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Amelie Noel

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THÈSE DE DOCTORAT D'AVIGNON UNIVERSITÉ

**École Doctorale N°536
Agrosciences et Sciences**

**Mention de doctorat :
Sciences agronomiques**

**Laboratoire Biologie et Protection de l'Abeille
UR 406 Abeilles et Environnement, INRAE**

Présentée par
Amélie Noël

**Ecologie chimique de la défense comportementale
de l'abeille domestique (*Apis mellifera*)
face au parasite *Varroa destructor***

Soutenue publiquement le 04/05/2023 devant le jury composé de :

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« Observer, c'est déjà quelque chose, mais ce n'est pas assez : il faut expérimenter, c'est-à-dire intervenir soi-même et faire naître des conditions artificielles qui mettent l'animal dans la nécessité de nous dévoiler ce qu'il ne dirait pas, livré au courant normal. Admirablement combinés pour atteindre la fin poursuivie, ses actes peuvent nous imposer sur leur réelle signification et nous faire admettre, de leur enchaînement, ce que nous dicte notre propre logique. Ce n'est pas la bête que nous interrogeons alors sur la nature de ses aptitudes, sur les ressorts primordiaux de son activité ; mais bien nos propres vues, donnant toujours réponse favorable aux idées que nous caressons. Comme je l'ai bien des fois démontré, l'observation seule et souvent un leurre : nous en traduisons les données d'après les exigences de nos systèmes. Pour en faire émerger le vrai, doit nécessairement intervenir l'expérimentation, seul capable de sonder le ténébreux problème de l'intellect chez la bête. On a parfois dénié à la zoologie d'être une science expérimentale. Le reproche serait fondé si la zoologie se bornait à décrire, à classer ; mais c'est le moindre côté de son rôle : elle a des visées plus hautes, et quand elle interroge l'animal sur quelque problème de la vie, son questionnaire est l'expérimentation. Dans ma modeste sphère, je me priverais de plus puissant moyen d'étude si je négligeais d'expérimenter. L'observation propose le problème ; l'expérimentation le résout, si toutefois il peut se résoudre ; du moins, impuissante à nous donner le plein jour, elle projette quelque clarté dans les flancs de l'impénétrable nuage. »

Jean-Henri Fabre (1823-1915)

Souvenir entomologiques : étude sur l'instinct et les mœurs des insectes

À mes grands-parents,

À Pierre,

R E M E R C I E M E N T S

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LISTE DES ABREVIATIONS

ABPV – *Acute Bee Paralysis Virus* : Virus de la paralysie aiguë de l'abeille.

BEP – *Brood Ester Pheromone* : Pheromone de couvain.

COVs – Composés Organiques Volatiles.

DWV – *Deformed Wing Virus* : Virus des ailes déformées.

IAPV – *Israeli Acute Paralysis Virus* : Virus israélien de la paralysie aiguë.

LVC – *Low Volatile Compounds* : Composés à faible volatilité.

MNR – *Mite Non-Reproduction* : Non reproduction du varroa impliqué due à différents comportements et caractères de résistances (SMR, VSH, réoperculation...).

PDMS-DVB – Polydimethylsiloxane divinylbenzène.

QMP – *Queen Mandibular Pheromone* : Pheromone mandibulaire de reine.

QRP – *Queen Retinue Pheromone* : Pheromone de reine.

SMR – *Suppressed Mite Reproduction* : Caractère de suppression de la reproduction du varroa par la nymphe d'abeille.

SPME – *Solid Phase Micro Extraction* : Technique d'extraction de composés en phase solide.

VDV – *Varroa Destructor Virus* : Virus de *Varroa destructor*.

VPS – *Varroa-Parasitized-Specific* : Composés chimiques spécifiques des alvéoles de couvain parasitées par varroa et déclenchant du comportement VSH.

VSH – *Varroa Sensitive Hygiene* : Comportement hygiénique orienté vers les alvéoles de couvain parasitées par varroa.

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CHAPITRE 1

INTRODUCTION GÉNÉRALE



L'effervescence de la colonie

© Anne Noel

I. La colonie d'abeilles domestiques : une vie sociale

La colonie d'abeilles domestiques (*Apis mellifera*) est un organisme fascinant par son organisation. L'activité intense au sein du nid peut paraître à première vue chaotique mais résulte en réalité d'une fine régulation dans l'organisation des tâches entre les individus qui composent la colonie. La colonie est un super-organisme ; un groupe d'individus génétiquement apparenté fonctionnant en une seule unité (Seeley, 1989; Wheeler, 1928). Dans ce type de groupe, l'individu seul ne peut pas survivre. *A. mellifera* appartient au groupe des insectes ayant développé le niveau d'existence coloniale le plus évolué ; l'eusocialité (Wheeler, 1928). L'eusocialité se traduit par un recouvrement des générations, une coopération dans les soins aux jeunes, une division et spécialisation des tâches et la présence de castes reproductrices et non-reproductrices (Wilson, 1971).

Le cycle de vie de la colonie suit le rythme des saisons. L'hiver, la colonie d'abeilles est réduite en nombre d'individus et les individus passent la plupart de leur temps à l'intérieur du nid. Au printemps, elle connaît une expansion en nombre d'individus et commence à stocker des ressources, nécessaires à sa survie, jusqu'à l'hiver suivant. Ses principales ressources sont l'eau, le nectar, le pollen et la propolis (Abou-Shaara, 2014). Une colonie d'abeilles se reproduit grâce à l'essaimage, en se séparant en deux ou plusieurs colonies. Lors de l'essaimage, la reine et une partie des ouvrières quittent le nid, formant ainsi une nouvelle colonie (Winston, 1987).

1. Les individus composant la colonie d'abeilles domestiques

a. Les adultes

La colonie est composée d'environ 20 000 à 50 000 individus : une reine, quelques milliers mâles et plusieurs dizaines de milliers d'ouvrières (Figure 1.1).



Figure 1.1 : Les trois différentes castes constituant la colonie d'abeilles domestiques *Apis mellifera*. Photos : © Laurent Guilbaud & Amélie Noël.

INTRODUCTION

La reine est la seule femelle ayant les ovaires développés et capable de pondre des œufs fécondés. Elle peut pondre jusqu'à 2 000 œufs par jour, principalement des œufs diploïdes qui donneront des femelles. Les œufs non-fécondés, haploïdes, donneront des mâles. La reine contrôle le comportement et la physiologie des ouvrières grâce à l'émission de phéromones (Slessor *et al.*, 1988; Winston *et al.*, 1991). Lorsque celle-ci est vieillissante, son bouquet phéromonal devient moins actif et les ouvrières commencent à élever une nouvelle reine (Butler, 1957). Une reine vit en moyenne 3 ans (Winston, 1987).

Les mâles, aussi appelés faux-bourçons, sont présents dans la colonie essentiellement lors de la saison de reproduction. Leur seule fonction est de féconder une reine non pondante ; ils ne participent à aucune autre tâche connue de la colonie (von Frisch, 1971). Une fois la saison de reproduction terminée, les ouvrières chassent les mâles du nid. Les mâles ont une durée de vie moyenne de six semaines (Winston, 1987).

Les ouvrières constituent la caste la plus abondante en nombre d'individus au sein de la colonie. Leur rôle est de maintenir l'homéostasie du nid. La répartition des tâches chez les ouvrières suit un polyéthisme d'âge (Seeley, 1982). Les plus jeunes se situent au centre du nid tandis que les plus âgées se retrouvent le plus souvent à l'extérieur de la ruche (Seeley, 1982) (Figure 1.2). Elles commencent par des comportements de soins, puis de magasinage, suivi de gardiennage de l'entrée du nid. Elles finiront leur vie par du butinage (Figure 1.2).



Figure 1.2 : Description du polyéthisme d'âge chez *Apis mellifera* (d'après Winston, 1987).

b. Le couvain

L'abeille est un insecte holométabole, chacun des individus de la colonie suit un développement en quatre stades : œuf, larve, nymphe et imago (adulte). Les individus en développement (œufs, larves, nymphes) sont appelés couvain. Le cycle de développement des différentes castes ne diverge que par sa durée. Le faux-bourdon a le cycle de développement le plus long, d'une durée de 26 jours. Ceux de l'ouvrière et de la reine durent respectivement 21 et 16 jours (Winston, 1987). Le couvain est complètement dépendant des soins apportés par les ouvrières. La reine pond un œuf dans une alvéole du nid. Au bout de 3 jours, une larve éclot de cet œuf. Elle se développe en passant par cinq stades larvaires. Au cours du dernier stade larvaire, l'alvéole de couvain est operculée par les ouvrières avec une fine pellicule de cire. L'opercule est poreux ce qui permet les échanges gazeux et la diffusion de molécules volatiles (Kubásek *et al.*, 2022). La larve se redresse au sein l'alvéole operculée et commence à tisser son cocon. Le couvain débute ensuite sa métamorphose, dont la première phase est le stade pré-nymphe. Le développement se poursuit avec neuf stades nymphaux, jusqu'à l'émergence de l'adulte (Winston, 1987) (Figure 1.3).

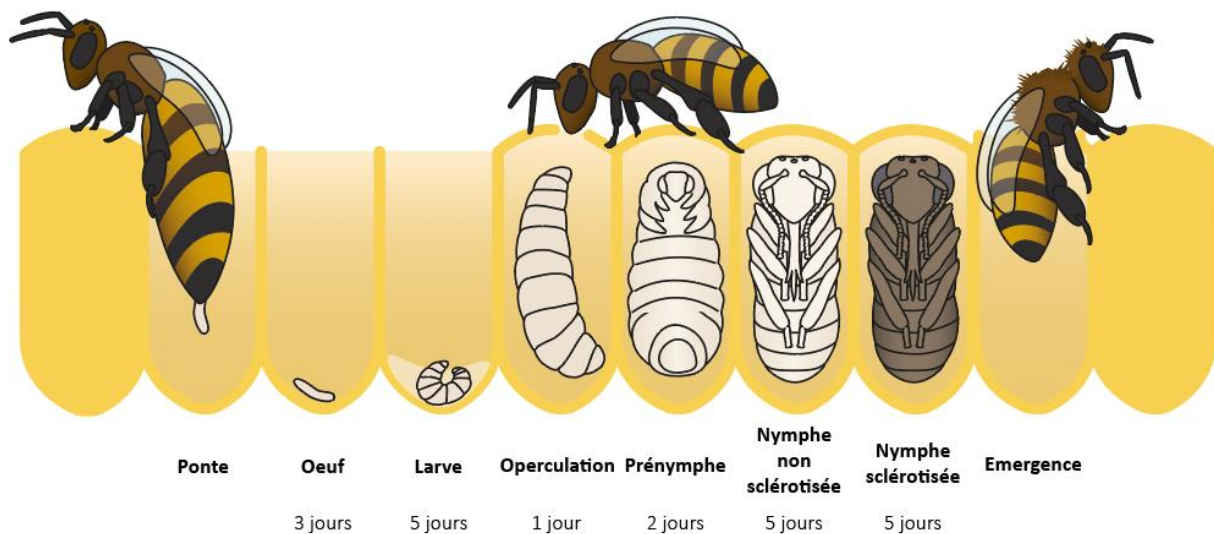


Figure 1.3 : Cycle de développement du couvain d'ouvrières d'*A. mellifera*. Le développement de l'ouvrière dure 21 jours.

2. La communication chez *A. mellifera*

a. Principe de la communication chimique chez les insectes sociaux

La communication est la clef des groupes sociaux. Elle permet de montrer l'appartenance au groupe, de coordonner les activités, d'identifier les castes et les congénères, ainsi que moduler leurs rôles dans la société. Elle peut s'opérer grâce à divers canaux à la fois vibro-acoustiques, tactiles, visuels et chimiques. Tous les systèmes de communication comprennent un individu émetteur du signal et un

individu receveur. La communication chimique est le type de communication le plus répandu dans le règne animal, jusqu'aux bactéries (Wilson, 1970; Wyatt, 2014), et le système de communication le plus utilisé chez les insectes sociaux (Leonhardt *et al.*, 2016; Wilson, 1970). L'échange d'informations par composés sémiachimiques peut se faire entre individus de la même espèce (phéromone) ou entre individus d'espèces différentes (allomone, kairomone ou synomone) (Figure 1.4). Karlson & Lüscher (1959) définissent le terme « phéromone » comme une substance émise par un individu et reçue par un autre individu de la même espèce, déclenchant une réaction spécifique telle qu'une modification physiologique ou un comportement spécifique. Dans le cas de phéromone composée de plusieurs molécules, le ratio de chaque composé est précis et fixe (Wyatt, 2014). Ces signaux chimiques peuvent être captés et utilisés par un individu d'une espèce différente au détriment de l'émetteur, et joueront le rôle de kairomones pour l'individu receveur (Figure 1.4). Cette communication inter-espèces peut aussi être bénéfique seulement pour l'émetteur (allomone) ou pour l'émetteur et le receveur (synomone) (Figure 1.4).

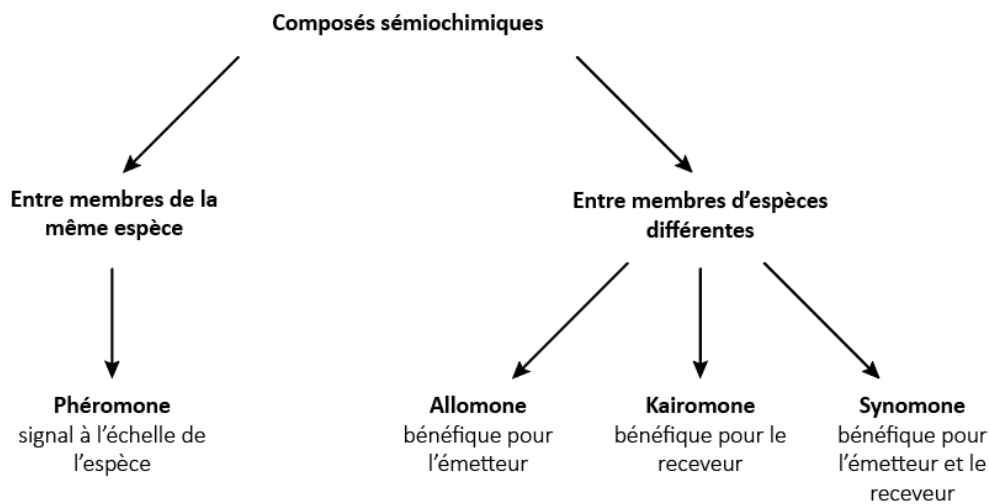


Figure 1.4 : Diagramme montrant les différents types de composés sémiachimiques. Modifié depuis Wyatt, 2014.

b. Le système de détection olfactif des molécules sémiachimiques de l'abeille

L'olfaction est l'un des sens majeurs de l'abeille domestique. L'olfaction est la traduction de l'environnement chimique en une activité neuronale cérébrale permettant la représentation sensorielle des odeurs (Sandoz, 2012). Chez l'abeille, les molécules odorantes sont détectées principalement au niveau des antennes. Les antennes des abeilles sont recouvertes de sensilles, des structures sensorielles poreuses renfermant les dendrites des neurones olfactifs (Figure 1.5). Les molécules entrent dans le système olfactif par les pores des sensilles et atteignent les récepteurs olfactifs présents sur les dendrites des neurones olfactifs en diffusant dans la lymphe sensillaire (Masson & Mustaparta, 1990). Tous les processus de traitement de l'information olfactive dépendent de la détection des molécules

odorantes à la périphérie, c'est-à-dire de la liaison de ces molécules aux récepteurs olfactifs exprimés par les neurones olfactifs. Des protéines impliquées dans la transduction du signal olfactif sont présentes dans cette lymph (Sandoz, 2012). C'est le cas des OBPs (*Odorant Binding Proteins*), des protéines de transport spécialisées impliquées dans le transport des molécules hydrophobes vers les récepteurs olfactifs (Figure 1.5). Chaque neurone exprime un récepteur olfactif particulier et le co-récepteur AmOR2 qui s'assemblent pour former un tétramère (Robertson & Wanner, 2006). Les récepteurs olfactifs peuvent être spécifiques à une seule molécule odorante (ex : une phéromone) ou peu spécifique (ex : des composés floraux). Une fois la molécule liée au récepteur, l'information électrique générée par la liaison de la molécule odorante sur le récepteur olfactif se propage le long de l'axone du neurone olfactif jusqu'au centre olfactif primaire, le lobe antennaire, où s'opère un pré-traitement de l'information. Ensuite, l'information olfactive est envoyée aux centres olfactifs cérébraux de second-ordre, les cornes latérales et les corps pédonculés, permettant ainsi le traitement complet de l'information et le déclenchement ou non de comportement ou de modification physiologique en réponse (Sandoz, 2012) (Figure 1.5).

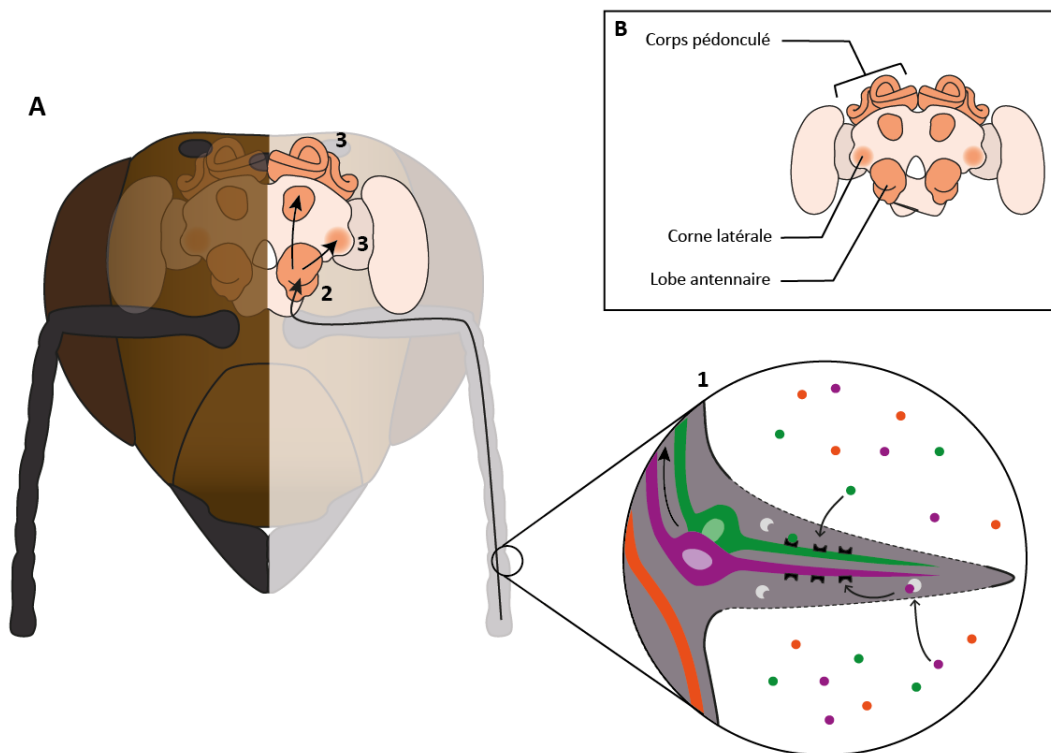


Figure 1.5 : Schématisation de l'olfaction chez l'abeille domestique (*Apis mellifera*). A. Traitement neuronal de l'information olfactive. (1) Les molécules odorantes (points orange, vert et violet) entrent dans le système olfactif par les pores des sensilles antennaires et se lient aux récepteurs olfactifs (noir) présents sur les neurones olfactifs (violet, vert, orange). Des protéines de transport (demi-lune grise) peuvent aider dans le transport des molécules vers les récepteurs olfactifs. L'information nerveuse est envoyée aux centres olfactifs. Elle est (2) pré-traitée par les lobes antennaires (3) puis traitée par les centres olfactifs de second-ordre (cornes latérales et corps pédonculés). Modifié depuis Sandoz, 2012. B. Les trois structures cérébrales impliquées dans le traitement de l'information olfactive.

c. Les principales phéromones et mélanges signature au sein de la colonie

Au sein de la colonie d'abeilles, les individus influent sur le comportement et la physiologie des congénères grâce à une régulation très fine de l'émission de composés phéromonaux. Les phéromones peuvent être modificatrices (influant sur la physiologie) ou incitatrices (influant sur le comportement). La communication chimique est très dépendante du contexte et du ratio de chaque composé chimique au sein du bouquet phéromonal. Cette communication phéromonale concerne tous les individus de la colonie, avec des échanges entre les faux-bourçons et la reine, la reine et les ouvrières, les ouvrières entre elles, le couvain et les adultes (Slessor *et al.*, 2005). Elle pourrait aussi concerner les faux-bourçons entre eux lorsqu'ils se regroupent pour se reproduire avec une reine non pondante. Actuellement, aucune phéromone de mâle n'a été identifiée mais les études tendent à montrer que les faux-bourçons sont attirés par les odeurs mandibulaires de mâles (Bastin *et al.*, 2017; Brandstaetter *et al.*, 2014; Mariette *et al.*, 2021; Villar *et al.*, 2017). Chez l'abeille, une phéromone est généralement constituée d'un mélange de composés chimiques où la proportion de chacun des composés est importante. La reconnaissance des congénères, de leur âge et de leur état de santé, ainsi que l'identification des intrus impliquent l'utilisation de composés phéromonaux (Dani *et al.*, 2005; Le Conte *et al.*, 1994b; Wyatt, 2014).

- *La phéromone royale*

La présence de la reine fécondée est signalée aux ouvrières par l'émission de phéromones. Le premier composé de la phéromone royale identifié fût l'acide (*E*)-9-oxo 2-décénoïque (Barbier & Lederer, 1960; Butler *et al.*, 1959; Pain, 1961). Quatre autres composés ont été identifiés par la suite ; les acides (*Z*)- et (*E*)-9-hydroxy décénoïque, le p-hydroxybenzoate de méthyle et l'alcool homovanillique. Cette phéromone composée de cinq composés synthétisés dans la glande mandibulaire de la reine fut nommée QMP (*Queen Mandibular Pheromone*) (Slessor *et al.*, 1988). La caractérisation de cette phéromone fut ensuite complétée par quatre autres composés : l'oléate de méthyle, alcool coniférylique, alcool palmitique et acide linoléique (Keeling *et al.*, 2003). Ces quatre nouveaux composés ne sont pas synthétisés par la glande mandibulaire ; le mélange des neuf composés est nommé QRP (*Queen Retinue Pheromone*) (QRP = QMP + l'oléate de méthyle + alcool coniférylique + alcool palmitique + acide linoléique) (Keeling *et al.*, 2003).

La QMP est impliquée dans le comportement de migration lors de l'essaimage (Avitabile *et al.*, 1975; Grozinger *et al.*, 2014). Elle inhibe aussi le développement ovarien des ouvrières et retarde le développement des ouvrières nourrices en butineuses (Hoover *et al.*, 2003; Pankiw *et al.*, 1998a). La QRP provoque le comportement de cour des ouvrières auprès de la reine (Keeling *et al.*, 2003). La phéromone de reine se répand au sein de la colonie principalement par contact entre les individus lors

du toilettage de la reine par les ouvrières ainsi que lors des échanges de nourriture par trophallaxie (Naumann, 1991). La réduction de la production de cette phéromone au sein de la colonie déclenche l'élevage par les ouvrières de nouvelles reines, dont l'une d'entre elles va remplacer la reine actuelle (Butler, 1957).

- *La phéromone d'aggrégation*

La cohésion des individus durant le processus d'essaimage est médiée par la phéromone de Nasonov. L'émission de cette phéromone par une ouvrière attire ses congénères sur la planche d'envol de la ruche ainsi que lors de l'essaimage (Free, 1987; Winston, 1987). Cette phéromone est constituée de sept composés volatiles ((Z,E)-citral, (E,E)-farnésol, géraniol, nérol, acide nérolique et acide gérannique) (Pickett *et al.*, 1980). Le signal de cette phéromone est complété par la présence de la reine au sein de l'essaim de telle sorte que les ouvrières se regroupent toutes dans une grappe contenant la reine (Avitabile *et al.*, 1975; Winston *et al.*, 1989).

- *La phéromone d'alarme*

La phéromone d'alarme est constituée de 25 composés qui sont synthétisés dans la glande de Koschevnikov, proche du dard (Free, 1987). Sa principale fonction est d'augmenter l'activité de vol, le recrutement et l'attaque aux cibles mouvantes. La modulation de chacun de ces comportements dépend de la variation de chaque composé dans le mélange phéromonal. Par exemple, l'isoamyle acétate, le composé iconique de la phéromone d'alarme à l'odeur de banane mûre, augmente chacun de ces trois comportements tandis que le 1-octanol va seulement augmenter le recrutement (Wager & Breed, 2000). La phéromone d'alarme est aussi constituée de composés provenant des glandes mandibulaires comme le 2-heptanone qui participe aussi au recrutement des ouvrières contre l'intrus (Shearer & Boch, 1965).

- *La reconnaissance des congénères par les hydrocarbures cuticulaires*

La reconnaissance des congénères de la même colonie se fait grâce à des hydrocarbures cuticulaires (CHC). Ces CHCs forment un mélange complexe d'*n*-alcanes et d'alcènes sur la cuticule de chaque individu qui sont reconnus par les abeilles (Châline *et al.*, 2005). Tous les individus d'une même colonie portent les mêmes CHCs. A l'émergence, la jeune abeille ne porte pas encore les CHCs de la cuticule. La reconnaissance des CHCs implique un apprentissage des odeurs de sa propre colonie. La modification d'un seul CHC peut être traduit par les ouvrières comme un signal d'intrusion et déclencher des comportements d'attaque (Dani *et al.*, 2005). Lors de maladie ou de vieillissement des ouvrières, le

mélange CHCs évolue et pourrait provoquer une ségrégation de l'individu (McDonnell *et al.*, 2013; Scholl & Naug, 2011).

d. L'influence du couvain sur le devenir de la colonie

La communication entre le couvain et les ouvrières est médiée par l'émission de phéromones. La première phéromone de couvain décrite est la BEP (*Brood Ester Pheromone*) ; elle est constituée d'un mélange complexe de 10 esters (oléate, linoléate, linoléate, palmitate et stéarate de méthyle et d'éthyle) et est sécrétée dans les glandes salivaires des larves (Conte *et al.*, 2006; Le Conte *et al.*, 1990b). La variation de la concentration de chacun des composés dans le message phéromonal émis par le couvain permet aux adultes de connaître l'âge des immatures et donc d'adapter leurs soins (Le Conte *et al.*, 1994a; Le Conte *et al.*, 1994b). Au dernier stade larvaire, c'est la quantité des quatre méthyles (linoléate, linoléate, oléate et palmitate) dans le mélange phéromonal qui induit l'operculation des alvéoles de couvain en prévision de la nymphose (Le Conte *et al.*, 1990b). Lors de l'élevage des reines, c'est le méthyle linoléate qui augmente l'acceptation des cellules royales par les ouvrières et le méthyle palmitate qui augmente la quantité de gelée royale donnée (Le Conte *et al.*, 1995). L'action de la BEP influence également l'homéostasie de la colonie dans sa globalité (Pankiw *et al.*, 1998b; Pankiw, 2004a; Sagili & Pankiw, 2009). La BEP a un effet inhibiteur sur le développement des ovaires des ouvrières, ce qui renforce le statut reproducteur de la reine (Le Conte *et al.*, 2001; Mohammedi *et al.*, 1998). La quantité de BEP émise au sein du nid accroît le butinage de pollen permettant l'augmentation des stocks nécessaires à la nutrition de la colonie (Metz *et al.*, 2010; Pankiw *et al.*, 1998b).

Une deuxième phéromone émise par les larves participe à la modulation du bouquet phéromonal du couvain, le composé volatil (*E*)- β -ocimène (Maisonasse *et al.*, 2009; Traynor *et al.*, 2014). L'(*E*)- β -ocimène participe à l'inhibition du développement des ovaires des ouvrières (Maisonasse *et al.*, 2009). L'émission de ce composé augmente le comportement de nourrissage des nourrices envers le couvain ainsi que la maturation comportementale des ouvrières (He *et al.*, 2016; Maisonasse *et al.*, 2010; Traynor *et al.*, 2015). L'émission de ce composé est aussi dépendante de l'âge du couvain et, couplée à la BEP, va permettre une fine régulation des comportements des ouvrières (Maisonasse *et al.*, 2010).

3. Les ouvrières face à un couvain malade

Un grand nombre de pathogènes et parasites peuvent affecter le couvain. Certains pathogènes peuvent entraîner la mort de l'immatrice, comme c'est le cas avec l'infection par le champignon *Ascosphaera apis* donnant le « couvain plâtré » (Heath, 1982), de la loque américaine (*Paenibacillus*

larvae spp.) (Hansen & Brødsgaard, 1999), de la loque européenne (*Melissococcus plutonius*) (Forsgren, 2010) et du virus SBV (*Sacbrood Virus*) (Wei *et al.*, 2022). D'autres pathogènes perturbent le développement du couvain et conduisent à l'émergence d'abeilles à la physiologie altérée, atrophiées, comme c'est le cas avec le parasitisme par *Varroa destructor* (Bowen-Walker & Gunn, 2001) ou encore avec l'infection au virus des ailes déformées (DWV – *Deformed Wing Virus*) (de Miranda & Genersch, 2010b).

Les colonies d'abeilles mellifères, comme tous les insectes eusociaux, ont développé un mécanisme d'immunité sociale permettant de combattre les parasites et pathogènes grâce à une coopération étroite entre le individu (Cremer *et al.*, 2007). Cette immunité sociale réside dans différents comportements qui permettent de freiner chaque étape de propagation du parasite ou pathogène : l'entrée dans la colonie est la première barrière (ex : reconnaissance d'individus malades ou parasités à l'entrée de la ruche), un contrôle de l'hygiène du nid réduit la possibilité de l'implantation de parasite et de pathogène au sein du nid (ex : sortie des morts du nid), sa prolifération dans la colonie est endigué par des comportements réduisant le contact des individus atteints (ex : cannibalisme du couvain infecté ou infesté) et la transmission verticale est évitée (ex : les soins à la reine ne sont pas donnés par des individus infectés ou infestés) (Cremer *et al.*, 2007; Cremer & Sixt, 2009). Ces comportements de défense sociale impliquent une étape commune, la détection de la maladie, de l'infection, du parasite ou d'un individu mort. Dans le cas du couvain, les colonies d'abeilles ont développé un comportement hygiénique consistant à identifier et vider entièrement les alvéoles de couvain touchées par des pathogènes ou parasites (Arathi *et al.*, 2000; Spivak, 1996; Spivak & Gilliam, 1998). Ce comportement participe à réduire la pression des parasites et pathogènes dans le développement de la colonie. Le comportement hygiénique a été montré comme étant efficace contre la loque américaine, le couvain plâtré et le parasitisme par varroa (Spivak & Danka, 2021). Néanmoins, le cannibalisme de nymphes infectées par DWV pourrait participer à propager le virus au sein de la colonie, par le comportement de trophallaxie entre adultes (Posada-Florez *et al.*, 2021). Cette infection coloniale pourrait être endiguée par d'autres mécanismes impliqués dans l'immunité sociale comme l'exclusion des individus infectés (Erez *et al.*, 2022).

II. L'acarien parasite *Varroa destructor* : menace biotique majeure des colonies d'abeilles domestiques

Cette partie de l'introduction a été adaptée d'une revue qui a été acceptée dans le journal *Emerging Topics for Life Science* (Noël *et al.*, 2020).

1. Historique – Le changement d'hôte de *Varroa destructor* vers *A. mellifera*, une expansion mondiale

La première description du genre *Varroa* date de 1904 avec la découverte de *Varroa jacobsoni* sur des abeilles de l'espèce *Apis cerana*, au nord de l'île de Java en Indonésie (Oudemans, 1904). La relation hôte-parasite entre ces deux espèces repose sur un équilibre dynamique depuis de nombreuses années (Rath, 1999). Le changement d'hôte de l'acarien vers *A. mellifera* est probablement apparu à la fin de la première moitié du XX^{ème} siècle. Il a été décrit sur *A. mellifera* en Russie en 1952, au Brésil en 1972 (Oldroyd, 1999), en Europe à la fin des années 70 (Ruttner & Ritter, 1980) avec une arrivée en France en 1982 (Colin *et al.*, 1983), aux Etats-Unis en 1987 (Wenner & Bushing, 1996). C'est en 2000 qu'il a été montré que le genre *Varroa* comprend en fait deux espèces : *Varroa jacobsoni* et *Varroa destructor* (Anderson & Trueman, 2000). Seul *V. destructor* se reproduit sur *A. mellifera*, cependant, il existe une confusion entre les deux espèces de *Varroa* dans les recherches scientifiques publiées avant 2000 (Traynor *et al.*, 2020). *V. destructor* a continué son expansion mondiale et est maintenant signalé dans la plupart des pays. Dans la dernière décennie, de nouvelles invasions ont été enregistrées : à Hawaï (Martin, 2010), à Madagascar (Rasolofoarivao *et al.*, 2013), à la Réunion (Esnault, 2018), à l'île Maurice (Esnault *et al.*, 2019) et sur le continent africain comme en Ouganda et en Ethiopie (Kugonza, 2020). Début 2023, seuls plusieurs pays d'Afrique et quelques îles n'ont toujours pas signalé la présence de *V. destructor*. L'Australie, jusqu'alors préservée de *V. destructor*, vient d'enregistrer les premières colonies d'*A. mellifera* parasitées en juin 2022 et lutte activement pour éradiquer le parasite.

2. La biologie de *V. destructor*

a. Le cycle de vie du parasite

Le cycle de vie de *Varroa destructor* peut être séparé en deux phases ; une phase dite « phorétique » et une phase reproductrice (Figure 1.6). La phase « phorétique » concerne seulement les femelles adultes. Elles utilisent les abeilles comme vecteur de transport et comme source alimentaire. Elles peuvent changer d'hôte et le choix de l'hôte se fait préférentiellement vers une abeille nourrice ce qui augmente les chances de trouver une alvéole à parasiter (Kuenen & Calderone, 1997).

Lors de cette phase, les abeilles participent à la dissémination du parasite au sein de leur colonie ainsi que des colonies alentour. La phase reproductrice commence lorsque la femelle adulte, appelée fondatrice, entre dans une alvéole de couvain prête à être operculée (Figure 1.6). Le varroa montre une préférence pour les alvéoles de couvain mâle par rapport aux alvéoles de couvain d’ouvrière (Fuchs, 1992). Une fois dans l’alvéole, la fondatrice pond un premier œuf non-fécondé donnant un mâle, suivi de plusieurs œufs fécondés donnant des femelles (Figure 1.6). Le premier œuf est pondu 60 heures après l’operculation de l’alvéole, puis les suivants sont pondus environ toutes les 30 heures, pour un maximum de sept œufs dans une alvéole de couvain mâle et de six œufs dans une alvéole de couvain d’ouvrières (Ifantidis, 1983) (Figure 1.6). Le développement du mâle varroa est de 6,25 jours tandis que celui de la femelle est de 5,5 jours (Martin *et al.*, 2001). Une fois mature, le mâle s’accouple avec ses sœurs arrivées à maturité (Donzé & Guerin, 1994). Au moment de l’émergence de la jeune abeille, une à deux filles varroa matures ainsi que la fondatrice sortent de l’alvéole de couvain (Figure 1.6). Elles commencent alors un cycle phorétique en se plaçant sur une abeille nourrice, ce qui permet de faciliter leur déplacement vers une nouvelle alvéole de couvain proche de l’operculation. Le mâle et les filles immatures meurent dans l’alvéole qui est nettoyée par les abeilles adultes (Figure 1.6). Récemment, il a été montré qu’une femelle vierge pouvait également commencer une phase phorétique (Häußermann *et al.*, 2019). Lorsque celle-ci est vierge au moment d’entrer dans l’alvéole de couvain, elle va pondre l’œuf mâle, puis se reproduire avec ce mâle une fois arrivé à maturation (Häußermann *et al.*, 2019). Cela confirme le système de reproduction parthénogénétique arrhénotoque de la femelle varroa, autrement dit la faculté de se passer d’accouplement pour pondre.

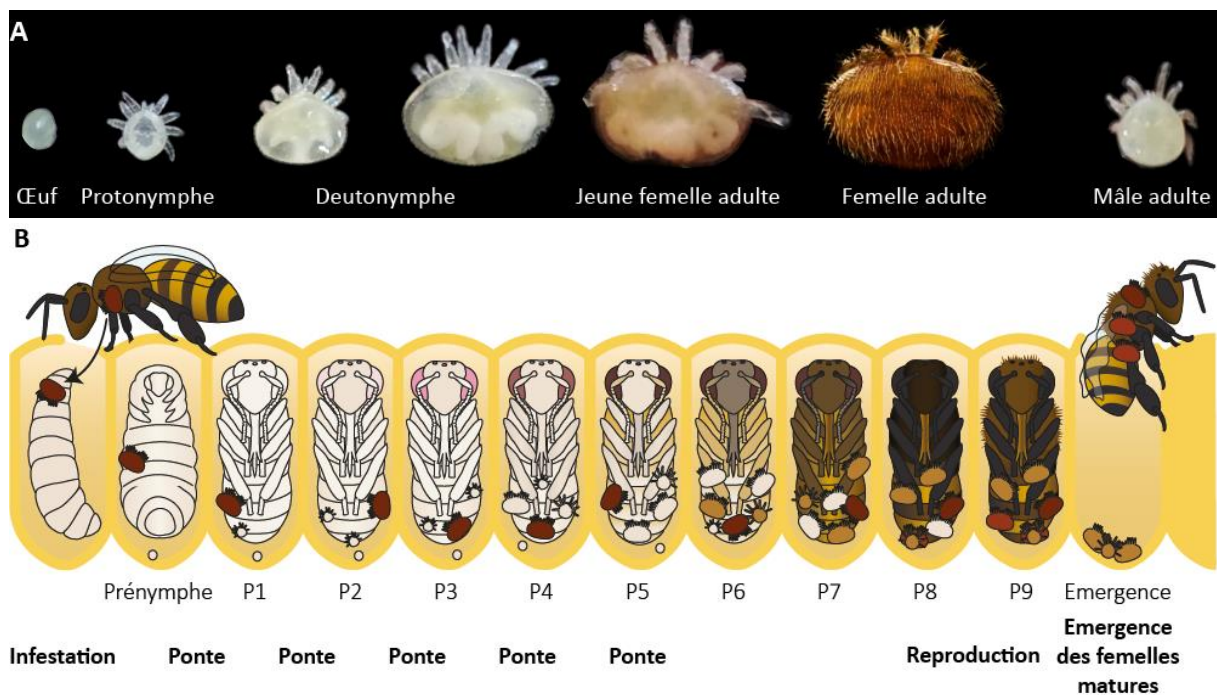


Figure 1.6 : Phase reproductrice de *Varroa destructor* au sein des alvéoles de couvain operculées d’*Apis mellifera*. A. Stades de développement du varroa. Photos : © Fanny Mondet & Amélie Noël. B. La fondatrice varroa

entre dans l'alvéole de couvain pour pondre au moment de l'operculation du couvain. Une fois à maturité, le mâle va se reproduire avec ses sœurs. La fondatrice (rouge foncé) et ses filles matures (rouge clair) sortent de l'alvéole au moment de l'émergence de l'abeille abimée. Les femelles immatures (ocre) et le mâle (ocre) meurent au fond de l'alvéole. Px : Stades nymphaux d'*A. mellifera* (9), gras : Etapes clef de la phase reproductrice de *V. destructor*.

b. Les modalités de nutrition de l'acarien

Il a longtemps été admis que le varroa se nourrissait seulement de l'hémolymphe de l'abeille, cependant des recherches récentes ont montré que le parasite se nourrit également du corps adipeux de la nymphe et de l'adulte (Ramsey *et al.*, 2019). La fondatrice perce le tégument de la nymphe afin d'y créer une ouverture pour avoir accès au fluide corporel de l'hôte (hémolymphe et corps gras). La dégradation des cellules du corps gras de la nymphe est induite par l'injection de la salive du varroa lors de la création du site de nutrition (Ramsey *et al.*, 2019). La création de ce site, au niveau du second segment abdominal de la nymphe, se fait approximativement en une heure et est très coûteuse en énergie pour le varroa (Donzé & Guerin, 1994). De plus, la progéniture est complètement dépendante de la fondatrice pour se nourrir car les chélicères des immatures ne sont pas assez puissantes pour pouvoir percer elles-mêmes le tégument de la nymphe. Il est donc important de maintenir le site de nutrition ouvert pour le succès reproducteur de la fondatrice. Les composés présents dans la salive du varroa perturbent la cicatrisation et maintiennent la blessure ouverte (Becchimanzi *et al.*, 2020; Yang & Cox-Foster, 2005). La maintenance du site de nutrition peut être considérée comme des soins parentaux d'un stade subsocial car cela sert aussi bien la mère que la progéniture (Donzé & Guerin, 1994).

c. Le système de détection de composés sémiocchimiques de l'acarien

Le varroa possède, lui aussi, un système de détection des molécules sémiocchimiques. La présence de sensilles sur les pièces buccales et sur les pattes avant, l'identification de récepteurs chémosensoriels sur les pattes antérieures de l'acarien, ainsi que l'abondance de protéine de transports des odorants vers les récepteurs olfactifs, confirment l'importance des composés chimiques dans la perception de son environnement (Eliash *et al.*, 2017; Iovinella *et al.*, 2018; Liu, 1988). De plus, des recherches en écologie chimique ont indiqué que le mâle varroa détecte les phéromones des femelles grâce à ses organes sensoriels situés sur ses pattes avant (Häußermann *et al.*, 2015; Ziegelmann *et al.*, 2013a; Ziegelmann *et al.*, 2013b). Le comportement reproducteur du mâle est déclenché par l'émission de la phéromone sexuelle par les femelles varroa (Ziegelmann *et al.*, 2013a; Ziegelmann *et al.*, 2013b). Celle-ci est composée d'ester éthylique aussi présent dans la BEP (palmitate, stéarate et oléate de méthyle) et d'acide gras (acide palmitique, acide stéarique, acide oléique) (Ziegelmann *et al.*, 2013a; Ziegelmann *et al.*, 2013b).

V. destructor a la capacité d'imiter les hydrocarbures cuticulaires de son hôte *A. mellifera* à différents stades de développement (Martin *et al.*, 2001). Il semble que ce mimétisme puisse s'adapter rapidement : le varroa est capable de modifier son profil cuticulaire en trois à neuf heures après changement d'hôte en adaptant la proportion en *n*-alcanes et alcènes de ses CHCs (Kather *et al.*, 2015b; Kather *et al.*, 2015a; Le Conte *et al.*, 2015). L'acarien est incapable de synthétiser les composés spécifiques de son hôte. Il doit avoir accès à la cuticule de l'abeille pour pouvoir imiter le profil chimique de l'abeille (Kather *et al.*, 2015b). Ce mimétisme est un mécanisme physico-chimique passif et l'adaptation des CHCs du varroa peut se faire même après sa mort (Kather *et al.*, 2015b).

Le varroa différencie les différents types d'alvéoles de couvain et évite les alvéoles de reine (Fuchs, 1992; Harizanis, 1991). Cet évitement des alvéoles de couvain de reine pourrait être expliqué par la plus forte émission d'éthyle oléate du couvain de reines par rapport au couvain d'ouvrière (Trouiller *et al.*, 1994). De plus, l'acide octanoïque, un composé présent dans la gelée royale donnée aux larves de reine, déclenche la répulsion du varroa (Nazzi *et al.*, 2009). *A contrario*, l'acarien est attiré par l'acide 2-hydroxy hexanoïque, un composé présent dans la bouillie larvaire, nourriture donnée aux couvains mâle et ouvrière (Nazzi *et al.*, 2004b). Les palmitate de méthyle et d'éthyle présents dans la BEP attirent aussi le varroa (Le Conte *et al.*, 1989; Rickli *et al.*, 1992). Le début de l'oviposition est stimulé par l'odeur des larves (Frey *et al.*, 2013; Garrido & Rosenkranz, 2004; Trouiller & Milani, 1999). Ces composés chimiques caractérisés comme phéromone dans la communication entre abeilles, constituent également des kairomones, par leur implication dans la communication abeille-varroa.

3. Effets pathologiques du varroa sur la colonie d'abeilles domestiques

a. Impact du varroa à l'échelle individuelle

Le parasitisme des abeilles mellifères par *V. destructor* diminue le poids corporel et la teneur en eau des jeunes abeilles émergentes (Bowen-Walker & Gunn, 2001). La baisse de poids de la future abeille adulte augmente avec le nombre de fondatrices d'acariens présentes au sein de l'alvéole (Duay *et al.*, 2003; Strauss *et al.*, 2016). Chez l'abeille domestique, le nombre de spermatozoïdes est corrélé à la taille du corps des faux-bourdons (Schlüns *et al.*, 2003). En diminuant la taille des bourdons, le varroa induit un déficit dans la production de spermatozoïdes, et donc dans la capacité de reproduction (Duay *et al.*, 2002). Le parasite altère également les capacités de vol, de repérage et d'orientation des butineuses ce qui limite l'efficacité de leur capacité à retourner et à collecter les ressources nécessaires au développement de la colonie (Kralj & Fuchs, 2006; Monchanin *et al.*, 2019). Le non-retour à la colonie pourrait aussi être considéré comme un mécanisme de défense contre le varroa. Les ouvrières parasitées pourraient se sacrifier en ne revenant pas au nid pour le bien de la colonie (Kralj & Fuchs,

2006). L'impact du varroa sur le comportement des abeilles peut s'expliquer par sa capacité à modifier les processus neuronaux (Kralj *et al.*, 2007), perturbant les capacités d'apprentissage non associatif de l'abeille hôte. En effet, les abeilles parasitées ont une plus faible sensibilité au sucre et une adaptation plus rapide à la stimulation olfactive, influençant le butinage (Kralj *et al.*, 2007). De plus, le varroa provoque une diminution de l'expression des gènes de l'immunité chez les adultes infestés émergents (Marche *et al.*, 2019), ainsi que des modifications protéomiques dans la réponse immunitaire de l'abeille (Erban *et al.*, 2019; Słowińska *et al.*, 2019; Surlis *et al.*, 2018; Zaobidna *et al.*, 2017). Par exemple, le varroa réduit le nombre d'hémocytes circulant dans l'hémolymphe, et diminue l'expression de la prophénol oxydase, impliquée dans la synthèse de la mélanine (Koleoglu *et al.*, 2018; Wegener *et al.*, 2016). Les hémocytes et la mélanine permettent tous deux l'encapsulation des agents pathogènes lors d'une infection ou d'une blessure, et jouent donc un rôle primordial dans la réponse immunitaire et les mécanismes de guérison des insectes (Kanost & Gorman, 2008). L'affaiblissement de l'immunité des abeilles pourrait être lié au fait que les acariens se nourrissent de corps gras (Annoscia *et al.*, 2019; Yang & Cox-Foster, 2005), organe jouant un rôle majeur dans l'immunité (Arrese & Soulages, 2010).

b. La dépendance de l'expansion de la population en varroa à la dynamique de population de son hôte

La taille de la population de *V. destructor* au sein de la colonie est directement corrélée à la quantité de couvain et, par extension, à la taille de la population de la colonie. L'augmentation du comportement d'essaimage, qui est à la base un comportement de reproduction de la colonie, peut être vue comme un mécanisme de défense adaptatif de la colonie d'abeilles à la surpopulation en acariens (Loftus *et al.*, 2016) (Figure 1.8). L'augmentation des périodes sans couvain induisent alors une diminution naturelle de la population en varroa. Le transfert de varroa est possible entre colonies. Les colonies peuvent être réinfestées lorsque les butineuses vont piller les réserves en nourriture des colonies mourantes ou lors de la dérive d'individus vers d'autres colonies (Peck & Seeley, 2019), et lorsque des faux-bourdons parasités se reposent dans des colonies étrangères en période de reproduction (Mortensen *et al.*, 2018). Une forte densité de colonies au sein d'un rucher pourrait aussi être un facteur avantageux pour la population de varroa (Frey & Rosenkranz, 2014).

c. Le varroa comme vecteur de virus de l'abeille

Les virus infectant les abeilles ont plusieurs voies de transmission au sein de la colonie, à la fois verticale (à la descendance), et horizontale (entre individus sans lien parents-descendants). La transmission horizontale peut se faire par contacts oraux et/ou corporels, lors des échanges

alimentaires ou de la reproduction, mais aussi par l'intermédiaire d'autres organismes (Yañez *et al.*, 2020). La relation hôte-parasite entre les abeilles domestiques et le varroa devrait plutôt être considérée comme une relation tripartite, car la présence de varroa au sein de la colonie est étroitement associée à la présence de plusieurs virus (Beaurepaire *et al.*, 2020; Martin *et al.*, 2012; Mondet *et al.*, 2014; Wilfert *et al.*, 2016). Le varroa est un vecteur de transmission horizontale très efficace (Amiri *et al.*, 2018; de Miranda *et al.*, 2013; Martin *et al.*, 2012; Posada-Flórez *et al.*, 2019). Il permet la réplication de certains virus (Gisder *et al.*, 2009; Posada-Flórez *et al.*, 2019), la transmission à l'abeille (Bowen-Walker *et al.*, 1999), ainsi que l'altération des barrières immunitaires de l'abeille favorisant ainsi la réplication du virus au sein de son hôte (Nazzi *et al.*, 2012; Yang & Cox-Foster, 2005). Depuis 2015, plusieurs virus et variants liés à varroa ont été découverts chez des abeilles mellifères infestées par l'acarien (de Miranda *et al.*, 2015; Herrero *et al.*, 2019; Levin *et al.*, 2016; Levin *et al.*, 2019; Mordecai *et al.*, 2016). L'association entre le DWV et l'acarien a été confirmée, mais uniquement pour le variant DWV-B (Campbell *et al.*, 2016; de Miranda & Genersch, 2010a; Posada-Flórez *et al.*, 2019; Santamaria *et al.*, 2018). C'est aussi le cas pour le complexe viral de la paralysie aiguë de l'abeille (ABPV – *Acute Bee Paralysis Virus*) (de Miranda *et al.*, 2010) et le virus israélien de la paralysie aiguë (IAPV – *Israeli Acute Paralysis Virus*) (Di Prisco *et al.*, 2011). De nouveaux variants et espèces virales ont également été décrits spécifiquement chez l'acarien, comme c'est le cas VDV (*Varroa Destructor Virus*) (Campbell *et al.*, 2016). Les variants VDV-2, VDV-3 et VDV-5 peuvent se répliquer chez *V. destructor* et non chez *A. mellifera*, ce qui suggère que leur présence chez l'abeille est due au comportement alimentaire du varroa (Herrero *et al.*, 2019). Il a été montré que les variants du DWV peuvent se recombinaison entre eux et pourraient donner des variants plus ou moins virulents pour l'abeille (Dalmon *et al.*, 2017).

Certains des virus infectant les acariens pourraient modifier le comportement du varroa, ce qui présente une nouvelle perspective pour trouver des cibles permettant de tuer l'acarien (Campbell *et al.*, 2016). De même que son impact sur la communauté de virus infectant les abeilles, l'acarien modifie également le bactériome de l'abeille domestique (Hubert *et al.*, 2017; Marche *et al.*, 2019), mais l'impact d'une telle association reste peu connu. De plus, le couple varroa-virus joue un rôle clé dans l'affaiblissement de la colonie (Barroso-Arévalo *et al.*, 2019). Les abeilles adultes émergentes qui ont été parasitées pendant le stade nymphal présentent un taux d'infection par le DWV plus élevé que les individus non parasités (Marche *et al.*, 2019). L'infection par le DWV induit des effets pathologiques tels que des ailes déformées et une réduction de la taille du corps, ce qui entraîne des troubles du comportement et la mort précoce des abeilles. Le titre du DWV chez les abeilles mellifères augmente à mesure que l'immunité de l'abeille diminue (Zhao *et al.*, 2019). Le virus DWV peut également immunodéprimer les abeilles, ce qui peut avoir un effet favorable sur la reproduction du varroa (Di Prisco *et al.*, 2016).

d. Les interactions néfastes entre différents facteurs de stress et le varroa sur la colonie d'abeilles mellifères

Le varroa peut interagir avec d'autres facteurs de stress biotiques et abiotiques, tel que des facteurs environnementaux, d'autres parasites, pathogènes ou pesticides. Le changement climatique, avec l'augmentation de la durée des périodes chaudes, peut induire de plus longues périodes de ponte et de butinage au sein de la colonie. L'allongement des périodes de présence du couvain au sein de la colonie engendre un plus grand nombre de cycles reproducteurs chez varroa ce qui amène à une augmentation de la population en acariens (Le Conte *et al.*, 2010). Une infection par *Nosema*, un champignon unicellulaire parasite de l'abeille, réduit l'efficacité de la défense contre varroa de l'abeille (Bahreini & Currie, 2015). Par ailleurs, les pesticides néonicotinoïdes et le varroa contribuent tous deux à la diminution de la population d'abeilles d'hiver au sein de la colonie (Straub *et al.*, 2019; van der Zee *et al.*, 2015). L'effet négatif des insecticides néonicotinoïdes, tels que l'imidaclopride et le thiaméthoxame, sur le vol et le comportement de retour des abeilles à la colonie est amplifié lorsqu'ils sont associés à une forte infestation en varroa (Blanken *et al.*, 2015; Monchanin *et al.*, 2019).

4. Les différentes méthodes de lutte contre l'infestation à varroa

Le contrôle de varroa s'est imposé comme une nécessité pour les apiculteurs depuis son introduction car une colonie infestée meurt en quelques mois voire quelques années, en l'absence de traitement (Fries *et al.*, 2006). Différentes approches sont utilisées par les apiculteurs afin de contrôler l'infestation en varroa au sein de leurs colonies. Des acaricides utilisés en agriculture conventionnelle, ainsi que certaines huiles essentielles et acides organiques montrent de bons résultats pour prévenir la perte des colonies et ces dernières représentent un outils intéressant pour la pratique de l'apiculture biologique (Haber *et al.*, 2019a). Tous les acaricides (excepté l'acide formique) tuent seulement le varroa en phase phorétique et ne peuvent atteindre les varroas en phase reproductrice cachés au sein des alvéoles de couvain operculées.

a. Le contrôle médicamenteux

Les méthodes de lutte conventionnelles à l'aide d'acaricides de synthèse contre varroa ont été utilisées depuis plus de 40 ans maintenant (Koeniger & Fuchs, 1989). Selon le pays, les méthodes peuvent différer en raison des différences dans les lois régissant l'utilisation des produits chimiques. Seulement une quantité limitée de molécules avec autorisation de mise sur le marché est disponible selon les pays (Figure 1.7). Elles comprennent les insecticides pyréthrinoïdes τ -fluvalinate (ex : Apistan[®], Klartan[®], Mavrik[®]) et fluméthrine (ex : Bayvarol[®], Polyvar yellow[®]), l'insecticide et acaricide

organophosphoré coumaphos (ex : Checkmite®, Asuntol®, Perizin®) ou l’insecticide et acaricide formamidine amitraze (ex : Apivar) (Mondet & Le Conte, 2014; Rosenkranz *et al.*, 2010). Les varroas deviennent de plus en plus résistants à certains acaricides (Colin *et al.*, 1997; González-Cabrera *et al.*, 2018; Martin, 2004; Milani, 1999; Panini *et al.*, 2019; Stara *et al.*, 2019). Une solution provisoire pour limiter et temporairement contourner cette résistance aux acaricides est d’alterner l’utilisation de composés chimiques ayant des cibles moléculaires différentes. Néanmoins, de plus en plus de données publiées montrent d’importantes quantités de résidus d’acaricides au sein des produits de la ruche (Kast *et al.*, 2019; Ostiguy *et al.*, 2019; Premrov Bajuk *et al.*, 2017).

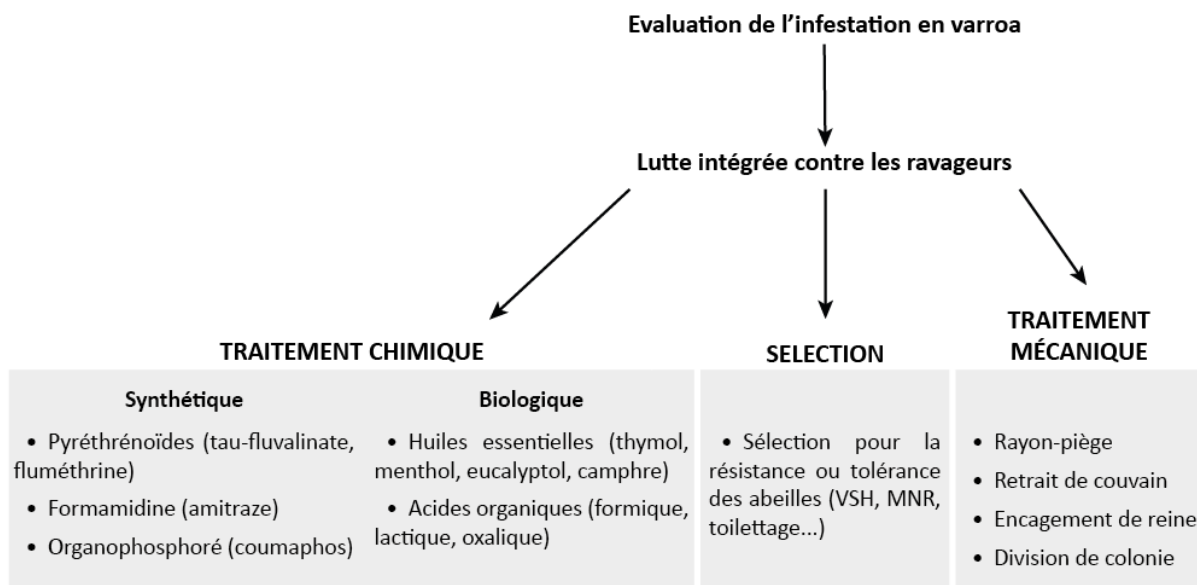


Figure 1.7: Méthodes actuellement utilisées pour traiter les colonies d'abeilles mellifères contre le parasitisme de *Varroa destructor*. Les méthodes peuvent être couplées dans le cadre d'un programme de lutte intégrée contre les ravageurs. VSH : *Varroa-Sensitive Hygiene* ; MNR : *Mite Non-Reproduction*.

A cause des défauts d’efficacité récurrents des acaricides synthétiques conventionnels ainsi qu’avec le développement de l’apiculture biologique, les apiculteurs ont de plus en plus recours à des molécules autorisées en agriculture biologique. Les composés utilisés les plus communs sont les huiles essentielles tel que le thymol, et des acides organiques tels l’acide oxalique ou l’acide formique (Mondet & Le Conte, 2014) (Figure 1.7). Les acides organiques sont naturellement présents dans les produits de la ruche et ont un risque amoindri de déclencher une résistance chez le varroa (Mondet & Le Conte, 2014). L’utilisation des acides organiques repose principalement sur des traitements flash lors des périodes sans couvain au sein de la colonie afin de tuer les varroas phorétiques. Ainsi, les applications de produits compatibles avec l’agriculture biologique associées à des méthodes mécaniques pour exclure le couvain

(engagement de reines, retrait de couvain) peuvent constituer une bonne solution de lutte contre le varroa (Giacomelli *et al.*, 2016; Gregorc *et al.*, 2017; Rademacher & Harz, 2006).

b. Un besoin de nouveaux composés actifs grandissant

Au vu de la multiplication des phénomènes de résistance, qui limite le nombre de molécules utilisables en apiculture, une solution peut résider dans la mise au point de nouveaux composés actifs. Une approche de criblage combinant le criblage *in silico* (criblage virtuel d'une chimiothèque de modèles de séquences d'homologie) avec des expériences *in vitro* pour rechercher une inhibition sélective de l'acétylcholinestérase du varroa a été développée avec succès, conduisant à la découverte de composés qui ont le potentiel de devenir de nouveaux traitements contre l'acarien (Riva *et al.*, 2019). Le chlorure de lithium a également été démontré comme un composé potentiel contre varroa (Ziegelmann *et al.*, 2018), ainsi que d'autres huiles essentielles et leurs combinaisons (Bendifallah *et al.*, 2018; Brasesco *et al.*, 2017). Cependant, il n'existe actuellement aucun produit homologué basé sur ces nouvelles sources bioactives. Le plus difficile dans la recherche de nouveaux acaricides pour lutter contre l'acarien est la nécessité d'avoir des composés inoffensifs pour les abeilles et pour l'homme et efficaces sur le terrain lors d'applications dans les colonies. Un atout pour cette recherche de composés serait de réussir à élever et à reproduire le varroa de façon *in vitro*. Actuellement, l'élevage de varroa en laboratoire n'est pas tout à fait au point.

c. L'utilisation d'approches alternatives aux traitements chimiques

Différentes techniques ont été décrites pour limiter l'infestation de varroa, comme la méthode du « rayon-piège » consistant à n'avoir qu'un nombre restreint de cadres de couvain pouvant être parasités par le varroa et les retirer de la colonie une fois operculés, ou la division des colonies, qui imite les phénomènes d'essaimage (Loftus *et al.*, 2016). Néanmoins, l'action de ces méthodes ne permet qu'une diminution temporaire de la charge parasitaire de la colonie ce qui n'est pas suffisant pour les considérer comme des méthodes de lutte à part entière. Des méthodes de lutte biologique ont montré des résultats probants lors de conditions contrôlées mais la plupart des expériences de terrain sur les colonies d'abeilles n'ont pas été couronnées de succès. C'est le cas des champignons parasites (Hamiduzzaman *et al.*, 2012), le genre de champignons entomopathogènes *Beauveria* (Sewify *et al.*, 2015), ainsi que le prédateur du varroa *Stratiolaelaps scimitus* (Rangel & Ward, 2018; Rondeau *et al.*, 2019). L'hyperthermie est utilisée depuis les années 70, et est basée sur la meilleure résistance à la chaleur de l'abeille en comparaison au varroa. Chauffer artificiellement les colonies stoppe la reproduction du varroa et tue le parasite, sans blesser les abeilles (Kablau *et al.*, 2019; Le Conte *et al.*,

1990a). Depuis 2012, l'utilisation de ARN interférant pour neutraliser des gènes spécifiques du varroa est étudiée et montre des résultats prometteurs (Campbell *et al.*, 2016; Huang *et al.*, 2019; Leonard *et al.*, 2020). A ce jour, il n'y a pas de produits disponibles sur le marché pour les apiculteurs.

d. La lutte intégrée contre les ravageurs

Une variété d'outils disponibles pour les apiculteurs rend possible le développement et l'utilisation du concept de lutte intégrée contre les ravageurs dans la lutte contre varroa (Figure 1.7) (Barzman *et al.*, 2015; Jack & Ellis, 2021). La lutte intégrée contre les ravageurs repose en partie sur la limitation de l'utilisation des pesticides au seuls cas où cela est nécessaire. Cela nécessite un suivi régulier des niveaux de population de varroa, afin de détecter les infestations critiques et de décider d'un traitement (Jack & Ellis, 2021). Des techniques efficaces existent pour diagnostiquer l'infestation en varroa (Mondet *et al.*, 2016b). Les acariens peuvent être comptés dans le couvain, sur les abeilles adultes ou dans les débris tombés sur le plancher de la ruche (Dietemann *et al.*, 2013; Kretschmar *et al.*, 2015; Mondet & Le Conte, 2014).

III. Lutter durablement contre varroa en utilisant la résistance de l'abeille

1. Historique – Le besoin de sélectionner des colonies d'*A. mellifera*

Parallèlement au développement de solutions de lutte médicamenteuse contre l'acarien, les généticiens quantitatifs et les sélectionneurs d'abeilles ont commencé à chercher une solution durable à plus long terme : sélectionner des populations d'abeilles mellifères capables de survivre à une infestation d'acariens sans traitement. Plusieurs populations d'abeilles survivantes ont été identifiées ou élevées dans le monde entier (voir les revues suivantes : Büchler *et al.*, 2010; Le Conte *et al.*, 2020; Locke, 2015; Mondet *et al.*, 2020a; Rinderer *et al.*, 2010). Les exemples d'utilisation à grande échelle de ces populations en apiculture sont encore rares (Danka *et al.*, 2016; Kefuss *et al.*, 2015). Le développement de la sélection d'abeilles survivantes est actuellement limité par le manque d'outils permettant de sélectionner les colonies d'abeilles survivantes sur le terrain.

Le développement de tels outils repose tout d'abord sur l'identification de phénotypes qui caractérisent ces populations. Pour ce faire, une meilleure compréhension des mécanismes qui régissent cette capacité à survivre avec une infestation en varroa est nécessaire. La survie peut être obtenue par l'expression de caractères de résistance ou de tolérance. La résistance implique une réduction de la croissance de la population en varroa, tandis que la tolérance réduit l'impact de

l'infestation parasitaire malgré des niveaux élevés de varroa (Schneider & Ayres, 2008). Une large gamme de caractères impliqués dans la survie de l'abeille vis-à-vis de varroa a été identifiée, dont la plupart sont des mécanismes de résistance (Mondet *et al.*, 2020a). La tolérance n'a été suggérée jusqu'à présent que dans les cas où les colonies sont capables de supporter la charge de varroa grâce à des mécanismes de tolérance aux virus qui sont associés aux infestations de varroa, comme, par exemple, au sein de la population d'abeilles de Gotland en Suède (Mordecai *et al.*, 2016; Thaduri *et al.*, 2019).

2. Les différents caractères de résistance des abeilles mellifères au varroa

Des études soulignent l'importance des défenses comportementales des populations d'abeilles mellifères résistantes à varroa (Figure 1.8) (revu par Mondet *et al.*, 2020). Différents caractères ont été montrés comme participant à la résistance au parasite. Certains sont liés au couvain, comme le caractère SMR (*Suppressed Mite Reproduction*) (Mondet *et al.*, 2016b; Page *et al.*, 2016) (voir III.2.a. pour description), une durée post-operculation plus courte (Moritz, 1985) ou encore une plus grande sensibilité du couvain (augmentation du taux de mortalité dû au parasitisme) (Guzmán-Novoa *et al.*, 1999). D'autres sont davantage liés à l'adulte, tel que le comportement VSH (*Varroa Sensitive Hygiene*) (Harbo & Hoopingarner, 1997) (voir III.2.b. pour description), le comportement de réoperculation (Oddie *et al.*, 2019b) (voir III.2.b. pour description), le toilettage auto- et allo-centré (Bahreini & Currie, 2015) ou encore la construction d'alvéoles de couvain de plus petite taille (Martin & Kryger, 2002; Message & Gonçalves, 1995; Oddie *et al.*, 2019a) (Figure 1.8). Néanmoins ce dernier caractère est controversé (Berry *et al.*, 2010; Ellis *et al.*, 2009; Seeley & Griffin, 2011). Au niveau de la colonie, l'essaimage peut renforcer la résistance des populations survivantes vivant dans la nature (Loftus *et al.*, 2016; Ratti *et al.*, 2016) (Figure 1.8). Dans l'ensemble, ces traits participent à la limitation de la croissance des populations d'acariens (Figure 1.8).

La non-reproduction du varroa (MNR, *Mite Non Reproduction*) est une caractéristique clef dans la résistance des colonies à varroa (Figure 1.8). Dans la littérature scientifique, il existe une confusion dans l'utilisation des termes SMR, MNR et VSH. Fin des années 90, il a été observé de faibles proportions d'acariens reproducteurs capables de produire une fille mature au sein de colonies (Harbo & Hoopingarner, 1997). La non-reproduction du varroa observée au sein de ces colonies résistantes fut supposée comme résultant de l'action du couvain et fut appelée SMR (Harbo & Harris, 1999b). Cette caractéristique héritable fut alors utilisée pour la sélection de colonies résistantes à varroa (Harbo & Harris, 1999a; Harbo & Harris, 2003). Il a ensuite été démontré que la capacité des colonies résistantes à empêcher la reproduction du varroa était principalement due au fort hygiénisme des colonies (Harbo & Harris, 2005). Les abeilles réalisant ce comportement hygiénique nettoyaient préférentiellement les alvéoles de couvain contenant du varroa reproducteur plutôt que les alvéoles de couvain sans

reproduction du varroa (Harbo & Harris, 2005; Ibrahim & Spivak, 2006). Cette capacité à nettoyer les alvéoles de couvain parasité chez les colonies sélectionnées sur le caractère SMR était supérieure à celles sélectionnées pour leur hygiénisme vis-à-vis du couvain tué par le froid (Ibrahim & Spivak, 2006; Spivak, 1996). Par la suite, cette caractéristique fut renommée VSH (Harris, 2007). Il est maintenant recommandé d'utiliser le terme SMR pour la capacité du couvain à perturber la reproduction du varroa et le terme MNR pour désigner les effets combinés du couvain et des ouvrières dans la non-reproduction du parasite (Mondet *et al.*, 2020a) (Figure 1.8).

Colonies sensibles

Colonies résistantes

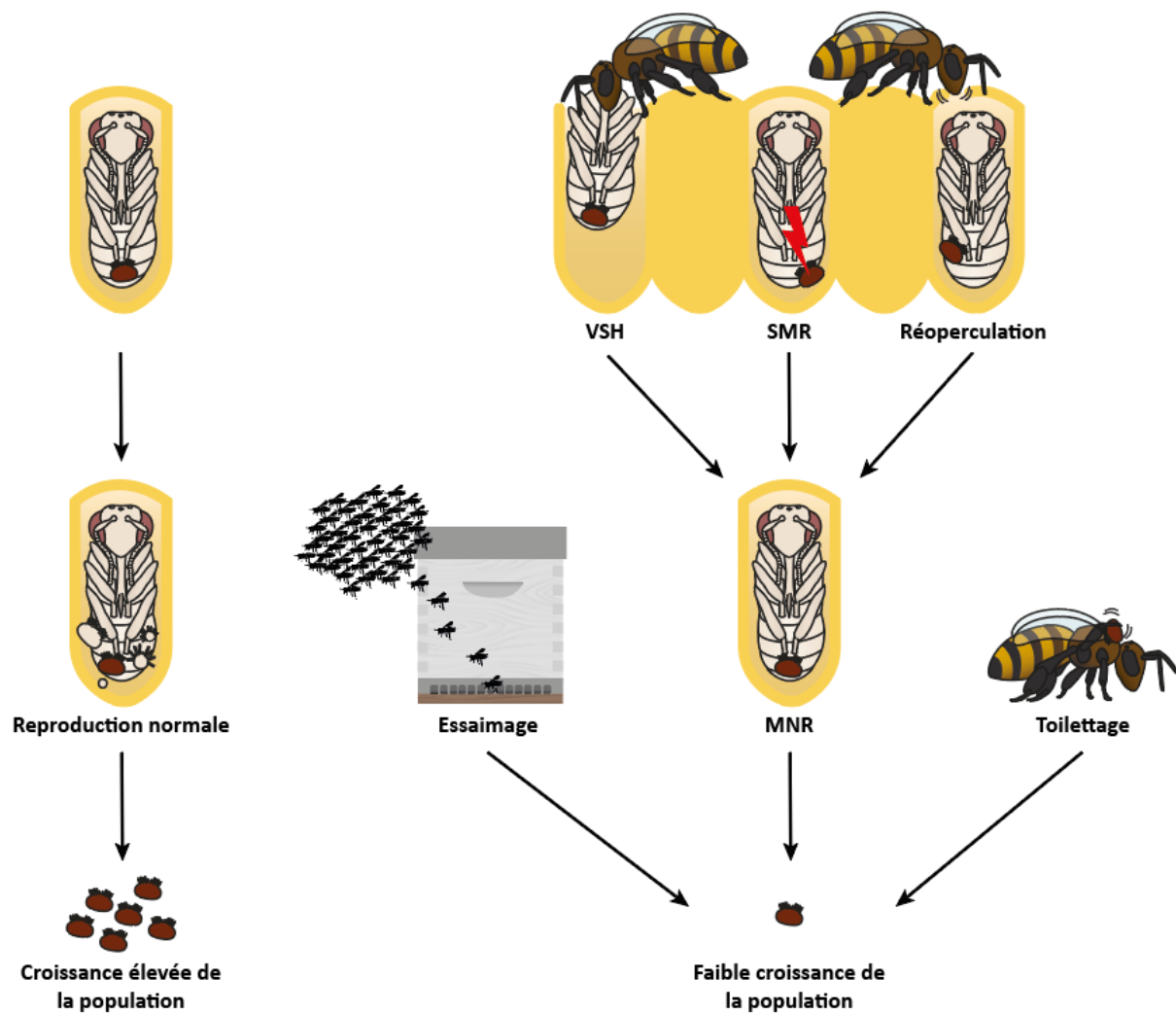


Figure 1.8 : Principaux traits comportementaux et physiologiques impliqués dans la résistance naturelle des abeilles mellifères au parasite varroa. Le comportement VSH (*Varroa Sensitive Hygiene*), le comportement de réoperculation et le caractère SMR (*Suppressed Mite Reproduction*) contribuent à la non reproduction du varroa (MNR : *Mite Non Reproduction*). Ensemble, ces caractères, associées au toilettage et à l'essaimage, permettent aux colonies de résister en limitant la croissance de la population d'acariens. D'après Noël *et al.*, 2020.

a. Le caractère SMR

Les caractéristiques du couvain peuvent donc être impliquées dans les capacités de résistance, si elles confèrent une hypersensibilité du couvain qui conduit à une mort accélérée ou à un taux d'élimination accru du varroa, empêchant ainsi sa propagation et sa reproduction (Mondet *et al.*, 2016b; Page *et al.*, 2016) ou si elles permettent de diminuer la reproduction du parasite (Mondet *et al.*, 2020b). Cette dernière s'illustre dans le caractère SMR (*Suppressed Mite Reproduction*) qui est la capacité de la nymphe à perturber la reproduction du varroa (Mondet *et al.*, 2020b). Une fois dans l'alvéole de couvain, la fondatrice ne se reproduit pas, ne pond qu'un mâle ou présente un retard dans son cycle de ponte. Cela a pour conséquence d'empêcher l'émergence de nouvelles varroa femelles matures lors de la sortie de la jeune abeille de l'alvéole. Cette capacité du couvain a été décrite à propos des colonies de l'île de Gotland (Suède) et d'Avignon (France) (Locke *et al.*, 2012). L'infertilité du varroa est due à l'émission de composés sémiocchimiques par le couvain parasité qui inhibe la reproduction du varroa (Frey *et al.*, 2013; Milani *et al.*, 2004; Nazzi *et al.*, 2002; Nazzi & Milani, 1996). Actuellement, le seul composé identifié comme émis par le couvain et qui perturbe la reproduction du varroa est le (Z)-8-heptadécène (Milani *et al.*, 2004).

b. Le comportement VSH et le comportement de réoperculation

Le comportement VSH est un comportement hygiénique ciblant spécifiquement les alvéoles de couvain operculées infestées par varroa (VSH, *Varroa Sensitive Hygiene*). Ce comportement est un trait majeur qui contribue à réduire la croissance des populations d'acariens dans les populations d'abeilles européennes et africaines (Cheruiyot *et al.*, 2018; Kirrane *et al.*, 2018; Mondet *et al.*, 2020a; Panziera *et al.*, 2017). L'ouvrière identifie l'alvéole de couvain parasitée, l'ouvre et la vide entièrement de son contenu, sacrifiant la nymphe (Figure 1.9). Les processus olfactifs ont une grande importance dans la réalisation du comportement VSH (Gramacho & Spivak, 2003; Le Conte *et al.*, 2011; Mondet *et al.*, 2015; Mondet *et al.*, 2021; Schöning *et al.*, 2012). Le comportement VSH est réalisée par des ouvrières spécialistes dites « VSH ». Il existe une différence dans les transcriptomes des antennes des abeilles réalisant le comportement VSH et celles ne le réalisant pas (Mondet *et al.*, 2015) ainsi que dans le cerveau (Le Conte *et al.*, 2011). L'expression des différences de comportement entre des abeilles VSH et des abeilles non-VSH ne s'expliquerait pas par une incapacité des abeilles non-VSH à détecter les odeurs, mais résiderait dans le traitement neuronal de l'information olfactive (Mondet *et al.*, 2021). Il semblerait que les abeilles non-VSH ne soient pas en capacité d'intégrer l'information olfactive afin de déclencher le comportement approprié (Mondet *et al.*, 2021). De plus, il existe une latéralisation antennaires chez les abeilles spécialisées dans le comportement hygiénique et dans le comportement

VSH, ce qui pourrait leur donner un avantage dans la réalisation de ces comportements (McAfee *et al.*, 2017; Mondet *et al.*, 2021; Swanson *et al.*, 2009).

Une variante du comportement VSH est le comportement de réoperculation (Martin *et al.*, 2020; Oddie *et al.*, 2019b). Cette fois, au lieu de vider l'alvéole, l'ouvrière referme l'opercule de cire (Figure 1.9). Cette désoperculation puis réoperculation de l'alvéole de couvain semble suffire à perturber le cycle de reproduction et baisser la fertilité de la fondatrice varroa (Guichard *et al.*, 2023; Oddie *et al.*, 2019a; Oddie *et al.*, 2021) (Figure 1.8). Néanmoins, ce constat fait toujours l'objet de débat compte tenu des résultats contradictoires trouvés par Sprau *et al.* (2021). La physiologie du varroa présent dans l'alvéole impacte la taille de l'aire réoperculée de l'opercule (Grindrod & Martin, 2021; Martin *et al.*, 2020). Contrairement au comportement VSH, le comportement de réoperculation reste peu connu. Ce comportement pourrait être une extension du comportement VSH, où les ouvrières ne détecteraient pas les signaux qui déclenchent le sacrifice de la nymphe et refermeraient l'alvéole. La présence d'ouvrières spécialisées dans le comportement de réoperculation au sein de la colonie est inconnue.

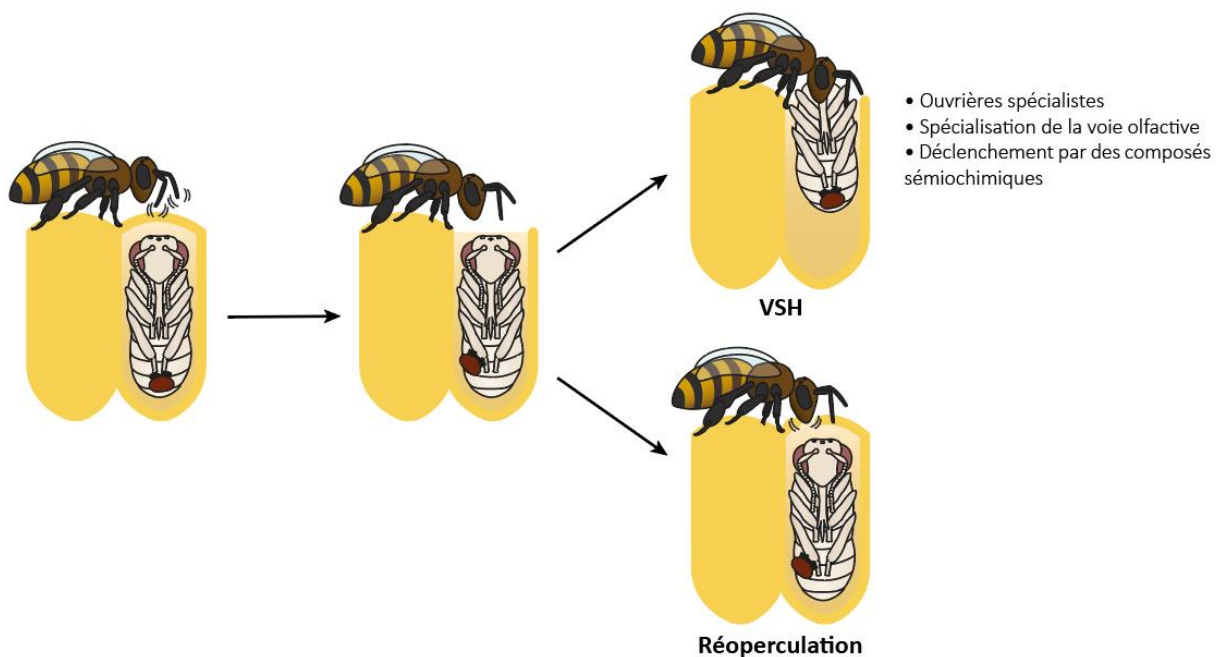


Figure 1.9 : Schéma représentation le comportement VSH (*Varroa Sensitive Hygiene*) et le comportement de réoperculation des alvéoles de couvain. Dans les deux comportements, l'ouvrière détecte l'alvéole de couvain parasitée par varroa et la désopercule. Lors du comportement VSH, l'ouvrière sacrifie la nymphe. Lors du comportement de réoperculation, l'ouvrière referme l'alvéole de couvain parasité.

3. Le rôle de la communication chimique dans la reconnaissance du parasitisme du couvain

La capacité de détection des alvéoles de couvain parasitées par varroa reposerait essentiellement sur la détection de substances sémiocchimiques associées à l'infestation par varroa (Liendo *et al.*, 2021;

Mondet *et al.*, 2016a; Nazi *et al.*, 2004a; Wagoner *et al.*, 2019; Wagoner *et al.*, 2020; Wagoner *et al.*, 2021). Dix molécules ont été identifiées chez le couvain parasité par *V. destructor* comme déclenchant le comportement VSH des ouvrières : un mélange de six composés VPS (*Varroa-Parasitization-Specific* : 2-tricosanone, 2-pentacosanone, tetracosyl acetate, 2-heptacosanone, hexacosyl acetate et 2-nonacosanone) (Mondet *et al.*, 2021), le (Z)-6-pentadecene (Nazi *et al.*, 2004a; Wagoner *et al.*, 2020), le (Z)-10-tritriacontene (Wagoner *et al.*, 2020), ainsi que l' α -pinène et l'hexanoate d'éthyle (Liendo *et al.*, 2021). Aucun composé n'est actuellement candidat au déclenchement spécifique du comportement de réoperculation.

La grande majorité des composés impliqués dans le déclenchement du comportement VSH sont des molécules à faible volatilité. Les composés organiques volatiles (COVs) sont des composés à haute volatilité et peuvent traverser rapidement l'opercule poreux des alvéoles de couvain. L'émission de COVs provoquant le comportement de nettoyage des alvéoles de couvain a déjà été montrée avec le (E)- β -ocimène chez le couvain mort (McAfee *et al.*, 2018), et avec l'acétate de phénéthyle, le 2-phényléthanol et l'alcool benzylique chez le couvain parasité par le champignon *Ascosphaera apis* (Swanson *et al.*, 2009). Il a aussi été constaté que le couvain infecté par la loque européenne (*Melissococcus plutonius*) émettait plus de γ -octalactone que le couvain sain, mais le rôle de composé dans le déclenchement d'un comportement hygiénique vis-à-vis du couvain infecté par la loque européenne n'a pas été confirmé (Kathe *et al.*, 2021). L'alvéole de couvain parasitée par varroa pourrait émettre une signalisation double avec l'émission de composés peu volatiles et de COVs qui pourrait déclencher le comportement VSH et/ou de réoperculation. L'identification de deux COVs (α -pinène et hexanoate d'éthyle (Liendo *et al.*, 2021)) déclencheurs du comportement VSH renforce l'hypothèse de signal en deux temps. Les COVs pourraient se répandre rapidement à travers l'opercule signalant la zone où se trouve le parasitisme et les composés moins volatiles pourraient permettre l'identification précise de l'alvéole de couvain parasitée. Une autre hypothèse serait que les COVs déclenchent le comportement de désoperculation et que la présence des composés plus lourds déclenche le nettoyage, ou *a contrario* leur absence déclencherait le comportement de réoperculation.

Les progrès dans la compréhension des mécanismes qui sous-tendent les capacités de résistance, ainsi que l'essor récent des outils génomiques, ouvrent la possibilité de concevoir un outil de diagnostic de la résistance basé sur le phénotype, directement accessible aux apiculteurs. Pour l'instant, les méthodes disponibles sont très fastidieuses et difficiles à appliquer sur un grand nombre de colonies, que ce soit pour les mesures de la non-reproduction d'acariens ou des comportements hygiéniques. Il est nécessaire de développer des outils simples pour aider les apiculteurs à phénotyper leurs colonies tels que l'utilisation de médiateurs chimiques et/ou de marqueurs moléculaires. Récemment, une synergie de composés déclenchant le comportement hygiénique a été brevetée dans l'optique d'aider

les apiculteurs à phénotyper leurs colonies en appliquant ce produit sur les alvéoles de couvain et en quantifiant le nettoyage des ouvrières (Wagoner & Rueppell, 2022). Les identifications de marqueurs moléculaires et protéiques de plusieurs traits liés à la résistance (toilettage, hygiène, VSH et SMR), bien que très limitées en termes de chevauchement entre les différentes études (Mondet *et al.*, 2020a), ouvrent la possibilité d'une sélection assistée par marqueurs (Boutin *et al.*, 2015; Conlon *et al.*, 2018; Gempe *et al.*, 2016; Hu *et al.*, 2016; Jones *et al.*, 2020; Kirrane *et al.*, 2015; Mondet *et al.*, 2015; Scannapieco *et al.*, 2017; Spötter *et al.*, 2016). Cela permettrait aux apiculteurs de sélectionner facilement leurs colonies sur la base de traits de résistance intéressants lorsque le phénotype est difficile à caractériser. A ce jour, aucun produit n'est disponible sur le marché et les recherches se poursuivent dans ces domaines.

IV. Contexte et problématique de la thèse

L'impact de *Varroa destructor* sur la colonie d'abeilles et sa résistance croissante aux traitements acaricides ont des conséquences néfastes pour la filière apicole et il devient urgent de sélectionner des colonies résistantes à l'acarien. Une meilleure connaissance des caractères de résistance de l'abeille au parasite permettrait de développer un système de phénotypage facile et rapide à mettre en place par les apiculteurs dans le but de faciliter la sélection des colonies afin d'améliorer la lutte contre le parasite et de diminuer l'utilisation d'acaricides.

L'objectif principal de cette thèse est d'approfondir les connaissances sur la résistance de l'abeille domestique à *V. destructor* en décryptant les mécanismes qui sous-tendent le comportement VSH et le comportement de réoperculation. Ici, le décryptage se fait par l'identification de COVs caractérisant les alvéoles parasitées et l'étude comportementale des ouvrières face à l'émission des composés candidats. L'évaluation de la réponse hygiénique des abeilles après l'application de ces composés candidats dans les colonies pourrait aboutir à un outil de terrain pratique pour phénotyper les colonies résistantes.

Les objectifs de cette thèse sont résumés par trois questions scientifiques :

(1) Quelles sont les différences d'émissions de COVs entre un couvain parasité par *V. destructor* et un couvain non-parasité ?

Afin de répondre à cette question, il était nécessaire de connaître les émissions de COVs d'un couvain sain. Au vu de l'absence de données continues disponibles sur ces émissions tout au long de son développement, des captures sur les 21 jours de développement du couvain d'ouvrière ont été réalisées afin de décrire les émissions et leurs variations au cours du développement (**Chapitre 2**). Par la suite, l'observation des potentielles variations d'émissions des alvéoles de couvain en fonction du

parasitisme à varroa a été réalisée sur les sept premiers jours après operculation, jours-clefs dans la réalisation du comportement VSH (**Chapitre 3**).

(2) Les composés associés au statut parasité du couvain déclenchent-ils le comportement chez l'adulte ?

L'observation de différences d'émissions de COVs entre le couvain parasité et le couvain non-parasité par varroa pose la question d'une réponse comportementale chez les ouvrières. Dans le **Chapitre 3**, des tests comportementaux ont été réalisés afin d'observer la réponse des adultes face à l'émission de COVs que nous avons identifiés comme caractérisant le couvain parasité. L'hypothèse d'un double signal avec l'émission de composés peu volatiles couplée à celle de COVs fait l'objet du **Chapitre 4**. Pour cela, les COVs identifiés dans le cadre de cette thèse (**Chapitre 3**) ont été testés avec les composés candidats publiés dans la littérature scientifique.

(3) Le comportement de réoperculation est-il déclenché par la présence de COVs et l'absence de composés moins volatiles dans les émissions d'une alvéole de couvain ?

Le comportement VSH et le comportement de réoperculation sont des séquences comportementales qui commencent tous les deux par la détection de l'alvéole de couvain parasitée suivi de la désoperculation de cette alvéole. La réoperculation plutôt que le sacrifice de l'alvéole de couvain pourrait être déclenchée par l'absence de composés peu volatiles. La réponse des ouvrières, en termes de comportements VSH et de réoperculation, face à des mélanges contenant des COVs et/ou des composés moins volatiles associés au parasitisme par *V. destructor* a été étudiée dans le **Chapitre 4**.

CHAPITRE 2

CARACTERISATION DES EMISSIONS DE COMPOSES ORGANIQUES VOLATILES DU COUVAIN AU COURS DE SON DEVELOPPEMENT



Le soin au couvain

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I. Avant-propos

Le couvain, lors de son développement, passe par des phases physiologiques très différentes nécessitant des comportements spécifiques de la part des adultes. Les soins des ouvrières au couvain sont médiés par l'émission de divers composés sémiochimiques. Deux phéromones de couvain ont été identifiées : la BEP (Le Conte *et al.*, 1990b) et le (*E*)- β -ocimène (Maisonnasse *et al.*, 2009). Ces phéromones ont divers effets au sein de la colonie, comme induire les comportements d'alimentation et de soins au couvain (He *et al.*, 2016; Sagili & Pankiw, 2009), réguler le comportement de butinage (Le Conte *et al.*, 2001; Pankiw, 2004a; Pankiw, 2004b; Traynor *et al.*, 2015) ou encore inhiber la maturation des ovaires des ouvrières (Maisonnasse *et al.*, 2009; Mohammedi *et al.*, 1998).

Les composés organiques volatiles peuvent se disperser facilement dans le nid, favorisant l'émission rapide et réversible d'un potentiel message phéromonal. Quelques études ont montré l'émission de COVs par le couvain au cours d'une partie spécifique ou lors de stades spécifiques du développement du couvain (Carroll & Duehl, 2012; Light *et al.*, 2020b; Maisonnasse *et al.*, 2010). Ces études participent à dresser le tableau des émissions de COVs des immatures, mais ne montrent qu'une vision parcellaire des émissions du couvain au cours de son développement.

L'étude présentée dans ce chapitre a pour but de décrire les émissions de COVs du couvain d'abeille de façon continue, de l'œuf à l'émergence. Un dispositif de capture quotidienne de COVs a en effet été mis au point, permettant ainsi de suivre les émissions en continu et de les relier aux moments clés du développement du couvain. Cette étude, décrivant le paysage olfactif du couvain au cours de son développement et notamment au cours de la phase operculée, a servi de contexte pour la suite des travaux de cette thèse.

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II. Article 1

Detailed chemical analysis of honey bee (*Apis mellifera*) worker brood volatile profile: from egg to emergence

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Abstract

Chemical communication is a widely used mode of communication for social insects and has been demonstrated to be involved in many behaviours and physiological processes such as reproduction, nutrition or the fight against parasites and pathogens. In the honey bee, *Apis mellifera*, the release of chemical compounds by the brood plays a role in worker behaviour, physiology, and foraging activities and colony health as a whole. Several compounds have already been described as brood pheromones, such as components of the brood ester pheromone and (*E*)- β -ocimene. Several other compounds originating from diseased or varroa-infested brood cells have been described as triggering the hygienic behaviour of workers. So far, studies of brood emissions have focused on specific stages of development and little is known about the emission of volatile organic compounds by the brood. In this study, we investigate the semiochemical profile of worker honey bee brood during its whole developmental cycle, from egg to emergence, with a specific focus on volatile organic compounds. We describe variation in emissions of thirty-two volatile organic compounds between brood stages. We highlight candidate compounds that are particularly abundant in specific stages and discuss their potential biological significance.

Keywords: Honey bee, brood, volatile collection, chemical communication, volatile organic compound, chemical ecology, pheromone

1. Introduction

Communication is fundamental to all life forms. Chemical communication, *i.e.* the emission of specific chemical compounds by an individual and received by another at a specific moment, is highly present in the living world. In eusocial insects, compounds emitted and perceived by individuals of the same species constitute a pheromonal message, which is part of the chemical language repertoire. Individuals receiving this chemical message will respond by adapting their behaviour or their physiology (Bell & Cardé, 1984; Karlson & Butenandt, 1959; Slessor *et al.*, 2005). This type of communication is key to the regulation of superorganism homeostasis, as it allows eusocial species to organise the colony and the division of labour (Bagnères & Hanus, 2015; Hölldobler, 1999; Winston, 1987).

In the honey bee, *Apis mellifera*, chemical communication is widely deployed across castes and individuals (Slessor *et al.*, 2005). It is well known that the brood communicates with the adults through the use of pheromones. An iconic example of brood chemical communication is the brood ester pheromone (BEP), composed of by a mixture of 10 fatty esters (methyl and ethyl oleate, methyl and ethyl linoleate, methyl and ethyl linolenate, methyl and ethyl palmitate, methyl and ethyl stearate) (Le Conte *et al.*, 1990b). Another well-known brood pheromone is (*E*)- β -ocimene (Maisonasse *et al.*, 2009). These two pheromones have both releaser (*i.e.* triggers an immediate behavioural response) and primer effects (*i.e.* changes the physiology of the receiver). As releaser effects, BEP triggers worker brood-rearing behaviour and cell-capping behaviour (closing brood cells with wax) (Le Conte *et al.*, 1990b; Sagili & Pankiw, 2009), and (*E*)- β -ocimene increases pollen foraging and brood-feeding behaviour (He *et al.*, 2016; Traynor *et al.*, 2015). A common primer effect of these two pheromones is the inhibition of worker ovarian growth (Maisonasse *et al.*, 2009; Mohammedi *et al.*, 1998).

Dead brood emit oleic acid and (*E*)- β -ocimene, which both trigger hygienic behaviour (detection of sick brood and cleaning of the brood cell), suggesting a death-signalling role for these two compounds (McAfee *et al.*, 2018). Hygienic behaviour is also performed on diseased brood, before it dies. This is the case for brood infected by chalkbrood (*Ascosphaera apis*). In order not to become a vector of contamination, adults must detect brood parasitized by the fungus before the spores reach the cuticle of the larva. The hygienic behaviour is then triggered by the emission of phenethyl acetate, 2-phenylethanol, and benzyl alcohol (Swanson *et al.*, 2009). A variant of hygienic behaviour, VSH (*Varroa Sensitive Hygiene*) behaviour, targets brood cells parasitized by the varroa mite and is triggered by compounds emitted by parasitized brood cells ((*Z*)-6-pentadecene, (*Z*)-10-tritriacontene, 6 VPS (*Varroa Parasitization-Specific*: tricosan-2-one, pentacosan-2-one, tetracosyl acetate, heptacosan-2-one, hexacosyl acetate and nonacosan-2-one), α -pinene and ethyl hexanoate) (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Wagoner *et al.*, 2020). Stressed or diseased brood emit other

compounds but potential behavioural responses of workers have not been assayed. Such is the case for γ -octalactone, a compound that larvae infected with the European foulbrood emit at higher levels than healthy brood (*Melissococcus plutonius*) (Kathe *et al.*, 2021). *Varroa destructor* infestation and *Deformed Wing Virus* infection increase the emission of ten chemical compounds in brood targeted by hygienic behaviour (pentacosane, hexacosane, 4-methyltetracosane, 9- and 11-methyltricosane, 11- et 13-methylpentacosane, 12- et 14-methylhexacosane, pentacosene, heptacosene, hentriacontene) (Wagoner *et al.*, 2019). With the exception of a few volatile organic compounds (VOC), the majority of chemical compounds already described as brood emissions that trigger adult behaviours are not very volatile. Little is known about highly volatile compounds of honey bee brood; studies have tended to focus on low-volatile compounds. Unlike cuticular hydrocarbons, little is known about VOC emission of brood in the other social insect (Bagnères & Hanus, 2015; Le Conte & Hefetz, 2008; Schultner *et al.*, 2017). The role of the brood on the behaviour (e.g. foraging regulation) and physiology (e.g. ovary maturation) of adults in social insects is well established. For example, in ants, brood has been shown to have a role in adult physiology and behaviour, but identification of this potential ant brood pheromone is still lacking (see review: Schultner & Pulliainen, 2020). In bumblebees, the pupal odour attracts the workers but has no impact on the physiology of the adult. The concept of a brood pheromone in the bumblebee is still discussed (Starkey *et al.*, 2019).

The emission of highly volatile chemical compounds by the brood allows information to be disseminated quickly and over a long distance within the nest. Studying emission of VOC by the brood would provide a better understanding of its chemical communication and development.

Thus far, no documentation of VOC emissions over the entire brood development is available, as studies describing VOC emissions from brood focus on specific stages of development and/or on specific compounds (Carroll & Duehl, 2012; Light *et al.*, 2020b; Maisonnasse *et al.*, 2010). Maisonnasse *et al.* (2010) showed the difference in (*E*)- β -ocimene emission between larvae and pupae (first larval stage to fifth pupal stage). Carroll & Duehl (2012) carried out *in situ* VOC captures on unstressed brood from hatching to the pre-pupal stage, in the presence of nurses on the frame. The authors showed changes in the emission of six VOC from the brood according to age (2-heptanone, 3-carene, β -ocimene, methyl benzoate, octanoic acid and decanal) and reported the presence of 22 VOC in total (2-heptanol, 2-heptanone, 2-pentanone, 3-carene, 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, α -pinene, citral, decanal, decane, ethyl benzoate, (*E*)- β -ocimene, geraniol, heptanal, hexanal, isobutyl acetate, isopentyl acetate, methyl benzoate, nerol, nonanal, octanal and *n*-nonane) (Carroll & Duehl, 2012). Light *et al.* (2020) also captured VOC *in situ* emitted by worker and drone brood at different developmental stages (last day of egg, first instar larvae, first, third and fifth instar pupae for drone brood, fifth instar pupae for cold-killed drone brood, fifth instar pupae for worker brood). They identified 75 VOC emitted by

worker and drone brood frames that triggered an electrophysiological response in *V. destructor*. These different studies give an indication as to the VOC emitted by bee brood but only provide a fragmented view of VOC emissions during its development.

Similarly to "-omic" studies (genomics, transcriptomics, proteomics, metabolomics and metagenomics), capturing VOC emissions of the brood throughout its development, from oviposition to emergence, makes it possible to observe and capture the essence of brood-adult chemical communication in its entirety. Studying VOC emissions has led to a better understanding of reproduction in beetles (Haberer *et al.*, 2014) and the estimation of larval and pupal age in blowflies (Frederickx *et al.*, 2012; Sharma *et al.*, 2021). In honey bees, VOC collection permits the study of the changes in larval food consumption and pheromonal emission over the course of their development (Carroll & Duehl, 2012; Maisonnasse *et al.*, 2010).

Bee brood development is characterised by a sequence of different physiological stages. The brood passes from an open brood state comprising the egg stage and five larval stages, to a capped brood state (*i.e.* brood cells closed with a wax cap by workers) before the emergence of the adult bee, comprising a sealed larval stage, a prepupal stage, and nine pupal stages (Winston, 1987). Physiological changes in the brood can be observed through the brood haemolymph proteome (do Nascimento *et al.*, 2004; Woltedji *et al.*, 2013). These important physiological changes can also be observed in brood-nurse interactions according to the developmental stage. Under the influence of brood pheromones, workers adapt the food they give to the brood according to the age of the larvae (Haydak, 1970; Le Conte *et al.*, 1995; Sagili & Pankiw, 2009), cap brood (Le Conte *et al.*, 1990b), modify their foraging behaviour (Le Conte *et al.*, 2001; Traynor *et al.*, 2015), and realise hygienic behaviour (McAfee *et al.*, 2018; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Swanson *et al.*, 2009; Wagoner *et al.*, 2020).

In this study, we develop an *in situ* experimental approach to analyse VOC emissions of healthy worker brood during the whole developmental cycle (21 days, from egg stage to adult bee emergence). Our target is to highlight the temporal changes in brood-characterising compound presence and abundance during the entire brood development. We highlight semiochemical compounds emitted by brood that may have a role in the communication between brood and workers.

2. Materials and methods

2.1. Honey bee colonies and experimental setup

This study was performed with nine local honey bee colonies (*A. mellifera*) installed at the INRAE (Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement) research centre in Avignon (France). As the aim of this study was to capture volatile organic compounds (VOC) in healthy brood, we were very careful to use colonies that were free of parasites and disease. The health of these colonies was closely monitored throughout the experiment: colonies were qualitatively screened by our beekeepers to confirm the absence of brood illness. This study was conducted from April to July 2021, when varroa infestation is at its lowest, to maximize chances that capped brood cells were not infested by the parasite.

To ensure working with brood of a homogeneous age, queens of the nine selected colonies were caged on an empty frame for 24h to allow for egg laying. After that period, the queen was removed from the cage and no longer allowed access to the laid frame (*brood frame*). An empty built frame (no brood, pollen or nectar) was concomitantly used as a control in each colony (*control frame*). On the first day of the experiment (following queen laying), circles containing about 130 cells were drawn on both brood and control frames to select cell patches to be monitored daily, for 21 days. Each day, brood and control frames were removed from their colonies and brought to the laboratory for chemical analysis. Experimental procedures were optimised to minimise any source of stress. To do so, adult bees were gently removed from the brood frame with a soft brush, and the brood was placed directly in a warm, moist box during the short journey from the hives to the experimental room. They were kept in a room with controlled temperature and humidity (34-35 °C, 65 % RH). The same patch of cells was monitored each day (Figure 2.1), and the content of each cell was recorded on a transparent plastic sheet.

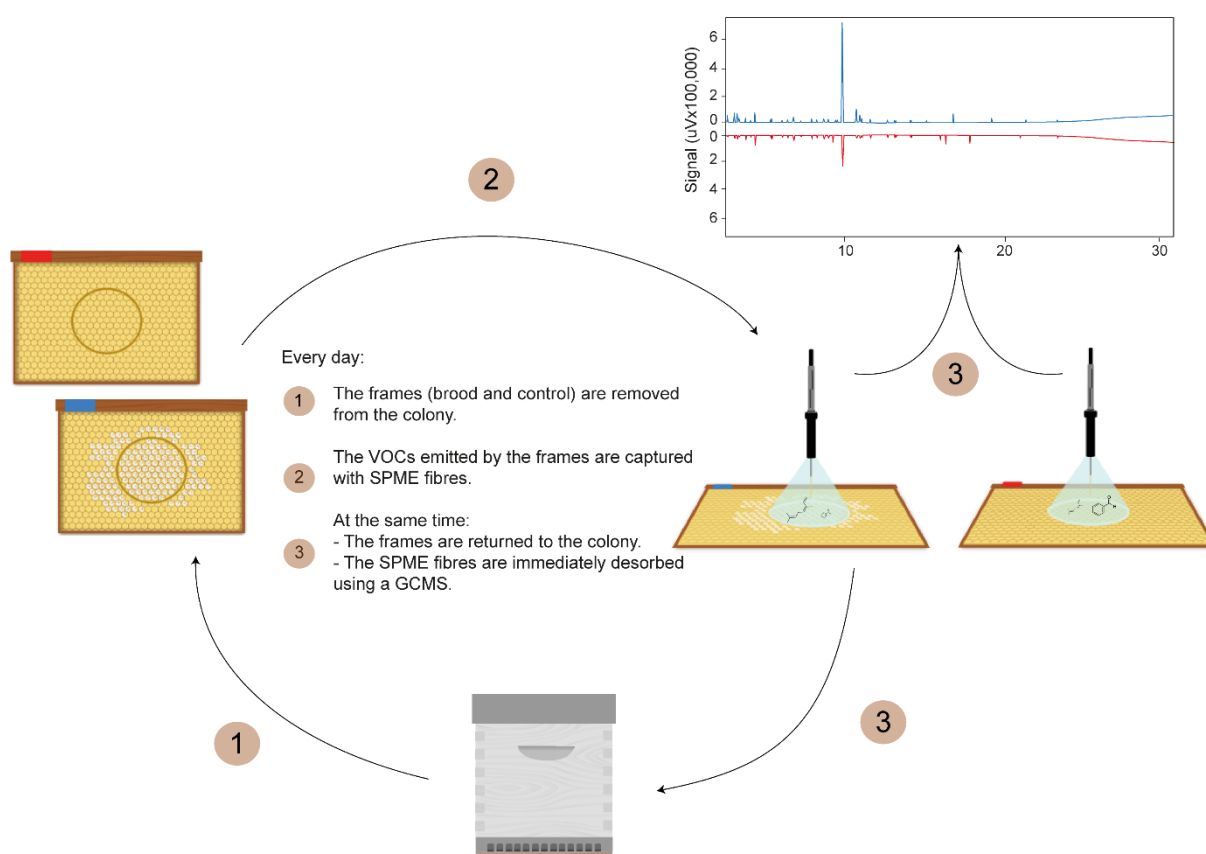


Figure 2.1: Experimental setup used for the *in situ* capture of the volatiles organic compounds (VOC) during honey bee brood development. Every day for 21 days, brood and control frames were removed from their colonies. VOC of the same patch of cells (brood and control) were captured for 20 minutes with a SPME fibre, in a room with controlled temperature and humidity (34-35 °C, 65 % RH). After capture, frames were immediately put back in their colony while fibres were desorbed on a GC-MS. Nine colonies were used, each with a control and a brood frame (371 captures in total).

2.2. Volatile organic compound capture and analysis

VOC capture was conducted passively, using a polydimethylsiloxane divinylbenzene (PDMS DVB) Solid Phase Micro Extraction (SPME) fibre (65 μm , manual holder). The fibre was inserted inside a glass funnel (\varnothing 70 mm) placed upside down on the patch of cells to be monitored (Figure 2.1). For each capture, the funnel was pressed against the waxy surface to form a hermetic junction between the sample (cell patch) and the capture system. Captures lasted for 20min, after which frames were returned to their colonies. Each capture of a brood sample was paired with that of a control sample (seven control samples could not be captured due to bad weather conditions on day 11 and 12), thus forming a total of 371 captures (9 control + 9 brood samples per day). Qualitative and quantitative analyses of VOC were performed on a gas chromatograph coupled with mass spectrometry (GC-MS). SPME fibres were desorbed into a GC-MS (Shimadzu GCMS-QP2010SE), equipped with an electronic impact ion source (70 eV) and a ZB 5 MS column (Zebtron ZB 5ms, 20 m x 0.18 mm, 0.18 μm thickness). Desorption was realised in splitless mode, with the constant column flow at 0.82 mL/min; the carrier

gas was helium. The injector temperature was set at 250 °C and the oven temperature program as follows: 45 °C isothermal held for 5 min, followed by temperature increases at a rate of 10 °C/min up to 250 °C. The oven was finally held at 250 °C for 4 min. The ion source was set at 200 °C and *m/z* scanned from 40 to 350 amu. Compound identification was performed by comparison of their mass spectra and retention indices with those found in the NIST 2017 library (v2.3). This identification was further confirmed by injection of the reference standards purchased from Sigma Aldrich (except for hexenyl acetate (s24) for which the location of the unsaturation remains unknown).

2.3. Statistical analyses

Statistical analyses were performed using R (version 4.0.5). To account for non-linear trends across time, generalized additive mixed models (GAMM) were fit on total peak area for each day and each sample group (brood, control) to investigate differences between control and brood samples and their evolution over time (R package “mgcv” v1.8-39; <https://cran.r-project.org/web/packages/mgcv/index.html> (Wood, 2011)).

Three redundancy analyses (RDA) were performed on three different phases of development, based on GAMM results (“Eggs”: day 1 to day 3; “Larvae and Non-Sclerotised Capped Brood”: day 4 to day 16; “Sclerotised Pupae”: day 17 to day 21), to identify compounds that characterise the brood (R package “vegan” v2.6-2; <https://cran.r-project.org/web/packages/vegan/index.html>). Day and sample group (brood and control) and their interaction were included as fixed factors in the RDA to identify brood-characterising compounds. To graphically represent the RDAs, we selected the two components which explain the largest proportion of the data variance. Compounds detected in at least five out of the nine replicates in brood samples for any given day were included in the analyses. From the 3 RDAs, a list of 32 brood-characterising compounds was compiled. We then split brood development into eight characteristic periods to study the compounds or chemical families that characterised each period, as well as those that covaried together. The four days around the capping of brood cells by the workers, represent a key moment in the development of the brood, both physiologically and behaviourally (Jay, 1963; Le Conte *et al.*, 1990b; Slessor *et al.*, 2005; Winston, 1987). For this reason, those four days were separated into four one-day periods. The eight periods were thus defined as follows: Eggs: days 1-3; 1st to 4th instar larvae (L1-L4): days 4-7; 5th instar larvae (L5): day 8; Capping: day 9; Sealed larvae (SL): day 10; Prepupae (PP): day 11; Non-sclerotised pupae (NSP): day 12-16; and Sclerotised pupae (SP): days 17-21.

In order to classify the data, we chose to group the compounds found according to their respective chemical families. We chose this presentation not because of the potential effects on

behaviour, but rather to highlight a classification based on the chemical structure of these molecules, in particular to evaluate shared patterns of variation and stage-specific emission.

As our target is to highlight the temporal changes in the presence and abundance of brood-characterising compounds during development, we normalised brood compound area values by those measured in the control (*i.e.* $(A_{\text{brood}} - A_{\text{control}})/A_{\text{control}}$) for each day, compound, and colony. To avoid division by zero, a constant ten times lower than the smallest non-zero value was added to the data. We then calculated the median normalised area of each compound across the nine colonies for each day. Negative median values, indicating a higher amount of the compound in control samples than in brood samples, were set at zero. Compounds were subsequently quantified relative to the highest median normalised value observed across the 21 capture days. We represented these relative median normalized values in a heatmap with a \log_{10} scale (adding a constant of 1). We similarly calculated normalised brood compound area by stage, analogous to that done by day. For two compounds, these stage-specific median normalised values were equal to 0 as they were observed on a single day of the four days comprising the SP stage. As before, compounds were quantified relative to the highest median value observed across the 8 developmental stages. The evolution across stages of relative median normalised compound emissions was visualized in another heatmap. We realised a clustering of the arcsine-transformed relative median normalised values per stage using a Gaussian mixture model to highlight compounds that shared stage-specific maximum emissions (R package “coseq” version 1.17.2; <https://bioconductor.org/packages/release/bioc/html/coseq.html> (Rau & Maugis-Rabusseau, 2017)). The number of clusters was identified based on the integrated completed likelihood (ICL) model selection criterion.

For comparisons with the scientific literature, we calculated the estimated emission per mg of individuals weight for (*E*)- β -ocimene. As the tracking of the individuals throughout the experiment did not allow us to weigh the brood, we instead used in-house brood weight data (first larval to fifth pupal stage, day 4 to day 15 in this paper, $n = 9$ per stage) obtained in a previous experiment on similar colonies.

3. Results

3.1. Total compound emission profile across development

A comparison of total peak area between control and brood samples revealed that all compounds (total peak area) are significantly more abundant in brood samples than in control samples (GAMM; Brood: estimate = 296,010, $p < 2e^{-16}$; control: estimate = 200,479, $p < 2e^{-16}$) (Figure 2.2). The quantity of

compounds fluctuates significantly from $2.6e^5$ (day 16) to $5.4e^5$ (day 11) of total compound area (GAMM; Brood over time: $F = 12.403$, $p < 2e^{-16}$; Control over time: $F = 0.078$, $p = 0.820$) (Figure 2.2). The first three days (egg stage) and the last five days (sclerotised pupal stages) are periods of low overall emissions, with small differences between brood and control samples (Figure 2.2). From day 4 (first instar larvae) to day 16 (fourth instar pupae), total compound abundance is quite variable and strikingly different between brood and control samples, with a notable peak of brood emission around days 10 and 11 (prepupae) (Figure 2.2). Based on these tendencies, and to improve the brood-characterising compound determination step, we separated our dataset into three groups to perform multivariate redundancy analyses (RDA): Egg (day 1 to day 3), Larvae and Non-Sclerotised Capped Brood (day 4 to day 16) and Sclerotised Pupae (day 17 to day 21).

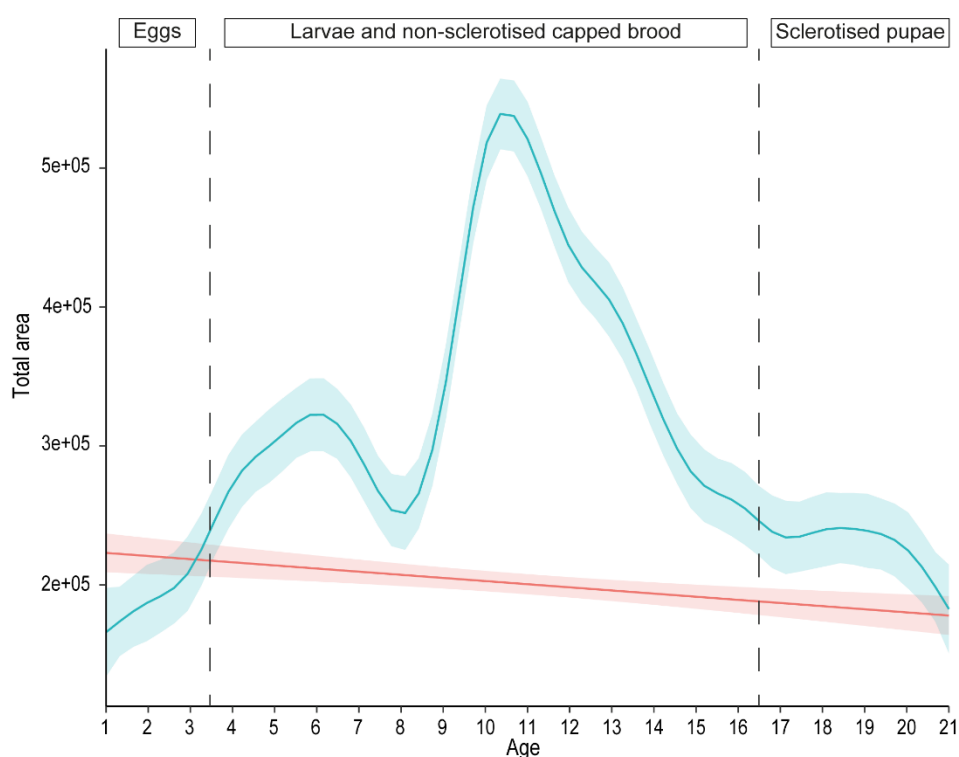


Figure 2.2: Evolution of the total peak area of all compounds across development (GAMM models). Lines show the mean total area and the surrounding 95% standard error, for brood (blue) and control (red) samples. Dotted lines separate the three groups: “Eggs” (day 1 to 3), “Larvae and non-sclerotised capped brood” (day 4 to 16) and “Sclerotised Pupae” (day 17 to 21) considered for the multivariate analyses.

3.2. Identification of brood-characterising compounds

Based on the total emission analysis and the identification of 3 distinct phases of compound emission, we performed three redundancy analyses (RDAs).

In the largest RDA (day 4 to day 16, “Larvae and non-sclerotised capped brood” group), the first two components of the RDA explained 56.82 % of the variance. Both day and sample group, as well as their interaction, were significant (Table 2.1). The two sample groups (brood and control) were well

separated on the first component (Figure 2.3). Based on this analysis, twenty-seven compounds characterising the brood samples were retained (s01, s02, s03, s05, s09, s12, s13, s15, s17, s18, s24, s27, s28, s29, s30, s32, s36, s37, s38, s41, s42, s47, s52, s54, s65, s71, s74, s75, s76) (Figure 2.3, Table 2.2).

Table 2.1: Results of the 3 RDAs showing the effects of Day and Sample groups on compound area. For each day: brood sample n = 9, control sample n = 9.

Groups	Factors	F	P
Eggs (day 1 to day 3)	Day	2.04	0.008
	Sample group	4.39	0.002
	Day : Sample group	0.49	0.994
Larvae and non-sclerotised capped brood (day 4 to day 16)	Day	2.34	0.001
	Sample group	22.92	0.001
	Day : Sample group	1.45	0.001
Sclerotised Pupae (day 17 to day 21)	Day	1.89	0.001
	Sample group	7.28	0.001
	Day : Sample group	0.73	0.950

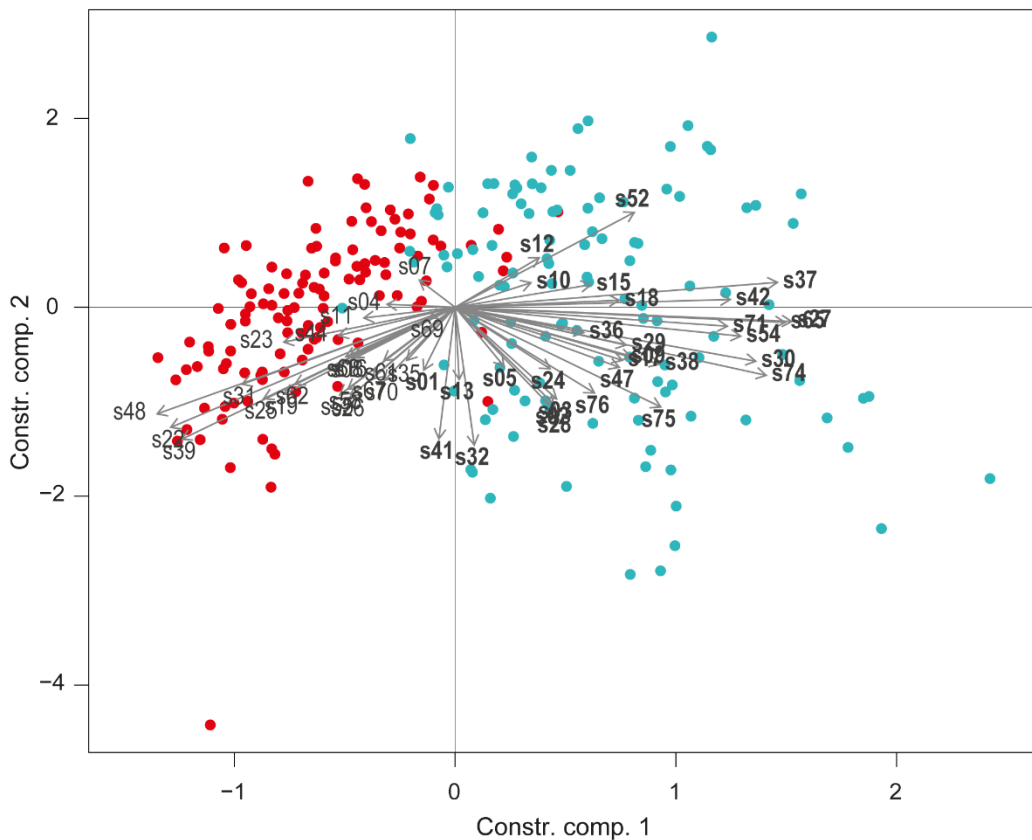


Figure 2.3: Redundancy Analysis (RDA) performed on chemical compounds captured on brood and control samples for the “larvae and non-sclerotised capped brood” group (day 5 to day 16). For each day: brood sample n = 9, control sample n = 9. Each dot represents a sample (blue: brood, red: control). Compounds are represented

by their IDs and grey arrows. The compounds characterising the brood and retained for the rest of the analysis are shown in bold.

Table 2.2: List of chemical compounds found as brood-characteristic during brood development (21 days). Days of detection represent periods where a given compound is found in at least 5 replicates in brood samples for a given day. *: Unsaturation remains unknown, **: Stereochemistry remains unknown.

ID	Compounds	Chemical families	Retention time	Retention index	Days of detection
s01	3-Methyl-2-buten-1-ol (Prenyl alcohol)	Alcohol	2.947	784	4 – 6, 9
s02	2,3-Butanediol ** (diastereomer)	Alcohol	3.112	789	8 – 16, 18 – 20
s03	2,3-Butanediol ** (diastereomer)	Alcohol	3.307	795	8 – 16, 18 – 20
s05	<i>n</i> -Octane	Alkane	3.447	800	3 – 5, 8 – 11, 14 – 21
s09	1-Hexanol	Alcohol	5.473	869	8 – 21
s10	Methylbutyl acetate (Isoamyl acetate)	Ester	5.735	877	1, 4 – 8, 10 – 12, 14, 16, 19 – 20
s12	2-Heptanone	Ketone	6.090	890	4 – 9
s13	<i>n</i> -Nonane	Alkane	6.435	900	5, 9 – 10, 13, 16 – 19
s15	α -Pinene	Terpene	7.273	934	2 – 9, 11 – 20
s17	1-Heptanol	Alcohol	8.260	973	12, 16, 18, 21
s18	β -Pinene	Terpene	8.345	977	3 – 4, 6, 11 – 20
s24	2-Hexenyl acetate **	Ester	9.385	1023	11
s27	(<i>Z</i>)- β -ocimene	Terpene	9.660	1036	6 – 18
s28	Phenylacetaldehyde	Aldehyde	9.750	1042	6, 9 – 13, 15, 17 – 21
s29	α -Ocimene	Terpene	9.800	1045	7, 11, 15, 17
s30	(<i>E</i>)- β -Ocimene	Terpene	9.886	1047	1 – 21
s32	1-Octanol	Alcohol	10.348	1072	9 – 20
s35	(<i>E</i>)-Linalool furanoxide	Alcohol	10.623	1086	19
s36	2-Nonanone	Ketone	10.713	1090	6 – 9
s37	Methyl benzoate	Ester	10.753	1092	1 – 21
s38	Linalool **	Alcohol	10.89	1099	9 – 14, 16 – 20
s41	Phenylethyl alcohol	Alcohol	11.085	1111	9 – 12, 18 – 20
s42	Allo-ocimene	Terpene	11.368	1127	6 – 7, 10 – 16
s45	(<i>E</i>)-Linalool pyranoxide	Alcohol	12.080	1170	19
s47	Methyl salicylate	Ester	12.428	1190	4 – 12, 14 – 16, 18 – 19
s52	γ -Octalactone	Ester	13.403	1253	1 – 15

s54	<i>n</i> -Tridecane	Alkane	14.105	1300	1, 5 – 21
s65	<i>n</i> -Pentadecane	Alkane	16.793	1500	4 – 21
s71	<i>n</i> -Heptadecane	Alkane	19.172	1700	1, 4 – 18
s73	Nonadecene *	Terpene	21.087	1880	19 – 21
s74	<i>n</i> -Nonadecane	Alkane	21.315	1900	4 – 21
s75	<i>n</i> -Eicosane	Alkane	23.262	2000	8 – 21

In the “Egg” RDA analysis (days 1-3), the first two components of the RDA explained 73.95 % of the variance. Both day and sample group were significant (Table 2.1), but the interaction was not, confirming that overall brood and control compound emissions maintain the same temporal trend. Both sample groups (brood and control) were also well separated on the first component (Figure S2.1). Five compounds characterising brood samples, all included in the previous list, were highlighted by the RDA (s15, s18, s30, s37, s52) (Figure S2.1, Table 2.2).

In the “Sclerotised pupae” RDA (day 17 to day 21), the first two components explained 64.07 % of the variance. Both single factors were significant, but their interaction was not (Table 2.1). The two sample groups were once again well separated on the first component (Figure S2.1). Twenty-eight compounds characterising the brood sample were retained, partially overlapping with the previous lists (s01, s02, s03, s05, s09, s10, s12, s13, s15, s17, s18, s27, s28, s30, s32, s35, s37, s38, s41, s45, s47, s54, s65, s71, s73, s74, s75, s76) (Figure S2.1, Table 2.2).

Using these three RDAs, we were able to compile a final list of 32 VOC characterising the brood samples (Figure S2.2, Table 2.2).

3.3. Evolution of brood-characterising compound emission during brood development

Each day of brood development was characterised by different emission of VOC. The 32 VOC could be classified into two groups; a group of compounds with a marked variation of emission over time and a group with a more constant emission. The group of compounds fluctuating over time included 15 compounds (in order of appearance in the figure 2.4, from top to bottom: s27, s30, s42, s52, s18, s10, s12, s47, s36, s29, s73, s03, s09, s02 and s01) (Figure 2.4). Except for α -pinene (s15), all terpenes found in this study were in this group (s27, s30, s42, s18, s29 and s73, Figure 2.4). The same pattern applied to the two ketones (s12 and s36, Figure 2.4).

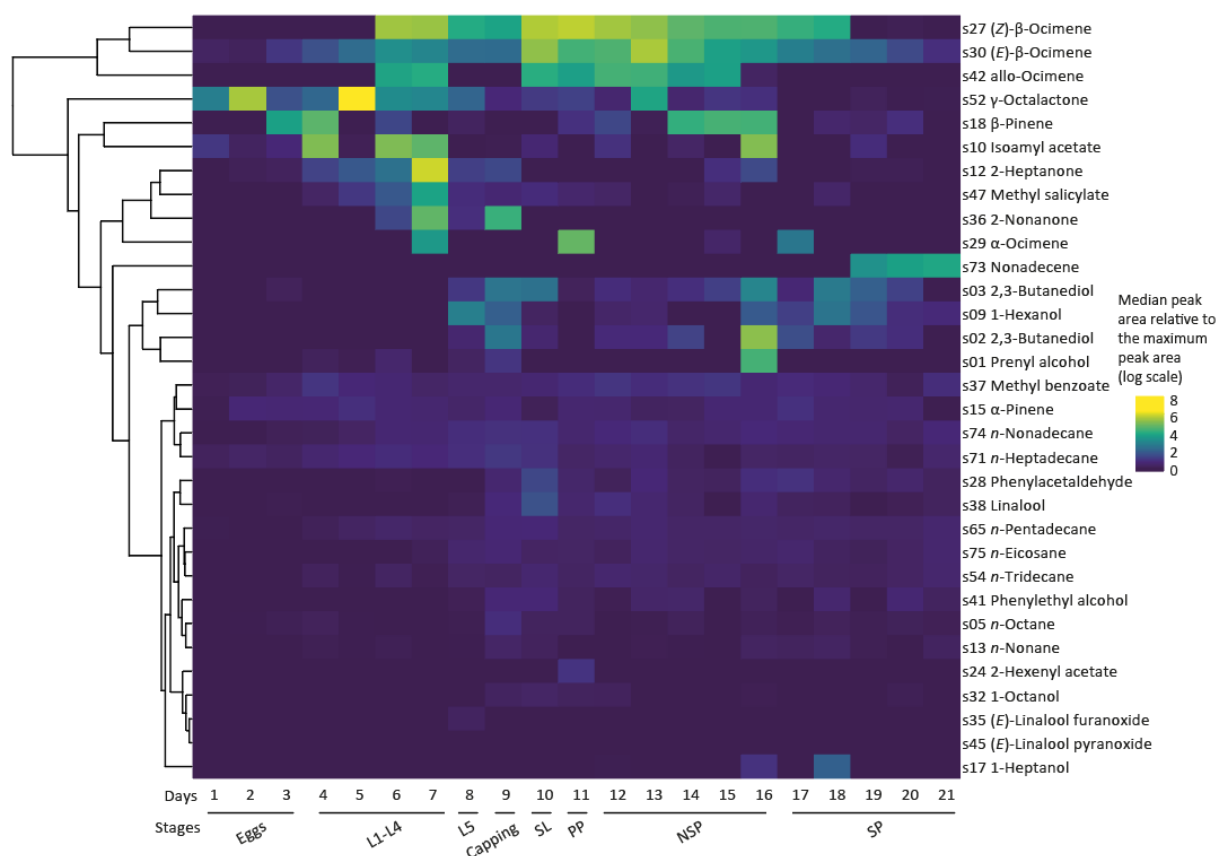


Figure 2.4: Heatmap showing the temporal evolution of the amount of the 32 compounds characterising brood throughout development. The temporal evolution of each compound area is represented in rows, with a colour gradient representing the median normalized peak area relative to the respective maximum for each compound (in log scale). The colour change represents a change in emission of the compound, with a maximum emission in yellow and a minimum in dark blue. Each column represents the age of brood development. Compounds are labelled with their IDs and chemical names. A dendrogram representing a hierarchical clustering (Euclidean distance, complete linkage) of compounds is indicated on the left.

The group of compounds with a more constant emission over time included 17 compounds (s37, s15, s74, s71, s28, s38, s65, s75, s54, s41, s05, s13, s24, s32, s35, s45 and s17) (Figure 2.4). The seven alkanes found in this study were in this group (s74, s71, s65, s75, s54, s05 and s13), as well as the aldehyde (s28) (Figure 2.4). For the alcohols and esters found in this study, their fluctuation over time depends on the compound. Four alcohols showed a marked change over time (s03, s09, s02 and s01), and six others displayed more constant emissions level (s38, s41, s32, s35, s45 and s17) (Figure 2.4). Two esters fluctuated strongly over time (s52, s10 and s47) and two others remained constant (s37 and s24) (Figure 2.4).

To study the compounds or chemical families that characterised each brood stage as well as those that covaried together, the 21 days of brood development have been divided into eight periods, as follow: Eggs: days 1-3; 1st to 4th instar larvae (L1-L4): days 4-7; 5th instar larvae (L5): day 8; Capping: day 9; Sealed larvae (SL): day 10, Prepupae (PP): day 11, Non-sclerotised pupae (NSP): days 12-16, Sclerotised pupae (SP): days 17-21. Different compounds were characteristic of the eight brood

developmental stages (Figure 2.5). None of the compounds had its maximum at the egg stage (days 1-3), but six compounds were found in greater quantities in brood containing eggs than in the control (s15, s18, s30, s37, s52 and s71) (Figure 2.5, Figure S2.1). None of these six compounds were specific to the eggs; their emission increased upon hatching. In the first larval instars (L1-L4, days 4-7), four compounds reached their maximum emission (s10, s47, s52 and s12), and two other compounds neared it (s15 and s37) (Figure 2.5). In the last larval stage (L5, day 8), the blend began to change drastically. Compounds with their greatest quantity in the L1-L4 stage decreased (s10, s47, s52, s12 and s37), while the quantity of other compounds began to increase (s09, s35, s74, s75, s71 and s54) (Figure 2.5). During the capping period (Capping, day 9), 11 compounds had their maximum emission peak (s15, s74, s75, s71, s36, s01, s05, s13, s03, s02 and s41) (Figure 2.5). All of these compounds decreased during the sealed larval stages (SL, day 10) (Figure 2.5). In this stage, seven compounds had their maximum emission peak (s65, s32, s38, s54, s28, s30 and s42) (Figure 2.5). In the prepupal stage (PP, day 11), only three compounds reached their maximum emission peaks (s27, s29 and s24) and two compounds were slightly under maximum emission levels (s15 and s37) (Figure 2.5). In the non-sclerotised pupal stage (NSP, days 12-16), two compounds had their maximum emission peaks (s37 and s18) and two others were slightly under maximum emission levels (s15 and s42) (Figure 5). In the last stage of brood development (SP, days 17-21), only one compound was at its maximum (s73) (Figure 2.5).

The total amount of (*E*)- β -ocimene (s30) was higher during capped brood stages (SL, PP, NSP and SP) than in larvae, especially just after capping. When the amount of (*E*)- β -ocimene (s30) was reported per mg of individual weight, its quantity was found to be higher in larval brood stages than in capped brood stages and reached its maximum in first instar larvae (day 4) (Figure S2.3).

Depending on their chemical families, the compounds highlighted in this study did not have their maximum emission peaks at the same stages of brood development. Ketones had their maximum emission peak at L1-L4 for s12, and Capping stages for s36 (clusters 1 and 3, Figure 2.6). Except for s37, which had its maximum peak at the NSP stage, and s24, which had its maximum peak at PP stage, the esters had their maximum emission peaks at the L1-L4 stage (Figure 2.6). The alcohols peaked at the late larval stages (L5, Capping and SL) (clusters 2, 4 and 6, Figure 2.6). Alkanes had their peaks at the capping and just after (Capping and SL) (clusters 3 and 5, Figure 2.6). The aldehyde had its peak at the SL stage (cluster 6, Figure 2.6). Finally, except for s15 which had a double peak at L1-L4 and Capping (cluster 11), all of the terpenes had their maximum emission peaks after capping of the brood cells (clusters 6, 7, 8, 9, 10 and 11, Figure 2.6).

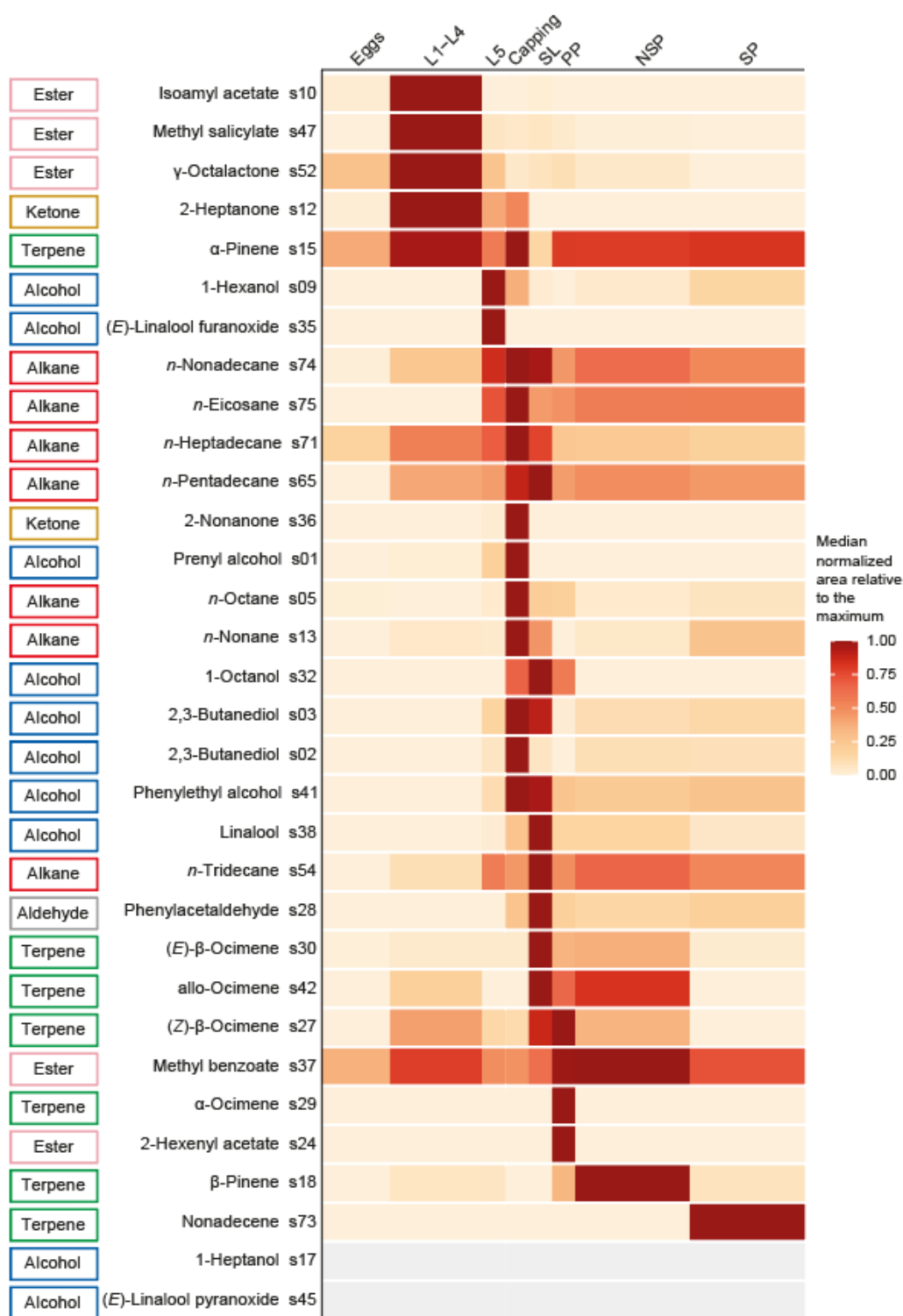


Figure 2.5: Heatmap showing the temporal evolution of the relative amount of the 32 brood characterising compounds, along developmental stages. The evolution of the quantity of each compound is represented in rows, with a colour gradient. The absence of compounds is represented in grey (here, both compounds are present only on a single day of the SP stage, resulting in a median of 0 for all stages). Each column represents a key stage of brood development (Eggs: day 1 to day 3; L1-L4: 1st to 4th instar larvae, day 4 to day 7; L5: 5th instar larvae, day 8; Capping: adult capping behaviour, day 9; LS: larvae spinning cocoon, day 10; PP: pre-pupae, day 11; NSP: non-sclerotised pupae, day 12 to day 16; SP: sclerotised pupae, day 17 to day 21). The width of each box is proportional to the duration of each stage. Compounds are labelled with their IDs, chemical names and chemical families.

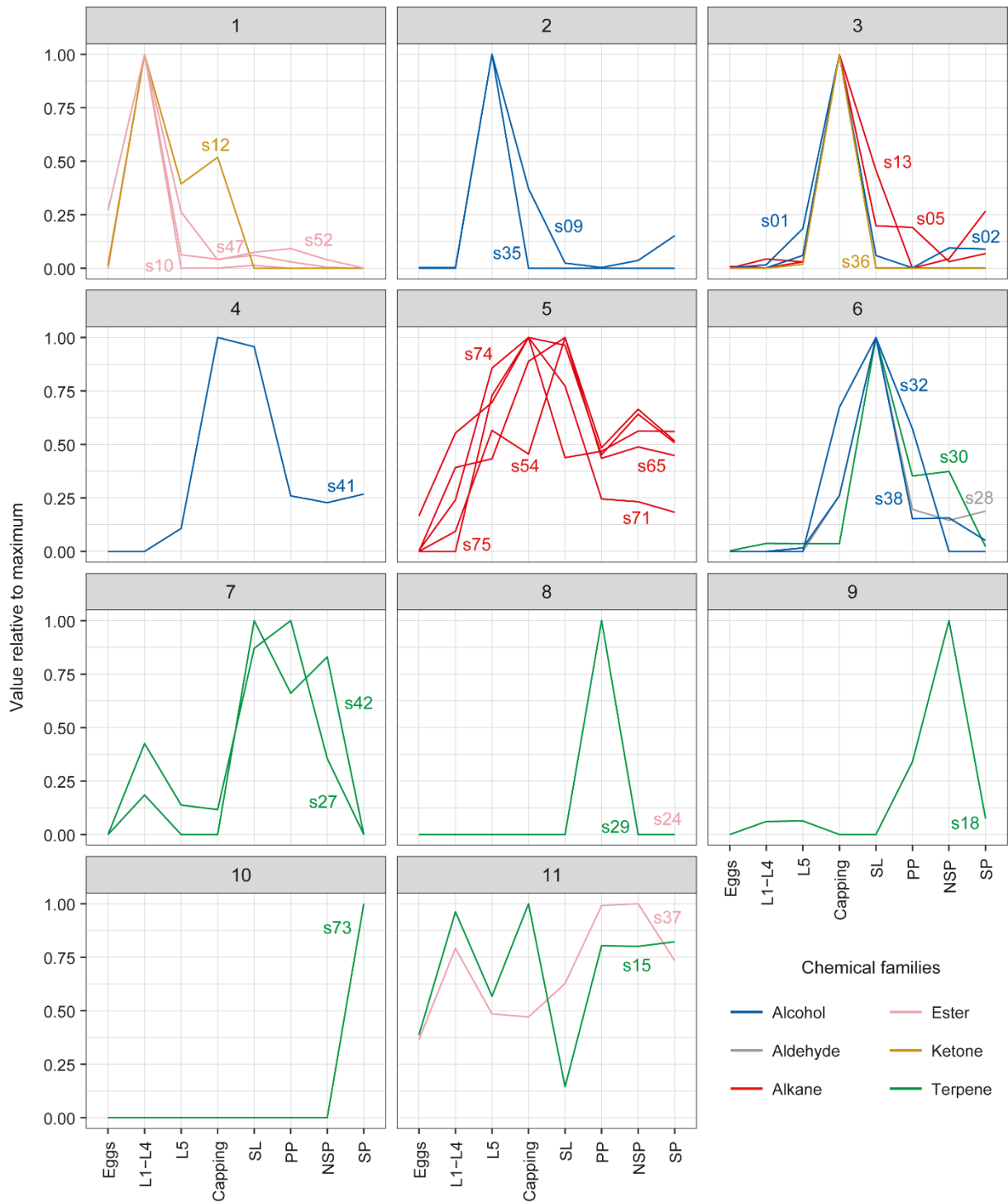


Figure 2.6: Clusters (Gaussian mixture models) showing the temporal variation of the relative (with respect to the maximum across stages) median normalized amount of the 32 brood-characterising compounds along developmental stages. Eggs: day 1 to day 3; L1-L4: 1st to 4th instar larvae, day 4 to day 7; L5: 5th instar larvae, day 8; Capping: adult capping behaviour, day 9, LS: larvae spinning cocoon, day 10, PP: pre-pupae, day 11, NSP: non-sclerotised pupae, day 12 to day 16; SP: sclerotised pupae, day 17 to day 21. Clusters group compounds with similarly timed emission peaks. Compounds are labelled with their IDs and coloured according to their chemical families.

4. Discussion

In this study, we analysed the VOC emitted by honey bee brood (*A. mellifera*) throughout its development. We identified 32 VOC characterising the brood, belonging to six different chemical families. The compounds did not have the same temporal dynamic during brood development and most of them appear to be stage-dependant. Chemical families were also specific to developmental stages.

Headspace capture is a common method for studying volatile emissions from bee brood (Liu *et al.*, 2022; Maisonnasse *et al.*, 2009; Maisonnasse *et al.*, 2010; McAfee *et al.*, 2018; Nazzi *et al.*, 2004a; Nazzi *et al.*, 2004b). The focus of our study is to describe the landscape of chemical emissions from bee brood so that it can serve as a base for future, more functional studies. Some compounds were not fully identified (e.g. s24 – 2-Hexenyl acetate for which its stereochemistry is not found or s73 – Nonadecene where the location of the double bond is still unknown) and would require further identification.

To date, this study presents for the first time the emission of VOC from honey bee brood during its whole development, from egg laying to emergence. Some previous studies showed the evolution of specific compounds during the larval or early pupal stages (Carroll & Duehl, 2012; Maisonnasse *et al.*, 2010) or the presence of compounds in larvae or pupae at specific ages (Haber *et al.*, 2019b; Light *et al.*, 2020b). A key point of our method was the monitoring of the same brood cells throughout development, with a non-invasive passive capture. No food resources (pollen, nectar, honey) were present on the brood frames, except for the larval jelly necessary for larval nutrition, which suggest that the compounds retained in this study are representative of the brood. Of the 32 compounds described in this study, 15 compounds are newly described in bees, with one compound never described either in bees or in the hive environment but has been described in floral emissions (Bergström *et al.*, 2018), and 14 of them already described as being present in the hive environment (wax, propolis, pollen, honey, ambient air) (Cellini *et al.*, 2019; Duru *et al.*, 2021; Isidorov *et al.*, 2012; Jánošková *et al.*, 2014; Najar *et al.*, 2021; Smith *et al.*, 2002). The other 17 compounds had already been described in bees (Butler *et al.*, 1959; Collins & Blum, 1983; Donzé *et al.*, 1998; Haber *et al.*, 2019b; Kathe *et al.*, 2021; Lee *et al.*, 2020; Light *et al.*, 2020b; Liu *et al.*, 2022; Maisonnasse *et al.*, 2009; Shearer & Boch, 1965; Wager & Breed, 2000; Wu *et al.*, 2019), with 13 already described has emitted by bee brood (Donzé *et al.*, 1998; Haber *et al.*, 2019b; Kathe *et al.*, 2021; Lee *et al.*, 2020; Light *et al.*, 2020b; Liu *et al.*, 2022; Maisonnasse *et al.*, 2009; Wu *et al.*, 2019).

4.1. Ocimene brood emission

(*E*)- β -ocimene (s30) was the compound that was captured in the greatest quantity throughout this experiment, four times higher than the other compounds. Thus, the total peak area of the full set of compounds largely reflected the dynamics of (*E*)- β -ocimene (s30) during brood development. (*E*)- β -ocimene (s30) was found every day during brood development, from the first day of the egg stage to the emergence of the adult bee. This compound has already been described in the scientific literature in the uncapped brood to early capped brood stages (Carroll & Duehl, 2012; Maisonnasse *et al.*, 2010). Its presence in the emission of VOC from eggs is described here for the first time. In our study, (*E*)- β -ocimene (s30) is more abundant in the capped stages than in the uncapped larval stages, with a peak in the SL stage. However, when the amount of compound is calculated according to the weight of the individuals, the peak of (*E*)- β -ocimene (s30) appears to be on day 4, at the first instar larvae. As a consequence, the amount of (*E*)- β -ocimene (s30) emitted by an individual is higher in the uncapped larval stages, especially in early larval stages, than in the capped brood stages. These results are in accordance with the results obtained by Maisonnasse *et al.* (2010), thus providing additional validation to our experimental set-up. The presence of this compound in stages other than uncapped and early-capped brood raises questions about its possible role in other stages of brood development and its involvement in chemical communication between the brood and the adults of the colony.

In addition to (*E*)- β -ocimene (s30), we identified three ocimene isomers: (*Z*)- β -ocimene (s27), α -ocimene (s29) and allo-ocimene (s42). Allo-ocimene (s42) was previously described as a putative pheromone emitted by young larvae, in a similar context to (*E*)- β -ocimene (Wu *et al.*, 2019). Here, we found the presence of allo-ocimene in more than five of our samples mainly in the 3rd and 4th instar larvae as well as from the SL stage to the end of the NSP stage. Similarly to (*E*)- β -ocimene, allo-ocimene triggers a rapid nervous response of the antenna (Wu *et al.*, 2019). In addition, the ocimene is processed by the antennal lobe (Locatelli *et al.*, 2016), the first brain region processing olfactory information sent by the antennal neurons (Sandoz, 2012). Neither of the two other ocimenes captured in this study have been described as part of brood pheromones. Nevertheless, the response of adults to (*E*)- β -ocimene and allo-ocimene, and the high similarity between these four ocimenes, suggest that nurses may detect (*Z*)- β -ocimene and α -ocimene. Like (*E*)- β -ocimene with the BEP, the emission of these three other ocimenes could have an additive or synergistic effect and trigger different behaviours in adults (Maisonnasse *et al.*, 2010; Traynor *et al.*, 2015). Further studies on behaviour and brain processing of olfactory information of nurse bees are necessary to highlight the potential discrimination between these four ocimenes, and the potential role of the (*Z*)- β -ocimene, the α -ocimene and the allo-ocimene.

4.2. Compounds known to transmit pheromonal information between adult bees

In our study, we found four compounds (1-hexanol (s09), isoamyl acetate (s10), 1-octanol (s32) and 2-heptanone (s12)) already described as part of pheromone alarm mixtures emitted and received by adult bees (Butler, 1966; Collins & Blum, 1983; Shearer & Boch, 1965; Wager & Breed, 2000). The alarm pheromone is composed of a cocktail of nearly twenty chemical compounds (Boch *et al.*, 1962; Slessor *et al.*, 2005; Wager & Breed, 2000).

The compound 2-heptanone is known to be synthesized by the mandibular glands (Butler, 1966; Shearer & Boch, 1965). During the larval stage, nurses feed the brood with royal jelly for the first stages and then with a mixture of pollen, honey and glandular extract from the mandibular and hypopharyngeal glands (Hartfelder & Engels, 1998; Peters *et al.*, 2010). The compound 2-heptanone has been described as a royal jelly VOC (Isidorov *et al.*, 2012) and from VOC of uncapped brood frames in the presence of adult workers (Carroll & Duehl, 2012). In our study, 2-heptanone (s12) was present in all uncapped larval stages, with a peak in 4th instar larvae (L1-L4, day 7). As the *in situ* capture of brood VOC did not allow us to separate brood emissions from potential emissions by larval food, our results suggest that the presence of 2-heptanone in our experiment during the early larval stages may be explained by the presence of the larval food in the brood cells. This would indicate that this compound is released from the larval food, even if we cannot rule out a possible emission by larvae.

The compound 1-hexanol (s09), isoamyl acetate (s10) and 1-octanol (s32) are synthesized in the Koschevnikov gland, close to the stinger (Collins & Blum, 1983; Wager & Breed, 2000), and are known to be part of the alarm pheromone of adult workers (Boch *et al.*, 1962; Slessor *et al.*, 2005; Wager & Breed, 2000). The main functions of the alarm pheromone for colony defence are increased flight activity, recruitment of individuals and behavioural responses to moving targets. Not all active chemical compounds trigger these three functions. The common function of isoamyl acetate, 1-hexanol and 1-octanol is their ability to recruit other adults (Wager & Breed, 2000). In our experiment, 1-hexanol (s09) had its emission peak at the 5th larval stage (L5, day 8) and remained abundant at the time of operculation (Capping, day 9) and at the last pupal stages (SP, days 17-18). The compound 1-octanol (s32) was captured mainly on days 8, 9 and 10 (L5, Capping, PP), with a peak at the time of operculation. Due to the synchronization between the emission of these compounds and the capping behaviour observed during the experiment, we hypothesise that 1-hexanol and 1-octanol emission by the brood may play a role in eliciting capping behaviour by workers. This hypothesis needs to be validated by behavioural assays, such as those already used to confirm the role of brood pheromone compounds as a capping signal (Le Conte *et al.*, 1990b).

The amount of isoamyl acetate in the Koschevnikov gland differs according to the age of the worker. Nurse bees displayed more isoamyl acetate in their gland than guards or foragers, suggesting another function in a context other than the defence of the colony (Camargos *et al.*, 2020). This compound had already been reported as part of VOC of uncapped brood frames in the presence of adult workers (Carroll & Duehl, 2012). In our study, we captured isoamyl acetate (s10) mainly during the larval stages (L1-L4). This suggests that broods may emit isoamyl acetate as a chemical signal for care to the nurse bees. This signal can have various meanings such as a signal that the brood is healthy, a call for feeding, for temperature or humidity control. Behavioural assays are needed to validate this hypothesis.

4.3. Compounds emitted by the brood but not described as pheromones

In this study, we found 32 compounds characterising the brood belonging to six chemical families, including ten alcohols (prenyl alcohol (s01), 2,3-butanediol (s02), 2,3-butanediol (s03), 1-hexanol (s09), 1-heptanol (s17), 1-octanol (s32), (*E*)-linalool furanoxide (s35), linalool (s38), phenylethyl alcohol (s41), (*E*)-linalool pyranoxide (s45)), one aldehyde (phenylacetaldehyde (s28)), seven alkanes (*n*-octane (s05), *n*-nonane (s13), *n*-tridecane (s54), *n*-pentadecane (s65), *n*-heptadecane (s71), *n*-nonadecane (s74), *n*-eicosane (s75)), five esters (isoamyl acetate (s10), hexenyl acetate (s24), methyl benzoate (s37), methyl salicylate (s47), γ -octalactone (s52)), two ketones (2-heptanone (s12), 2-nonanone (s36)) and seven terpenes (α -pinene (s15), β -pinene (s18), (*Z*)- β -ocimene (s27), α -ocimene (s29), (*E*)- β -ocimene (s30), allo-ocimene (s42), nonadecene (s73)). As previously mentioned, only five compounds have been described as pheromonal compounds in bees. Within the remaining 27 compounds, 13 have already been described in brood, either as being emitted by the brood (*n*-octane (s05), *n*-nonane (s13), α -pinene (s15), (*E*)-linalool furanoxide (s35), 2-nonanone (s36), linalool (s38), methyl benzoate (s37), allo-ocimene (s42), methyl salicylate (s47), γ -octalactone (s52), *n*-tridecane (s54), *n*-heptadecane (s71), *n*-nonadecane (s74), *n*-eicosane (s75)) (Carroll & Duehl, 2012; Donzé *et al.*, 1998; Kathe *et al.*, 2021; Lee *et al.*, 2020; Light *et al.*, 2020b; Liu *et al.*, 2022; Wu *et al.*, 2019), or by dead brood extracts (*n*-octane (s05), *n*-nonane (s13), *n*-eicosane (s75) *n*-tridecane (s54)) (Haber *et al.*, 2019b). Our results are in accordance with these studies. The 14 other compounds found to characterize the brood (prenyl alcohol (s01), 2,3-butanediol (s02), 2,3-butanediol (s03), 1-heptanol (s17), β -pinene (s18), hexenyl acetate (s24), (*Z*)- β -ocimene (s27), phenylacetaldehyde (s28), α -ocimene (s29), phenylethyl alcohol (s41), allo-ocimene (s42), (*E*)-linalool pyranoxide (s45), *n*-pentadecane (s65), nonadecene (s73)) have already been described as being present in the VOC of hive products (honey, propolis, larval jelly, pollen) or floral extracts (Duru *et al.*, 2021; Isidorov *et al.*, 2012; Jánošková *et al.*, 2014; Lee *et al.*, 2015; Najar *et al.*, 2021; Starowicz *et al.*, 2021; Wu *et al.*, 2019), but we described them in honey bee brood for the first time. Nevertheless, α -pinene (s15) and β -pinene (s18) have been described as part of the odours of

termite guardians (Quintana *et al.*, 2003) or as being present in Dufour's gland of ants (Morgan, 2008). The presence of VOC previously measured in hive products in brood emissions is not surprising as hive products are involved in nutrition (honey, larval jelly and pollen) and protection (honey, propolis, larval jelly) against various pathogenic agents of all the individuals in the colony (Borba & Spivak, 2017; Di Pasquale *et al.*, 2013; Haydak, 1970; Klaudiny *et al.*, 2012; Kwakman *et al.*, 2010; Silici & Kutluca, 2005). These chemical compounds could be brought to the uncapped brood cells by nurses when they visit brood cells during caring behaviours. Then, compounds can be trapped in and emitted from the wax of brood cells, or released at specific times by the brood as a means of communication with adults.

4.4. Egg compounds

We found that eggs emitted a blend of five VOC: α -pinene (s15), β -pinene (s18), (*E*)- β -ocimene (s30), methyl benzoate (s37) and γ -octalactone (s52). Even if all of these compounds are maximally abundant at stages of brood development other than the egg stage, this study is the first to describe these 5 compounds as characteristic of eggs. The compound β -ocimene (s30) and methyl benzoate (s37) have already been found on VOC from frames containing eggs and one-day-old larvae (Carroll & Duehl, 2012). Here, we confirm the presence of (*E*)- β -ocimene and methyl benzoate in egg brood cells and suggest that (*E*)- β -ocimene and methyl benzoate take part in the egg odour blend. *n*-Heptadecane (s71) had already been found in VOC from frames containing fourth and fifth instar larvae (Light *et al.*, 2020b). We found *n*-heptadecane on the first day of eggs. Here, we suggest that *n*-heptadecane takes part in the newly laid egg odour blend. α -pinene (s15) and β -pinene (s18) can be found in the VOC hive atmosphere (Smith *et al.*, 2002). α -pinene has also been found in VOC of larvae and pupae (Light *et al.*, 2020b), and in VOC from brood frame with adult workers (Carroll & Duehl, 2012). β -pinene has been found in VOC of honey (Duru *et al.*, 2021). In our study, frames were empty of bees and captures were done outside the hive. These two compounds were higher in brood samples than in control wax samples, thus suggesting that they could be part of the blend of VOC emitted by eggs or by cells polished by the workers for the queen to lay eggs.

Beginning our captures on the first day of the egg stage allowed us to discover that honey bee eggs and healthy larvae emit γ -octalactone (s52). γ -octalactone has been described in *Apis dorsata* as an alarm pheromone (Li *et al.*, 2014), and was recently described for the first time in *A. mellifera* larvae (Kathe *et al.*, 2021). The authors found that the amount of γ -octalactone increased with the infection by European foulbrood (*M. plutonius*). This compound has also been described as stimulating oviposition in flies (Kempraj *et al.*, 2019; Pagadala Damodaram *et al.*, 2014), and as being present in the metapleural glands of ants (Morgan, 2008), glands that allow for the marking of the nest (Cammaerts & Cammaerts, 2001). Both of these observations corroborate our observation of γ -octalactone in eggs,

and suggest that it may also be involved, along the other four compounds, in egg marking by the queen or polished cell marking by the workers. A comparison between queen and worker eggs would be interesting to solve this question.

4.5. Description of brood VOC emissions at key moments of development

Chemical compounds described in this study had their maximum emission at different times of brood development. Nevertheless, most of these compounds had their peaks during the capping and capped larval stage of the brood (day 9 to 10: Capping, SL).

Larvae have a constant need to communicate with workers in order to be fed. The modulation of the pheromone signal of larvae during their development allows an adaptation of the feeding behaviour of the worker bees (Le Conte *et al.*, 1990b; Maisonnasse *et al.*, 2010; Sagili & Pankiw, 2009). Here, four compounds had their maximum emission at the L1-L4 stage (isoamyl acetate (s10), 2-heptanone (s12), γ -octalactone (s52) and methyl salicylate (s47)) and two at the L5 stage (1-hexanol (s09) and (*E*)-linalool furanoxide (s35)). The modulation of VOC emitted between the L1-L4 stage and the L5 stage could allow for modulation of worker feeding behaviour, but perhaps more importantly signal the need for capping preparation.

The capping of brood cells is a key moment in brood development. Workers close the brood cell around the 9th day after egg laying. The brood is no longer fed by workers, but the larvae feed on the jelly left on the bottom of the cell and then start to weave their cocoon and prepare for metamorphosis (Winston, 1987). It has already been shown that the BEP, and particularly methyl esters present in this pheromone, trigger this capping behaviour (Le Conte *et al.*, 1990b). Here, we found 11 compounds at their maximum at the time of brood capping (day 9) (prenyl alcohol (s1), 2,3-butanediol (s02 and s03), *n*-octane (s05), *n*-nonane (s13), α -pinene (s15), 2-nonanone (s36), phenylethyl alcohol (s41), *n*-heptadecane (s71), *n*-nonadecane (s74), *n*-eicosane (s75)). A complex mixture of VOC and BEP could trigger the capping of brood cells by workers. More research is necessary to determine if, and which of, these compounds alone trigger capping behaviour. The complexity of the mixture captured at this key day in brood development opens up new perspectives in understanding chemical communication between brood and adults.

After capping, the larva stretches out and starts weaving its cocoon (day 11, SL) (Winston, 1987). At this time, we found seven compounds at their maximum emission (phenylacetaldehyde (s28), (*E*)- β -ocimene (s30), 1-octanol (s32), linalool (s38), allo-ocimene (s42), *n*-tridecane (s54) and *n*-pentadecane (s65)). Then the larva starts its metamorphosis (day 12, PP) (Winston, 1987); we found three compounds at their maximum release on this day (hexenyl acetate (s24), (*Z*)- β -ocimene (s27), α -ocimene (s29)).

Communicating at the beginning of metamorphosis could allow the workers to monitor the health of the brood. A modification of those compounds emitted by the brood just after capping, for example because of *Varroa* or *Nosema* parasitism, may trigger the cleaning behaviour by the adults (Kathe *et al.*, 2021; Nazzi *et al.*, 2004a; Spivak & Gilliam, 1998).

The pupal stages were characterised by the peaking of two terpene compounds, one during NSP, and one during SP. β -pinene (s18) had its maximum emission in the early pupal stages (NSP), mainly on days 14 to 16. Nonadecene (s73) is the only compound that had its peak of emission during the last stage of brood development (SP), especially during the last 3 days before adult emergence. At the end of metamorphosis, the brood is not fed and not subject to hygienic behaviour (Winston, 1987). Here, we described for the first time an emission of VOC from the brood before the emergence of the adult bee. When it could just be part of the chemical process of development and given off as a by-product, it also suggests a potential emergence signal, with a possible role in the acceptance of newly emerged workers by adults.

Interestingly, the emission of chemical families correlates with the age of the brood. Uncapped broods emitted mainly ketones, esters and alcohols, while capped broods emitted alkanes and terpenes. The emission of volatile esters (isoamyl acetate (s10), methyl benzoate (s37), methyl salicylate (s47), γ -octalactone (s52)) in larval stages is consistent with the emission of BEP from the brood at these stages (Le Conte *et al.*, 1990b). These compounds could be complementary to the BEP. In addition, the brood emitted several highly volatile alkanes. Adults recognise their kin with cuticular hydrocarbon mixtures of low volatile alkanes and alkenes (Blomquist & Bagnères, 2010; Dani *et al.*, 2005). It has been shown that long carbon chain alkanes are differentially recognised by adult honey bees (Châline *et al.*, 2005), and it would be interesting to see if short carbon chain alkanes are also involved in nestmate recognition.

This study is the first study of VOC emissions of brood covering the entire honey bee developmental cycle, from egg laying to adult emergence. Here, we show the complexity of the volatile chemical messages emitted by the brood at different developmental stages. When Martin *et al.* (Martin *et al.*, 2004) suggested low volatile compound as egg marking signal, we describe the olfactory signature made of volatile compounds of *A. mellifera* eggs that could also have a potential implication in the marking of queen eggs. We also show a potential emergence signal. While the compounds identified could have a potential involvement in the chemical recognition of brood and its needs by the workers, some of the compounds identified could also be the result of metabolic by-products. Our suggestions need to be assessed using appropriate biological tests. This study opens up new perspectives on the

intricate relationship between brood and adult bees, and the behaviour of nurse bees towards the brood. Brood emission of molecules already described as part of chemical communication in the adult reinforces the idea that chemical communication in the bee is highly context-dependent and based on the quantity of each molecule emitted.

5. Acknowledgments

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III. Conclusion

Dans ce chapitre, l'étude des émissions de COVs du couvain sain a été réalisée en capturant les molécules émises par le couvain en période de faible infestation par varroa et dans son milieu naturel (dans les cadres de couvain). L'article 1 illustre la dynamique des émissions de chaque composé identifié, tout au long du développement du couvain. Cette étude descriptive est la première à détailler les émissions de COVs du couvain du premier jour de l'œuf à l'émergence de l'adulte, et elle met en avant la grande diversité de COVs émis par les alvéoles de couvain.

La découverte de molécules déjà connues comme faisant partie du bouquet phéromonal des adultes appuie le fait que la communication chimique chez l'abeille peut être extrêmement dépendante du contexte et de la dose de chaque composé. La présence d'un composé ne suffit pas forcément à émettre un message précis. Sa fluctuation et la présence d'autres composés dans le message olfactif peuvent être importantes. L'émission d'un composé dans un contexte inadéquate et/ou en mauvaise proportion dans un mélange odorant peut déclencher des comportements spécifiques chez l'adulte, comme le comportement hygiénique. Ce travail sert de base au chapitre suivant, qui a pour objectif de mettre en lumière les COVs impliqués dans la détection des alvéoles de couvain parasitées par varroa.

CHAPITRE 3

CARACTERISATION DES EMISSIONS EN COMPOSES ORGANIQUES VOLATILES
DU COUVAIN PARASITE PAR *V. DESTRUCTOR*



L'odeur du couvain

I. Avant-propos

La colonie d'abeilles combat les pathogènes et les parasites du couvain grâce à un système d'immunité sociale (Cremer *et al.*, 2007). Plusieurs comportements sont directement liés à l'immunité sociale de la colonie, l'un des comportements clef est le comportement hygiénique (Evans & Spivak, 2010; Wilson-Rich *et al.*, 2009). Le comportement hygiénique implique la détection des alvéoles de couvain malade ou parasité par les ouvrières qui, par la suite, les nettoient de leur contenu (Arathi *et al.*, 2000). Dans le cas particulier du parasitisme à varroa, le comportement hygiénique, appelé comportement VSH, est directement lié à la résistance des colonies d'abeilles envers le parasite (Spivak, 1996; Spivak & Gilliam, 1998). Une variante du comportement VSH est le comportement de réoperculation. Une fois que l'alvéole de couvain parasité par varroa est identifiée, les ouvrières la désoperculent puis la réoperculent sans toucher à son contenu, ce qui suffit à perturber la reproduction du varroa (Oddie *et al.*, 2018; Oddie *et al.*, 2021). Ces deux comportements contribuent à réduire la pression du parasite sur la colonie (Mondet *et al.*, 2020a).

L'étape primordiale au comportement hygiénique, comportement VSH et comportement de réoperculation est la détection des alvéoles de couvain malade ou parasité. Plusieurs molécules ont été identifiées comme impliquées dans le déclenchement le comportement hygiénique et VSH. Le (*E*)- β -ocimène couplé à l'acide oléique est un signal de couvain mort (McAfee *et al.*, 2018), les six VPS, le (*Z*)-6-pentadecene, le (*Z*)-10-tritriacontene, l' α -pinène et l'hexanoate d'éthyle déclenchent le comportement de nettoyage des alvéoles de couvain parasitées par le varroa (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazi *et al.*, 2004a; Wagoner *et al.*, 2020). Contrairement au comportement VSH, aucun composé n'a été identifié comme déclenchant le comportement de réoperculation. Diverses molécules ont aussi été décrites comme étant émises en plus grande quantité lorsque le couvain est parasité, infecté par des virus, stressé ou malade. Néanmoins, la plupart de ces molécules sont peu volatiles. L'émission de COVs permettrait de signaler rapidement l'état du couvain au sein du nid et, de fait, agirait comme un signal primaire dans la détection des alvéoles de couvain malade ou parasité.

L'étude réalisée dans ce chapitre a pour but d'étudier les émissions de COVs caractéristiques du couvain parasité. Les comportements VSH et de réoperculation des ouvrières ont été observés vis-à-vis de ces composés.

II. Article 2

Characterisation of volatile organic compounds involved in the detection of varroa-infested brood cells by worker honey bees

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Abstract

Varroa destructor is well known as a worldwide threat for honey bee colonies (*Apis mellifera*). A colony infested with the mite can collapse within a few months. Varroa infestation increase with an exponential tendency along the beekeeping season, and without treatment colony survival is compromised in most cases. However, some honey bee colonies have developed resistance to the parasite and are able to control the mite infestation rate. To achieve this, they display specific behaviours, such as VSH (*Varroa Sensitive Hygiene*), a form of hygienic behaviour that is directed towards varroa-infested brood cells. Bees performing this behaviour detect parasitized brood cells, uncap and clean their contents, thus disrupting mite reproduction. Several semiochemicals have been shown to be released from varroa-infested brood cells targeted by VSH behaviour and to trigger this behaviour. These chemicals harbour long carbon chains and a low volatility. In this study, we focus on the study of volatile organic compound (VOC) emissions from these same cells, as such highly volatile semiochemicals could allow the information about parasitism to be disseminated on a large scale in the nest. Under this hypothesis, compounds with lower volatility would complement VOC role by helping refine the specific detection of parasitized cells. We compared VOC emissions by *Varroa destructor* parasitized versus non-parasitized brood cells in a non-targeted approach, using solid phase microextraction (SPME) methods and gas chromatography coupled with mass spectrometry (GC-MS). We identified several VOCs that characterise the health status of parasitized and non-parasitized brood cells. These candidate VOCs were then tested in the field to characterise their hygienic behaviour triggering ability in workers. These findings increase our understanding of how honey bees fight brood diseases such as varroosis with hygienic behaviour.

Keywords: *Varroa destructor*, chemical ecology, Volatile organic compounds, VSH, recapping.

1. Introduction

The negative impact of *Varroa destructor* on *Apis mellifera* colonies is well known. Its worldwide prevalence, with its very recent arrival in Australia, places it among the main threats to beekeeping (Nazzi *et al.*, 2012). Control of the varroa population in the honey bee colony is mainly conducted using chemical control, but acaricides, whether synthetic or biological, can have a detrimental effect on bees (Noël *et al.*, 2020). *V. destructor* is becoming increasingly resistant to synthetic acaricides, making its control ever more complex (González-Cabrera *et al.*, 2018; Martin, 2004; Mitton *et al.*, 2022). One promising option for mite control is the use of varroa-tolerant or varroa-resistant honey bee populations. Resistance is the ability of a host to reduce the growth of the parasite population, while tolerance is the ability to limit the damage done to the host without reducing the parasite population (Schneider & Ayres, 2008). Such resistant honey bee colonies are able to survive with a basal level of varroa mites into the colony, thus reducing the need to use of acaricides. Resistance to varroa can be explained by several traits, one of the most prominent being Varroa Sensitive Hygiene (VSH) behaviour (Mondet *et al.*, 2020a). VSH is a hygienic behaviour specifically directed towards parasitized brood cells (Harbo & Harris, 2005). VSH consists into the detection, uncapping and cleaning of infested brood cells by worker bees (Harbo & Harris, 2005). A variant of VSH behaviour is the recapping behaviour, where bees detect, uncap and recap the infested brood cell without touching its content (Aumeier *et al.*, 2000; Boecking & Spivak, 1999; Corrêa-Marques & De Jong, 1998). This recapping of the parasitized brood cell could be sufficient to disrupt varroa reproduction (Oddie *et al.*, 2019a).

The first step in these behaviours relies on the ability of worker bee to specifically detect varroa-infested brood cells. It has been shown that this detection is mainly based on olfactory cues (Mondet *et al.*, 2015; Nazzi & Le Conte, 2016). Ten compounds have been described as triggering VSH behaviour; (*Z*)-6-pentadecene, (*Z*)-10-tritriacontene, 6 varroa-parasitization-specific (VPS), α -pinene and ethyl hexanoate (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Wagoner *et al.*, 2019). In contrast to VSH behaviour, no compounds have yet been shown to trigger recapping behaviour. To date, it is not known whether specific signals trigger VSH and recapping behaviour, or whether a common signal triggers the uncapping followed by a specific signal triggering cleaning or recapping of the brood cell. Most of the compounds identified as VSH triggers are not very volatile and only α -pinene and ethyl hexanoate can be considered as volatile organic compounds (VOCs). It can be assumed that compounds of different volatility, both low and high volatility, are highly involved in VSH and recapping behaviours. The VOCs could spread rapidly through the cell to signal parasitism and the less volatile compounds could allow the precise identification of the parasitized brood cell. This seems to be the case with the hygienic behaviour against dead brood, being triggered by the oleic acid and the (*Z*)- β -ocimene (McAfee *et al.*, 2018). The recent identification of two VOCs from varroa-parasitized brood cells, after the

identification of low-volatile compounds, supports a similar signalling-pathway. Another hypothesis would be that VOCs trigger the uncapping behaviour, the presence of low-volatile compounds the cleaning behaviour, and their absence the recapping behaviour (Martin *et al.*, 2020). Regardless of these two hypotheses, VOCs are highly likely to play a central role in the triggering of one of several steps of hygienic behaviours towards varroa infested brood cells.

The aim of this study is to improve our knowledge of the function of VOCs in VSH and recapping behaviour triggering. We have identified five VOCs characteristic of parasitized brood that trigger hygienic behaviour. The blend of these five VOCs triggers also recapping behaviour.

2. Materials and methods

2.1. *In vitro* rearing of *Apis mellifera* brood

This experiment was conducted using ten colonies present at the INRAE research centre of Avignon, France. Uncapped larvae in their last larval stage (L5) were passively placed in food-grade gelatine capsules (\emptyset 6.1 mm, LGA, Laboratoire des Gélules et des Azymes, France)). To do so, the capsules were pre-pierced with nine holes at both ends and 27 holes along the body with a sewing pin to favour gas exchange. The bodies of the open capsules were placed on the brood cell to allow the larvae to move into the capsules on their own. Brood frames were then placed horizontally so that the larvae descend by themselves into the capsule bodies. After three hours, the capsules were collected, and closed with the capsule head. For each colony, one Non-Parasitized (NP) and one Parasitized (P) group was made, composed of between 20 and 30 capsules each. For the P group, each capsule was infested with a varroa mite. *Varroa* came from three donor colonies and were collected using the icing sugar method (Dietemann *et al.*, 2013). The capsules were placed horizontally in an incubator in order to mimic the orientation of the cells on a brood frame in the hive. After 12h of rearing (D1), because of the shrinkage of the capsules due to the ambient humidity, the holes in the capsules were gently punched with a sewing needle so that the volatiles could escape the cells, taking care not to injure the individual(s) inside (larvae or larvae with varroa). The brood were reared in capsule for 8 days at 34°C and 75% relative humidity. On day 8, the capsules were checked for brood stage.

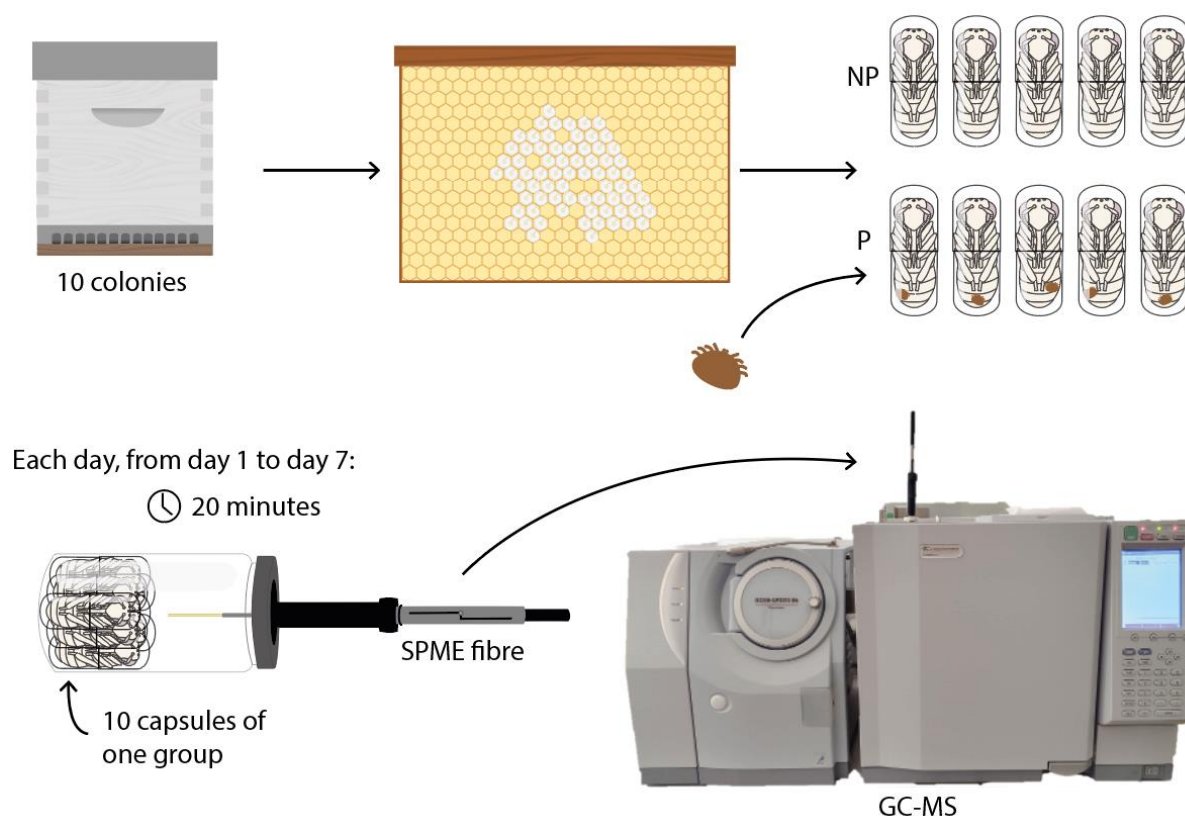


Figure 3.1: Experimental setup used for the capture of Volatiles Organics Compounds (VOCs) on Varroa parasitized (P) and non-parasitized (NP) honey bee brood. Brood was reared in gelatine capsules from day 1 after capping to day 7 in an incubator (34°C, 65% RH). Every day, during seven days, VOCs of ten capsules of P and NP group were captured during 20 minutes with a SPME fibre, in an incubator (34°C, 65% RH). After capture, fibres were desorbed on a GC-MS. Ten colonies were used in each P and NP groups.

2.2. VOCs capture and analysis

For the VOCs captures, 10 capsules per colony for each group (P and NP) were placed in an open glass vial (10 mL). VOCs were captured every 24 hours. At the moment of the capture, the vial containing the 10 individuals was sealed with a Teflon septum cap. The septum was pierced in order to insert a PDMS-DVB (polydimethylsiloxane divinylbenzene) SPME (Solid Phase Micro Extraction) fibre (65 µm, manual holder). For each capture, the vial and fibre were held horizontally and kept in an incubator (at 34°C, 75% RH). Captures lasted for 20 minutes, after which the vial containing individuals was re-open. Each NP group capture was coupled with the P group capture from the same colony. Captures were carried out from day 1 to day 7 (D1 to D7) after capping, thus forming a total of 140 captures (10 for each group per day). Captures on empty capsules were made in the same way every day. Qualitative and quantitative analyses of VOCs were performed by gas-chromatography coupled with mass spectrometry (GC-MS) following the protocol of Noël *et al.* (2023). In brief, desorption was realised in splitless mode, with the constant column flow at 0.82 mL/min; the carrier gas was helium. The injector temperature was set at 250 °C and the oven temperature program as follows: 45 °C isothermal held for

5 min, followed by temperature increases at a rate of 10 °C/min up to 250 °C. The oven was finally held at 250 °C for 4 min. The ion source was set at 200 °C. Identification of compounds of interest was performed using the NIST library (v.2.3) and confirmed by injection of standards purchased from Sigma Aldrich. Five out of the nine compounds of interests were successfully identified.

2.3. Solution preparations

Solutions containing the identified compounds alone and a solution containing all compounds together were prepared. For the single-compound solutions, the compound was diluted in isohexane to a concentration of 10% w/w to test a dose high enough avoiding a lack of response which could be due to a too low concentration. For the mixture, each of compound were at 10% w/w in the final solution containing the five compounds and the solvent. As a negative controls, two solutions containing compounds with two carbons less than two of the compounds of interest found, but being of the same chemical family, were used at 10% w/w.

2.4. Behavioural assays

Behavioural assays were conducted on 11 honey bee colonies, during July to August 2022 at the INRAE research centre of Avignon, France. For each behavioural assay, 1µL of VOC(s) solution was injected into a brood cell at the white to pink eye stage using a Hamilton syringe. On each brood frame, 15 cells per treatment were injected and their position marked on a transparent plastic sheet. The brood cell opening and cleaning were checked at 6h, 24h and 48h after injection. At 48h, the cap of the remaining cells was checked to score the recapping behaviour.

2.5. Statistical analyses

Statistical analyses were performed using R (v4.1.3). For VOC captures, compounds detected less than six times on the ten replicates in each sample for each day were removed from the analysis. Day 1 was really different from the other days (early pre-pupae, capsules that shrunk, totally different VOC emissions), so it was taken out of the rest of the analyses. To identify compounds characterising NP and P samples, a redundancy analysis (RDA) was performed on day 2 to day 7, with days and sample type (NP or P) and their interaction included as fixed factors. ANOVA were performed for each compound on day 2 to day 7, with days and sample type and their interaction included as fixed factors. Pairwise comparisons were performed for each compound on day and sample variables.

To study the response of workers to the solution injected into the brood cells, the data were analysed using a generalised linear mixed model, with the proportion of brood cells cleaned as a function of treatment (fixed factor) and colonies (random factor). To study the recapping behaviour triggered by the solutions, the data were analysed using a generalised linear mixed model, with the proportion of brood cells recapped in comparison to the remaining cells as a function of treatment (fixed factor) and colonies (random factor). (R packages 'lme4: Linear Mixed-Effects Models using Eigen' and 'S4': <https://cran.r-project.org/web/packages/lme4/index.html>, and 'car: Companion to Applied Regression': <https://cran.r-project.org/web/packages/car/index.html>).

3. Results

3.1. Identification of varroa-parasitized characterising compounds

To identify volatile organic compounds (VOCs) potentially involved in VSH and recapping behaviours, the emission profiles of *Varroa destructor* parasitized brood (P) reared in capsules were compared with those of non-parasitized brood (NP). A total of 111 compounds were detected in the GC-MS profiles. None of these compounds was specific (presence/absence) to a sample group (P or NP). Comparison of total peak areas over time between the P and NP groups shows no difference in total abundance (GAMM; NP: estimate = 2,911,042, P: estimate = 2,385,984, $p = 9.49e^{-1}$). The fluctuation of the two groups over time is also not significant (GAMM; NP over time: $F = 0.379$, $p = 0.615$; P over time: $F = 0.051$, $p = 0.852$). A RDA was performed to identify the most characteristic compounds of the parasitized group, in order to perform behavioural tests in which only a limited number of compounds can be tested. The first three components of the RDA explained 84.89% of the variance. Day, sample group and their interaction were significant (RDA; Day: $F = 5.93$, $p = 0.001$; Sample group: $F = 12.07$, $p = 0.001$; Day:Sample group: $F = 1.40$, $p = 0.026$). The two sample groups (P and NP) were separated on the component 2. To select the most characteristic compounds of group P, and in order to perform behavioural tests, we considered the results of the RDA, significant results of the ANOVA and at least 3 significant days in the pairwise comparisons (Figure 3.2, Table 3.1., Figure 3.3). We selected nine parasitized characterising compounds (s43, s48, s54, s60, s67, s76, s81, s85, s86) (Figure 3.3). The nine compounds were emitted in significantly greater quantities in the P group than in the NP group each day, except for compounds s43, s81 and s86 on the 2nd, 3rd and 4th days of rearing, and for compounds s54 and s60 on the 4th day of rearing (Figure 3.3). Of the nine compounds, five were successfully identified (octanoic acid (s43), benzothiazole (s48), *n*-tetradecane (s67), dodecanoic acid (s81), *n*-hexadecane (s85)).

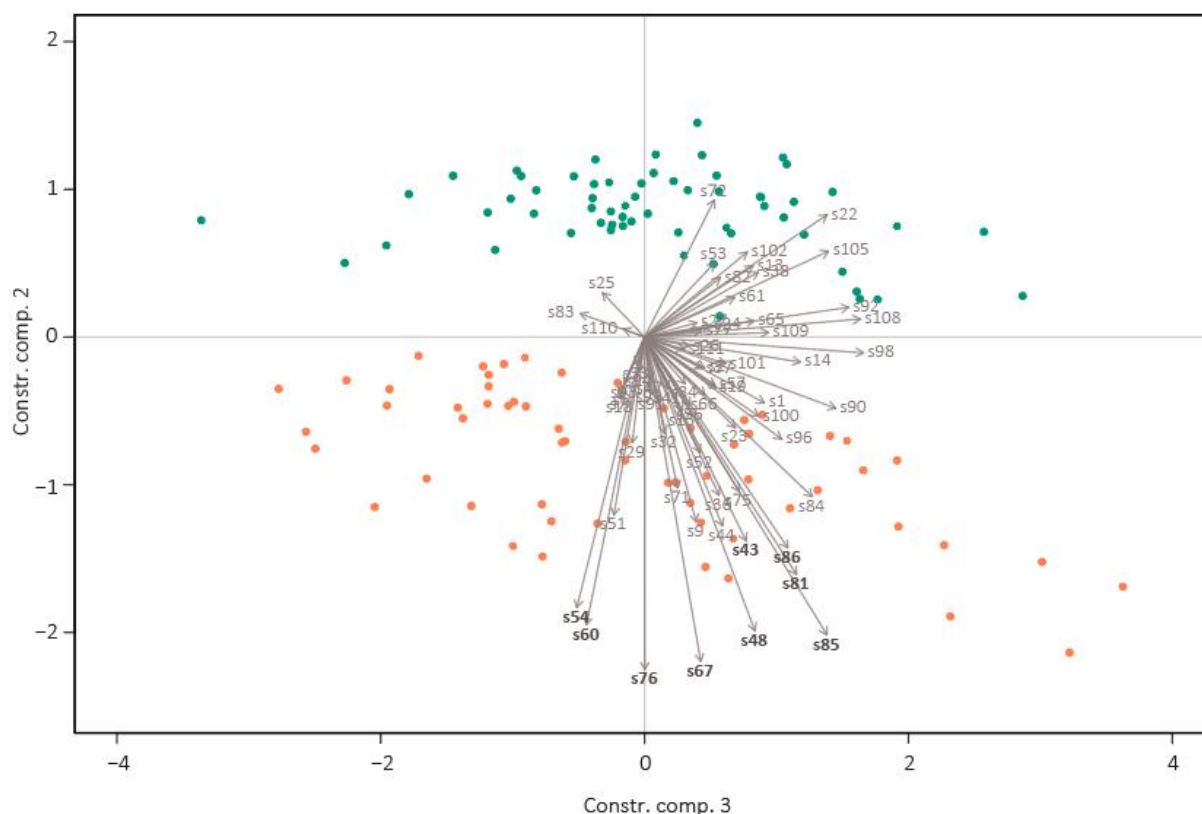


Figure 3.2: Redundancy Analysis (RDA) performed on chemical compounds captured on parasitized (P) and non-parasitized (NP) brood reared in capsule, day 2 to day 7. For each day: (P) n = 10; (NP) n = 10. Each dot represents a group sample (P: orange, NP: green). Compounds are represented by their IDs and grey arrows. The compounds characterising the P group and retained for the rest of the analysis are shown in bold. s43: octanoic acid, s48: benzothiazole, s54: unidentified, s60: unidentified, s67: *n*-tetradecane, s81: dodecanoic acid, s85: *n*-hexadecane, s46: unidentified.

Table 3.1: Results of the Anova showing the effects of Sample groups on compounds area of the six compounds of interest. For each group sample: n = 10. s43: octanoic acid, s48: benzothiazole, s54: unidentified, s60: unidentified, s67: *n*-tetradecane, s81: dodecanoic acid, s85: *n*-hexadecane, s46: unidentified.

	s43	s48	S54	S60	s67	s76	s81	s85	S86
Chisq	51.05	335.03	200.64	182.75	2372.90	177.34	39.30	166.18	60.46
Pr(>Chisq)	9.006e ⁻¹³	< 2.2e ⁻¹⁶	< 2e ⁻¹⁶	< 2e ⁻¹⁶	< 2.2e ⁻¹⁶	< 2e ⁻¹⁶	3.640e ⁻¹⁰	< 2.2e ⁻¹⁶	7.528e ⁻¹⁵

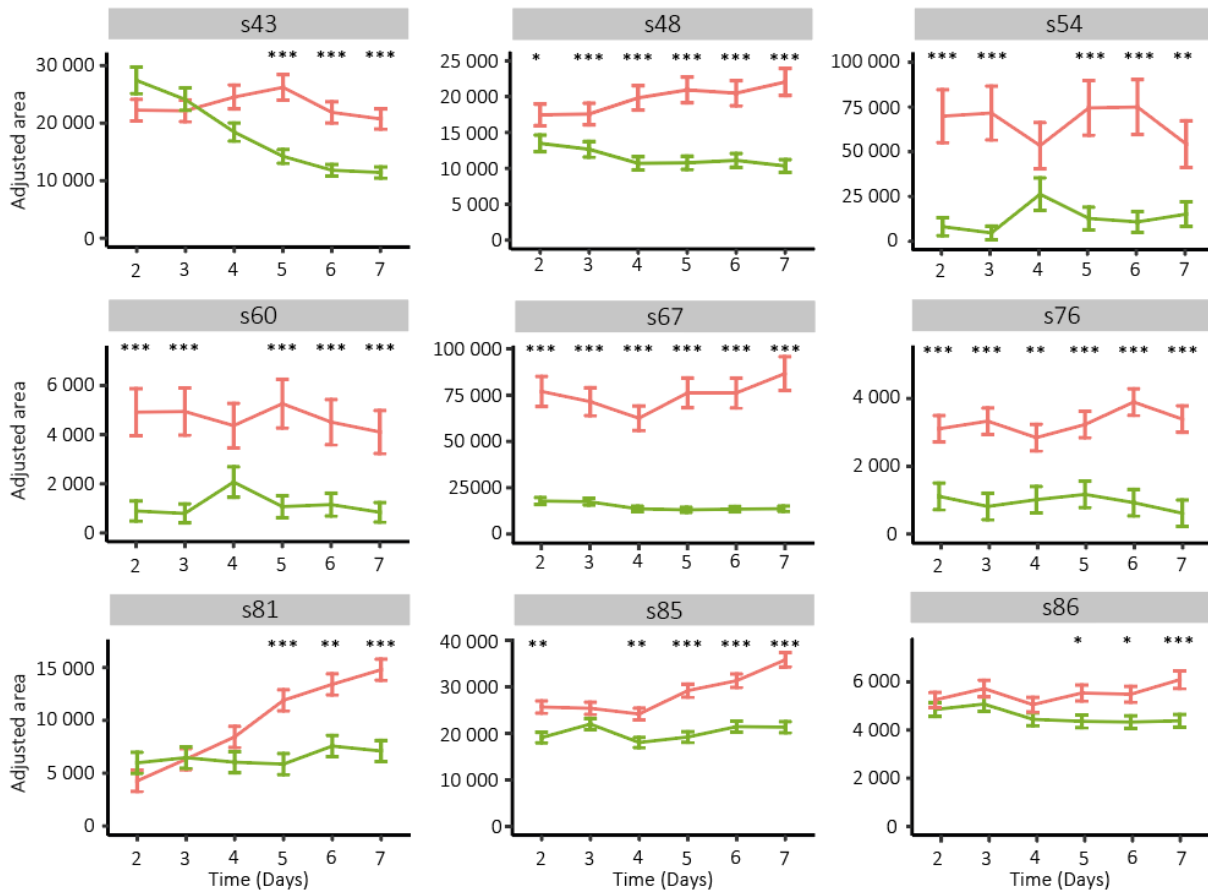


Figure 3.3: Adjusted means areas (+/- SE) for the nine compounds of interest per 10 parasitized (P) and non-parasitized (NP) brood samples reared in capsule, for the day 2 to day 7. P: orange, NP: green. For each day and each group, n = 10 colonies. Tukey-adjusted P values from pairwise comparisons of estimated marginal means. s43: octanoic acid, s48: benzothiazole, s54: unidentified, s60: unidentified, s67: *n*-tetradecane, s81: dodecanoic acid, s85: *n*-hexadecane, s46: unidentified.

3.2. Adult worker behaviour on COV treated brood cells

In order to observe the response of worker bees to these varroa parasitization-characterising compounds, each solution containing one or all compounds of interest was injected into brood cells (n = 11 colonies). There was a significant difference between treatments ($\chi^2 = 183.77$, d.f. = 7, $P < 2.2e^{-16}$) (Figure 3.4). Brood cells treated with solutions containing one or more VOCs were more visited (cleaned, uncapped or recapped) than cells treated with the solvent control or those that received no treatment (Figure 3.4). The cells visited by bees in the solvent and untouched controls were mainly recapped (Figure 3.4). Due to the small proportion of cleaned brood cells for the untreated brood cells control, this control was not considered in the analysis of the cleaning behaviour. There was a significant treatment effect in brood cell cleaning behaviour (6h: $\chi^2 = 99.161$, d.f. = 6, $P < 2.2e^{-16}$; 24h: $\chi^2 = 124.38$, d.f. = 6, $P < 2.2e^{-16}$; 48h: $\chi^2 = 143.2$, d.f. = 6, $P < 2.2e^{-16}$). Each solution triggered a response with a different temporal dynamic. The response to the s67 solution (*n*-tetradecane) was greater than for all other treatments 6h after injection (Figure 3.5). 24h after injection, the response to the mix solution was

greater than for the other treatments (Figure 3.5). At 48h after injection, the responses to all of the treatments was greater than the control solvent, but no difference between the treatments themselves was observed (Figure 3.5). In comparing the response of the workers to the candidate compounds and their respective control compounds (NC1: octadecane vs. s67: *n*-tetradecane and s85: *n*-hexadecane; NC2: decanoic acid vs. s43: octanoic acid and s81: dodecanoic acid), we found a significant effect of the treatments (NC1-s67-s85: 6h: $\chi^2 = 92.131$, d.f. = 3, $P < 2.2e^{-16}$; 24h: $\chi^2 = 88.966$, d.f. = 3, $P < 2.2e^{-16}$; 48h: $\chi^2 = 100.85$, d.f. = 3, $P < 2.2e^{-16}$; NC2-s43-s81: 6h: $\chi^2 = 20.404$, d.f. = 3, $P = 0.00014$; 24h: $\chi^2 = 83.337$, d.f. = 3, $P < 2.2e^{-16}$; 48h: $\chi^2 = 100.6$, d.f. = 3, $P < 2.2e^{-16}$) (Figure S3.1). NC1 triggered a smaller response than each of the two compounds of the same chemical family at 6h and 24h but not at 48h (Figure S3.1A). NC2 triggered a smaller response than s81 at 6h and each of the two compounds of the same chemical family at 24h. The response to NC2 did not differ from the response to s43 and s81 at 48h after injection (Figure S3.1B).

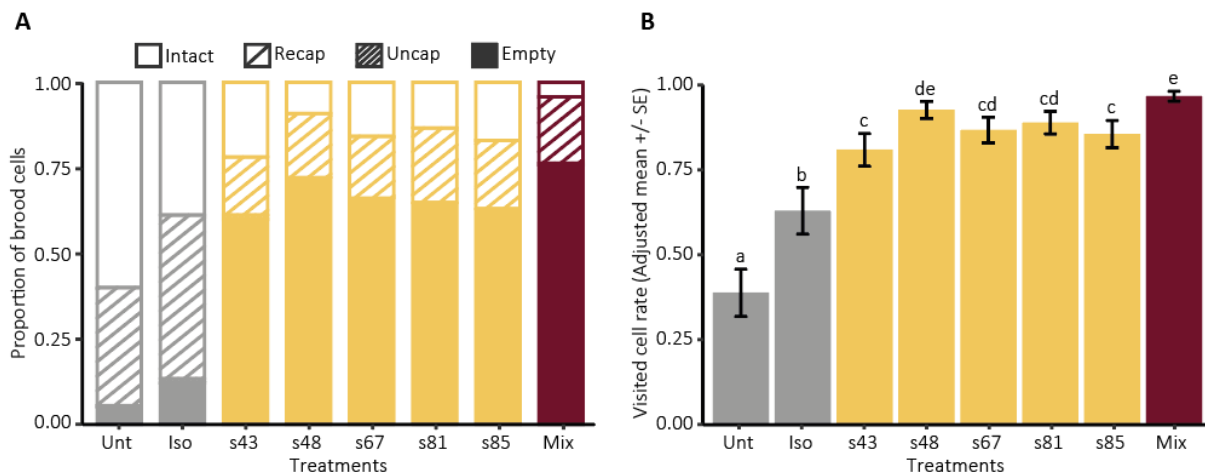


Figure 3.4: Hygienic response of workers to brood cells treated with different treatments, 48h after injection. **A.** Proportion of brood cells in each status (Intact: untouched cells, Recap: recapped cells, Uncap: uncapped cells, Empty: fully cleaned cells). **B.** Adjusted visited cell rate means (+/- SE) of brood cells according to the different treatments at 48h. A cell has been visited when it has been cleaned, uncapped or recapped. For each treatment, $n = 11$ colonies. Intact: intact cells; Recap: recapped cells; Uncap: uncapped cells, Empty: fully cleaned cells. Unt: untreated cells, Iso: isohexane, s43: octanoic acid, s48: benzothiazole, s67: *n*-tetradecane, s81: dodecanoic acid, s85: *n*-hexadecane, Mix: all of the compounds.

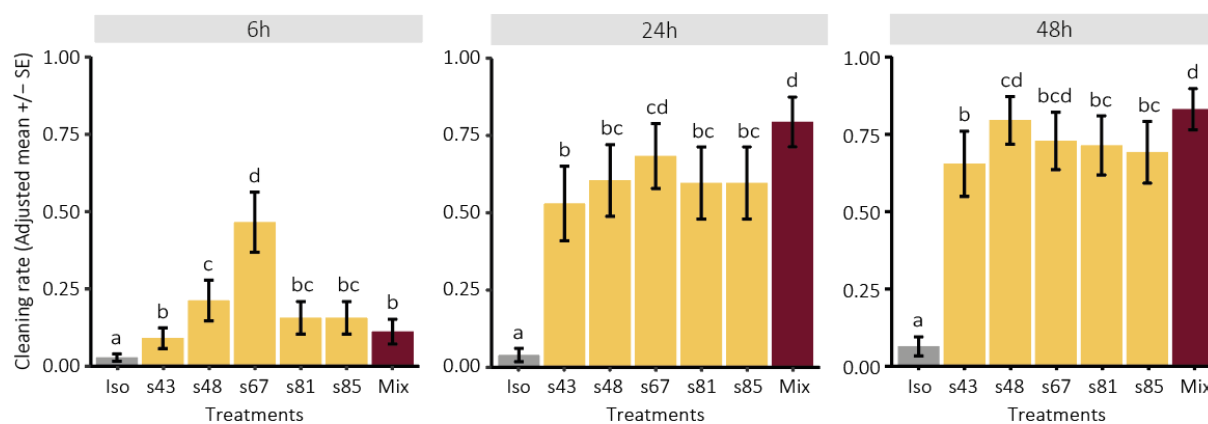


Figure 3.5: Adjusted cleaning rate means (+/- SE) of injected brood cells according to the different treatments, 6h, 24h and 48h after injections. For each treatment, n = 11 colonies. Iso: isohexane, s43: octanoic acid, s48: benzothiazole, s67: *n*-tetradecane, s81: dodecanoic acid, s85: *n*-hexadecane, Mix: all of the compounds.

The recapping rate of the remaining capped brood cells after 48h was also monitored. There was a significant difference in recapping rates between treatments ($\chi^2 = 17.389$, d.f. = 6, $P = 0.007956$). Compounds presented alone did not trigger a different recapping behaviour than the solvent control (Figure 3.6). The mix solution triggered the highest response and was different to the controls, and the s43 and untreated cells triggered the lowest response (Figure 3.6).

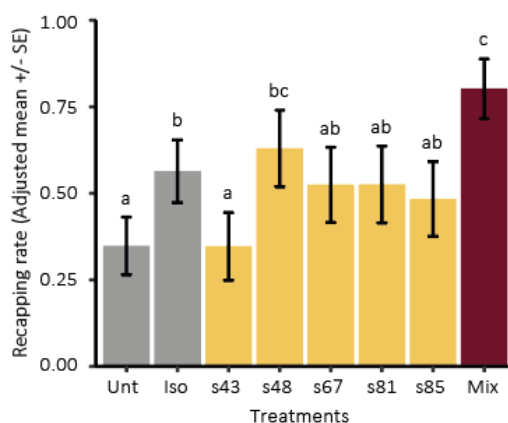


Figure 3.6: Adjusted recapping rate means (+/- SE) of remaining injected capped brood cells. For each treatment, n = 11 colonies. Untreated: untreated cells, Solvent: solvent, s43: octanoic acid, s48: benzothiazole, s67: *n*-tetradecane, s81: dodecanoic acid, s85: *n*-hexadecane, Mix: all of the compounds.

4. Discussion

We analysed the VOCs emissions of *Varroa destructor*-infested and non-infested honey bee brood (*Apis mellifera*) reared in gelatine capsules, to identify candidate compounds specific of varroa infested cells that could trigger VSH and/or recapping behaviours. To our knowledge, this is the first time that VOCs of varroa-parasitized bee brood have been captured on brood reared in gelatine capsules on the same individuals for several days in a row. We have highlighted nine compounds characteristic of varroa-infested brood cells, of which five were identified and tested in the field. These

five VOCs triggered worker cleaning and one compound triggered a faster response than the others. Recapping of the cells was more triggered by the mixture of these five compounds than by the compounds alone.

Rearing bee brood in gelatine capsules makes it possible to be completely free of wax odours while keeping the brood in an environment physically close to the wax cell (diameter, depth, volume available...). This device allowed the capture of 111 compounds over the seven days of experimentation, which illustrates a rich olfactory landscape of the brood. Compounds found in the study of Noël *et al.* (2023) as being emitted in the pre-pupal and non-sclerotized pupal stages were all found here, except for hexenyl acetate. This rearing assay ensures that the vast majority of the compounds captured come from the brood and/or the parasite. No difference was observed in the total amount of compounds emissions between parasitized and non-parasitized groups, suggesting a subtle modulation of brood emissions due to varroa-parasitism and confirming the ability of varroa to mimic the olfactory profile of its host (Kather *et al.*, 2015b; Kather *et al.*, 2015a; Le Conte *et al.*, 2015).

The VOCs emissions showed quantitative differences between parasitized and non-parasitized brood samples for several compounds, but no compound was emitted only by one of the two groups. Amongst the compounds that were more abundant in the P group, we identified the nine compounds that characterized the most the parasitized status, in order to perform behavioural assays. Chemical compounds are strongly involved in the recognition of disease and parasites among insects (Milutinović & Schmitt, 2022). Several compounds have already been found to be emitted by varroa-infested brood cells (Liendo *et al.*, 2021; Martin *et al.*, 2002; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Schöning *et al.*, 2012; Wagoner *et al.*, 2019; Wagoner *et al.*, 2020). The method used here allowed the capture of short carbon chain compounds such as compounds highlighted by Schöning *et al.* (2012) and Liendo *et al.* (2021), and therefore did not allow the capture of larger compounds as found by Martin *et al.* (2002), Nazzi *et al.* (2004a), Wagoner *et al.* (2019, 2020) and Mondet *et al.* (2021). Schöning *et al.* (2012) analysed the odours of non-parasitised varroa and parasitised brood with high and low DWV infection rates. They placed pupae of interest in a vial and performed compound captures using Tenax TA cartridges during 1h at $34.5 \pm 1^\circ\text{C}$ (Schöning *et al.*, 2012). Nine of the 39 compounds found by Schöning *et al.* (2012) in the headspace of the parasitized and non-parasitized pupae were found in our captures (2-methylpropanoic acid, 2,3-butanediol (diastereomers), isoamyl acetate, benzaldehyde, (*Z*)- β -ocimene, (*E*)- β -ocimene, octanoic acid and methyl benzoate). In both studies, no marked difference was found between the two groups. Liendo *et al.* (2021) captured VOCs from the headspace of parasitized (no varroa present) and non-parasitized pupae at the pre-pupal (in our study: day 1), light pink-eyed pupae (in our study: day 4) and black-eyed pupae (in our study: day 6) stages in a glass aeration chamber. The captures were made during 24 hours, at room temperature, with HayeSep Q cartridges. They found

α -pinene and ethyl hexanoate to be specific to parasitized brood (Liendo *et al.*, 2021). We did not find ethyl hexanoate in our captures. α -pinene was present in trace amounts and could not be quantified. A reason for the differences in the compounds found in these two studies and in our study can be explained by the different properties of the adsorbents used. Each adsorbent has its own volatile affinity (Agelopoulos & Pickett, 1998). Differences in the time and temperature of capture as well as the environment of the (pre-)pupa for capture (vial, microscope slide or gelatine capsule) could also have an effect on the compounds emitted and captured. These studies are complementary in the comprehension of the emissions from *V. destructor* parasitized and non-parasitized brood, but our study presents the advantage to illustrate emissions over 7 continuous days and thus to highlight temporal evolutions in the profiles.

Interestingly, the temporal evolution in compound emission between the parasitized and non-parasitized groups differed according to the compounds. Seven compounds (s48, s5, s60, s67, s76, s85 and s86) had a higher emission in the parasitized group than in the non-parasitized group regardless of the day of capture. On day 4, two of these compounds (s54 and s60) showed a smaller difference of emission between the two groups. Two compounds had a higher emission for the non-parasitized group on days 2 and 3 of the captures (late pre-pupal and first pupal stage) followed by a higher emission in the parasitized group from day 4 (second pupal stage). The second pupal stage may be a key moment in the detection of parasitism. Further studies are needed to understand the full characteristics of this stage.

The compounds identified in this study had either been found in trace amounts or were hidden by wax compounds in the study of VOC capture on brood frames (Noël *et al.*, 2023). Because SPME fibres have different affinities between volatiles (Roberts *et al.*, 2000), the emission of wax compounds may have saturated the fibres, preventing the capture of compounds emitted in small quantities or with a lower affinity to the fibre. The capture of VOCs from the emissions of the brood reared in capsules provided the opportunity to avoid the interfering odours of the wax.

The five compounds identified in this study belong to three different chemical families: alkanes (*n*-tetradecane and *n*-hexadecane), carboxylic acids and more particularly fatty acids (octanoic acid and dodecanoic acid) and benzothiazoles (benzothiazole). Alkanes are strongly involved in social recognition and changes in alkane emissions by an individual can trigger aggressive behaviour (Blomquist & Bagnères, 2010; Dani *et al.*, 2005). *n*-Hexadecane is emitted from dead brood extracts and triggers an increase in aggressive behaviour towards individuals treated with this compound (Breed & Stiller, 1992; Haber *et al.*, 2019b). *n*-Tetradecane triggers an electrophysiological response from the varroa mite (Light *et al.*, 2020b). Benzothiazoles have a broad spectrum of effect in pharmacology (Ali & Siddiqui,

2013). Their anti-microbial properties could play a major role in the defence against pathogens in the social spider *Stegodyphus dumicola* (Lammers *et al.*, 2021). Benzothiazole also triggers an electrophysiological response from varroa (Light *et al.*, 2020b). The two fatty acids, octanoic acid and dodecanoic acid, identified have already been shown to be present in total lipids extracted of varroa and varroa-parasitized brood (Dmitryjuk *et al.*, 2015). Dodecanoic acid (also known as lauric acid) was found to be present on the cuticle of the varroa foundress (Martin *et al.*, 2002). This compound was present in greater quantities in total lipids extracts of parasitized drone pre-pupae than varroa mite (Dmitryjuk *et al.*, 2015). Octanoic acid (also known as caprylic acid) was only found in the total lipid extracts of parasitized pre-pupae drones (Dmitryjuk *et al.*, 2015). This VOC has already been shown to be emitted from uncapped brood frames and is a known compound in larval jelly (Carroll & Duehl, 2012; Nazzi *et al.*, 2009), and to be repellent to varroa mites (Nazzi *et al.*, 2004a; Nazzi *et al.*, 2009). With our capture system, it is not possible to identify the emitter of the VOCs between the brood and the parasite. These five compounds, in addition to being involved in the detection of varroa-infested cells by the workers, could be a response of the pupa in an attempt to disrupt the varroa survival and/or reproduction.

The five VOCs triggered hygienic behaviour in the field bioassay. We expected that the mixture of the five compounds would trigger a faster and stronger response than the solutions containing a single compound. However, we observed a faster response from the *n*-tetradecane compared to all other solutions tested, when measured 6h post treatment. Similar responses have already been shown in the scientific literature with different compounds. For example, phenethyl acetate triggers a more important hygienic behaviour than a mixture containing this compound and two others (Swanson *et al.*, 2009). Oleic acid triggers a cleaning rate equivalent to that obtained from the mixture of oleic acid and β -ocimene (McAfee *et al.*, 2018). Similarly, ethyl hexanoate triggers an equivalent cleaning rate as that triggered by a mix of ethyl hexanoate and α -pinene (Liendo *et al.*, 2021). This rapid response to *n*-tetradecane solutions suggests a prioritisation of the message by the workers, for this compound.

We tested two control compounds for alkanes and fatty acids. The chosen compounds have the same chemical structure as our compounds of interest, with two carbon more or less. The purpose of testing similar compounds to the candidate compounds is to support the specificity of the activity of the latter (Nazzi *et al.*, 2004a; Wagoner *et al.*, 2019; Wagoner *et al.*, 2020). These two control compounds trigger a weaker response 6h and 24h after injection than our candidate VOCs. The lack of difference 48h after injection between our control compounds and our candidate VOCs may be explained by the time between the injection and the observation. At 48 h after injection, the workers may have finished cleaning the major signals and, therefore, have time to clean anything that is slightly different from the normal olfactory bouquet.

Amongst all the parasitism related compounds, several have already been shown to trigger hygienic responses (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazi *et al.*, 2004a; Wagoner *et al.*, 2019). Most of these are long-chain carbon compounds (Mondet *et al.*, 2021; Nazi *et al.*, 2004a; Wagoner *et al.*, 2019). It has already been suggested that highly volatile compounds coupled with less volatile compounds may permit the workers to better target the cells to be cleaned (McAfee *et al.*, 2018; Mondet *et al.*, 2021). The VOCs described in this study could complement the message of the two VOCs found by Liendo *et al.* (2021). Also, the rapid activity of *n*-tetradecane supports this hypothesis and that this compound may have a central role in VSH behaviour triggering.

As the concentration used was high, almost 70% of the injected cells had been cleaned 48h after treatment, leaving only a small quantity of brood cells to study the recapping behaviour. However, only the mix triggers a significant response compared to the solvent control. This suggests that the signal that triggers recapping is complex and requires the presence of several molecules. Currently, it is unknown which triggers the first step in recapping behaviour, *i.e.* the opening of the brood cell. However, this could potentially provide access to low-volatile chemical cues to assess the infestation of the brood cell (Grindrod & Martin, 2021; Martin *et al.*, 2020). The VOCs would attract the workers while the less volatile compounds would allow the workers to target the cell. This hypothesis needs to be confirmed with new behavioural assays coupling the chemicals compounds (VOCs and low volatiles) described as characteristic of varroa-infected brood.

This study reveals that five newly identified VOCs may have a key role in triggering VSH and recapping behaviour by workers. These five compounds were released in greater quantities in parasitized brood than in non-parasitized brood. These VOCs triggered brood cell cleaning and one compound, *n*-tetradecane, triggered it faster than the others. This compound may play a key role in the early detection of parasitized cells. VOCs appeared to be involved in recapping behaviour. It would be interesting to study the VSH and recapping behaviour of workers to these five VOCs mixed with compounds identified in the scientific literature as characteristic of varroa-infested brood.

5. Acknowledgments

We thank J. Sénéchal for giving access to the honey bee colonies. This work was supported by a PRIMA grant (EU H2020, #1812, "Plant-B").

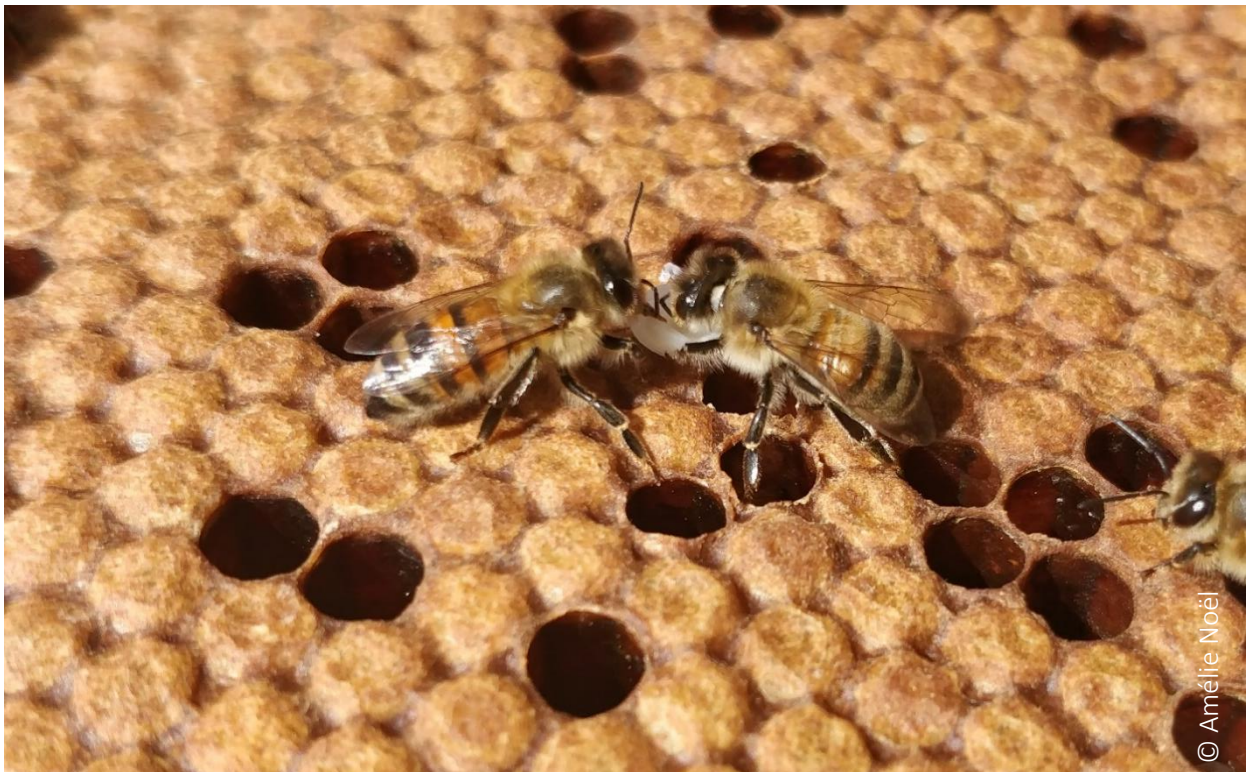
III. Conclusion

Dans cette étude, les différences d'émissions des COVs émis par un couvain parasité et non-parasité par varroa ont été mises en évidence à l'aide de captures réalisées sur du couvain élevé *in vivo* (en gélules). L'article 2 montre neuf COVs, dont cinq identifiés, qui sont caractéristiques du couvain parasité par *V. destructor*. Le comportement des ouvrières face à ces composés a été testé en injectant des solutions contenant un seul ou un mélange de ces composés.

Les COVs décrits dans cette étude pourraient compléter le message des deux COVs déjà décrits comme impliqués dans le déclenchement du comportement de nettoyage des alvéoles de couvain (Liendo *et al.*, 2021). La découverte d'un composé (tétradécane) émis en plus grande quantité par le couvain parasité que les huit autres COVs et qui déclenche un nettoyage des alvéoles de couvain plus rapide laisse penser que ce composé joue un rôle particulièrement central dans le déclenchement du comportement VSH. La faible quantité d'alvéoles encore operculées 48h après injection (~70% de nettoyage) a rendu difficile l'étude de la réoperculation. Néanmoins, le comportement de réoperculation accru face aux alvéoles de couvain traitées par le mélange des cinq COVs suggère que ce comportement est déclenché par un mélange de composés. Des analyses supplémentaires sont nécessaires afin d'étudier le rôle de ces cinq COVs sur le comportement VSH et le comportement de réoperculation lorsqu'ils sont mélangés avec des composés identifiés dans la littérature scientifique impliqués également dans le déclenchement du comportement VSH.

CHAPITRE 4

EFFETS DES COMPOSES CARACTERISTIQUES DU PARASITISME PAR *V. DESTRUCTOR* SUR LE COMPORTEMENT DES OUVRIERES *A. MELLIFERA*



Le sacrifice d'une nymphe

I. Avant-propos

Dix composés ont été mis en évidence dans la littérature scientifique comme impliqués dans le déclenchement du comportement VSH, et les cinq composés mis en évidence dans le chapitre 3 s'ajoutent à cette liste (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Noël *et al.*, Unpublished; Wagoner *et al.*, 2020). Les protocoles de ces cinq études diffèrent de par les concentrations utilisées ainsi que le mode de traitement des alvéoles de couvain (injection de solution, dépôt de solution sur un papier filtre inséré dans l'alvéole et dépôt sur l'opercule), ce qui rend la comparaison de l'efficacité des composés difficile.

La communication chimique de l'abeille peut être très dépendante du ratio de chaque composé dans un mélange chimique (Le Conte *et al.*, 1994b). Les 15 composés potentiellement impliqués dans le déclenchement du comportement VSH pourraient constituer le même signal de parasitisme. Le mélange de ces composés pourrait augmenter ou inhiber le comportement des ouvrières. De plus, les COVs et les composés peu volatiles pourraient déclencher des étapes différentes du comportement de nettoyage (désoperculation, sacrifice ou réoperculation).

Dans ce chapitre, une étude comportementale a été réalisée afin d'étudier les comportements hygiénique et de réoperculation des ouvrières vis-à-vis des 15 molécules décrites comme impliquées dans le déclenchement de l'activité hygiénique, à l'aide d'un protocole harmonisé permettant de comparer les réponses obtenues avec les différents composés. Cette étude a aussi pour but de mettre en évidence d'éventuels effets additifs ou synergiques de ces composés sur le comportement des abeilles.

II. Article 3

Role of highly or less volatile organic compounds on the varroa sensitive hygiene and recapping behaviours in the honey bee (*Apis mellifera*)

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Abstract

Understanding the resistance mechanisms of honey bee colonies (*Apis mellifera*) to their main parasite *Varroa destructor* is a major challenge for beekeeping. Indeed, the mite has a harmful effect on colonies and can kill a colony in a few months without treatment. The increasing resistance of varroa mites to chemical acaricide treatments makes the control of the parasite more and more difficult. The development of resistance traits in some colonies provides an ideal avenue for long-term sustainable control of the parasite. Two key behaviours of this resistance are VSH (Varroa Sensitive Hygiene) and recapping behaviour. These behaviours consist in the detection and opening of brood cells. In the case of VSH behaviour, the pupa is sacrificed and the cell cleaned of its contents, whereas in the case of recapping behaviour, the pupa is left untouched and the brood cell will be recapped. These behaviours are mediated by olfaction. Currently, fifteen compounds have been described as triggering VSH behaviour: eight low volatile compounds and seven volatile organic compounds (VOCs). No compound has been identified as triggering recapping behaviour. The aim of this study is to investigate the hygienic behaviour of workers in response to the fifteen chemical signals identified in the scientific literature, through the cleaning and recapping of brood cells. The effect of the compounds on both behaviours was studied in relation to the volatile nature of the compounds. Simple (one compound) and complex (two to fifteen compounds) treatments were tested. Finally, the effect of the treatments on the VSH behaviour was compared with the ability of the colonies to perform the VSH behaviour. This study contributes to a better understanding of the mechanisms underlying VSH and recapping behaviour.

Keywords: *Varroa destructor*, resistance, honey bee, VSH, recapping, semiochemical compounds.

1. Introduction

The honey bee (*Apis mellifera*) is a social insect, living in colonies of an average of 50,000 individuals. Living in large groups has the advantage of simplifying progeny care, improving food collection and protection, and reducing predator pressure (Keller & Chapuisat, 2010). On the other hand, the promiscuity of individuals makes it easier for pathogens and parasites to spread. Fortunately, social insects like the honey bee have a collective defence system called social immunity, which is a set of traits that reduce the spread of parasites and pathogens at the colony level (Cremer *et al.*, 2007; Simone-Finstrom, 2017). In honey bees, various behaviours are used by the workers to control pathogens and parasites, one of the key behaviours of this social immunity being hygienic behaviour (Evans & Spivak, 2010; Wilson-Rich *et al.*, 2009). Hygienic behaviour consists in identifying cells containing dead, abnormal or diseased brood and removing their contents, thus reducing the pressure of pathogens or parasites on colony health and growth (Arathi *et al.*, 2000; Spivak, 1996; Spivak & Gilliam, 1998). This behaviour is achieved both on larval and (pre-)pupal stages. Some colonies have developed a specific hygienic behaviour on capped brood cells parasitized by the parasite *Varroa destructor*, called Varroa Sensitive Hygiene (VSH) (Mondet *et al.*, 2020a).

Varroa represents one of the greatest threats to honey bee colonies (Rosenkranz *et al.*, 2010). The life cycle of *V. destructor* is separated into two phases: the phoretic phase, where it is present on adult bees, and a reproductive phase where it enters into a brood cell just before it is capped. Inside the brood cell, the varroa foundress lays a male egg followed by several female eggs (reviewed by Mondet & Le Conte, 2014). Once mature, the male mates with his mature sisters. When the young bee emerges, the mite foundress and one or several daughters emerge from the brood cell and start a new life cycle. This life cycle results in an exponential growth of the varroa population within the colony (Mondet *et al.*, 2016b). By feeding on both pupae and adults, varroa has a detrimental effect on the development of the pupa, and acts as a vector in the transmission of viruses (de Miranda *et al.*, 2010; de Miranda & Genersch, 2010b; Martin & Brettell, 2019; Mondet *et al.*, 2014).

VSH behaviour has been confirmed as an effective natural solution for reducing the growth of mite populations in honey bee colonies (Cheruiyot *et al.*, 2018; Kirrane *et al.*, 2018; Mondet *et al.*, 2020a; Panziera *et al.*, 2017). Workers identify the parasitized brood cell, open it and empty it completely of its content, thus sacrificing the pre-pupa or pupa. A variant of this behaviour is the recapping behaviour (Aumeier *et al.*, 2000; Boecking & Spivak, 1999; Harris *et al.*, 2012; Martin *et al.*, 2019; Oddie *et al.*, 2019b). In this behaviour, instead of emptying the cell, workers reclose the cap of the cell once uncapped. This opening and recapping of the brood cell could be sufficient to disrupt the reproductive cycle of the varroa foundress (Guichard *et al.*, 2023; Oddie *et al.*, 2018; Oddie *et al.*, 2021)

but this is still debated (Sprau *et al.*, 2021). So far, it remains unknown whether these two behaviours are indeed distinct or two variations of the same defence strategy.

Chemical communication plays an important role in the detection of these parasitized brood cells in the VSH behaviour (Mondet *et al.*, 2015; Nazzi & Le Conte, 2016; Rosenkranz *et al.*, 1993). This detection could be done in two steps. The first cue could be the emission of volatile organic compounds (VOCs) that would easily pass through the cap of the brood cell to signal the area where the parasitized cell is located (Kim *et al.*, 2018). The second cue could involve low volatile compounds (LVCs) that the worker would detect when it is close to the brood cell or when opening it. The search for compounds that are characteristic of *Varroa* parasitism and trigger VSH behaviour has recently gained considerable interest. Currently eight low volatile compounds and seven volatile organic compounds have been identified as triggering VSH behaviour (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Noël *et al.*, Unpublished; Wagoner *et al.*, 2020). These compounds, produced by varroa parasitized cells, are recognised by the workers when they are artificially introduced into or applied on the brood cells and trigger the cleaning of the brood cell (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Noël *et al.*, Unpublished; Wagoner *et al.*, 2020). However, compounds that trigger recapping behaviour rather than the sacrifice of the immature are still unknown but it is suggested that the lack of specific cues emitted by the open brood cell may trigger recapping (Martin *et al.*, 2020).

As the different compounds have been identified by different authors using different approaches, the aim of this study was to investigate the hygienic behaviour of workers in response to these 15 chemical signals with a standardised protocol. The effect of the compounds on the two behaviours was studied based on the volatility characteristics of the compounds (VOCs or LVCs). The hygienic behaviour was studied through the cleaning behaviour as well as the recapping behaviour of the treated brood cells, and with regards to the VSH status of the tested colonies.

2. Materials and methods

2.1. Bioassay design for the evaluation of VSH and recapping behaviours

The bioassays tested the effect of different treatments containing compounds characteristic of varroa-infested brood cells on VSH and recapping behaviours. This study was carried out on 31 honey bee colonies, located in the apiary of the INRAE research centre in Avignon (France), between September and October 2022. Homogeneous brood patches with brood cells containing white-eyed to pink-eyed pupae, stages where VSH behaviour is most performed (Harris, 2007), were injected with 1µL of solution using a Hamilton syringe just below the cap of the cells and positions of the injected cells

were marked on a transparent plastic sheet. For each treatment, 30 cells were injected. The status of the cells (emptied, uncapped) was observed at 6h, 24h and 48h after injections. At 48 hours, the recapping of the cells was additionally checked by gently opening the remaining brood cells.

2.2. Extract preparations of volatiles organic compounds and low volatile compounds

The large number of compounds identified in the scientific literature as triggering VSH behaviour did not allow to test all possible mixtures. Compounds were mixed in order to obtain increasingly complex combinations until the final mixture containing all 15 compounds tested, to observe potential synergy or additivity (Figure 4.1). Simple solutions were composed of the compounds identified together in the same publication ("A", "B", "C" and "D", Figure 4.1). *n*-Tetradecane was tested alone due to its rapid effect on hygienic behaviour (Noël *et al.*, Unpublished) ("a", Figure 4.1). Tetracosyl acetate was tested alone for the high concentration modality because the 6 VPS are immiscible at high concentrations ("d", Figure 4.1). These six solutions were used as controls to observe the impact of more complex mixtures on worker behaviour. Solutions containing all VOCs ("A+B", Figure 4.1) and LVCs ("C+d" and "C+D", Figure 4.1) were tested. The final solution was constituted of VOCs and LVCs ("A+B+C+D", Figure 4.1). Two concentrations were tested, one at 10% w/w concentration (high concentration) (n = 16 colonies) with the aim of reproducing what had already been done in the scientific literature and a 10 equivalents concentration (physiological concentration) (n = 15 colonies) to test a concentration closer to the biological conditions found in the brood cells. One equivalent corresponds to the amount of a given compound emitted by one parasitized brood cell. With the exception of the five VOCs identified in the study in chapter 3 (Noël *et al.*, Unpublished), the equivalents were calculated from the scientific literature (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Salvy, 1999; Salvy *et al.*, 2000). The determination of an equivalent for the VOCs is described below. For all solutions, compounds were diluted in isohexane, except for the 10% w/w mixture containing all the ten compounds ("A+B+C+d", Figure 4.1) where no solvent was used. Due to solubility problems at high concentrations, the mixture of the 6 VPS was replaced by only one of the VPS (TCA: tetracosyl acetate) for the 10% concentration (Figure 4.1).

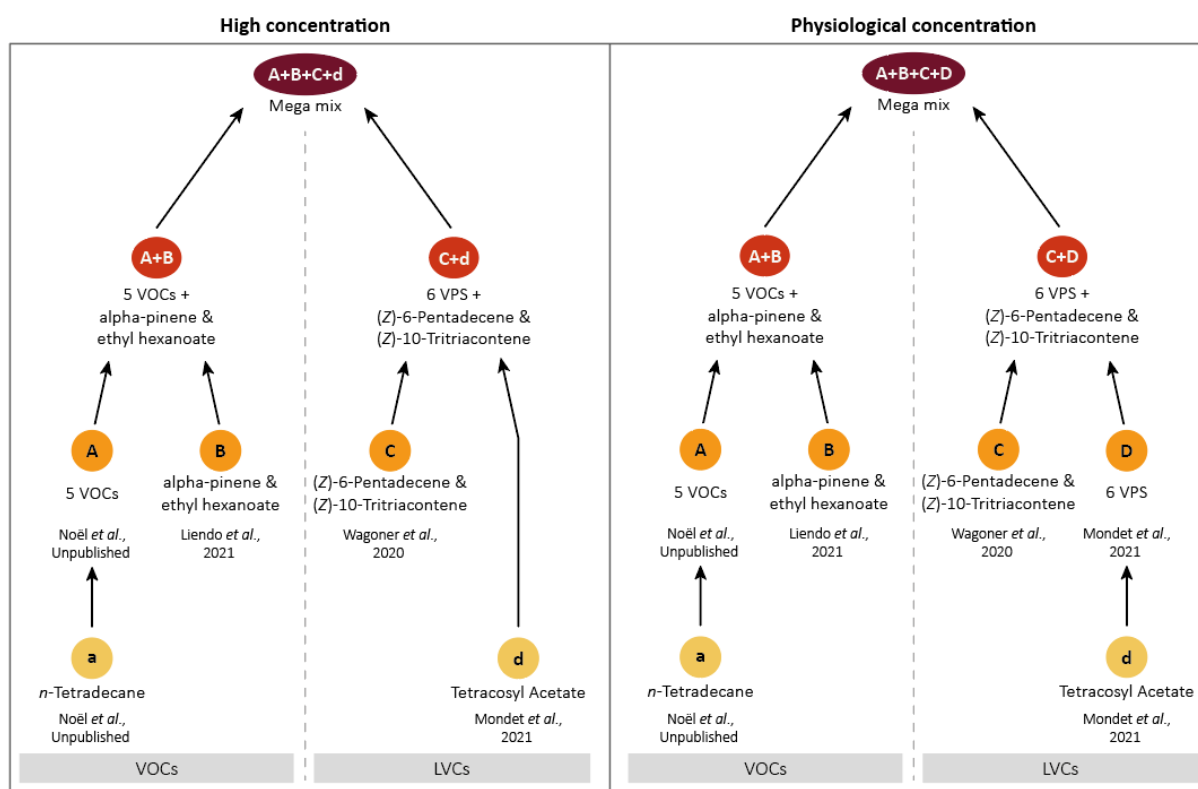


Figure 4.1: Scheme of the different mixtures used, for the two concentrations. Due to solubility issues at high concentrations, only one of the 6 VPS compounds was used for the high concentration. High concentration: 10%w/w; physiological concentration: 10 equivalents. VOCs: Volatile Organic Compounds, LVCs: Low Volatile Compounds.

2.3. Determination of an equivalent of parasitized brood cell for 5 of the VOCs

In order to determine the emission of each of the 5 VOCs (Noël *et al.*, Unpublished) from a parasitized brood cell, calibration curves were performed for the quantification of the five VOCs identified as specific to parasitism (Noël *et al.*, Unpublished). Each compound alone was diluted in isohexane and then 10µL was placed in a sealed vial (10 mL). The septum was pierced to allow passage of a PDMS-DVB (polydimethylsiloxane divinylbenzene) SPME (Solid Phase Micro Extraction) fibre (65 µm, manual holder). Captures lasted 20 minutes. Quantitative analysis of the VOCs was carried out using a gas-chromatography coupled with mass spectrometry (GC-MS) following the protocol of Noël *et al.* (2023). In brief, desorption was realised in splitless mode, with the constant column flow at 0.82 mL/min; the carrier gas was helium. The injector temperature was set at 250 °C and the oven temperature program as follows: 45 °C isothermal held for 5 min, followed by temperature increases at a rate of 10 °C/min up to 250 °C. The oven was finally held at 250 °C for 4 min. The ion source was set at 200 °C.

2.4. Determination of the VSH score of the colonies

In each colony tested, one brood frame containing last instar larvae was collected, and cells being capped were marked on a transparent plastic sheet and put back in the honey bee colony. After 6 hours, these brood cells just capped were artificially infested with varroa mites previously collected with icing sugar from heavily infested colonies. Eight days later, the cleaning of the artificially infested brood cells was checked. The VSH score was calculated as the number of cleaned brood cells over the number of artificially infested brood cells (Tison *et al.*, 2022). Colonies where the VSH score could not be determined (not enough brood of the right stage) were not included in the VSH score analysis (High concentration: n = 12, physiological concentration: n = 11).

2.5. Statistical analyses

Statistical analyses were performed using R (v4.1.3). To study the effect of each solution on the number of cells visited (fully cleaned, uncapped and recapped), the data were analysed using a generalised linear mixed model (GLMM), with the proportion of brood cells visited in comparison to the remaining cells as a function of treatment (fixed factor) and colonies (random factor). To study the effect of each solution on the number of cells fully cleaned, cleaning rate was analysed using a GLMM in which the proportion of cells targeted by hygienic behaviour was explained by the treatment (fixed factor) and colonies (random factor). The effect of the solutions on the recapping behaviour of the workers was analysed in the same way. Due to the non-linearity of the data, generalised additive mixed models (GAMMs) were fitted on the proportion of brood cells cleaned as a function of the VSH score of the colony, for each treatment. A threshold was determined from these GAMMs to separate colonies in two groups of VSH performance (“VSH+”: good recognition and cleaning varroa-infested cells, “VSH-”: poor recognition and cleaning varroa-infested cells) (R package “mgcv” v1.8-39; <https://cran.r-project.org/web/packages/mgcv/index.html> (Wood, 2011)). Colonies with a VSH score close to this threshold ($\pm 1\%$) are removed from the analysis in order to have a net limit (one colony for the 10% concentration). To study whether colonies with a high VSH score responded faster to compounds than those with a low VSH score, generalized linear models were performed to analyse the effect of treatments on cleaning of injected cells by workers at 6h, according to the two VSH groups (VSH-/VSH+), with the proportion of brood cells cleaned as a function of VSH groups (fixed factor). (R packages “mgcv: Mixed GAM Computation Vehicle with Automatic Smoothness Estimation” v1.8-41: <https://cran.r-project.org/web/packages/mgcv/index.html> and “lme4: Linear Mixed-Effects Models using 'Eigen' and S4” v1.1-28: <https://cran.r-project.org/web/packages/lme4/index.html>).

3. Results

3.1. Effects of compounds characteristic of varroa-infested brood on brood cell visit by workers

Brood cells treated with solutions containing compounds characteristics of *Varroa*-parasitized brood cells were significantly more visited (cleaned, uncapped and recapped) than solvent-treated (control) or untreated brood cells (10%: $\chi^2 = 789.71$, d.f. = 9, $P < 2.2e^{-16}$; 10 equivalents: $\chi^2 = 422.89$, d.f. = 10, $P < 2.2e^{-16}$) (Figure 4.2). Due to the small proportion of cleaned brood cells for the untreated brood cells control, this control was not considered in the analysis of the cleaning behaviour.

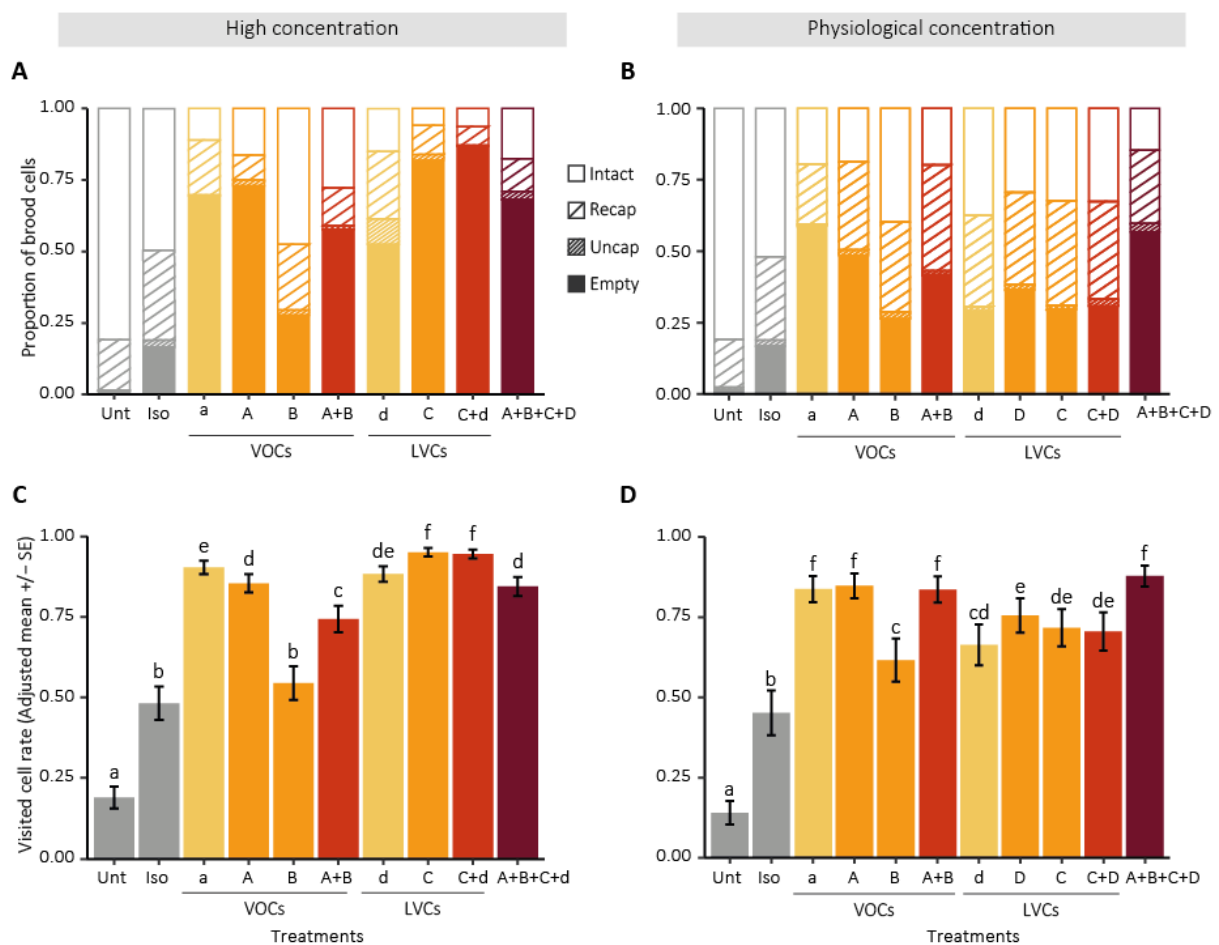


Figure 4.2 : Hygienic response of workers to brood cells treated with different treatments, at two different concentrations, 48h after injection. **A.** Proportion of brood cells in each status (Intact: untouched cells, Recap: recapped cells, Uncap: uncapped cells, Empty: fully cleaned cells). **B.** Adjusted visited cell rate means (+/- SE) of brood cells according to the different treatments. A cell has been visited when it has been cleaned, uncapped or recapped. For each treatment, high concentration (10% w/w): n = 16 colonies, physiological concentration (10 equivalents): n = 15 colonies. Unt: untreated cells, Iso: isohexane, a: *n*-tetradecane, A: five VOCs of Noël *et al.* (Unpublished) (*n*-tetradecane, *n*-hexadecane, benzothiazole, octanoic acid & dodecanoic acid), B: α -pinene & ethyl hexanoate, C: (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene, d: tetracosyl acetate, D: six VPS (2-tricosanone, 2-pentacosanone, tetracosyl acetate, 2-heptacosanone, hexacosyl acetate & 2-nonacosanone). VOCs: Volatile Organic Compounds, LVCs: Low Volatile Compounds. Grey: control, yellow: treatments with one compound, orange: treatments containing compounds found in the same study, red: treatments containing all VOCs or all LVCs, dark red: treatment containing all of the compounds.

3.2. Cleaning behaviour towards characteristic compounds of varroa-infested brood

For the high concentration (10% v/v), there was a significant treatment effect (6h: $\chi^2 = 629.2$, d.f. = 8, $P < 2.2e^{-16}$; 24h: $\chi^2 = 651.34$, d.f. = 8, $P < 2.2e^{-16}$; 6h: $\chi^2 = 629.2$, d.f. = 8, $P < 2.2e^{-16}$; 48h: $\chi^2 = 633.82$, d.f. = 8, $P < 2.2e^{-16}$). All treatments triggered more brood cell cleaning than the solvent control but the *n*-tetradecane solution (“a”), the mix of (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene + tetracosyl acetate (“mix C+d”) and the mix of (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene (“mix C”) triggered it more rapidly than others (Figure 4.3). At 6h and 24h after injection, the highest cleaning rates were triggered by the mix of (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene (“mix C”), the *n*-tetradecane solution (“a”) and the mix of (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene + tetracosyl acetate (“mix C+d”) (Figure 4.3). At 24h, the response to the *n*-tetradecane solution (“a”) and all the solutions containing the five VOCs of Noël *et al.* (five VOCs: “mix A”, five VOCs + α -pinene & ethyl hexanoate: “mix A+B”, and the Mega mix: “mix A+B+C+d”) exceeded 25% of cleaning rate (Figure 4.3). At 48h after injections, all solutions exceeded 50% of cleaning rate, except for the solution of α -pinene & ethyl hexanoate (“mix B” - Figure 4.3). The response triggered by the solution of α -pinene & ethyl hexanoate was still higher than the solvent control but lower than all other solutions (Figure 4.3).

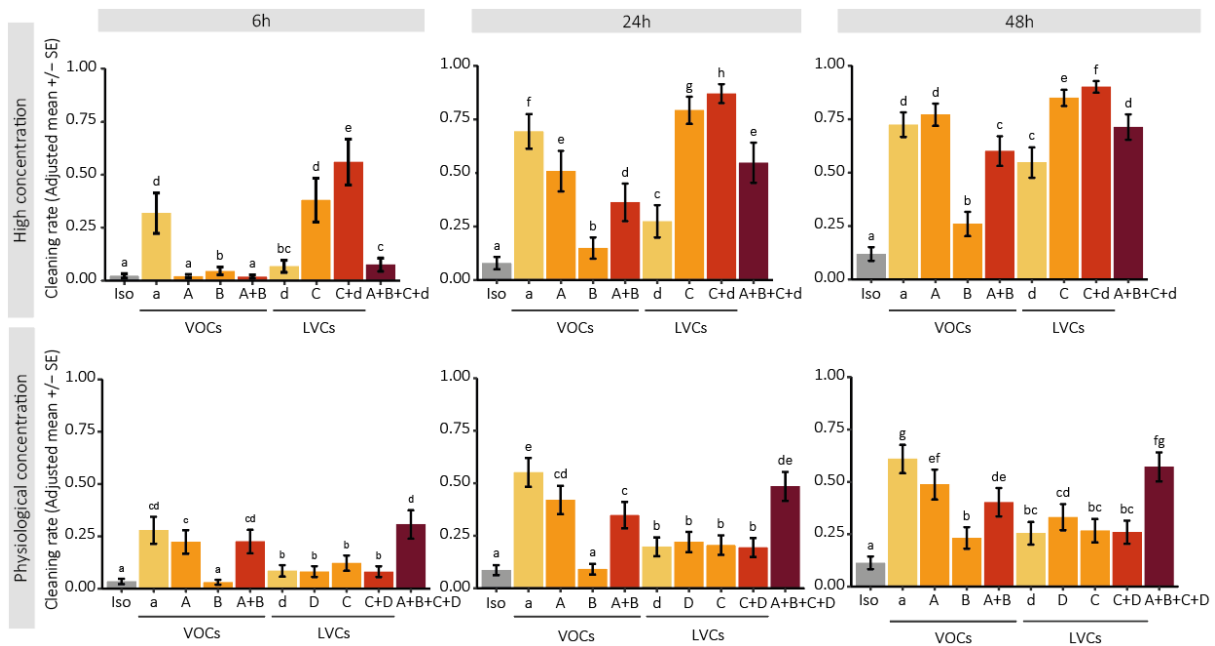


Figure 4.3 : Adjusted cleaning rate means (+/- SE) of injected brood cells according to the different treatments, 6h, 24h and 48h after injections. For each treatment, high concentration (10% w/w): n = 16 colonies, physiological concentration (10 equivalents): n = 15 colonies. Iso: isohexane, a: *n*-tetradecane, A: five VOCs of Noël *et al.* (Unpublished) (*n*-tetradecane, *n*-hexadecane, benzothiazole, octanoic acid & dodecanoic acid), B: α -pinene & ethyl hexanoate, C: (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene, d: tetracosyl acetate, D: six VPS (2-tricosanone, 2-pentacosanone, tetracosyl acetate, 2-heptacosanone, hexacosyl acetate & 2-nonacosanone). VOCs: Volatile Organic Compounds, LVCs: Low Volatile Compounds. Grey: control, yellow: treatments with one compound, orange: treatments containing compounds found in the same study, red: treatments containing all VOCs or all LVCs, dark red: treatment containing all of the compounds.

At physiological concentration (10 equivalents), the modalities triggered significantly different cleaning responses (6h: $\chi^2 = 216.64$, d.f. = 9, $P < 2.2e^{-16}$; 24h: $\chi^2 = 296.11$, d.f. = 9, $P < 2.2e^{-16}$; 6h: $\chi^2 = 629.2$, d.f. = 8, $P < 2.2e^{-16}$; 48h: $\chi^2 = 254.6$, d.f. = 9, $P < 2.2e^{-16}$) (Figure 4.3). The only solution that did not trigger a higher cleaning rate than the solvent control was the solution of α -pinene & ethyl hexanoate, 6h and 24h after injection (“mix B” - Figure 4.3). At 6h, 24h and 48h after injections, the highest cleaning rates were triggered by all solutions containing *n*-tetradecane (*n*-tetradecane: “solution a”, five VOCs: “mix A”, five VOCs + α -pinene & ethyl hexanoate: “mix A+B”, and the Mega mix: “mix A+B+C+d” - Figure 4.3). At 48h after injections, all solutions triggered at least a 25% cleaning rate (Figure 4.3).

3.3. Recapping behaviour towards compounds characteristic of varroa-infested brood

In our study, almost 90% of the injected brood cells were emptied by the workers for several treatments at the 10% concentration, leaving only a small proportion of cells available to measure recapping. The physiological concentration appeared to be more relevant to study this behaviour, as it triggered less brood cell cleaning (40% on average). Recapping of the brood cells varied according to the treatment ($\chi^2 = 202.68$, d.f. = 10, $P < 2.2e^{-16}$) and treated brood cells triggered more recapping than untreated brood cells (Figure 4.4), except for the mix of α -pinene & ethyl hexanoate (“mix B” - Figure 4.4). The highest recapping rates was triggered by all the solutions that contain *n*-tetradecane (*n*-tetradecane: “solution a”, five VOCs: “mix A”, five VOCs + α -pinene & ethyl hexanoate: “mix A+B”, and the Mega mix: “mix A+B+C+d” - Figure 4.4).

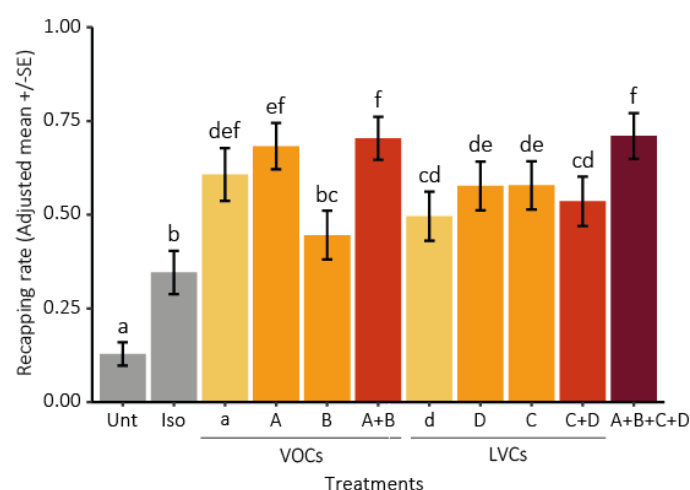


Figure 4.4 : Adjusted recapping rate means (+/- SE) of remaining injected capped brood cells to physiological concentration treatments (10 equivalents). n = 15 colonies. Unt: untreated cells, Iso: isohexane, a: *n*-tetradecane, A: five VOCs of Noël *et al.* (Unpublished) (*n*-tetradecane, *n*-hexadecane, benzothiazole, octanoic acid & dodecanoic acid), B: α -pinene & ethyl hexanoate, C: (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene, d: tetracosyl acetate, D: six VPS (2-tricosanone, 2-pentacosanone, tetracosyl acetate, 2-heptacosanone, hexacosyl acetate & 2-nonacosanone). VOCs: Volatile Organic Compounds, LVCs: Low Volatile Compounds. Grey: control, yellow:

treatments with one compound, orange: treatments containing compounds found in the same study, red: treatments containing all VOCs or all LVCs, dark red: treatment containing all of the compounds.

3.4. Hygienic response of colonies to the treatments depending on the colony VSH scores

Cleaning of injected brood cells was also analysed according to the capacity of colonies to perform VSH (Varroa Sensitive Hygiene) behaviour, 6h after injection. The threshold allowing to classify the colonies in two groups (VSH- and VSH+) is set at 47% VSH in order to place it at the most optimum point in the trend changes of the curves (Figure S4.1). For the high concentration, except for the solvent control, the five VOCs (“mix A”) solution and the α -pinene & ethyl hexanoate (“mix B”), all solutions triggered a different response according to the ability of the colonies to perform the VSH behaviour (Figure 4.5). VSH+ colonies responded more than VSH- colonies. The most pronounced difference between the two groups (VSH+/VSH-) was triggered by the *n*-tetradecane solution (“a”) and the (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene solution (“mix C”) (Figure 4.5). For the physiological concentration, solutions containing tetracosyl acetate (tetracosyl acetate: “d”, VPS: “mix D”, (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene + VPS: “mix C+D” and the Mega mix: “mix A+B+C+D”) and VOCs treatments that do not contain α -pinene & ethyl hexanoate (*n*-tetradecane: “a” and five VOCs: “mix A”) triggered significantly different rates of brood cell cleaning depending on the VSH capacity (Figure 4.5).

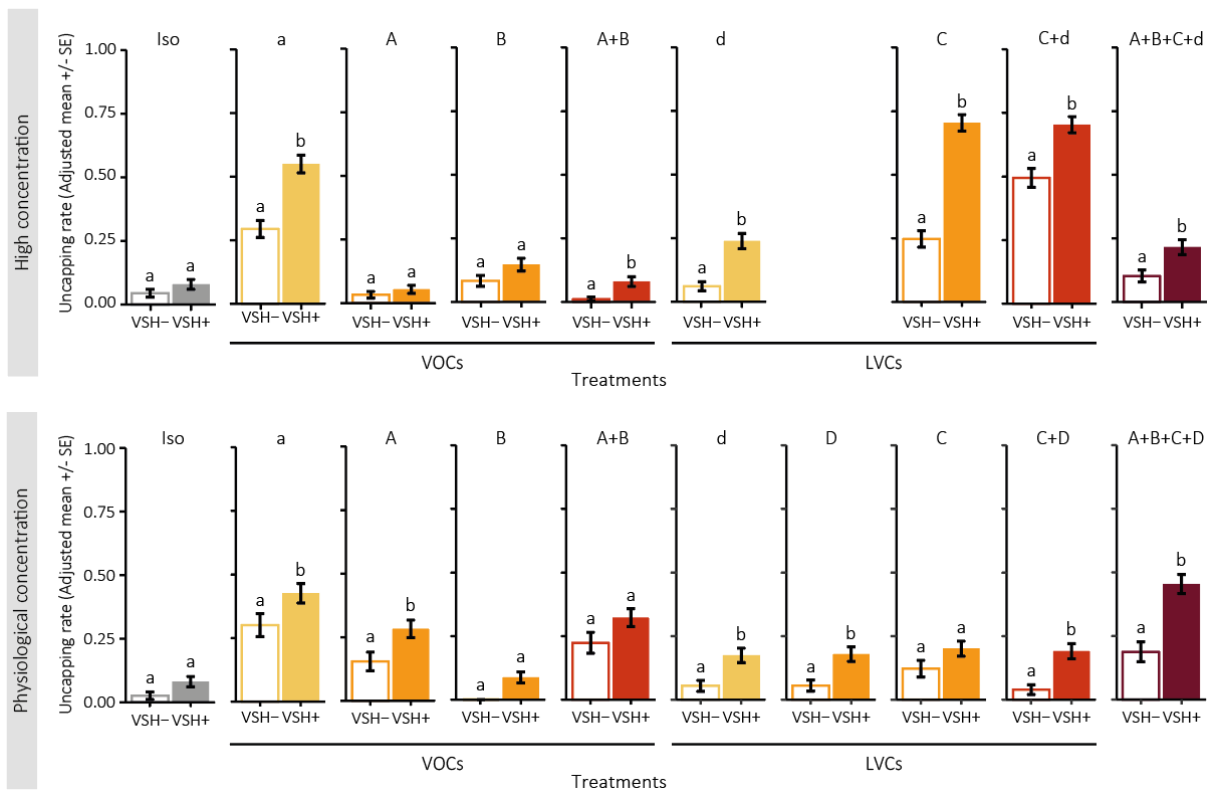


Figure 4.5: Adjusted cleaning rate means (+/- SE) of injected brood cells according to the two VSH groups (VSH+/VSH-) and the different treatments, 6h after injections. For each treatment, for the high concentration (10%

w/w), VSH+: n = 5 colonies, VSH-: n = 7 colonies; for the physiological concentration (10 equivalents), VSH+: n = 6 colonies, VSH-: n = 5 colonies. Iso: isoheptane, a: *n*-tetradecane, A: five VOCs of Noël *et al.* (Unpublished) (*n*-tetradecane, *n*-hexadecane, benzothiazole, octanoic acid & dodecanoic acid), B: α -pinene & ethyl hexanoate, C: (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene, d: tetracosyl acetate, D: six VPS (2-tricosanone, 2-pentacosanone, tetracosyl acetate, 2-heptacosanone, hexacosyl acetate & 2-nonacosanone). VOCs: Volatile Organic Compounds, LVCs: Low Volatile Compounds. Grey: control, yellow: treatments with one compound, orange: treatments containing compounds found in the same study, red: treatments containing all VOCs or all LVCs, dark red: treatment containing all of the compounds.

4. Discussion

In this study, we compared the response of workers to different mixtures of compounds described as specific to parasitized brood cells and triggering a hygienic response. Compounds used can be classified into two categories: volatile organic compounds (VOCs) with a high volatility (Liendo *et al.*, 2021; Noël *et al.*, Unpublished) and low volatile compounds (LVCs) with a lower volatility (Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Wagoner *et al.*, 2020). Using solutions containing one to fifteen compounds, we looked at the cleaning and recapping behaviour of the workers. The cleaning behaviour of each solution was also studied according to the ability of the colonies to perform the VSH behaviour. We found a greater effect on brood cell cleaning with the treatment containing VOCs and LVCs at physiological concentration. VOCs were more involved in recapping behaviour than LVCs, especially the VOCs identified by Noël *et al.* (Unpublished) (*n*-tetradecane, *n*-hexadecane, benzothiazole, octanoic acid, dodecanoic acid). The cleaning behaviour according to the treatments differed with the ability of the colony to perform the VSH behaviour.

4.1. Effects of compounds on worker cleaning behaviour

Compounds inducing a behavioural response such as pheromones or other semiochemicals are often optimal at a specific dose and no longer induce responses at lower or higher doses (Wyatt, 2014). Therefore, we decided to use two different concentrations to study the behaviour induced by the characteristic compounds of parasitized brood: a physiological concentration (10 equivalents) and a high concentration (10% w/w) of compounds. The use of a high concentration to test worker behaviour to compounds is commonly used in the scientific literature (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Noël *et al.*, Unpublished; Wagoner *et al.*, 2021). The use of this concentration ensures that potential worker non-response is not due to a low concentration. A physiological dose approaches the natural emissions of *Varroa destructor* parasitized brood cells. The dose of 10 equivalents was selected according to the results found by Mondet *et al.* (2021). In the latter study, the response of workers to VPS treatment at 10 equivalents was low enough to observe potential synergistic or additive effects with treatments containing VPS and other compounds. At this concentration, 48h

after injection, our results agree with those found by Mondet *et al.* (2021) (“Mix D”: Mondet *et al.*, 2021: 40% cleaning, here: 38% cleaning).

All the compounds tested have been previously described as triggering hygienic behaviour (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Noël *et al.*, Unpublished; Wagoner *et al.*, 2021), but the mixture of these compounds on triggering hygienic behaviour had never been tested before. LVCs alone (“d”, “mix D”, “mix C”, “mix C+D”) triggered a stronger response from workers at high concentrations than at physiological concentrations. The lower response of workers to LVCs at physiological concentration could reflect a lower perception of these compounds by the workers. Since these compounds are not very volatile, increased concentration may favour the passage of compounds through the waxy cap and promote perception of this chemical message by the workers. Also, at high concentrations, a greater effect can be observed when the LVCs are all present (“mix C+d”), which was not observed at the physiological concentration. This effect can be seen as early as 6h after injection. This suggests a prioritisation of the workers to a more complex message. This strong response of workers to LVCs confirmed the crucial role of these compounds in cleaning behaviour (Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Wagoner *et al.*, 2020; Wagoner *et al.*, 2021). The treatment with *n*-tetradecane alone (“a”) triggered a faster response from the workers compared to all other treatments containing VOCs. This prioritisation of cleaning for this compound has already been observed in Noël *et al.* (Unpublished). In contrast to LVCs, increase the complexity of the VOCs mixture did not necessarily increase the cleaning of the cells. With *n*-tetradecane (“a”, “A”, “A+B”), the more complex the mixture, the lower the response of the workers to the treatments. A chemical compound alone may trigger one behaviour, while mixing it may trigger another. This is the case when the odours of two host plants are presented separately, they have an attractive power for female gravid hawkmoths (*Manduca sexta*), while when these two odours are mixed, they are repulsive (Späthe *et al.*, 2013). This difference in worker response to mixtures of VOCs could be explained by a brain process of combinatorial coding (Haverkamp *et al.*, 2018). At physiological dose, the mixture of all compounds triggered a greater cleaning response than the mixture of LVCs and the mixture of VOCs. It has already been suggested that highly volatile compounds coupled with less volatile compounds can be used as chemical cues by workers to identify brood cells and then to remove it (McAfee *et al.*, 2018; Mondet *et al.*, 2021). The present study confirms that VOCs could be used by the workers to target the cell to be uncapped. Uncapping would allow the workers to access the LVCs and thus to trigger cleaning of the brood cell. This is also confirmed at high concentration, where the passage of LVCs through the cap would be sufficient to identify the cells to be cleaned, even before the uncapping event.

4.2. Differential hygienic response depending on the protocol used

The low cleaning rate of brood cells injected with α -pinene & ethyl hexanoate (“mix B”) observed in our study is not in accordance with the results found by Liendo *et al.* (2021). The authors showed that the combination of these two compounds triggered a cleaning rate of 70% (Liendo *et al.*, 2021). In our study, this cleaning rate did not exceed 25%, even at the highest concentration. Nevertheless, our two tested concentrations were below the concentration tested by Liendo and co-workers (this study: 10% w/w maximum; Liendo *et al.*, 2021: 2 μ L of pure compounds). In addition, the brood cell treatment methods were different between the two studies. Liendo *et al.* (2021) applied the compounds on a filter paper that was inserted into the brood cell, whereas we injected our mixtures directly into the brood cell to be able to compare with other already published compounds and to have a standardised protocol. These differences in protocols could explain the different results.

4.3. A more important role of VOCs than LVCs in recapping behaviour

Brood cell cleaning was more extensive with the high concentration treatments. Recapping behaviour is not easy to study as the injection of compounds into the brood cells triggered hygienic behaviour thereby reducing the number of cells available to quantify recapping. To be able to truly measure it, it would be necessary to inhibit the removal of pupae from brood cells by the workers. We thus decided to study the recapping behaviour of the remaining cells 48 hours after injection only for the lowest concentration, where more uncleaned cells were present. The highest recapping rates were triggered by all solutions containing VOCs found by Noël *et al.* (“a”, “mix A”, “mix A+B” and “mix A+B+C+D”) (Noël *et al.*, Unpublished). It has been assumed that highly volatile compounds would trigger the opening of the brood cell and the lack of less volatile compounds would trigger its recapping (Grindrod & Martin, 2021; Martin *et al.*, 2020). In this hypothesis, we should have observed marked differences in recapping between solutions containing only VOCs (“a”, “mix A”, “mix B” and “mix A+B”) and solutions containing only LVCs (“mix C”, “d”, “mix D” and “mix C+D”). However, as the recapping rate of the total mixture (“mix A+B+C+D”) was similar to the recapping rate of the VOC mixture (“mix A+B”) and lower than that induced by LVCs (“mix C+D”), this suggests that VOCs may play a more important role than LVCs in the recapping behaviour.

4.4. Relationship between treatments effect and colony VSH behaviour performance

The relationship between treatment response and VSH score differed between treatments and doses. All treatments containing VPS compounds (“d”, “D”, “mix C+D”, “mix C+d”, “mix A+B+C+D”) triggered more hygienic behaviour in VSH+ colonies than in VSH- colonies, regardless of the dose tested.

This is also the case for the treatment containing only *n*-tetradecane. To date, only the six VPS have been shown to be strongly related to the ability of the colony to perform VSH behaviour (Mondet *et al.*, 2021). Our results confirm the effect of VPS and show that a VOC can also be linked to the VSH behaviour of colonies. The effect of certain compounds on the hygienic behaviour in VSH+/- colonies was dose dependent. The mixture of all VOCs ("mix A+B"), as well as the mixture of (Z)-10-tritriacontene & (Z)-6-pentadecene ("mix C"), was only related to VSH behaviour at high concentrations. It was shown that the cleaning of brood cells treated with (Z)-10-tritriacontene and with (Z)-6-pentadecene alone was performed in the same way in colonies selected for their VSH character, colonies selected for their hygienic capacity and non-selected colonies (Wagoner *et al.*, 2020). The observation that the (Z)-10-tritriacontene & (Z)-6-pentadecene mixture ("mix C") only triggered a difference in cleaning at high doses between VSH+ and VSH- colonies suggests that another signal was missing in the chemical message for VSH+ colonies at physiological doses. The mixture of the five VOCs identified in Noël *et al.* (Unpublished) ("mix A") was only related to VSH behaviour at physiological doses. However, since the treatments were tested at the same time on the same brood frames, prioritization of workers for other treatments may explain this. Indeed, regardless of colony type (VSH-/+), three treatments were intensively cleaned ("a", "C", and "C+d") compared to the treatment containing all five VOCs ("A"). To make these results stronger, it would be interesting to increase the number of colonies tested to see if the link between certain compounds and the VSH capacity of the colony is confirmed under different environments.

5. Conclusion

This study shows the impact of a blend of compounds on the VSH behaviour. Workers use VOCs coupled with LVCs to precisely detect parasitized cells. Additive and synergic effects of compounds on cleaning and recapping behaviours have not been observed. One VOC, *n*-tetradecane, as well as tetracosyl acetate (Mondet *et al.*, 2021) appear to be key cues in the achievement of VSH behaviour. We show here that the detection of these two compounds could be particularly linked to the ability of the colony to perform VSH defences. Further studies are needed to fully understand the mechanisms underlying recapping behaviour, but this study shows the importance of the presence of VOCs in the performance of this behaviour.

6. Acknowledgments

We thank G. Costagliola for his advice in the determination of parasitized brood cell equivalent. This work was supported by a PRIMA grant (EU H2020, #1812, "Plant-B").

III. Conclusion

Dans ce chapitre, le comportement VSH et de réoperculation des ouvrières a été testé vis-à-vis des quinze molécules identifiées comme déclenchant une activité d'hygiène dans la littérature ou dans le cadre de cette thèse. L'article 3 illustre l'impact de ces composés sur le comportement des adultes avec un protocole uniformisé. Le test de deux concentrations différentes permet de se rapprocher des protocoles réalisés dans la littérature (forte concentration) et de tester une concentration plus proche des conditions biologiques (concentration physiologique). Les composés ont été présentés en mélange de COVs, de composés à faible volatilité et avec un mélange regroupant les COVs et les composés à volatilité faible pour observer l'effet de la volatilité des composés sur les deux comportements de résistance. Nous avons confirmé l'effet des quinze molécules sur le comportement hygiénique des ouvrières.

Le message de parasitisme est un message complexe composés de plusieurs molécules et les résultats de cette étude permettent de mieux comprendre l'impact des signaux chimiques sur le comportement VSH et de réoperculation. L'observation d'un nettoyage plus important des alvéoles de couvain en présence de COVs et de composés faiblement volatiles illustre la dépendance de la communication chimique vis-à-vis de la présence d'un complexe de plusieurs composés. L'émission de composés faiblement volatiles par l'alvéole de couvain joue un rôle plus important dans le nettoyage que dans la réoperculation des alvéoles de couvain. Ceci confirme l'importance des COVs dans la détection des alvéoles de couvain et l'importance des composés faiblement volatiles dans le sacrifice de la nymphe parasitée. Les COVs semblent liés à la capacité des colonies à réaliser le comportement VSH. Pour les composés de parasitisme VPS, seules les ouvrières réalisant le comportement VSH arrivent à discriminer les odeurs de couvain sain couplé aux VPS des odeurs de couvain sain (Mondet *et al.*, 2021). Un avantage similaire dans la discrimination des odeurs pourrait exister pour les autres composés. Des études supplémentaires sont nécessaires afin de comprendre les mécanismes mis en jeu dans le traitement neuronal de ces messages chimiques chez les individus réalisant le comportement VSH.

CHAPITRE 5
DISCUSSION GÉNÉRALE



La réopercolation des alvéoles

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I. Conclusions

Le comportement hygiénique vis-à-vis du couvain, qu'il soit dirigé contre du couvain malade ou mort, nécessite trois étapes : (i) l'émission d'un message sémiachimique par l'alvéole de couvain infesté ou malade, (ii) la détection et le traitement neuronal de ce message par les ouvrières, (iii) la réponse comportementale des ouvrières. L'objectif de cette thèse était d'approfondir les connaissances sur le comportement hygiénique impliqué dans la résistance des colonies d'abeilles domestiques envers le parasite *Varroa destructor*, et particulièrement en participant au décryptage du comportement VSH (*Varroa Sensitive Hygiene*) et du comportement de réoperculation. Pour cela, la première et la troisième étapes du comportement hygiénique ont été plus spécifiquement abordées avec :

- ▶ Une approche de chimie visant à étudier les émissions de COVs (Composés Organiques Volatiles) du couvain sain lors de son développement ainsi que du couvain parasité par varroa. Cette approche a été exposée au **Chapitre 2** et **Chapitre 3**.
- ▶ Une approche comportementale des ouvrières vis-à-vis des composés caractérisant le couvain parasité par *V. destructor*. Pour cela, le comportement VSH et celui de réoperculation ont été mesurés vis-à-vis de composés présentant différents degrés de volatilité. Cette approche a fait l'objet du **Chapitre 3** et du **Chapitre 4**.

1. La fluctuation des émissions du couvain et le parasitisme par varroa

Afin de mieux comprendre l'impact du parasitisme par *V. destructor* sur les émissions du couvain, il est crucial de bien connaître les émissions de l'hôte, *A. mellifera*, dans un contexte de développement sain. Dans cette thèse, les émissions de COVs du couvain sont présentées pour un couvain sain tout au long de son développement ainsi que pour un couvain parasité ou non par varroa durant les sept premiers jours du développement operculé (période durant laquelle les ouvrières réalisent des comportements hygiéniques). L'un des atouts des captures de COVs réalisées dans ces deux études est le suivi répété des émissions au cours du développement. Le fait de réaliser les captures toutes les 24h permet d'obtenir une bonne vision de la dynamique des émissions de chaque composé au fil de la maturation du couvain avec et sans présence de varroa.

a. Les émissions de volatiles par le couvain : de potentiels composés phéromonaux dans la communication couvain-adultes et kairomonaux pour varroa ?

Les signaux olfactifs sont primordiaux pour les nourrices afin qu'elles puissent adapter leur comportement en fonction de l'âge des immatures ; de plus, les émissions volatiles du couvain varient considérablement entre chaque stade du développement du couvain. Actuellement, deux phéromones du couvain ont été identifiées ; la BEP (*Brood Ester Pheromone*) constituée d'un mélange de dix esters et le (*E*)- β -ocimène (Le Conte *et al.*, 1990b; Maisonnasse *et al.*, 2009). Contrairement aux composés constituant la BEP, le (*E*)- β -ocimène est très volatile. Aucun autre COV émis par le couvain n'a été montré comme ayant une activité phéromonale, mais plusieurs études ont décrit des émissions de COVs durant le développement larvaire et nymphale (Carroll & Duehl, 2012; Haber *et al.*, 2019b; Light *et al.*, 2020b; Maisonnasse *et al.*, 2010; Schöning *et al.*, 2012). Dans cette thèse, nous avons identifié 32 COVs émis par le couvain sain au cours de son développement, dont la phéromone (*E*)- β -ocimène. Les travaux réalisés dans le chapitre 2 peuvent servir de base exploratoire pour investiguer le rôle de chacune des molécules qui apparaissent avec des profils temporels très marqués. Par exemple, le composé émis en fin de développement, le nonadécène, pourrait être un indice d'augmentation à venir du nombre d'ouvrières naissantes au sein de la colonie et ainsi avoir un effet sur divers comportements comme la ponte ou le butinage (Figure 5.1). Les cinq COVs émis par les œufs (α -pinène, β -pinène, (*E*)- β -ocimène, benzoate de méthyle et γ -octalactone) pourrait être impliqué dans le marquage des œufs par les reines et, par conséquent, pourrait permettre aux ouvrières de distinguer les œufs pondus par la reine de ceux pondus par les ouvrières (Ratnieks, 1995) (Figure 5.1). C'est au moment de l'operculation que l'émission de COVs captés est la plus variée en termes de composés et de familles chimiques. Ces résultats suggèrent que ces COVs pourraient avoir un rôle dans le comportement d'operculation des ouvrières (Figure 5.1), à l'image des esters méthyliques de la BEP qui déclenchent le comportement d'operculation des alvéoles de couvain par les ouvrières (Le Conte *et al.*, 1990b).

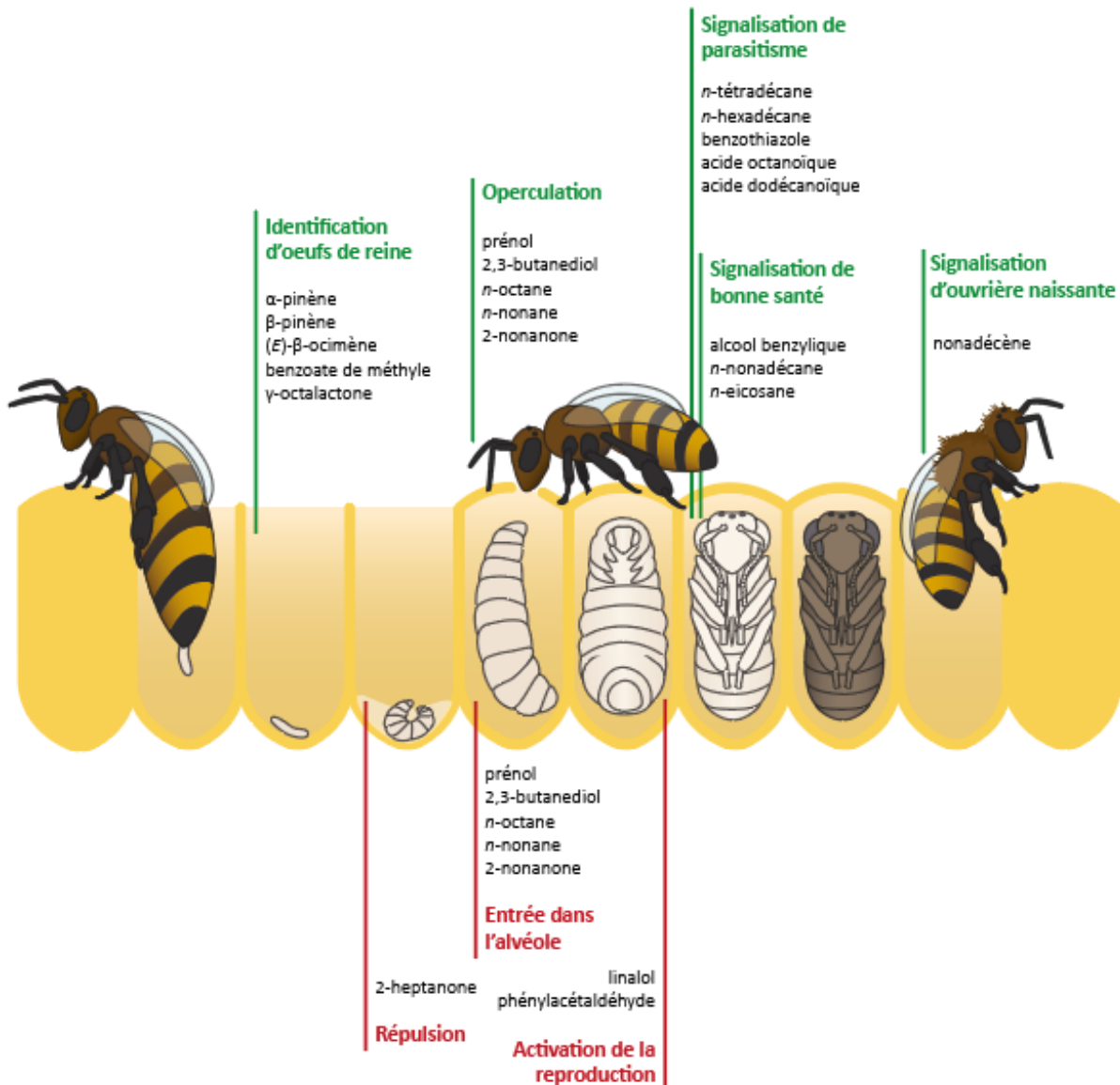


Figure 5.1: Composés organiques volatiles identifiés dans le cadre de cette thèse qui pourraient potentiellement jouer un rôle dans la communication entre le couvain et les ouvrières (vert) et entre le couvain et *Varroa destructor* (rouge).

Le changement d'émissions du couvain au cours du développement pourrait aussi être utilisé par le varroa afin de détecter les alvéoles à parasiter ou d'autres étapes de sa physiologie comme sa reproduction. Dans la BEP, les palmitate de méthyle et d'éthyle sont des kairomones pour *V. destructor* (Le Conte *et al.*, 1989; Liu *et al.*, 2022). Ce dernier utilise les signaux émis par la larve pour s'orienter et identifier les alvéoles en operculation dans lesquelles il vient se reproduire. Il serait intéressant de tester l'attraction ou la répulsion du varroa envers les COVs émis par la larve, notamment ceux émis au moment de l'operculation (Figure 5.1). Il est à noter que quatre composés captés lors des captures sur les émissions du COVs du couvain au cours de son développement sont des phéromones d'ouvrière : le 2-heptanone, le 1-octanol, le 1-hexanol et l'acétate d'isoamyle. Le 2-heptanone est sécrété par les

glandes mandibulaires des abeilles mellifères (Butler, 1966; Shearer & Boch, 1965). Ce composé participe au recrutement des ouvrières lorsqu'il est émis dans le bouquet de la phéromone d'alarme (Shearer & Boch, 1965). Il est aussi présent dans les émissions de COVs de la gelée royale (Isidorov *et al.*, 2012). Il a déjà été montré que le gelée royale avait un effet répulsif pour le varroa (Nazzi *et al.*, 2009). Des enregistrements par électrotarsogrammes ont montré que *V. destructor* répondait au 2-heptanone (Light *et al.*, 2020a). Ce composé a été capté à tous les stades larvaires avec un pic au 4^{ème} stade. De ce fait, il pourrait participer à l'évitement du *Varroa* pour les larves trop jeunes (Figure 5.1). Les pics d'émission du 1-hexanol et du 1-octanol ont été captés au 5^{ème} stade larvaire, au moment de l'operculation ainsi qu'au stade larve scellée. Le parasite *Varroa* s'infiltré dans les alvéoles de couvain au moment de l'operculation. Néanmoins, ces deux alcools ont un effet répulsif sur le *Varroa* lors de tests de choix d'adulte ouvrière à parasiter (Kraus, 1990). Du fait de l'émission de ces composés au moment de l'operculation, l'effet répulsif de ces deux alcools pourrait donc dépendre du contexte, de la dose ou du mélange de composés émis. L'isoamyle acetate a été montré comme étant une kairomone d'agrégation pour le petit coléoptère des ruches (*Aethina tumida*) (Torto *et al.*, 2007), une espèce invasive parasite des colonies d'abeilles qui pond ses œufs dans les alvéoles de couvain (Neumann *et al.*, 2016). Aucun effet kairomonal pour varroa n'a été rapporté pour ce composé. Il n'a d'ailleurs pas d'effet attractif, ni répulsif (Kraus, 1990). La reproduction du varroa est activée dans les 12h à 36h après operculation, par des composés de la BEP (palmitate, stéarate et oléate d'éthyle) émis par la larve operculée (Frey *et al.*, 2013; Garrido & Rosenkranz, 2004; Trouiller & Milani, 1999). Les composés trouvés ayant leur pic d'émission au stade larve operculée (ex : linalol et phénylacétaldéhyde), lors des captures de COVs sur le couvain sain, pourraient aussi avoir une action sur la reproduction de l'acarien (Figure 5.1). Le linalol a déjà été montré comme stimulant la ponte chez *Chrysopa phyllochroma* ainsi que chez le sphinx du tabac (*Manduca sexta*) (Reisenman *et al.*, 2010; Riddick, 2020). Le phénylacétaldéhyde, quant à lui, stimule la reproduction du chrysiope *Chrysoperla carnea* (Riddick, 2020). Des études supplémentaires sont nécessaires afin de déterminer si ces deux COVs sont impliqués dans l'activation de la ponte chez la fondatrice varroa.

b. Quel impact du varroa sur les émissions de COVs du couvain ?

L'étude des émissions du couvain parasité et non-parasité a mis en évidence neuf composés (dont cinq identifiés) émis en plus grande quantité chez le couvain parasité. Ces composés sont répartis en trois familles chimiques : deux alcanes (tétradécane et hexadécane), deux acides carboxyliques (acide octanoïque et acide dodécanoïque) et un benzothiazole (benzothiazole) (Figure 5.1). Cette étude a également permis de mettre en évidence quatre composés de la famille des alcanes, chacun étant émis en plus grande quantité par le couvain sain que par le couvain parasité, dont trois ayant pu être identifiés

(alcool benzylique, nonadécane, *n*-eicosane) (Figure 5.1). Ces deux derniers composés sont émis par les nymphes non sclérotisées et pourraient être considérés comme des composés de bonne santé (Noël *et al.*, 2023).

La source d'émission des quatre composés caractéristiques du couvain parasité n'a pas été identifiée. Il pourrait s'agir aussi bien des émissions de la (pré)-nymphe, du varroa ou bien du couple (pré)-nymphe et varroa. *A contrario*, les potentiels composés de bonne santé sont bien émis par le couvain. Cette baisse d'émission de composés en cas de parasitisme suggère une potentielle altération physiologique due à l'acarien. Les changements dans les mélanges chimiques émis par le couvain avec ou sans parasitisme sont principalement décrits lors du parasitisme (Liendo *et al.*, 2021; Martin *et al.*, 2002; Mondet *et al.*, 2016a; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Salvy *et al.*, 2000; Schöning *et al.*, 2012; Wagoner *et al.*, 2019; Wagoner *et al.*, 2020). Il est connu que, chez l'abeille mellifère, la composition relative de chaque composé (la proportion de chaque molécule d'un bouquet d'odeurs) caractérise des messages phéromonaux (Engels, 1986; Free, 1987; Slessor *et al.*, 2005). Par exemple, les différences dans les ratios des esters de la BEP dans le bouquet phéromonal émis par les larves sont impliquées dans la reconnaissance de l'âge des larves par les ouvrières (Le Conte *et al.*, 1994b). Ainsi, le message de parasitisme émis par l'alvéole de couvain pourrait aussi bien être constitué de composés émis seulement (ou en plus grande quantité) lors de la présence de varroa au sein de l'alvéole et de composés émis en plus faible quantité (voire complètement absents). La reproduction de la fondatrice varroa ainsi qu'une nymphe à la physiologie altérée déclenche une augmentation des émissions de BEP et de VPS par rapport à une nymphe saine (Mondet *et al.*, 2016a; Mondet *et al.*, 2021). Une infection virale par le KBV ou le DWV modifie les émissions du couvain (Mondet *et al.*, 2016a; Schöning *et al.*, 2012; Wagoner *et al.*, 2019). Il n'est pas possible de savoir si l'émission de ces COVs identifiés comme caractéristiques du couvain parasité est due à un état physiologique altéré tel qu'une infection à un virus, une plaie infligée par la nutrition du varroa, ou encore à une réponse de la nymphe au parasitisme. Des études complémentaires sont nécessaires afin de montrer une relation entre les émissions de ces COVs du couvain, les infections virales et le parasitisme.

2. Comment les émissions de COVs influent le comportement des ouvrières ?

Les ouvrières réalisant le comportement VSH sont capables de détecter les alvéoles de couvain parasitées par *V. destructor*, de les ouvrir et de les nettoyer de leur contenu. Cette détection passe principalement par des stimuli olfactifs (Le Conte *et al.*, 2011; Liendo *et al.*, 2021; Mondet *et al.*, 2015; Mondet *et al.*, 2016a; Navajas *et al.*, 2008; Nazzi *et al.*, 2004a; Wagoner *et al.*, 2019; Wagoner *et al.*, 2020; Wagoner *et al.*, 2021).

DISCUSSION

Les deux approches abordées dans les tests comportementaux de cette thèse apportent un aperçu intéressant quant aux composés olfactifs qui permettent la détection des alvéoles parasitées. Lors des premiers tests, le comportement des ouvrières a été testé face aux cinq composés spécifiques du parasitisme de varroa identifiés dans cette thèse (tétradécane, hexadécane, benzothiazole, acide octanoïque et acide dodécanoïque) ainsi qu'à un mélange de ces composés. Ces résultats montrent que les COVs participent à la détection des alvéoles de couvain parasité. La réponse de nettoyage rapide des ouvrières face à un composé précis, le tétradécane, le classe comme un signal potentiellement capital dans cette détection. De plus, le mélange de ces cinq COVs semble jouer un rôle dans le comportement de réoperculation, particulièrement lorsqu'ils sont testés en mélange. Les COVs identifiés dans le cadre de cette thèse ont ensuite été testés avec des composés décrits dans la littérature scientifique comme impliqués dans le déclenchement du comportement VSH. Des solutions contenant un à quinze composés décrits impliqués dans le déclenchement du comportement VSH chez les ouvrières ont été testées. Deux concentrations ont été utilisées, pour permettre d'étudier le comportement de abeilles face à une dose forte (10% p/p) et une dose physiologique (10 équivalents). Comme il n'était pas réalisable de tester toutes les combinaisons possibles, nous avons choisi de tester des mélanges en fonction du caractère volatile des composés : des mélanges contenant les COVs, des mélanges contenant les composés moins volatiles et un mélange contenant tous les composés. Les résultats confirment l'activité des COVs identifiés dans cette thèse (Noël *et al.*, Unpublished), ainsi que les COVs (dans une moindre mesure) et composés faiblement volatiles identifiés dans la littérature scientifique (Liendo *et al.*, 2021; Mondet *et al.*, 2021; Nazzi *et al.*, 2004a; Wagoner *et al.*, 2020; Wagoner *et al.*, 2021) sur le comportement de nettoyage des alvéoles de couvain. Tandis que la présence de composés faiblement volatiles et de COVs semble importante dans le déclenchement du nettoyage des alvéoles de couvain, les COVs semblent induire une activité plus importante que les composés faiblement volatiles dans le cadre de la réoperculation (Figure 5.2).

DISCUSSION

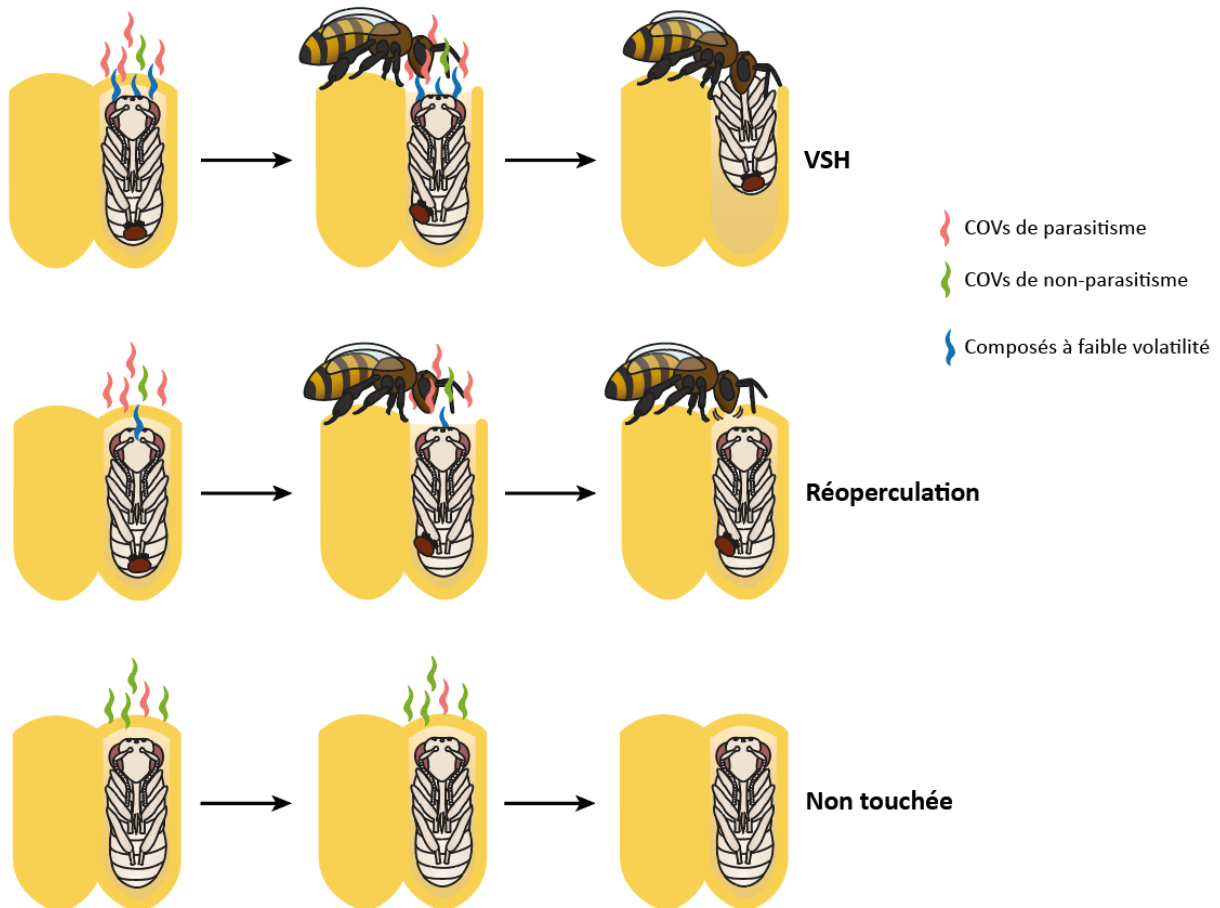


Figure 5.2 : Représentation schématique de la détection par les ouvrières des alvéoles de couvain parasitées par varroa. La santé du couvain serait identifiée grâce à de subtiles changements dans ses émissions de COVs (Composés Organiques Volatiles) qui permettraient aux ouvrières d'identifier les alvéoles de couvain parasitées. La présence de composés à faible volatilité aurait un rôle prépondérant dans le déclenchement du comportement VSH (*Varroa Sensitive Hygiene*), alors que les COVs induiraient une activité plus importante dans le comportement de réoperculation que les composés à faible volatilité.

L'un des objectifs de cette thèse est de décrypter les mécanismes du comportement de réoperculation, et plus particulièrement de savoir si le comportement de réoperculation est une composante du comportement VSH ou bien un comportement à part entière. Le début de leurs séquences comportementales est identique : (i) les abeilles détectent puis (ii) ouvrent les alvéoles, c'est dans la troisième étape que le devenir du contenu de l'alvéole diffère. Dans le comportement VSH, (iii-a) l'alvéole est complètement vidée de son contenu. Dans le cas du comportement de réoperculation, (iii-b) l'alvéole est refermée sans que la (pré)-nymphe et le parasite ne soient touchés. Ce comportement perturbe la reproduction du varroa, limitant ainsi le développement de la population d'acariens dans la colonie, tout en préservant la nymphe (Oddie *et al.*, 2021). Ces dernières années, l'étude de ce comportement a connu un essor et a été décrite dans un grand nombre de colonies (Grindrod & Martin, 2021; Hawkins & Martin, 2021; Luis *et al.*, 2022; Oddie *et al.*, 2018; Oddie *et al.*, 2021). Ce comportement ne serait pas déclenché par la reproduction du varroa (Sprau *et al.*, 2021).

Cependant, la raison pour laquelle certaines alvéoles sont nettoyées et d'autres refermées n'est pas encore élucidée et le choix pourrait bien être induit par des composés sémiocchimiques comme c'est le cas pour le comportement hygiénique en général. Deux hypothèses sont faites :

- ▶ L'hypothèse de deux comportements distincts part du postulat que l'entière séquence comportementale soit déclenchée par des stimuli différents. Dans ce cas, des ouvrières spécialisées dans le comportement de réoperculation des alvéoles parasitées pourraient être présentes au sein des colonies résistantes, à l'image des individus spécialistes du comportement VSH. Le comportement VSH est réalisé par des ouvrières spécialistes qui présentent une sensibilité olfactive et neuronale accrue (Hu *et al.*, 2016; Le Conte *et al.*, 2011; Mondet *et al.*, 2015).
- ▶ L'hypothèse d'un même comportement avec deux finalités distinctes signifierait qu'un stimulus déclenche l'ouverture de l'alvéole et que d'autre(s) stimulus/stimuli déclenche(nt) le nettoyage ou la réoperculation. L'émission de COVs pourrait déclencher l'ouverture de l'alvéole et la présence de composés moins volatiles spécifiques déclencherait le nettoyage, quand leur absence déclencherait la réoperculation (Grindrod & Martin, 2021; Martin *et al.*, 2020).

Les résultats des différentes études de cette thèse tendent à supporter la deuxième hypothèse. Le faible nettoyage des alvéoles de couvain traitées avec des composés à faible volatilité à concentration physiologique par rapport à la concentration haute illustre le fait que les composés diffusent difficilement à travers l'opercule. Lorsque l'alvéole de couvain émet à la fois ces composés faiblement volatiles et des COVs, le nettoyage des alvéoles de couvain par les ouvrières est alors plus important. Cela évoque que (i) la présence d'un message plus volatile est nécessaire dans la détection puis (ii) dans l'ouverture de l'alvéole et que (iii-a) la présence de molécules faiblement volatiles déclenche le nettoyage. Le taux de réoperculation est plus important lorsque l'alvéole de couvain émet des COVs que lorsqu'elle n'émet que des composés à faible volatilité, suggérant (iii-b) la nécessité d'un message très volatile dans le processus de réoperculation des alvéoles de couvain. Néanmoins, tous les COVs utilisés dans l'étude 3 ne déclenchent pas de comportement de réoperculation. Le taux de réoperculation est augmenté lorsque les cinq COVs identifiés dans cette thèse (tétradécane, hexadécane, benzothiazole, acides octanoïque et dodécanoïque) sont émis par l'alvéole, ce qui n'est pas le cas lorsque ces composés sont testés seuls, ou en présence d' α -pinène et d'hexanoate d'éthyle. Cela suggère la nécessité d'un message très volatile complexe. Les COVs induisent également le nettoyage. Si l'émission de COVs sans composés peu volatiles induisait seulement le comportement de réoperculation, nous n'aurions dû observer qu'une absence ou qu'un faible taux de nettoyage des alvéoles de couvain traitées avec les COVs. Ce nettoyage pourrait être dû à un message volatile incomplet. Il ne faut pas oublier que seulement cinq des neuf COVs de parasitisme ont été identifiés

avec succès et donc testés en comportement. Les quatre COVs inconnus pourraient être une pièce manquante au puzzle du message volatile de parasitisme. L'ouverture des alvéoles de couvain pourrait être médiée par une combinaison spécifique de COVs et l'absence ou la présence de certains composés pourrait déclencher une inhibition ou un changement comportemental, tel qu'une diminution du comportement de réoperculation et une augmentation du comportement de nettoyage (Haverkamp *et al.*, 2018).

Lors des expérimentations réalisées dans cette thèse, l'aire (surface de réoperculation de l'alvéole) de la réoperculation sur l'opercule des alvéoles pouvait varier en fonction du composé injecté. Ces observations ont été remarquées en cours d'expérimentation, elles n'ont pas été prises en compte dans le traitement des données. Des différences dans la taille des aires réoperculées sur l'opercule de couvain parasitée varient en fonction de l'état physiologique du varroa (Martin *et al.*, 2020). L'aire réoperculée est plus importante lorsque le varroa est mort ou vivant dans l'alvéole que lorsqu'il est mort coincé entre le cocon de la nymphe et la paroi de l'alvéole (Martin *et al.*, 2020). Martin et collaborateurs (2020) suggèrent que de petites réoperculations seraient dues à des erreurs dans la détection d'alvéoles parasitées. L'alvéole serait entrouverte de 1 à 2 mm, puis, si l'infestation est avérée, ouverte de façon plus large (3-4 mm) et ainsi permettrait l'accès à d'autres signaux volatiles ou non-volatiles déclenchant (ou non) le nettoyage (Martin *et al.*, 2020). L'aire réoperculée est aussi de petite taille lorsque l'alvéole de couvain n'est pas parasitée (Grindrod & Martin, 2021). Il est intéressant de noter que ce type de réoperculation a été observé pour des traitements contenant des COVs, et ne laisse pas à penser à une ouverture hasardeuse de la part des ouvrières, mais bien à une réponse face au traitement. En effet, en fonction du traitement, la réoperculation pouvait atteindre jusqu'à 100% des alvéoles injectées. Il a été montré que la réoperculation des alvéoles de couvain serait réalisée dans des clusters d'alvéoles (Grindrod & Martin, 2021). Autrement dit, lorsqu'une alvéole est réoperculée, les alvéoles alentour ont tendance à se faire inspecter aussi par les ouvrières (Grindrod & Martin, 2021). Cela serait aussi le cas pour les alvéoles nettoyées lors du comportement VSH (Kim *et al.*, 2018). L'effet des traitements sur les alvéoles de couvain aux alentours des alvéoles traitées est inconnu car elles n'ont pas fait l'objet de contrôle. Des études supplémentaires sont donc nécessaires afin de confirmer l'effet de COVs sur la taille de la réoperculation et la spatialisation des alvéoles réoperculées et nettoyées.

II. Limite méthodologique des captures de COVs

Lors de l'étude des émissions du couvain *in situ* au cours de son développement, 78 composés ont été captés (groupe couvain et groupe contrôle cire confondus) dont 32 composés appartenant à 6 familles chimiques ont été retenus comme caractérisant le couvain. Dans les captures des émissions du couvain élevé en gélules, 111 composés ont été captés (groupe parasité, groupe non-parasité et groupe

contrôle gélules confondus). Les captures de COVs ont été réalisées à l'aide de fibres SPME (*Solid Phase Micro Extraction*), un système de capture passif constitué d'une aiguille creuse d'où sort une fibre de silice fondue, sur lequel est déposé un adsorbant (Shirey, 2012). L'utilisation de fibre SPME est une solution simple d'utilisation, sensible, précise et causant peu de perturbations pour capturer les émissions de volatiles *in vivo* (Musteata & Vuckovic, 2012). Néanmoins, certains composés peuvent avoir plus d'affinité avec la fibre que d'autres. L'augmentation du nombre de composés captés dans la deuxième expérimentation pourrait s'expliquer par la plus grande proximité de la fibre des échantillons dans un volume plus restreint. Toutefois, ces composés ne représentent dans tous les cas potentiellement qu'une fraction des COVs pouvant être émis par le couvain d'abeilles mellifères. En effet, les composés captés dépendent de l'adsorbant utilisé (Shirey, 2012). En outre, il peut exister une compétition dans l'adsorption par la fibre entre les composés (Roberts *et al.*, 2000). Dans cette thèse, la nature chimique des COVs recherchés était inconnue. Il était primordial d'avoir une fibre SPME polyvalente. Le choix s'est donc porté sur du polydiméthylsiloxane divinylbenzène (PDMS-DVB) qui a la particularité de pouvoir capter à la fois les composés polaires et les composés apolaires de haute à moyenne volatilité (Shirey, 2012). De plus, des tests préliminaires de capture de COVs sur larves ont été réalisés avec différents adsorbants (non publiés), l'adsorbant PDMS-DVB montrait une plus grande polyvalence de familles chimiques captées et un plus grand nombre de composés chimiques adsorbés, confirmant notre choix d'utilisation de ce type de fibre SPME.

III. Perspectives

1. Un signal de bonne santé impliqué dans la reconnaissance du couvain parasité ?

Les captures réalisées sur le couvain élevé en gélule ont permis de mettre en évidence des composés émis en plus grande quantité chez le couvain non-parasité que chez le couvain parasité par *varroa*.

La détection de composés émis par le couvain lors d'infestation et infection déclenchant le comportement de nettoyage est bien documenté chez l'abeille (Kathe *et al.*, 2021; Lee *et al.*, 2020; Liendo *et al.*, 2021; McAfee *et al.*, 2018; Mondet *et al.*, 2016a; Mondet *et al.*, 2016a; Nazi *et al.*, 2004a; Swanson *et al.*, 2009; Wagoner *et al.*, 2019). Cette détection de composés caractéristiques de mauvaise santé du couvain est aussi démontrée comme impliquée dans le comportement d'ouverture du cocon des nymphes infectées par le champignon *Metarhizium brunneum*, un comportement proche du comportement hygiénique de l'abeille, chez la fourmi sociale *Lasius neglectus* (Pull *et al.*, 2018), ainsi que dans le comportement hygiénique chez la fourmi *Ectatomma tuberculatum* vis-à-vis de couvain parasité (Pérez-Lachaud *et al.*, 2015). La reconnaissance du couvain en bonne santé pourrait aussi jouer

un rôle dans les comportements hygiéniques du couvain chez les hyménoptères sociaux (Spivak & Danka, 2021), en particulier par la modification des ratios des composés dans les odeurs de couvain. Cette modification des ratios permet la reconnaissance de l'âge des larves ainsi que de la caste du couvain chez *A. mellifera* (Le Conte *et al.*, 1990b; Le Conte *et al.*, 1994a; Le Conte *et al.*, 1995) et la reconnaissance des conspécifiques chez les hyménoptères sociaux (Leonhardt *et al.*, 2016; Pérez-Lachaud *et al.*, 2015). Les odeurs de couvain sont aussi impliquées dans la reconnaissance de l'âge des larves, de la caste des œufs et de couvain étranger chez la fourmi (Schultner & Pulliainen, 2020). Les captures des émissions du couvain, au cours du développement ainsi que dans le cas de parasitisme et non-parasitisme à varroa, ont illustré les importantes fluctuations des composés au fil de la maturation du couvain. La majeure partie des composés identifiés dans cette thèse ont été émis en continu pendant le développement mais avec des changements dans les quantités de composés émis. Ainsi, la communication entre couvain et ouvrière pourrait s'apparenter à une « syntaxe » chimique. La détection de couvain malade ou parasité ne serait alors pas un système binaire de présence/absence de composés de mauvaise santé mais une fluctuation subtile des ratios des molécules émises (Figure 5.2).

Au vu de l'hypothèse de composés de bonne santé, deux composés volatiles (nonadécane et alcool benzylique) trouvés comme plus émis par le couvain non parasité ont été testés dans le but d'inhiber le nettoyage des alvéoles de couvain. Pour ce faire, deux traitements ont été testés contenant chacun un COV de bonne santé dilué à 10% p/p. Les injections de ces traitements ont été faites dans des alvéoles de couvain tué par pin-test. Ces essais n'ont pas été concluants car les alvéoles de couvain ont été nettoyées de la même façon que les alvéoles contrôles de couvain tué par pin-test. Les tests des molécules de bonne santé ont très vite été arrêtés en vue de se concentrer sur les molécules de parasitisme. Toutefois, ces composés olfactifs pourraient jouer un rôle important dans la reconnaissance des alvéoles parasitées. Dans le cas des tests réalisés, la mort du couvain par pin-test pourrait renvoyer un message très spécifique déclenchant le nettoyage, tel que l'émission de β -ocimène et d'acide oléique impliqués dans la reconnaissance du couvain mort (McAfee *et al.*, 2018), masquant ainsi tout autre message. Un moyen d'éviter de tuer le couvain pour tester ces composés serait d'utiliser du couvain qui a été infesté artificiellement avec *V. destructor*. Une fondatrice pourrait être déposée dans une alvéole de couvain fraîchement operculée, puis le cadre de couvain remis dans la colonie. Les injections des traitements contenant ces composés de bonne santé se feraient ensuite sur du couvain arrivé aux stades P1 à P3, stades où le comportement VSH est le plus réalisé (Harris, 2007) (Figure 1.4). Une autre possibilité serait d'utiliser des leurres en paraffine. L'operculation des alvéoles peut être déclenchée avec des leurres en paraffine contenant de la BEP (Le Conte *et al.*, 1990b). Trois jours après operculation, différentes solutions de COVs serait injectées à travers l'opercule dans ces leurres. Des

solutions comprenant seulement des composés de bonne santé ou des composés de parasitisme, ainsi que des solutions intermédiaires contenant des mélanges de ces composés à différentes concentrations pourraient être testées afin d'observer le comportement des ouvrières.

2. Une spécialisation du système olfactif possible dans le comportement VSH

Il a été montré dans cette thèse que des COVs pouvaient caractériser le parasitisme à varroa et déclencher à la fois le nettoyage et la réoperculation des alvéoles de couvain. Les potentielles différences dans la capacité d'identification de ces COVs et dans leur traitement neuronal par les ouvrières spécialistes et non-spécialistes du VSH restent inconnues. Seules les ouvrières VSH arrivent à discriminer les odeurs de couvain sain couplé aux composés de parasitisme VPS des odeurs de couvain sain (Mondet *et al.*, 2021). Les ouvrières VSH présentent une latéralisation antennaire dans la discrimination des composés VPS (Mondet *et al.*, 2021) comme les ouvrières hygiéniques pour le β -ocimène (McAfee *et al.*, 2017). Les comportements antagonistes ont tendance à être exacerbés lors de communication avec l'antenne gauche tandis que l'antenne droite contrôlerait les comportements sociaux (Rogers *et al.*, 2013). De plus, lors de tests de mémoire à long-terme, les abeilles stimulées par l'antenne gauche réussissent mieux les tâches de rappel que lorsqu'elles sont stimulées par l'antenne droite (Rogers & Vallortigara, 2008). Ce biais de l'antenne gauche pour les comportements agressifs se retrouve aussi chez l'abeille sauvage *Osmia bicornis* (Rogers *et al.*, 2016). Il serait intéressant de voir si une latéralisation antennaire s'opère aussi avec les COVs illustrés dans cette thèse et pouvant donner un avantage aux abeilles VSH dans la détection des alvéoles de couvain parasité. Comme l'olfaction ne se résume pas à l'antenne et est traitée au niveau central pas les lobes antennaires, les corps pédonculés et les cornes latérales (Paoli & Galizia, 2021), et au vu des résultats trouvés pour les composés VPS (Mondet *et al.*, 2021), la spécialisation des ouvrières pourrait très probablement se traduire par des différences cérébrales dans le traitement du signal. La présence d'un moins grand nombre de sensilles sur l'antenne gauche par rapport à l'antenne droite (Frasnelli *et al.*, 2010), renforce l'hypothèse d'une spécialisation des neurones olfactifs associés au comportement VSH. De plus, dans une étude recherchant les gènes influençant le comportement VSH à l'aide de cartographie de loci de caractères quantitatifs (QTL – *Quantitative trait loci*), il a été mis en évidence une association entre le récepteur D2 de la dopamine *Amdop3* et le comportement d'ouverture et de nettoyage des alvéoles de couvain (Tsuruda *et al.*, 2012). Chez la drosophile et l'abeille, la dopamine est impliquée dans le comportement d'apprentissage aversif (Tedjakumala & Giurfa, 2013; Vergoz *et al.*, 2007; Waddell, 2013; Wright, 2011), et il est suggéré que le récepteur *Amdop3* soit impliqué dans des fonctions olfactives par sa présence au niveau des lobes antennaires (Beggs *et al.*, 2005; Beggs & Mercer, 2009). En revanche, la spécialisation des nourrices dans la détection des COVs de parasitisme à varroa décrits dans cette thèse

paraît peu probable car ces composés sont très communs, en particulier dans les émissions florales (Knudsen *et al.*, 2006).

3. Potentielles applications des résultats de l'étude pour l'apiculture

a. Un outil pour quantifier l'infestation au sein des colonies

Actuellement, la quantification de l'infestation par varroa au sein des colonies se fait principalement par comptage de varroas phorétiques et est un processus relativement chronophage (Dietemann *et al.*, 2013). L'identification de composés caractéristiques du couvain parasité par varroa pourrait permettre de mettre en place un système de détection rapide et spécifique du varroa présent au sein des alvéoles de couvain. Il est aussi possible d'imaginer un nez électronique portable tel que le NeOse Pro qui permet de détecter, de mettre en mémoire et de reconnaître des profils de COVs en espace de tête (Claus *et al.*, 2022; Demarigny *et al.*, 2021). Dans l'alimentaire, ce capteur est capable de différencier des morceaux de bœuf et saumon stockés sous diverses atmosphères (Claus *et al.*, 2022) ainsi que la fermentation d'un yaourt (Demarigny *et al.*, 2021). On peut imaginer que ce type de capteurs puisse différencier du couvain operculé parasité et non parasité, après création d'une base de données olfactive spécifique au parasitisme. Les résultats seraient obtenus rapidement et facilement. Ce type de capteur étant coûteux pour un apiculteur, il pourrait être détenu par les ADA (Associations de Développement de l'Apiculture) qui interviendraient dans les ruchers pour quantifier l'infestation des colonies en varroa.

b. Un système de phénotypage du comportement VSH accessible aux apiculteurs

La finalité de la caractérisation des traits de résistance à *Varroa* chez l'abeille mellifère est de donner les moyens aux apiculteurs de lutter contre l'acarien. L'utilisation d'abeilles résistantes à varroa est une solution durable et écologique permettant de s'affranchir des traitements de luttés chimiques qui s'accumulent dans les matrices apicoles (Kast *et al.*, 2019; Murcia-Morales *et al.*, 2022; Ostiguy *et al.*, 2019; Premrov Bajuk *et al.*, 2017), qui sont toxiques pour les abeilles (Sabová *et al.*, 2022; Tihelka, 2018; Tosi *et al.*, 2022) et engendrent des résistances chez varroa (Colin *et al.*, 1997; González-Cabrera *et al.*, 2018; Martin, 2004; Milani, 1999; Mitton *et al.*, 2022; Panini *et al.*, 2019; Stara *et al.*, 2019). Cette solution d'utilisation de la résistance naturelle des colonies passe par la sélection et élevage sur ce critère. Actuellement, le phénotypage de la résistance des colonies à varroa est fastidieux et difficile à appliquer sur un cheptel de grande taille. Il est principalement réalisé par mesure de l'infestation à varroa, de la croissance de la population en acariens et des comportements hygiénique et VSH (Noël *et al.*, 2020). Les recherches génomiques, transcriptomiques et protéomiques montrent des résultats

prometteurs quant à la caractérisation du comportement VSH (Mondet *et al.*, 2020a), mais actuellement aucun outil n'est disponible pour les apiculteurs afin de phénotyper les caractères spécifiques de résistance à varroa de façon simple et efficace (VSH, SMR, MNR, réoperculation). La découverte de nouveaux composés pourrait permettre de développer un système de phénotypage du comportement VSH et/ou de réoperculation des colonies à varroa, facile d'utilisation par les apiculteurs. Cela pourrait se faire sous forme de solution en spray à pulvériser sur une partie du couvain à l'image de celui en développement par Wagoner *et al.* (2021). Le phénotypage de la colonie passerait alors par une observation du comportement de nettoyage par les ouvrières d'alvéoles de couvain traitées. Un tel dispositif serait facile d'utilisation et permettrait d'être utilisé sur un grand nombre de colonies.

Les abeilles évoluent dans un environnement olfactif très riche. Les COVs sont des composés très présents dans l'univers sensoriel des abeilles, que ce soit dans l'environnement de la ruche (cire, propolis, pollen, miel) (Cellini *et al.*, 2019; Duru *et al.*, 2021; Isidorov *et al.*, 2012; Jánošková *et al.*, 2014; Najar *et al.*, 2021; Smith *et al.*, 2002), dans l'émission des plantes butinées pour les ressources alimentaires (Knudsen *et al.*, 2006) ou dans les messages phéromonaux (Maisonnasse *et al.*, 2009; Pickett *et al.*, 1980; Shearer & Boch, 1965; Slessor *et al.*, 2005; Veith *et al.*, 1984). Cette étude apporte un nouveau regard sur les émissions du couvain, que ce soit pendant son développement sain ou dans le cas du parasitisme à varroa, et elle montre l'importance des émissions de COVs dans l'environnement olfactif du couvain. Les COVs semblent jouer un rôle de premier plan dans la détection des alvéoles parasitées par varroa. Couplés à des composés moins volatiles, ils déclenchent le nettoyage de l'alvéole. Contrairement aux composés moins volatiles, la présence de COVs semble primordiale dans le comportement de réoperculation. Le comportement de réoperculation n'apparaît pas comme un comportement à part entière mais bien comme faisant partie de l'éthogramme du comportement VSH. Ainsi, les ouvrières identifieraient les alvéoles de couvain parasitées grâce aux COVs. Ils permettraient de déclencher l'ouverture des alvéoles de couvain. La présence de composés faiblement volatiles au sein de l'alvéole de couvain déclencherait le sacrifice de la nymphe. Un message complexe de COVs déclencherait le comportement de réoperculation. Des études supplémentaires sont nécessaires pour pouvoir comprendre les mécanismes en jeu dans la détection et le traitement neuronal des composés spécifiques des alvéoles parasitées par les ouvrières spécialisées dans le VSH, afin de comprendre plus profondément les modalités des comportement VSH et de réoperculation. Cette étude est un pas en avant dans la compréhension de la résistance des colonies contre varroa grâce à l'illustration détaillée du répertoire olfactif des ouvrières face à un couvain (non-)parasité par varroa.

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ANNEXES

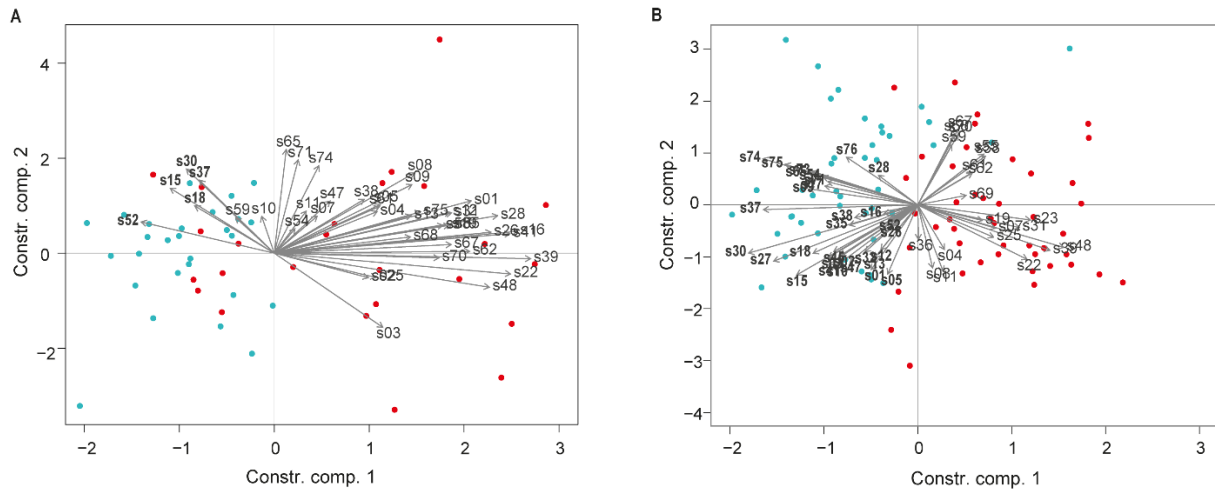


Figure S2.1: Redundancy Analysis (RDA) performed on chemical compounds captured on brood and control samples (A) for “Eggs” group (day 1 to day 3), (B) “Sclerotised Pupae” group (day 17 to day 21). For each day: brood sample $n = 9$, control sample $n = 9$. Points represent samples (blue: brood, red: wax). Compounds are represented by their IDs and grey arrows. The compounds characterising the brood that are retained for the rest of the analysis are shown in bold.

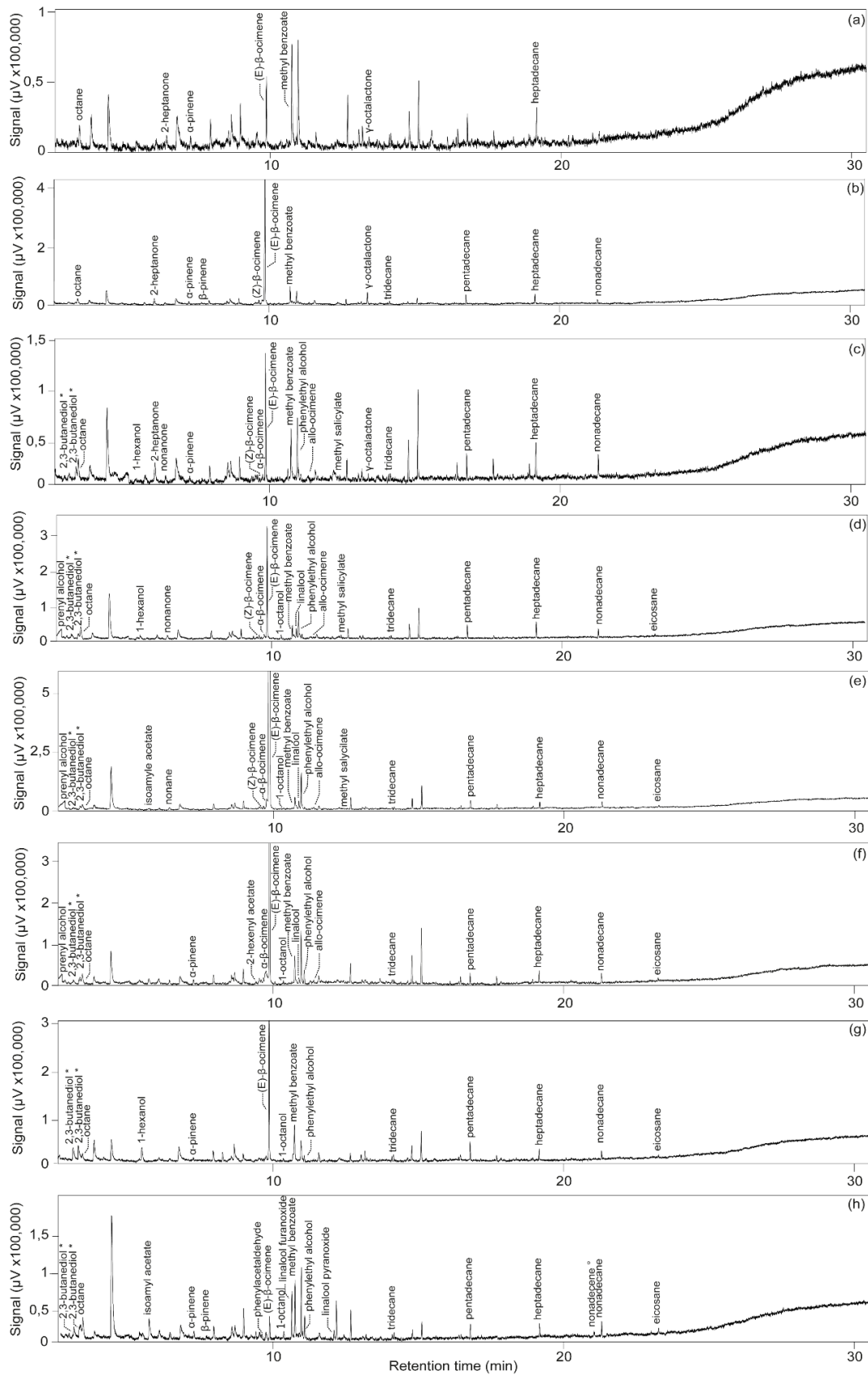


Figure S2.2: Representative gas chromatography profiles of each brood stage. The compounds listed are those identified as brood specific. The other peaks represent fibre peaks or peaks specific to the wax control. (a) Eggs, (b) L1-L4: 1st to 4th instar larvae, (c) L5: 5th instar larvae, (d) Capping: adult capping behaviour, (E) LS: larvae spinning cocoon, (f) PP: pre-pupae, (g) NSP: non-sclerotised pupae, (h) SP: sclerotised pupae. °: Unsaturation remains unknown, *: Stereochemistry remains unknown.

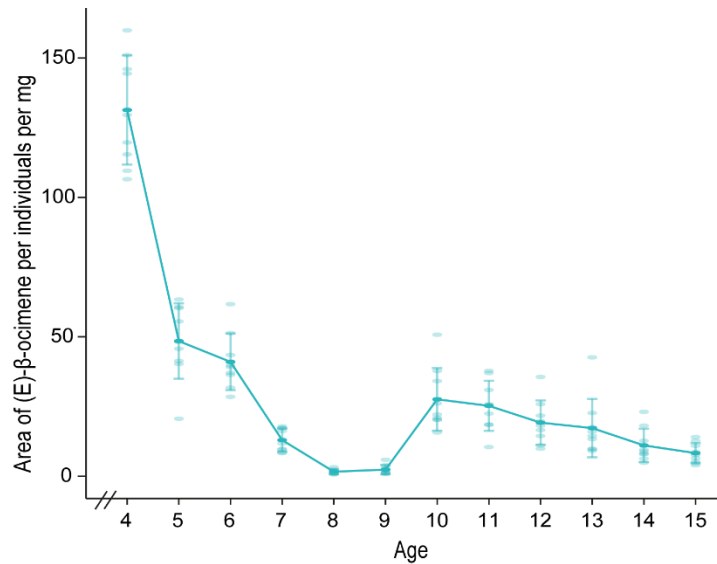


Figure S2.3: Quantification of (E)-β-ocimene emission for an individual from the 1st instar larval to the 5th pupal stage. Weight data are from previous experiments. Line represents mean values and bars represent standard deviation. For each day, n = 9.

Table S2.1: Peak areas of the 78 compounds found for each group (brood or wax), at each day and for each colony. Full data available at <https://doi.org/10.1371/journal.pone.0282120.s004>.

Hive #	Sample	Day	s01	s02	s03	s04	s05	s06	s07	s08	s09	s10	s11	s12	s1
H428	brood	D01	642	0	0	1308	3175	8559	11038	5442	0	7587	5257	3220	
H127	brood	D01	696	0	0	1937	5751	20641	17245	11273	0	9534	7387	2301	
H428	wax	D01	1063	0	0	0	4066	8022	3205	8499	0	4199	6678	2662	
H127	wax	D01	0	0	0	0	8983	0	0	2628	0	3484	0	0	
H164	brood	D01	2605	0	0	8212	6912	14495	108077	10433	0	3534	5094	2170	
H164	wax	D01	2594	0	0	4098	4341	28314	31509	12284	0	2001	3190	0	
H74	wax	D01	6491	3795	9488	18512	10335	54992	11186	13660	0	0	3841	5195	
H74	brood	D01	3894	2900	6035	7727	9723	52593	11574	7570	0	2735	2956	2192	
H120	wax	D01	681	11477	16214	4461	7344	54003	33901	5231	0	0	3097	2642	
H120	brood	D01	960	4025	8840	1813	4040	59053	12992	6100	0	902	2294	0	
H41	wax	D01	3472	6474	19187	2307	4187	72224	12177	4031	0	1508	1832	4979	
H41	brood	D01	0	0	0	0	0	97257	1363	0	0	0	0	0	
H32	wax	D01	3173	3762	7576	5451	6912	32916	19714	9435	587	1599	3618	3027	
H32	brood	D01	2907	7089	15365	1935	3853	45803	9621	3957	0	4542	1718	2525	
H191	wax	D01	7909	4590	8143	8781	9130	12857	46023	19713	4106	0	6688	5710	
H191	brood	D01	830	0	3551	2553	9757	21400	11859	6577	0	4151	2806	2165	
H98	wax	D01	3173	3762	7576	5451	6912	32916	19714	9435	587	1599	3618	3027	
H98	brood	D01	3106	2865	7925	4544	16751	24405	19304	10914	0	8144	3214	3128	
H127	brood	D02	2320	0	0	0	5996	15527	25648	10280	0	4172	6750	3141	
H164	wax	D02	4464	0	0	17522	4615	25438	429128	11016	0	23587	6208	2004	
H164	brood	D02	3380	0	0	4973	6250	15061	41829	10855	0	4687	9725	2531	
H428	wax	D02	772	0	0	16252	4056	8989	51731	15666	0	0	3007	1566	
H428	brood	D02	361	0	0	17185	4749	9422	49748	10021	0	2410	7461	2469	
H127	wax	D02	0	0	0	2590	0	24572	44220	14152	0	1943	1901	0	
H98	wax	D02	8114	6179	0	13417	18282	29641	34258	22366	5554	4759	4183	7259	
H98	brood	D02	3437	3752	0	12117	14483	32424	22506	9905	1694	4265	2463	2745	
H74	wax	D02	4702	3831	0	9813	16483	60353	16648	13567	0	3745	3395	5130	
H74	brood	D02	2803	3036	0	12007	15898	72405	18213	7958	0	4849	2692	2374	
H191	wax	D02	10069	4158	0	6067	12748	18651	27388	15205	7153	2969	6550	6036	
H191	brood	D02	945	4184	125	4141	13698	17070	29286	7684	0	3312	2654	1999	
H41	wax	D02	3476	3450	0	3710	8106	60948	29846	13220	0	0	4871	2562	
H41	brood	D02	2567	6233	323	1888	5258	81995	11747	8808	3437	2583	2430	3860	
H32	wax	D02	2973	8726	234	3372	6269	39867	26385	8537	0	0	3477	2245	
H32	brood	D02	5566	0	0	0	4373	44654	22714	6189	0	0	1927	1918	
H120	wax	D02	1065	4151	324	1502	7932	48372	33860	7952	0	0	2650	2176	

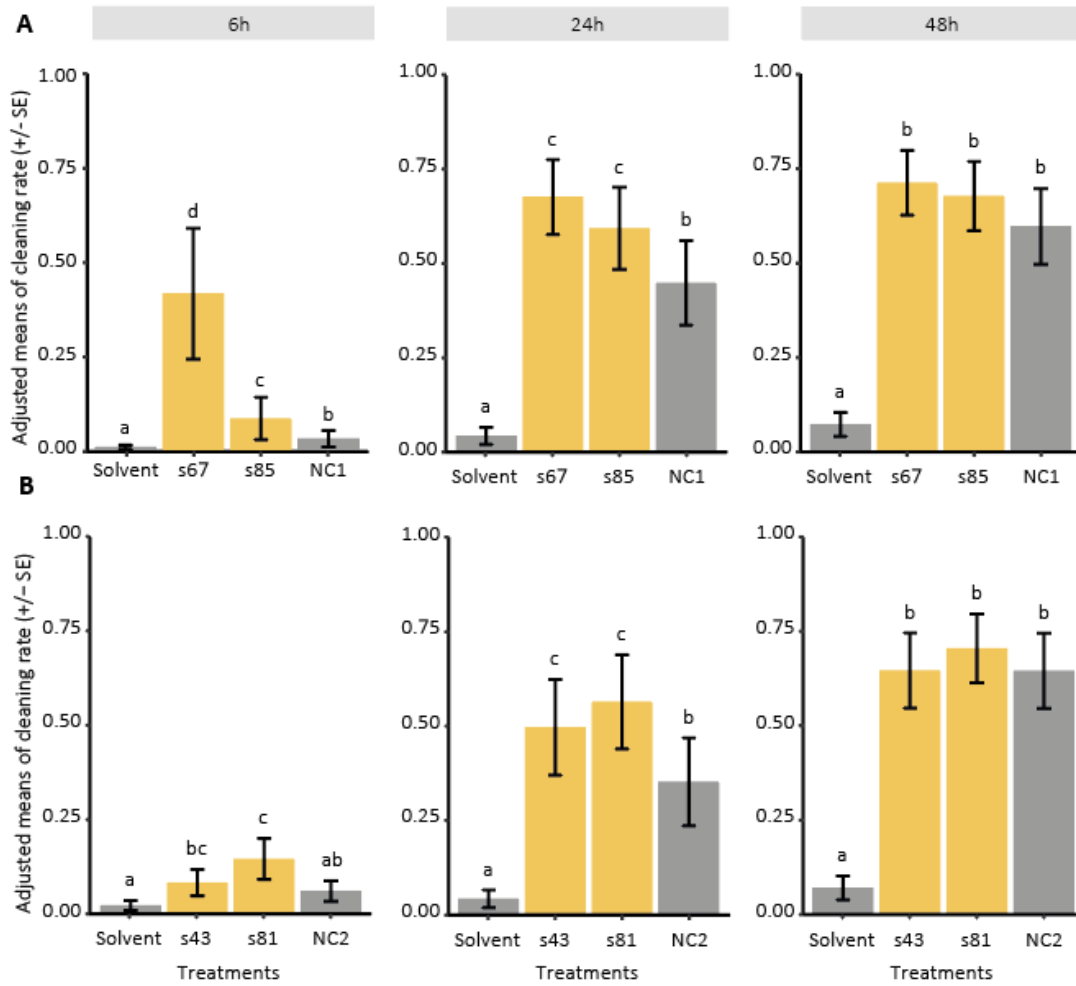


Figure S3.1: Adjusted means of cleaning rate (+/- SE) of injected brood cells according to the different treatments, 6h, 24h and 48h after injections. (A) Treatment of alkanes characteristic of parasitic brood cells (s67 and s85) and alkane control (NC1); s67: *n*-tetradecane, s85: *n*-*n*-hexadecane, NC1: *n*-octadecane. (B) Treatment of carboxylic acids characteristic of parasitic brood cells (s43 and s81) and carboxylic acid control (NC2); s43: octanoic acid, s81: dodecanoic acid, NC2: decanoic acid. For each treatment, *n* = 11 colonies.

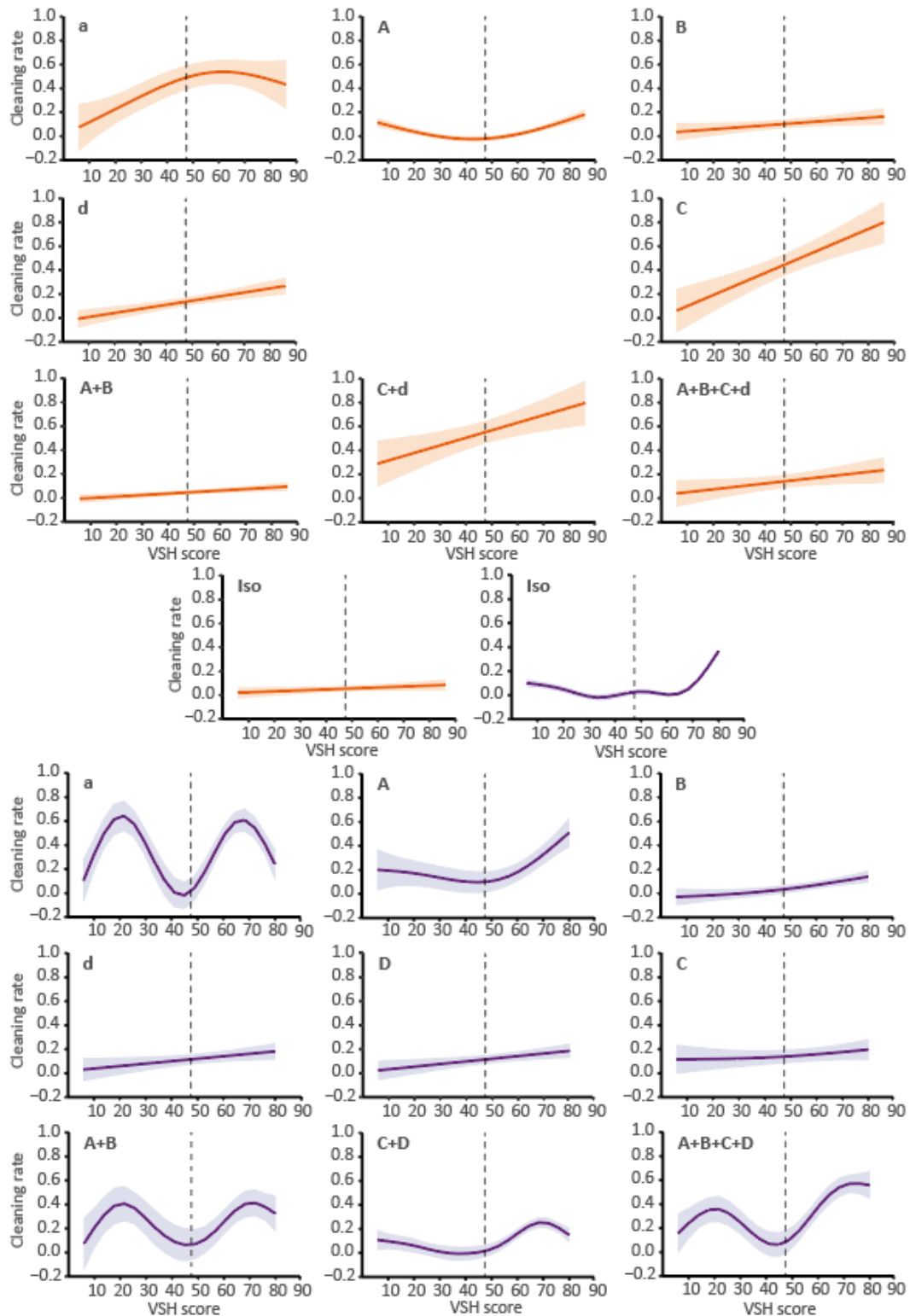


Figure S4.1: Evolution of the cleaning rate for the different treatments injected in brood cells according to VSH score of colonies (GAMM models). Lines show the adjusted means of cleaning rate and the surrounding 95% standard error. Dotted lines separate the two groups: “VSH-” (VSH score below 47) and “VSH+” (VSH score above 47) considered for the analysis of cleaning rate according to the VSH score. For each treatment, high concentration (10% w/w – orange): $n = 13$ colonies; physiological concentration (10 equivalents – purple): $n = 11$ colonies. Iso: isohexane, a: *n*-tetradecane, A: five VOCs of Noël *et al.* (Unpublished) (*n*-tetradecane, *n*-*n*-hexadecane, benzothiazole, octanoic acid & dodecanoic acid), B: α -pinene & ethyl hexanoate, C: (*Z*)-10-tritriacontene & (*Z*)-6-pentadecene, d: tetracosyl acetate, D: six VPS (2-tricosanone, 2-pentacosanone, tetracosyl acetate, 2-heptacosanone, hexacosyl acetate & 2-nonacosanone).

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Review Article

Varroa destructor: how does it harm *Apis mellifera* honey bees and what can be done about it?

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Since its migration from the Asian honey bee (*Apis cerana*) to the European honey bee (*Apis mellifera*), the ectoparasitic mite *Varroa destructor* has emerged as a major issue for beekeeping worldwide. Due to a short history of coevolution, the host–parasite relationship between *A. mellifera* and *V. destructor* is unbalanced, with honey bees suffering infestation effects at the individual, colony and population levels. Several control solutions have been developed to tackle the colony and production losses due to *Varroa*, but the burden caused by the mite in combination with other biotic and abiotic factors continues to increase, weakening the beekeeping industry. In this synthetic review, we highlight the main advances made between 2015 and 2020 on *V. destructor* biology and its impact on the health of the honey bee, *A. mellifera*. We also describe the main control solutions that are currently available to fight the mite and place a special focus on new methodological developments, which point to integrated pest management strategies for the control of *Varroa* in honey bee colonies.

Introduction

The mite, *Varroa destructor*, has been the subject of thorough investigations, since the 1980s after its introduction in Europe to *Apis mellifera* honey bee populations [1]. *V. destructor* continues its worldwide expansion and has now been reported in most countries. In the last 10 years, new invasions were noted in Hawaii [2], Reunion Island [3], Madagascar [4], Mauritius Island [5] and also African countries such as Uganda [6] and Ethiopia [7]. In early 2020, only Australia, several countries in Africa and a few islands have yet to report the presence of *V. destructor* in their *A. mellifera* populations [8,9]. Despite the extensive literature on the mite (see [10–12] for reviews), its biology and impact on its honey bee host remain partially unknown. The scientific community has also put many efforts into the development and validation of control methods to fight the deadly mite in *A. mellifera* colonies. This review highlights the main findings revealed by the rather large corpus of literature published between 2015 and early 2020 on *V. destructor* biology, the mite's pathogenic effects, and solutions developed to fight the mite.

Biology of *Varroa destructor*

Between 2015 and 2020, the behaviour of *V. destructor*, its genetics and physiology, have become more precisely understood. Its life cycle can be separated into two phases: the phoretic phase and the reproductive phase. The phoretic phase only concerns the female mite which uses the adult bee as a 'transportation vector' and food source. During this phase, bees unintentionally take part in the spreading of *Varroa* within and between honey bee colonies. The reproductive phase begins when the mite enters into an unsealed brood cell containing a 5th stage bee larva, to lay eggs. Recently, Häußermann *et al.* revealed the possibility of a virgin mite beginning the phoretic phase. In that case, the mite invades a brood cell, lays an unfertilised egg in a new comb, and mates with its male offspring [13]. This finding confirms that female *Varroa* do not need to mate to lay eggs, and thus, have an arrhenotokous parthenogenetic reproduction system. While it was first thought that *Varroa* feeds

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on honey bee haemolymph, recent research shows that the parasite also feeds on the honey bee fat body [14]. Analyses of the whole-life transcriptome and proteome of the mite have helped to understand the gene expression and protein variations during its different life stages and have provided data for further investigations of mite physiology [15,16]. The morphology of *Varroa*'s heart has been described, its heartbeats have been recorded [17] and the sensory pit organs of the male have been scanned by electron microscopy [18], complementing the morphological research on female pit organs [19]. The identification of the chemosensory receptors on *Varroa* forelegs confirms the importance of their front legs in understanding their chemical environment [20]. Iovinella and collaborators performed the proteomic analysis of the chemosensory organs of the mites. This study identified novel semiochemical proteins, including odorant-binding proteins which are particularly abundant in mouths and forelegs [21]. Further chemical ecology investigations have indicated that the male *Varroa* detects female pheromones with its sensory pit organs present on their front legs [18]. All these findings are important to better understand *V. destructor* mating behaviour, detection and invasion of brood cells close to be capped. We know that *V. destructor* has the ability to mimic the cuticular hydrocarbons of their *A. mellifera* hosts at different stages [22]. It seems that this mimicry can rapidly adapt as *Varroa* is able to harbour the cuticular hydrocarbon profile of a new, artificially-given host [23,24]. Furthermore, *V. destructor* from *A. cerana* are better in mimicking new hosts than *V. destructor* from *A. mellifera* [23]. To do so, *Varroa* adapts its *n*-alkane:alkene ratio to fit with the honey bee's chemical profile [25]. This mimicry stands as a passive ability, because even when dead, *Varroa* continues to imitate host cuticular hydrocarbons [24]. Nevertheless, the mite needs to have access to the cuticular hydrocarbon of the host in order to adapt its own cuticular hydrocarbons [24].

***Varroa destructor* genetics**

V. destructor infestation of *A. mellifera* was described as two partially-isolated clones in early 2000 [26], but it now seems that the genetic population of *Varroa* is more variable than expected [27]. Different haplotypes of *V. destructor* can be found within a given apiary or colony, ensuring the genetic flow in the mite population [28,29]. *V. jacobsoni* and *V. destructor* show very different evolutionary trajectories since their divergence [30]. Despite these differences, it seems that the two subspecies have a potential to hybridise [31]. If that is the case, hybridisation events could result in a new species of *Varroa*, possibly more detrimental, that would become a greater risk for honey bees.

Varroa-virus duo

The host-parasite relationship between honey bees and *Varroa* should actually be considered as a three-way relationship, as *Varroa* presence is closely associated with several bee viruses in colonies [32]. However, clear vectoring by *Varroa* has only been described for two viral species, deformed wing virus (DWV) and acute bee paralysis virus complex (ABPV) [33–35]. Bee viruses have several routes of vertical and horizontal transmission within the colony, but the vectoring ability of *Varroa* opens up new, very effective horizontal routes of transmission [36,37]. In the last 5 years, new viruses and variants linked to *Varroa* have been discovered in honey bees infested by the mite [38–42], and the association between DWV and the mite has been confirmed, but only for specific DWV variants [2,32]. New variants and viral species have also been described specifically in the mite, as demonstrated by VDV replication in the mite [43], in which, VDV-5, VDV-3 and VDV-2 can replicate in *V. destructor* and not in *A. mellifera*, suggesting that their presence in honey bees is due to the *Varroa*'s feeding behaviour [38].

It is thought that some of the viruses infesting mites can change *Varroa* behaviour, which presents a new perspective for finding targets to kill the mite [43]. Similarly to its impact on the virus community infecting bees, the mite also alters the honey bee's bacteriome [44,45], but the impact of such an association remains unknown.

What can it do?

Impact of the mite at the individual level

The parasitism of honey bees by *V. destructor* decreases the body weight and water content of young emerging bees [46]. The lowered weight of the future adult bee increases with the number of mite foundresses [47,48]. In honey bees, the number of spermatozooids is correlated with drone body size [49]. By decreasing the size of drones, *Varroa* induce a deficit in sperm production, and thus, in reproductive fitness [50]. *Varroa* also alters

flying, homing and orientation abilities in foragers [51], which in turn, limits efficiency in their ability to collect resources needed for colony development. Non-returning to the colony can also be considered as a mechanism of defence from parasitised bees. The impact of *Varroa* on bee behaviour may be explained by its ability to alter neural processes [52], disrupting the bee-host's non-associative learning abilities. Indeed, parasitised bees have a lower sugar responsiveness and a faster habituation to olfactory stimulation [52]. Moreover, *Varroa* provokes the down-regulation of immune gene expression in emerging infested adults [45], as well as proteomic changes in the honey bee's immune response [53–55]. It disrupts the bee's immune response by interfering in the cascade immune response [56]. For instance, *Varroa* reduces the number of haemocytes circulating in the haemolymph, and lowers the expression of prophenol oxidase involved in the synthesis of melanin [57,58]. Both haemocytes and melanin permit the encapsulation of pathogens during infection or wounding, and thus play a role in insect immune response and healing mechanisms [59]. The weakening of honey bee immunity [60,61] may be linked to the finding that mites feed on fat bodies, given that these organs play a major role in immunity [62].

The impact of *Varroa* coupled with other stressors of the colony

Varroa can interact with other biotic and abiotic stressors, such as environmental factors, other parasites and pathogens, pesticides or viruses. Climate change induces longer periods of brood rearing in honey bee colonies and foraging because of longer warm seasons. Longer brood period means more *Varroa* reproduction cycles and may lead to an increase in mite populations [63]. *Nosema* infection reduces the effectiveness of bee defences against the mite. In addition, neonicotinoid pesticides and *Varroa* both contribute to the decrease of winter honey bee population of the colony [64,65]. Together with another neonicotinoid, imidacloprid, *Varroa* decreases the bee's flying ability [66]. Monchanin *et al.* [67] demonstrate the negative impact of *Varroa* coupled with the neonicotinoid insecticide thiamethoxam on honey bee homing behaviour. Also, the *Varroa*-virus duo plays a key role in the weakening of the colony [68]. Emerging adult bees that were parasitised during the pupal stage show a higher infection rate of DWV compared with non-parasitised individuals [45]. DWV infection induces pathological effects such as crumpled wings and reduced body size leading to behavioural impairment. The DWV titer in honey bees increases as the immunity of the bee decreases [69]. DWV virus can also immunocompromise bees, which may result in a beneficial effect on *Varroa* reproduction [70].

As colonies are constantly exposed to different stressors at the same time, further research needs to focus on the interaction between two or more stressors at the same time to investigate their combined impact on honey bee fitness. For example, to determine interactions between co-occurring viruses and *Varroa* on honey bee health.

Honey bee colony dynamics favours *Varroa* population growth

The population of *V. destructor* in a colony is directly related to the amount of brood and, by extension, the colony's population size. One way for a colony to decrease the mite population is to create a broodless period. To do so, an increase in the frequency of the reproductive behaviour of swarming can be an adaptive defence mechanism of the honey bee colony against the overpopulation of mites. [71] (Figure 1).

Moreover, the number of colonies within an apiary can be advantageous for mite populations. Indeed, colonies of high-density apiaries have a greater infestation rate than that of low-density apiaries [72]. Colonies treated for *Varroa* can be reinfested when foragers rob food stores from a dying colony or drift into another colony [73], and when drones rest in foreign colonies during the mating period [74].

What can be done?

Different approaches are used by beekeepers to control *Varroa* infestation of their colonies. Synthetic and organic acaricides as well as essential oils provide good results to prevent colony losses and the latter, represent interesting tools for organic beekeeping [75]. Depending on the country, methods may differ due to variations in the laws governing the use of chemicals.

Chemical control

Conventional control using synthetic miticides has been used for more than 40 years against *V. destructor* [76]. However, only a limited number of molecules are available (Figure 2). They include the pyrethroids tau-fluvalinate (e.g. Apistan®, Klartan®, Mavrik®) and flumethrin (e.g. Bayvarol®, Polyvar yellow®), the formamidine amitraz (e.g. Apivar®, Apitraz®) or the organophosphorus coumaphos (e.g. Checkmite®, Asuntol®, Perizin®)

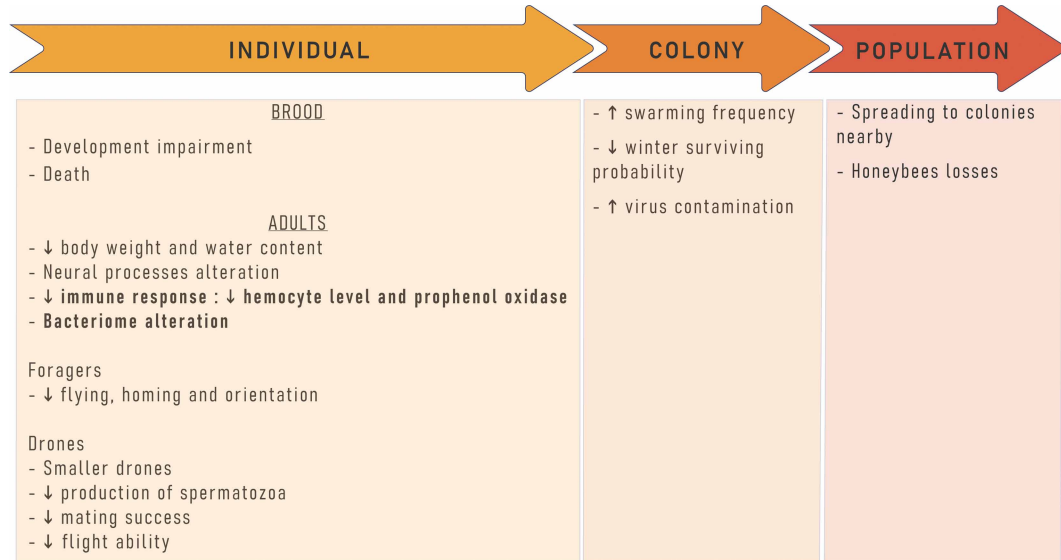


Figure 1. Impact of *Varroa destructor* parasitism on *Apis mellifera* honey bees.

Varroa's impact can be described at individual, colony and population levels. Bold terms correspond to findings published between 2015 and 2019.

[10,77]. All miticide products kill only *Varroa* on adult bees, as they cannot target the reproducing mites hidden in the capped brood. To overcome this shortcoming, products made of strips releasing the acaricide compound over time have been successfully developed and mites are killed when they emerge from the brood cells. These strips are easy to use and efficient but have important limitations, like the resistance developed by the mite and the drawback of accumulating as residues in bee products [78].

Mites are becoming increasingly resistant to acaricides [79–81]. *Varroa* resistance to fluvalinate is now widespread [82], due to DNA mutations [83,84]. Molecular tools have been developed to detect this resistance in *Varroa* populations [85,86]. A provisional solution to limit and temporally bypass *Varroa* resistance to

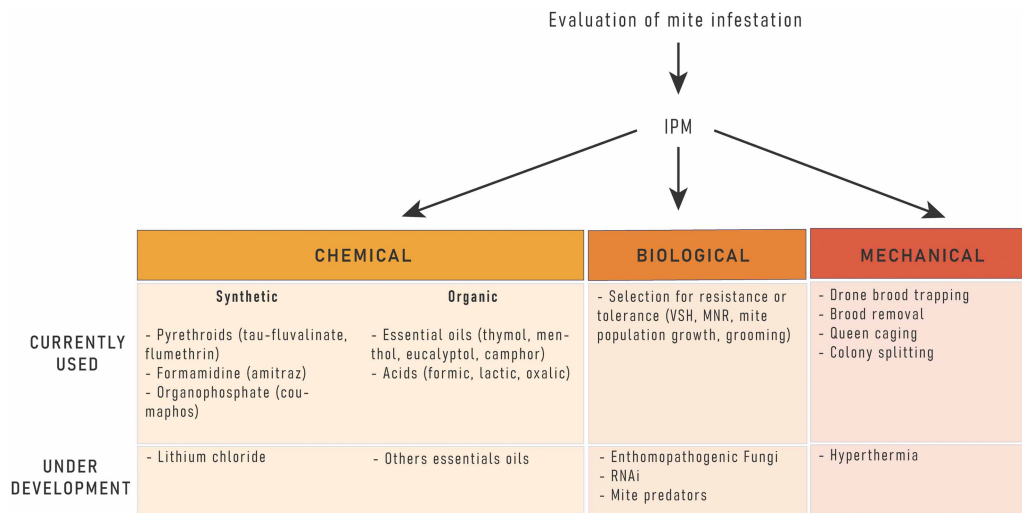


Figure 2. Methods currently used or under development to treat honey bee colonies against *Varroa destructor* parasitism.

Methods can be coupled within an integrated pest management scheme (IPM). VSH: *Varroa*-sensitive hygiene; MNR: Mite non-reproduction.

miticides is to switch between molecules with different molecular targets. Synthetic acaricides could then provide good results for preventing colony losses [75]. Growing data have been published on acaricide residues in honey bee colony matrices, particularly in wax, which have the chemical properties to store the used lipophilic acaricides at concentrations that could even be toxic to the bees [78,87,88].

Because of the adverse impact that conventional synthetic acaricides have on bees and bee products worldwide, beekeepers are increasingly using organic control methods. Organic methods are usually less efficient compared with conventional synthetic acaricide treatment, but still effectively control mite populations [89]. The most common are essential oils such as thymol, and organic acids like oxalic acid and formic acid [77] (Figure 2). Organic acids are naturally found in bee products and have lower risk for triggering resistance in mites [77], but can still have some negative effects on bees, such as decreasing worker populations, increasing capped brood removal or decreasing drone sperm quality [90].

The use of organic control mostly involves flash treatments and thus needs a broodless colony to be efficient in killing phoretic *Varroa*. As such, organic product applications coupled with mechanical methods to exclude the brood can provide a good *Varroa* control solution [91,92]. Queen caging or brood removal can artificially create a broodless colony that keeps the mites on adult bees, making them accessible to acaricides [91,93]. A recent study suggests that while brood removal may result in a decrease in honey production, this loss can be compensated for by avoiding the use of acaricides, enabling a better price of hive products to be achieved [94]. Recently, a new formulation, to be used when brood is present and based on strips releasing oxalic acid, was tested successfully for *Varroa* control, creating a real opportunity in the organic control of *Varroa* [95].

Need for new active compounds

Although several products that efficiently control the mite are available, there is an urgent need for new active compounds because of the risk of *Varroa* resistance. A screening approach combining *in silico* screening (virtual screening of a chemolibrary of homology sequence models) with *in vitro* experiments to search for selectively inhibiting *Varroa* acetylcholinesterase was successfully developed leading to the discovery of new compounds that have the potential to become new treatments against the mite [96]. Lithium chloride has also been demonstrated as a potential compound against *Varroa* [97], as well as other essential oils and their combinations [98]. Recently, Bendifallah *et al.* [99] demonstrated the biological activity of sage essential oil as a *Varroa* control. However, currently there are no registered products available based on these new bioactive sources. The difficult part about the search of new acaricides to control the mite is that the compounds need to be safe for the bees. Development of *in vitro* rearing of *Varroa* under laboratory conditions will provide an efficient platform for rapidly screening activity of new compounds which are potentially useful in *Varroa* control.

Alternative approaches to the use of chemical treatments

Different techniques have been previously described to limit *Varroa* infestation, such as the ‘trapping comb technique’ or the use of screen bottom boards to trap the mites. A recent study showed that splitting colonies, which mimic swarming events that can control *Varroa* growth, could be an effective method for decreasing the mite populations [71].

Biological control methods using parasitic fungi have been developed successfully in controlled experiments [100,101]. Hamiduzzama *et al.* [100] showed varying virulence of entomopathogenic fungi on *V. destructor*. Unfortunately, most field experiments on honey bee colonies were not yet successful as the fungi can cause detrimental effects on brood development, queens and worker mortality as well as decreasing of weight in newly emerged adult bees [100]. Another experiment using a *Beauveria* strain showed an effect on *Varroa* mortality in the field but no visible negative effect on honey bee health [101]. To date, there is no available method on the market for beekeeping using this technique.

The use of predators has shown mixed results and was unsuccessful when applied to honey bee colonies, as was recently the case in an evaluation of the predatory mite *Stratiolaelaps scimitus*, which demonstrated interesting results in vials but no effect on honey bee colonies [102].

Hyperthermia has been used since the 1970s, and is based on the better heat resistance of the bees compared with *Varroa* [103]. Artificially heating the hive stops *Varroa* reproduction and kills the parasite, without harming the bees, because *Varroa* reproduction is significantly compromised at 36.5°C and mites die at 38°C [104,105]. Heating systems have been proposed to control the mites in different parts of the world, and there are a few systems available on the market at the moment [103,106].

One other option is to use RNA interference to knock down specific genes of *Varroa*, which has been studied since 2012 with successful results [43,107,108]. To date, there is no product available for beekeepers [109] (Figure 2).

We have the tools for integrated pest management (IPM)!

A variety of tools available for beekeepers make the development and use of the IPM concept in *Varroa* control possible (Figure 2). IPM is partly based on limiting pesticide use to when it is only necessary. This requires regular monitoring of the *Varroa* population levels, in order to detect critical infestations and decide on a treatment. As critical infestation levels differ worldwide, these must be defined for specific regions and biotopes.

Efficient but time-consuming techniques exist to diagnose a *Varroa* infestation [110]. Mites can be counted in the brood, on adults or on debris at the bottom of the hive. Brood examination consists of opening capped cells to verify *Varroa* infestation by removing the pupae and counting mites [77,111]. Mite count on adult bees is better documented and increasingly used. It consists of collecting 200–300 bees, separating the mites from bees with a surfactant substance, such as chloroform, alcohol, icing sugar or acaricide treatment, and counting the removed mites [77]. Debris examination can be operated with a sticky sheet placed on the floor of the hive with a thin wire mesh on top of it to prevent bees from cleaning out the fallen mites. Fallen mites stick to the board and honey bees are not able to remove the parasite from the hive [77]. As it is a time consuming and tedious method, a stratified method has been proposed to make an accurate estimation of the mites [112].

New techniques, using technological developments such as gas sensors or computer vision systems, are being tested to estimate *Varroa* infestation [113,114]. They have yet to be transferred to the field and to the beekeeping community.

Selective breeding of naturally resistant or tolerant honey bee populations

In parallel to the development of control solutions to fight the mite, quantitative geneticists and bee breeders have started to search for a longer-term, sustainable solution: selecting honey bee populations that can survive mite infestation without treatments. Several surviving honey bee populations have been identified or bred throughout the world (for a review of these efforts, see [115–117]). Examples of large-scale use of such populations in beekeeping are scarce [118,119] and currently limited by the lack of tools that allow selection of surviving honey bee colonies in the field.

Such tool development relies on the identification of specific phenotypes that characterise these populations. To do so, a better understanding of the mechanisms that undergird the ability to survive is necessary. Surviving can occur through the expression of resistance or tolerance traits, with resistance involving a reduction in *Varroa* growth, while tolerance reduces parasitic burden despite similar levels of *Varroa* growth [120]. A wide range of traits involved in honey bee survival to *Varroa* have been identified, and mainly relate to resistance mechanisms [121]. Tolerance has so far been suggested only in cases (e.g. in the Gotland population in Sweden) where colonies are able to support *Varroa* burden due to mechanisms of tolerance or resistance to the viruses that are associated with *Varroa* infestations [42,122].

Recent investigations highlight the importance of behavioural defences displayed by *Varroa* resistant honey bee populations (Figure 3). Hygienic behaviour specifically targeting *Varroa*-infested capped brood cells (VSH, *Varroa*-sensitive hygiene) has been confirmed as a major trait contributing to reduced mite population growth in European and African bee populations [123–125]. Two other adult bee traits, grooming [126] and recapping [127,128], have been confirmed as important mechanisms for *Varroa* resistance. At the colony level, swarming can enhance resistance in surviving populations living in the wild [71,129]. Brood traits could also be involved in resistance abilities, if they confer hypersensitivity of the brood that leads to the accelerated death or an increased rate of removal, thereby preventing the spread and reproduction of *Varroa* [110,130]. Altogether, these traits participate in limiting mite population growth as characterised by high levels of mite non-reproduction within the brood.

Research has mostly focused on host traits to explain the survival of untreated colonies, but the parasite itself may play a central role too. Indeed host fitness can directly be affected by parasite fitness, as suggested by studies showing that *Varroa* infesting surviving colonies are genetically distinct from *Varroa* infesting neighbouring susceptible colonies [131,132].

Progress in understanding the mechanisms that underlie resistance abilities along with the recent boost in genomic tool development has opened the possibility of devising a diagnostic tool of resistance based on

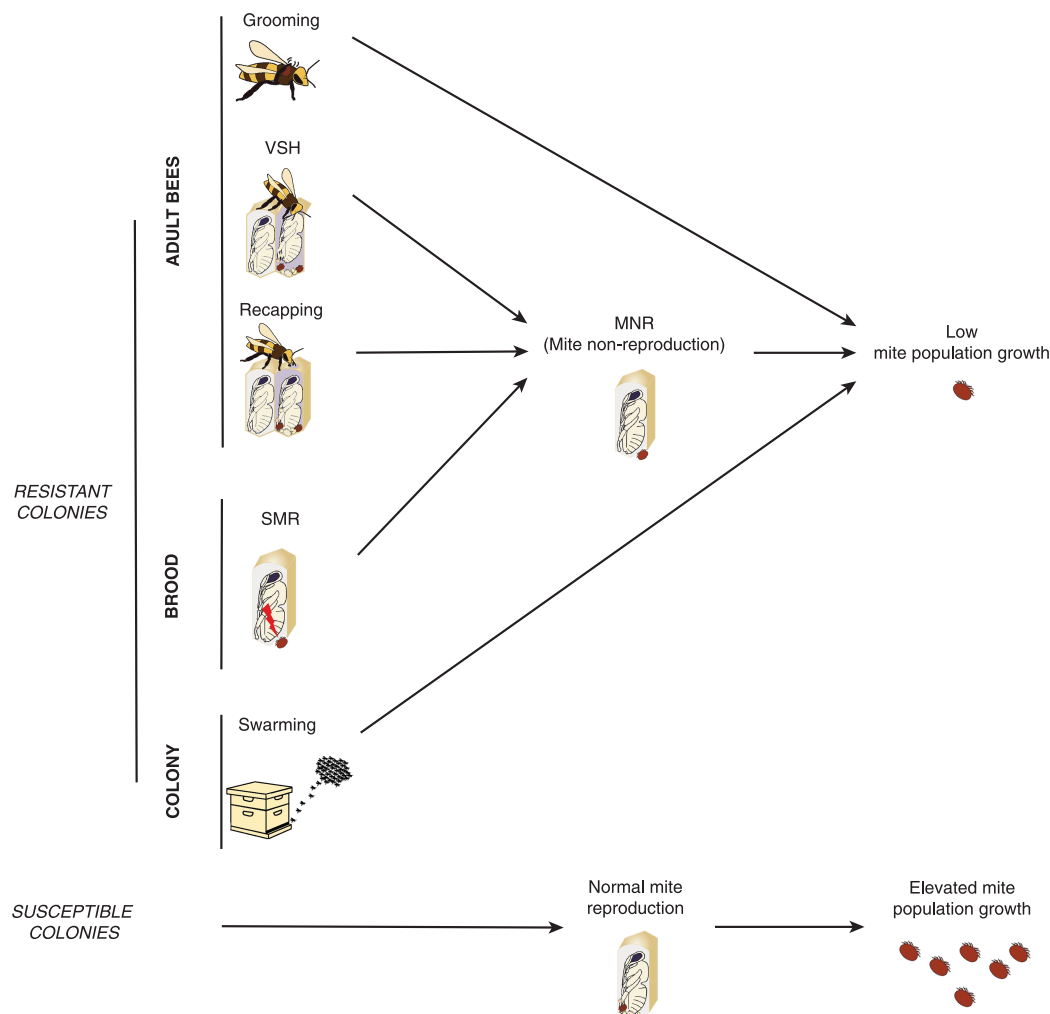


Figure 3. Main behavioural and physiological traits involved in the natural resistance of honey bees to the parasite *Varroa*.

VSH (*Varroa*-sensitive hygiene), recapping and SMR (suppressed mite reproduction) contribute to MNR (mite non-reproduction). Together with grooming and swarming these traits lead to colony resistant through low mite population growth.

phenotype, directly accessible to beekeepers. So far, available methods are very tedious and difficult to apply in the field on large numbers of colonies. This is true for measures of mite population growth, mite non-reproduction or hygienic behaviours. The recent identifications of molecular and protein markers of several traits related to resistance (grooming, hygiene, VSH and mite non-reproduction), although very limited in overlap between different studies [121], opens up the possibility for marker-assisted selection [133–140]. It would allow beekeepers to easily select their colonies for on the basis of interesting resistance traits when the phenotype are difficult to characterise. To date, there are no products available on the market and research is continuing in this area.

Another perspective for the development of selection tools originates specifically from a detailed understanding of the mechanisms of VSH behaviour, a trait in which honey bees are able to specifically detect *Varroa*-infested brood. Strong evidence suggests that the recognition step involves the detection of *Varroa* infestation-associated semiochemicals [16,141–144]. Evaluation of the bee response following application of such candidate compounds in colonies, by acting as a reliable proxy of the VSH activity of the colony, could result in a practical field tool to phenotype resistant colonies.

Conclusion

Despite the amount of research done on the *A. mellifera*–*V. destructor* host-parasite model, *Varroa* remains a major issue for beekeeping throughout the world. Future developments, both in fundamental and applied research, are necessary to generate sustainable control solutions for this deadly parasite.

Summary

- The latest findings on the physiology of *Varroa* and its behaviour provide a better understanding of its negative impact on bee health.
- IPM methods can be used to limit the use of acaricides to control *Varroa*. The selection of resistant or tolerant honey bee populations could bring a sustainable mite control solution for beekeeping and wild honey bee populations.
- Research needs to focus on the development of control methods, especially new active compounds to counter the mite's resistance against acaricides and to efficiently fight *V. destructor*.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

A.N., F.M. and Y.L.-C. designed and wrote the manuscript.

Abbreviations

ABPV, acute bee paralysis virus complex; DWV, deformed wing virus; IPM, integrated pest management; VSH, *Varroa*-sensitive hygiene.

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RESEARCH ARTICLE

Detailed chemical analysis of honey bee (*Apis mellifera*) worker brood volatile profile from egg to emergence

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Abstract

Chemical communication is a widely used mode of communication for social insects and has been demonstrated to be involved in many behaviours and physiological processes such as reproduction, nutrition or the fight against parasites and pathogens. In the honey bee, *Apis mellifera*, the release of chemical compounds by the brood plays a role in worker behaviour, physiology, and foraging activities and colony health as a whole. Several compounds have already been described as brood pheromones, such as components of the brood ester pheromone and (E)- β -ocimene. Several other compounds originating from diseased or varroa-infested brood cells have been described as triggering the hygienic behaviour of workers. So far, studies of brood emissions have focused on specific stages of development and little is known about the emission of volatile organic compounds by the brood. In this study, we investigate the semiochemical profile of worker honey bee brood during its whole developmental cycle, from egg to emergence, with a specific focus on volatile organic compounds. We describe variation in emissions of thirty-two volatile organic compounds between brood stages. We highlight candidate compounds that are particularly abundant in specific stages and discuss their potential biological significance.

Introduction

Communication is fundamental to all life forms. Chemical communication, *i.e.* the emission of specific chemical compounds by an individual and received by another at a specific moment, is highly present in the living world. In eusocial insects, compounds emitted and perceived by individuals of the same species constitute a pheromonal message, which is part of the chemical language repertoire. Individuals receiving this chemical message will respond by adapting their behaviour or their physiology [1–3]. This type of communication is key to the

regulation of superorganism homeostasis, as it allows eusocial species to organise the colony and the division of labour [4–6].

In the honey bee, *Apis mellifera*, chemical communication is widely deployed across castes and individuals [3]. It is well known that the brood communicates with the adults through the use of pheromones. An iconic example of brood chemical communication is the brood ester pheromone (BEP), composed of by a mixture of 10 fatty esters (methyl and ethyl oleate, methyl and ethyl linoleate, methyl and ethyl linolenate, methyl and ethyl palmitate, methyl and ethyl stearate) [7]. Another well-known brood pheromone is (E)- β -ocimene [8]. These two pheromones have both releaser (*i.e.* triggers an immediate behavioural response) and primer effects (*i.e.* changes the physiology of the receiver). As releaser effects, BEP triggers worker brood-rearing behaviour and cell-capping behaviour (closing brood cells with wax) [7, 9], and (E)- β -ocimene increases pollen foraging and brood-feeding behaviour [10, 11]. A common primer effect of these two pheromones is the inhibition of worker ovarian growth [8, 12].

Dead brood emit oleic acid and (E)- β -ocimene, which both trigger hygienic behaviour (detection of sick brood and cleaning of the brood cell), suggesting a death-signalling role for these two compounds [13]. Hygienic behaviour is also performed on diseased brood, before it dies. This is the case for brood infected by chalkbrood (*Ascosphaera apis*). In order not to become a vector of contamination, adults must detect brood parasitized by the fungus before the spores reach the cuticle of the larva. The hygienic behaviour is then triggered by the emission of phenethyl acetate, 2-phenylethanol, and benzyl alcohol [14]. A variant of hygienic behaviour, VSH (*Varroa Sensitive Hygiene*) behaviour, targets brood cells parasitized by the varroa mite and is triggered by compounds emitted by parasitized brood cells ((Z)-6-pentadecene, (Z)-10-tritriacontene, 6 VPS (*Varroa-Parasitization-Specific*: tricosan-2-one, pentacosan-2-one, tetracosyl acetate, heptacosan-2-one, hexacosyl acetate and nonacosan-2-one), α -pinene and ethyl hexanoate) [15–18]. Stressed or diseased brood emit other compounds but potential behavioural responses of workers have not been assayed. Such is the case for γ -octalactone, a compound that larvae infected with the European foulbrood emit at higher levels than healthy brood (*Melissococcus plutonius*) [19]. *Varroa destructor* infestation and *Deformed Wing Virus* infection increase the emission of ten chemical compounds in brood targeted by hygienic behaviour (pentacosane, hexacosane, 4-methyltetracosane, 9- and 11-methyltricosane, 11- et 13-methylpentacosane, 12- et 14-methylhexacosane, pentacosene, heptacosene, hentriacontene) [20]. With the exception of a few volatile organic compounds (VOC), the majority of chemical compounds already described as brood emissions that trigger adult behaviours are not very volatile. Little is known about highly volatile compounds of honey bee brood; studies have tended to focus on low-volatile compounds. Unlike cuticular hydrocarbons, little is known about VOC emission of brood in the other social insect [4, 21, 22]. The role of the brood on the behaviour (e.g. foraging regulation) and physiology (e.g. ovary maturation) of adults in social insects is well established. For example, in ants, brood has been shown to have a role in adult physiology and behaviour, but identification of this potential ant brood pheromone is still lacking [see review: 23]. In bumblebees, the pupal odour attracts the workers but has no impact on the physiology of the adult. The concept of a brood pheromone in the bumblebee is still discussed [24].

The emission of highly volatile chemical compounds by the brood allows information to be disseminated quickly and over a long distance within the nest. Studying emission of VOC by the brood would provide a better understanding of its chemical communication and development.

Thus far, no documentation of VOC emissions over the entire brood development is available, as studies describing VOC emissions from brood focus on specific stages of development and/or on specific compounds [25–27]. Maisonnasse *et al.* [27] showed the difference in

(E)- β -ocimene emission between larvae and pupae (first larval stage to fifth pupal stage). Carroll & Duehl [25] carried out *in situ* VOC captures on unstressed brood from hatching to the pre-pupal stage, in the presence of nurses on the frame. The authors showed changes in the emission of six VOC from the brood according to age (2-heptanone, 3-carene, β -ocimene, methyl benzoate, octanoic acid and decanal) and reported the presence of 22 VOC in total (2-heptanol, 2-heptanone, 2-pentanone, 3-carene, 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, α -pinene, citral, decanal, decane, ethyl benzoate, (E)- β -ocimene, geraniol, heptanal, hexanal, isobutyl acetate, isopentyl acetate, methyl benzoate, nerol, nonanal, octanal and nonane) [25]. Light *et al.* [26] also captured VOC *in situ* emitted by worker and drone brood at different developmental stages (last day of egg, first instar larvae, first, third and fifth instar pupae for drone brood, fifth instar pupae for cold-killed drone brood, fifth instar pupae for worker brood). They identified 75 VOC emitted by worker and drone brood frames that triggered an electrophysiological response in *V. destructor*. These different studies give an indication as to the VOC emitted by bee brood but only provide a fragmented view of VOC emissions during its development.

Similarly to "-omic" studies (genomics, transcriptomics, proteomics, metabolomics and metagenomics), capturing VOC emissions of the brood throughout its development, from oviposition to emergence, makes it possible to observe and capture the essence of brood-adult chemical communication in its entirety. Studying VOC emissions has led to a better understanding of reproduction in beetles [28] and the estimation of larval and pupal age in blowflies [29, 30]. In honey bees, VOC collection permits the study of the changes in larval food consumption and pheromonal emission over the course of their development [25, 27].

Bee brood development is characterised by a sequence of different physiological stages. The brood passes from an open brood state comprising the egg stage and five larval stages, to a capped brood state (*i.e.* brood cells closed with a wax cap by workers) before the emergence of the adult bee, comprising a sealed larval stage, a prepupal stage, and nine pupal stages [6]. Physiological changes in the brood can be observed through the brood haemolymph proteome [31, 32]. These important physiological changes can also be observed in brood-nurse interactions according to the developmental stage. Under the influence of brood pheromones, workers adapt the food they give to the brood according to the age of the larvae [9, 33, 34], cap brood [7], modify their foraging behaviour [10, 35], and realise hygienic behaviour [13–17].

In this study, we develop an *in situ* experimental approach to analyse VOC emissions of healthy worker brood during the whole developmental cycle (21 days, from egg stage to adult bee emergence). Our target is to highlight the temporal changes in brood-characterising compound presence and abundance during the entire brood development. We highlight semiochemical compounds emitted by brood that may have a role in the communication between brood and workers.

Materials and methods

Honey bee colonies and experimental setup

This study was performed with nine local honey bee colonies (*A. mellifera*) installed at the INRAE (Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement) research centre in Avignon (France). As the aim of this study was to capture volatile organic compounds (VOC) in healthy brood, we were very careful to use colonies that were free of parasites and disease. The health of these colonies was closely monitored throughout the experiment: colonies were qualitatively screened by our beekeepers to confirm the absence of brood illness. This study was conducted from April to July 2021, when varroa infestation is at its lowest, to maximize chances that capped brood cells were not infested by the parasite.

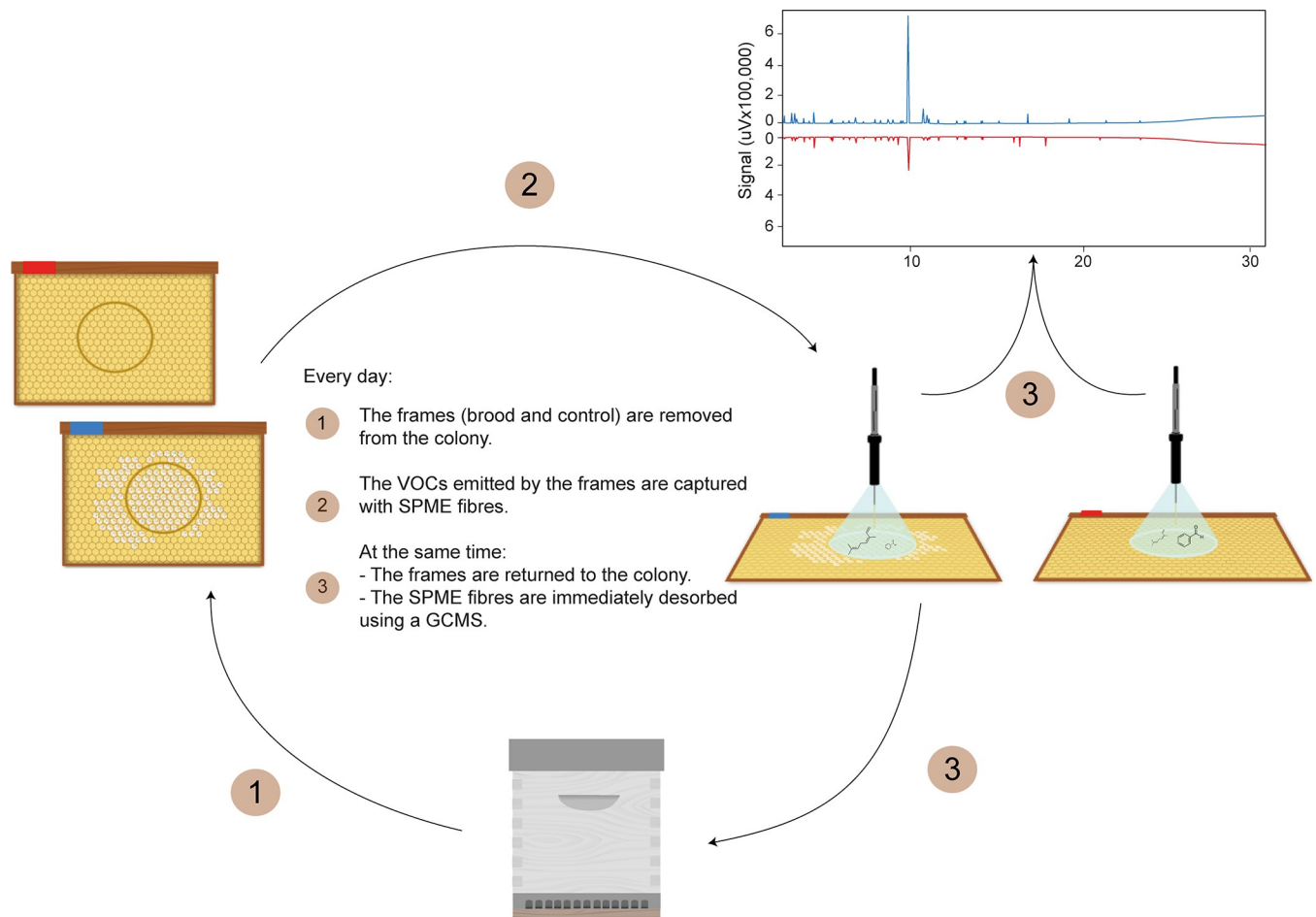


Fig 1. Experimental setup used for the *in situ* capture of the volatiles organic compounds (VOC) during honey bee brood development. Every day for 21 days, brood and control frames were removed from their colonies. VOC of the same patch of cells (brood and control) were captured for 20 minutes with a SPME fibre, in a room with controlled temperature and humidity (34–35°C, 65% RH). After capture, frames were immediately put back in their colony while fibres were desorbed on a GC-MS. Nine colonies were used, each with a control and a brood frame (371 captures in total).

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To ensure working with brood of a homogeneous age, queens of the nine selected colonies were caged on an empty frame for 24h to allow for egg laying. After that period, the queen was removed from the cage and no longer allowed access to the laid frame (*brood frame*). An empty built frame (no brood, pollen or nectar) was concomitantly used as a control in each colony (*control frame*). On the first day of the experiment (following queen laying), circles containing about 130 cells were drawn on both brood and control frames to select cell patches to be monitored daily, for 21 days. Each day, brood and control frames were removed from their colonies and brought to the laboratory for chemical analysis. Experimental procedures were optimised to minimise any source of stress. To do so, adult bees were gently removed from the brood frame with a soft brush, and the brood was placed directly in a warm, moist box during the short journey from the hives to the experimental room. They were kept in a room with controlled temperature and humidity (34–35°C, 65% RH). The same patch of cells was monitored each day (Fig 1), and the content of each cell was recorded on a transparent plastic sheet.

Volatile organic compound capture and analysis

VOC capture was conducted passively, using a polydimethylsiloxane divinylbenzene (PDMS DVB) Solid Phase Micro Extraction (SPME) fibre (65 μm , manual holder). The fibre was inserted inside a glass funnel (\O 70 mm) placed upside down on the patch of cells to be monitored (Fig 1). For each capture, the funnel was pressed against the waxy surface to form a hermetic junction between the sample (cell patch) and the capture system. Captures lasted for 20 min, after which frames were returned to their colonies. Each capture of a brood sample was paired with that of a control sample (seven control samples could not be captured due to bad weather conditions on day 11 and 12), thus forming a total of 371 captures (9 control + 9 brood samples per day). Qualitative and quantitative analyses of VOC were performed on a gas chromatograph coupled with mass spectrometry (GC-MS). SPME fibres were desorbed into a GC-MS (Shimadzu GCMS-QP2010SE), equipped with an electronic impact ion source (70 eV) and a ZB 5 MS column (Zebron ZB 5ms, 20 m x 0.18 mm, 0.18 μm thickness). Desorption was realised in splitless mode, with the constant column flow at 0.82 mL/min; the carrier gas was helium. The injector temperature was set at 250°C and the oven temperature program as follows: 45°C isothermal held for 5 min, followed by temperature increases at a rate of 10°C/min up to 250°C. The oven was finally held at 250°C for 4 min. The ion source was set at 200°C and m/z scanned from 40 to 350 amu. Compound identification was performed by comparison of their mass spectra and retention indices with those found in the NIST 2017 library (v2.3). This identification was further confirmed by injection of the reference standards purchased from Sigma Aldrich (except for hexenyl acetate (s24) for which the location of the unsaturation remains unknown).

Statistical analyses

Statistical analyses were performed using R (version 4.0.5). To account for non-linear trends across time, generalized additive mixed models (GAMM) were fit on total peak area for each day and each sample group (brood, control) to investigate differences between control and brood samples and their evolution over time (R package “mgcv” v1.8–39; <https://cran.r-project.org/web/packages/mgcv/index.html> [36]).

Three redundancy analyses (RDA) were performed on three different phases of development, based on GAMM results (“Eggs”: day 1 to day 3; “Larvae and Non-Sclerotised Capped Brood”: day 4 to day 16; “Sclerotised Pupae”: day 17 to day 21), to identify compounds that characterise the brood (R package “vegan” v2.6–2; <https://cran.r-project.org/web/packages/vegan/index.html>). Day and sample group (brood and control) and their interaction were included as fixed factors in the RDA to identify brood-characterising compounds. To graphically represent the RDAs, we selected the two components which explain the largest proportion of the data variance. Compounds detected in at least five out of the nine replicates in brood samples for any given day were included in the analyses. From the 3 RDAs, a list of 32 brood-characterising compounds was compiled. We then split brood development into eight characteristic periods to study the compounds or chemical families that characterised each period, as well as those that covaried together. The four days around the capping of brood cells by the workers, represent a key moment in the development of the brood, both physiologically and behaviourally [3, 6, 7, 37]. For this reason, those four days were separated into four one-day periods. The eight periods were thus defined as follows: Eggs: days 1–3; 1st to 4th instar larvae (L1–L4): days 4–7; 5th instar larvae (L5): day 8; Capping: day 9; Sealed larvae (SL): day 10; Prepupae (PP): day 11; Non-sclerotised pupae (NSP): day 12–16; and Sclerotised pupae (SP): days 17–21.

In order to classify the data, we chose to group the compounds found according to their respective chemical families. We chose this presentation not because of the potential effects on behaviour, but rather to highlight a classification based on the chemical structure of these molecules, in particular to evaluate shared patterns of variation and stage-specific emission.

As our target is to highlight the temporal changes in the presence and abundance of brood-characterising compounds during development, we normalised brood compound area values by those measured in the control (*i.e.* $(A_{\text{brood}} - A_{\text{control}}) / A_{\text{control}}$) for each day, compound, and colony. To avoid division by zero, a constant ten times lower than the smallest non-zero value was added to the data. We then calculated the median normalised area of each compound across the nine colonies for each day. Negative median values, indicating a higher amount of the compound in control samples than in brood samples, were set at zero. Compounds were subsequently quantified relative to the highest median normalised value observed across the 21 capture days. We represented these relative median normalized values in a heatmap with a \log_{10} scale (adding a constant of 1). We similarly calculated normalised brood compound area by stage, analogous to that done by day. For two compounds, these stage-specific median normalised values were equal to 0 as they were observed on a single day of the four days comprising the SP stage. As before, compounds were quantified relative to the highest median value observed across the 8 developmental stages. The evolution across stages of relative median normalised compound emissions was visualized in another heatmap. We realised a clustering of the arcsine-transformed relative median normalised values per stage using a Gaussian mixture model to highlight compounds that shared stage-specific maximum emissions (R package “coseq” version 1.17.2; <https://bioconductor.org/packages/release/bioc/html/coseq.html> [38]). The number of clusters was identified based on the integrated completed likelihood (ICL) model selection criterion.

For comparisons with the scientific literature, we calculated the estimated emission per mg of individuals weight for (E)- β -ocimene. As the tracking of the individuals throughout the experiment did not allow us to weigh the brood, we instead used in-house brood weight data (first larval to fifth pupal stage, day 4 to day 15 in this paper, $n = 9$ per stage) obtained in a previous experiment on similar colonies.

Results

Total compound emission profile across development

A comparison of total peak area between control and brood samples revealed that all compounds (total peak area) are significantly more abundant in brood samples than in control samples (GAMM; Brood: estimate = 296,010, $p < 2e^{-16}$; control: estimate = 200,479, $p < 2e^{-16}$) (Fig 2). The quantity of compounds fluctuates significantly from $2.6e^5$ (day 16) to $5.4e^5$ (day 11) of total compound area (GAMM; Brood over time: $F = 12.403$, $p < 2e^{-16}$; Control over time: $F = 0.078$, $p = 0.820$) (Fig 2). The first three days (egg stage) and the last five days (sclerotised pupal stages) are periods of low overall emissions, with small differences between brood and control samples (Fig 2). From day 4 (first instar larvae) to day 16 (fourth instar pupae), total compound abundance is quite variable and strikingly different between brood and control samples, with a notable peak of brood emission around days 10 and 11 (prepupae) (Fig 2). Based on these tendencies, and to improve the brood-characterising compound determination step, we separated our dataset into three groups to perform multivariate redundancy analyses (RDA): Egg (day 1 to day 3), Larvae and Non-Sclerotised Capped Brood (day 4 to day 16) and Sclerotised Pupae (day 17 to day 21).

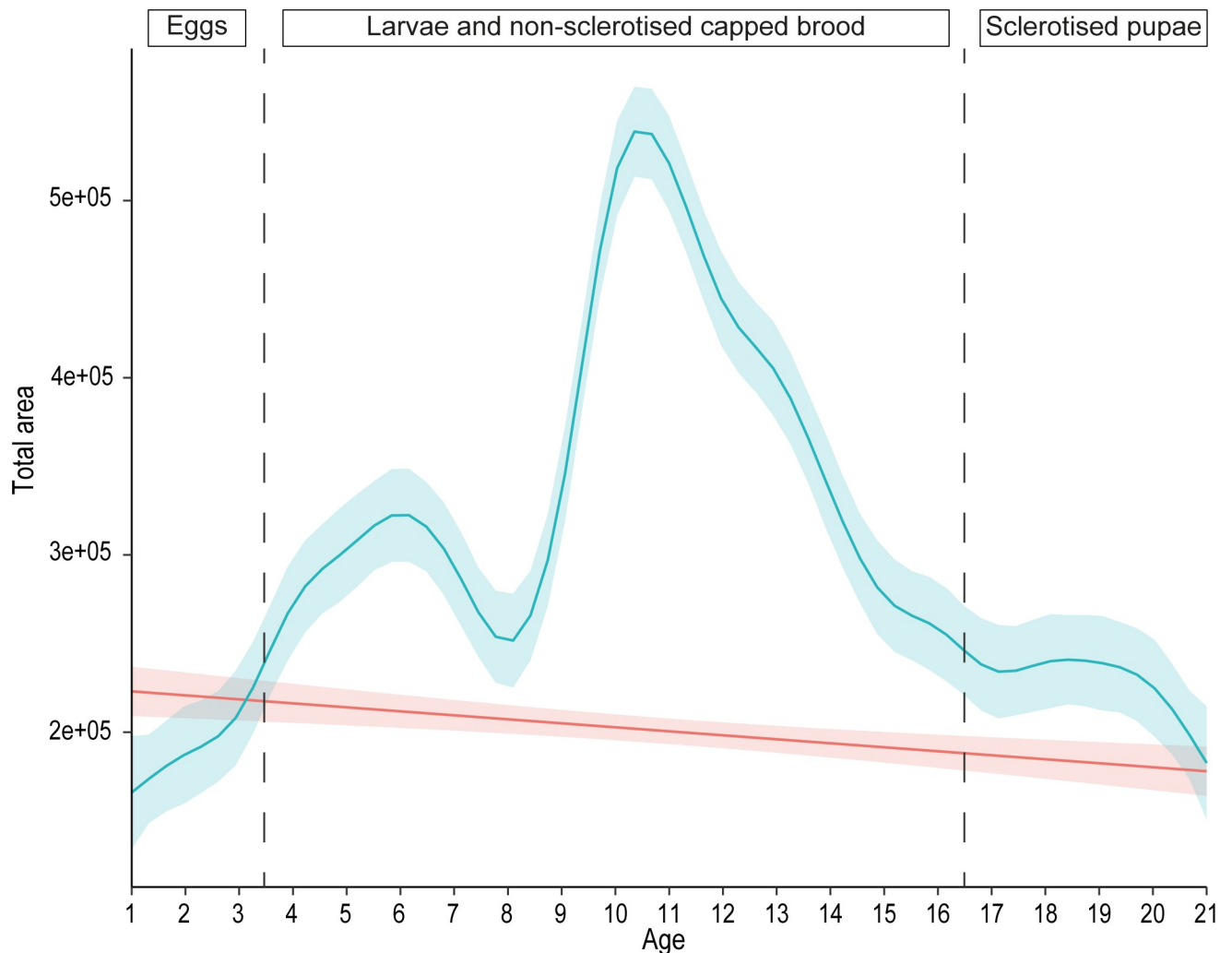


Fig 2. Evolution of the total peak area of all compounds across development (GAMM models). Lines show the mean total area and the surrounding 95% standard error, for brood (blue) and control (red) samples. Dotted lines separate the three groups: “Eggs” (day 1 to 3), “Larvae and non-sclerotised capped brood” (day 4 to 16) and “Sclerotised Pupae” (day 17 to 21) considered for the multivariate analyses.

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Identification of brood-characterising compounds

Based on the total emission analysis and the identification of 3 distinct phases of compound emission, we performed three redundancy analyses (RDAs).

In the largest RDA (day 4 to day 16, “Larvae and non-sclerotised capped brood” group), the first two components of the RDA explained 56.82% of the variance. Both day and sample group, as well as their interaction, were significant (Table 1). The two sample groups (brood and control) were well separated on the first component (Fig 3). Based on this analysis, twenty-seven compounds characterising the brood samples were retained (s01, s02, s03, s05, s09, s12, s13, s15, s17, s18, s24, s27, s28, s29, s30, s32, s36, s37, s38, s41, s42, s47, s52, s54, s65, s71, s74, s75, s76) (Fig 3, Table 2).

In the “Egg” RDA analysis (days 1–3), the first two components of the RDA explained 73.95% of the variance. Both day and sample group were significant (Table 1), but the interaction was not, confirming that overall brood and control compound emissions maintain the

Table 1. Results of the 3 RDAs showing the effects of day and sample groups on compound area. For each day: brood sample n = 9, control sample n = 9.

Groups	Factors	F	P
Eggs (<i>day 1 to day 3</i>)	Day	2.04	0.008
	Sample group	4.39	0.002
	Day: Sample group	0.49	0.994
Larvae and non-sclerotised capped brood (<i>day 4 to day 16</i>)	Day	2.34	0.001
	Sample group	22.92	0.001
	Day: Sample group	1.45	0.001
Sclerotised Pupae (<i>day 17 to day 21</i>)	Day	1.89	0.001
	Sample group	7.28	0.001
	Day: Sample group	0.73	0.950

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same temporal trend. Both sample groups (brood and control) were also well separated on the first component (S1 Fig). Five compounds characterising brood samples, all included in the previous list, were highlighted by the RDA (s15, s18, s30, s37, s52) (S1 Fig, Table 2).

In the “Sclerotised pupae” RDA (day 17 to day 21), the first two components explained 64.07% of the variance. Both single factors were significant, but their interaction was not (Table 1). The two sample groups were once again well separated on the first component (S1 Fig). Twenty-eight compounds characterising the brood sample were retained, partially overlapping with the previous lists (s01, s02, s03, s05, s09, s10, s12, s13, s15, s17, s18, s27, s28, s30, s32, s35, s37, s38, s41, s45, s47, s54, s65, s71, s73, s74, s75, s76) (S1 Fig, Table 2).

Using these three RDAs, we were able to compile a final list of 32 VOC characterising the brood samples (S2 Fig, Table 2).

Evolution of brood-characterising compound emission during brood development

Each day of brood development was characterised by different emission of VOC. The 32 VOC could be classified into two groups; a group of compounds with a marked variation of emission over time and a group with a more constant emission. The group of compounds fluctuating over time included 15 compounds (in order of appearance in the Fig 4, from top to bottom: s27, s30, s42, s52, s18, s10, s12, s47, s36, s29, s73, s03, s09, s02 and s01) (Fig 4). Except for α -pinene (s15), all terpenes found in this study were in this group (s27, s30, s42, s18, s29 and s73, Fig 4). The same pattern applied to the two ketones (s12 and s36, Fig 4).

The group of compounds with a more constant emission over time included 17 compounds (s37, s15, s74, s71, s28, s38, s65, s75, s54, s41, s05, s13, s24, s32, s35, s45 and s17) (Fig 4). The seven alkanes found in this study were in this group (s74, s71, s65, s75, s54, s05 and s13), as well as the aldehyde (s28) (Fig 4). For the alcohols and esters found in this study, their fluctuation over time depends on the compound. Four alcohols showed a marked change over time (s03, s09, s02 and s01), and six others displayed more constant emissions level (s38, s41, s32, s35, s45 and s17) (Fig 4). Two esters fluctuated strongly over time (s52, s10 and s47) and two others remained constant (s37 and s24) (Fig 4).

To study the compounds or chemical families that characterised each brood stage as well as those that covaried together, the 21 days of brood development have been divided into eight periods, as follow: Eggs: days 1–3; 1st to 4th instar larvae (L1-L4): days 4–7; 5th instar larvae (L5): day 8; Capping: day 9; Sealed larvae (SL): day 10, Prepupae (PP): day 11, Non-sclerotised pupae (NSP): days 12–16, Sclerotised pupae (SP): days 17–21. Different compounds were

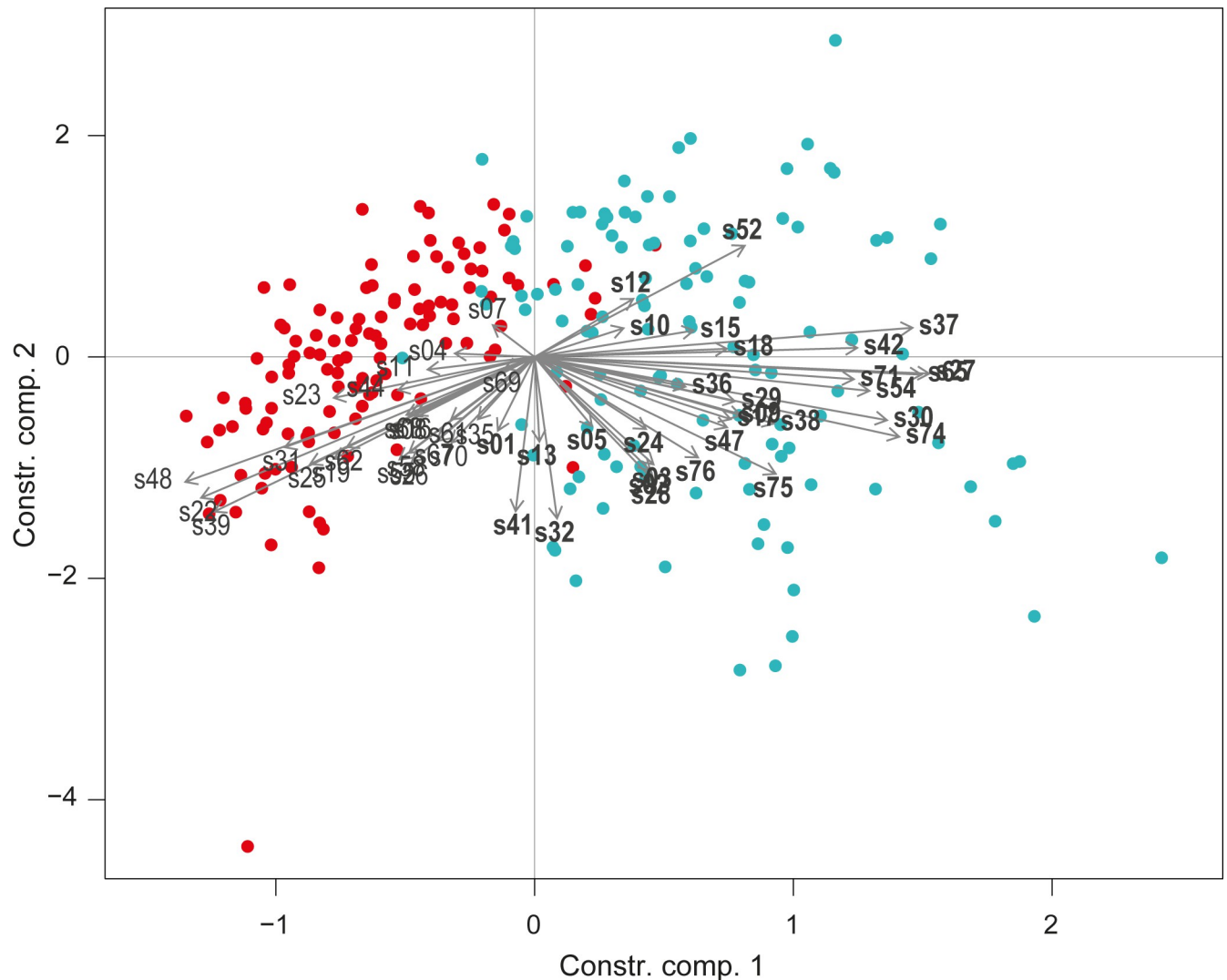


Fig 3. Redundancy analysis (RDA) performed on chemical compounds captured on brood and control samples for the “larvae and non-sclerotised capped brood” group (day 5 to day 16). For each day: brood sample $n = 9$, control sample $n = 9$. Each dot represents a sample (blue: brood, red: control). Compounds are represented by their IDs and grey arrows. The compounds characterising the brood and retained for the rest of the analysis are shown in bold.

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characteristic of the eight brood developmental stages (Fig 5). None of the compounds had its maximum at the egg stage (days 1–3), but six compounds were found in greater quantities in brood containing eggs than in the control (s15, s18, s30, s37, s52 and s71) (Fig 5 and S1 Fig). None of these six compounds were specific to the eggs; their emission increased upon hatching. In the first larval instars (L1–L4, days 4–7), four compounds reached their maximum emission (s10, s47, s52 and s12), and two other compounds neared it (s15 and s37) (Fig 5). In the last larval stage (L5, day 8), the blend began to change drastically. Compounds with their greatest quantity in the L1–L4 stage decreased (s10, s47, s52, s12 and s37), while the quantity of other compounds began to increase (s09, s35, s74, s75, s71 and s54) (Fig 5). During the capping period (Capping, day 9), 11 compounds had their maximum emission peak (s15, s74, s75, s71, s36, s01, s05, s13, s03, s02 and s41) (Fig 5). All of these compounds decreased during the sealed larval stages (SL, day 10) (Fig 5). In this stage, seven compounds had their maximum

Table 2. List of chemical compounds found as brood-characteristic during brood development (21 days). Days of detection represent periods where a given compound is found in at least 5 replicates in brood samples for a given day. *: Unsaturation remains unknown, **: Stereochemistry remains unknown.

ID	Compounds	Chemical families	Retention time	Retention index	Days of detection
s01	3-Methyl-2-buten-1-ol (Prenyl alcohol)	Alcohol	2.947	784	4–6, 9
s02	2,3-Butanediol ** (diastereomer)	Alcohol	3.112	789	8–16, 18–20
s03	2,3-Butanediol ** (diastereomer)	Alcohol	3.307	795	8–16, 18–20
s05	Octane	Alkane	3.447	800	3–5, 8–11, 14–21
s09	1-Hexanol	Alcohol	5.473	869	8–21
s10	Methylbutyl acetate (Isoamyl acetate)	Ester	5.735	877	1, 4–8, 10–12, 14, 16, 19–20
s12	2-Heptanone	Ketone	6.090	890	4–9
s13	Nonane	Alkane	6.435	900	5, 9–10, 13, 16–19
s15	α -Pinene	Terpene	7.273	934	2–9, 11–20
s17	1-Heptanol	Alcohol	8.260	973	12, 16, 18, 21
s18	β -Pinene	Terpene	8.345	977	3–4, 6, 11–20
s24	2-Hexenyl acetate **	Ester	9.385	1023	11
s27	(Z)- β -ocimene	Terpene	9.660	1036	6–18
s28	Phenylacetaldehyde	Aldehyde	9.750	1042	6, 9–13, 15, 17–21
s29	α -Ocimene	Terpene	9.800	1045	7, 11, 15, 17
s30	(E)- β -Ocimene	Terpene	9.886	1047	1–21
s32	1-Octanol	Alcohol	10.348	1072	9–20
s35	(E)-Linalool furanoxide	Alcohol	10.623	1086	19
s36	2-Nonanone	Ketone	10.713	1090	6–9
s37	Methyl benzoate	Ester	10.753	1092	1–21
s38	Linalool **	Alcohol	10.89	1099	9–14, 16–20
s41	Phenylethyl alcohol	Alcohol	11.085	1111	9–12, 18–20
s42	Allo-ocimene	Terpene	11.368	1127	6–7, 10–16
s45	(E)-Linalool pyranoxide	Alcohol	12.080	1170	19
s47	Methyl salicylate	Ester	12.428	1190	4–12, 14–16, 18–19
s52	γ -Octalactone	Ester	13.403	1253	1–15
s54	Tridecane	Alkane	14.105	1300	1, 5–21
s65	Pentadecane	Alkane	16.793	1500	4–21
s71	Heptadecane	Alkane	19.172	1700	1, 4–18
s73	Nonadecene *	Terpene	21.087	1880	19–21
s74	Nonadecane	Alkane	21.315	1900	4–21
s75	Eicosane	Alkane	23.262	2000	8–21

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emission peak (S65, s32, s38, s54, s28, s30 and s42) (Fig 5). In the prepupal stage (PP, day 11), only three compounds reached their maximum emission peaks (s27, s29 and s24) and two compounds were slightly under maximum emission levels (s15 and s37) (Fig 5). In the non-sclerotised pupal stage (NSP, days 12–16), two compounds had their maximum emission peaks (s37 and s18) and two others were slightly under maximum emission levels (s15 and s42) (Fig 5). In the last stage of brood development (SP, days 17–21), only one compound was at its maximum (s73) (Fig 5).

The total amount of (E)- β -ocimene (s30) was higher during capped brood stages (SL, PP, NSP and SP) than in larvae, especially just after capping. When the amount of (E)- β -ocimene (s30) was reported per mg of individual weight, its quantity was found to be higher in larval brood stages than in capped brood stages and reached its maximum in first instar larvae (day 4) (S3 Fig).

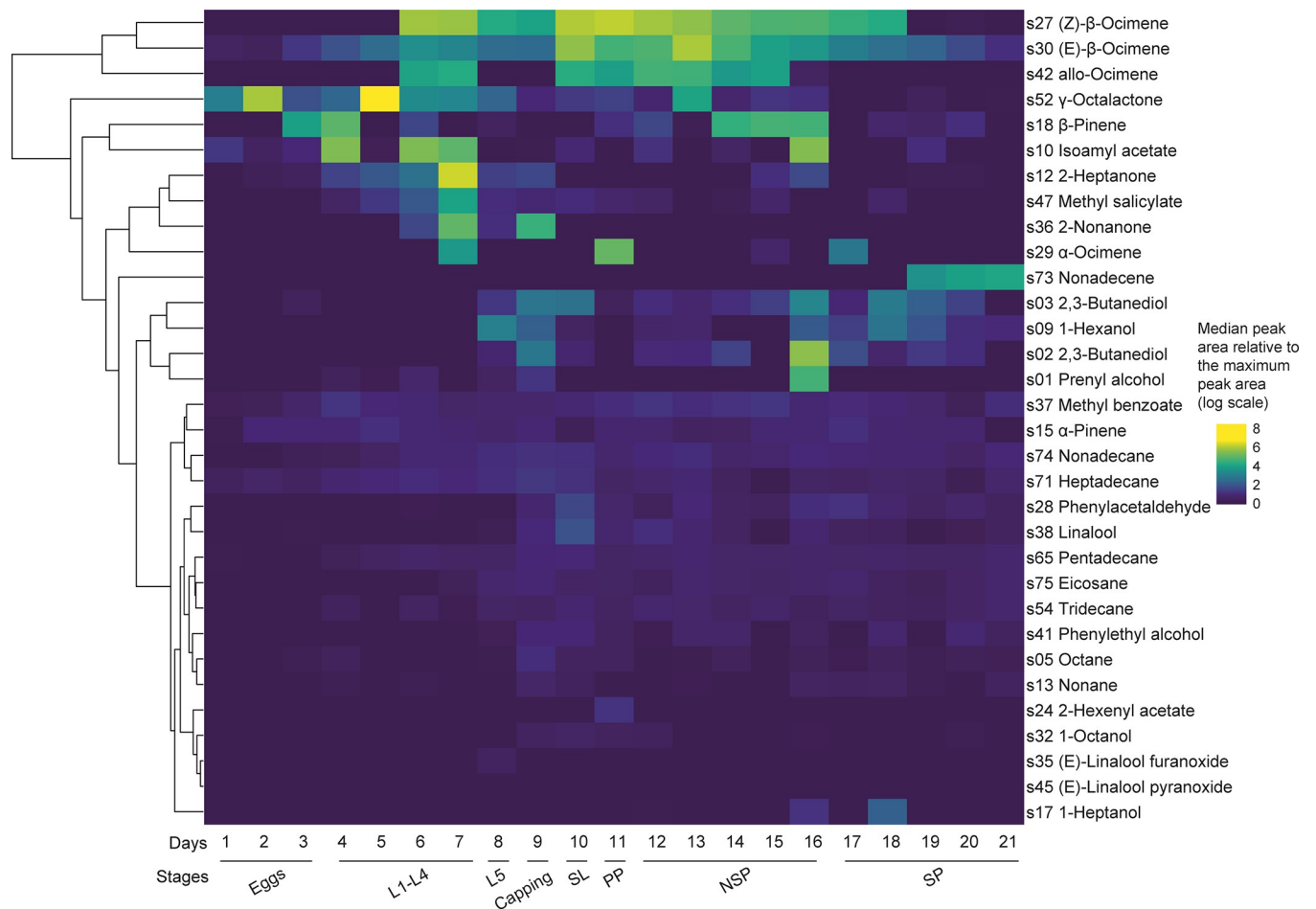


Fig 4. Heatmap showing the temporal evolution of the amount of the 32 compounds characterising brood throughout development. The temporal evolution of each compound area is represented in rows, with a colour gradient representing the median normalized peak area relative to the respective maximum for each compound (in log scale). The colour change represents a change in emission of the compound, with a maximum emission in yellow and a minimum in dark blue. Each column represents the age of brood development. Compounds are labelled with their IDs and chemical names. A dendrogram representing a hierarchical clustering (Euclidean distance, complete linkage) of compounds is indicated on the left.

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Depending on their chemical families, the compounds highlighted in this study did not have their maximum emission peaks at the same stages of brood development. Ketones had their maximum emission peak at L1-L4 for s12, and Capping stages for s36 (clusters 1 and 3, Fig 6). Except for s37, which had its maximum peak at the NSP stage, and s24, which had its maximum peak at PP stage, the esters had their maximum emission peaks at the L1-L4 stage (Fig 6). The alcohols peaked at the late larval stages (L5, Capping and SL) (clusters 2, 4 and 6, Fig 6). Alkanes had their peaks at the capping and just after (Capping and SL) (clusters 3 and 5, Fig 6). The aldehyde had its peak at the SL stage (cluster 6, Fig 6). Finally, except for s15 which had a double peak at L1-L4 and Capping (cluster 11), all of the terpenes had their maximum emission peaks after capping of the brood cells (clusters 6, 7, 8, 9, 10 and 11, Fig 6).

Discussion

In this study, we analysed the VOC emitted by honey bee brood (*A. mellifera*) throughout its development. We identified 32 VOC characterising the brood, belonging to six different

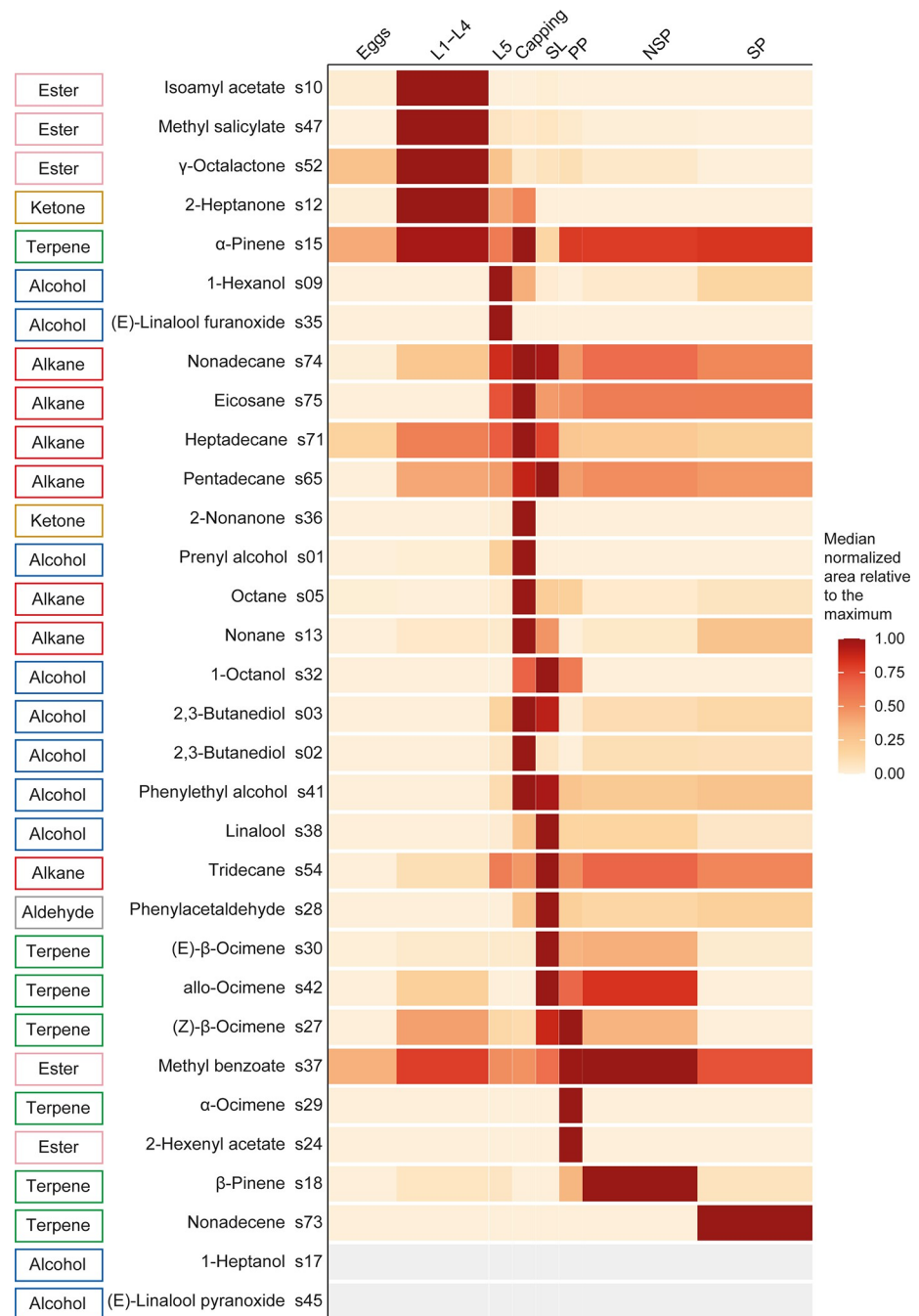


Fig 5. Heatmap showing the temporal evolution of the relative amount of the 32 brood characterising compounds, along developmental stages. The evolution of the quantity of each compound is represented in rows, with a colour gradient. The absence of compounds is represented in grey (here, both compounds are present only on a single day of the SP stage, resulting in a median of 0 for all stages). Each column represents a key stage of brood development (Eggs: day 1 to day 3; L1-L4: 1st to 4th instar larvae, day 4 to day 7; L5: 5th instar larvae, day 8; Capping: adult capping behaviour, day 9; LS: larvae spinning cocoon, day 10; PP: pre-pupae, day 11; NSP: non-sclerotised pupae, day 12 to day 16; SP: sclerotised pupae, day 17 to day 21). The width of each box is proportional to the duration of each stage. Compounds are labelled with their IDs, chemical names and chemical families.

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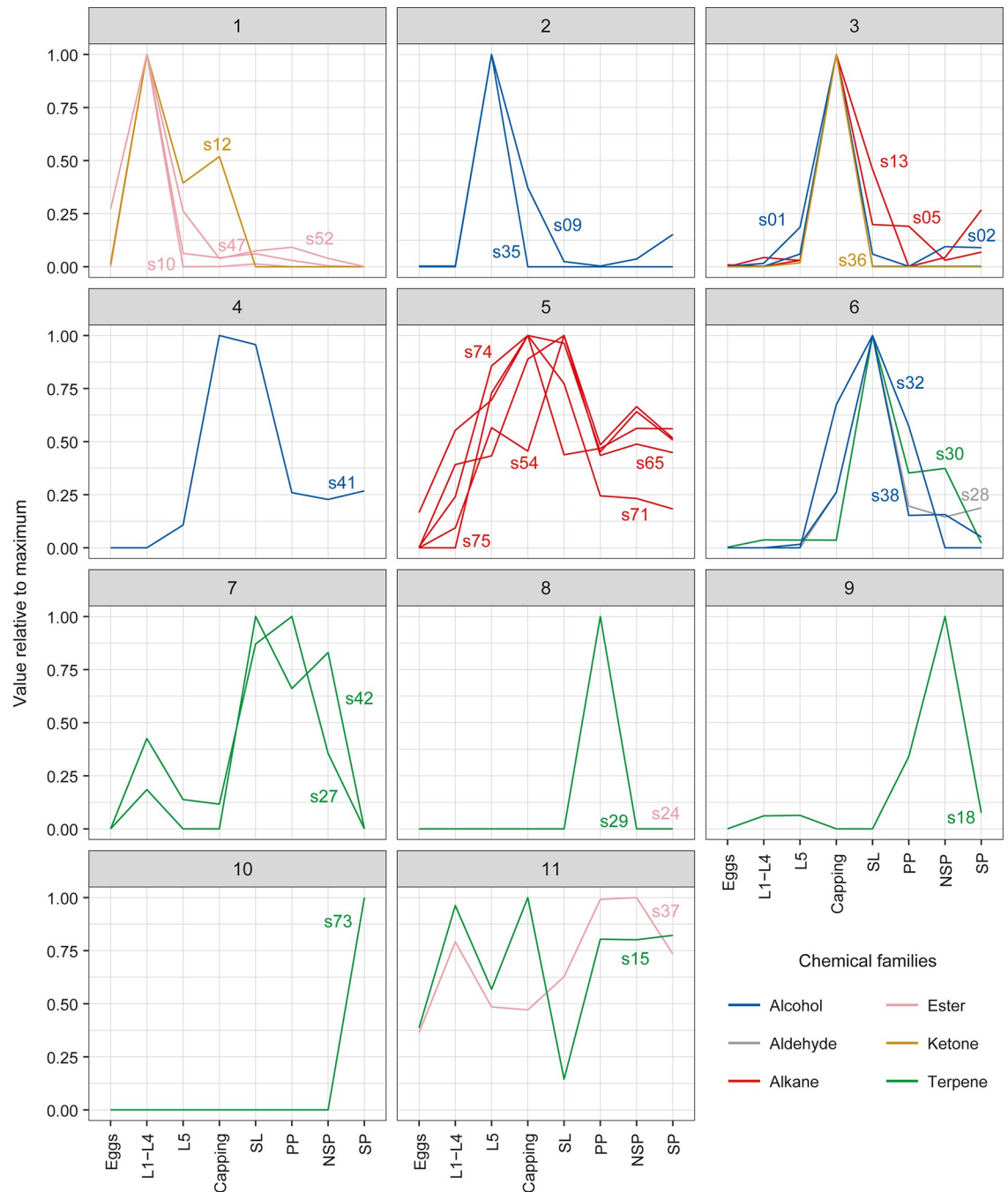


Fig 6. Clusters (Gaussian mixture models) showing the temporal variation of the relative (with respect to the maximum across stages) median normalized amount of the 32 brood-characterising compounds along developmental stages. Clusters group compounds with similarly timed emission peaks. Compounds are labelled with their IDs and coloured according to their chemical families. Eggs: day 1 to day 3; L1-L4: 1st to 4th instar larvae, day 4 to day 7; L5: 5th instar larvae, day 8; Capping: adult capping behaviour, day 9, SL: larvae spinning cocoon, day 10, PP: pre-pupae, day 11, NSP: non-sclerotised pupae, day 12 to day 16; SP: sclerotised pupae, day 17 to day 21.

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chemical families. The compounds did not have the same temporal dynamic during brood development and most of them appear to be stage-dependant. Chemical families were also specific to developmental stages.

Headspace capture is a common method for studying volatile emissions from bee brood [8, 13, 16, 27, 39, 40]. The focus of our study is to describe the landscape of chemical emissions from bee brood so that it can serve as a base for future, more functional studies. Some compounds were not fully identified (e.g. s24–2-Hexenyl acetate for which its stereochemistry is not found or s73 –Nonadecene where the location of the double bond is still unknown) and would require further identification.

To date, this study presents for the first time the emission of VOC from honey bee brood during its whole development, from egg laying to emergence. Some previous studies showed the evolution of specific compounds during the larval or early pupal stages [25, 27] or the presence of compounds in larvae or pupae at specific ages [26, 41]. A key point of our method was the monitoring of the same brood cells throughout development, with a non-invasive passive capture. No food resources (pollen, nectar, honey) were present on the brood frames, except for the larval jelly necessary for larval nutrition, which suggest that the compounds retained in this study are representative of the brood. Of the 32 compounds described in this study, 15 compounds are newly described in bees, with one compound never described either in bees or in the hive environment but has been described in floral emissions [42], and 14 of them already described as being present in the hive environment (wax, propolis, pollen, honey, ambient air) [43–48]. The other 17 compounds had already been described in bees [8, 19, 26, 40, 41, 49–55], with 13 already described has emitted by bee brood [8, 19, 26, 40, 41, 51, 52, 55].

Ocimene brood emission

(E)- β -ocimene (s30) was the compound that was captured in the greatest quantity throughout this experiment, four times higher than the other compounds. Thus, the total peak area of the full set of compounds largely reflected the dynamics of (E)- β -ocimene (s30) during brood development. (E)- β -ocimene (s30) was found every day during brood development, from the first day of the egg stage to the emergence of the adult bee. This compound has already been described in the scientific literature in the uncapped brood to early capped brood stages [25, 27]. Its presence in the emission of VOC from eggs is described here for the first time. In our study, (E)- β -ocimene (s30) is more abundant in the capped stages than in the uncapped larval stages, with a peak in the SL stage. However, when the amount of compound is calculated according to the weight of the individuals, the peak of (E)- β -ocimene (s30) appears to be on day 4, at the first instar larvae. As a consequence, the amount of (E)- β -ocimene (s30) emitted by an individual is higher in the uncapped larval stages, especially in early larval stages, than in the capped brood stages. These results are in accordance with the results obtained by Maisonnasse *et al.* [27], thus providing additional validation to our experimental set-up. The presence of this compound in stages other than uncapped and early-capped brood raises questions about its possible role in other stages of brood development and its involvement in chemical communication between the brood and the adults of the colony.

In addition to (E)- β -ocimene (s30), we identified three ocimene isomers: (Z)- β -ocimene (s27), α -ocimene (s29) and allo-ocimene (s42). Allo-ocimene (s42) was previously described as a putative pheromone emitted by young larvae, in a similar context to (E)- β -ocimene [55]. Here, we found the presence of allo-ocimene in more than five of our samples mainly in the 3rd and 4th instar larvae as well as from the SL stage to the end of the NSP stage. Similarly to (E)- β -ocimene, allo-ocimene triggers a rapid nervous response of the antenna [55]. In addition, the ocimene is processed by the antennal lobe [56], the first brain region processing olfactory information sent by the antennal neurons [57]. Neither of the two other ocimenes captured in this study have been described as part of brood pheromones. Nevertheless, the response of adults to (E)- β -ocimene and allo-ocimene, and the high similarity between these

four ocimenes, suggest that nurses may detect (Z)- β -ocimene and α -ocimene. Like (E)- β -ocimene with the BEP, the emission of these three other ocimenes could have an additive or synergistic effect and trigger different behaviours in adults [10, 27]. Further studies on behaviour and brain processing of olfactory information of nurse bees are necessary to highlight the potential discrimination between these four ocimenes, and the potential role of the (Z)- β -ocimene, the α -ocimene and the allo-ocimene.

Compounds known to transmit pheromonal information between adult bees

In our study, we found four compounds (1-hexanol (s09), isoamyl acetate (s10), 1-octanol (s32) and 2-heptanone (s12)) already described as part of pheromone alarm mixtures emitted and received by adult bees [50, 53, 54, 58]. The alarm pheromone is composed of a cocktail of nearly twenty chemical compounds [3, 54, 59].

The compound 2-heptanone is known to be synthesized by the mandibular glands [53, 58]. During the larval stage, nurses feed the brood with royal jelly for the first stages and then with a mixture of pollen, honey and glandular extract from the mandibular and hypopharyngeal glands [60, 61]. The compound 2-heptanone has been described as a royal jelly VOC [45] and from VOC of uncapped brood frames in the presence of adult workers [25]. In our study, 2-heptanone (s12) was present in all uncapped larval stages, with a peak in 4th instar larvae (L1-L4, day 7). As the *in situ* capture of brood VOC did not allow us to separate brood emissions from potential emissions by larval food, our results suggest that the presence of 2-heptanone in our experiment during the early larval stages may be explained by the presence of the larval food in the brood cells. This would indicate that this compound is released from the larval food, even if we cannot rule out a possible emission by larvae.

The compound 1-hexanol (s09), isoamyl acetate (s10) and 1-octanol (s32) are synthesized in the Koschevnikov gland, close to the stinger [50, 54], and are known to be part of the alarm pheromone of adult workers [3, 54, 59]. The main functions of the alarm pheromone for colony defence are increased flight activity, recruitment of individuals and behavioural responses to moving targets. Not all active chemical compounds trigger these three functions. The common function of isoamyl acetate, 1-hexanol and 1-octanol is their ability to recruit other adults [54]. In our experiment, 1-hexanol (s09) had its emission peak at the 5th larval stage (L5, day 8) and remained abundant at the time of operculation (Capping, day 9) and at the last pupal stages (SP, days 17–18). The compound 1-octanol (s32) was captured mainly on days 8, 9 and 10 (L5, Capping, PP), with a peak at the time of operculation. Due to the synchronization between the emission of these compounds and the capping behaviour observed during the experiment, we hypothesise that 1-hexanol and 1-octanol emission by the brood may play a role in eliciting capping behaviour by workers. This hypothesis needs to be validated by behavioural assays, such as those already used to confirm the role of brood pheromone compounds as a capping signal [7].

The amount of isoamyl acetate in the Koschevnikov gland differs according to the age of the worker. Nurse bees displayed more isoamyl acetate in their gland than guards or foragers, suggesting another function in a context other than the defence of the colony [62]. This compound had already been reported as part of VOC of uncapped brood frames in the presence of adult workers [25]. In our study, we captured isoamyl acetate (s10) mainly during the larval stages (L1-L4). This suggests that broods may emit isoamyl acetate as a chemical signal for care to the nurse bees. This signal can have various meanings such as a signal that the brood is healthy, a call for feeding, for temperature or humidity control. Behavioural assays are needed to validate this hypothesis.

Compounds emitted by the brood but not described as pheromones

In this study, we found 32 compounds characterising the brood belonging to six chemical families, including ten alcohols (prenyl alcohol (s01), 2,3-butanediol (s02), 2,3-butanediol (s03), 1-hexanol (s09), 1-heptanol (s17), 1-octanol (s32), (E)-linalool furanoxide (s35), linalool (s38), phenylethyl alcohol (s41), (E)-linalool pyranoxide (s45)), one aldehyde (phenylacetaldehyde (s28)), seven alkanes (octane (s05), nonane (s13), tridecane (s54), pentadecane (s65), heptadecane (s71), nonadecane (s74), eicosane (s75)), five esters (isoamyl acetate (s10), hexenyl acetate (s24), methyl benzoate (s37), methyl salicylate (s47), γ -octalactone (s52)), two ketones (2-heptanone (s12), 2-nonanone (s36)) and seven terpenes (α -pinene (s15), β -pinene (s18), (Z)- β -ocimene (s27), α -ocimene (s29), (E)- β -ocimene (s30), allo-ocimene (s42), nonadecene (s73)). As previously mentioned, only five compounds have been described as pheromonal compounds in bees. Within the remaining 27 compounds, 13 have already been described in brood, either as being emitted by the brood (octane (s05), nonane (s13), α -pinene (s15), (E)-linalool furanoxide (s35), 2-nonanone (s36), linalool (s38), methyl benzoate (s37), allo-ocimene (s42), methyl salicylate (s47), γ -octalactone (s52), tridecane (s54), heptadecane (s71), nonadecane (s74), eicosane (s75)) [19, 25, 26, 40, 51, 52, 55], or by dead brood extracts (octane (s05), nonane (s13), eicosane (s75) tridecane (s54)) [41]. Our results are in accordance with these studies. The 14 other compounds found to characterize the brood (prenyl alcohol (s01), 2,3-butanediol (s02), 2,3-butanediol (s03), 1-heptanol (s17), β -pinene (s18), hexenyl acetate (s24), (Z)- β -ocimene (s27), phenylacetaldehyde (s28), α -ocimene (s29), phenylethyl alcohol (s41), allo-ocimene (s42), (E)-linalool pyranoxide (s45), pentadecane (s65), nonadecene (s73)) have already been described as being present in the VOC of hive products (honey, propolis, larval jelly, pollen) or floral extracts [44–47, 55, 63, 64], but we described them in honey bee brood for the first time. Nevertheless, α -pinene (s15) and β -pinene (s18) have been described as part of the odours of termite guardians [65] or as being present in Dufour's gland of ants [66]. The presence of VOC previously measured in hive products in brood emissions is not surprising as hive products are involved in nutrition (honey, larval jelly and pollen) and protection (honey, propolis, larval jelly) against various pathogenic agents of all the individuals in the colony [33, 67–71]. These chemical compounds could be brought to the uncapped brood cells by nurses when they visit brood cells during caring behaviours. Then, compounds can be trapped in and emitted from the wax of brood cells, or released at specific times by the brood as a means of communication with adults.

Egg compounds

We found that eggs emitted a blend of five VOC: α -pinene (s15), β -pinene (s18), (E)- β -ocimene (s30), methyl benzoate (s37) and γ -octalactone (s52). Even if all of these compounds are maximally abundant at stages of brood development other than the egg stage, this study is the first to describe these 5 compounds as characteristic of eggs. The compound β -ocimene (s30) and methyl benzoate (s37) have already been found on VOC from frames containing eggs and one-day-old larvae [25]. Here, we confirm the presence of (E)- β -ocimene and methyl benzoate in egg brood cells and suggest that (E)- β -ocimene and methyl benzoate take part in the egg odour blend. Heptadecane (s71) had already been found in VOC from frames containing fourth and fifth instar larvae [26]. We found heptadecane on the first day of eggs. Here, we suggest that heptadecane takes part in the newly laid egg odour blend. α -pinene (s15) and β -pinene (s18) can be found in the VOC hive atmosphere [48]. α -pinene has also been found in VOC of larvae and pupae [26], and in VOC from brood frame with adult workers [25]. β -pinene has been found in VOC of honey [44]. In our study, frames were empty of bees and captures were done outside the hive. These two compounds were higher in brood samples

than in control wax samples, thus suggesting that they could be part of the blend of VOC emitted by eggs or by cells polished by the workers for the queen to lay eggs.

Beginning our captures on the first day of the egg stage allowed us to discover that honey bee eggs and healthy larvae emit γ -octalactone (s52). γ -octalactone has been described in *Apis dorsata* as an alarm pheromone [72], and was recently described for the first time in *A. mellifera* larvae [19]. The authors found that the amount of γ -octalactone increased with the infection by European foulbrood (*M. plutonius*). This compound has also been described as stimulating oviposition in flies [73, 74], and as being present in the metapleural glands of ants [66], glands that allow for the marking of the nest [75]. Both of these observations corroborate our observation of γ -octalactone in eggs, and suggest that it may also be involved, along the other four compounds, in egg marking by the queen or polished cell marking by the workers. A comparison between queen and worker eggs would be interesting to solve this question.

Description of brood VOC emissions at key moments of development

Chemical compounds described in this study had their maximum emission at different times of brood development. Nevertheless, most of these compounds had their peaks during the capping and capped larval stage of the brood (day 9 to 10: Capping, SL).

Larvae have a constant need to communicate with workers in order to be fed. The modulation of the pheromone signal of larvae during their development allows an adaptation of the feeding behaviour of the worker bees [7, 9, 27]. Here, four compounds had their maximum emission at the L1-L4 stage (isoamyl acetate (s10), 2-heptanone (s12), γ -octalactone (s52) and methyl salicylate (s47)) and two at the L5 stage (1-hexanol (s09) and (E)-linalool furanoxide (s35)). The modulation of VOC emitted between the L1-L4 stage and the L5 stage could allow for modulation of worker feeding behaviour, but perhaps more importantly signal the need for capping preparation.

The capping of brood cells is a key moment in brood development. Workers close the brood cell around the 9th day after egg laying. The brood is no longer fed by workers, but the larvae feed on the jelly left on the bottom of the cell and then start to weave their cocoon and prepare for metamorphosis [6]. It has already been shown that the BEP, and particularly methyl esters present in this pheromone, trigger this capping behaviour [7]. Here, we found 11 compounds at their maximum at the time of brood capping (day 9) (prenyl alcohol (s1), 2,3-butanediol (s02 and s03), octane (s05), nonane (s13), α -pinene (s15), 2-nonanone (s36), phenylethyl alcohol (s41), heptadecane (s71), nonadecane (s74), eicosane (s75)). A complex mixture of VOC and BEP could trigger the capping of brood cells by workers. More research is necessary to determine if, and which of, these compounds alone trigger capping behaviour. The complexity of the mixture captured at this key day in brood development opens up new perspectives in understanding chemical communication between brood and adults.

After capping, the larva stretches out and starts weaving its cocoon (day 11, SL) [6]. At this time, we found seven compounds at their maximum emission (phenylacetaldehyde (s28), (E)- β -ocimene (s30), 1-octanol (s32), linalool (s38), allo-ocimene (s42), tridecane (s54) and pentadecane (s65)). Then the larva starts its metamorphosis (day 12, PP) [6]; we found three compounds at their maximum release on this day (hexenyl acetate (s24), (Z)- β -ocimene (s27), α -ocimene (s29)). Communicating at the beginning of metamorphosis could allow the workers to monitor the health of the brood. A modification of those compounds emitted by the brood just after capping, for example because of *Varroa* or *Nosema* parasitism, may trigger the cleaning behaviour by the adults [16, 19, 76].

The pupal stages were characterised by the peaking of two terpene compounds, one during NSP, and one during SP. β -pinene (s18) had its maximum emission in the early pupal stages

(NSP), mainly on days 14 to 16. Nonadecene (s73) is the only compound that had its peak of emission during the last stage of brood development (SP), especially during the last 3 days before adult emergence. At the end of metamorphosis, the brood is not fed and not subject to hygienic behaviour [6]. Here, we described for the first time an emission of VOC from the brood before the emergence of the adult bee. When it could just be part of the chemical process of development and given off as a by-product, it also suggests a potential emergence signal, with a possible role in the acceptance of newly emerged workers by adults.

Interestingly, the emission of chemical families correlates with the age of the brood. Uncapped broods emitted mainly ketones, esters and alcohols, while capped broods emitted alkanes and terpenes. The emission of volatile esters (isoamyl acetate (s10), methyl benzoate (s37), methyl salicylate (s47), γ -octalactone (s52)) in larval stages is consistent with the emission of BEP from the brood at these stages [7]. These compounds could be complementary to the BEP. In addition, the brood emitted several highly volatile alkanes. Adults recognise their kin with cuticular hydrocarbon mixtures of low volatile alkanes and alkenes [77, 78]. It has been shown that long carbon chain alkanes are differentially recognised by adult honey bees [79], and it would be interesting to see if short carbon chain alkanes are also involved in nest-mate recognition.

This study is the first study of VOC emissions of brood covering the entire honey bee developmental cycle, from egg laying to adult emergence. Here, we show the complexity of the volatile chemical messages emitted by the brood at different developmental stages. When Martin *et al.* [80] suggested low volatile compound as egg marking signal, we describe the olfactory signature made of volatile compounds of *A. mellifera* eggs that could also have a potential implication in the marking of queen eggs. We also show a potential emergence signal. While the compounds identified could have a potential involvement in the chemical recognition of brood and its needs by the workers, some of the compounds identified could also be the result of metabolic by-products. Our suggestions need to be assessed using appropriate biological tests. This study opens up new perspectives on the intricate relationship between brood and adult bees, and the behaviour of nurse bees towards the brood. Brood emission of molecules already described as part of chemical communication in the adult reinforces the idea that chemical communication in the bee is highly context-dependent and based on the quantity of each molecule emitted.

Supporting information

S1 Fig. Redundancy analysis (RDA) performed on chemical compounds captured on brood and control samples (A) for “Eggs” group (day 1 to day 3), (B) “Sclerotised Pupae” group (day 17 to day 21). For each day: brood sample n = 9, control sample n = 9. Points represent samples (blue: brood, red: wax). Compounds are represented by their IDs and grey arrows. The compounds characterising the brood that are retained for the rest of the analysis are shown in bold.

(TIF)

S2 Fig. Representative gas chromatography profiles of each brood stage. The compounds listed are those identified as brood specific. The other peaks represent fibre peaks or peaks specific to the wax control. (a) Eggs, (b) L1-L4: 1st to 4th instar larvae, (c) L5: 5th instar larvae, (d) Capping: adult capping behaviour, (e) LS: larvae spinning cocoon, (f) PP: pre-pupae, (g) NSP: non-sclerotised pupae, (h) SP: sclerotised pupae. °: Unsaturation remains unknown, *: Stereochemistry remains unknown.

(TIF)

S3 Fig. Quantification of (E)- β -ocimene emission for an individual from the 1st instar larval to the 5th pupal stage. Weight data are from previous experiments. Line represents mean values and bars represent standard deviation. For each day, n = 9.

(TIF)

S1 Table. Peak areas of the 78 compounds found for each group (brood or wax), at each day and for each colony.

(CSV)

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ABSTRACT

The parasite *Varroa destructor* is a global threat to the honey bee (*Apis mellifera*) and to beekeeping worldwide. *Varroa* infestation of a colony can compromise its survival within a few months if left untreated. However, some colonies have developed resistance to the parasite and are able to regulate the growth of the *Varroa* population within the colony. This resistance is based on various traits, including VSH (Varroa Sensitive Hygiene) and brood cell recapping behaviours. These two hygienic behaviours involve (i) the detection of parasitized brood cells, (ii) the opening and then (iii) a choice in the destiny of these brood cells. Depending on this choice, the brood will be sacrificed and the cell emptied of its content (VSH) or it will be recapped leaving the brood intact (recapping). The mechanisms underlying the choice between these behaviours are not well understood but they require the emission of a semiochemical message, the detection and neural processing of this message by the workers as well as the appropriate behavioural response. In the scientific literature, ten compounds have been identified as triggering VSH behaviour, and no compound has been identified as triggering recapping behaviour. Only two of these compounds can be defined as Volatile Organic Compounds (VOCs). It is assumed that compounds of different volatility (VOCs and low volatile compounds) are involved in VSH and recapping behaviour. VOCs are thought to propagate rapidly through the wax cap thus signalling parasitism, while less volatile compounds would enable precise identification of the parasitized cell. The objective of this thesis is to improve our knowledge of the hygienic behaviour involved in the resistance of honey bee colonies to the *Varroa destructor* parasite, and in particular by participating in deciphering the differences between VSH and recapping behaviours. To this end, VOC emissions were studied in the context of healthy brood development and varroa parasitism, and worker behaviour towards all compounds identified as characteristic of parasitized brood was tested. This thesis described the emission of 32 VOCs during healthy brood development and nine VOCs characteristic of varroa parasitism, of which five could be identified. Behavioural results highlight the importance of VOCs in the detection, opening and recapping of brood cells, while low volatile compounds seems to play a particularly central role in the sacrifice of the pupa. This study opens new perspectives in the understanding of these two resistance behaviours of honey bees against their main parasite *Varroa destructor*.

Key-words: *Varroa destructor*, chemical communication, volatile compounds, resistance, VSH, recapping.

RESUME

Varroa destructor est une menace mondiale pour l'abeille domestique (*Apis mellifera*) et l'apiculture. L'infestation d'une colonie par varroa peut, en quelques mois et en l'absence de traitement, compromettre sa survie. Cependant, certaines colonies ont développé une résistance au parasite et sont capables de réguler la croissance de la population de varroa au sein de la colonie. Cette résistance s'appuie sur divers caractères, dont le comportement VSH (*Varroa Sensitive Hygiene*) et le comportement de réoperculation des alvéoles de couvain. Ces deux comportements de type hygiénique impliquent (i) la détection des alvéoles de couvain parasité, (ii) l'ouverture puis (iii) le choix dans le devenir de ces alvéoles de couvain. En fonction de ce choix, le couvain est sacrifié et l'alvéole vidée de son contenu (VSH) ou elle est réoperculée laissant intact le couvain (réoperculation). Les mécanismes sous-jacents à ce choix entre ces comportements restent assez méconnus mais ils nécessitent l'émission d'un message sémi chimique, la détection et le traitement neuronal de ce message par les ouvrières ainsi que la réponse comportementale adéquate. Dans la littérature scientifique, dix composés ont été identifiés comme déclenchant le comportement VSH, et aucun composé n'est identifié comme déclenchant le comportement de réoperculation. Seulement deux de ces composés sont caractérisés comme Composés Organiques Volatiles (COVs). Il est supposé que des composés de volatilité différente (COVs et composés faiblement volatils) participent aux comportements VSH et de réoperculation. Les COVs se propageraient rapidement à travers l'opercule de cire signalant le parasitisme et les composés moins volatils permettraient l'identification précise de l'alvéole parasitée. L'objectif de cette thèse est d'approfondir les connaissances sur le comportement hygiénique engagé dans la résistance des colonies d'abeilles domestiques envers le parasite *Varroa destructor*, et particulièrement en participant au décryptage du comportement VSH et du comportement de réoperculation. Pour cela, les émissions de COVs sont étudiées dans le cadre du développement sain du couvain et dans le cadre du parasitisme à varroa, et les comportements des ouvrières vis-à-vis de tous les composés identifiés comme étant caractéristiques du couvain parasité sont testés. Cette thèse a permis de mettre en évidence l'émission de 32 COVs au cours du développement sain du couvain et neuf COVs caractéristiques du parasitisme à varroa dont cinq ont pu être identifiés. Les résultats comportementaux soulignent l'importance des COV dans la détection, l'ouverture et la réoperculation des alvéoles de couvain, tandis que les composés faiblement volatils semblent jouer un rôle particulièrement central dans le sacrifice de la nymphe. Cette étude ouvre de nouvelles perspectives dans la compréhension de ces deux comportements de résistance des abeilles mellifères face à leur principal parasite *Varroa destructor*.

Mots-clefs : *Varroa destructor*, communication chimique, composés volatiles, résistance, VSH, réoperculation.