

REVIEW

The defensive response of the honeybee *Apis mellifera*

Morgane Nouvian^{1,2,*}, Judith Reinhard¹ and Martin Giurfa²**ABSTRACT**

Honeybees (*Apis mellifera*) are insects living in colonies with a complex social organization. Their nest contains food stores in the form of honey and pollen, as well as the brood, the queen and the bees themselves. These resources have to be defended against a wide range of predators and parasites, a task that is performed by specialized workers, called guard bees. Guards tune their response to both the nature of the threat and the environmental conditions, in order to achieve an efficient trade-off between defence and loss of foraging workforce. By releasing alarm pheromones, they are able to recruit other bees to help them handle large predators. These chemicals trigger both rapid and longer-term changes in the behaviour of nearby bees, thus priming them for defence. Here, we review our current understanding on how this sequence of events is performed and regulated depending on a variety of factors that are both extrinsic and intrinsic to the colony. We present our current knowledge on the neural bases of honeybee aggression and highlight research avenues for future studies in this area. We present a brief overview of the techniques used to study honeybee aggression, and discuss how these could be used to gain further insights into the mechanisms of this behaviour.

KEY WORDS: Honeybee, Defence, Aggression, Alarm pheromones, Neurobiology

Introduction

The honeybee (*Apis mellifera*) is a eusocial insect. Central to their society is the nest, which contains all the resources of the colony: the queen (the only reproductive female), the brood (attended by nurse bees), the honey produced from nectar collected by foragers, the pollen stores and also the wax combs constructed when the initial swarm moved into the colony housing. Thus, defending this nest (and the main foraging paths emanating from it; Lecomte, 1961) is of prime importance. Yet, with sociality comes the challenge of coordinating the actions of thousands of bees to achieve an efficient response to potential threats, without depleting the colony of too much of its workforce (Rivera-Marchand et al., 2008). The aims of this Review are to: (1) describe how honeybees [of European lineages; see Breed et al. (2004b) for information about Africanized bees] defend their colony at an individual and a collective level, (2) highlight the fine-tuning of this behaviour and (3) review our current knowledge about the neurobiology of this response. In doing so, we hope to provide a framework for future investigations of the mechanisms regulating this complex behaviour, which will provide tools to better manage this domestic species. Indeed, with

approximately 3% of the general population (and 14–43% of beekeepers) being allergic to bee venom, the defensive behaviour of honeybees is an important public health issue (Bilo et al., 2005).

In this Review, after identifying the bee castes involved in colony defence, we describe their behaviour towards different intruders at the hive entrance. Next, we review our current knowledge on alarm pheromones as the key coordinating signals of this social behaviour, before discussing open questions and new research avenues in the study of honeybee aggression, in particular its neural bases. As this analysis requires controlled laboratory assays to study individual aggression, we conclude by presenting the protocols available to study this behaviour.

Division of labour during colony defence

Honeybee colonies are organized into castes according to a temporal polyethism, with individuals of different ages having different roles in sustaining the community (Winston, 1987). Two populations of bees that perform nest defence have been described: guards and soldiers or stingers. Here, we will use these denominations for simplicity; however, the most striking feature of these populations is that they are not well defined. In contrast to other eusocial species (e.g. some ants and termites), in which defensive individuals can be highly specialized, guard and soldier bees are not morphologically different from other bees. Furthermore, nest defence is a very transient behaviour of honeybees and strongly overlaps with other tasks, particularly foraging; hence, the identity of the defensive bees is constantly changing.

Guarding is typically performed by bees during the transition period from inside duties to foraging. Guards can vary greatly in age but are usually 2 to 3 weeks old, and they consistently become foragers after or between guarding bouts. Guards are commonly seen sitting at the hive entrance in a characteristic stance, their forelegs off the ground and their antennae pointing forward (Fig. 1A), or, when very excited, with their mandibles open and their wings held away from their body, ready to fly towards any intruder (Fig. 1B) (Breed and Rogers, 1991; Butler and Free, 1952; Free, 1954; Moore et al., 1987; Paxton et al., 1994). The main roles of guards (described in more detail below) are to check whether incoming bees are their nestmates, and to alert the colony to the presence of a predator. The number of bees allocated to guarding is fairly small – only 10 to 15% of workers become guards (Moore et al., 1987) – and usually they guard for no more than a day. However, this number increases after a disturbance or when more intruders are trying to enter the hive (Breed et al., 1992; Butler and Free, 1952). Colonies displaying a stronger overall defensive response tend to allocate more workers to guarding, and these guards remain active for a longer period (Arechavaleta-Velasco and Hunt, 2003; Breed et al., 1989; Guzman-Novoa et al., 2004).

The number of guards at the hive entrance correlates with the defensive response of a colony to a disturbance; however, only a small fraction of guards actually participates in the stinging response (Arechavaleta-Velasco and Hunt, 2003). Thus, the main function of guards may be the detection and signalling of threats. There is some

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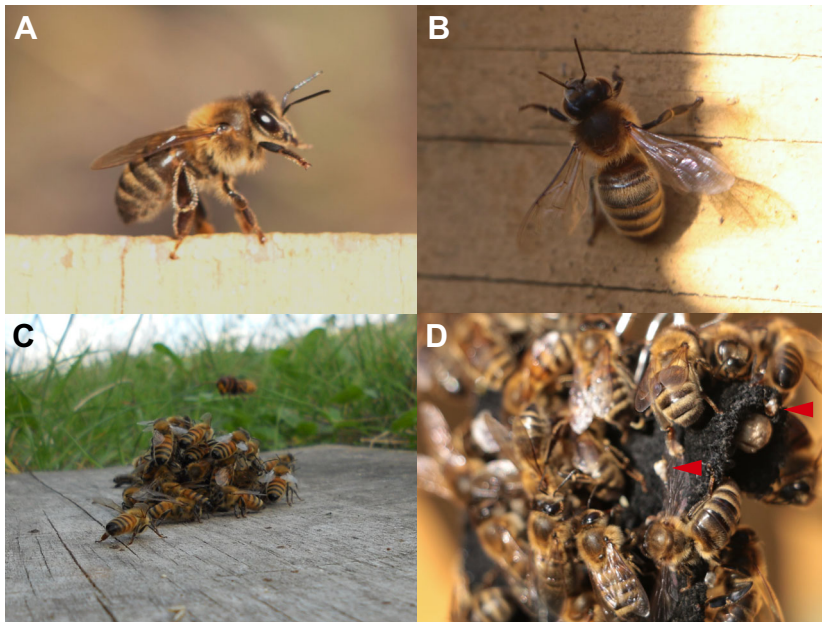


Fig. 1. Behavioural responses of honeybees to different threats. (A) Guard in the characteristic stance, forelegs off the ground and antennae pointing forward. (B) Alerted bee ready to fly off toward the intruder. (C) Honeybees engulfing a hornet in a 'hot bee ball'. A second hornet (*Vespa crabro*) is visible in the background. (D) Guards recruit nestmates to sting large intruders (here the leather flag used as decoy during a field assay). Sting autotomy is evidenced by the stingers (red arrowheads) remaining embedded in the leather. Photos are courtesy of David Vogel, Centre de Recherche sur la Cognition Animale (CRCA) (A,B,D), and David Baracchi, CRCA (C).

evidence that another population of bees – referred to as ‘soldier bees’ – is responsible for harassing any intruders, but this remains a subject of debate. The degree of wear of soldiers’ wings is significantly lower than that of foragers of the same age, so it has been suggested that these bees spend more time inside the hive, where they can be quickly mobilized to the entrance (Breed et al., 1990, 1992). In addition, the propensity to sting is regulated by both genetic factors and age, with older bees being more likely to sting (Giray et al., 2000). Indeed, a number of studies have also demonstrated a patrilineal effect, and have mapped quantitative trait loci that are associated with guarding, stinging or both behaviours (Arechavaleta-Velasco and Hunt, 2004; Breed et al., 2004b; Guzman-Novoa et al., 2002; Hunt, 2007; Hunt et al., 1998; Lenoir et al., 2006; Robinson and Page, 1988; Shorter et al., 2012). More recently, a transcriptional ‘signature’ of aggression has been identified in the bee brain (Alaux et al., 2009; Chandrasekaran et al., 2011). In this Review, we will not include further detail regarding the genetics of honeybee aggression, as this has been extensively reviewed previously (Breed et al., 2004b; Hunt, 2007).

At first glance, the overall defensiveness of a colony correlates with the individual response of its members to noxious stimuli (Avalos et al., 2014), but the link between defensiveness at the individual and at the colony level is far from simple. Complex interactions between individuals are also at play, as evidenced by cross-fostering experiments showing that bees from an aggressive genetic background tend to take over guarding when raised in more gentle colonies, and inversely, gentle bees are less likely to guard when placed in aggressive colonies (Breed and Rogers, 1991). In parallel, cross-fostered bees seem to adopt the propensity to sting of their host colony to some extent (Guzman-Novoa and Page, 1994; Paxton et al., 1994), which suggests that guarding and stinging are differentially regulated but both dependent on colony environment. Finally, when the most aggressive bees of a population are removed, the remaining ones then take over defensive tasks (Lecomte, 1951). This strongly suggests that some kind of defence homeostasis is maintained within the colony. Overall, these studies highlight the sensitivity of guarding and stinging behaviours to both internal (individually based) and environmental factors, and suggest that these behaviours are regulated by interactive, complex and subtle

mechanisms. These mechanisms, which have to take place at the individual level, are largely still to be discovered.

Defence of the hive entrance

Intra-specific defence

When another bee lands at the hive entrance, guards quickly approach and antennate it in order to check whether it is a nestmate. Nestmate recognition is based on the perception of chemical cues carried by the arriving bee (cuticular hydrocarbons, especially alkenes) (Dani et al., 2005; Pradella et al., 2015). These cuticular cues have both a genetic component (Breed, 1983; Getz and Smith, 1983; Page et al., 1991) and an environmental component acquired inside the hive by contact with the comb wax (Breed et al., 1995, 1998; d’Ettorre et al., 2006; Downs and Ratnieks, 1999). Interestingly, emerging bees present a ‘blank slate’ protecting them from expulsion during the short delay before they are endowed with the proper cues (Breed et al., 2004c).

The task of guards is thus to compare the chemical profile of incoming bees with that of their own colony. Current theories posit that guards have an ‘internal template’ of the colony odour, although the exact nature of this template remains debated (Breed et al., 2004a; Ozaki and Hefetz, 2014; Page et al., 1991). Because the colony odour can change (e.g. when a new queen takes over, after swarming or when different patrilines are produced), guards continuously update their internal template and accept other bees in accordance with their chemical similarity (Breed et al., 2004a; d’Ettorre et al., 2006). Surprisingly, increased acceptance of non-nestmates after a comb transfer between two hives seems to rely on guards quickly adopting a new template rather than on a change of the bees’ odour, suggesting that guards retrieve this information directly from the combs rather than from their kin (Couvillon et al., 2007). Inspections by guard bees are usually very quick (1–5 s), and most of the bees examined do not even stop while they are antennated by guards (Butler and Free, 1951). Sometimes, however, inspections are much longer, up to 30 s or more. On such occasions, the examinee adopts a submissive posture and heats up its thorax, probably to enhance chemical evaporation to facilitate its identification (Butler and Free, 1951; Stabentheiner et al., 2002). If an incoming bee is recognized as an intruder, it is mauled by the

guards and dragged away, while remaining in the submissive posture. Even if they gain access to the colony, intruders may still be examined on the combs and dragged back to the hive entrance, suggesting that guard bees are also present inside the hive (Butler and Free, 1951; Stabentheiner et al., 2002).

The sequence of behaviours described above is mainly directed at returning nestmates or non-nestmate foragers that accidentally land at the wrong hive. Yet bees might also try to steal honey from other colonies' stores. These 'robber bees' are identified even before they land because they exhibit a characteristic swaying flight, moving to-and-fro in front of the hive 'as though watching for an opportunity to alight unseen by guard bees' (Butler and Free, 1951). Guard bees dart towards the would-be robber as soon as it lands and start mauling it without any apparent need for an olfactory inspection, although they will release a bee that carries their own colony odour. A robber caught in this way will immediately try to escape and, if successful, will resume its swaying flight. However, if the guards succeed in maintaining their grip, a one-on-one fight ensues in which a guard and the robber try to sting each other (Free, 1954). Such fights end with the death of the opponent that is successfully stung, with the guard and the robber having a similar probability of winning.

There are a variety of factors that influence the defensive behaviour of honeybees to non-nestmates. The amount of resources available to the colony has a strong influence on the behaviour of guards. They are rarely aggressive to non-nestmates landing at the hive entrance when the colony has sufficient resources; however, under conditions of food shortage, they reject or even kill non-nestmates (Butler and Free, 1951; Ribbands, 1954). This effect could be mediated by the presence of empty combs in the nest, which has been linked to a significant increase in colony defensiveness (Collins and Rinderer, 1985). By contrast, guarding is decreased, along with foraging, under high predation pressure (Rittschof and Robinson, 2013). In addition, guards will more readily reject non-nestmates with activated ovaries; honeybee workers start laying eggs (producing haploid males) if their colony has been deprived of a queen for too long.

The presence or absence of a queen has a strong effect on honeybee defensive behaviour. Without a queen, all bees become generalists and participate in nest defence (Naeger et al., 2013). Furthermore, they reject all non-nestmates to prevent reproductive parasitism (Chapman et al., 2009). However, the prolonged absence of a queen actually causes colonies to become more docile, suggesting that the queen exerts a direct influence on hive defence in order to ensure her own survival (Delaplane and Harbo, 1987).

Defence against other insects

Honey stores also attract other insects, such as ants. When confronted with these pedestrian invaders, the bees at the hive entrance exhibit a stereotyped behaviour: they first turn away from the ants and then blow these small insects off the landing board by fanning their wings at a very high frequency (275 Hz on average, exceeding the wing-beat frequency during flight) (Spangler and Taber, 1970; Yang et al., 2010). Ants are faster than bees on foot and do not hesitate to bite them, so this strategy successfully removes the ants while avoiding direct contact. Different subspecies of bees exhibit slight variations of this pattern: *Apis mellifera ligustica* completes this behaviour by kicking its hind legs to strike ants (Spangler and Taber, 1970) but also beetles (Atkinson and Ellis, 2011), whereas *Apis mellifera capensis* performs alternating circles in clockwise and anticlockwise directions to ensure that a large area is covered (Yang et al., 2010). Although defence against ants is

rarely observed, the occurrence of this behaviour in two different bee subspecies suggests that it may be widespread.

While ants are mostly opportunistic, other insects have developed strong parasitic associations with honeybee colonies. These pests, well-known to beekeepers, include the mite *Varroa destructor*, the greater wax moth, *Galleria mellonella*, and the small hive beetle, *Aethina tumida*. Defence of the colony against *V. destructor* relies on grooming and hygienic behaviours rather than on active guarding, and will therefore not be addressed here (see Rosenkranz et al., 2010 for a recent review on this topic). Wax moths enter the hive through unscreened top entrances and lay eggs in cracks, out of the reach of bees. The emerging larvae feed on wax and hive debris, tunnelling just under the cell caps and feeding on discarded cocoons, thus destroying the combs. Honeybees remove wax moth larvae by biting and dragging them out of the nest (Papachristoforou et al., 2012; Yang et al., 2010). Intruding beetles are usually mauled by several guard bees. However, the small hive beetle has evolved a shielding exoskeleton and reduced appendages that it can retract under its body in a turtle-like manner. This body shape and behaviour make it difficult for guards to grasp or sting this beetle, which often finds a small, out-of-reach place to hide in the hive. Guard bees will surround this area, confining the beetles to it. Nevertheless, the beetles are still able to survive under such conditions because they can trick their hosts into feeding them through trophallaxis, a mouth-to-mouth food exchange (Atkinson and Ellis, 2011; Ellis and Hepburn, 2006). Within the natural range of this beetle, honeybees (*Apis mellifera capensis*) will further encapsulate the beetles with propolis (Neumann et al., 2001). If the beetle infestation becomes overwhelming, the bees will abscond, a specific form of swarming during which they leave their nest all at once (Ellis et al., 2003). Interestingly, honeybees show a heightened defensive response towards the specialized small hive beetle compared with other beetle species that can accidentally occur within hives, suggesting that they have developed an adaptive strategy towards this specific intruder (Atkinson and Ellis, 2011).

Finally, honeybees also have to face predatory hornets. These large insects prey on adult honeybees, usually hovering near the hive entrance and swooping on returning foragers. A few workers of the Japanese giant hornet *Vespa mandarinia* can exterminate a large honeybee colony within a single day, and later feed on the pupae and larvae (Matsuura and Sagakami, 1973). Because of the hornets' hard cuticles, it is nearly impossible for honeybees to sting them. Thus, the bees' defensive behaviour during such attacks first involves forming large aggregations at the hive entrance. The bees cling to each other to form a 'carpet' and try to catch the hornet with their front legs and mandibles. If successful, they will then quickly trap the hornet within a dense ball of bees (Baracchi et al., 2010). Interestingly, this behaviour is widespread throughout the *Apis* genus but has evolved to fit the particular interactions of each honeybee species/subspecies with the corresponding local species of hornet. *Apis cerana* honeybees, which originate from Asia, where there are six species of hornets, are particularly efficient in recruiting over 30 workers to form a 'living ball' inside which the hornet is trapped and killed by the high core temperature of approximately 45°C. Bees achieve this increase in temperature by contracting their thoracic muscles. The temperature in the centre of the ball is above the thermal limit of the hornet, yet it is harmless for the bees themselves, which have a thermal limit of approximately 50°C (Ken et al., 2005; Matsuura and Sagakami, 1973). *Apis mellifera ligustica* also use this strategy to confront *Vespa crabro*, a mild predator that

occurs in the native range of this subspecies, although only 15 to 20 workers are involved (Fig. 1C), and they raise the ball temperature to 44°C only (Baracchi et al., 2010; Ken et al., 2005). Another subspecies, *Apis mellifera cypria*, is confronted by *Vespa orientalis*, which has a thermal limit similar to that of honeybees. Consequently, these bees block the hornet's respiration by inhibiting the pumping movements of its abdomen in addition to increasing the temperature, thus asphyxiating it (Papachristoforou et al., 2007). Alternatively, some colonies of this subspecies retreat behind propolis walls with narrow, easy-to-guard openings at the hive entrance and never try to engulf the hornet (Papachristoforou et al., 2011). The reason for the co-existence of these different strategies remains unknown.

Honeybees have been reported to produce piping sounds or 'hisses' when hornets are around, also described as 'shimmering' (Baracchi et al., 2010; Papachristoforou et al., 2008). Hissing seems to be an innate response to noxious stimuli, as this behaviour is also produced in response to electric shocks (Wehmann et al., 2015). Whether these sounds are used as an alarm signal to the colony, as a threat to hornets (which are known to use high-frequency sounds for communication) or are just distress sounds remains to be determined.

Defence against large predators

Guards are also the first defensive line against larger predators, such as birds, mice, raccoons, bears and humans. They will fly to check on any disturbance occurring near the hive (Moore et al., 1987), and are mostly triggered by dark colours, rapid movements, mammalian scents and rough textures (Free, 1961). When confronted with a large predator, some guards immediately fly towards it, while others extrude their sting, raise their abdomen and run inside the hive fanning their wings (Collins et al., 1980; Maschwitz, 1964), releasing the alarm pheromones produced by their stinger apparatus (see below), and thus alerting their nestmates to the potential threat. Indeed, guards cannot handle large predators alone. Therefore, the defence of the colony relies on the recruitment of a larger number of bees (Fig. 1D).

Once recruited, a bee will start searching for the possible target. They are primarily attracted by the animal's movement (Wager and Breed, 2000). However, a study of the number and pattern of stings left in two moving targets presented simultaneously revealed that the alarm pheromones left by previous defenders is a powerful attractant, causing the bees to quickly focus on the single most stung target (Millor et al., 1999). Most bees do not actually sting the localized enemy (Cunard and Breed, 1998), but instead harass it by flying rapidly around it and often bumping into it with a characteristic high-pitched buzz (Collins et al., 1980), in what is thought of as a threatening manner. Because mammalian tissue is elastic, when a bee does sting, the barbed lancets of her stinger (Fig. 2A) – along with its weak connection to the rest of the abdomen – cause this apparatus and the associated muscles to stay in the wound even if the bee itself is quickly removed (Hermann, 1971). This increases the quantity of venom injected into the wound, a single sting thus being equivalent to many injections. This phenomenon, which is followed by the death of the mutilated bee, is called sting autotomy and is found only among eusocial insects where loss of a sterile worker does not have a direct effect on its reproductive fitness (Shorter and Rueppell, 2012). In addition, and contrary to common belief, the stinging bee does not die right away but lives 18 to 114 h after losing her sting (Haydak, 1951), thus conserving some value as a defender through pursuing, harassing, biting and hair pulling (Collins et al., 1980; Cunard and Breed, 1998).

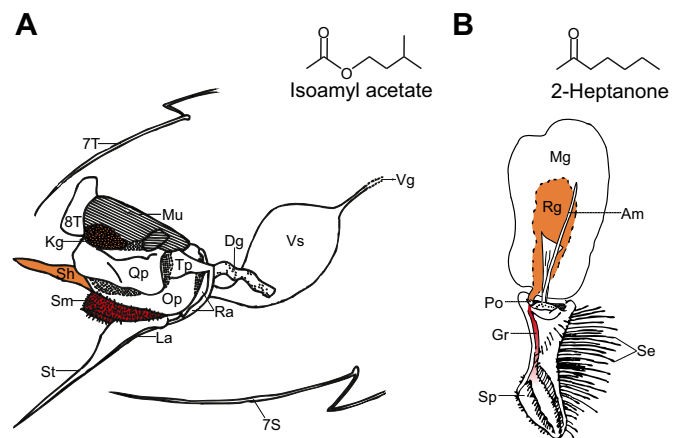


Fig. 2. Organs producing and dispersing the alarm pheromones. Organs producing the alarm pheromones are shown in orange, and those that disperse the pheromones are shown in red. (A) The sting apparatus and the chemical structure of the main component of the sting alarm pheromone, isoamyl acetate. 7S, seventh sternum; 7T, seventh tergum; 8T, eighth tergum; Dg, Dufour gland; Kg, Koschewnikow gland; La, lancet; Mu, muscle; Op, oblong plate; Qp, quadrate plate; Ra, rami; Sh, sheath lobe (in this image it is abnormally folded upwards instead of laying along the stylet); Sm, setaceous membrane; St, stylet; Tp, triangular plate; Vg, venom gland; Vs, venom sac. (B) The mandible and its gland. The chemical structure of the alarm compound 2-heptanone is shown in the upper right corner. Am, apodeme of the adductor muscle; Gr, groove; Mg, mandibular gland; Po, pore; Rg, reservoir of the mandibular gland; Se, setae; Sp, spatula. Adapted from Lensky and Cassier (1995) and Snodgrass (1956) with permission.

Communication in a defensive context: alarm pheromones

Pheromones are chemicals used for communication between individuals of the same species (Karlson and Luscher, 1959). Two types of pheromones are commonly distinguished: releaser pheromones that cause immediate and short-term responses, and primer pheromones that cause long-term physiological changes, eventually leading to behavioural modifications (Wyatt, 2003). The role of these molecules is especially important for colony cohesion in social insects, and the defensive behaviour of honeybees is no exception. Below, we discuss two pheromones that are important for the defensive behaviour of honeybees.

The sting alarm pheromone

Production and dispersal

As mentioned above, one of the key elements in the defensive behaviour of honeybees is a pheromonal blend that signals threats to the whole colony. Beekeepers are familiar with this characteristic banana-like scent emanating from the hive whenever the bees are disturbed. Early research demonstrated that the sting apparatus itself carries an alarm pheromone that can alert and attract bees and provokes stinging attacks (Free, 1961; Ghent and Gary, 1962). Anatomical studies showed that the sting alarm substance is produced by both the Koschewnikow glands and the proximal part of the sting sheaths (Fig. 2A, orange) (Cassier et al., 1994; Grandperrin and Cassier, 1983). The secreted blend flows into the sting chamber, where it accumulates on the setaceous membrane (Fig. 2A, red) (Mauchamp and Grandperrin, 1982). Abundant setae on this structure provide a large surface area, thus enabling a quick discharge of pheromone whenever the sting is extruded (Lensky et al., 1995). Newly emerged bees do not produce iso-amyl acetate (IAA) (Fig. 2A), the main component of this pheromonal blend, until they are 3 days old, and levels remain very low for up to a week, although they can already perceive it (Allan et al., 1987).

When the bee becomes older, however, the volume of alarm pheromone produced rapidly increases to reach approximately 4–5 μg per sting before stabilizing around 2 μg per sting. Interestingly, this peak period of production corresponds to the onset of foraging and guarding behaviours, independent of the age of the bee (Allan et al., 1987; Boch and Shearer, 1966). No correlation was found between production of this alarm pheromone and the overall defensive behaviour of a colony, suggesting that aggressive colonies have a lowered response threshold to the pheromone rather than an increased pheromone production (Boch and Rothenbuhler, 1974).

Composition

As mentioned above, the first identified and main component of the sting alarm pheromone is IAA (also called isopentyl acetate or IPA). A stationary object marked with IAA at the hive entrance attracts and alerts the guards (Boch et al., 1962), but only a moving object releases their stinging behaviour (Free, 1961; Ghent and Gary, 1962). Although honeybees react strongly to IAA, this odorant does not account on its own for the full response observed with sting extracts (Boch et al., 1962; Free and Simpson, 1968). A second compound present in similar quantities was later identified: (Z)-11-eicosen-1-ol. This compound attracts bees to a moving target but not to a stationary one, unlike IAA. A mixture of these two molecules is sufficient to trigger a full response, and prolongs IAA activity on stationary items (Pickett et al., 1982). However, over 40 other compounds have been identified as part of this pheromonal blend (Blum et al., 1978; Collins and Blum, 1983; Pickett et al., 1982). The reason for such complexity is unknown, although it could serve to create a unique signature of this pheromone (Sandoz et al., 2007; Wang et al., 2008).

Functions: alert and orientation

All alarm compounds are releaser pheromones and, as such, trigger fast responses. Table 1 presents some of the molecules identified and their efficiencies in causing agitation of young cage-reared bees (data pooled from Collins and Blum, 1983; Collins and Blum, 1982). Wager and Breed (2000) further tested the functions of some of these molecules by placing them on moving or stationary targets in front of the hive entrance. They found that some were strictly involved in recruiting more defenders (e.g. 1-butanol, 1-octanol), whereas others were orienting the bees towards the target (octyl acetate), and some had both properties (IAA, 1-hexanol, butyl acetate). IAA was the only compound tested that increased flight activity (Wager and Breed, 2000). Interestingly, the alerting function of the sting alarm pheromone seems to be restricted to encounters with other species: contrary to what could be expected, guard bees do not reject non-nestmates more readily when IAA is blown at the hive entrance (Couvillon et al., 2010). The quick decrease in the guards' acceptance threshold observed after a high number of non-nestmate bees have been trying to enter the hive must therefore rely on another mechanism which remains to be determined.

Long-term exposure and primer effects

A few studies have investigated the consequences of long-term exposure to IAA on behaviour and physiology. First, it was demonstrated that bees adapt to their own alarm pheromones. When a dispenser containing synthetic alarm substances is placed into a hive, within 1 h the bees become less inclined to sting and do not differentiate between scented and control targets (Al-Sa'ad et al., 1985; Free, 1988). Under natural conditions, however, the high volatility of IAA makes adaptation very unlikely. Second, it is known that disturbed colonies remain aroused for a long period.

Indeed, repeatedly stimulating a colony with IAA caused the number of bees recruited to the entrance to increase over trials before reaching a plateau (Alaux and Robinson, 2007). IAA also induces the expression of the immediate early gene and transcription factor *c-Jun* in the antennal lobes, which suggests that it has a role as a primer pheromone, prompting long-term changes in brain gene expression (Alaux and Robinson, 2007).

Finally, IAA has been reported to induce analgesia through activation of an opioid system (Núñez et al., 1998), an effect that would ensure that the recruits are unlikely to withdraw from the fight. This could play an important role in social coordination, facilitating recruitment during intense and/or long-lasting defensive events. In addition, prolonged exposure to IAA impairs appetitive learning for up to 24 h (Urlacher et al., 2010). This could be part of a general mechanism priming the bees for defence by causing them to focus on stimuli that would be relevant for colony defence rather than on appetitive stimuli. Interestingly, honeybees exposed to IAA while foraging in a food patch stop other bees from recruiting foragers to this particular location when they return to the hive (Nieh, 2010; Srinivasan, 2010). We recently discovered that appetitive floral odours can, in turn, prevent the bees from stinging in response to IAA (Nouvian et al., 2015). This blocking effect of floral odours could thus be part of an adaptive, long-term strategy to avoid predator-infested areas: by preventing bees from engaging in defence – and potentially dying – this mechanism makes sure that they come back to the colony and communicate the danger at the foraging site.

The mandibular alarm pheromone

Composition and production

Another compound with alarm function is stored in the worker mandibular glands (Shearer and Boch, 1965). Identified as 2-heptanone, this substance is produced in relatively high amounts (15–23 μg per bee). A pore on the internal face of the mandibles allows this secretion to flow out of the mandibular glands, and a groove directs it towards the sharp edges at the tip of the spatula (Fig. 2B, red) (Papachristoforou et al., 2012; Vallet et al., 1991). Young bees produce very small amounts of 2-heptanone, but as they get older this quantity slowly increases. Bees performing indoor tasks have the lowest level of production of mandibular alarm pheromone, guards show intermediate levels and it peaks in foragers (Vallet et al., 1991), which has raised some doubts about the postulated defensive role of this substance (see below). Although a correlation between high levels of 2-heptanone and stinging behaviour has been reported (Kerr et al., 1974), later studies showed no such link (Lensky and Cassier, 1995; Vallet et al., 1991). This may be because the first study used related colonies (Kerr et al., 1974), so the results might simply indicate a genetic linkage between these two elements.

Functions

The efficacy of 2-heptanone as an alarm pheromone has been much debated. When applied on corks at the hive entrance, it elicits defensive behaviour in guard bees (Shearer and Boch, 1965). Similarly, other studies found that it causes agitation in young cage-reared bees (Table 1) (Collins and Blum, 1982), that it increases sensitivity (measured by the sting extension) to electric shocks (Balderrama et al., 2002) and that bees preferentially attack a ball treated with 2-heptanone over a control one (Free and Simpson, 1968). However, the dose of 2-heptanone required is 20 to 70 times larger than the dose of IAA necessary to trigger similar behaviours (Balderrama et al., 2002; Boch et al., 1970). Only one study found

Table 1. Effectiveness of individual compounds in eliciting an alarm response in young caged bees

Chemical	Alarm response					Total	Alarm score	Statistical group	
	No	Weak	Medium	Strong	Very strong			1982	1983
2-Heptanone (M)	2	2	22	42	4	72	2.583		a
2-Nonanol (St)	2	1	29	32	8	72	2.569		a
1-Hexanol (St)	1	7	31	32	10	81	2.519	a	
<i>n</i> -Hexyl acetate (St)	1	3	33	31	4	72	2.458		a
IAA (St)	2	13	102	89	10	216	2.417	b	a
<i>n</i> -Butyl acetate (St)	5	5	31	27	4	72	2.208		a
Benzyl acetate (St)	5	7	34	25	1	72	2.069		a
2-Heptanol (St)	0	16	43	13	0	72	1.958	b	
Iso-pentyl alcohol (St)	17	29	70	33	4	153	1.745	b	b
1-Acetoxy-2-nonene (St)	0	32	29	11	0	72	1.708	c	
1-Butanol (St)	6	24	35	7	0	72	1.514	d	
2-Nonyl acetate (St)	53	96	65	9	2	144	1.444	e	
1-Octanol (St)	6	24	30	3	0	63	1.381	d	
<i>n</i> -Octyl acetate (St)	17	12	28	15	0	72	1.333		b
2-Heptyl acetate (St)	10	26	30	6	0	72	1.306	d,e	
1-Acetoxy-2-octene (St)	14	43	15	0	0	72	0.819	f	
<i>n</i> -Decyl acetate (St)	35	5	28	4	0	72	0.528		c
Benzyl alcohol (St)	37	12	19	4	0	72	0.347		c
1-Decanol (St)	57	11	14	7	2	81	0.136	h	
Phenol (St)	41	24	7	0	0	72	−0.042	g	
Trans-cinnamaldehyde (C)	40	28	4	0	0	72	−0.056	g	
Methyl benzoate (C)	46	23	3	0	0	72	−0.236	g	
Beta-ionone (C)	61	11	0	0	0	72	−0.694	g	

M, compounds from the mandibles; St, compounds from the sting; C, control chemicals not produced by the bees. For comparison purposes, we created an alarm score. Alarm score = $(N_{\text{weak}} + 2 \times N_{\text{medium}} + 3 \times N_{\text{strong}} + 4 \times N_{\text{very strong}} - N_{\text{no}}) / N_{\text{total}}$. The compounds marked by the same group letter in the 'Statistical group' column elicited similar reactions in the original studies. The alarm score closely matches the original statistics run by Collins and Blum. Data are taken from Collins and Blum, (1982, 1983).

that 2-heptanone and IAA had similar efficiency: when presented simultaneously on two moving balls, the bees did not attack either one preferentially (Free and Simpson, 1968). In fact, in some studies, 2-heptanone acts as a repellent or does not elicit any reaction from the guard bees (Butler, 1966; Papachristoforou et al., 2012; Vallet et al., 1991).

Recently, a different function for 2-heptanone in the context of colony defence was revealed: when this substance is injected into parasites through biting, it causes local anaesthesia and paralysis, facilitating their removal from the hive. The injection of 2-heptanone can even kill small parasites such as *Varroa* mites (Papachristoforou et al., 2012). This finding, together with the weak efficacy of 2-heptanone as a recruiting pheromone in simulated mammalian attacks, suggests that this molecule may be more important in the context of defence against other insects. For example, it could help to recruit nestmates not to sting but to remove parasites, or be released as a threat to non-nestmates trying to enter the hive.

Finally, 2-heptanone is used differently in a foraging context. There, it serves as a forage-marking pheromone, repelling foragers from flowers that were just visited and depleted of nectar, thereby saving them time and energy. This allows a bee to forage efficiently in a patch of flowers and to coordinate its activity with the other workers (Giurfa, 1993; Giurfa and Núñez, 1992). This function of 2-heptanone is consistent with its peak production in foragers, and may indicate that its recruiting role in colony defence is of secondary importance, thus explaining the discrepancies between the results of previous studies.

Neurobiology of honeybee aggression

Olfactory processing of alarm pheromones

Neurophysiological studies have analyzed how odorants and their individual components are processed in the olfactory circuits of the

bee brain (Sandoz, 2011). Odorants are first detected by olfactory receptor neurons (ORNs) located within specialized structures on the antennae. ORNs send their projections to the brain, where they contact local interneurons and projection neurons within specific subunits (termed glomeruli) of the primary olfactory centre, the antennal lobe. The number of glomeruli corresponds to the number of olfactory receptors existing in the bee genome (around 160), because all ORNs carrying the same molecular receptor converge within a single glomerulus. As olfactory receptors tend to be broadly tuned (i.e. responsive to a wide range of odorants), odours are encoded in the antennal lobe as specific spatio-temporal patterns of glomerular activation (Galizia, 2014; Sandoz, 2011). The olfactory message is then conveyed to higher-order structures, the mushroom bodies and the lateral horn, via parallel tracts (Carcaud et al., 2015).

In contrast to ants, in which a cluster of five 'alarm-sensitive' glomeruli has been identified (Mizunami et al., 2010), no specific brain structure dedicated to alarm pheromones has been found in the honeybee so far. Rather, components of these pheromones seem to be processed like general odours (Carcaud et al., 2015; Sandoz et al., 2007). Nonetheless, there are some distinctions between the processing of alarm pheromones and that of other odorants. In the antennal lobe, the representation of a mixture of general odours can be predicted based on the linear combination of responses to its individual components (elemental processing) (Deisig et al., 2006, 2010), yet this is not the case for components of the sting alarm pheromone (Wang et al., 2008). This supports the hypothesis that the large number of compounds found in this pheromone could serve to create a unique signature. Little information is available about alarm pheromone processing beyond the antennal lobe. However, one study found that pheromone components elicited patterns of activity in the lateral horn that were similar for compounds carrying the same message (e.g. alarm, aggregation, presence of the queen or of brood) (Roussel et al., 2014). This is in

agreement with current views positing that this structure is a pre-motor centre mediating fast, innate responses in insects (Galizia, 2014; Parnas et al., 2013).

Central and peripheral control

Our recent results show that honeybees integrate all stimuli – relevant ones, such as the alarm pheromone, but also contextual odours – before taking the decision to engage in stinging, thus suggesting that this process is more complex than previously thought (Nouvian et al., 2015). However, the central neural network controlling aggression is still unknown. More is known about peripheral control, particularly about the regulation of the movements of the stinger by the terminal abdominal ganglion. This structure contains a central pattern generator consisting of two loosely connected oscillators, each controlling the thrusting movement of one of the stinger's lancets. The activity of each oscillator is further regulated by afferent inputs from proprioceptors located throughout the sting apparatus: campaniform sensilla, which detect the stress and strain in the cuticle of the stylet and lancets (Fig. 2A), and hairplates between the cuticular plates, which provide information about the relative position of the different elements of the stinger (Ogawa et al., 2011; Shing and Erickson, 1982). The rhythmic movements produced simultaneously bury the stinger deep into the tissue and push the venom towards the tip of the sting, thus maximizing venom delivery (Ogawa et al., 1995). Severing the ventral nerve cord either behind the head or behind the thorax produces activity in the sting muscles (Burrell and Smith, 1994) and triggers the release of alarm pheromones (Balderrama et al., 1996), thus revealing a general inhibitory effect from the brain.

Biogenic amines

Biogenic amines are small molecules synthesized by the nervous system that play a variety of roles, from local neurotransmitters and neuromodulators to peripheral neurohormones (Farooqui, 2012; Libersat and Pflueger, 2004; Scheiner et al., 2006). Using isolated abdominal preparations, it was shown that octopamine reduces the rhythmic activity of the stinger (Burrell and Smith, 1995), but the nature of the effectors (muscles or neurons) remains unknown. Studies of other invertebrate species also suggest that central biogenic amines may play a crucial role in shaping aggression

(Alekseyenko et al., 2013; Hunt, 2007; Kravitz and Huber, 2003; Zhou et al., 2008). Indeed, the serotonergic system has been linked to the fight-or-flight response in crustaceans (Edwards and Kravitz, 1997; Livingstone et al., 1980). More recently, the molecular tools available in the fruit fly *Drosophila melanogaster* enabled the localization of subsets of serotonergic (Alekseyenko et al., 2010; Dierick and Greenspan, 2007), dopaminergic (Alekseyenko et al., 2013) and octopaminergic neurons (Dierick, 2008; Hoyer et al., 2008; Zhou et al., 2008), functional alteration of which caused significant changes in the aggressive behaviour displayed by male flies. Activation of the octopaminergic system has also been linked to a transient increase in aggressiveness in crickets (Rillich et al., 2011; Rillich and Stevenson, 2011; Stevenson et al., 2005, 2000). In the honeybee, the sting extension reflex, an innate response elicited by noxious stimuli, has been coupled with injections of biogenic amine antagonists in the bee brain (Fig. 3A) in an attempt to determine whether and how these amines modulate stinging responsiveness. Dopamine and serotonin antagonists upregulate responsiveness (Fig. 3B,C). It has been proposed that both amines act on attention processes, avoiding excessive responsiveness to irrelevant stimuli (Tedjakumala et al., 2014). Overall, these studies strongly suggest that biogenic amines are main regulators of invertebrate aggression, and that studying their involvement in honeybee aggression in more detail would be an important first step towards the identification of the underlying neural mechanisms.

Brain metabolism

The first hint that the brain metabolism of honeybees was altered during aggressive bouts came from a transcriptomic study identifying functional clusters of genes that were consistently upregulated or downregulated in the brains of aggressive bees (Alaux et al., 2009). These results were confirmed recently by studies revealing that mitochondrial oxidative phosphorylation is inhibited in the brain of aggressive bees in favour of aerobic glycolysis (Barros et al., 2015; Chandrasekaran et al., 2015; Li-Byarlay et al., 2014; Rittschof et al., 2015b). This holds true when comparing genetically aggressive bees with gentle ones, but also when comparing bees from the same background before and after exposure to IAA (Chandrasekaran et al., 2015). Direct manipulation of the brain metabolism of bees confirmed this

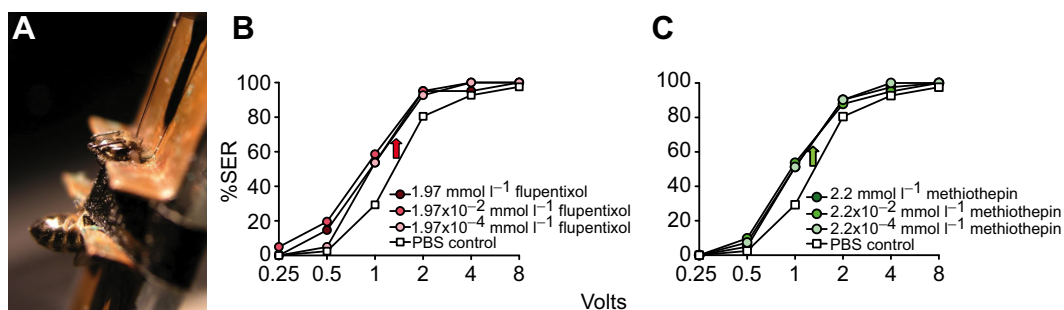


Fig. 3. Dopamine and serotonin modulate the honeybee's responsiveness to noxious stimuli. (A) Brain injection via the ocellar tract in a honeybee harnessed on a shock delivery setup. A tiny hole was pricked into the cornea of the median ocellus to allow the insertion of a Hamilton syringe located above the bee. The syringe allows delivery of the drug to be tested into the median ocellar tract, which runs medially and caudally from the dorsal margin of the head capsule into the protocerebrum. (B) Effects of dopamine blocking on stinging responsiveness. Three different groups of bees were injected with three different concentrations of the dopamine antagonist flupentixol (1.97 mmol l^{-1} , $n=41$; $1.97 \times 10^{-2} \text{ mmol l}^{-1}$, $n=41$; $1.97 \times 10^{-4} \text{ mmol l}^{-1}$, $n=41$). A fourth group was injected with PBS as a control ($n=41$). Sting responsiveness [sting extension reflex (SER)] was measured in response to increasing voltages during shock trials. All three flupentixol concentrations induced an increase in responsiveness to electric shocks compared with that of PBS controls. (C) Effects of serotonin blocking on aversive responsiveness. Three different groups of bees were injected with three different concentrations of the serotonin antagonist methiothepin (2.2 mmol l^{-1} , $n=41$; $2.2 \times 10^{-2} \text{ mmol l}^{-1}$, $n=41$; $2.2 \times 10^{-4} \text{ mmol l}^{-1}$, $n=41$). Each methiothepin concentration induced a significant increase in stinging responsiveness with respect to the PBS control. Figure adapted from Tedjakumala et al. (2014).

relation to be causal, because inhibiting oxidative phosphorylation increased the aggressiveness of treated bees (Li-Byarlay et al., 2014). How this shift in energy metabolism is acting is unknown. It may involve the accumulation of metabolic by-products, including neurotransmitter precursors, thus increasing neuronal excitability. Another hypothesis is that aerobic glycolysis, although less energy efficient than oxidative phosphorylation, may be faster, thus providing the aroused bee with a more immediate supply of energy to cope with this short, energy-demanding state.

Assessing aggression experimentally

A major constraint to the study of the elements modulating and underlying the honeybee's defensive behaviour has been the lack of reliable methods to quantify this behaviour (Guzman-Novoa et al., 1999; Kolmes and Fergusson-Kolmes, 1989; Shorter and Rueppell, 2012; Uribe-Rubio et al., 2008). Field assays are influenced by numerous uncontrolled environmental conditions,

causing huge variability across trials (Guzman-Novoa et al., 1999, 2003; Southwick and Moritz, 1987). They are, however, necessary to study aggression in its natural context. Laboratory-based assays are better controlled and provide more detailed information about the behaviour of individual bees, but they sometimes use stimuli that are difficult to relate to those occurring in the field, such as electric shocks (Kolmes and Fergusson-Kolmes, 1989; Núñez et al., 1983). Here, we summarize these techniques and suggest ways to improve the assessment of aggression in order to facilitate its study.

Colony assays

The majority of assays developed over the years to assess the defensive response of a honeybee colony use a moving target (flag or ball covered in leather) jerked above or in front of the hive, the alarm pheromone IAA or a combination of both: Table 2 presents a number of these methods, and the variables used to measure the bees' response. This overview reveals the lack of consensus on a single

Table 2. Colony-level assays of aggressive traits

Bee containment	Prior disturbance	Target	Measures	First described in ^a	
Whole hive (field)	None	Ball moving at the hive entrance	Number of stings in gloves	Free, 1961; Stort, 1974	
			Number of stings in ball		
			Time before first sting		
			Time before fierce		
			Pursuit distance		
		Suede flag waved at the hive entrance	Number of stings	Villa, 1988	
			Time before first sting		
	Brick dropped on the hive		Number of stings	Giray et al., 2000	
			Time before first sting		
		Honeybee temper tester ^b	Number of hits	Guzman-Novoa et al., 1999	
			Time before hits		
	Puff of breath		Number of hits	Spangler et al., 1990	
	Opening of the hive		Suede flag passed above top frames	Number of stings	Delaplane and Harbo, 1987
	Opening of the hive+alarm pheromone			Number of stings	Moritz et al., 1987
Alarm pheromone(s)	None		Number of bees recruited	Breed and Rogers, 1991	
		Ball in front of the hive entrance	Number of stings	Moritz et al., 1985	
Alarm pheromone(s)+marble shot		Suede flag waved at the hive entrance	Number of bees recruited	Collins and Kubasek, 1982; Collins and Rinderer, 1985	
			Time before recruitment		
			Number of stings		
			Time before first sting		
Opening of the hive+manipulation of brood frames+smoke	None		Ratings of the tendency to run, fly, hit and sting	Guzman-Novoa et al., 2003	
Transparent box at the hive entrance	None	Moving suede flag	Number of stings	Guzman-Novoa et al., 2003	
			Time before first sting		
Cages (laboratory based)	Odours	None	Agitation of young bees	Collins and Blum, 1982	
	None	Live bee or moving dummy	Frequency of attack	Lecomte, 1951	
	Alarm pheromone(s)	None	Metabolic rate	Moritz et al., 1985	

^aTo the best of our knowledge.

^bThe honey bee temper tester is a black bottle containing a small microphone recording the noise made by the bees impacting it.

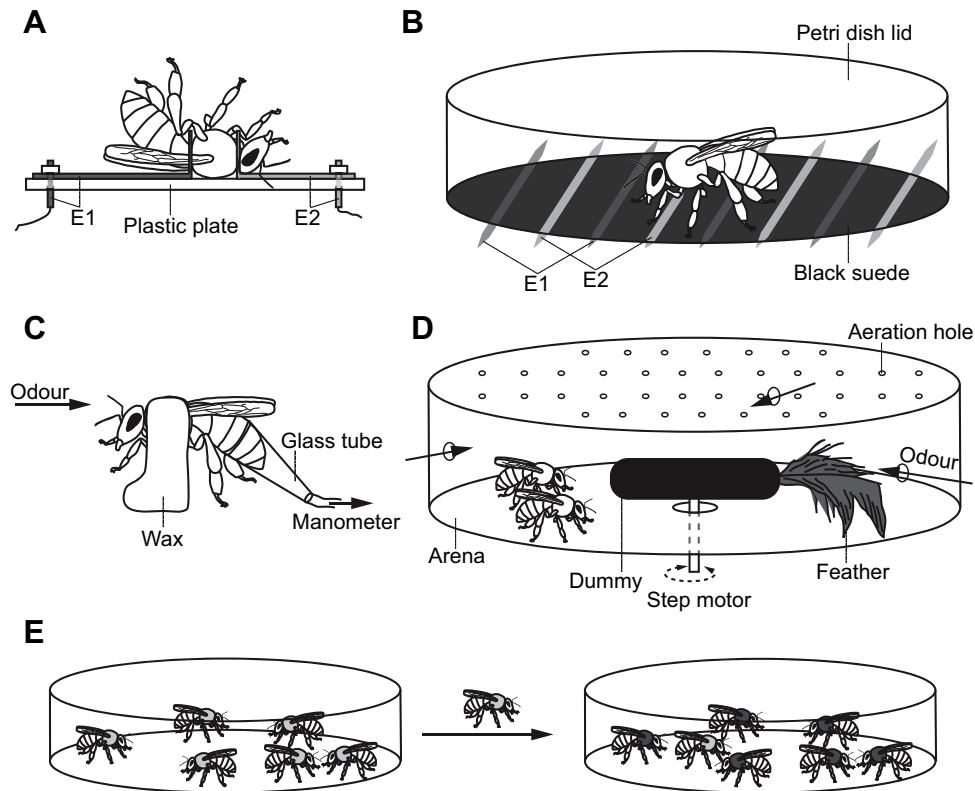


Fig. 4. Individual assays of defensive behaviour. (A) In the sting extension reflex assay (Núñez et al., 1983), the bee is completely restrained in a holder made of two stainless steel plates connected to a power unit that delivers the electric shocks. The extension of the bee's sting in response to the shocks is analyzed. E1, E2: electrodes. (B) The Petri dish assay involves parallel wires set upon a black suede patch. Adjacent wires are connected to opposite poles of the DC power unit (E1, E2) such that a connection between them will cause a short circuit. The honeybee is placed on this surface under a Petri dish cover. The bee receives a shock when she contacts adjacent wires simultaneously (while walking), and can react by stinging the suede patch (Kolmes and Fergusson-Kolmes, 1989). (C) Setup to assess the stinging response to alarm pheromones (from Tel-Zur and Lensky, 1995). The tip of the bee abdomen is sealed into a glass tube and abdominal contractions are recorded by a manometer as the differences in air pressure inside the tube. (D) In the rotating dummy assay, the bees are placed in a small arena and confronted with a rotating dummy to which a light feather is attached. Stinging of the dummy is the response assessed. A continuous air flow can deliver odours into the arena (Nouvian et al., 2015). (E) In the intruder assay, groups of bees are placed in small containers. After some time, individuals from one group are then introduced into recipient groups, and the aggressive behaviour (mauling, biting, stinging) that resident bees display against this intruder is scored (Breed, 1983).

assay to measure aggression in the field, a fact that renders comparisons between studies difficult. This diversity of approaches to measuring aggression may allow precise dissection of the different traits underlying this behaviour (such as responsiveness to the alarm pheromone, responsiveness to visual stimuli, propensity to sting, etc.). However, we believe that the study of honeybee aggression would also benefit from a more careful design of field assays. In many cases, the bees are disturbed before the presentation of the moving target (Table 2). As pointed out by Collins and Kubasek (1982), such prior disturbances affect different components of the defensive sequence, so they can create confounding effects. In particular, the use of alarm pheromones circumvents all the initial regulatory steps during which guards detect and signal a threat.

Individual assays

Honeybees are rarely aggressive when they are alone and away from the hive. Thus, measuring aggressiveness at the individual level has proved challenging. To our knowledge, only five assays are available. In the sting extension reflex assay (Fig. 4A) (Núñez et al., 1983), as well as in its free-walking equivalent, the Petri dish assay (Fig. 4B) (Kolmes and Fergusson-Kolmes, 1989), stinging is provoked by electric shocks. These assays can be used to measure a threshold voltage at which the bees start responding (Balderrama et al., 2002; Kolmes and Njehu, 1990; Núñez et al., 1998; Paxton

et al., 1994). Alternatively, at a constant voltage, the frequency of response (Núñez et al., 1983), degree of response (Lenoir et al., 2006) or time needed for the bee to respond can be recorded (Uribe-Rubio et al., 2008). Because current, not electrical tension, has physiological impacts, improved versions of these assays should benefit from technological progress and control this parameter rather than voltage. A third assay was developed in order to measure the stinging response of a honeybee exposed to alarm pheromones (Tel-Zur and Lensky, 1995). In this assay, a bee is placed in an apparatus made of a delivery compartment and a recording chamber, in which the abdominal contractions of the bee are recorded upon alarm-pheromone stimulation (Fig. 4C). A fourth assay, recently introduced by our laboratory, measures the aggressiveness of honeybees confronted with a dummy rotated by a step motor (Fig. 4D) (Nouvian et al., 2015), based on a previous version in which the dummy was moved manually (van der Burg et al., 2014). We also added a feather that touched the bees during dummy rotation, which reliably induces a stinging response. This assay uses the same stimuli as in field assays, hence providing a new opportunity to investigate the mechanisms regulating honeybee aggression at the individual level. In addition, in this assay the bees exhibit their full defensive repertoire, including lower-level behaviours such as threatening, chasing and 'hair-pulling' (of the feather), so it could also be used to study these responses. Finally,

the intruder assay (Fig. 4E) rates the aggressiveness of small groups of bees towards an intruding conspecific (Breed, 1983). This assay was originally designed for the study of nestmate recognition, but in recent years it has been adapted to investigate how honeybee aggression is affected by different treatments such as early-life experience (Rittschof et al., 2015a), metabolic manipulation (Li-Byarlay et al., 2014) and compromised immunity (Richard et al., 2012). The various assays described here provide interesting possibilities for characterizing honeybee aggression, as they cover different aspects of this behaviour. Thus, combining them may be a good way forward in the study of honeybee aggression.

Conclusions

Despite thousands of years of honeybee domestication, managing the defensive responses of this insect is still a current issue. A wealth of knowledge on this behaviour has been accumulated over the decades: not only has the behaviour been described in detail, but many details on the sensory triggers, environmental factors and pheromonal regulation of the behaviour have been reported. However, there is still a need to uncover the biochemical and neural mechanisms regulating aggression in honeybees. Knowledge of these mechanisms may allow us to understand at what level environmental factors act on individual responsiveness to potential threats, and may facilitate the development of new tools to manage aggressive colonies or the selection of lines with desirable physiological or neural traits in order to improve colony handling.

The defensive behaviour of honeybees requires sophisticated multisensory integration, involving olfactory, visual and mechanosensory cues. It constitutes, therefore, an interesting case study in terms of multimodal analysis and decision-making. The dissection of its neural bases offers a rich opportunity to understand how neural circuits mediate coordinated behaviour, and the resulting coordination between individuals producing a collective defensive response provides an appropriate framework for studies on collective intelligence and adaptive evolution. The availability of cutting-edge technology and techniques to study cellular and molecular mechanisms in the honeybee brain, combined with the appropriate behavioural assays, will allow us to take on these challenges.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Writing – original draft preparation: M.N.; Writing – review and editing: M.N., J.R. and M.G.

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