $n = 18$, and (b) *B. (Pyrobombus) impatiens* (testes) $n = 18$. Scale bar = $1\mu\text{m}$. From Owen (1983).

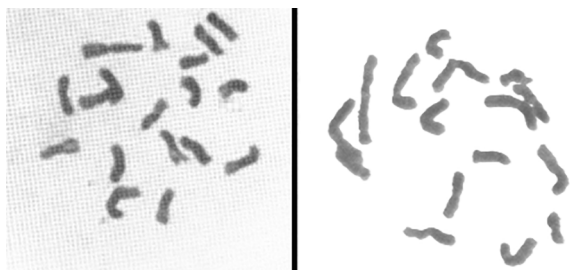


FIGURE 6

Left: Chromosomes of *Lasioglossum (Evylaeus) cooleyi* from the testes of a male pink-eyed pupa showing the haploid chromosome number, $n = 18$. From: Packer and Owen (1989); Right: *Bombus appositus* (male), $n = 16$ from Owen et al. (1995). Both preparations were made using the air-drying technique of Imai et al. (1977) followed by Giemsa staining.

3.2 Variation in size and number of chromosomes

There is great interspecific variation in the number and the size of chromosomes within a taxonomic group, (White, 1977) which are generally inversely correlated because if we view this as a resulting from "...merely in packaging, if we regard chromosomes as packages of genetic material." (Hsu and Mead, 1969). Gokhman (2009) points out this applies equally well to the Hymenoptera. Hymenopteran chromosomes at metaphase have an average length of 3–5 μm , but range in size from 0.5–17 μm (Gokhman, 2009). A dramatic example of size variation is seen in the ant subfamily Myrmeciinae where the Dinosaur Ant, *Nothomyrmecia macrops*, with $n=47$ has chromosomes of sizes 1–4 μm (Imai et al., 1988, 1990) whereas *Myrmecia croslandi* (Crossland and Crozier, 1986) a most unusual species in the same subfamily, has a single pair of chromosomes (the minimum possible haploid number, $n=1$) of length 17 μm (Gokhman, 2009). However, although the total genome size may be about the same in species with very different chromosome numbers and sizes there is no doubt that karyotypes are of adaptive significance and not just differences in "packaging" (White, 1977). Having an n of 1 may be risky since any chromosomal change such as a deletion however small could be lethal, whereas individuals with higher chromosome numbers might be able to tolerate this. Indeed *M. croslandi* is known from only a few locations and is one of a set of sibling species with various chromosome numbers (Crossland and Crozier, 1986; Taylor, 1991). Ants themselves exhibit the total range of chromosome number as found in the Hymenoptera as a whole (Imai et al., 1990; Cardoso and Cristiano, 2021) from the Australian *M. croslandi* with $n=1$ to the Brazilian giant ant *Dinoponera lucida* with $n=60$ (Mariano et al., 2008). Bees show a smaller range of variation with the lowest found in the andrenid *Andrena togashii* ($n=3$) and the highest ($n=28$) of colletid *Hylaeus* sp. 2, both from Japan (Hoshiba and Imai, 1993).

The range of bee haploid chromosome numbers is shown in Figure 7 with data taken from the Bee Chromosome Database (www.bees.ufop.br) established by Cunha et al. (2021) which

"... is an online resource to gather information on chromosome number and nuclear genome size on bee species from all over the world. Considering the importance of cytogenetic studies for

taxonomy, phylogeny, genetics, systematics, conservation, and evolution, the main goal of this database is to outline what has been done in the field of bee cytogenetics over the last century."

This is a very valuable initiative and it is to be hoped that as new studies are published that the authors will upload their results to the database.

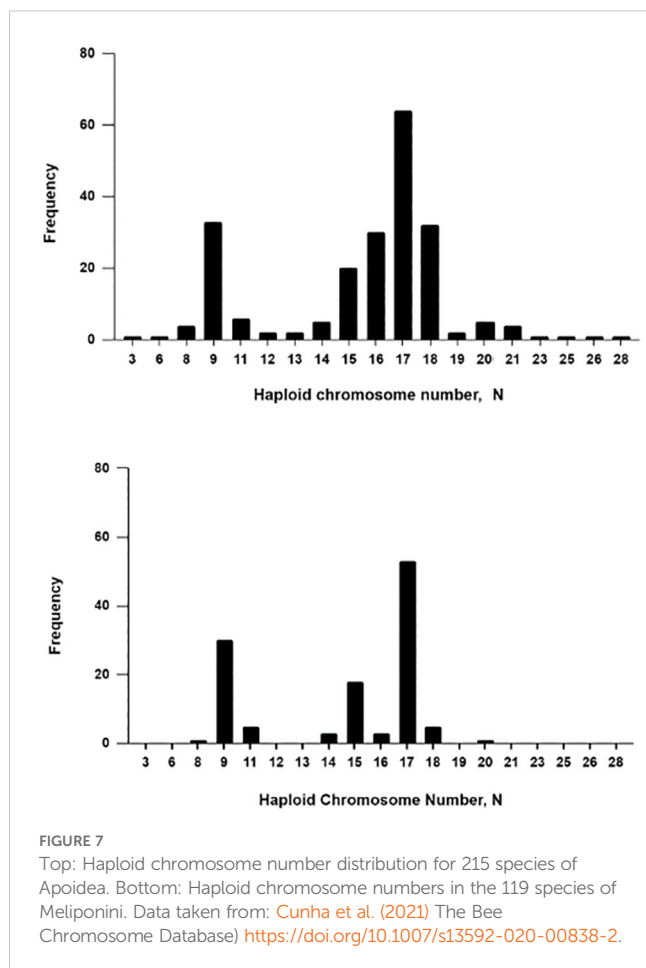
Although relatively few chromosome counts are available for bees (Apoidea) - only 215 species out of a total of 1846 hymenopterans (Table 1) and over 20,000 species of bees - it is possible that most of the entire range of chromosome numbers has been discovered (Figure 7). However, the frequency distribution may not be completely representative, since over half of the counts (119) are of meliponid species which have a distinct clade with an $n=9$ (Cunha et al., 2023) as seen in Figure 7. It would appear that the modal haploid number for the Apoidea as a whole is 17 and the second mode of 9 is exaggerated given the number of meliponid species that have been studied; but this is not to say that this is not of significance nor of importance. Higher chromosome numbers may be found, since ants have a maximum n value of 60 (Table 1) numbers this high could occur in bees also.

In addition to the standard chromosome complement of a species there may be supernumerary or B chromosomes in some (or many) individuals in a population, which as defined by White (1977) are "ones additional to the normal karyotype and not homologous, or only partly homologous to members of the regular set". They are usually heterochromatic and there can be geographical variation among populations (White, 1977). B chromosomes are found in some Hymenoptera; various species of ants, a sphecid wasp (Gokhman, 2009) and the meliponid bees *Melipona quinquefasciata*, *Tetragonisca fiebrigi* (Cunha et al., 2023), *Partamona cupira* and *P. helleri* (Costa et al., 1992; Brito et al., 1997).

Returning to the question of variation in chromosome size, it is clear that the cellular mechanisms, such as spindle formation, etc, and the processes of mitosis and meiosis, are intimately related to the number and morphology of the chromosomes (White, 1977). There are also genetic implications, for example it is well known that recombination rates increase with increasing chromosome number and not just total map length (White, 1977; Sherman, 1979; Templeton, 1979). There will be genetic consequences of chromosomal fusions as this inevitable results in some loss of genes (White, 1977). Within taxonomic groups there is variation of total chromosome complement length as well as variation in size of chromosomes in some species. For example, the bumble bee *Bombus pennsylvanicus* shows little variation in individual chromosome length while *B. fervidus* shows somewhat more (Figure 8). The mean haploid total complement lengths (TCL) being 24.00 μm and 23.3 μm respectively (Owen et al., 1995). Owen et al. (1995) did find significant, but not great, differences in TCL among different bumble bee species (see also Table 2).

3.3 Chromosome evolution in bees (Apoidea); overall trends

An early attempt to describe and understand chromosome evolution in bees was made by Kerr and Silveira (1972) and they advanced the idea that this had proceeded by various rounds of polyploidy (Figure 4). In their own words:



“The chromosome numbers published for all bees ... suggest that polyploidy originated independently at least 5 times: (1) From an ancestor of *Augochloropsis sparsilis* $n=8$, to *Pseudoaugochloropsis graminea* $n=16$; (2) from an ancestor of *Leurotrigona muelleri* $n=8$ to *Frieseomelitta* (3 species) $n=15$; (3) from an ancestral Trigonini $n=9$ to *Plebeia* (6 species) $n=18$; (4) from an ancestor of *Melipona quadrifasciata*, *Melipona arginate* and other species $n=9$ to *Melipona quinquefasciata* $n=18$; (5) from an ancestor of *Apis florea* $n=8$, to *Apis cerana* and *Apis mellifera* $n=16$.”

At the time they were writing this was plausible given the data available at that time, however even soon after it was published it was regarded skeptically (Crozier, 1975) and is no longer accepted. There is no evidence of widespread polyploidy, and where it has rarely been found it is quite obviously unusual, as already discussed earlier with the triploid gall wasp (Sanderson, 1988) and sawfly (Naito and Inomata, 2006). Also, some of the chromosome numbers were incorrect; as we have seen it was not until 1977 that it was established that all *Apis* species had an $n=16$ (Fahrenhorst, 1977), also Kerr's (1972) count of 16 for *Melipona quinquefasciata* was incorrect; it is in fact just 8 (Cunha et al., 2023).

Treating the bees as the superfamily Apoidea and the major groups as families (Table 1) rather than as subfamilies as done by Cunha et al. (2021), we can map chromosome numbers on a phylogeny (Bossert et al., 2019), as shown in Figure 9. This gives the modal haploid chromosome numbers for the major families of

bees with the Apidae split into the five recognized tribes (Bossert et al., 2019).

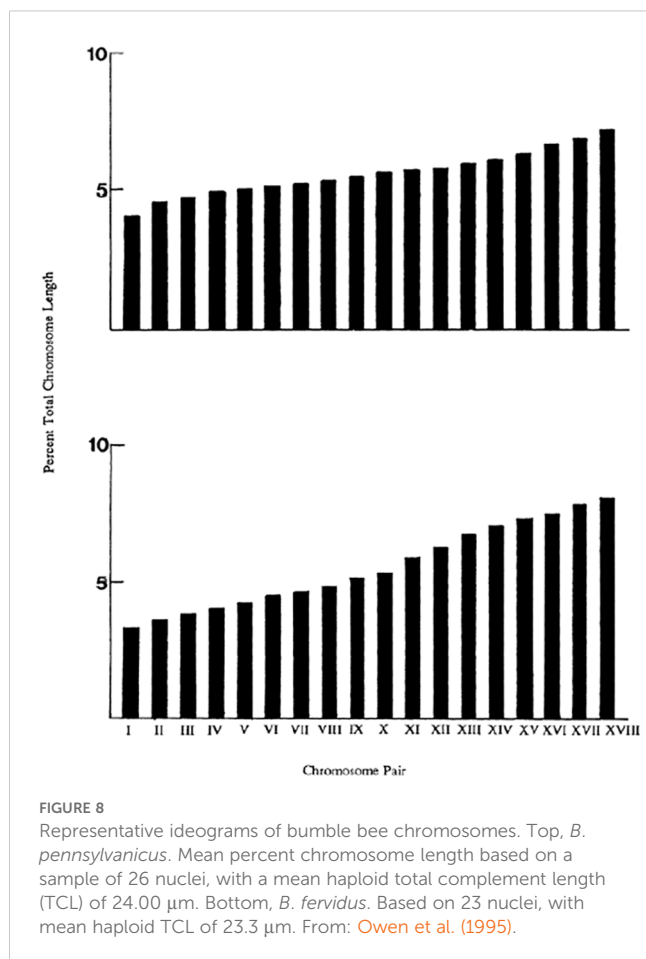
As mentioned earlier, Hoshiba and Imai (1993) and Gokhman and Quicke (1995) suggest that the ancestral chromosome number of the Apoidea was low, $n=8$ or less. Thus, according to this hypothesis, chromosome evolution in the Apoidea has proceeded by fission to generate the higher chromosome numbers in the families Halictidae, Megachilidae and the Apidae (Figure 9) corresponding to the general predictions of the minimum interaction theory of Imai et al. (1986). Reduction in haploid number would have occurred in the Andrenidae. Although the overall trends of the MIT seem to be met it does not necessarily mean that within groups chromosome evolution always follow the MIT, and this is particularly true with the meliponid bees (Cunha et al., 2023).

3.4 Karyotypic variation in the major taxa of bees

Relatively few species (28 total) have been karyotyped in the families Andrenidae (1), Colletidae (5), Halictidae (14), and Megachilidae (8). The only andrenid bee karyotyped, *Andrena togashii* with $n=3$, just happens to have a particularly low chromosome number. It clearly is of importance to obtain more chromosome counts for the Andrenidae. However, it must be noted that haploid numbers of two other *Andrena* species are given by Goodpasture, (1974¹, unpublished doctoral dissertation) but which are used by Ross et al. (2015) and given in their Supplementary table of data. These are *A. duboisi* with $n=3$, and an *Andrena* sp. with $n=10$ (Goodpasture, 1974). These are not included in the Bee Chromosome Database (Cunha et al., 2021) and Cunha et al. (2021) do point out that only one andrenid bee (*A. togashii*) has been karyotyped. Since these observations were not published in the primary literature that is good reason to exclude them, nevertheless this does suggest a low n in this group but still with some variation. Also, it is important to recognize that Goodpasture (1974) published high quality chromosome preparations of five species of eumenid wasps so there is no reason to doubt his results for the *Andrena* species. The two of the three chromosomes of *A. togashii* are relatively long with lengths (when measured from Figure 6J of Hoshiba and Imai (1993) using their 5 μm as reference) of about 8.9 μm and 6.7 μm , the other being 3.3 μm (Hoshiba and Imai, 1993). In contrast all the 28 chromosomes of *Hylaeus* sp. 2 are of shorter length being about 3.3 μm (Figure 6I of Hoshiba and Imai, 1993). This does illustrate the inverse relationship between size and number of chromosomes (Gokhman, 2009).

The five species of the Colletidae show the range of haploid numbers from 8 to the highest in the bees of 28, with a mode of 16. Again, is unlikely to be a coincidence that one of the five species examined happened to have the highest n of the bees, so one might

¹ Goodpasture, C. (1974). Cytological data and classification of the Hymenoptera. University of California, Davis. Unpublished Ph.D.



expect other species to also have high haploid numbers. Since *Hylaeus* is a large genus with 47 subgenera and over 650 described species globally (Michener, 2007; Almeida and Danforth, 2009), much chromosome variation is likely. It would be particularly interesting to look at the 60 Hawaiian species since they form a single clade, although the arrival of the common ancestor and the subsequent rapid adaptive radiation only occurred about 0.4–0.7 MYBP (Magnacca and Danforth, 2006). It would also be very informative to karyotype bees in the closely allied genus of Chilean bees, *Xeromelissa* (Almeida and Danforth, 2009), some of which, such as *X. rozeni*, have extremely long tongues as a result of extreme ecological adaptation (Miklasevskaja and Packer, 2015).

The 14 Halictidae karyotyped have a mode of $n = 16$, but range of 6–20 (Figures 6, 9). A count of 21 for the former *Nomia nevadensis angelesia* (Cockerell, 1910), now a subspecies of *Dieunomia nevadensis*, was reported by Goodpasture (1974a) and is not included by Cunha et al. (2021) in the bee chromosome database and is not included “officially” here.

Chromosome counts have been made for eight leafcutter bees (Megachilidae) all of which have $n = 16$. However, another 12 species were also karyotyped by Goodpasture (1974a) and of these all had $n = 16$ except for one with $n = 15$ and another with $n = 17$.

Turning now to the five tribes of the Apidae. The modal haploid chromosome number of each tribe where known, and number of species on which this is based is shown in Figure 9.

3.4.1 Xylocopini

The nine Xylocopini species karyotyped do not show great variation in chromosome number. Small carpenter bees, *Ceratina*: $n = 17$ (5 spp.), $n = 14$ (1 spp.); large Carpenter bees, *Xylocopa*: $n = 16$ (1 spp.), $n = 17$ (1 spp.) and *Exoneura robusta* $n = 13$ (Bousjeun et al., 2019).

3.4.2 Euglossini

Orchid bees are essential pollinators in the neotropics with hundreds of species, often very abundant and many of which are endangered (Roubik et al., 2021). Although allozyme and microsatellite variation is quite well known in these bees (e.g. López-Urbe et al., 2007; Soro et al., 2017; Souza et al., 2007, 2010) only six species have been examined cytogenetically. Of these four have $n = 21$, one $n = 15$, *Eufriesea violacea* (Gomes et al., 1998) and the other, *Eg. hyacinthine* $n = 20$ (Eltz et al., 1997). Intriguingly, Fernandes et al. (2013) found high heterochromatin content in *Euglossa carolina* with euchromatin only at the chromosome ends, while *Eg. townsendi* had low heterochromatin content throughout, both species had $n = 21$. Gomes et al. (1998) found a similar distribution of chromatin in *Eu. violacea*, in which the long arm of 13 of the chromosome pairs consisted of constitutive heterochromatin, whereas in two pairs the end of the long arm was more euchromatic. Fernandes et al. (2013) suggest that these high and low degrees of heterochromatinization represent a different mechanism of chromosome evolution in these solitary bees than in other Hymenoptera and which is not consistent with the MIT of Imai et al. (1986).

3.4.3 Apini

The tribe Apini consists of a single genus *Apis* comprised of three recognized clades or subgenera; *Apis* – the cavity-nesting species (*A. mellifera*, *A. cerana*, *A. koschevnikovi*, *A. nigrocincta*, *A. nuluensis*); *Megapis* – the Giant bees (*A. dorsata*, *A. laboriosa*), and *Micrapis* – the Dwarf bees (*A. florea*, *A. andreniformis*), thus nine species are formally distinguished (Gupta, 2014; Shanas et al., 2022). However, Shanas et al. (2022) recently described a new species, *Apis karinjodian*, endemic to the Western Ghats Mountain range (a biodiversity hotspot and UNESCO World Heritage Site) which runs north to south in southwestern India. Shanas et al. (2022) used both morphometrics and mitochondrial COI and COII sequence data to identify this as a new species. Their analysis also suggested that the specific status of *A. indica* Fabricius, 1798 be restored (Shanas et al., 2022) as it is currently treated as synonym of *A. cerana* (Radloff et al., 2010). Eleven therefore, would be the total number of distinct honeybee species. There are numerous subspecies or races of most species also identified on the basis of morphology, behavior, ecology and genomic sequences (Ruttner, 1986; Le Conte and Navajas, 2008; Gupta, 2014; Carr, 2023). Interestingly there is still considerable debate over the origins of *A. mellifera*; either out of Africa, out of Asia, from the Middle East to Europe (Gupta, 2014), or a sole European origin (Carr, 2023).

Much is known about honeybee genetics from the pioneering work of Sladen (1913) on the Mendelian inheritance of body colour to the current genome sequencing. For example, complete

TABLE 2 Chromosome lengths in bumble bee (*Bombus* spp.) arranged and numbered according to subgenus (Williams et al., 2008).

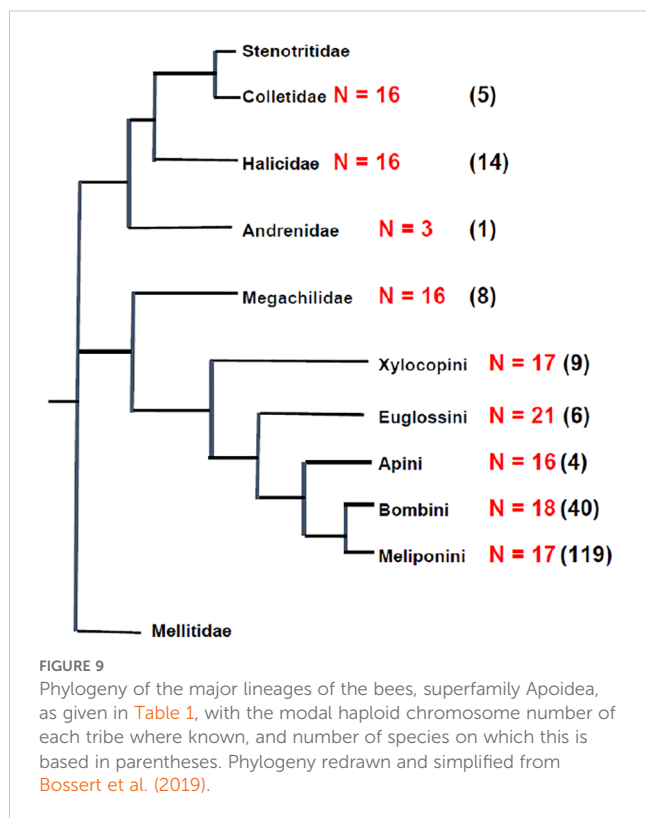
Subgenus		Species		Technique		Estimated		Reference
				Measurement	Genome assembly			
				$\bar{X} \pm SE \mu m$	Total chromo. size, Mb			
4	<i>Orientalibombus</i>	<i>haemorroidalis</i>	18	M: 15.06 $\pm$ 1.14	—	—	—	Chauhan et al., 2015.
				F: 29.18 $\pm$ 0.070	—	—	—	Chauhan et al., 2015.
				—	240.54	942	21.07	Sun et al., 2021.
5	<i>Subterraneobombus</i>	<i>borealis</i>	18	F: 24.22 $\pm$ 0.203	—	1073	—	Owen et al., 1995.
7	<i>Thoracobombus</i>	<i>fervidus</i>	18	F: 22.53 $\pm$ 0.262	—	1007	—	Owen et al., 1995.
8	<i>Psithyrus</i>	<i>citrinus</i>	26	F: 25.51 $\pm$ 0.789	—	1140	—	Owen et al., 1995.
		<i>turneri</i>	25	—	243.33	952	21.29	Sun et al., 2021
9	<i>Pyrobombus</i>	<i>bifarius</i>	18	—	266.80	1045	23.58	Koch et al., 2024
		<i>bimaculatus</i>	18	F: 30.90 $\pm$ 0.562	—	1381	—	Owen et al., 1995.
		<i>huntii</i>	18		317.40	1245	30.59	Koch et al., 2024
		<i>hypnorum</i>	12		297.30	1166	28.65	Crowley and Sivell, 2023
		<i>impatiens</i>	18	F: 25.25 $\pm$ 0.346		1128		Owen et al., 1995.
					242.00	949	21.22	Koch et al., 2024
		<i>perplexus</i>	12	F: 24.15 $\pm$ 0.100	—	1073	—	Owen et al., 1995.
		<i>vagans</i>	18	F: 24.22 $\pm$ 0.203	—	1073	—	Owen et al., 1995.
		<i>vancouverensis</i>	18		282.10	1106	27.19	Heraghty et al., 2020
		<i>vosnesenskii</i>	18		275.60	1081	26.56	Heraghty et al., 2020
11	<i>Bombus</i> s.s.	<i>ignitus</i>	18	—	242.57	949	21.22	Sun et al., 2021.
		<i>terrestris</i>	18	—	274 Mb $\equiv$ 1073cM	—	24.00	Gadau et al., 2001.
		<i>terrestris</i>	18	—	249	—	24.00	Sun et al., 2021
		<i>terricola</i>	18	F: 24.22 $\pm$ 0.203	—	1073	—	Owen et al., 1995.
12	<i>Alpigenobombus</i>	<i>breviceps</i>	18	—	248.12	971	21.72	Sun et al., 2021.
13	<i>Melanobombus</i>	<i>pyrosoma</i>	18	—	254.80	995	22.25	Sun et al., 2021.
15	<i>Cullumanobombus</i>	<i>griseocollis</i>	18	F: 24.22 $\pm$ 0.203	—	1073	—	Owen et al., 1995.
		<i>rufocinctus</i>	18	F: 24.22 $\pm$ 0.203	—	1073	—	Owen et al., 1995.

Nine of the 15 subgenera are represented. Total complement length given in centi-Morgans (cM). The factor used to convert Mb to cM to μ m is based on data from Gadau et al. (2001) and is shown in bold. The values for *B. impatiens* are given in bold italics, as these allow the accuracy of the conversion factors used to be checked (see text for details).

mitochondrial DNA sequences are known for 21 of the 25 subspecies (Le Conte and Navajas, 2008) of *A. mellifera* alone (Carr, 2023). Although much cytogenetic work has been done on honeybees (e.g. Deodikar et al., 1959; Fahrenhorst, 1977; Hoshiba and Kusanagi, 1978; Milne, 1986; Stanimirovic et al., 2005) it is surprising that actual chromosome counts have only been made for four species: *A. mellifera*, *A. dorsata*, *A. cerana* and *A. florea*, and all have $n=16$ (Figure 9). Although sectioning techniques are not ideal, Deodikar et al. (1959) did achieve good preparations with both diploid (worker destined) and haploid eggs and larvae using this method with *A. indica*. Their photographs clearly show 16 and 32 chromosomes for haploid and diploid eggs respectively (Deodikar et al., 1959). Brito and Oldroyd (2010) refined the technique for

preparing karyotypes from eggs and found that three-day old eggs yielded cells in metaphase with clearly seen chromosomes.

As mentioned earlier, Fahrenhorst (1977) demonstrated that all four *Apis* species all had the same haploid number of chromosomes. He used testes of white eyed drone pupae and a maceration and evaporation method which gave excellent results with the sister chromatids clearly visible in his preparations (Fahrenhorst, 1977). Hoshiba and Kusanagi (1978) using male and female gonadal tissue and drone head ganglia, provided a more detailed analysis and classified the *A. mellifera* chromosomes as consisting of 8 metacentric (m) and 8 submetacentric (sm) pairs with lengths of 1.3-4.3 μ m. Later, Hoshiba (1984a, b) using tissue (testes) from young larvae of haploid and diploid males and C- and G-banding



refined the classification to 4 m and 12 sm pairs, and also found that each chromosome had a unique banding pattern. Some comparison in banding pattern of chromosomes between honeybee species was done by Hoshiba and Imai (1993) where they found different c-banding patterns in *A. cerana japonica* and *A. mellifera ligustica* in at least six of their chromosomes as shown in their Figures 3F, G.

Stanimirovic et al. (1999a, b, 2005) in their extremely detailed studies of chromosomal variation in *A. mellifera carnica*, found differences in length of some chromosomes and in G-banding patterns among different ecotypes in Serbia. They studied three populations corresponding to three ecotypes²; Banat (B), Timok (T) and Syenichko – Peshterski (S-P), distributed roughly north to south in present day Serbia (Figure 1 in Stanimirovic et al., 2005). Stanimirovic et al. (2005) consider that “...honey bees of each ecotype investigated ... are adapted to specific microclimatic and floristic conditions of the region they inhabit.” That these represent semi-isolated populations is very likely since Stanimirovic et al. (2005) sampled small apiaries at least 7 km distant from any others, and which had been established for at least 50 years. Moreover, traditional honeybee keeping practices had been followed and requeening of colonies was strictly natural (Stanimirovic et al., 2005). Stanimirovic et al. (1999a) observed significant differences in the relative chromosome and arm lengths between bees from the B

and S-P ecotypes; chromosomes 12, 2, 3, 1 and 6 being longer in the S-P ecotype, while chromosomes 15, 14 and 11 were longer in the B ecotype (Stanimirovic et al., 1999a). G-bands of chromosomes 2, 4, 11 and 13 showed different patterns in T and B ecotypes, and for the T and S-P ecotypes, there were differences for chromosomes 1, 12, 15 and 16 Stanimirovic et al. (1999b, 2005). Overall, the B and S-P ecotypes showed the largest differences in G-band number and distribution for chromosomes 1, 2, 4, 11, 12, 13, 15 and 16 Stanimirovic et al. (2005). All these differences are clearly illustrated in Figures 7 and 8 of Stanimirovic et al. (2005). Muñoz et al. (2012) followed this up with mitochondrial DNA analysis to look for other evidence of genetic differentiation among the ecotypes and for any indication of hybridization between the subspecies *A. mellifera carnica* and *A. m. macedonica*. Both of these belong to the East Mediterranean or carnica (C-branch) as defined morphometrically (Ruttner, 1986). Muñoz et al. (2012) analyzed the tRNA^{leu}-cox2 gene and identified seven mt haplotypes of the C-branch present, including two new ones; C2o and C2p restricted to the regions B and S-P respectively. Only the C2d haplotype was present throughout the country at frequencies 0.615, 0.500, 0.400 and 0.750 in the regions B, T, S-P and SE (Southeast³) respectively (Muñoz et al., 2012). Since C2d is found in *A. m. macedonica* in Greece and in countries neighbouring Serbia, this suggests introgression from *A. m. macedonica* into *A. m. carnica* (Muñoz et al., 2012). Similarly, C1a, present in *A. m. carnica* (region T), implies introgression from *A. m. ligustica* (Muñoz et al., 2012). Comparisons with surrounding honeybee populations suggest a hybrid situation between *A. m. carnica* and *A. m. macedonica* and also introgression from *A. m. ligustica*.

Inversions in some populations of honeybees have been detected, not through classical cytogenetics but by genomics. Wallberg et al. (2017) and Christmas et al. (2019) found genomic regions on chromosomes 7 and 9 that differed between highland and lowland populations of the honeybee *A. mellifera* in Kenya in East Africa. These were 573kb and 1639kb in length, and dated at ages 3.2MYBP and 1.28MYBP for chromosomes 7 and 9 respectively (Wallberg et al., 2017). These blocks are interpreted as inversions and presumably maintained by balancing selection in each region as each retain genes in a complex coadapted to the differing environmental conditions in the cooler highlands and warmer lowlands (Wellenreuther and Bernatchez, 2018). Also, one of the genomic regions contains octopamine receptor genes which may regulate differences in foraging behavior between the bees in the two habitats (Wallberg et al., 2017). Since different highland populations share the same inversions (Wallberg et al., 2017; Christmas et al. (2019), they may have had the same initial origin and then as Westram et al. (2022) suggest, have “travelled” across areas where they are maladaptive; a process consistent with some theoretical models (Westram et al., 2022).

3.4.4 Bombini

Of the 289 currently recognized species of bumble bees (Williams et al., 2022), chromosome numbers of 40 have been

² The concept of an ecotype was introduced by Turesson (1922) and a revised version by Le Moan et al. (2016) who define ecotypes as “...populations of the same species which have evolved heritable physiological, morphological, behavioral or life history differences that are closely associated with environmental variation”.

³ another region added in this study.

reported (Kerr and Silveira, 1972; Garófalo, 1973; Owen, 1983; Hoshiba and Imai, 1993; Hoshiba et al., 1995; Owen et al., 1995; Ayabe et al., 2004; Chauhan et al., 2015). Ideograms and c-banding patterns have determined for some species (Hoshiba and Imai, 1993; Owen et al., 1995; Chauhan et al., 2015). Given that the modal number of 18 is most likely to be the ancestral chromosome number for *Bombus*, it is not surprising that there is relatively little variation among subgenera (Figure 10), with the exception of *Psithyrus*, Figures 10, 11), which will be discussed in more detail shortly. Haploid numbers range from $n = 12$ in *B. Pyrobombus perplexus* (Figure 3), and *B. hypnorum* Koch et al. (2024) to $n = 26$ in *B. (Psithyrus) citrinus* with a mode of 18 (26 species, Owen et al., 1995).

Given that the divergence of the Bombini from the Meliponini is estimated to be about 34 MYBP (Hines, 2008) the n of 18 found in most species of bumble bees may represent a stable chromosome number that is optimal for this taxon. However, there is some interesting variation within and among subgenera (Figure 10). The subgenus *Thoracobombus* shows variation at the higher end of the scale, with n values from 17–23. The higher values being 20 in *B. atratus* and *B. morio* (Kerr and Silveira, 1972) and *B. deuteronymus* with $n = 23$ (Hoshiba and Imai, 1993). These higher chromosome numbers must have resulted from fission of some chromosomes. Fusion of chromosomes likely resulted in the n of 16 for the two *Subterraneobombus* species *appositus* and *borealis* (Owen et al., 1995) and certainly for the exceptionally low n in *B. perplexus* and *B. hypnorum* (Figure 3, Table 2).

Chromosome length has been measured directly in some species (Owen et al., 1995; Chauhan et al., 2015), and in others

chromosome size in megabases (*Mb*, where 1 *Mb* = 1 million bases) has been assessed using genomic techniques (Sun et al., 2021), or through linkage analysis for *B. terrestris* (Gadau et al., 2001). These different estimates are given in Table 2.

Where possible I have shown the equivalents using the conversion factor derived from the data in Gadau et al. (2001) on the genome of *B. terrestris*. They determined the physical size of haploid genome to be 274*Mb*, and the total minimum recombination size to be 1073 *cM*, thus 1 *cM* is about equal to 255 kb. Similarly, using the rough estimate that 1 *Mb* $\approx$ 1 *cM* (true for the human genome) we can convert the chromosome sizes given by Sun et al. (2021) to *cMs* and total complement lengths in μm . It is important to realize that these conversion factors appear inconsistent because those based on linkage analysis depend on the underlying recombination rate of that particular genome, so as Gadau et al. (2001) point out in the honeybee 1 *cM* = 50 *kb* due to the five times higher recombination rate in *Apis* as compared to *Bombus*. However, Stolle et al. (2011) provided a second generation linkage map for *B. terrestris* and estimated the size of the genome to be 433 *Mb*, and the total corrected map length to be 2047 *cM*, giving 1 *cM* $\approx$ 210 *kb*. Stolle et al. (2011) do quote another (unpublished) estimate of genome assembly size of 250 *Mb* from the Baylor College of Medicine Human Genome Sequencing Center, thus I have used the earlier estimate of Gadau et al. (2001). For *B. impatiens*, we do have an actual measurement of mean total complement length of 25.25 μm from Owen et al. (1995), and a genome assembly size of 242.00 *Mb* from Koch et al. (2024). These two independent estimates allow us to check the accuracy of the conversions. Converting the *Mb* back to μm gives a length of 21.22 μm (Table 2) which is reasonably close to 25.25 μm . Thus, looking at Table 2, we can see that even with these approximations, there is considerable consistency among these measurements and estimates

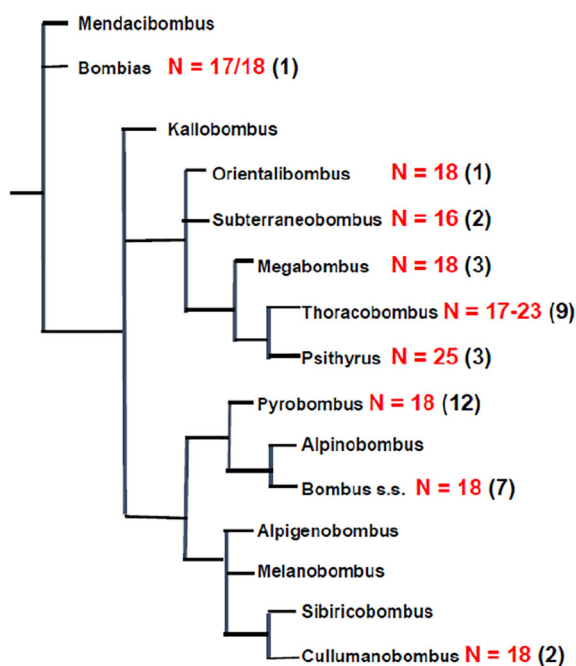


FIGURE 10
The simplified subgenera of bumble bees (*Bombus*) as proposed by Williams et al. (2008) with the modal haploid chromosome numbers shown for each subgenus, and number of species on which this is based in parentheses.

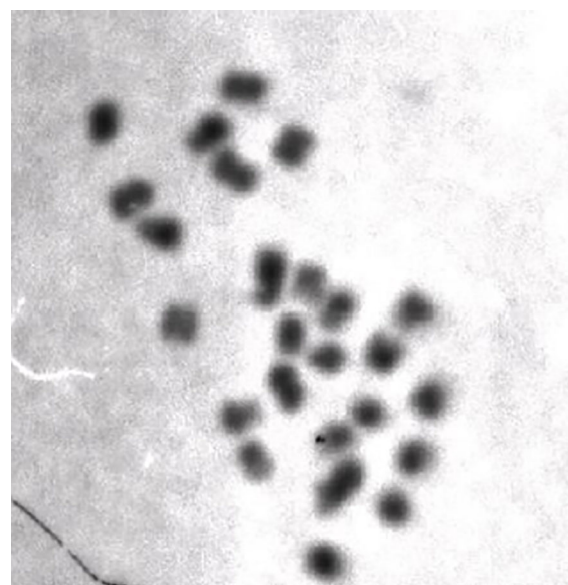


FIGURE 11
Bombus (Psithyrus) ashtoni male, $N = 25$. Photograph and preparation by A. Wilkes, previously unpublished.

of bumble bee chromosome sizes and total complement lengths. The latter ranges from about 21 to 30 μm , total chromosome size is about 240 Mb, and total map length about 1000 cM. The only anomaly, apart from the revised linkage map estimate of Stolle et al. (2011), is the observation by Chauhan et al. (2015) of the shorter chromosome complement length (15 μm) in male *B. haemorroidalis* as compared to that in females of 29 μm . This is difficult to understand. Although total chromosome lengths are reasonably consistent among species there is some variation of chromosome size within species (Figures 8, 3).

Relatively little has been done on other types of chromosome variation in bumble bees. *B. deuteronymus* ($n=23$) has one pair that has polymorphic chromosomes (Hoshiba and Imai, 1993). The recent molecular work of Sun et al. (2021), in addition to confirming the basic 18 chromosome complement of the genus (with the exception of the subgenus *Psithyrus*) estimated some chromosomal rearrangement (inversion) rates. These ranged from 0.0016 to 0.0075 inversions/Mb/My, which as they point out are much lower than rates in various Diptera, thus in this regard bumble bee chromosome evolution is relatively slow (Sun et al., 2021).

The high haploid number of the species in the subgenus *Psithyrus*, as compared to the rest of the *Bombus* is of considerable interest. It certainly has occurred by fission of some of the chromosomes. As Owen et al. (1995) point out the total complement length of *B. citrinus* at 25.51 μm is no longer than that of other species (Table 2). Moreover, Sun et al. (2021) have shown by genomic analysis, that the 25 chromosomes of *B. turneri* have arisen from fission, fusion and conservation of ancestral chromosomes.

The subgenus *Psithyrus* (originally classified as a separate genus of bumble bees), is a very well defined monophyletic group on the basis of many characters (Plowright and Stephen, 1973; Williams, 1985; Pamilo et al., 1987; Sun et al., 2021) and is comprised of obligate socially parasitic bees. The queens infiltrate eusocial *Bombus* colonies, and kill the host queen, or cohabit with the host queen and use chemical mimicry to “blend-in” and not be recognized by the host workers (Fisher, 1985; Martin et al., 2010; Lhomme and Hines, 2019; Dozier et al., 2023). Three of the 27 *Psithyrus* species have been karyotyped; *B. (P.) ashtoni* $n = 25$ (Owen, 1983), *B. (P.) turneri* $n = 25$ (Sun et al., 2021), *B. (P.) citrinus* $n = 26$ (Owen et al., 1995). Note that *B. (P.) ashtoni* may be conspecific with *B. (P.) bohemicus*, similarly *B. fernaldae* and *B. flavidus* are also probably conspecific (Cameron et al., 2007; Hines, 2008) which would reduce the number of species to 25. When I initially observed (Owen, 1983) the *B. (P.) ashtoni* chromosome number I regarded it as a possible outlier, however the finding of equally high n 's in the two other species suggests that an $n = 25$ is possibly ancestral in this subgenus. This is likely since all three species are quite well separated phylogenetically within this subgenus (Cameron et al., 2007). The following hypotheses could account for the high n of *Psithyrus*: (i) the common ancestor of the subgenera *Thoracobombus* and *Psithyrus* (Figure 10) could have had an n of 25, which has subsequently been reduced in *Thoracobombus* but retained in *Psithyrus*, (ii) the common ancestor of *Psithyrus* could have had an n of 25 which has been retained in some species but lost in others, (iii) the common

ancestor of *Psithyrus* could have had an n of 25 which has been retained in all species. Clearly it is essential to determine haploid numbers of other *Psithyrus* species to help distinguish among these possibilities. For hypotheses (i) and (iii) if the n of 25 has been retained, then there are at least two obvious sub-hypotheses; either (a) there has not yet been enough time for subsequent fusion of chromosomes to occur as expected under the minimum interaction theory (Imai et al., 1986), which assumes fission-fusion cycles, or just fusion as other theories assume (King, 1993). Since the subgenus diverged quite recently, about 9 MYBP compared to other bumble bee subgenera (Hines, 2008) this is plausible. Alternatively (b) there may a selective advantage for this higher n which actively maintains it, and this is what I will now explore.

As is well known, Darwin (1859) saw the existence of sterile workers in social insect colonies as a very real problem for his idea of natural selection⁴, and he solved this by invoking the idea of family or colony-level selection, which although relevant in other contexts (Owen, 1986) is not really a satisfactory solution for the evolution of eusociality and altruism. However, other later authors, even R.A. Fisher (1930) pursued similar models (see Owen, 2014), even relatively recently (Nowak et al., 2010). The problem with this group-selection type approach is that the model itself implicitly assumes the outcome. Hamilton (1964) solved this conundrum with his formalization of the concept of inclusive fitness and also suggested that haplodiploidy was a major driver social evolution in the Hymenoptera since the $\frac{3}{4}$ degree of relatedness among workers would lower the cost/benefit ratio required for an altruistic allele to spread. Subsequently this “kin-selection” has become the dominant paradigm for the evolution of eusociality (Wilson, 1971; Nowak et al., 2010; Ratnieks et al., 2011; Owen, 2014). Early on Sherman (1979) pointed out that although the average relatedness among workers was $\frac{3}{4}$, the actual fraction of alleles shared, those identical by descent (IBD) will vary due to Mendelian segregation and the rules of meiosis. Thus, if workers can recognize and preferentially assist the siblings to which they are most similar, this will reduce the reproductive success of their mother (Sherman, 1979). However, as Sherman (1979) also argued, if the same number of gene loci, including ones with recognition alleles, were distributed over a large number of chromosomes then the actual number of alleles IBD would approach the average. Thus, he predicted that eusocial species should have higher chromosome numbers ($2n$) than their solitary counterparts, and tested this by comparing eusocial and solitary Hymenoptera, and did find a significant effect of n on eusociality (Sherman, 1979). However, since then the association has broken down and there appears to be no association *per se* between chromosome number and sociality, although eusocial taxa may show increased recombination rates (Kent and Zayed, 2013; Ross et al., 2015).

However, we can turn this around and can use this reasoning to postulate how high chromosome numbers may be of selective advantage to the social parasite bees, *Psithyrus*. Templeton (1979)

⁴ In fact, he saw the evolution of different morphological castes within the same colony as the real difficulty (Ratnieks et al., 2011).

extended Sherman's (1979) reasoning to account for chromosomes of different lengths and derived the variance in the coefficient of kinship among full sisters in haplodiploid species as:

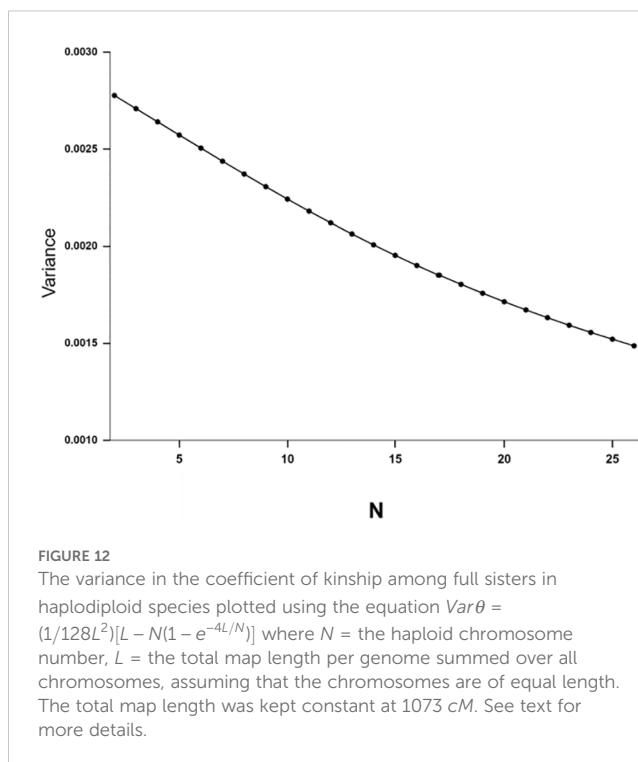
$$\text{Var}\theta = \frac{1}{128L^2} \left(4L - N + \sum_{j=1}^N e^{-4l_j} \right) \quad (1)$$

where N = the haploid chromosome number, l_j = the map length (Morgans) of chromosome j , L = the total map length per genome summed over all chromosomes. If it is assumed that the chromosomes are of equal length ($l_j = L/N$ for all j) then Equation 1 reduces to,

$$\text{Var}\theta = (1/128L^2)[L - N(1 - e^{-4L/N})] \quad (2)$$

which approaches zero as N increases (Templeton, 1979). This is a generalization of Sherman's (1979) demonstration that the variance in relatedness among siblings decreases as chromosome numbers increase. Although the equations apply specifically to the coefficient of kinship, since this is measure of alleles IBD, it will apply to all genes. This means that as chromosome number and length increases offspring will become more and more uniform. Cuticular hydrocarbons provide *Psithyrus* species with the means of chemical mimicry with which to evade chemical recognition by the host species (Martin et al., 2010; Kather and Martin, 2015). If they are produced by many genes [(although not polygenic inheritance *sensu* quantitative genetics (Owen, 1989)] we can expect these loci to be spread out over the chromosomes so the young queen offspring of the successful *Psithyrus* queen will be more uniform in cuticular hydrocarbon composition the higher the chromosome number. Therefore, my hypothesis is that it will of selective advantage to a *Psithyrus* queen who has successfully invaded a *Bombus* nest to have female offspring with a chemical profile similar to hers, as their success is also more likely. The higher chromosome number will help to achieve this by reducing the variance of genes IBD, and so a larger proportion of the female offspring will have a more advantageous profile than if n was lower. Of course, the original queen will mate with a male who will probably carrying genes for a different profile (unless there is also some assortative mating as well) and the female offspring carry only half of the queen's genes. Monoandry is the usual condition in bumble bees (Payne et al., 2003; Owen and Whidde, 2013). However, even given this, the slight, but definite selective advantage may be enough to help maintain this high chromosome number found in this subgenus. To quantify this, I have plotted Equation 2 for various n (Figure 12) assuming that the chromosomes are of equal length, and total map length constant at 1073 cM, typical of that found in bumble bees (Table 2). Increasing the number of chromosomes from 18 to 25 decreases the variance among progeny considerably, from 0.001804 to 0.001521, or 15.7%.

Given that *Psithyrus* species, as are all parasites, are under intense selective pressure to evade their host defenses (Lhomme and Hines, 2019; Dozier et al., 2023) as part of the co-evolutionary "arms-race" between host and parasite (Wurdack et al., 2015) this additional selective advantage may help to retain the high n in this subgenus. The hypothesis predicts that *Psithyrus* offspring in a colony will be very uniform in their cuticular hydrocarbon profile, more so than their hosts. The hypothesis can be disproved if this is

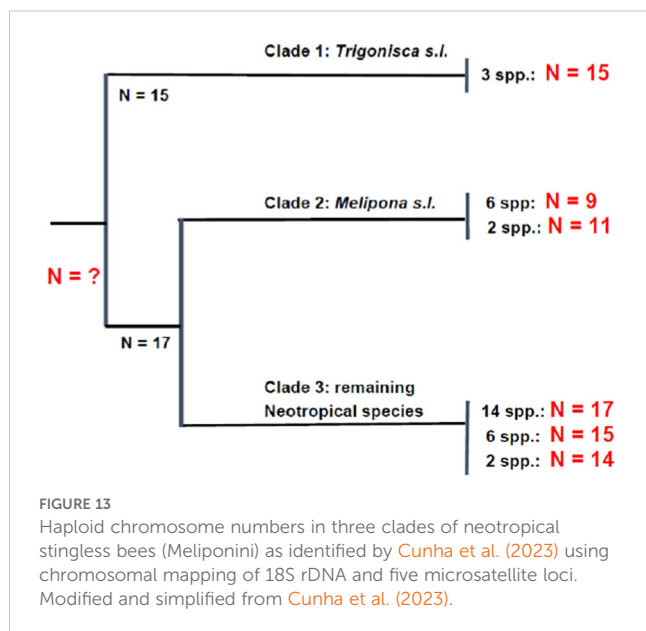


not the case, also, as pointed out earlier, if not all species in this subgenus retain the high n of 25 then this will be a disproof of the hypothesis. There still has to be variation among *Psithyrus* females to keep up with the constant selection for increased discrimination on the part of the host species, and there is variation in *Psithyrus* cuticular hydrocarbon composition (see Figure 5 in Martin et al., 2010), so presumably there must be an optimal balance between variation and uniformity, which possibly occurs at this number of chromosomes. I do admit that this hypothesis is somewhat tenuous, but it is an attempt to explain the high chromosome number found in this subgenus, which is considerably greater than those of other bumble bee subgenera, and is unlikely to be coincidental.

3.4.5 Meliponini

Extensive work has been done on the chromosomes of the stingless bees (Rocha et al., 2002; Costa et al., 1992; Tavares et al., 2017, 2021; Pereira et al., 2021; Cunha et al., 2023) and at least 119 species have been karyotyped (Figure 7). Most of the species are from South America (Brazil) with a few Afrotropical and Indo-Malayan/Australasia species examined (Tavares et al., 2017). Although only 32 of the 54 extant genera of Meliponini have been karyotyped and chromosome numbers vary from $n=8-20$ (Tavares et al., 2017). Three main clades occur with $n = 9, 15$ and 17 (Tavares et al., 2017; Cunha et al., 2023) as shown in Figure 13.

It is the number of meliponid species with $n = 9$ that accounts for the bimodal distribution of haploid numbers in the bees as a whole (Figure 7). Assuming that the ancestral chromosome number of the Meliponini was relatively high ($n = 17-18$) as for the other Apidae (Figure 9) then clearly reduction by fusion has occurred to yield the chromosome numbers in clade 2 (Cunha et al. (2023). Tavares et al. (2017) discuss chromosome evolution in stingless bees



as it relates to the Minimum Interaction Theory (MIT). They point out that for some taxa it applies quite well, but not for others. For example, Pompolo and Campos (1995) found that it explained quite well the karyotypic difference between two *Leurotrigona* species, whereas it does not satisfactorily account for chromosome evolution in some species of *Melipona* (Rocha and Pompolo, 1998).

Under MIT ancestral chromosome numbers should be low, but then would increase by a series of fissions, followed by accumulation of heterochromatin in one chromosome arm (Hoshiba and Imai, 1993; Rocha et al., 2002). In most genera of Meliponini, this is seen, but not in *Melipona* because of its lower chromosome numbers ($n = 9, 11$, Figure 13) and the position of the heterochromatin in some species (Rocha et al., 2002).

Similarly, it is difficult to account for the clade with $n = 9$ purely on the basis of the MIT, and Tavares et al. (2017) conclude that chromosome evolution in the stingless bees cannot simply be explained by a single process.

The Meliponini are notable for various types of chromosomal variation (Tavares et al., 2017). For example, 12 different B chromosomes occur in *Partamona helleri*, with geographical variation between populations (Martins et al., 2009). Tavares et al. (2021) found geographical karyotypic variation in *Trigona spinioes* in Brazil. Although chromosome number of $2n = 34$ was constant there was variation in chromosome type (i.e. metacentric vs submetacentric, etc.), and in the localization of rDNA clusters and of a repetitive DNA sequence (Tavares et al., 2021). Meliponid bees have been studied using sophisticated techniques such as FISH, and chromosomal mapping (Figure 13) of 18S rDNA and microsatellites (Cunha et al., 2023).

4 The minimum interaction hypothesis as applied to bees

The Minimum Interaction Theory or MIT, proposed and elaborated by Imai et al. (1977, 1986, 2001) postulates that

karyotypes evolve to minimize deleterious interactions between chromosomes. This occurs in cycles; centric fissions first increase the number of chromosomes, which reduces their size and the interactions between them. Subsequently there will be an increase in heterochromatin in one of the chromosomal arms to restore the stability of the telomeres. Initially the karyotypes of a group consist of a small number of large chromosomes which would evolve to give a larger number of smaller acrocentric chromosomes resulting from fissions. In many groups of bees the general trends predicted by the MIT appear to hold, increasing from a low chromosome number ($n=2-8$) as seen in the Halictidae and Andreniidae (Figure 9) and to higher numbers of 17-18 (Hoshiba and Imai, 1993). However, this trend certainly does not hold in all clades, for example in the stingless bees (Tavares et al., 2017). Nevertheless, the MIT is a very helpful theoretical framework with which to view chromosomal evolution in the Hymenoptera.

The karyograph method, devised by Imai et al. (1977, 1986) shows these changes visually, and allows actual changes to be plotted. Chromosomes are constrained within the two border lines K_A and K_M where K_A are karyotypes having only acrocentric chromosomes and K_M are those with only metacentric chromosomes. Summarizing from Imai et al. (2001), a haploid karyotype, K is defined as $K = aA + mM$ with “a” numbers of A-chromosomes and “m” numbers of M-chromosomes. The haploid chromosome number (n) is $n = a + m$. Since the number of euchromatin arms in each A- and M-chromosome is, respectively, one and two, the haploid arm number (AN, the total arm number in K) is $AN = a + 2m$. Karyotypes having only A- or M-chromosomes are denoted, respectively, as acrocentric karyotypes (K_A) and metacentric karyotypes (K_M) as special cases.

Actual chromosome evolution is plotted on the karyograph, as done by Hoshiba and Imai (1993) by following a series of steps: (1) classify A and $\bar{M}$ group chromosomes and arrange these by frequency, (2) list the chromosomal rearrangements showing morphological alterations between A and $\bar{M}$ groups (see Figure 2), i.e. centric fissions, fusions, inversions, etc., (3) list the various transitions that can occur, e.g. $\bar{M}-(fis) \rightarrow t-(C^+) \rightarrow A$, (4) reconstruct chromosomal networks (not shown here, Figure 12 in Hoshiba and Imai, 1993), (5) rank each chromosomal alteration by frequency of the chromosome types involved. The results can then be plotted on the karyograph (Figure 13, in Hoshiba and Imai, 1993). This is a complicated method and requires detailed C-banding of chromosomes from many species, but does give a convincing pattern of chromosome evolution for many taxa of Hymenoptera, for example ants (Lorite and Palomeque, 2010) and many wasps (Hoshiba and Imai, 1993) and bees.

Imai et al. (2001) have modeled chromosome evolution under the MIT. They view chromosome evolution as a stochastic process and used Monte Carlo methods to simulate mass-karyotype evolution, and were able to generate theoretical karyographs similar to those derived from empirical data (Hoshiba and Imai, 1993). The MIT, although not necessarily applicable for all taxa is a very useful theoretical framework, but nevertheless does put selective limits on the extent of chromosomal rearrangements.

5 Conclusions

Although much is known about the chromosomes of bees there is still much to learn about overall trends in haploid number and chromosome organization. In this review I have focused on largely on chromosome number – the most basic aspect of all, but we are still lacking this information for many important families of bees. Only 28 species in total have been karyotyped for the families Andrenidae, Colletidae, Halictidae, and Megachilidae. The only andrenid bee karyotyped, *A. togashii* has the low n of 3, so we certainly need to know which other species in these families have low chromosome numbers to see if this is an exception and to further test the MIT prediction of the evolutionary increase in chromosome number. The potential adaptive value of chromosome number *per se* is of great interest. I propose a hypothesis to account for the high ($n=25$) chromosome number found in the social parasitic bumble bee subgenus *Psithyrus*. Straightforward counts of additional species would help resolve this question. More sophisticated techniques beyond chromosome counting and karyotyping using C-banding, yields much more detailed information about chromosomal rearrangements as shown by the work on the neotropical meliponid bees by the Brazilian cytogeneticist (Rocha et al., 2002; Costa et al., 2004; Tavares et al., 2017, 2021; Pereira et al., 2021; Cunha et al., 2023). When these techniques are applied to other taxa of bees they will undoubtedly reveal features of great interest. Genomic approaches are starting to identify chromosomal rearrangements such as inversions and this holds much potential to explore their adaptive significance.

Author contributions

RO: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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